JOURNAL OF VIROLOGY, May 1991, p. 2457–2466
0022-538X/91/052457-10$02.00/0
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Vol. 65, No. 5

Regulation of the Herpesvirus Saimiri (HVS) Delayed-Early 110-Kilodalton Promoter by HVS Immediate-Early Gene Products and a Homolog of the Epstein-Barr Virus R *trans* Activator†

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Received 28 November 1990/Accepted 7 February 1991

We have reported previously the detection of two stable immediate-early (IE) transcripts that accumulate in cycloheximide-treated cells infected with herpesvirus saimiri (HVS). These are the 1.6-kb mRNA from the 52-kDa gene (which is homologous to the BSLF2-BMLF1 gene of Epstein-Barr virus) and the 1.3-kb mRNA from the *Hin*dIII-G fragment of virus DNA. In order to study the roles of the HVS IE gene products in the progression of a lytic infection, the promoter region of the delayed-early 110-kDa gene of HVS was sequenced, the transcription initiation site was mapped by RNase protection, and the promoter sequences were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene. Sequences between −447 and +37 (relative to the 110-kDa transcription initiation site) were sufficient for response to HVS superinfection of transfected cells, but the 110-kDa promoter was activated only poorly by the 52-kDa and *Hin*dIII-G IE (IE-G) proteins in cotransfection experiments. However, a distinct region of the genome, *Eco*RI-D (15 kbp), was able to activate 110-kDa-CAT expression relatively efficiently in similar experiments. A 4.7-kbp *Pst*I fragment encoding this function was isolated and sequenced, and further subcloning identified the gene encoding the *Eco*RI-D *trans* activator. This gene, which we now designate HVS.R, is homologous to the BRLF1-encoded transcriptional effector of Epstein-Barr virus.

Herpesvirus saimiri (HVS) establishes asymptomatic latent infections in T lymphocytes of its natural host, the squirrel monkey (*Saimiri sciureus*), but causes fatal lymphomas and lymphoproliferative diseases in other species of New World primates. HVS has been classified as a gammaherpesvirus on the basis of its biological properties, and recent sequencing studies have shown it to be closely related to the human gammaherpesvirus Epstein-Barr virus (EBV) (1, 7, 17, 18, 23, 31). HVS productively infects epithelial and fibroblastic cells in culture, and this property has allowed the identification of more than 30 virus-specified proteins and the analysis of HVS gene regulation. As in other herpesviruses, the pattern of gene expression occurs in the three main temporal phases: immediate-early (IE), delayed-early (DE), and late. Two stable HVS IE transcripts that map to the 52-kDa and *Hin*dIII-G genes have been identified (31, 32). These genes are transcribed in the absence of de novo protein synthesis and thus require only cellular proteins for their expression. DE gene expression requires the prior synthesis of at least one of the IE gene products, whereas late gene expression occurs maximally after the onset of virus DNA replication (6, 32, 35). Thus, the viral IE genes encode the *trans*-acting proteins required for activation of DE gene expression and onset of the lytic cycle.

In EBV, three *trans* activators have been identified. These are the products of the BZLF1, BSLF2-BMLF1, and BRLF1 genes (4, 9, 10, 20, 28, 33, 42, 45). The HVS IE 52-kDa gene is homologous to the BMLF1 reading frame, and previous studies have shown that the 52-kDa protein is able to activate chloramphenicol acetyltransferase (CAT) expression from heterologous promoter-CAT constructs in transient transfection assays (31, 32). However, the role of the IE 52-kDa protein in the regulation of a productive HVS infection has not been determined. Similarly, no function for the product of the *Hin*dIII-G IE gene (IE-G) in the regulation of HVS gene expression has been demonstrated. In this report, we describe experiments designed to investigate the regulation of an HVS DE promoter, that of the 110-kDa gene (5, 6, 17, 35), by the HVS IE gene products. Our results show that although the 110-kDa promoter is weakly induced by the 52-kDa and IE-G proteins, the promoter is activated very efficiently by a gene product encoded by the *Eco*RI-D fragment of HVS L-DNA. The *Eco*RI-D *trans* activator is homologous to the EBV BRLF1 gene product. Therefore, of the three IE/*trans*-activating genes of EBV and HVS, two are structurally and functionally conserved. These results further demonstrate the similarities between these two gammaherpesviruses.

MATERIALS AND METHODS

DNA sequencing. The 110-kDa N-terminal coding and promoter regions were isolated as a 1.9-kbp *Kpn*I-*Pst*I fragment from p*Kpn*I-F (25). This fragment was sonicated, end repaired, and cloned into the *Sma*I site of M13mp18 as previously described (3). Clones were sequenced by the dideoxy-chain terminator procedure of Sanger et al. (41) to produce overlapping stretches of sequence which were assembled as previously described (7). The 4.7-kbp *Pst*I fragment from *Eco*RI-D (dD4.7) was sequenced by using the same procedures.

RNA preparation. Confluent monolayers of Vero cells (Flow Laboratories, Inc., Irvine, United Kingdom) grown at 37°C in Dulbecco modified Eagle medium containing 10%

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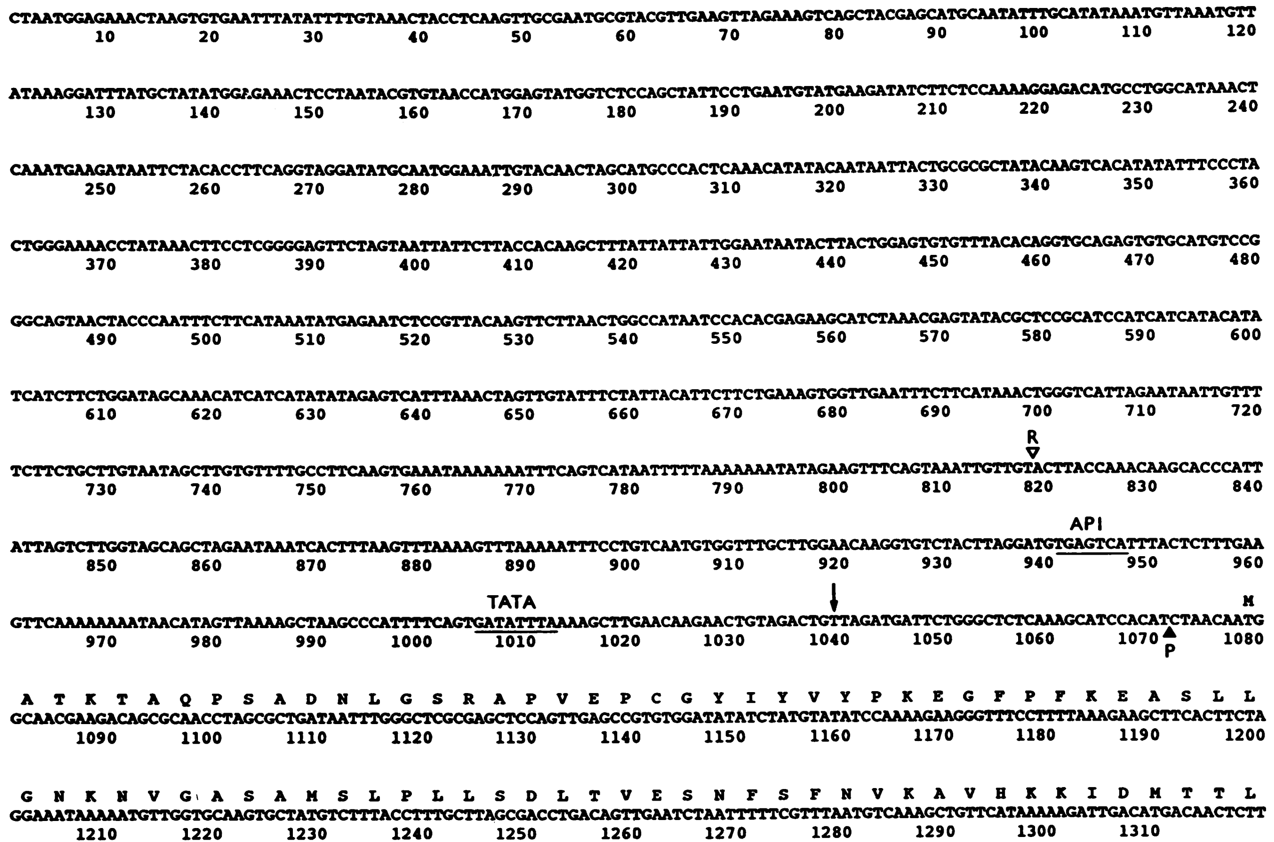

FIG. 1. Nucleotide sequence of the N-terminal coding and upstream region of the 110-kDa gene. The translation of the 5′ coding sequence is shown (nucleotides 1078 to 1320) in single-letter code, beginning at the predicted initiation methionine. Also indicated on the nucleotide sequence are the transcription initiation site (arrow, position 1039), putative AP1 binding site, and TATA box. The position of a *Pst*I cleavage site (P), introduced by mutagenesis and used to clone promoter sequences into CAT vectors (see Materials and Methods), and a *Rsa*I site (R), utilized for the generation of an SP6 probe for RNase protection mapping, are indicated by closed and open arrowheads, respectively.

newborn calf serum were infected with HVS strain 11(0) (14) at 50 PFU per cell or mock infected in the presence or absence of cycloheximide (50 μg/ml) as previously described (37). Cells were harvested at 20 h postinfection, and total RNA was prepared (11) and treated with DNase I (RQ1 DNase; Promega).

SP6 RNase protection. An M13 clone (m110K) containing a sonicated DNA fragment (nucleotides 1 to 1208 of the 110-kDa sequence [Fig. 1]) was digested with *Bam*HI and *Eco*RI (flanking sites in mp18 polylinker) to remove the insert, which was then cloned into the SP6 transcription vector pSP72 (Promega) to create pSP110K. [α-^{32}P]UTP-labeled antisense transcripts were made from the SP6 promoter, hybridized to 1 μg of total RNA, RNase digested, and analyzed on 6% acrylamide–8 M urea gels (41) essentially as described by Melton et al. (30), except that hybridizations were done in 60% formamide at 42°C because of the AT-rich nature of the HVS genome (22).

Plasmids. The CAT expression vector pUCATPV (gift from P. Vize) was constructed by cloning a 1.63-kb blunt-ended *Hin*dIII-*Bam*HI fragment from pSV0-cat (19) into the *Hin*dIII site of the pUC19 polylinker by using *Hin*dIII linkers. Plasmids p110CAT.A and p110CAT.AR were constructed by cloning a 484-bp *Sfa*NI end-repaired fragment (nucleotides 592 to 1075 of the 110-kDa sequence [Fig. 1]) into the *Sma*I site of pUCATPV. The p110CAT.A construct contains the 110-kDa promoter in the same transcriptional orientation as the CAT gene, whereas p110CAT.AR contains 110-kDa sequences in reverse orientation relative to the CAT gene. Plasmid p110CAT.B was constructed by cloning a 1.08-kb *Pst*I-*Sac*I fragment from the M13 clone m110kpst into pUCATPV. Clone m110kpst was generated by oligonucleotide site-directed mutagenesis (46) of m110K to insert a *Pst*I recognition site (CTGCAG) 6 bp 5′ of the 110K ATG codon, between nucleotides 1071 and 1072 of the 110-kDa sequence (Fig. 1). Digestion with *Pst*I and *Sac*I (in mp18 polylinker) generates a fragment containing nucleotides 1 to 1071 of the 110-kDa sequence. The plasmid pKS110CAT contains these same sequences cloned between the *Sac*I and *Pst*I sites of pKSSVOCAT (43).

For construction of IE expression clones, nucleotides 701 to 2220 of the 52-kDa gene sequence (31) and nucleotides 521 to 1555 of the *Hin*dIII-G gene sequence (32) were amplified by polymerase chain reaction with appropriate oligonucleotide primers. The amplified DNA fragments (containing the 52-kDa and IE-G coding sequences and 3′ RNA processing signals) were end repaired and cloned into the end-repaired *Eco*RI site of a modified pKSV-10 (Pharmacia) vector (*Bgl*II cloning site replaced by *Eco*RI site; a gift from E. Manet) to create pKSV52 and pKSVHG, respectively. The plasmid p*Hin*dIII-G contains the 3.0-kbp *Hin*dIII fragment from p*Eco*RI-F (25) cloned into the *Hin*dIII site of pUC8. The 52-kDa expression plasmid p*Eco*RI-(I/E′) contains 52-kDa gene sequences cloned into pUC8 (31). The plasmid

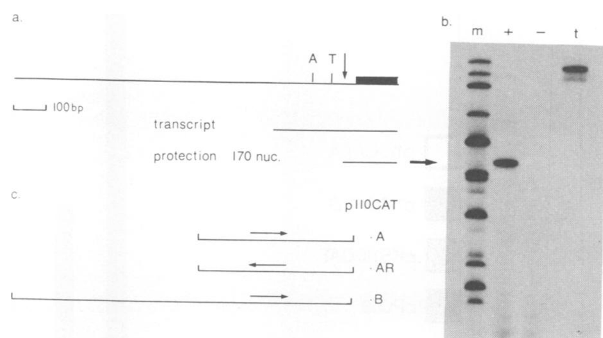

FIG. 2. Mapping the 110-kDa transcription initiation site. (a) Diagrammatic representation of the 110-kDa promoter and N-terminal coding regions. The AP1 motif (TGAGTCA) (A) and TATA box (T) are indicated, together with the transcription initiation site (arrow) determined by RNase protection assays by using a 443-nucleotide SP6-generated riboprobe (transcript). (b) This probe was annealed to RNA from HVS-infected (+) or uninfected (−) Vero cell monolayers before digestion with RNase A and fractionation of protected products on a sequencing gel alongside the SP6 transcript (t) and size markers (m; denatured ^{32}P-labeled pUC8 DNA cut with *Hpa*II). By using RNA from HVS-infected cells, a 170-nucleotide protected fragment was obtained, thus mapping the mRNA cap site to position 1039 of the sequence in Fig. 1. (c) The indicated promoter sequences were cloned into the vector pUCAT.PV to generate p110CAT.A, p110CAT.AR, and p110CAT.B (see Materials and Methods). Promoter sequences used to generate p110CAT.B were also cloned into the vector pKSSVOCAT to give pKS110CAT (see Materials and Methods).

p*Eco*RI-D contains the *Eco*RI-D fragment of HVS L-DNA cloned into the *Eco*RI site of pACYC184 (25). Subfragments of *Eco*RI-D were generated by restriction endonuclease digestion and cloned into pUC19 to generate pdD4.7, pdD3.0, pdD5.4, pdD9.6, and pdD1.9 (see text for details).

Transfection assays. Cells were transfected with plasmid DNA essentially as described previously (19). Calcium phosphate precipitates were applied for 10 h prior to changing the medium, and cells were harvested either 24 to 36 h (*Eco*RI-D and *Eco*RI-D subclone cotransfections) or 60 to 72 h (52-kDa and *Hin*dIII-G cotransfections) after changing the medium. Cells were assayed for CAT activity as described by Gorman et al. (19), and percentages of acetylation were calculated by the scintillation counting of appropriate regions of the chromotography plate.

Nucleotide sequence accession number. The 110-kDa and dD4.7 sequences reported in this article will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers M60849 and M60850, respectively.

RESULTS

Mapping the 110-kDa promoter. The location of the 110-kDa transcription start site was determined by SP6 RNase protection (30). The plasmid pSP110K (see Materials and Methods) was digested with *Rsa*I (Fig. 1), and a 443-nucleotide transcript was generated from the SP6 promoter. Following hybridization with HVS-infected and uninfected Vero cell RNA samples and RNase digestion, a 170-nucleotide protected fragment was generated (Fig. 2). This maps the 110-kDa gene cap site to nucleotide 1039, 39 bp 5′ to the ATG initiation codon. This was confirmed by additional protection experiments with different probes (data not shown).

Analysis of the promoter sequence reveals a consensus AP1 site (TGAGTCA) (26) at −98 and a TATA sequence at −34 from the cap site (Fig. 1). AP1 sites are also found in the promoter regions of the homologous EBV and varicella-zoster virus (VZV) genes and occur in analogous positions relative to putative TATA box elements (2, 13). A conserved AP1 site has also been found in the HVS IE 52-kDa promoter (31), and their functional relevance in the regulation of EBV gene expression (15, 44) suggests that they may play an analogous role in HVS.

Response of the 110-kDa promoter to HVS superinfection. Two fragments of the 110-kDa promoter containing sequences −447 to +37 and −1038 to +33 were cloned into the CAT vector pUCATPV (as described in Materials and Methods) to generate p110CAT.A and p110CAT.B, respectively. The −447 to +37 fragment was also cloned in reverse orientation relative to the direction of transcription of the CAT gene to generate p110CAT.AR (Fig. 2c). The constructs were transfected into Vero cells, superinfected with HVS, and assayed for CAT activity. The constructs had little activity in the absence of superinfection, but p110CAT.A and p110CAT.B were activated efficiently by HVS infection (Fig. 3A), indicating that sequences required for viral response are contained between −447 and +33. Since p110CAT.AR also responded to superinfection (albeit at reduced levels relative to those of p110CAT.A and p110CAT.B), these promoter sequences may contain an enhancer capable of activating transcription from a divergent cryptic promoter. The presence of an HVS-inducible promoter serving a divergent gene is a less likely explanation since the only open reading frame (ORF) found immediately 5′ to the 110-kDa gene is in the same orientation. The 3′ end of this 5′ ORF lies at nucleotide 488 of the sequence shown in Fig. 1.

Response of the 110-kDa promoter to HVS IE gene products. Two HVS IE genes have been previously identified; these are the 52-kDa and the *Hin*dIII-G genes (31, 32, 38). The 52-kDa gene, when used in cotransfection assays, is able to activate expression from heterologous promoter-CAT constructs (31, 31a). To determine if the products of the 52-kDa and *Hin*dIII-G genes could activate expression from the 110-kDa DE promoter and to enable a direct comparison of the relative efficacies of these proteins in *trans* activation, the 52-kDa and *Hin*dIII-G coding regions were placed under the control of the simian virus 40 (SV40) early promoter (see Materials and Methods) to create pKSV52 and pKSVHG, respectively. These plasmids, together with p52K [p*Eco*RI-(I/E′)] and p*Hin*dIII-G (containing the 52-kDa and *Hin*dIII-G genes cloned into pUC8), were used in cotransfection experiments with the target plasmids p110CAT.A, p110CAT.B, and pKS110CAT (see Materials and Methods). The results of these experiments are shown in Fig. 3B. Small increases in CAT activity were detected from each of the target plasmids in response to p52K, pKSV52, and pKSVHG, but no significant activation was detected with p*Hin*dIII-G. This is consistent with recent results from this laboratory mapping the initiation site of *Hin*dIII-G IE transcription to a position only 23 nucleotides from the right end (conventional HVS orientation) of *Hin*dIII-G (9a). Thus, one might expect poor expression of the IE-G protein from p*Hin*dIII-G. All four effector plasmids, however, were able to *trans* activate the herpes simplex virus type 1 (HSV-1) IE 175K promoter in the plasmid pPO13 (34) relatively efficiently, demonstrating that all of these plasmids are capable of expressing levels

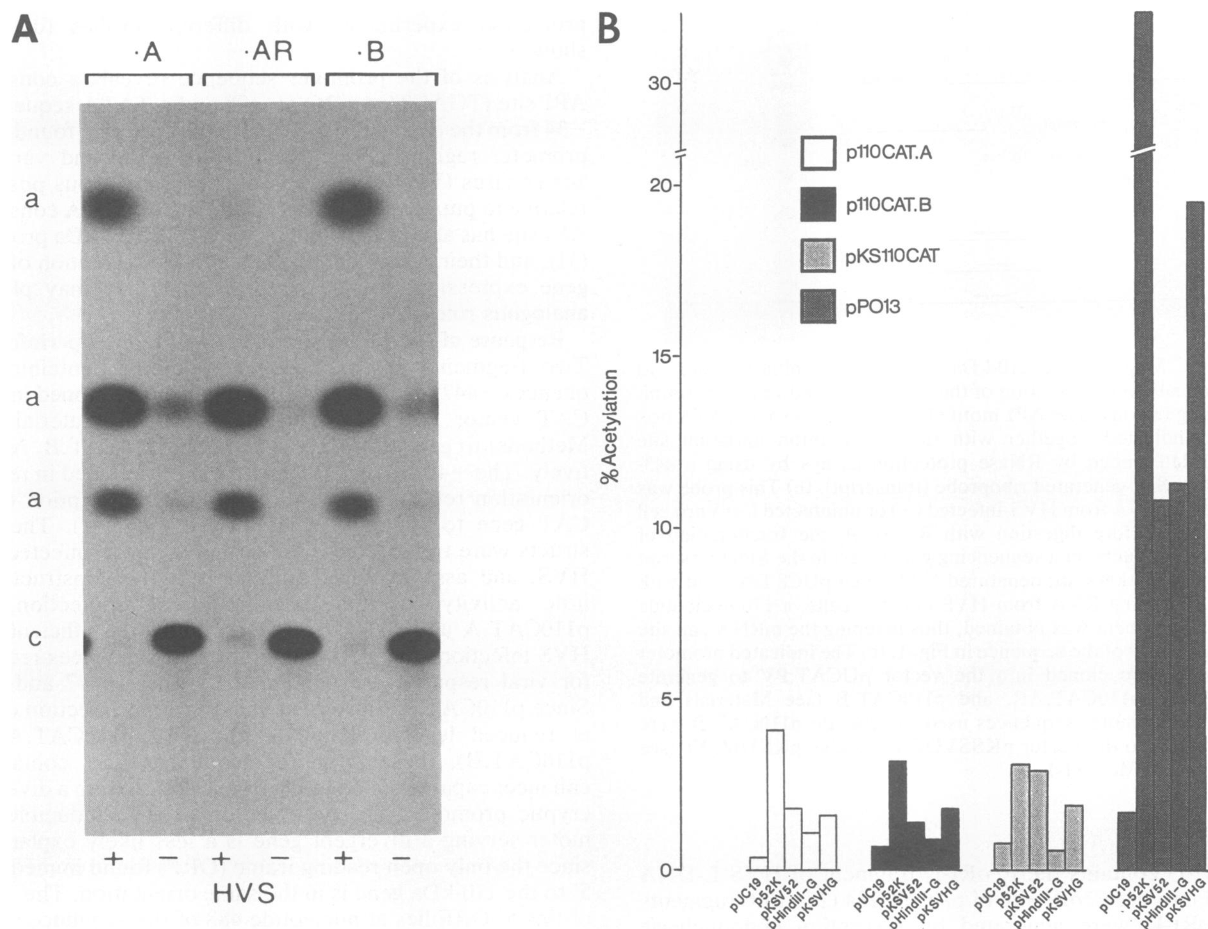

FIG. 3. Activation of the 110-kDa promoter by HVS superinfection (A) and HVS IE gene products (B). (A) Vero cell monolayers were transfected with 2 μg of p110CAT.A, p110CAT.AR, or p110CAT.B and subsequently were either superinfected with approximately 50 PFU of HVS per cell (+) or not infected (−). Cell extracts were made and CAT activities were assayed as described previously (19). The autoradiogram of the chromatogram showing the separated acetylated (a) and unacetylated (c) [^{14}C]chloramphenicol products is shown. (B) Results of cotransfections of p110CAT.A, p110CAT.B, pKS110CAT, and pPO13 (HVS IE 175K-CAT) (34) target plasmids with pUC19 or the IE 52-kDa or IE-G expression plasmids indicated. Amounts of target and effector plasmids used were 5 μg and 10 μg, respectively. The targets pKS110CAT and p110CAT.B both contain 110-kDa promoter sequences between −1038 and +33 relative to the transcription initiation site, whereas p110CAT.B contains sequences between −447 and +33.

of IE 52-kDa or IE-G protein sufficient for high levels of induction and that poor activation of the DE 110-kDa promoter cannot be explained merely by inadequate expression of these *trans* activators. Using various amounts of target and effector or using pKSV52 and pKSVHG in combination did not result in efficient activation of the 110-kDa promoter (data not shown). These results suggested, therefore, that additional HVS products provided by viral superinfection may be required for full activation.

***trans* activation function in *Eco*RI-D.** As no major IE transcripts other than the 52-kDa and *Hin*dIII-G mRNAs have been detected (32), we attempted to locate additional regulatory genes by comparing the locations of IE genes of the gammaherpesvirus EBV. Of the herpesviruses analyzed to date, HVS is most similar to EBV in the conservation of gene sequences and the arrangement of homologous genes within the genome (17). In addition to the BSLF2-BMLF1 gene (homolog of the HVS 52-kDa gene), EBV encodes two other known *trans* activators, the BZLF1 and BRLF1 gene products. It was predicted that homologs of these genes, if they were present, would be located in the *Eco*RI-D fragment of HVS. Consistent with this, cotransfection of the plasmid p*Eco*RI-D (25) with pKS110CAT, p110CAT.A, and p110CAT.B resulted in increased CAT activity (Fig. 4). To localize the sequences within *Eco*RI-D which were responsible for *trans* activating these 110-kDa-CAT target plasmids, we utilized known restriction endonuclease cleavage sites within the region (22a) to subclone fragments of *Eco*RI-D (Fig. 5A). Two *Eco*RI-*Bam*HI fragments of 5.4 kbp and 9.6 kbp, a 4.7-kbp *Pst*I fragment spanning the *Bam*HI site, and a deleted version of this *Pst*I fragment (*Xba*I deletion, 3.0 kbp) were cloned into pUC19 by using the appropriate restriction sites within the polylinker. Each of the resulting plasmids (10 μg) was cotransfected with pKS110CAT (5 μg) into Vero cells. The results of these experiments are shown in Fig. 5B. Neither of the two *Eco*RI-*Bam*HI clones (pdD5.4 and pdD9.6) were active, and using both of these plasmids together did not result in activation (data not shown). However, pdD4.7, containing sequences spanning the *Bam*HI site in *Eco*RI-D, was able to activate expression from pKS110CAT (approximately eightfold stimulation). The deletion of 1.7 kbp between the *Xba*I

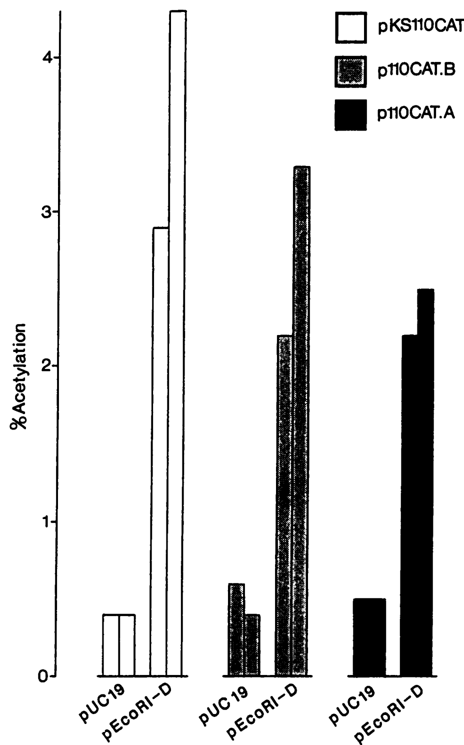

FIG. 4. Assays of CAT activities in extracts of Vero cells cotransfected with p*Eco*RI-D (10 μg) and pKS110CAT, p110CAT.B, or p110CAT.A (5 μg). The results of these cotransfections and of controls using pUC19 (10 μg) are shown here in duplicate. The 110-kDa promoter plasmids pKS110CAT and p110CAT.B contain sequences between −1038 and +33 relative to the transcription initiation site, whereas p110CAT.A contains sequences between −447 and +37.

and right *Pst*I sites (Fig. 5A, pdD3.0) abolished activity. Therefore, sequences within the 1.7-kbp *Xba*I-*Pst*I fragment were important for *trans* activation.

Identification of the *trans*-regulatory gene within *Eco*RI-D. As the 4.7-kbp *Pst*I fragment was sufficient for *trans* activation of the 110-kDa promoter, we sequenced this region (see Materials and Methods). The nucleotide sequence determined is shown in Fig. 6 and contains three ORFs (RF1, RF2, and RF3 in Fig. 5A). As predicted from their positions and orientations within the HVS genome relative to EBV, RF1 and RF3 are homologous to the EBV BRLF1 and BRRF1 genes, respectively. Thus, these genes and their relative locations are conserved between HVS and EBV. Although the BRLF1 gene product has been identified as a transcriptional activator, the function of the BRRF1-encoded protein is unknown. Alignments between the proteins encoded by these ORFs and their homologs in HVS are shown in Fig. 7. It is important to note that the first methionine codon in RF1 occurs far into the ORF (amino acid position 131), although the similarity between the RF1- and BRLF1-encoded proteins begins near their N termini. We would therefore predict splicing of the primary transcript from the RF1 gene, allowing the utilization of an initiator methionine codon from sequences upstream of this ORF, as is the case for the IE 52-kDa mRNA (9a, 31). The translation product of RF2, which is in the predicted position and orientation for the BZLF1 homolog, shows little similarity to the product of this ORF. However, the transcript from the BZLF1 gene is spliced, and this alters the predicted amino acid sequence in the C-terminal region of the protein (4). This contains the basic and coiled-coil domain involved in DNA binding and protein-protein dimerization, features which are characteristic of the bZIP family of transcription factors (8, 27, 36). It is possible that the mRNA from the RF2 gene is also spliced, which may reveal sequence similarities between the RF2 and BZLF1 proteins in their C-terminal domains.

As described above, the deletion of sequences between the *Xba*I site and the right *Pst*I site in pdD4.7 to give pdD3.0 (Fig. 5A) abolished *trans* activation of the 110-kDa promoter. Also, using the *Bam*HI-*Eco*RI subclones pdD9.6 and pdD5.4 either alone or in combination in cotransfections with pKS110CAT did not result in increased CAT activity. Thus, the *Xba*I and *Bam*HI sites occur in regions of the 4.7-kbp *Pst*I fragment important for *trans* activation, regions which are encompassed by RF1. We isolated and cloned RF1 and flanking sequences into pUC19 by utilizing the *Hin*dIII restriction sites present in the 4.7-kbp *Pst*I fragment (lower part of Fig. 5A). The resulting plasmid, pdD1.9, was used in cotransfections with pKS110CAT and was found to *trans* activate 110-kDa-CAT expression with high efficiency (32-fold) (Fig. 5B). Thus, RF1, the homolog of the BRLF1 gene of EBV, encodes the *trans*-acting function present in *Eco*RI-D. Because of the structural and functional conservation between the EBV R *trans* activator and the RF1 gene product, we now designate the HVS gene HVS.R.

DISCUSSION

In this report, we have investigated the roles of the IE 52-kDa and IE-G proteins in the regulation of HVS gene expression. For these studies, we chose to use the 110-kDa DE gene which specifies the HVS major DNA binding protein, the homolog of the ICP8, BALF2, and RF29 genes of HSV, EBV, and VZV, respectively. The promoter and N-terminal coding regions of the 110-kDa gene were sequenced and the transcriptional initiation site was determined, data which were then used to clone the 110-kDa promoter upstream of the CAT coding sequences. Promoter sequences from −447 to +37 (present in p110CAT.A) were found to be sufficient for activation of CAT expression by HVS superinfection of transfected cells and to be functional in inverse orientation relative to the CAT gene, suggesting the presence of an enhancer within this fragment. It is intriguing that the HVS 110-kDa promoter contains a sequence corresponding to a binding site for the AP1 transcription factor, as AP1 sites are also found in the promoter regions of the homologous EBV and VZV genes and occur in analogous positions relative to putative TATA box elements (2, 13). Sequence elements identical or closely related to AP1 sites have been shown to be important in *trans* activation of target promoters by the BZLF1-encoded protein, these motifs forming functional binding sites for this *trans* activator (8, 15, 16, 21, 27, 36).

Although the 110-kDa promoter responded well to HVS superinfection, it was activated only weakly when cotransfected with the two HVS IE genes, IE 52-kDa and IE-G, either alone or in combination. Unlike pKSVHG (containing the IE-G ORF under the control of the SV40 early promoter), the plasmid p*Hin*dIII-G did not effect significant activation of CAT activity in these cotransfection experiments, the most likely explanation being the lack of promoter sequences upstream of +22 relative to the IE-G transcriptional

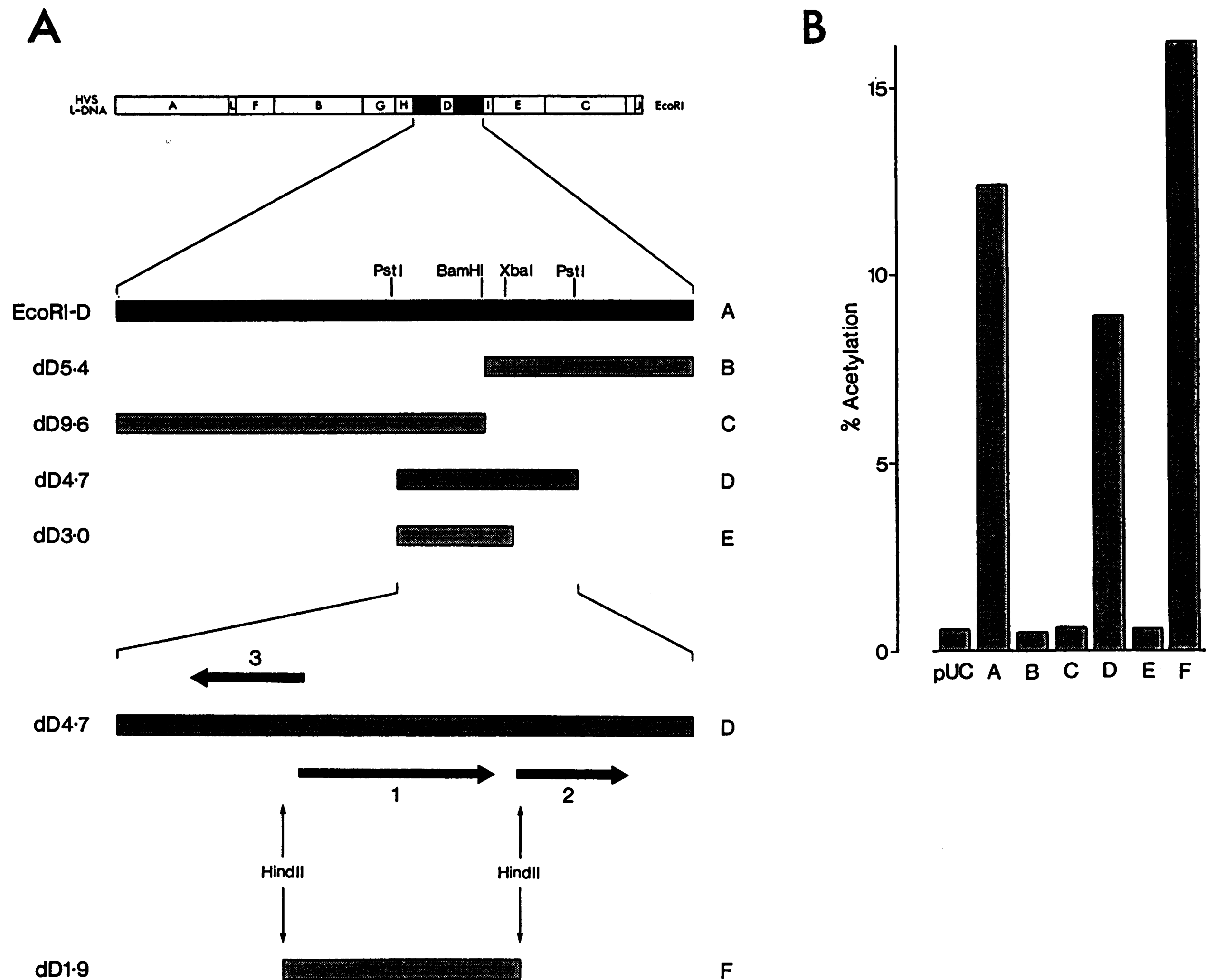

FIG. 5. Identification of the *trans*-acting gene in *Eco*RI-D. (A) The location of the 15-kbp *Eco*RI-D fragment of HVS DNA within the L-DNA component of the genome is shown relative to other *Eco*RI fragments (conventional orientation of HVS). Indicated within the *Eco*RI-D fragment (A) are the *Pst*I, *Bam*HI, and *Xba*I sites used for the subcloning of portions of *Eco*RI-D (B through E) into pUC19. The 4.7-kbp *Pst*I fragment (dD4.7) (D) was sequenced, and three complete ORFs (RF1, RF2, and RF3) were identified. RF1 was subcloned as a 1.9-kbp *Hin*dIII fragment (dD1.9) (F) into the *Hin*dIII site of pUC19. (B) Results of cotransfections of pKS110CAT with pUC19 (pUC), p*Eco*RI-D (A), and the subclones of *Eco*RI-D (B through F) shown in panel A. Cotransfections were performed in triplicate by using 5 μg of pKS110CAT and 10 μg of effector plasmid, and average values, normalized to take account of the sizes of the effector plasmids relative to that of pdD1.9, are shown.

initiation site (9a) and consequent poor expression of the IE-G mRNA. However, this plasmid, together with p52K and the SV40 early promoter expression plasmids pKSV52 and pKSVHG, was able to support activation (approximately 6.5-fold) of the HSV IE 175K promoter, demonstrating that IE-G protein was being expressed from p*Hin*dIII-G.

The low levels of 110-kDa *trans* activation by the 52-kDa and *Hin*dIII-G gene products, relative to the levels of activation seen using these genes in cotransfection with heterologous promoter-CAT targets, led us to investigate the possibility that additional viral factors are involved in activation of the DE 110-kDa promoter. As mentioned previously, the arrangement of homologous genes between HVS and EBV is conserved (1, 17). In addition to the EBV 52-kDa homolog, EB2 (BSLF2-BMLF1 encoded) (31), EBV encodes two other *trans* activators, Z (BZLF1 encoded) and R (BRLF1 encoded). These genes appear to be essential for the reactivation of latent EBV genomes. The BZLF1 gene

FIG. 6. Nucleotide sequence of the 4,689-bp *Pst*I fragment of *Eco*RI-D and translations (single-letter code) of RF1 and RF2. The sequence is shown in the conventional HVS orientation (i.e., as displayed in Fig. 5A), and RF1 and RF2 occur between nucleotide positions 1491 and 3095 and positions 3280 and 4125, respectively. RF3 corresponds to sequences between positions 1532 and 621 on the negative strand (not shown). The first methionine of the RF2 translation product (amino acid position 14) is boxed. Putative polyadenylation signals (AATAAA) downstream of RF1 (position 3229) and RF2 (positions 4124 and 4568) are underlined.

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A    1 NQRIPLWLVRSTHCLILLFQDDVQVRKSCLEPFLFLSPERKREIHQLLVA 50
     1 ......................MRPKKDGLEDFLRLTPEIKKQLGSLVSD 28

    51 FNQSLVT..PTQDEEKILSDIQRACLQIAEDLKHLNPFTGLLLDLNLYTL 98
    29 YCNVLNKEFTAGSVEITLRSYKICKAFINEAKAHGREWGGLMATLNICNF 78

    99 WTLLRNYKTKQRSQPVNSTVVSRYAHHVVKYIMQRLVYTTDRLFLTAPTS 148
    79 WAILRNNRVRRRAENAGNDACSIACPIVMRYVLDHLIVVTDRFFIQAPSN 128

   149 GIVLPVPLANAIFNLLSHCRKKCTGLWRNYGTEKSVLMGLGKEITLCYQA 198
   129 RVMIPATIGTAMYKLLKHSRVRAYTYSKVLGVDRAAIMASGKQVVEHLNR 178

   199 LNESGIVSTTLAAFIKLSFPTISIPNLFKPMFQ................. 231
   179 MEKEGLLSSKFKAFCKWVFTYPVLEEMFQTMVSSKTGHLTDDVKDVRALI 228

   232 ........SCKGNQDNFPDICTQGSVIRRPHQGVFGDTFPIPDPLMRE.. 271
   229 KTLPRASYSSHAGQRSYVSGVLPACLLSTKSKAVETPILVSGADRMDEEL 278

   272 ISENSFKKFSTANISTLLQNPKEILEMDPFDPRIGGFPLNKEETATPLKD 321
   279 MGNDGGASHTEARYSESGQFHAFTDELESLPSPTMPLKPGAQSADCGDSS 328

   322 SSFSNPTFINTGAANTLLPAASVTPALESLFSPTHFPCMSDESIASTSHV 371
   329 SSSSDSGNSDTEQSEREEARAEAPRLRAPKSRRTSRPNRGQTPCPSNAAE 378

   372 PLDNNIS...........LPTLVKTNF..PLKRKRQSRNI.......... 398
   379 PEQPWIAAVHQESDERPIFPHPSKPTFLPPVKRKKGLRDSREGMFLPKPE 428

   399 ............DPNTPRRPRG.RPKGSKTKKRPTCSPALFQSSDIPTDS 435
   429 AGSAISDVFEGREVCQPKRIRPFHPPGSPWANRPL..PASLAPT..PTGP 474

   436 LHVKCPEMLPTVPQNEFCDSSNIQPCTSSSVLENDNLVPINEAETDDNIL 485
   475 VHEPVGSLTPAPVPQPLDPAPAVTP.EASHLLEDPDEETSQAVKALREMA 523

   486 ATILQ........DLYDLPAPPVLCSHENQTLEIDNNVDIEDLGLSFPMS 527
   524 DTVIPQKEEAAICGQMDLSHPPPRGHLDELTTTLESM..TEDLNLDSPLT 571

   528 ......LQDFLNDE.................... 535
   572 PELNEILDTFLNDECLLHAMHISTGLSIFDTSLF 605

B    1 MSRPYQPQRYSLISELHKNF..HYVDVSVIQSEFKNVILKTVVPKLSQPA 48
     1 MASSNRGNARPLKSFLHELYLKHYPEVGDVVHLLNTIGVDCDLPP.SHPL 49

    49 THLEKGDFLLKICQLLMIHREEEQQILNKVKSNIIYFLNELWSAEYGKVQ 98
    50 LTAQRGLFLARVLQAVQQHKLLEDTIVPKILKKLAYFLELLSYYSPKDEQ 99

    99 EQVKNILCEVKLDKTDSELSTYLAQEIPKLTVLKYPTHFKVCEETIPNGR 148
   100 RDIAEVLDHLKTNR.DLGLDDRLWALIRKLRQDRHHASVNVLMPGSDYTA 148

   149 WCLHNLLGIEQYYKDFSNIVLHDPETSLGSVQAYSRLSKLLFWCDSFMNK 198
   149 VSLQYYDGISIGMRKVIADVCRSGYASMPSMTATHNLSHQLLMASGPSEE 198

   199 IYPCNAFNSSINQVVLWSTMFHFYSVAHCNDCISESISFTEALLKQEVSA 248
   199 PC...AWRGFFNQVLLWTVALCKFRRCIYYNYIQGSIATISQLLHLEIKA 245

   249 FYEWCLEEE....YEEDRMAKFMKFSADQITILSTHTDLQNLAEYIYSYK 294
   246 LCSWIISQDGMRLFQHSRPLLTLWESVAANQEVTDAITLPDCAEYIDLLK 295

   295 KCLINRRFE........ 303
   296 H..TKHVLENCSAMQYK 310
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FIG. 7. Comparisons of amino acid sequences of the RF1 (A) and RF3 (B) translation products with those of BRLF1 and BRRF1, respectively. The HVS sequences are shown on the upper lines, and the EBV sequences are shown on the lower lines. The RF1 (HVS.R) translation product is shown in its entirety, but the RF3 amino acid sequence begins at the first methionine (amino acid position 14). Alignments were made by using the "gap" algorithm in the Genetics Computer Group (GCG) sequence analysis software package, with gap and length weights set at 3.0 and 0.1, respectively. The percent similarities and identities are 43 and 23% for RF1 and BRLF1 and 43 and 22% for RF2 and BRRF1. In the alignments, similar residues are indicated by a single dot or a pair of dots (joining well-conserved amino acids) and identical residues are indicated by vertical lines.

product has been defined as the initial trigger for the lytic cycle (10, 39, 40, 42), but its function is dependent on the presence of the BRLF1-encoded protein (9, 12, 24). We predicted that the homologs of these genes in HVS, if they were present, would occur within the *Eco*RI-D (15-kbp) fragment of HVS L-DNA (Fig. 5A). In cotransfections with 110-kDa-CAT targets, we were able to show that an effector function was indeed encoded by *Eco*RI-D, and by subcloning and subsequent sequencing of the appropriate region of *Eco*RI-D this function was localized to a single gene. This gene, which we now designate HVS.R, is homologous to the EBV R *trans* activator. The leftward ORF (RF3) (Fig. 5A) upstream of HVS.R (RF1) is homologous to the BRRF1 reading frame of EBV (2), but the function of the encoded protein is unknown. Significant amino acid sequence similarities between the product of the downstream ORF, RF2, and its counterpart in EBV, BZLF1, were not detected, but the BZLF1 mRNA is known to be spliced, changing and extending the C-terminal portion of the encoded protein (4). We do not yet have data on the structure of the RF2 mRNA. It seems clear, however, that whereas the BRLF1 protein is encoded by a bicistonic mRNA containing BRLF1 and BZLF1 coding sequences (4, 29), the HVS.R transcription unit appears to span only the HVS.R ORF and to be contained within the 1.9-kbp *Hin*dIII fragment of *Eco*RI-D (Fig. 5A). This contains a poly(A) signal 133 nucleotides 3′ to the HVS.R ORF; this signal must be functional in our transfection assays and presumably is used also during HVS infections in vivo. Thus, in addition to the lack of detectable homology between the BZLF1 and RF2 genes, there appear to be significant differences in the structures of the BRLF1 and HVS.R mRNAs.

Homologs of the EBV BMLF1 gene have now been found in HSV (IE 63K, ICP27), VZV (RF4), and HVS (IE 52-kDa), but homologs of the BRLF1 gene have not previously been reported. The presence of a BRLF1 homolog in HVS, in addition to the 52-kDa gene, further illustrates the genetic similarities between these two gammaherpesviruses. Our present functional and structural characterization of the HVS.R gene product makes it possible to identify conserved protein domains between HVS.R and the BRLF1 protein. On the basis of the alignment of the two proteins shown in Fig. 7A, we would suggest that the N-terminal regions of the proteins are likely to be functionally important. Of particular note are the well-conserved sequences between amino acids 26 and 44 and between amino acids 126 and 146 of the HVS.R translation product and amino acids 4 and 22 and amino acids 106 and 126 of the BRLF1 protein (50 and 43% identity and 72 and 86% similarity, respectively). Also of interest is the highly basic region of the HVS.R protein between positions 390 and 418 of the RF1 amino acid sequence (50% basic residues over a 28-amino-acid stretch), which is partly conserved in the BRLF1 protein. The precise roles of these gene products in the biology of HVS and EBV in relation to the activities of the other *trans* activators encoded by these viruses remain to be established. However, the presence of apparently unique *trans* activators in both viruses (IE-G in HVS and the BZLF1 protein in EBV), together with likely differences in temporal expression of the HVS.R and BRLF1 gene products during lytic infections (4, 32), suggests that despite the similarities of gene organization and gene structure between HVS and EBV, there may be significant differences in the regulation of gene expression during the initial phases of lytic infection by these gammaherpesviruses.

REFERENCES

1. **Albrecht, J.-C., and B. Fleckenstein.** 1990. Structural organisation of the conserved gene block of herpesvirus saimiri coding for DNA polymerase, glycoprotein B, and major DNA binding protein. Virology **174**:533–542.
2. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
3. **Bankier, A. T., and B. G. Barrell.** 1983. Shotgun DNA sequencing, p. 1–33. *In* R. A. Flavell (ed.), Techniques in the life sciences, vol. B5. Elsevier, Dublin.
4. **Biggin, M., M. Bidescot, M. Perricaudet, and P. Farrell.** 1987. Epstein-Barr virus gene expression in P3HRI-superinfected Raji Cells. J. Virol. **61**:3120–3132.
5. **Blair, E. D., and R. Honess.** 1983. DNA-binding proteins specified by herpesvirus saimiri. J. Gen. Virol. **64**:2697–2715.
6. **Blair, E. D., R. E. Randall, and R. W. Honess.** 1986. Evidence that the major delayed-early DNA-binding proteins of herpesvirus saimiri are bound to DNA *in vivo*. J. Gen. Virol. **67**:759–764.
7. **Cameron, K. R., T. Stamminger, M. Craxton, W. Bodemer, R. W. Honess, and B. Fleckenstein.** 1987. The 160,000-M_r virion protein encoded at the right end of the herpesvirus saimiri genome is homologous to the 140,000-M_r membrane antigen encoded at the left end of the Epstein-Barr virus genome. J. Virol. **61**:2063–2070.
8. **Chang, Y.-N., D. L.-Y. Dong, G. S. Hayward, and S. D. Hayward.** 1990. The Epstein-Barr virus Zta transactivator: a member of the bZIP family with unique DNA-binding specificity and a dimerization domain that lacks the characteristic heptad leucine zipper motif. J. Virol. **64**:3358–3369.
9. **Chevallier-Greco, A., E. Manet, P. Chavrier, C. Mosnier, J. Daillie, and A. Sergeant.** 1986. Both Epstein-Barr virus (EBV)-encoded *trans*-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J. **5**:3243–3249.

9a. **Coles, L. S., and R. W. Honess.** Unpublished data.

10. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. USA **82**:4085–4089.
11. **Cox, R. A.** 1968. Use of guanidinium chloride in the isolation of nucleic acids. Methods Enzymol. **12**:120–129.
12. **Cox, M. A., J. Leahy, and J. M. Hardwick.** 1990. An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators. J. Virol. **64**:313–321.
13. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759–1816.
14. **Falk, L. A., L. G. Wolfe, and F. Deinhardt.** 1972. Isolation of herpesvirus saimiri from blood of squirrel monkeys (Saimiri sciureus). J. Natl. Cancer Inst. **48**:1499–1505.
15. **Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides.** 1989. Epstein-Barr virus BZLFI *trans*-activator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. **8**:127–132.
16. **Flemington, E., and S. H. Speck.** 1990. Identification of phorbol ester response elements in the promoter of Epstein-Barr virus putative lytic switch gene BZLF1. J. Virol. **64**:1217–1226.
17. **Gompels, U. A., M. A. Craxton, and R. W. Honess.** 1988. Conservation of gene organization in the lymphotropic herpesviruses herpesvirus saimiri and Epstein-Barr virus. J. Virol. **62**:757–767.
18. **Gompels, U. A., M. A. Craxton, and R. W. Honess.** 1988. Conservation of glycoprotein H (gH) in herpesviruses: nucleotide sequence of the gH gene from herpesvirus saimiri. J. Gen. Virol. **69**:2819–2829.
19. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
20. **Hardwick, J. M., P. M. Lieberman, and S. D. Hayward.** 1988. A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. J. Virol. **62**:2274–2284.
21. **Holley-Guthrie, E. A., E. B. Quinlivan, E.-C. Mar, and S. Kenney.** 1990. The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. J. Virol. **64**:3753–3759.
22. **Honess, R. W.** 1984. Herpes simplex and "the herpes complex": diverse observations and a unifying hypothesis. J. Gen. Virol. **65**:2077–2107.

22a. **Honess, R. W.** Unpublished data.

23. **Honess, R. W., U. A. Gompels, B. G. Barrell, M. Craxton, K. R. Cameron, R. Staden, Y.-N. Chang, and G. S. Hayward.** 1989. Deviations from expected frequencies of CpG dinucleotides in herpesvirus DNAs may be diagnostic of differences in the states of their latent genomes. J. Gen. Virol. **70**:837–855.
24. **Kenney, S., E. Holley-Guthrie, E.-C. Mar, and M. Smith.** 1989. The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. J. Virol. **63**:3878–3883.
25. **Knust, E., S. Schirm, W. Dietrich, W. Bodemer, E. Kolb, and B. Fleckenstein.** 1983. Cloning of herpesvirus saimiri DNA fragments representing the entire L-region of the genome. Gene **25**:281–289.
26. **Lee, W., A. Haslinger, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49**:741–752.
27. **Lieberman, P. M., and A. J. Berk.** 1990. In vitro transcriptional activation, dimerization, and DNA-binding specificity of the Epstein-Barr virus Zta protein. J. Virol. **64**:2560–2568.
28. **Lieberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward.** 1986. Promiscuous *trans*-activation of gene expression by an Epstein-Barr virus-encoded early nuclear protein. J. Virol. **60**:140–148.
29. **Manet, E., H. Gruffat, M. C. Trescol-Biemont, N. Morono, P. Chambard, J. F. Glot, and A. Sergeant.** 1989. Epstein-Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional *trans*-activators. EMBO J. **8**:1819–1826.
30. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
31. **Nicholas, J., U. A. Gompels, M. A. Craxton, and R. W. Honess.** 1988. Conservation of sequence and function between the product of the 52-kilodalton immediate-early gene of herpesvirus saimiri and the BMLF1-encoded transcriptional effector (EB2) of Epstein-Barr virus. J. Virol. **62**:3250–3257.

31a. **Nicholas, J., and R. W. Honess.** Unpublished data.

32. **Nicholas, J., E. P. Smith, L. Coles, and R. Honess.** 1990. Gene expression in cells infected with gammaherpesvirus saimiri: properties of transcripts from two immediate-early genes. Virology **179**:189–200.
33. **Oguro, M. O., N. Shimizu, Y. Ono, and K. Takada.** 1987. Both the rightward and leftward open reading frames within the *Bam*HI M DNA fragment of Epstein-Barr virus act as *trans*-activators of gene expression. J. Virol. **61**:3310–3313.
34. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
35. **O'Hare, P., and R. W. Honess.** 1983. Identification of a subset of herpesvirus saimiri polypeptides synthesized in the absence of DNA replication. J. Virol. **46**:279–283.
36. **Packham, G., A. Economou, C. M. Rooney, D. T. Rowe, and P. J. Farrell.** 1990. Structure and function of the Epstein-Barr virus BZLF1 protein. J. Virol. **64**:2110–2116.
37. **Randall, R. E., R. W. Honess, and P. O'Hare.** 1983. Proteins specified by herpesvirus saimiri: identification and properties of virus-specific polypeptides in productively infected cells. J. Gen. Virol. **64**:19–35.
38. **Randall, R. E., C. Newman, and R. W. Honess.** 1984. A single major immediate-early virus gene product is synthesized in cells

productively infected with herpesvirus saimiri. J. Gen. Virol. 65:1215–1219.

39. **Rooney, C., N. Taylor, J. Countryman, H. Jenson, J. Kolman, and G. Miller.** 1988. Genome rearrangements activate the Epstein-Barr virus gene whose product disrupts latency. Proc. Natl. Acad. Sci. USA **85**:9801–9805.
40. **Rooney, C. M., D. T. Rowe, T. Ragot, and P. J. Farrell.** 1989. The spliced BZLF1 of Epstein Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J. Virol. **63**:3109–3116.
41. **Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
42. **Takada, K., N. Shimizu, S. Sakuma, and Y. Ono.** 1986. *trans*-activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. J. Virol. **57**:1016–1022.
43. **Tsonis, P. A., T. Manes, J. L. Millan, and P. F. Geotinck.** 1988. CAT constructs with convenient sites for cloning and generating deletions. Nucleic Acids Res. **16**:7445.
44. **Urier, G., M. Buisson, P. Chambard, and A. Sergeant.** 1989. The Epstein-Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites. EMBO J. **8**:1447–1453.
45. **Wong, K.-M., and A. J. Levine.** 1986. Identification and mapping of Epstein-Barr virus early antigens and demonstration of a viral gene activator that functions in *trans*. J. Virol. **60**:149–156.
46. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.