

Transcription of Epstein–Barr Virus-Encoded Nuclear Antigen 1 Promoter Qp Is Repressed by Transforming Growth Factor- β via Smad4 Binding Element in Human BL Cells

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In Epstein–Barr virus (EBV)-infected BL cells, the oncogenic EBV-encoded nuclear antigen 1 (EBNA 1) gene is directed from the latent promoter Qp. Yeast one-hybrid screen analysis using the -50 to -37 sequence of Qp as the bait was carried out to identify transcriptional factors that may control Qp activity. Results showed that Smad4 binds the -50 to -37 sequence of Qp, indicating that this promoter is potentially regulated by TGF- β . The association of Smad4 with Qp was further confirmed by supershift of EMSA complexes using Smad4-specific antibody. The transfection of a Qp reporter construct in two EBV(+) BL cell lines, Rael and WW2, showed that Qp activity is repressed in response to the TGF- β treatment. This repression involves the interaction of a Smad3/Smad4 complex and the transcriptional repressor TGIF, as determined by cotransfection assay and coimmunoprecipitation analysis. Results suggest that TGF- β may transcriptionally repress Qp through the Smad4-binding site in human BL cells. © 2000 Academic Press

Key Words: EBV; BL cells; Qp; TGF- β ; Smad4; TGIF.

INTRODUCTION

Epstein–Barr virus (EBV) is a herpesvirus associated with many malignant diseases, including Africa Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease, and T-cell lymphoma (Rickinson and Kieff, 1996). In these infected tumor cells, the expression of EBV latent genes is limited. For example, EBNA 1 is the only antigen expressed in BL cells (Rowe *et al.*, 1986). Transgenic mice specifically expressing EBNA 1 in B cells show B-cell neoplasia (Wilson *et al.*, 1996) and overexpression of EBNA 1 enhances the malignant progression of human NPC cells, indicating an important role of EBNA 1 in EBV-associated malignancies (Sheu *et al.*, 1996). EBNA 1 is a DNA-binding protein that binds to the *ori-P* region of the EBV genome and allows the viral genome to be present as an episome in infected cells (Yates *et al.*, 1985). Transcription of the EBNA 1 gene in BL and NPC cells is initiated from a latent promoter, Qp (Tsai *et al.*, 1995). Several studies show that Qp expression can be

regulated by interferon regulatory factors (IRFs) through interaction with the basal transcription machinery (Nonkwelo *et al.*, 1995, 1997) and Qp is positively regulated by JAK/STAT activation through two potential STAT-binding sites (Chen *et al.*, 1999), indicating that Qp is potentially regulated by cytokines. Xu *et al.* (1999, 2000) also reported that the levels of TGF- β in serum samples from patients with EBV-associated BL and NPC are elevated compared to those from healthy individuals. TGF- β is a cytokine that plays important regulatory roles in cell growth, morphogenesis, cell differentiation, and apoptosis (Massague, 1998).

Following TGF- β stimulation, the constitutively active type II receptor kinase (T β RII) phosphorylates and activates the kinase T β RI and its downstream signaling mediators, or Smads. Activated Smad2/Smad3 forms a heteromeric complex with Smad4 and is then translocated into the nucleus to affect the transcriptional activity of target genes (Zhang *et al.*, 1997). Smad complexes participate in transactivation by association with a coactivator, the p300/CREB-binding protein, through the interaction between Smad3 and the C-terminal fragment of p300 in a temporal and phosphorylation-dependent manner (Feng *et al.*, 1998). On the other hand, upon activation, the Smad complex can associate with a repressor (or corepressor) to

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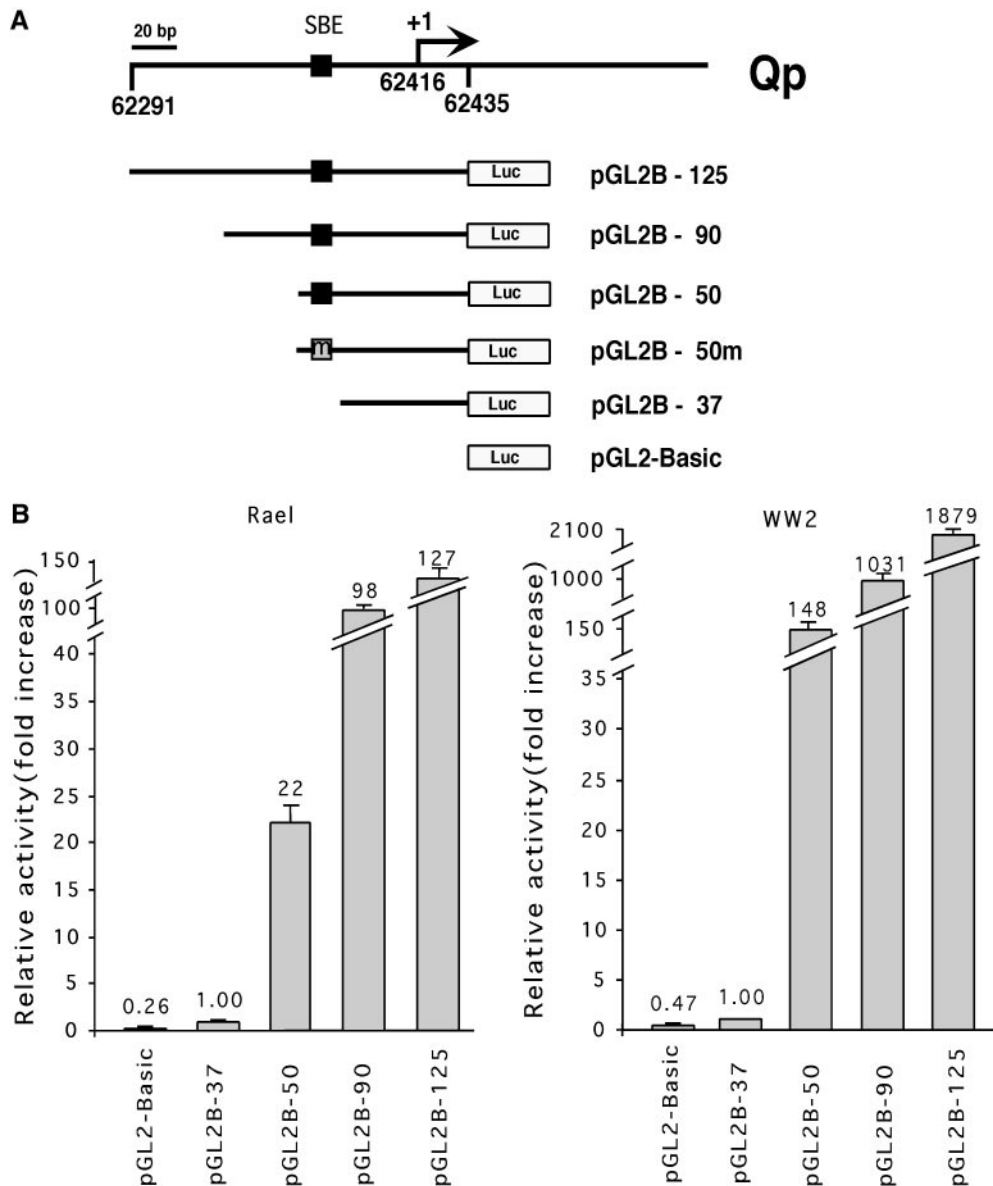


FIG. 1. Schematic representation of the Qp-containing reporter constructs and their activities in type 1 BL cells. (A) Qp-containing promoter construct and its mutants. pGL2B-50m contained mutations (5'-GTCTGGTC-3' to 5'-ATGTAGTC-3') in the SBE of Qp. pGL2B-37 was generated as a deletion mutant, which did not contain SBE. pGL2-Basic is the vector control. The transcription initiation site of Qp is marked as "+1", which corresponds to 62415 on the EBV genome of B95-8 strain. (B) Qp-containing reporter gene constructs were transfected into Rael and WW2 cells. The promoter activity was determined by the luciferase activity. pGL2-Basic was used as the negative control plasmid. Relative activity (fold activation) was measured by dividing the luciferase activity of each construct with that of pGL2B-37. Data were the average of six independent experiments. The standard deviations (SD) are shown by vertical bars.

down-regulate the transcription. To date, several corepressors including TGT-interacting factor (TGIF) (Wotton *et al.*, 1999) have been identified. In the course of determining Qp regulation in BL cells, we found Qp was repressed through the Smad4-binding element (SBE) located at the region between -49 and -45 of Qp in response to TGF- β treatment. The Qp repression was caused by the TGF- β -induced association of the Smad3/Smad4 complex with TGIF, a transcription corepressor.

RESULTS

TGF- β effector protein Smad4 is identified as a Qp-interacting protein

To determine the transcription factors that may regulate EBNA 1 promoter Qp, we first identified the shortest promoter sequence of Qp that possesses a promoter activity. Qp constructs (Fig. 1A) containing various lengths of a previously identified 125-bp region (Tsai *et al.*, 1995) were introduced into two BL cell lines, Rael and

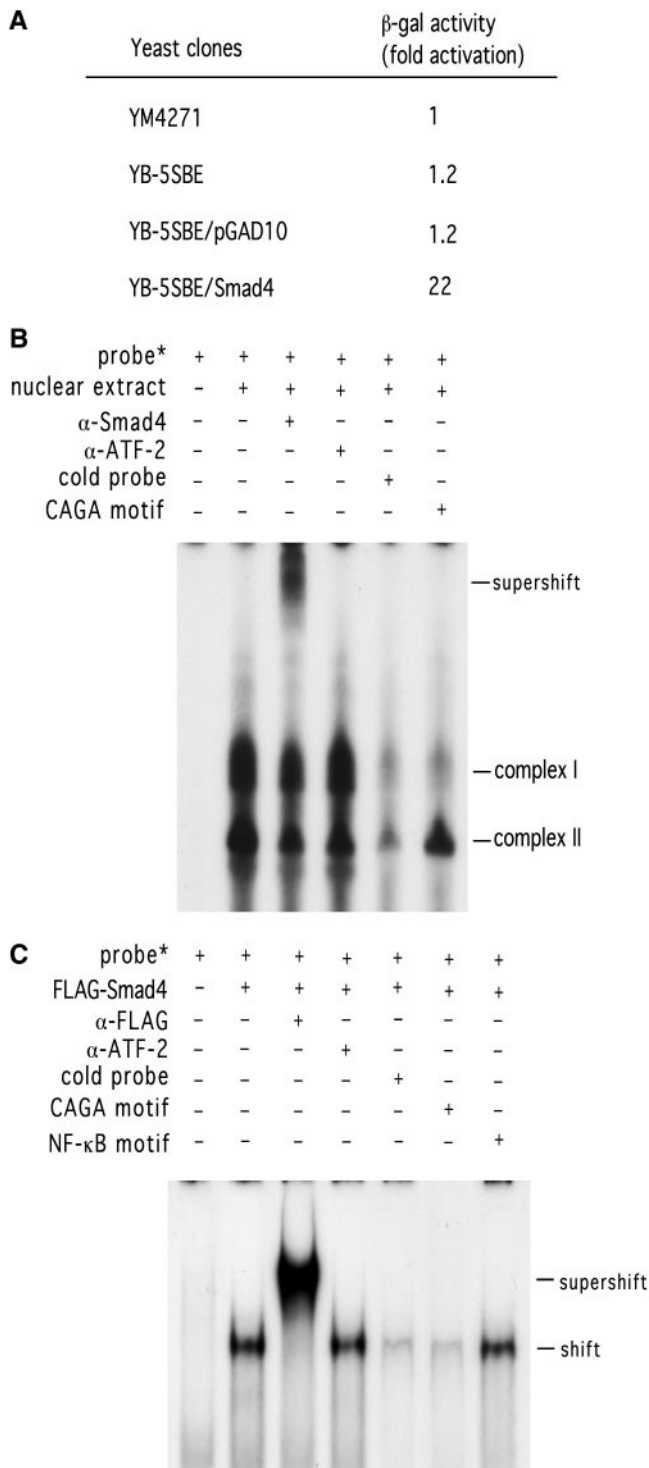


FIG. 2. Association of Smad4 with Qp determined by yeast one-hybrid screen and as analyzed by electrophoretic mobility shift assay (EMSA). (A) The sequence between -50 and -37 (5'-TGTCGTGCTCGCTAG-3') relative to the transcription initiation site of Qp was used as the bait in a yeast one-hybrid screen. The interaction of Smad4 and Qp was examined by β -galactosidase activity in the yeast one-hybrid system. YM4271 is the parental yeast strain. YB-5SBE indicates the yeast cells harboring the LacZ reporter plasmid pLacZ-5SBE, which contains five copies of the -50 to -37 region of Qp. YB-5SBE/pGAD10 indicates the yeast cells harboring both plasmid pLacZ-5SBE and the vector control pGAD10. YB-5SBE/Smad4 indicates the yeast cells har-

WW2. As shown in Fig. 1B, pGL2B-50 possessed a promoter activity, which was at least 20 times higher than the basal activity of pGL2B-37 in Rael cells. In addition, pGL2B-90 and pGL2B-125 showed at least 100-fold higher activity. A similar, but more obvious, effect was observed in WW2 cells, in which pGL2B-50 showed ~ 150 -fold higher activity than pGL2B-37, and pGL2B-90 and pGL2B-125, more than 1000-fold higher activity. Thus, five copies of the sequence between -50 and -37 were used as the bait in yeast one-hybrid screen. From 47 transformants grown on SD/Ura⁻/Leu⁻ plates, three clones showed positive reactions. DNA sequence analysis revealed that one of three clones contained an insert whose sequence was identical to nt $+134$ to $+1427$ of Smad4, an effector in TGF- β signal transduction. This cDNA clone was designated as pGAD/Smad4. The binding specificity of pGAD/Smad4 was measured by β -galactosidase activity assay in the yeast one-hybrid system. Yeast cells (YB-5SBE/Smad4) harboring both pLacZ-5SBE and pGAD/Smad4 showed an activity that was 22-fold higher than that of the parental yeast cells (YM4271), yeast cells (YB-5SBE) harboring pLacZ-5SBE alone, or yeast cells (YB-5SBE/pGAD10) harboring both pLacZ-5SBE and pGAD10 vector (Fig. 2A). Smad4 is a DNA-binding protein, which recognizes the SBE site (Zawel *et al.*, 1998). Sequence analysis of the -50 to -37 region of Qp reveals a potential SBE site between -49 and -45 . Therefore, identification of Smad4 binding to Qp suggests the function of TGF- β in regulation of EBNA 1 gene expression.

To test whether endogenous Smad4 recognized the potential SBE site of Qp, the sequence between -54 and -40 of Qp was used as the probe in the electrophoretic mobility shift assay (EMSA). As shown in Fig. 2B, using nuclear extracts from TGF- β -treated Rael cells, two complexes were formed. Both complexes were affected by unlabeled probe, whereas only Complex I was completely competed off by the $200 \times$ CAGA box, a Smad4-

boring both the reporter plasmid pLacZ-5SBE and the Smad4 cDNA clone pGAD10/Smad4. The fold activation was determined as described under Materials and Methods. (B) Binding of SBE of Qp with the endogenous and ectopically expressed Smad4. TGF- β -treated Rael cell extracts were reacted with 5'-end-labeled oligonucleotide (-54 to -40 of Qp). For the supershift experiment, the anti-Smad4 antibody or anti-ATF-2 antibody (as a negative control) was used. For competition assay, the unlabeled probe or CAGA sequence (which is a Smad4 consensus-binding sequence) was used as the competitor. (C) For binding of SBE of Qp with ectopically expressed Smad4, 293 cells were infected with adenovirus carrying a Flag-tagged Smad4 gene and then treated with TGF- β . Nuclear extracts isolated from treated cells were incubated with 5'-end-labeled oligonucleotide (-54 to -40 of Qp). For the supershift experiment, the anti-Flag antibody or anti-ATF-2 antibody (as a negative control) was used, respectively. For competition assay, the unlabeled CAGA sequence or NF- κ B motif was used as the competitor. The products were analyzed on 4% polyacrylamide gel. These results are representative of at least three independent experiments.

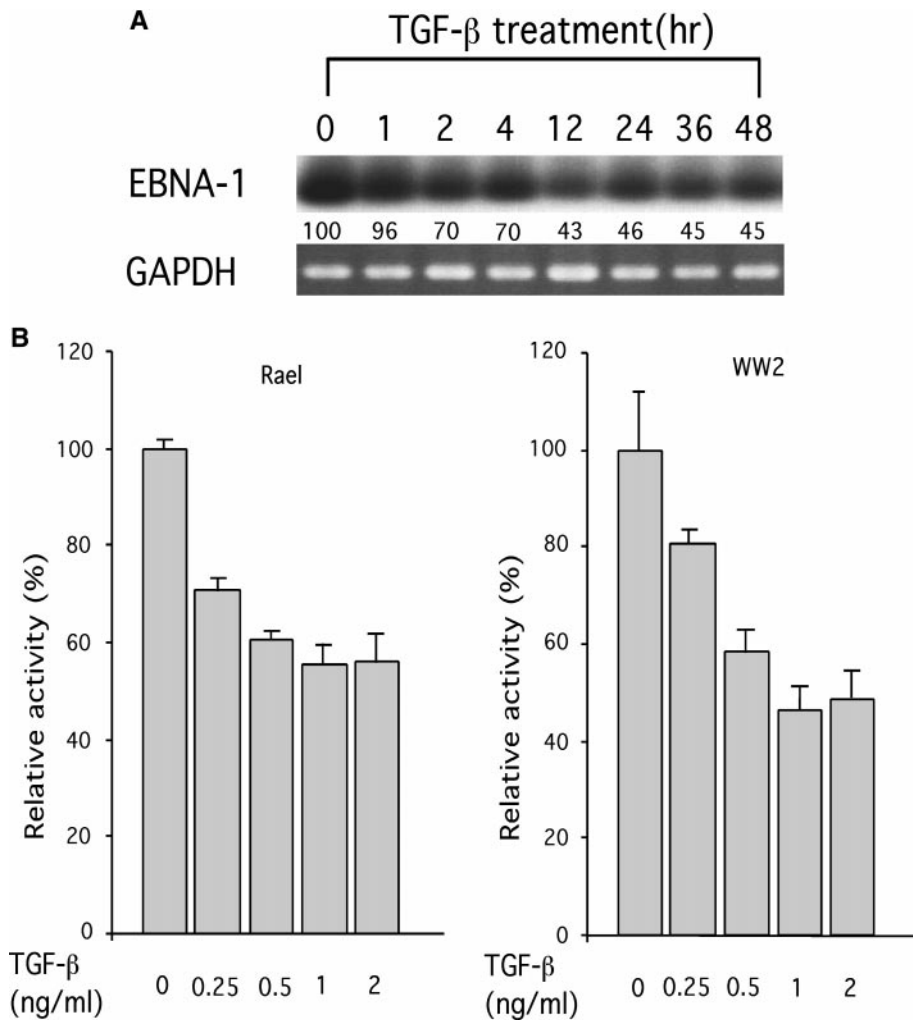


FIG. 3. TGF- β represses EBNA 1 gene expression in type 1 BL cells. (A) Type 1 BL cells Rael were treated with TGF- β at 1 ng/ml and total RNA was isolated from the treated cells at 1, 2, 4, 12, 24, 36, and 48 h, respectively, after the treatment. RT-PCR was carried out with RNA from treated cells and EBNA 1 gene-specific primers. The RT-PCR products were then subjected to Southern blot analysis for detection of EBNA 1 gene expression. The percentage decrease of EBNA 1 transcripts is shown at the bottom. Expression of GAPDH gene is used as the internal control. (B) Qp-containing reporter construct pGL2B-50 was electroporated into Rael and WW2 cells, respectively. Cells were treated without or with TGF- β at 0.25, 0.5, 1, and 2 ng/ml, respectively. Relative activity was determined by dividing the luciferase activity from each transfection assay after treatment with the indicated concentrations of TGF- β with that of pGL2B-50 without the TGF- β treatment (as 100%). Data were the average of at least six independent experiments. Vertical bars show the standard deviations (SD).

binding site. Furthermore, an anti-Smad4 antibody supershifted and reduced the intensity of Complex I. On the other hand, anti-ATF2 antibody (as a negative control) had no effect on either complex. A similar pattern was detected, but less obviously, when nuclear extracts were isolated from Rael cells without TGF- β treatment (data not shown). To further confirm the interaction of Smad4 and Qp, the Qp probe was incubated with the nuclear extract isolated from 293 cells, which was infected with a recombinant adenovirus carrying the Flag-tagged Smad4 gene. As shown in Fig. 2C, only one complex was detected, which was competed off by the unlabeled probe and the CAGA box, but not by the NF- κ B-binding motif. Furthermore, this complex was supershifted by anti-Flag antibody, but not by anti-ATF-2 antibody. Taken

together, these results indicated that Smad4, induced endogenously or introduced exogenously, interacts with the SBE site of Qp.

TGF- β represses EBNA 1 gene expression and its promoter activity in type 1 BL cells

To investigate the effect of TGF- β on Qp, BL cells were treated with TGF- β and RNA was extracted from cells at 1, 2, 4, 12, 24, 36, and 48 h, respectively, after the treatment. The presence of EBNA 1 transcripts was determined by RT-PCR and EBNA 1 gene-specific primers. As shown in Fig. 3A, TGF- β reduced the expression of EBNA 1 transcripts in Rael cells by \sim 30% at 1 h after the treatment, and by \sim 50% 12 to 48 h after the treatment.

To test whether TGF- β affects Qp activity, pGL2B-50 was introduced into Rael and WW2 cells, which were then treated with TGF- β . As shown in Fig. 3B, the Qp activity was repressed by \sim 20% upon TGF- β treatment at 0.25 ng/ml, by \sim 40% at 0.5 ng/ml, and by \sim 50% at 1 ng/ml or higher concentrations, as compared to the activity in the absence of TGF- β in both Rael and WW2 cells. Similar results were detected when pGL2B-125 and pGL2B-90 were examined (data not shown). In this set of experiments, an almost eightfold activation by TGF- β treatment at 1 ng/ml was observed for p3TP-Lux (Keeton *et al.*, 1991), a well-studied TGF- β -inducible reporter construct, in both cell lines (data not shown). Taken together, these results indicated that Qp is specifically repressed by TGF- β in BL cells.

TGIF is involved in TGF- β repression of Qp activity in type 1 BL cells

A homeoprotein TGIF has been identified as a Smad2/Smad3-binding protein and functions as a corepressor for TGF- β -inducible transcription (Wotton *et al.*, 1999). To determine whether TGIF was involved in TGF- β repression of Qp activity, Flag-tagged TGIF was cotransfected with pGL2B-50 into Rael cells and transfected cells were then treated with TGF- β (1 ng/ml). As shown in Fig. 4A, cotransfection of cells with Smad3 and Smad4 showed an almost 20% reduction of Qp activity, which was further reduced to \sim 75% after TGF- β treatment. Cotransfection of Smad3/Smad4 and TGIF-expressing vector resulted in more than \sim 40% reduction of Qp activity and \sim 85% reduction after treatment with TGF- β . This TGF- β -induced repression was inhibited by cotransfection of a Smad4 dominant-negative mutant. These results suggest that TGIF and Smad3/Smad4 cooperatively repress the Qp activity in response to TGF- β treatment.

Association of Smad3/Smad4 with TGIF was then confirmed by the coimmunoprecipitation method. Flag-tagged TGIF-expressing vector was cotransfected with Myc-tagged Smad3- and Smad4-expressing vectors into 293 cells, followed by TGF- β treatment. As shown in Fig. 4B, the complex immunoprecipitated by anti-Flag antibody (for TGIF) contained Smad3 and Smad4, as detected by the subsequent Western blot analysis using anti-Myc antibody. Similarly, the anti-Smad4 antibody was able to immunoprecipitate the Flag-tagged TGIF. The ectopic expression of TGIF, Smad3, and Smad4 was confirmed by Western blot analysis of the whole-cell lysates using anti-Flag and anti-Myc antibody, respectively (Fig. 4B). Results confirmed the physical association of TGIF and Smad proteins in this study.

Histone deacetylase (HDAC) is involved in TGF- β -repressed Qp activity in type 1 BL cells

A recent report showed that the TGF- β -induced inhibition of the TGF- β /activin response element (ARE)-con-

taining promoter (A3-Lux) activity results from the recruitment of a histone deacetylase (HDAC) by TGIF (Wotton *et al.*, 1999). To determine whether HDAC participates in TGF- β repression of Qp activity, Rael cells were transfected with pGL2B-50 and pGL2B-50m, and then treated with either 1 ng/ml of TGF- β or 1 ng/ml of TGF- β plus 100 ng/ml of trichostatin A (TSA), a specific inhibitor for HDAC. Plasmid pGL2B-50 contains -50 to $+17$ sequence of Qp and plasmid pGL2B-50m contains mutations at the SBE site of pGL2B-50. As shown in Fig. 4C, pGL2B-50m showed elevated activity and showed loss of responsiveness to TGF- β repression (gray bars) as compared to that of pGL2B-50. Treatment with TSA alone (data not shown) or combined treatment with TGF- β and TSA (striped bars) also resulted in the release of Qp repression. Importantly, the promoter activity of pGL2B-50m was elevated more than threefold over that of pGL2B-50 in the presence of TGF- β , and this activity was not affected by the addition of TSA. These results suggest that HDAC is involved in TGF- β repression of Qp activity and this repression is mediated through the SBE site within Qp. Similar results were obtained using WW2 cells (data not shown).

DISCUSSION

EBV is known to infect and immortalize human B cells into continuously growing LCLs *in vitro* and is associated with many human malignant diseases. Earlier studies demonstrated that EBV and its gene products can induce TGF- β production and secretion in human cells (Cayrol and Flemington, 1995). In sera of patients with EBV-associated BL and NPC, the concentration of TGF- β is higher than that of healthy individuals (Xu *et al.*, 1999, 2000), thus suggesting a role of this cytokine in the pathogenesis of EBV-associated diseases. In this study, we identified a transcription factor Smad4, which binds the SBE site within Qp. We also found that Smad proteins cooperated with TGIF to repress the Qp via the SBE site in type 1 BL cells in response to TGF- β stimulation.

Qp is a latent promoter, selectively used in type 1 BL cells. Upon TGF- β stimulation, this promoter was repressed through the SBE site. Mutation of the SBE site, therefore, released the TGF- β -induced repression (Fig. 4C). The SBE site is located within the previously identified positive element QRE-1. QRE-1 is the potential binding site of the cellular transcription factor LBP-1, which shows significant promoter activity in conjunction with the interferon regulatory factor (IRF) binding site QRE-2 located between -12 and $+2$ of Qp (Nonkwelo *et al.*, 1995, 1997). In this study, we found that pGL2B-50 showed an almost 20-fold higher promoter activity than that of pGL2B-37. Promoter construct pGL2B-50 contains both the QRE-1 and the QRE-2 sites, while promoter construct pGL2B-37 contains only the QRE-2 site. As discussed above, both QRE-1 and QRE-2 sites are re-

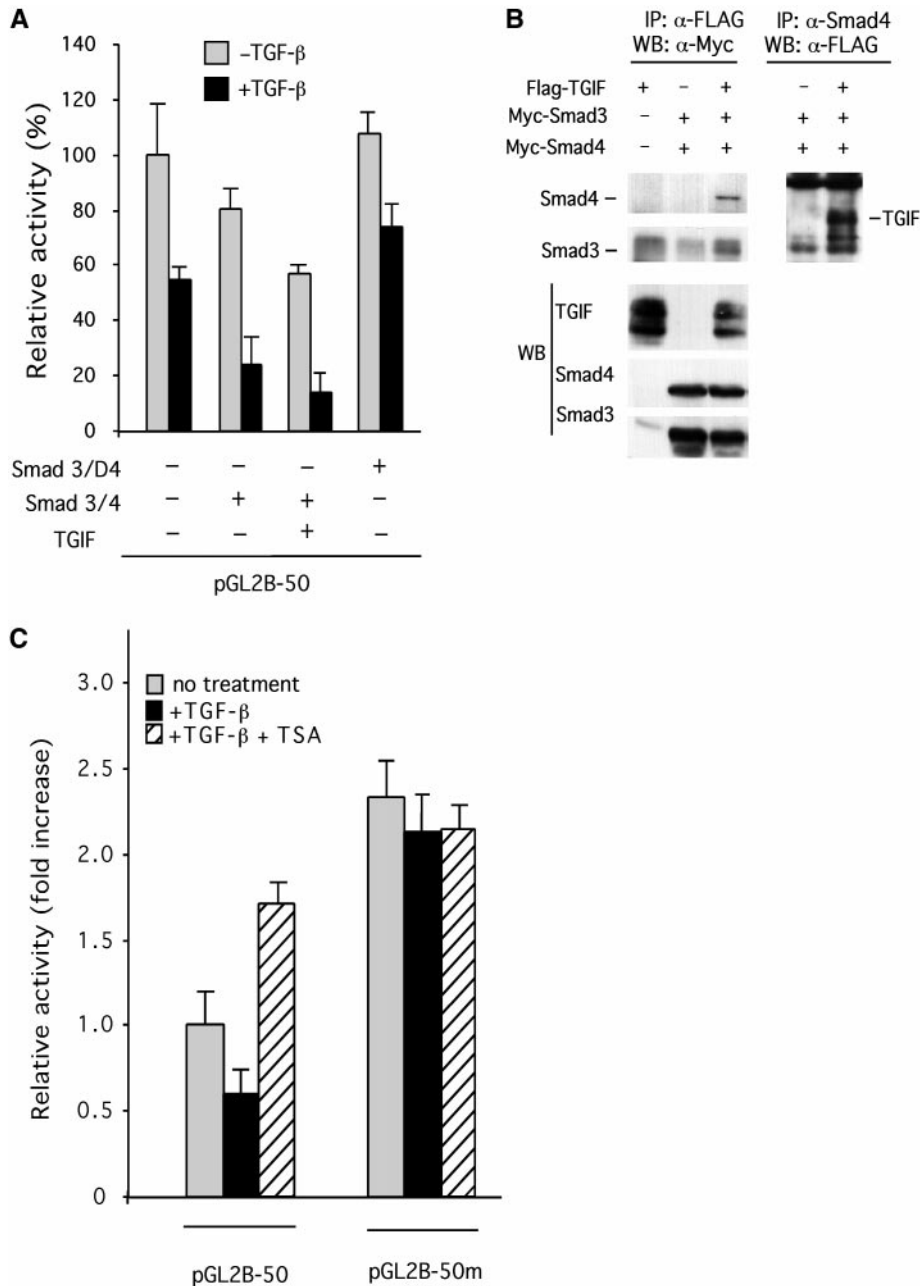


FIG. 4. Transcriptional repression of Qp by cooperation of TGF- β -inducible Smads and TGIF. (A) Transcriptional repression of Qp by Smad3/Smad4 and TGIF. Rael cells were cotransfected with pGL2B-50 and 1 μ g each of the expressing plasmids of Smad3 and Smad4 with or without TGIF-expressing vector, and then treated with or without 1 ng/ml of TGF- β . The plasmid pcDNA3 was used as the vector control. (B) Interaction of TGIF with Smad3/Smad4 by immunoprecipitation analysis. Flag-tagged TGIF and Myc-tagged Smad3- and Smad4-expressing vectors were cotransfected into 293 cells. Cells were first immunoprecipitated with anti-Flag antibody and analyzed by Western blot analysis with anti-Myc antibody, or first precipitated with anti-Smad4 antibody and then analyzed by Western blot analysis with anti-Flag antibody. The bands corresponding to Smad3, Smad4, and TGIF are indicated by horizontal bars. Lower panel: total cell lysates were directly subjected to Western blot analysis using anti-Flag for Flag-TGIF identification and anti-Myc antibody for both Myc-Smad3 and Myc-Smad4 identification. (C) Effect of TSA and TGF- β on Qp activity. Qp construct pGL2B-50 and its mutant pGL2B-50m were individually electroporated into Rael cells and transfected cells were treated with 100 ng/ml of TSA, 1 ng/ml of TGF- β , or both. Relative activity (fold increase) was measured by dividing the luciferase activity of each treatment with that of pGL2B-50 without any treatment. Results were the average of six independent transfections. Vertical bars show the standard deviations (SD).

quired for a significant promoter activity. This may explain the low promoter activity of pGL2B-37 observed in this study (Fig. 1B).

The QRE-2- or IRF-binding site within Qp is also over-

lapped with two STAT sites (-15 to -6 and +1 to +9 of Qp), which are important for positive regulation of Qp by the JAK/STAT pathway (Chen *et al.*, 1999). However, this activation can be repressed by Zta, which up-regulates

p53 gene expression, and in turn leads to p53-mediated interference of JAK/STAT signaling (Chen *et al.*, 1999). It is also known that Zta expression is activated by TGF- β . Thus, Qp can be negatively regulated through the Zta-mediated interference of the JAK/STAT signaling pathway. Therefore, TGF- β might activate Zta, which, in turn, represses Qp through the STAT sites. However, the promoter activity of pGL2B-50 was still repressed by TGF- β in two EBV-negative BL cell lines, Ramos and Akata(-), to a level similar to that observed in EBV(+) BL cells (data not shown). This eliminates the possibility that Qp repression was the result of induction of the Zta protein by TGF- β . It is also true that p53 is often mutated in BL cells. Therefore, Qp repression examined in this study was more likely a direct effect in response to TGF- β . Physically, the potential IRF- and STAT-binding sites are near the transcription initiation site of Qp, whereas the SBE identified in this study is located ~30 nt upstream from these sites. It is conceivable that TGF- β -induced Smad4 binding interferes with the LBP-1/IRF interaction at the transcriptional level and vice versa. This may suggest that the regulatory actions, either by STAT and IRF or by LBP-1 and Smad4, are mutually exclusive.

Transcriptional control in eukaryotic cells is involved in multiple layers of cooperativity (Cayrol and Flemington, 1995). TGF- β -induced transcription regulation is not limited to interactions only between Smad proteins. Association of Smad proteins with different transcriptional factors may contribute to the opposite effect, either transcriptional activation or transcriptional repression. Transcriptional repression is often a result of recruitment of histone deacetylase (HDAC) to transcriptional corepressors such as TGIF (Wotton *et al.*, 1999; this study) by forming a specific transcription complex. In this study, Qp repression was caused by recruitment of HDAC to the Smad/TGIF complex. Therefore, this supports a model in which TGIF is recruited to TGF- β -targeted promoters such as Qp, and functions as an adapter to link Smad to HDAC 1, leading to transcriptional repression. However, we cannot rule out that other repressors or unidentified factors may be also involved in this Qp repression. EBNA 1 is an EBV-encoded oncogene, which plays an important role in maintaining the viral episomes in infected cells. Qp is the key promoter in regulating EBNA 1 expression in EBV-associated tumors. Down-regulation of Qp by agents such as Smad4 and transcriptional corepressors might be a possible therapeutic approach to eradicate the EBV genome from those tumors.

Recent reports by Xu *et al.* (1999, 2000) indicated that the levels of TGF- β in serum samples from patients with EBV-associated BL and NPC are elevated compared to those from healthy individuals. Levels of this cytokine could correlate with the severity of NPC (Xu *et al.*, 1999), although it is not clear whether this is also true for BL patients. Furthermore, other cytokines may regulate the EBV lytic cycle in addition to TGF- β . It has been reported

before that TGF- β induces latent EBV to enter into the lytic cycle (di Renzo *et al.*, 1994). Currently, we are testing whether the BZLF1 gene, a latent-to-lytic switching gene, is also regulated by TGF- β at the transcriptional level. Study of the regulation of EBV genes by TGF- β will be important to further understand the role of EBV in the development of tumors.

MATERIALS AND METHODS

Cell lines

Rael and WW2 are EBV-positive type 1 Burkitt's lymphoma (BL) cell lines. These cells were maintained at 37°C in RPMI 1640 medium containing 15% (v/v) fetal bovine serum (FBS), 100 μ g/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine. The 293 cells were maintained in DMEM supplemented with 10% (v/v) FBS, 100 μ g/ml streptomycin, and 100 μ g/ml penicillin.

RNA extraction, RT-PCR, and Southern blot hybridization

Total RNA was isolated from Rael cells treated with 1 ng/ml of TGF- β by using REzol C&T protocol (PROtech Technology, Taiwan, ROC). A 5- μ g sample of total RNA was heated at 70°C for 10 min and rapidly cooled on ice. Reverse transcription was carried out in a reaction containing 1 μ M of gene-specific 3' primer, 3'K 5'-CCATT-TCCAGGTCCTGTA-3' for EBNA 1, 0.125 mM dNTP, 8 units of avian myeloblastosis virus reverse transcriptase (AMV-RT; Promega, Madison, WI), and 20 units of RNase inhibitor (Promega) at 42°C for 1 h. PCR assays were carried out by adding 10 pmol of 5' primer 5' Qp 5'-GCGTTTCTTGAGCTT-3' for EBNA 1 transcripts and 2.5 units of *Taq* DNA polymerase (Promega) to one-third of the reverse transcription reaction mixture (Tsai *et al.*, 1995). RT-PCR products were separated by electrophoresis in 2% agarose gel and then transferred to a nitrocellulose membrane. The membrane was fixed in an XL-1000 UV CROSSLINKER (Spectronics, Westbury, NY) before hybridization with [α -³²P]dCTP-labeled RT-PCR products. After the hybridization reaction, the membranes were washed in washing buffer containing 0.2 \times SSC and 0.1% SDS for 30 min before exposure to X-ray films.

Yeast one-hybrid system

Five copies of -50 to -37 (5'-TGTCTGGTTCGCTAG-3') of EBV Qp was inserted into the *Sma*I site of pLacZi and pHisi-1 (Clontech, Palo Alto, CA) to generate pLacZi-5SBE and pHisi-5SBE. Plasmid pLacZi-5SBE was linearized by *Nco*I and plasmid pHisi-5SBE was digested with *Xho*I. The linearized plasmids were transformed into yeast YM4271, to establish two stable clones, YB-5SBE and YH-5SBE. YH-5SBE yeast strain was transformed with the metastasized NPC biopsy cDNA library (2.6 \times

10^6 individual colonies) generated with pGAD10 (custom-made by Clontech) and then selected on SD/His⁻/Leu⁻ plates with 45 mM 3-amino-1,2,4-triazole (3-AT; Sigma, St. Louis, MO). A total of 47 clones was obtained from the 2.6×10^6 colonies of the cDNA library. YB-5SBE yeast cells were transformed with the purified plasmid DNA and selected on SD/Ura⁻/Leu⁻ plates. The clones that showed positive β -galactosidase (β -gal) activity were isolated and the plasmid DNAs were purified before being subjected to DNA sequence analysis. The β -gal activity was assayed with *n*-nitrophenyl- β -D-galactopyranoside (ONPG) as the substrate by using the β -galactosidase enzyme assay system (Promega).

Plasmid construction

Qp-containing reporter plasmids were constructed as follows. Various regions of the Qp (spanning nts 62291 to 62416 of the EBV genome) were individually generated by PCR and then cloned into the pGL2-Basic vector (Promega). Briefly, PCR products for pGL2B-125, -90, -50, -50m, and -37 were generated by Q3/Fb, Q5(5'-GAAATTGGGTGACCACTG-3')/Fb, Q4(5'-TGTCTGGTCGCTAGATGG-3')/Fb, Q4m(5'-TATGTAGTCGCTAGATGG-3')/Fb, and Q2/Fb, respectively, and were inserted into *HincII*-digested pUC18 (Gibco BRL, Life Technologies, Paisley, UK). The recombinant plasmids digested with *HindIII* and *BamHI* were eluted and subcloned into *HindIII*- and *BglII*-treated pGL2-Basic (Promega). These reporter plasmids were designated as pGL2B-125, -90, -50, -50m, and -37, respectively (Fig.1) (Tsai *et al.*, 1995).

The expression vectors of pcDNA3-Flag-Smad3, Smad4, and dominant-negative Smad4 mutant (SmadD4) were described previously (Zhang *et al.*, 1997).

The full-length TGIF cDNA was generated by RT-PCR using primers TGIF-F (5'-AATGAAAGGCCAAAGGT-3') and TGIF-R (5'-TTAAGCCGTAAGTTTTGCC-3'). This RT-PCR product was digested with *BglII* and *EcoRI*, and then cloned to the *BglII*- and *EcoRI*-digested pFlag-CMV2 (Kodak, Rochester, NY). The plasmid was designated as pFlag-TGIF.

The sequences of all the constructs were confirmed by Sanger's dideoxy chain-termination method (Sanger *et al.*, 1977), using the T7 sequence version 2.0 DNA sequencing kit (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's protocol.

DNA transfection and TGF- β treatment

Reporter plasmids (2.5 μ g) and the indicated amounts of expression plasmids were introduced into Rael and WW2 cells by electroporation at a capacitance of 960 μ F and a voltage of 0.22 kV using the Gene Pulser (Bio-Rad, Richmond, CA). After transfection, cells were harvested and lysed in 50 μ l of lysis buffer (Promega). The concentration of the cell lysates was measured by using Bio-Rad

protein assay reagent. A 50- μ g sample of protein extracts was used to assay the luciferase activity in an illuminometer (autolumat model LB953; Berthold, Germany).

For TGF- β treatment, cells were first transfected with indicated plasmid DNA and cultured in RPMI 1640 supplemented with 0.2% FBS for 12 h, prior to treatment with 1 ng/ml of recombinant human TGF- β 1 (R&D Systems, Minneapolis, MN) for 24 h. All of the plasmid DNAs were purified by the cesium chloride/ethidium bromide gradient centrifugation method.

Western blot analysis and immunoprecipitation

For the Western blot analysis, cells were washed three times with phosphate-buffered saline (PBS) before being harvested and lysed in the buffer containing 10 mM Tris-HCl, pH 7.1, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1% Triton X-100. Cells were disrupted by sonication, after which the cell debris was removed by centrifugation at 10,000 *g*. The supernatants of the whole-cell lysates were then separated by NaDodSO₄-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes and immunoblotted with the indicated antibodies. Anti-Flag antibody (Kodak), anti-Myc antibody, and anti-Smad4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained commercially. The blot was then subjected to the ECL system (Amersham, Arlington Heights, IL) for the detection of TGIF or Smad3/Smad4 proteins.

Coimmunoprecipitation of Smads and TGIF was performed as previously described by Wotton *et al.* (1999). The immunoprecipitates were analyzed by SDS-PAGE, followed by the Western blot analysis as described above.

Electrophoretic mobility shift assay

The double-stranded oligonucleotide containing two copies of the -54 to -40 sequence of Qp promoter was used as the probe in the electrophoretic mobility shift assay (EMSA). In brief, the oligonucleotide was labeled with T4 polynucleotide kinase and [γ -³²P]ATP. Nuclear extracts prepared from either Rael cells or 293 cells infected by a recombinant adenovirus carrying Flag-tagged Smad4 (kindly provided by Dr. Kohei Miyazono) were incubated with 15,000 cpm of [γ -³²P]-labeled oligonucleotide for 10 min at room temperature in a buffer containing 0.13 μ g/ μ l poly dl:dC, 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5. For competition assay, a 200-fold excess of the individual cold double-stranded oligonucleotides was added into the reaction mixture before addition of the [γ -³²P]-labeled probe. After a 30-min incubation at room temperature, the samples were loaded onto 4% nondenaturing acrylamide gel and electrophoresis was carried out in 0.5 \times TBE at 100 V for 2.5-3 h. For

the supershift assay, the reaction mixture was incubated at 4°C with 4 µg of anti-Smad4 antibody (Santa Cruz Biotechnology), anti-ATF-2 antibody (Santa Cruz Biotechnology), and anti-Flag antibody (Kodak), respectively. The gel was subsequently dried and exposed to Kodak BioMax film.

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