行政院國家科學委員會專題研究計畫　成果報告

EB 病毒潛伏性基因的Q啟動子在巴克氏淋巴瘤中之研究

計畫類別： 個別型計畫
計畫編號： NSC92-2321-B-040-002-
執行期間： 92 年 11 月 01 日至 93 年 07 月 31 日
執行單位： 中山醫學大學醫學系微生物及免疫學科

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報告類型： 精簡報告

處理方式： 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 93 年 10 月 28 日

中文摘要

EB 病毒感染細胞後其基因表現甚爲複雜，其中 EBNA 1 是唯一會在第一型潛伏性巴克氏淋巴瘤[Burkitt's lymphoma (BL)]中表現的基因，並且此基因只會藉由Q啓動子來啓動轉錄反應。暗示著 EBNA 1 蛋白質對於維持第一型潛伏細胞可能扮演重要角色，所以針對 EBNA 1 蛋白質在第一型潛伏細胞調控機制的研究，可更深入了解其角色。經由前人研究結果顯示，在 BL 細胞中有某些蛋白質會與Q啓動子相互結合。這些與其結合的蛋白質可能會影響 EBNA 1 啓動子的調控，但尚未有證據證實。在我們初步研究結果得知，利用酵母菌 one-hybrid 的方法可以找到一個能與Q啓動子上游區域結合的轉錄因子，這個轉錄因子稱爲 HIV-enhancer binding protein 2 (HIV-EP2)。然而目前對於 HIV-EP2 是否會影響Q啓動子的活性仍然不清楚。爲了能夠清楚瞭解 HIV-EP2 與Q啓動子之間的關係，我們使用 electrophoretic mobility shift assay(EMSA)的方法，再次証明 HIV-EP2 在哺乳動物細胞會與Q啓動子區域結合。此外，爲了更瞭解 HIV-EP2 在細胞角色，我們偵測了在人體組織中 HIV-EP2 分布情形。發現其主要表現在淋巴系統器官。此結果表示 HIV-EP2 可能與 B 細胞或 T 細胞生長調控有關，並藉此影響到 EB 病毒Q啓動子活性。綜合以上的研究，我們初步了解 HIV-EP2 如何在第一型潛伏細胞中調控Q啓動子的活性，但是還需要更多研究予以證實。此外，我們更希望能從中找到治療 EB 病毒所引起的腫瘤及控制 EB 病毒感染的新方法。

關鍵詞：EB 病毒, Q啓動子, 巴克氏淋巴瘤

英文摘要

EBNA 1 is one of Epstein-Barr virus (EBV) latent genes, which is only expressed in type I latency of Burkitt's lymphoma (BL) cells. This implicates that EBNA 1 may probably play an important role in type I latency cells. EBNA 1 transcript in these type I latency cells is specifically initiated from Q promoter (Qp). The investigation of regulation of Qp by cellular factors is the best way to explore the role of EBNA 1 in type I latency cells. Previous data showed that some cellular proteins in type I BL cells are bound to the promoter region of Qp. Whether these proteins are to be candidates for the regulation of EBNA 1 Qp activity in type I latency cells remains unknown. In present study, we have identified a transcription factor, HIV-enhancer binding protein 2 (HIV-EP2), which was associated with the upstream region of Qp, through the yeast one-hybrid system. In addition, we also demonstrated the potential HIV-EP2 associated with the region of Qp by using EMSA. To further understand the role of HIV-EP2 in cells, we detected the distribution of HIV-EP2 in human tissues. The result indicated that the expression of HIV-EP2 mainly occurs in lymphatic organs. These observations showed us that HIV-EP2 could be a component involved in lymphocyte regulation. In addition, this process of HIV-EP2 mediated lymphocyte regulation possibly, directly or indirectly affects on Qp activity in type I latency cells. Taken together, that EBV Qp regulation in type I latency cells is activated or inhibited by HIV-EP2 is under way until now. We still need more evidences to further clarify the unknown relationship of Qp and HIV-EP2.

Key words： EBV, HIV-EP2, Qp, Burkitt's lymphoma

Introduction

Epstein-Barr Virus (EBV) is a human herpesvirus, which infects both lymphocytes and squamous epithelial cells *in vivo* (Epstein *et al.*, 1964; Klein *et al.*, 1974). This virus has been considered to be associated with human malignant diseases such as endemic Africa Burkitt's lymphoma (BL)(de-The *et al.*, 1978) and nasopharyngeal carcinoma (NPC) (Henle *et al.,* 1970; Henle *et al.,* 1976; Chang *et al.,* 1990). Expression of EBV genes in these two malignant tumors was distinctly different from that in EBV-immortalized lymphoblastoid cell lines (LCL) (Brooks *et al.,* 1992; Rowe *et al.,* 1986). EBV genes expressed in the LCLs included six EBV-encoded nuclear antigens (EBNA 1, 2, 3A, 3B, 3C, and LP); two latent membrane proteins, LMP 1 and LMP 2; and two abundantly transcribed small RNAs (EBERs) (reviewed in Kieff, 1996). This pattern of viral gene expression is referred to as latency III (Gregory *et al.*, 1990; Rowe *et al.*, 1992). In contrast to EBV gene expression in LCL, EBNA 1 was the only antigen expressed in BL biopsies and BL cells which retained the cellular phenotype of the original tumors (Dambaugh *et al*., 1979; Rowe *et al.,* 1986; Rowe *et al.,* 1987; Rowe *et al*., 1992). This pattern of viral gene expression belongs to latency I. EBNA 1 is essential for virus replication and maintenance of the virus DNA, which associated with chromosomes during mitosis (Grogan *et al*., 1983). EBNA 1 is also a DNA binding protein which binds to three specific binding sites 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 within 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 Qp initiation site. The binding of EBNA 1 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 the *Bam*HI C promoter (Cp) (Reisman and Sugden, 1986) and ED-L1 promoter (LMP1 promoter)(Gahn and Sugden, 1995). On the contrary, the binding of EBNA 1 to the *Bam*HI Q locus auto-regulates the Qp-initiated EBNA 1 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, in BL and NPC cells, Cp is methylated and kept silent (Robertson *et al*., 1995). Instead, EBNA 1 gene is transcribed from another latent promoter, Qp, which is ~40 kbp downstream region of Cp (Tsai *et al*., 1995; Schaefer *et al.*, 1995). Several studies showed that Qp expression could be regulated by interferon regulatory factors (IRFs) through interaction with the basal transcription machinery (Nonkwelo *et al*., 1995). Recently our reports also showed that Qp is negatively regulated by TGF-β signal pathway through the Smad 4 binding site of its promoter region (Liang *et al*., 2000). TGF-β is not only suppressed the Qp activity but also activated the immediate early promoter Zp, which drived a Zta protein responsible for EBV lytic infection (Liang *et al*., 2002).

As described above, EBV obviously adopts different latent promoters in different cellular environments to regulate EBNA 1 gene expression (Kenney *et al.,* 1989; Sample *et al.,* 1991; Schaefer *et al.,* 1991; Smith and Griffin, 1992) For example, in the environment of type I latency cells, the transcript of EBNA 1 was only initiated from the Qp, which was different from the conventional Cp and Wp (Tsai *et al*., 1995; Schaefer *et al.,* 1995; Woisetchlaeger *et al.,* 1990). This implicated that the promoter

usage of EBNA 1 in type I latency cells might be controlled by specific cellular factors distinct from that in type III latency. Therefore, it is worth to investigate the underlying mechanism of regulation of EBNA 1 promoter by cellular factors.

The minimal promoter region of Qp contains a 125-bp fragment, which remains active in type I latency of cells, including some Burkitt's lymphoma cell lines (Fig 1,Tsai *et al.,* 1995). Previous data have showed that the upstream sequence of Qp was associated with some cellular proteins. It is interesting to see whether those Qp binding proteins have functions on regulation of promoter activity of Qp and the promoter usage in type I latency cells. In order to clarify the role of the Qp-binding cellular factors in the regulation of EBNA 1 Qp activity in type I BL cells, we initially identify a human immunodeficiency virus- enhancer binding protein 2 (HIV-EP2), which associated with Qp in the yeast cells and mammalian cells. In the meantime, we further explore the effect of EP2 on the expression of EBNA1 in the EBV infected cells by over-expression. This study will help us to understand how to regulate EBNA 1 Qp by those cellular factors in type I latent infection.

Results

Identification of the Qp binding cellular protein

EBNA 1 is the only EBV protein that is expressed in type I BL tumor cells. EBNA 1 transcript in these cells is derived from Qp, which is not active in EBV-immortalized lymphoblastoid cell line (LCL). This implicated that the Qp of EBNA 1 is specifically activated and regulated by unknown mechanisms in type I latency of cells. Furthermore, sequence analysis indicated that the upstream region of Qp contained two NF-κB-like binding sites located at -84 to -75 and -71 to -62, relative to the initiation site of Qp (Fig 1). These data imply that the NF-κB motif binding factors could be candidates for Qp regulation. In order to search for which cellular factors bound to the NF-κB-like region of Qp and involved in its promoter activity in BL cells, we prepared five copies of Qp sequence, which located at –90 and –50 relative to initiation site of Qp, as a bait to screen a cDNA library derived from Rael cells, a type 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 in β-gal activity assay as compared to the vector control, the other two positive clones, clone 2 and 3 only increase one fold (Fig. 2). 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 human immunodeficiency virus type I enhancer-binding protein 2 (HIV-EP2) (Nomura *et al.,* 1991). This implicated that HIV-EP2 protein in BL cells may play a role in Qp regulation through association of the –90 to –50 region of Qp promoter.

The association of HIV-EP2 with Qp *in vitro* in mammalian cells

The above data showed that the DNA binding domain of HIV-EP2 was interacted with the upstream region of Qp in yeast cells (Fig 2). Whether this interaction also occurs in mammalian cells remains to be investigated. To prove this, 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 3A). 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. 3B, the *in vitro* translated HIV-EP2 protein formed a DNA-protein complex with the probe (-90 to -50) and this complex was competed away by unlabelled probe. In addition, the Myc-HIV-EP2 was used as an expression vector for transfection of 293T cells. The over-expressed HIV-EP2 associated with the –50 to –90 region of Qp to form the complex (Fig 3C, lane2). The complex also shifted up by an anti-Myc antibody against HIV-EP2 (Fig3C, lane3). However the non-specific antibody (anti-ATF2) was not able to shift the complex (Fig3C, lane4). These results of two experiments indicated that the HIV-EP2 was indeed able to bind to the region of –90 to –50 of Qp.

Characterization of the distribution of HIV-EP2 in human tissue

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. 4). This RNA is preferentially expressed in the brain, skeletal muscle, lymph node, spinal cord, thyroid, trachea and adrenal gland. In contrast, expression of this RNA was very low in the heart, lung, liver, kidney, pancreas, stomach and bone marrow (Fig. 4). This implicated that HIV-EP2 could be a component involved in lymphocyte growth control.

Discussion

Transcription of EBNA1 gene in type I BL cells is driven by a promoter (Qp) distinct from that (Cp or Wp) used in type III BL cells, implicating that the promoter usage could be regulated by the cellular proteins existed in type I BL cells. In present study, we have used the region of –90 to –50 relative to initiation site of Qp as a bait in the yeast one-hybrid screen, a transcription regulator HIV-EP2 protein was identified as Qp binding protein through the –90 to –50 region. HIV-EP2 was first described in 1991 as a member of HIV-1-enhancer binding proteins, which binds to the enhancer region within the HIV-1 long terminal repeat (LTR)(Nabel and Baltimore, 1987; Nomura *et al*., 1991). It is a protein of 1833 amino acids with a molecular weight of ~210 kDa, which is mapped on the chromosome 6q23-6q24 (Sudo *et al*., 1992), and whose mRNA is variable in different cells but is induced in the activated T cells (Nomura *et al*., 1991). HIV-EP2 belongs to a family of rel oncoproteins and metal-finger proteins, respectively (Nomura *et al*., 1991). Several known members of this family are HIV-EP1, KBP-1, MBP 1 (also named as PRDII-BF1, AT-BP2 or a-ACRYBP1) and MBP-2 (MBP, MHC-binding protein). Three regions, the potential nuclear localization signal followed by a Ser/Thr-rich region, the DNA-binding domain consisting of a metal-finger structure and a cluster of acidic amino acids are highly conserved between HIV-EP2 and other members of this family (van'T Veer *et al*., 1992). This family of proteins binds to related enhancer motifs, although the binding affinity for the different enhancers varies. For example, HIV-EP2 binds to a 10-nucleotide NF-kB motif, while HIV-EP1 binds to 7 out of 10 nucleotides as assayed by the DNAse I footprinting analysis (Nomura *et al.,* 1991). The Qp containing two

potential NF-kB-like binding motifs within the region of -90 to –50 was also recognized by HIV-EP2 as assayed by the EMSA. However I did not rule out the possibility that MBP-1 and other members of this family can recognize these sites within Qp in Rael cells.

HIV-EP2 is highly expressed in lymph node tissues, indicating its potential role in cells of lymphoid organ. Nomura *et al.* (1991) reported that expression of HIV-EP2 was induced by mitogen and phorbal ester in T cell line, Molt 4. Therefore, it has been implied as a regulatory component of T cell growth control involved in HIV-EP2 mediated Qp activity. Whether the expression of HIV-EP2 mRNA in BL cells is induced by mitogen and phorbol ester is worth further investigating. It is also interesting to find out if agents that activate NF-κB activity also activate Qp, or prevent the association of HIV-EP2 to the predicted sequence.

In summary, 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 have been identified. The –49 to –45 region of Qp is a TGF-β inducible element associated with Smad4, which recruits a transcriptional repressor TGIF and result in transcriptional repression. On the contrary, Zp of 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 found that the –90 to –50 region of Qp is associated with HIV-EP2. However this promoter-protein interaction functions as a positive regulator or negative repressor in type I BL cells remains to be investigated. The following experiments are therefore to examine the effect of HIV-EP2 on Qp activity in the EBV infected cells 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 the mechanism that control gene expression. Furthermore, we will establish the new strategies for therapy of EBV associated tumors based on these studies.

Materials and Methods

Cell culture

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

Plasmid construction

Four overlapping fragments covering the full-length HIV-EP2 cDNA are generated by RT-PCR with RNA isolated from Rael cells and four sets of primers. Using these primers sets and PCR, four RT-PCR products are amplified individually. These four cDNA fragments are 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 pGEM4Z/EP2 is used to *in vitro* transcription/translation. The EP2 expression construct, pCMVMyc/EP2, is generated by insetion of EP2 into pCMVMyc plasmid. This expression plasmid is subjected to luciferase assay and EMSA

Yeast one-hybrid system

Five copies of the –90 to –50 of EBV Qp was inserted into the *Sma* I site of pLacZi and pHisi-1 (Clontech, USA) to generate pLacZi-5κB and pHisi-5κB. The pLacZi-5κB was linearlized by *Nco* I and pHisi-5kB was digested with *Xho* I. The linearlized plasmids were transformed into yeast YM4271 to establish stable clones, YB-5kB and YH-5SkB. YH-5kB yeast cells were transformed with Rael cDNA library plasmids (1x 10^6 individual colonies) and then selected on SD/His^-/Leu^- plates with 3-AT. Three clones showed positive β-gal activity among 37 clones grown on plates. The clones showed positive β-gal activity were isolated and the plasmid DNAs were purified before subjecting to DNA sequence analysis. The quantitative b-gal activity was assayed with n-nitrophenyl β-D galactopyranoside (ONPG) as the substrate by using β-galactosidase enzyme assay system (Promega, USA).

Northern blotting

The Multiple Tissue Northern Blots (Clontech, USA) were used to perform northern blotting for the human tissues according to Clontech manufecturer' s instruction.Briefly, the probes corresponding to the part of the full-length HIV-EP2 cDNA or to the part of actin cDNA were labeled using random priming kit (Amersham, England). The membrane was prehybridized for 2 hr at 42°C in 50% formamide, 5X SSC, 0.1% SDS, 50uM PBS, 1uM EDTA, 2.5X Denhard' s solution, 200ug/ml single-stranded DNA. Hybridization was performed at 42°C for 18 hr by adding >~10^6 cpm/ml ^{32}P-labeled probes in the prehybridization solution. The membrane was washed for 20 mim at RT in 2X SSC, 0.1% SDS, followed by two washes of 20 min each in 0.2X SSC, 0.1% SDS at 55°C.

Electrophoretic mobility shift assay

The proper region of Qp is used as a probe in electrophoretic mobility shift assay (EMSA). In brief, the fragment is labeled with T4 polynucleotide kinase and [γ^{32}P]ATP. *In vitro* translated protein lysate or nuclear extract is 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 is added in the reaction before addition of the ^{32}P-labelled probe. After 30 min of incubation at RT, the samples are loaded onto a 4% nondenaturing acrylamide gel and electrophoresis is performed in 0.5X TBE at 100V for 3 hr. For the supershift assay, the reaction mixture is incubated with 4 μg of antibody and applied to gel. The gels subsequently are dried and exposed to Kodak BioMax film.

In vitro transcription and translation

The reaction of transcription and translation of the pGEM-EP2 is performed by using a TNT coupled reticulocyte lysate system (Promega, USA) to generate HIV-EP2 protein. The reaction is carried out in a reaction mixture of 50μl including 1mg of circular pGEM-EP2, pGEM or luciferase control plasmid (Promega, USA) respectively, and 25μl rabbit reticulocyte lysate, 1X TNT reaction buffer, 20mM 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°C incubation for 90min, products are analyzed with 5% SDS -polyacylamide gel electrophoresis (SDS-PAGE) and subjected to EMSA.

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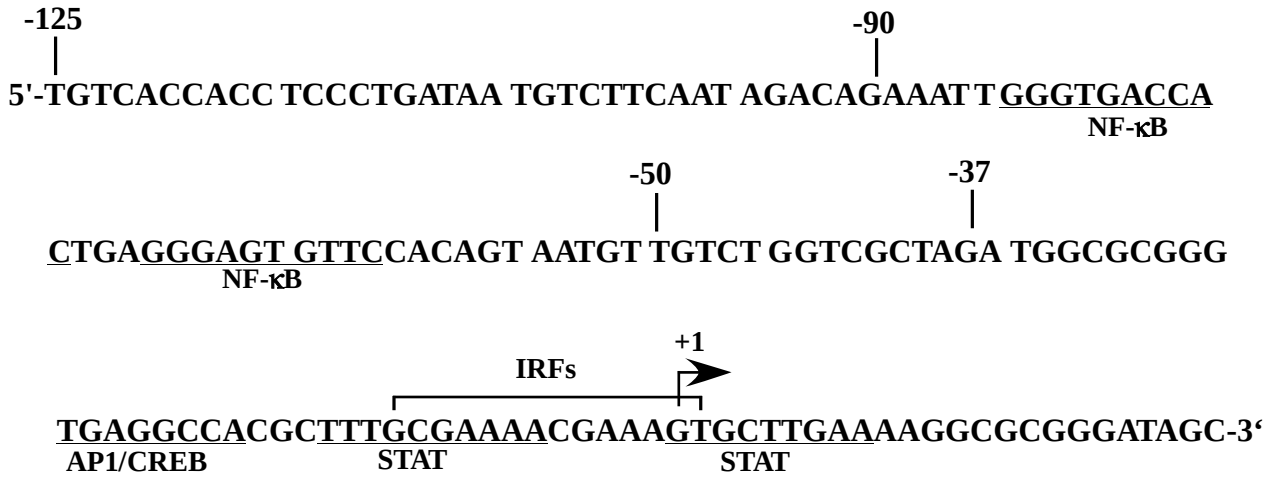

Fig 1 The sequence of Q promoter (Qp)(-125 to +24) The Number represents the position of promoter sequence relative to initiation site(+1). The position of IRF, AP1 and STAT are shown as the above. The two NF-κB motifs are located at the -84 to -75 and -71 to -62 regions of Qp

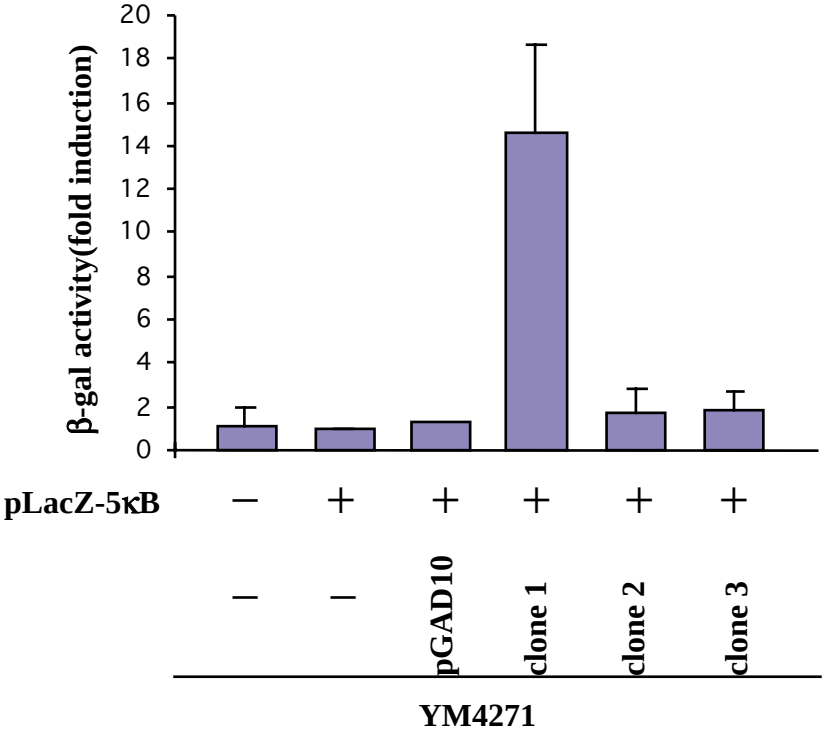

Fig 2 Specific interaction of the –90 to –50 region within Qp with clone 1 cDNA by yeast one-hybrid assay. The YM4271 is a parental yeast cells, which were used as host for yeast one-hybrid assay. The YM4271 yeast cells were cotransformed with the different sets of plasmids, as indicated above. The pLacZ-5kB is a β-galactosidase reporter plasmid, which contains a 5 copies of the –90 to –50 region of Qp located at the upstream of β-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).

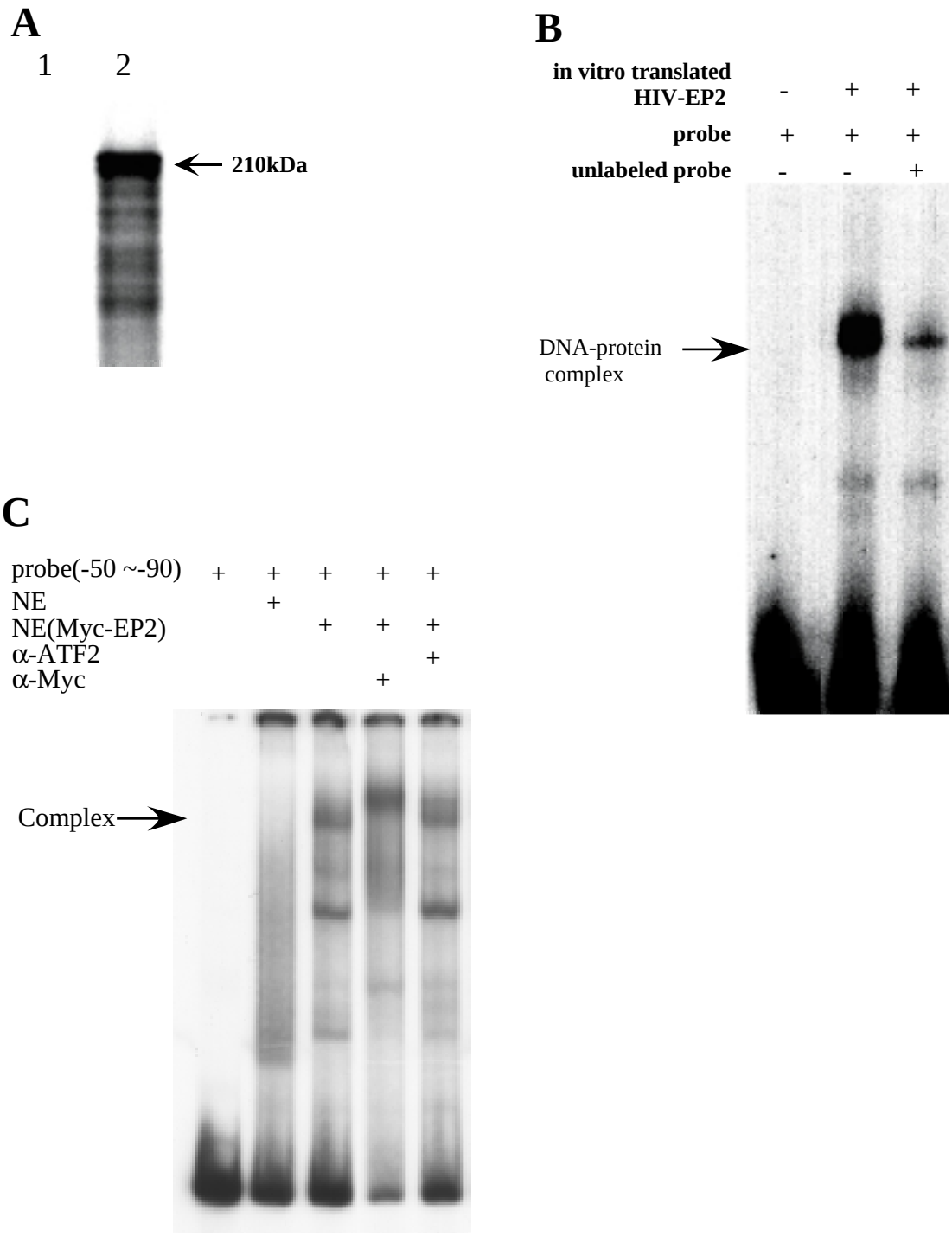

Fig 3 DNA binding activity of *in vitro* translated HIV-EP2. (A) A 5% SDS-polyacrylamide protein gel of ^{35}S-methionine labeled full-length(lane2) EP2. The pGEM4Z as control was used in *in vitro* translation system(lane1). The arrows indicated the molecular weight of translated proteins. (B) EMSA analysis of *in vitro* translated EP2 with 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).

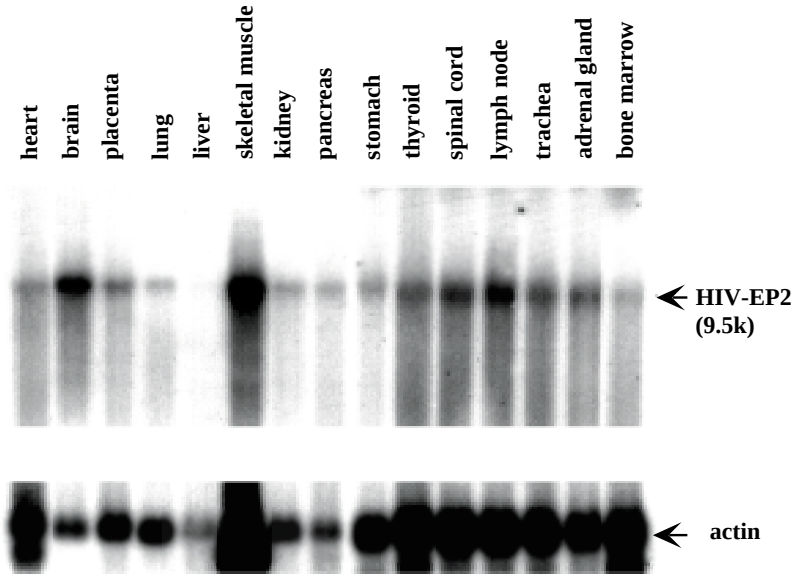

Fig 4 The distribution of HIV-EP2 mRNA in human tissues and cell lines. The HIV-EP2 mRNA from human tissues were determined by using northern blot analysis. The arrows represent the positions of HIV-EP2 and actin mRNA. Actin acts as internal control.

成果自評

1. We have no change of the content of project. The content of this project very similar to that of the original project. The progress of this project is up to about 60 percent, because of the lack of human and financial resources. If NSC continues to support this project without any condition, this project will be finished soon.
2. There are some experimental blocks which need to be removed in this project, such as the expression of plasmid in Burkitt's lymphoma cell lines. If these experimental blocks are broken, it will promote progress of this project.
3. If the progress of this project is up to 100 percent, we will not only publish our project on the science Journal but also establish a set of new therapeutic methods for EBV induced diseases.