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Kaposi's Sarcoma-Associated Herpesvirus *ori-Lyt*-Dependent DNA Replication: *cis*-Acting Requirements for Replication and *ori-Lyt*-Associated RNA Transcription

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Herpesvirus lytic DNA replication requires both the *cis*-acting element, the origin, and *trans*-acting factors such as virally encoded origin-binding protein and DNA replication enzymes. Recently, the origins of lytic DNA replication (*ori-Lyt*) in Kaposi's sarcoma-associated herpesvirus (KSHV) have been identified and a virally encoded bZip protein, K8, has been shown to specifically bind to the origin. To map *cis*-acting elements within KSHV *ori-Lyt* that are required for DNA replication function and to define the nature of K8 bZip protein binding to the origin, we constructed consecutive internal deletion mutations across the core domain of a KSHV *ori-Lyt* and tested them for DNA replication function in a transient replication assay. This mutagenesis study allowed the identification of four components within the *ori-Lyt*, and all were indispensable for *ori-Lyt* function. The first component contains eight CCAAT/enhancer binding protein (C/EBP) binding motifs that organize as four spaced C/EBP palindromes. Each palindrome contains two head-to-head CCAAT consensus motifs that are separated by a 13- or 12-bp space sequence. Substitution mutagenesis of these C/EBP motifs showed that these C/EBP palindromes are required for both K8 binding and *ori-Lyt*-dependent DNA replication. The second component is an 18-bp AT palindrome, which is essential for *ori-Lyt* function. The third component was determined to be a 32-bp previously unidentified sequence and is required for DNA replication. The last component consists of an open reading frame 50 (ORF50)/Rta responsive element (RRE) and a TATA box. We showed that the binding of an ORF50/Rta protein to the RRE was essential for *ori-Lyt*-dependent DNA replication. The presence of a functional RRE and a downstream TATA box suggested that this region serves as an ORF50/Rta-dependent promoter and a transcription event may be necessary for *ori-Lyt*-dependent DNA replication. Using a luciferase reporter system, we demonstrated that the region of the RRE and TATA box constitutes an ORF50/Rta-dependent promoter. Furthermore, a polyadenylated RNA of 1.4 kb was identified downstream of the promoter.

As a gammaherpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV) has two alternative life cycles, namely latent and lytic replication cycles (14, 16). During latency, only a limited number of viral genes are expressed and no infectious virus is produced. In latently infected cells, multiple copies of the viral genome are maintained as extrachromosomal episomes (plasmids) and are replicated in synchrony with cell division (3). The terminal repeat sequence in the KSHV genome is necessary and sufficient for episome persistence and likely serves as the origin of latent plasmid replication (*ori-P*) (2). Virally encoded latency-associated nuclear antigen specifically binds to *cis*-acting terminal repeat DNA and acts in *trans* on the *ori-P* to mediate episome persistence (2). When latency is disrupted, KSHV switches to a lytic life cycle (14, 16). In the lytic phase, the virus expresses most or all of its genes and viral DNA is amplified by a replication mechanism distinct from that for latent viral DNA replication (9, 21). In general, lytic DNA replication of herpesviruses differs in two aspects from latent DNA replication. First, in contrast to latent DNA replication that is in synchrony with the host cell for a stable amount of viral episomal DNA in each cell, viral DNA is amplified 100- or even 1,000-fold in lytic replication via a rolling circle mechanism. Second, in contrast to latent DNA replication that depends on host cellular DNA polymerase and accessory factors, viral lytic replication utilizes its own DNA polymerase and other factors.

Lytic cycle DNA replication of herpesviruses is initiated from an origin (*ori-Lyt*) and requires many viral gene products. The origin region is bound by a virus-specified origin-binding protein (OBP) that recruits the core replication machinery. Recently, two duplicated copies of the lytic DNA replication origin [referred to as *ori-Lyt* (L) and *ori-Lyt* (R)] were identified in the KSHV genome in our laboratory and another laboratory (1, 11). These *ori-Lyt* are located in the KSHV genome between K4.2 and K5 and between K12 and open reading frame 71 (ORF71), respectively. In addition, we also found that a KSHV-encoded bZip protein, namely K8 (also referred to as replication-associated protein), was associated with a 500-bp essential core segment of the KSHV *ori-Lyt* (11). This finding, together with the observation that K8 is incorporated into the KSHV viral DNA replication compartments (21), suggests that K8 may be an OBP, similar to the Zta protein of Epstein-Barr virus (EBV) (8).

The structural and functional associations of KSHV *ori-Lyt*

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appear to be complex. Two *ori-Lyt* share an almost identical 1.1-kb sequence and 600-bp GC-rich repeats that are represented as 20- and 30-bp tandem arrays. Previous data showed that the whole 1.7-kb DNA sequences are necessary and sufficient as a *cis*-acting signal for KSHV replication (11). The first 465-bp sequence is 100% identical between these two *ori-Lyt*. Many interesting motifs are found in the highly conserved region, including two long AT-rich palindromes, four multiple short repetitive motifs containing XcaI and PvuII sites that are found in EBV *ori-Lyt*, two FspI/SphI motifs that are found within cytomegalovirus *ori-Lyt*, and clustered consensus binding motifs for AP-1, CCAAT/enhancer binding protein (C/EBP), and ATF (11, 15). Among them, two AT palindromes are extremely intriguing. AT palindromes are often found in eukaryotic cellular DNA replication origins, and initiation of replication generally involves local unwinding at an AT palindromic sequence (5). Another remarkable feature is that eight C/EBP motifs are found in a 200-bp segment between two AT palindromes, and they are arrayed as four unusually spaced palindromes. However, the roles of these motifs in initiating DNA replication have not been experimentally tested.

The goal of this study was to understand the structure and function of KSHV *ori-Lyt* and identify *cis*-acting elements that are essential for origin function. A systematic mutational analysis was performed on the core domain of a KSHV *ori-Lyt* for *cis*-acting constituents essential for the origin function. The ability of each *ori-Lyt* mutant to support lytic DNA replication was examined in a transient replication assay. By analyzing more than 50 *ori-Lyt* mutants, we defined several components in the *ori-Lyt*. First, we found that the palindromically arrayed C/EBP motifs are absolutely essential for both K8 binding to the *ori-Lyt* and lytic DNA replication. Second, mutation of the 18-bp AT palindrome completely abolished the origin function. Third, an ORF50 (Rta) responsive element (RRE) and a TATA box consensus sequence are found to be essential for *ori-Lyt*-dependent DNA replication. We demonstrated that the RRE and TATA box constitute an ORF50-dependent promoter, which directs transcription of a 1.4-kb polyadenylated RNA. Our data implied that a transcription activity is associated with KSHV lytic DNA replication.

MATERIALS AND METHODS

Cell culture. The primary effusion lymphoma cell line BCBL-1, which carries latently infected KSHV and was established by Renne and colleagues (16), was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The cells were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Gibco-BRL). BC-1 cells, which carry KSHV and EBV and were established by Cesarman et al. (4), were purchased from the American Type Culture Collection and grown in RPMI 1640 medium supplemented with 15% fetal bovine serum. BJAB cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. 293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. All cultures contained penicillin-streptomycin (50 U/ml) and Fungizone (1.25 μg of amphotericin B/ml and 1.25 μg of sodium deoxycholate/ml).

Plasmids and DNA transfection. Plasmid pOri-A was constructed by cloning an EcoRI-PstI fragment (nucleotides 22409 to 26491, according to the numbering of Russo et al. [17]) of KSHV DNA in pBluescript at the EcoRI/PstI site. The internal deletion mutants of pOri-A (pOri-ID1 and pOri-ID2, etc.) were generated by using a PCR-based mutagenesis system, namely ExSite (Stratagene). In brief, a pair of phosphorylated oligonucleotides toward opposite directions was used in a high-fidelity PCR with pOri-A plasmid as a template. After PCR, the template DNA (wild-type pOri-A) was removed by complete digestion with DpnI, which does not degrade PCR-synthesized DNA. The PCR products were self-ligated and used to transform *Escherichia coli*-competent cells. Thirty deletion mutants of pOri-A are listed and described in Table 1. The nucleotide substitution mutants of pOri-A were also engineered by using the ExSite mutagenesis system. Twenty-one substitution mutants are listed and described in Table 1.

pCR3.1-ORF50 was constructed by cloning the cDNA sequence of the ORF50 coding region into the pCR3.1 vector (Invitrogen). The construct was described in detail by Lin et al. (11). The expression vectors for Gal4 fusion proteins (pSG-C50 and pSG-424) were described by Lukac et al. (13).

To transfect cells, 5 μg of pOri-A or its mutant plasmids and 5 μg of pCR3.1-ORF50 (or pCR3.1 vector) were mixed with 10^7 BCBL-1 cells in OPTI-MEM medium (Gibco-BRL) and electroporated (200 V, 960 μF) with a Genepulser II (Bio-Rad, Hercules, Calif.). Electroporated cells were then transferred to RPMI 1640 medium supplemented with 10% serum and grown for 72 h.

DNA replication assay. Extrachromosomal DNAs were prepared from cells by using the Hirt DNA extraction method as follows. Cells were lysed in 700 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, and 0.6% sodium dodecyl sulfate). Chromosomal DNA was precipitated at 4°C overnight by adding 5 M NaCl to the final concentration of 0.85 M. Cell lysates were centrifuged at 4°C at 14,000 rpm (Eppendorf 5417R) for 30 min. The supernatant containing extrachromosomal DNA was subjected to phenol-chloroform extraction, followed by ethanol precipitation. The DNA was treated with RNase A at 25°C for 30 min and then with proteinase K at 50°C for 30 min. Five micrograms of DNA was digested with KpnI/SacI or KpnI/SacI/DpnI (New England Biolabs). The DNAs were separated by electrophoresis on 1% agarose gels and transferred onto GeneScreen membranes (Perkin Elmer, Boston, Mass.). The Southern blots were hybridized with ^{32}P-labeled pBluescript plasmid in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2× Denhardt's solution, 1% sodium dodecyl sulfate, and 50 μg of denatured salmon sperm DNA/ml at 68°C.

In vitro DNA binding assay. Various DNA fragments were synthesized by PCR with pOri-A DNA or its mutants as templates and two oligonucleotides as primers. The two oligonucleotides were ori-2.1F (5′-AGGTCGCCGAGGTGG CAAGGTGACT-3′) and ori-7R (5′-ACTGGAATAGGGGCTGCGATGACT C-3′). One of the oligonucleotides was biotinylated at its 5′ end. The resultant biotinylated PCR fragments were coupled to streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway) and then incubated with nuclear extracts prepared from tetradecanoyl phorbol acetate (TPA)-induced (and uninduced) BCBL-1 cells for 45 min at 25°C. The bound material was washed four times in D150 buffer (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 150 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.05% NP-40) and then progressively eluted with D300 (same as above, except 300 mM KCl), D500 (500 mM KCl), and D1000 (1 M KCl). The affinity-purified materials were assayed by Western blot analysis with anti-K8 antibody.

Reporter plasmids and luciferase assays. Reporter plasmid 4F and its mutants (4FΔRRE, 4FΔ7, 4F-Gal4, 4F-2xGal4, and 4FΔTATA) were constructed by cloning the appropriate PCR-generated fragments into the SmaI/NcoI-digested pGL3-Basic vector (Promega). These fragments were generated by PCR with oligonucleotides 4F (5′-CCCATAATCCTCTGCCCCGCTCCCAT-3′) and OriT-2 (5′-CATGCCATGGTGCTGCCGGGGCTCCTCGTTAC-3′) as primers and pOri-A or its mutants (pOri-ID15.4, pOri-ID15.7, pOri-Gal4Δ6, pOri-2xGal4, and pOri-16.2) as templates, followed by digestion with NcoI. 13F and its mutants (13FΔRRE, 13FΔ7, 13F-Gal4, 13F-2xGal4, and 13FΔTATA) were generated by digesting plasmid 4F and its mutants with restriction endonucleases AscI and MluI and religating these plasmids. The inserts of these constructs have been illustrated (see Fig. 7A).

Ten micrograms of luciferase reporter construct, 1 μg of pRL-TK plasmid, and 3 μg of pCR3.1-ORF50 (or pCR3.1 empty vector) were mixed with 10^7 BJAB or BCBL-1 cells in OPTI-MEM medium (Gibco-BRL) and electroporated (250 V, 960 μF) with a Genepulser II (Bio-Rad). Electroporated cells were then transferred to RPMI 1640 medium supplemented with 10% serum and grown for 48 h. The pRL-TK plasmid was included as an internal control which constitutively expresses *Renilla* luciferase. The dual luciferase reporter assay system (Promega) was used to examine the responsiveness of the promoters to ORF50/Rta. Transfected cells were washed once with 1× phosphate-buffered saline and suspended in 400 μl of 1× passive lysis buffer. Cells were freeze-thawed once and centrifuged in a microcentrifuge for 1 min. Supernatants were assayed for firefly luciferase and *Renilla* luciferase activities by using a TD-20/20 luminometer with a dual autoinjector (Turner Designs). The luciferase assays were carried out according to the manufacture's instructions (Promega).

Northern blotting and hybridization. Total RNA was isolated from BC-1 cells with Trizol reagent (Gibco-BRL), and poly(A^+) mRNA was purified by using the

TABLE 1. Characteristics of a KSHV *ori-Lyt* plasmid and its mutants

Construct	Characteristics
pOri-A (wt)	EcoRI-PstI fragment (nucleotides 22409–26491) of KSHV DNA cloned in pBluescript at EcoRI/PstI site
pOri-ID1	185 bp (nucleotides 22944–23128) deleted from pOri-A
pOri-ID2	49 bp (nucleotides 23150–23198) deleted from pOri-A
pOri-ID3	73 bp (nucleotides 23203–23275) deleted from pOri-A
pOri-ID4	33 bp (nucleotides 23276–23308) deleted from pOri-A
pOri-ID5	40 bp (nucleotides 23291–23330) deleted from pOri-A
pOri-ID6	76 bp (nucleotides 23332–23407) deleted from pOri-A
pOri-ID7	110 bp (nucleotides 23407–23516) deleted from pOri-A
pOri-ID8	56 bp (nucleotides 23517–23572) deleted from pOri-A
pOri-ID9	45 bp (nucleotides 23555–23599) deleted from pOri-A
pOri-ID10	50 bp (nucleotides 23587–23636) deleted from pOri-A
pOri-ID11	68 bp (nucleotides 23637–23704) deleted from pOri-A
pOri-ID12	86 bp (nucleotides 23705–23790) deleted from pOri-A
pOri-ID13	117 bp (nucleotides 23782–23898) deleted from pOri-A
pOri-ID14	125 bp (nucleotides 23896–24020) deleted from pOri-A
pOri-ID15	134 bp (nucleotides 24024–24157) deleted from pOri-A
pOri-ID16	96 bp (nucleotides 24157–24252) deleted from pOri-A
pOri-ID13.1	26 bp (nucleotides 23782–23807) deleted and replaced by GGATCC in pOri-A
pOri-ID13.2	33 bp (nucleotides 23805–23837) deleted and replaced by GGATCC in pOri-A
pOri-ID13.3	34 bp (nucleotides 23838–23871) deleted and replaced by GGATCC in pOri-A
pOri-ID13.4	29 bp (nucleotides 23872–23900) deleted and replaced by GGATCC in pOri-A
pOri-ID15.1	36 bp (nucleotides 24025–24060) deleted and replaced by GGATCC in pOri-A
pOri-ID15.2	26 bp (nucleotides 24061–24086) deleted and replaced by GGATCC in pOri-A
pOri-ID15.3	24 bp (nucleotides 24086–24109) deleted and replaced by GGATCC in pOri-A
pOri-ID15.4	17 bp (nucleotides 24104–24120) deleted and replaced by GGATCC in pOri-A
pOri-ID15.5	27 bp (nucleotides 24111–24137) deleted and replaced by GGATCC in pOri-A
pOri-ID15.6	27 bp (nucleotides 24138–24164) deleted and replaced by GGATCC in pOri-A
pOri-ID15.7	54 bp (nucleotides 24111–24164) deleted and replaced by GGATCC in pOri-A
pOri-ID16.1	25 bp (nucleotides 24165–24189) deleted and replaced by GGATCC in pOri-A
pOri-ID16.2	25 bp (nucleotides 24190–24214) deleted and replaced by GGATCC in pOri-A
pOri-ID16.3	38 bp (nucleotides 24215–24252) deleted and replaced by GGATCC in pOri-A
pOri-Gal4	22 bp (nucleotides 24104–24125) deleted and replaced by GATCCGGAGGACTGTCCTCCGG in pOri-A
pOri-Gal4Δ6	58 bp (nucleotides 24104–24161) deleted and replaced by GATCCGGAGGACTGTCCTCCGG in pOri-A
pOri-2xGal4	58 bp (nucleotides 24104–24161) deleted and replaced by two copies of Gal4 binding motif in pOri-A
pOri-M1	CCAAT (nucleotides 23524–23520) mutated to CGTCT in pOri-A
pOri-M2	CCAAT (nucleotides 23538–23542) mutated to GTCAT in pOri-A
pOri-M1M2	Combined M1 and M2 mutations in pOri-A
pOri-M3	CCAAT (nucleotides 23453–23449) mutated to CGTCT in pOri-A
pOri-M4	TCAAT (nucleotides 23466–23470) mutated to GTCAT in pOri-A
pOri-M3M4	Combined M3 and M4 mutations in pOri-A
pOri-M1M2M3M4	Combined M1, M2, M3, and M4 mutations in pOri-A
pOri-M5	CCAAT (nucleotides 23400–23396) mutated to CGTCT in pOri-A
pOri-M6	CCAAT (nucleotides 23414–23418) mutated to GTCAT in pOri-A
pOri-M5M6	Combined M5 and M6 mutations in pOri-A
pOri-M1M2M5M6	Combined M1, M2, M5, and M6 mutations in pOri-A
pOri-M7	CCAAT (nucleotides 23359–23355) mutated to CGTCT in pOri-A
pOri-M8	TCAAT (nucleotides 23372–23376) mutated to GTCAT in pOri-A
pOri-M7M8	Combined M7 and M8 mutations in pOri-A
pOri-M1M2M7M8	Combined M1, M2, M7, and M8 mutations in pOri-A
pOri-M9	18-bp palindrome TATATATATATATATAAT mutated to TACCTACCTATATATAAT in pOri-A
pOri-M10	18-bp palindrome TATATATATATATATAAT mutated to TATATATATACCTACCAT in pOri-A
pOri-M9M10	18-bp palindrome TATATATATATATATAAT mutated to TACCTACCTACCTACCAT in pOri-A

PolyAtract mRNA isolation system (Promega). The mRNA was separated by electrophoresis in 1% agarose–6% formaldehyde gel in 20 mM morpholinepropanesulfonic acid (MOPS) buffer, pH 7.0. Each lane was loaded with mRNA from 2×10^7 cells. The RNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.) and hybridized with a single-stranded ^{32}P-labeled probe. The probe was prepared by asymmetric PCR with PmlI-digested pOri-A plasmid as the template and the oligonucleotide OriT-1(5′-TGTTTATTTCAA GAGCCTATGCTCG-3′, nucleotides 25428 to 25403) as the primer. The labeling reaction was performed in 15 μl of reaction solution (1× *Taq* polymerase buffer: 16.67 μM [each] dATP, dGTP, and dTTP, 1.67 μM dCTP, 5 μl of [α-^{32}P]dCTP [800 Ci/mmol, 10 μCi/μl; Amersham], 100 ng of DNA, 20 pmol of primer, and 2.5 U of *Taq* polymerase). The PCR was initiated with a denaturing step of 2 min at 94°C, followed by 15 cycles of sequential steps of 1 min at 94°C, 1 min at 50°C, and 3 min at 74°C. Finally, the reaction was extended for 10 min at 74°C. The RNA loading equivalence was controlled by probing with β-actin cDNA. A 0.24- to 9.5-kb RNA ladder (Gibco-BRL) was included in each agarose-formaldehyde gel and detected in Northern blots by hybridization with labeled λ DNA.

RACE. A rapid amplification of cDNA ends (RACE)-ready cDNA pool was generated by using the Marathon cDNA amplification kit with Advantage cDNA polymerase mix (Clontech, Palo Alto, Calif.). Polyadenylated RNA was isolated from BC-1 cells that had been treated with sodium butyrate for 24 h. Double-stranded cDNA was synthesized with avian myeloblastosis virus reverse transcriptase and cDNA synthesis primer [a modified lock-docking oligo(dT) primer; Clontech]. The cDNAs were ligated with the Marathon cDNA adapter (Clontech).

To obtain the 5′ RACE fragment of *ori-Lyt*-associated transcript, the RACE-ready cDNA pool was amplified first with oriT-RACE2 primer (5′-GAGGGTG GCGCGCCGGGGCT-3′) and adaptor primer AP-1 (provided in the Marathon cDNA kit). The PCR products were then amplified with oriT-RACE3 (5′-GG

GTGCTGCCGGGGCTCCTCGTTAC-3′) and nested adaptor primer AP-2 from the kit. Similarly, the 3′ portion of the cDNA was obtained through two PCRs. RACE4 (5′-TGGATTTACCCGTAACGAGGAGC-3′) and AP-1 primers were used in the first reaction, and RACE1 (5′-AGCCCCGGCGCGCCAC CCTC-3′) and AP-2 were used in the second. DNA fragments generated in the RACE reactions were cloned into the T/A type PCR cloning vector pCR2.1 (Invitrogen) and sequenced.

RESULTS

Mapping of *cis*-acting elements for *ori-Lyt* function. Although both duplicated *ori-Lyt* are able to mediate KSHV lytic DNA replication (11), we focused on the *ori-Lyt* (L) (located in the KSHV genome between K4.2 and K5) for further analysis. Previous study with progressive deletion mutants of an *ori-Lyt* plasmid established minimal *ori-Lyt* boundaries (11). According to these data, serial internal deletion mutants across the KSHV *ori-Lyt* core domain (nucleotides 23137 to 24290) were constructed by using a PCR-based mutagenesis system, namely ExSite (Stratagene), and designed as scanning mutations (Fig. 1A). Deletion mutants were generated in pOri-A, which contains a 2.6-kb insert of KSHV *ori-Lyt* sequence (nucleotides 22409 to 25063) in a pBluescript backbone (11). Each mutant contains a small internal deletion (33 to 134 bp), and the whole set of mutants was used to scan the entire *ori-Lyt* core domain. Replication of pOri-A and its derivatives was measured in BCBL-1 cells, which are latently infected with KSHV and support the lytic replication of KSHV when the virus is induced to lytic phase by either chemical induction or expressing virally encoded transcription activator ORF50 (Rta). The cells were cotransfected with the wild-type or mutant *ori-Lyt* plasmids and the ORF50 expression vector (pCR3.1-ORF50). ORF50 (Rta) induced the lytic cycle in these cells to provide KSHV lytic replication function in *trans*. At 72 h posttransfection, DNAs were isolated from the cotransfected cells and digested with KpnI/SacI and KpnI/SacI/DpnI. The replicated plasmid DNA can be distinguished from the input plasmid by DpnI restriction digestion, which cleaves input DNAs that have been methylated in dam-positive *E. coli* strains but leaves DNA intact that has been replicated in at least one round in eukaryotic cells. In other words, newly replicated DNA in BCBL-1 cells was resistant to DpnI digestion. The cleaved DNAs were separated on agarose gels and subjected to Southern hybridization with ^{32}P-labeled pBluescript plasmid probe. Then each deletion mutant was scored for its ability to be replicated in the viral lytic phase. As illustrated in Fig. 1, the majority of deletion mutants displayed reduced replication function in comparison with that of the wild-type *ori-Lyt* plasmid (pOri-A). But some mutants had greater effects on *ori-Lyt*-dependent DNA replication than others, such as ID6, ID7, ID8, ID9, ID15, and ID16, in which the *ori-Lyt* function was apparently completely abolished. To focus on the most critical elements in the *ori-Lyt* in our initial phase of the study, we decided to choose those mutants that retained less than 10% of wild-type *ori-Lyt* function for further analysis. This cutoff value let us continue to analyze the regions that were deleted in the mutants ID6, ID7, ID8, ID9, ID13, ID15, and ID16.

Unusually spaced C/EBP palindromic motifs are essential for the association of K8 to the *ori-Lyt* and DNA replication. The *ori-Lyt* functions were severely impaired or completely lost in mutants ID6, ID7, ID8, and ID9 (Fig. 1). Among the four mutants, ID9 had an 18-bp AT palindromic sequence deleted, and the AT palindrome has been shown to be very crucial for *ori-Lyt*-dependent DNA replication (11). The region covered by ID6, ID7, and ID8 lies between two AT palindromic motifs and is also critical for *ori-Lyt* function. Interestingly, this 240-bp region between nucleotides 23332 and 23572 falls within the region that has been shown to be required for association of the K8 protein with the *ori-Lyt* (11). We wondered whether the sequences that were deleted in ID6, ID7, and ID8 contain *cis*-acting requirements for K8 protein binding. Therefore, we examined the effects of these deletion mutants on K8 binding to *ori-Lyt* DNA. Biotinylated DNA fragments were synthesized by PCR with pOri-A DNA and serial deletion mutants (ID4, ID5, ID6, ID7, ID8, ID9, and ID10) as templates. The DNAs were coupled to streptavidin-conjugated magnetic beads and then incubated with nuclear extract of TPA-induced BCBL-1 cells. The bound materials were washed with 150 mM KCl-containing buffer and eluted with 300 mM KCl. Each eluted fraction was assayed by Western blotting with anti-K8 antibody. The result, as illustrated in Fig. 2, showed that K8 failed to bind to the *ori-Lyt* DNA with ID6, ID7, ID8, and ID9 deletions. Thus, the sequences that were deleted in these mutants (between nucleotides 23332 and 23599) are critical for K8 binding to the *ori-Lyt*, and the losses of *ori-Lyt* function in ID6, ID7, ID8, and ID9 were possibly attributed to failure of K8 protein binding to the *ori-Lyt* DNA.

Then we attempted to define the *cis*-acting elements in the sequences that were deleted in ID6, ID7, and ID8 for K8 binding and *ori-Lyt*-dependent DNA replication. Inspection of the 240-bp sequence revealed eight putative C/EBP binding sites. Interestingly, these eight C/EBP sites organize as four paired palindromic sequences. All four palindromes contain two head-to-head CCAAT motifs that are separated by a 13- or 12-bp space sequence (approximately equivalent to one full turn of a DNA helix). We designated these eight C/EBP binding sites as C/EBP-1 to C/EBP-8 (Fig. 3A). To investigate the contribution of these C/EBP palindromic motifs to *ori-Lyt*-dependent DNA replication and K8 binding to DNA, each CCAAT consensus sequence in an *ori-Lyt* plasmid was mutated to GTCAT or CGTCT. These mutants were introduced into BCBL-1 cells with an ORF50/Rta expression vector and tested for *ori-Lyt* function in a transient replication assay (Fig. 3B). We also examined the effects of these C/EBP point mutations on K8 association to the *ori-Lyt* by using an in vitro binding assay (Fig. 3C). The results showed that mutation of C/EBP-1, -2, and -6 completely abolished *ori-Lyt*-dependent DNA replication. The *ori-Lyt* function was partially or fully retained with mutations on the rest of the C/EBP motif. However, every double mutation, which altered two individual C/EBP in a palindrome, led to the loss of *ori-Lyt* function, suggesting that all four of the C/EBP palindromes are required for *ori-Lyt*-dependent DNA replication (Fig. 3B). The in vitro K8 binding assay showed that K8 protein can bind to the region of the *ori-Lyt* when one C/EBP palindrome is mutated. However, K8 binding to the DNA became very weak when two C/EBP palindromes were mutated (Fig. 3C). Recently, Hayward and his colleagues reported that two of these four C/EBP α palindromic structures, i.e., C/EBP-1 and -2 and C/EBP-5 and -6, were able to be bound by C/EBP α in vitro. Furthermore, they also showed that C/EBP α can physically interact

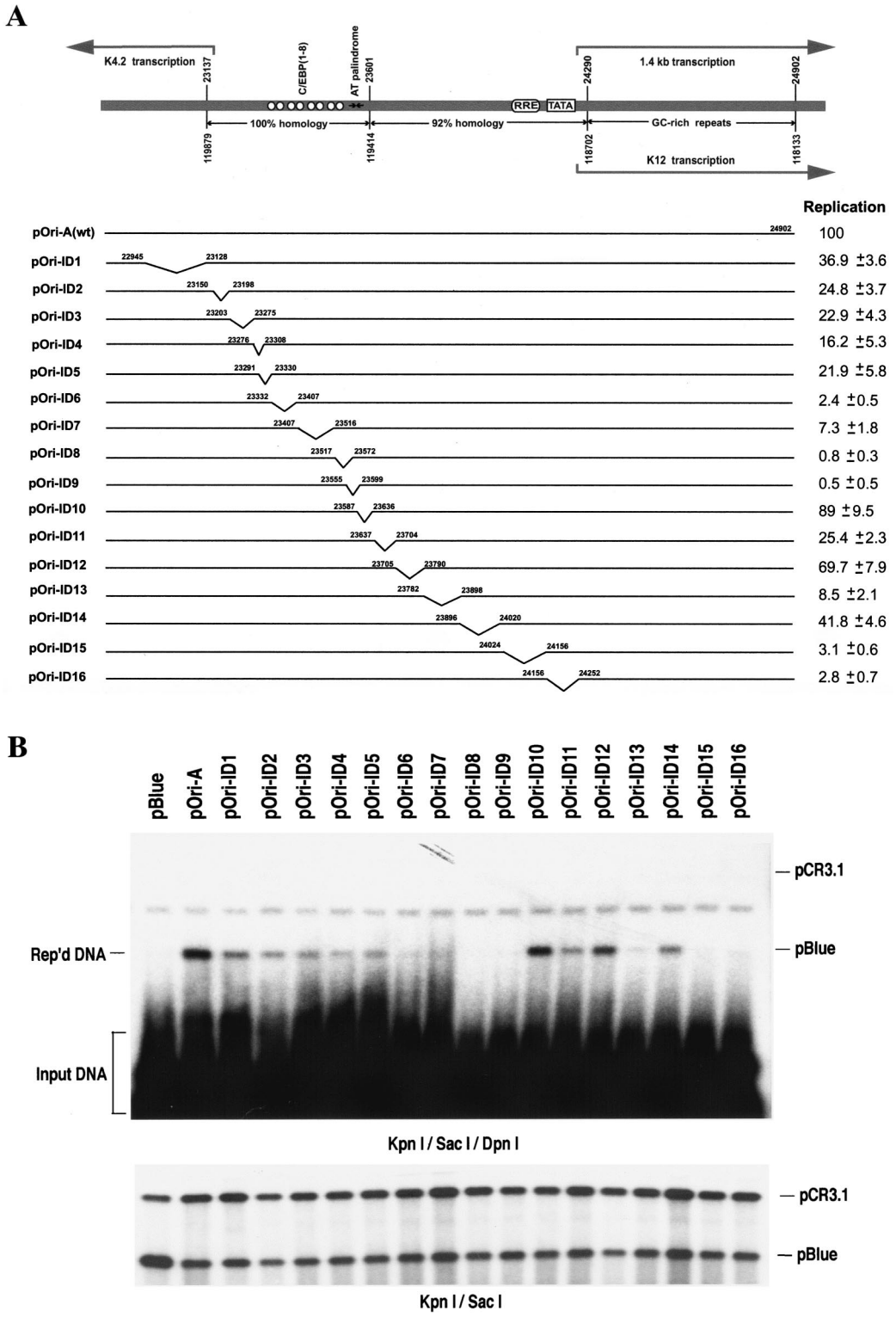

FIG. 1. Scanning mutation analysis of KSHV *ori-Lyt* for *cis*-acting elements. (A) Schematic diagram of two KSHV *ori-Lyt* and plasmid constructs of the wild-type (wt) origin and deletion mutants. The sequence elements in the *ori-Lyt* are illustrated by symbols including the CCAAT boxes, RRE, TATA box, and AT palindromic sequences. The homology between two duplicated *ori-Lyt* is indicated. The positions of wild-type *ori-Lyt* and each deletion are indicated with the nucleotide numbers of the KSHV genome (the numbers are according to the method of Russo et al. [17]). (B) The abilities of these constructs to mediate DNA replication were tested in BCBL-1 cells by a transient replication assay as described in Materials and Methods. KSHV lytic replication is induced by expression of ORF50 (Rta). Extrachromosomal DNAs were prepared by the Hirt extraction method and used for the assay. DpnI-resistant products of DNA replication were detected by Southern blotting with ^{32}P-labeled pBluescript plasmid. The replicated DNAs were quantitated by PhosphorImager scanning and normalized with their total plasmid DNAs (without DpnI digestion). The replication rate of each mutant relative to that of wild-type pOri-A was calculated by comparing the intensity of the replicated (Rep'd) DNA band with that of wild type pOri-A. Each number is the average of the results from two independent experiments.

with K8 protein (22). Taken together, the data from our laboratory and Hayward's laboratory suggest that the four C/EBP motifs are indeed the sites for K8 binding to *ori-Lyt* DNA and that the K8 binding may occur by interacting with the C/EBP α, which are bound to four C/EBP motifs. K8 can bind to *ori-Lyt* DNA only when all four of the C/EBP motifs have been bound by C/EBP α.

It is interesting that the binding of K8 protein to *ori-Lyt* DNA was also greatly impaired in the ID9 mutant (Fig. 2) in which the 18-bp AT palindrome was deleted but all eight C/EBP binding sites were intact. This result suggests that these C/EBP motifs are required but not sufficient for K8 binding to the *ori-Lyt* DNA. Other sequences including the 18-bp AT palindrome have influences on K8 association with the *ori-Lyt* DNA.

An AT palindrome sequence in *ori-Lyt* is required for lytic DNA replication. In agreement with previous data (11), two AT palindromes in the *ori-Lyt* contribute to the *ori-Lyt*-dependent DNA replication. The 18-bp AT palindrome is more critical for the *ori-Lyt* function because deletion of the palindromic sequence in *ori-Lyt* plasmid ID9 completely abolished *ori-Lyt*-dependent DNA replication (Fig. 1). To further define the role of the 18-bp AT palindrome in *ori-Lyt* function, three nucleotide substitution mutations were generated by introducing G-C pairs into the AT palindromic sequence. When four G-C pairs were introduced into the first half of palindrome (pOri-M9), the *ori-Lyt* replication was completely lost. However, when the second half of the AT palindrome was interrupted by G-C pairs, the mutant *ori-Lyt* (pOri-M10) was still able to support lytic DNA replication (Fig. 4). No DNA replication was detected in a combined mutant of M9 and M10 (pOri-M9M10). This result confirmed the importance of the AT palindromic sequence in *ori-Lyt* function and also suggests that the AT palindrome can be shortened to the first 10 bp, in which the *ori-Lyt* function still remains.

An RRE and a TATA box are essential for *ori-Lyt* function. Mutant plasmids pOri-ID13, ID15, and ID16 identified two discontiguous regions that appear to be absolutely required for DNA replication (Fig. 1). These regions were further analyzed by using smaller deletions to identify *cis*-acting elements required for *ori-Lyt* function in these regions. Four smaller deletion mutants were constructed to scan the 108-bp region of pOri-ID13. These four deletion plasmids were introduced into BCBL-1 cells for transient transfection and replication assay (Fig. 5). Among these four mutants, pOri-ID13.2 had the greatest effect on DNA replication and resulted in an 85% loss of DNA replication activity. Thus, this study narrowed down the DNA sequence required for *ori-Lyt* function to a 32-bp region (5′-CTACCCCCAACTGTATTCAACCCTCCTTTGTTT-3′). Six smaller deletion mutants were designed to scan the region between nucleotides 24025 to 24164 that was shown to be critical for DNA replication by mutant pOri-ID15. These six deletion plasmids were introduced into BCBL-1 cells for transient replication assay (Fig. 5). The *ori-Lyt* function was greatly impaired in mutants pOri-ID15.3, ID-15.4, ID-15.5, and ID-15.6, suggesting that the sequence that was missing in these mutants harbors component(s) important for *ori-Lyt* function. Inspection of the sequences that were deleted in these four mutants revealed that a putative RRE, which is identical to the RRE in the K12 promoter and similar to the RRE in poly(A)

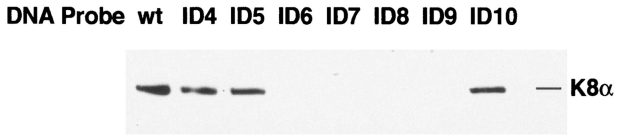

FIG. 2. Effects of deletion mutations on binding of K8 protein to KSHV *ori-Lyt*. Biotinylated DNA fragments were prepared by PCR with pOri-A (wild type [wt]) DNA template and scanning mutations as described in Materials and Methods. TPA-induced BCBL-1 nuclear extract was incubated with the DNA fragments conjugated on magnetic beads, washed, and eluted with D300 elution buffer. Samples were assayed by Western blotting with anti-K8 antibody (K8α).

nuclear RNA (PAN) or PAN RNA promoter (6, 20), lies in the sequence. The transient DNA replication assay showed that the putative RRE is essential for the *ori-Lyt* function, and partial or entire deletion of the RRE severely impaired *ori-Lyt*-dependent DNA replication. However, the 32-bp sequence downstream of the RRE consensus sequence also appears to be required for efficient DNA replication, as shown with the mutant ID15.6.

To confirm that the RRE is bound by the ORF50/Rta protein and that ORF50/Rta binding is required for *ori-Lyt*-dependent DNA replication, we mutated the putative RRE by replacing 22-bp RRE consensus sequence (5′-AACATGGGTGGCTAACGCCTAC-3′) with a yeast Gal4 binding motif (5′-GATCCGGAGGACTGTCCTCCGG-3′). The mutant, designated pOri-Gal4, was introduced into BCBL-1 cells with pCR3.1-ORF50 for a transient DNA replication assay. As shown in Fig. 6C, *ori-Lyt* function was greatly impaired in the pOri-Gal4 plasmid, again confirming a critical role of the RRE in *ori-Lyt*-dependent DNA replication. When a construct of the Gal4-ORF50 fusion protein (SG-C50), in which the activation domain of ORF50/Rta (205 carboxy-terminal amino acids of ORF50) is fused with the DNA binding domain of yeast protein Gal4, was included in the cotransfection, the DNA replication was found to be totally restored (Fig. 6C). It was shown previously that SG-C50 retained the full transcription function of ORF50 when targeted to DNA as a Gal4 fusion (13). As a control, pSG424, which expresses Gal4 alone, was not able to restore the DNA replication (Fig. 6C). Our result suggested that binding of ORF50/Rta to the *ori-Lyt* is necessary and critical for *ori-Lyt*-dependent DNA replication.

Since the 32-bp sequence downstream of the consensus RRE was also found to contribute to *ori-Lyt*-dependent DNA replication, we sought to determine, if the 32-bp sequence is deleted, whether an ORF50 or a fusion protein binding to the *ori-Lyt* is able to support the DNA replication. We replaced the RRE and the following 32-bp sequence (54 bp in total) with a Gal4 binding motif (designated pOri-Gal4Δ6) and examined it in a transient DNA replication assay in the presence and absence of SG-C50 fusion protein. The results showed that the SG-C50 fusion protein could not restore the DNA replication of pOri-Gal4Δ6 (Fig. 6D). However, DNA replication was restored when two copies of Gal4 binding motifs were used to replace the 54-bp sequence (designated pOri-2XGal4) and the SG-C50 fusion protein was included in the replication assay (Fig. 6D). This result suggested that the 32-bp sequence may serve as another RRE or a binding motif for another transac-

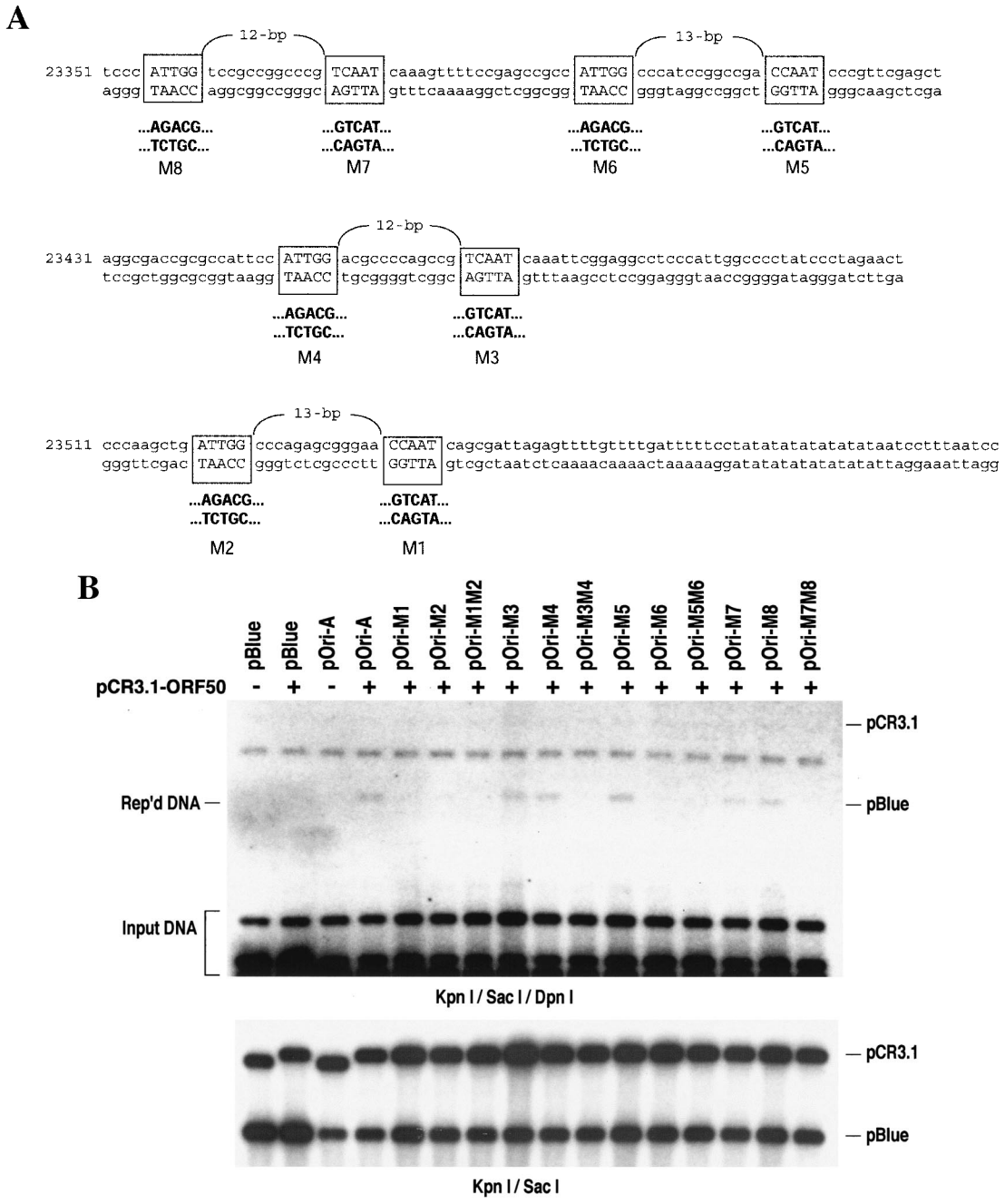

FIG. 3. Identification of four unusually spaced C/EBP palindromic motifs in KSHV *ori-Lyt* that are critically required for DNA replication. (A) Schematic diagram of the sequences and locations of eight C/EBP binding sites that are organized as four spaced palindromes as well as site-specific mutations on these C/EBP motifs. (B) Effects of the mutations of these C/EBP binding sites on *ori-Lyt*-dependent DNA replication. A transient replication assay was performed on these mutants in BCBL-1 cells, and DpnI-resistant products of DNA replication were detected by Southern blotting. The replication rate of each mutant relative to that of pOri-A was calculated by measuring the intensities of the replicated (Rep'd) DNA bands normalized with total plasmid DNA (without DpnI digestion). The experiment was repeated three times with similar results. +, present; −, absent. (C) A K8 binding assay was performed with biotinylated DNA fragments with point mutations of C/EBP binding sites (Table 1 and Fig. 3A). Samples were assayed by Western blotting with anti-K8 antibody (K8α). C, control with an irrelevant DNA; Wt, wild type.

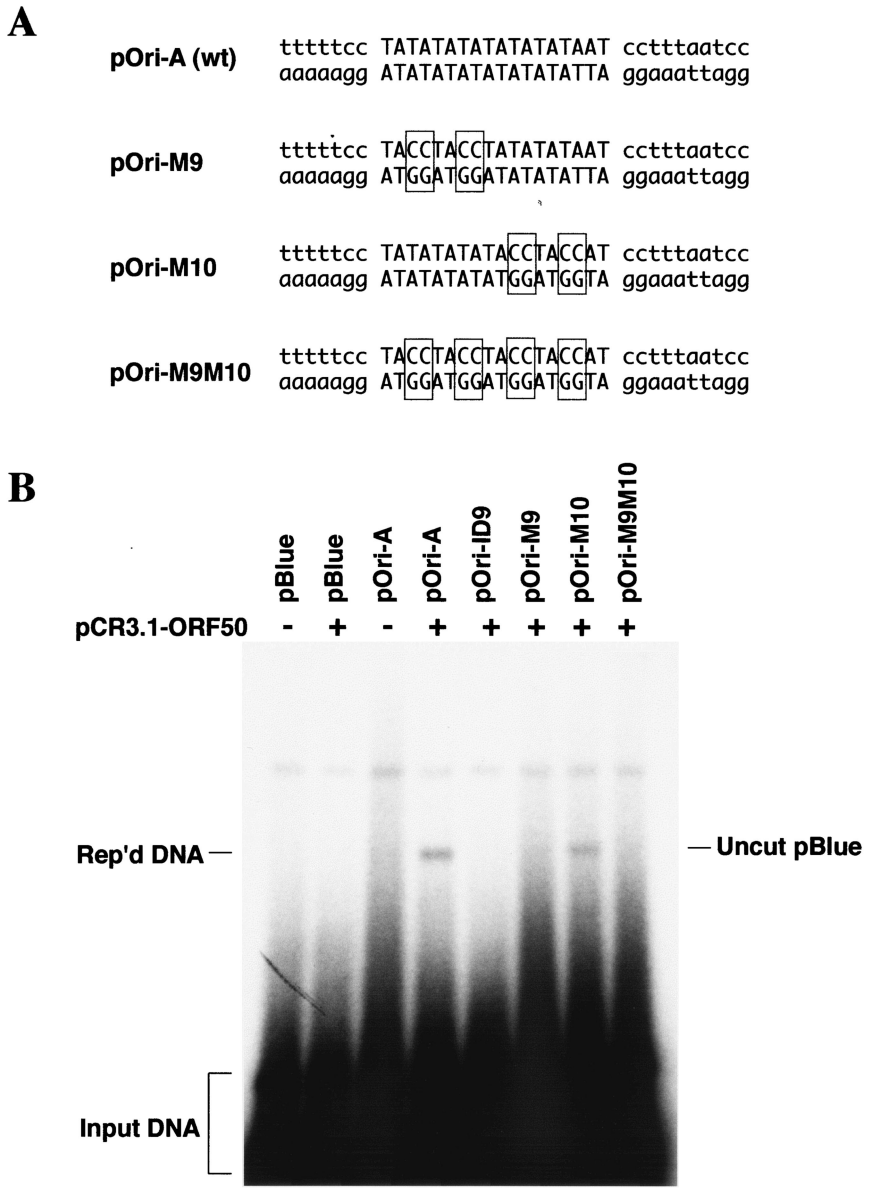

FIG. 4. The 18-bp AT palindrome is essential for KSHV *ori-Lyt* replication. (A) Nucleotide sequence of the wild-type (wt) 18-bp AT palindrome in the *ori-Lyt* and its mutants. (B) The wild-type and mutant plasmids were examined in BCBL-1 cells by a transient replication assay for their abilities to support lytic-phase DNA replication. KSHV lytic replication is induced by expression of ORF50 (Rta). Extrachromosomal DNAs were prepared by the Hirt extraction method and used for the assay. Replicated DNAs were distinguished from input DNAs by DpnI digestion and detected by Southern blotting with ^{32}P-labeled pBluescript plasmid. The replication rate of each mutant relative to that of pOri-A was calculated by measuring the intensities of the replicated (Rep'd) and input DNA bands. +, present; −, absent.

tivator and that KSHV DNA replication needs an ORF50/Rta dimer, either homodimer or heterodimer, binding to the 54-bp bipartite RRE sequence.

Similarly, three smaller deletion mutants were designed to scan the region between nucleotides 24165 and 24252 that was identified by mutant pOri-ID16. A transient DNA replication assay with these three mutants showed that removal of the 25-bp sequence from nucleotide 24190 to 24224 (pOri-ID16.2) resulted in the complete loss of *ori-Lyt* function. The most remarkable sequence in the 25-bp region is a TATA consensus motif. It is likely that this TATA box is indispensable for *ori-Lyt* function.

Identification of transcription promoter within KSHV *ori-Lyt* and its transcript. The presence of an RRE and a downstream TATA box within the *ori-Lyt* suggests that there is an ORF50/Rta-dependent promoter. We can further speculate that a transcription event may be necessary for *ori-Lyt*-dependent DNA replication. To examine whether the RRE and TATA box indeed constitute an ORF50/Rta-dependent promoter, we made reporter constructs in which various *ori-Lyt*

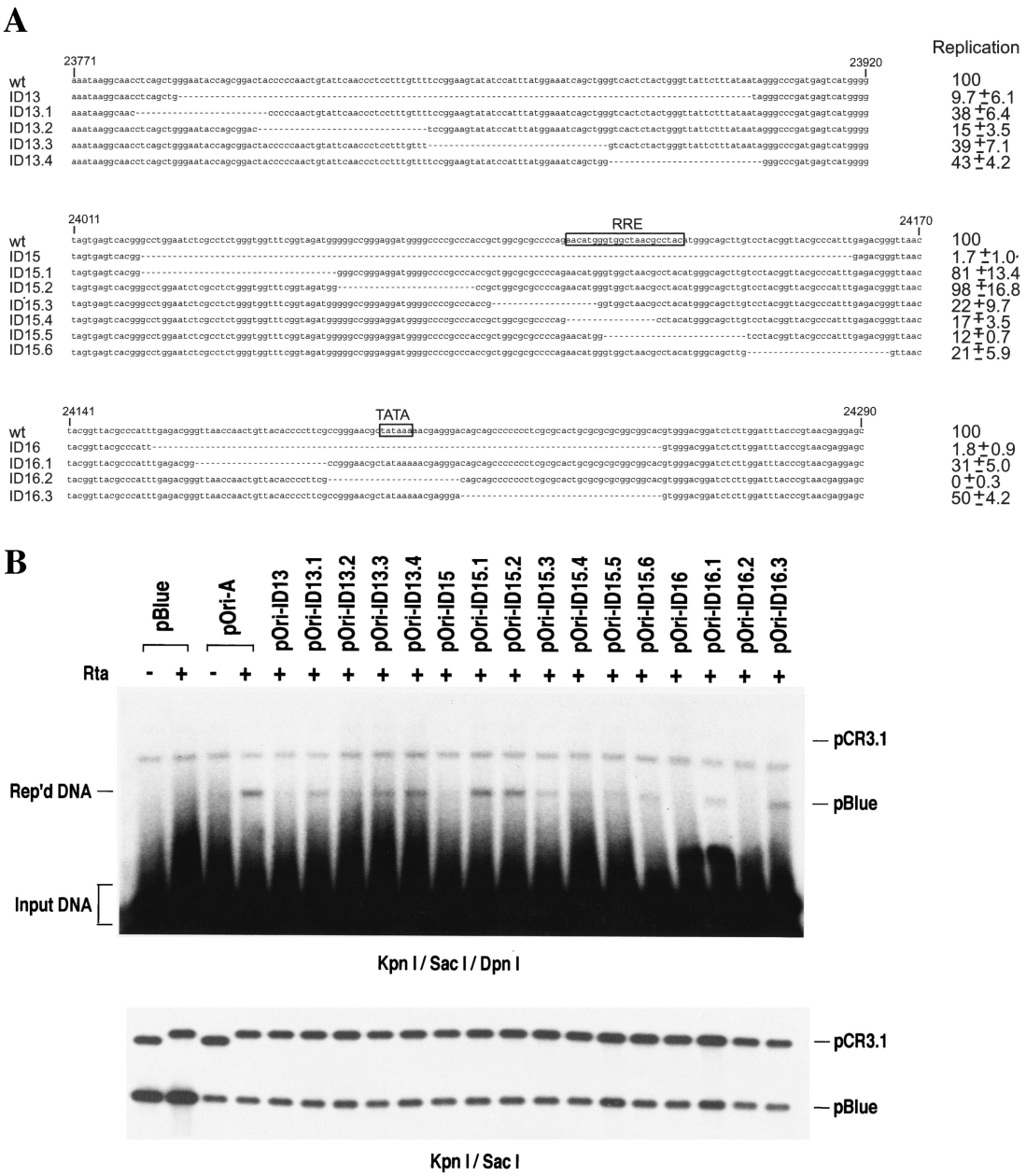

FIG. 5. Mutations in two discontiguous essential regions. (A) A series of small deletion mutants were constructed across the regions that were identified by ID13 and ID15-ID16. wt, wild type. (B) Each mutant plasmid was assayed in BCBL-1 cells for its ability to support lytic-phase DNA replication. KSHV lytic replication is induced by expression of ORF50 (Rta). Extrachromosomal DNAs were prepared by the Hirt extraction method and used for the assay. Replicated (Rep'd) DNAs were distinguished from input DNAs by DpnI digestion and detected by Southern blotting with ^{32}P-labeled pBluescript plasmid. The replicated DNAs were quantitated by PhosphorImager scanning and normalized with their total plasmid DNAs (without DpnI digestion). The replication rate of each mutant relative to that of wild-type pOri-A was calculated by comparing the intensity of the replicated DNA band with that of wild-type pOri-A. Each number is the average of the results from two independent experiments.

DNA sequences that contain the RRE, TATA box, and 100-bp downstream sequence were cloned into the pGL3-basic vector right in front of the firefly luciferase coding sequence (Fig. 7A). These constructs were cotransfected into KSHV-negative BJAB and KSHV-positive BCBL-1 cells with pCR3.1-ORF50 or empty pCR3.1 plasmids for ORF50-dependent promoter activities. The results showed that in the presence of ORF50/Rta, the transcription activities of 4F, which contained an *ori-Lyt* sequence between nucleotides 23331 and 24342, were 236-fold higher than that of empty vector pGL3 in BJAB cells (Fig. 7B). Similarly, in BCBL-1 cells, the transcription activities of 4F were 2,056-fold higher than that of pGL3 (Fig. 7C). The promoter activities of the constructs were ORF50-inducible because its transcription activities were elevated up to 20- and 12-fold by ORF50/Rta in BJAB and BCBL-1 cells, respectively (Fig. 7). Progressive deletion of the *ori-Lyt* sequence from the

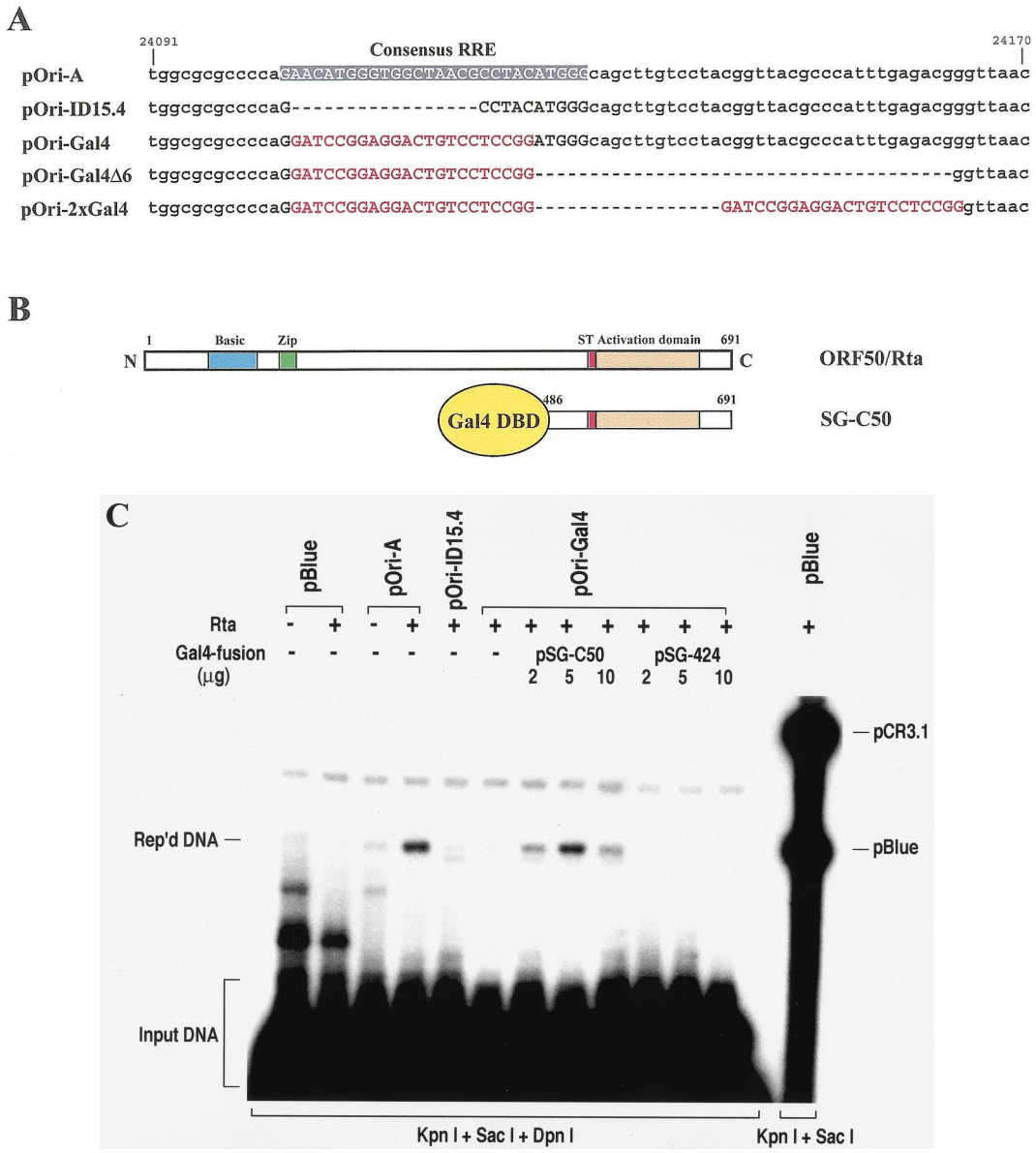

FIG. 6. Restoration of *ori-Lyt* function by Gal4-ORF50 fusion protein—evidence that association of ORF50/Rta with KSHV *ori-Lyt* is required for DNA replication. (A) Sequence of the consensus RRE in KSHV *ori-Lyt* and the mutants in which the consensus RRE has been deleted or replaced with one or two copies of yeast Gal4 binding motif (in red). (B) Schematic illustration of ORF50 structure and domains and a fusion protein in which the ORF50/Rta activation domain fused N-terminally to the DNA binding domain of yeast Gal4 protein (adapted from Lukac et al. [13]). (C) Wild-type pOri-A and pOri-Gal4 were introduced into BCBL-1 cells in the absence (−) and presence (+) of Gal4 fusion proteins (SG-C50 and SG424) in various amounts (2, 5, and 10 μg) and ORF50 expression vectors. The pOri-Gal4 plasmid was assayed for its ability to support lytic-phase DNA replication. (D) Wild-type pOri-A, pOri-Gal4Δ6, and pOri-2xGal4 were introduced into BCBL-1 cells. DNA replication in the absence and presence of the SG-C50 fusion protein and ORF50/Rta was analyzed by a DNA replication assay. Replicated (Rep'd) DNAs were distinguished from input DNAs by DpnI digestion and detected by Southern blotting with ^{32}P-labeled pBluescript plasmid.

5′ end up to nucleotide 24093 (10F, 12F, and 13F) had a trivial effect on the promoter activity and the responsiveness to ORF50/Rta, suggesting that the sequence upstream of the RRE (including the K8 binding region) is not involved in the transcription activity. To further characterize the *ori-Lyt*-associated promoter, several mutants were constructed based on 13F as illustrated in Fig. 7A. When the TATA box was deleted from 13F plasmid (13FΔTATA), the majority of transcription activity was lost in both BJAB and BCBL-1 cells (Fig. 7B and C). Deletion of the 17-bp RRE consensus sequence (AACAT GGGTGGCTAACG) (13FΔRRE) resulted in a reduction of luciferase activity by 57% in BJAB cells and 71% in BCBL-1 cells, but the mutant still displayed considerable responsiveness to ORF50-Rta induction (5-fold induction in BJAB cells, 11-fold induction in BCBL cells). However, removal of the RRE and its following 32-bp sequence (13FΔ7) brought about greater reduction in both luciferase activities (93% in both BL41 and BCBL-1 cells) and ORF50 responsiveness. The data

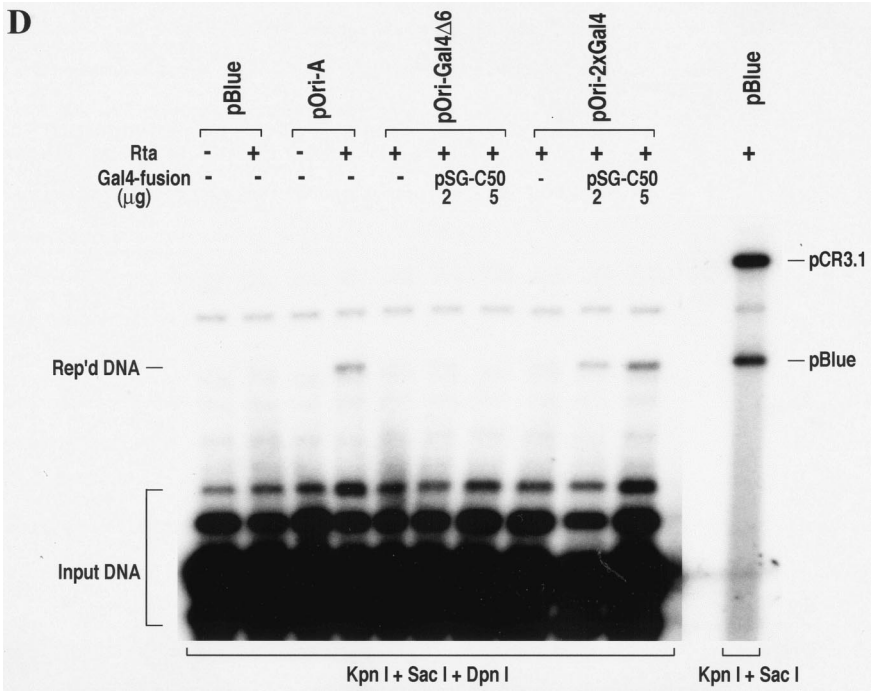

FIG. 6—*Continued*

strongly suggest that the RRE is not restricted in the 22-bp consensus sequence but extends to the whole 54-bp sequence. Replacement of the whole RRE (54 bp) with one or two copies of yeast Gal4 binding sequence (GATCCGGAGGACTGTCC TCCGG) caused 90 to 96% loss in both BJAB and BCBL-1 cells of ORF50-dependent transcription activities. When a Gal4-ORF50 fusion protein (SG-C50) was included in a co-transfection, the transcription activity of 13F-Gal4 was induced 51-fold in BJAB cells and 120-fold in BCBL-1 cells in the absence of ORF50/Rta. However, the presence of ORF50 brought about additional three- and fivefold increases in transcription activities in BCBL-1 and BJAB cells, respectively (Fig. 7). In addition, SG-C50 caused a greater induction of luciferase activity of the construct in which the RRE has been replaced with two copies of Gal4 binding motif (13F-2xGal4). In the absence of ORF50, SG-C50 induced the transcription activities of the 13F-2xGal4 construct 2,298-fold in BJAB cells and 2,566-fold in BCBL-1 cells. Obviously, two SG-C50 molecules synergistically stimulated transcription of the promoter. In summary, the data from the luciferase assays demonstrated that the region, which was identified to be critical for *ori-Lyt* function by mutants ID-15 and ID-16, serves as an ORF50/Rta-dependent promoter and may direct an *ori-Lyt*-associated transcription. It was also suggested that there are two RRE motifs in the 54-bp region which are bound by two ORF50/Rta molecules or an ORF50 dimer. Taken together with the transient replication assays with *ori-Lyt* mutants and Gal4-ORF50 fusion proteins, our data indicate that this ORF50-dependent promoter is critical for lytic DNA replication.

To examine whether there is a transcription event occurring downstream of the ORF50-dependent promoter, we carried out a Northern analysis of mRNAs isolated from virally latent and reactivated BC-1 cells. The Northern blot was hybridized with a single-stranded DNA probe from nucleotide 25600 to 24250. As shown in Fig. 8A, the Northern analysis identified a rightward RNA transcript of around 1.4 kb which is absent in the latent phase but induced in the viral lytic life cycle. Interestingly, the transcription of the RNA was inhibited by phosphonoacetic acid (PAA), suggesting the transcription is coupled with viral lytic DNA replication. In contrast, the transcription of the K8 gene was not affected by PAA (Fig. 8B).

The full-length cDNAs for the 1.4-kb *ori-Lyt*-associated RNA were obtained by a PCR-based cDNA amplification procedure and then cloned and sequenced. The 5′ and 3′ ends of the RNA were determined by using a RACE approach. The results revealed that the 5′ end of the RNA is at or near nucleotide 24243, 30 bp downstream of the TATA box. The RNA terminates at nucleotide 25442, 16 nucleotides downstream of a consensus AAUAAA polyadenylation signal, and is followed by a poly(A) tail (Fig. 9). The sequence of the RNA consists of the whole GC-rich direct repeat region and is followed by an ORF of 75 amino acids (Fig. 9).

DISCUSSION

Multipartite and complex structure of KSHV *ori-Lyt*. The goal of this study is to dissect the KSHV *ori-Lyt* and determine the *cis*-acting components that are essential for *ori-Lyt* function. Initially, 16 deletion mutants were engineered across the 1.1-kb core domain of a KSHV *ori-Lyt* and tested for the origin function. This assay led to the identification of four regions that are absolutely required for *ori-Lyt*-dependent DNA replication. Then these regions were further analyzed by using smaller deletions, and some notable motifs within these regions were further examined by substitution mutations. This study allows us to identify *cis*-acting elements of the origin and

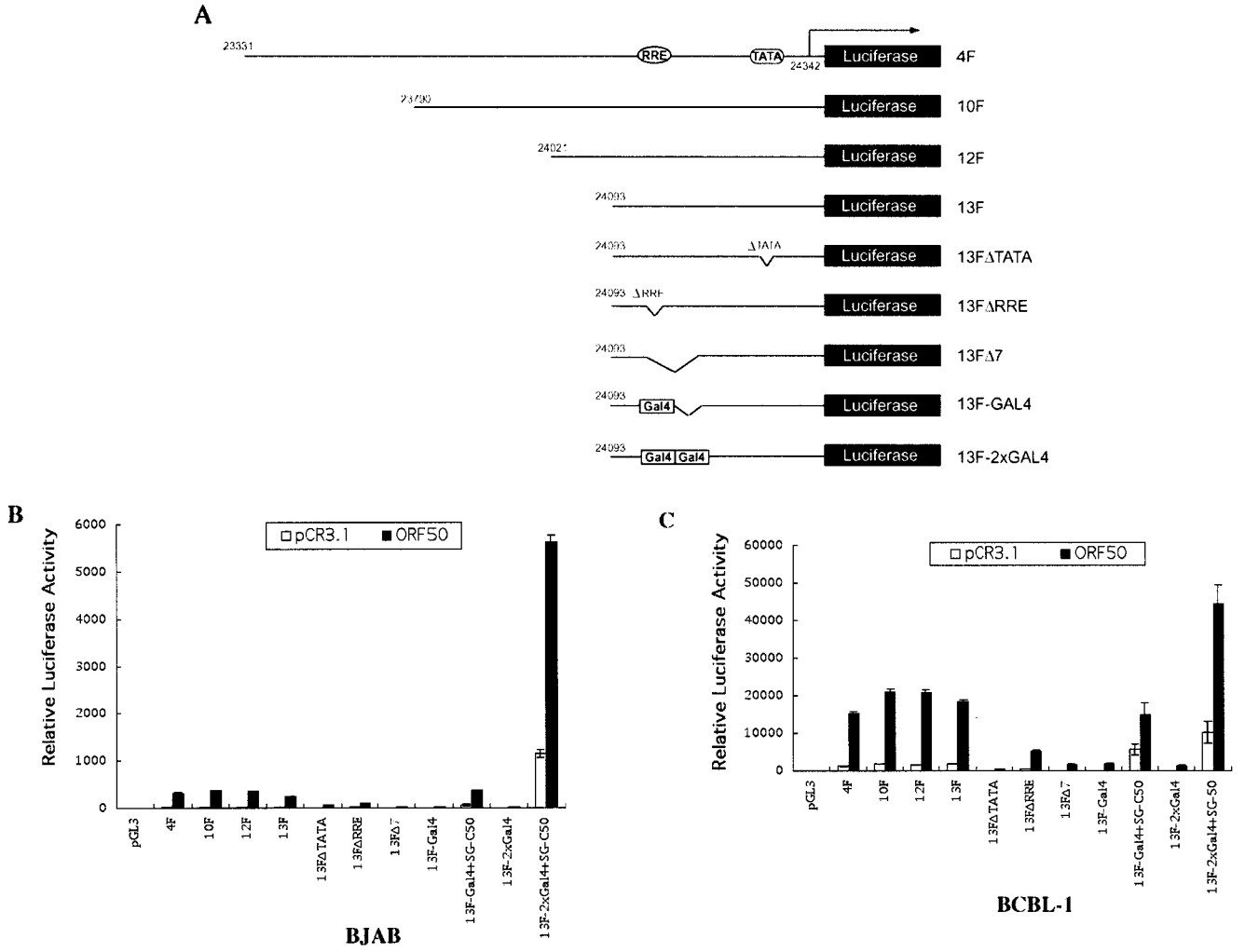

FIG. 7. Transcription activation of putative *ori-Lyt*-associated promoter by ORF50/Rta. (A) Schematic illustration of reporter plasmids containing the firefly luciferase gene under the control of the wild-type *ori-Lyt* sequence or its mutants where the RRE and TATA box have been deleted or mutated. These reporter plasmids were used to cotransfect BJAB or BCBL-1 cells with pCR3.1-ORF50 or empty pCR3.1 vector by electroporation. *Renilla* luciferase plasmid was included in each transfection as an internal control. At 48 h posttransfection, dual luciferase assays were performed with the cell lysates of transfected BJAB cells (B) and BCBL-1 cells (C). Relative luciferase activities were calculated by dividing the normalized firefly luciferase activity of each reporter by that of the pGL3 plasmid in pCR3.1-transfected cells.

to assign functions to some of the elements. The most critical *cis*-acting elements are as follows: (i) eight C/EBP binding motifs, arranged as four spaced palindromes, that appear to be associated with K8 protein through interacting with C/EBP α; (ii) an 18-bp AT palindromic sequence that is believed to involve local unwinding of double-stranded DNA during the initiation of replication; (iii) a 32-bp sequence that had no previously assigned function; and (iv) an ORF50/Rta-dependent promoter which is composed of an RRE closely resembling the RRE in the K12 and PAN RNA promoters and a TATA box.

In addition to the mutants that had any of the four crucial *cis*-acting elements deleted, many other deletions also displayed reduced *ori-Lyt* replication activity (Fig. 1). The regions, represented by the deletion mutants that produced significantly reduced (but not abolished) *ori-Lyt* activities, determine the relative efficiency of *ori-Lyt* function. These DNA sequences may either have auxiliary functions for *ori-Lyt*-dependent DNA replication or simply provide space requirements between two components of the *ori-Lyt*. The nature of our scanning deletion mutants could not distinguish these two possibilities. Thus, to focus our study on the most critical *cis*-acting elements, we decided to set up a cutoff value in which only those mutants that retained 10% *ori-Lyt* function or less would be further analyzed in detail. The other regions that only had moderate effects on *ori-Lyt* function were left for future studies.

Association of K8 protein with *ori-Lyt* through C/EBP binding motifs. A virally encoded bZip protein, namely K8, was found to bind to the KSHV *ori-Lyt*. Further analysis showed that a stable K8 binding to DNA requires an extended DNA sequence between nucleotides 23128 and 23635 (11). The region that is required for K8 binding to *ori-Lyt* DNA is 100% conserved between two KSHV *ori-Lyt*. K8 protein displays significant similarity to the Zta protein of EBV, which is known to be an OBP of EBV (8). Therefore, it is suggested that K8 may function as an OBP of KSHV.

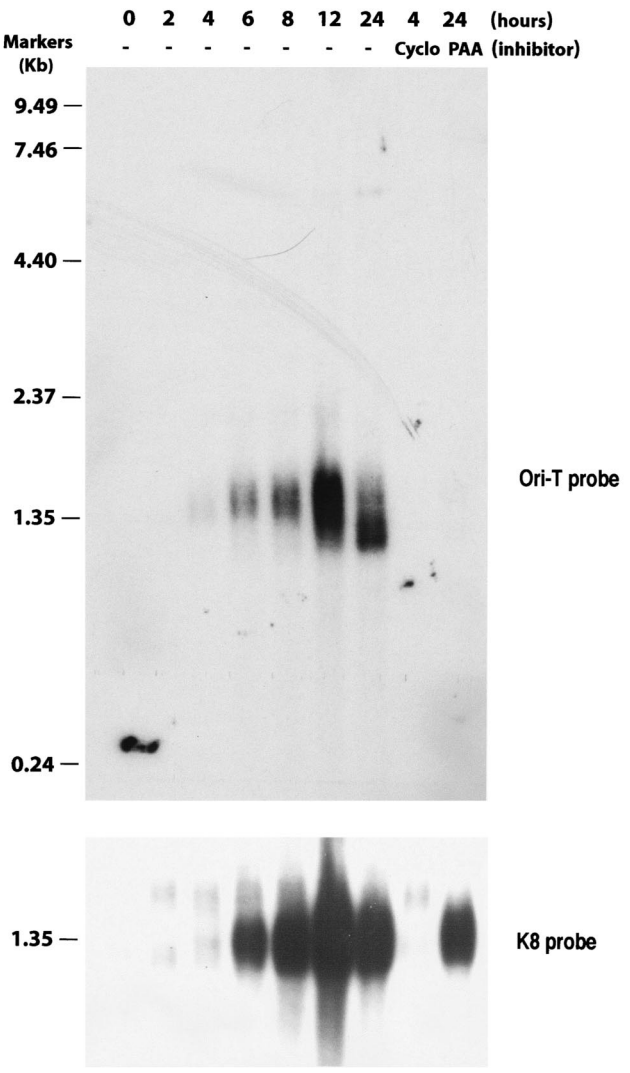

FIG. 8. Northern analysis of transcripts downstream of the *ori-Lyt*-associated promoter. Poly(A^+) RNAs were isolated from BC-1 cells that had been treated with sodium butyrate for various times in the absence (−) or presence (+) of cycloheximide (cyclo) and PAA as indicated above each lane. These RNAs were separated on a 1.0% agarose–formaldehyde gel and transferred onto Nytran membranes. The membranes were probed with ^{32}P-labeled single-stranded DNA probes that were complementary to the sequence from nucleotide 25428 to 24250 (5′ to 3′) (A) and K8 mRNA (B). Molecular marker, 0.24- to 9.5-kb RNA ladder.

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24243   gcggcggcacgtgggacggatctcttggatttacccgtaacgaggagccccggcagca
24301 ccccaggagccccggcagcaccccaggagccccggcagcaccccaggagccccggcagca
24361 ccccaggagccccggcagcaccccaggagccccggcagcaccccaggagccccggcagca
24421 ccccaggagccccggcagcaccccaggagccccggcagcaccccaggagccccggcagca
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                                                              M
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25261 ttcaaagagggcacagaggattcacattgtttagagagagtttttcttagtcaccattcc
      F  K  E  G  T  E  D  S  H  C  L  E  R  V  F  L  S  H  H  S
25321 atacttgggcagtattggcctacgatttgggcgacgtttcaggctggtctattctccgtc
      I  L  G  Q  Y  W  P  T  I  W  A  T  F  Q  A  G  L  F  S  V
25381 cacttttccccggctattctgtcccagcataggctcttgaaataaacaatgtttaccgag
      H  F  S  P  A  I  L  S  Q  H  R  L  L  K  *
25441 taaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

FIG. 9. Nucleotide sequence of the *ori-Lyt*-associated transcript and its predicted ORF. The amino acid sequence deduced from the cDNA sequence is depicted beneath each line of nucleotide sequence.

The most striking structural feature in the 500-bp sequence that K8 binds is eight C/EBP consensus motifs that are arrayed as four spaced palindromic pairs. The mutagenesis studies showed that three of these C/EBP sites (C/EBP-1, -2, and -6) are essential for *ori-Lyt* function and that mutations in any of these three C/EBP motifs completely abolished *ori-Lyt*-dependent DNA replication. In addition, every C/EBP palindrome is indispensable for *ori-Lyt* function. In parallel, deletion or mutation of these C/EBP motifs also impaired the binding of K8 to the *ori-Lyt*, suggesting that K8 may bind to the *ori-Lyt* through these C/EBP motifs. However, in spite of great efforts, we were unable to demonstrate any direct binding of K8 protein to the region that harbors these C/EBP motifs by electrophoretic mobility shift assay (11). Recently, Wu et al. (22) showed that C/EBP α can bind to four of these C/EBP sites (C/EBP-1, -2, -5, and -6). Furthermore, they also demonstrated that K8 protein specifically associated with C/EBP α. Taken together, these data implied that K8 binds to KSHV *ori-Lyt* through interacting with DNA-bound C/EBP α. We do not know whether K8 binds to DNA through C/EBP α in a piggybacking fashion or whether K8 can directly bind DNA while C/EBP α stabilizes the binding. But it is clear that both K8 and C/EBP α are important components for KSHV *ori-Lyt*-dependent DNA replication.

In EBV, seven Zta-responsive elements (ZREs) were found in its *ori-Lyt* domain. Among them, four ZREs (designated ZRE1 to ZRE4) are necessary and sufficient to activate lytic replication. The other three (ZRE5 to ZRE7) have no or only very marginal influence on the replication efficiency (18). Similarly, there are eight C/EBP motifs in KSHV *ori-Lyt* that are the sites for K8 and C/EBP α binding. They were also shown to be essential for *ori-Lyt*-dependent DNA replication. Although Zta of EBV can bind to its ZREs efficiently by itself while K8 binds to C/EBP sites in the aid of C/EBP α molecules, once they bind to *ori-Lyt*, they are likely to exercise the same function for the activation of *ori-Lyt*. In both viruses, the biochemical basis for the activation of *ori-Lyt* by Zta or K8 has not been elucidated yet. It was hypothesized that binding of Zta to four sites within the EBV *ori-Lyt* may cause linking and distortion of the origin which could be a prerequisite for *ori-Lyt*-dependent DNA replication (19). We think that K8 may play the same

role as Zta does in *ori-Lyt* activation. We further hypothesize that four K8 molecules or a K8 tetramer binds four C/EBP sites by interacting with C/EBP α proteins and causing a structure change in the *ori-Lyt* domain. As a result, DNA replication compartments form in the altered structure of the *ori-Lyt* and DNA replication begins.

Role of AT palindromes in *ori-Lyt*-dependent DNA replication. There are two perfect AT palindromic sequences in a KSHV *ori-Lyt*, i.e., a 16-bp AT palindrome and an 18-bp AT palindrome. Although both of them were found to contribute to maximal DNA replication of the *ori-Lyt*, the 18-bp AT palindrome is more critical for the *ori-Lyt* function because deletion or mutation of the AT palindrome abolished the *ori-Lyt* function completely (Fig. 1 and 4). An AT-rich palindrome is a common feature of both cellular and viral DNA replication origins (5, 12). It is believed that an AT-rich palindrome facilitates DNA unwinding and enhances helicase activity during replication. Our mutagenesis study showed that introduction of G-C pairs into the first half of the 18-bp AT palindrome completely abolished *ori-Lyt*-dependent DNA replication while mutations in the second half of the palindrome had little effect on the *ori-Lyt* function. There are two implications in this result: (i) the AT palindrome can be shortened to 10 bp and its function remains and (ii) besides the possible function as a DNA unwinding region, the AT palindrome may have an additional function such as serving as a protein binding site. The second implication came from three considerations as follows. (i) The different effects of the mutations in the first and second halves of the palindrome suggested that facilitating DNA unwinding may not be the only function of the AT-rich sequence. (ii) Our result showed that the 18-bp AT palindrome had an influence on K8 binding to the *ori-Lyt* (Fig. 2C). (iii) In the adenovirus origin, an AT-rich sequence is the DNA binding site for proteins involved in the initiation of DNA replication (7).

RRE in the origin and *ori-Lyt*-associated RNA transcription. The mutagenesis study revealed the requirement of an RRE and a downstream TATA box for lytic DNA replication. Using a Gal4-ORF50 fusion protein and pOri-Gal4 mutants, where the RRE consensus sequence has been replaced by a Gal4 binding motif, we provided evidence that binding of ORF50/Rta to the RRE in the *ori-Lyt* is necessary and essential for DNA replication. It was also shown by using a luciferase reporter system that the RRE and the TATA box constitute a promoter for a transcription in the *ori-Lyt* [*ori-Lyt* (L)]. A similar sequence in the other *ori-Lyt* [i.e., *ori-Lyt* (R)] was known to serve as a promoter for K12 (6). In addition, the luciferase reporter assay and transient replication assay consistently suggested that an ORF50/Rta dimer, either homodimer or heterodimer, may bind an extended RRE region (54 bp). Binding of two ORF50 or fusion proteins to the bipartite element synergistically stimulates transcription of the promoter. Also, binding of two ORF50 or fusion proteins to the extended RRE region is required for *ori-Lyt*-dependent DNA replication.

The *ori-Lyt*-associated transcript was identified as a 1.4-kb rightward polyadenylated RNA by Northern analysis. The full-length cDNAs of the RNA were generated by using a PCR-based cDNA amplification strategy. The most striking feature of this RNA is the GC-rich tandem repeat sequences in its 5′ half of the molecule. An ORF of 75 amino acids is seen in the 3′ approximate sequence of the RNA. We do not know whether the ORF in the 1.4-kb RNA is translated or not.

A promoter is present within the EBV *ori-Lyt* and is essential for lytic DNA replication. When the promoter was deleted, the *ori-Lyt* function was lost. However, when the promoter is replaced with a human cytomegalovirus immediate-early promoter, the EBV *ori-Lyt* function was restored (10). The EBV *ori-Lyt* promoter controls the transcription of BHLF1 RNA, which encompasses the entire GC-rich tandem repeat region. The function of the transcript is unknown. Overall, the KSHV *ori-Lyt* promoter and related transcript share some similarity with those found in the EBV *ori-Lyt*. The roles of these promoters in KSHV and EBV *ori-Lyt*-dependent DNA replication need to be explored.

The importance of the RRE and TATA box for the *ori-Lyt* function strongly suggested that a transcription event is necessary for *ori-Lyt*-dependent DNA replication. However, it should be noted that transient assays may not accurately reflect regulation in the context of the viral genome, and the role of the RRE in that context remains to be established. In addition, it is interesting that the *ori-Lyt*-associated transcription is inhibited in the presence of viral DNA polymerase inhibitor PAA (Fig. 8). Since PAA acts on viral DNA polymerase and does not inhibit ORF50/Rta-dependent transcription of viral delayed-early genes such as K8 (as shown in Fig. 8), the role of ORF50/Rta in transcription and DNA replication appears to be more sophisticated than simply serving as a transcription activator. It appears that there is a reciprocal interaction between DNA replication and transcription, and ORF50/Rta is directly involved in both events. Besides being a transcription activator, ORF50/Rta may also participate in directing the assembly of replication complexes that remodel the chromosome context and/or in recruiting specific proteins required for initiation of DNA synthesis, which could be an antecedent event to activation of transcription in the viral genome context.

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