cis-Acting Elements in the Lytic Origin of DNA Replication of Epstein-Barr Virus

ALOYS SCHEPERS, DAGMAR PICH, JOACHIM MANKERTZ, AND WOLFGANG HAMMERSCHMIDT*

Institut für Klinische Molekularbiologie und Tumorgenetik, GSF-Forschungszentrum für Umwelt und Gesundheit, GmbH, Marchioninistrasse 25, D-8000 München 70, Germany

oriLyt, the cis-acting element of Epstein-Barr virus, mediates viral DNA replication in the lytic phase of the virus's life cycle. Oligonucleotide-directed in vitro mutagenesis of oriLyt plasmids allowed the identification of two noncontiguous components within the complex structure of oriLyt. Both components were indispensable for DNA replication of this origin. The upstream component colocalized with the promoter of the viral BHLF1-encoding gene, and mutants affecting DNA replication affected RNA transcription, too. The second component crucial for oriLyt function was determined to be 40 bp long and positioned approximately 530 bp downstream. It was dispensable for transcriptional transactivation but it was absolutely required for replication. Thus, the overall design of oriLyt has striking similarity to multipartite regulatory elements of transcription, consisting of proximal promoters and distal enhancers, but special elements are exclusively dedicated to DNA replication.

Epstein-Barr virus (EBV) is a human gammaherpesvirus which infects and immortalizes human primary B lymphocytes in vitro. The viral genome is maintained as extrachromosomal, multiple copies in immortalized cells. The plasmid origin of DNA replication, oriP, which is required for replication of the EBV genome in dividing B cells, has been identified together with the viral trans-acting element (43). Immortalized cells are latently infected with EBV; i.e., only a very limited number of viral genes are expressed, and no virus is produced. Within the population of latently infected cells, a minor proportion of cells can support the productive, lytic cycle of EBV. During this phase of EBV's life cycle, most or all of the approximately 100 viral genes are expressed, herpesviral DNA is amplified, and viral structural proteins are made. As a consequence, mature virions are released and the cells die. The cis-acting element oriLyt, which is required for lytic-phase DNA amplification, has been identified (21). This origin is functionally and structurally very different from oriP. Thus, EBV requires two genetic elements to replicate its DNA genome in its bipartite life cycle (see references 8 and 20 for reviews).

The structure of oriLyt differs from that of oriP. The plasmid origin of DNA replication consists of two components, a dyad symmetry element and a family of closely related 30-bp repeats which are both needed for replication (37). The latter component scores as a transcriptional enhancer in conjunction with the only viral gene, EBNA1, which is required to activate oriP in trans (36). In contrast, the lytic origin of DNA replication, oriLyt, has several regions which are required for its complete function. oriLyt consists of two essential or core elements and several nonessential or auxiliary elements. The core elements, which have been defined operationally, comprise the minimal oriLyt sequence. oriLyt consists of an upstream region which is 321 bp long (nucleotides 52,623 to 52,944 of EBV prototype strain B95-8) (3) and of a downstream region which is 374 bp long (nucleotides 53,207 to 53,581; Fig. 1A and B) (21). The two regions of the core element are flanked

by poorly defined EBV sequences which are nonessential but influence the efficiency with which oriLyt-containing plasmids replicate in transient experiments. Since removal of these sequence stretches decreased the efficiency of replication, they score as auxiliary regions (11, 21).

The functional association of oriLyt appears to be complex. It colocalizes with a locus of the EBV genome which has been identified as an efficient promoter and enhancer element that functions during the early events in the lytic phase of EBV's life cycle (27). This locus, which is called the duplicate segment, is present twice in most EBV strains and encompasses a promoter which lies within the upstream region of oriLyt. This promoter, which drives the BHLF1-encoding gene, adjacent to the left duplicate segment, is transactivated directly by the viral BZLF1 gene product, a sequence-specific DNA-binding protein (Fig. 1A) (9). The BZLF1 transcription factor has similarities to c-Fos of the AP1 family (14) and binds as a homodimer to specific DNA motifs which have been identified within the duplicate segment. Four BZLF1-binding sites, which are also called ZREs, have been mapped close to the TATA and CCAAT boxes of the BHLF1 promoter (14, 28, 31, 40). A fifth ZRE site with high affinity to BZLF1 resides within the downstream region of oriLyt (Fig. 1B).

The BZLF1 gene product, which also constitutes the key switch to activate the lytic phase of EBV's life cycle (10, 39), is not the only viral transcriptional activator that interacts with sequences essential for oriLyt function. The R enhancer factor binds specifically to two sequence motifs located within the downstream region of oriLyt (Fig. 1B). Together with R, they make up a generic enhancer element (18). This arrangement of sequences indicates that elements required for RNA transcription might be intimately linked with elements required for DNA replication. Moreover, it is possible that BZLF1, R, or other nonviral transcription factors play a role in activating viral DNA replication directly.

With this possible association in mind, we set out to dissect the core elements of oriLyt. Owing to its complex structure, we constructed mutant oriLyt plasmids in which limited, systematic alterations within the upstream and
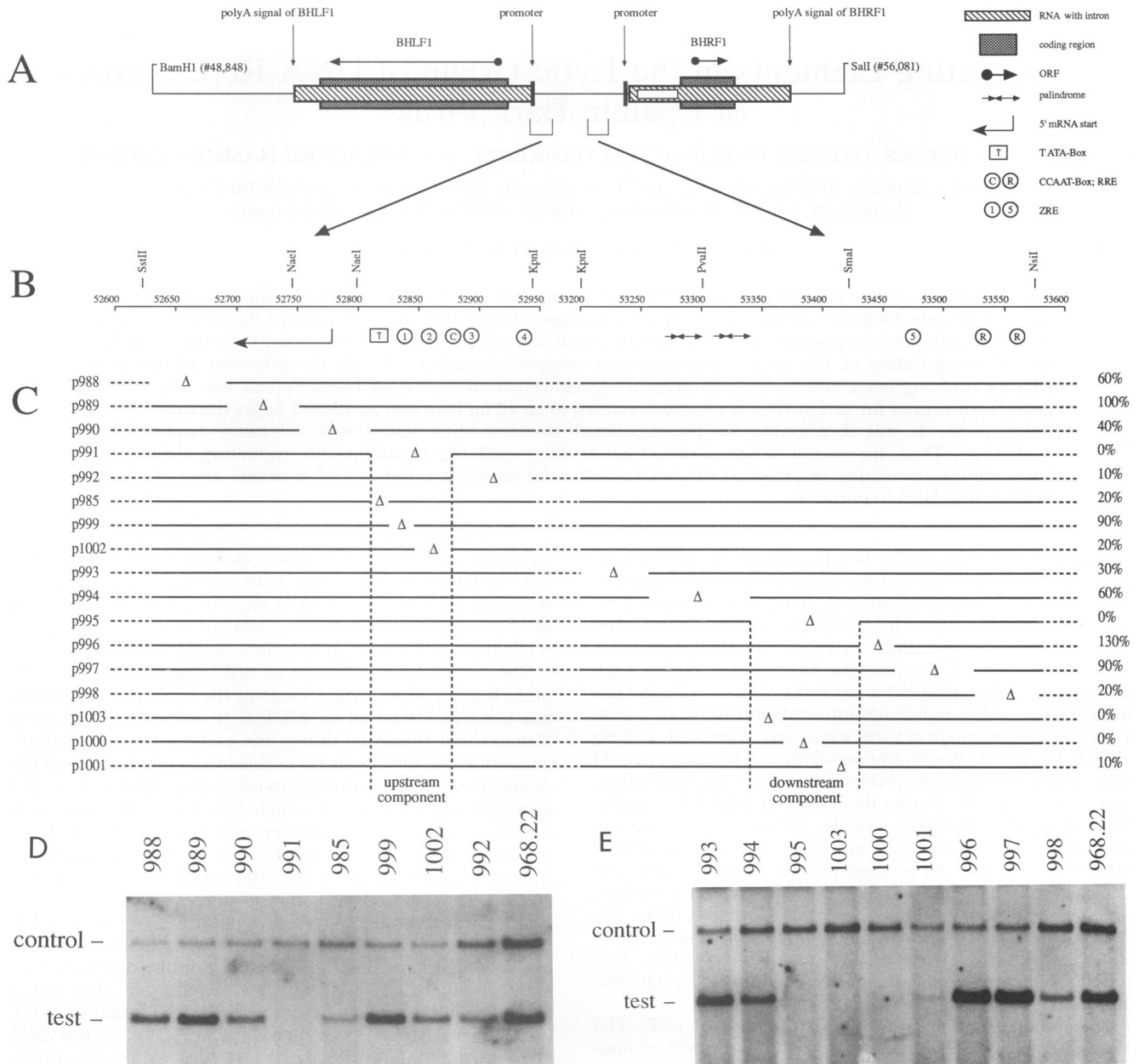
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* Corresponding author.

FIG. 1. Structure of the lytic origin of DNA replication of EBV. (A) The BamHI-to-SalI fragment encompassing oriLyt within the left duplicated segment is schematically shown. The coordinates of this fragment are based on reference 3 and represent the situation of the B95-8 strain of EBV. This fragment was cloned into a pUC vector which was called p968.22. It spans the wild-type lytic origin of EBV, including the core and auxiliary regions. The divergent genes for BHLF1 and BHRF1 are shown together with their promoter localizations, mRNA structures, and open reading frames (ORFs). The horizontal brackets delineate the sequence elements described in reference 21 which encompass the minimal oriLyt element. (B) Enlarged view which shows the upstream (from SstII to KpnI) and downstream (from KpnI to NsiI) regions of oriLyt together with the nucleotide sequence map of B95-8. Known sequence elements are indicated by symbols, including binding sites for BZLF1 (ZREs), the TATA and CCAAT boxes, and obvious secondary structures which include direct and inverted repeats. (C) Relevant parts of oriLyt mutant plasmids derived from p968.22 are indicated by horizontal lines, and the localization of the deletions can be inferred from the nucleotide map. The replication efficiencies of the different deletion mutants in the oriLyt replication assay, compared with the replication efficiency of p968.22, which was set to 100%, are summarized at the right. The deletion in p991 and p995 abolished replication completely and gives the boundaries of the essential upstream and downstream components, respectively. (D and E) Two autoradiograms which present the original data of one set of transient replication assays focusing on mutations of the upstream and downstream regions, respectively. The position of the control signals gives the position of wild-type oriLyt plasmid p526 (21), which served as an internal standard to correct for transfection efficiency in the individual samples. The position of the test signals gives the signal strength, which is a relative measurement of the efficiency with which the mutated oriLyt plasmid replicated. The deletions in the mutated oriLyt plasmids removed the following nucleotides: 52,630 to 52,692 in p988, 52,693 to 52,752 in p989, 52,753 to 52,810 in p990, 52,811 to 52,877 in p991, 52,878 to 52,945 in p992, 52,811 to 52,825 in p985, 52,826 to 52,846 in p999, 52,847 to 52,877 in p1002, 53,202 to 53,256 in p993, 53,257 to 53,340 in p994, 53,341 to 53,428 in p995, 53,429 to 53,459 in p996, 53,460 to 53,526 in p997, 53,527 to 53,589 in p998, 53,341 to 53,368 in p1003, 53,369 to 53,401 in p1000, and 53,402 to 53,428 in p1001.

downstream regions of oriLyt could be tested in the context of core and auxiliary elements. By using this strategy, the overall structure of oriLyt was unchanged and preserved in all mutants. The function of the mutant oriLyt plasmids was tested in transient replication assays and quantitated to ensure reproducibility. In parallel, transcriptional activation of the flanking or integral promoter regions was measured in the mutated plasmids in two different EBV-positive cell lines. By analyzing more than 60 mutant oriLyt plasmids, two novel components within the lytic origin of DNA replication were identified. One component residing in the upstream region of oriLyt encompassed the BHLF1 promoter element, and the second was found within the downstream region. The upstream component seemed to constitute mainly transcription factor-binding sites, including binding sites for the BZLF1 gene product. The downstream component had no previously assigned function. The upstream component of oriLyt was crucial for both DNA replication and RNA transcription. The downstream component was absolutely required for replication but had no significant impact on transcription. Thus, at least two components with different yet interacting functions are required for DNA replication of oriLyt.

MATERIALS AND METHODS

Cell lines. D98 HR1 cells were derived from a somatic cell hybrid between EBV genome-positive Burkitt's lymphoma cell line P3HR1 and human epithelial cell line D98 (17). This adherent cell line contains approximately 20 copies of the EBV genome (data not shown) and was maintained in Dulbecco's modified Eagle's medium containing 5% fetal and 5% newborn calf sera. HH514 is a het-free cell clone of the P3HR1 Burkitt's lymphoma cell line (23) which was grown in RPMI1640 medium supplemented with 5% fetal and 5% newborn calf sera.

Recombinant plasmids. Plasmid p968.22 was constructed by ligating a 7.2-kbp BamHI-SalI fragment from EBV strain B95-8 (nucleotides 48,848 to 56,084) (3) into a BamHI-SalI-cut pUC8 vector plasmid. This plasmid clone was further modified to contain two linker insertions at positions 52,385 and 54,465 to carry additional XhoI and SalI sites, respectively, for cloning. These alterations did not affect the oriLyt function of this plasmid (data not shown). Mutants of p968.22 were constructed in three steps. (i) A SacI-BglII fragment (EBV nucleotides 52,385 to 54,360) was inserted into appropriately cleaved M13mp18 or pBluescriptIIsk(−) plasmids. (ii) The single-stranded DNAs derived from these vector plasmids were mutated in accordance with the oligonucleotide-directed in vitro mutagenesis protocol (26) with a commercially available kit (M13 in vitro mutagenesis kit; Bio-Rad) supplemented with T4 gene 32 and T4 DNA polymerase from Boehringer Mannheim. (iii) The mutated fragment was reinserted into the p968.22 plasmid to provide the oriLyt flanking sequences. All mutations were confirmed by DNA sequencing with the chain termination method (38). The synthetic oligonucleotides needed for mutagenesis and the sequencing primers 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. All deletion, randomization, and substitution mutants of oriLyt relevant to this study are graphically presented in the Fig. 1 to 5, which include sequence information on specific mutations.

To perform the transient transcription assays, the 3.8-kbp

SacII-EcoRI fragment of p968.22 (from position 52,623 of EBV to the multiple cloning site in p968.22) carrying the BHLF1 open reading frame was replaced by the luciferase gene, which was used as a reporter gene. Similarly, the 1.7-kbp BglII-SalI fragment (from position 54,360 of EBV to the multiple cloning site in p968.22) carrying the BHRF1 open reading frame was replaced by the luciferase gene.

Plasmid pCMV-BZLF1 is an expression vector which efficiently induces the lytic phase of EBV's life cycle. The BZLF1-encoding gene is driven in this retroviral vector construct by the promoter of the immediate-early genes of the human cytomegalovirus as previously described (21).

Transfection of plasmid DNA. Transfections of plasmid DNA were performed with an electroporator (Bio-Rad). We used cuvettes (0.4-cm gap) containing 5×10^6 cells in a volume of 250 μl of cell culture medium supplemented with serum. The cells were electroporated with 180 (D98HR1 cells) or 220 (HH514 cells) V.

Transient replication assays. Ten micrograms of oriLyt plasmid DNA was cotransfected with 1 μg of p526 and 10 μg of pCMV-BZLF1 into D98HR1 cells as described previously (21). Plasmid p526 has been described (21) and contains a wild-type oriLyt fragment. It was used as an internal control for standardization for each mutant plasmid to be tested. Concomitant introduction of plasmid pCMV-BZLF1 efficiently induces the lytic cycle of EBV. Two days after cotransfection, DNA was prepared, input plasmid DNA was digested with DpnI and BamHI, and the efficiency with which the oriLyt plasmids replicated was quantitated by scanning the specific fragment signals detected after Southern blot hybridization and autoradiography as described previously (21).

Transient transcription assays. To measure relative transcriptional activation from the divergent promoter elements of the BHLF1- and BHRF1-encoding genes in different mutant oriLyt plasmids (Fig. 1A), 10 μg of plasmid DNA containing the luciferase reporter gene at the appropriate position was cotransfected together with 10 μg of pCMV-BZLF1 and 0.5 μg of pCMV-β-gal into D98HR1 and HH514 cells as described above. Plasmid pCMV-β-gal was used as an internal standard to normalize for transfection efficiency. Luciferase activity was measured as previously described (12), with minor modifications. Transfected cells were harvested 2 days after transfection, washed once in phosphate-buffered saline, and lysed in 100 μl of 100 mM K_2HPO_4 (pH 7.8)–1 mM dithiothreitol–1% Triton X100. The lysate was transferred into an Eppendorf tube, vortexed for 1 min, and centrifuged for 15 min at $15,300 \times g$ at 4°C. Ten microliters of the supernatant was added to 350 μl of ice-cold 25 mM glycyl-glycine–5 mM rATP–15 mM $MgSO_4$. An LB9501 luminometer (Berthold) was programmed to inject 100 μl of luciferin (0.3 mg/ml in 0.5 M K_2HPO_4, pH 7.8). Relative light units were determined during a 10-s period. Each sample was analyzed in duplicate. To normalize for efficiency of transfection, the β-galactosidase activity expressed from cotransfected plasmid pCMV-β-gal (32) was measured as described in reference 24, with slight modifications. The chemiluminescent substrate for β-galactosidase was 3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl-β-D-galactopyranoside (AMPGD; Serva) at 10 μg/ml, and 5 μl of the supernatant of each sample was measured in 150 μl of 100 mM Na_2HPO_4 and 0.1 mM $MgCl_2$. After 15 to 30 min at room temperature, the reaction was stopped with the injection of 100 μl of 0.2 M NaOH and 10% Emerald Enhancer (Serva). Two seconds after injection of

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MUTANT                                                                                                          REPLICATION
PLASMID                                                                                                          EFFICIENCY
                                            TATA-BOX              ZRE1           ZRE2            CCAAT-BOX
         52780  +1      .          .           .        .           .      .       .        .      .     52880
         I      I                                                                                         I
         CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTTTGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    100%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT
              ←___|

p991     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC ————————————————————— Δ ———————————————————— GGTTA     0%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG                                                   CCAAT

p985     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC ——— Δ ——— GGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    20%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG           CCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p980     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTCTCTTTTTTCTTAGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    35%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAGAGAAAAAAGAATCCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p981     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCATTTCTTTCCTTTTTGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    45%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGTAAAGAAAGGAAAAACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p982     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCATAAATAAAAAAAATTAGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    75%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGTATTTATTTTTTTTAATCCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p983     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTAATTTTTTTTATTTATGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    90%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGATTAAAAAAAATAAATACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p984     CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCAAAAAGAGGATAAAAGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    60%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGTTTTTCTCCTATTTTCCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p979     #52630 ——————— Δ ——————— TTTTATCCTCTTTTTGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA    90%
                                  AAAATAGGAGAAAAACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT

p1055    CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC ——— Δ ——— GGGGTCTCTGTGTAATACTTT ——————— Δ ——————— GGTTA     0%
         GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG           CCCCAGAGACACATTATGAAA                        CCAAT

                                          L_____|

                                                  upstream component
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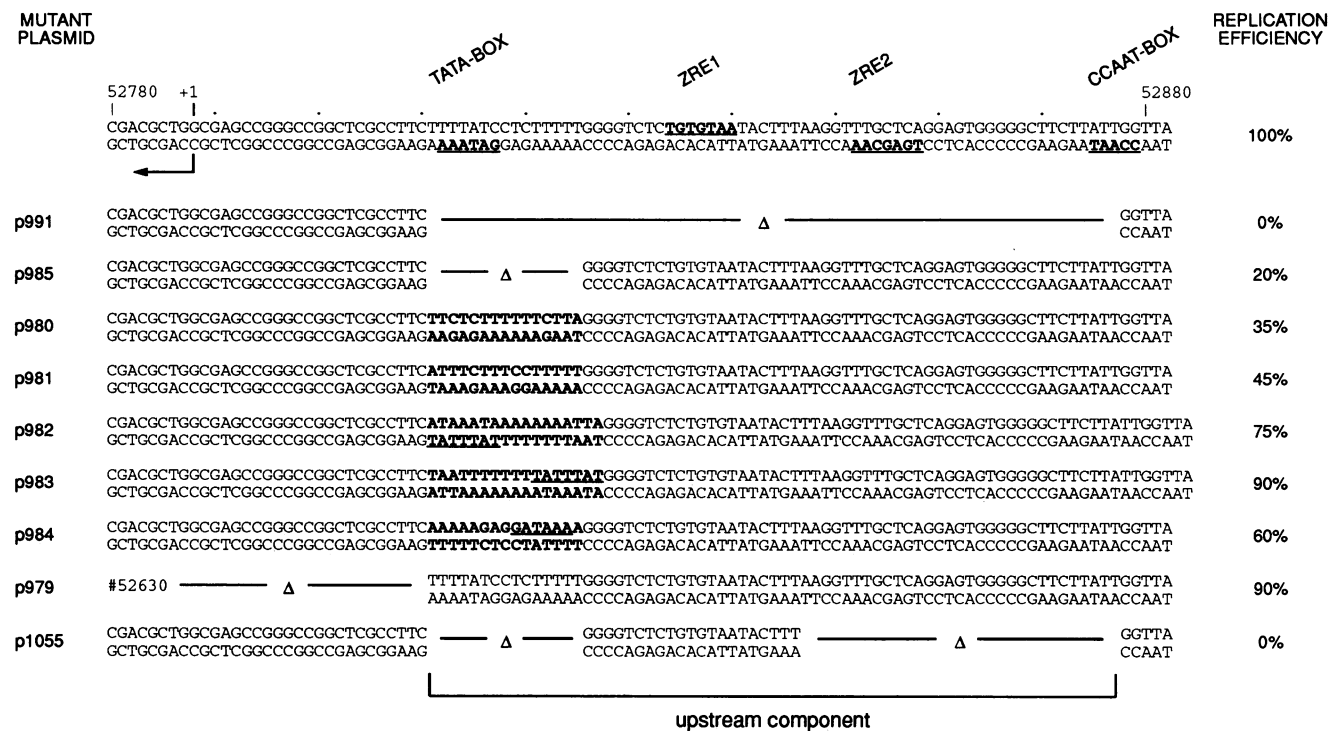
FIG. 2. Mutations in the TATA box of the BHLF1 promoter modulate the efficiency of oriLyt replication. The top line gives the structure of the BHLF1 promoter with known hallmarks of this element, including the 5' end of the mRNA; the TATA box motif (27); BZLF1-binding sites ZRE1 and ZRE2 (28), to which BZLF1 binds (4, 25); and the CCAAT box motif. These sequence elements are underlined and indicated in bold together with the strand specificity. The mutants are based on wild-type plasmid p968.22, and the deletions and substitutions are given in detail in the lower section together with the mutated oriLyt plasmids (left column) and their relative replication efficiencies (right column). In p980 and p981, the original sequence was randomized arbitrarily, p982 and p983 carry the simian virus 40 early promoter TATA box (5), and p984 carries an inversion of the BHLF1 TATA box shown in the top line. The consensus TATA box is underlined, and the mutated nucleotides are in bold.

the stop mixture, the emitted photons were measured for 5 s. All reactions were performed in duplicate.

RESULTS

Fine mapping of sequence elements required for oriLyt function. Replication of oriLyt plasmids was measured in D98HR1 cells, which are latently infected with EBV and support the lytic phase of EBV's life cycle. The cells were cotransfected with three different plasmids. (i) pCMV-BZLF1 induced the lytic cycle in these cells to provide EBV functions in *trans*, (ii) p526 carries a fully functional oriLyt sequence and served as an internal control for standardization, and (iii) p968.22 or a mutant oriLyt plasmid to be tested. Two days after electroporation, DNA was prepared and digested with *Bam*HI to cleave the oriLyt plasmids once or twice. The cell DNA was digested in parallel with *Dpn*I, which cleaves input DNA that had been methylated in a *dam*+ *Escherichia coli* host into small fragments but leaves DNA intact that has gone through at least one round of DNA replication in eukaryotic cells. The cleaved DNAs were separated on gels, and the oriLyt plasmid DNAs were detected with radioactively labeled prokaryotic sequences after Southern blotting as previously described (21).

Previously, two minimal regions within oriLyt had been identified by using progressive deletion mutants of oriLyt plasmids (Fig. 1B) (21). This information was the basis for dissection of these minimal regions in more detail. Small

deletions (from 25 to 90 bp long) were introduced into oriLyt and designed as scanning mutations, as shown in Fig. 1C. The mutations were generated by oligonucleotide-directed mutagenesis on single-stranded DNA templates. All oriLyt mutant plasmids encompassed EBV sequences from coordinates 48,848 to 56,081 (3) (Fig. 1A), and they all were partially sequenced to confirm the intended mutations and to ensure the integrity of flanking regions. The results of the transient replication assays are summarized in Fig. 1C, and examples are shown in Fig. 1D and E. The relative replication efficiencies, compared with that of wild-type oriLyt, given in Fig. 1C and elsewhere in this report, are mean values of at least three independent experiments.

Within the upstream region of the minimal oriLyt, mutant plasmid p991 identified a 67-bp sequence which was absolutely required for DNA replication (Fig. 1C). Likewise, mutant plasmid p995 allowed identification of an 88-bp sequence in the downstream region which was essential for replication. For clarity, these sequences were termed upstream and downstream components of oriLyt (Fig. 1C). Other mutations merely affected the efficiency with which oriLyt mutant plasmids replicated. Some deletions reduced the efficiency of oriLyt replication by a factor of 10 (p992); others had no measurable effect (p989). In this study, the two components identified by oriLyt plasmids p991 and p995 were investigated in detail.

The 67-bp upstream component was further analyzed for sequence elements required for oriLyt replication in *cis*.
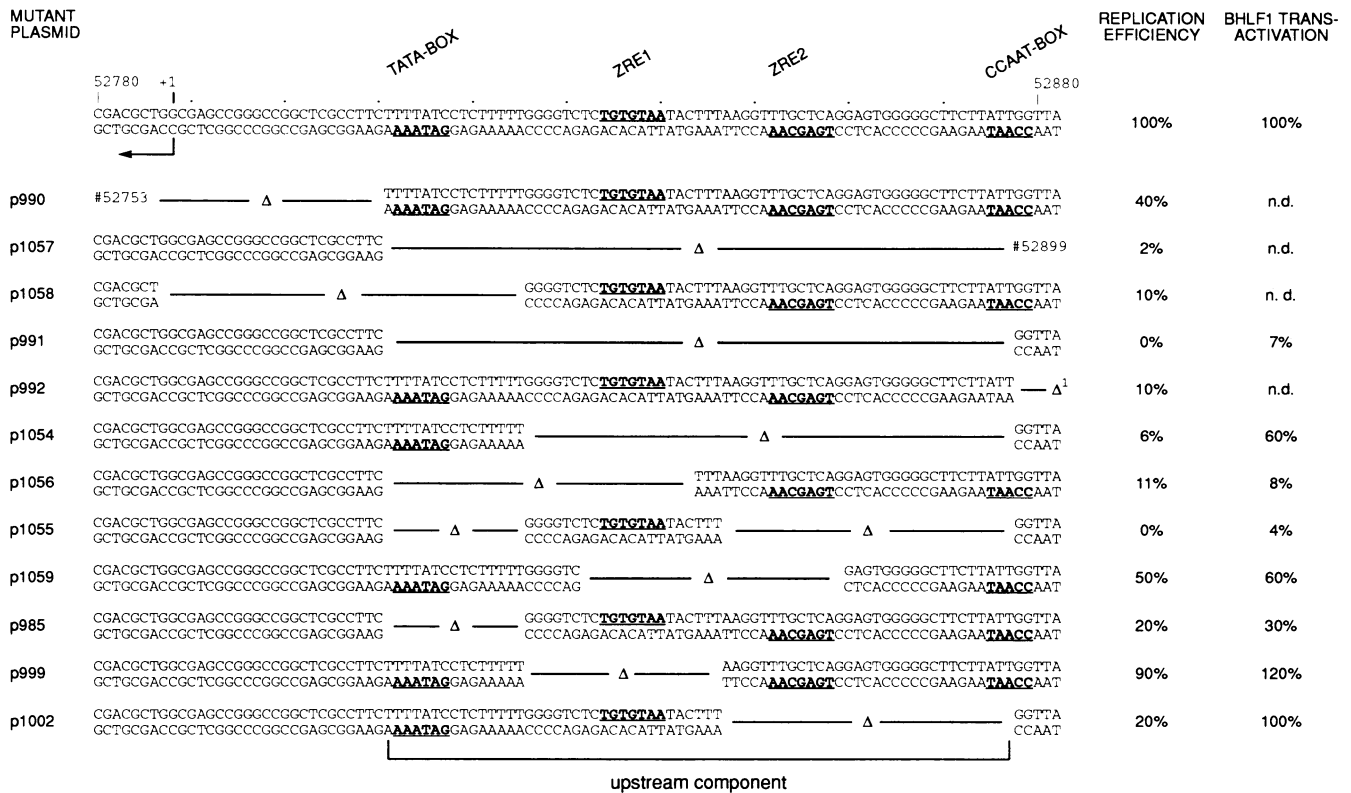
MUTANT
PLASMID

TATA-BOX   ZRE1   ZRE2   CCAAT-BOX   REPLICATION EFFICIENCY   BHLF1 TRANS-ACTIVATION

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52780  +1                                                                                                      52880
CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTTGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA
GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT          100%         100%
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		Replication Efficiency	BHLF1 Trans-activation
p990	#52753 ——— Δ ——— TTTTTATCCTCTTTTTGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA / AAAATAGGAGAAAAACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT	40%	n.d.
p1057	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG ———————— Δ ———————— #52899	2%	n.d.
p1058	CGACGCT / GCTGCGA ——— Δ ——— GGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA / CCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT	10%	n.d.
p991	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG ———————— Δ ———————— GGTTA / CCAAT	0%	7%
p992	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTTGGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATT __ Δ¹ / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAACCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAA	10%	n.d.
p1054	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTT / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAA ——————— Δ ——————— GGTTA / CCAAT	6%	60%
p1056	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG ——— Δ ——— TTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA / AAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT	11%	8%
p1055	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG — Δ — GGGGTCTCTGTGTAATACTTT / CCCCAGAGACACATTATGAAA —— Δ —— GGTTA / CCAAT	0%	4%
p1059	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTTGGGGTC / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAACCCCAG ——— Δ ——— GAGTGGGGGCTTCTTATTGGTTA / CTCACCCCCGAAGAATAACCAAT	50%	60%
p985	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTC / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAG — Δ — GGGGTCTCTGTGTAATACTTTAAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA / CCCCAGAGACACATTATGAAATTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT	20%	30%
p999	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTT / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAA ——— Δ ——— AAGGTTTGCTCAGGAGTGGGGGCTTCTTATTGGTTA / TTCCAAACGAGTCCTCACCCCCGAAGAATAACCAAT	90%	120%
p1002	CGACGCTGGCGAGCCGGGCCGGCTCGCCTTCTTTTTATCCTCTTTTTGGGGTCTCTGTGTAATACTTT / GCTGCGACCGCTCGGCCCGGCCGAGCGGAAGAAAATAGGAGAAAAACCCCAGAGACACATTATGAAA ——— Δ ——— GGTTA / CCAAT	20%	100%

upstream component

FIG. 3. Cooperative elements mediate oriLyt replication in the upstream component. The top line is identical to Fig. 2, which gives the structure of the BHLF1 promoter with important elements, including the TATA box motif (27), BZLF1-binding sites ZRE1 and ZRE2 (28), and the CCAAT box motif. Single and double deletion mutants in the background of wild-type oriLyt plasmid p968.22 are indicated together with the sequence elements that were removed in the single constructs. Relative efficiency in terms of DNA replication is indicated (first column on the right side), together with the effects on transcriptional transactivation of the BHLF1 promoter in luciferase reporter constructs (second column on the right side). The boundaries of the upstream component are given at the bottom.

Surprisingly, all three deletion mutants that removed parts of the upstream component reduced the efficiency of DNA replication but did not abolish it completely (Fig. 1C and E, p985, p999, p1002). This initial finding indicated that elements affected by smaller deletions might cooperate with other sequence elements that reside within the upstream component. In contrast, the refinement of the downstream component identified a contiguous sequence element which was nonfunctional like p995 (Fig. 1C to E).

TATA box mutations alone have a minor effect on DNA replication. First, we wanted to dissect the upstream component even further. We speculated that the TATA box might be a good candidate to start with, since its deletion in the BHLF1 promoter decreased the efficiency of p985 replication by 80% (Fig. 1). We wondered whether this effect was caused by removal of a generic TATA box-binding factor motif or by a spacing problem. Two substitution mutants which had the same A·T content but a randomized sequence composition (p980 and p981) partially restored activity to 35 and 45% of the wild-type level, respectively, compared with deletion mutant p985 (Fig. 2). Introduction of the TATA box motif from the simian virus 40 early promoter (5) in either orientation (p982 and p983) improved replication almost to the wild-type level, whereas inversion of the BHLF1 TATA box and its flanking region had an intermediate effect (p984; Fig. 2). In all, a functional TATA box motif seemed to improve the efficiency of oriLyt replication but was not an absolute requirement.

At least two promoter elements cooperate for oriLyt function. The modulation of DNA replication seen with TATA box mutants of oriLyt led us to look for synergistic or at least cooperative effects with other elements. Known transcription factors that bind within the BHLF1 promoter might represent such candidates which cooperate with TATA box-binding proteins (29) and could play a role in DNA replication, too. Binding motifs for the BZLF1 gene product (ZRE1 and ZRE2) are located between the TATA box and the CCAAT box consensus sequence, and each of them was inactivated in oriLyt null mutant p991 (Fig. 1 and 3). Consequently, we analyzed oriLyt mutants in which these individual sequence elements were deleted in various combinations (Fig. 3). Deletion of sequences downstream of the TATA box that removed ZRE1, ZRE2, and the CCAAT box had a pronounced effect (6%; p1054), very similar to that of combinatorial deletions of the TATA box together with one ZRE element (11%; p1056) or one ZRE element plus the CCAAT box motif (0%; p1055). Removal of the two ZRE-binding motifs alone had an intermediate effect (50%; p1059) which is partly due to a spacing problem which brings downstream sequences closer to the TATA box sequence motif (data not shown).

RNA transcription and DNA replication are dependent on shared sequence elements. Since the upstream component encompasses the well-defined BHLF1 promoter (27, 30, 31), it was tempting to speculate that the elements required for DNA replication are identical to elements required for RNA
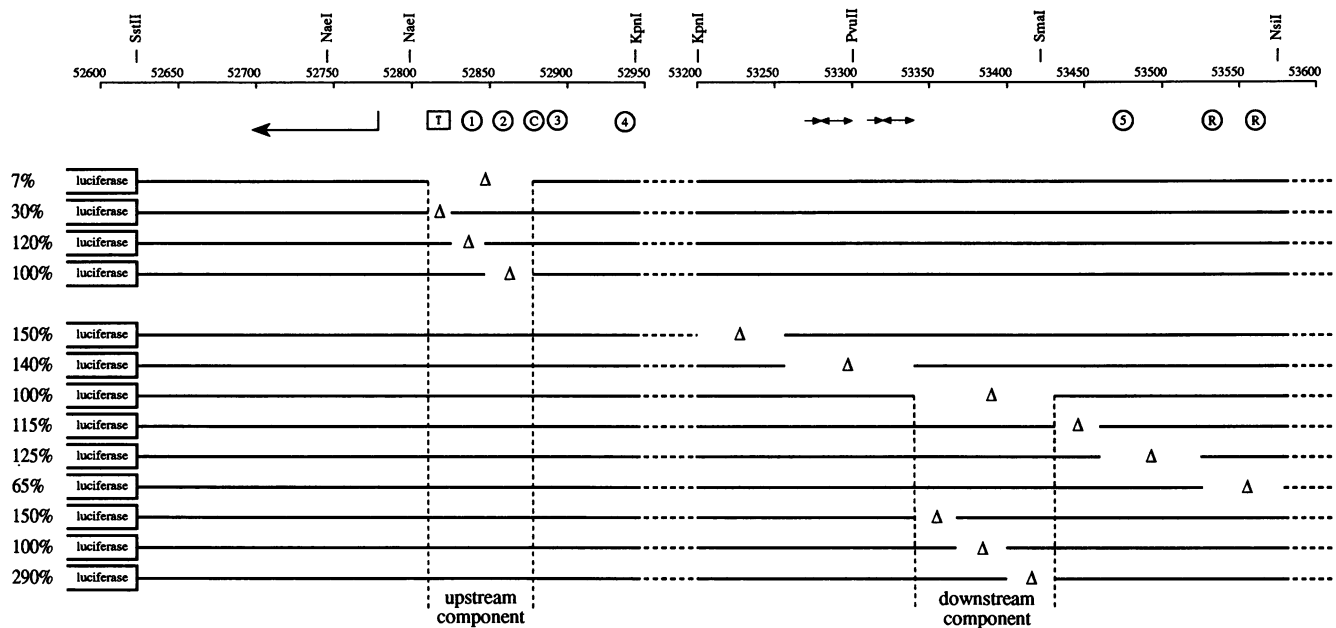
FIG. 4. Some, but not all, of the elements involved in transcription and replication colocalize in oriLyt. oriLyt mutants were analyzed for transcriptional transactivation of the BHLF1 promoter in luciferase reporter constructs. The deletion mutants used were identical to the ones shown in Fig. 1C, including the features and annotations of the minimal oriLyt regions in the top line. Removal of the complete upstream component reduced the level of the promoter activity to 7% of the wild-type configuration (left column) but did not abolish it completely. In contrast, smaller deletion mutants of the upstream component partly relieved this effect. Scanning deletion mutants of the downstream region, including the downstream component, had only marginal effects on BHLF1 promoter activity, with one exception (bottom line) which caused threefold activation of the BHLF1 promoter.

transcription. To concentrate on this possibility, we constructed a number of reporter plasmids based on oriLyt deletion mutants. The luciferase reporter gene replaced the BHLF1 coding sequences, which allowed quantitation of BHLF1 promoter activity in the different mutants. The plasmids are schematically depicted in Fig. 3 and 4 together with their relative levels of BHLF1 promoter activity. From this analysis, it appeared that most mutations had comparable effects on transcription and replication. Deletion of the TATA box, alone or in conjunction with other binding sites for promoter factors, paralleled the effects seen with single and combinatorial oriLyt mutants in general. However, there were two remarkable exceptions to this rule: mutants p1002 and p1054 (Fig. 3), which had very much reduced levels of DNA replication and nearly wild-type levels of RNA transcription. Although we do not know the molecular basis for this finding, it indicates that transcription and replication are not necessarily intimately linked (see below).

The downstream component is a novel motif in complex herpesviral origins. The downstream component of oriLyt was defined by oriLyt mutant p995 (Fig. 1). Attempts to delineate further the sequences crucial for DNA replication were undertaken with 3 deletion mutants (Fig. 1C, p1001, p1002, and p1003) and 19 substitution mutants (Fig. 5a). The latter were arranged in partly overlapping fashion and contained randomized stretches (usually 10 bp) within the downstream component. Again, the mutations were embedded into the BamHI-SalI fragment of EBV depicted in Fig. 1A. In transient replication assays, the oriLyt mutant plasmids identified a unique sequence element spanning EBV coordinates 53,355 to 53,395 which was extremely sensitive to any

mutation introduced (Fig. 5). Further downstream, a second site was identified with oriLyt mutants p1030 and p1031. Randomized substitution of 10 to 20 bp was sufficient to reduce the efficiency of oriLyt DNA replication substantially. A nucleotide sequence comparison with the regions from coordinates 53,350 to 53,430 revealed similarly conserved regions which were reiterated in the simian cytomegalovirus lytic origin of replication (data not shown; 2). Whether this homology merely reflects the very high GC content of this area or is functionally important remains to be demonstrated.

RNA transcription and DNA replication are not always linked in oriLyt. Since the upstream component of oriLyt showed an impressive interaction with known sequence elements involved in RNA transcription, we wanted to know whether this finding might hold true for the downstream element. Others identified enhancer-like structures in this region which included two binding sites for viral transcription factor R (18, 30). oriLyt mutants spanning the downstream region of the minimal origin were constructed to measure transcriptional transactivation from the BHLF1 (Fig. 4) or BHRF1 promoter in two different cell lines (D89HR1 and HH514; data not shown). The mutants were found to be only marginally affected with respect to transcription. Whereas transcription from the BHRF1 promoter was generally reduced to about 30 to 50% of the wild-type level irrespective of the mutation, BHLF1 promoter activity was partly diminished by one mutant but stimulated almost threefold by another (Fig. 4). The former removed the two binding sites for transcription factor R, and the latter had a sequence removed which was found to be part of the downstream component. Neither deletion of a fifth BZLF1-
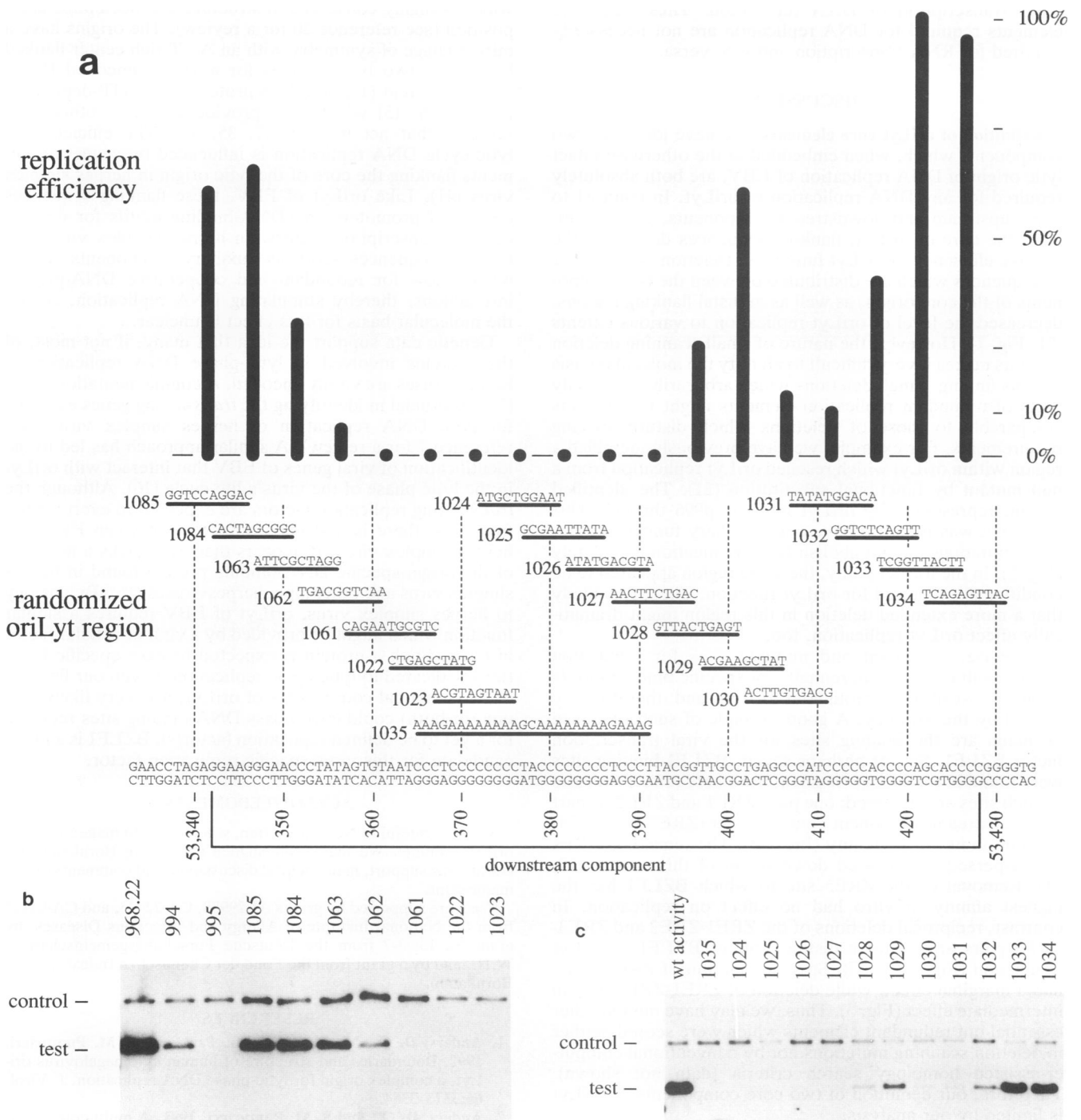
a

replication efficiency

— 100%

— 50%

— 10%
— 0%

randomized oriLyt region

1085 GGTCCAGGAC
1084 CACTAGCGGC
1063 ATTCGCTAGG
1062 TGACGGTCAA
1061 CAGAATGCGTC
1022 CTGAGCTATG
1023 ACGTAGTAAT

1024 ATGCTGGAAT
1025 GCGAATTATA
1026 ATATGACGTA
1027 AACTTCTGAC
1028 GTTACTGAGT
1029 ACGAAGCTAT
1030 ACTTGTGACG
1035 AAAGAAAAAAAAGCAAAAAAAAGAAAG

1031 TATATGGACA
1032 GGTCTCAGTT
1033 TCGGTTACTT
1034 TCAGAGTTAC

GAACCTAGAGGAAGGGAACCCTATAGTGTAATCCCTCCCCCCCCTACCCCCCCCTCCCTTACGGTTGCCTGAGCCCATCCCCCACCCCAGCACCCCGGGGTG
CTTGGATCTCCTTCCCTTGGGATATCACATTAGGGAGGGGGGGGGATGGGGGGGGAGGGAATGCCAACGGACTCGGGTAGGGGGTGGGGTCGTGGGGCCCCAC

53,340 — | 350 | 360 | 370 | 380 | 390 | 400 | 410 | 420 | 53,430 —

downstream component

b

968.22 994 995 1085 1084 1063 1062 1061 1022 1023

control —
test —

c

wt activity 1035 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034

control —
test —

FIG. 5. Fine mapping of the downstream component reveals a novel element involved in oriLyt replication. Deletion scanning mutants of the downstream region were tested for DNA replication in the transient oriLyt replication assay. In part a, the replication efficiencies of the single mutants are given in a histogram in which that of wild-type oriLyt plasmid p968.22 was set to 100%. The sequence stretches mutated by random substitutions are given in the lower section together with the context of oriLyt spanning the downstream component from nucleotides 52,342 to 53,428 of EBV (3). Again, the mutant plasmids are based on p968.22 to provide all of the other flanking regions shown in Fig. 1A. Panels b and c present the original data on these mutants in two autoradiograms. The mutated plasmids are arranged in identical order, and plasmids p994 and p995, which are also shown in Fig. 1, are included.

binding site (ZRE5 in Fig. 4) nor removal of obvious secondary structure sequences had any significant impact on RNA transcription or DNA replication. Thus, sequence elements required for DNA replication are not necessarily required for RNA transcription and vice versa.

DISCUSSION

Definition of oriLyt core elements. We have identified two components which, when embedded in the otherwise intact lytic origin of DNA replication of EBV, are both absolutely required for any DNA replication of oriLyt. In contrast to these upstream and downstream components, which comprise the core of oriLyt, flanking sequences determine the relative efficiency of oriLyt functions. Deletion of a number of sequences which are distributed between the two components of the core origin, as well as in distal flanking regions, decreased the level of oriLyt replication to various extents (21; Fig. 1). However, the nature of small scanning deletion mutants makes it very difficult to identify the molecular basis for this finding. Small deletions which arbitrarily affect only parts of redundant replication elements might have effects comparable to those of deletions which disturb spacing requirements. For example, we have previously identified a region within oriLyt which rescued oriLyt replication from a null mutant by functional substitution (21). The identified region, represented by oriLyt mutants p996 through p998 (Fig. 1C), was found to have only auxiliary functions here, since mutations did not abolish DNA replication completely (Fig. 1). In the former study, the same region appeared to be conditionally required for oriLyt function. Thus, it is likely that a more extended deletion in this region might dramatically affect oriLyt replication, too.

Likewise, redundant and multiple sites for replication factors with certain requirements for specific binding motifs might be scattered throughout oriLyt and therefore be missed by this strategy. A good example of such sequence elements are the binding sites for the viral transcription factor BZLF1. Six, or perhaps seven, BZLF1-binding sites were found in the oriLyt fragment used here (28). Two pairs of such sites are clustered; one pair (ZRE1 and ZRE2) is part of the upstream component, and the other (ZRE3 and ZRE4) is found in its close vicinity (Fig. 1B). The remaining ZREs are dispersed and located downstream of this cluster (19, 31). Removal of the ZRE5 site to which BZLF1 has the highest affinity in vitro had no effect on replication. In contrast, reciprocal deletions of the ZRE1-ZRE2 and ZRE3-ZRE4 pairs argued for a distinct role of BZLF1 in oriLyt replication (Fig. 1C to E). Specific deletion of ZRE1 alone had a marginal effect, while deletion of ZRE1-ZRE2 had an intermediate effect (Fig. 3). Thus, we may have missed other essential but redundant elements which were scored neither in deletion scanning mutations nor by conventional computer-assisted homology search criteria (data not shown). Therefore, our definition of two core components of oriLyt is limited by our analysis.

oriLyt is distinct from the lytic origins of the alphaherpesvirus and betaherpesvirus subgroups. The overall structure of oriLyt, its multipartite organization, and its colocalization with elements of transcription make it very different from other herpesviral origins. Although the structure of two lytic origins of replication from members of the betaherpesvirus subgroup is similarly complex (1, 2, 22, 33), it does not share any obvious extended homology with oriLyt of EBV. Herpes simplex virus and other members of the alphaherpesvirus subgroup, on the other hand, appear to be simple with

respect to their lytic-phase origins of DNA replication. These generally consist of one element of less than 150 bp which is highly conserved in structure and nucleotide composition (see reference 20 for a review). The origins have a mirror image of symmetry with an A · T-rich center flanked by one or two binding sites for a virally encoded DNA-binding protein (13, 34). This protein is an ATP-dependent helicase (6, 15) which may provide a link to other viral proteins that act in *trans* (7, 35, 42). The efficiency of lytic-cycle DNA replication is influenced by sequence elements flanking the core of the lytic origin in herpes simplex virus (41). Like oriLyt of EBV, these flanking sequences consist of promoters and DNA-binding motifs for viral or cellular transcription factors. In herpes simplex virus, the flanking sequences score as auxiliary components, which might allow for redundant and cooperative DNA-protein interactions, thereby stimulating DNA replication. Again, the molecular basis for this effect is unclear.

Genetic data support the idea that many, if not most, of the proteins involved in lytic-phase DNA replication of herpesviruses are virally encoded. A complementation assay (7) was crucial in identifying the *trans*-acting genes essential for lytic DNA replication of herpes simplex virus (see reference 7 for a review). A similar approach has led to the identification of viral genes of EBV that interact with oriLyt in the lytic phase of the virus's life cycle (16). Although the *trans*-acting replication factors are expected to exert similar functions, there is a striking difference between EBV and herpes simplex virus. It appears that EBV lacks a homolog of the origin-specific DNA-binding protein found in herpes simplex virus and other alphaherpesviruses (16). By analogy to herpes simplex virus, oriLyt of EBV requires a similar function which might be provided by a viral or cellular factor in *trans*. Such a protein is expected to have specific functions dedicated to lytic-cycle replication. Given our findings of two essential components of oriLyt, it is very likely that one (or both) could encompass DNA-binding sites required for a yet to be defined replication factor(s). BZLF1 is a likely candidate for this virus-specific replication factor.

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