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RAPID COMMUNICATION

Reactivation of Kaposi's Sarcoma-Associated Herpesvirus Infection from Latency by Expression of the ORF 50 Transactivator, a Homolog of the EBV R Protein

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Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), or human herpesvirus 8, is a lymphotropic virus strongly linked to several AIDS-related neoplasms. The primary reservoir of infection consists of latently infected B lymphocytes and possibly other mononuclear cells. Viral reactivation from latency and spread from this lymphoid reservoir is presumably required for development of nonlymphoid tumors like KS. Here we show that deregulated expression of a single viral gene, ORF 50, which encodes a transactivator able to selectively upregulate delayed-early viral genes, suffices to disrupt latency and induce the lytic gene cascade in latently infected B cells. The identification of this gene opens the way to studies of the physiologic mechanisms controlling reactvation of KSHV from latency.

Kaposi's sarcoma (KS) is the most common neoplasm in persons infected with the human immunodeficiency virus (HIV), striking 15–25% of homosexual and bisexual men with AIDS (Beral, 1991). Recently, a novel human herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8), has emerged as a key factor in KS pathogenesis (*9*; see *10, 34*, for review). Infection by the virus is uncommon in the general population, but strikingly more prevalent in groups at high risk for KS (*13, 16, 35*). In HIV-positive subjects, prior KSHV infection predicts a strongly increased risk of KS tumorigenesis (*24, 27*); in the resulting tumors, the virus can be found in a latent state in the majority of tumor cells (*37*). Current evidence suggests that KSHV is necessary for KS tumor development: in the absence of KSHV infection, KS rarely if ever occurs. However, since approximately 5% of the general population is KSHV-seropositive (*8*) yet has a vanishingly small KS risk, other cofactors—most notably HIV infection—are also required for tumorigenesis.

Based on nucleotide sequence analysis (*33*), KSHV has been classified as a member of the lymphotropic (or gamma) subfamily of the Herpesviridae, which also includes the transforming viruses Epstein–Barr virus (EBV) and *Herpesvirus saimiri* (HVS). In keeping with this classification, virologic evidence supports the inference that the primary target of viral infection is the B cell (*1, 25, 40*) (though some other mononuclear cells may also be susceptible to infection (*4*). Also consistent with primary lymphotropism is the fact that KSHV infection is also tightly linked to two AIDS-related lymphoproliferative disorders—primary effusion lymphoma (PEL) (*6*) and multicentric Castleman's disease (*36*). But KS is an endothelial, not a lymphoid, neoplasm, raising the issue of how such endothelial cells are targeted during KS pathogenesis. Since latent KSHV infection is established well before the onset of KS (*24, 29*), most authorities believe that a necessary antecedent step in KS development is reactivation of productive (lytic) KSHV infection from the latently infected lymphoid reservoir. Efficient or sustained reactivation—facilitated, for example, by HIV-induced immunosuppression—would be expected to elevate KSHV viral load, with resulting enhancement of the opportunity for spread to extralymphoid targets like endothelium. Consistent with this notion, clinical studies have shown that KSHV nucleic acid is infrequently found in peripheral blood lymphocytes (PBLs) of AIDS patients before tumor development, but is detectable in at least 50% of patients following the onset of disease (*1, 40*), and the viral load in circulating PBLs in those with AIDS or AIDS-KS is higher than that seen in HIV-negative subjects (*3*). Thus, active KSHV replication is strongly correlated with increased risk of progression to KS.

Little is known about the switch from latency to lytic reactivation in KSHV infection, but this subject has received considerable attention in the distantly related EBV (*17, 26*). As in most DNA viruses, expression of the lytic cycle genes in EBV unfolds in a strict temporal sequence and is governed by virus-encoded regulatory proteins. The earliest class of such regulators is the so-called

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immediate-early (IE) class, which in EBV includes three proteins known as Z (or BZLF1), R (or BRLF1), and M (or BSMLF1). The Z and R proteins are known transcriptional activators, while M appears to have complex pleiotropic posttranscriptional effects. In classical experiments, Miller and colleagues (*12*) demonstrated that ectopic expression of the viral Z gene disrupts latency in B cells and triggers the switch to the lytic gene cascade. Based on these and other experiments, Z is now established as the switch governing the latency-to-lytic transition in EBV-infected B cells.

Examination of the KSHV genome sequence (*33*) reveals homologs of R and M, encoded by ORFs 50 and 57, respectively. Here we report that the ORF 50 gene product of KSHV, which is expressed very early in the lytic cycle, can selectively activate the expression of downstream (delayed-early or DE) genes. More importantly, forced expression of this gene, but not of a panel of other candidate IE and DE genes, can trigger reactivation from an established latent infection, establishing the KSHV R protein as a critical regulator of the lytic cycle and an important determinant of the natural history of KSHV infection.

The studies presented here utilize a cell line called BCBL-1, a B lymphocyte line derived from a PEL specimen and latently infected with KSHV (*18, 31, 32*). During latency, viral gene expression is highly restricted, and the cells do not produce virus (although 1 to 5% of the cell population displays spontaneous reactivation). Upon treatment with TPA or sodium butyrate, a lytic cascade of viral gene expression is activated, resulting in viral genomic replication, virion production, and cell lysis (*31*).

We have identified four candidate switch genes by searching for the first genes to be expressed after cells are stimulated by TPA. These include the genes ORF 50 (encoding the KSHV R protein), ORF 57 (M protein), K3, and K5. The roles of R and M in EBV lytic induction have been described above. K3 and K5 are homologs of each other and distant homologs of BHV-4 immediate early genes; they contain RING finger motifs known to participate in protein–protein interactions. All of the messages for these proteins appear within 6 h after stimulation of viral reactivation in BCBL-1 cells by TPA (Lukac and Ganem, manuscript in preparation; unpublished data of R. Renne and J. Kirshner). Expression of these genes precedes the activation of known DE genes, which are defined as genes that are expressed prior to viral DNA replication and whose expression is dependent upon expression of viral IE genes. DE KSHV RNAs are typically detected at 8–24 h postinduction and include such genes as DNA polymerase (DNA pol) and the single-strand DNA binding protein (DBP). Delayed early gene expression is followed by viral DNA replication, which is a prerequisite for viral late gene expression. Therefore, the very early kinetics of R, M, K3, and K5 expression suggested that any or all of these proteins may play a role in initiating the lytic program of gene expression required for successful viral replication. Unfortunately, the toxicity of cycloheximide in BCBL-1 cells has made it impossible to rigorously establish that the transcripts of these genes accumulate in the absence of *de novo* protein synthesis, which is the formal definition of an immediate-early gene product.

We focused initially on the ORF 50 (R) protein for two reasons: (i) in EBV reactivation in B cells, the R gene is coregulated with the switch gene (Z) and its product can coordinately upregulate target DE genes when coexpressed with Z (*17, 26*), and (ii) the EBV R protein has been suggested to serve as a molecular switch for EBV reactivation in some nonlymphoid cells, e.g., certain cultured epithelial cells (*41*).

Expression of KSHV ORF 50 (R). The genomic region surrounding KSHV ORF 50 encodes multiple, abundantly expressed messages, a detailed characterization of which (by cDNA cloning, Northern blotting, primer extension, nuclease protection, 5′ RACE, and RT–PCR) will be presented elsewhere (Lukac and Ganem, manuscript in preparation). Figure 1 presents a summary of the features of ORF 50 expression pertinent to the present study. ORF 50 message (Fig. 1A) is approximately 3.4 kilobases (kb) in length and is spliced: the 53-nucleotide (nt) exon 1 is separated by a 959-nt intron from exon 2. Exon 2 then extends through the remainder of ORF 50 and includes coding sequences from the downstream K8 and K8.1 genes. Although there are no additional splices within ORF 50, the transcript undergoes three splices within ORFs K8 and K8.1 and is polyadenylated at a canonical poly(A) signal 3′ to ORF K8.1. Although ORF 50 mRNA production commences within 6 h of TPA addition to BCBL-1 cells, its levels continue to rise thereafter, reaching a maximum at 48 h, when viral DNA replication is also maximal.

The ORF 50 transcript is predicted to encode a 691-amino-acid (aa) protein; inspection of its sequence reveals a number of domains that suggest it is a transcription factor (Fig. 1B). ORF 50 encodes two putative nuclear localization signals (NLSs), and indirect immunofluorescence of BCBL-1 cells induced to viral replication by TPA reveals that R is indeed localized to the nucleus (Fig. 1C). ORF 50 also encodes an amino-terminal basic domain (with 19% basic residues) followed by a potential leucine zipper (ZIP domain) and a carboxy-terminal domain containing multiple acidic and bulky hydrophobic aas with homology to the transcriptional activation domains (ADs) of many mammalian, viral, and yeast transcription factors. Two independent regions of KSHV R share significant homology to the R proteins encoded by the other gammaherpesviruses (Fig. 1B). The N-terminal 238 aas of KSHV R, including the basic domain, contain a region 20–32% identical and 41–52% similar to the R proteins of EBV, HVS, bovine herpesvirus-4 (BHV-4), equine herpesvirus (EHV), alcelaphine herpesvirus, and mouse herpes-

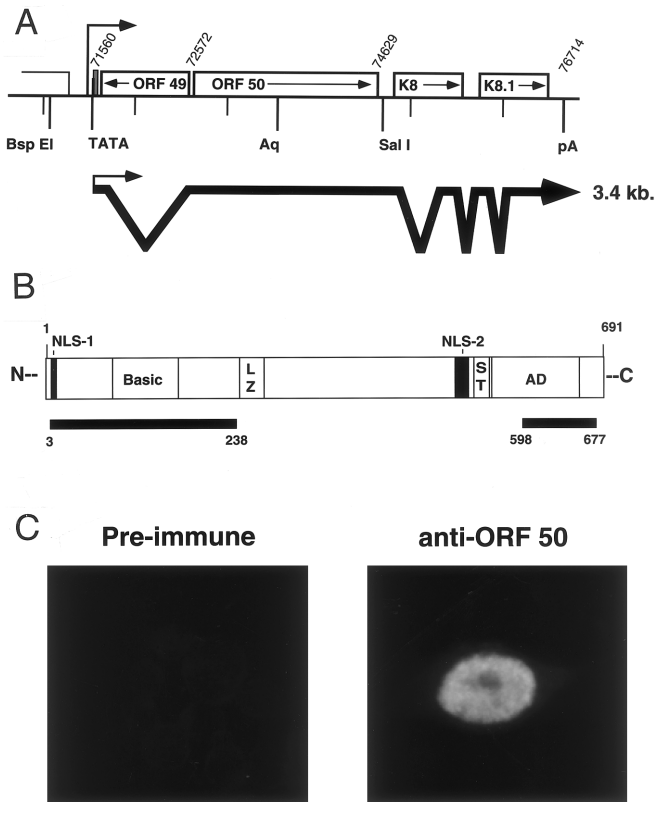

FIG. 1. Expression of KSHV ORF 50 R protein. (A) The ORF 50 genomic locus. The top of the figure shows the ORFs located between the indicated nucleotides in the KSHV genome. The large arrow indicates the start site of transcription of ORF 50. The large bars below the genomic locus designate the major transcript across ORF 50 as detected by Northern blotting. (B) ORF 50 is homologous to EBV BRLF 1. This diagram schematically depicts the putative structural and functional domains of ORF 50. NLS, nuclear localization signal; LZ, leucine zipper; ST, serine/threonine-rich; AD, activation domain. The bars underneath the ORF delineate the boundaries of the two regions of highest homology to the EBV R protein. (C) The ORF 50 R protein is localized to the nucleus. These panels show results of immunofluorescence staining of BCBL-1 cells at 48 h after TPA addition, using sera from rabbit before (left) or after (right) immunization with a recombinant ORF 50 fragment (aa 484 to 691) expressed in *E. coli.* The reactivity with anti-ORF 50 was detected using a FITC-conjugated goat anti-rabbit antibody.

virus-68 (MHV-68). In fact, this homologous region in the EBV protein mediates dimerization and DNA binding of EBV R (*14*). Likewise, 79 amino acids near the carboxy terminus of KSHV R are 25–66% identical and 37–71% similar to domains found in the cognate EBV, HVS, BHV-4, and EHV proteins. This domain is included in a region of EBV R which functions as an activation domain when fused to the DNA binding domain of the yeast Gal4 protein (*15*). Thus, the two major functional domains of the EBV R protein (DNA binding/dimerization and transcriptional activation) are conserved with the KSHV R protein, suggesting that the KSHV protein may function similarly to its EBV counterpart in the viral life cycle.

KSHV R protein encodes a transactivator. In order to express the R protein, we subcloned the 4.2-kb *Bsp*EI to *Sal*I fragment containing KSHV ORF 50 (see Fig. 1A) from a KSHV genomic DNA library into the mammalian expression vector pcDNA3. The ORF 50 protein made from this vector was expressed in the nuclei of transfected CV-1 cells, confirming its proper subcellular localization (not shown). To explore the potential of this protein to regulate the expression of KSHV lytic cycle genes, we examined its ability to transactivate a selection of KSHV DE and late gene promoters in transient transfection assays. These promoters included those for the DE genes nut-1, thymidine kinase (TK), DBP, and DNA pol and the late genes encoding assembly protein (AP) and glycoprotein B (gB). We also examined its effects on the promoter of the kaposin B gene, a latent gene whose transcripts are also known to be further upregulated with DE kinetics during the lytic cycle. For each of these genes, we fused DNA fragments bearing 5′ regulatory sequences to the luciferase coding region of plasmid pGL3 (see Materials and Methods). Each of these plasmids was cotransfected into CV-1 cells either with the control pcDNA3 vector or with increasing amounts of pcDNA3-ORF 50. Figure 2A shows a representative analysis of the nut-1 promoter. This promoter was activated to over 40-fold at the lowest amount of ORF 50 plasmid tested and reached a maximum of 45-fold when cotransfected with 3 μg of pcDNA3 ORF 50.

Figure 2B summarizes our transfection data for all of the tested promoters. Each was analyzed similarly to the experiment shown in Fig. 2A; in Fig. 2B, we have depicted the maximal point on the titration curve for each reporter. In these assays, the promoters of nut-1, TK, kaposin, and DBP were all activated by ORF 50; nut-1 was activated to the highest value by ORF 50, although TK activation reached 40-fold. Interestingly, nut-1 is known to be the most highly expressed RNA in the virus lytic cycle; in fact, this 45-fold induction of the nut-1 promoter exceeds the fold induction of steady-state levels of endogenous nut-1 transcript after TPA-treatment of BCBL-1 cells (*38, 42*). Of the delayed early promoters tested, only the DNA pol promoter was not activated. The inability of KSHV R to activate the DNA pol promoter contrasts with the strong activation of the EBV DNA pol promoter by EBV R (*23*). (We are currently dissecting the promoter requirements for activation by KSHV ORF 50, which may suggest explanations for this discrepancy.) Further experiments revealed that ORF 50 did not activate the AP or gB promoters, both promoters of the late class of RNAs (Fig. 2B). Thus, the ORF 50 (R) protein activates delayed-early but not late KSHV promoters, a pattern that is highly consistent with a proposed role for this transactivator in regulating the cascade of lytic gene induction. However, we note that results with transiently expressed herpesviral reporter genes do not always reproduce the patterns of regulation observed in the context of viral replication *in vivo.*

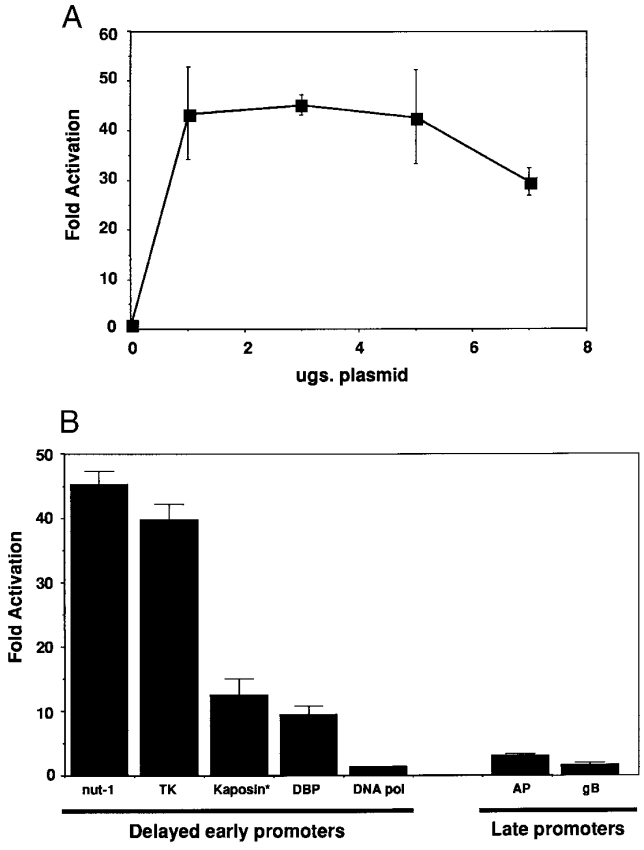

FIG. 2. The KSHV ORF 50 R protein is a transcriptional transactivator. (A) The R protein activates the nut-1 promoter. CV-1 cells were cotransfected with the indicated amounts of pcDNA3-ORF 50 and a constant amount of pGL3-nut 1. Cells were harvested at 42 h posttransfection, and luciferase activity was measured. Values are plotted as fold activation relative to the luciferase activity found in cells similarly cotransfected with pGL3-nut 1 and the control pcDNA3 vector; values were normalized to transfection efficiency, determined by cotransfection of a B-galactosidase-expressing plasmid. Vertical bars represent standard deviations of results from transfections performed in triplicate. (B) The KSHV R protein transactivates viral delayed-early but not late promoters. Transfections were performed with varying amounts of pcDNA3-ORF 50 and fixed amount of luciferase reporter constructs driven by the indicated promoters, as in A; the maximum amount of transactivation observed in each dose–response experiment is plotted here. Error bars are as in A. See text for construction of all reporter plasmids.

KSHV R protein expression triggers reactivation from latency. To determine whether the ORF 50 gene product can indeed disrupt latency and trigger the KSHV lytic cycle, we examined the effects of ectopic expression of KSHV R protein (and of the other candidate activator proteins) upon markers of viral replication in BCBL-1 cells. Several technical considerations are relevant to analysis of this experiment. First, although BCBL-1 cells are latently infected, even in the absence of TPA 1–3% of BCBL-1 cells spontaneously enter the lytic cycle (this is not unique to BCBL-1; other B cell lines bearing latent KSHV (*28*) or EBV (*17*) genomes all display such spontaneous reactivation to various extents). This represents the background level of reactivation against which *de novo* induction must be measured. Second, for reasons that are unclear, even the most potent chemical inducers of lytic replication—e.g., TPA—result in detectable induction in only 10–25% of BCBL-1 cells (again, this is also true of all other latently infected B cell lines; many EBV producer lines display only 5–10% infected cells post phorbol treatment (*17*)). Third, the transfection of BCBL-1 cells is very inefficient, with optimal methods typically delivering the exogenous DNA to only ~1% of exposed cells. Therefore, even if the transfected switch gene were highly effective as an inducer, the low frequency of delivery and the high spontaneous background of reactivation would make lytic induction difficult to observe in mass culture, especially using biochemical methods (e.g., Northern or immunoblotting) that do not examine single cells. Accordingly, in our experiments, candidate switch genes were transfected into BCBL-1 cells together with an expression vector for an irrelevant protein (here, hepatitis delta antigen, HDAg) whose expression was followed 48–84 h posttransfection by immunofluorescence assay (IFA) to mark successfully transfected cells. In each experiment, at least 1000 HDAg-positive cells were scored by IFA for expression of two markers of KSHV lytic reactivation: (i) ORF 59 protein, a DNA polymerase-associated processivity factor expressed as a delayed-early gene (*7, 22*); and (ii) K8.1 protein, a viral envelope glycoprotein expressed with late kinetics (*30*). Each of these two markers was detected with monoclonal antibodies specific for the corresponding protein.

Figure 3A shows the collated results of several such experiments, in which BCBL-1 cells were transfected in parallel with expression vectors for ORF 50 (R), ORF 57 (M), K3, or K5, the four candidate switch genes we had earlier identified based on their patterns of expression and homologies with known IE proteins of other herpesviruses. We also tested the impact of expression of the K1 gene, a virally encoded membrane protein and potent signal-transducing molecule that is also upregulated during lytic infection (*19, 20, 21*). As a positive control, BCBL-1 cells were transfected with the pcDNA3 and HDAg vectors and induced with TPA; cells similarly transfected but not treated with TPA served as controls for spontaneous reactivation. Examination of the results of the positive and negative control experiments was instructive. Although in untransfected cultures, 1–3% of cells were in the lytic cycle pre-TPA and 10–20% post-TPA, in transfected cells the numbers were substantially reduced, to 0.3–0.6 and 3–3.5% at 48 h posttransfection, respectively, as judged by ORF 59 staining (Fig. 3A, left). This most likely reflects the adverse impact of electroporation and transfection on cell physiology and is not surprising when one considers that ca. 50% of treated cells die following electroporation. By 60 h posttransfection (Fig. 3A, right), up to 6–7% of the transfected cells are expressing ORF 59. This ca. 10-fold induction of replica-

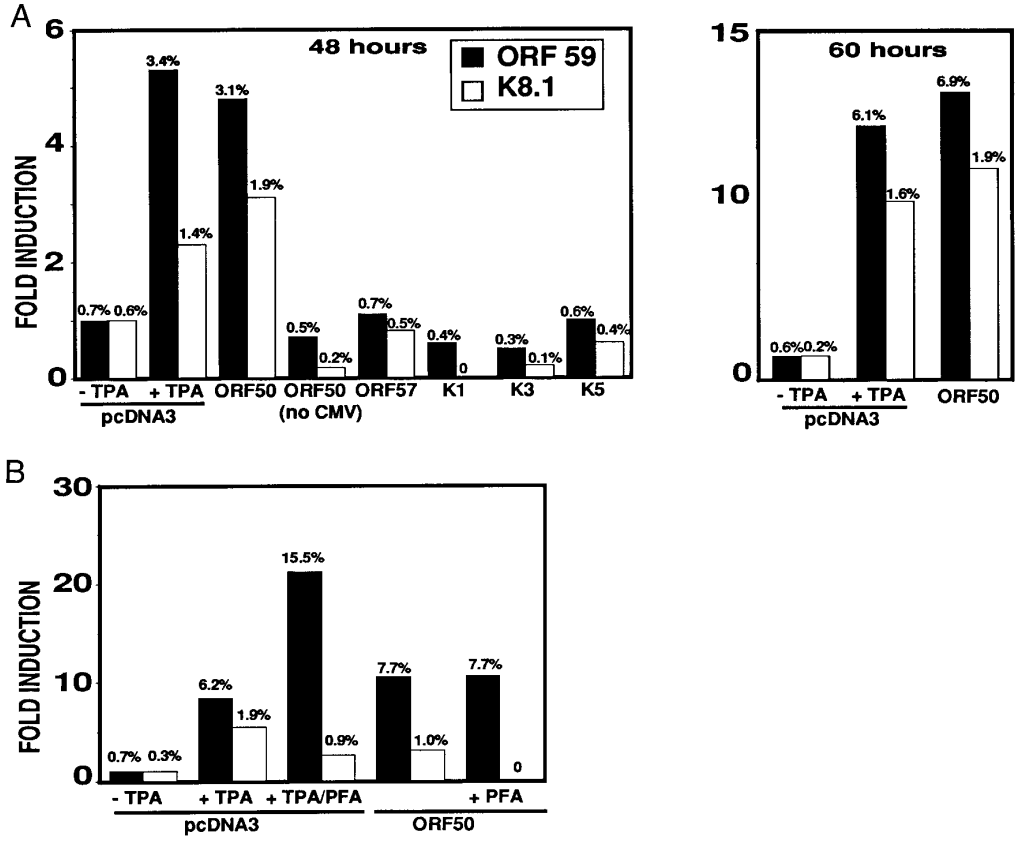

FIG. 3. KSHV R protein expression induces the KSHV lytic cascade in BCBL-1 cells. (A) (Left) The KSHV ORF 50 R protein induces the lytic antigens ORF 59 and K8.1 as efficiently as TPA. Cells were electroporated with vectors expressing the indicated proteins and hepatitis delta antigen (HDAg). Successfully transfected cells, identified by positive staining for HDAg, were scored 48 h posttransfection for expression of either ORF 59 or K8.1; 1000 to 2500 transfected cells were counted for each electroporation; the percentage of transfected cells expressing the respective KSHV lytic marker is indicated above each bar. The bars indicate the fold induction of the lytic markers relative to cells electroporated in the absence of TPA with the control pcDNA3 vector. Note: in columns ORF 50 no CMV, cells were transfected with a clone bearing ORF 50 coding and promoter sequences in the absence of an exogenous CMV promoter/enhancer. (Right) Induction of KSHV lytic genes by deregulated ORF 50 expression in transfected BCBL-1 cells. Experiment performed exactly as in A (left), except that staining was carried out at 60 h posttransfection. (B) Late but not DE gene expression is blocked by phosphonoformate (PFA). BCBL-1 cells were transfected with the indicated plasmid plus an HDAg expression vector and then incubated in either the absence or the presence of TPA; one parallel culture was exposed to both TPA and PFA, as indicated. At 60 h posttransfection, HDAg-positive cells were scored for expression of ORF 59 (dark bars) or K8.1 (light bars). Number atop each bar denote the percent of scored cells positive for each marker; height of bars denotes fold induction over that induced by transfection of pcDNA3 control vector in the absence of TPA.

tion by TPA is highly reproducible and is similar in magnitude to the induction ratio produced by TPA in cultures not exposed to electroporation. In addition, we note that staining for the DE ORF 59 protein was consistently 2-fold more sensitive that staining for the late K8.1 antigen. This might be due to the different affinities of the two mAbs used for detection or could reflect possible abortive lytic induction in some cells, in which DE genes are triggered without progression to DNA synthesis and late gene expression.

Figure 3A (left) shows that, of the tested genes, only expression of ORF 50 led to induction of detectable lytic cycle markers: the M, K1, K3, and K5 expression vectors had no impact on lytic reactivation. Importantly, when examined quantitatively, the induction of replication by KSHV R protein in transfected cells was comparable in efficiency to that induced by TPA treatment (see also Fig. 3A, right). Although for the reasons outlined above, analysis of DNA replication in bulk cultures was not possible here, strong indirect evidence points to the induction by KSHV R protein of viral DNA replication as well as gene expression. Figure 3B shows that when ORF 50 transfection was performed in the presence of the viral DNA polymerase inhibitor foscarnet (phosphonoformate, PFA), DE (ORF 59) gene upregulation was unaffected but late (K8.1) gene expression was profoundly inhibited. Since late gene (but not DE) expression is dependent on DNA replication, this experiment provides strong evidence that authentic genomic replication was ongoing in transfected cells. Taken together, the evidence strongly indicates that the full program of KSHV reactivation was induced by ectopic R expression. Importantly, a genomic construct carrying R coding seqeunces and 5′ regulatory elements but no exogenous promoter to drive deregu-

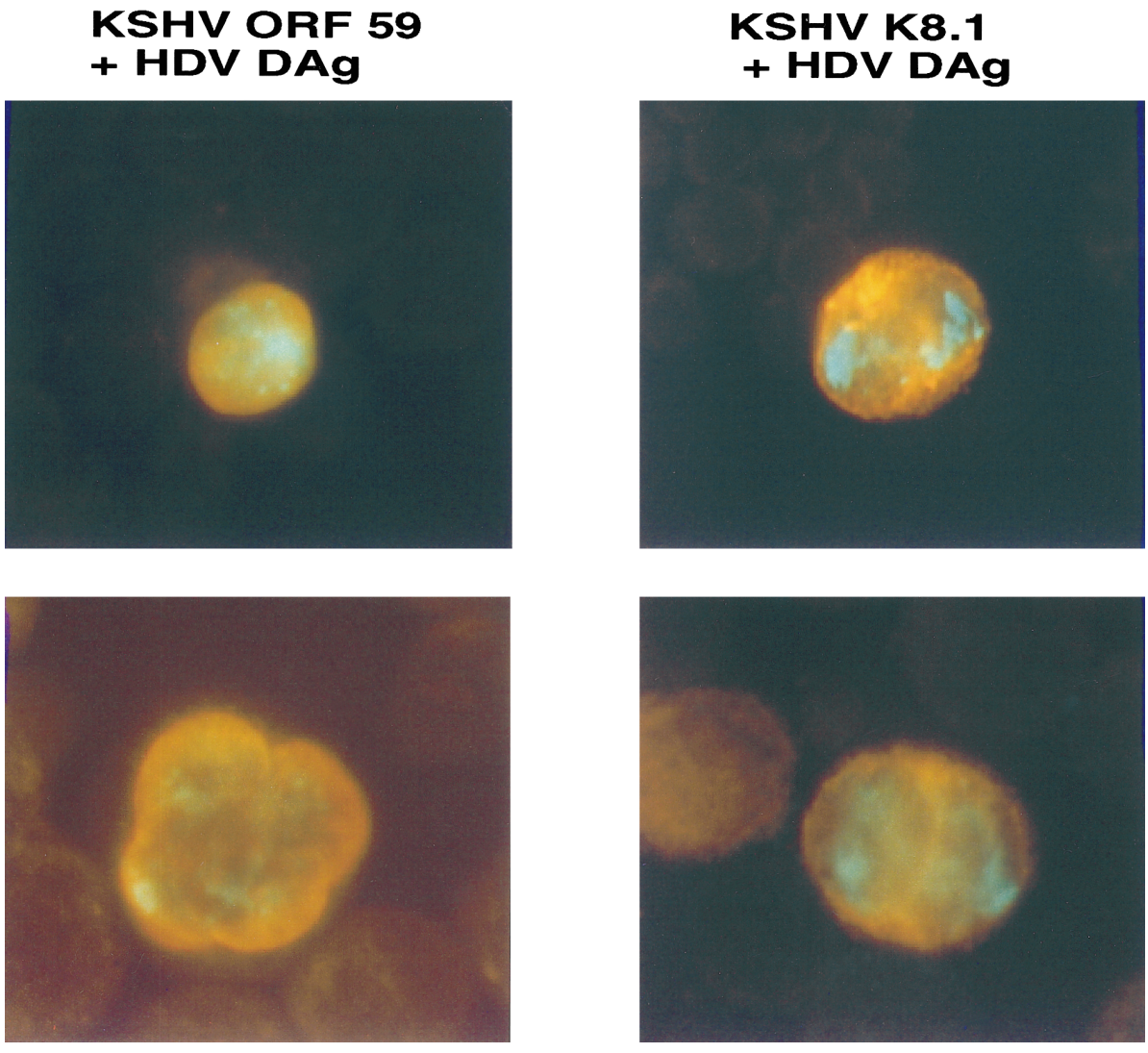

FIG. 4. Induction by KSHV R protein expression of bi- and multinucleate cells expressing lytic cycle markers. Cells transfected with expression vectors for HDAg and ORF 50 were examined at 48 h posttransfection by staining for HDAg (green) and either ORF 59 (orange, left panels) or K8.1 (orange, right panels). (Top left) Typical single cell staining for both HDAg and ORF 59; (bottom left) ORF 59-positive syncytium containing 3–4 HDAg-positive nuclei; (top and bottom right) two different binucleate cells expressing nuclear HDAg and cytoplasmic/membrane K8.1 (see text for details of staining).

lated expression (Fig. 3A, left, labeled "ORF 50 no CMV") did not induce lytic reactivation. This is consistent with the notion that some cellular stimulus (e.g., TPA-induced activation of host transcription factors controlling the ORF 50 promoter) is required for the physiologic induction from latency. Finally, additional evidence for lytic induction comes from analysis of the cytopathic effect of lytic induction by R protein expression. As shown in Fig. 4, R-transfected cells in which ORF 59 or K8.1 expression was induced frequently showed bi- or multinucleate syncytium formation, a characteristic cytopathic effect in many herpesvirus infections.

Our results—and similar recent findings of Sun and colleagues (*39*)—are formally analogous to those that established the EBV Z gene as the molecular switch governing lytic reactivation in that virus (*12*) and point strongly to a similar role for the R protein in KSHV reactivation. If so, then study of the regulation of the R promoter by cellular factors may allow definition of the host machinery that governs reactivation and thereby influences subsequent pathogenetic events in infected patients.

Plasmids. All plasmids were propagated in *Escherichia coli* DH5α and were purified by alkaline lysis and double banding in cesium chloride. All genomic sequence positions are according to Russo *et al.* (*33*). The source of starting genomic DNA for all clones was our KSHV genomic lambda library (*42*). Plasmids used for expression of KSHV proteins and their construction are as follows: pcDNA3-ORF 50 was constructed by subcloning the 7-kb *Sal*I fragment spanning nt 67861 to 74810 into *Sal*I-digested pBluescript II (KS^+), generating the

plasmid pBS KS-Sal 7. The ORF 50 genomic expression vector was then constructed in two steps. Exon 2, containing the body of ORF 50, was first subcloned using the unique *Spe*I site at nt 72223 and the 3′ *Spe*I site contributed by the pBluescript polylinker. This fragment was cloned into pcDNA3 which had been linearized with *Xba*I, generating the plasmid pcDNA3-ORF 50 short. The entire ORF 50 locus was then reconstructed by digesting and blunting pBS-Sal 7 at the *Bsp*E1 site at nt position 71251 and then removing the 1316-nt fragment using *Bsu*36I. This was cloned into pcDNA3-ORF 50 short, which had been digested with *Eco*RV and *Bsu*36 I, to create pcDNA3-ORF 50.

pBS-ORF 50 was constructed by digesting pBS-Sal 7 with *Nsi*I at genomic position 70703 and then blunting with Klenow polymerase. The KSHV sequences upstream of that site were then removed by cutting with *Xho*I in the polylinker, blunting with T4 DNA polymerase, and then religating the plasmid. The bovine growth hormone poly(A) addition sequence was then removed from pcDNA3 by digesting it with *Eco*RI/*Pvu*II. This fragment was ligated into the ORF 50 construct that had been digested with *Xba*I, blunted with Klenow, and then digested with *Eco*RI.

pRSET 0.8 was generated by subcloning the *Sma*I/*Sac*I fragment from the 3′ terminus of ORF 50 into pRSET B (Invitrogen) that had been digested with *Pvu*II/*Sac*I.

pcDNA3.1-ORF 57-Hygro was constructed by subcloning the 1496-nt *Apa*LI to *Bst*98I fragment, after Klenow blunting, into pcDNA 3.1 that was linearized with *Eco*RV.

The construction of pcDNA- ORF K1 is described in Lagunoff *et al.* (*20*).

pcDNA3-K3 and pcDNA3-K5 were constructed by PCR amplification of the respective ORFs using primers that introduced 5′ *Bam*HI and a 3′ *Eco*RI restriction sites. These PCR products were digested with those enzymes and cloned into the corresponding sites in pcDNA3.

Numerous reporter plasmids were constructed using the luciferase reporter plasmid pGL3-Basic (Promega). In most cases, we attempted to use the native initiating AUG for each KSHV gene to control translation of luciferase. The reporters and construction are as follows: pGL3-TK was constructed by PCR amplification of a 1456-nt fragment from genomic positions 33927 to 35383, using a primer that introduced an *Nco*I site at the initiation codon of TK. Digestion of this fragment with *Nco*I and *Hin*dIII yielded two fragments: one ended with only *Nco*I sites and the other ended with *Nco*I and *Hin*dIII sites. The *Nco*I/*Hin*dIII fragment was first cloned into pGL3-Basic that had been digested with both enzymes, to yield pGL3-TK N/H. This was followed by insertion of the *Nco*I fragment at the *Nco*I site in this plasmid.

pGL3-gB was constructed by PCR amplification of a 1216-nt fragment from genomic positions 7483 to 8699. One primer introduced an *Nco*I site at the initiation codon of gB. The PCR product was digested with *Nco*I and *Hin*dIII and subcloned into *Nco*I/*Hin*dIII-digested pGL3-Basic.

pGL3-pol was constructed by PCR amplification of the 1569-nt fragment from genomic position 9794 to 11363. One primer introduced an *Nco*I site at the initiation codon of DNA pol. This product was digested with *Eco*RV and *Nco*I and cloned into pGL3-Basic which had been digested with *Sma*I and *Nco*I.

pGL3-DBP was constructed by subcloning the 1326-nt *Bam*HI/*Kpn*I fragment from genomic positions 1883 to 3209 into pBluescript (SK^+) to generate pBS-DBP. The promoter was then liberated from this plasmid using *Sac*I and *Nco*I donated by the pBS polylinker and cloned into pGL3-Basic that had been digested with the same enzymes.

pGL3-nut1 was constructed by cloning the 1737-nt *Bgl*II/*Nco*I fragment from genomic positions 27199 to 28936 into pGL3-Basic that had been digested with the same enzymes.

pGL3-B3 contains the kaposin promoter and was constructed by cloning the 1434-nt *Xho*II/*Kpn*I fragment from genomic positions 120163 to 118729 into pGL3-Basic that had been digested with *Bgl*II and *Kpn*I.

pGL3-AP was constructed by cloning the 696-nt *Sac*I/*Eco*RV fragment from genomic positions 32362 to 31666 into pGL3-Basic that had been digested with *Sma*I. The *Sal*I site was blunted with Klenow polymerase. The initiating ATG of the AP ORF (nt position 31687) was then changed to TTG using PCR mutagenesis.

pnut24 expresses histidine-tagged HDV LDAg from pcDNA3.

PcDNA 3.1-lac Z expresses B-galactosidase and was used as an internal control for all transfections.

Cell lines and transfections. The cell line BCBL-1 was propagated and maintained as in Renne *et al.* (*32*). For electroporation, cells were split to a density of 4×10^5/mL 18–24 h prior to electroporating. On the day of electroporation, cells were washed twice in 1× PBS and then resuspended in minimal RPMI 1640 media (supplemented with 10 mM dextrose in Tris-buffered saline/0.1 mM dithiothreitol) at a density of 1×10^7 cells/mL. Cells were aliquotted in 0.3-mL volumes to electroporation cuvettes (0.42 cm), and DNA was added to the cells. For induction experiments, 3 μg of each respective KSHV expression vector and 2 μg of pnut24 were electroporated with 960 mF, 150 mV. Electroporated cells were then transferred to fresh growth media or media containing 20 ng/mL TPA and/or 0.5 mM PFA. Cells were harvested at 48 to 84 h postelectroporation and analyzed by IFA.

CV-1 cells were propagated and maintained in DME-H21 media supplemented with 10% fetal calf serum and pennicillin/streptomycin. For transfections, low-passage cells were plated at 3×10^5 cells per 60-mm tissue culture dish and grown overnight. On the day of transfection, monolayers were fed 3 h before DNA addition.

DNA amounts used were as listed in the appropriate figures; pcDNA3 was used to normalize total DNA amounts for each transfection. DNA was precipitated using the calcium phosphate procedure with the *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) modification (*11*). The precipitate was left on the cells for 6 h, then the cells were shocked with 15% glycerol (in BES-buffered saline) for 1 min, and fresh medium added. Cells were harvested at 42 to 48 h post glycerol shock for luciferase and β-galactosidase assays. Data shown in Fig. 3 are the result of at least two experiments performed in triplicate for each reporter.

Luciferase and β-galactosidase assays. Cells were washed twice in 3 mL 1× PBS at room temperature and then incubated for 30 min with rocking with 0.2 mL reporter lysis buffer (Promega). Cells were scraped and collected, and then debris was removed by centrifugation for 1 min at 16,000 rpm in a microcentrifuge. Supernatants were transferred to new tubes, and 20- and 50-μL aliquots were analyzed by luciferase or β-galactosidase assays, respectively, according to the manufacturer's instructions (Promega).

Antibodies and IFA. Antibody 733 vs HDAg was as described in Brazas and Ganem (*5*). Rabbit antiserum D3861 (Animal Pharm Services) was generated vs the His-tagged protein expressed from pRSET-ORF 50. For IFA, cells were washed twice in 1× PBS, and then 2.5×10^5 cells were applied in 0.2-mL volumes to individual wells of a six-well cell staining slide (CelLine Associates). Cells were allowed to adhere for 30 min, after which the remaining liquid was removed by suction. Slides were immediately fixed in 50% methanol/50% acetone, which had been precooled to −20°C. The slides were kept covered at −20°C for 10 min and then removed and air dried under a tissue culture hood for 5 min. Slides were then stored at −70°C or analyzed immediately. For immunofluorescence staining, cells were rehydrated in 0.2 mL of 3% bovine serum albumin (BSA) for 15 min. This was removed by suction, and cells were blocked in 3% BSA/1% glycine (blocking buffer) for 30 min. This was removed, and cells were incubated with the anti-DAg, at a dilution of 1:300, and either of the KSHV monoclonal antibodies (1H10 vs ORF 59 and 19B4 vs K8.1; kindly provided by B. Forghani) at a dilution of 1:1000. Antibodies were diluted in blocking buffer, and incubation was for 1 h. This was followed by two 30-min washes in 1× PBS/4% Tween 20, and one 30-min wash in 1× PBS. Excess PBS was aspirated, and the cells were subjected to the secondary stain. This step utilized FITC-conjugated goat anti-rabbit whole antibody and TRITC-conjugated goat anti-mouse $F(ab)_2$ fragments, both diluted 1:300. Secondary reagents were incubated with the cells for 1 h and then washed as above.

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