The EMBO Journal vol.15 no.11 pp.2748–2759, 1996

The Epstein–Barr virus bZIP transcription factor Zta causes G_0/G_1 cell cycle arrest through induction of cyclin-dependent kinase inhibitors

C.Cayrol[1] and E.K.Flemington[2]

Division of Tumor Virology, Division of Neoplastic Disease Mechanisms, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA

[1]Present address: Laboratory of Bernard Ducommun, Laboratoire de Pharmacologie et de Toxicologie Fondamentales (LPTF), 205, route de Narbonne, 31077 Toulouse, France

[2]Corresponding author

While oncoproteins encoded by small DNA tumor viruses and Epstein–Barr virus (EBV) latent antigens facilitate G_1/S progression, the EBV lytic switch transactivator Zta was found to inhibit growth by causing cell cycle arrest in G_0/G_1 in several epithelial tumor cell lines. Expression of Zta results in induction of the tumor suppressor protein, p53, and the cyclin-dependent kinase inhibitors, p21 and p27, as well as accumulation of hypophosphorylated pRb. Up-regulation of p53 and p27 occurs by post-transcriptional mechanisms while expression of p21 is induced at the RNA level in a p53-dependent manner. Inactivation of pRb by transient overexpression of the human papillomavirus E7 oncoprotein indicates that pRb or pRb-related proteins are key mediators of the growth-inhibitory function of Zta. These findings suggest that EBV plays an active role in redirecting epithelial cell physiology to facilitate the viral replicative program through a Zta-mediated growth arrest function.

Keywords: EBV/growth arrest/p53/pRB/Zta

Introduction

Epstein–Barr virus (EBV) is a human herpes virus that infects B-lymphocytes as well as oropharyngeal and cervical epithelial cells (Rickinson, 1986; Sixbey *et al.*, 1986; Klein, 1994). EBV is the causal agent of infectious mononucleosis and is associated with the development of both B-cell and epithelial cell malignancies, including the endemic form of Burkitt's lymphoma, post-transplantation lymphoproliferative diseases, AIDS-associated lymphomas, Hodgkin's disease and undifferentiated nasopharyngeal carcinoma (Kieff and Liebowit, 1990; Miller, 1990).

Infection of primary B-lymphocytes *in vitro* results in efficient immortalization of the infected cells such that they grow indefinitely in culture (Kieff and Liebowit, 1990; Klein, 1994). In these cells, little viral replication takes place and the ~100 EBV lytic replication-associated genes encoding early antigens (EAs), viral capsid antigens (VCAs) and membrane antigens (MAs) are not expressed. Instead, EBV establishes a restricted pattern of gene expression (referred to as latency) involving only those genes that are required for immortalization and for maintaining the viral genomic episome. These genes include that for the EBV nuclear antigen (EBNA-1) which is necessary and sufficient for maintenance of the EBV genome (Yates *et al.*, 1985), and EBNA-2, EBNA-3a, EBNA-3c, EBNA-LP and the latent membrane antigen, LMP1, which co-operate to immortalize infected B cells (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993; Sinclair *et al.*, 1994; Kempkes *et al.*, 1995). While insight into the mechanisms involved in driving proliferation by these EBV-encoded antigens have only begun to be understood recently (Henkel *et al.*, 1994; Alday *et al.*, 1995; Hsieh and Hayward, 1995; Mosialos *et al.*, 1995), it is clear that EBV immortalization is achieved through mechanisms that are distinct from those employed by small DNA tumor viruses. For example, while SV40, human papilloma virus (HPV) and adenovirus functionally inactivate p53, EBV immortalizes human B-cells without neutralizing the function of p53 (Alday *et al.*, 1995).

Stimulation of the EBV replicative cycle in latently infected B-lymphocytes by reagents that mimic B-cell terminal differentiation signals results in a switch in the viral genetic program, leading to the ordered cascade of lytic gene expression which culminates in virus production (Miller, 1990). Although the EBV lytic cycle can be activated in B-cells, replication of EBV *in vivo* occurs primarily in epithelial cells of the oropharynx, salivary glands and uterine cervix (Sixbey *et al.*, 1984, 1986; Wolf *et al.*, 1984). Full EBV replication appears to be highly dependent on the differentiated state of the epithelium since it is observed in the upper spinous layer which contains cells that have stopped dividing, but not in the basal, mitotically active layer (Wolf *et al.*, 1984; Becker *et al.*, 1991; Young *et al.*, 1991). In agreement with these observations of EBV replication in growth-arrested, differentiated cells *in vivo*, it has been shown *in vitro* that replication of EBV, as well as other herpes viruses, can occur in cells treated with agents shown previously to arrest cell cycle progression (Shadan *et al.*, 1994). In contrast, replication of small DNA viruses such as SV40 and papillomavirus is inhibited by these reagents (Shadan *et al.*, 1994). Since large DNA viruses, such as EBV, encode proteins involved in DNA synthesis (DNA polymerase, DNA metabolic enzymes and other replication factors), they are less dependent on the host cell DNA replication machinery than small DNA viruses, which have a strict requirement for inactivation of growth inhibitory signals provided by the tumor suppressor proteins pRb and p53 in order to promote S-phase progression (Vousden, 1995).

Although EBV efficiently promotes immortalization of human B-lymphocytes, this is clearly a function of latency-associated viral gene expression. Unlike SV40 or papillomavirus replication, EBV lytic replication is associated with cellular growth arrest and/or differentiation. In

the oral epithelium, the production of virus in the outer spinous layer probably results in the efficient shedding of virus into the saliva where it can be transmitted from host to host. Therefore, the association of lytic replication with terminal differentiation plays an important role in the viral life cycle. Despite the link between EBV lytic replication and cellular growth arrest, it is unknown presently whether onset of the lytic cycle is purely a response to terminal differentiation signals or whether EBV plays a role in altering cell cycle progression.

The immediate-early lytic switch transactivator Zta (also called BZLF1, EB1, Zebra) is known to play a crucial role in the initiation of the lytic cascade, since ectopic expression of Zta in latently infected cells is sufficient to activate the entire EBV replicative cycle (Countryman *et al.*, 1987; Grogan *et al.*, 1987). Zta is a sequence-specific DNA binding protein, related to the bZip family of transcription factors, which transactivates several early lytic cycle viral promoters via *cis*-acting AP-1 or ZRE (Zta-responsive elements) sites (Farrell *et al.*, 1989; Kenney *et al.*, 1989; Lieberman *et al.*, 1989; Urier *et al.*, 1989; Flemington and Speck, 1990a). Zta contains a carboxy-terminal domain that mediates homodimerization through a coiled-coil interaction (Chang *et al.*, 1990; Flemington and Speck, 1990c; Kouzarides *et al.*, 1991), a basic region sharing sequence homology with the DNA binding domain of members of the AP1 family of transcription factors (Farrell *et al.*, 1989) and a long amino-terminal domain that plays a role in activation of transcription (Lieberman and Berk, 1990; Giot *et al.*, 1991; Flemington *et al.*, 1992; Chi and Carey, 1993).

In addition to its role in promoting viral transcription (Grogan *et al.*, 1987) and replication (Schepers *et al.*, 1993), several studies have suggested that Zta might also be involved in altering host cell gene transcription, at least in part, through binding to cellular AP-1 promoter elements (Farrell *et al.*, 1989; Flemington and Speck, 1990b; Cayrol and Flemington, 1995). Other groups have reported that Zta can interact physically with cellular transcription factors involved in cell proliferation including the p65 subunit of NF-κB (Gutsch *et al.*, 1994), the retinoic acid receptor (RAR) (Sista *et al.*, 1993) and the tumor suppressor protein, p53 (Zhang *et al.*, 1994). These studies strongly suggest that Zta plays an active role in altering cellular gene expression pathways.

Here, we demonstrate that Zta can induce a G_0/G_1 cell cycle block in certain epithelial tumor cell lines. Employing a tightly controlled expression system regulated by tetracycline (Gossen and Bujard, 1992; Cayrol and Flemington, 1995), we demonstrate that growth suppression does not require strong overexpression of Zta. Zta-mediated G_0/G_1 growth arrest correlates with the post-transcriptional induction of p53, and is observed most readily in tumor cell lines that express wild-type p53 and where the defect in the p53 pathway occurs as a result of HPV E6-mediated destabilization of p53 (Scheffner *et al.*, 1990). We also observe a concordant induction of the p53-responsive, 'universal' cyclin-dependent kinase (CDK) inhibitor p21/WAF-1/CIP-1 (for a review, see Sherr and Roberts, 1995), as well as a post-transcriptional induction of the CDK inhibitor p27/KIP-1 (Sherr and Roberts, 1995). In line with the observed elevation of these CDK inhibitors is the accumulation of the hypophosphorylated form of the retinoblastoma tumor suppressor protein, pRb, which is a known target of CDKs. Moreover, the accumulation of hypophosphorylated pRb is likely to play an active role in growth suppression since overexpression of wild-type HPV E7 oncoprotein, but not a mutant E7 which is defective for binding to pRb, can override Zta-mediated growth arrest. Together, these results demonstrate that Zta can have profound effects on cell cycle progression, and further implicate Zta in altering host cell regulatory processes.

Results

Zta inhibits cell proliferation

A conditional expression system was used to generate a HeLa epithelial cell line, HeLa-Zta, in which Zta expression is controlled by a tetracycline-regulated promoter (Gossen and Bujard, 1992; Cayrol and Flemington, 1995). Western blot analysis revealed that expression of Zta is undetectable in HeLa-Zta cells incubated in the presence of tetracycline, but is detectable 48, 72 and 96 h after withdrawal of tetracycline from the growth media (Figure 1A). In parallel with this experiment, HeLa-Zta cells were counted 24, 48, 72 and 96 h after culturing in the presence or absence of tetracycline (Figure 1B). HeLa-Zta cells cease to accumulate between 48 and 96 h following tetracycline withdrawal. In contrast, HeLa-Zta cells grown in the presence of tetracycline continue to proliferate normally. The parental cells, HeLa-HtTA, served as a control to demonstrate that the growth arrest observed in HeLa-Zta cells was due to the induction of Zta expression and not to non-specific effects of tetracycline on cell growth (Figure 1B). The timing of cell growth inhibition immediately follows the onset of Zta expression (Figure 1A and B), further suggesting that growth arrest is a specific result of Zta expression. The loss of cell proliferation is not due to non-specific toxic effects of Zta or apoptosis since the presence of floaters or non-trypan blue-excluding cells in Zta-expressing cultures was not observed, and fluorescence activated cell sorting (FACS) analysis of propidium iodide-stained HeLa-Zta cells induced for 4 days did not reveal evidence of DNA fragmentation (data not shown).

S-phase progression in Zta-expressing cells was assessed by staining cells for bromodeoxyuridine (BrdU) incorporation. HeLa-Zta cells were cultured for 3 days in the presence or absence of tetracycline, incubated for 90 min with BrdU and stained with an anti-BrdU antibody. As shown in Figure 1C, BrdU incorporation is clearly observed in HeLa-Zta cells cultured in the presence of tetracycline. In contrast, most HeLa-Zta cells expressing Zta do not show BrdU incorporation (Figure 1C). As shown in Table I, the percentage of cells in S phase falls from 24 to 3% in cells expressing Zta. Therefore, Zta expression is linked to a loss of S-phase progression.

Zta blocks cell cycle progression in G_0/G_1

To address at what point in the cell cycle Zta-induced growth arrest occurs, Zta-expressing cells were monitored for DNA content employing propidium iodide staining. HeLa-HtTA or HeLa-Zta cells cultured in the presence or absence of tetracycline for 24, 48, 72 and 96 h were stained with propidium iodide and separated based on

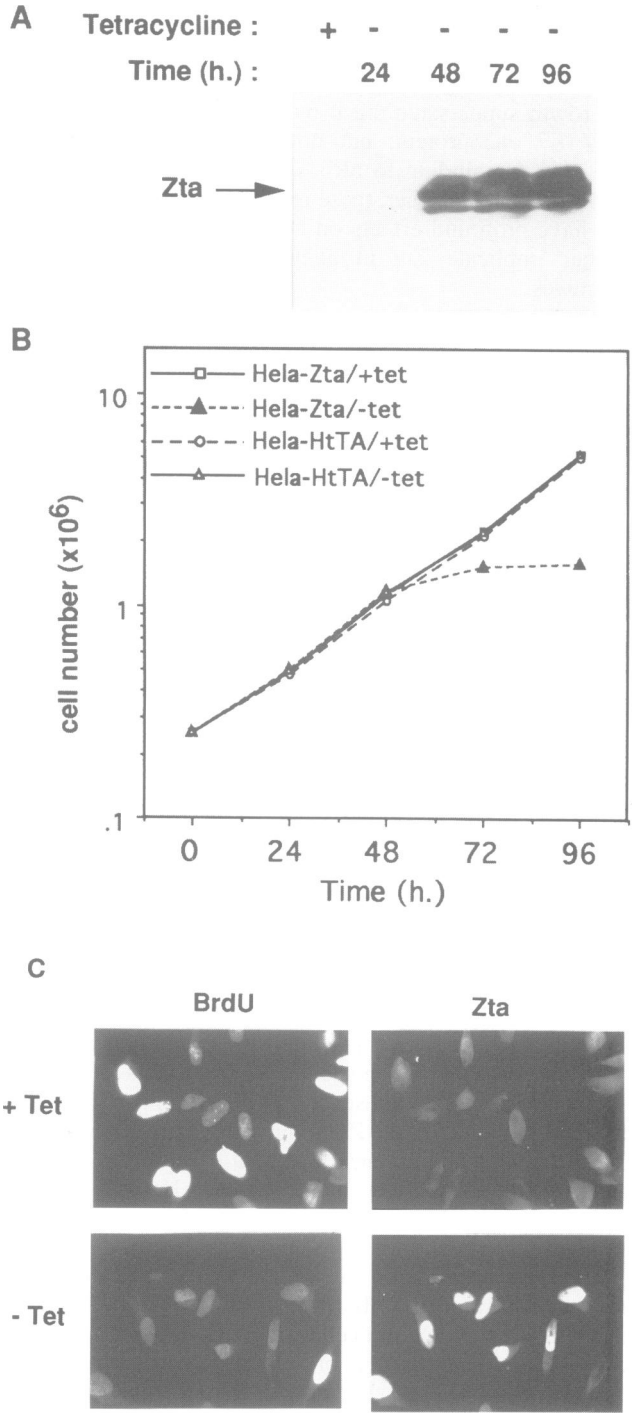


Fig. 1. Zta inhibits cell proliferation. (**A**) Expression of Zta is regulated by tetracycline in HeLa-Zta transfectants. HeLa-Zta cells were grown in the presence (+) or absence (–) of tetracycline for the indicated times. Expression of HA-tagged Zta protein was determined by Western blot analysis of whole cell lysates, using the anti-HA monoclonal antibody, 12CA5F. (**B**) Zta inhibits the growth of HeLa cells. HeLa-Zta transfectants and parental non-transfected HeLa-HtTA cells were plated at a density of 1.5×10^5 per 60 mm diameter plate on day 0 and maintained in the presence (+) or absence (–) of tetracycline. At the indicated time, cells were detached and counted with a hemocytometer. (**C**) Inhibition of DNA synthesis by Zta. HeLa-Zta cells were cultured in the presence (top panels) or in the absence (bottom panels) of tetracycline for 3 days. DNA synthesis was monitored by incubating cells with BrdU followed by immunostaining as described in Materials and methods. Cells were visualized for BrdU incorporation (left panels) and Zta expression (right panels).

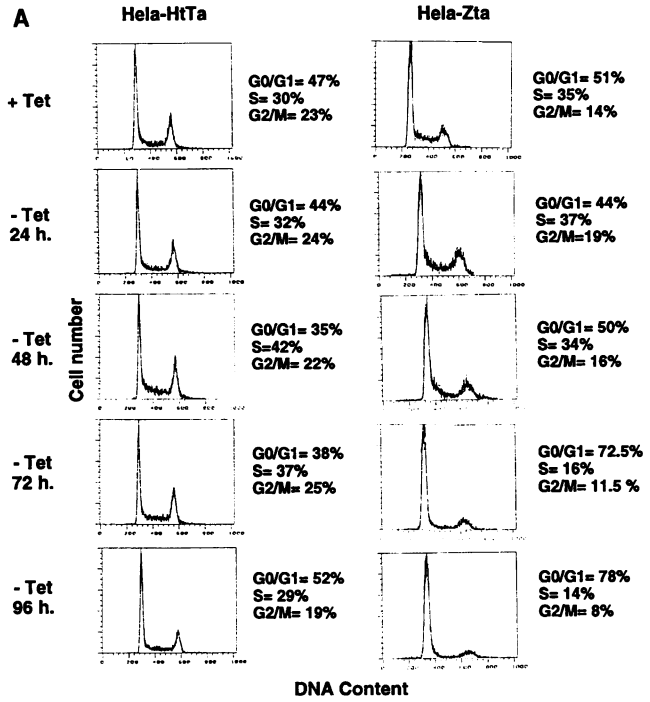
Table I. Inhibition of DNA synthesis in cells expressing Zta

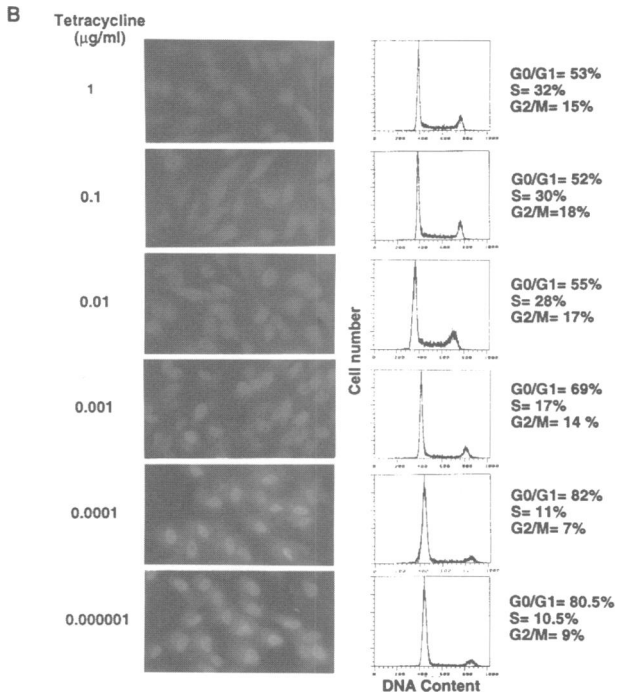
HeLa-Zta	No. of cells	$BrdU^+$	S phase (%)
+Tet	919	229	24
–Tet	831	25	3

HeLa-Zta cells cultured in the presence (+Tet; not expressing Zta) or absence of tetracycline for 3 days (–Tet; expressing Zta) were pulse-labeled with BrdU for 90 min. FITC-conjugated anti-BrdU antibody was used to identify cells in the S phase of the cell cycle. The percentage of cells in S phase was determined by dividing the number of $BrdU^+$ cells by the total number of cells.

DNA content by FACS (Figure 2A). At 72 and 96 h following tetracycline withdrawal, there is a decrease of S and G_2/M phase cells and an accumulation of G_0/G_1 phase cells in HeLa-Zta but not HeLa-HtTA cells (Figure 2A). The cell cycle profile shows that ~ 80% of HeLa-Zta cells are in G_0/G_1 after 96 h of Zta induction (Figure 2A, bottom right). Notably, the onset of the observed cell cycle redistribution at day 3 (72 h) is 1 day after Zta expression is first observed (Figure 1A) and is therefore consistent with growth arrest being mediated by Zta. In contrast, the cell cycle profile of HeLa-HtTA cells cultured in the absence of tetracycline for an identical period shows that ~50% of the cells are in either S or G_2/M phase, indicating continued cell growth (Figure 2A, bottom left).

We also tested whether low level Zta expression was sufficient to mediate a G_0/G_1 growth arrest. It was reported previously that intermediate expression levels could be obtained with the tetracycline-controlled expression system by culturing cells with low amounts of tetracycline (Gossen and Bujard, 1992). Therefore, a dose–effect experiment was performed to link Zta expression levels with G_0/G_1 growth arrest. HeLa-Zta cells were incubated for 3 days in media containing different concentrations of tetracycline, and Zta expression was monitored by immunofluorescence staining. In addition, a fraction of the cells were assayed for DNA content employing FACS separation of propidium iodide-stained cells. As expected, bright Zta staining was observed in cells cultured with 0.000001 or 0.0001 μg/ml of tetracycline and a corresponding G_0/G_1 growth arrest was observed (Figure 2B). Cells cultured in the presence of 0.001 μg/ml of tetracycline exhibited predominantly low level Zta expression but growth arrest was still observed. At higher concentrations of tetracycline, no Zta could be detected and, correspondingly, no cellular growth arrest was observed. This experiment strengthens the intimate correlation between Zta expression and cell growth arrest and demonstrates that growth arrest is observed even at apparently low concentrations of Zta. Notably, the level of Zta expression observed in HeLa-Zta cells cultured in the presence of 0.001 or 0 μg/ml of tetracycline is similar to the level of Zta expression observed in two EBV-positive Burkitt's lymphoma cell lines, Akata and P3HR1, which were treated with agents that activate the lytic cascade (Figure 2C). Therefore, the observed Zta-mediated growth arrest is not due to non-specific effects resulting from high level Zta expression.





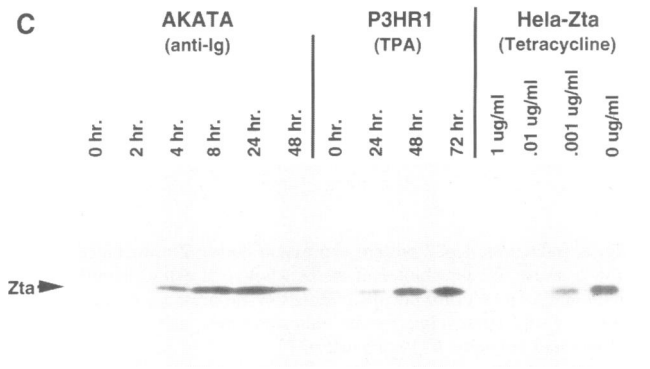


Fig. 2. Zta blocks cell cycle progression in G_0/G_1. (**A**) Zta induces cell cycle arrest in G_0/G_1. HeLa-HtTA and HeLa-Zta cells, cultured in the presence (+) or absence (–) of tetracycline for the indicated times, were fixed, stained with propidium iodide and their DNA content was assessed by flow cytometry as described in Materials and methods. The percentages of cells in the different G_0/G_1, S and G_2/M phases are indicated on the right of each cell cycle profile. (**B**) G_0/G_1 growth arrest is elicited by relatively low levels of Zta expression. Immunofluorescence analysis of Zta expression in HeLa-Zta cells cultured for 3 days with different concentrations of tetracycline (left panels). Cell cycle distribution was assessed by FACS analysis, after propidium iodide staining and the percentage of cells in G_0/G_1, S and G_2/M for each profile is shown (right panel). (**C**) Comparison of Zta expression in induced HeLa-Zta cells with Zta expression in anti-Ig- or TPA-treated Akata or P3HR1 cells, respectively. Akata and P3HR1 cells cultured in the presence of anti-Ig or TPA for the indicated times, a fraction of HeLa-Zta cells from the experiment shown in (B) (1 μg/ml, 0.01 μg/ml, 0.001 μg/ml tetracycline) and HeLa-Zta cells cultured without tetracycline for 3 days were assayed by Western blot analysis employing the anti-Zta antibody, M47 (Flemington *et al.*, 1992). Equal amounts of total cell protein were loaded in each lane.

Induction of the cyclin-dependent kinase inhibitors p21, p27 and the tumor suppressor, p53, during Zta-mediated growth arrest

To investigate the mechanism of Zta-mediated growth arrest, we analyzed the expression of p21, p27 and p16, three low molecular weight CDK inhibitors that play essential roles in arresting cell cycle progression (Sherr and Roberts, 1995), in HeLa-Zta cells following Zta induction. Northern blot analysis revealed a significant increase in the steady-state levels of p21 mRNA after induction of Zta expression (Figure 3A). In contrast, p16 and p27 mRNA levels were not affected significantly. To determine whether induction of p21 also occurs at the protein level, Western blot analysis was performed. Expression of Zta was found to result in a strong increase in p21 protein levels (Figure 3B). Since p21 has been isolated as a major target of p53 (El-Diery *et al.*, 1993) and is up-regulated by p53 in response to DNA-damaging agents (Dulic *et al.*, 1994; El-Diery *et al.*, 1994), we addressed whether p21 induction might be related to a p53-dependent response in HeLa-Zta cells undergoing G_1 arrest. As shown in Figure 3B, a marked increase in p53 protein levels is observed following Zta induction. The increase in p53 levels occurs through a post-transcriptional mechanism, since no change in p53 mRNA levels is detectable by Northern blot analysis (Figure 3C).

Further support for a p53-mediated response in these cells is provided by the observed induction of another known p53 target gene, *mdm2* (Wu *et al.*, 1993) (Figure 3C). These data suggest that the increase in p21 expression could be the result of increased amounts of functional p53. To address this issue specifically, we tested whether overexpression of SV40 large T antigen or HPV E6 oncoproteins, which are known to inactivate p53 function (Lane and Crawford, 1979; Linzer and Levine, 1979; Werness *et al.*, 1990), can override the induction of p21 in HeLa cells expressing Zta. HeLa-HtTA cells were co-transfected with a Zta expression vector and either a control plasmid or expression vectors containing the HPV E6 or SV40 T antigen genes. While Zta expression alone

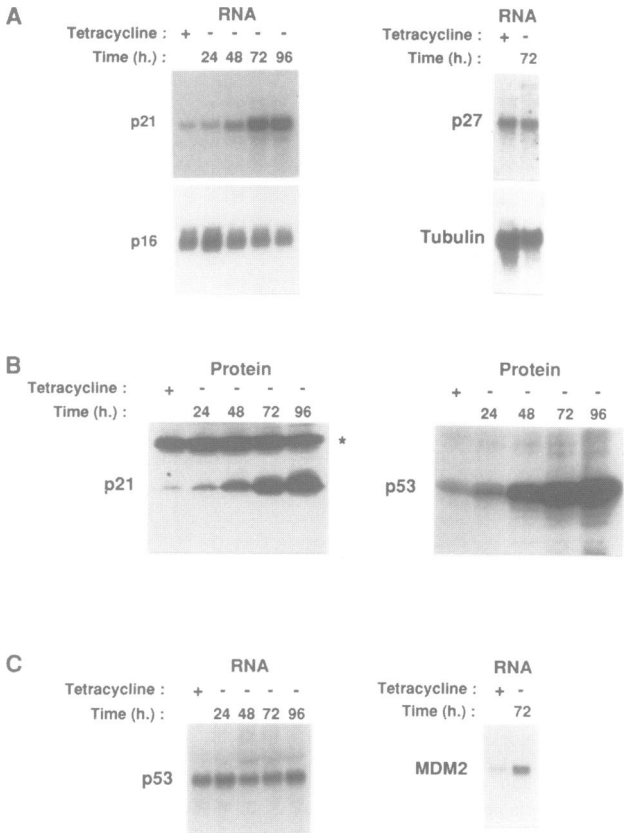


Fig. 3. Induction of p21 during Zta-mediated G_1 growth arrest. (**A**) Northern blot analysis of p21, p16 and p27 mRNA expression in HeLa-Zta transfectants. Total RNAs were isolated from HeLa-Zta cells cultured in the presence (+) or absence (–) of tetracycline, for the indicated period of time. After electrophoresis and transfer, RNAs were hybridized with a ^{32}P-labeled probe for p21. The same blot was stripped and re-probed with a ^{32}P-labeled probe derived from p16 cDNA (left panels). Polyadenylated RNAs (3 μg) from HeLa-Zta cells grown with (+) or without (–) tetracycline for 3 days were hybridized sequentially with radiolabeled p27 and tubulin probes (right panel). (**B**) Western blot analysis of p21 and p53 protein expression in HeLa-Zta cells maintained in complete medium with tetracycline (+) or after withdrawal of tetracycline (–) for the indicated times, using the monoclonal antibodies, 187 (p21) or DO-1 (p53). The asterisk marks the position of a protein recognized non-specifically by the antibody. (**C**) Northern blot analysis of p53 mRNA levels in HeLa-Zta transfectants. The blot used for p21 and p16 Northern blots (A, left panel) was stripped and re-probed with ^{32}P-labeled p53 cDNA. The blot used for p27 and tubulin Northern blots was stripped and re-probed with ^{32}P-labeled *mdm2* cDNA to analyze *mdm2* mRNA expression (A, right panel).

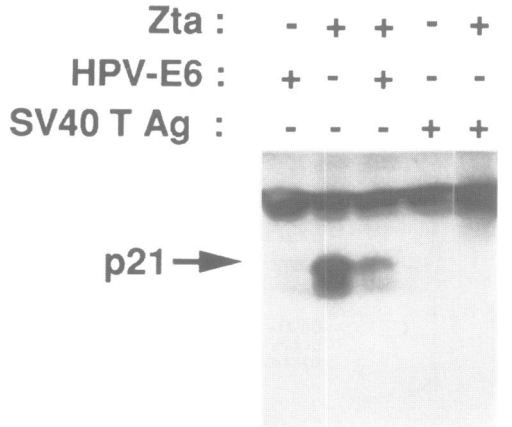


Fig. 4. Expression of HPV E6 or SV40 large T antigen overcomes p21 induction in cells expressing Zta. HeLa cells were transiently co-transfected with 5 μg of Zta expression plasmid (+) or control vector (–) together with 25 μg of HPV E6 (+) or 2 μg of SV40 T antigen (+) expression plasmid or control vectors (–). Whole cell lysates were separated by SDS–PAGE and probed with the antibody 187 against p21.

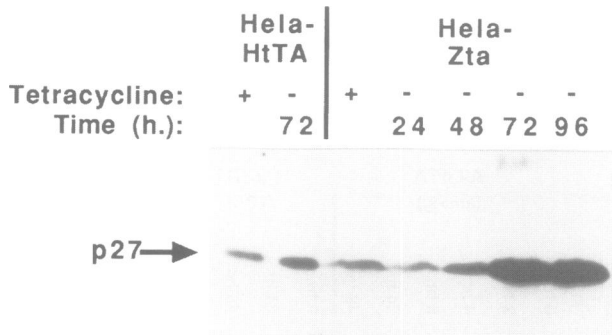


Fig. 5. Induction of p27 protein expression during Zta-mediated G_1 growth arrest. Western blot analysis of whole cell extracts from HeLa-HtTA and HeLa-Zta cells cultured in the presence (+) or absence (–) of tetracycline for the indicated times, using the monoclonal antibody, C-19, against p27.

leads to elevated levels of p21 expression, co-transfection with HPV E6 or SV40 T antigen expression results in marked decreases in Zta-induced p21 expression (Figure 4). These results suggest that p53 plays a direct role in the induction of p21 expression during Zta-mediated G_0/G_1 arrest.

Although p27 mRNA levels were not affected by Zta expression (Figure 3A), p27 is known to be regulated at the post-transcriptional level in other systems (Polyak *et al.*, 1994a; Pagano *et al.*, 1995). We therefore analyzed p27 expression by Western blot analysis. A significant increase in the amount of p27 protein is observed in HeLa-Zta cells 72 and 96 h after tetracycline withdrawal (Figure 5). In contrast, p27 levels were not affected in the parental HeLa-HtTA cell line, indicating that the increase in p27 is due to the induction of Zta expression and not to non-specific effects of tetracycline. Therefore, expression of Zta results in the induction of p27 by a post-transcriptional mechanism.

Zta-mediated G_1 growth arrest in different nasopharyngeal cell lines

EBV lytic replication *in vivo* is known to occur in epithelial cells of the nasopharynx. We therefore tested several nasopharyngeal cell lines for their susceptibility to Zta-mediated growth arrest. For these studies, a transient transfection assay was employed. The Zta gene was cloned into an expression vector (pMARK) designed to co-express the gene of interest and a signal transduction-defective CD7 cell surface marker (Frangioni *et al.*, 1994). Cells were transfected with pMARK-Zta or the progenitor plasmid, pMARK, and the cell cycle distribution of CD7-positive and CD7-negative populations was analyzed. Consistent with the results obtained employing the stable HeLa-Zta transfectants (see above), a G_0/G_1 cell cycle arrest is observed in the CD7+ population from pMARK-Zta transfected HeLa cells [(CD7-Zta)$^+$] but not in the

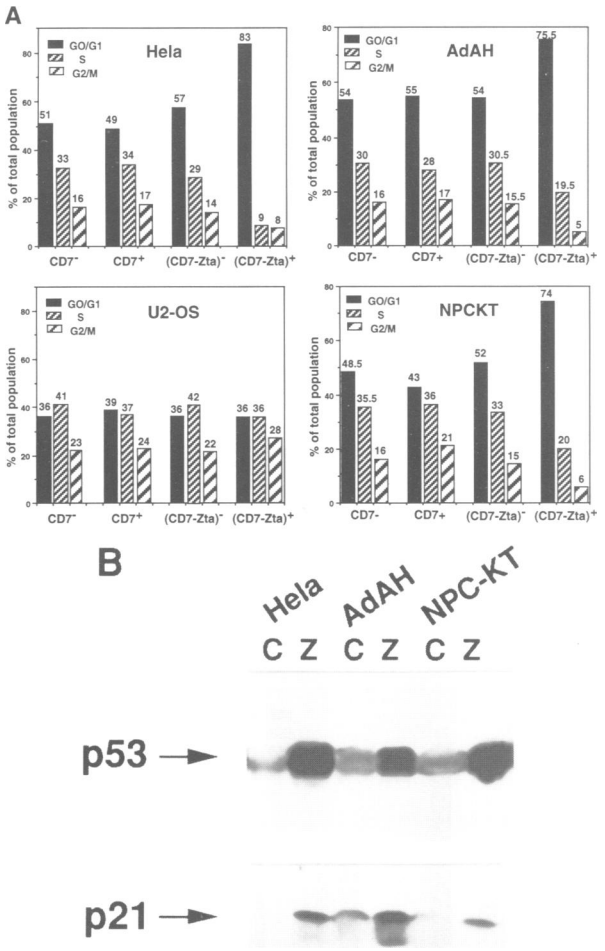


Fig. 6. Zta induces G_1 growth arrest in different nasopharyngeal cell lines. (**A**) Effect of Zta on cell cycle distribution in HeLa cells, AdAH (adenoid epithelial cell line), NPC-KT (EBV+ epithelial cell line) and U2-OS (osteosarcoma cell line). All cell lines were transfected with pMARK (control vector) encoding only cell surface CD7 marker or pMARK-Zta encoding both CD7 marker and Zta, and were stained with propidium iodide. DNA content was determined by cytofluorometry on both transfected cells [CD7+; $(CD7\text{-}Zta)^+$] and non-transfected cells [CD7–; $(CD7\text{-}Zta)^-$]. The histograms represent the percentage of cells found in each phase (G_0/G_1, S and G_2/M) of the cell cycle. For each cell type, analysis was performed at least three times with similar results. (**B**) Induction of p53 and p21 by Zta in transiently transfected HeLa, AdAH and EBV+ NPC-KT cell lines. Cells were transfected with 5 μg of control vector (C) or Zta expression vector (Z). Total cell lysates were subjected to Western blot analysis, using the antibodies DO1 against p53 and 187 against p21.

CD7– population [$(CD7\text{-}Zta)^-$] (Figure 6A). In addition, no cell cycle arrest is observed in either the CD7+ or CD7– population of pMARK-transfected HeLa cells. In the EBV-negative nasopharyngeal cell line, AdAH, and the EBV-positive AdAH derivative, NPC-KT, Zta was found to cause a G_0/G_1 cell cycle arrest. In both cell lines, an increase in the G_0/G_1 population and a decrease in the S and G_2/M populations are observed in the CD7+ population from pMARK-Zta-transfected cells [$(CD7\text{-}Zta)^+$] but not in the CD7– population (Figure 6A). In contrast, in the osteosarcoma cell line, U2-OS, no G_0/G_1 cell cycle arrest is observed in the CD7+ population of pMARK-Zta-transfected cells, although in some experiments an increase in the G_2/M population is observed (Figure 6A).

Table II. BrdU incorporation in different cell lines expressing Zta

Cell lines	Zta	No. of cells	$BrdU^+$	S-phase (%)
NPCKT	–	644	172	27
	+	216	7	3
AdAH	–	535	109	20
	+	213	3	1
Fadu	–	1382	711	51
	+	218	22	10
D98	–	953	345	36
	+	110	16	14
C33A	–	875	343	39
	+	76	33	43
HaCAT	–	733	401	55
	+	46	24	52

Cells were transiently transfected with Zta expression vector, pulse-labeled with BrdU for 90 min and stained with FITC-conjugated anti-BrdU and Zta antibodies as described in Materials and methods. The percentage of transfected (Zta^+) or untransfected (Zta^-) cells in the S phase of the cell cycle was determined by dividing the number of $BrdU^+$ cells by the total number of cells.

The ability of Zta to induce growth arrest in various cell lines was also assayed by transfecting cells with a Zta expression vector and co-staining for BrdU incorporation and Zta expression by indirect immunofluorescence. The number of BrdU-positive cells in the Zta^+ and Zta^- populations were counted (Table II). As expected, a dramatic reduction of BrdU-positive (S phase) cells was observed in Zta-expressing NPC-KT and AdAH cells (3 and 1%, respectively) compared with the corresponding Zta-negative cells (27 and 20% respectively). In addition, a reduction in the number of S-phase cells is also apparent in the Zta-positive population of the EBV-negative nasopharyngeal epithelial cell line, FaDu, as well as the HeLa subclone, D98. On the other hand, no apparent growth arrest is observed with the cervical carcinoma cell line, C33A, or the keratinocyte cell line, HaCAT, which both contain mutated p53 genes (Scheffner *et al.*, 1991).

The inability of Zta to arrest cell growth in C33A and HaCAT cells further suggests the involvement of p53 in eliciting Zta-induced growth arrest. Notably, a G_0/G_1 growth arrest was not observed in U2-OS cells which express wild-type p53. However, the p53 pathway in these cells is known to be disrupted downstream from p53 by high level expression of mdm2 (Florenes *et al.*, 1994) which inhibits p53-mediated transcriptional activation (Wu *et al.*, 1993).

Western blot analysis of p53 in NPC-KT and AdAH cells did not reveal abnormal migration suggestive of mutant p53 expression; however, expression was found to be low level (Figure 6B and data not shown), providing evidence that p53 might be subjected to destabilization. Moreover, these cell lines were found to express HPV E6 as shown by Western blot analysis [data not shown, several recent studies have reported the identification of HPV in oral epithelial tissues (Giannoudis *et al.*, 1995; Lin *et al.*, 1995; MacDonald *et al.*, 1995)]. In line with this observation, we found that transient expression of Zta in AdAH and NPC-KT cell lines resulted in elevated levels of both p53 and p21 proteins (Figure 6B). These data suggest that p53 is a key component of the Zta-mediated growth arrest

and that this might occur through stabilization of p53. Importantly, this is not due to an effect on HPV E6 expression, since steady-state levels of E6 are not affected by Zta expression in induced HeLa-Zta cells (data not shown).

Interestingly, an apparent growth arrest is observed in FaDu cells which do not express HPV E6 (data not shown), but instead express a mutant p53 allele (Reiss *et al.*, 1992; Kim *et al.*, 1993). Therefore, Zta may be able to elicit growth arrest through certain p53 mutants. Alternatively, Zta might affect cell cycle control pathways through induction of p27 levels or through other as yet unknown mechanisms.

pRb or pRb-related proteins are key mediators of the growth arrest induced by Zta

p21 and p27 have been shown to cause G_0/G_1 arrest by inhibiting G_1 cyclin–CDK kinase activities (Harper *et al.*, 1993; Xiong *et al.*, 1993; Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994), thereby suppressing pRb phosphorylation and causing accumulation of pRb in its hypophosphorylated form. In order to determine whether Zta-induced cell cycle arrest is mediated through pRb, Western blot analysis was performed using a monoclonal antibody that recognizes both the hypophosphorylated (pRb) and hyperphosphorylated (ppRb) forms of pRb. At 72 or 96 h after induction of Zta expression, HeLa-Zta cells were found to accumulate pRb in its hypophosphorylated form (Figure 7A). In contrast, no change in pRb phosphorylation was observed in the parental HeLa-HtTA cell line after culturing in the presence or absence of tetracycline for 72 h. This indicates that the accumulation of hypophosphorylated pRb is induced specifically by Zta and is not due to effects of tetracycline. Therefore, the mechanism of G_0/G_1 growth arrest induced by Zta is consistent with a model in which both pRb and the CDK inhibitors p21 and p27 play essential roles.

To determine whether pRb or pRb-related proteins are absolutely required for Zta-mediated G_0/G_1 arrest, we tested whether Zta-mediated growth arrest could be inhibited in HeLa cells by high level expression of wild-type HPV E7 oncoprotein, which binds and functionally inactivates pRb as well as the pRb-related proteins, p107 and p130 (Dyson *et al.*, 1989; Munger *et al.*, 1989; Davies *et al.*, 1993). Importantly, significant amounts of pRb are found complexed to the transcription factor E2F in uninduced HeLa cells, as determined by electrophoretic mobility shift analysis, although the levels are somewhat lower than the levels observed in non-transformed cells (data not shown). This suggests that HPV E7 is limiting in these cells and that a substantial fraction of pRb is subjected to regulation by normal cell cycle control mechanisms. Therefore, if the increase in hypophosphorylated pRb observed in Zta-expressing HeLa cells plays a role in mediating Zta-induced growth arrest, high level expression of E7 should be able to overcome this cell cycle block. For these studies, pMARK and pMARK-Zta expression vectors were used in transient transfection assays. The G_0/G_1 growth arrest observed in the CD7+ population from pMARK-Zta-transfected HeLa cells is abrogated when the pMARK-Zta plasmid is co-transfected with an expression vector containing a wild-type HPV E7 gene (Figure 7B), showing that overexpression of E7 can

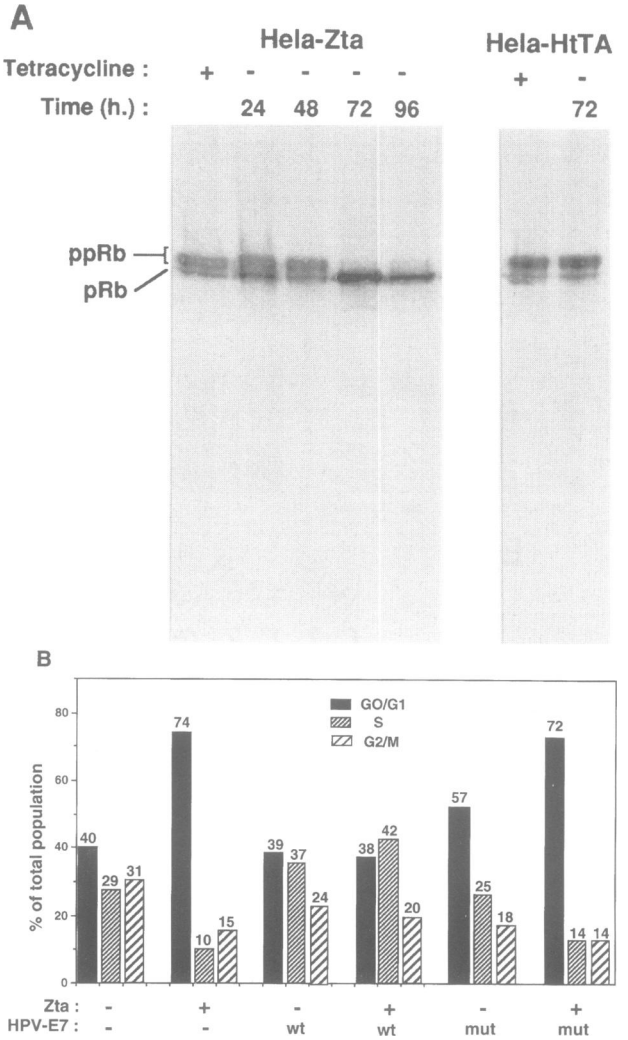


Fig. 7. Involvement of pRb or pRb-related proteins in Zta-mediated G_1 growth arrest. (**A**) The phosphorylation status of pRb is altered in cells expressing Zta. Similar amounts of cellular extracts from HeLa-HtTA and HeLa-Zta cells cultured in the presence (+) or absence (–) of tetracycline for the indicated times, were subjected to Western blot analysis, using the anti-pRb G3-245 antibody. Slower migrating bands are hyperphosphorylated forms of pRb and are indicated as ppRb; the faster migrating band is the hypophosphorylated form and is indicated as pRb. (**B**) Reversion of Zta-induced-growth arrest by HPV E7. HeLa cells were transiently co-transfected with 5 μg of control pMARK (–) encoding only cell surface marker CD7 or pMARK-Zta (+) vector encoding both CD7 marker and Zta, together with 25 μg of control (–), wild-type (wt) or mutant (mut) E7 expression plasmid. The proportion of CD7+ cells in the G_1, S or G_2/M stages of the cell cycle was assessed by propidium iodide staining and FACS analysis. The histograms represent the percentage of cells found in each phase of the cell cycle.

overcome the growth-inhibitory function of Zta. This effect is not observed with a mutant of E7 that is defective for binding of pRb and pRb-related proteins (Figure 7B). These observations indicate that pRb or pRb-related proteins are key downstream mediators in the pathway leading to a Zta-mediated G_0/G_1 growth arrest.

Discussion

The results presented here demonstrate that the bZIP transcription factor, Zta, can induce a G_0/G_1 growth arrest in several human epithelial cell lines. The observed growth

inhibition is elicited by relatively low levels of Zta expression and is not due to non-specific toxic effects of Zta, since growth-arrested cells remain viable, are blocked at a specific point in the cell cycle (G_0/G_1) and do not show any signs of apoptosis. Zta-mediated growth inhibition occurs in the EBV-positive nasopharyngeal carcinoma-derived cell line NPC-KT, but is independent of the expression of other EBV-encoded genes since it is also observed in several EBV-negative epithelial cell lines (HeLa, D98, AdAH and FaDu).

Zta-mediated growth arrest is likely to be elicited through induction of the potent, universal CDK inhibitors, p21 and p27, which have been shown to cause cell cycle arrest in different mammalian cell lines (reviewed by Sherr and Roberts, 1995). Evidence that this is true is suggested by the observed loss of phosphorylated forms of the CDK target protein, pRb, in cells expressing Zta. Moreover, accumulation of hypophosphorylated pRb follows induction of p21 and coincides with the induction of p27, suggesting that the loss of pRb phosphorylation is a downstream event. The finding that overexpression of the HPV E7 oncoprotein, which binds and inactivates pRb, can abrogate Zta-mediated G_0/G_1 growth arrest indicates that pRb or other pRb-related proteins that bind HPV E7 are key participants in the Zta-induced G_1 arrest.

The p21 gene has been identified as a major transcriptional target of the p53 tumor suppressor gene (El-Diery *et al.*, 1993). Accordingly, DNA-damaging agents that stabilize and thereby increase p53 levels induce p21 synthesis (Dulic *et al.*, 1994; El-Diery *et al.*, 1994). It has also been shown, however, that activation of p21 expression by some effectors, such as transforming growth factor (TGF)-β and MyoD, can occur through p53-independent pathways (Datto *et al.*, 1995; Havely *et al.*, 1995). Several lines of evidence suggest that the induction of p21 in Zta-expressing cells is elicited by p53. First, p53 levels are increased markedly by Zta expression. Second, expression of another p53-responsive gene, *mdm2*, is also activated in Zta-expressing cells. Third, Zta-mediated induction of p21 can be inhibited by the overexpression of two different oncoproteins, HPV E6 and SV40 large T antigen, which are known to be negative regulators of p53 (Sarnow *et al.*, 1982; Scheffner *et al.*, 1990). Together, these results argue strongly that p53 is a key mediator of p21 induction in this system. This observation is in apparent contradiction to a previous report showing that Zta inhibits p53-mediated transcriptional activation of reporter plasmids containing p53 response elements through a physical interaction between these two factors (Zhang *et al.*, 1994). We have performed similar experiments with reporter plasmids containing synthetic p53 response elements or the natural p21 promoter and have been unable to demonstrate an increase in promoter activity in Zta-expressing HeLa cells (data not shown). These results may suggest that chromatin assembly influences the ability of p53 to activate transcription in the presence of Zta.

While it is clear that certain viral proteins, such as SV40 large T antigen and the adenovirus E1B proteins, are able to stabilize the short-lived p53 protein (Sarnow *et al.*, 1982), these interactions result in functional inactivation of p53 and consequently allow cells to escape from p53-mediated cell cycle arrest signals. Zta is thus the first viral protein shown to up-regulate levels of functional p53 and to induce p21 expression by a p53-dependent mechanism.

The increase in p53 levels in Zta-expressing cells clearly occurs through a post-transcriptional mechanism, since no changes in mRNA levels are detectable. p53 is a short-lived protein that is highly sensitive to proteolytic degradation by the ubiquitin-dependent pathway (Oren *et al.*, 1981; Reihsaus *et al.*, 1990; Scheffner *et al.*, 1990), and mutant cell lines deficient in this proteolytic system have been shown to accumulate p53 (Chowdary *et al.*, 1994). Therefore, Zta may, directly or indirectly, protect p53 from ubiquitin-mediated protein degradation. Zhang *et al.* (1994) similarly observed an increase in p53 levels in Zta-expressing D98 cells and suggested that binding of Zta to p53 could result in the direct masking of p53 sequences that are required for targeted degradation. Alternatively, Zta may protect p53 from ubiquitin-dependent degradation by an indirect mechanism. For example, Zta could inhibit the expression of genes involved in the ubiquitination pathway, such as ubiquitin-activating enzymes, ubiquitin-conjugating enzymes and ubiquitin-protein ligases, or Zta could activate the expression of de-ubiquitinating enzymes. In support of such possibilities is the observation that, like p53, induction of p27 by Zta occurs through a post-transcriptional mechanism. Moreover, p27 has been shown recently to be regulated by the ubiquitin-dependent proteolytic pathway (Pagano *et al.*, 1995) and the level of p27 induction observed in cells expressing Zta is similar to the level of p27 observed in cell lines that are mutant in the ubiquitin pathway or treated with inhibitors of the ubiquitin–proteasome pathway (Pagano *et al.*, 1995). Assessing whether these or other possibilities are relevant to the observed increase in p53 and p27 levels in Zta-expressing cells is the subject of an ongoing investigation.

Here we have established a strong correlation between Zta-mediated growth arrest and tumor cell lines expressing wild-type p53 and the HPV-encoded E6 oncoprotein. A high percentage of tumors arise, in part, due to mutations in p53 (Zambetti and Levine, 1993). In HPV-negative tumor cell lines that express a wild-type p53 protein, there is typically some other abnormality in the p53 pathway such as overexpression of the p53 inhibitor mdm2, as has been observed in U2-OS cells (Florenes *et al.*, 1994). Therefore, HPV-associated tumors appear to be unique in that both p53 alleles are wild-type and pathways downstream of p53 are left intact. Although little is known regarding specific mechanisms involved in regulating p53 *in vivo*, it is clear that, as in HPV-derived tumors, p53 is regulated by protein degradation and is subjected to rapid turnover in primary tissues (Oren *et al.*, 1981; Reihsaus *et al.*, 1990). The studies described here suggest, therefore, that Zta might play a role in altering cell cycle progression *in vivo* at least in part through elevating levels of functional p53.

While Zta can inhibit cell proliferation and block cell cycle progression in G_0/G_1, the EBV latent antigens, EBNA2 and EBNA-LP, facilitate entry into G_1/S during immortalization of B-lymphocytes by EBV (Sinclair *et al.*, 1994; Kempkes *et al.*, 1995). Therefore, Zta and EBV latent antigens may have opposite effects on cell cycle progression. Although latency-associated viral factors are

clearly associated with activation of cellular proliferation pathways, unlike small DNA tumor viruses, activation of cell cycle progression by the EBV latent program is not linked to viral production but is instead involved in expanding the infected B-cell population. On the other hand, the EBV lytic replication cycle is associated with terminal differentiation, and full EBV replication is only observed in the outer growth-arrested/differentiated layers. This may have evolved, in part, to facilitate efficient shedding of virus into the saliva where it can be transmitted readily from host to host. Since the EBV genome is large, it has the capacity to encode enzymes that are involved in generating nucleotide precursors as well as several major components of the replication machinery. EBV therefore does not rely as heavily on cellular replication machinery as do small DNA tumor viruses. As a result, the ability of EBV to replicate in growth-arrested cells might favor a more robust viral replication since resources are not diverted to accommodate replication of cellular DNA. Our results suggest that EBV has evolved mechanisms to alter specifically key cell cycle and/or differentiation control pathways during the lytic cycle in a way that might favor viral replication. Being able to promote growth arrest might allow EBV to escape a dependency on the normal progression of cellular differentiation. This could be particularly important during times of prolific EBV replication, such as during initial infection, in order to rapidly expand the pool of infected cells. This activity might also be relevant to progression of the high level EBV lytic activity which drives the pathology observed in the AIDS-associated epithelial lesion, oral hairy leukoplakia (OHL) (Resnick *et al.*, 1988; Greenspan, 1989).

Our studies have thus far been limited to the effects of Zta expression in cells of epithelial origin. At this time, it remains unknown whether Zta can elicit growth arrest in B-lymphocytes. Can Zta reverse the immortalizing functions of Group III latency-associated EBV gene products? Does Zta elicit growth arrest in Burkitt's lymphoma cell lines containing mutant p53 proteins? While these remain open questions, accumulating data suggest that Zta may elicit multiple independent growth arrest signals. For example, while induction of p21 expression is dependent on p53, p27 is likely to be activated through a p53-independent pathway. Also, we have shown previously that Zta induces the expression of two secreted factors, TGF-β1 and TGF-βigh3, with known growth inhibitory activities (Cayrol and Flemington, 1995). Two experiments suggest that although these factors elicit cell growth inhibition in some milieux, they may not contribute significantly to Zta-mediated growth arrest in HeLa cells. First, in transient transfection experiments with the pMARK-Zta plasmid, no growth arrest is observed in the CD7/Zta$^-$ cell population, indicating that soluble factors alone are not sufficient to elicit growth arrest (see Figure 6A). Second, cell dilution experiments with HeLa-Zta cells indicate that growth arrest occurs equally well at low and high cell densities (data not shown), indicating that soluble factors are not likely to be essential for growth arrest. It is possible, however, that these factors might contribute to growth arrest in other cell systems. For example, many Burkitt's lymphoma cell lines are known to express functional TGF-β1 receptors (Renzo *et al.*, 1994). Finally, other investigators have demonstrated functional interactions between Zta and other growth regulatory factors [RAR (Sista *et al.*, 1993) and NF-κB (Gutsch *et al.*, 1994)]. While some cell lines appear to be defective for Zta-mediated G_0/G_1 growth arrest pathways (U2OS, C33A and HaCAT), it is possible that these or other pathways might elicit Zta-mediated growth arrest in still other cell systems.

In conclusion, our results provide additional support for the idea that Zta is a multifunctional protein that is involved not only in activating viral transcription and replication, but also in altering host cell regulatory pathways. Further investigations into the Zta-mediated cell cycle alterations should not only lead to a better understanding of the virus–host relationship but should also reveal additional insights into cell cycle control mechanisms.

Materials and methods

Cell lines

The stable cell lines HeLa-HtTA and HeLa-Zta (Cayrol and Flemington, 1995) were maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; GIBCO) and tetracycline (1 μg/ml, unless specified; Sigma) in a 5% CO_2 environment. For growth curve assays, HeLa-HtTA and HeLa-Zta cells were seeded at a density of 1.5×10^5 per 60 mm diameter plate in the presence or absence of tetracycline. At the indicated time, cells were trypsinized, stained with trypan blue and counted with a hemocytometer. The percentage of living cells, determined by trypan blue exclusion, was similar for HeLa-Zta or HeLa-HtTA cells cultured in the presence or absence of tetracycline. The experiment was performed twice with identical results. AdAH [adenoid epithelial cells derived from the human nasopharynx (Takimoto *et al.*, 1984)], NPC-KT [generous gift from Takeshi Sairenji; hybrid cell line derived from the fusion of AdAH cells and EBV genome-positive NPC epithelial explant culture cells (Takimoto *et al.*, 1984)], FaDu (head and neck squamous cell carcinoma), D98 (human carcinoma cell line, HeLa variant), C33A (human cervical carcinoma), HaCAT (human keratinocyte cell line) and U2-OS (human osteosarcoma cell line), were grown in DMEM (Cellgro) supplemented with 10% FBS (GIBCO).

Plasmids and constructions

pMARK vector-encoding cell surface marker CD7 was a generous gift from Seth Alper. The Zta gene was subcloned into pMARK downstream from the SV40 promoter, by using *Xba*I and *Sma*I restriction sites. The Zta expression vector used in transient transfection assays for DNA synthesis analysis (BrdU experiment) was derived from the previously described Zta expression vector (Flemington *et al.*, 1994) containing the SV40 early promoter and enhancer upstream from the genomic *BZLF1* gene, with an in-frame hemagglutinin (HA)-tag sequence. The HPV-16 E6 expression plasmid (1221) and the corresponding control vector (1213) were obtained from Peter Howley, Harvard Medical School, Boston. The Rous sarcoma virus (RSV)-E7 plasmids (wild-type and mutant) which carry the HPV E7 gene driven by the RSV long terminal repeat were provided by William Sellers, Dana-Farber Cancer Institute, Boston. For the E7 mutant, the amino acids 21–24 contained within the pRb binding motif were deleted. The pCMV-mdm2 expression plasmid was a generous gift from Arnold Levine, Princeton. The vector carrying large T antigen and the corresponding control vector were provided by James DeCaprio, Dana-Farber Cancer Institute, Boston.

Western blot analysis

Cellular lysates were subjected to SDS–PAGE on 8–15% polyacrylamide gels. Proteins were electrotransferred for 1 h at 0.8 mA/cm^2 (Multiphor II, LKB Pharmacia) onto nitrocellulose membrane (Bioblot-NC; Costar). The blots were blocked for 30 min in Tris-buffered saline (TBS) containing 5% milk powder and 1% FBS, incubated in this blocking buffer with the primary antibody overnight at 4°C and washed four times for 15 min in TBS buffer containing 0.5% (v/v) Tween-20 (TBST). The blots were then incubated with the secondary sheep anti-mouse horseradish peroxidase-conjugated antibody (dilution 1/2000; Amersham) in blocking buffer, for 3 h at room temperature before being washed four times for 15 min in

TBST buffer. Proteins were detected using an enhanced chemiluminescence detection system (ECL, Amersham), according to the manufacturer's recommendations, and filters were exposed to Kodak XR film. For the Zta time course experiment, mouse purified 12CA5F monoclonal antibody (mAb) against the HA epitope (BAbCO) was used at a concentration of 1 mg/ml. The purified mouse mAbs against p21 (187), p27 (C-19) and p53 (DO-1) (0.1 μg/ml), were purchased from Santa-Cruz Biotech. For the pRb Western blot experiment, we used purified mAb G3-245 (0.5 μg/ml) from PharMingen.

Cell cycle analysis by flow cytometry

For cell cycle analysis of HeLa-HtTA and HeLa-Zta cells, 1×10^6 cells were collected, washed in phosphate-buffered-saline (PBS), fixed with 70% cold ethanol for at least 30 min, washed with PBS, treated for 30 min at 37°C with RNase A (0.1 mg/ml) and stained with propidium iodide (69 mM) (Sigma) in 38 mM Na citrate. Cell cycle analysis was carried out with a fluorescence-activated cell sorter (FACScan; Beckton-Dickinson). Analysis were performed three times on 40 000 cells with similar results. The effects of Zta on cell cycle distribution in different cell lines were determined as previously described (Polyak *et al.*, 1994a) with modifications. Briefly, cells were co-transfected with 5 μg of pMARK-Zta plasmid and with 25 μg of pGL2Basic (Promega) carrier plasmid, using a standard calcium phosphate precipitation method (Qin *et al.*, 1992). Control experiments were performed using 5 μg of pMARK plasmid encoding only CD7 marker and 25 μg of pGL2Basic carrier plasmid. Three days after transfection, cells were collected, washed in PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated CD7 mAb (3A1, diluted 1/10; Sigma) for 1 h on ice. Cells were washed prior to propidium iodide DNA staining as described above, and analyzed by FACS for both DNA content and CD7 staining. The same procedure was adopted for cell cycle analysis after co-transfection of pMARK-Zta plasmid and the HPV E7 (control, wild-type and mutant) expression plasmid.

Analysis of DNA synthesis by bromodeoxyuridine staining

Subconfluent cells were co-transfected with 5 μg of an SV40 promoter/enhancer-driven HA-tagged Zta expression plasmid and 25 μg of pGL2Basic carrier plasmid, using a standard calcium phosphate precipitation method (Qin *et al.*, 1992). Three days after the transfection, cells were labeled for 90 min with 10 mM BrdU (Sigma), washed with PBS, fixed for 15 min with 3.7% formaldehyde in PBS, treated with NH_4Cl (50 mM in PBS), permeabilized for 13 min with 0.1% Triton X-100 and stained with anti-HA mAb (12CA5F, 1 μg/ml) for 1 h, followed by incubation for 45 min with Texas red-labeled sheep anti-mouse immunoglobulin (Amersham). Cells were fixed again with formaldehyde/NH_4Cl, denatured in 2 M HCl for 1 h at 37°C and incubated with 0.1 M sodium tetraborate, pH 8.5 for 5 min. Cells were then stained with a purified FITC-conjugated polyclonal rabbit antibody against BrdU (dilution 1/4; Beckton-Dickinson), washed in PBS and mounted on glass slides with Mowiol.

Immunofluorescence microscopy

For analysis of Zta expression, indirect immunofluorescence was performed on HeLa-Zta cells cultured with different concentrations of tetracycline (0.000001–1 μg/ml). Cells, grown on cover slips, were fixed for 15 min with formaldehyde (3.7% in PBS), neutralized with NH_4Cl (50 mM in PBS, 10 min), permeabilized with Triton X-100 (0.01% in PBS), blocked with FBS (20% in PBS, 15 min) and incubated with the anti-HA monoclonal antibody 12CA5F (12.5 μg/ml, 1 h), followed by incubation with fluorescein-linked sheep anti-mouse immunoglobulin G (Amersham). Slides were mounted with Mowiol and cells were visualized under a fluorescence microscope (Zeiss). The photographs were taken using a Nikon microflex and Kodak T Max 400 film.

Northern blot analysis

Northern blot experiments were carried out as previously described (Cayrol and Flemington, 1995). Briefly, total RNAs or poly(A)$^+$ RNAs from HeLa-HtTA and HeLa-Zta cells grown in the presence or absence of tetracycline were isolated by acid guanidine isothiocyanate–phenol–chloroform extraction (Coffman *et al.*, 1989). Poly(A)$^+$ RNAs were prepared by passage over an oligo(dT)–cellulose column (Collaborative Research). RNAs [3 μg of poly(A)$^+$ RNAs or 20 μg of total RNAs] were denatured, fractionated by electrophoresis through 1% agarose–formaldehyde gels and transferred to nylon membranes (Hybond-N; Amersham). RNAs were cross-linked to the membranes by UV irradiation. Blots were pre-hybridized for 2 h and hybridized with the corresponding probes overnight at 42°C in 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's solution, 10 mM EDTA (pH 8) and 100 μg of salmon sperm DNA per ml. Blots were washed with 2× SSC, 0.5% SDS at room temperature for 2 min, then with 2× SSC, 0.1% SDS for 15 min and with 0.2× SSC, 0.5% SDS for 2 h at 55°C. Blots were exposed to autoradiography film (X-Omat; Kodak) at –70°C for 1–3 days. Probes were ^{32}P-labeled by random priming (GIBCO BRL).

Acknowledgements

We would like to thank Roger Brent, Mark Ewen, Joan Massagué, Bert Vogelstein, William Sellers, James DeCaprio, Arnold Levine and Seth Alper for kindly providing expression plasmids for p21, p16, p27, p53, E7 wild-type and mutant, SV40 T antigen, mdm2 and pMARK-CD7 respectively. We thank Takeshi Sairenji for the gift of the NPC-KT cell line. We are grateful to Miguel Campanero, Brian Schaefer, Sam Speck, William Sellers, Ana Borras and Joyce Fingeroth for helpful discussions and reading of the manuscript. We also thank Drs Jack Strominger and David Livingston for their continued support and encouragement. This work was supported by NIH grant CA47554, a Fondation pour la Recherche Medicale (FRM) fellowship (to C.C.), NIH first award R29 GM48045 (to E.K.F.) and a Leukemia Society of America Special fellowship (to E.K.F.).

References

Alday,M.J., Sinclair,A., Parker,G., Crawford,D.H. and Farrell,P.J. (1995) Epstein–Barr virus efficiently immortalizes human B cells without neutralizing the function of p53. *EMBO J.*, **14**, 1382–1391.

Becker,J., Leser,U., Marschall,M., Langford,A., Jilg,W., Gelderblom,H., Reichart,P. and Wolf,H. (1991) Expression of proteins encoded by Epstein–Barr virus trans-activator genes depends on differentiation of epithelial cells in oral hairy leukoplakia. *Proc. Natl Acad. Sci. USA*, **88**, 8332–8336.

Cayrol,C. and Flemington,E.K. (1995) Identification of cellular target genes of the Epstein–Barr virus transactivator Zta: activation of TGF-βigh3 and TGF-β1 *J. Virol.*, **69**, 4206–4212.

Chang,Y.-N., Dong,D.L.-Y., Hayward,G.S. and Hayward,S.D. (1990) The Epstein–Barr virus Zta transactivator: a member of the bZIP family with unique DNA binding specificity and dimerization domain that lacks the characteristic heptad leucine zipper motif. *J. Virol.*, **64**, 3358–3369.

Chi,T. and Carey,M. (1993) The ZEBRA activation domain: modular organization and mechanism of action. *Mol. Cell. Biol.*, **13**, 7045–7055.

Chowdary,D.R., Dermody,J.J., Jha,K.K. and Ozer,H.L. (1994) Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. *Mol. Cell. Biol.*, **14**, 1997–2003.

Coffman,R.L., Lebman,D.A. and Shrader,B. (1989) Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.*, **170**, 1039–1044.

Cohen,J.I., Wang,F., Mannick,J. and Kieff,E. (1989) Epstein–Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proc. Natl Acad. Sci. USA*, **86**, 9558–9562.

Countryman,J., Jensen,H., Seibel,R., Wolf,H. and Miller,G. (1987) Polymorphic proteins encoded within BZLF1 of defective and standard Epstein–Barr viruses disrupt latency. *J. Virol.*, **61**, 3672–3679.

Datto,M.B., Li,Y., Panus,J.F., Howe,D.J., Xiong,Y. and Wang,X.-F. (1995) Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc. Natl Acad. Sci. USA*, **92**, 5545–5549.

Davies,R., Hicks,R., Crook,T., Morris,J. and Vousden,K.H. (1993) HPV16 E7 associates with a histone H1 kinase activity and p107 through sequences necessary for transformation. *J. Virol.*, **67**, 2521–2528.

Dulic,V., Kaufmann,W.K., Wilson,S.J., Tisty,T.D., Lees,E., Harper,J.W., Elledge,S.J. and Reed,S.I. (1994) p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G_1 arrest. *Cell*, **76**, 1013–1023.

Dyson,N., Howley,P.M., Munger,K. and Harlow,E. (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, **143**, 934–936.

El-Diery,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W. and Vogelstein,B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

El-Diery,W.S. *et al.* (1994) WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. *Cancer Res.*, **54**, 1169–1174.

Farrell,P.J., Rowe,D.T., Rooney,C.M. and Kouzarides,T. (1989) Epstein–Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos. *EMBO J.*, **8**, 127–132.

Flemington,E. and Speck,S. (1990a) Autoregulation of the Epstein–Barr virus putative lytic switch gene *BZLF1*. *J. Virol.*, **64**, 1227–1232.

Flemington,E. and Speck,S. (1990b) Epstein–Barr virus BZLF1 trans activator induces the promoter of a cellular cognate gene, c-*fos*. *J. Virol.*, **64**, 4549–4552.

Flemington,E. and Speck,S.H. (1990c) Evidence for coiled-coil dimer formation by an Epstein–Barr virus transactivator that lacks a heptad repeat of leucine residues. *Proc. Natl Acad. Sci. USA*, **87**, 9459–9463.

Flemington,E., Borras,A.M., Lytle,J.P. and Speck,S. (1992) Characterization of the Epstein–Barr virus BZLF1 protein trans-activation domain. *J. Virol.*, **66**, 922–929.

Flemington,E.K., Lytle,J.P., Cayrol,C., Borras,A.M. and Speck,S.H. (1994) DNA-binding defective mutants of the Epstein–Barr virus lytic switch activator Zta transactivate with altered specificities. *Mol. Cell. Biol.*, **14**, 3041–3052.

Florenes,V.A., Maelandsmo,G.M., Forus,A., Andreassen,A., Myklegbost,O. and Fostad,O. (1994) MDM2 gene amplification and transcript levels in human sarcomas: relationship to TP53 gene status. *J. Natl Cancer Inst.*, **86**, 1297–1302.

Frangioni,J.V., Moghal,N., Stuart-Tilley,A., Neel,B.G. and Alper,S.L. (1994) The DNA binding domain of retinoic acid receptor beta is required for ligand-dependent suppression of proliferation. *J. Cell Sci.*, **107**, 827–838.

Giannoudis,A., Ergazake,M., Segas,J., Giotakis,J., Adamopoulos,G., Gorgoulis,V. and Spandidos,D. (1995) Detection of Epstein–Barr virus and human papillomavirus in nasopharyngeal carcinoma by the polymerase chain reaction technique. *Cancer Lett.*, **89**, 177–181.

Giot,J.-F., Mikaelian,I., Buisson,M., Banet,E., Joab,I., Nicolas,J.-C. and Sergeant,A. (1991) Transcriptional interference between the EBV transcription factors EB1 and R: both DNA binding and activations of EB1 are required. *Nucleic Acids Res.*, **19**, 1251–1258.

Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.

Greenspan,J.S. (1989) Oral hairy leukoplakia: diagnosis and management. *Oral Surg., Oral Med., Oral Pathol.*, **67**, 396–403.

Grogan,E.J., Jenson,J., Countryman,J., Heston,L., Gradoville,L. and Miller,G. (1987) Transfection of a rearranged viral DNA fragment, WZhet, stably converts latent Epstein–Barr virus infection to productive infection in lymphoid cells. *Proc. Natl Acad. Sci. USA*, **84**, 1332–1336.

Gutsch,D.E., Holley-Guthrie,E.A., Zhang,Q., Stein,B., Blanar,M.A., Baldwin,A.S. and Kenney,S.C. (1994) The bZIP transactivator of Epstein–Barr virus, BZLF1, functionally and physically interacts with the p65 subunit of NF-κB. *Mol. Cell. Biol.*, **14**, 1939–1948.

Hammerschmidt,W. and Sugden,B. (1989) Genetic analysis of immortalizing functions of Epstein–Barr virus in human B lymphocytes. *Nature*, **340**, 393–397.

Harper,J.W., Adami,G.R., Wei,N., Deyomarsi,K. and Elledge,S.J. (1993) The p21 cdk-interacting protein cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Havely,O., Novitch,B.G., Spicer,D.B., Skapek,S.X., Rhee,J., Hannon,G.J., Beach,D. and Lassar,A.B. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 and MyoD. *Science*, **267**, 1018–1021.

Henkel,T., Ling,P.D., Hayward,S.D. and Peterson,M.G. (1994) Mediation of Epstein–Barr virus EBNA2 transactivation by recombination signal-binding protein Jk. *Science*, **265**, 92–95.

Hsieh,J.J.-D. and Hayward,S.D. (1995) Masking of the CBF/RBPJκ transcriptional repression domain by Epstein–Barr virus EBNA2. *Science*, **268**, 560–563.

Kaye,K.M., Izumi,K.M. and Kieff,E. (1993) Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl Acad. Sci. USA*, **90**, 9150–9154.

Kempkes,B., Spitkovsky,D., Jansen-Durr,P., Ellwart,J.W., Kremmer,E., Delecluse,H.-J., Rottenberger,C., Bornkamm,G.W. and Hammerschmidt,W. (1995) B-cell proliferation and induction of early G_1-regulating proteins by Epstein–Barr virus mutants conditional for EBNA2. *EMBO J.*, **14**, 88–96.

Kenney,S., Holley-Guthrie,E., Mar,E.C. and Smith,M. (1989) The Epstein–Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. *J. Virol.*, **63**, 3878–3883.

Kieff,E. and Liebowit,D. (1990) Epstein–Barr virus and its replication. In Fields,B.N. and Knipe,D.M. (eds), *Virology*. Raven Press, New York, pp. 1889–1920.

Kim,M.S., Li,S.L., Bertolami,C.N., Cherrick,H.M. and Park,N.H. (1993) State of p53, Rb and DCC tumor suppressor genes in human oral cancer cell lines. *Anticancer Res.*, **13**, 1405–1413.

Klein,G. (1994) Epstein–Barr virus strategy in normal and neoplastic B cells. *Cell*, **77**, 791–793.

Kouzarides,T., Packham,G., Cook,A. and Farrell,P. (1991) The BLZF1 protein of EBV has a coiled coil dimerisation domain without a heptad leucine repeat but with homology to the C/EBP leucine zipper. *Oncogene*, **6**, 195–204.

Lane,D. and Crawford,L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature*, **278**, 261–263.

Lieberman,P. and Berk,A. (1990) *In vitro* transcriptional activation, dimerization, and DNA-binding specificity of the Epstein–Barr virus Zta protein. *J. Virol.*, **64**, 2560–2568.

Lieberman,P.M., Hardwick,J.M. and Hayward,S.D. (1989) Responsiveness of the Epstein–Barr virus *Not*I repeat promoter to the Z transactivator is mediated in a cell-type-specific manner by two independent signal regions. *J. Virol.*, **63**, 3040–3050.

Lin,C.-T., Dee,A.N., Chen,W. and Chan,W.-Y. (1995) Association of Epstein–Barr virus, human papilloma virus, and cytomegalovirus with nine nasopharyngeal carcinoma cell lines. *Lab. Invest.*, **71**, 731–736.

Linzer,D.I.H. and Levine,A.J. (1979) Characterization of a 54 Kdalton cellular SV40 tumor antigen in SV40 transformed cells. *Cell*, **17**, 43–52.

MacDonald,M.R., Le,K.T., Freeman,J., Hui,M.F., Cheung,R.K. and Dosch,H.-M. (1995) A majority of inverted sinonasal papillomas carries Epstein–Barr virus genomes. *Cancer*, **75**, 2307–2312.

Mannick,J., Cohen,J.I., Birkenbach,M., Marchini,A. and Kieff,E. (1991) The Epstein–Barr virus nuclear protein encoded by the leader or the EBNA-RNAs (EBNA-LP) is important in B lymphocyte transformation. *J. Virol.*, **65**, 6826–6837.

Miller,G. (1990) Epstein–Barr virus. In Fields,B.N. and Knipe,D.M. (eds), *Virology*. Raven Press, New York, pp. 1921–1958.

Mosialos,G., Birkenbach,M., Yalamanchili,R., VanArsdale,T., Ware,C. and Kieff,E. (1995) The Epstein–Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell*, **80**, 389–399.

Munger,K., Weerness,B., Dyson,N., Phelps,W.C., Harlow,E. and Howley,P.M. (1989) Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.*, **8**, 4099–4105.

Oren,M., Maltzman,W. and Levine,A.J. (1981) Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. *Mol. Cell. Biol.*, **1**, 101–110.

Pagano,M., Tam,S.W., Theodoras,A.M., Beer-Romero,P., Del Sal,G., Chau,V., Yew,P.R., Draetta,G.F. and Rolfe,M. (1995) Role of the ubiquitin–proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*, **269**, 682–685.

Polyak,K., Kato,J., Solomon,M.J., Sherr,C.J., Massague,J., Roberts,J.M. and Koff,A. (1994a) p27Kip1, a cyclin–cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes Dev.*, **8**, 9–22.

Polyak,K., Lee,M.-H., Erdjument-Bromage,H., Koff,A., Roberts,J.M., Temps,P. and Massague,J. (1994b) Cloning of p27/Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, **78**, 59–66.

Qin,X., Chittenden,T., Livingston,D.M. and Kaelin,W.G. (1992) Identification of a growth suppression domain within the retinoblastoma gene product. *Genes Dev.*, **6**, 953–964.

Reihsaus,E., Kohler,M., Kraiss,S., Oren,M. and Montenarh,M. (1990) Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. *Oncogene*, **5**, 137–145.

Reiss,M., Brash,D.E., Munoz-Antonia,T., Simon,J.A., Ziegler,A., Vellucci,V.F. and Zhou,Z.L. (1992) Status of the p53 tumor suppressor gene in human squamous carcinoma cell lines. *Oncol. Res.*, **4**, 349–357.

Renzo,L.D., Altiok,A., Klein,G. and Klein,E. (1994) Endogenous TGF-β contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines. *Int. J. Cancer*, **57**, 914–919.

Resnick,L., Herbst,J.S. and Ablashi,D. (1988) Regression of oral hairy leukoplakia after orally administered acyclovir therapy. *J. Am. Med. Assoc.*, **259**, 384–388.

Rickinson,A.B. (1986) Cellular immunological responses to infection by the virus. In Epstein,M.A. and Achong,B.G. (eds), *The Epstein–Barr Virus: Recent Advances*. William Heinemann, London, pp. 75–125.

Sarnow,P., Ho,Y.S., Williams,J. and Levine,A.J. (1982) Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell*, **28**, 387–394.

Scheffner,M., Werness,B.A., Huibregtse,J.M., Levine,A.J. and Howley,P.M. (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129–1136.

Scheffner,M., Munger,K., Byrne,J.C. and Howley,P.M. (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl Acad. Sci. USA*, **88**, 5523–5527.

Schepers,A., Pich,D. and Hammerschmidt,W. (1993) A transcription factor with homology to the AP-1 family links RNA transcription and DNA replication in the lytic cycle of Epstein–Barr virus. *EMBO J.*, **12**, 3921–3929.

Shadan,F.F., Cowsert,L.M. and Billarreal,L.P. (1994) *n*-Butyrate, a cell cycle blocker, inhibits the replication of polyomaviruses and papillomaviruses but not that of adenoviruses and herpesviruses. *J. Virol.*, **68**, 4785–4796.

Sherr,C.J. and Roberts,J.M. (1995) Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.*, **9**, 1149–1163.

Sinclair,A.J., Palmero,I., Peters,G. and Farrell,P.J. (1994) EBNA-2 and EBNA-LP cooperate to cause G_0 to G_1 transition during immortalization of resting human B lymphocytes by Epstein–Barr virus. *EMBO J.*, **13**, 3321–3328.

Sista,N.D., Pagano,J.S., Liao,W. and Kenney,S. (1993) Retinoic acid is a negative regulator of the Epstein–Barr virus protein (BZLF1) that mediates disruption of latent infection. *Proc. Natl Acad. Sci. USA*, **90**, 3894–3898.

Sixbey,J.W., Nedrud,J.G., Raab-Traub,N., Hanes,R.A. and Pagano,J.S. (1984) Epstein–Barr virus replication in oropharyngeal epithelial cells. *N. Engl. J. Med.*, **310**, 1225–1230.

Sixbey,J.W., Lemon,S.M. and Pagano,J.S. (1986) A second site for Epstein–Barr virus shedding: the uterine cervix. *Lancet*, **ii**, 1122–1124.

Takimoto,T., Kamide,M. and Umeda,R. (1984) Establishment of Epstein–Barr virus (EBV) associated nuclear antigen (EBNA) positive nasopharyngeal carcinoma hybrid cell line (NPC-KT). *Arch. Otor.*, **239**, 87–92.

Tomkinson,B., Robertson,E. and Kieff,E. (1993) Epstein–Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J. Virol.*, **67**, 2014–2025.

Toyoshima,H. and Hunter,T. (1994) p27, a novel inhibitor of G_1 cyclin–cdk protein kinase activity, is related to p21. *Cell*, **78**, 67–74.

Urier,G., Buisson,M., Chambard,P. and Sergeant,A. (1989) The Epstein–Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites. *EMBO J.*, **8**, 1447–1453.

Vousden,K.H. (1995) Regulation of the cell cycle by viral oncoproteins. *Semin. Cancer Biol.*, **6**, 109–116.

Werness,B.A., Levine,A.J. and Howley,P.M. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*, **248**, 76–79.

Wolf,H., Haus,M. and Wilmes,E. (1984) Persistence of Epstein–Barr virus in the parotid gland. *J. Virol.*, **51**, 795–798.

Wu,X., Bayle,J.H., Olson,D. and Levine,A.J. (1993) The p53–mdm-2 autoregulatory feedback loop. *Genes Dev.*, **7**, 1126–1132.

Xiong,Y., Hannon,G., Zhang,H., Casso,D., Kobayashi,R. and Beach,D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704.

Yates,J.L., Warren,N. and Sugden,B. (1985) Stable replication of plasmids derived from Epstein–Barr virus in a variety of mammalian cells. *Nature*, **313**, 812–815.

Young,L.S., Lau,R., Rowe,M., Niedobotek,G., Packham,G., Shanahan,F., Rowe,D.T., Greenspan,D., Greenspan,J.S., Rickinson,A.B. and Farrell,P.J. (1991) Differentiation associated expression of the Epstein–Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. *J. Virol.*, **65**, 2868–2874.

Zambetti,G.P. and Levine,A.J. (1993) A comparison of the biological activities of wild-type and mutant p53. *FASEB J.*, **7**, 855–865.

Zhang,Z., Gutsch,D. and Kenney,S. (1994) Functional and physical interaction between p53 and BZLF1: implications for Epstein–Barr virus latency. *J. Virol.*, **14**, 1929–1938.

Received on December 1, 1995; revised on January 23, 1996