



# The enhanced transcriptional activity of the V-val subtype of Epstein-Barr virus nuclear antigen 1 in epithelial cell lines

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**Abstract.** The Epstein-Barr virus encoded nuclear antigen 1 (EBNA1) is required for the replication and maintenance of the episomal EBV genome and for the transactivation of viral gene expression. EBNA1 has been classified into five subtypes, among which the V-val subtype was reported to be associated with nasopharyngeal carcinoma (NPC). Here we report a higher transcriptional activity of the V-val subtype of EBNA1 than for the prototype derived from B95.8 cells to transactivate FR-containing luciferase plasmid, which was mainly a consequence of the mutations in the carboxy-terminus of EBNA1. This interpretation was further supported by the finding that the variant form of EBNA1 has a higher binding affinity for the FR sequence than the prototype by electrophoretic mobility shift assays. The functional advantage of the V-val EBNA1 investigated in this study may contribute to the oncogenesis of NPC.

## Introduction

Nasopharyngeal carcinoma (NPC) is a human malignancy derived from epithelial cells that is rare in most parts of the world but is common in Southern China, Southeast Asian, and some North African countries. It has been convincingly shown to be an Epstein-Barr virus (EBV)-associated cancer via evidence that EBV is harbored in almost every undifferentiated NPC tumor irrespective of its geographic distribution. Despite the extremely variable incidence of NPC that is dependent on the geographic region, EBV is a ubiquitous human herpes virus that infects more than 90% of people worldwide such that many of these infected individuals

remain lifelong carriers of the virus without showing any overt or serious consequences of the disease. Thus, it has been hypothesized that specific EBV strains prevalent in NPC-high incidence areas may have higher transforming potentials that contribute to the development of NPC.

Infection in EBV-associated tumors is strictly latent, with only a limited range of viral genes being expressed by the cells. EBV encoded nuclear antigen 1 (EBNA1) is the only EBV protein consistently expressed in all infected proliferating cells, and it plays several important roles in EBV latent infection, including the initiation of EBV DNA replication, the mitotic segregation of the EBV genomes, and the transcriptional activation of other EBV latency proteins by binding directly to specific recognition sites in the latent replication origin region, *oriP*, of EBV DNA as a homodimer (1). EBV DNA replication initiates within the dyad symmetry (DS) element of *oriP*, which contains four EBNA1 binding sites (2,3). The partitioning of the episomes involves EBNA1 binding to the family-of-repeat (FR) elements of *oriP*, which contains 20 EBNA1 recognition sites (4). FR is also a transcriptional enhancer through which EBNA1 supports the activity of the EBV latent membrane protein promoter and the Cp promoter (5-7). The EBNA1 protein also autoregulates the Qp promoter, which is the promoter for EBNA1 gene expression during latency I and II (8).

The predicted amino acid sequence of EBNA1, which consists of 641 amino acid residues, can be separated into unique amino-terminal (residues 1-89) and carboxy-terminal (residues 328-641) domains joined by an internal glycine/alanine-rich short repeat (GAR) sequences (9). EBNA1 residues that mediate DNA binding and dimerization have been localized to the carboxy-terminal portion of the protein between amino acids 459 and 607. Amino acids 1-89 and 322-379 cooperatively mediate the association with cell chromosomes and are essential for episome persistence and transcription (10), whereas amino acids 379 through 386 form a nuclear localization sequence, and amino acids 605 through 641 comprise an acidic activating domain.

Based on the variation of the amino acid at residue 487, EBNA1 was classified into two prototypes (P) and three variant (V) subtypes: P-ala (B95.8 prototype), P-thr (amino acid 487 ala to thr), V-val, V-leu and V-pro (11). A close relationship of V-val EBNA1 with NPC was suggested by its preference to infect NPC cells. V-val was the only subtype

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of EBNA-1 observed in a total of 20 cases of Chinese NPC biopsies in the study reported by Snudden *et al* (12) and Wang *et al* (13). In our previous study, 96.55% (84/87) of the NPC specimens contained the V-val subtype as the sole species of EBNA1, while multiple EBNA-1 subtypes, which included V-val, P-ala and/or V-thr EBNA1, were simultaneously presented in the peripheral blood lymphocytes of 77.08% (111/144) of EBV healthy carriers from the Guangzhou area in Southern China (14,15). Therefore, it is reasonable to infer some additional risk for those individuals with V-val EBNA1 over the other subtypes in regard to the development of NPC. According to a comparison of the sequencing results of the V-val and the prototype EBNA1 coding region, most of the variations of V-val EBNA1 would lead to substantial amino acid alterations in apparently functionally important regions, which may result in significant biological differences between the V-val and the prototype EBNA1.

In our previous study, we have investigated the functional difference between V-val and the prototype EBNA1 in a human epithelial cell line. The coding regions of the P-ala and the V-val subtypes of EBNA1 were cloned into the pGFP-C2 vector and transfected into 293 cells. The results of flow cytometry analyses indicated that the relative average intensity of fluorescence after 14 days of G418 selection in the V-val-EBNA1/293 cells was statistically higher than that observed for the P-ala-EBNA1/293 cells ( $P < 0.05$ , t-test), which indicated an enhanced maintenance and/or trans-activation function of V-val EBNA1 over the prototype (16).

In order to confirm the functional advantage of V-val EBNA1 and further locate the region responsible for this statistically significant difference, the EBNA1 mutants with their amino- and carboxy-terminal derived from the V-val or from the prototype were constructed and cotransfected with an FR-containing luciferase reporter plasmid in the epithelial cell lines used in the study presented herein. The DNA binding affinity to the FR sequence was compared between the V-val and the prototype EBNA1 using an electrophoretic mobility shift assay. Our results indicate a stronger transcriptional activity of V-val EBNA1 compared with the prototype, which may be due to the higher DNA binding affinity to the FR sequence. Moreover, this functional advantage may contribute to the oncogenesis of NPC.

## Materials and methods


**Cell lines.** The spontaneously transformed human keratinocyte cell line HaCaT that was derived from normal human trunk skin was used in this study. The poorly differentiated NPC cell line CNE-2 and human embryonic kidney epithelial cell line 293, were used to confirm the results of the reporter assays. All cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum and incubated at 37°C in an incubator with 5% CO<sub>2</sub>. The NPC biopsies were collected from the Cancer Center at Sun Yat-sen University.

**Construction of mutants of EBNA1 lacking amino acids 91 to 321.** In order to compare the expression and activity of the prototype and the mutated EBNA1 proteins, a series of pcDNA3.1+ vectors were created using the same expression

cassette that encodes different EBNA1 genes. The coding region of the truncated forms of EBNA1 containing a deletion of most of the Gly-Ala repeat region (amino acids 91 through 321) was included, since a previous study showed that this region can be removed with little or no effect on the function of EBNA1 (17). The EBNA1 derivatives expressed from these plasmids were predicted to have a molecular weight of 43 kDa.

The prototype and the V-val subtype of EBNA1 were amplified using template DNA extracted from the B95.8 cell line and the NPC biopsy. The amino-terminal (amino acids 1-90) region of the EBNA1 gene was amplified by two rounds of polymerase chain reaction (PCR) amplification using primers that introduced a 6-His tag, a Kozark sequence and the *Bam*HI and *Hind*III restriction sites (sense primer for first round of PCR: 5'-GAACACCACCATCACCATCACA TGTCTGACG-3', antisense primer for first and second round of PCR: 5'-ACTAGTAAGATTTCCTGTTCCACCGTGG-3' and a sense primer for second round of PCR: 5'-ATACTC GAGGGATCCACCATGGAACACCAC-3'). The carboxy-terminus (amino acids 322-641) of EBNA1 was amplified using primers with *Hind*III and *Eco*RI restriction sites (sense primer: 5'-ACTAGTAAGCTTGCAGGAGCAGGAGGT GGA-3' and an antisense primer: 5'-TACTCGAGGAATT CTCACTCCTGCCCTT-3'). Pyrobest DNA polymerase (Takara Biotechnology, Dalian, China) with proof-reading activity was used for the amplification step. The prototype and variant amino- and carboxy-terminal region of the EBNA1 fragments were purified from agarose gels, digested with *Hind*III, and subsequently ligated. In order to test the different effects of the amino- and carboxy-terminal variations on the transcriptional activity of EBNA1, the fragments derived from the prototype and the V-val subtype were ligated. PCR was performed using the ligation mixture as a template with the EBNA1 amino- and carboxy-terminal primers. PCR products were gel purified, digested with *Bam*HI and *Eco*RI, and ligated into the *Bam*HI and *Eco*RI sites of pcDNA3.1+. The resulting constructs expressed EBNA1 mutants as amino- and carboxy-terminal fusion proteins that lacked the Gly-Ala repeat region (amino acids 91-321) and instead were replaced with the 6 nucleotide sequence AAGCTT (*Hind*III restriction site). Four EBNA1 mutants were subsequently constructed and included pp-EBNA1 (derived from B95.8 cells, p, prototype), vv-EBNA1 (derived from NPC, v, variant), pv-EBNA1 (amino-terminus derived from B95.8 and carboxy-terminus derived from NPC), and vp-EBNA1 (amino-terminus derived from NPC and carboxy-terminus derived from B95.8). These desired constructs were verified by sequencing analysis and further amplified and purified using QIAGEN maxi prep protocols.

**Construction of reporter gene plasmids.** The FR element of *ori*P (EBV coordinates 7421-8042) was amplified by PCR using a sense primer (5'-TGTGGATCCGGGTATCATATG CTGAC-3'), an antisense primer (5'-CACGGATCCTAAT GTTGCCATGGGTA-3'), and DNA extracted from B95.8 cells as a template. The resulting fragment contained two engineered *Bam*HI sites at positions 7421 and 8042. The FR fragment was then digested with *Bam*HI and was used for ligation into the site of the pGL3-SV40 promoter vector. In

 SPANDIDOS PUBLICATIONS ing construct, referred to here as pSV40-luciferase-FR element is positioned 510 bp downstream from the luciferase reporter gene. Recombinants were confirmed by enzyme digestion and sequencing analyses.

EBV Cp (-121 to +98 relative to the Cp start site, coordinates 11215-11434 in the B95-8 genome) and LMP1-TR (-123 to +221 relative to the LMP1-TR promoter start site, coordinates 169976-170319 in the B95-8 genome) promoter sequences were amplified from B95-8 DNA by PCR and used to substitute the simian virus 40 (SV40) promoter in the pSV40-luciferase-FR plasmid. The pCp-FR and pLMP1-TR-FR constructs were confirmed by sequencing.

**Western blot analysis.** HaCaT cells in 100-mm dishes were transfected with 10  $\mu$ g of pcDNA3.1+ vector, pc3-pp-EBNA1, pc3-pv-EBNA1, pc3-vp-EBNA1 and pc3-vv-EBNA1 plasmids, respectively. The culture medium was removed 48 h post-transfection, and the cells were washed once in PBS. The cells in each dish were then lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and a Protease Inhibitor Cocktail (Sigma). The cell debris was removed by centrifugation at 14,000 g for 5 min. The protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce). The cellular protein was used for the Western blot analysis and electrophoretic mobility shift assay. For the Western blot assay, the lysate supernatant equivalent of 40  $\mu$ g of protein was separated by electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene fluoride membrane (Roche). After blocking, the membranes were incubated with anti-His tag Mouse Monoclonal Antibody (Novagen, Madison, WI), followed by an HRP-conjugated secondary antibody. The proteins were detected by incubation with LumiGLO™ (Cell Signaling Technology, Beverly, MA) and captured on Kodak X-ray film.

**Reporter assays.** HaCat cells were plated in 24-well plates at a density of  $5 \times 10^4$  cells/well and reached 70-80% confluence 24 h later. Subsequently, 0.4  $\mu$ g of pcDNA3.1+ plasmid expressing pp-EBNA1, pv-EBNA1, vp-EBNA1, vv-EBNA1, or no EBNA1 was combined with 0.1  $\mu$ g of the pSV40-luciferase-FR reporter construct and 10 ng of pRL-TK (Promega, Madison, WI) in 1  $\mu$ l of the FuGENE 6 reagent diluted in 10  $\mu$ l of serum-free medium. The experiments were performed in three replicate wells for each transfection. The transfection reagent: DNA complex was incubated for 15 min at room temperature and then was added dropwise to cells. After incubation for 12-16 h at 37°C, the cells were given fresh medium. After 48 h of incubation at 37°C, the dual-luciferase reporter assay was performed using the luciferase assay system (Promega) according to the manufacturer's instructions. The cells were washed with 1X PBS and lysed with 100  $\mu$ l 1X PLB for each well. Subsequently, 20  $\mu$ l of the cell lysate was mixed with 50  $\mu$ l of LARII, and the luminescence was immediately measured using a Sirius Luminometer V3.1 (Berthold Detection Systems, Germany) that recorded the firefly luciferase activity measurements (F-value). The sample was then removed from the luminometer

and added to 50  $\mu$ l of the 1X Stop&Glo reagent, and the Renilla luciferase activity measurement (R-value) was recorded again. The relative light units (RLU) were calculated by dividing the R-value by the F-value and represented the transcriptional activity with the normalized transfection efficiency of each group. The reporter assay results are presented as an increase (n-fold) in the level of transcription. The increase in transcription was calculated by dividing the RLU obtained from cells transfected with the various derivatives of EBNA1 by the RLU obtained from cells transfected with the empty vector. The experiment was performed independently at least three times.

The experiment was performed by repeating the same procedure as above to confirm the result of reporter assay using the CNE-2 and 293 cell lines. The EBV-negative NPC cell line (CNE-2) was chosen because of its lack of endogenous EBNA1 expression. Prior to using the luciferase assay, either pp- or vv-EBNA1 was cotransfected with pSV40-luciferase that lacked the inserted FR element in HaCat cells in order to confirm the necessity of the FR element in EBNA1-mediated transactivation.

The EBNA1-mediated transcriptional activation of the EBV promoter was measured by replacing the SV40 promoter in the pGL3-FR plasmid with Cp or LMP1-TR. Fixed amounts (0.4  $\mu$ g) of the EBNA1 derivatives were cotransfected with 0.1  $\mu$ g of pCp-FR or pLMP1-TR-FR into HaCat cells, and the luciferase activity was measured 48 h after cotransfection. For each group, the experiments were performed in 3 replicate wells.

To evaluate the dynamic changes in luciferase activity during 4-day culture, the HaCat cells were plated in 24-well plates at a low density of  $1.5 \times 10^4$  cells/well and transfected with the same procedure as described above 24 h after the cells reached 30-40% confluence. These experiments were performed in 12 replicate wells for each transfection. Specifically, 0.4  $\mu$ g of pcDNA3.1+ plasmid expressing pp-EBNA1, pv-EBNA1, vp-EBNA1, vv-EBNA1, or no EBNA1 was combined with 0.1  $\mu$ g of the pSV40-luciferase-FR reporter construct and 10 ng of pRL-TK in 1  $\mu$ l of the FuGENE 6 reagent. In addition to the empty vector, a mock control with a non-sense mutation in the beginning of the pp-EBNA1 sequence was also included. These cells were grown for 4 days, collected, and screened for the presence of luciferase activity at 24, 48, 72, and 96 h post-transfection.

**Electrophoretic mobility shift assay.** Seven of the amino acid mutations identified in V-val EBNA1 occur within the DNA binding and dimerization domain. In order to determine whether the changes have an effect on the binding affinity, we performed electrophoretic mobility shift assays (EMSA) to compare the ability of the prototype and V-val EBNA1 to bind DNA. The biotin end-labeled single-stranded oligonucleotide (5'-Biotin-TAGCATATGCTACCCGGATACAGATTAGGATAGCATATACTACCCAGATATAGATTAGGA-3') and its complementary oligomer (3'-ATCGTATACGATGGGCCTATGTCTAATCCTATCGTATATGATGGGTCTATATCTAATCCT-Biotin-5'), which corresponded to the EBNA1 binding site in the FR element, were synthesized (Shanghai Invitrogen Biotech Co., Ltd., Shanghai, China) and subjected to double-stranded annealing. The

whole cellular proteins from the pp-EBNA1 or vv-EBNA1 transfected HaCat cells were extracted as described above. EMSA was performed using the LightShift® Chemiluminescent EMSA Kit according to the manufacturer's protocol (Pierce). The binding reaction was carried out in a total volume of 10  $\mu$ l containing 3  $\mu$ g of cellular extract, 10 fmol of biotin-labeled oligonucleotides and 1X binding buffer comprising 10 mM Tris, 50 mM KCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 2.5% glycerol, 0.05% NP-40 and 0.5  $\mu$ g poly(dI·dC). The protein/DNA complexes were incubated for 20 min at room temperature and then subjected to vertical electrophoresis in a 6% Tris-borate-EDTA polyacrylamide gel. The gel was electrophoresed at 100 V in 0.5X Tris-borate-EDTA buffer until the bromophenol blue dye had migrated approximately 2/3 to 3/4 of the length of the gel. Transfer was performed at 380 mA for 30 min. The transferred DNA was cross-linked to membrane and detected by chemiluminescence and then exposed to X-ray film.

Supershift experiments were carried out as described above except that the samples were preincubated for 1 h at 4°C in the presence of 1  $\mu$ g of anti-His tag antibody (Novagen). For the competition binding assay, unlabeled double-stranded oligonucleotides were used at a 50-fold excess of biotin-labeled probe.

**Statistics.** Data are presented as the mean  $\pm$  SD. The differences were assessed by analysis of variance with double-sided t-test for comparisons within multiple groups. Statistical significance was defined as  $P < 0.05$ .

## Results

**The expression of EBNA1 derivatives in the HaCat cell line.** The comparative expression level and molecular weight of the EBNA1 derivatives were analyzed in the HaCat transformants via Western analysis using the anti-His tag antibody. The total protein fraction was prepared and equal amounts of this fraction were separated using SDS-polyacrylamide gel electrophoresis. As illustrated in Fig. 1, the EBNA1 proteins from the four derivatives pp-EBNA1, pv-EBNA1, vp-EBNA1 and vv-EBNA1 were detectable at equivalent levels (Fig. 1, lanes 3-6). In this set of experiments, the untransfected HaCat cells (Fig. 1, lane 1) and cells transfected with pcDNA3.1+ (Fig. 1, lane 2) were used as controls. The molecular weights of the recombinant EBNA1 proteins were predicted to be 43 kDa.

**The transactivation of the FR-containing luciferase gene is higher in vv-EBNA1-expressing cells.** The different derivatives of EBNA1 and the empty vector pcDNA3.1+ were co-transfected with pSV40-luciferase-FR and the internal control pRL-TK into cells. The relative light units (RLU) were calculated using the F/R value obtained after quantification of the luciferase activity in the transfected cells. We analyzed four derivatives of EBNA1 in order to compare the different contribution of the fragments to the transcription of the FR-plasmid.

The mean luciferase results that were corrected for the transfection efficiency obtained from at least three independent transfections performed in duplicate were depicted graphically (Fig. 2A). The increases in the transcription level

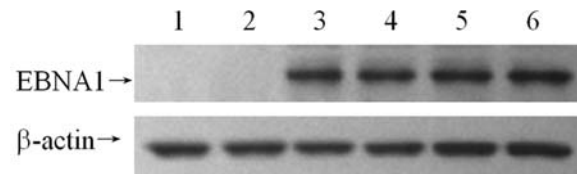


Figure 1. Western blot assays demonstrating the expression of the various derivatives. Forty micrograms of protein extracted from the transfected HaCat cells were resolved by 10% SDS-polyacrylamide gels and probed with the anti-His tag primary antibody (Upstate). The antibody did not detect a signal in untransfected cells (lane 1) or in cells transfected with the empty vector (lane 2). Western blot analysis shows that the expression of pp-EBNA1 (lane 3), pv-EBNA1 (lane 4), vp-EBNA1 (lane 5) and vv-EBNA1 (lane 6) with a putative molecular weight of 43 kDa. Notably,  $\beta$ -actin was used as the internal control.

over that of the empty vector mediated by pp-EBNA1 [mean increase (n-fold) over the empty vector, 10.78 ( $P=0.0004$ )], pv-EBNA1 [mean increase (n-fold) over the empty vector, 14.16 ( $P=6.99 \times 10^{-5}$ )], vp-EBNA1 [mean increase (n-fold) over the empty vector, 10.98 ( $P=0.0004$ )], and vv-EBNA1 [mean increase (n-fold) over the empty vector, 14.57 ( $P=2.28 \times 10^{-5}$ )] are statistically significant. The apparent increases in transcription over the prototype-type EBNA-1 as mediated by the pv-EBNA1 and vv-EBNA1 derivatives were statistically significant ( $P < 0.05$ ). However, the derivatives of pp-EBNA1 and vp-EBNA1 were found to show no statistically significant difference between their levels of transcription ( $P > 0.05$ ). These results suggest that variations in the carboxy-terminal region of V-val EBNA1 enhanced the transactivation activity.

The experiment was repeated by using the CNE-2 (Fig. 2B) and 293 (Fig. 2C) cell lines using the same procedure. Interestingly, similar results were observed for these two cell lines. When cotransfected with the pSV40-luciferase without the FR in HaCat cells, the transactivating activity of pp-EBNA1 and vv-EBNA1 was found to be equivalent to that of the empty vector, demonstrating the requirement of the FR element in EBNA1-mediated transactivation (Fig. 2D). As the SV40 promoter in luciferase reporter was replaced by EBV promoter Cp or LMP1-TR, similar results were obtained from the reporter assay. The vv-EBNA1-mediated transcriptional activities of pCp-FR and pLMP1-TR-FR were 2.56 and 2.15 times higher than the corresponding pp-EBNA1-mediated transcriptional activities, respectively (Fig. 2E and 2F). The differences were statistically significant ( $P < 0.05$  for both differences, t-test).

The luciferase activity was measured at 24, 48, 72, and 96 h after transfection of the EBNA1 derivatives. The RLU detected in the empty pcDNA3.1+ vector transfected cells was very low and decreased from  $0.56 \pm 0.05$  to  $0.11 \pm 0.01$  over a period of 3 days, while the RLU detected in EBNA1 transfected cells increased during the same period of time (from  $2.34 \pm 0.36$  to  $5.98 \pm 1.25$  for pp-EBNA1, from  $3.52 \pm 0.56$  to  $7.83 \pm 0.71$  for pv-EBNA1, from  $4.00 \pm 0.48$  to  $6.16 \pm 0.49$  for vp-EBNA1, from  $4.81 \pm 0.42$  to  $12.61 \pm 0.31$  for vv-EBNA1). Interestingly, only low levels of luciferase activity could be detected in the mock group over this period (from  $0.56 \pm 0.19$  to  $0.90 \pm 0.21$ ). The increase in transcription calculated by dividing the RLU obtained from cells trans-

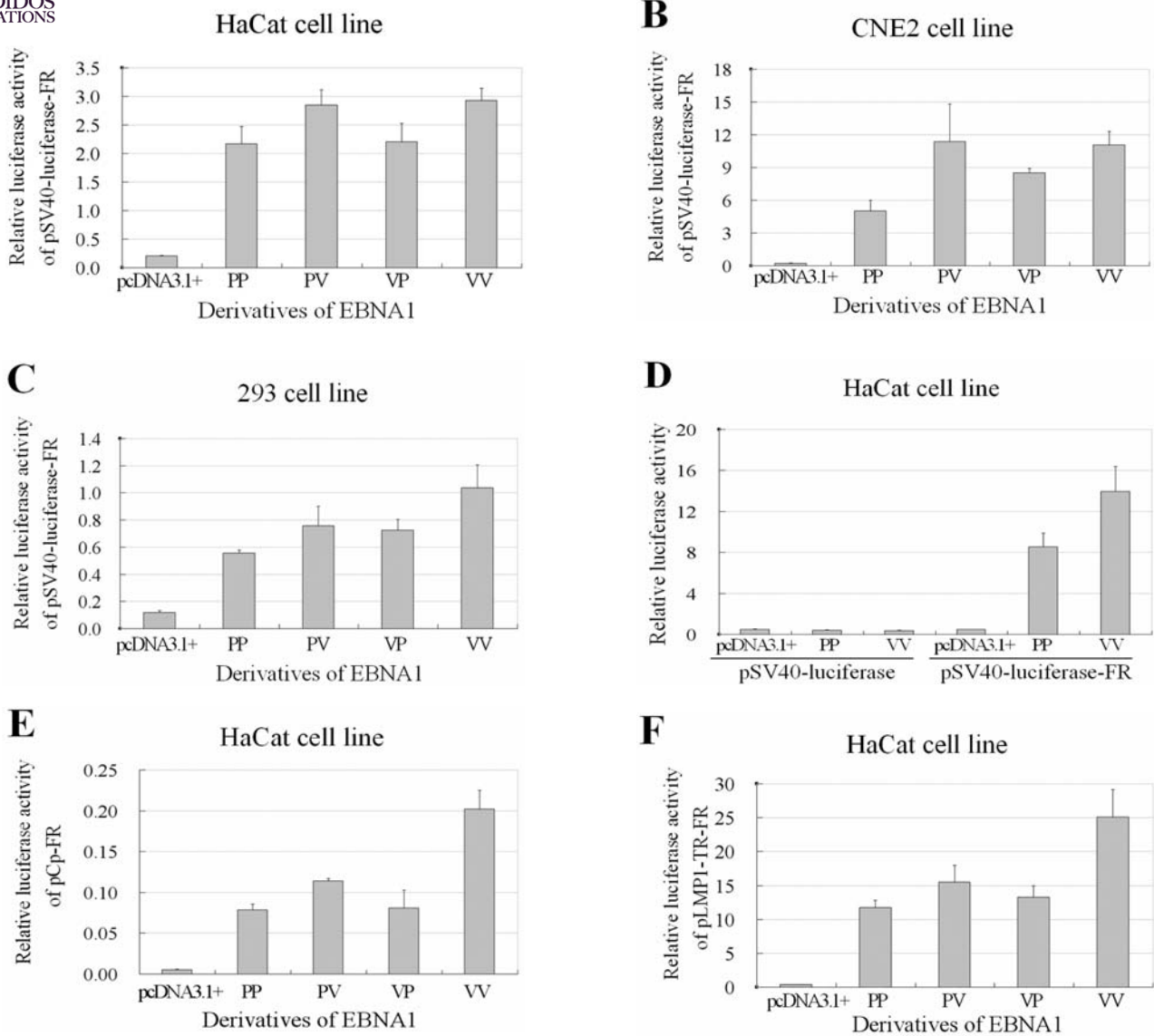


Figure 2. Derivatives of EBNA1 support transcription through the FR to different degrees in epithelial cell lines. (A) Plasmids expressing the indicated EBNA1 derivatives or the empty vector were cotransfected with pSV40-luciferase-FR in HaCat cells and the luciferase activity was subsequently measured 48 h later. (B and C) The reporter assay was repeated in CNE-2 (B) and 293 (C) cell lines following the exact same procedure. In the two aforementioned cell lines, the transactivating activity of vv-EBNA1 and pv-EBNA1 was significantly higher than that observed for pp-EBNA1 ( $P < 0.05$ , t-test), and pv-EBNA1 was consistently more active than vp-EBNA1. (D) A cotransfection of  $0.4 \mu\text{g}$  of pcDNA3.1<sup>+</sup>, pc3-pp-EBNA1, or pc3-vv-EBNA1 with  $0.1 \mu\text{g}$  of the pSV40-luciferase-FR reporter or pSV40-luciferase vector without insertion of the FR element was performed. The luciferase reporter assay was then carried out 48 h post-transfection using the luciferase assay system according to the manufacturer's instructions. Neither pp-EBNA1 nor vv-EBNA1 promoted considerable transcription of the pSV40-luciferase plasmid. (E and F) Plasmids expressing the indicated EBNA1 derivatives or the empty vector were cotransfected with Cp (E)- or LMP1-TR (F)-derived FR-containing luciferase reporter plasmid in HaCat cells by using the procedure described above. The results above were normalized for *Renilla* luciferase derived of the plasmid expressing *Renilla* luciferase. The mean  $\pm$  SD of three independent experiments was shown.

fectured with the various derivatives of EBNA1 by the RLU obtained from cells transfected with the empty vector is shown in Fig. 3. The luciferase activity for the vv-EBNA1/HaCat cells was consistently higher than that observed for the pp-EBNA1 over the three-day period.

*vv-EBNA1 protein binds the FR sequence with higher affinity.* The DNA binding abilities of the prototype EBNA1 and the V-val EBNA1 were compared via the EMSA by using a synthetic 60-mer oligonucleotide containing the FR binding site and the cellular protein extracted from the pp-EBNA1 and vv-EBNA1 transfected HaCat cell lines as the substrate

(Fig. 4). Notably, the addition of an excess of cold probe inhibited the DNA-protein complex formation ability. Moreover, the addition of the anti-His tag antibody effectively inhibited the interaction between the cellular protein and the biotin-labeled FR probe as evidenced by a dramatic shift of the band. The EMSA bands were scanned and quantified densitometrically using the Bio-Rad Quantity One software. In 3 separate experiments, the intensities of the FR-binding band with vv-EBNA1 were 1.25, 1.39, and 1.5 times stronger than the intensities of the corresponding bands for pp-EBNA1. Although the difference was modest, it was consistent in the replicated assays, thereby indicating that the DNA-binding

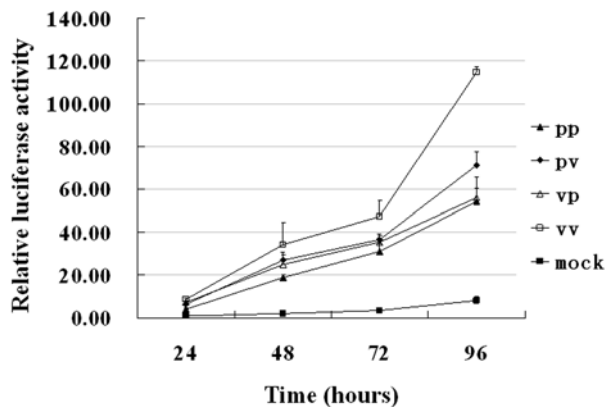


Figure 3. Dynamic changes in the luciferase activity of EBNA1-expressing cells during a four-day culture period. HaCat cells were cotransfected with the pSV40-luciferase-FR reporter plasmid and with pcDNA3.1+ plasmids expressing no EBNA1 (empty vector), pp-EBNA1 (solid triangles), pv-EBNA1 (solid diamonds), vp-EBNA1 (open triangles), vv-EBNA1 (open squares) or a mock control (solid squares) that contained a non-sense mutation in the beginning of the pp-EBNA1 sequence. Equal amounts of lysate were prepared 24, 48, 72, and 96 h post-transfection and were used for the measurement of the luciferase activity. The value of the relative luciferase activity indicated in the ordinate showed the increased transcription level as mediated by the EBNA1 derivatives over that of the empty vector. The error bars indicated the standard error of the mean for each group.

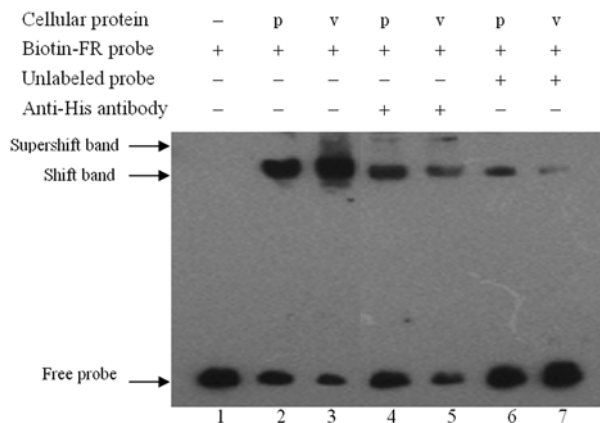


Figure 4. The higher DNA binding affinity of vv-EBNA1 to the FR sequence upon comparison to that of pp-EBNA1. EMSA experiments were performed using the total cellular extract of HaCat cells after transfection of pp-EBNA1 or vv-EBNA1 and the biotin labeled FR probes, which were used as substrate bait. The DNA-protein complex formation was shown in lanes 2 and 3, and the complex was competed out (with cold oligonucleotides) by a 50-fold molar excess of unlabeled oligonucleotide (lanes 6 and 7). The supershifted band was observed with antibodies against the His tag (lanes 4 and 5). The panel shows a representative autoradiograph from three independent experiments. The band intensity of pp-EBNA1 (lane 2) and vv-EBNA1 (lane 3) was determined using densitometric scanning and the ratio of vv-EBNA1/pp-EBNA1 was calculated to be 1.5.

affinity of vv-EBNA1 was higher than that of pp-EBNA1. The representative result is shown in Fig. 4.

## Discussion


Interestingly, while EBV infects most of the global population, only a small percentage of infected individuals develop EBV-associated malignancies, one of which is known as

NPC. NPC occurs at a high frequency in Asian populations, especially among people of Cantonese ancestry. We identified a unique EBV strain GD1 (GenBank accession no. AY961628) that notably differs from the B95.8 prototype and is widespread in Guangdong (18). The contribution of the GD1 substrain of EBV to NPC development is therefore certainly worthy of further investigation.

The V-val subtype of the EBNA1 strain is identical to the EBV strain GD1 and has been determined to be closely associated with the incidence of NPC in the Asian region, which suggests a functional advantage of V-val EBNA1 over the other subtypes regarding the oncogenesis of NPC. According to our previous study, a higher transactivation ability was observed for V-val EBNA1 when compared to the prototype by using GFP as an indicator (16), which was a result later supported by a study published by Do *et al* (19).

In order to evaluate the contribution of the EBNA1 fragments to the enhanced transactivation function, constructs containing a variety of amino- and carboxy-terminal EBNA1 fragments were examined herein for their ability to transactivate the FR containing luciferase reporter plasmid epithelial cell lines. The transcription of the luciferase gene can be promoted by all four EBNA1 derivatives by a factor greater than 10-fold over that observed for the empty vector. The increases in the level of transcription as mediated by vv-EBNA1 and pv-EBNA1 were significantly higher than that observed for pp-EBNA1, which supports the hypothesis that V-val EBNA1 is more likely to be responsible for the enhanced transactivation ability than the prototype. We suggest that the enhanced transactivation ability of V-val-EBNA1 was mainly contributed by the carboxy-terminus of EBNA-1 due to the observation that the luciferase activity was consistently higher in the pv-EBNA1-expressing cells than that observed for the vp-EBNA1-expressing cells in separate experiments. This conclusion is reasonable since the sequences that are essential for the EBNA1 transcriptional activation function were mainly located in the carboxy-terminus. Specifically, EBNA1 amino acid residues 459-607 are responsible for the DNA binding and dimerization abilities, and the structural basis for DNA recognition and dimerization has been revealed through X-ray crystallography studies (20). It was reported that the deletion of amino acid residues 325 through 376 of EBNA1 severely affects the transactivation activity, indicating a major transcriptional role for this Gly-Arg-rich region (21,22). Interestingly, 7 and 1 amino acid mutations were detected in amino acid residues 459 through 607 and 325 through 376 of V-val EBNA1, respectively. Furthermore, the dynamic changes in luciferase activity were detected over four consecutive days after the transfection of the EBNA1 mutants into HaCaT cells, where a significant and continued increase in reporter activity was observed. This increase in the level of expression may be due to the persistent ability of the plasmid to express the EBNA1 coupled with the selection of EBNA1-expressing cells in the culture, which would lead to the contribution of EBNA1 to the overall cell growth.

EBNA1 exerts its functions by binding to FR and DS in the origin of the viral plasmid replication (*oriP*) in the EBV genome. EBNA1 binds to sites in the DS in order to support

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be responsible for efficient segregation, mitotic-chromosome attachment of the DNA constructs, and the transcriptional activation ability of EBNA1 (23,24). The binding ability to the DS domain has been examined by gel shift assays that indicated no major differences between P-ala and V-val (12). Thus, we determined the binding affinity of EBNA1 to the FR element that is known to contribute to plasmid maintenance and the overall transcriptional activation ability. The mechanism of the enhanced transactivation ability of V-val EBNA1 as indicated by the luciferase reporter assays performed herein was revealed by the EMSA which demonstrated that vv-EBNA1 has a higher affinity for the FR sequence than pp-EBNA1. The increased binding affinity of V-val EBNA1 to the FR would also improve its ability to maintain the plasmid, which may contribute to the outgrowth of the V-val viral substrain in NPC cells and lead to stable latent infection of EBV in nasopharyngeal mucosa.

EBNA1 activates the transcription of EBV Cp and the LMP1 promoter. As shown in our results, in the presence of the FR enhancer, the variant-EBNA1-mediated transactivations of Cp and the LMP1-TR promoter were significantly higher than those mediated by the prototype EBNA1. Although Cp is hypermethylated and silenced in NPCs (25), V-val EBNA1 might increase the Cp transcription in normal PBMCs and contribute to the persistence of latent EBV infection. The transactivation of the LMP1 promoter by EBNA1 might contribute to the transformation of host cells. In addition to the modulation of the EBV latent promoters (26), multiple reports have shown a correlation between EBNA1 expression and the activation of prominent cellular targets such as the anti-apoptotic bcl-2 family members, the V(D)J recombinase-activating genes RAG1 and RAG2, in addition to CD25 (27-29). These proteins may contribute to genetic instability and cellular transformation. Gene profiling analysis of EBNA1-expressing carcinoma cells revealed that EBNA1 can influence the expression of a range of cellular genes including those involved in translation, transcription, and cell signaling, resulting in effects that may contribute to the development of EBV-associated tumors (30). These reports indicate that the transactivation ability of EBNA1 may have important functional significance in the pathogenesis of EBV associated tumors.

It is likely that EBNA1 contributes to cell transformation by guaranteeing extrachromosomal viral maintenance in order to establish the latent status of EBV and foster the transcription of viral latent genes in addition to a bevy of cellular genes. We present here evidence to suggest the functional advantage of V-val EBNA1 over the prototype, may be helpful in the elucidation of the possible oncogenic potential of EBNA1.

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