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RAZ, an Epstein-Barr Virus Transdominant Repressor That Modulates the Viral Reactivation Mechanism

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Epstein-Barr virus (EBV) is associated with the development of several types of human cancers and is an important cause of lymphomas in immunocompromised hosts. Expression of the EBV BZLF1 immediate-early gene product (Z) triggers disruption of latency in EBV-infected cells. Z is a member of the b-Zip family of proteins and binds to AP-1-like sites in early viral promoters. Here we show that a viral RNA related to Z, in which there is replacement of the transactivation domain of Z by fusion through alternate splicing with a portion of another EBV transactivator, BRLF1 (R), can repress Z function. This differentially spliced mRNA is predicted to express a novel chimeric protein which we call RAZ for R and Z. RAZ retains the dimerization and DNA-binding domains of Z but loses its transactivation domain. We show that in vitro the RAZ protein acts transdominantly to repress transactivation of early promoters by Z. Repression is produced by dimerization of RAZ with Z resulting in RAZ:Z heterodimers that can no longer bind to Z-binding sites despite retention of the DNA-binding domains in both proteins. Deletion of the R domain of RAZ restores the ability of the truncated RAZ homodimers and RAZ:Z heterodimers to bind to DNA. A biologic effect of RAZ was shown by cotransfection of latently infected Raji cells with Z and RAZ expression clones; RAZ diminished viral reactivation induced by Z, as indicated by amount of early replicative antigens (EA-D) detected. The RAZ protein presents a model for transcriptional control unique among the herpesviruses and distinct from analogous viral and cellular repressors. RAZ, by limiting the availability of Z protein, is likely to modulate EBV reactivation.

Epstein-Barr virus (EBV) is an oncogenic human herpesvirus that infects two cell types, epithelial cells and lymphocytes. The virus immortalizes B lymphocytes, producing in these proliferating cells a latent infection in which only a subset of the approximately 100 viral genes is expressed (for a review, see reference 31). In such latently infected cells, viral replication and cytolysis are prevented. However, although there is considerable information on the mechanism by which viral reactivation is triggered, how reactivation is suppressed is poorly understood and equally important.

Viral reactivation in vitro is produced by treatment of latently infected cell lines with agents such as phorbol esters (12), sodium butyrate (40), or anti-immunoglobulin (11, 55), all of which have in common the ability to induce the expression of the key immediate-early gene, BZLF1, used in the switch from latency to cytolytic replication (9). The product of BZLF1, Z (also referred to as ZEBRA, Zta, and EB1), when overexpressed by transfection into latently infected cells is sufficient to trigger viral reactivation (7, 9, 51). In cotransfection experiments, Z is able to transactivate several early promoters in EBV-infected lymphoblastoid cells and in EBV-negative HeLa cells (6, 10, 26, 28, 38, 51) as well as to autoregulate its own promoter (17). Deletional and mutational analysis of these promoters has revealed a consensus Z-responsive element (ZRE) (T[G/T][A/T]G[T/C][G/C/A]A) (32, 36, 47) which conveys Z inducibility when placed upstream of a heterologous promoter.

Z is a member of the b-Zip family of transcription factors and has significant amino acid homology within the basic DNA-binding and dimerization domains of c-Fos (15) and C/EBP (32). Z functions as a homodimer and binds to authentic AP-1 sites (T[G/T][A/C][G/C]TCA) (15), as well as to consensus C/EBP binding sites (T[T/G]NNG[C/T]AA[T/G]) (32) and somewhat degenerate AP-1 sites (5, 17, 36, 47).

Another EBV immediate-early protein, the BRLF1 gene product (R) (also known as Rta), is also a sequence-specific DNA-binding protein and transcriptional transactivator (24, 25, 41). In addition, in certain cell types, R can act synergistically with Z to produce transcriptional transactivation (10, 26). However, the possibility that the Z transactivation effect might also be negatively regulated by viral gene products has until now not been studied.

The immediate-early region of the EBV genome generates Z and R viral transactivators by the use of two promoters and differential splicing as shown in Fig. 1A (41). Both the pZ and pR promoters play a role in disruption of latency (18). pZ gives rise to a 1-kb transcript encoding the Z transactivator. Z is composed of three exons generating a 245-amino-acid (aa) protein that migrates at 38 kDa on sodium dodecyl sulfate (SDS) gels. Z has well-characterized transactivation, basic–DNA-binding, and dimerization domains (16, 32, 36) (see Fig. 1B) and has recently been shown to interact with TFIID (37). pR is responsible for three transcripts of 4.0, 3.3, and 0.8 kb (41). The 4.0- and 3.3-kb transcripts are bicistronic and encode both the BZLF1 and the BRLF1 transactivators.

In this paper, we report the characterization of the alternately spliced 0.8-kb transcript generated from pR first detected by Manet et al. (41) but never characterized. This transcript is predicted to encode a chimeric protein consisting of the dimerization and DNA-binding domains of Z and an

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amino-terminal domain derived from R. In vitro, this protein, designated RAZ (BRLF1 and BZLF1), can transdominantly inhibit Z transactivation by forming RAZ:Z heterodimers that are unable to bind to ZREs.

Alternate splicing is an efficient mechanism for the generation of a transdominant repressor from the same gene that encodes a transcriptional transactivator (for a review, see reference 20). Structurally, many transdominant repressors contain deletions or domain substitutions within the transactivation domain resulting in a protein that competes with the activator for binding to the same site and thus blocks activation of transcription (8, 14, 19, 34, 45, 46, 56). RAZ represents a new type of an alternately spliced transdominant repressor whose action is to inhibit DNA binding through heterodimerization with the target transactivator protein. Biologically, RAZ may play an important role in curtailing viral reactivation in EBV-transformed cells.

MATERIALS AND METHODS

Cell lines. The EBV-positive Akata (11, 55) and Raji (48) cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum. The human cervical epithelial cell line HeLa was maintained as monolayers in Dulbecco's modified Eagle medium H supplemented with 10% fetal calf serum. Cells were maintained at 37°C in a 5% CO_2 environment. Virus replication was induced in Akata cells by treatment with 100 μg of anti-human immunoglobulin G (IgG) per ml of medium (Sigma). Virus production was monitored by indirect immunofluorescence for early antigen and viral capsid antigen by using high-titer EBV-positive sera from patients with nasopharyngeal carcinoma and fluorescein isothiocyanate-conjugated goat anti-human antibody.

Plasmids and DNA transfections. The plasmid pEA-BS-CAT was made by inserting the *Xba*I-*Bam*HI fragment of pEA-CAT (which contains the complete BMRF1 promoter sequences [positions 79,537 to 79,898], the chloramphenicol acetyltransferase [CAT] gene and the simian virus 40 polyadenylation site) into the *Xba*I-*Bam*HI site of the M13 Bluescript SK^+ plasmid (Stratagene) (26). pPolCAT contains the DNA-polymerase promoter in a 1.29-kb *Bal*I fragment (EBV nucleotides 156,859 to 158,149) cloned into the *Hin*cII site upstream of the CAT gene of pBS.CAT (21). pBHRF1-CAT (a gift from Q. Zhang) contains the EBV sequences from nucleotides 52,799 to 53,819 cloned into the *Hin*cII site of pBS.CAT. The pEBV-ZIE plasmid contains the *Bam*HI Z gene fragment (EBV nucleotides 101,741 to 103,947) in the pGEM2-based vector pHD1013 (13), so that the BZLF1 gene is under the control of the human cytomegalovirus immediate-early promoter. The pEBV-R/Z plasmid contains both the BRLF1 and BZLF1 transactivators under the control of the cytomegalovirus immediate-early promoter. This plasmid has the 4,061-bp *Bgl*II-*Bgl*II fragment (EBV nucleotides 101,351 to 105,412) inserted into the *Bam*HI site of the vector pHD1013. Plasmid DNA was purified either by two sequential cesium-chloride gradients or by the Quiagen system. DNA was transfected into both lymphoid and epithelial cell lines by electroporation (26). For each condition, 10^7 cells were shocked at 1,500 V with the Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin). Epithelial cells were harvested and resuspended into RPMI medium for electroporation.

CAT assays. At 48 h after transfection, cell extracts were prepared from electroporated cells and incubated at 37°C with [^{14}C]chloramphenicol and acetyl coenzyme A (23). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by scintillation counting or scanning of the chromatography plates by scanner (AMBIS, Inc.) for 10 h. If results were not in the linear range, CAT assays were repeated with less extract.

cDNA synthesis and PCR amplification. Mock- or anti-IgG-induced Akata cells were harvested, pelleted, and washed in phosphate-buffered saline (PBS); total cellular RNA was extracted with guanidinium isothiocyanate (53). For RNA-based PCR analysis, 1 μg of total cellular RNA was incubated at 42°C for 1 h in a total volume of 20 μl containing 500 ng of oligo(dT) primer, 1 mM each deoxynucleoside triphosphate, 1 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 U of RNase inhibitor, and 2 μl of Superscript RNase H^- RT (Life Technologies; 200 U/μl). After first-strand cDNA synthesis, the reaction mixture was diluted to 50 μl with Tris-EDTA (TE). A 1-μl aliquot of each diluted reaction mixture was used in a 50-μl PCR mixture containing 1× Promega *Taq* DNA polymerase buffer, 1 mM each primer, 250 mM (each) deoxynucleotide triphosphates and 2.5 U of *Taq* DNA polymerase (Promega). Thermal cycling conditions for RAZ and R/Z cDNAs were 2 initial cycles at 94°C for 2 min, 55°C for 2 min, and 72°C for 3 min, followed by 23 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplification for actin cDNA was essentially the same except that the total number of cycles was varied at 15, 20, and 25 cycles to achieve a subplateau range of amplification. Primers specific for R/Z bicistronic cDNA were A (CAGAATCGC ATTCCTCCAGCGA) and B (GAATGTCTGCTGCATGCC ATGC); for RAZ-specific cDNA amplification, primers were A and C (ATGACGAGGGATCCTCTAGAGCCATGAGG CCTAAAAAGGATGG); and for actin cDNA amplification, primers were Act-1 (CCTTCCTGGGCATGGAGTC) and Act-2 (GGAGCAATGATCTTGATCTTC). PCR products were resolved on a 1% agarose gel, blotted onto nitrocellulose filters, fixed by heat, and prehybridized for 1 h at 37°C in prehybridization solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.15% sodium PP_i, 100 μg of salmon sperm DNA per ml, and 0.1% SDS. Hybridization was performed in the same solution at 37°C with a ^{32}P-end-labelled R/Z- and RAZ-specific oligonucleotide probe, AGGTGCTTCTCCCCCGGCTTGGTTAG TCTGTTGATTCTG, or actin-specific probe, CTGTGTTG GCGTACAGGTCTTTGCGGATGT. Filters were washed at 50°C in 0.1× SSC–0.1% SDS, and products were visualized by autoradiography.

DNA mobility shift assays. For mobility shift assays performed with programmed rabbit reticulocyte lysates, 3 μl of each translation product was mixed with 20,000 cpm of a ^{32}P-labelled ZRE-containing EBV-DNA probe from the *Bam*HI A fragment (coordinates 156,860 to 157,006) (21) in 25 μl of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9)–10% (vol/vol) glycerol–60 mM KCl–0.1 mM EDTA–0.25 mM DTT–2 μg of poly(dI · dC). Binding reactions were carried out for 20 min at 27°C and loaded onto 4% nondenaturing acrylamide gels, which were electrophoresed in 0.25× Tris-borate-EDTA, dried, and visualized by autoradiography.

RAZ cDNA cloning. RAZ-specific cDNAs were PCR amplified from a λgt10 cDNA library prepared from EBV-positive B95-8 cells (22). Briefly, total lambda DNA was prepared from 10^7 library PFU (53) and resuspended in 100 μl of TE. One microliter of this stock was PCR amplified with a RAZ 3′ primer (ATCACGAGGGATCCAAACCACACGATGCTAA GAGAGCCGACAGGAAGA) and primer C. RAZ cDNAs were cloned into phagemid pBS^+ (Stratagene), sequenced, and compared with the published B95-8 sequence (1) for any mutations.

Construction of RAZ mutants and in vitro expression. RAZΔDIM was constructed by PCR mutagenesis. Briefly, 2 μg of pBScRAZ single-stranded phagemid DNA was annealed in 1× annealing buffer (40 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$, 50 mM NaCl) with 4 pmol of kinased primer (CC GGGGGATAATGGAGTGCTGCAGCAGTTG) that was discontinuous with RAZ cDNA sequence and annealed to 15 bases on either side of the dimerization domain (aa 207 to 235). Second-strand DNA was synthesized with the addition of 5 U of T7 DNA polymerase (Stratagene), 1× reaction buffer (10 mM Tris-HCl [pH 7.5], 2 mM DTT, 0.5 mM deoxynucleoside triphosphates, 1 mM ATP), and 3 U of T4 DNA ligase (Promega), and synthesis was allowed to proceed at 27°C for 1 h. Subsequently, the reaction mixture was diluted to 100 μl with TE, and PCR amplification was performed with the RAZ 3′ primer and primer C. For generation of RAZΔR, aa 87 to 252 were PCR amplified from pBScRAZ with primers RAZΔR′ (ATCACGAGGGATCCTCTAGAGCCACCATG CCTGCTCCTGAGAATGCTTATC) and RAZ 3′. Both PCR-generated mutants were cloned into pBS^+ and fully sequenced. For in vitro transcription and translation, pBS^+cRAZ, RAZΔR, and RAZΔDIM plasmid DNAs were linearized with *Eco*RI (Promega) transcribed with T7 RNA polymerase, and transcripts were used to program rabbit reticulocyte lysates (Promega). pSP64Z was linearized with *Eco*RI, and SP6 RNA polymerase was used to generate Z transcripts. For cotranslations, transcripts were mixed and added to reticulocyte lysates with or without [^{35}S]methionine. ^{35}S-labelled proteins were resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE) and quantitated by densitometry.

GST-RAZ and GST-Z bacterial expression and dimerization assays. cRAZ was recovered from the pBScRAZ vector with *Bam*HI and cloned into the same site of pGEX2T (Pharmacia) to create pGEX2T-cRAZ. The Z cDNA was recovered from pSP64Z with *Bam*HI and *Eco*RI, blunt ended with Klenow enzyme, and cloned into the *Sma*I site of pGEX3X to create pGST-Z. *Escherichia coli* BL21DE3-pLysS (Novagen) was transformed with pGEX2T-cRAZ and pGST-Z, and fusion-protein expression was induced from mid-log-phase cultures with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h at 30°C. To prepare bacterial lysates, 10 ml of induced cultures was pelleted and resuspended in 1 ml of PBS, sonicated, and cleared of bacterial debris by centrifugation at 12,000 × *g*. A total of 100 μl of glutathione-Sepharose 4B beads (Pharmacia) was added to the cleared lysate to purify fusion proteins, and this addition was followed by three centrifugations and washing in 1-ml aliquots of PBS and a final resuspension in 100 μl of ELB^+ (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0], 5 mM EDTA [pH 8.0], 0.5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). For dimerization experiments, 20-μl aliquots of fusion protein bound to beads were incubated with 5 μl of programmed reticulocyte lysates in 1 ml of ELB^+ for 2 h at 4°C. Bead complexes were washed five times in 1 ml of ELB^+ buffer, pelleted, and boiled in Laemmli sample buffer. Bound proteins were resolved on SDS–10% polyacrylamide gels and visualized by fluorography and autoradiography.

Immunoblotting. Raji cells were transfected as described above, and cells were harvested 24 h after transfection, washed twice with PBS, and resuspended in 200 μl of ELB^+ buffer. Cells were then lysed by sonication for 10 s, and the lysate was cleared by centrifugation for 10 min at 4°C. Protein concentration of the samples was determined by the Bradford method. Samples were resuspended in Laemmli sample buffer, boiled, and loaded onto an SDS–8% PAGE gel. Following electrophoresis, the gel was transferred to polyvinylidene difluoride membrane (Schleicher & Schuell) for 2 h at 140 mA and then immunoblotted with EA-D monoclonal antibody (Dupont no. 9240) and visualized by the chemiluminescence method as specified by the manufacturer (Amersham).

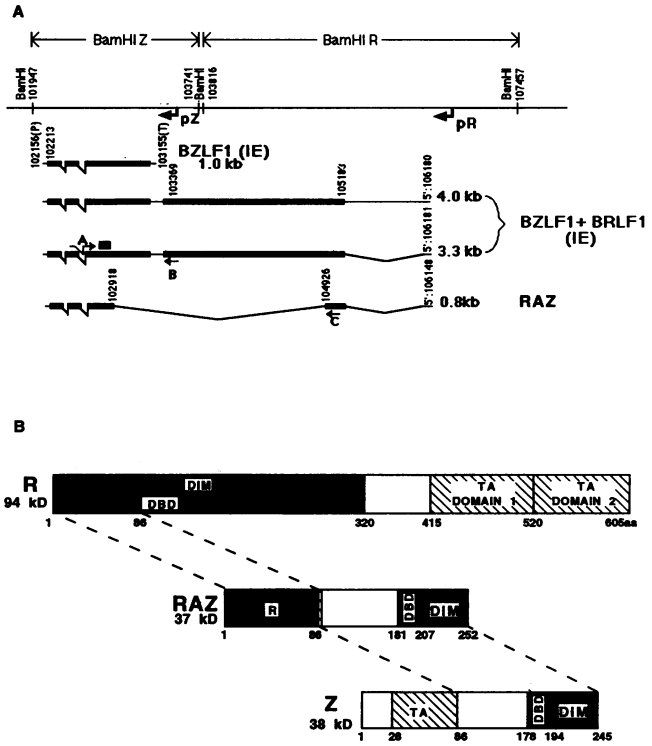

FIG. 1. EBV viral transactivator domain structures predicted by transcription and differential splicing in the EBV immediate-early region. (A) Leftward transcripts derived from promoters pR and pZ (41) with nucleotide coordinates according to the B95-8 strain of EBV (1); shown are the polyadenylation site (P), TATA box (T), and primers A, B, and C used in RNA-based PCR for identification of the RAZ-specific splice versus the BRLF1-BZLF1 bicistronic transcripts. (B) Functional domains of the R, Z, and putative RAZ proteins with transactivation (TA), DNA-binding (DBD), and dimerization (DIM) domains.

RESULTS

The 0.8-kb transcript initiating at pR (Fig. 1A) results from alternate splicing of the 3.3-kb bicistronic transcript and yields an mRNA with a portion of the amino terminus of BRLF1 and the carboxy terminus of BZLF1. The predicted protein (RAZ) would include the DNA-binding and coiled-coil dimerization domains of BZLF1 (located in exons 3 and 4, respectively) (Fig. 1A), linked to the first 86 aa of BRLF1 to generate a 252-aa chimeric protein. The amino acids of R contain parts of its DNA-binding and dimerization domains (Fig. 1B); however, deletional analysis has demonstrated that these 86 aa alone are insufficient for either of these functions (25, 42). The first 80 aa of Z, which contain most of the Z transactivation domain (16), are not present in RAZ.

RAZ-specific RNA appears during EBV reactivation. To study the role of RAZ in the EBV cytolytic cycle, the induction of RAZ mRNA was assessed by RNA-based PCR in the Burkitt lymphoma cell line Akata. This line contains latent EBV genomes that can be reactivated synchronously into the

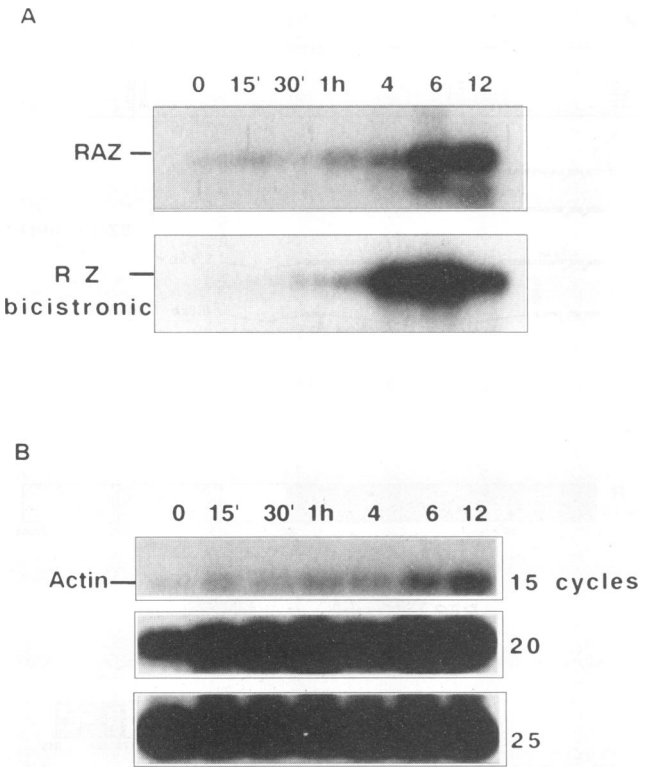

FIG. 2. Transcription of RAZ mRNA follows that of R/Z bicistronic RNA. (A) Akata cells were induced with anti-human IgG, and total RNA was harvested at various time points. First-strand cDNAs were generated from 1 μg of total cellular RNA primed with oligo(dT). Amplimer pairs indicated in Fig. 1A, A:C and A:B, were used in separate reactions for RAZ and R/Z-specific PCR amplification, respectively. cDNA products were detected by oligonucleotide Southern blot hybridization. (B) Actin amplification was performed to establish a subplateau level that could be used to estimate the initial amount of first-strand cDNA used in each PCR. PCR amplification of RAZ and the R/Z bicistronic cDNAs was performed multiple times with various Akata cell inductions and always yielded similar expression kinetics.

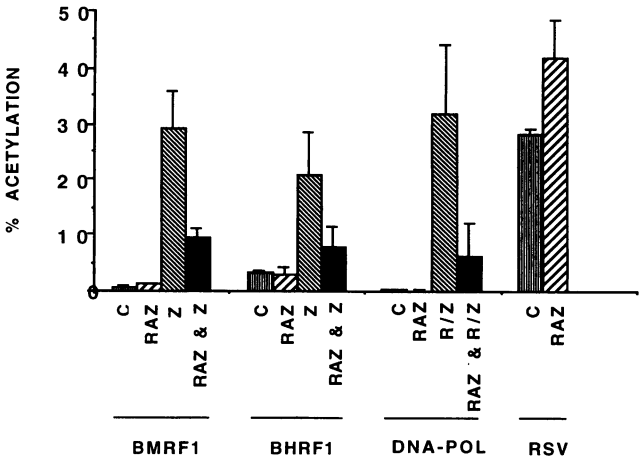

FIG. 3. Expression of RAZ protein suppresses transactivation of early promoters by Z protein in transient assays. The promoter-CAT construct pEA-BS-CAT (BMRF1), pBHRF1-CAT (BHRF1), pPOL-CAT (DNA-POL), or pRSV-CAT (RSV) was cotransfected with a control plasmid (pHD1013 [C]) or expression clone pCMV-RAZ (RAZ), pEBV-ZIE (Z), or pEBV-R/Z (R/Z) or with both RAZ and Z (RAZ & Z or RAZ & R/Z). The results are presented as average percent acetylation (indicated on the ordinate). The bars above the columns indicate the range of the percent acetylation. The experiments with the BMRF1, BHRF1, and Rous sarcoma virus promoters were performed in HeLa cells with 5 μg of each promoter construct and 5 μg of pEBV-ZIE and pCMV-RAZ. The experiments with the DNA-POL promoter were performed in EBV-positive Raji cells with 10 μg of pPOL-CAT, 2.5 μg of pCMV-R/Z, and 2.5 μg of pCMV-RAZ. For all assays, the amount of the DNA transfected was kept constant by addition of pHD1013 or salmon sperm DNA in the transfection.

virus-productive cycle by treatment with anti-human IgG (52). PCR primers specific for either the 0.8-kb RAZ transcript or the 4.0- and 3.3-kb BZLF1-BRLF1 bicistronic transcripts are indicated in Fig. 1A and are predicted to produce amplification products of 553 and 768 bp, respectively. As shown in Fig. 2A, amplification of cDNA specific for the 4.0- and 3.3-kb R/Z bicistronic transcripts reveals that one or both are first induced about 1 h postinduction, reach a plateau at 6 h, and have decreased at 12 h. In contrast, cDNA specific for RAZ RNA appears to be induced later and persists longer. The apparent persistence of RAZ message at later times during disruption of latency may suggest that its function involves inhibition of Z activity or possibly activation of late promoters. Actin mRNA amplification at a limited number of cycles indicated that nearly equal amounts of RNA were employed at each time point to generate the cDNAs (Fig. 2B). However, determination of the kinetics of induction of RAZ RNA requires direct RNA measurements, which are in process.

RAZ inhibits transactivation of Z-responsive promoters. Since RAZ could in theory dimerize with Z and alter its DNA-binding and its transactivational capabilities, the RAZ cDNA (cRAZ) was amplified and fully sequenced from a λgt10 library made from the prototype EBV cell line, B95-8 (22). cRAZ was cloned into the pHD1013 expression vector driven by the cytomegalovirus immediate-early promoter (13), designated pCMV-RAZ, and used for transient expression in promoter-CAT assays. The effects of cRAZ expression on Z transactivation were determined with several EBV early promoters that respond to Z and contain ZREs (21, 26, 28, 38). The BMRF1 and BHRF1 promoters linked to CAT were cotransfected with a Z-expression vector, pEBV-ZIE (28), into EBV-negative HeLa cells, resulting in activation of the two promoters (Fig. 3). This transactivation was inhibited on average 70% by the addition of pCMV-RAZ in the transfection. This result was duplicated in EBV-positive cells with the BMRF1 promoter (data not shown). The EBV DNA polymerase promoter, which can be activated by an R-plus-Z genomic expression clone (21), was also inefficiently activated in the presence of RAZ (Fig. 3). Therefore, RAZ inhibits Z transactivation of at least three early viral promoters, and inhibition is observed in both EBV-positive and -negative cells. The RAZ-dependent decrease in promoter activity is specific for the promoters assayed since pCMV-RAZ does not decrease the activity of the Rous sarcoma virus long terminal repeat (Rous sarcoma virus-CAT). A mutant of RAZ, RAZΔDIM, which deletes the dimerization domain (Fig. 4), did not affect activation of the BMRF1 promoter by Z (Fig. 5). The effect on promoter activity of a second mutant, RAZΔR (Fig. 4), is described subsequently.

The RAZ R domain negatively affects DNA-binding ability. Since RAZ differs from Z only at the amino terminus (Fig. 1B), RAZ protein might still be expected to bind to a consensus ZRE. RAZ DNA-binding ability was analyzed by

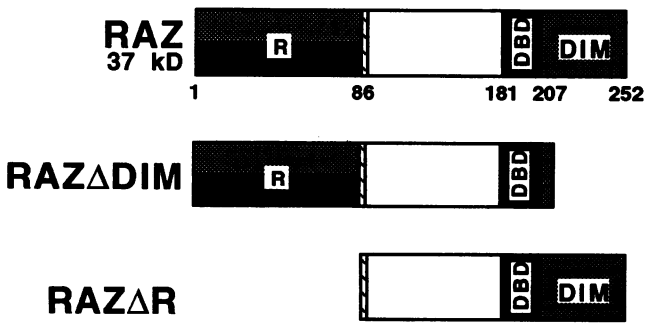

FIG. 4. Structure of RAZ mutants. The R domain (aa 1 to 86) and dimerization domain (aa 207 to 235) of RAZ were deleted to create RAZΔR and RAZΔDIM, respectively.

cloning the RAZ cDNA into the pBS$^+$ vector (Stratagene) (pBScRAZ), and RNA transcribed from this vector was used to program rabbit reticulocyte lysates. RAZ protein thus produced did not bind to the ZRE-containing probe (Fig. 6, lane 2) whereas Z protein produced from a similar vector (pSP64Z, a gift from P. Farrell [51]) bound readily (lane 3). Removal of the RAZ R domain (aa 1 to 86 from pBScRAZ) results in a protein (RAZΔR) that contains mainly the DNA-binding and the dimerization domains of Z (Fig. 4). As shown in Fig. 6 (lane 4), RAZΔR gained the ability to bind to the ZRE-containing probe. Thus, the R domain of RAZ is responsible for inhibiting either protein dimerization or DNA-binding activity. The carboxy-terminal portion of the Z protein (which is identical to the carboxy-terminal region of RAZ) is sufficient for Z to bind to DNA (16).

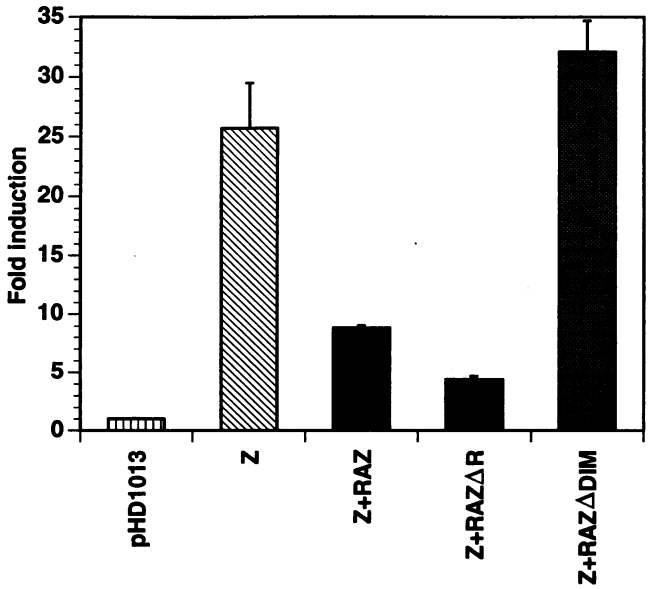

FIG. 5. Effect of expression of mutated RAZ on an early promoter activated by Z. Five micrograms of pEA-BS-CAT (BMRF1) was cotransfected in HeLa cells with a control plasmid (pHD1013), expression plasmid pEBV-ZIE (Z), or Z plus pCMV-RAZ, pCMV-RAZΔR, or pCMV-RAZΔDIM. The results are shown as average fold activation in duplicate assays. The bars above the columns indicate the range.

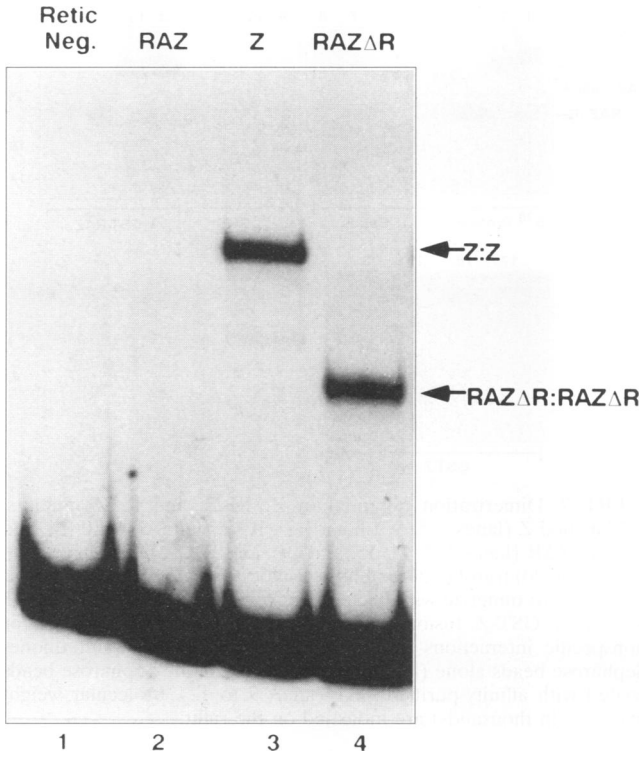

FIG. 6. The R domain of RAZ abolishes DNA binding. Shown are results of an electrophoretic mobility-shift assay using ^{32}P-end-labelled ZRE-containing probe and rabbit reticulocyte lysate (lane 1) or lysates programmed with RAZ RNA (lane 2), Z RNA (lane 3), or RAZΔR RNA (lane 4).

The RAZ R domain does not impair protein dimerization. To test the ability of RAZ protein to homodimerize and to heterodimerize with Z, cRAZ was subcloned into pGEX2T for overexpression in *E. coli*. ^{35}S-labelled Z, RAZ, or RAZΔR proteins generated in vitro (Fig. 7, lanes 1 to 3) were incubated with affinity-purified glutathione-*S*-transferase-RAZ (GST-RAZ) fusion protein. A mutant of RAZ lacking the protein-dimerization domain (aa 207 to 235), RAZΔDIM, served as a negative control for dimerization (Fig. 4). GST complexes resolved by SDS-PAGE showed that Z, RAZ, and RAZΔR could interact with GST-RAZ protein (Fig. 7, lanes 13 to 15). In contrast, the mutant RAZΔDIM did not bind GST-RAZ protein (lane 16). Neither glutathione-Sepharose beads alone nor beads coated with GST bound the ^{35}S-labelled proteins (lanes 5 to 12). In a reciprocal experiment, GST-Z fusion protein (a gift from D. Gutsch) and labelled RAZ proteins (wild type and mutants) resulted in equivalent dimerization (lanes 17 to 20). Thus, not only can RAZ homodimerize, it can also heterodimerize with Z, suggesting that the effect of the R sequence substituted in RAZ for the Z transactivation domain is to inhibit DNA binding rather than dimerization.

RAZ sequesters Z into non-DNA-binding heterodimeric complexes. Since RAZ could heterodimerize with Z, we next addressed directly whether RAZ:Z or RAZ:RAZΔR heterodimers result in a decrease in DNA-binding activity. A fixed amount of BZLF1 transcript was cotranslated in vitro with increasing amounts of either RAZΔR or RAZ transcripts (Fig. 8, lanes 1 to 5), and cotranslation was followed by densitometry to determine the molar ratio of the variable protein relative to the constant protein. Analysis of binding to DNA of the

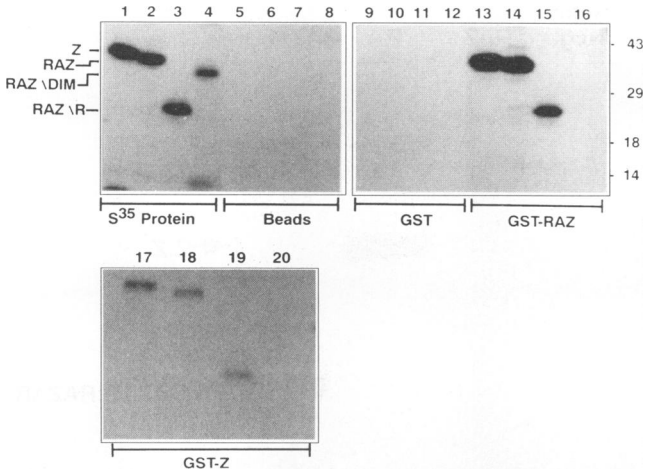

FIG. 7. Dimerization potential of Z, RAZ, and RAZ mutants. ^{35}S-labelled Z (lanes 1, 5, 9, 13, and 17), RAZ (lanes 2, 6, 10, 14, and 18), RAZΔR (lanes 3, 7, 11, 15, and 19), and RAZΔDIM (lanes 4, 8, 12, 16, and 20) translated in rabbit reticulocyte lysates were assayed for the ability to dimerize with bacterially expressed GST-RAZ (lanes 13 to 16) or GST-Z fusion proteins (lanes 17 to 20). Controls for nonspecific interactions with ^{35}S-labelled proteins are glutathione-Sepharose beads alone (lanes 5 to 8) or glutathione-Sepharose beads coated with affinity-purified GST (lanes 9 to 12). Molecular weight markers (in thousands) are indicated on the right.

cotranslated products showed that increasing amounts of RAZΔR caused a decrease in binding of Z homodimers (Fig. 9A; compare lanes 2 and 3 with lane 1). With this decrease was an increase in binding of Z:RAZΔR heterodimers and RAZΔR homodimers to DNA (lanes 2 and 3). Next, the effect of increasing amounts of RAZ was tested. As expected, Z binding decreased, presumably because of RAZ:Z heterodimers that are unable to bind DNA (Fig. 9A; compare lanes 4 and 5 with lane 1).

Since the sizes of RAZ and Z proteins are nearly identical, it is difficult to know whether cotranslation of RAZ with Z RNA caused a decrease in translation of the latter, which is then reflected in an artifactual decrease in DNA binding by Z. To answer this question, the truncated mutant, RAZΔR, which can bind to ZREs, was used in a similar cotranslation–DNA-binding experiment. RAZΔR RNA was kept as the constant RNA in cotranslations with increasing amounts of Z or RAZ RNA (Fig. 8, lanes 6 to 10). Increasing the amount of Z protein caused a decrease in RAZΔR homodimer binding (Fig. 9B, lanes 6 to 8) and the appearance of RAZΔR:Z heterodimers (lanes 7 and 8) and Z homodimers (lane 8). When an increasing amount of RAZ RNA was used, RAZΔR homodimer binding decreased (Fig. 9B; compare lanes 9 and 10 with lane 6) without the appearance of RAZΔR:RAZ heterodimers. Therefore, although such heterodimers form (Fig. 7), they do not bind DNA. Quantitation of the decreases in RAZΔR and Z DNA binding as a result of heterodimerization to RAZ is shown graphically in Fig. 9C. The decrease in DNA binding by RAZΔR:RAZ heterodimers is entirely due to complex formation rather than to a decrease in translational efficiency of RAZΔR as determined by densitometric quantitation of ^{35}S-labelled RAZΔR protein (Fig. 8, lanes 6 to 10).

Finally, in transient assays, RAZΔR was able to interfere with activation of the BMRF1 promoter by Z (Fig. 5, fourth lane). This result is expected since Z:RAZΔR heterodimers (although nonfunctional) can bind to ZREs and compete with the functional Z:Z homodimers for binding to ZREs.

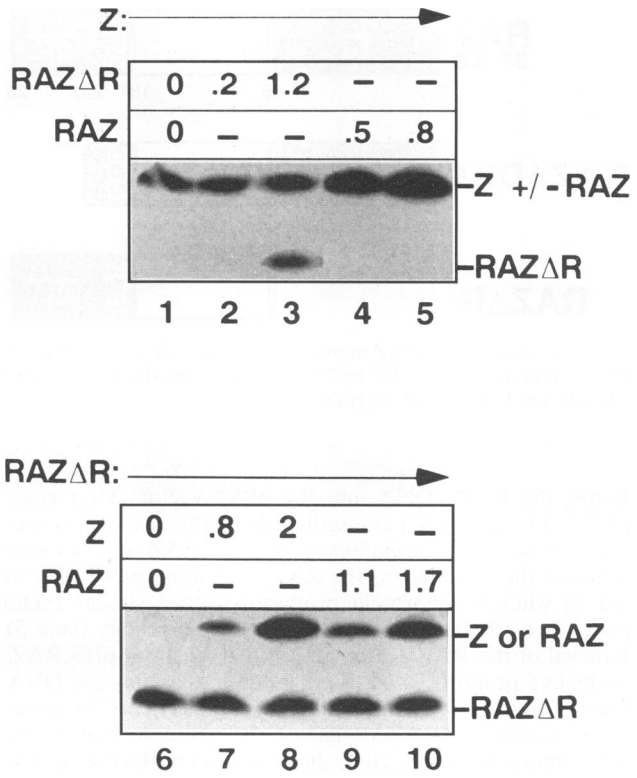

FIG. 8. Cotranslation of Z, RAZΔR, and RAZ in vitro transcripts. A fixed amount of Z transcript was translated in the presence of [^{35}S]methionine either alone (lane 1), with increasing amounts of RAZΔR transcript (lanes 2 and 3), or with increasing amounts of RAZ transcript (lanes 4 and 5). A fixed amount of RAZΔR transcript was translated either alone (lane 6), with increasing amounts of Z transcript (lanes 7 and 8), or with increasing amounts of RAZ transcript (lanes 9 and 10). Densitometry was performed on SDS-PAGE-resolved translation products and normalized to methionine content. Molar amounts of the proteins relative to the constant proteins are indicated above each gel. Since RAZ and Z comigrate, the molar amount of RAZ in lanes 4 and 5 was estimated by subtracting the amount of Z protein that was translated in lane 1.

RAZ diminishes early replicative antigens induced by Z in latently infected Raji cells. The biologic importance of Z was first indicated by showing that transfection of Z het DNA into Raji cells caused viral reactivation (9). We carried out a similar experiment in Raji cells transfected with both RAZ and Z expression clones. The index of viral reactivation was detection of early antigen-diffuse (EA-D) complex by Western blots (immunoblots). As shown in Fig. 10, viral reactivation induced by Z could be reduced or prevented by expression of RAZ. This result correlates well with the reversal produced by RAZ of transactivation of the BMRF1 (EA-D) promoter by Z shown in Fig. 3. Under these conditions, RAZ has a striking biologic effect on latent infection which is the reciprocal of that produced by Z.

Thus, in vitro RAZ acts as a transdominant repressor of Z transactivation by forming heterodimers which are unable to bind to ZREs. This is the first demonstration of such a repressor mechanism among the herpesviruses and stands unique among the alternately spliced transdominant repressors whose usual inhibitory mechanism involves direct binding to specific DNA sites and blocking of transactivation.

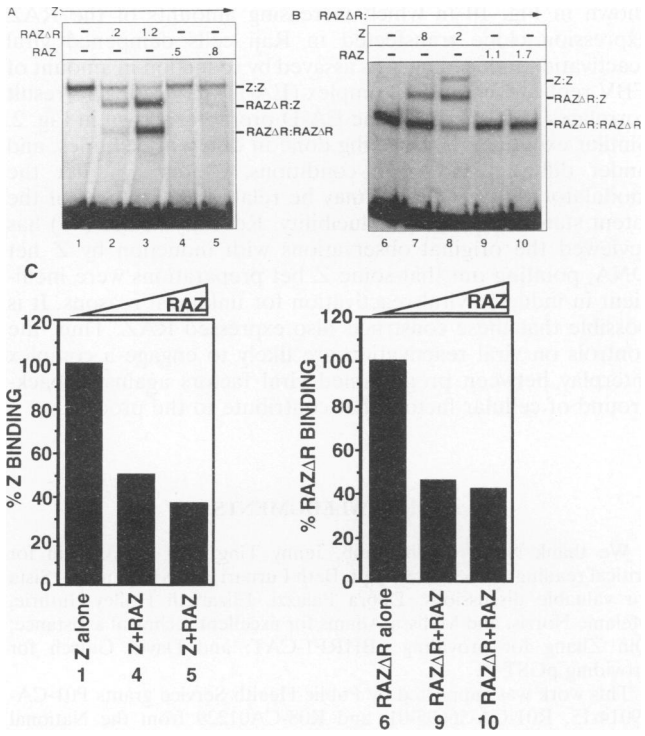

FIG. 9. Analysis of DNA-binding potential of RAZ:Z and RAZ:RAZΔR heterodimers. For electrophoretic mobility shift assays, equal amounts of unlabelled programmed reticulocyte lysate from Fig. 8 were reacted with the ^{32}P-end-labelled ZRE-containing probe. (A) Z protein alone (lane 1) and with increasing amounts of RAZΔR protein (lanes 2 and 3) or increasing amounts of RAZ protein (lanes 4 and 5). (B) RAZΔR protein alone (lane 6) and with increasing amounts of Z protein (lanes 7 and 8) or increasing amounts of RAZ protein (lanes 9 and 10). The molar amounts of RAZΔR, RAZ, or Z relative to the constant proteins are indicated above the respective bars. (C) Decreases in Z or RAZΔR DNA binding as a result of heterodimerization with RAZ, relative to the controls in panels A and B, were measured by densitometry and are presented graphically. Lanes used for each densitometry sample are indicated below each column and refer to lanes shown in panels A and B. Triangles above the bars indicate increasing molar amounts of RAZ. The exact amounts of RAZ used are indicated above the corresponding lanes in panels A and B.

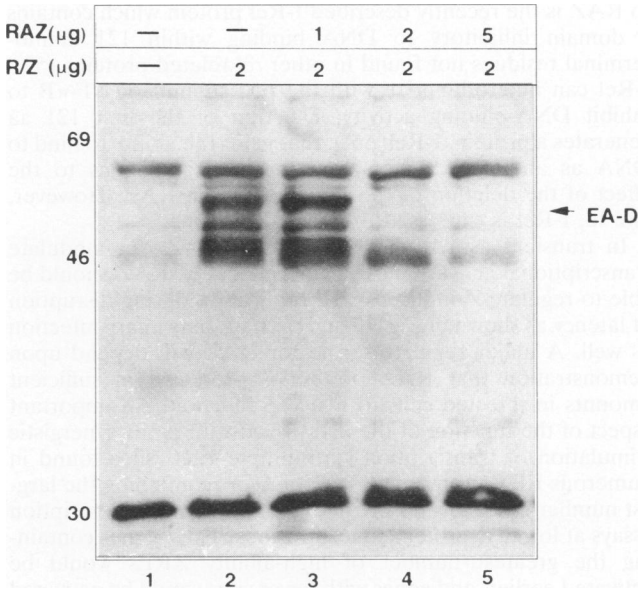

FIG. 10. RAZ inhibits expression of early antigen (EA-D) in induced Raji cells. Raji cells were transfected with a control plasmid (pHD1013, lane 1) or expression clone pEBV-R/Z (lanes 2 to 5) and increasing amounts of pCMV-RAZ (lanes 3 to 5). The cells were harvested 24 h after transfection, and equal amounts of protein were loaded onto an SDS-PAGE gel. The gel was transferred to a polyvinylidene difluoride membrane and then blotted with 1:40 EA-D monoclonal antibody (Dupont). Molecular weight markers (in thousands) are indicated on the left.

DISCUSSION

In this paper, we demonstrate that a previously detected but uncharacterized EBV gene product, RAZ, can modulate in vitro the function of the single viral protein, Z, that initiates viral reactivation. Moreover, RAZ has a biologic effect, as shown by the ability of RAZ to curtail or prevent viral reactivation induced by Z in latently infected cells. Although viral proteins that augment the reactivation cascade are known (7, 10, 26, 29), until now a virally encoded negative regulator of Z function has not been proposed.

RAZ presents a new mechanism for transdominant repression of transcriptional activation. The predicted viral protein, which has yet to be detected in vivo, is the result of alternate splicing between the open reading frames encoding the immediate-early transactivators, R and Z. We have shown that in vitro RAZ acts as an inhibitor of Z activity by forming RAZ:Z heterodimeric complexes which are unable to bind specific DNA-response elements. This RAZ-dependent inhibition of DNA binding is the result of an 86-aa domain contributed to the protein by R, replacing the Z transactivation domain.

Elements of such a transdominant mechanism have been described for both viral and cellular systems (20). The bovine papillomavirus E2 transactivator is differentially spliced to yield two repressors, E2-TR and E8/E2 (8, 34, 35), both of which share C-terminal DNA-binding and dimerization domains with E2. Whereas E2-TR is a truncated form of E2, E8/E2 contains the E8-coding sequence replacing the transactivation domain of E2, a situation analogous to the generation of RAZ transcripts. However, unlike RAZ the E8 open reading frame does not inhibit E8/E2 homodimers or E8/E2:E2 heterodimers from binding to DNA-recognition sites.

A similar strategy is used in cytolytic infections such as those produced by adenovirus. The adenovirus E1A region contains a single open reading frame that encodes both a positive and a negative transcription regulatory factor. These two proteins differ by 46 aa as a result of differential mRNA splicing (39). However, unlike bovine papillomavirus E2 proteins, adenovirus E1A does not bind DNA but mediates transcriptional regulation through cellular transcription factors.

RAZ-like inhibition of DNA binding has been demonstrated with the cellular protein Id (inhibitor of DNA binding), which associates with the basic helix-loop-helix proteins, MyoD, E12, and E47, involved in muscle differentiation (2, 27). Unlike RAZ, Id does not result from alternate splicing but is expressed from a unique gene; furthermore, Id exerts its poisoning effect because it lacks the basic domain region adjacent to the helix-loop-helix domain that is essential for DNA binding.

A transcriptional regulatory protein such as RAZ which combines inhibition of DNA binding with alternate splicing has not been described previously. The closest cellular counterpart

to RAZ is the recently described I-Rel protein which contains a domain inhibitory to DNA binding within 121 amino-terminal residues not found in other *rel*-related proteins (52). I-Rel can heterodimerize with the p50 subunit of NF-κB to inhibit DNA-binding activity. Deletion of the first 121 aa generates a protein, I-Rel(Δ5′), that gains the ability to bind to DNA as an I-Rel(Δ5′):p50 heterodimer, analogous to the effect of the deletion of the R domain from RAZ. However, like Id, I-Rel is expressed from a unique gene.

In transient assays, the viral protein RAZ can modulate transcriptional activation by Z quite precisely. RAZ should be able to regulate Z-induced EBV replication during disruption of latency as shown in Fig. 10 and perhaps in primary infection as well. A major regulatory role for RAZ will depend upon demonstration that RAZ protein is produced in sufficient amounts in infected cells to affect Z function. An important aspect of the function of the Z transactivator is the synergistic stimulation of transcription via multiple ZRE sites found in numerous EBV promoters (4). Promoters containing the largest number of ZRE sites are activated in in vitro transcription assays at lower Z-protein concentrations. Thus, genes containing the greatest number of high-affinity ZREs would be activated earlier, and genes with fewer sites would be activated later as Z concentrations rise during the cytolytic cycle. The results presented here have biologic implications for the EBV reactivation mechanism in that transcriptionally active concentrations of Z can be altered by titration with RAZ into inactive heterodimers. Thus, promoters which would be most sensitive to Z concentration variations would contain one or two ZREs, perhaps of low affinity.

Expression of RAZ RNA is governed by the same promoter used for the bicistronic expression of the R and Z proteins (41). The effect of the Z transactivator is first augmented by the appearance of the R transactivator (10, 26) expressed from the bicistronic mRNAs. An early RNA species having a size similar to that of RAZ was detected by Biggin et al. (3). The production of RAZ at a later time point after the early promoters have been activated may then serve to limit potential toxicity of the Z protein and to promote the transition from early to late gene expression and completion of the viral cycle. Similar roles have been proposed for herpes simplex virus ICP4 protein, which autoregulates its own expression by blocking transcription initiation (44, 50) and for the herpes simplex virus virion host shutoff function, which downregulates immediate-early and early gene expression by destabilizing mRNA (33, 49). Another possibility being studied is that RAZ, since it retains its DNA-binding domain, may bind to a late promoter.

Not understood are the cellular and viral functions that modulate the kinetics and relative amounts of RAZ versus Z RNAs generated by differential splicing. It is likely that the mechanisms regulating splicing of the RAZ versus BRLF1-BZLF1 bicistronic messages are dependent on cell type. In certain cells, RAZ mRNA may be generated earlier or in greater abundance than the bicistronic messages. In these situations, it is possible that the ability of RAZ to inhibit Z-induced activation of early EBV promoters may have the effect of maintaining viral latency. The effect of Z can also be modulated by cellular factors such as c-*myb*, a positive regulator (30), and retinoic acid receptors, which are negative regulators of Z (54). We have not yet confirmed which EBV-infected cell types express RAZ protein because of the current unavailability of high-affinity antibodies that distinguish between Z and RAZ. We are in the process of preparing RAZ- and Z-specific antibodies that will allow us to determine the biologic role of RAZ in EBV infection states.

A biologic role for RAZ is strongly suggested by results shown in Fig. 10 in which increasing amounts of the RAZ expression clone transfected in Raji cells dampened viral reactivation induced by Z as assayed by reduction in amount of EBV replicative antigen complex (EA-D) induced. This result correlates with effects on the EA-D promoter shown in Fig. 2. Similar experiments are being done in different cell lines, and under different induction conditions, to test whether the modulatory effect of RAZ may be related to tightness of the latent state and ease of inducibility. Recently Miller (43) has reviewed the original observations with induction by Z het DNA, pointing out that some Z het preparations were inefficient in inducing viral reactivation for unknown reasons. It is possible that these constructs also expressed RAZ. Thus, the controls on viral reactivation are likely to engage a complex interplay between programmed viral factors against a background of cellular factors that contribute to the process.

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