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Activation of *oriLyt,* the Lytic Origin of DNA Replication

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oriLyt, the *cis*-acting element of the lytic origin of DNA replication of Epstein–Barr virus, is activated by the viral transactivator BZLF1 which belongs to the extended bZIP class of transcription factors. Seven binding sites for BZLF1, so-called ZRE sites, are located within *oriLyt.* By mutational analysis of individual ZRE sites, we found that lytic DNA replication is dependent on only four of these sites which colocate with the promoter of the BHLF1 gene. The remaining three ZRE sites distal to the BHLF1 promoter were dispensable for DNA replication and did not contribute to long-range transcriptional activation of this promoter by BZLF1. This finding indicated that a similar set of ZRE sites is involved in DNA replication and transcriptional activation. To determine the function of BZLF1 in DNA replication, BZLF1 mutants with successive deletions in the transactivation domain were analyzed in replication assays. Unexpectedly, most BZLF1 mutants which failed to support DNA replication were found to be equally defective in transcriptional activation. Therefore, similar *trans*-acting domains of BZLF1 are involved both in replication and in transcription.

INTRODUCTION

In eukaryotic origins of DNA replication, transcriptional *cis*- and *trans*-acting elements are involved in the regulation of replication (DePamphilis, 1993; Heintz, 1992; for reviews). The link between transcription and replication is unclear but the two mechanisms utilize particular enhancer-binding proteins to activate both processes. One role for enhancer-binding proteins in regulating replication and transcription could be their effect on the chromatin structure (Felsenfeld, 1992; for a review). A second role for this class of proteins could be to interact with the basal replication as well as the basal transcription apparatus. Both functions involve distinct families of general factors, each of them responsible for the assembly of a multicomponent protein complex at initiation sites (Conaway and Conaway, 1993; Kornberg and Baker, 1992; Stillman, 1989; for reviews).

The ability of transcription factors to activate viral or cellular DNA replication is well documented. A number of transcription factors activate DNA replication under certain conditions; these include NF1/CTF (Cheng and Kelly, 1989), AP-1 (Guo and DePamphilis, 1992), OTF-1 (Oct-1) (O'Neill *et al.,* 1988), VP16 (Haigh *et al.,* 1990), Gal4 (Bennett-Cook and Hassell, 1991), E2 (Kuo *et al.,* 1994; Melendy *et al.,* 1995; Ustav *et al.,* 1993; Yang *et al.,* 1991), and ABF1 (Diffley and Stillman, 1989). The mode by which the transcription factors interact with the different eukaryotic replication systems is different (DePamphilis, 1993; van der Vliet, 1991; for reviews) but it is still unclear how transcriptional elements facilitate replication, although an interaction between the transcription factors VP16 and Gal4 and replication factor A has been described recently (He *et al.,* 1993; Li and Botchan, 1993).

We are interested in the origin of DNA replication which drives the amplification of the herpesvirus genome of Epstein–Barr virus (EBV) during its lytic life cycle (Hammerschmidt and Mankertz, 1991; for a review). This origin, called *oriLyt,* is structurally complex and is made up of two core components and several auxiliary regions which are only poorly defined (Hammerschmidt and Sugden, 1988; Schepers *et al.,* 1993b). *oriLyt* is located within the promoter regions of the BHLF1 and BHRF1 genes, which are transcriptionally activated during the early lytic phase of EBV by viral transcription factors (Chavrier *et al.,* 1989; Chevallier-Greco *et al.,* 1989, 1986; Cox *et al.,* 1990; Gruffat *et al.,* 1990; Hardwick *et al.,* 1988; Laux *et al.,* 1985; Lieberman *et al.,* 1989, 1990). A crucial factor for this activation is the product of the viral BZLF1 gene which is also called EB1, zta, Z, or ZEBRA. One of the two essential components of *oriLyt,* the upstream component (Fig. 1), is nearly identical with the BHLF1 promoter which is transactivated directly by BZLF1 (Chavrier *et al.,* 1989; Chevallier-Greco *et al.,* 1989, 1986; Cox *et al.,* 1990; Gruffat *et al.,* 1990; Hardwick *et al.,* 1988; Laux *et al.,* 1985; Lieberman *et al.,* 1989, 1990). BZLF1 belongs to the bZIP class of transcription factors with homologies to the AP-1 family (Chang *et al.,* 1990; Farrell *et al.,* 1989; Flemington and Speck, 1990; Flemington *et al.,* 1992;

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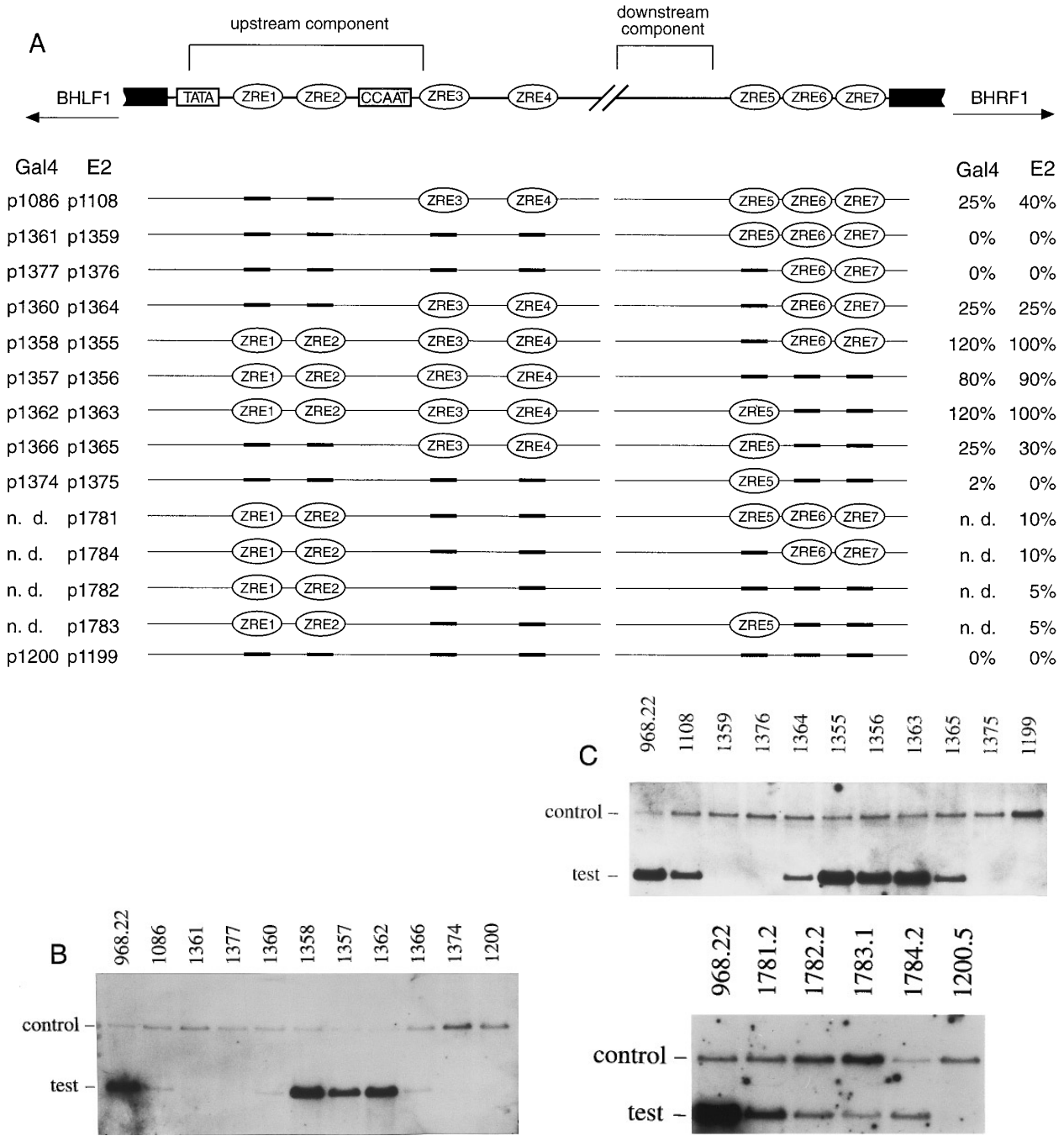

FIG. 1. Replication efficiency of *oriLyt* mutant plasmids with different combinations of functional ZRE sites. (A) *oriLyt* is located in a divergently transcribed promoter region which is shown schematically at the top. The fine structure of *oriLyt* encompassing the functional *cis*-acting elements is depicted. The flanking genes BHLF1 and BHRF1 are schematically shown on the left and right (black boxes). The upstream component (nucleotides 52,811 to 52,877; Baer *et al.,* 1984) consists of a cluster of functional redundant elements in *cis,* including basal promoter elements of the BHLF1-gene (TATA box, CCAAT box) and two binding sites for the EBV transactivator BZLF1 (ZRE1 and ZRE2). Two further ZRE sites are located in close vicinity (ZRE3 and ZRE4). The downstream component of *oriLyt* (nucleotides 53,341 to 53,428) is located 530 bp downstream and contains two binding sites for the ubiquitous transcription factor Sp1 or Sp1-related proteins (Gruffat *et al.,* 1995). Within the downstream component a 40-bp-long region could be identified (nucleotides 53,555 to 53,595), which shows in transient replication assays a remarkable sensitivity to point mutations (Gruffat *et al.,* 1995; Schepers *et al.,* 1993b). Between the downstream component and the BHRF gene are three additional binding sites for BZLF1 (ZRE5 to ZRE7) and two binding motifs for the EBV transactivator R and one for the cellular transcription factor MLTF (Ryon *et al.,* 1993) (not shown). The different mutants of *oriLyt* in which combinations of certain ZRE sites were replaced by Gal4- or E2-binding sites are presented underneath; the black bars indicate either Gal4 or E2 sites. As a consequence, each mutant plasmid exists in two versions carrying either Gal4 or E2 sites as listed in two columns on the left. Mean values of the relative replication efficiency of *oriLyt* mutant plasmids are summarized in the two columns on the right, the standard deviation was about ±20% of the mean values. Examples of the experimental data for the Gal4 and E2 replacement mutants are shown in B and C, respectively. On the left of the autoradiograms, the replication activity of the wild-type *oriLyt* plasmid p968.22 is shown. As an internal control which was used to correct for transfection efficiency the wild-type *oriLyt* plasmid p526 (Hammerschmidt and Sugden, 1988) was used in all transfections and its position on the autoradiograms is indicated. The signal strength of the individual *oriLyt* plasmids is a relative measure of the efficiency with which the *oriLyt* mutants replicated. The number of the plasmids refer to the mutant plasmids listed in A.

Taylor *et al.,* 1991) and acts as a homodimer and binds to specific DNA sequence motifs, mostly called ZRE sites (BZLF1-*r*esponsible-elements) (Chang *et al.,* 1990; Farrell *et al.,* 1989; Kouzarides *et al.,* 1991; Lieberman *et al.,* 1990; Taylor *et al.,* 1991). In the *oriLyt* region, BZLF1 binds to seven sites, ZRE1 to ZRE7 (Fig. 1). Two of them (ZRE1 and ZRE2) are located in the upstream component, between the TATA box and the CCAAT box of the BHLF1 promoter, ZRE3 and ZRE4 are located directly adjacent, whereas ZRE5 to ZRE7 are located beyond the downstream component of *oriLyt* (Gruffat *et al.,* 1995; Schepers *et al.,* 1993b) and near the promoter of the BHRF1 gene (Fig. 1). The consensus sequence of BZLF1 is quite variable (Chang *et al.,* 1990; Kouzarides *et al.,* 1991) and consequently the binding affinity of BZLF1 to these ZRE sites differs (Giot *et al.,* 1991; Gruffat *et al.,* 1990; Lieberman *et al.,* 1990). One function of BZLF1 is to interact with the general transcription factor TFIID that binds to the TATA box of the BHLF1 promoter (Chi and Carey, 1993; Chi *et al.,* 1995; Lieberman and Berk, 1991). In addition, BZLF1 interacts with TFIID-associated factors (TAFs) and stabilizes the formation of the transcription initiation complex (Lieberman *et al.,* 1994). Moreover, BZLF1 is the essential molecular switch for the induction of the lytic cycle of EBV's life cycle (Countryman and Miller, 1985; Takada *et al.,* 1986) and is also indispensable for the activation of *oriLyt* (Fixman and Hayward, 1995; Schepers *et al.,* 1993a).

The binding of BZLF1 to the ZRE sites within *oriLyt* has been found to be crucial for *oriLyt* function (Fixman and Hayward, 1995; Schepers *et al.,* 1993a). This observation indicated that the molecular basis of BZLF1 function could be the formation of a higher order complex to activate *oriLyt.* Such a complex could consist of BZLF1 molecules which link the seven ZRE sites over a distance of approximately 900 bp to distort the origin similar to what had been demonstrated for the plasmid origin of DNA replication of EBV (Frappier and O'Donnell, 1991; Mackey *et al.,* 1995). In order to test this hypothesis we mutated and replaced the ZRE sites within *oriLyt* with different combinations of nonfunctional sites. DNA replication was found to be dependent on four sites (ZRE1 to ZRE4), exclusively, which form a cluster within or in very close vicinity to the upstream component of *oriLyt.* Therefore, it is unlikely that BZLF1 activates lytic DNA replication by a simple conformational change of *oriLyt.* Rather it appears that the cluster of four BZLF1 binding sites and a yet to be identified factor (or factors) at the upstream component are crucial to provide functions which turn BZLF1 into a replication factor.

We have shown previously that the transactivation domain of BZLF1 is important to activate *oriLyt* DNA replication, indicating that this domain might contact other *trans*-acting factors (Schepers *et al.,* 1993a). To determine potential protein–protein interaction motifs, we designed a set of chimeric BZLF1 mutants with successive deletions in the transactivation domain. Unexpectedly, we found that regions critical for BZLF1-mediated DNA replication were nearly identical with regions which have been identified previously to be crucial for transcriptional activation of BZLF1-dependent promoters. This finding indicates that BZLF1 might utilize the same cofactors for DNA replication and transcriptional activation.

MATERIAL AND METHODS

Cell lines

D98HR1 cells were derived from a somatic cell hybrid between the EBV-genome-positive Burkitt's lymphoma cell line P3HR1 and the human epithelial cell line D98 (Glaser and Nonoyama, 1974). This adherent cell line contains approximately 20 copies of the EBV genome (data not shown) and was maintained in Dulbecco's modified Eagle's medium containing 5% fetal and 5% newborn calf serum. DG75 is an EBV-negative Burkitt lymphoma cell line (Ben Bassat *et al.,* 1977) which was grown in RPMI 1640 medium supplemented with 5% fetal and 5% newborn calf medium.

Recombinant plasmids

The plasmid p968.22 carrying wild-type *oriLyt* was constructed by ligating a 7.2-kb *Bam*HI/*Sal*I fragment from the EBV strain B95-8 (nucleotide coordinates 48,848 to 56,084) (Baer *et al.,* 1984) into a *Bam*HI/*Sal*I-cut pUC8 vector plasmid as described (Schepers *et al.,* 1993b). The plasmid pCMV-BZLF1 is an expression vector which efficiently induces the lytic phase of EBV's life cycle. The BZLF1 gene is expressed in this retroviral vector construct from the promoter of the immediate early genes of the human cytomegalovirus as described (Hammerschmidt and Sugden, 1988). Mutants of p968.22 were constructed by oligonucleotide-directed mutagenesis as described in detail (Schepers *et al.,* 1993b). All mutations were confirmed by DNA sequencing. Synthetic oligonucleotides for mutagenesis were purchased and purified on reverse-phase columns through high-pressure liquid chromatography. To perform the transient transcription assays, the 3.8-kb *Sac*II/*Eco*RI fragment of p968.22 (from 52,623 of EBV to the multiple cloning site in p968.22) carrying the BHLF1 open reading frame was replaced by the luciferase gene which was used as a reporter gene (de Wet *et al.,* 1987).

The BZLF1:E2 chimeric transcription factor which consists of the transactivation domain of BZLF1 (aa 1 to 169 of BZLF1) and the DNA binding and dimerization domain of the bovine papillomavirus factor E2 (aa 218 to 410 of E2) was cloned under the control of the human cytomegalovirus immediate early promoter as described previously (pCMV-BZLF1:E2) (Schepers *et al.,* 1993a). Similarly, the chimeric transcription factor Gal4:BZLF1 consisting of the Gal4 DNA binding domain (aa 1 to 141)

fused to the transactivation domain of BZLF1 (aa 3 to 169) is expressed from the same promoter (pCMV-Gal4:BZLF1). Internal deletion mutants of BZLF1:E2 and Gal4:BZLF1 were generated by oligonucleotide-directed mutagenesis. An additional *Spe*I site was introduced to replace the deleted region and to monitor the deletion. The mutants and their deletions are depicted in Fig. 3A.

Transient replication assays

Transient replication assays were performed in D98HR1 cells by cotransfection of 10 μg of *oriLyt* wild-type (p968.22) or mutant plasmid DNA together with an internal *oriLyt* standard plasmid (p526, 1 μg) and 10 μg of pCMV-BZLF1 which efficiently induces the lytic cycle of EBV. In order to analyze BZLF1 mutants, the chimeric pCMV-BZLF1:E2 plasmid or a mutant was cotransfected as a fourth plasmid. Two days after plasmid transfection cellular DNA was isolated and input plasmid DNA was digested with *Dpn*I and *Bam*HI (Hammerschmidt and Sugden, 1988; Schepers *et al.,* 1993b). The efficiency with which the *oriLyt* plasmids replicated was determined after Southern blot hybridization and autoradiography by scanning the specific fragment signals with the aid of a Phosphormager (Fuji). The quantification of the replication assays was based on at least three independent experiments.

Transient transcription assays

To measure the transcriptional activation of the BHLF1 promoter in different *oriLyt* mutants, 10 μg of plasmid DNA containing the luciferase reporter gene at the position of the BHLF1 gene was cotransfected into D98HR1 cells together with 10 μg pCMV-BZLF1 and 0.5 μg of pCMV-β-gal. The luciferase and β-galactosidase activities were measured in cell extracts as described in detail (Schepers *et al.,* 1993b). All reactions were performed at least in quadruplicate.

RESULTS

A cluster of four BZLF1 binding sites is required to activate *oriLyt*-mediated DNA replication

Seven binding sites for BZLF1 are located within *oriLyt* (Fig. 1A) and lytic DNA replication was abolished when all seven ZRE sites were rendered nonfunctional (Schepers *et al.,* 1993a). The biochemical basis for the activation of *oriLyt* by BZLF1 is unknown. We hypothesized that binding of BZLF1 to multiple sites within a stretch of 1.0 kb might cause linking and distortion of the origin which could be a prerequisite for its activation. To address this possibility, we constructed a series of *oriLyt* mutants carrying different combinations of functional and nonfunctional ZRE elements. By oligonucleotide-directed mutagenesis we replaced BZLF1 binding sites with either GAL4- or E2-binding sites in two otherwise identical series of mutants (Fig. 1A). These *oriLyt* plasmids were tested in a transient replication assay in D98HR1 cells which are latently infected with EBV. Together with the *oriLyt* mutant plasmid an *oriLyt* wild-type plasmid of different size (p526) and a BZLF1 expression plasmid (pCMV-BZLF1) were cotransfected. pCMV-BZLF1 induces the lytic phase of EBV's life cycle to provide all viral functions *in trans.* BZLF1 also binds to ZRE sites within *oriLyt;* however, wild-type BZLF1 is unable to interact with either Gal4- or E2-binding sites. Two days after electroporation, low molecular weight DNA was prepared and digested with *Bam*HI and *Dpn*I to linearize the plasmid DNA and to discriminate between input DNA and newly replicated DNA. The replication activity of each *oriLyt* mutant was detected by Southern blot hybridization with radioactively labeled prokaryotic sequences as described previously (Hammerschmidt and Sugden, 1988; Schepers *et al.,* 1993b).

Examples of the transient replication assays which were performed in triplicate are shown in Fig. 1B (ZRE sites replaced with Gal4 sites) and Fig. 1C (ZRE sites replaced with E2 sites). The replication efficiencies of 24 mutants are summarized in Fig. 1A. As expected, the Gal4 and E2 series of *oriLyt* mutants gave almost identical results which demonstrated that the BZLF1-binding sites ZRE1 to ZRE4 alone are necessary and sufficient to activate lytic replication. Mutations which render ZRE5 to ZRE7 nonfunctional have no or only very marginal influence on the replication efficiency, whereas mutations of the BZLF1-binding sites ZRE1 to ZRE4 reduce *oriLyt*-dependent replication (p1086 and p1108, p1781, p1782). DNA replication was completely undetectable in mutants which had no functional ZRE1 to ZRE4 sites (p1361 and p1359, 1377 and 1376, 1374 and 1375; Figs. 1B and 1C). ZRE5 to ZRE7 sites did not contribute to replication functions when ZRE1 to ZRE4 were mutated (p1361 and p1359, p1199 and p1200, p1377 and p1376, p1374 and p1375). The data also suggested that ZRE3 and ZRE4 are, together, at least as important for *oriLyt*-mediated DNA replication as are ZRE1 and ZRE2, if not more important.

Transcription and DNA replication of *oriLyt* are activated by similar combinations of BZLF1-binding sites

Lytic DNA replication and transcriptional activation of the BHLF1 and BHRF1 promoters are two independent processes (Schepers *et al.,* 1993a). Transcriptional activation of either of the two promoter elements (BHLF1 or BHRF1) within *oriLyt* is not a prerequisite for DNA replication. Nevertheless, both activities share common *cis*-acting elements and are dependent on BZLF1 (Chang *et al.,* 1990; Chavrier *et al.,* 1989; Fixman and Hayward, 1995; Gruffat *et al.,* 1990; Hardwick *et al.,* 1988; Laux *et al.,* 1985; Lieberman *et al.,* 1989, 1990; Schepers *et al.,* 1993a,b).

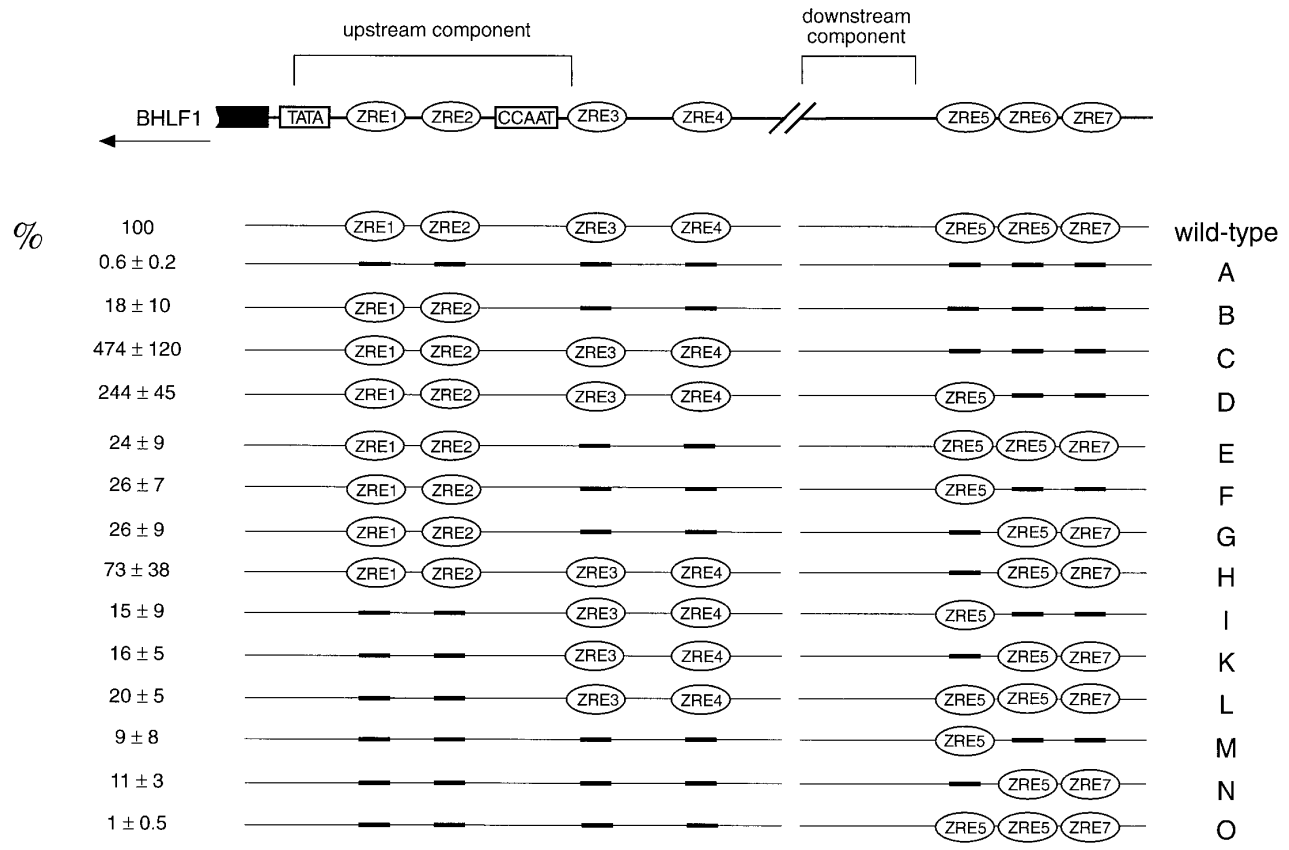

FIG. 2. Relative transcription activity of the BHLF1 promoter in different ZRE-replacement mutants of *oriLyt.* The set of *oriLyt* mutants contains different combinations of E2- and ZRE-binding sites; a functional site is indicated by ZRE1 through ZRE7, a nonfunctional site is indicated by a black bar. The *oriLyt* plasmid which contains seven ZRE sites represents the wild-type origin; mutant A lacks any functional ZRE sites, as shown. Each mutant was tested in a separate series of experiments with the native BZLF1 protein expressed from the human cytomegalovirus promoter/enhancer. The results are summarized in the column on the left. Mean values (%) and standard deviations were calculated from at least four independent experiments.

Since the seven ZRE sites within *oriLyt* form two clusters near the promoters of BHLF1 (ZRE1 to 4) and BHRF1 (ZRE5 to 7) (Fig. 1A), we argued that these clusters act as separate modules. *oriLyt*-mediated DNA replication was found to be dependent on the cluster of four ZRE sites (ZRE1 to 4), which supports this assumption. To address this question, we indirectly measured the promoter activity of the ZRE mutants by replacing the flanking BHLF1 gene with the luciferase reporter gene. Transcriptional activation of the BHLF1 promoter was determined in transient transcription assays in the presence of wild-type BZLF1 expressed from the cytomegalovirus early promoter and enhancer (Schepers *et al.,* 1993a). The same set of *oriLyt* mutants which had been used to analyze DNA replication (Fig. 1) was the basis for this experiment. Only *oriLyt* mutants which carried different combinations of ZRE- and E2-binding sites were employed and the results are summarized in Fig. 2. In general, the results demonstrated that transcriptional activation of the BHLF1 promoter is dependent on ZRE sites which had also been found to be essential for DNA replication. The four proximal sites ZRE1 to ZRE4 are critical; mutation of ZRE5 to ZRE7 even caused a fourfold stimulation of the BHLF1 promoter compared with wild-type (mutant C). As indicated by the mutants E and L, pairwise mutations of ZRE1 and ZRE2 or ZRE3 and ZRE4 resulted in a much reduced activity on the BHLF1 promoter. Again, the more distal sites ZRE5 to ZRE7 did not contribute significantly to the activation of the BHLF1 promoter (compare mutant B with mutants E through G, or mutant I with mutants K and L). Little activation from the distal ZRE sites could be detected with mutants lacking ZRE1 to ZRE4 (compare mutant A with mutants M through O). The observation that mutants with four functional ZRE elements (mutants C and D) are much more active than the wild-type plasmids with seven functional ZRE sites could mean that the two clusters of ZRE1 to ZRE4 and ZRE5 to ZRE7 sites are regulating each other negatively. An alternative interpretation is that the reduction of the number of functional binding sites for BZLF1 ensures the occupation of the remaining motifs. This possibility seems to be unlikely since even small amounts of different BZLF1-expression plasmids (CMV-BZLF1 and SV40-BZLF1) are able to support the full BHLF1-promoter activity (data not shown).

These data confirmed that BZLF1 has properties of a genuine promoter factor which is a well-documented characteristic (Chi and Carey, 1993; Lieberman and Berk, 1991; Lieberman *et al.,* 1994). These data show that BZLF1 cannot co-operate over a fairly long distance, a function which is reminiscent of BZLF1's contributions to *oriLyt*-mediated DNA replication (Fig. 1). Long-range co-operativity seems to be irrelevant for *oriLyt*-mediated DNA replication, suggesting that the upstream component of *oriLyt* with ZRE1 to ZRE4 can now be considered a functional unit for *oriLyt* replication.

Similar domains support replication and transcription

BZLF1 plays a dual role in replication as well as in transcription. Its transactivation domain is specifically required for DNA replication of *oriLyt* and cannot be replaced by transactivation domains of other transcription factors which, in contrast, can substitute for BZLF1's transcription functions (Schepers *et al.,* 1993a). The regions on the transactivation domain of BZLF1 which support transcription have been analyzed extensively (Baumann *et al.,* 1993; Flemington *et al.,* 1992; Giot *et al.,* 1991; Lieberman and Berk, 1991; Miller *et al.,* 1993). Although the results of the independent experiments are not identical, these investigations demonstrated that two regions of the transactivation domain are important for transcription: the region located between aa 28 and aa 78, and between aa 140 and aa 165, the latter containing a nuclear localization signal (Fig. 3A for a summary).

To determine whether specific regions of the BZLF1 transactivation domain are important for lytic DNA replication, we created a set of chimeric BZLF1:E2 and Gal4:BZLF1 deletion mutants (Fig. 3A) which were expressed from the human cytomegalovirus immediate early promoter. Successive deletions, each comprising 27 aa, were introduced into the transactivation domain of BZLF1. These chimeras were tested in replication assays with *oriLyt* mutant plasmids in which all seven ZRE sites were replaced by either E2- or Gal4-binding sites (plasmids p1199 and p1200 in Fig. 1). The results of the transient replication assays indicated that the core region of BZLF1 which supports DNA replication localizes to a domain from aa 27 to aa 78. Deletion from aa 78 to aa 103 of the BZLF1:E2 chimera resulted in a dramatically reduced activation of DNA replication (Figs. 3A and 3B). The region between aa 27 and aa 103 within the BZLF1 transactivation domain could be confirmed in a second independent series of experiments with Gal4:BZLF1 chimeras, although the results are not identical: Gal4:BZLF1 chimeras with deletions in the BZLF1 transactivation domain up to aa 140 were greatly impaired in their capacity to support DNA replication (Figs. 3A and 3C, and data not shown). The discrepancy between BZLF1:E2 and Gal4:BZLF1 could be explained by the completely different design of the functional modules of the two chimeras (Fig. 3A) which might cause misfolding of the BZLF1 part in case of certain carboxy-terminal mutations. These structural constraints could also result in proteins having different binding affinities which would translate into apparent differences in the replication assay. Since the DNA-binding domain of Gal4 resides within the first 74 amino-terminal residues (Keegan *et al.,* 1986) and the Gal4:BZLF1 fusion proteins encompass 141 amino acids of the yeast transcription factor, this assumption seems to be unlikely. Similarly, the nuclear localization signals of BZLF1 which reside between aa 140 and aa 165 of BZLF1 (Mikaelian *et al.,* 1993) should not be required in our BZLF1:E2 and Gal4:BZLF1 mutants since the respective DNA-binding domains of either E2 or Gal4 (Silver *et al.,* 1984) contain functional nuclear localization signal sequences. The BZLF1 chimeras showed the expected sizes and were expressed at about the same levels as indicated in Western blot experiments (data not shown).

In general, these results indicated that similar regions within the BZLF1 transactivation domain are functionally involved in transcription as well as in DNA replication.

DISCUSSION

In this work, three novel aspects of the role of the viral transactivator BZLF1 could be described: (i) the cluster of four BZLF1-binding sites proximal to the BHLF1 promoter is essential and sufficient for *oriLyt*-mediated DNA replication; (ii) the domains of the BZLF1 protein required for replication are similar to those required for transcriptional activation; and (iii) BZLF1 has properties reminiscent of a proximal promoter factor which does not show significant long-range cooperation in the context of *oriLyt.*

oriLyt-mediated DNA replication is specifically activated by ZRE1 to ZRE4

Both the lytic origin of DNA replication and the BHLF1 promoter have *cis*- and *trans*-acting elements in common. Both functions, replication and transcription, are dependent on the EBV-specific transactivator BZLF1 which binds seven times within the region of *oriLyt* (Lieberman and Berk, 1990, 1991; Lieberman *et al.,* 1990). We have demonstrated earlier that lytic DNA replication is dependent on a specific function that resides in the transactivation domain of BZLF1. In contrast, transcriptional activation of the BHLF1 promoter is promiscuous, indicating that DNA replication has more stringent requirements *in trans* than does mRNA synthesis (Schepers *et al.,* 1993a). In this report, we show that this specificity also acts *in cis.* The analysis of *oriLyt* mutants, with various combinations of functional and nonfunctional ZRE sites, revealed that *oriLyt*-mediated DNA replication depends entirely on ZRE1 to ZRE4 (Fig. 1A). Mutational inactivation of the BZLF1-binding sites ZRE5 to ZRE7 has

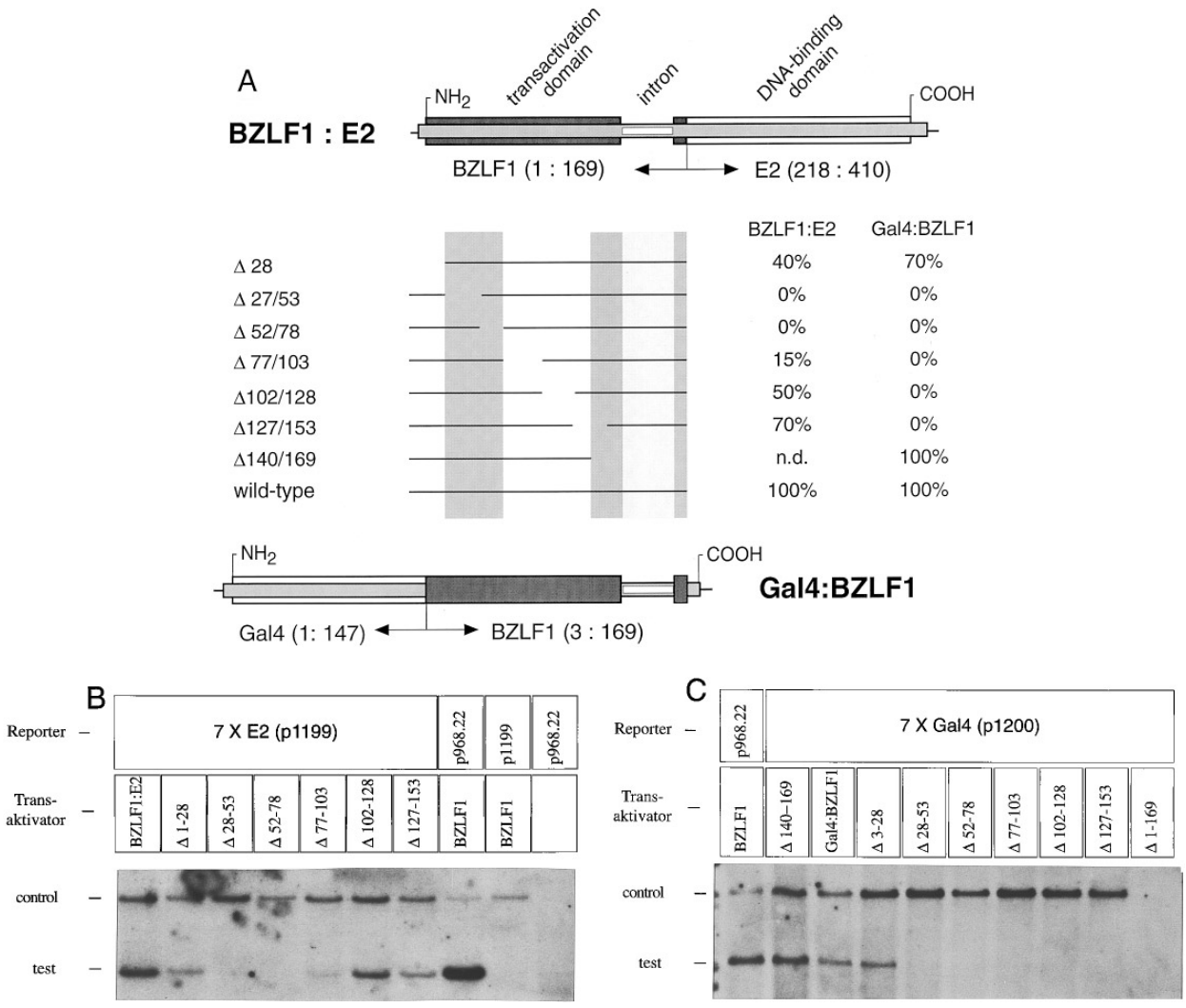

FIG. 3. *oriLyt* replication assays with deletion mutants of the chimeric BZLF1:E2 and Gal4:BZLF1 proteins. (A) To determine specific regions of the BZLF1-transactivation domain which are important for *oriLyt*-mediated DNA replication, a set of chimeric BZLF1:E2 and Gal4:BZLF1 deletion mutants was created which were expressed from the immediate early promoter/enhancer of the human cytomegalovirus. Successive deletions of about 27 aa were introduced into the transactivation domain of the two chimeric transactivators. All chimeric proteins were tested in replication assays with the aid of two *oriLyt* mutant plasmids which carry either seven E2-binding sites (p1199) or seven Gal4-binding sites (p1200) (Schepers *et al.,* 1993a). For comparison, the regions which have been described as important for transcriptional transactivation by BZLF1 are marked with gray areas ranging from aa 28 to aa 78 and aa 140 to aa 165. The latter region contains a functionally important nuclear localization signal (Flemington *et al.,* 1992; Giot *et al.,* 1991; Lieberman and Berk, 1991; Miller *et al.,* 1993). (B and C) Transient replication assays with the deletion mutants of the chimeric BZLF1:E2 and Gal4:BZLF1 fusion proteins. The Southern blots which resulted from transient replication assays with the *oriLyt* plasmids p1199 and p1200 and the chimeric BZLF1 deletion mutants indicate the region of BZLF1 which supports DNA replication. An example of the results with BZLF1:E2 chimeras is shown in B, the analogous results with Gal4:BZLF1 chimeras are shown in C. The results of three independent experiments were quantitated and the mean values are shown in A. The domain which is essential for replication is located between aa 27 and aa 103 for BZLF1:E2. The results obtained with the Gal4:BZLF1 mutants indicate that the important domain which supports lytic replication may extend to aa 140. The data were obtained with (B) the *oriLyt* mutant plasmid p1199 carrying seven E2-binding sites in the presence of the chimeric BZLF1:E2 protein or with (C) the *oriLyt* mutant plasmid p1200 with seven Gal4-binding sites and the chimeric Gal4:BZLF1 protein as transactivator. The control signals of the *oriLyt* wild-type plasmid p526 (Hammerschmidt and Sugden, 1988) were used as an internal standard to correct for transfection efficiencies.

only a marginal influence on DNA replication. This finding could mean that BZLF1 is not able to act over a longer distance and needs to be present in a cluster of binding sites that is part of the upstream component of *oriLyt.* This assumption also holds true for mRNA synthesis because the activity of the BHLF1 promoter is dependent on the same combination of ZRE sites. As in *oriLyt*-mediated replication ZRE1 to ZRE4 are mandatory; distal ZRE sites contribute little (Fig. 2). For transcriptional activation of BHLF1, no obvious hierarchy exists between ZRE1/ZRE2 versus ZRE3/ZRE4 sites despite the fact that they display remarkably different binding affinities for BZLF1 (Lieberman and Berk, 1990; Lieberman *et al.,* 1990). For DNA replication, however, ZRE3/ZRE4 which have the lowest

binding affinity among the ZRE sites of *oriLyt* are more important than ZRE1/ZRE2 (Fig. 1).

The comparison between *oriLyt*-mediated DNA replication and activation of the BHLF1 promoter points to an important finding. It appears that BZLF1 acts as a replication factor only when it is bound to the upstream component of *oriLyt.* Deletion analysis of this region indicated that other DNA sequence motifs besides the four BZLF1-binding sites might be influential on DNA replication (Schepers *et al.,* 1993b). This assumption is supported by the observation that an artificial cluster of BZLF1-binding sites with high affinity is nonfunctional in terms of *oriLyt*-mediated DNA replication even in the context of the basal BHLF1 promoter (to be published elsewhere). It appears likely that the upstream component of *oriLyt* promotes the assembly of a replication complex in which BZLF1 is associated with yet another replication factor(s) which happens to interact with the upstream component. We have preliminary evidence for a cellular factor which fulfills these criteria: it interacts with the transcriptional activation domain of BZLF1 and contacts DNA sequence motifs within the upstream component of *oriLyt* (data not shown). Another possibility is that the BZLF1 cluster of the upstream component provides a DNA structure that is necessary for the initiation of lytic DNA replication.

BZLF1 domains for replication and transcription activation are similar

The analysis of BZLF1's transactivation domain showed that transcription and lytic DNA replication are supported by similar regions of BZLF1. Transient replication assays with chimeric BZLF1:E2 and Gal4:BZLF1 deletion mutants identified the part between aa 28 and aa 103 of BZLF1 as essential for replication. Other regions located further downstream but adjacent to this region are also important although discrepancies between the two different BZLF1 chimeras, BZLF1:E2 and Gal4:BZLF1, exist (Fig. 3). The region is nearly identical with the domain which was identified to support transcription (aa 27–78) (Baumann *et al.,* 1993; Flemington *et al.,* 1992; Giot *et al.,* 1991; Lieberman and Berk, 1991; Miller *et al.,* 1993), presumably by interacting with the basal transcription factor TFIID as well as TAFs (Chi and Carey, 1993; Lieberman and Berk, 1991; Lieberman *et al.,* 1994). To our knowledge, BZLF1 possesses a unique dual function in that it is an essential replication *and* transcription factor. The coincidence of nearly identical requirements for both processes again indicates that the dual function of BZLF1 most likely requires a different protein (or proteins) which happens to interact via the same region of the transactivation domain of BZLF1. Such a "modulator" protein could be TFIID and/or its associated factors when BZLF1 functions as a transcription factor (Chi and Carey, 1993; Lieberman and Berk, 1991; Lieberman *et al.,* 1994). Whether a similar *trans*-acting factor exists which has modulator property specific for DNA replication remains to be demonstrated. It appears that BZLF1 has evolved as an omnipotent factor to combine multiple functions during the productive lytic phase of EBV's life cycle. Moreover, the transcriptional activation domain of BZLF1 seems to be the key element which most likely is engaged in different protein–protein interactions.

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