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Cellular transcription factors recruit viral replication proteins to activate the Epstein–Barr virus origin of lytic DNA replication, *oriLyt*

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DNA replication of Epstein–Barr virus (EBV) during the productive phase of the life cycle of this herpesvirus depends on the *cis*-acting element *oriLyt*. It consists of two essential domains, the upstream and the downstream component. Whereas the upstream component contains several DNA-binding motifs for the viral activator protein BZLF1, the downstream component is known to be the binding site of several cellular proteins. We identified cellular transcription factors that bind synergistically to a functionally relevant subsequence of the downstream component, the TD element. Two of these transcription factors, ZBP-89 and Sp1, stimulate replication as shown by protein fusions with the GAL4 DNA-binding domain and a single GAL4 DNA-binding motif inserted into the TD element. In protein binding assays, we observed an interaction of Sp1 and ZBP-89 with the viral DNA polymerase and its processivity factor. Our data indicate that cellular transcriptional activators tether viral replication proteins to the lytic origin via direct protein–protein interactions to assemble the viral replication complex at *oriLyt*.

Keywords: EBV/replication/Sp1/transcription factor/ZBP-89

Introduction

Studies on DNA tumor virus model systems strongly suggest that both *cis*- and *trans*-acting transcriptional elements are involved in the initiation of DNA replication (DePamphilis, 1993). This finding has led to the hypothesis that transcriptional activators play similar roles at promoters and origins since viral and cellular replication origins contain binding sites for transcription factors, which have been found to increase the initiation frequency up to 1000-fold (de Villiers *et al.*, 1984; Cheng and Kelly, 1989; Guo *et al.*, 1989; Ustav and Stenlund, 1991; Nguyen-Huynh and Schaffer, 1998). The molecular basis for the activation of replication and transcription by these proteins seems to rely on at least two mechanisms. One indirect mechanism involves the remodeling of chromatin. Transcriptional activators may stimulate both transcription and replication by enhancing the ability of initiation factors to displace histones and thereby improve the access to promoter or origin structures (Li and Botchan, 1994; Kadonaga, 1998; Hu *et al.*, 1999). Secondly, like the basal transcription machinery, the replication apparatus consists of distinct general factors that are responsible for the assembly of a functional activation complex at the initiation zones. This is achieved by the interaction of transcription factors with components of the basal machinery. For instance, some enhancer proteins that stimulate DNA replication have been shown to target essential replication factors such as replication protein A (RPA) or the DNA polymerase α-primase, thus helping to form the replication complex (Dornreiter *et al.*, 1992; He *et al.*, 1993; Li and Botchan, 1993).

In latently infected B cells, the Epstein–Barr virus (EBV) genome replicates synchronously with the cellular genome via the plasmid origin of DNA replication, *oriP* (Yates, 1996), but virions are only produced in the lytic phase of infection. The onset of the lytic phase is controlled by transcriptional activation of the viral transcription factor BZLF1 (Countryman and Miller, 1985), which initiates an expression cascade of the lytic genes and activates the lytic replication origin, *oriLyt* (Lieberman *et al.*, 1990; Schepers *et al.*, 1993a). *oriLyt* is located within the divergent promoter regions of the *BHLF1* and *BHRF1* genes and consists of two essential core elements, which constitute the minimal origin of DNA replication: the upstream and the downstream component (Hammerschmidt and Sugden, 1988; Schepers *et al.*, 1993b). The upstream component is nearly identical to the *BHLF1* promoter and harbors specific binding motifs for BZLF1, termed ZREs. Several cellular proteins were found to bind to a functional subsequence of the downstream component, the TD element, which is extremely sensitive to mutations with regard to *oriLyt*-dependent replication (Schepers *et al.*, 1993b; Gruffat *et al.*, 1995). These factors include Sp1, but their role has remained obscure.

Six EBV-encoded gene products are required to execute lytic DNA replication at the level of DNA synthesis: the DNA polymerase (BALF5); the polymerase processivity factor (BMRF1); the single-stranded DNA-binding protein (BALF2); the primase (BSLF1); the primase-associated protein (BBLF2/3); and the helicase (BBLF4) (Fixman *et al.*, 1992, 1995). In transfected cells, these six viral gene products together with BZLF1 are sufficient for the replication of *oriLyt*-containing plasmids. The EBV lytic replication proteins are homologous to and have similar biological properties to those required for herpes simplex virus-1 (HSV-1) lytic replication (Challberg, 1996; Yates, 1996), indicating that EBV and HSV-1 may utilize a similar mechanism during viral lytic DNA replication.

Table I. Proteins that bind the TD element in *S.cerevisiae* one-hybrid screens

Isolated clone	No. of isolates	Homology with known proteins	% homology[a]
α24-5	1	mouse c-Krox, I49603[b]	56
Ω9	3	human MAZi, P56270	69
VIII41	1	human ZBP-89, AF039019	99.5

[a]The percentage homology with known proteins that showed the highest score of similarity with the isolated clones based on amino acid sequence comparisons.
[b]The alphanumerical codes are the DDBJ/EMBL/GenBank accession numbers.

Numerous interactions between these proteins have been documented, consistent with the existence of a replication complex that is essentially viral in nature (Tsurumi *et al.*, 1993; Zeng *et al.*, 1997; Gao *et al.*, 1998).

We are interested in the functional characterization of the downstream component and sought to identify cellular proteins that bind to *oriLyt* and mediate DNA replication. Cellular transcription factors were isolated in a yeast one-hybrid screen and analyzed for their contribution to lytic replication. We show here that Sp1 and ZBP-89 bind to *oriLyt* and promote *oriLyt*-dependent replication. In protein-binding assays, both proteins interact with the viral DNA polymerase–processivity factor complex, supporting the idea that the contact between cellular transcription factors and viral replication proteins at the downstream component is essential for lytic DNA synthesis of EBV.

Results

Isolation of transcription factors that bind to the TD element of oriLyt

We used a yeast one-hybrid screen to identify factors that bind to the TD element of *oriLyt*. In this assay, a yeast strain was constructed that contains multimerized TD elements in the context of the *HIS3* locus and an artificial promoter driving *lacZ*. This yeast strain was transformed with a plasmid cDNA library derived from EBV-immortalized B cells in which cDNAs are fused to the transactivation domain (TAD) of GAL4 (Durfee *et al.*, 1993) to form chimeric transcription factors with potential DNA-binding characteristics. Both markers, *HIS3* and *lacZ*, allow the simple detection of positive transformants using histidine prototrophy and a blue colony phenotype in the presence of X-gal. From 7×10^6 primary transformants, five cDNAs could be isolated representing three different genes (Table I). These cDNAs scored positive upon testing in the original yeast strain but not in a control strain with mutated TD motifs (data not shown).

We obtained partial cDNAs encoding the DNA-binding domains (DBDs) of cellular zinc finger-containing proteins. While the cDNAs of α24-5 and Ω9 show a limited homology to the transcription factors c-Krox and MAZi, respectively, clone VIII41 is identical to the human transcriptional repressor ZBP-89. As the candidate cDNAs only coded for the C-terminal part of the corresponding proteins, we identified the remaining N-terminus. In the case of VIII41, this was done by RT–PCR of HeLa cell RNA with primers specific for the known sequence of human ZBP-89. Since some initial information could be obtained about the rat and the mouse homologs of ZBP-89, we concentrated on the analysis of human ZBP-89 and its function with respect to *oriLyt* activation.

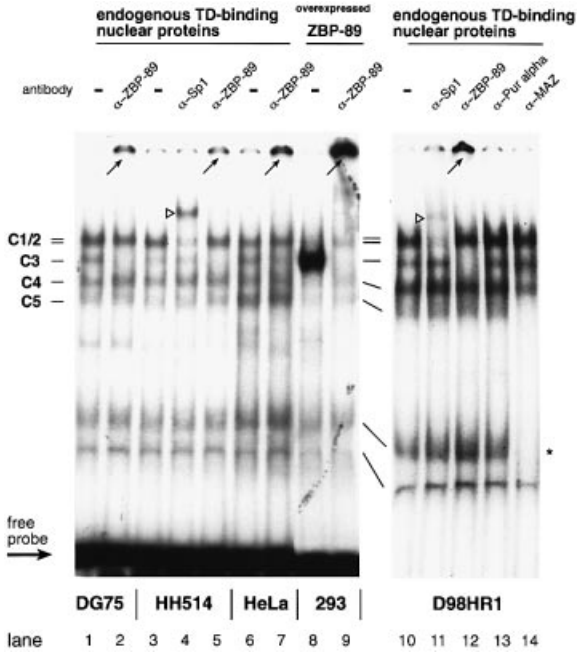

Fig. 1. Gel retardation assay with nuclear extracts. Nuclear lysates were incubated with a labeled oligonucleotide encompassing the TD element with or without specific antibodies directed against Sp1, ZBP-89, Pur alpha or MAZ, respectively. In lanes 8 and 9, nuclear extracts of 293 cells transfected with an expression plasmid for ZBP-89 were incubated with the labeled TD DNA probe prior to electrophoresis. The positions of the different complexes formed with the TD element are indicated as C1–C5. Black arrows mark the position of C3 supershifted with a polyclonal rabbit anti-ZBP-89 antibody. The position of C1/C2 supershifted with a monoclonal anti-Sp1 antibody (sc-59x by Santa Cruz Biotech.) is indicated by white arrowheads. The asterisk marks a faster migrating complex that disappears after incubation with the MAZ-specific antibody, likewise complex C5. In the right hand panel, the front of unbound probe DNA is not shown.

Sp1 and ZBP-89 are nuclear cellular proteins that bind to the TD element in vitro

We employed electrophoretic mobility shift assays (EMSAs) with nuclear extracts from various cell lines in order to examine whether ZBP-89 interacts with the TD element. A characteristic migration pattern of at least five cellular complexes C1–C5 (Figure 1) can be detected regularly (Gruffat *et al.*, 1995). Incubation with a ZBP-89-specific antibody resulted in the disappearance of complex C3 and a strong signal that did not enter the gel (Figure 1, lanes 2, 5, 7, 9 and 12). When 293 cells were transfected with a ZBP-89 expression plasmid, a shift complex could be detected which migrated with the same mobility as complex C3 (Figure 1, lane 8). Incubation with an antibody against Sp1 (Figure 1, lanes 4 and 11) showed that it is part of complex C1/C2, as has been reported previously (Gruffat *et al.*, 1995). We also tested an antibody against the MAZ protein (Tsutsui *et al.*, 1996) that led to the disappearance of complex C5 and a faster migrating complex (Figure 1, lane 14). Although we were

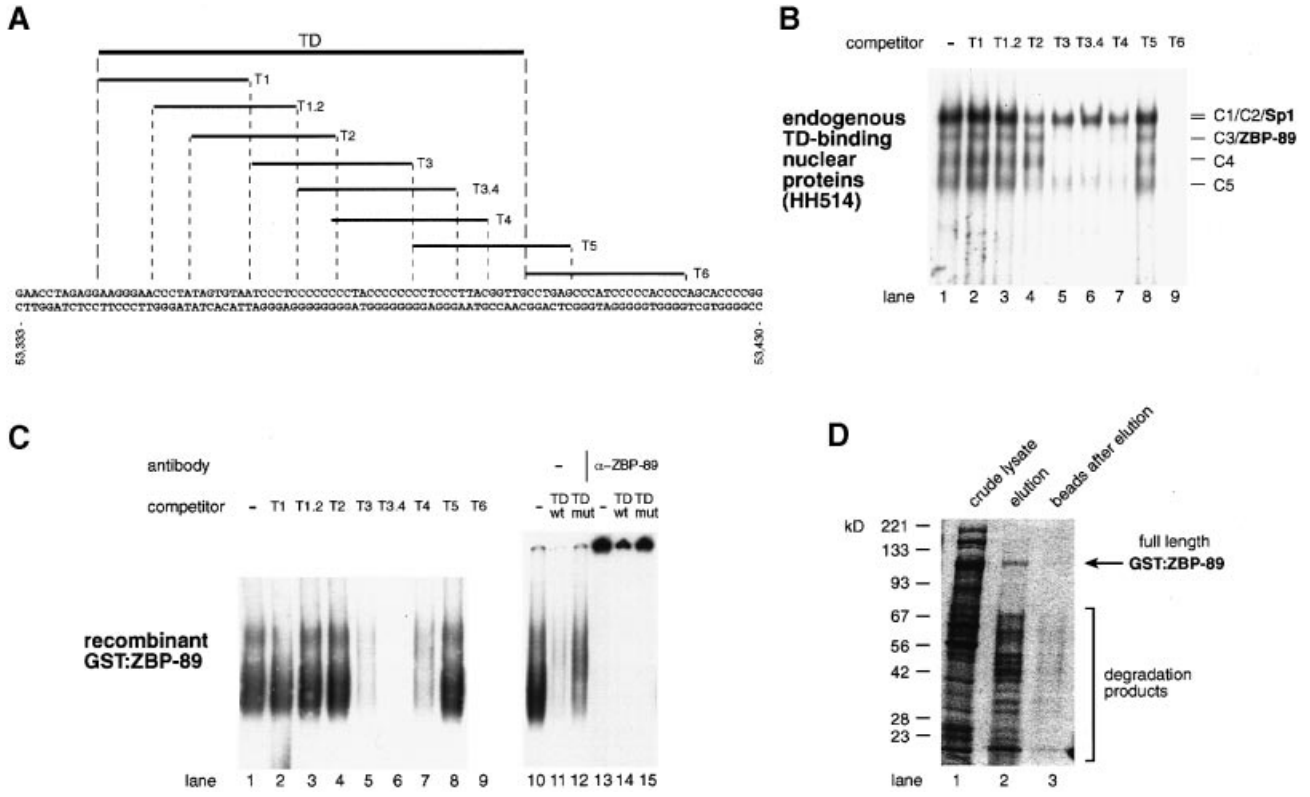

Fig. 2. Competitive gel shift. (**A**) Nucleotide sequence of the *oriLyt* downstream component of the EBV strain B95-8 (Baer *et al.*, 1984) with the positions of subsequences T1–T6 shown. (**B**) Gel shift analysis. Nuclear extracts were derived from the EBV-positive cell line HH514 and incubated with the TD probe in the presence of a 200-fold molar excess of unlabeled competitor oligonucleotides T1–T6. (**C**) Gel shift analysis. GST:ZBP-89 protein was mixed with the TD probe prior to incubation with a 200-fold molar excess of unlabeled T1–T6, TD-wt or TD-mut as competitor oligonucleotides. In lanes 13, 14 and 15, samples were incubated with a ZBP-89-specific antibody. The smeary appearance of the shifted complex presumably results from degradation products of GST:ZBP-89 as judged from analysis of preparations on Coomassie Blue-stained SDS gels [shown in (D)]. (**D**) Coomassie Blue-stained gel of the corresponding GST:ZBP-89 preparation used for gel shift analysis presented in (C). GST:ZBP-89 was produced in *E.coli* and purified from bacterial lysates (lane 1) by binding to a glutathione–Sepharose matrix. Bound GST fusion proteins were eluted by the addition of glutathione (lane 2). Lane 3 shows the Sepharose beads after elution by glutathione.

unable to detect any supershifted band, this finding argues for the capacity of MAZ(-related) proteins to bind the TD element *in vitro*. In contrast, incubation with an antibody against Pur alpha, a protein known to bind to purine-rich strands found in initiation zones of both cellular and viral DNA replication (Jurk *et al.*, 1996), had no effect (Figure 1, lane 13). Thus, ZBP-89, Sp1 and MAZ or a MAZ-related protein are among those nuclear proteins that can bind to the TD element *in vitro*.

Next, competitive gel shift assays were performed using the TD subsequence oligonucleotides depicted in Figure 2A. The Sp1-containing complex C1/C2 is competed selectively with a 200-fold excess of T2, T3, T4 and T6 subsequences, indicative of preferential binding of Sp1 to these parts of TD (Figure 2B, lanes 4, 5, 7 and 9) (Gruffat *et al.*, 1995). The ZBP-89-containing complex C3 was competed efficiently with the oligonucleotides T3, T3.4, T4 and T6 (Figure 2B, lanes 5–7 and 9), all of which have a similar base composition. This finding suggests that the two TD-interacting factors bind to overlapping regions. Recombinant GST:ZBP-89 showed an almost identical competition pattern (Figure 2C, lanes 1–9), supporting the assumption that ZBP-89 binds to G/C-rich regions within the downstream region of *oriLyt* and in fact constitutes complex C3. Competition analysis with a cold excess of wild-type TD (TD-wt) versus a mutated TD (TD-mut) harboring C to G transversions (Figure 2C, compare lanes 11 and 12) confirmed the results obtained with the yeast one-hybrid screen. ZBP-89 interacts only weakly with an oligonucleotide containing the replication-defective TD sequence (TD-mut).

The interaction of Sp1 and ZBP-89 with the TD element was then examined by DNase I footprinting to define the binding regions more precisely. The assays were performed with recombinant proteins and a DNA fragment encompassing the *oriLyt* downstream component. Two prominent protected regions were observed for Sp1 or ZBP-89 alone (Figure 3, sites A and B for Sp1, and sites I and II for ZBP-89). The coordinates of the Sp1 sites A and B were in good agreement with the positions mapped previously (Gruffat *et al.*, 1995). The ZBP-89 footprint pattern overlapped and exceeded that of Sp1, and almost complete protection of the entire T6 element could be observed (Figure 3, lanes 5–7). The ZBP-89 footprint produced within the TD element (site I) was somewhat heterogeneous in that a strong protection was observed from nucleotides 53 359 to 53 379 but downstream nucleotides (53 380–53 390) were less well protected. Sites I and II were separated by a DNase I-hypersensitive spacer of ~10 bases, which is approximately the length of a single DNA helix turn, suggesting that ZBP-89 binds 2-fold to one face of the DNA. The pattern of protein–DNA interaction was also examined in footprinting assays when both proteins were present in various combinations

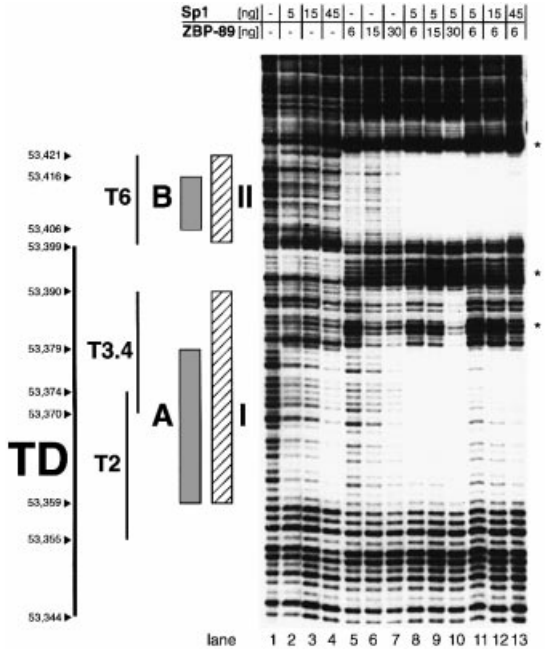

Fig. 3. DNase I protection assay. Recombinant Sp1 protein and/or ZBP-89 cleaved off from GST were incubated with ^{32}P-labeled probe DNA followed by limited digestion with DNase I. Regions of DNase I protection are indicated by stippled rectangles A and B for Sp1 and hatched rectangles I and II for ZBP-89. Hypersensitive bases are indicated by asterisks. The relative position of the TD element together with the subsequences T2 and T3.4 and the T6 element, all of which were used in the competition experiments in Figure 2, are depicted. Numbers indicate the position of the residues within the *oriLyt* sequence.

(Figure 3, lanes 8–10 and 11–13). The footprint was significantly enhanced and dominated by the ZBP-89-specific protection (Figure 3, compare lanes 10 and 7 at nucleotide positions 53 380–53 390). The protection of the Sp1 site A in the presence of a low concentration of ZBP-89 was improved (Figure 3, compare lane 11 with lane 2 at nucleotide positions 53 359–53 379).

These results suggest that Sp1 and ZBP-89 are not only able to bind simultaneously to the TD element, but do so in a synergistic manner. Since the two factors do not appear to interact directly with each other (data not shown), we hypothesize that this co-operation might result from an altered DNA conformation introduced by one protein, which facilitates the binding of the other.

Sp1 and ZBP-89 activate replication of oriLyt plasmids

ZBP-89 has been characterized as a bona fide transcriptional repressor, but a function in DNA replication was not reported (Merchant *et al.*, 1996). Therefore, we investigated whether ZBP-89 stimulates *oriLyt*-mediated DNA replication in transient replication assays. An expression plasmid encoding full-length ZBP-89 was co-transfected together with the plasmid 968.22 encompassing *oriLyt* and an expression vector for BZLF1 to activate the lytic cycle in a latently EBV-infected cell line as described (Hammerschmidt and Sugden, 1988; Schepers *et al.*, 1993a). As shown in Figure 4A, ZBP-89 enhanced the signal of the *oriLyt* plasmid whereas overexpression of Sp1 resulted only in a slight increase. It appears that under these experimental conditions, ZBP-89 is limiting due to the co-transfection of substantial amounts of *oriLyt* plasmid DNA. Co-expression of ZBP-89 might correct for this phenomenon as indicated by shift experiments in Figure 1, lane 8. This effect is less obvious with Sp1, which seems to be expressed at higher levels. Although these results might support a role for ZBP-89 and Sp1 in *oriLyt*-dependent DNA replication, overexpression of transcription factors could also lead to an indirect activation of *oriLyt*. Therefore, we sought to investigate the role of Sp1 and ZBP-89 in terms of *oriLyt* activation in an unambiguous manner.

First, *oriLyt* plasmids were constructed that contain a single yeast GAL4-binding site at different positions within the TD element shown in Figure 4B. Secondly, we asked whether a single GAL4-binding motif at various positions has consequences for the binding of cellular proteins to the TD element. When fragments generated from the individual *oriLyt* mutant plasmids (Figure 4B) were used as probes in gel retardation assays (Figure 4C), complex formation was significantly altered compared with the wild-type *oriLyt* sequence. The formation of the Sp1 complex C1/C2 was much weaker with DNA probes from 2204 to 2206, and 2208 (Figure 4C, lanes 3, 4, 5 and 7). The intensity of complex C3 containing ZBP-89 was strongly diminished in 2203 and was completely abolished with the DNA probes prepared from plasmids 2205–2208 (Figure 5C, lanes 2, 4, 5, 6 and 7). Furthermore, the intensity of complexes with a higher mobility, C4 and C5, vanished from 2205 through 2208. Thirdly, the replication capacity of the GAL4-inserted mutant *oriLyt* plasmids was measured in transient replication assays. The insertion of a single GAL4 site impaired *oriLyt* activity severely (Figure 4D). Only the *oriLyt* plasmid 2203 kept to ~5–10% residual replication activity in comparison with the wild-type *oriLyt* plasmid 968.22 (Figure 4D, compare lane 1 with 2), while the remaining mutant plasmids did not replicate detectably (Figure 4D, lanes 3–7), suggesting a crucial functional correlation between the replication capacity of *oriLyt*, its overall structure and the correct assembly of all cellular proteins on the TD element. Fourthly, we constructed plasmids that express chimeric polypeptides consisting of the TAD of Sp1 or ZBP-89 fused to the GAL4 DBD. The fusion proteins bound exclusively to GAL4-inserted DNA templates and not to the wild-type plasmid 968.22 in gel retardation assays (data not shown). To investigate the ability of Sp1 and ZBP-89 to rescue replication-deficient *oriLyt* plasmids, we tested the fusion proteins in transient replication assays. Both GAL4:Sp1(N) and GAL4:ZBP-89(C) were able partially to restore the replication activity of the *oriLyt* mutant plasmid 2203 to ~50% of the wild-type level (Figure 4E, lanes 5, 6, 18 and 19). We also found a small but reproducible rescue of the *oriLyt* plasmid 2204 of ~5% of 968.22 activity (Figure 4E, lanes 9 and 10). Transfection of a plasmid encoding only the GAL4 DBD did not result in replication (Figure 4E, lanes 7 and 17, and data not shown). We were also unable to see any *oriLyt* activity with an expression plasmid encoding the TAD of VP16 coupled to the GAL4 DBD (Figure 4E, lanes 4, 8 and 11) although GAL4:VP16 proved to be a potent activator of replication in other viral systems (He *et al.*, 1993; Li and Botchan, 1993) and a much better transcriptional activator than GAL4:Sp1(N) or

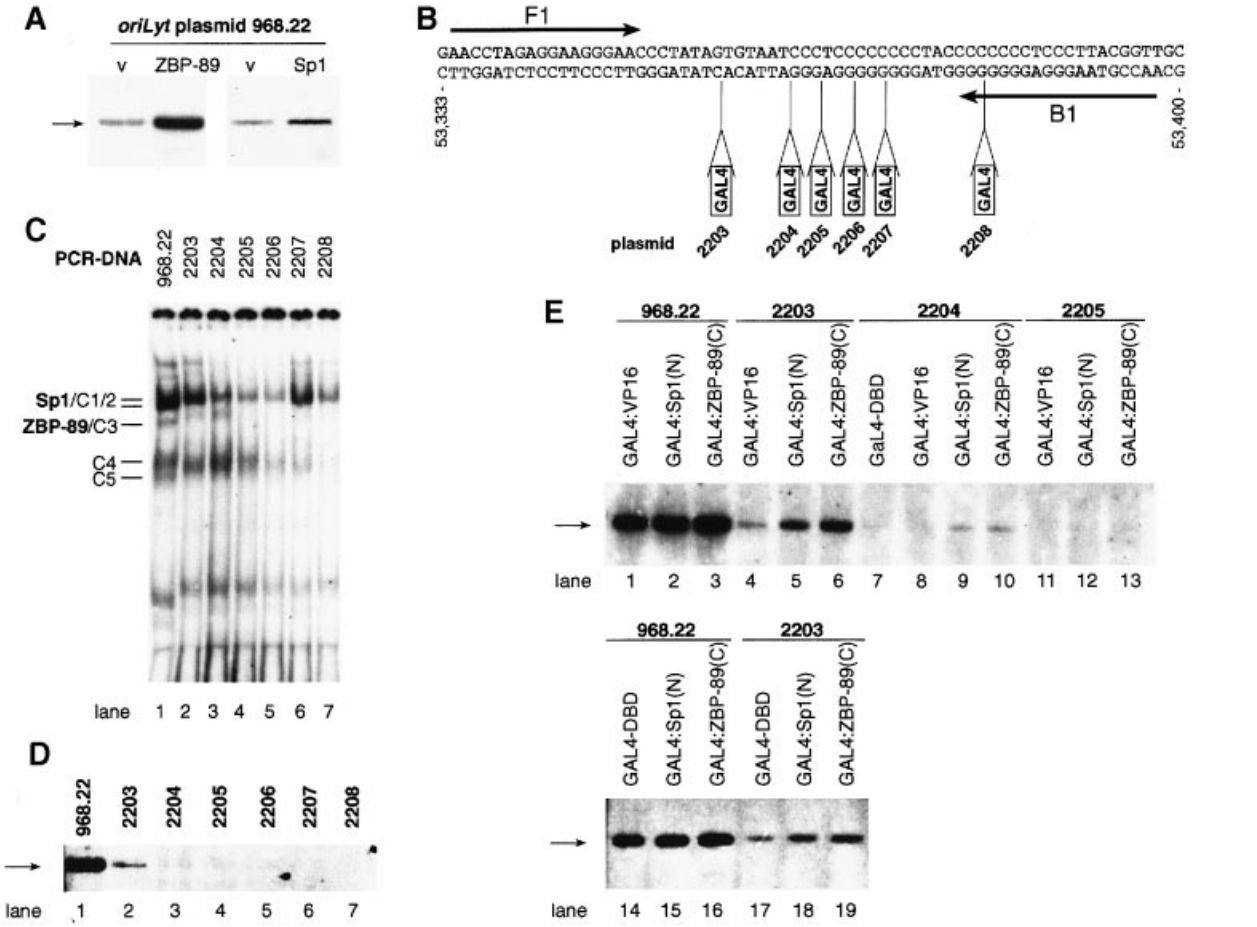

Fig. 4. Enhancement of *oriLyt* replication by Sp1 and ZBP-89. (**A**) Transient replication assay. Constitutive expression plasmids encoding ZBP-89 or Sp1 or a control vector (v) were co-transfected into D98HR1 cells together with p968.22. Newly replicated *oriLyt* plasmid DNA is indicated by an arrow. (**B**) Nucleotide sequence of the *oriLyt* downstream component from nucleotide coordinates 53 333 to 53 400. Insertions of a single GAL4-binding site at different positions are indicated by rectangles with their designations below. The positions of the PCR primers F1 and B1 which were used to generate DNA probes for gel retardation assays are marked. (**C**) Gel shift with nuclear extracts derived from the EBV-positive cell line HH514. DNA probes were generated by PCR amplification of the wild-type plasmid 968.22 and the mutant plasmids 2203–2208 as indicated in (B). (**D**) Transient replication assay with 968.22 in comparison with the GAL4-inserted *oriLyt* plasmids 2203–2208. (**E**) Transient replication assay with the reporter plasmids 968.22 and 2203–2205 after co-transfection of expression vectors encoding the GAL4 DBD or chimeras of the GAL4 DBD with the TAD of Sp1, ZBP-89 or VP16.

GAL4:ZBP-89(C) (data not shown). Hence, when tethered to the origin, the TADs of Sp1 or ZBP-89 can compensate to some extent for the loss of C1/C2 or C3 binding activity in GAL4-inserted *oriLyt* mutant plasmids 2203 and 2204, resulting in partial rescue of origin function.

A plausible explanation for the failure of mutants 2205–2208 to replicate in conjunction with Sp1 or ZBP-89 fusion proteins (Figure 4E, lanes 12 and 13, and data not shown) might be the lack of the TD-binding complexes C4 and C5 on the mutated TD elements (Figure 4C). It appears that the loss of C4/C5 binding activity cannot be complemented by GAL4:Sp1(N) or GAL4:ZBP-89(C), suggesting that the presence of these cellular factors is also necessary for origin function.

In summary, the formation of protein–DNA complexes at TD correlates with the efficiency of *oriLyt*-dependent replication. This finding argues in favor of a role for these factors in *oriLyt*-mediated DNA replication. Whereas the loss of binding of certain TD-binding proteins to mutant TD motifs is correlative only, the functional rescue of two *oriLyt* mutant plasmids indicates a role for both Sp1 and ZBP-89 in *oriLyt* function.

EBV replication proteins interact with Sp1 and ZBP-89

The viral transcription factor BZLF1, which is indispensable for DNA replication, has been shown to interact with components of the EBV helicase–primase complex (Gao *et al.*, 1998) as well as with BMRF1, the accessory factor of the viral DNA polymerase BALF5 (Zhang *et al.*, 1996). These interactions are likely to contribute to DNA replication at the upstream component of *oriLyt*. We were interested to know if similar interactions occur at the downstream component since BMRF1 has been proposed to transcriptionally activate the downstream component of *oriLyt* (Zhang *et al.*, 1996). Although BMRF1 does not bind specifically to the downstream component, BMRF1 could be tethered indirectly to the downstream component by factors which do bind to *oriLyt* (Zhang *et al.*, 1997).

To test whether Sp1 or ZBP-89 interact with the viral DNA polymerase complex, we co-expressed BMRF1, BALF5 or BALF2 together with BZLF1 in 293 cells. Cell extracts were subjected to immunoprecipitation with polyclonal antibodies directed against BMRF1, BALF2 and BALF5. Immunoprecipitated complexes were then

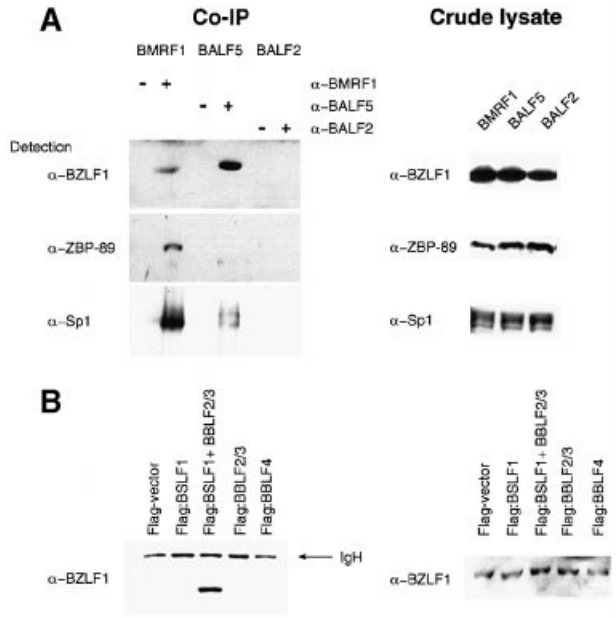

Fig. 5. Interaction of Sp1, ZBP-89 and BZLF1 with lytic replication proteins of EBV *in vivo*. (**A**) Left panels: *in vivo* co-immunoprecipitation. 293 cells were transiently transfected with expression plasmids for BMRF1, BALF5 or BALF2 together with an expression vector for BZLF1. Cell lysates were incubated with protein A–agarose beads coupled to specific antibodies against BMRF1, BALF5 and BALF2. As negative controls, the specific antibodies were omitted. Precipitated BZLF1, Sp1 and ZBP-89 were visualized by Western blotting. Right panels: immunodetection of BZLF1, Sp1 and ZBP-89 in the crude cell lysates prior to immunoprecipitation demonstrated equal amounts of the indicated proteins. (**B**) Left panel: *in vivo* co-immunoprecipitation. To precipitate Flag:BSLF1, Flag:BBLF2/3 and Flag:BBLF4, the corresponding plasmids were transfected into 293 cells together with an expression plasmid for BZLF1. Where indicated, cells were co-transfected with Flag:BSLF1 and BBLF2/3 plus BZLF1. Cell extracts were mixed with anti-Flag antibody bound to agarose beads. Precipitated BZLF1 was visualized by Western blotting. Right panel: equal amounts of BZLF1 in the crude cell lysates prior to immunoprecipitation were demonstrated by immunodetection.

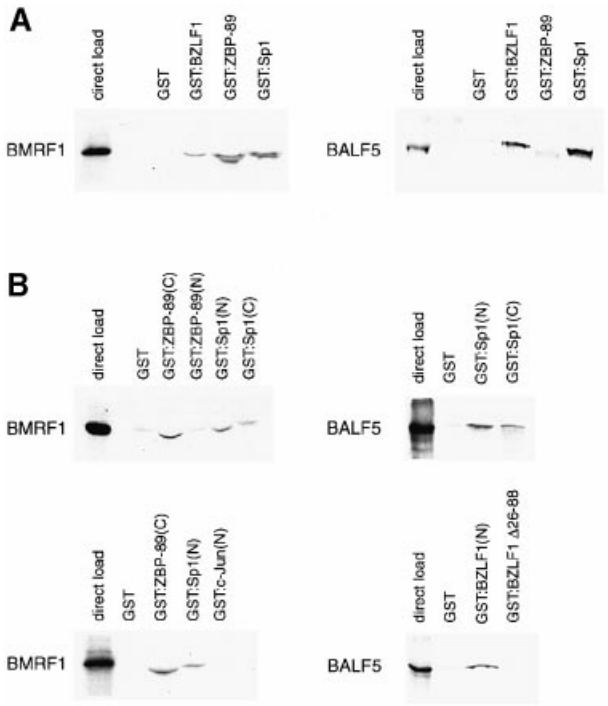

Fig. 6. Interaction of Sp1, ZBP-89 and BZLF1 with lytic replication proteins of EBV in GST pull-down assays. (**A**) Glutathione–Sepharose affinity beads were prepared with comparable amounts of GST alone or GST fusion proteins containing either full-length BZLF1, ZBP-89 or Sp1. GST proteins were incubated with [^{35}S]methionine-labeled BMRF1 or BALF5 followed by SDS–PAGE and autoradiography. Ten percent of the amount of translation products used for the *in vitro* binding assay were directly loaded as a control. (**B**) Derivatives of ZBP-89 or Sp1 GST fusion proteins containing either the TAD of ZBP-89 or Sp1 [GST:ZBP-89 (C) or GST:Sp1(N)] or the DBD of ZBP-89 or Sp1 [GST:ZBP-89 (N) or GST:Sp1(C)] were treated as in (A). GST alone and GST:c-Jun(N), which consists of GST in fusion with the TAD of c-Jun (amino acids 1–165), served as negative controls. GST:B2LF1(N) consists of GST in fusion with the TAD of BZLF1, whereas GST:BZLF1Δ26-88 harbors an internal deletion in the TAD of BZLF1.

immunoblotted with antibodies directed against BZLF1, ZBP-89 or Sp1 (Figure 5A). All three transcription factors could be detected in BMRF1-specific immunoprecipitates. In the case of BZLF1 and Sp1, we observed an additional interaction with the DNA polymerase BALF5. Since no specific antibodies were available for the three helicase–primase complex proteins, we co-transfected Flag epitope-tagged BSLF1, BBLF2/3 and BBLF4 together with BZLF1 into 293 cells. The only interaction found was between BZLF1, BSLF1 and BBLF2/3, but only when both BSLF1 and BBLF2/3 were present concomitantly (Figure 5B). When transfected individually, no interaction was observed, confirming findings by Gao and colleagues that the interaction between BZLF1, BSLF1 and BBLF2/3 requires a ternary complex (Gao *et al.*, 1998). No interaction could be identified between the protein of the helicase–primase complex and Sp1 or ZBP-89 (data not shown).

Next, a protein affinity assay was performed with GST fusion proteins of BZLF1, Sp1 and ZBP-89. [^{35}S]methionine-labeled BALF5 and BMRF1 proteins were incubated with equal amounts of immobilized GST fusions. An interaction of BZLF1 and Sp1 with BALF5 and BMRF1 was observed, while ZBP-89 showed binding to BMRF1 only (Figure 6A). In addition to the *in vivo* co-immunoprecipitation assays, these data support direct interactions. While complex formation between Sp1 and BALF5 appeared strong *in vitro*, the interaction of the two proteins was relatively weak *in vivo*, suggesting that only a fraction of Sp1 is complexed with BALF5 or an unstable Sp1–BALF5 complex throughout the immunoprecipitation reaction.

The TADs of Sp1 and ZBP-89 should be involved in the direct binding to BALF5 and BMRF1 if these protein–protein interactions were the molecular basis for our observations with the chimeric factors GAL4:Sp1(N) and GAL4:ZBP-89(C) (Figure 4E). Therefore, GST fusion proteins were constructed with the DBD or the TAD of Sp1 and ZBP-89 and examined in protein affinity assays with ^{35}S-labeled BALF5 and BMRF1 (Figure 6B). A GST fusion protein containing the TAD of c-Jun was also included in these assays as a negative control (Figure 6B, lower left panel). The immediate-early transcriptional activator c-Jun shares some homology with BZLF1 (Farrell *et al.*, 1989) but does not contribute to *oriLyt* function (Schepers *et al.*, 1993a). As shown in Figure 6B (upper left panel), BMRF1 bound preferentially to the activator domain of ZBP-89, supporting the assumption that the

TAD of ZBP-89 recruits the viral DNA polymerase complex to the downstream element of *oriLyt*. However, the region of Sp1 interacting with BALF5 or BMRF1 was not confined exclusively to the N-terminal activation domain (Figure 6, upper left and right panel). Small amounts of the C-terminus of Sp1 bound reproducibly to both viral replication factors, which might be due to specific interactions with the very C-terminus of Sp1 which contains an additional activation domain (Courey and Tjian, 1988). In a similar assay, we were able to map the interaction domain of BZLF1 and BALF5 to the TAD of BZLF1 (Figure 6B, lower right panel). This interaction seems to involve a region between amino acids 26 and 88 in the TAD of BZLF1, as a deletion of this domain significantly reduced binding to BALF5. Interestingly, this part of BZLF1 is essential for replication, as has been reported recently (Schepers *et al.*, 1996).

Taken together, these findings argue for the tethering of EBV replication proteins to essential components of *oriLyt* with the aid of the activation domains of transcription factors.

Discussion

Identification of cellular oriLyt-binding proteins

The lytic replication origin of EBV, *oriLyt*, is composed of multiple elements, some of which are also present in other herpesviruses. These elements include two core domains, the upstream and downstream component, which are indispensable under all circumstances. The upstream component of *oriLyt* constitutes a strong promoter activated by BZLF1 (Lieberman *et al.*, 1990). BZLF1 binds to four sites within the promoter, and *oriLyt* function requires this interaction whereas additional binding sites for BZLF1 are dispensable for origin function (Schepers *et al.*, 1996). It is clear from domain-swapping experiments that the transactivation domain of BZLF1 is responsible and unique in being able to activate *oriLyt*, since several unrelated transcription factors are incapable of supporting replication although they transactivate the *oriLyt* promoter effectively (Schepers *et al.*, 1993a). In contrast, little is known about how the downstream component contributes to origin function. The downstream component is critical for replication and contains the TD element that is extremely sensitive to mutations, suggesting a strict requirement for specific protein–DNA interactions or a specific DNA structure (Schepers *et al.*, 1993b; Gruffat *et al.*, 1995; Portes-Sentis *et al.*, 1997). Cellular proteins, among them Sp1, are known to bind to the TD element, but their function in lytic replication has remained unclear (Gruffat *et al.*, 1995).

In a search for putative TD-binding proteins by a yeast one-hybrid approach, we isolated homologs of the transcription factors ZBP-89, MAZ and c-Krox, which have been shown to play important roles in transcriptional activation and repression but have not been linked to DNA replication. Sp1 and ZBP-89 (and presumably a MAZ-related protein) were found to participate in protein complexes binding to TD *in vitro*, and Sp1 as well as ZBP-89 enhance *oriLyt* activity in transient replication assays. The ability of Sp1 and ZBP-89 to activate *oriLyt* seems to rely on their transactivation domains. Our data establish that the transactivation domains of Sp1 and ZBP-89, although necessary, are not sufficient to activate *oriLyt*, and argue for an essential role for additional cellular proteins assembled at the TD element. According to the reactivation experiments with *oriLyt* mutants (Figure 4E), Sp1 or ZBP-89 can only rescue those mutants that have lost part of their specific binding capacity for either Sp1 or ZBP-89. Mutations that affected the stability of the complexes C4 and C5 could not be compensated with Sp1 and/or ZBP-89 or a combination of both (Figure 4E and data not shown).

The TD element forms a triple helix that could be functionally relevant (Portes-Sentis *et al.*, 1997). In this structure, a DNA strand from one half of the sequence folds back into the major groove, leaving the complementing strand in a single-stranded state. There is a strong correlation between the ability of the downstream component to adopt such a non-B-DNA structure *in vitro* and the potential of *oriLyt*-dependent replication (Portes-Sentis *et al.*, 1997). As members of the MAZ protein family are known to bind preferentially to single-stranded DNA (Tsutsui *et al.*, 1996), mutations that interfere with formation of a triple helix structure may abrogate the binding of these factors, resulting in a non-functional origin.

oriLyt's downstream component acts as a scaffold for viral replication proteins recruited by transcription factors

The protein interactions demonstrate that the activation domains of the cellular transcription factors Sp1 and ZBP-89 bind directly to the viral replication proteins BMRF1 and BALF5. These findings support the assumption that the downstream component acts as a binding platform for components of the EBV replication machinery, which are guided to *oriLyt* by virtue of their interaction with TD-binding transcription factors (Figure 7). It is conceivable that additional TD-binding proteins, as represented by shift complexes C4 and C5, also participate in recruiting viral proteins or serve to stabilize the DNA–protein complex.

HSV-1 *oriS* was the first herpesvirus origin whose function was shown to be enhanced via transcription factors (Nguyen-Huynh and Schaffer, 1998). However, the molecular basis for this effect is unknown. To our knowledge, a mechanism that involves the tethering of viral replication proteins by cellular transcription factors has only been reported for adenovirus so far. Two cellular transcription factors, NFI and Oct-1, that bind to the adenoviral origins are crucially involved in stimulation of adenovirus DNA replication (Nagata *et al.*, 1982; Leegwater *et al.*, 1985; Pruijn *et al.*, 1986; O'Neill *et al.*, 1988). Initiation of DNA replication is dependent on the recruitment of the viral precursor terminal protein (pTP) and the viral DNA polymerase (pol), forming a pTP–pol heterodimer complex, by protein–protein interactions with NFI as well as Oct-1. Hence, in contrast to SV40, papillomavirus and polyomavirus, which utilize viral factors to tether components of the cellular replication machinery (Dornreiter *et al.*, 1992; Park *et al.*, 1994), EBV and adenovirus seem selectively to target virus-encoded replication factors to their replication origins with the aid of cellular enhancer proteins.

Numerous cellular and viral promoters contain binding

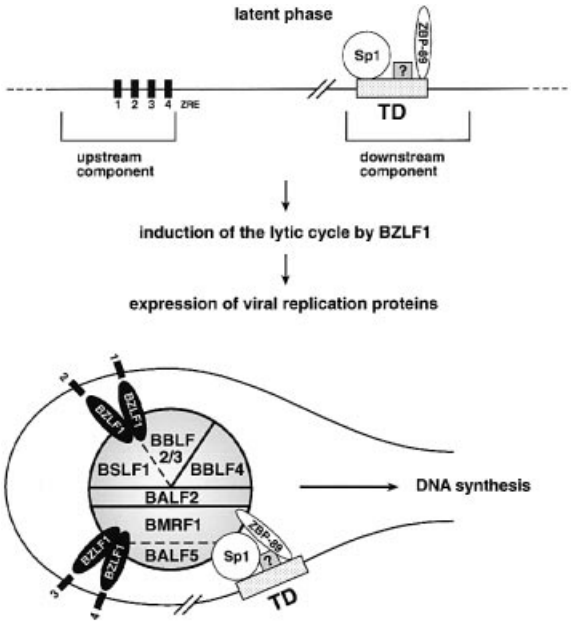

Fig. 7. Model of replication complex formation at *oriLyt* after induction of the lytic phase of EBV's life cycle. Ubiquitiously expressed Sp1 and ZBP-89 presumably bind to the TD element during the latent as well as lytic phase of EBV's life cycle. In contrast, viral lytic proteins cannot be detected in the latent phase, but stimuli, such as TPA, lead to the activation of the lytic activator BZLF1, which in turn results in the induction of viral lytic genes including viral DNA replication proteins (reviewed in Speck *et al.*, 1997). BZLF1 is also capable of activating *oriLyt* directly by binding to DNA motifs, so-called BZLF1-responsive DNA elements (ZRE), located in the upstream component (Lieberman *et al.*, 1990; Schepers *et al.*, 1993a, 1996; Sarisky *et al.*, 1996; Askovic and Baumann, 1997). Protein–protein interactions have been demonstrated to exist between the DNA-binding domain of BZLF1 and the DNA polymerase accessory factor (BMRF1) (Zhang *et al.*, 1996), and between the transactivation domain of BZLF1 and both the viral helicase (BBLF4) and the primase subcomplex (composed of BSLF1 and BBLF2/3) (Gao *et al.*, 1998). The single-stranded DNA-binding protein BALF2 may in turn contact the helicase–primase subcomplex (Gao *et al.*, 1998). Our data provide evidence for additional interactions of the viral DNA polymerase complex composed of the catalytic subunit BALF5 and BMRF1 (Kiehl and Dorsky, 1991) with the cellular transcription factors Sp1 and ZBP-89. Furthermore, BZLF1 seems to interact through its activation domain with BALF5. The different components of the viral replication complex and the multitude of protein–protein interactions could lead to the stabilization of a replication initiation complex and the beginning of *oriLyt*-dependent DNA synthesis.

sites for Sp1, ZBP-89, MAZ and alternative GC box-binding proteins (Parks and Shenk, 1996; Law *et al.*, 1998), which could all provide potential docking sites for replication factors of EBV. This assumption raises the question of how *oriLyt* is defined and selected among many potential elements since EBV, in contrast to α-herpesviruses, appears to lack a specific viral origin-binding protein. It now appears likely that *oriLyt*'s activation is dependent on the concomitant interaction of the enzymatic viral DNA replication machinery with viral (BZLF1) and cellular transcription factors (Sp1, ZBP-89 and others) at the upstream and downstream component, respectively (Figure 7). In turn, this could lead to the formation of a looping structure as has been observed for the EBV latent origin of replication, *oriP*, and the origin *oriS* of HSV (Frappier and O'Donnell, 1991; Makhov *et al.*, 1996).

Alternative mechanisms of oriLyt activation

In latently infected B cells, the EBV replicon is packaged into chromatin, which is very likely to repress lytic gene expression and replication (Dyson and Farrell, 1985). Nothing is known about the chromatin structure and the presumed chromatin remodeling at *oriLyt*. Unlike SV40, there is no cell-free replication system for herpesviruses that would allow study of the activation of DNA synthesis of *oriLyt* templates packaged into chromatin *in vitro*. Since at least Sp1 has been reported to be involved in nucleosome reconfiguration at cellular replication origins (Dimitrova *et al.*, 1996), we cannot exclude the possibility that such a mode of action may also exist for *oriLyt*. However, chromatin remodeling by transcription factors seems to be independent of a functional origin of replication (Hu *et al.*, 1999), again raising the question of specificity.

An additional mode for the regulation of *oriLyt* activity might involve its methylation state, as has been discussed for *oriP* (Falk *et al.*, 1998), since hypermethylation of the divergent promoters of *oriLyt* seems to suppress its transcription and lytic replication (Nonkwelo and Long, 1993). Accordingly, it has been shown that Sp1 elements protect a CpG island from *de novo* methylation (Brandeis *et al.*, 1994) and that CpG islands may serve as initiation sites for both transcription and DNA replication (Delgado *et al.*, 1998). Therefore, a link between DNA replication and replication-coupled demethylation of *oriLyt* might exist, although our data point to a direct contribution of Sp1 and ZBP-89 to the mechanics of DNA replication.

A general mode of herpesviral reactivation by cellular enhancer proteins

Our finding of cellular transcription factors that act as bridging proteins for components of the viral replication machinery is new in the field of herpesviruses. Interestingly, the HSV-1 origin of DNA replication, *oriS*, also contains transcriptional regulatory elements including an Sp1 consensus site. Observations from mutational analysis suggest that binding sites for cellular proteins facilitate *oriS*-dependent DNA replication (Nguyen-Huynh and Schaffer, 1998). Therefore, it seems possible that the proposed model of EBV lytic DNA replication holds true for other members of the herpesvirus family. Despite the fact that an origin-specific binding protein with helicase activity is encoded by α-herpesviruses (in contrast to EBV), origin-dependent DNA replication with purified replication factors has not yet been demonstrated (Challberg, 1996). This failure might be due to the lack of a cellular factor(s) that might be instrumental in origin activation of all herpesviruses.

Future studies will include mutational analysis of Sp1 and ZBP-89 to define the specific interaction regions of both transcription factors with the EBV replication proteins BALF5 and BMRF1. As predicted by our *oriLyt* replication model, mutations that compromise the binding of Sp1 and ZBP-89 to BALF5 and BMRF1 should also reduce the ability of Sp1 and ZBP-89 to activate EBV DNA replication. In addition, the nature and the role of the TD-binding complexes C4 and C5 need to be addressed to provide a clear picture of the downstream component of *oriLyt* as a model of a complex eukaryotic origin of DNA replication.

Materials and methods

Yeast one-hybrid screen

The plasmid pHISi-1 (Clontech) was altered to contain a tandem repeat of three TD-wt elements in front of the *HIS3* gene. This cassette was recombined into the defective *HIS3* locus in *Saccharomyces cerevisiae* strain RH1533 (MATα; *leu2-3,-112; ura3-52; his3-del200; trp1-del901; lys2-801; suc2-del9;* MCI$^-$) to generate the yeast strain MAB1. MAB1::*lacZ* was constructed by integrating the *lacZ* gene encoding β-galactosidase into the *URA* locus of MAB1. The expression of *lacZ* is also under the control of three multimerized TD-wt elements. Likewise, we constructed yeast strain YAS1750.9 containing an oligomerized TD-mut element (described below) in front of the *HIS3* promoter. A cDNA library from an EBV-immortalized B lymphocyte cell line in the pACT shuttle vector (Durfee *et al.*, 1993) was transformed into yeast. Transformants were selected for growth in the absence of histidine at 30°C in the presence of 80 mM 3-amino-1,2,4-triazole. Colonies were plated on 5-bromo-4-chloro-3-indolyl β-galactoside (X-gal) plates to test for relative β-galactosidase activity. Plasmid DNAs recovered from blue colonies were transformed into *Escherichia coli* DH5α. The plasmids were transformed back into MAB1::*lacZ* and YAS1750.9 to confirm the phenotype, followed by sequence analysis of the respective plasmids.

Eukaryotic plasmids

The missing 5′ end of human ZBP-89 cDNA was reconstructed by reverse transcription of HeLa cell RNA following PCR amplification with ZBP-89-specific primers. ZBP-89 full-length cDNA was reconstructed and cloned into the expression plasmid pBKCMV (Stratagene). pCMV:Sp1 (amino acids 66–778) was used as an expression plasmid for Sp1. The plasmid pCMV:BZLF1 is an expression vector which efficiently induces the lytic phase of EBV's life cycle (Hammerschmidt and Sugden, 1988). pGAL4:Sp1(N) encodes a fusion of GAL4(1–147) (containing the DBD and dimerization domain) and the N-terminal TAD (amino acids 68–611) of Sp1. Similarly, pGAL4:ZBP-89(C) encodes the C-terminal TAD of ZBP-89 (amino acids 445–794) as a fusion with GAL4(1–147). pGAL4:VP16 has been described (Sadowski and Ptashne, 1989). All of the GAL4 fusion polypeptides are produced under the control of the SV40 early promoter.

The plasmid 968.22 with wild-type *oriLyt* has been described (Schepers *et al.*, 1993b). PCR-directed mutagenesis with appropriate PCR primers was performed to introduce a GAL4 DNA-binding site (5′-CGGAGTACTGTCCTCCG-3′) into 968.22. The insertion of the GAL4 DNA-binding site was confirmed by DNA sequencing, and corresponding *oriLyt* plasmids were designated 2203–2208. Expression vectors for the lytic replication proteins of EBV coding for BSLF1, BALF2, BALF5, BMRF1, BBLF2/3 and BBLF4 have been described (Fixman *et al.*, 1995). All of these genes are cloned into pSG5 (Stratagene). The cDNAs of BSLF1, BBLF2/3 and BBLF4 were introduced into pCMV2:Flag (Kodak) to allow their detection as Flag epitope-tagged full-length proteins.

Bacterial expression constructs

The full-length cDNAs of Sp1 and ZBP-89 were expressed as GST fusions in pGEX-KG (Guan and Dixon, 1991) in the *E.coli* strain DH5α. A 5′ fragment of the ZBP-89 cDNA encompassing the DBD (amino acids 1–444) was cloned into pGEX-KG and the vector was designated pGST:ZBP-89(N). pGEX-KG was also used to insert the 3′ end of the ZBP-89 cDNA encoding the C-terminal TAD (amino acids 445–794), generating pGST:ZBP-89(C). pGST:Sp1(N) encoding a fusion of GST and the TAD of Sp1 (amino acids 68–611) and pGST:Sp1(C) encoding a fusion of GST and the DBD of Sp1 (amino acids 612–778) were generated by insertion of the corresponding fragments into pGEX-KG. Procedures for production and purification of fusion polypeptides were carried out as described (Baumann *et al.*, 1998). GST:ZBP-89 bound to glutathione–Sepharose beads (Amersham Pharmacia) was eluted by the addition of 20 mM glutathione or was cleaved with 1 U of thrombin (Sigma) to separate ZBP-89 from GST.

Cell lines

D98HR1 cells were derived from a somatic cell hybrid between the EBV-positive Burkitt's lymphoma (BL) cell line P3HR1 and the human epithelial cell line D98 (Glaser and Nonoyama, 1974). This cell line was maintained in a 1:1 mixture of RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). HH514, an EBV-positive BL cell line, DG75, an EBV-negative BL cell line, and human embryonic kidney 293T cells were grown in RPMI 1640 medium supplemented with 10% FCS, whereas the cervix carcinoma cell line HeLa was cultivated in DMEM containing 10% FCS.

Preparation of nuclear extracts

Cell cultures were split in a 1:3 ratio 2 days before extraction. Where indicated, 5×10^6 293 cells were transfected with 5 μg of pBKCMV:ZBP-89 and grown for 2 days. After washing three times with phosphate-buffered saline (PBS), cells were resuspended in 300 μl of hypotonic buffer A [10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), protease inhibitors: 0.5 μg/ml leupetin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM benzamidine, 1 mM pefabloc, 0.5 μg/ml aprotinin] and incubated on ice for 30 min. Permeabilized nuclei were recovered by brief centrifugation, and the crude nuclear pellet was resuspended in 200 μl of hypertonic buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 25% glycerol, protease inhibitors as mentioned above) followed by incubation on ice for 30 min. After centrifugation, the supernatant was used for gel retardation assays.

Gel mobility shift assays

For these experiments, a TD DNA probe encompassing the EBV genome sequence from coordinates 53 344 to 53 399 (Baer *et al.*, 1984) was chiefly used. The replication-deficient DNA probe TD-mut harbors C to G transversions at coordinates 53 365, 53 366, 53 385 and 53 386. Alternatively, DNA probes were prepared by PCR amplification of the different *oriLyt* plasmids 968.22 and 2203–2208 using the primers F1 and B1. The PCR products containing the TD element were ^{32}P-labeled using T4 polynucleotide kinase, and protein preparations were mixed with labeled oligonucleotides (30 000 d.p.m. per sample) in 25 μl of 10 mM HEPES pH 7.9, 10% glycerol (v/v), 60 mM KCl, 0.1 mM EDTA, 0.25 mM DTT and 2 μg of calf thymus DNA and poly(dA–dT). Binding reactions were carried out at room temperature for 40 min in the presence or absence of specific antibodies as indicated and separated on a 4.5% polyacrylamide gel (20:1 acrylamide–bisacrylamide) in 0.2× TBE (1× TBE is 90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA pH 8.3).

DNase I footprinting

The 179 bp *Pvu*II–*Pvu*II fragment from nucleotide coordinates 53 308–53 486 of the EBV strain B95.8, which contains the *oriLyt* downstream component, was subcloned into pBluescript II SK(–) and then excised as a 242 bp fragment which was used for the DNase I footprinting experiments. The DNA fragment was 5′-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The 50 μl binding reactions contained human, recombinant Sp1 (Promega) and/or ZBP-89 protein, as specified in the figure legends, 25 mM Tris–HCl pH 8.0, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT and 20 000 d.p.m. of labeled probe DNA. Reactions were incubated at room temperature for 10 min, then 50 μl of a solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ were added, and the reaction was incubated for 1 min at room temperature. DNase I (0.2 U; Promega) was added and the incubation was continued for 1 min at room temperature. The reaction was stopped by adding 90 μl of buffer containing 200 mM NaCl, 30 mM EDTA, 1% SDS, 100 μg/ml yeast tRNA (Sigma). Reaction products were purified by phenol–chloroform extraction and precipitation, and analyzed on DNA sequencing gels with chemical sequencing reactions of probe DNA as markers.

Transient replication assay

A total of 5×10^6 D98HR1 cells were electroporated with 10 μg of the *oriLyt* plasmid 968.22 together with 5 μg of pCMV:BZLF1 and 10 μg of pCMV:Sp1 or pBKCMV:ZBP-89. To determine the rescue of the mutated *oriLyt* plasmids 2203–2208, 10 μg of each plasmid were transfected with 10 μg of pGAL4-DBD, pGAL4:Sp1(N), pGAL4:ZBP-89(C) or pGAL4:VP16, and 5 μg of pCMV:BZLF1. Two days after transfection, DNA was extracted according to the Hirt method. Following restriction with *Bam*HI and *Dpn*I, the efficiency with which *oriLyt* plasmids replicated was quantified according to the specific signals detected after Southern blot hybridization and autoradiography by standard procedures as described elsewhere (Schepers *et al.*, 1993b).

Protein-binding assays

In vitro binding assays. A 100 μl aliquot of bacterial lysates containing GST fusion proteins was mixed with 15 μl of glutathione–Sepharose beads (Amersham Pharmacia). Following incubation for 1 h, the resin was washed with binding buffer (20 mM HEPES pH 7.7, 75 mM KCl,

0.1 mM EDTA, 25 mM $MgCl_2$, 10 mM DTT, 0.15% NP-40) and *in vitro* translated BALF5 and BMRF1 were added. After incubation for 2 h at 4°C, the resin was washed with binding buffer and the bound fraction was eluted by boiling in Laemmli buffer, analyzed by SDS–PAGE and visualized by autoradiography.

In vivo interaction assays. A total of 5×10^6 293 cells were transiently transfected with 5 µg of pCMV-BZLF1 together with 10 µg of expression plasmids encoding various EBV replication proteins. At 30 h post-transfection, cells were lysed in 1 ml of PBS plus 1% bovine serum albumin (BSA) and 1% Triton X-100. Cell lysates were incubated with 30 µl of protein A–agarose beads (Boehringer Mannheim) coupled with either the anti-BALF5 antibody, the anti-BALF2 antibody or the anti-BMRF1 antibody. As a negative control, an aliquot of each cell lysate was incubated with protein A–agarose beads. A 10 µg aliquot of pCMV2:Flag:BSLF1, pCMV2:Flag:BBLF2/3 and pCMV2:Flag:BBLF4 was transfected into 293 cells together with 5 µg of pCMV-BZLF1, and cell extracts were mixed with agarose beads covalently attached to the anti-Flag antibody M2 (Kodak). Incubation was performed overnight at 4°C. After washing with lysis buffer, bound proteins were released from beads by boiling in Laemmli buffer, separated by SDS–PAGE and blotted onto a nitrocellulose membrane. Precipitated BZLF1, Sp1 and ZBP-89 were visualized by Western blotting.

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