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# The EBNA2 Polyproline Region Is Dispensable for Epstein-Barr Virus-Mediated Immortalization Maintenance

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**Epstein-Barr virus nuclear antigen 2 (EBNA2) is required for EBV-mediated immortalization of primary human B cells and is a direct transcriptional activator of viral and cellular genes. The prototype EBNA2 protein contains a unique motif in which 43 out of 45 amino acids are prolines (polyproline region [PPR]). Previous genetic analysis has shown that deletion of the PPR resulted in viruses unable to immortalize B cells, although the protein did appear transcriptionally functional (R. Yalamanchili, S. Harada, and E. Kieff, J. Virol. 70:2468-2473, 1996). The PPR's uniqueness and requirement for immortalization make it an attractive therapeutic target. However, the role of this highly unusual motif for immortalization remains enigmatic. We have recently developed a transcomplementation assay that allows both genetic and functional analyses of EBNA2 in EBV-mediated immortalization maintenance (A. V. Gordadze, R. Peng, J. Tan, G. Liu, R. Sutton, B. Kempkes, G. W. Bornkamm, and P. D. Ling, J. Virol. 75:5899-5912, 2001). Surprisingly, we found that ΔPPR-EBNA2 was able to support B-cell proliferation similar to that of wild-type EBNA2 in this assay, indicating that deletion of the PPR from EBNA2 does not result in a loss of function required for immortalization maintenance. Further analysis of this mutant EBNA2 revealed that it consistently activated the viral LMP1 and LMP2A promoters severalfold better than wild-type EBNA2 in transient cotransfection assays. In addition, one striking difference between lymphoblastoid cell lines expressing wild-type EBNA2 from those expressing ΔPPR-EBNA2 is that the latter cells have significantly reduced EBV genomic levels. The data are consistent with a model in which lower EBNA2 target gene dosage may be selected for in ΔPPR-EBNA2-dependent cell lines to compensate for hyperactive stimulation of viral genes, such as LMP-1, which is cytostatic for B cells when overexpressed. It is conceivable that the hyperactivity rather than the loss of function, as hypothesized previously, could be responsible for the inability of recombinant ΔPPR-EBNA2 EBVs to immortalize B cells.**

Epstein-Barr virus (EBV) is a ubiquitous human pathogen associated with both lymphoid and epithelial malignancies (44). EBV is also a common cause of infectious mononucleosis (44). EBV efficiently immortalizes primary human B lymphocytes in vitro in a process that requires several EBV-encoded proteins characteristically expressed during latency (3, 31). EBV nuclear antigen 2 (EBNA2) is a direct transcriptional activator of both viral and cellular genes in immortalized cells and is required for EBV-mediated immortalization (6, 8, 19, 24, 26, 30). EBV-infected cells expressing EBNA2 and with a lymphoblastoid phenotype characteristic of EBV-immortalized lymphoblastoid cell lines (LCLs) are found during infection in vivo (2). This observation suggests that EBNA2 function is important for EBV pathogenesis. Being a principal regulator of the immortalized cell phenotype, EBNA2 is a plausible target for therapeutic intervention. EBNA2 serves as an important modulator of viral latency gene expression by stimulating the viral latency C promoter, the latent membrane protein 2A (LMP2A), and latent membrane protein 1 (LMP1) promoters (47, 49, 50, 61). The LMP1 promoter is the only viral EBNA2 target essential for immortalization, since the viral latency C promoter can functionally be replaced by the EBNA2-independent W promoter and the LMP2A protein is dispensable for immortalization altogether (4, 34, 48). Regulation of LMP1 by EBNA2 is likely to be especially crucial, since LMP1 has transforming functions and is also required for immortalization (27, 32). Regulation of cellular genes by EBNA2 may also be important for immortalization (53, 58). Consistent with this idea, EBNA2 has been found to directly up-regulate transcription of the cellular proto-oncogene c-myc (26). c-myc activity is important for cell cycle progression in B cells (10, 42).

EBNA2 cannot bind DNA by itself and is recruited to its target viral promoters by means of physical interaction with a sequence-specific cellular DNA-binding protein, CBF1 (17, 23, 38, 60). EBNA2-CBF1 complex formation is absolutely required for EBNA2 to maintain EBV-induced immortalization (16). Another cellular protein, SKIP, interacts with both EBNA2 and CBF1 and appears to be critical for transcriptional activation by EBNA2 (56). A number of other cellular proteins have been implicated in mediation of EBNA2 function, including PU.1, AUF1, PP1/PP2A-like protein, DP103, and hSNF5/Ini1 (12, 14, 18, 25, 35, 52, 55). However, the functional significance of interactions between these proteins and EBNA2 for immortalization remains to be demonstrated. The prototype EBNA2 from a type 1 EBV isolate is a 490-amino-acid protein that contains an acidic transcriptional activation domain and a canonical nuclear localization signal in the carboxy-terminal third of the protein (6, 9, 39). A highly unusual region in which 43 out of 45 amino acids are prolines is found in the amino-terminal part of EBNA2 (amino acids 59

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to 103) and is commonly referred to as the polyproline region (PPR) (9). Several functional domains have been identified in EBNA2 that are required for transcriptional activity and/or the ability to support immortalization (6, 7, 53). The domains map to several evolutionarily conserved regions found in EBNA2 proteins from human and primate lymphocryptoviruses (37). Conserved region 6 (CR6) mediates binding to CBF1, while CR5 mediates interaction with SKIP and CR8 is important for activation domain function (6, 23, 38, 39, 54, 57). While these regions are located in the carboxy-terminal half of EBNA2, much of the amino-terminal half of the protein has been shown to be dispensable for both transcriptional activity and immortalization (21, 53). Within this region, however, the highly unusual polyproline motif was required for immortalization in marker rescue experiments (53). Curiously, deletion of the PPR did not abolish EBNA2's ability to transactivate the viral LMP1 promoter in transient transfection assays, nor did deletion of this region abolish EBNA2's ability to interact with CBF1 or disrupt a proposed multimerization function mediated by the amino terminus (20, 53). All EBNA2 homologues cloned so far contain PPRs of considerable lengths. EBNA2 from type 2 EBV has a 16 (14P)-amino-acid-long PPR, while in baboon and rhesus lymphocryptoviruses the PPRs are of intermediate length, with 24 and 28 proline residues, respectively (5, 39, 43). To our knowledge, no other primate protein has been identified possessing such a high number of consecutive or near-consecutive prolines. The PPR's uniqueness and the fact that it appears to be essential for immortalization by EBV make the putative PPR-mediated function an attractive candidate for therapeutic intervention, yet the role of the PPR in mediating EBNA2 function remains a mystery. The experiments used to establish the requirement for PPR in immortalization relied on generating recombinant EBVs by a marker rescue strategy carrying the immortalization-incompetent ΔPPR-EBNA2 genes and failed to yield any information as to the consequences of the PPR deletion for the immortalization (53). On the basis of previous studies we hypothesized that ΔPPR-EBNA2 would be defective in one or more EBNA2 functions essential for immortalization and involving cellular rather than viral targets.

As a first step towards development of a model to dissect the mechanism of the effect of PPR deletion on EBNA2 function, we have analyzed the phenotypic properties of ΔPPR-EBNA2 in the context of an EBV-immortalized cell line. A transcomplementation assay for EBNA2 function in immortalization was recently developed (16). In this assay the EREB cell line is used, which is immortalized by an EBV with a conditional EBNA2 allele (30). EBNA2 function in this cell line is rendered strictly dependent on estrogen in the medium as a result of a fusion of the estrogen-receptor-binding domain (ERLBD) to EBNA2 (EREBNA2). In the absence of estrogen, EREBNA2 function is inactivated and EREB cells undergo growth arrest and apoptosis and eventually die out (29, 30). We have demonstrated that lentiviral transduction of a wild-type EBNA2 protein rescued cellular growth in the absence of estrogen quite readily (16). In contrast, an immortalization-incompetent mutated EBNA2 gene encoding a protein unable to interact with CBF1 (WW326SR) failed to rescue the EREB cells after estrogen withdrawal (16). Thus, the transcomplementation approach can be used to study the effect of mutations in EBNA2 on cellular growth and/or phenotype.

There are important differences between the transcomplementation approach and the previously utilized marker rescue assay employed by other investigators to analyze the immortalizing ability of ΔPPR-EBNA2 (7, 8, 16, 21, 53). In marker rescue experiments, the ability of recombinant viruses carrying ΔPPR-EBNA2 genes to initiate and maintain immortalization of primary B cells was assessed. The process of immortalization is a complex phenomenon involving many stages, including initial infection, EBV genome circularization, activation of B cells accompanied by entry into cell cycle, amplification of EBV genomes in the infected cells, latency promoter usage switch, oligoclonalization of outgrowing LCLs, and finally, maintenance of the immortalized state (3, 31, 45). In the transcomplementation assay, however, only the ability of ectopically expressed EBNA2 proteins to rescue the maintenance of the immortalized state can be assessed.

**Ectopic expression of ΔPPR-EBNA2 replaces EREBNA2 function in proliferation maintenance of EREB cells.** An EBNA2 open reading frame without the PPR was generated by PCR and subcloned into the SG5 expression vector (Stratagene) and lentiviral vector pLIG as described previously for wild-type EBNA2 (16, 43). EREB cell pools were prepared expressing wild-type EBNA2, the mutant ΔPPR-EBNA2, or the lentiviral vector alone and were grown as described previously (16). Briefly, the EREB cells were transduced with the corresponding recombinant lentiviruses followed by enrichment for cells expressing green fluorescent protein by using fluorescence-activated cell sorting (FACS). Two independently generated cell pools were prepared for each provirus. The transduced and sorted cells were expanded in the presence of estrogen for 2 weeks. Subsequent FACS analysis showed that the resulting cell pools had similar mean fluorescence intensities and were approximately 90% green fluorescent protein positive (data not shown). After 2 weeks of expansion in the presence of estrogen, the cell pools were further analyzed for their ability to survive and proliferate under estrogen-free medium conditions that result in inactivation of endogenous EREBNA2 function (16). The cell pools were maintained in a logarithmic phase immediately before estrogen withdrawal. After estrogen withdrawal, the survival and proliferation of the cells was monitored by using the MTT assay with Cell Proliferation Kit I (Molecular Roche) as recommended by the manufacturer (41). Unexpectedly, the ΔPPR-EBNA2-expressing cells survived and proliferated as well as the wild-type EBNA2-expressing cells, while the empty-vector-expressing cells quickly died out (Fig. 1). Once the sorted cell pools were expanded for 2 weeks in the presence of estrogen, long-term estrogen-independent LCLs were readily established from EBNA2 and ΔPPR-EBNA2-expressing EREB cells, but not from the empty-vector-transduced cells, after being switched to estrogen-free medium during routine passaging. After 6 weeks in culture in estrogen-free media, such EBNA2- and ΔPPR-EBNA2-rescued estrogen-independent LCLs expressed EBNA2 proteins at similar levels and proliferated at a similar rate (Fig. 2). Invariably, in the EBNA2-transduced cells lower-molecular-weight EBNA2-specific bands were detected by Western blotting (Fig. 1B). These EBNA2-specific bands were present even in the long-term wild-type or mutant EBNA2-

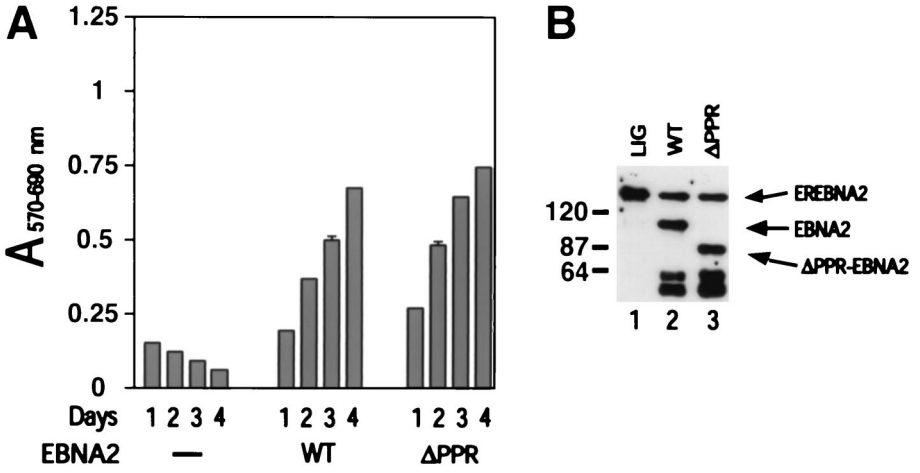


FIG. 1. ΔPPR-EBNA2 supports survival and proliferation of EREB cells similar to that of wild-type EBNA2. (A) EREB cell pools transduced with the pLIG vector alone (−), pLIG.EBNA2 (WT), or pLIG.ΔPPR-EBNA2 (ΔPPR) lentiviruses after enrichment by FACS were grown in the presence of 1 μM estrogen (β-estradiol; Sigma) for 2 weeks prior to the experiment. At day 0 the cells were washed of estrogen and placed in the estrogen-free medium. On the same day an equal aliquot of cells was also processed for Western blot analysis (see below). Twenty-four hours later the cell pools were plated in 200-μl aliquots of estrogen-free medium in 96-well plates at $2 \times 10^4$ cells/well and were monitored for survival and proliferation by MTT assay daily over the course of 4 days, starting with day 1 (day of plating) after estrogen withdrawal. Absorbance at 570-nm ($A_{570}$) and 690-nm ($A_{690}$) wavelengths of completed MTT reactions were determined to calculate the $A_{570-690}$ values by subtracting $A_{690}$ from $A_{570}$. (B) Western blot analysis of pLIG (lane 1), pLIG.EBNA2 (lane 2), and pLIG.ΔPPR-EBNA2 (lane 3) transduced cell pools analyzed in panel A by using an anti-EBNA2 monoclonal antibody, PE2. Equal amounts of total cellular protein were analyzed in each lane. Molecular weight markers are shown in kilodaltons on the left.

rescued estrogen-independent LCLs (Fig. 2B). Some possible explanations for these lower-molecular-weight forms are that they are degradation products or are polypeptides translated from alternatively spliced mRNAs derived from the lentiviral vector. Since wild-type EBNA2 expressed from the lentiviral vector readily substitutes for inactivated EREBNA2 protein and the resulting cell lines are similar in growth rate and phenotype to the parental cells, these lower-molecular-weight polypeptides appear not to affect EBNA2 function in the immortalized cells. The relative intensities of the lower-molecular-weight bands in both the wild-type and mutant EBNA2-transduced cells were similar, suggesting that the underlying phenomenon is not likely to contribute to the cellular phenotypes observed in our experiments (16). Interestingly, in the estrogen-independent cell lines the EREBNA2 protein was barely detectable (Fig. 2B). In the EREB cells the EREBNA2

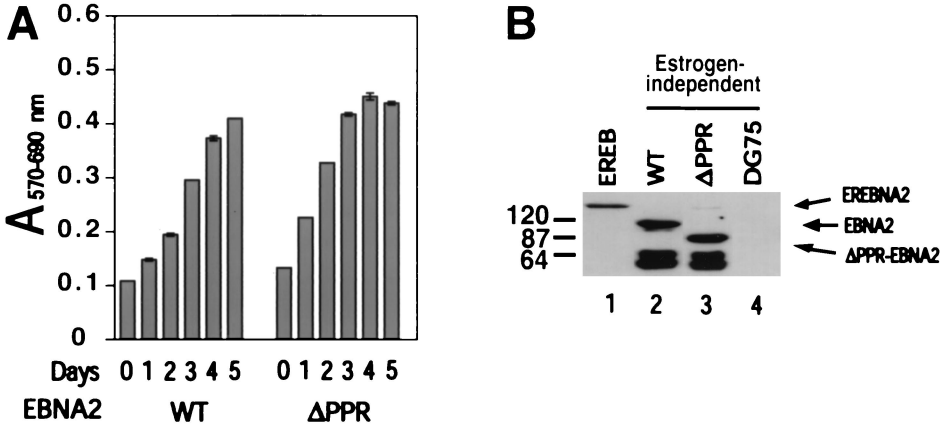


FIG. 2. Estrogen-independent EREB-derived LCLs expressing ΔPPR-EBNA2 or wild-type EBNA2 exhibit similar growth rates. (A) Upon initial expansion after enrichment by FACS in the presence of estrogen for 2 weeks, the cell pools were grown in estrogen-free medium for an additional 6 weeks, resulting in the outgrowth of the estrogen-independent wild-type EBNA2-expressing (WT) and ΔPPR-EBNA2-expressing (ΔPPR) LCLs. Proliferation of the rescued estrogen-independent cell lines was monitored by MTT assay (as described for Fig. 1) in estrogen-free medium over a course of 5 days, starting with day 0 (day of plating). On the same day an equal aliquot of cells from the same cell pool was also processed for Western blot analysis (see below). Absorbance at 570-nm ($A_{570}$) and 690-nm ($A_{690}$) wavelengths of completed MTT reactions were determined to calculate the $A_{570-690}$ values by subtracting $A_{690}$ from $A_{570}$. (B) Western blot analysis of wild-type (lane 2) and ΔPPR-EBNA2 (lane 3) protein expression in the LCLs analyzed for panel A was done as described in the legend to Fig. 1. The parental EREB cells transduced with vector alone and grown in the presence of estrogen (lane 1) and DG75 (lane 4), an EBV-negative Burkitt's lymphoma cell line, were used as controls. Equal amounts of protein were analyzed in each lane. Molecular weight markers are shown in kilodaltons on the left.

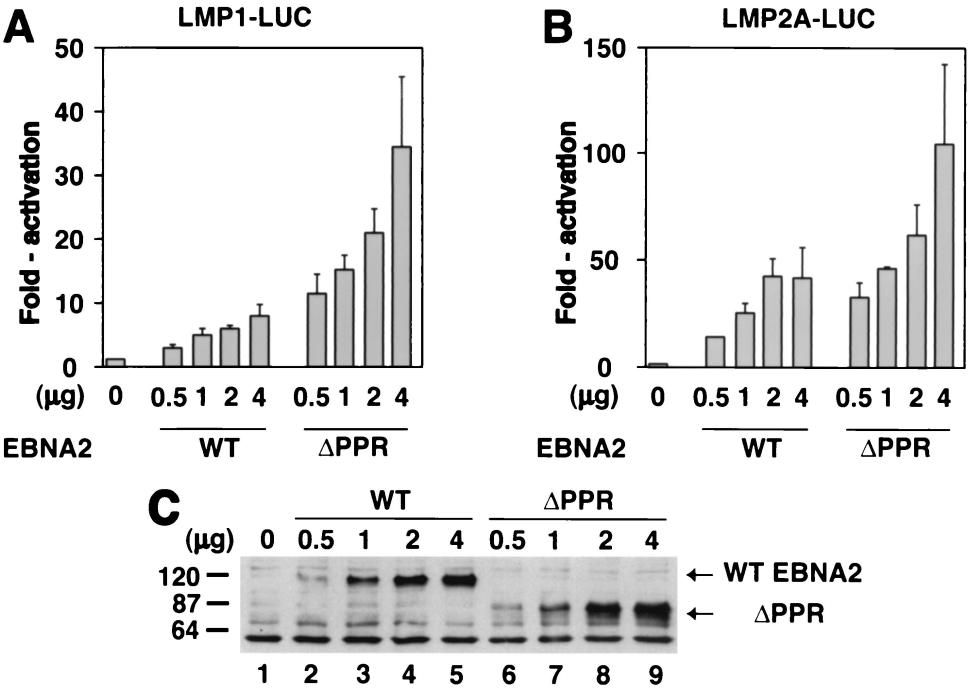


FIG. 3. ΔPPR-EBNA2 transactivation of viral latency promoter constructs. (A) An LMP1 promoter-luciferase reporter plasmid (0.5 μg) was cotransfected into DG75 cells with the indicated amounts of wild-type EBNA2 (WT) or ΔPPR-EBNA2 (ΔPPR) expression plasmids. The results are presented as fold-activation of the promoter in the presence of the effector. Average transactivation efficiencies representing three independent experiments are presented. (B) Transactivation of the LMP2A promoter reporter plasmid. An LMP2A promoter-luciferase reporter plasmid (0.5 μg) was cotransfected with the indicated amounts of the wild-type EBNA2 (WT) or ΔPPR-EBNA2 (ΔPPR) expression plasmids. The results are presented as fold-activation of the promoter in the presence of the effector. Average transactivation efficiencies representing three independent experiments are presented. The reporter plasmids used in these experiments were described previously (36, 59). (C) A representative Western blot analysis of wild-type and ΔPPR-EBNA2 expression in samples from the transient transfection experiments described for panel A. Forty-eight hours after transfection cells transfected with the indicated amounts of wild-type EBNA2 (WT, lanes 2 to 5) or ΔPPR-EBNA2 (ΔPPR, lanes 6 to 9) expression plasmids were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis as described in the legend to Fig. 1. Cell lysate aliquots containing equal amounts of total cellular protein were loaded in each lane. Molecular weight markers are shown in kilodaltons on the left. Arrows on the right indicate migration of WT and ΔPPR proteins.

protein is encoded by a mini-EBV plasmid replicating separately from the EBV P3HR1 genome (30). Southern blot analysis with a mini-EBV-specific probe (ERLBD) revealed that the EREBNA2 gene had largely been lost from the estrogen-independent cells, suggesting that the mini-EBV plasmids are rapidly disappearing from the immortalized cells once they are no longer required for survival and growth (data not shown).

Our result refines the previously reported inability of the ΔPPR-EBNA2 recombinant viruses to immortalize B cells (53). The simplest interpretation of this discrepancy is that the PPR is required at early stages of immortalization, which are not analyzed with the transcomplementation assay. However, the possibility that the PPR is still required for immortalization maintenance when EBNA2 is expressed in the natural regulatory context of the episomal EBV genome cannot be excluded. Since both wild-type and mutant EBNA2 proteins are expressed at similar levels in our experiments, we conclude that, contrary to our original hypothesis, ΔPPR-EBNA2 is not severely impaired in any of its interactions with cellular targets and/or processes relevant to immortalization maintenance. Thus, we considered the possibility that rather than being defective, perhaps the ΔPPR-EBNA2 was an overly active protein. Expression of this protein away from the context of the EBV genome would allow for some novel compensatory mechanisms that would otherwise have been constrained. The recent discovery that LMP1 protein overexpression was cytostatic would also be consistent with this idea (13, 28). Therefore, we reevaluated the ability of ΔPPR-EBNA2 to transactivate the LMP1 and other EBNA2-responsive viral promoters in transient cotransfection assays.

**Comparison of ΔPPR-EBNA2 and wild-type EBNA2 activation of viral EBNA2-responsive promoters.** An LMP1 promoter (−327 to +40) luciferase reporter construct, LMP1LUC0, was cotransfected into DG75 cells with the EBNA2 and ΔPPR-EBNA2 expression plasmids at different concentrations, as described previously (16). Luciferase activity in the cell extracts was measured 48 h posttransfection with the Dual-Luciferase Reporter Assay System (Promega) as recommended by the manufacturer. The results indicate that ΔPPR-EBNA2 stimulated the LMP1 promoter 3.5 times higher on average than the wild-type EBNA2 protein (Fig. 3A). Western blot analysis of the transfected cell extracts revealed that both proteins accumulated to similar levels, indicating that differences in transactivation were not due to differences in protein levels (Fig. 3C). This effect was more dramatic on the LMP1 promoter as ΔPPR-EBNA2 stimulated the LMP2A promoter (−804 to +59) only twice as well as wild-type EBNA2 (compare Fig. 3A and B), and both proteins

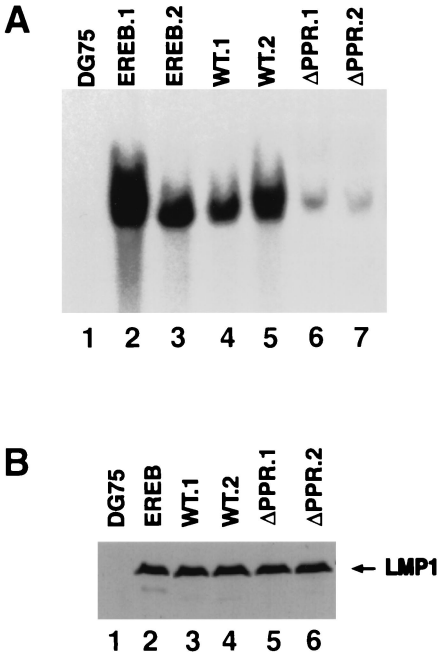


FIG. 4. (A) Analysis of LMP1 expression and relative EBV genomic DNA content. (A) Total genomic DNA from two subclones of the parental cell lines (EREB.1 and EREB.2), two independently derived wild-type EBNA2-rescued LCLs (WT.1 and WT.2), two independently derived ΔPPR-EBNA2-rescued LCLs (ΔPPR.1 and ΔPPR.2), and EBV-negative cell line DG75 was isolated and digested with *Eco*RI and *Hin*dIII restriction enzymes. Ten micrograms of each digested DNA was run on a 0.8% agarose gel. Southern blot analysis was performed with a radioactively labeled LMP1 cDNA probe. (B) Western blot analysis of LMP1 expression in the cell lines described above. Western blots were performed as described in the legend to Fig. 1, except that the S12 monoclonal antibody specific for LMP1 was used (40).

showed similar activity on the viral C promoter (data not shown). We conclude that ΔPPR-EBNA2 possesses a higher specific transcriptional activity towards the LMP1 and LMP2A promoters than the wild-type protein.

This result suggests that ΔPPR-EBNA2 may stimulate higher LMP1 expression levels than wild-type EBNA2 in EBV-infected cells. Thus, the hypothesis that overexpression of LMP1 at cytostatic levels during the immortalization process by ΔPPR-EBNA2 recombinant viruses may at least in part be responsible for the immortalization-null phenotype of the virus deserves certain merit. The transcomplementation assay permits testing one prediction, namely, that EBV genomic DNA content might be down-regulated in the estrogen-independent ΔPPR-EBNA2-rescued EREB cells compared to that in wild-type EBNA2-rescued cells to compensate for LMP1 overexpression.

**Analysis of relative viral genomic DNA content in the ΔPPR-EBNA2- and wild-type EBNA2-expressing estrogen-independent LCLs.** To compare relative genomic DNA content, total genomic DNA from the ΔPPR-EBNA2- and EBNA2-expressing estrogen-independent LCLs grown in the absence of estrogen as well as the parental EREB cells was prepared, digested with *Eco*RI and *Hin*dIII, and subjected to Southern blot analysis by using a full-length LMP1 cDNA probe followed by quantification with a phosphorimager. EBV genomic DNA content in the ΔPPR-EBNA2-rescued cells was 7.5 times lower on average than that in the wild-type EBNA2-rescued cells and was 18 times lower than that in the parental estrogen-dependent EREB cells, while LMP1 protein expression levels were similar (Fig. 4). Next we wanted to confirm that the differences in EBV genomic DNA levels were indeed determined by the differences in latent EBV genome content rather than by lytically replicated EBV DNA as a result of a potential difference in permissivity for lytic replication. To do this we took advantage of the fact that lytically replicated EBV DNA is linear, while latently replicated EBV DNA is circular. We employed a Gardella gel technique, which allows differentiation between the circular and linear EBV DNA (15). We determined that higher EBV genomic DNA content in the wild-type EBNA2-rescued cells is indeed due to differences in circular, that is, latent, EBV genomic DNA levels (Fig. 5).

In summary, to gain insight into the role that the unique PPR might play in mediating EBNA2 function we assayed the ability of ΔPPR-EBNA2 to rescue EBV-immortalized cell growth in a recently developed transcomplementation assay for EBNA2 function (16). ΔPPR-EBNA2 was able to support the immortalized phenotype of EBV-infected cells in a manner similar to that for wild-type EBNA2. However, the ΔPPR-EBNA2-expressing LCLs were distinctly different from wild-type EBNA2-expressing LCLs in that they harbored significantly lower EBV genomic DNA levels. In addition, the ΔPPR-EBNA2 protein consistently induced the latent membrane promoters, particularly LMP1, to higher levels than wild-type EBNA2 in transient cotransfection assays.

The data suggest that ΔPPR-EBNA2-expressing cells with higher EBV genome copy numbers have a selective growth disadvantage. One possible reason for this effect may be due to viral gene products whose expression is induced by EBNA2. The ΔPPR-EBNA2-transduced cells with more EBV genomes may express higher levels of EBNA2-induced genes, which could be inhibitory for proliferation. Reduction of target gene dosage would be one mechanism to compensate for these effects. A candidate viral protein potentially capable of mediating such an effect is LMP1, which is required for proliferation yet is cytostatic when overexpressed in B-cell lines (13, 28). In agreement with this hypothesis, ΔPPR-EBNA2 does appear to transactivate the LMP1 promoter to significantly higher levels than wild-type EBNA2 (Fig. 3A). However, our results do not exclude the possibility that other EBNA2 viral target gene products, such as LMP2A, could be responsible for the observed phenomenon.

A previous study indicated that deletion of the PPR from EBNA2 resulted in recombinant viruses unable to immortalize B cells and appears to contrast with our observations (53). The difference in outcomes between our study and the previous one is likely to be due to differences in assay conditions and certain features of the transcomplementation assay. The very fact that the ΔPPR-EBNA2-expressing LCLs grow out in our assay may be due to the inherent heterogeneity within LCLs in many parameters, including EBV genome copy numbers per individual cell (1, 51). At any given time a significant percentage of an LCL population possesses EBV DNA levels so low that cellular proliferation is reduced, as is expression of EBNA2 target

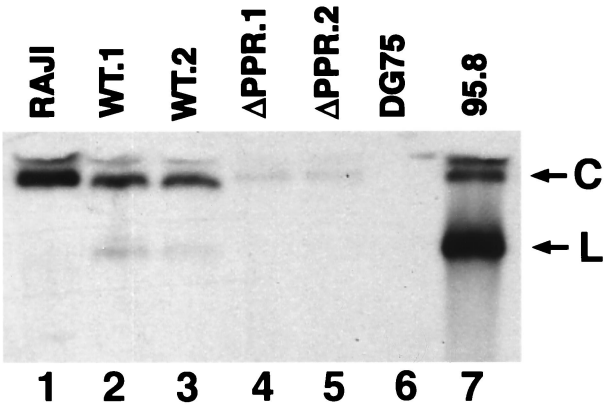


FIG. 5. (A) Analysis of circular and linear EBV genomic DNA content by Gardella gel technique. (A) Two independently derived wild-type EBNA2-rescued LCLs (WT.1 and WT.2) and two independently derived ΔPPR-EBNA2-rescued LCLs (ΔPPR.1 and ΔPPR.2) were analyzed on a Gardella gel. Raji is a latently EBV-infected nonproducer cell line defective in lytic viral replication and, thus, possesses circular but lacks linear viral DNA (22). B95.8 is a marmoset-derived LCL that supports both latent and lytic infection by EBV and in which both linear and circular EBV genomes can be detected. DG75 is an EBV-negative lymphoma cell line. Cells ($5 \times 10^6$) from each cell line were loaded per lane. The cells were then subjected to lysis by sodium dodecyl sulfate and digestion with proteinase K followed by electrophoresis in 1% agarose. Southern blot analysis was performed by using a radioactively labeled LMP1 cDNA probe. Migrations of circular (C) and linear (L) forms of EBV genomic DNA are indicated by arrows on the right.

genes (51). Therefore, upon transduction with the lentiviruses carrying the wild type or ΔPPR-EBNA2, different subpopulations with compatible EBV genome numbers may have a selective growth advantage. On the other hand, EBV-derived *oriP*-containing plasmids show a propensity for quick loss from cells in which they are stably maintained if marker selection is lifted (33, 46). Therefore, in the transcomplementation assay changes in viral genome load can quickly be selected for and can compensate for the effect of the PPR deletion. Compensation for EBNA2 hyperactivity through reduction of EBV genome levels may not be possible during immortalization by a recombinant ΔPPR-EBNA2 EBV when the EBNA2 and LMP1 genes reside on the same chromosome. A block in proliferation might be expected to occur shortly upon infection of B cells with a recombinant ΔPPR-EBNA2 virus due to overexpression of cytostatic levels of LMP-1. Interestingly, even the wild-type EBNA2-expressing estrogen-independent LCLs appear to have 2.4-fold lower average EBV genome content than the parental EREB cells (Fig. 4A). While the reason for this is unknown, it would be consistent with the idea that in the immortalized cell lines EREBNA2 protein function may be negatively affected by the fusion and may require greater genome numbers for optimal viral gene expression.

Our results are consistent with a model in which hyperactivity of ΔPPR-EBNA2 in stimulating one or more viral promoters rather than loss of function, as hypothesized previously, may be responsible for the inability of recombinant ΔPPR-EBNA2 EBVs to immortalize B cells. Replacement of EBNA2-responsive elements in EBNA2 target promoters with regulatable heterologous elements conferring EBNA2 independence would allow further testing of this hypothesis. Recent development of the bacterial artificial chromosome-EBV system now allows this to be technically feasible (11).

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## REFERENCES

1. **Altmeyer, A., R. C. Simmons, S. Krajewski, J. C. Reed, G. W. Bornkamm, and S. Chen-Kiang.** 1997. Reversal of EBV immortalization precedes apoptosis in IL-6-induced human B cell terminal differentiation. Immunity **7:**667–677.
2. **Babcock, J. G., D. Hochberg, and A. D. Thorley-Lawson.** 2000. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. Immunity **13:**497–506.
3. **Bornkamm, G. W., and W. Hammerschmidt.** 2001. Molecular virology of Epstein-Barr virus. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356:**437–459.
4. **Brielmeier, M., J. Mautner, G. Laux, and W. Hammerschmidt.** 1996. The latent membrane protein 2 gene of Epstein-Barr virus is important for efficient B cell immortalization. J. Gen. Virol. **77:**2807–2818.
5. **Cho, Y. G., A. V. Gordadze, P. D. Ling, and F. Wang.** 1999. Evolution of two types of rhesus lymphocryptovirus similar to type 1 and type 2 Epstein-Barr virus. J. Virol. **73:**9206–9212.
6. **Cohen, J. I., and E. Kieff.** 1991. An Epstein-Barr virus nuclear protein 2 domain essential for transformation is a direct transcriptional activator. J. Virol. **65:**5880–5885.
7. **Cohen, J. I., F. Wang, and E. Kieff.** 1991. Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation. J. Virol. **65:**2545–2554.
8. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86:**9558–9562.
9. **Dambaugh, T., K. Hennessy, L. Chamnankit, and E. Kieff.** 1984. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. Proc. Natl. Acad. Sci. USA **81:**7632–7636.
10. **de Alboran, I. M., R. C. O'Hagan, F. Gartner, B. Malynn, L. Davidson, R. Rickert, K. Rajewsky, R. A. DePinho, and F. W. Alt.** 2001. Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity **14:**45–55.
11. **Delecluse, H. J., T. Hilsendegen, D. Pich, R. Zeidler, and W. Hammerschmidt.** 1998. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc. Natl. Acad. Sci. USA **95:**8245–8250.
12. **Fahraeus, R., L. Palmqvist, A. Nerdstedt, S. Farzad, L. Rymo, and S. Lain.** 1994. Response to cAMP levels of the Epstein-Barr virus EBNA2-inducible LMP1 oncogene and EBNA2 inhibition of a PP1-like activity. EMBO J. **13:**6041–6051.
13. **Floettmann, J. E., K. Ward, A. B. Rickinson, and M. Rowe.** 1996. Cytostatic effect of Epstein-Barr virus latent membrane protein-1 analyzed using tetracycline-regulated expression in B cell lines. Virology **223:**29–40.
14. **Fuentes-Panana, E. M., R. Peng, G. Brewer, J. Tan, and P. D. Ling.** 2000. Regulation of the Epstein-Barr virus C promoter by AUF1 and the cyclic AMP/protein kinase A signaling pathway. J. Virol. **74:**8166–8175.
15. **Gardella, T., P. Medveczky, T. Sairenji, and C. Mulder.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. **50:**248–254.
16. **Gordadze, A. V., R. Peng, J. Tan, G. Liu, R. Sutton, B. Kempkes, G. W. Bornkamm, and P. D. Ling.** 2001. Notch1IC partially replaces EBNA2 function in B cells immortalized by Epstein-Barr virus. J. Virol. **75:**5899–5912.
17. **Grossman, S. R., E. Johannsen, X. Tong, R. Yalamanchili, and E. Kieff.** 1994. The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein. Proc. Natl. Acad. Sci. USA **91:**7568–7572.
18. **Grundhoff, A. T., E. Kremmer, O. Tureci, A. Glieden, C. Gindorf, J. Atz, N. Mueller-Lantzsch, W. H. Schubach, and F. A. Grasser.** 1999. Characterization of DP103, a novel DEAD box protein that binds to the Epstein-Barr virus nuclear proteins EBNA2 and EBNA3C. J. Biol. Chem. **274:**19136–19144.
19. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340:**393–397.
20. **Harada, S., R. Yalamanchili, and E. Kieff.** 2001. Epstein-Barr virus nuclear protein 2 has at least two N-terminal domains that mediate self-association. J. Virol. **75:**2482–2487.

21. **Harada, S., R. Yalamanchili, and E. Kieff.** 1998. Residues 231 to 280 of the Epstein-Barr virus nuclear protein 2 are not essential for primary B-lymphocyte growth transformation. J. Virol. **72:**9948–9954.
22. **Hatfull, G., A. T. Bankier, B. G. Barrell, and P. J. Farrell.** 1988. Sequence analysis of Raji Epstein-Barr virus DNA. Virology **164:**334–340.
23. **Henkel, T., P. D. Ling, S. D. Hayward, and M. G. Peterson.** 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. Science **265:**92–95.
24. **Hofelmayr, H., L. J. Strobl, C. Stein, G. Laux, G. Marschall, G. W. Bornkamm, and U. Zimber-Strobl.** 1999. Activated mouse notch1 transactivates Epstein-Barr virus nuclear antigen 2-regulated viral promoters. J. Virol. **73:**2770–2780.
25. **Johannsen, E., E. Koh, G. Mosialos, X. Tong, E. Kieff, and S. R. Grossman.** 1995. Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by Jκ and PU.1. J. Virol. **69:**253–262.
26. **Kaiser, C., G. Laux, D. Eick, N. Jochner, G. W. Bornkamm, and B. Kempkes.** 1999. The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2. J. Virol. **73:**4481–4484.
27. **Kaye, K. M., K. M. Izumi, and E. Kieff.** 1993. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc. Natl. Acad. Sci. USA **90:**9150–9154.
28. **Kaykas, A., and B. Sugden.** 2000. The amino-terminus and membrane-spanning domains of LMP-1 inhibit cell proliferation. Oncogene **19:**1400–1410.
29. **Kempkes, B., M. Pawlita, U. Zimber-Strobl, G. Eissner, G. Laux, and G. W. Bornkamm.** 1995. Epstein-Barr virus nuclear antigen 2-estrogen receptor fusion proteins transactivate viral and cellular genes and interact with RBP-J kappa in a conditional fashion. Virology **214:**675–679.
30. **Kempkes, B., D. Spitkovsky, P. Jansen-Durr, J. W. Ellwart, E. Kremmer, H. J. Delecluse, C. Rottenberger, G. W. Bornkamm, and W. Hammerschmidt.** 1995. B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2. EMBO J. **14:**88–96.
31. **Kieff, E.** 1996. Epstein-Barr virus and its replication, p. 107–172. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
32. **Kilger, E., A. Kieser, M. Baumann, and W. Hammerschmidt.** 1998. Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. EMBO J. **17:**1700–1709.
33. **Kirchmaier, A. L., and B. Sugden.** 1995. Plasmid maintenance of derivatives of *oriP* of Epstein-Barr virus. J. Virol. **69:**1280–1283.
34. **Konishi, K., S. Maruo, H. Kato, and K. Takada.** 2001. Role of Epstein-Barr virus-encoded latent membrane protein 2A on virus-induced immortalization and virus activation. J. Gen. Virol. **82:**1451–1456.
35. **Laux, G., B. Adam, L. J. Strobl, and F. Moreau-Gachelin.** 1994. The Spi-1/PU.1 and Spi-B ets family transcription factors and the recombination signal binding protein RBP-J kappa interact with an Epstein-Barr virus nuclear antigen 2 responsive cis-element. EMBO J. **13:**5624–5632.
36. **Laux, G., F. Dugrillon, C. Eckert, B. Adam, S. U. Zimber, and G. W. Bornkamm.** 1994. Identification and characterization of an Epstein-Barr virus nuclear antigen 2-responsive *cis* element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes. J. Virol. **68:**6947–6958.
37. **Ling, P. D., and S. D. Hayward.** 1995. Contribution of conserved amino acids in mediating the interaction between EBNA2 and CBF1/RBPJκ. J. Virol. **69:**1944–1950.
38. **Ling, P. D., D. R. Rawlins, and S. D. Hayward.** 1993. The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein. Proc. Natl. Acad. Sci. USA **90:**9237–9241.
39. **Ling, P. D., J. J. Ryon, and S. D. Hayward.** 1993. EBNA-2 of herpesvirus papio diverges significantly from the type A and type B EBNA-2 proteins of Epstein-Barr virus but retains an efficient transactivation domain with a conserved hydrophobic motif. J. Virol. **67:**2990–3003.
40. **Mann, K. P., D. Staunton, and D. A. Thorley-Lawson.** 1985. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. J. Virol. **55:**710–720.
41. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. **65:**55–63.
42. **Pajic, A., D. Spitkovsky, B. Christoph, B. Kempkes, M. Schuhmacher, M. S. Staege, M. Brielmeier, J. Ellwart, F. Kohlhuber, G. W. Bornkamm, A. Polack, and D. Eick.** 2000. Cell cycle activation by c-myc in a Burkitt's lymphoma model cell line. Int. J. Cancer **87:**787–793.
43. **Peng, R., A. V. Gordadze, E. M. Fuentes Panana, F. Wang, J. Zong, G. S. Hayward, J. Tan, and P. D. Ling.** 2000. Sequence and functional analysis of EBNA-LP and EBNA2 proteins from nonhuman primate lymphocryptoviruses. J. Virol. **74:**379–389.
44. **Rickinson, A. B., and E. Kieff.** 1996. Epstein-Barr virus, p. 2397–2446. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
45. **Sugden, B., M. Phelps, and J. Domoradzki.** 1979. Epstein-Barr virus DNA is amplified in transformed lymphocytes. J. Virol. **31:**590–595.
46. **Sugden, B., and N. Warren.** 1988. Plasmid origin of replication of Epstein-Barr virus, oriP, does not limit replication in cis. Mol. Biol. Med. **5:**85–94.
47. **Sung, N. S., S. Kenney, D. Gutsch, and J. S. Pagano.** 1991. EBNA-2 transactivates a lymphoid-specific enhancer in the *Bam*HI C promoter of Epstein-Barr virus. J. Virol. **65:**2164–2169.
48. **Swaminathan, S.** 1996. Characterization of Epstein-Barr virus recombinants with deletions of the BamHI C promoter. Virology **217:**532–541.
49. **Tsang, S. F., F. Wang, K. M. Izumi, and E. Kieff.** 1991. Delineation of the *cis*-acting element mediating EBNA-2 transactivation of latent infection membrane protein expression. J. Virol. **65:**6765–6771.
50. **Wang, F., S. F. Tsang, M. G. Kurilla, J. I. Cohen, and E. Kieff.** 1990. Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. J. Virol. **64:**3407–3416.
51. **Wendel-Hansen, V., W. Tao, M. Ericson, G. Klein, and A. Rosen.** 1992. Cell phenotype (CD23)-dependent variation in EBV genome copy numbers within lymphoblastoid cell lines (LCL). Int. J. Cancer **50:**589–592.
52. **Wu, D. Y., G. V. Kalpana, S. P. Goff, and W. H. Schubach.** 1996. Epstein-Barr virus nuclear protein 2 (EBNA2) binds to a component of the human SNF-SWI complex, hSNF5/Ini1. J. Virol. **70:**6020–6028.
53. **Yalamanchili, R., S. Harada, and E. Kieff.** 1996. The N-terminal half of EBNA2, except for seven prolines, is not essential for primary B-lymphocyte growth transformation. J. Virol. **70:**2468–2473.
54. **Yalamanchili, R., X. Tong, S. Grossman, E. Johannsen, G. Mosialos, and E. Kieff.** 1994. Genetic and biochemical evidence that EBNA 2 interaction with a 63-kDa cellular GTG-binding protein is essential for B lymphocyte growth transformation by EBV. Virology **204:**634–641.
55. **Zhao, B., and C. E. Sample.** 2000. Epstein-Barr virus nuclear antigen 3C activates the latent membrane protein 1 promoter in the presence of Epstein-Barr virus nuclear antigen 2 through sequences encompassing an spi-1/Spi-B binding site. J. Virol. **74:**5151–5160.
56. **Zhou, S., M. Fujimuro, J. J. Hsieh, L. Chen, and S. D. Hayward.** 2000. A role for SKIP in EBNA2 activation of CBF1-repressed promoters. J. Virol. **74:**1939–1947.
57. **Zhou, S., M. Fujimuro, J. J. Hsieh, L. Chen, A. Miyamoto, G. Weinmaster, and S. D. Hayward.** 2000. SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function. Mol. Cell. Biol. **20:**2400–2410.
58. **Zimber-Strobl, U., B. Kempkes, G. Marschall, R. Zeidler, C. Van Kooten, J. Banchereau, G. W. Bornkamm, and W. Hammerschmidt.** 1996. Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. EMBO J. **15:**7070–7078.
59. **Zimber-Strobl, U., E. Kremmer, F. Grasser, G. Marschall, G. Laux, and G. W. Bornkamm.** 1993. The Epstein-Barr virus nuclear antigen 2 interacts with an EBNA2 responsive cis-element of the terminal protein 1 gene promoter. EMBO J. **12:**167–175.
60. **Zimber-Strobl, U., L. J. Strobl, C. Meitinger, R. Hinrichs, T. Sakai, T. Furukawa, T. Honjo, and G. W. Bornkamm.** 1994. Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless. EMBO J. **13:**4973–4982.
61. **Zimber-Strobl, U., K. O. Suentzenich, G. Laux, D. Eick, M. Cordier, A. Calender, M. Billaud, G. M. Lenoir, and G. W. Bornkamm.** 1991. Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene. J. Virol. **65:**415–423.