JOURNAL OF VIROLOGY, Mar. 1992, p. 1795–1798
0022-538X/92/031795-04$02.00/0

Vol. 66, No. 3

A Chimera of EBNA1 and the Estrogen Receptor Activates Transcription but Not Replication

TIM MIDDLETON* AND BILL SUGDEN

McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, Wisconsin 53706

Received 3 September 1991/Accepted 7 December 1991

DNA replication from the plasmid origin of replication of Epstein-Barr virus requires one viral protein, EBNA1. This protein also acts as a transcriptional activator. Mutational analyses of EBNA1 have led to the conclusion that it supports transcription and DNA replication similarly. Such analyses have not probed the DNA-binding domain of EBNA1. To test whether domains of EBNA1 specifically required for either transcription or replication lie within its DNA-binding domain, we constructed a functional transcriptional activator by placing the EBNA1 DNA-binding domain in the context of the activation domains of the estrogen receptor. This hybrid protein did not support DNA replication, which indicates that the DNA-binding domain does not contain a replication-specific domain that can function along with heterologous transcriptional activating domains.

Infection of B lymphocytes by Epstein-Barr virus usually results in replication of the viral DNA in a fashion similar to that of mammalian chromosomal replicons. The viral DNA is replicated once per cell cycle in S phase and maintains its copy number stably for many cell generations in human somatic cells (1, 2, 22). The origin of DNA replication, *oriP*, consists of a family of direct repeats (FR), and an element of dyad symmetry (DS; 7, 15, 16, 23; Fig. 1A). Both FR and DS are made up of multiple binding sites for viral protein EBNA1. EBNA1 is the only viral protein required for *oriP* function (12, 23); the rest of the replication proteins are contributed by the cell.

In addition to supporting DNA replication, EBNA1 activates transcriptional enhancement from FR, both with heterologous promoters (15) and from a promoter located about 3 kbp away in Epstein-Barr virus (18). There is remarkable similarity in the function of the enhancer element for DNA replication and transcription. Unlike a number of transcription-replication enhancer elements (6), the FR element supports initiation of DNA replication at DS with the same independence of position and orientation with which it activates transcription (5, 15). This similarity between the effect of EBNA1 on DNA replication and its effect on transcriptional enhancement is heightened by the findings of extensive mutational analyses of EBNA1. No mutations have been found in EBNA1 that affect one of these processes without affecting the other (13, 21). This apparent overlap in function is intriguing because it suggests that understanding transcriptional activation by EBNA1 may indicate how EBNA1 also supports DNA replication. These mutational analyses would not reveal replication-specific functions in the region of EBNA1 required for DNA binding, however, because mutations in this region abolish all of the EBNA1 activities that have been studied. This region alone, which constitutes approximately one-third of the protein, does not support either transcription or replication (13, 21), but it might supply an activity (e.g., binding of a helicase or other replication-specific proteins) which, in conjunction with a transcriptional activator domain, supports DNA replication. We tested for such an activity in the DNA-binding domain by fusing this domain to heterologous domains from a transcriptional activator protein that does not appear to be associated with DNA replication. This hybrid protein is capable of activating transcription via the FR enhancer but does not support *oriP*-mediated DNA replication. This finding indicates that the DNA-binding region of EBNA1 does not encode activities that need only a transcriptional activating domain to support replication.

We constructed a hybrid protein consisting of the terminal third of EBNA1 in place of the DNA-binding domain of the estrogen receptor (ER-EBNA1, Fig. 1B). ER-EBNA1 should be capable of supporting *oriP*-mediated replication if binding of any transcriptional activator to *oriP* will suffice for activation of replication, or if a replication-specific function resides in the region required for DNA binding, with the rest of the protein supplying generic functions that are needed for both transcription and DNA replication. The human estrogen receptor (10) and EBNA1 open reading frames (4) have been described previously. The estrogen receptor was derived from plasmid HE63 (provided by Pierre Chambon). The *Sma*I site at amino acid 450 of EBNA1 was converted to a *Kpn*I site, and the *Sac*II site at amino acid 619 was converted to an *Xho*I site. The *Kpn*I-to-*Xho*I fragment was used to replace the *Kpn*I-to-*Xho*I fragment of HE63 to generate ER-EBNA1. The DNAs encoding EBNA1, ER-EBNA1, and the estrogen receptor were inserted into expression plasmid p763. p763 was constructed by making the following modifications to pHEBo (13). (i) The promoter-enhancer from the immediate-early region of human cytomegalovirus (19) was inserted into the *Cla*I and *Hin*dIII sites, (ii) the polyadenylation sites from simian virus 40 (*Bam*HI to *Bcl*I) were inserted into the *Bam*HI site, and (iii) the *Hin*dIII site was converted to an *Xho*I site.

Vectors that express resistance to hygromycin B plus either EBNA1, the estrogen receptor, or ER-EBNA1 were separately introduced into human osteosarcoma cell line 143. This cell line supports both efficient EBNA1-mediated activation of transcription and EBNA1-mediated DNA replication from *oriP*. Hygromycin B resistant clones of cells that express the desired proteins were selected. Expression of EBNA1 or ER-EBNA1 in these clones was assayed by immunoblot analysis using antibodies that recognize the EBNA1 DNA-binding domain, which is common to both

* Corresponding author.

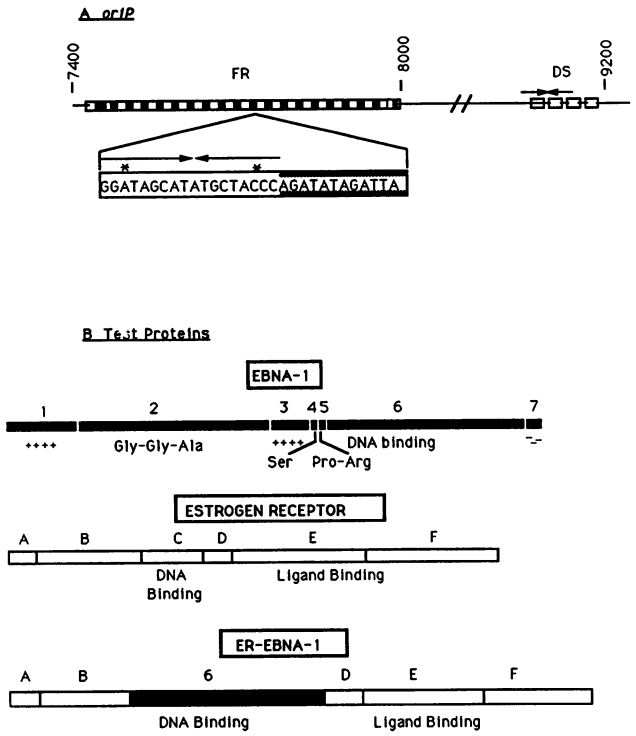

FIG. 1. (A) EBV *oriP* origin of replication. The two *cis*-acting elements of the origin are indicated as FR, the family of repeats which can function as an enhancer, and DS, which contains the dyad symmetry element within which DNA replication probably initiates (7). FR consists of 20 copies of a 30-bp repeated sequence to which EBNA1 binds; a consensus sequence is indicated in the enlargement. This consensus sequence is composed of an 18-bp palindrome (arrows) with one mismatch (asterisks) and 12 nonpalindromic bp (shaded). DS contains four degenerate copies of the consensus sequence found in FR. The arrows above DS denote a 65-bp dyad symmetry element that covers a region containing two copies of the 30-bp repeated sequence (boxes). The numbers correspond to the sequence of EBV DNA according to reference 4. (B) Structures of the test proteins. Some of the structural characteristics of EBNA1 (as determined from the DNA sequence [4]) that may be functionally important are shown (adapted from reference 21). Regions 1 and 3 have a high basic amino acid content. They are interrupted by a region composed entirely of glycine and alanine, which is dispensable for transcriptional activation and replication. Regions 4 and 5 are rich in serine and proline plus arginine, respectively. In addition to the DNA-binding domain (region 7), portions of regions 1, 3, and 4 are required for EBNA1 function (21). The estrogen receptor is depicted as indicated in reference 10. The letters indicate domains of the protein some of which have identified functions. The region of EBNA1 shown to be sufficient for binding to *oriP* DNA (3, 9, 14) (EBV bp 109,298 to 109,802 according to reference 4, indicated as region 6) was used to replace amino acids 185 to 250 of the human estrogen receptor (10) (region C) to generate the ER-EBNA1 gene.

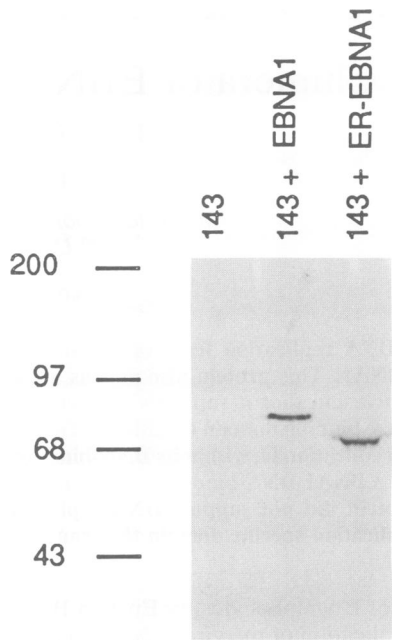

FIG. 2. Expression of EBNA1 and ER-EBNA1 in 143 cells. EBNA1 and ER-EBNA1 genes were placed in a plasmid containing the cytomegalovirus immediate-early enhancer-promoter, and the hygromycin phosphotransferase gene (8). Plasmids were transfected into 143 cells by electroporation, and clones of cells were selected for resistance to hygromycin B. Hygromycin B-resistant colonies were expanded, cells were solubilized, and proteins were resolved on sodium dodecyl sulfate–10% polyacrylamide gels, electrophoretically transferred to nitrocellulose, and probed with antibodies that recognize the portion of EBNA1 that is common to EBNA1 and ER-EBNA1 (17). Stained molecular weight markers consisted of myosin (heavy chain), phosphorylase *b*, bovine serum albumin, and ovalbumin. Their sizes are shown to the left in thousands.

proteins. Figure 2 shows that in the clones used for the following experiments, EBNA1 and ER-EBNA1 were expressed at comparable levels. There is an apparent discrepancy between the predicted and apparent sizes of EBNA1 and ER-EBNA1. ER-EBNA1 contains 702 amino acids, and EBNA1 contains 641 amino acids. However, this discrepancy can be explained by the fact that EBNA1 migrates with an apparent mobility of 75 to 80 kilodaltons in sodium dodecyl sulfate-polyacrylamide gels. Expression of the estrogen receptor was monitored by the ability of cells to bind [^{3}H]estradiol and by activation of a reporter gene downstream of an estrogen-responsive element (data not shown).

These clones were used as recipients for introduction of reporter plasmids to test for activation of transcription and replication. First, reporter plasmids that express a firefly luciferase gene from a herpes simplex virus (HSV) thymidine kinase (TK) promoter and either carry the FR enhancer of *oriP* (FR-TK Luc) or lack this enhancer (TK-Luc, Table 1) were used to test for transcriptional activation. In these recipient cells, EBNA1 stimulated luciferase activity approximately 25-fold with a reporter plasmid containing the FR enhancer, relative to the equivalent plasmid lacking the enhancer ($P < 0.01$). The level of expression appeared to be decreased slightly by addition of estradiol, but this decrease was not statistically significant.

ER-EBNA1 also stimulated luciferase activity from this plasmid, but only in the presence of estradiol ($P < 0.01$). The apparent fold stimulation observed with ER-EBNA1 is lower than that seen with EBNA1 because the basal level of synthesis is about 20-fold higher than that in the EBNA1-expressing cell line. It is not clear whether the difference in basal expression between the two cell lines represents clonal variation or an effect of ER-EBNA1 on the TK promoter in the absence of estradiol. Nevertheless, these experiments demonstrated that ER-EBNA1 activates transcription upon binding to FR in the presence of estradiol.

We also confirmed that ER-EBNA1 can productively

TABLE 1. Activation of transcription by EBNA1 and ER-EBNA1

Reporter plasmid	Estradiol concn (μM)	Avg RLU ± SD[a]	
		EBNA1[b]	ER-EBNA1[c]
TK-luciferase[d]	0	1.0 ± 0.6	1.0 ± 0.6
TK-luciferase	10	0.8 ± 0.3	1.0 ± 0.3
FR-TK-luciferase[e]	0	26.8 ± 16.1	1.0 ± 0.9
FR-TK-luciferase	10	8.4 ± 2.6	3.7 ± 1.0
DS-TK-luciferase[f]	0	9.0 ± 1.1	0.4 ± 0.1
DS-TK-luciferase	10	8.3 ± 1.8	1.4 ± 0.2

[a] Results are expressed as average relative light units (RLU) per one-third of the cells from a 90-mm-diameter tissue culture dish. Values are normalized to those from the TK-luciferase plasmid in the absence of estradiol. Actual values for the TK-luciferase plasmid were 28,000 and 645,000 relative light units for the cell lines carrying EBNA1 and ER-EBNA1, respectively. Treatments for which differences are statistically different, as determined by the Wilcoxon rank-sum test, are noted in the text. The results shown are based on duplicate samples from four experiments with luciferase reporter genes. Equivalent results were obtained with additional experiments done with reporter plasmids containing chloramphenicol acetyltransferase as the reporter gene.

[b] 143-derived cell line expressing EBNA1.

[c] 143-derived cell line expressing ER-EBNA1.

[d] HSV TK promoter upstream of the firefly luciferase gene.

[e] TK-luciferase plus the FR enhancer element of *oriP*.

[f] TK-luciferase plus five copies of the DS element of *oriP*.

interact with DS. It is known that multiple copies of DS can function as a transcriptional enhancer (20). A reporter plasmid containing five copies of the DS element upstream of the HSV TK promoter and the luciferase gene was transfected into 143 cells expressing EBNA1 or ER-EBNA1 (Table 1). In cells synthesizing EBNA1, luciferase activity was stimulated about eightfold by the presence of the multiple DS elements ($P = 0.05$). In cells with ER-EBNA1, the basal activity in the absence of estradiol is actually decreased relative to that seen in the absence of the DS elements ($P < 0.01$). However, addition of estradiol results in a threefold increase in luciferase activity ($P < 0.01$). Thus, ER-EBNA1 is capable of binding to the DS element and stimulating transcriptional activity.

We determined with two kinds of experiments whether ER-EBNA1, in the presence of its ligand, can support DNA replication mediated by *oriP*. First, the derivatives of 143 cells that express EBNA1, the estrogen receptor, or ER-EBNA1 were used as recipients for a plasmid containing *oriP*. The extent of replication of the introduced *oriP* plasmids was measured 72 h after their introduction by extracting nonchromosomal DNAs from these cells and digesting the unreplicated input DNAs with the *Dpn*I endonuclease. The DNA was resolved electrophoretically and transferred to nylon membranes, and *oriP* plasmid DNA was detected by nucleic acid hybridization. In this short-term experiment, only EBNA1 supported detectable *oriP*-mediated DNA replication (Fig. 3). The design of the second experiment took advantage of the fact that 143 cells lack TK. An *oriP* plasmid containing the TK gene of HSV type 1 was introduced in the presence of estradiol into the derivatives of 143 cells which express EBNA1, the estrogen receptor, or ER-EBNA1. TK-positive cells were selected, and the state of the introduced *oriP*-TK plasmids was ascertained after the cells had been propagated for greater than 20 generations. *oriP*-mediated DNA replication would permit the introduced DNA to be maintained extrachromosomally. Failure of *oriP*

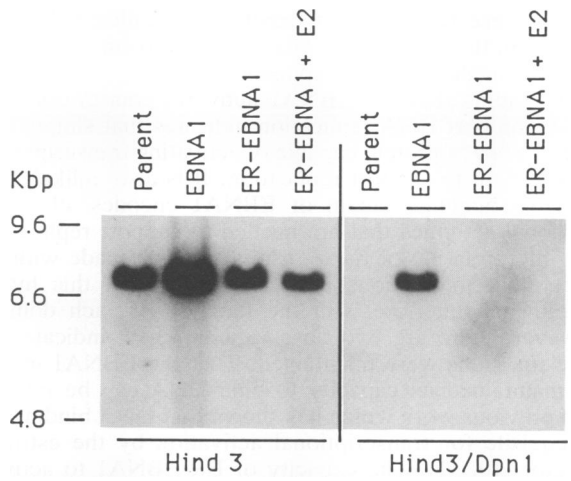

FIG. 3. Transient *oriP*-mediated DNA replication. Parental 143 cells or cells expressing EBNA1, ER-EBNA1, or the estrogen receptor were transfected with an *oriP*-containing plasmid. Small extrachromosomal DNAs were selectively extracted 72 h after their introduction into the cells and analyzed by Southern transfer for DNAs that had replicated. The left panel shows total extrachromosomal DNAs, while the right panel represents replicated DNA after digestion of the unreplicated input DNA. Cell lines were transfected by electroporation with plasmid pHEBO (16), and extrachromosomal DNAs were isolated by selective Hirt extraction. The extracted, purified DNA was digested with *Hin*dIII (left panel) or *Hin*dIII and *Dpn*I, separated on a 0.7% agarose gel, transferred to nylon membranes, and hybridized to ^{32}P-labeled Epstein-Barr virus DNA (bp 4163 to 7315 according to reference 4).

to function would permit the introduced DNAs to be maintained in the recipient cells only by being integrated into the host cell chromosomes. Only the 143 cells expressing EBNA1 supported extrachromosomal maintenance of the *oriP* plasmid (Fig. 4). Extrachromosomal maintenance of the *oriP* plasmid by ER-EBNA1 at a level 50-fold lower than that supported by EBNA1 would have been detectable by this

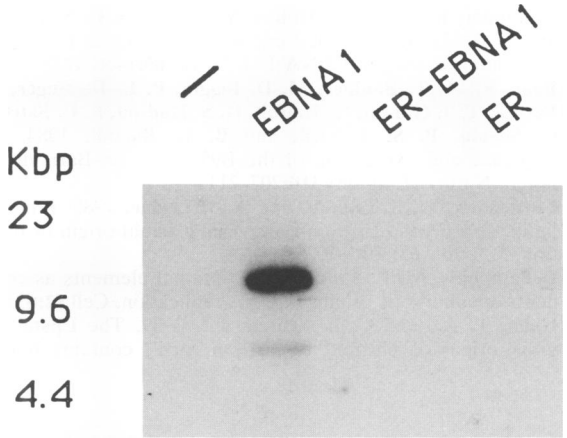

FIG. 4. Maintenance of an *oriP*-containing plasmid extrachromosomally. 143 cells or 143 cells expressing EBNA1, ER-EBNA1, or the estrogen receptor (lanes 1 to 4, respectively) were transfected with a plasmid containing *oriP* and the TK gene of HSV. TK-positive cells were selected, propagated for 20 generations, and tested for the presence of extrachromosomal *oriP* DNA by probing DNA isolated from Hirt extracts as described in the legend to Fig. 3.

assay. These two kinds of experiments indicate that ER-EBNA1 in the presence of estradiol fails to support detectable *oriP*-mediated DNA replication.

The finding that ER-EBNA1 activates transcription but fails to support DNA replication indicates that simply supplying to *oriP* a protein capable of activating transcription is not sufficient to support replication. It is also unlikely that the DNA-binding domain of EBNA1 encodes all of the additional activities that are needed to support replication. Any interpretation of these results must be made with the caveat that interactions between domains of this hybrid protein may interfere with the function of each domain. However, there are two observations which indicate that these functions were maintained. That the EBNA1 moiety has maintained its capacity to bind DNA can be inferred from previous work which has shown that DNA binding is a prerequisite for transcriptional activation by the estrogen receptor (10, 11). The capacity of ER-EBNA1 to activate transcription in a hormone-dependent manner also indicates that the functions of the estrogen receptor were maintained.

An *oriP*-binding protein that activates transcription but not replication provides a model to define more carefully the portions of EBNA1 that are required to support replication. Providing additional portions of EBNA1 to ER-EBNA1 to generate a protein that again supports DNA replication may identify in more detail the regions of EBNA1 that interact with cellular proteins to support replication.

We thank P. Chambon, G. Ryffel, and S. Subramani for providing pHE63, pA2(−260/−87)tkCAT8$^+$ and pA2(−331/−87)tkCAT8$^+$, and the luciferase gene, respectively, and N. Drinkwater, P. Farnham, J. Jones, P. Lambert, A. Poland, H. Temin, and our fellow laboratory members for reviewing the manuscript.

This work was supported by Public Health Service grants CA-22443 and CA-07175 from the National Cancer Institute (B.S.), by American Cancer Society grant IN-35-31-19 (T.M.), and by a fellowship from the Cancer Research Institute (T.M.).

REFERENCES

1. **Adams, A.** 1987. Replication of latent Epstein-Barr virus genomes in Raji cells. J. Virol. **61**:1743–1746.
2. **Adams, A., T. C. Pozos, and H. V. Purvey.** 1989. Replication of latent Epstein-Barr virus genomes in normal and malignant lymphoid cells. Int. J. Cancer **44**:560–564.
3. **Ambinder, R. F., M. A. Mullen, Y. N. Chang, G. S. Hayward, and S. D. Hayward.** 1991. Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. J. Virol. **65**:1466–1478.
4. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
5. **Chittenden, T., S. Lupton, and A. J. Levine.** 1989. Functional limits of *oriP*, the Epstein-Barr virus plasmid origin of replication. J. Virol. **63**:3016–3025.
6. **DePamphilis, M. L.** 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell **52**:635–638.
7. **Gahn, T. A., and C. L. Schildkraut.** 1989. The Epstein-Barr virus origin of plasmid replication, oriP, contains both the initiation and termination sites of DNA replication. Cell **58**:527–535.
8. **Gritz, L., and J. Davies.** 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in Escherichia coli and Saccharomyces cerevisiae. Gene **25**:179–188.
9. **Inoue, N., S. Harada, T. Honma, T. Kitamura, and K. Yanagi.** 1991. The domain of Epstein-Barr virus nuclear antigen 1 essential for binding to oriP region has a sequence fitted for the hypothetical basic-helix-loop-helix structure. Virology **182**:84–93.
10. **Kumar, V., S. Green, G. Stack, M. Berry, J. R. Jin, and P. Chambon.** 1987. Functional domains of the human estrogen receptor. Cell **51**:941–951.
11. **Kumar, V., S. Green, A. Staub, and P. Chambon.** 1987. Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. EMBO J. **5**:2231–2236.
12. **Lupton, S., and A. J. Levine.** 1985. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence in Epstein-Barr virus-derived plasmids in human cells. Mol. Cell. Biol. **5**:2533–2542.
13. **Polvino-Bodnar, M., J. Kiso, and P. A. Schaffer.** 1988. Mutational analysis of Epstein-Barr virus nuclear antigen 1 (EBNA-1). Nucleic Acids Res. **16**:3415–3435.
14. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42**:859–868.
15. **Reisman, D., and B. Sugden.** 1986. *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6**:3838–3846.
16. **Reisman, D., J. Yates, and B. Sugden.** 1985. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting components. Mol. Cell. Biol. **5**:1822–1832.
17. **Sternås, L., T. Middleton, and B. Sugden.** 1990. The average number of molecules of Epstein-Barr virus nuclear antigen 1 per cell does not correlate with the average number of Epstein-Barr virus (EBV) DNA molecules per cell among different clones of EBV-immortalized cells. J. Virol. **64**:2407–2410.
18. **Sugden, B., and N. Warren.** 1989. A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. J. Virol. **63**:2644–2649.
19. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.
20. **Wysokenski, D. A., and J. L. Yates.** 1989. Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within *oriP* of Epstein-Barr virus. J. Virol. **63**:2657–2666.
21. **Yates, J. L., and S. M. Camiolo.** 1988. Dissection of DNA replication and enhancer activation functions of Epstein-Barr virus nuclear antigen 1. Cancer Cells **6**:197–205.
22. **Yates, J. L., and N. Guan.** 1991. Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. J. Virol. **65**:483–488.
23. **Yates, J. L., N. Warren, and B. Sugden.** 1985. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature (London) **313**:812–815.