行政院國家科學委員會專題研究計畫 成果報告

HIV-EP2在其感染細胞中對EB病毒基因調控之研究(第2年)

研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2320-B-040-019-MY2
執 行 期 間 ：96年08月01日至97年12月31日
執 行 單 位 ：中山醫學大學醫學系微生物及免疫學科

計 畫 主 持 人：梁熾龍

計畫參與人員：學士級-專任助理人員：許文俐
大專生-兼任助理人員：黃冠鈞

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2年後可公開查詢

中 華 民 國 98年03月31日

目錄

1. Introduction

Epstein-Barr Virus (EBV) is a human herpesvirus, which infects both lymphocytes and squamous epithelial cells (Epstein *et al.*, 1964; Klein *et al.*, 1974). This virus has an instinct, which can immortalize the primary B-lymphocytes into lymphoblastoid cell lines (LCLs) *in vitro*, a process that is named as growth transformation. In the LCLs, all EBV latent genes, including six EBV-encoded nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP); three latent membrane proteins, LMP 1, LMP 2A and 2B; and two abundantly transcribed small RNAs (EBERs), are consistently expressed (Kieff and Rickinson, 2001). This kind of EBV gene expression pattern in LCLs is referred to as latency III (Gregory *et al.*, 1990; Rowe *et al.*, 1992). In the clinical studies, EBV has been considered to be associated with many human malignant tumors, especially endemic Africa Burkitt's lymphoma (BL)(de-The *et al.*, 1978) and nasopharyngeal carcinoma (NPC) (Henle *et al.,* 1970; Henle *et al.,* 1976). These two malignant tumors distinctly show different EBV gene expression pattern in contrast to that in LCL (Brooks *et al.,* 1992; Chang *et al.,* 1990; Rowe *et al.,* 1986). The BL retaining the cellular phenotype of the original tumors displays latency I form, in which only the EBV latent gene, EBNA1 is expressed (Dambaugh *et al*., 1979; Rowe *et al.,* 1986; Rowe *et al.,* 1987; Rowe *et al*., 1992). EBNA1, which associates with chromosomes during mitosis, is essential for virus replication and maintenance of the virus DNA (Grogan *et al*., 1983). Moreover, EBNA1 is also a DNA binding protein, which may bind to three specific domains within EBV genome, including the family of tandem repeat (FR), dyad symmetry (DS) and the *Bam*HI Q locus. The first two binding sites are located at the ori-P region of EBV genome (Ambinder *et al*., 1990; Wysokenski and Yates, 1989). The last one is located at the downstream region of Q promoter (Qp) initiation site. The binding of EBNA1 to ori-P region not only allows the viral genome to be an episomal form (Yates *et al*., 1985) but also be a transcriptional enhancer for viral promoters (Gahn and Sugden, 1995; Reisman and Sugden, 1986). On the contrary, the binding of EBNA1 to the *Bam*HI Q locus auto-regulates the Qp-driven EBNA1 gene expression (Sample *et al*., 1992; Tsai *et al*., 1995).

The regulation of transcription of EBNA1 gene in infected cells is very complex. In LCLs, transcription of EBNA 1 gene is initiated from Cp (Woisetschlaeger *et al*., 1990), which can be up-regulated by EBNA 2 (Ling *et al*., 1993). However, Cp activity in both BL and NPC cells usually keeps silent because its promoter region is

more methylated (Robertson *et al*., 1995). Instead, another EBNA 1 promoter Qp, which is located at ~ 40 kbp downstream region of Cp is activated and responsible for EBNA1 transcription (Tsai *et al*., 1995; Schaefer *et al*., 1995). In addition, several previous studies have demonstrated that Qp activation could be regulated by interferon regulatory factors (IRFs) through interaction with the basal transcription machinery (Nonkwelo *et al*., 1995). In our previous studies, we have clarified that Qp activity is regulated by TGF-β signal pathway, which not only suppressed the Qp activity but also activated the immediate early promoter Zp, which drove a Zta protein responsible for EBV lytic infection (Liang *et al*., 2000; Liang *et al*., 2002)

The human immunodeficiency virus type 1-enhancer binding protein 2 (HIV-EP2) was first described in 1991. It is a large transcription protein with two separate zinc finger domains and can bind to the enhancer region within the HIV-1 long terminal repeat (LTR) (Nabel and Baltimore, 1987; Nomura *et al*., 1991). HIV-EP2 is a protein of 1833 amino acids with a molecular mass of ~210 kDa, which is mapped on the chromosome 6q23-6q24 (Sudo *et al*., 1992). Most of HIV-EP2 mRNAs distribute in the brain, heart and spleen tissues (Ron *et al*., 1991; Makino *et al*., 1994; Campbell and Levitt, 2003). It belongs to the family of rel oncoproteins and metal-finger proteins, respectively (Nomura *et al*., 1991). This family at least has three members, including HIV-EP1 (also named as KBP-1, MBP1, PRDII-BF1 and Schnurri-1 [Shn-1]), HIV-EP2 (also named as MBP-2, MIBP1 and Shn-2) and HIV-EP3 (also named as KRC and Shn-3). Previous studies have found that HIV-EP2 mRNAs mostly distribute in the lymphatic tissues (Ron *et al*., 1991; Makino *et al*., 1994; Campbell and Levitt, 2003) and is induced in activated T cells (Nomura et al., 1991). Recently, more accumulating evidences have revealed that HIV-EP2 was involved in T cell development and adipocyte differentiation via the transcriptional repression and activation of its target genes (Takagi *et al*., 2001; Jin *et al*., 2006). Moreover, the Shn-3 (HIV-EP3) deficient mice may lead to a defect in thymocyte development (Allen *et al*., 2002). These results implicate that HIV-EP2 could control and contribute to T cell development. However, the precise function of HIV-EP2 still remains unclear so far.

Here, we have identified a Qp binding cellular protein, HIV-EP2, which was associated with the promoter region of EBV Qp using yeast one-hybrid system and EMSA. Therefore, the main purpose of this research plan is to explore the relationship of HIV-EP2 to regulation of EBV and cellular gene expression in the

infected cells. First of all, we observed that the level of HIV-EP2 RNA was preferentially expressed in the tissues of brain, spinal cord, and lymph node. This implicates that HIV-EP2 could be involved in growth regulation of lymphatic and neuronal cells. In addition, the overexpression of HIV-EP2 significantly reduced not only the expression of EBNA1 but also that of Zta protein. By contrary, HIV-EP2 increased the expression of EBV oncogene, LMP1 up to threefold. That means that HIV-EP2 supports EBV latent infection. Furthermore, overexpression of HIV-EP2 resulted in the increase of expression and activity and of telomerase, a hallmark of tumor cells, and cell DNA repair through the increase of c-Myc and inhibition of p53. Taken together, those results significantly provide a new mechanism by which HIV-EP2 expression is prone to EBV latent infection, and to contribute to cell immortalization through transcriptional or translational activation and repression of EBV genes and cellular genes.

2. Results

2.1 Identification of a Qp binding cellular protein, HIV-EP2

EBNA1 is the only EBV protein that is expressed in the latency I BL cells. EBNA1 transcript in these cells is derived from Qp, which is not active in the latency III LCL. This implicated that the activation of Qp in the latency I cells could be due to the interaction of specific cellular proteins and the regulatory region of Qp. In order to identify these Qp binding proteins, we prepared five copies of Qp sequence, which span the region of the -90 to -50 relative to initiation site of Qp (Fig. 1A), as a bait to screen a cDNA library derived from Rael cells, a latency I BL cell line by using the yeast one-hybrid system. The result showed that three positive clones were identified as the Qp binding protein.

Under β-gal binding assay, clone 1 showed ~15-fold increase as compared to the vector control, the other two positive clones, clone 2 and 3 only increased one fold (Fig. 1B). This indicated that clone 1 was more strongly associated with Qp sequence. Furthermore, the sequence analysis of clone 1 showed a cDNA of 607 bp, which encoded a 200-amino acid peptide with two zinc finger domains and one acidic domain and was identical to the partial region (aa1117 to aa1316) of HIV-EP2 (Nomura *et al.,* 1991). This implicated that HIV-EP2 protein in BL cells may play an important role in Qp regulation.

2.2 Association of HIV-EP2 with Qp *in vitro* in mammalian cells

The results as shown in Fig. 1B demonstrated that the DNA binding domain of HIV-EP2 was interacted with the -90 to -50 region of Qp in yeast cells. Whether this interaction also occurs in mammalian cells remains to be further investigated. To prove this point, the two plasmids of Myc-tagged HIV-EP2 and pGEM-HIV-EP2 were constructed. The pGEM-HIV-EP2 was used as a DNA template to produce the *in vitro* translated HIV-EP2 protein with a molecular weight of 210 kDa (Fig. 2A). The *in vitro* translated full-length protein was first used to react with ^{32}P-ATP-labelled -90 to -50 fragment and then the reaction mixture was analyzed in a 4% nondenaturing acrylamide gel. As shown in Fig. 2B, the *in vitro* translated HIV-EP2 protein formed a DNA-protein complex with the probe (the -90 to -50 fragment of Qp) and this complex was competed away by unlabelled probe. In addition, the Myc-HIV-EP2 was used as an expression vector for transfection of 293 cells. The over-expressed HIV-EP2 associated with the –50 to –90 region of Qp to form the complex

(Fig. 2C, lane2). The complex obviously shifted up by an anti-Myc antibody against HIV-EP2 (Fig. 2C, lane3), whereas the non-specific antibody (anti-ATF2) was not able to shift the complex (Fig 2C, lane4**).** These results of two experiments suggested that the HIV-EP2 was significantly able to bind to the region of -90 to -50 of Qp.

2.3 Distribution of HIV-EP2 expression in human tissues

To further explore the function of HIV-EP2 on the regulation of EBV infected cells, the distribution of HIV-EP2 in human tissues need to be characterized. The human RNA blots probed with ^{32}P-labelled DraIII-ClaI fragment of HIV-EP2 gene were used to perform Northern Blot analysis. A single band of RNA with an estimated size of 9.5 kb was detected and thought to be a mRNA of HIV-EP2 (Fig. 3). The HIV-EP2 RNA is preferentially expressed in the brain, skeletal muscle, lymph node, spinal cord, thyroid, trachea and adrenal gland, especially lymph node and brain. In contrast, expression of this RNA was very low in the heart, lung, liver, kidney, pancreas, stomach and bone marrow (Fig. 3). This result may support the speculation that HIV-EP2 could be a regulator involved in lymphocytes and neuron cells.

2.4 Effect of HIV-EP2 on the expression of EBV genes

Previous data have indicated that HIV-EP1 and 2 functioned as an enhancer element for HIV transcriptional regulation through a NF-κB binding site located within the HIV-1 long terminal repeat (LTR) (Maekawa *et al*., 1989; Kieran *et al*., 1990; Ghosh *et al*., 1990; Nomura *et al*., 1991). However, whether HIV-EP2 also functions on transcriptional regulation of EBV genes is unknown. In order to test the possibility, HIV-EP2 expression plasmid was individually cotransfected into 293 cells with the luciferase reporter constructs of EBV gene promoters, Qp, Zp and LMP1p (LMP1 promoter). As shown in Fig. 4A, overexpression of HIV-EP2 resulted in an about 50% inhibition of EBNA1 and Zta promoter activity. In contrast, LMP1 promoter activity was increased to about threefold as compared to vector control. Moreover, overexpression of HIV-EP2 in EBV positive cells, P3HR-1 cells, resulted in the repression of endogenous mRNA of both EBNA1 and Zta (Fig. 4B). On the contrary, HIV-EP2 increased the both mRNA and protein expression of EBV oncogene, LMP1 (Fig. 4C). This data obviously is consistent with the result of Luciferase assay (Fig. 4A), suggesting that HIV-EP2 supports the maintenance of EBV latent infection.

2.5 HIV-EP2 up-regulates the telomerase activity

Telomerase in human contains two subunits, the RNA moiety and the reverse transcriptase encoded by hTR and hTERT genes respectively, that is responsible for

maintenance of telomeres and continuous proliferation of neoplastic cells. Telomerase is specifically activated in most malignant tumors but usually inactivated in normal cells in which the telomeres are progressively shortened during cell division (Kim *et al*., 1994). Therefore, the telomerase activity may be a hallmark or critical step in cell immortalization and oncogenesis. The above result shown in Fig. 4 indicated HIV-EP2 provided a suitable environment for EBV latent infection. It is worth investigating that whether HIV-EP2 activates the telomerase activity to induce cell transformation. To address this possibility, a telomerase promoter, hTERT212 was cotransfected into 293 cells with 0, 1, 2 and 3 μg of HIV-EP2 expression plasmid. 24 hr after transfection, the cellular protein extract from those transfected cells was used to measure the luciferase activity of firefly and Renilla constructs. Results indicated that HIV-EP2 significantly activated the promoter activity of telomerase (hTERT212) in a dose-dependent manner (Fig.5A).

In addition, we had to further test whether HIV-EP2 activated the enzymatic activity of endogenous telomerase. To confirm this, the 293 cells were transfected with 0, 1 and 3 μg of HIV-EP2 expression plasmid for 24 hr. 24 hr post transfection, the cell lysate extracted from the transfected cells was used to measure the endogenous telomerase activity by using the TRAP assay described in Materials and Methods. The data of TRAP assay revealed that the telomerase activity was trigged along with the increase of HIV-EP2 (Fig. 5B). That implicated that HIV-EP2 indeed activated the telomerase activity in dose-dependent manner.

2.6 HIV-EP2 represses DNA repair of cells

Tumorigenesis induced by oncogenic viruses can be attributed to several factors, increase of cell proliferation, activation of oncogenes, inhibition of tumor suppressor genes and interference with apoptosis. Several evidences suggested that genome instability may be a common feature in the development of tumorigenesis (Kamada et al., 1992; Macera et al., 1996). The inhibition of DNA repair significantly resulted in the accumulation of unrepaired DNA and genomic instability, finally leading to the development of tumorigenesis. To investigate whether HIV-EP2 disrupted the DNA repair system to contribute to tumorigenesis, we respectively cotransfected 293 or NPC cells with HIV-EP2 and UV-damaged or non-damaged pCMV-Luc reporter construct. In this assay, the 293 cells with HIV-EP2 resulted in a 28% repression of

the DNA repair (Fig. 6A). However, the NPC cells with HIV-EP2 notably repressed its DNA repair (a 85% repression of DNA repair) (Fig. 6B).

Previously, both p53 and c-Myc has been shown to be involved in DNA repair (Smith and Seo, 2002; Zurer *et al*., 2004; Jin *et al*., 2006). The p53 mediated DNA repair may play an essential role in maintenance of genomic stability. Whereas, c-Myc is thought to induce Bcl-2 mediated suppression of DNA repair (Jin *et al*., 2006). Therefore, we examined whether HIV-EP2 repressed the DNA repair is due to the involvement of p53 and c-Myc. In other to address this, the HIV-EP2 expression plasmid or vector control was respectively transfected into 293 cells. After 24 hr, the cell lysate extracted from the transfected cells was used to perform western blot analysis. The data indicated that overexpression of HIV-EP2 resulted in the increase of c-Myc protein expression, but less expression of p53 (Fig. 6C). The results implied that HIV-EP2 suppresses DNA repair by involvement of p53 and c-Myc.

3. Discussion

Qp is an EBV latent promoter, which is active in EBV-associated tumor cells such as type I BL and NPC. By analysis of the cellular factors controlling Qp activity, several regions of Qp have been identified. The -49 to -45 region of Qp is a TGF-β inducible motif associated with Smad4, which recruits a transcriptional repressor TGIF, resulting in transcriptional repression. On the contrary, BZLF1 gene encoding Zta protein is activated by TGF-β. The activation is mediated through Smad4 binding to the SBE site and cooperation of Smad proteins and the AP1 complex (Liang *et al*., 2000; Liang *et al*., 2002). In present study, we have used the region of -90 to -50 relative to initiation site of Qp as bait to carry out the yeast one-hybrid screen, a transcription regulator, HIV-EP2 screened from cDNA library was identified as Qp binding protein. The Qp containing two potential NF-κB-like binding motifs within the region of -90 to -50 was also recognized by HIV-EP2 as assayed by the EMSA. However we did not rule out the possibility that other members of HIV-EP2 family can recognize these sites within Qp in EBV infected cells.

HIV-EP2, also named as the vertebrate homolog of Shn-2, is a large zinc finger transcription factor that may function in the regulation of cellular development. Though HIV-EP2 was originally identified as the NF-κB binding protein (Maekawa *et al*., 1989), it also entered the nucleus and recruited to *PPARγ2* (a key gene involved in adipogenesis) promoter through interaction with Smad1 and C/EBPα upon stimulation of TGF-β/BMP/activin (Jin *et al*., 2006). This implicates that HIV-EP2 may not only directly bind to promoter but also serve as scaffold protein to form ternary complex with various transcription factors, eventually leading to synergistically regulate gene transcription. Therefore, the HIV-EP2 mediated regulation is most probably involved in EBV gene expression and life cycle in infected cells. Moreover, the present study also demonstrates that HIV-EP2 represses Zta protein, a key switch from EBV latent to lytic infection, but activates LMP1, an oncogenic latent gene, which is essential for the EBV transformation in latently infected cells. These results markedly provide the speculation that HIV-EP2 may prevent from EBV reactivation by Zta and help LMP1 to establish the B-cell transformation. Interestingly, HIV-EP2 also represses the expression of another latent gene EBNA1, which always expresses in all EBV infected cells. The result seems to be against the principle of EBV latent infection. However, the reason of HIV-EP2

mediated EBNA1 repression may probably minimize the EBNA1 expression in infected cells and avoid the surveillance of immune system. This concept seems to be similar to the previous report that self-inhibition of EBNA1 prevents antigen presentation on MHC class I molecule and avoids cytotoxic T cell recognition (Yin *et al*., 2003). Thus, EBV can keep alive via escaping from host immune system in infected cells.

Telomerase is a cellular reverse transcriptase that mediates the extension of telomeres. This enzyme is usually activated in malignant tumors but inactivated in normal cells (Greider *et al*., 1989; Kim *et al*., 1994). In general, the increase of telomerase activity represents the extension of cell lifespan. About 90% of human cancer *in vivo* expresses the presence of telomerase activity. Telomerase activity is therefore a rate-limiting or critical step in cellular immortalization and oncogenesis. The present result shows that HIV-EP2 not only activates an EBV oncogene, LMP1 but also cellular telomerase, a hallmark of tumor cells (Fig. 5). This results means in addition to LMP1, cellular telomerase may be a new key regulator maintaining EBV immortalization in latently infected cells via HIV-EP2 activation. On the other hand, expression of HIV-EP2 was induced by mitogen and phorbal ester in T cell line, Molt 4 (Nomura *et al*., 1991). It has been therefore implied HIV-EP2 mediated regulation is possibly involved in T cell growth control. In northern blot analysis, HIV-EP2 is highly expressed in lymph node tissues (Fig. 3), further indicating its potential role in lymphoid cells. Taken together, the future work is therefore to examine the effect of HIV-EP2 on the EBV infected cells upon stimulation of TGF-β and find out which signal pathway involved in gene regulation. Understanding the critical response elements within promoters of key genes of EBV should help us to elucidate how the cellular proteins cooperate with EBV gene expression to regulate EBV life cycle. Furthermore, we can establish the new strategies for therapy of EBV associated tumors based on the role of HIV-EP2 in EBV infection.

4. Materials and methods

Cell culture

Rael, an EBV-positive Burkitt's lymphoma (BL) cell line, was maintained in RPMI 1640 (Sigma, USA) supplemented with 15% (v/v) fetal bovine serum (FBS). C33-A (ATCC HTB31) was cultured in Delbecco's modified Eagle medium (DMEM) with 10% FBS. 293T and MCF-7 (ATCC HTB22) was maintained in DMEM medium (Sigma, USA) with 10% FBS.

Plasmid construction

Four overlapping fragments covering the full-length HIV-EP2 cDNA were generated by RT-PCR with RNA isolated from Rael cells and four sets of primers. These four cDNA fragments were first digested with appropriate restriction enzymes and then ligated as a full-length cDNA of HIV-EP2 before inserted into the *Eco*RI site of pGEM4Z (Promega,USA) . The resulting plasmids designated as pGEM-HIV-EP2 was used to *in vitro* transcription/translation. The EP2 expression construct, pMyc-HIV-EP2, was generated by insertion of the full-length EP2 into pCMVMyc plasmid. This expression plasmid was subject to luciferase assay and EMSA.

RNA extraction and northern blot analysis

RNA was extracted from the cells according to the manufacturer's instructions. The 50μg of total RNA was separated on 1% formaldehyde agarose gel. The gel was transferred to NC paper and hybridized with the specific probes as described. After washing of non-specific binding, the membrane was subjected to auto-radiography.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Five μg of total RNA of Rael cells was annealed with 1μM 3'primer and then reverse transcribed using 200U SuperScript II (Life Technologies, USA) at 42^{o}C for 1 hr to generate the cDNA products. One third of the cDNA products were added into the reaction mixture containing 0.2μM 3'primer, 0.2μM 5'primer, 1X PCR buffer, 125 μM dNTP and 3u Expand high fidelity Taq polymerase to a final volume of 100μl. PCR was carried out at 28 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec and 68^{o}C for 8 min. The PCR products were analyzed on agarose gel in 1X TAE buffer.

Electrophoretic mobility shift assay

The proper region of Qp was used as a probe in electrophoretic mobility shift assay (EMSA). In brief, the fragment was labeled with T4 polynucleotide kinase and [γ^{32}P]ATP. *In vitro* translated protein lysate or nuclear extract was incubated with

15,000cpm of each the ^{32}P-labelled probe in the reaction buffer containing 0.1μg/ul poly dI:dC, 4% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl and 10mM Tris-HCl, pH 7.5. For the competition assay, a 200-fold excess of the cold probe was added in the reaction before addition of the ^{32}P-labelled probe. After 30 min of incubation at RT, the samples were loaded onto a 4% nondenaturing acrylamide gel and electrophoresis was performed in 0.5X TBE at 100V for 3 hr. For the supershift assay, the reaction mixture was incubated with 4 μg of antibody and applied to gel. The gels subsequently were dried and exposed to Kodak BioMax film.

In vitro transcription and translation

The reaction of transcription and translation of the pGEM-EP2 was performed by using a TNT coupled reticulocyte lysate system (Promega, USA) to generate HIV-EP2 protein. The reaction was carried out in a reaction mixture of 50μl including 1μg of circular pGEM-EP2, pGEM or luciferase control plasmid (Promega, USA) respectively, and 25μl rabbit reticulocyte lysate, 1X TNT reaction buffer, 20μM methionion-free amino acid mixture, 4μl ^{35}S-methionine (3,000Ci/mmol, Amersham, England), 40u RNasin ribonuclease inhibitor and 1u T7 RNA polymerase. After 30^{o}C incubation for 90min, products were analyzed with 5% SDS -polyacylamide gel electrophoresis (SDS-PAGE) and subjected to EMSA.

DNA transfection and luciferase assays

The indicated amount of expression plasmids were introduced into the suspension cells ($1x10^7$) by electroporation at 960 μF and 0.22 kV using the Gene Pulser (Bio-Rad, USA) or the epithelial cells ($1x10^6$) by Lipofectamine^{TM}2000 (Invitrogen) and the transfected cells were incubated for 24 hr. The cells were then harvested and lysed in 50 μl of lysis buffer (Promega, USA). The protein concentration of the cell lysate was measured by using a Bio-Rad protein assay reagent (Bio-Rad, USA). A 50μg sample of protein extract was used to measure the luciferase activity in an illuminometer (Berthold autolumat model LB953, Germany).

Immunoprecipitation and western blotting

Immunoprecipitation assays were performed in 293 cells. Cleared lysates were incubated overnight with the specific antibodies and immobilized to protein G-Sepharose beads (Amersham Pharmacia Biotech) to immunoprecipitate the proteins. Samples were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred onto PVDF membranes (Millipore). Immunoreactive

protein bands were visualized with an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology Inc.). Western blot analysis was carried out by incubation of cell lysates with the indicated antibody. Briefly, the cells were lysed for 30 min. on ice in 0.5% NP-40 lysis buffer containing 50mM HEPES (pH 7.4) 250mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, 2μg / ml aprotinin, 2μg / ml pepstatin and 2μg / ml leupeptin. Cell debris was removed by centrifugation at 10,000Xg for 10 min. at 4^{o}C. The protein concentration of cell lysate was measured by Bradford method (Bio-Red, Richmond CA). The lysate was applied to 10% SDS-PAGE gel and then transferred to the nitrocellulose membrane. The membrane was incubated with polyclonal antibody of anti-Myc (Santa Cruz Biotechnology Inc.) or anti-S antibody and reacted with the horseradish peroxidase-conjugated anti-rabbit antibodies as secondary antibody.

DNA repair assay

DNA repair assay was previously described by using a luciferase reporter plasmid (Tran *et al*., 2002; Liu *et al*., 2004). Briefly, the luciferase plasmid (pCMV-Luc) either UV-treated or mock treated and GFP plasmid as internal control were cotransfected into HIV-EP2 stable cells or the cells without HIV-EP2 with Lipofectamine 2000 (Invitrogen). 24 hr post transfection, the lysates from different treatments of cells were prepared to assay the activity of luciferase. The luciferase assay was followed as the procedure described by Liu *et al*., 2004.

Telomerase activity assay

Telomerase activity was measured with the modified telomere repeat amplification protocol (TRAP) assay (14,26). The cells were lysed with 100μL of 1X CHAPS lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EGTA, 0.5% CHAPS, 10% [v/v] glycerol, 5 mM β-2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride), incubated on ice for 30 min and centrifuged (13,000g, 4^{0}C, 30 min). The protein concentration of cell extracts was determined with a BSA Protein Assay Kit (BioRad). The cellular proteins (50ng) of each sample mixed with a set of primers (TS, 5'-AAT CCGTCGAGCAGAGTT-3'; CXA, 5'–GCGCGGCTTA CCCTTACCC TTACCCT AACC-3') were performed to telomerase-mediated extension (30^{0}C, 30 min) and followed by heating (85^{0}C, 15min) for inactivating. After telomerase-mediated extension, Taq polymerase was added to each reaction to perform 27 cycles of polymerase chain reaction (PCR) amplification (94^{0}C for 30 s, 55^{0}C for 30 s, and

72^0C for 90 s) in a DNA thermal cycler (GeneAMP, PCR System 9700). TRAP products of each sample were resolved by 12.5% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE) and visualized by staining with ethidium bromide.

5. References

Allen, C. E., Muthusamy, N., Weisbrode, S. E., Hong, J. W., and Wu, L. C. (2002). Developmental anomalies and neoplasia in animals and cells deficient in the large zinc finger protein KRC. Genes Chromosomes Cancer *35*, 287-298.

Ambinder, R. F., Shah, W. A., Rawlins, D. R., Hayward, G. S., and Hayward, S. D. (1990). Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein-Barr virus DNA. Journal of Virology *64*, 2369-2379.

Brooks, L., Yao, Q. Y., Rickinson, A. B., and Young, L. S. (1992). Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. Journal of Virology *66*, 2689-2697.

Campbell, D. B., and Levitt, P. (2003). Regionally restricted expression of the transcription factor c-myc intron 1 binding protein during brain development. J Comp Neurol *467*, 581-592.

Chang, Y. S., Shih, L. Y., Peng, C. L., Chen, S. H., and Liu, S. T. (1990). Characterization of two newly established EBV-containing lymphoblastoid cell lines from patients with myeloid leukemias. Leukemia Research *14*, 309-320.

de-The, G., Geser, A., Day, N. E., Tukei, P. M., Williams, E. H., Beri, D. P., Smith, P. G., Dean, A. G., Bronkamm, G. W., Feorino, P., and Henle, W. (1978). Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature *274*, 756-761.

Epstein, M. A., Achong, B. G., and Barr, Y. M. (1964). Virus particles in cultured lymphocytes from Burkitt's lymphoma. Lancet *1*, 702-703.

Fahraeus, R., Fu, H. L., Ernberg, I., Finke, J., Rowe, M., Klein, G., Falk, K., Nilsson, E., Yadav, M., and Busson, P. (1988). Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. International Journal of Cancer *42*, 329-338.

Gahn, T. A., and Sugden, B. (1995). An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. Journal of Virology *69*, 2633-2636.

Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. Nature *344*, 678-682.

Gregory, C. D., Rowe, M., and Rickinson, A. B. (1990). Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. Journal of General Virology *71*, 1481-1495.

Greider, C. W., and Blackburn, E. H. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature *337*, 331-337.

Grogan, E. A., Summers, W. P., Dowling, S., Shedd, D., Gradoville, L., and Miller, G. (1983). Two Epstein-Barr viral nuclear neoantigens distinguished by gene transfer, serology, and chromosome binding. Proc Natl Acad Sci USA *80*, 7650-7653.

Henle, G., and Henle, W. (1976). Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. International Journal of Cancer *17*, 1-7.

Henle, W., Henle, G., Ho, H. C., Burtin, P., Cachin, Y., Clifford, P., de Schryver, A., de-The, G., Diehl, V., and Klein, G. (1970). Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms, and control groups. Journal of the National Cancer Institute *44*, 225-231.

Hitt, M. M., Allday, M. J., Hara, T., Karran, L., Jones, M. D., Busson, P., Tursz, T., Ernberg, I., and Griffin, B. E. (1989). EBV gene expression in an NPC-related tumour. EMBO Journal *8*, 2639-2651.
Jepsen, K., Hermanson, O., Onami, T. M., Gleiberman, A. S., Lunyak, V., McEvilly, R. J., Kurokawa, R., Kumar, V., Liu, F., Seto, E.*, et al.* (2000). Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell *102*, 753-763.
Jin, W., Takagi, T., Kanesashi, S. N., Kurahashi, T., Nomura, T., Harada, J., and Ishii, S. (2006a). Schnurri-2 controls BMP-dependent adipogenesis via interaction with Smad proteins. Dev Cell *10*, 461-471.
Jin, Z., May, W. S., Gao, F., Flagg, T., and Deng, X. (2006b). Bcl2 suppresses DNA repair by enhancing c-Myc transcriptional activity. J Biol Chem *281*, 14446-14456.
Kamada, N., Sakurai, M., Miyamoto, K., Sanada, I., Sadamori, N., Fukuhara, S., Abe, S., Shiraishi, Y., Abe, T., Kaneko, Y., and et al. (1992). Chromosome abnormalities in adult T-cell leukemia/lymphoma: a karyotype review committee report. Cancer Res *52*, 1481-1493.
Kenney, S., Kamine, J., Holley-Guthrie, E., Lin, J. C., Mar, E. C., and Pagano, J. (1989). The Epstein-Barr virus (EBV) BZLF1 immediate-early gene product differentially affects latent versus productive EBV promoters. Journal of Virology *63*, 1729-1736.
Kieff, E., and Rickinson, A. (2001). Epstein-Barr virus and its replication., 4rd edn (New York: Racen Press).
Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israel, A. (1990). The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. Cell *62*, 1007-1018.
Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.
Klein, G., Wiener, F., Zech, L., zur Hausen, H., and Reedman, B. (1974). Segregation of the EBV-determined nuclear antigen (EBNA) in somatic cell hybrids derived from the fusion of a mouse fibroblast and a human Burkitt lymphoma line. International Journal of Cancer *14*, 54-64.
Liang, C. L., Chen, J. L., Hsu, Y. P., Ou, J. T., and Chang, Y. S. (2002). Epstein-Barr virus BZLF1 gene is activated by transforming growth factor-beta through cooperativity of Smads and c-Jun/c-Fos proteins. J Biol Chem *277*, 23345-23357.
Liang, C. L., Tsai, C. N., Chung, P. J., Chen, J. L., Sun, C. M., Chen, R. H., Hong, J. H., and Chang, Y. S. (2000). Transcription of Epstein-Barr virus-encoded nuclear antigen 1 promoter Qp is repressed by transforming growth factor-beta via Smad4 binding element in human BL cells. Virology *277*, 184-192.
Ling, P. D., Rawlins, D. R., and Hayward, S. D. (1993). The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein. Proceedings of the National Academy of Sciences of the United States of America *90*, 9237-9241.
Macera, M. J., Hyde, P., Peddanna, N., Szabo, P., Gogineni, S. K., and Verma, R. S. (1996). T-cell receptor J beta 1/J beta 2 locus rearrangements in an HTLV-1-positive T-cell lymphoma with complex chromosomal aberrations. Am J Hematol *52*, 53-57.

Maekawa, T., Sakura, H., Sudo, T., and Ishii, S. (1989). Putative metal finger structure of the human immunodeficiency virus type 1 enhancer binding protein HIV-EP1. Journal of Biological Chemistry *264*, 14591-14593.
Makino, R., Akiyama, K., Yasuda, J., Mashiyama, S., Honda, S., Sekiya, T., and Hayashi, K. (1994). Cloning and characterization of a c-myc intron binding protein (MIBP1). Nucleic Acids Res *22*, 5679-5685.
Nabel, G., and Baltimore, D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells.[erratum appears in Nature 1990 Mar 8;344(6262):178]. Nature *326*, 711-713.
Nomura, N., Zhao, M. J., Nagase, T., Maekawa, T., Ishizaki, R., Tabata, S., and Ishii, S. (1991). HIV-EP2, a new member of the gene family encoding the human immunodeficiency virus type 1 enhancer-binding protein. Comparison with HIV-EP1/PRDII-BF1/MBP-1. Journal of Biological Chemistry *266*, 8590-8594.
Nonkwelo, C., Henson, E. B., and Sample, J. (1995). Characterization of the Epstein-Barr virus Fp promoter. Virology *206*, 183-195.
Nonkwelo, C., Ruf, I. K., and Sample, J. (1997). Interferon-independent and -induced regulation of Epstein-Barr virus EBNA-1 gene transcription in Burkitt lymphoma. Journal of Virology *71*, 6887-6897.
Reisman, D., and Sugden, B. (1986). trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Molecular & Cellular Biology *6*, 3838-3846.
Robertson, K. D., Hayward, S. D., Ling, P. D., Samid, D., and Ambinder, R. F. (1995). Transcriptional activation of the Epstein-Barr virus latency C promoter after 5-azacytidine treatment: evidence that demethylation at a single CpG site is crucial. Molecular & Cellular Biology *15*, 6150-6159.
Ron, D., Brasier, A. R., and Habener, J. F. (1991). Angiotensinogen gene-inducible enhancer-binding protein 1, a member of a new family of large nuclear proteins that recognize nuclear factor kappa B-binding sites through a zinc finger motif. Mol Cell Biol *11*, 2887-2895.
Rowe, D. T., Rowe, M., Evan, G. I., Wallace, L. E., Farrell, P. J., and Rickinson, A. B. (1986). Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. EMBO J *5*, 2599-2607.
Rowe, M., Evans, H. S., Young, L. S., Hennessy, K., Kieff, E., and Rickinson, A. B. (1987). Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. Journal of General Virology *68*, 1575-1586.
Rowe, M., Lear, A. L., Croom-Carter, D., Davies, A. H., and Rickinson, A. B. (1992). Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. Journal of Virology *66*, 122-131.
Sample, J., Brooks, L., Sample, C., Young, L., Rowe, M., Gregory, C., Rickinson, A., and Kieff, E. (1991). Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. Proceedings of the National Academy of Sciences of the United States of America *88*, 6343-6347.
Sample, J., Henson, E. B., and Sample, C. (1992). The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. Journal of Virology *66*, 4654-4661.
Schaefer, B. C., Strominger, J. L., and Speck, S. H. (1995a). Redefining the Epstein-Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription

initiation site in group I Burkitt lymphoma cell lines. Proceedings of the National Academy of Sciences of the United States of America *92*, 10565-10569.
Schaefer, B. C., Strominger, J. L., and Speck, S. H. (1995b). The Epstein-Barr virus BamHI F promoter is an early lytic promoter: lack of correlation with EBNA 1 gene transcription in group 1 Burkitt's lymphoma cell lines. Journal of Virology *69*, 5039-5047.
Schaefer, B. C., Woisetschlaeger, M., Strominger, J. L., and Speck, S. H. (1991). Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proceedings of the National Academy of Sciences of the United States of America *88*, 6550-6554.
Sen, R., and Baltimore, D. (1986). Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell *47*, 921-928.
Smith, M. L., and Seo, Y. R. (2002). p53 regulation of DNA excision repair pathways. Mutagenesis *17*, 149-156.
Smith, P. R., and Griffin, B. E. (1992). Transcription of the Epstein-Barr virus gene EBNA-1 from different promoters in nasopharyngeal carcinoma and B-lymphoblastoid cells. Journal of Virology *66*, 706-714.
Sudo, T., Ozawa, K., Soeda, E. I., Nomura, N., and Ishii, S. (1992). Mapping of the human gene for the human immunodeficiency virus type 1 enhancer binding protein HIV-EP2 to chromosome 6q23-q24. Genomics *12*, 167-170.
Sung, N. S., Wilson, J., Davenport, M., Sista, N. D., and Pagano, J. S. (1994). Reciprocal regulation of the Epstein-Barr virus BamHI-F promoter by EBNA-1 and an E2F transcription factor. Molecular & Cellular Biology *14*, 7144-7152.
Takagi, T., Harada, J., and Ishii, S. (2001). Murine Schnurri-2 is required for positive selection of thymocytes. Nat Immunol *2*, 1048-1053.
Tsai, C. N., Liu, S. T., and Chang, Y. S. (1995). Identification of a novel promoter located within the Bam HI Q region of the Epstein-Barr virus genome for the EBNA 1 gene. DNA & Cell Biology *14*, 767-776.
Woisetschlaeger, M., Yandava, C. N., Furmanski, L. A., Strominger, J. L., and Speck, S. H. (1990). Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proceedings of the National Academy of Sciences of the United States of America *87*, 1725-1729.
Wysokenski, D. A., and Yates, J. L. (1989). Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus. Journal of Virology *63*, 2657-2666.
Yates, J. L., Warren, N., and Sugden, B. (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature *313*, 812-815.
Yin, Y., Manoury, B., and Fahraeus, R. (2003). Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. Science *301*, 1371-1374.
Young, L. S., Dawson, C. W., Clark, D., Rupani, H., Busson, P., Tursz, T., Johnson, A., and Rickinson, A. B. (1988). Epstein-Barr virus gene expression in nasopharyngeal carcinoma. Journal of General Virology *69*, 1051-1065.
Zhang, L., and Pagano, J. S. (1997). IRF-7, a new interferon regulatory factor associated with Epstein-Barr virus latency. Molecular & Cellular Biology *17*, 5748-5757.
Zhang, L., and Pagano, J. S. (1999). Interferon regulatory factor 2 represses the Epstein-Barr virus BamHI Q latency promoter in type III latency. Molecular & Cellular Biology *19*, 3216-3223.

Zurer, I., Hofseth, L. J., Cohen, Y., Xu-Welliver, M., Hussain, S. P., Harris, C. C., and Rotter, V. (2004). The role of p53 in base excision repair following genotoxic stress. Carcinogenesis *25*, 11-19.

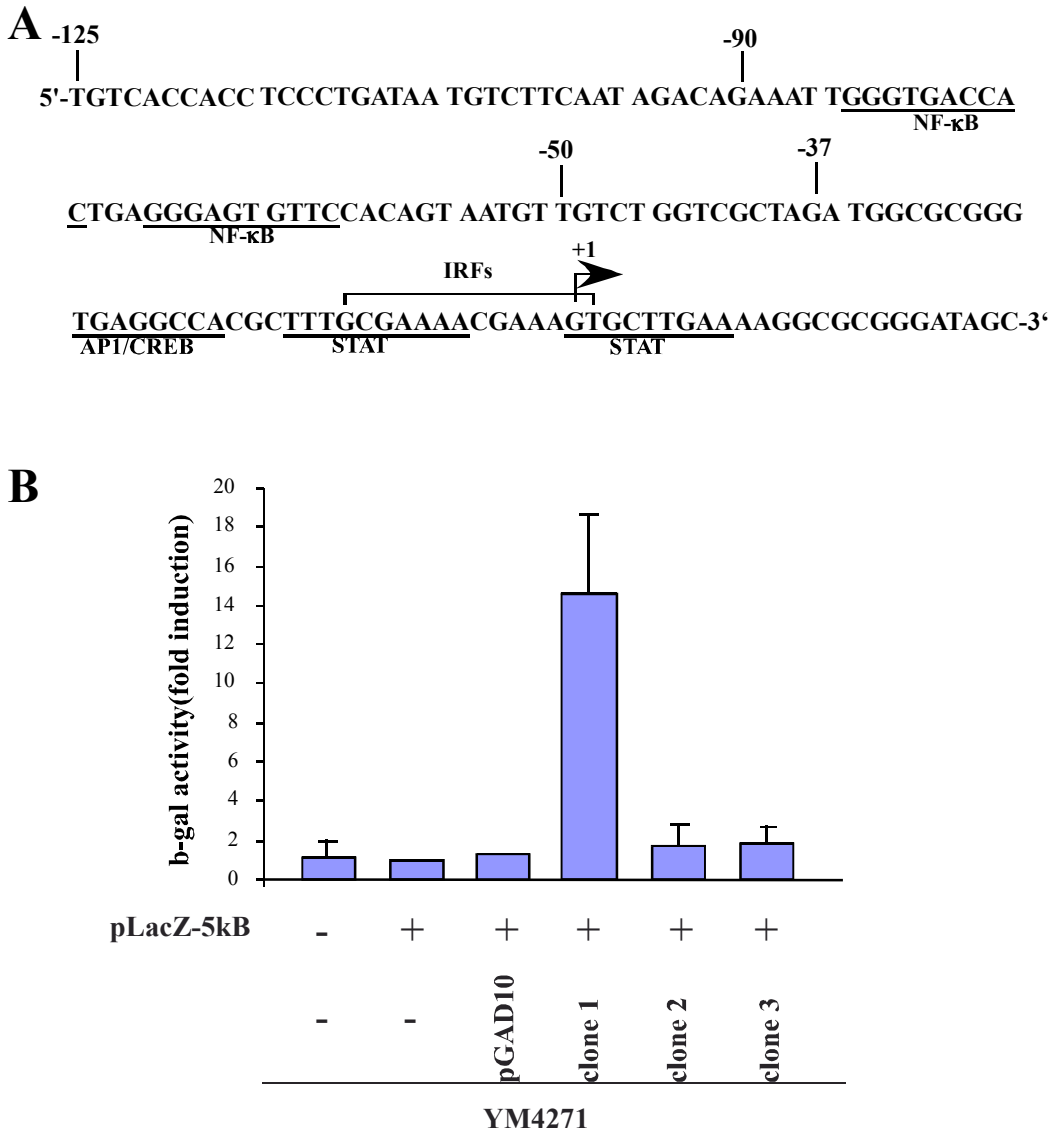

Fig. 1 Identification of Qp binding protein, HIV-EP2. (A) The sequence of Q promoter (Qp)(-125 to +24)The Number represents the position of promoter sequence relative to initiation site(+1). The position of IRF, AP1 and STAT are shown as the above. The two NF-κB motifs are located at the -84 to -75 and -71 to -62 regions of Qp **(B)** Specific interaction of the -90 to -50 region within Qp with clone 1 cDNA by yeast one-hybrid assay. The YM4271 is a parental yeast cells, which were used as host for yeast one-hybrid assay. The YM4271 yeast cells were cotransformed with the different sets of plasmids, as indicated above. The pLacZ-5kB is a b-galactosidase reporter plasmid, which contains a 5 copies of the –90 to –50 region of Qp located at the upstream of b-galactosidase gene. Clone 1, 2 and 3 are the positive cDNA clones for binding to Q promoter. The pGAD10 is used as vector control. B-gal activity was determined as fold induction compared to the activity of YM4271 yeast cells (as 1 fold).

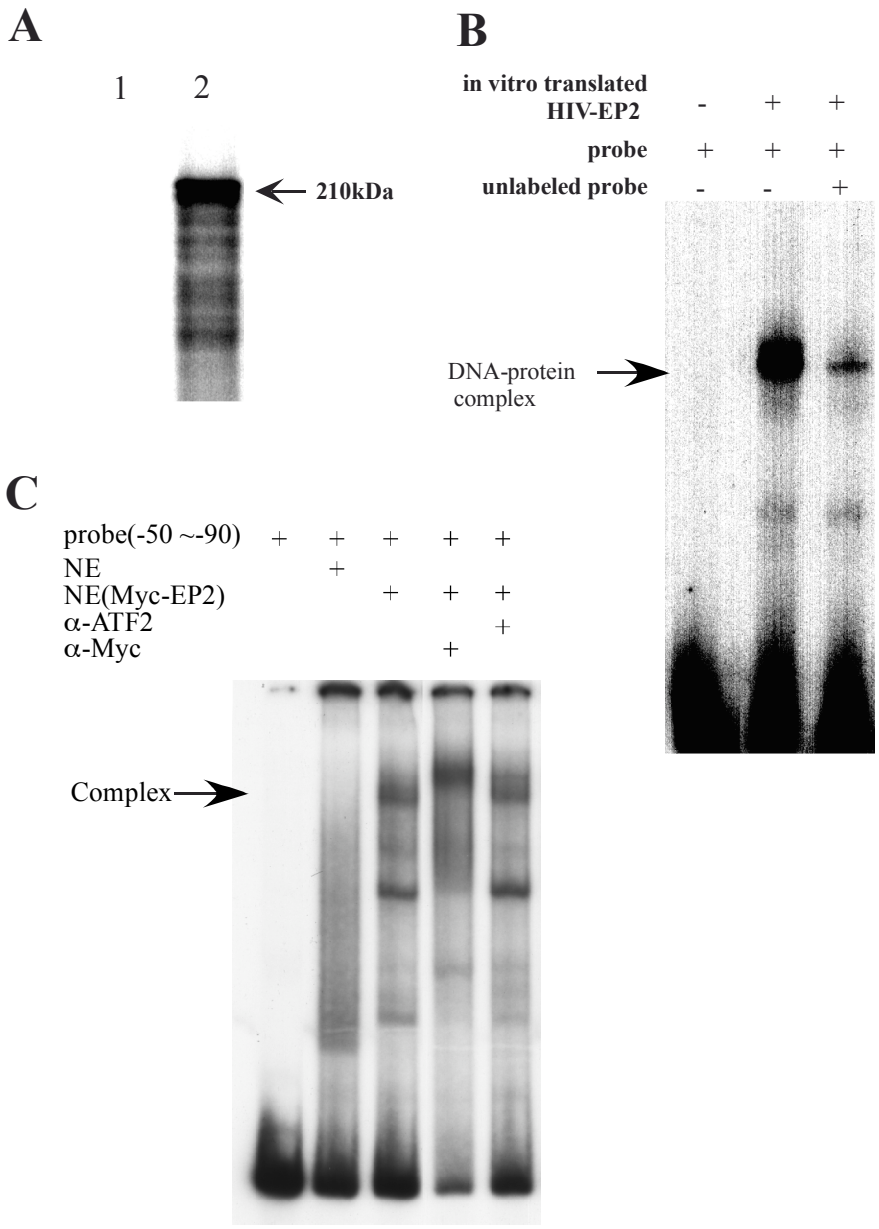

Fig 2 Association of HIV-EP2 with EBV Qp *in vitro.* (A) A 5% SDS-polyacrylamide protein gel of the *in vitro* full-length EP2 labeled with ^{35}S-methionine(lane2). The pGEM4Z as control was used in *in vitro* translation system (lane1). The arrows indicated the molecular weight of translated proteins. **(B)** EMSA analysis of *in vitro* translated EP2 with the end-labeled probe, which spans from -90 to -50 within Qp **(C)** EMSA analysis of over-expressed HIV-EP2 with the same probe(-90 to -50) in 293 cells.NE: nuclear extract, NE(Myc-EP2): nuclear extract with HIV-EP2.

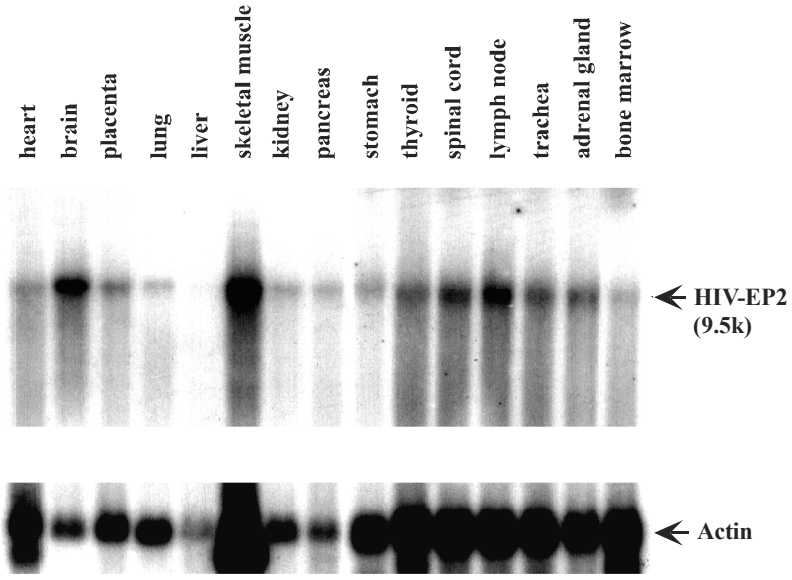

Fig 3 The distribution of HIV-EP2 mRNA in human tissues and cell lines. The HIV-EP2 mRNA from human tissues were determined by using northern blot analysis. The arrows represent the positions of HIV-EP2 and actin mRNA. Actin acts as internal control.

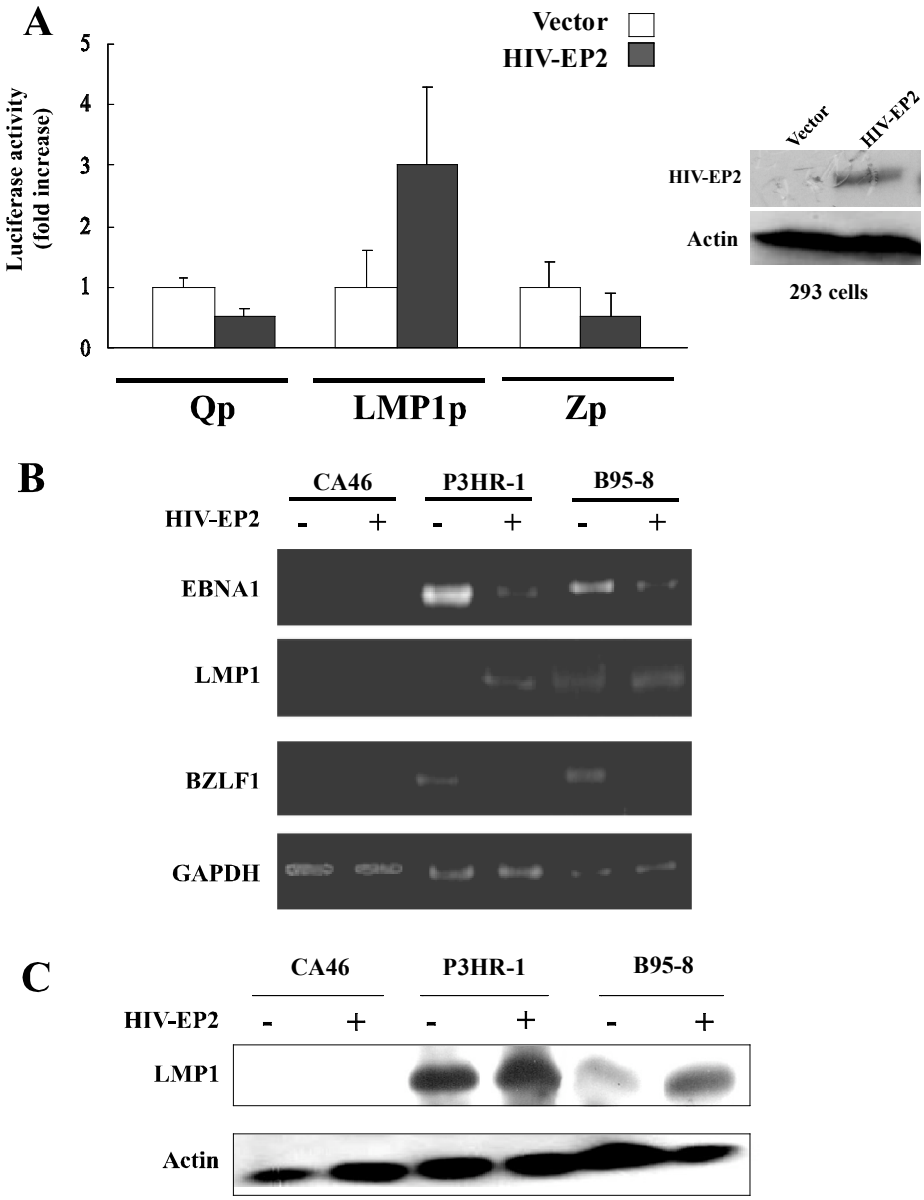

Fig. 4 Effect of HIV-EP2 on EBV gene expression (A) HIV-EP2 affects the transcriptional activity of EBV genes. The promoter luciferase plasmids, Qp, LMP1p and Zp were individually cotransfected with HIV-EP2 or vector control in 293 cells. Luciferase activity was analyzed 24hr post transfection. Error bars represent the mean ±SD (n=3). The transfected cell lysate with HIV-EP2 or vector was analyzed by western blotting with antibody to c-Myc (left panel). Qp: EBNA1 promoter luciferase plamid; LMP1p: LMP1 promoter luciferase plsmid; Zp: Zta promoter luciferase plasmid. **(B)** HIV-EP2 affects the RNA expression of EBV genes. The cells, EBV positive cells (P3HR-1 and B95-8) or EBV negative cells (CA46) were transfected with HIV-EP2 or vector plamid. 24hr post transfection, the total RNA from those transfected cells was used to carry out the RT-PCR with the specific primer sets of EBV, including EBNA1, LMP1 and BZLF1. GADPH acts as internal control. **(C)** HIV-EP2 affects the protein expression of EBV LMP1. The protocol of cell transfection was same as panel **B**. 24hr after treatment, the cell lysate extracted from the transfected cells was used to carry out the western blotting with antibody against EBV LMP1 antibody (S12). Actin acts as internal control.

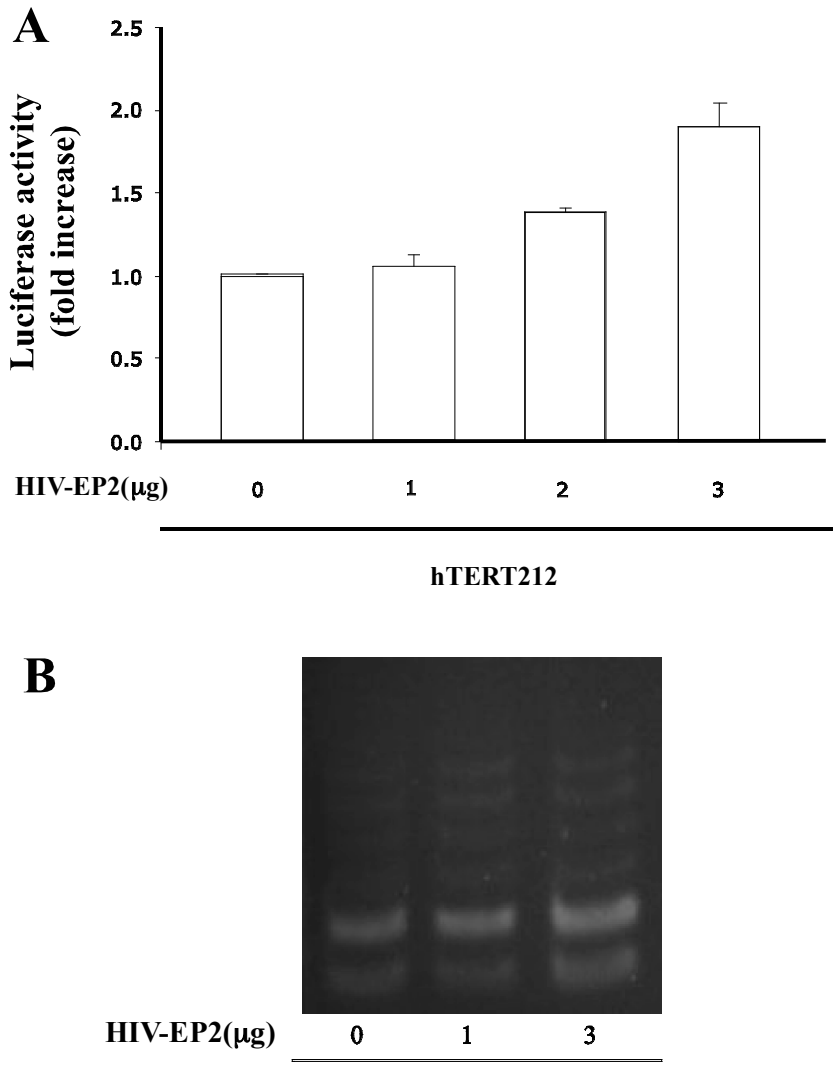

Fig. 5 HIV-EP2 up-regulates the telomerase activity. (A) Transcriptional activity of telomerase is activated by HIV-EP2. The luciferase reporter construct of telomerase promoter, hTERT212 was respectively cotransfected with 0μg, 1μg, 2μg and 3μg of HIV-EP2 using lipofectamine^{TM}2000. The luciferase activity was measured 24hr post transfection. The data represents the mean ± SD of three indenpendent experiments. **(B)** Up-regulation of telomerase activity by HIV-EP2. The 293 cells were transfected with different dosage (0, 1 and 3μg) of HIV-EP2. Telomerase activity in each treatment was determined by using TRAP assay described in Materials and Methods. The data represents the result of three independent experiments.

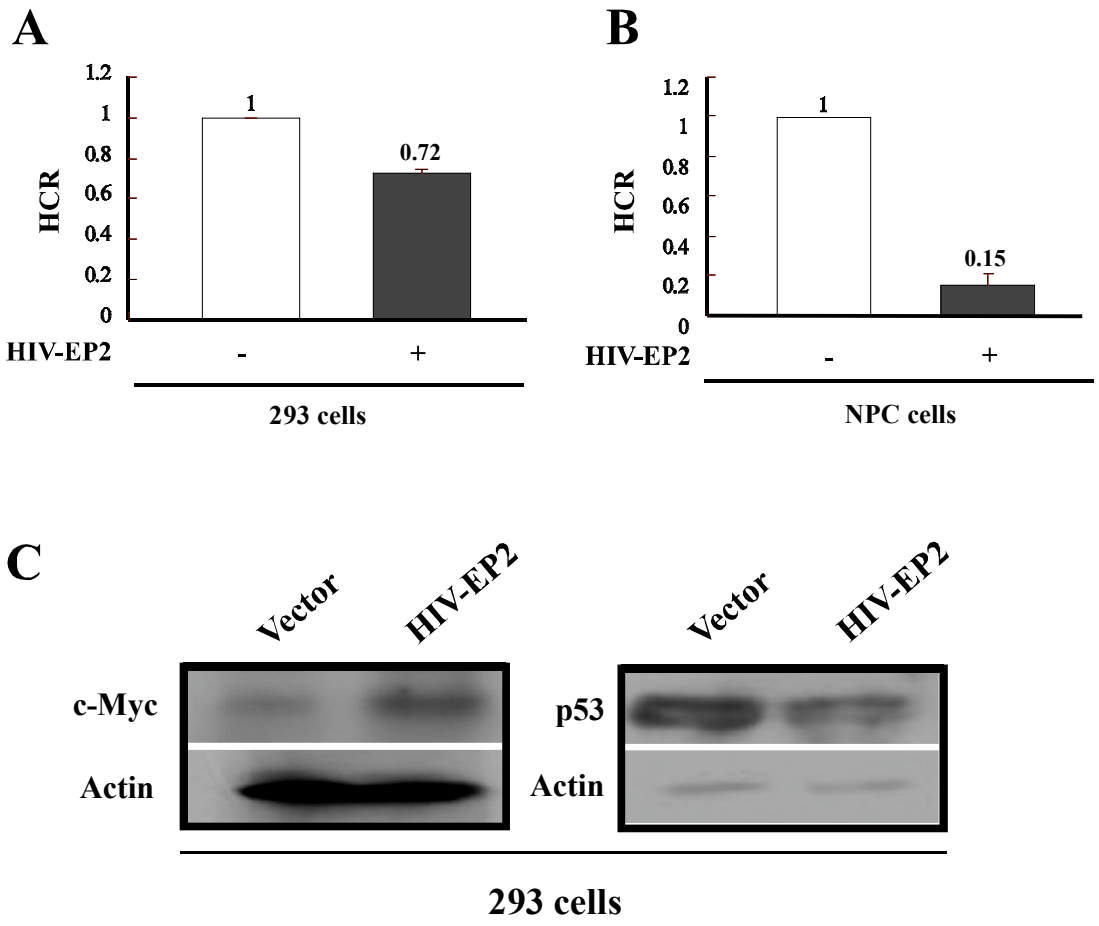

Fig. 6 HIV-EP2 represses the DNA repair of cells. The HIV-EP2 was individually cotransfected into the 293 **(A)** or NPC **(B)** cells with UV-damaged (2000J/m^2) or non-damaged pCMV-Luc reporter construct and pRL-CMV as internal control. 24hr post transfection, the cell extracts from the transfected cells were collected to detect the luciferase activity of firefly and Renilla. The fold of HCR represents the DNA repair, calculated by using the value of luciferase activity as described in Materials and Methods. The data represents the means and SD of at least three independent experiments.**(C)** HIV-EP2 affects the expression of c-Myc and p53 genes. 293 cells were transfected with HIV-EP2 expression plasmid or vector construct using Lipofectamine^{TM}2000. The cell lysate extracted from the transfected cells was used to perform western blotting with antibodies against to c-Myc, p53 and Actin 24hr post transfection. Actin acts as internal control.

成果自評

1. 本計畫乃依據原計畫步驟進行，但實驗過程中總有不如預期。但我們會依據實驗初步結果做為步驟與方向調整，目前已有初步結果與方向。
2. 研究助理總是實驗進度快慢重要原因之一，若往後貴會在經費充裕時，應每年至少編列一位助理，協助主持人進行實驗，我認為將有助於計畫進展。
3. 本計畫已有初步結果，但未能勾勒出完整圖像。若貴會能鼎力支持，將會發展出新方向與進展。
4. 2008年英國著名的「羅素集團」大學成員們發表了一項研究報告，分析該集團大學所進行的82項研究計畫中，純理論的基礎研究 (blue-skies research) 成果帶來了四千多萬英鎊的商業收益，是應用型研究計畫 (applied research) 產出的兩倍，本計畫屬於EB病毒基礎研究，若能完成將有助於人類細胞癌化的機制。所以本計畫適合在學術期刊發表以及往後的應用。就如“羅素集團“研究報告所述。