行政院國家科學委員會專題研究計畫成果報告

計畫編號名稱：利用酵母菌雙混種系統鑑定與愛滋病毒 VPR 蛋白直接作用之細胞蛋白(第二年)

計畫編號：NSC 88-2314-B-002-141
執行期限：87 年 8 月 1 日至 88 年 7 月 31 日

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一、中文摘要

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(tat、rev、nef、vpr、vpu、vif)。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，因此已成為學界研究重點。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。我們將運用酵母菌雙混種系統以鑑定蛋白與蛋白作用。在第二年中我們選定 HAX-1 研究其與 Vpr 的關係。至今已確定 Vpr 與 HAX-1 確實有交互作用(interaction)。在構成 Vpr 的三個單元(domain)中，第一次元是與 HAX-1 作用最重要者，不過如加上第二次元二則交互作用最強。目前正在進行 Vpr 加上 HAX-1 後對細胞週期 (cell cycle) 的影響研究。

關鍵詞：愛滋病毒，酵母菌雙混種系統，Vpr, HAX-1

Abstract

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 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). 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 has been shown to be a powerful tool in disclosing protein-protein interaction and will be adopted in this study. We have successfully cloned whole Vpr cDNA and cDNAs coding for various Vpr domains into expression plasmids and are screening for cellular interacting proteins using yeast system. A few cellular proteins have been identified to be reacting with Vpr. In this year of study we have reconfirmed the existence of interaction between Vpr and HAX-1. Our results also indicated that the first domain of Vpr is critical for this interaction. Moreover, domain 1 and domain 2 together bring about the strongest interaction. The effect of HAX-1 on the cell cycle arresting function of Vpr is being analyzed now.

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

二、緣由與目的

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 & Hay, 1998) and we believe it is worth trying this system to study HIV-1 accessory genes.

In the previous year, 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. 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 is aimed to address the subsequent issues. These include verification of the interaction by independent biochemical assay and genetic analysis of Vpr as well as the interacting cellular proteins. Finally, we need to figure out the functions of Vpr in vivo and the impact of this interaction at cellular level.

三、結果

(1) Screening for cellular proteins interacting with Vpr

本實驗經由酵母菌雙混種系統篩檢出的細胞蛋白有 HHR23A, HAX-1, MRJ-1, SMA (spinal muscular atrophy protein), 以及一些未知的蛋白(表格一所示). SMA 基因(spinal muscular atrophy-determining gene)異常會導致人體肌肉運動神經失常, 產生漸進性的痲痺(progressive paralysis), 為體染色體異常的隱性遺傳疾病(fatal autosomal recessive disorder) (Cell 1995;80:155-165). HHR23A protein 主要是參與 DNA 修補酵素系統 (DNA repair enzyme system)核甘酸移除複合體 (nucleotide excision repair complex)中的一員，在 1997 年已有文獻陸續證明它確實會與 Vpr 作用，並參與細胞週期停滯功能(J. Virol. 1997;71(12):9732-9742)。HAX-1 蛋白被發現會與 HS1 蛋白連結(associate), 而 HS1 蛋白又是 Src 酪氨酸激脢家族的受質蛋白(a substrate of Src family tyrosine kinase). HAX-1/HS1 complex 可能會與 HIV-Vpr 作用而參與調控淋巴球細胞訊息的傳遞 (signal transduction)功能, 也許透過這樣的機轉, Vpr 蛋白在愛滋病毒對人體致病力的相關性上扮演了某種重要的功能。在白血球細胞內的蛋白質摺疊(folding)與組合(assembly)作用主要靠 Hsp-70 protein family 的各不同成員蛋白協力完成, DanJ 或 DanJ-like protein(MRJ 蛋白)會與 Hsp-70 作用參與這方面的功能 (Trends Biochem Sci 1994;19:176-81)。Vpr 蛋白是否透過對 MRJ 蛋白的作用而影響宿主細胞蛋白質的合成 抑或是還有其他有趣重要的功能？也值得我們進一步去研究發現。目前本實驗系統篩檢到的已知或未知蛋白都有異於其他相關論文的新發現，在未來的深入研究中若能證明其功能與角色，相信可幫助瞭解 Vpr 蛋白並進而明瞭愛滋病毒致病機轉。基於 HAX-1 是首先得到全長基因序列(full length cDNA)的候選蛋白，又可能參與免疫細胞訊息傳遞功能，優先選擇它執行進一步分析實驗。

(2) Mapping domains of HAX-1 which interact with Vpr

我們將蛋白區分成三段次元：第一次元包括 1-42 a.a.；第二次元包括 43-82 a.a；第三次元包括 77-96 a.a。我們構築了全長、一、二、三及一到二、二到三次元等 6 種不同的 Vpr deletion mutants，在酵母菌雙混種系統中找出與 HAX-1 作用

區域，結果如表格三。Vpr 為一多效性蛋白，具 5 種重要功能：virion incorporation, immune suppression, nuclear localization, cell cycle arrest (at G_2/M phase), transactivation function。第一次元主要與 virion incorporation，nuclear localization 有關；第二次元主要參與 nuclear import 及 G_2/M arrest 功能；第三次元為富含鹼性氨基酸區域，功能不明確，可能具 nuclear localization signal；一到二次元(1-82a.a.)都參與 virion incorporation, transactivation function。在這一部分實驗中，我們發現第一次元 (domain I) 對 Vpr---HAX-1interaction 具決定性，包含一、二次元區域(Vp1+2)比全長 Vpr 對 HAX-1 更具 binding affinity (參看表格三)。

(3) In vitro protein-protein interaction studies (GST pull down assay)

分別將 Vpr 與 HAX-1 構築在含 GST 蛋白的質體中，由大腸桿菌系統大量表達 GST 融合蛋白經分析純化後，固定 (immobilized) 在 glutathione-agarose beads。同時又利用 "In vitro transcription and translation" 系統 (TNT kit, Promega)將放射性元素標定在蛋白上(^{35}S-methionine label protein)。讓 ^{35}S-Vpr 與 GST-HAX-1(或 ^{35}S- HAX-1 與 GST-Vpr)進行 binding assay，並附加 GST control，分析結果如圖形二。基於此實驗結果，在體外系統中又再度證明篩選到的細胞蛋白 HAX-1 會跟 HIV-1 Vpr 作用。

(4) Detection of Vpr-medicated cell cycle arrest

HAX-1 究竟是參與在 Vpr 哪一項功能方面，截至目前還是未知數，這是因為目前文獻對於 HAX-1 功能研究方面仍屬不祥 (J. Immunol. 1997, 158: 2736-44)，實際上從 97 年首次發表 clone 到此基因蛋白後，未曾再有後續研究了。因而我們只能從 Vpr 的功能上去推測研究。Vpr 為多功能性蛋白，在它諸多功能中建立最完整的分析系統是關於 G_2/M arrest 功能。我們利用 lipofetamine (Gibco BRL)將 Vpr DNA construct 送到 HeLa 細胞株去表現，經 72 小時培養後，收集細胞用 PI (propidium iodide)染色，以流式細胞儀(Flow cytometry)分析 DNA 含量界定細胞週期，如圖形三所示，over-express Vpr 的確造成細胞週期停滯，4N stage 的細胞數量增加(第二個 peak)。此為 Vpr-medicated G_2/M arrest 功能分析系統的初步建立，未來將同時加入 HAX-1 construct 及 anti-sense HAX-1construct，探討過量及抑制 HAX-1 蛋白表現，對既有的 G_2/M arrest 功能是否有加強或消減影響。

四、討論

Vpr 為多功能性蛋白(virion incorporation, immune suppression, nuclear localization, cell cycle arrest, transactivation)，而 HAX-1 又功能不祥。我們利用 Vpr 這已知蛋白去推測研究 HAX-1 參與在 Vpr 哪一項功能方面，目前最重要的工作在於建立分析 Vpr 功能的穩定系統。G_2/M arrest 功能可利用 PI (propidium iodide)染色分析 DNA 測定 4N stage 的細胞數量是否增加，同時還可觀察週期激素(cyclin)的變化。雖然我們實驗已初步觀察到 4N 增加現象，基於 lipofetamine (Gibco BRL)將 Vpr DNA 送到 HeLa 細胞的效率無法達 100%，考慮用 dual plasmid system 將載有 Vpr 的 DNA plasmid 同時帶上 GFP 螢光蛋白當做標記，結合 cell sorting 技術將 Vpr+的細胞區分收集進行分析，將得到更顯著的結果。目前已架構好 Vpr and GFP in dual plasmid，預定 co-transfect HAX-1DNA 及 anti-sense HAX-1，探討過量及抑制 HAX-1 蛋白表現，對既有的 G_2/M arrest 功能是否有加強或消減影響。HeLa 與 293 細胞適合用於觀察 Vpr-mediated G_2/M arrest 功能，而 Jurkat (CD4+ T cell line)與 U-87 MG (human astrocytic glial cell line) 則適合用於分析 Vpr-induced activation

of HIV-1 LTR。我們也將 LTR-CAT construct 與 CMV-Vpr plasmid co-transfect 到 Jurkat，著手進行 CAT assay，近期應有結果，此套分析系統的建立有助日後探討 HAX-1 是否參與 Vpr transactivation 功能。

Nuclear localization 的實驗分析，可藉由螢光共軛焦顯微鏡(confocal laser microscopy)，觀察送入的 Vpr 與 HAX-1 蛋白分佈在細胞內的位置(nuclear or cytoplasmic，將選用 COS-7 細胞的優點在於易表現外來蛋白、適用螢光抗體染色、及不具 endogenous HAX-1 protein 干擾。初步構想是先做出 Vpr mutants: E25K、A30F (loss nuclear localization)，由 two-hybrid system 決定與 HAX-1 binding affinity，再置換到細胞內觀察 co-localization 特性喪失與否，用以推測 HAX-1 是不是協助 Vpr nuclear import function，Confocal 分析還可以提供細胞骨架型態及細胞核的變化等進一步的觀察。

由我們目前實驗知道 Vpr domain I(1-42a.a.)對 HAX-1 binding 具關鍵性，L23F、E25K、A30F 分別會喪失不同功能，如同下表，也將被帶到此研究計畫做下階段的分析研究。關於其他功能的分析系統也會努力嘗試建立起來，期待這艱困工作的完成可運用在我們 two-hybrid 所找到的 novel protein 鑑定分析功能方面。

五、計畫成果自評

已確定 HAX-1 與 Vpr之交互作用。也確定 Vpr 與 HAX-1 作用之次元，功能研究症進行中，第二年的成果達到原先的預期。

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表格三:

I. Mapping of Vpr domain that interacts with candidate proteins:

protein	clone	Vpr	Vp1	Vp2	Vp3	lamin
SMA	1-2	+++	-	-	-	-
	1-6	+++				
	4-15	+				
HHR23A	1-5	±	-	-	-	-
	1-16	+				
	3-11	+				
HAX-1	1-30	+	-	-	-	-
	2-3	+				
MRJ	3-13	+	-	-	-	-
	3-25	+				

+, growth ; −, no growth in His minus plate

II.

protein	clone	Vpr	Vp1	Vp2	Vp3	Vp1+2	Vp2+3	lamin
HHR23A	3-11	+	-	-	-	++	-	-
HAX-1	1-30	±	-	-	-	+	-	-
	2-3	+	-	-	-	++	-	-
MRJ	3-25	-	±	-	-	±	±	-
	3-13		-	-	-	+	-	-

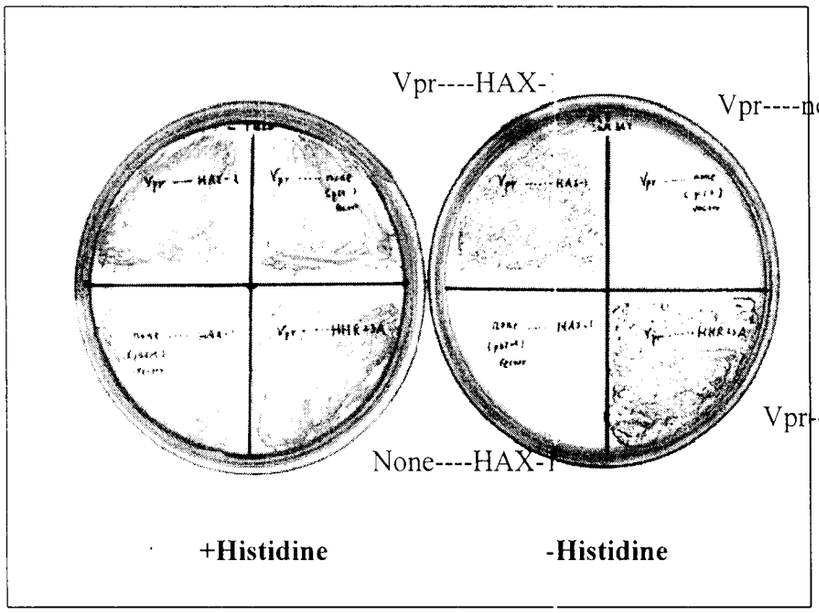
* VpI (1-42a.a.) VpII (43-82a.a.) VpIII (77-96a.a.)

* Vp1+2 (1-82 a.a.) Vp2+3 (43-96 a.a.)

III. In vivo interaction of Vpr mutants with HAX-1 in two-hybrid system

Vpr mutant		β-gal activity
WT (1-96 a.a.)	————————	+
Vp I (1-42 a.a.)	———	–
Vp II (43-82 a.a.)	———	–
Vp I (77-96 a.a.)	——	–
Vp I+II (1-82 a.a.)	————————	+++
Vp II+III (43-96 a.a.)	————————	–

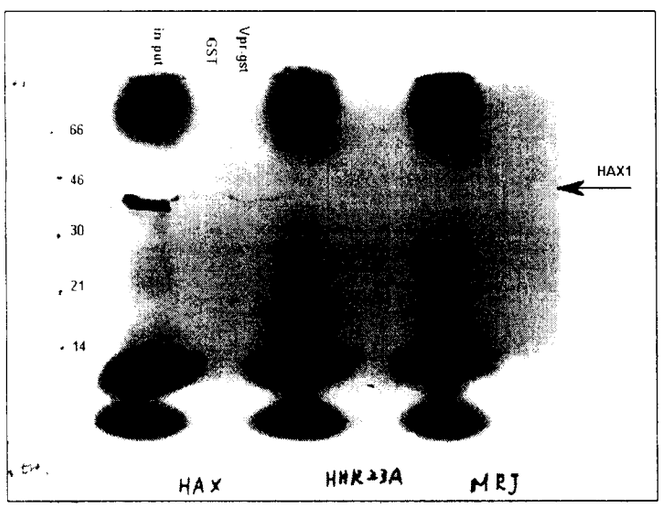
圖形一



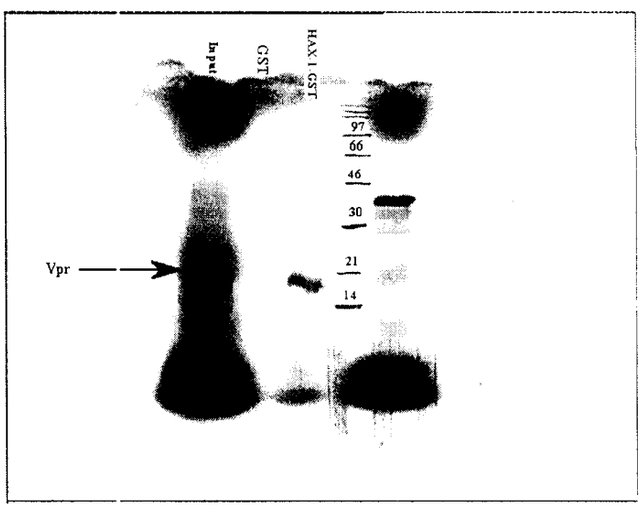
A. Specific interaction of Vpr with HAX-1

圖形二

a. Binding of HAX-1 to GST-Vpr

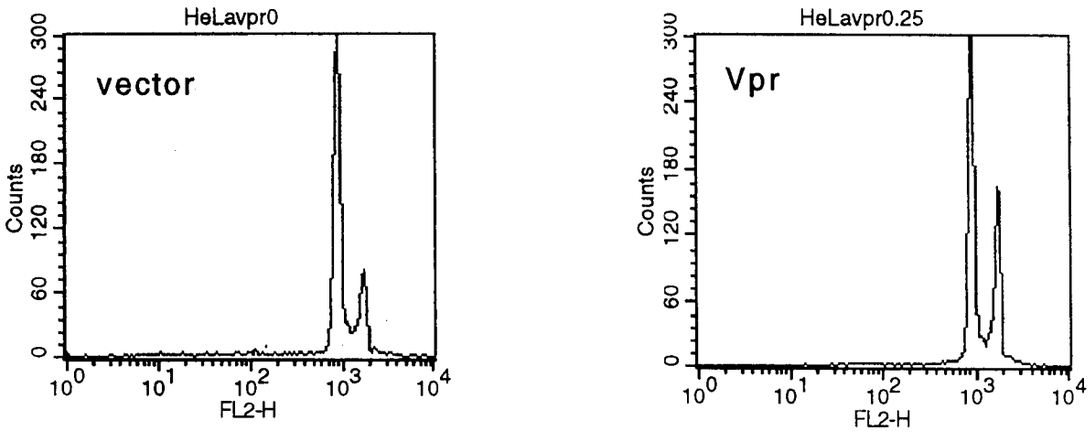


b. Binding of Vpr to GST-HAX-1

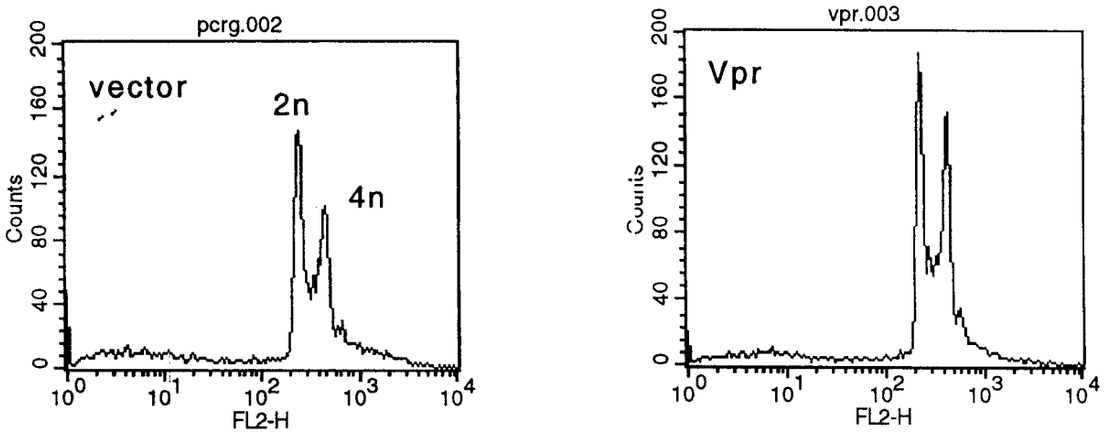


B. In vitro protein-protein interaction

圖形三 **Detection of Vpr-medicated cell cycle arrest**



a. HeLa cells post-transfection 72 hr



b. 293T cells post-transfection 72 hr

附表一:**Summary of Vpr mutants phenotypes**

mutants	Stability (% of w.t.)	Nuclear localization	Virion Incorporation (% of w.t.)	G2-arrest	HIV-1 LTR transactivation (% of w.t.)
Wild-type	100	N (nuclear)	100	++	100
L23F	85	N	20	++	90
E25K	70	P (prenuclear)	25	++	100
A30F	80	P	20	+/-	44
V57L	100	P	75	++	100
R62P	80	P	70	++	92
I63F	100	P	90	++	69
I63K	45	P	65	++	78
LI68/70RK	80	P	Not determined	-	30
R80A	100	N	100	-	34

表格二: **Summary of Vpr deleted mutants phenotypes**

mutant	stability	Nuclear localization	Virion Incorporation
△73-96	-		
△78-96	+	+/-	
△78-87	++	+--	
△84-96	+		+/-
△88-96	++	+--	
△85-88	++	+/-	
△79-82	++	+	