A transcription factor with homology to the AP-1 family links RNA transcription and DNA replication in the lytic cycle of Epstein–Barr virus

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oriLyt, the lytic origin of DNA replication of Epstein–Barr virus (EBV), ensures viral DNA amplification during the productive or lytic phase of the virus' life cycle. To understand the contribution of *cis*- and *trans*-acting elements involved in DNA replication of oriLyt, a detailed mutational analysis was undertaken which defined BZLF1, a viral transcriptional activator, as an essential replication factor. The BZLF1 protein belongs to the extended fos/jun family of transcription factors and binds to specific BZLF1 binding motifs within oriLyt, as well as to consensus AP-1 sites. Recombinant, chimeric transcription factors identified the transcriptional activation domain of BZLF1 as being necessary to mediate DNA replication, a function which could not be substituted by any other transcription factor tested, including jun, E2, myc or VP16.

Key words: BZLF1/DNA replication/Epstein–Barr virus/oriLyt/transcriptional activator

Introduction

One of the fascinating features of DNA viruses is their capacity to replicate viral DNA independently of the restriction which regulates cellular DNA replication. Despite the fact that the central control of mammalian replicons is bypassed, viral DNA replication during lytic or productive infection is a highly regulated process. It requires coordination of enzymatic steps of viral gene products involved in DNA replication, as well as viral elements acting in *cis*. The essential enzymatic activities involved in DNA replication have been found to be conserved in bacteria, bacteriophages and DNA viruses of mammalian cells [Challberg and Kelly (1989) and Stillman (1989) for reviews]. Although replication activities of SV40 and adenoviruses can be reconstructed *in vitro* and genetic studies have identified most, if not all, viral proteins involved in DNA amplification of herpesviruses, it is not yet clear how these processes are regulated and at which level cellular and viral DNA replication are distinct.

The identification of several *cis*-acting sequences that function as origins of DNA replication in eukaryotic systems has revealed complex structures with a somewhat modular design. A unifying concept was proposed by DePamphilis (1988) who defined two different, yet interacting, components of origins of replication. The core component determines the binding of basic replication factors, the

assembly of the replication protein complex, and the site and direction of the initiation of DNA synthesis. One or multiple auxiliary components that flank the core component facilitate or enhance origin functions, but are dispensable under certain conditions. These auxiliary components encompass promoters and/or enhancer elements which are involved in RNA transcription. The mode by which these transcriptional elements interact with origins of DNA replication can be functionally diverse in different eukaryotic systems [DePamphilis (1988) and van der Vliet (1991) for reviews] and it is still unclear how transcriptional elements facilitate origin functions.

To assess the interaction of *cis*- and *trans*-acting factors of a complex eukaryotic origin, we focused on the lytic origin of DNA replication of Epstein–Barr virus (EBV). This origin, termed oriLyt, amplifies herpesviral DNA several hundred fold relative to cell DNA in cells that support the lytic phase of EBV's life cycle (Hammerschmidt and Sugden, 1988). oriLyt is made up of core regions and non-essential or auxiliary regions which were defined operationally (Figure 1) (Hammerschmidt and Sugden, 1988; Schepers *et al.*, 1993). The core of oriLyt is absolutely required for DNA replication and consists of two components, an upstream and downstream component, which are ~530 bp apart. The two non-contiguous components are flanked by loosely defined auxiliary regions which increase the efficiency with which oriLyt replicates (Hammerschmidt and Sugden, 1988) (Figure 1). These sequences are dispensable under certain conditions, but the omission of most or all of them decreases oriLyt-mediated replication by a factor of several hundred (Hammerschmidt and Sugden, 1988). The lytic origin of EBV is present in two copies in most EBV strains and the left copy of oriLyt colocalizes with a locus in the EBV genome which has been identified as a complex array of two divergent promoter/enhancer elements which are highly active during the lytic phase of EBV's life cycle (Figure 1). These promoter elements are activated by viral transcriptional activators (Laux *et al.*, 1985; Chevallier-Greco *et al.*, 1986, 1989), one of which is called BZLF1 (EB1, zta, ZEBRA, Z are synonyms). BZLF1 belongs to the bZIP class of transcription factors and is related to the AP-1 family of regulatory proteins with transcription factor activity (Farrell *et al.*, 1989). BZLF1 forms homodimers (Kouzarides *et al.*, 1991) which bind to specific DNA sequence motifs mostly called ZRE sites (for BZLF1-responsive elements) and activates transcription from viral, cellular and recombinant templates containing ZREs. The carboxy-terminal region of BZLF1 which mediates sequence-specific DNA binding is similar to the functional equivalents of c-fos, c-jun, c/EBP and GCN4 (Farrell *et al.*, 1989; Kouzarides *et al.*, 1991) and consequently BZLF1 binds to TPA-responsive elements (TRE) of the AP-1 transcription factor c-fos/c-jun with high affinity (Uriet *et al.*, 1989; Lieberman *et al.*, 1990). Although TREs and ZREs display sequence homologies, ZRE sites do not serve as recognition

Fig. 1. Structure of the lytic origin of DNA replication of EBV. An EBV subgenomic fragment encompassing oriLyt within the left duplicated segment is shown schematically in the top part of the figure. The divergent genes BHLF1 and BHRF1 are depicted together with their promoters (black bars) and mRNA structures (hatched rectangles). The open rectangles delineate the essential sequence elements of oriLyt which encompass the minimal oriLyt (Schepers *et al.*, 1993). The shaded areas represent poorly defined regions which score as auxiliary elements of oriLyt. In most EBV strains, a part of oriLyt is present twice in two functional copies which are > 100 kbp apart. This duplicated segment of oriLyt is shown underneath. An enlarged view of oriLyt is given at the bottom of the figure, including known functional sequence elements which are indicated by icons representing seven binding sites for BZLF1 (ZRE1–7), one cluster of binding sites for the viral enhancer factor R (R), and the TATA and CCAAT boxes. The essential upstream component encompasses the promoter of the BHLF1 gene, but no specific structural element has been assigned to the downstream component yet (Schepers *et al.*, 1993).

sites for the cellular AP-1 transcription complex (Lieberman *et al.*, 1990). Clearly, BZLF1 has a more relaxed target specificity compared with that of AP-1.

The amino-terminal domain of BZLF1 confers transcriptional activation when bound to promoter targets of different viral genes (Farrell *et al.*, 1989; Urier *et al.*, 1989; Giot *et al.*, 1991; Flemington *et al.*, 1992). One function of the amino-terminal activation domain of BZLF1 is its stable interaction with the basic transcription factor TFIID that binds to the TATA-box element found in most eukaryotic promoters (Lieberman and Berk, 1991). The BZLF1 gene is highly regulated and repressed during the latent phase of the bipartite life of EBV [Kieff and Liebowitz (1991) and Middleton *et al.* (1991) for reviews]. This is because the BZLF1 gene product is sufficient to activate the lytic or productive phase of EBV's life cycle (Countryman and Miller, 1985; Takada *et al.*, 1986).

Several BZLF1 binding sites (ZRE) are located within oriLyt (Figure 1). Four BZLF1 binding sites have been mapped to lie close to the TATA and CCAAT boxes of the BHLF1 promoter which is transactivated by BZLF1 bound to these four sites. Three additional ZREs have been identified by functional analysis and consensus search criteria (Figure 1). The sequence composition of these seven ZREs differs and consequently BZLF1 binds with different affinities to these sites (Gruffat *et al.*, 1990b; Lieberman *et al.*, 1990; Giot *et al.*, 1991). BZLF1 is not the only viral transcription factor which interacts with sequences crucial for oriLyt function. The R enhancer factor binds specifically to two R-responsive sequence motifs which are located

between ZRE5 and ZRE6 (Figure 1). This region has been identified to carry enhancer characteristics (Gruffat *et al.*, 1990a; Giot *et al.*, 1991) and it seemed possible that R, BZLF1, or other non-viral transcription factors could play a role in oriLyt DNA replication.

In this study, we ask whether the BZLF1 transcriptional activator contributes to DNA replication and, if so, how. Our approach was two fold. First, *cis*-acting elements were identified by mutating and replacing known BZLF1 binding sites within the lytic origin of DNA replication of EBV, oriLyt. To replace the BZLF1 binding sites, synthetic oligonucleotides were used that bind the AP-1 transcription complex c-fos/c-jun, the yeast transcription factor GAL4, or the bovine papillomavirus factor E2. Second, we designed *trans*-acting chimeric factors consisting of the specific DNA binding domains of GAL4 and E2, and various transcriptional activation domains which were expressed with appropriate vectors in cotransfection experiments. oriLyt DNA replication was measured in transient replication assays, as was transcriptional activation from two divergent promoters within oriLyt. The data presented here prove that the transactivation domain of BZLF1 is required to promote oriLyt DNA replication. In contrast, other chimeric transcription factors do not support DNA replication, but do stimulate efficient RNA transcription from oriLyt promoters. On this basis, we conclude that BZLF1 has a novel dual property which links RNA transcription and DNA replication *in trans*, probably through its direct interaction with the cellular transcription machinery and other viral replication proteins.

Fig. 2. Mutations in the BZLF1 binding sites within the BHLF1 promoter modulate the efficiency of oriLyt replication. (A) The top line gives the structure of the BHLF1 promoter with known hallmarks of this element, including the 5' end of the mRNA, the TATA box motif, the two BZLF1 binding sites ZRE1 and ZRE2, and the CCAAT box. These sequence elements are underlined and indicated in bold together with their strand specificity. The oriLyt mutants are based on the wild-type plasmid p968.22, spanning several kilobases of EBV which included the BHLF1 and BHRF1 open reading frames (Schepers *et al.*, 1993). Base substitutions are given in detail with the respective mutated oriLyt plasmids (left column). In p1060, the original sequences of the two ZRE1 and ZRE2 sites were randomized arbitrarily to make the BZLF1 binding sites non-functional, p1077 carries two ZRE5 sites which probably have a higher affinity for BZLF1 compared with ZRE1 and ZRE2 (Lieberman and Berk, 1990), p1078 carries two ΔZRE5 sites which have no affinity to BZLF1 (Lieberman and Berk, 1990), p1079 carries two TRE sites to which BZLF1 and the cellular AP-1 transcription complex bind (Farrell *et al.*, 1989). The other mutant oriLyt plasmids carry various combinations of these binding site substitutions. The sequence-specific DNA binding sites are indicated in bold letters, the mutated nucleotides are shown in lower case. The phenotype of the mutants and their replication efficiencies compared with wild-type p968.22 plasmid are summarized in the two columns on the right. The relative replication efficiencies with which the mutant oriLyt plasmids replicated represent the mean of at least three independent experiments (see Materials and methods). (B) gives an example of one set of original data with the oriLyt mutants shown in (A). The position of the 'control' signals gives the position of the wild-type oriLyt plasmid p526 (Hammerschmidt and Sugden, 1988) which served as an internal standard to correct for transfection efficiency in the single samples. The position of the 'test' signals gives the signal strength, which is a relative measure of the efficiency with which the mutated oriLyt plasmids replicated. The test plasmids are identical to the ones shown in (A). p968.22 indicates the replication efficiency of wild-type oriLyt.

Results

Mutations of BZLF1 binding sites modulate oriLyt activity

First, we set out to investigate the contribution of promoter elements to influence oriLyt's activity. From previous work, it was known that deletions removing the TATA box and

two ZRE sites (ZRE1 and ZRE2) of the BHLF1 promoter markedly reduced oriLyt-mediated replication (Figure 1) (Schepers *et al.*, 1993). Since the role of BZLF1 in DNA replication was uncertain, we postulated that BZLF1 might contribute to oriLyt activity. If this hypothesis were to be correct, we expected to modulate oriLyt DNA synthesis by changing the affinity with which BZLF1 binds to the BHLF1

promoter. Using oligonucleotide-directed mutagenesis, we replaced ZRE1 and ZRE2 with randomized sequences or with two ZRE mutants (Δ ZRE5) with no affinity for BZLF1 (Lieberman *et al.*, 1990). In addition, we introduced binding sites for BZLF1 with a different, probably higher affinity (ZRE5), or with consensus TRE sites to which the AP-1 transcription complex and BZLF1 can bind. The oriLyt mutants were tested in a transient DNA replication assay in D98HR1 cells which are latently infected with EBV and support the lytic phase of EBV's life cycle upon induction. These cells were cotransfected with three plasmids. The mutant oriLyt plasmid was mixed with a wild-type oriLyt plasmid of different size to allow for internal standardization, and with the plasmid pCMV-BZLF1 which expresses the viral BZLF1 gene under the control of a heterologous promoter (Hammerschmidt and Sugden, 1988). pCMV-BZLF1 induces the lytic phase of EBV's life cycle in D98HR1 cells to provide all EBV functions needed *in trans* for oriLyt replication. Two days after cotransfection, DNA was extracted and cleaved with *Bam*HI and *Dpn*I to linearize the oriLyt plasmids and to distinguish input DNA from DNA replicated in eukaryotic cells. The DNAs were detected after Southern blot hybridization with radioactively labeled prokaryotic sequences as described previously (Hammerschmidt and Sugden, 1988; Schepers *et al.*, 1993).

The first set of ZRE mutants is shown in Figure 2B; the nature of the various mutations and the efficiency with which the mutants replicated are given in Figure 2A. Two mutations abrogating the ZRE1 and ZRE2 sites reduced the efficiency of oriLyt replication to about one-quarter, which was roughly the same effect seen with two dysfunctional Δ ZRE5 sites. Single mutations eliminating one ZRE site at a time had an intermediate effect (50–70% compared with wild-type level activity). On the contrary, exchanging ZRE1 and ZRE2 for ZRE5 or TRE sites enhanced oriLyt replication. The stimulation above wild-type level was probably a reflection of BZLF1 binding tighter to ZRE5 and TRE sites than to ZRE1 and ZRE2 (Lieberman *et al.*, 1990). The results also suggested that the cellular AP-1 transcription complex might stimulate oriLyt synthesis when bound to oriLyt. However, it was obvious that efficient BZLF1 binding to the upstream component of oriLyt is critical for oriLyt activity.

BZLF1 binding is absolutely required for oriLyt DNA replication

Seven BZLF1 binding sites are located within the lytic origin, but only ZRE1 and ZRE2 colocalize with the essential upstream component of oriLyt. Since the mutation of these two sites revealed their direct or indirect contribution to oriLyt activity, we asked whether the remaining five sites might mediate a similar effect. To address this question, we mutated all seven ZRE sites in oriLyt to eliminate BZLF1 binding and to confer different binding specificities. The binding sites for the yeast transactivator GAL4 (Giniger *et al.*, 1985) and for the specific DNA binding factor E2 of bovine papilloma virus (Androphy *et al.*, 1987; Moskaluk and Bastia, 1987) were introduced for all ZRE sites. Clearly, GAL4 and E2 proteins are absent in D98HR1 cells and these mutations disrupt BZLF1 interacting with oriLyt. As a control, we mutated ZRE1 and ZRE2 sites to either GAL4 or E2 binding sites as in the previous set of experiments.

As expected, the mutations of ZRE1 and ZRE2 to GAL4

or E2 sites reduced the oriLyt activity to ~20% (Figure 3), similar to the ZRE Δ 5 or nonsense mutations shown in Figure 2 and presented above. Mutagenesis of all seven BZLF1 binding sites abolished oriLyt replication (Figure 3) independent of the nature of the substitutions introduced in their place. Thus, binding of BZLF1 to oriLyt is absolutely required to mediate its DNA replication. However, we do not know whether the binding of BZLF1 to specific or to all ZRE sites is essential.

Rescue of oriLyt functions with chimeric BZLF1 proteins

To examine further the role of BZLF1 in oriLyt replication, we constructed chimeric molecules in which the DNA binding and dimerization domain of BZLF1 was substituted with the DNA binding and dimerization domain of either E2 (McBride *et al.*, 1989) or of GAL4 (Keegan *et al.*, 1986). The BZLF1 domain known to be required for transcriptional transactivation (Lieberman and Berk, 1990; Giot *et al.*, 1991; Flemington *et al.*, 1992) was cloned at the amino-terminus of the DNA binding domain of E2 (BZLF1:E2) or at the carboxy-terminus of the DNA binding domain of GAL4 (GAL4:BZLF1). The BZLF1:E2 chimera contained amino acids 1–169 of BZLF1 in frame with amino acids 128–410 of E2, the GAL4:BZLF1 chimera contained amino acids 1–147 of the GAL4 protein and 3–169 of BZLF1 (Figure 4A). The BZLF1 chimeras were expressed from the SV40 early promoter (SV-GAL4:BZLF1) in a conventional expression plasmid (Sadowski and Ptashne, 1989) or from the stronger immediate early promoter of the human cytomegalovirus (CMV-GAL4:BZLF1, CMV-BZLF1:E2) in a retroviral expression plasmid background (Stinski and Roehr, 1985; Hammerschmidt and Sugden, 1988). The BZLF1 chimeric molecules shown schematically

Fig. 3. Binding of BZLF1 to ZRE sites in oriLyt is absolutely required for its activity. The autoradiogram represents the results of oriLyt mutants which had the ZRE1 and ZRE2 sites replaced by two GAL4 or two E2 binding sites (1086 and 1108, respectively) or which carried no ZRE sites, but only GAL4 or E2 binding sites (1200 and 1199). The former pair of mutants showed a reduced efficiency of DNA replication compared with wild-type oriLyt, the latter pair had no residual DNA replication activity at all. The nature of the mutation (E2 or GAL4 binding sites) was irrelevant for the phenotype of the mutant, indicating that the binding of BZLF1 to specific sites within oriLyt is essential for DNA replication.

in Figure 4A were functional in conjunction with recombinant reporter constructs containing multiple GAL4 or E2 binding sites. The reporter constructs showed an ~1000-fold activation in the presence of the chimeric BZLF1 proteins, as measured by luciferase activity in cotransfection experiments (data not shown).

In the transient oriLyt replication assay, all BZLF1

chimeric proteins rescued DNA replication from oriLyt mutants which either contained seven E2 or seven GAL4 binding sites. These oriLyt mutants were completely unable to replicate in the presence of native BZLF1 expressed from pCMV-BZLF1 (Figure 4B), as shown before (Figure 3). The level of DNA replication of the oriLyt mutants did not reach that of wild type (Figure 4B). This diminution may

Fig. 4. oriLyt null mutants can be rescued by chimeric BZLF1 transcription factors. **(A)** At the top, the modular structure of BZLF1 is shown which consists of three coding exons (black bars) for the transcriptional activation domain, the DNA binding domain and the dimerization domain, respectively. Two introns (white rectangles) separate the three domains at the level of the genomic DNA. The stippled area indicates the length of the primary transcript for BZLF1. The middle and the bottom parts of the figure show the structure of the recombinant chimeric transcription factors encompassing the transcriptional activation domain of BZLF1 fused to the DNA binding domains of GAL4 (GAL4:BZLF1) and E2 (BZLF1:E2). The amino acid coordinates are given in parentheses to indicate the length of the different components that make up the chimeras. It is obvious from this figure that BZLF1:E2 closely resembles native BZLF1, whereas GAL4:BZLF1 has its structure inverted with respect to the DNA binding and transactivation domains. **(B)** The oriLyt mutants 1199 and 1200 (also shown in Figure 3) carry seven E2 or GAL4 binding sites, respectively, which replace the seven ZRE sites and abolish oriLyt replication (- signs). In the presence of the chimeric transcription factors BZLF1:E2 and GAL4:BZLF1 (+ signs), these mutants regained DNA replication functions, although the level of DNA replication did not reach wild-type activity. In all transfection experiments, native BZLF1 expressed from the pCMV-BZLF1 plasmid was present.

have resulted from four cotransfected plasmids competing for various cellular transcription factors (data not shown). On the other hand, the DNA binding and dimerization domain of BZLF1 might carry unknown functions that cannot be substituted by E2 or GAL4 domains. Clearly, the DNA binding domains of BZLF1, E2 and GAL4 have otherwise similar properties with respect to site-specific DNA binding, dimerization and nuclear localization. Taken together, the results obtained with the hybrid BZLF1 proteins suggested that crucial functions of BZLF1 needed for oriLyt DNA replication are encoded in the same domain needed for transactivation of transcription (Lieberman and Berk, 1990; Giot *et al.*, 1991; Flemington *et al.*, 1992).

The transactivation domain of BZLF1 is involved in DNA replication and cannot be replaced by that of other transcription factors

BZLF1 protein, when bound to appropriate sites in oriLyt, could carry out its function by recruiting other replication factors. The data obtained so far indicated that the transcriptional activation domain of BZLF1 which interacts with the TATA-box binding factor TFIID is crucial for this function. Since this protein-protein interaction is not unique to the activation domain of BZLF1, we wanted to investigate whether the transcriptional domain of any generic transcription factor might support DNA replication of oriLyt. Towards this end, we tested a number of native and recombinant proteins which bind to GAL4 or E2 sites. Specifically, we tested the E2 transcription factor of bovine papilloma virus which binds to E2 sites in the upstream regulatory region of the papilloma virus genome. The full-length E2 protein constitutes a transcriptional transactivator (E2-TA) when bound to E2 sites (Spalholz *et al.*, 1987), whereas an amino-terminally truncated E2 protein (E2-TR) only binds to E2 sites as a transcriptional repressor (Lambert *et al.*, 1987). Moreover, full-length E2 protein was documented to participate in DNA replication of bovine papilloma virus by stimulating the binding of the viral E1 replication protein to the origin (Yang *et al.*, 1991), although a contradictory report exists (Seo *et al.*, 1993). An expression vector for the chimeric transcription factor c-jun:E2 has the same specificity for DNA binding and stimulates transcription on various reporter constructs with multiple E2 sites (Baichwal and Tjian, 1990; data not shown). Similarly, we included an equivalent construct GAL4:c-jun in which the transcriptional activation domain of jun is linked to the GAL4 binding domain (Baichwal and Tjian, 1990). An expression vector coding for the extremely potent activation domain of VP16 fused to the GAL4 binding domain (GAL4:VP16) was included too (Sadowski *et al.*, 1988).

The different plasmids expressing either GAL4 or E2 fusion proteins were cotransfected together with the appropriate oriLyt mutant carrying seven GAL4 or E2 binding sites, pCMV-BZLF1 and an internal control plasmid. In such experiments, oriLyt DNA replication was only rescued with chimeras expressing the activation domain of BZLF1. The plasmids expressing the native E2 transcriptional activator (E2-TA) or the transcriptional repressor (E2-TR) failed to activate oriLyt-mediated replication and the c-jun:E2 chimera also failed in this assay (Figure 5 and data not shown). The same held true for its GAL4 counterpart GAL4:c-jun, the GAL4:VP16 transcrip-

Fig. 5. The replication function of the BZLF1 transactivation domain cannot be substituted by other transcription factors. The oriLyt mutant plasmids 1199 and 1200, which carry seven E2 or GAL4 binding sites, were tested for replication activity in the presence of a number of recombinant and native transcription factors. Only the expression plasmids SV-GAL4:BZLF1 and CMV-GAL4:BZLF1 were able to rescue DNA replication of the oriLyt null mutant 1200, the GAL4 DNA binding domain (SV-GAL4:φ) alone, the GAL4 chimeras SV-GAL4:VP16 and SV-GAL4:c-jun were inactive. Similarly, neither E2TR, nor E2TA, nor c-jun:E2 (not shown), was able to rescue the null oriLyt mutant 1199 which was partly activated by CMV-BZLF1:E2 (Figures 3 and 4B).

tion factor, a GAL4:c-myc chimera (data not shown) and the plasmid expressing the GAL4 DNA binding domain only (GAL4:φ; Figure 5). Taken together, these results indicate that oriLyt DNA replication is dependent on BZLF1 binding to this origin.

RNA transcription is not a prerequisite for DNA replication

Our data suggested that a specific activity located in the transcriptional activation domain mediates BZLF1's function as a replication factor. As a consequence, one might expect that RNA transcription and DNA replication are tightly linked, i.e. a high level of transcription is paralleled by efficient DNA replication and vice versa. If our failure to rescue oriLyt DNA replication with chimeric transcription factors other than BZLF1 resulted from their failure to activate transcription from oriLyt, one could easily explain BZLF1's dual role as reflecting its peculiar contribution to transcription. To address this possibility, we indirectly measured the promoter activities in oriLyt mutants in the presence of the chimeric transcription factors and compared it with wild-type levels. We cloned the firefly luciferase gene as a reporter in front of either the BHLF1 or the BHRF1 promoter. As oriLyt constructs, we used (i) wild-type oriLyt, (ii) oriLyt carrying two E2 or GAL4 binding sites at the position of ZRE1 and ZRE2, and (iii) the oriLyt mutants harboring seven E2 or GAL4 but no ZRE sites. These

Fig. 6. RNA transcription is neither sufficient nor obligatory for DNA replication of oriLyt. Two pairs of oriLyt mutants were tested for transcriptional activation of the BHLF1 and BHRF1 promoters with different transcription factors interacting with E2 or GAL4 binding sites. The results are compiled in histograms which give the relative transcriptional activation (% , mean value and SD) compared with wild-type oriLyt carrying seven ZRE binding sites (Figure 1) in the presence of BZLF1. The transcriptional activation was measured with the luciferase reporter gene which was either cloned at the position of the BHLF1 gene (large arrows at the top of the histograms pointing to the left) or at the position of the BHRF1 gene (large arrows at the top of the histograms pointing to the right). The oriLyt mutants p1132 and p1134 had ZRE1 and ZRE2 replaced with two E2 or GAL4 sites, respectively, all other mutants had the seven ZRE sites replaced with seven E2 or GAL4 binding sites. The exchange of all seven binding sites caused a dramatic reduction in transcriptional activation of both promoters (reporter plasmids p1215, 1216, 1221, 1222), whereas the replacement of both ZRE1 and ZRE2 had a moderate effect only (reporter plasmids p1132 and p1134). The chimeric transcription factors SV-GAL4:BZLF1, CMV-GAL4:BZLF1 and BZLF1:E2, which were capable of rescuing DNA replication, activated RNA transcription, but only BZLF1:E2 was capable of activating the BHLF1- (reporter plasmid p1215) and the BHRF1-promoters (reporter plasmid p1221) from one-third up to almost wild-type activity. The difference between GAL4:BZLF1 and BZLF1:E2 with respect to transcriptional activation might be related to the different structure of the two chimeric factors (Figure 4A). In contrast, transcription factors which were inactive with respect to DNA replication (GAL4:VP16, c-jun:E2) showed a similar activation level of the BHLF1 promoter (reporter plasmids p1215 and p1216) and the BHRF1 promoter (reporter plasmid p1221).

reporter constructs were cotransfected with pCMV-BZLF1 alone or in conjunction with the chimeric transcription factors carrying DNA binding specificity for either GAL4 or E2 sites which we had tested for their capacity to support replication of oriLyt. The basic oriLyt reporter constructs are schematically depicted in Figure 6 together with their relative transcriptional activity when cotransfected with the plasmids expressing the various chimeric molecules.

Quite unexpectedly, we found that (i) activation of RNA transcription was not a prerequisite to support oriLyt replication and (ii) transcriptional activation on its own was not sufficient to support DNA replication. Specifically, oriLyt mutants carrying seven E2 or GAL4 binding sites were not transactivated by wild-type BZLF1 alone, as expected, or in conjunction with E2(TA), E2(TR), GAL4:Ø and GAL4:c-jun (Figure 6). Vectors expressing chimeric BZLF1 proteins which successfully rescued DNA replication of oriLyt also failed to activate transcription completely (reporter plasmid p1216 with GAL4:BZLF1 chimeras) or

were inefficient (reporter plasmid p1222 with BZLF1:E2 and GAL4:BZLF1 chimeras, respectively). On the other hand, BZLF1:E2 transactivated the BHLF1 promoter to wild-type levels (reporter plasmid p1215) as did c-jun:E2, but only the former chimera supported DNA replication. GAL4:VP16 efficiently stimulated the BHLF1 promoter (reporter plasmid p1216), but did not stimulate the BHRF1 promoter (reporter plasmid p1222). Nevertheless, GAL4:VP16 failed to support oriLyt replication.

The transcriptional activation domain of BZLF1 efficiently activates transcription under certain conditions (reporter plasmid p1215). However, this function of BZLF1 *per se* was not required for oriLyt replication since a transcriptionally silent BZLF1 chimera (SV-GAL4:BZLF1 with reporter plasmid p1216 in Figure 6) can efficiently support DNA replication in the authentic context (Figures 3–5). Thus, the contribution to DNA replication by BZLF1 appears to be distinct from its contribution to transcriptional activation of oriLyt, suggesting that both functions might

be separable. It is also clear that transcriptional activation from either of the two oriLyt promoters alone is not sufficient to promote DNA replication of this origin.

Discussion

The transcriptional activation domain of BZLF1 is required for oriLyt function

In this report, we identified BZLF1 as a *trans*-acting factor which is absolutely required for oriLyt-mediated DNA replication. One of the functions of the BZLF1 transcription/replication factor is encoded in its DNA binding domain. In oriLyt DNA replication, the site-specific binding of BZLF1 to ZRE sites was found to be a prerequisite to activate DNA replication. Although we do not have direct evidence which ZRE sites must be occupied by BZLF1 to promote replication, BZLF1 might interact with multiple sites. The affinity of BZLF1's binding to certain sites affects oriLyt replication (Figure 2), indicating that DNA binding of BZLF1 itself could be rate limiting. Nevertheless, the DNA binding domain of BZLF1 could be functionally replaced by either the GAL4 or E2 DNA binding domains to promote oriLyt function. This finding indicates that the transcriptional activation domain of BZLF1 is absolutely required for DNA replication of oriLyt.

It appears likely that the transcriptional activation domain of BZLF1 has to interact with other proteins to exert its function in DNA replication. This assumption is underscored by our finding that the deletion of its entire upstream component abolished oriLyt DNA replication, while the deletion or non-functional substitution of the two ZRE sites which are resident in the upstream component only reduced the efficiency of oriLyt-mediated DNA replication. This somewhat puzzling finding could give a clue to BZLF1's dual role in mediating RNA transcription and DNA replication. Certain sequence elements contained within the upstream component of the oriLyt core are required in addition to ZRE1 and ZRE2 for DNA replication. These sequence elements, or proteins bound to them, might act downstream from BZLF1's binding to ZRE1 and ZRE2. Potential candidates include factors which are known or expected to interact with sequence elements present in the upstream component. They include the TFIID- and CCAAT-box binding factors, for example, or other proteins from the RNA transcription initiation complex which are recruited to the BHLF1 promoter. TFIID is known to bind to the transcriptional activation domain of BZLF1, which in turn stabilizes TFIID at the promoter and stimulates transcription (Lieberman and Berk, 1991).

What does BZLF1 contribute to DNA replication as compared with other transcription factors?

Eukaryotic origins of DNA replication usually contain auxiliary components which consist of transcription factor binding sites. The function of the auxiliary component is that of a replication enhancer analogous to enhancer elements involved in RNA transcription. Although it appears that the auxiliary components do not map to the locus of initiation of DNA synthesis, they determine the efficiency of this step. The detailed analysis of the origin components of small DNA viruses has revealed several features of transcription factors which functionally interact with different auxiliary elements (Cheng and Kelly, 1989; Wasylyk *et al.*, 1990; Baru *et al.*,

1991; Bennett-Cook and Hassell, 1991; Murakami *et al.*, 1991; Nilsson *et al.*, 1991; Cheng *et al.*, 1992; Guo and DePamphilis, 1992; Hoang *et al.*, 1992). (i) Auxiliary origin components are activated only by transcription factors carrying the activation domain that is also involved in stimulating RNA transcription. Recombinant transcription factors consisting of the specific DNA binding domain are inactive. (ii) The nature of the activation domain is usually irrelevant, even yeast GAL4 protein can facilitate initiation of SV40 or polyoma virus DNA synthesis. Thus, a specific requirement for a domain involved in mediating this effect is not apparent. (iii) Domains responsible for site-specific DNA binding are functionally interchangeable. (iii) Stimulation of DNA synthesis by natural or recombinant transcription factors reflects their potency to stimulate RNA transcription in most, but not in all cases.

Our study suggests that BZLF1 does not fall into any of these categories. Although BZLF1 is a potent transcription factor, its role in DNA replication seems to be different from that of other transcription factors which interact with auxiliary components of eukaryotic origins. This is because BZLF1 is absolutely required to mediate DNA synthesis from oriLyt. BZLF1's role in DNA replication resides within its transcriptional activation domain, which cannot be substituted by that of other transcription factors, even though some of them are known to bind TFIID (VP16) (Lin *et al.*, 1991), as does BZLF1 (Lieberman and Berk, 1991). It may be that (i) BZLF1 possesses an inherent enzymatic activity similar to the helicase activity of other viral origin-specific DNA proteins, and (ii) that BZLF1 provides a mechanical link to the DNA replication complex of oriLyt, but these possibilities remain to be tested.

Materials and methods

Cell line

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 ~20 copies of the EBV genome (data not shown) and was maintained in Dulbecco's modified Eagles's medium containing 5% fetal and 5% newborn calf serum.

Recombinant plasmids

The plasmid p968.22, carrying wild-type oriLyt, was constructed by ligating a 7.2 kbp *Bam*HI–*Sal*I fragment from the EBV strain B95-8 (nucleotide coordinates # 48 848 – # 56 084; Baer *et al.*, 1984) into a *Bam*HI/*Sal*I cut pUC8 vector plasmid as described previously (Schepers *et al.*, 1993). The plasmid pCMV-BZLF1 is an expression vector which efficiently induces the lytic phase of EBV's life cycle. The BZLF1 gene is driven in this retroviral vector construct by the promoter of the immediate early genes of the human cytomegalovirus as described by Hammerschmidt and Sugden (1988). Mutants of p968.22 were constructed as described in detail by oligonucleotide-directed mutagenesis (Schepers *et al.*, 1993). All mutations were confirmed by DNA sequencing. The synthetic oligonucleotides needed for the mutagenesis and the sequencing protocols were purchased (University of Wisconsin, Biotechnology Center) or synthesized on an oligonucleotide synthesizer (Pharmacia) and purified on reverse-phase columns through high-pressure liquid chromatography. To perform the transient transcription assays, the 3.8 kbp *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 (de Wet *et al.*, 1987). Similarly, the 1.7 kbp *Bgl*II–*Sal*I fragment (from # 54 360 of EBV to the multiple cloning site in p968.22) carrying the BHRF1 open reading frame was replaced by the luciferase gene. The BZLF1 chimeric transcription factors were cloned under the control of either the SV40 early promoter (SV-GAL4:BZLF1) or under the control of the human cytomegalovirus immediate early promoter (CMV-GAL4:BZLF1 and CMV-BZLF1:GAL4). The expression plasmid SV-GAL4:BZLF1 was

made in the following steps. In the first step, by oligonucleotide-directed mutagenesis two bases were exchanged at nucleotide coordinates # 120 522 and # 120 523 of EBV in a recombinant plasmid that contained the open reading frame of the BZLF1 gene. The purpose of this manipulation was to create a new *SnaI* site within the very beginning of the second exon of the BZLF1 gene. In the second step, a partial *AvaII*–*SnaI* fragment encompassing nucleotide coordinates # 102 524–# 103 149 of EBV was cloned in frame into the *Bam*HI cut and blunt-ended plasmid pSG424 (Sadowski and Ptashne, 1989). This resulted in the vector SV-GAL4:BZLF1 which expressed a chimeric fusion protein consisting of amino acids 1–147 of GAL4 and amino acids 3–169 of BZLF1. This open reading frame was transferred to an expression vector similar to CMV-BZLF1 (Hammerschmidt and Sugden, 1988) to express the chimeric transcription factor under the control of a stronger promoter. To generate CMV-BZLF1:E2, several cloning steps were necessary. The final plasmid contained a fused open reading frame which consisted of amino acids 1–169 of BZLF1 and amino acids 128–410 of the bovine papilloma virus E2 transcription factor. The former was derived from the plasmid pCMV-BZLF1 (Hammerschmidt and Sugden, 1988); the latter was taken from the plasmid pML142.6 which was a kind gift from Paul Lambert. Other native and recombinant transcription factors expressed from various vector plasmids [SV-GAL4:c-jun; SV-GAL4:VP16; SV-GAL4:φ(pSG424); E2 TA, E2 TR; CMV-c-jun:E2] were generous gifts from Vijai Baichwal, Mark Ptashne and Paul Lambert (Yang *et al.*, 1985; Lambert *et al.*, 1987; Sadowski *et al.*, 1988; Sadowski and Ptashne, 1989; Baichwal and Tjian, 1990).

Transient replication assays

Ten micrograms of oriLyt plasmid DNA were cotransfected with 1 µg of p526 and 10 µg of pCMV-BZLF1 into D98HR1 cells as described previously (Schepers *et al.*, 1993). The plasmid p526 has been described previously (Hammerschmidt and Sugden, 1988) and contains a wild-type oriLyt fragment. It was used as an internal control for standardization for each mutant plasmid to be tested. The concomitant introduction of the plasmid pCMV-BZLF1 efficiently induces the lytic cycle of EBV. As a fourth plasmid, expression vectors were cotransfected to provide the various chimeric and native transcription factors interacting with GAL4 or E2 binding sites. Two days after cotransfection, DNA was prepared, input plasmid DNA was digested with *DpnI* and *Bam*HI, and the efficiency with which the oriLyt plasmids replicated was quantitated by scanning the specific fragment signals detected after Southern blot hybridization and autoradiography. The quantification of the replication assays was based on at least three independent experiments and the evaluation of the data was obtained indirectly by recording the autoradiograms with a CCD camera and scanning the digitalized images.

Transient transcription assays

To measure the transcriptional activation from the two promoter elements in different mutant oriLyt plasmids, 10 µg of plasmid DNA containing the luciferase reporter gene at the position of either the BHRF1 or BHLF1 gene were cotransfected together with 10 µg pCMV-BZLF1, 10 µg of a vector plasmid expressing one of the native or chimeric transcription factors and 0.5 µg of pCMV-β-gal into D98HR1 cells. The luciferase activity was measured in cell extracts as described in detail by Schepers *et al.* (1993). All reactions were performed at least in quadruplicate.

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