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Gene Expression from the ORF50/K8 Region of Kaposi's Sarcoma-Associated Herpesvirus

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The ORF50 gene of Kaposi's sarcoma (KS)-associated herpesvirus, or human herpesvirus 8 (KSHV), activates viral replication and is weakly homologous to the herpesvirus family of R transactivators; therefore, the transcription and translation events from this region of KSHV are key events in viral reactivation. We demonstrate that ORF50 is expressed in a bicistronic message after induction of the viral lytic cycle. ORF50 migrated as a series of polypeptides: the major ones as 119 and 101 kDa, respectively. Using 3′ rapid amplification of cDNA ends, RT-PCR, and cDNA library screening, we demonstrate that the major ORF50 transcript also encodes K8. The ORF50/K8 transcript was resistant to cyclohexamide, whereas the K8 transcript was only partially resistant to cyclohexamide at early timepoints. Both transcripts showed partial resistance after 12 h of phorbol ester induction. Using a GAL4-ORF50 fusion protein expression vector, we demonstrate that the transactivation domain of ORF50 resides within a 160-amino-acid region of the carboxyl portion of the ORF. Upstream regions of both ORF50 and K8 have basal promoter activity in KSHV-infected cells. K8, which had sequence homology to Bzip proteins, did not activate either promoter. However, both promoters were activated after cotransfection of ORF50 in BCBL-1 cells.

INTRODUCTION

Kaposi's sarcoma (KS)-associated herpesvirus, or human herpesvirus 8 (KSHV), is present in >90% of all forms of KS (Ambroziak et al., 1995; Buonoguuro *et al.,* 1996; Chang *et al.,* 1994; de Lellis *et al.,* 1995; Dupin *et al.,* 1995; Ekman *et al.,* 1995; Moore and Chang, 1995). Sequencing of KSHV reveals a novel herpes virus homologous to gamma herpesviruses saimiri and Epstein–Barr virus (EBV), two viruses that are strongly associated with malignant transformation (Kieff, 1996; Meinl *et al.,* 1995; Rickinson and Kieff, 1996). The KSHV DNA is recovered from all stages of lesions, early as well as at end stage (Aluigi *et al.,* 1996). Active gene expression of the identified genes has been detected within lesions (Reed *et al.,* 1998; Zhong *et al.,* 1996). KSHV in the peripheral blood of patients with KS is detectable years before the development of overt disease (Moore *et al.,* 1996; Quinlivan *et al.,* 1997; Whitby *et al.,* 1995). In addition, analysis of stored blood samples revealed that the presence of KSHV in the blood is a predictor for the subsequent development of KS (Whitby *et al.,* 1995). Low KSHV detection rates in various samples from control populations have substantiated the strong correlation of this virus with KS and have suggested a role for this virus in other neoplastic processes (Cesarman and Knowles, 1997).

KSHV genes include those with sequence and functional homology to many cellular genes controlling cell proliferation (Neipel *et al.,* 1997; Russo *et al.,* 1996; Sarid *et al.,* 1998). The expression of many of these genes occurs predominantly during lytic replication. Studies of viral gene expression in KS have revealed that latency-associated transcripts are present in the majority of cells in KS lesions. However, lytic transcripts (those found in KSHV-positive cell lines after induction of viral replication) are found in a subset of cells (Zhong *et al.,* 1996). The lytic transcripts are present in the malignant cells (i.e., those with spindle morphology). Productively infected monocytes are also present within KS lesions (Blasig *et al.,* 1997). An elegant study by Orenstein *et al.* (1997) demonstrated mature herpesvirus virions in splenic KS lesions within spindle-shaped cells. Thus replication is likely to play a role in KSHV pathogenesis.

Virally encoded control mechanisms to which KSHV replication is subjected are likely to be complex. EBV encodes two immediate-early viral proteins (BZLF1; ZEBRA; Zta, or *Z*; and BRLF1, Rta, or *R*) that regulate initiation of the viral replicative pathway (Kieff, 1996). The derived amino acid sequence of ORF50 gene has homology with the EBV lytic switch BRLF1 protein (Russo *et al.,* 1996). The KSHV ORF50 gene product activates lytic KSHV replication (Lukac *et al.,* 1998; Sun *et al.,* 1998). The

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other members of the gamma herpesvirus group also encode proteins that are homologous to the prototype EBV R (Bermudez-Cruz, 1997; Whitehouse *et al.,* 1997). Initial genomic analysis of these gamma herpesviruses did not reveal a homolog to the other EBV lytic switch protein (Bublot *et al.,* 1992). Recently, identification of a KSHV ORF with sequence homology to Z has been reported (Lin *et al.,* 1999). In this study, we further characterized gene expression in the ORF50/K8 region.

RESULTS

Kinetics of tetradecanoyl phorbol ester acetate induction of ORF50

To examine the kinetics of ORF50 expression, the latently infected B cell line BCBL-1 was induced with tetradecanoyl phorbol ester acetate (TPA). Twelve hours after TPA induction, multiple transcripts were dramatically increased (Fig. 1A) as detected by Northern blot analysis using a probe spanning both ORF50 and K8 sequences (Fig. 2). The major transcripts were ~4.9, ~3.7, and ~1.5 kb. All three transcripts accumulated until 48 h. The accrual of the 4.9- and 3.7-kb transcripts was threefold at 24 h and peaked with a sevenfold increase at 48 h (Fig. 1B). The 1.5-kb transcript was increased at 24 h with a sixfold accumulation but peaked with a 20-fold increase at 48 h. These results show that transcription in this region after TPA stimulation is rapid and complex.

To correlate the protein production with the accumulation of ORF50 mRNA, nuclear and cytoplasmic extracts were prepared from BCBL-1 cells after treatment with TPA. ORF50 protein was detected by Western blot analysis using a rabbit polyclonal anti-ORF50 antibody (Lukac *et al.,* 1998; gift of D. Ganem). This antibody recognized a predominant 119-kDa protein in nuclear and cytoplasmic extracts (Fig. 1C). A basal level of the 119-kDa ORF50 protein could be detected in both nuclear and cytoplasmic extracts of DMSO-treated cells. After TPA induction, a 101-kDa protein was also detected. A progressive increase was seen in both the nuclear ORF50 119- and 101-kDa proteins throughout the TPA treatment. To determine whether the presence of ORF50 protein in nuclear extracts was due to contamination with cytoplasmic proteins, antibodies (Santa Cruz Biotechnology, Inc.) to IκB-α (a strictly cytoplasmic protein; Baeuerle and Baltimore, 1988) did not detect IκB-α in the nuclear extracts (data not shown). The predominant forms were much larger than the predicted size of 74-kDa Peptide Sort (GCG). Smaller forms (predominantly 89 and 72 kDa) of ORF50 were also present in cytoplasmic extracts.

KSHV ORF50 is expressed as a bicistronic message, encoding K8

Northern blot analysis was performed using poly(A)$^+$ RNA isolated from TPA-treated BCBL-1 cells to identify specific ORF50 and K8 transcripts. An antisense riboprobe (Fig. 2) complementary to the 5′ end of ORF50 cDNA (Sun *et al.,* 1998) hybridized predominantly with the 3.7-kb transcript from TPA-treated BCBL-1 cells (Fig. 3A). An antisense riboprobe from the 5′ end of the K8 ORF detected the 3.7-kb mRNA as well as a 1.5-kb transcript (Fig. 3B).

Efficient transcription from the ORF50/K8 region requires protein synthesis

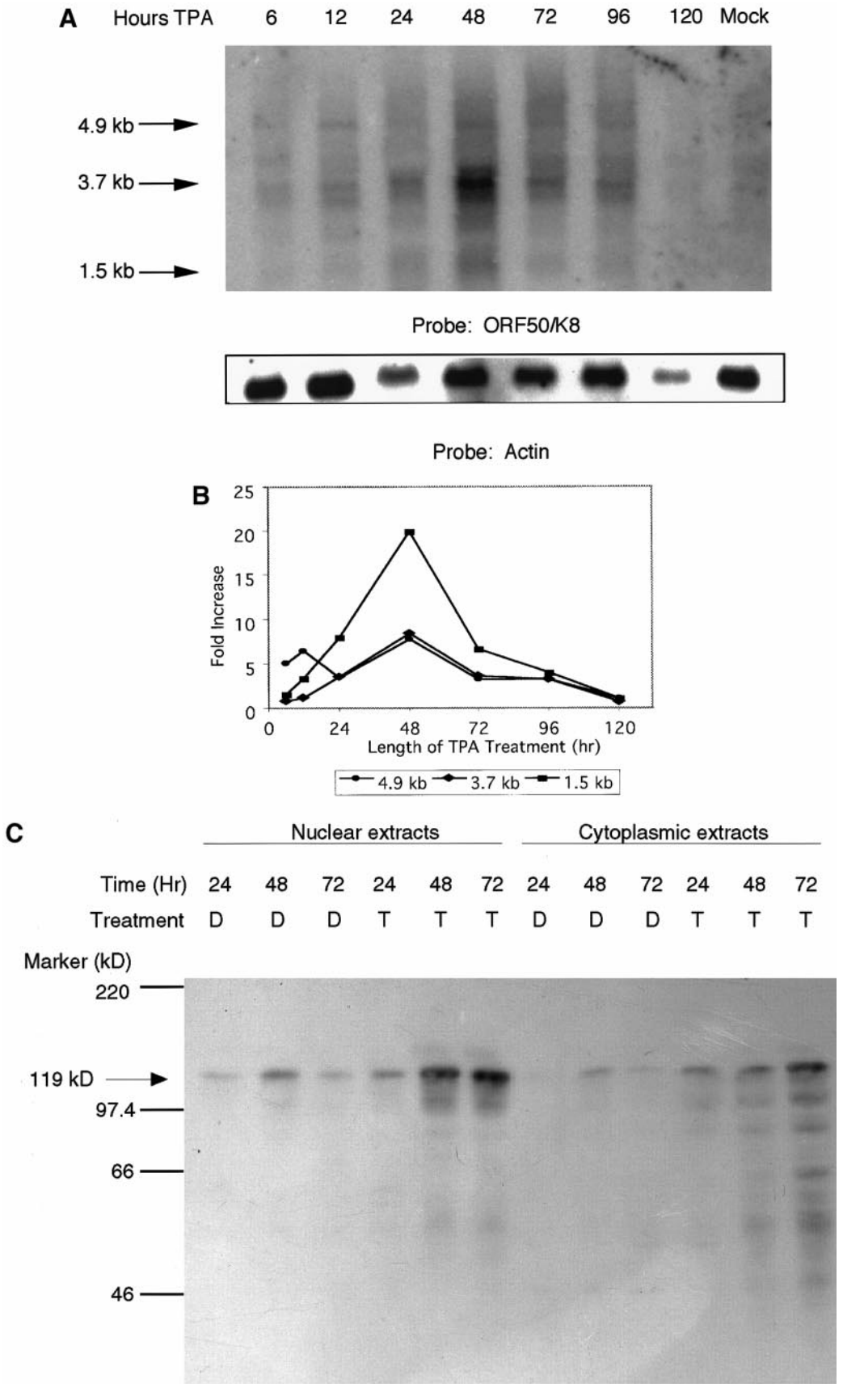
To characterize these transcripts, we used chemical inhibition of translation [cyclohexamide (Cyc)] and herpesvirus DNA replication [phosphonoacetic acid (PAA)]. The loading of RNA was normalized by determining the density of the GAPDH hybridization signal. Densitometry of the 3.7-kb transcript revealed a rapid doubling of the amount of transcript as early as 1 h after TPA induction. Transcript accumulation was steady through 12 h of TPA treatment. After this transcript, accrual continued through 48 h with a maximum 4.5-fold increase. Neither Cyc nor PAA inhibited the first phase of production. The second phase (12–48 h) was inhibited by Cyc but not PAA. This characterized the 3.7-kb transcript ORF50 as an immediate-early mRNA.

A similar analysis was performed on the 1.5-kb transcript. The kinetics of this transcript showed an early and a late phase of accumulation (before and after 12 h). No inhibition by PAA was seen. Cyc blunted the first phase of accumulation as well as preventing the second phase. The blunting of the earlier phase was in contrast to an absence of an effect of Cyc on the 3.7-kb transcript.

To determine the downstream structure of ORF50 transcripts, we performed rapid amplification of cDNA ends (RACE) (Fig. 2). Sequence analysis revealed that the 3′RACE product began at the gene specific primer site (74313) and extended to a splice donor site in the K8 ORF at nucleotide (nt) 75323 (Fig. 4A). The splicing removed a 149-bp intron, but the K8 reading frame was maintained. A second splice removed the region between nt 75838 and 76433, including the K8.1 translational start site. The sequence terminated at nt 76740 after the poly(A)$^+$ signal located at nt 76714. This was the first identifiable poly(A)$^+$ signal downstream of ORF50. A poly(A)$^+$ tail was present at the 3′ end of the RACE product (nt 76738).

K8 is expressed as two spliced forms and is weakly homologous with the EBV lytic switch protein Z

We screened a cDNA library to obtain clones that would confirm the splicing pattern of the 3′ RACE product. Four positive clones were isolated by this screen. All four clones had an identical 3′ end (nt 76738), with a poly(A)$^+$ tail implying an authentic transcript (Fig. 4A). The poly(A)$^+$ signal used was located 20–25 bp upstream from the 3′ terminus in all these clones (nt 76714). The 5′ ends were variable (74537, 74862, 75077, and one hybrid with non-KSHV sequence). The splicing pattern


A
Hours TPA 6 12 24 48 72 96 120 Mock
4.9 kb
3.7 kb
1.5 kb
Probe: ORF50/K8
Probe: Actin
B
Fold Increase
25
20
15
10
5
0
0 24 48 72 96 120
Length of TPA Treatment (hr)
4.9 kb 3.7 kb 1.5 kb
C
Nuclear extracts
Cytoplasmic extracts
Time (Hr) 24 48 72 24 48 72 24 48 72 24 48 72
Treatment D D D T T T D D D T T T
Marker (kD)
220
119 kD
97.4
66
46

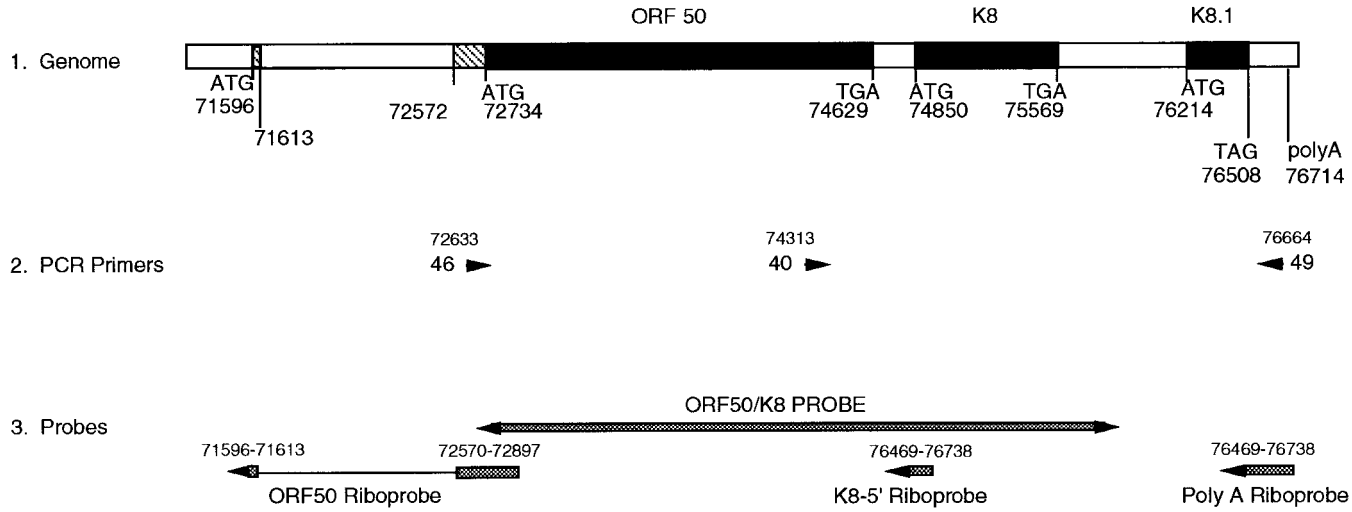


FIG. 2. Map of primers and probes. (1) Map of the ORF50 region of the KSHV genome. Genomic ORFs are indicated by filled boxes. The first exon of ORF50 is indicated by the hatched area. (2) Arrows indicate primer sites used for 3′ RACE and RT-PCR. (3) Probes used for Northern blots are indicated. Bidirectional bar indicates the random prime labeled probe; unidirectional bars indicate riboprobes.

included the two splices previously identified in the 3′ RACE clone. An additional splice was also present that produced a frameshift and extends the K8 ORF for an additional 48 amino acids, creating an alternatively spliced form of K8, K8B. Splicing downstream of K8 removes the translational start site of the recently described K8.1 (Chandran *et al.,* 1998; Raab *et al.,* 1998).

Recognition that K8 was transcribed in concert with ORF50 suggested that it was a homolog of the EBV lytic switch protein, Z, which has a colinear position in the EBV genome. We used computer alignment programs to determine the sequence homology between KSHV K8 and EBV Z (Blast, Pileup, and Bestfit; GCG). Overall, the sequence homology is very low, and the BLAST retrieval software did not identify homology between EBV Z and HHV K8. However, Bestfit and Pileup programs were used to search the K8A and K8B sequences for regions homologous to identified regions of weak homology (range of identity, 3/16 to 5/18 amino acids) to the Z transactivator and Bzip signature (domains activation and DNA binding domains, respectively) (Flemington *et al.,* 1992; Kouzarides *et al.,* 1991) (Fig. 4B). A region weakly homologous with the activator domain is found in exon 1 and is present in both K8A and K8B. A second region homologous with the Z transactivator domain is located in the third exon of K8B but is not present in K8A. A basic amino acid region with homology to Bzip signature domains as identified in exon 2 (present in both K8A and K8B). However, the leucine zipper motif is present only in the third exon of K8. The spliced forms of K8 do have limited homology with EBV Z functional domains.

To confirm the presence of the spliced transcripts identified by 3′ RACE and cDNA library screening, RT-PCR was performed with gene-specific primers and RNA isolated from TPA-induced BCBL-1 cells (Fig. 4C). The primers used in these experiments were designed to span the identified splice sites. The cloned 3′ RACE product was used as a positive control. Amplification of genomic DNA was seen with primers 40 and 49 (product size 2351) in the RT− lane. A 1600-bp product is present in the RT+ lane, consistent with the predicted spliced transcript size of 1611. A second set of primers (46 and 49) was also designed that spanned the entire genomic ORF50 (72633–76664). This time, the genomic DNA product (expected size, 4031) was not seen, but a 3300-bp product is seen in the RT+ lanes, consistent with the predicted spliced bicistronic transcript size of 3291 bp.

ORF50 encodes a proline-rich transactivator function

We constructed a fusion protein expression vector with ORF50 linked to the yeast GAL4 DNA binding do-

FIG. 1. (A) Northern blot of ORF50 transcripts after induction with TPA treatment. A blot of poly(A)$^+$ RNA from TPA-treated BCBL-1 cells was probed with the ORF50/K8 random primed probe (top panel) and actin (bottom panel). Time in hours after induction is indicated above each lane. "Mock" refers to cells mock treated with DMSO and harvested at 120 h. Approximate sizes of the major transcripts are indicated at the left. (B) Time of peak transcript accumulation. Quantification of the predominant transcripts identified in the blot shown in panel A was performed using phosphorimaging (ImageQuant; Molecular Dynamics). The *y* axis represents the fold increase, and the *x* axis represents the time after induction. (C) ORF50 protein levels increase in TPA-activated BCBL-1 cells. Nuclear (N) and cytoplasmic (C) extracts were prepared from BCBL-1 cells after 24, 48, and 72 h of treatment with DMSO (D) or TPA (T). Equal amounts of total nuclear protein (10 μg) and cytoplasmic protein (50 μg) were loaded onto wells, separated by SDS–PAGE, and electroblotted onto nitrocellulose filter paper. ORF50 was identified with a polyclonal rabbit anti-ORF50 antibody (gift of D. Ganem).

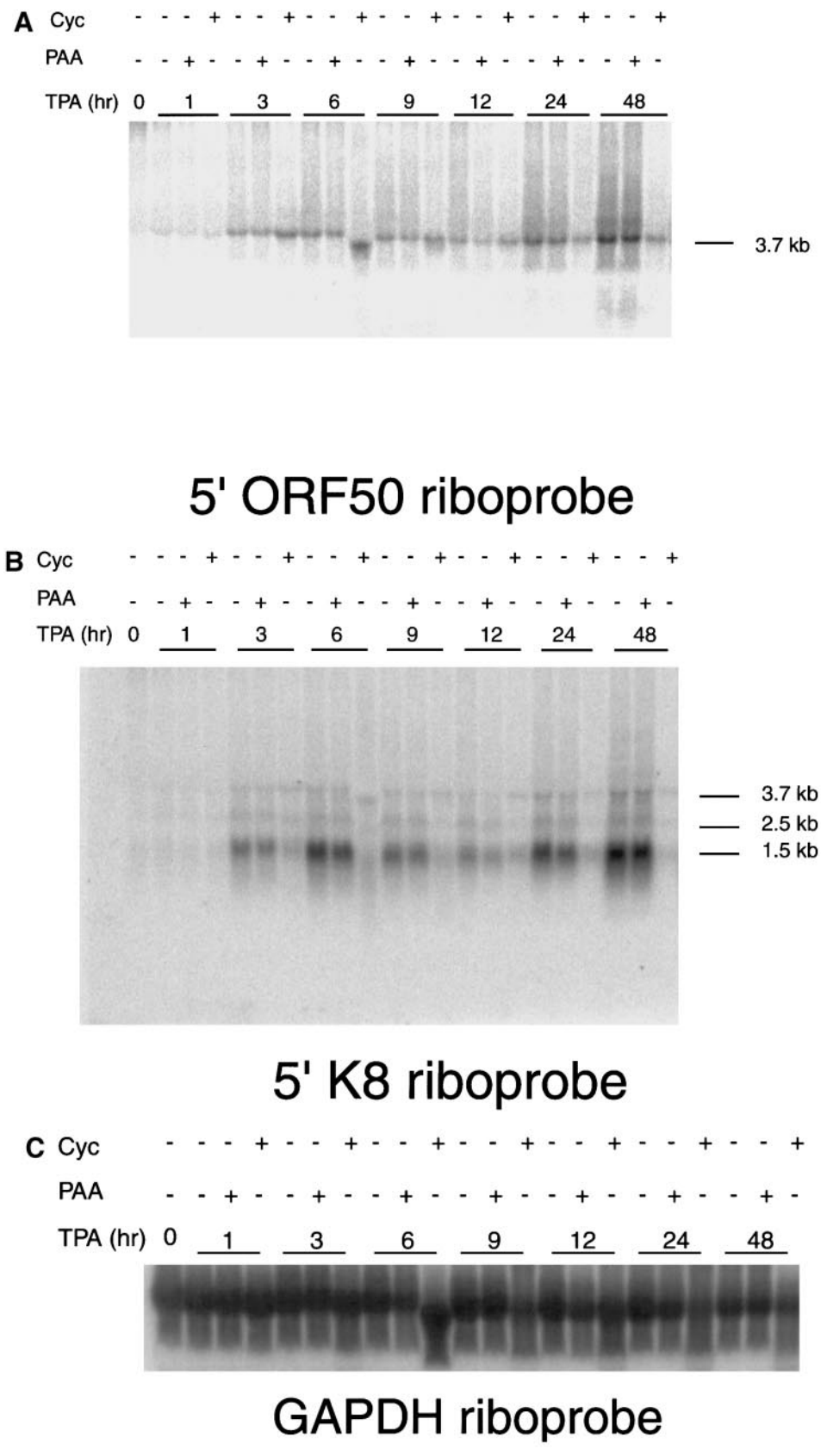


FIG. 3. Northern blot analysis of ORF50 RNA after TPA activation. Total RNA was isolated from BCBL-1 cells treated with TPA, Cyc (CYC), or PAA for the times indicated above each lane. RNA (10 μg) was electrophoresed and blotted to nylon membrane. The blot was stripped after each of the following hybridizations: (A) ORF50 riboprobe (complement 72570–72887), (B) K8-5′ riboprobe (complement 74810–74930), (C) GAPDH, (D) densitometry of 3.7-kb transcript, and (E) densitometry of 1.5-kb transcript (see Fig. 1 legend).

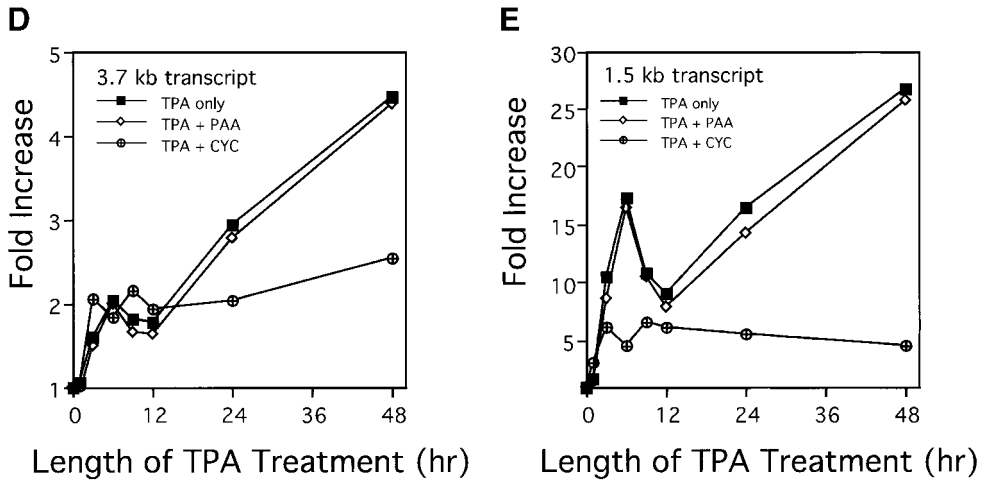


FIG. 3—*Continued*

main. BCBL-1 cells cotransfected with the construct encoding the GAL4-ORF50 fusion protein and a chloramphenicol acetyltransferase (CAT) reporter gene construct with upstream GAL4 binding sites (pGAL4-CAT) had significantly increased activity (Fig. 5A) compared with cells cotransfected with vector and pGAL4-CAT. These results indicated that the C-terminus of ORF50 contained a transactivator domain. Activation was present to a lesser degree in KSHV-negative cells. To further characterize the activation domain in ORF50, we constructed a series of deletion mutants (Fig. 5B). Deletion of amino acids 627–691 (d13) did not reduce activation at all. However, extending the deletion to amino acid 526 (d12) completely abolished activation, indicating the amino acid sequences between 526–626 were involved. Amino acids 548–626 and 524–626 restored activation to 10% and 40%, respectively, of that seen with the GAL4-ORF50 construct. The amino acids 450–626 were key to ORF50-mediated activation.

Regions upstream of ORF50 and K8 are responsive to ORF50 but not K8

To determine the regulatory potential of ORF50 in the control of gene expression in the ORF50/K8 region, additional transfection studies were performed (Fig. 6). Regions upstream of the ORF50 and K8 start sites were cloned upstream of a CAT reporter gene in both sense and antisense orientations (Lin *et al.,* 1999; Sun *et al.,* 1998). CAT expression from these constructs was compared with that from a promoterless construct cotransfected with an expression vector, pcDNA3. In the background of KSHV-1 latently infected cells, both K8 and ORF50 upstream regions had basal promoter activity. The K8 promoter had threefold activity in either sense or antisense orientation, whereas the ORF50 promoter only had activity in the sense orientation (>fourfold).

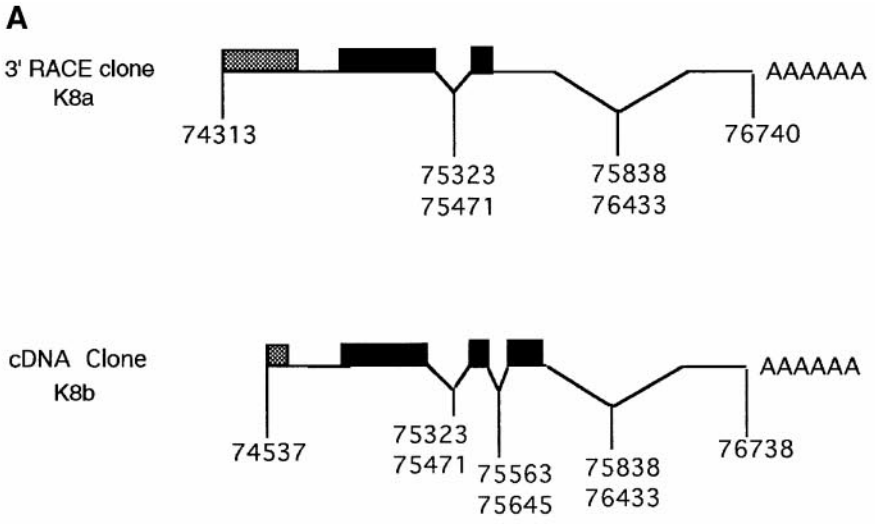
Cotransfection with a CMV expression vector directing ORF50 or K8b expression was also performed. The K8b construct did not produce activation from any of the K8 or OF50 promoter constructs. ORF50 expression produced a marked increase in K8 promoter activity (19-fold). Activation of the ORF50 promoter was also seen, although not to the degree seen with the K8 promoter (12-fold). The antisense K8 promoter construct also demonstrated responsiveness to ORF50 in some experiments, but this observation was not a consistent finding.

DISCUSSION

ORF50 has recently been shown to reactivate KSHV from latency (Lukac *et al.,* 1998; Sun *et al.,* 1998). Our results and those of others (Lin *et al.,* 1999; Lukac *et al.,* 1998; Sun *et al.,* 1998) provide the formal basis for understanding events that occur in the ORF50 region of KSHV genome after chemical induction. Rapid induction of complex transcription from the ORF50/K8 region was seen after TPA treatment of a KSHV-infected cell line. A concomitant increase in the level of nuclear-associated ORF50 protein was observed after TPA induction. The molecular masses of the predominant forms of ORF50 are larger than the predicted masses; posttranslational processing is the likely explanation. Phosphorylation may provide the extra mass as is the case with Z (Baumann *et al.,* 1998) and R (unpublished data). The smaller forms may represent translation products from truncated mRNAs. It is likely that the posttranslational modifications of ORF50 control specific functions of the protein.

ORF50/K8 transcription is both sensitive and resistant to Cyc treatment

At timepoints of 1–12 h, only the 3.7-kb transcript was resistant to Cyc treatment. This characterized only the 3.7-kb transcript as immediate-early type. After 12 h, the accumulation of both transcripts was sensitive to Cyc.



B

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                                                                EBV Z Activation domain
                                                    PDPYQVPFVQ AFDQATRVYQDLGGPSQA PLPCVLWPVL
                                                    |  :|  :     |   |  | |     ||| |
K8a   MPRMKDIPTKSSPGTDNSEKDEAVIEEDLSLNGQPFFTDNTDGGENEVSWTSSLLSTYVGCQPPAIPVCE TVIDLTAPSQS.GAPGDE HLPCSLNAETKF
K8b   MPRMKDIPTKSSPGTDNSEKDEAVIEEDLSLNGQPFFTDNTDGGENEVSWTSSLLSTYVGCQPPAIPVCE TVIDLTAPSQS.GAPGDE HLPCSLNAETKF

                                                                  EBV Z Bzip signature domain
                                                      SLEECDSELE IKRYKNRVASRKCRAK FKQ
                                                      | ||        | . |      :|  :|
K8a   HIPDPSWTLSHTPPRGPHISTLSHTPPRGPHISQQLPTRRSKRRLHRKFEEERLCTKAKQGAGRPVPASVVKAEVCDQSHS PTRKQGRYGRVSSKAY TRQ
K8b   HIPDPSWTLSHTPPRGPHISTLSHTPPRGPHISQQLPTRRSKRRLHRKFEEERLCTKAKQGAGRPVPASVVKAEVCDQSHS PTRKQGRYGRVSSKAY TRQ

      (Bzip cont.)
        LLQHYREVAAAKSSENDRLRLLLKQMCP SLDVDSIIPRTPDVLHED LLNF*
        | |   |  |        ||      |       |          |        |
K8a     LQQV*
K8b     LQQALEEKDAQLCFLAARLEAHKEQIIF LRDMLMRMCQQPASPTDA PLPPC*
                                .   :    |   |. |    |. | || ||
                              PDPYQVPFVQ AFDQATRVYQDLGGPSQA PL.PCLWPVL
                                        EBV Z Activation domain
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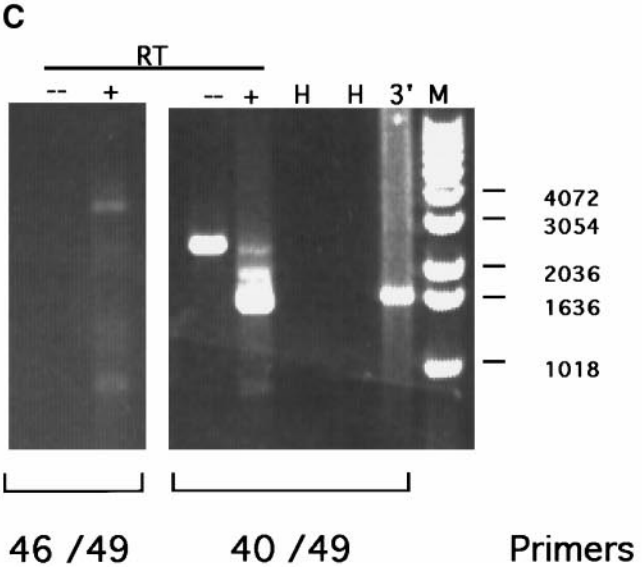

This indicates that efficient production of both transcripts requires protein synthesis and occurs in the early phase of the lytic cyle. In the BC-1 cells (Sun *et al.,* 1999), even 10 μM Cyc markedly inhibits ORF50 and K8 transcription at early timepoints. The induction of the ORF50/K8 message (3.7 kb) is modest (~fivefold) in the BCBL-1 cells compared with the marked increase seen by Sun *et al.* (1999). The BC-1 cell line is inducible with *n*-butyrate, but in the absence of induction, this cell line is tightly latent (Sarid *et al.,* 1998). The BCBL-1 cells, on the other hand, have an appreciable rate of spontaneous lytic replication (Lennette *et al.,* 1996). The differences in ORF50/K8 transcription may explain in part the different rates of spontaneous lytic replication seen in the two cell lines.

Functional domains of ORF50 are colinear to functional domains in R

A sequence analysis of ORF50 reveals two weakly conserved regions at either end of the amino acid sequence. The functional domains of EBV R have proved to be difficult to map (Hardwick *et al.,* 1992). The DNA binding domain is located in the amino half of the protein but is readily disturbed by further deletions. The entire region is unlikely to have contact with the DNA recognition site; instead two or more separate subdomains within the functional domain may be required. Alternatively, the entire region may be required before a complicated tertiary structure can hold the contact region exposed for DNA recognition. The R activation domain is very large and complex with two proline-rich and two acidic domains. Dissection of these regions reveals a strong transcriptional activator that can function when one or more of these regions are absent. Similar stretches of proline-rich regions occur in the carboxyl region of ORF50 (amino acids 385–600). The deletional analysis that we performed revealed that the amino acids centered around amino acid 580 were required before significant activation was seen. Thus a series of proline-rich regions in ORF50 encode a complex activation domain that contribute to ORF50 activation.

Reporter gene assays identified ORF50 and K8 promoter elements capable of increasing transcription twofold to fourfold in a KSHV-positive cell line. The basal level of ORF50 promoter activity was greater than that of K8, suggesting that K8 expression is more tightly regulated in these cells. ORF50 activated both the ORF50 and the K8 promoter elements. Similarly, EBV R is able to activate its own promoter through a nonbinding mechanism (Zalani *et al.,* 1992). Promoters that are much more strongly activated by the EBV R such as the BMLF1 require DNA binding (Gruffat *et al.,* 1992). Although no basal or induced activation was found with the ORF50 promoter in the antisense orientation, the basal CAT activity of the K8 promoter was independent of orientation. Additionally, K8 promoter in the antisense orientation was activated by ORF50 in some experiments. These observations suggest the presence of an ORF50-responsive enhancer element or bidirectional promoter located within this region. ORF50 induced CAT activity using the K8 upstream region. The level of activation seen here is quite striking. The EBV R is also capable of up-regulating Z promoter expression (Holley-Guthrie *et al.,* 1990; Kenney *et al.,* 1989; Zalani *et al.,* 1996). Additionally, ORF50 was capable of inducing expression using sequences 5′ to the ORF50 transcription start site. This is also similar to EBV R, which is capable of inducing activity from its own promoter. EBV Z is a known transactivator, but the KSHV K8B product expressed downstream of the CMV IE promoter did not induce CAT gene expression from the ORF50 promoter. However, this by no means excludes the possibility that K8B is a transcriptional activator. The activation may require additional DNA target sites that were not provided in our constructs.

In contrast, differences between EBV and HHV-8 transcripts were detected. Initial characterization of the 3′ RACE PCR product and the cDNA clones revealed that both ORF50 and K8 coding sequences are present in the same transcript. This is analogous to the bicistronic transcript encoding EBV R and Z (Manet *et al.,* 1989) Both EBV proteins can be translated from this bicistronic mRNA. In addition, EBV Z is translated from a monocistronic transcript that does not contain R coding sequence. The 1.5-kb K8 transcript is analogous to this transcript; however, the homologous region in EBV produces a transcript with a fusion ORF consisting of amino acids from both R and Z DNA binding domains. This transcript has an alternative splicing arrangement and encodes a fusion of R and Z (RAZ) (Furnari *et al.,* 1994). RAZ has been shown to repress the function of Z. A homologous transcript from KSHV was not identified, but the truncated form of K8 (K8A), which does not include exon 3, may perform a similar negative regulatory function. Because the organization of these transcripts is

FIG. 4. (A) Maps of the ORF50 3′ RACE product and the cDNA library clone. ORFs are indicated by filled boxes: ORF50 (gray) and K8 (black). The acceptor and donor splice sites are indicated beneath each figure. (B) Sequence alignment of the 3′ RACE and cDNA library transcripts. The amino acid sequences of the K8 transcripts identified by 3′RACE (K8a) and cDNA library screening (K8b) are aligned with the activation and Bzip domains of the EBV Z protein (underlined). Conserved amino acids are indicated (|). Leucines in a potential leucine zipper motif are indicated in bold. (C) ORF50 RT-PCR. Lanes with (+) and without (−) RT are indicated above the lanes; H indicates water, and M indicates bp marker. 3′ indicates lane with cloned 3′ RACE product used as a template. Primers used are indicated below the figure and molecular mass marker sizes are indicated on the right.

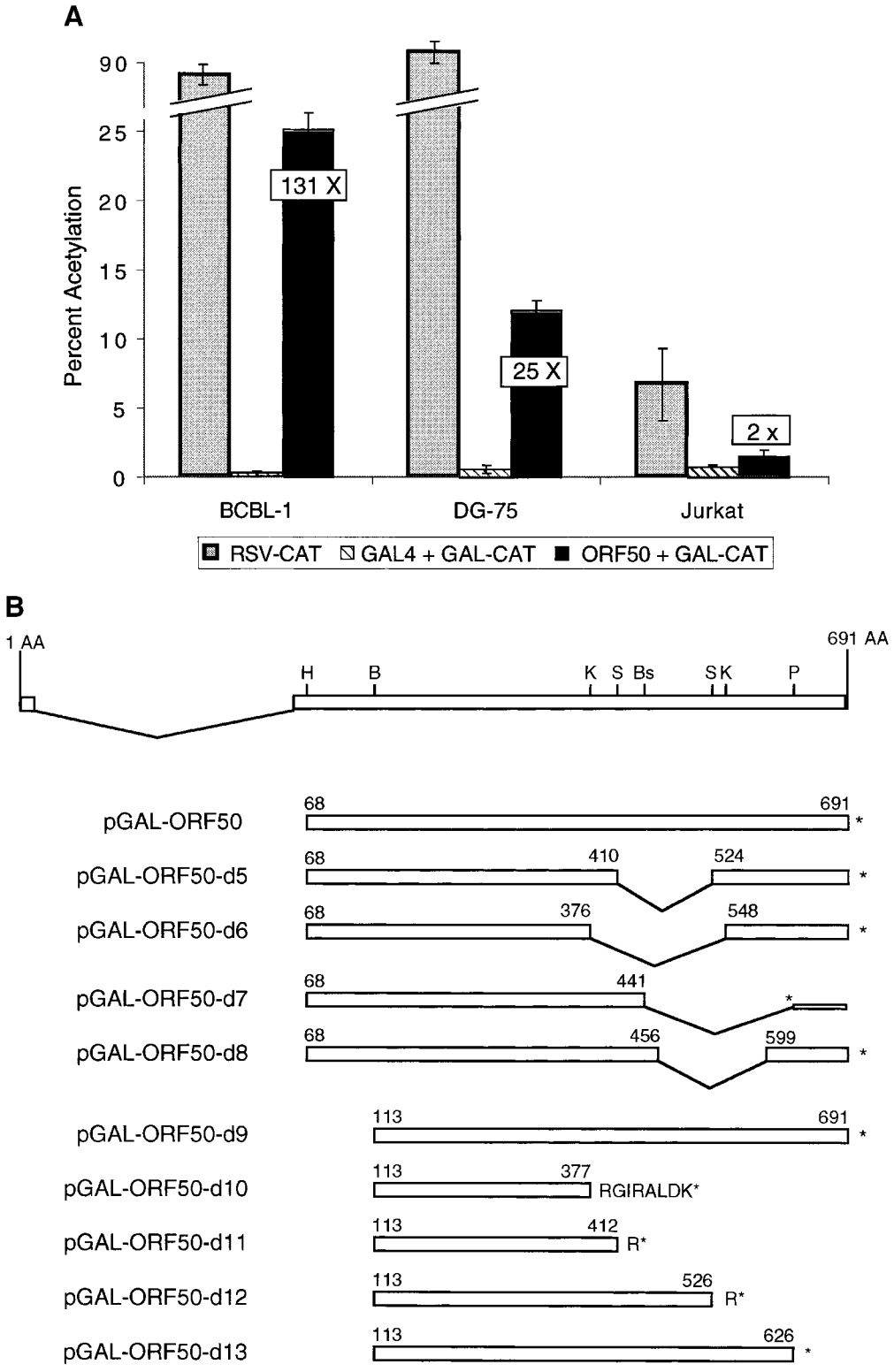

FIG. 5. (A) Transactivation of a minimal promoter construct containing five GAL4 response elements in BCBL-1 cells by gal4-ORF 50. A construct containing five GAL4 response elements upstream of a minimal adenovirus E1B promoter linked to CAT (GAL4-CAT) was cotransfected with the following plasmids: pGAL4 expresses a protein with the GAL-4 DNA binding domain without a transactivator domain (GAL4), pGAL4-ORF50 expresses a chimerical protein with the GAL-4 DNA binding domain fused to the ORF 50 of HHV8 (ORF50), and for RSV-CAT, Rous sarcoma virus LTR is linked to CAT. The cell lines used are indicated at the bottom of the figure (BCBL-1, DG75, Jurkat). The *y*-axis indicates percent acetylation. Fold activation by pGAL-ORF50 is indicted over each column. The error bars indicate the standard deviation. (B) GAL4-ORF50 deletion mutants. The region included in each construct is indicated by a box. Numbers refer to amino acids of full-length ORF50. *Stop codon. H indicates *Hin*dIII; B, *Bam*HI; K, *Kpn*I; S, *Sac*I; Bs, *Bst*EII; and *P*, *Pvu*I. (C) Activity of GAL4-ORF50 deletion mutants. The construct used is indicated on the *x*-axis. Percent acetylation is indicated on the *y*-axis. Fold activation by each construct is indicated over each column. If fold activation is not indicated, the activation was no greater than the activity seen with the GAL4 vector alone (1×).

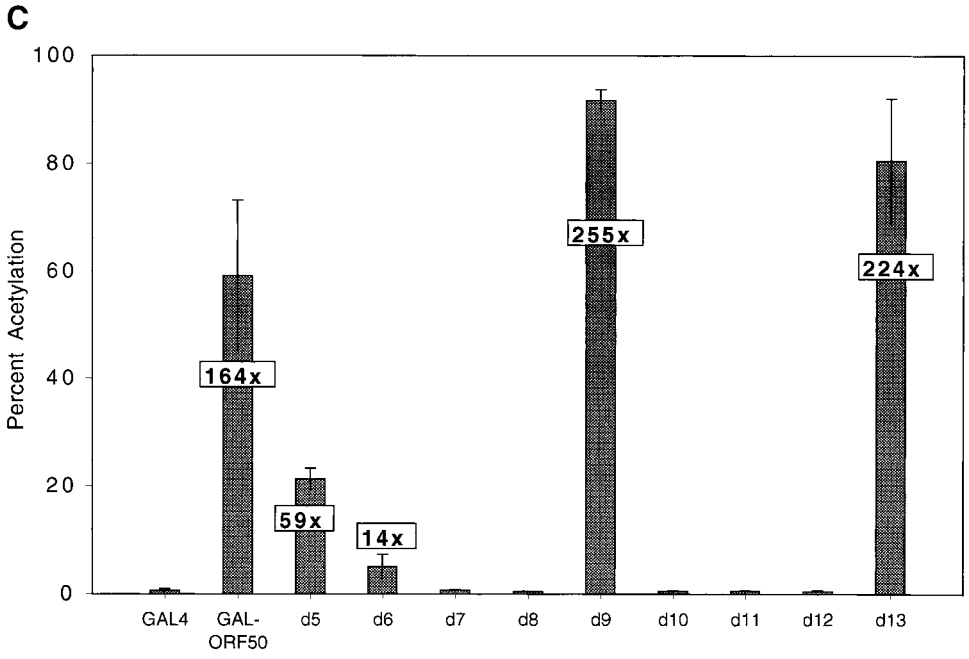

FIG. 5—*Continued*

somewhat different from that of EBV R and Z mRNAs, it is tempting to speculate that the encoded proteins perform alternate functions for KSHV. Further experiments will be required to determine whether this is a possibility.

We characterized the expression from the ORF50/K8 region to identify early expression events after KSHV reactivation. A preliminary description of early events after KSHV reactivation may now be attempted. ORF50 and TPA have both been shown to reactivate KSHV expression. Here we show that the region upstream to the ORF50 coding sequence has basal promoter activity as well as ORF50-inducible activity. After TPA treatment, ORF50 transcripts are amplified, leading to increased ORF50 protein accumulation. Transfection of ORF50 increases K8 promoter-directed transcription in the context of KSHV-infected cells. To a lesser degree, ORF50 also positively autoregulates its own expression, potentially amplifying viral reactivation in the absence of intervening events. Experiments using chemical inhibition of protein and DNA synthesis characterized the ORF50 transcript as immediate-early mRNA with a second accumulation phase. The second phase of ORF50 production as well as the K8 transcription may depend on ORF50 protein production. ORF50 then activates expression from the K8 promoter and, to a lesser extent, from its own promoter. At present, the functional role of K8 and additional events downstream of ORF50 expression are not known. Characterization of cell-type specific functions of ORF50, interactions between ORF50 and K8, and the individual roles these proteins play in the cascade of replicative gene expression await further experiments.

MATERIALS AND METHODS

Cell lines

Jurkat is a human T cell line, HeLa is human papillomavirus-infected cervical carcinoma cell line, DG75 is a human B cell line, and BCBL-1 (gift of D. Ganem) (Renne *et al.,* 1996) is a KSHV-infected human primary effusion lymphoma cell line. All cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin. Induction of KSHV transcripts was accomplished by treating BCBL cells with 20 ng/ml TPA (Sigma).

Plasmids

The plasmid pCMV-50 was created by inserting a 2-kb fragment (72888–74930; gift of Yuan Chang and Patrick Moore) into the *Bam*HI–*Not*I sites of pcDNA3, producing pSGORF50. ORF5–5′ (71596–71613/72572–72590; 5′-cccgggatccatggcgcaagatgacaag GGTAAGAAGCTTCGGCGGT-3′) and ORF50–3′ (complement of 72933–72953; 5′-cccgctgcatgcggctgtca-3′) oligonucleotides were synthesized at the UNC-CH Nucleic Acid Core Facility. ORF50–5′ and ORF50–3′ were used to PCR amplify the 5′ end of the ORF50 cDNA (Sun *et al.,* 1998) with BCBL-1 DNA as a template. After amplification, the PCR fragment was digested with *Bam*HI and inserted into the *Bam*HI site of pSGORF50 to obtain pCMV-50. Sequencing was performed to confirm that the open reading frame was maintained.

A K8 expression vector (pCMV-K8) was constructed by placing full-length K8 cDNA under the transcription con-

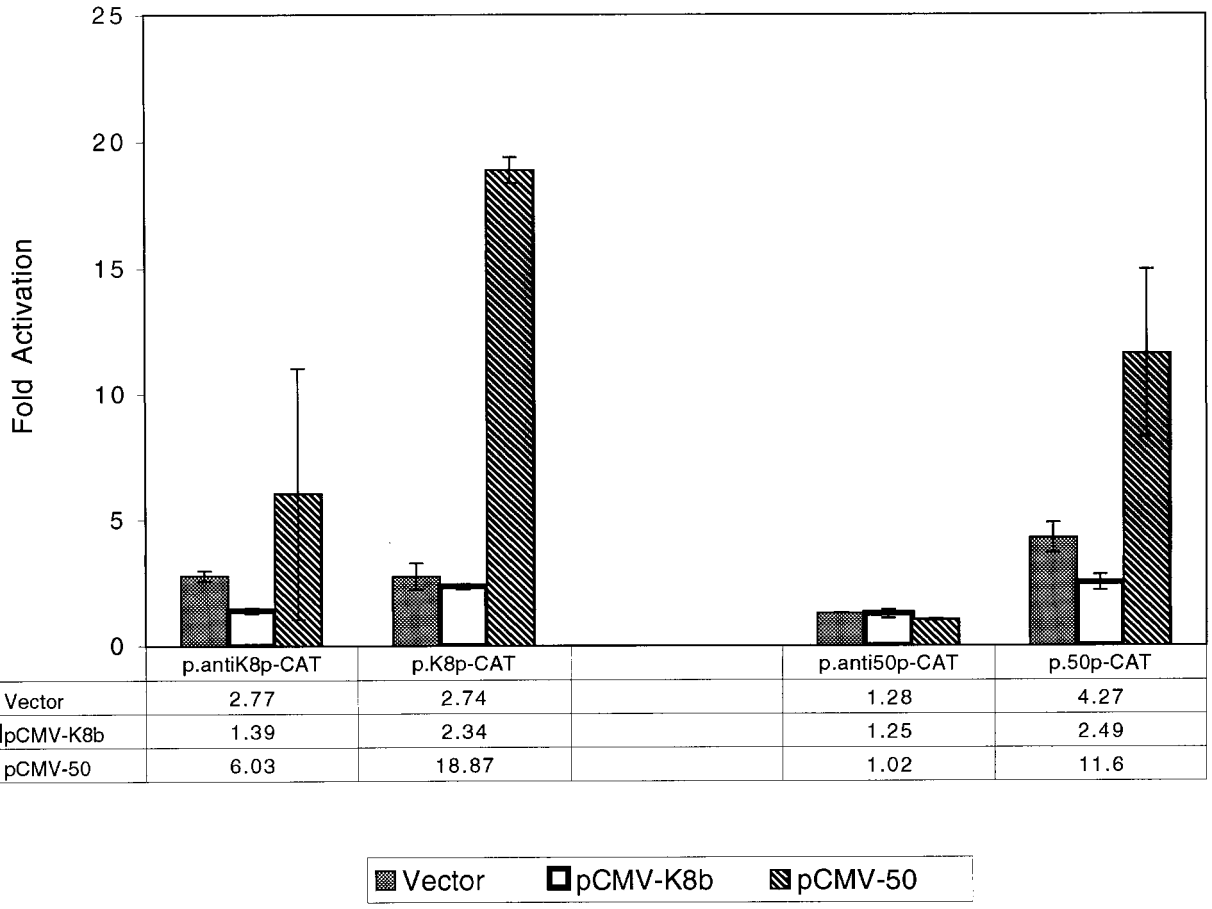

FIG. 6. Identification of the ORF50 and K8 promoter regions. BCBL-1 cells were transfected with vector, pCMV-K8b or pCMV-ORF50, and CAT reporter genes under the control of K8 upstream region in the antisense (p.anti-K8p-CAT) or sense (p.K8p-CAT) orientation or the upstream ORF50 region in the antisense (p.antiORF50p-CAT) or sense (p.ORF50p-CAT) orientation. Fold activity was compared with that seen with cotransfection of the expression vector and a promoterless CAT construct.

trol of the CMV promoter. A 1000-bp *Bgl*II–*Xba*I (nt 74930–75323/75471–75563/75645–75838/76432–76738) fragment was isolated from a clone identified by cDNA library screening. A 120-bp *Sal*I–*Bgl*II fragment (74810–74930) was isolated from 3′ RACE clone. This 120-bp fragment contains the remaining K8 coding sequence. These two fragments were inserted into pcDNA3 to produce the pCMV-K8 construct. The construction was confirmed by sequencing.

The promoterless CAT plasmid pGCAT-A (Frebourg and Brison, 1988) was used to construct reporter plasmids containing putative HHV-8 promoters. A 655-bp fragment (70934–71589) 5′ to the ORF50 transcription start site (71560) was PCR amplified from TPA-treated BCBL-1 cells using the primers PORF50–5′ (5′-cccggatcctgtgtctcccgtgggacaaa-3′) and PORF50–3′ (5′-cccggatccggctgcctggacagtattct-3′). The PCR product was digested with *Bam*HI and inserted into the *Bam*HI site of pGCAT-A in the sense and antisense orientations to obtain p.50p-CAT and p.anti50p-CAT, respectively. A 557-bp *Dde*I–Sa1I fragment (74253–74810) from pORF50 was ligated into the *Bam*HI site of pGCAT-A in the sense and antisense orientations to obtain p.K8p-CAT and p.antiK8p-CAT, respectively.

A *Hin*dIII fragment (72749–75791) (Russo *et al.,* 1996) from pORF50 was inserted into the pSG424 vector (Ma and Ptashine, 1987) in frame with the yeast GAL4 DNA binding domain to yield pGAL-ORF50. The pGAL4-CAT contains five GAL4 recognition sites upstream of a minimal adenovirus E1b promoter. Deletions in the ORF50 coding sequence were made as follows: pGAL-ORF50-d5 was created with *Sac*I digestion and inframe religation, removing nt 73787–74129; pGAL-ORF50-d6 was created with *Kpn*I digestion and inframe religation, removing nt 73683–74196; pGAL-ORF50-d7 was created with *Bst*EII digestion and out-of-frame religation, removing nt 73876–74451; and pGAL-ORF50-d8 was created with partial *Apa*I digestion and inframe religation removing nt 73927–74348. The pGAL-ORF50-d9 was constructed by inserting the *Bam*HI (72888)–*Bgl*II (74930) fragment from pcDNAORF50 into the *Bam*HI–*Xba*I site of pSG424. The plasmid pGAL-ORF50-d10 was constructed by inserting the *Bam*HI (72888)–*Kpn*I (73683) fragment from pcDNAORF50 into the *Bam*HI–*Kpn*II site of pSG424.

The plasmid pGAL-ORF50-d11 was constructed by inserting the *Bam*HI (72888)–*Eco*ICRI (73787) fragment from pcDNAORF50 into the *Bam*HI–*Eco*ICRI site of pSG424. The plasmid pGAL-ORF50-d12 was constructed by inserting the *Bam*HI (72888)–*Kpn*I (74196) fragment from pcDNAORF50 into the *Bam*HI–*Kpn*II site of pSG424. The plasmid pGAL-ORF50-d13 was constructed by inserting the *Bam*HI (72888)–*Pvu*II (74428) fragment from pcDNAORF50 into the *Bam*HI–EcoICRI site of pSG424. *Eco*ICRI is a neoschizomer of *Sac*I and leaves a blunt end. All plasmids contained the GAL4 nuclear localization domain (Silver *et al.,* 1984). All plasmids were sequenced to verify the ligation site, and only the *Bst*EII deletion produced a frameshift.

Northern blots

Poly(A)$^+$ RNA was extracted using the Ultraspec RNA Isolation System (Biotecx) and then running the total RNA over an Oligotex mRNA Mini Kit (Qiagen). The RNA was electrophoresed and transferred to a nylon membrane. The blot was baked at 80°C for 1 h, followed by UV cross-linking. Random primed ORF50 probe spanned 72749–75791 nt. A 1700-bp *Eco*RI–*Xho*I human actin cDNA random primed probe was used to detect actin mRNA. Antisense RNA probes were prepared using a Promega Riboprobe kit. ORF50 riboprobe spanned the complement of 71596–71613 and 72570–72887. K8-5′ riboprobe spanned the complement of 74810–74930. GAPDH hybridization was used to correct for variations in RNA loading. The blot was incubated with the following prehybridization solution for 5 h at 45°C: 5× standard saline citrate (SSC), 7% SDS, 50 mM sodium phosphate, pH 7.0, 50% formamide, and 2% blocking reagent Boehringer-Mannheim). The probe was denatured by incubation at 80°C for 10 min and then was allowed to hybridize to the membrane overnight at 45°C. The probe was removed, and the blot was washed twice with 2× SSC containing 0.1% SDS (15 min/wash) at room temperature, followed by washing twice with 0.5× SSC containing 0.1% SDS (15 min/wash) at 65°C. Quantification of the transcripts was performed by phosphorimaging of the blot (ImageQuant; Molecular Dynamics).

Chemical treatments of cells and Northern blotting

BCBL-1 cells were treated with 20 nM TPA. For PAA treatment, cells were incubated simultaneously with 20 nM TPA and 100 μM PAA. For Cyc treatment, cells were incubated simultaneously with 20 nM TPA and 50 μg/ml Cyc. Total RNA was isolated using Trizol reagent (GIBCO BRL) according to the manufacturer's specifications. Ten micrograms of total RNA from each sample was electrophoresed on a 1% agarose gel containing 1% formaldehyde. RNA was blotted to nylon membrane (Nytran; Schleicher & Schuell) using 10× SSC. The blot was incubated with prehybridization solution (5× SSC, 50% formamide, 50 mM sodium phosphate, pH 7.0, 7.0% SDS, 2% Boehringer-Mannheim blocking reagent) at 65°C. The blot was hybridized with riboprobes at 65°C in prehybridization solution. The blot was washed twice with 2× SSC at room temperature and twice with 0.5× SSC at 65°C. Hybridized probes were detected by phosphorimaging.

3′ RACE

Poly(A)$^+$ RNA was prepared as described above and subjected to a first-strand RT-PCR (Superscript) using an oligo(dT) primer. The second-strand cDNA was made using a poly(T) oligomer and the 3′ RACE primer (primer 40, ATCAGAGTCTATTCGCCC). The product was gel purified and cloned into pCRII vector. Sequencing of the insert was performed by automated sequencing (ABI 377).

RT-PCR

RT-PCR was performed according to the manufacturer's instructions (Superscript; GIBCO BRL). Primer sites used for these experiments are indicated in Fig. 2A. Primer 46 was CCCAACTCTACCAGTGTGTGCT, and primer 49 was CGTGGAACGCACAGGTAAAG.

cDNA library screening

Construction of the library in the ZAP express vector from TPA-stimulated BCP-1 cells and screening of the clones were performed according to the manufacturer's protocol (Stratagene, La Jolla, CA). The inserts were sequenced by automated DNA sequencing (ABI 377 sequencer).

Sequencing

DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (Perkin–Elmer, Applied Biosystems Division) using the ABI PRISMDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin–Elmer, Applied Biosystems Division)

Preparation of nuclear and cytoplasmic extracts

BCBL-1 cells (5×10^6) were treated with TPA at a final concentration of 20 ng/ml in DMSO for 24, 48, and 72 h. As a negative control, an equal amount of BCBL-1 cells were treated with an equal volume of DMSO only. After treatment, BCBL-1 cells were washed twice with PBS and pelleted by centrifugation at 3000 *g*. Cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM dithiothreitol) and centrifuged for 2 min at 4°C. The cells were gently resuspended in buffer A containing 0.1% Nonidet P-40 and 2 mM phenylmethylsulfonyl fluoride and incubated at 0°C for 10 min. Nuclei were pelleted by centrifugation at 3000 *g* for 5 min at 4°C. The supernatant was removed and saved as cytoplasmic extract. The nuclear pellet was washed

once with buffer A, and nuclei were pelleted by centrifugation at 3000 *g* for 5 min at 4°C. Nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 25% glycerol, 2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. Debris was removed by centrifugation at 12,000 *g* for 10 min at 4°C. The supernatant was removed and used as nuclear extract. Protein concentration were determined by the Bradford assay.

Western blot analysis of ORF50 protein

Nuclear and cytoplasmic extracts from BCBL-1 cells were subjected to SDS–PAGE analysis. For each timepoint, 10 μg of total nuclear protein and 50 μg of total cytoplasmic protein were loaded on the gel. Proteins were transferred to nitrocellulose membranes using a Fisher semidry blotting apparatus. Electroblotting was performed at 175 mA constant current for 30 min in Western blotting buffer (20 mM Tris, 150 mM glycine, 20% methanol). After the transfer, the membrane was incubated in 1× TBS (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and 1% blocking reagent (Boehringer-Mannheim) for 1 h. ORF50 protein was detected by incubating the blot with a 1:1000 dilution of rabbit polyclonal anti-ORF50 antibody (gift of D. Ganem) in 1× TBS. The blot was incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer-Mannheim) in 1× TBS. Protein was detected by ECL (Amersham) followed by autoradiography.

Transfections and CAT assays

Briefly, 10^7 cells were resuspended in 0.5 ml of RPMI 1640. Total DNA (20 μg) was added, and cells were electroporated with a BioRad gene pulser. The capacitor was charged to 950 F, and the cells received 0.25 V. After electroporation, the cells were resuspended in RPMI 1640 and 10% FCS. The cells were incubated at 37°C for 48 h, and protein extraction and CAT assays were performed as previously described (Quinlivan *et al.,* 1990). HeLa cells were transfected according to the method of Chen and Okayama (1987). Briefly, HeLa cells were seeded onto 60-mm plates at 6.5×10^5 cells/plate 24 h before transfection. Then 0.5 ml of transfection cocktail was prepared by combining 0.25 ml of 250 mM $CaCl_2$ with 0.25 ml of 2× BBS [50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2PO_4, pH 6.95]. Activator plasmid (3.5 μg) and reporter plasmid (3.5 μg) were added to the transfection cocktail. Cells were overlaid with the transfection cocktail, followed by incubation for 20 h at 35°C in 3% CO_2. Cells were washed twice with PBS, overlaid with DMEM and10% FCS, and incubated at 37°C in 5% CO_2. At 48 h after the start of transfections, the cells were washed twice with PBS and removed from the plates with a rubber policeman. Cell pellets were obtained by centrifugation at 3000 *g* for 5 min. Cells were suspended in 250 mM Tris, pH 7.5. Lysates were prepared by three cycles of freezing and thawing followed by centrifugation at 14,000 *g* for 5 min. Supernatants were recovered and used to determine CAT activity. Total protein was determined by the Bradford assay. Quantification was performed by phosphorimaging (ImageQuant; Molecular Dynamics).

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