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The Epstein-Barr Virus R Transactivator (Rta) Contains a Complex, Potent Activation Domain with Properties Different from Those of VP16

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Rta, encoded by Epstein-Barr virus (EBV), is a potent activator of transcription via enhancer sequences located upstream of several viral genes. To identify the domains of Rta that facilitate transcription by interacting with cellular transcription factors, different segments of Rta were linked to the DNA binding domain of yeast transactivator GAL4 (residues 1 to 147). These GAL4-Rta fusion proteins were tested in transfected cells for their ability to activate the adeno E1b promoter with an upstream GAL4 DNA binding site. The acidic C-terminal domain of Rta (amino acids 520 to 605) was a potent activator but behaved differently from VP16 in dose-response and competition experiments. A subterminal domain of Rta (amino acids 416 to 519) linked to GAL4 had weak activation activity. Deletion of these domains from native Rta showed that the C-terminal domain was required for transactivation, but the subterminal domain was required only in B cells. The C-terminal activation domain of Rta contains a pattern of positionally conserved hydrophobic residues shared with VP16 and other transactivators. Substitution of several conserved hydrophobic amino acids in Rta severely impaired transactivation. The importance of hydrophobic residues was further substantiated by comparing EBV Rta with that of herpesvirus saimiri, which revealed little sequence similarity except for a few acidic residues and the positionally conserved hydrophobic amino acids. The C-terminal domain of EBV Rta contains three partially overlapping copies of this hydrophobic motif. Mutational analysis indicated that all three copies were required for full activity. However, two of the three copies appeared to be sufficient to produce full activity on a target promoter with multiple binding sites, suggesting that these motifs are functional subdomains that can synergize.

Epstein-Barr virus (EBV) can establish a latent state in B cells and subsequently reactivate to the lytic cycle. EBV-infected cells and tissues derived from various sources, including Burkitt's lymphomas, nasopharyngeal carcinomas, central nervous system lymphomas, and lymphoblastoid cell lines, have been used to study EBV gene expression during latency. Depending on the source, latently infected cells express between 3 and 10 genes (including EBNAs, LMPs, and EBERs), while the remaining 80 or so EBV genes are silent (20, 41, 42, 58, 61). When the virus is triggered to reactivate from latency, the lytic cycle ensues and virtually all EBV genes are expressed.

Reactivation may occur when the infected cell, as a result of stimulation or differentiation, induces expression of the EBV transactivator Zta (EB1 or ZEBRA, encoded by BZLF1). Zta is a member of the bZip family of transactivators, along with Jun/Fos, because it contains the basic DNA binding domain and zipper dimerization domain shared with other members of this family (4, 17, 52). Although Zta binds to AP1 sites on DNA (to which Jun/Fos binds), it also binds efficiently to a related set of motifs, ZREs, which are much more prevalent in the EBV genome than AP1 sites (4, 16, 26, 35, 38). Zta activates the expression of many EBV genes including other transactivators, Rta (BRLF1), Mta (BMLF1), and Ita (BI'LF4) (18, 25, 31, 37, 45, 57, 66). While some studies are consistent with this hypothesis (19, 33), others have reported that under some circumstances a subset of these transactivators (Zta, Rta, and perhaps Ita) are expressed simultaneously at immediately early times (2, 45, 64).

Although Rta alone can not activate the lytic cycle when transfected into latently infected cells, a property possessed only by Zta, it is likely to have an essential role in reactivation (6, 9, 10, 12). Rta can activate the expression of most EBV promoters tested; however, it is a particularly potent activator of two genes, the transactivator Mta and BHRF1 (25). BHRF1 encodes an intracellular membrane protein with sequence homology to the bcl-2 oncogene (7, 25, 53). The DNA sequences which are required for activation by Rta have been mapped to enhancer elements (5, 12, 30, 60). Reportedly, Rta binds as a homodimer to DNA sequences within the enhancers (22, 23, 43). Although these enhancers also contain binding sites for Zta, they respond poorly to Zta alone. However, the combination of Rta and Zta activates synergistically (12).

The enhancer located upstream of the BHRF1 gene is also a required component of the EBV lytic origin of replication (24). Thus, Rta is likely to have an important role in lytic cycle replication. A second copy of this origin-enhancer domain (without the flanking BHRF1 gene) is present in some strains of EBV and also responds to Rta (5).

Transactivators contain at least two essential domains: one directs the protein to its target (e.g., DNA binding domain), and a second facilitates initiation of RNA transcription (activation domain) presumably by contacting other

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cellular factors at the promoter (49, 54). Direct contacts between basal transcription factors and the activation domains of viral transactivators VP16, E1A, and Zta have recently been demonstrated (28, 34, 36, 40, 63).

Deletion of sequences near the C-terminal end of Rta abolished transactivation activity without affecting its ability to bind DNA (25, 43). These data suggest that the domain of Rta which contacts cellular transcription factors to facilitate initiation of RNA transcription is located at the C terminus. To identify activation domains of Rta, we transferred segments of Rta to a heterologous DNA binding domain and tested for the ability to activate gene expression. Rta appears to contain a complex acidic activation domain composed of subdomains, as well as an adjacent domain which confers cell type specificity.

MATERIALS AND METHODS

Plasmid constructions. Plasmids GAL4-VP16 and GAL4 (1–147) were obtained from Sadowski and Ptashne (59), and modified GAL4(1–147) with either 8-, 10-, or 12-mer *Bgl*II linkers inserted between *Sma*I sites was obtained from Gary Hayward. Restriction fragments of pMH48 (25) plus linkers were ligated to the appropriately modified GAL4(1–147) plasmid to generate the following plasmids: pMH208 (*Xmn*I [amino acid {aa} 11] to downstream *Bgl*II of pMH48), pMH236 (*Xmn*I [aa 11] to *Sst*I [aa 276]), pLT13 (*Sst*I [aa 279] to *Bsu*36I [aa 415]), pLT15 (*Bsu*36I [aa 416] to *Bsu*36I [aa 519]), pMH210 (*Nae*I [aa 493] to downstream *Bgl*II in pMH48), pMH212 (*Bsu*36I [aa 520] to downstream *Bgl*II); pMH264 (aa 520 to 548) was generated by inserting an *Xba*I triple terminator linker at the *Msc*I site of pMH212, and pMH266 (aa 520 to 592) was generated by inserting an *Xba*I triple terminator linker at the *Sph*I site of pMH212. pLT18 contains the *Xba*I-to-*Hin*cII fragment of herpesvirus saimiri pD1.9 (50) inserted into GAL4(1–147). Target promoters E1b-CAT, $(GAL4)_1$E1b-CAT, and $(GAL4)_5$E1b-CAT were obtained from Michael Green (39).

To generate mutants of native Rta, *Xba*I triple terminators were inserted into pMH48 at the first *Bsu*36I site to delete both domains in pLT25 or at the second *Bsu*36I site to delete domain 2 in pLT24. To delete domain 1, the *Bsu*36I fragment was dropped out (pLT26/27). The EBV enhancer-CAT construct (pMH103) has been described previously (12) and contains the 258-bp enhancer region of *Bam*HI-H inserted into A10CAT.

Amino acid substitutions in pMH212 (aa 520 to 605) were generated by recombinant polymerase chain reaction (27) with primers of the sequence CTTAATGAAATCGGGGA TACAGGTGGAAATGATGAATGT and its complement for glycine substitutions in domain 2a, or ATGACAGAGG ATGGGAATGGGGACTCCCCCGGGACCCCCGAACTT and its complement for glycine substitutions in domain 2b. Outside primers were complementary to the vector. All new constructs were sequenced to verify reading frames and mutations.

Transfections and CAT assays. Vero cells were maintained in Dulbecco modified Eagle minimal essential medium with 10% fetal calf serum (GIBCO) and passed 1:30 once or twice a week. The day before transfection, six-well dishes were seeded at 5×10^5 cells per 35-mm well. Cells were fed 4 h before transfection. Unless indicated otherwise, cells were transfected with 2 μg of target plasmid and 1 μg of activator plasmid per well. The HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-calcium phosphate transfection method was described in detail previously (25). The only alteration to this protocol was that cells were glycerol shocked the following morning. IB4 cells (human B-lymphoid cells latently infected with EBV) (32) and DG75 cells (EBV-negative Burkitt-like B-lymphoma cells) (56) were maintained in RPMI 1640 with 10% fetal calf serum and were transfected (5×10^6 per sample) with DEAE-dextran as previously described (25).

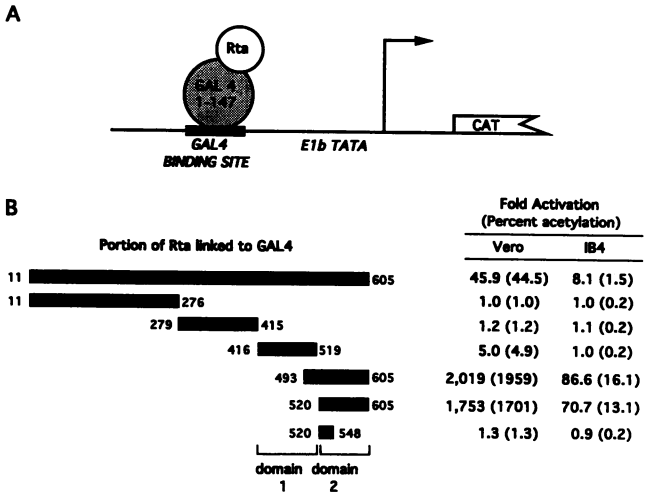


FIG. 1. Mapping activation domains of Rta. (A) Plasmids encoding GAL4-Rta fusion proteins were cotransfected into Vero or IB4 cells with a second plasmid containing the illustrated target promoter. Arrow represents transcription start site. (B) GAL4-Rta fusion proteins consisted of GAL4 aa 1 to 147 with the indicated domains of Rta linked in frame at the C-terminal end. Fold activations are the mean of six experiments and were calculated as the fold increase in percent acetylation over that obtained with a control plasmid (pBR322). For potent activators, CAT assays were repeated on 1:10 and 1:100 dilutions of cell lysates to obtain data within the linear range of the experiment, and the percent acetylation obtained was multiplied by the dilution factor.

Immunofluorescence. Vero cells were seeded into two-well slides at 1.25×10^5 cells per well and transfected as described above except that the precipitate was mixed with the medium by pipeting with a micropipettor. Cells were fixed with −20°C acetone or methanol and stained with a 1:500 dilution of rabbit anti-GAL4 serum (Michael Green) as described previously (25).

RESULTS

To identify amino acid sequences of Rta that could function as transcriptional activation domains, portions of Rta were substituted for the activation domain of yeast transactivator GAL4. The GAL4-Rta chimeras were composed of the GAL4 DNA binding domain (aa 1 to 147) followed by the regions of Rta indicated in Fig. 1B. The GAL4 DNA binding domain served to target the fusion proteins to the promoter, and Rta contributed the activation domain. The target promoter consisted of the adenovirus E1b TATA with a single upstream GAL4 binding site (Fig. 1A). These constructs were transfected into Vero cells or IB4 B-lymphoid cells, and fold activation over basal levels was quantitated from a standard CAT assay.

Activation domains of Rta. When all except the first 10 amino acids of Rta (residues 11 to 605) were linked to GAL4, a 46-fold activation was observed over basal levels of expression in Vero cells. Rta segments 11 to 276 and 279 to

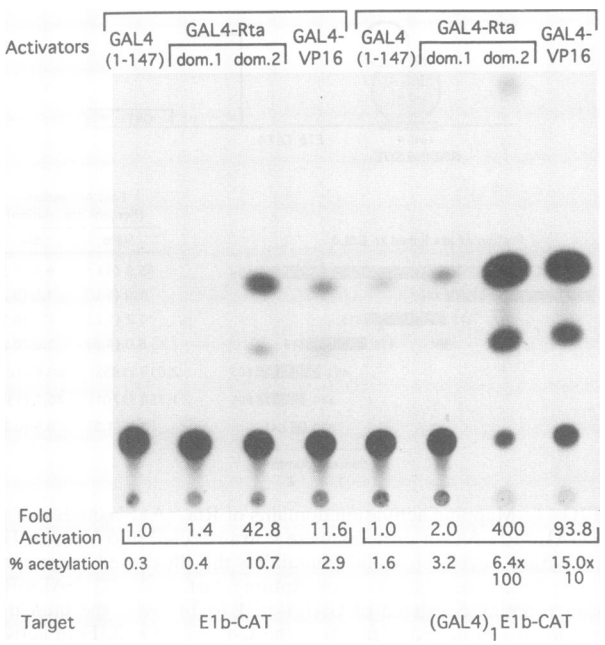


FIG. 2. CAT assay showing that activation by GAL4-Rta was enhanced by a GAL4 binding site. Plasmids encoding potential activators were cotransfected into Vero cells with a target promoter lacking a GAL4 binding site, E1b-CAT, or with a single GAL4 binding site, $(GAL4)_1$E1b-CAT. Fold activations reflect the increase in activity over that obtained with GAL4(1–147). Dilutions of cell lysates were made when necessary to accurately calculate fold activations as described in the legend to Fig. 1.

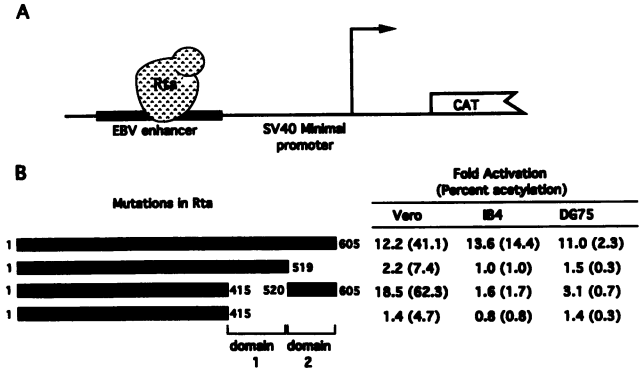


FIG. 3. Role of Rta domains 1 and 2 in transactivation by native Rta. (A) A plasmid (pMH48) expressing Rta or its derivatives was cotransfected into Vero, IB4 or DG75 cells with a target plasmid (pMH103) containing the 258-bp EBV enhancer from BamHI-H. (B) Inclusive amino acid numbers of Rta and its mutants are indicated. Fold activations are the means of four to eight CAT assays (except for DG75 results, which are from a single experiment), reflecting the increase in percent acetylation over that of a control plasmid (pBR322).

415 had essentially no activity. aa 416 to 519 consistently activated approximately fivefold, suggesting the presence of a weak activation domain (domain 1, Fig. 1B). The C-terminal third of domain 1 contains several acidic residues typically found in a number of other activation domains. In contrast to this weak domain, the C-terminal 86 residues (domain 2) of Rta was a potent activator, producing over a thousandfold activation in Vero cells. Deletion of the C-terminal 57 amino acids from domain 2, leaving only residues 520 to 548, essentially abolished activity. The C-terminal 113 aa (493 to 605) contain the acidic portion of domain 1 as well as domain 2 and had slightly greater activity than domain 2 alone.

When the GAL4-Rta chimeras were tested in IB4 B-lymphoid cells, similar results were observed except that the fold activations were reduced (Fig. 1B). However, fold activations in B cells were assumed to be underestimated since basal levels of expression were below detection. Domain 1 was either inactive in B cells or below the limits of detection. (Similar results [not shown] were obtained in DG75 cells). Fold activations were determined relative to the basal levels of expression with a control plasmid, pBR322, rather than GAL4(1–147) which reportedly has a cryptic transactivation domain (3, 44) (see Fig. 7). This cryptic activity was apparently not expressed when present in a chimera as reflected by the observation that all nonactive domains of Rta had lower levels of activity than GAL4(1–147) alone; therefore, it was not used as the control in this experiment.

All GAL4-Rta constructs were sequenced to confirm the correct reading frame and were screened in an immunofluorescence assay with anti-GAL4 antibody to verify the production of a stable product that was localized to the nucleus (data not shown). Some chimeras were exclusively nuclear while others were both nuclear and cytoplasmic. The staining pattern did not correlate with transactivation activity but may have reflected the presence or absence of an Rta nuclear localization signal. Based on staining intensity, the least abundant fusion proteins were those with the greatest activity (aa 493 to 605 and 520 to 605). Therefore, the weak activation by domain 1 and the lack of activity by other constructs cannot be explained in these experiments by insufficient levels of protein expression.

DNA-dependent activation. To determine that activation by the GAL4-Rta chimeras was dependent on the GAL4 DNA binding domain, constructs were tested for activation of target promoters that contained one or no GAL4 binding site (Fig. 2). Strikingly, GAL4-Rta2 (domain 2) activated 42-fold in the absence of a binding site. As has been noted by others, GAL4-VP16 also activated in the absence of a binding site. GAL4-VP16 was composed of GAL4(1–147) plus the C-terminal 78 amino acids of the herpes simplex virus transactivator VP16 (59). When appropriate dilutions of cell lysates were assayed, the calculated fold activations indicated that GAL4-Rta2 and GAL4-VP16 were approximately 10-fold more active in the presence of a GAL4 binding site. [Fold activations were calculated relative to GAL4(1–147) and therefore are lower than that shown in Fig. 1.]. Although these data are consistent with a second indirect mechanism of activation, a simpler explanation is that low-affinity DNA interactions are sufficient for potent activators to exert their effects.

Deletion of activation domains from Rta. The importance of domains 1 and 2 for transactivation by native Rta was determined by constructing mutants of Rta which lacked domain 1, domain 2, or both. These mutants were tested in a CAT assay for their ability to activate the minimal simian virus 40 (SV40) promoter with an EBV enhancer located upstream (Fig. 3A). This enhancer contains Rta-responsive elements and regulates the expression of BHRF1 in the EBV genome. CAT assay results indicated that deletion of domain 2 substantially reduced transactivation activity in all cell types tested, and a slightly greater diminution was observed

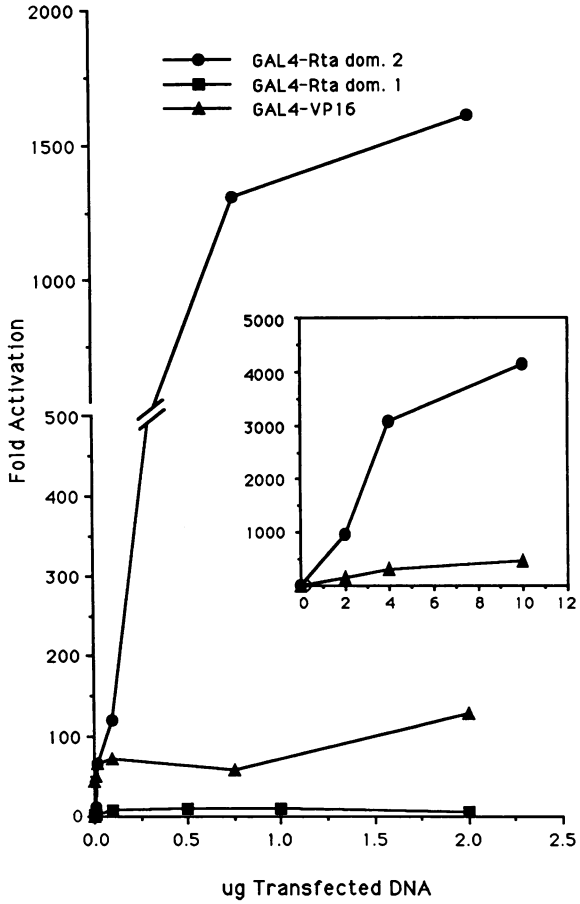


FIG. 4. Dose-response curves for different GAL4 derivatives. Concentrations of the indicated activator plasmids ranging from 0.0001 to 10 μg were tested for activation of the target promoter containing a single GAL4 binding site in cotransfected Vero cells. Fold activations (increase in percent acetylation) compared with a control plasmid (pBR322) are the means of three experiments.

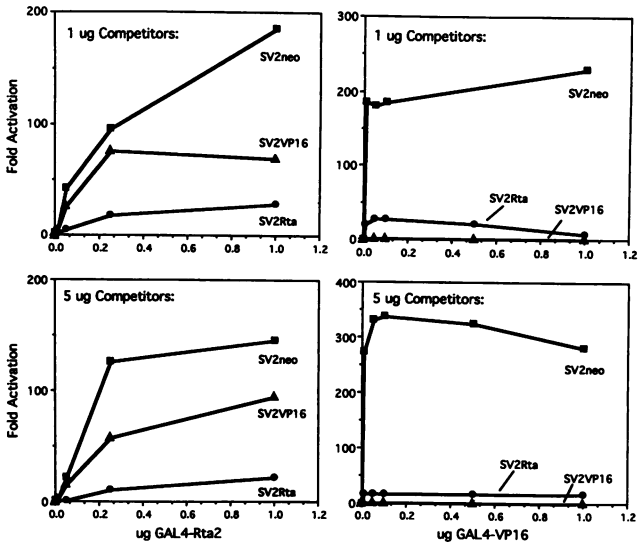


FIG. 5. Competition experiment with Rta and VP16. Increasing concentrations of GAL4-Rta2 or GAL4-VP16 were cotransfected with either 1 or 5 μg of the indicated competitors (SV40-driven Rta, VP16, or the neomycin resistance gene) and the target plasmid, $(GAL4)_1$E1b-CAT. Fold activations were calculated as described in the legend to Fig. 1. This experiment was done with increasing concentrations of the activator rather than competitors because increasing concentrations of each competitor produced a different profile which would have to be subtracted from the activator effect.

with deletion of both domains (Fig. 3B). Deletion of domain 1 had an apparent stimulatory effect in Vero cells but severely impaired activity in B-lymphoid cells. Similar results were obtained in the EBV-infected IB4 cells and the uninfected DG75 cells, indicating that the presence of latent EBV had no effect on the requirement for domain 1. The inability of domain 2 (without domain 1) to activate was surprising since domain 2 functioned well in B cells when linked to GAL4. Domain 1 could be required to relieve the effect of a negative B-cell factor rather than for direct interactions with TATA box-associated factors. Rta minus domain 1 worked well in Vero cells, arguing that the protein is functionally intact.

Comparison of Rta and VP16 activation domains. To compare the activation domains of Rta and VP16, their respective GAL4 constructions were tested in a dose-response experiment in Vero cells on a target promoter containing a single GAL4 binding site. Although immunofluorescence assays showed that increasing amounts of transfected plasmid produced increasing amounts of protein (not shown), GAL4-VP16 activity reached a plateau before detectable levels of protein were achieved (Fig. 4). In contrast, GAL4-Rta2 transactivation activity continued to increase with increasing amounts of transfected plasmid, commensurate with protein expression. The activation profiles of Rta2 and VP16 activation domains suggest that they have different modes of action. We also learned from this experiment that the 1 μg of input plasmid used for the experiments shown in Fig. 1 corresponds to the maximum response for the Rta1 and VP16 domains but may be an underestimate of the maximal level for Rta2.

Rta2 and VP16 were further compared in a reciprocal competition experiment in which SV40-driven wild-type VP16 or Rta (1 or 5 μg of transfected plasmid) was tested for the ability to inhibit transactivation by GAL4 chimeras (Fig. 5). Native VP16 was more efficient at inhibiting activation by GAL4-VP16 than by GAL4-Rta2. Conversely, native Rta was a more efficient inhibitor of GAL4-Rta2 than GAL4-VP16. The fact that neither wild-type activator was able to completely inhibit the other's activation domain suggests that they interact with different subsets of cellular factors. The SV40-driven neomycin resistance gene was included as a control to correct for any competition by the SV40 promoters used to drive the expression of the competitors.

Homologies among activation domains. Activation domain 2 of Rta is highly acidic like that of VP16. Extensive mutagenesis studies on the VP16 activation domain have suggested that the acidic residues contribute to its activity but are second in importance compared with the intervening hydrophobic residues (13). The phenylalanine of VP16 at position 442 (which is in a position analogous to that of the phenylalanine in Rta domain 2b) was found to be critical for transactivation activity as well as direct interaction with cellular transcription factors TFIIB and TFIID (13, 29, 40). Cress and Triezenberg (13) noted that activation domains of a number of yeast (GAL4, HAP4, and GCN4) and mammalian (VP16, SP1, and CTF) proteins exhibit a conserved pattern of hydrophobic residues. Domain 2 of Rta also

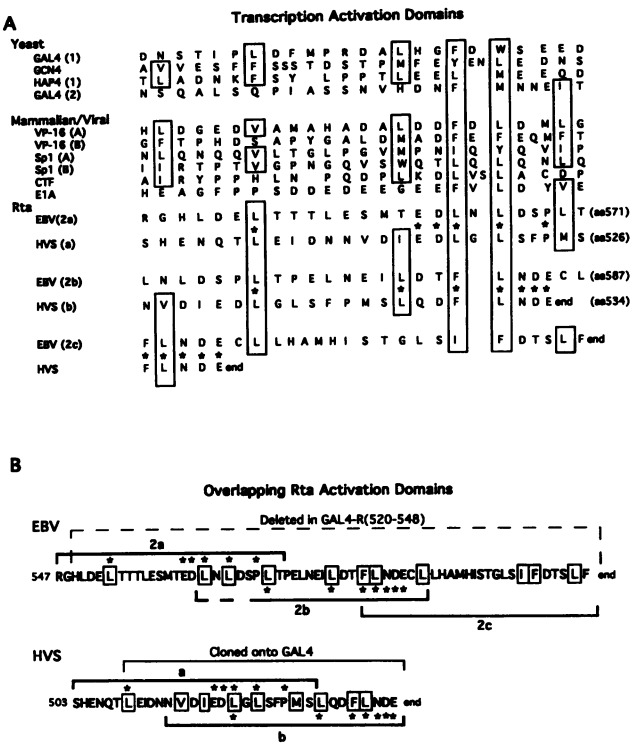


FIG. 6. Comparison of the primary sequences of different activation domains. (A) Alignment of hydrophobic residues (boxed) of Rta (EBV and HVS) and E1A (30) activation domains compared to the alignment of VP16, Sp1, CTF, and several yeast activators, as reported by Cress and Triezenberg (13). *, amino acid identity between Rtas of EBV and HVS. (B) The subdomains of Rta are shown on the linear sequence to illustrate their overlapping relationship. Conserved hydrophobic residues (boxed) and identical amino acids between Rtas (*) are indicated.

appears to maintain most of these positionally conserved hydrophobic amino acids (Fig. 6A).

The frequent occurrence of hydrophobic residues within activation domains sometimes permits multiple alignments. Such is the case for domain 2 of Rta which has three overlapping segments, 2a, 2b, and 2c, each with a similar hydrophobic motif. The frequency of hydrophobic residues within activation domains could suggest that their alignment is fortuitous and that the spacing between hydrophobics is not an important factor for activity. However, comparison of EBV Rta with analogous sequences from herpesvirus saimiri (HVS) Rta (50) suggested that this conserved pattern of hydrophobics may be significant. Although the predicted activation domain of HVS Rta has only 20% identity with EBV, the aligned hydrophobic residues are largely conserved (Fig. 6A). Of the 13 identical amino acids, there are 4 acidic residues, a proline, and an asparagine, and the remaining 7 correspond to the positionally conserved hydrophobics. HVS Rta differs from EBV Rta in that it appears to lack the third hydrophobic motif and the first two motifs overlap more extensively (Fig. 6B).

To determine if the predicted activation domain of HVS Rta had activity, the C-terminal 27 residues were fused to GAL4(1–147) and tested in a CAT assay for activation from a single GAL4 binding site. The HVS C-terminal domain exhibited transactivation activity comparable to that of the EBV GAL4-Rta2 lacking domain 2c (Fig. 7).

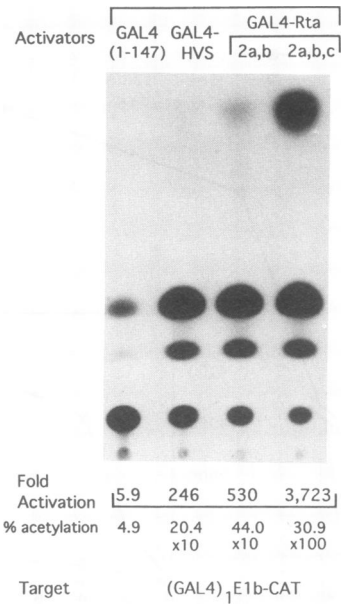
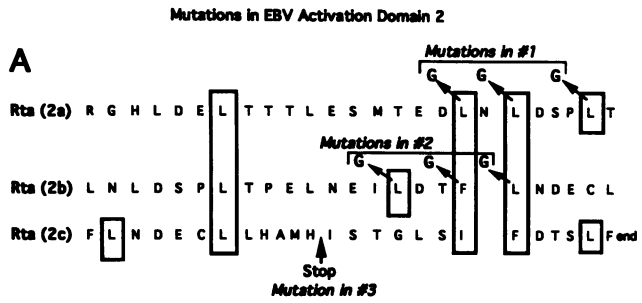


FIG. 7. CAT assay showing that the C-terminal 27 amino acids of HVS Rta also encode an activation domain. Plasmids encoding potential activators were tested on a target promoter containing a single GAL4 binding site in cotransfected Vero cells. Activator 2a,b is the same as mutant 3 in Fig. 8, and 2a,b,c contains the 86-residue domain 2 of EBV Rta (GAL4-Rta2). Fold activations (over pBR322) were calculated as described in the legend to Fig. 1.

Mutations in domain 2 of Rta. To determine the importance of positionally conserved hydrophobic amino acids for transactivation function, mutants of GAL4-Rta2 were generated in which glycines replaced three hydrophobic amino acids of 2a (mutant 1) or of 2b (mutant 2) (Fig. 8A). When these mutants were tested for transactivation activity on a single GAL4 binding site in Vero cells, their activity was significantly impaired (Fig. 8B). Mutations in domain 2a reduced activity by 98 to 99%, while mutations in 2b resulted in 80 to 90% loss of activity, indicating that these hydrophobic residues were required for full activity. Similar results were obtained in IB4 cells.

Mutation of the hydrophobic motifs in 2a and 2b of Rta also indicated that both motifs were required for full activity. To determine the importance of motif 2c, a third mutant which deleted a large portion of 2c by insertion of a stop codon was generated (mutant 3) (Fig. 8A). This mutant had activity similar to that of mutant 2 when assayed on a single binding site, a reduction by 80 to 90% compared with the wild type (Fig. 8B). These results could be interpreted to mean that 2a, 2b, and 2c are inherent parts of one domain. In contrast, each motif may constitute a functional subdomain. Despite the significant loss of activity, the mutated activators still produced 5- to 50-fold activations. If 2a, 2b, and 2c are unit length subdomains, then the leucine-to-glycine mutations introduced into 2a might be expected to disrupt domain 2b as well (since all three substitutions also lie within 2b). This mutant had the lowest activity of the three; perhaps it contains only one functional subdomain, 2c. Whether these regions represent a single domain or distinct functional subdomains, the results suggest that sequences within all three regions are important for transactivation of a target with a single binding site.

Evidence for synergism between subdomains. When the



B

		Fold Activation			
		1 binding site		5 binding sites	
Activator	Domains	Vero	IB4	Vero	IB4
GAL4-Rta2	2abc	331.3	80.0	565	462
GAL4-mut.#1	2(b)c	4.5	1.1	7.7	1.5
GAL4-mut.#2	2ac	45.1	1.6	956	236
GAL4-mut.#3	2ab	46.7	1.6	924	404
GAL4-VP16	AB	108	43.2	990	1,787

FIG. 8. Mutations in the Rta2 activation domain impair activity. (A) Using recombinant polymerase chain reaction (27), glycines were substituted for conserved hydrophobic residues (arrows) in Rta activation domain 2 linked to GAL4. Only 59 of the 86 residues in domain 2 are shown. Boxes indicate positionally conserved hydrophobic residues. (B) The mutants indicated in panel A were tested for activation of a target promoter containing one or five GAL4 binding sites in cotransfected Vero or IB4 cells. Fold activations (over pBR322) are the mean of several experiments and were calculated as described in the legend for Fig. 1.

mutants of activation domain 2 were tested on a target promoter with 5 GAL4 binding sites, very different results were obtained (Fig. 8B). Mutants with a defective 2b or 2c completely recovered their activity with multiple binding sites. In contrast, mutant 1 with a defective 2a (and probably 2b as well) could not recover activity with multiple binding sites. These results provide support for the possibility that 2a, 2b, and 2c are functional subdomains because they appear to synergize on multiple binding sites. When all three subdomains are intact (GAL4-Rta2), only additive, or less than additive, activity was observed on multiple binding sites suggesting that these subdomains are already synergizing when bound to a single site. The activation domain of VP16 contains at least two copies of the hydrophobic motif (Fig. 6A) but differs from Rta2 in that it synergizes on multiple binding sites rather than on a single binding site. This synergism was even more dramatic in IB4 cells.

DISCUSSION

Activation domains of transcriptional transactivators are those protein domains that interact with cellular proteins, either basal factors, cofactors, or both, to facilitate initiation of RNA synthesis (8, 15, 47, 68). By joining different domains of the EBV transactivator Rta onto the DNA binding domain of GAL4, we have identified a potent activation domain in the C-terminal 86 amino acids (domain 2). This domain contains 3 overlapping copies of a motif of positionally conserved hydrophobic amino acids found in other transactivators. Site-directed mutagenesis indicated that the hydrophobic residues are important for activity, and that sequences within all three copies of the motif are required for full activity. Amino acid sequence comparison with Rta from a second herpesvirus, HVS, revealed little similarity except for a few acidic residues and the positionally conserved hydrophobics, supporting the hypothesis put forth by Cress and Triezenberg (13) that these hydrophobic residues are essential components of activation domains. It is interesting to note that the Rtas from EBV and HVS share considerable homology in their N-terminal halves which presumably function as DNA binding domains, yet they have very little conservation in the activation domains (43, 50).

The occurrence of three copies of the hydrophobic motif within the Rta activation domain suggested the possibility of three functional subdomains. This was supported by the observation that mutations affecting only one motif (in GAL4-Rta2) severely impaired activation of a target promoter containing a single GAL4 binding site. However, these mutants had full activity when the target contained multiple binding sites, suggesting that the remaining subdomains could synergize when bound at multiple sites. If VP16 has only two subdomains and only one of these is active per molecule, then VP16 would be expected to synergize with multiple binding sites but not on a single site; this was the result observed. In contrast, Rta, with three potential subdomains, appears to synergize on a single binding site. But when only two subdomains were intact, it behaved like VP16, synergizing only on multiple binding sites. These results suggest that more than one subdomain may be required to stabilize the interaction with a cell factor, or that subdomains contact different cell factors, thereby facilitating synergism. Subcloning these motifs and studying their activities in tandem and in combination will help delineate their mechanisms of action.

It has been reported that GAL4(1–147) and its derivatives do not bind DNA cooperatively (1). Although we think that it is unlikely, we can not eliminate the possibility that cooperative binding of the Rta fusion proteins plays a role in synergism. We favor the hypothesis that the synergism observed on multiple binding sites was due to protein-protein interactions rather than DNA binding cooperativity.

A subterminal domain of 114 amino acids, domain 1, had minimal activation activity in Vero cells and almost no detectable activity in B cells when linked to GAL4. These results are consistent with domain 1 having either an auxiliary or a direct role in activation. The requirement of domain 1 for activation by native Rta in B cells suggests the possibility that it relieves the effect of a negative factor, perhaps one that is important for maintaining latency. Alternatively, domain 1 could be a positive effector and serve a function similar to that of the positionally homologous domain of VP16 that appears to contact Oct-1 (62). A third possibility is that domain 1 is a true activation domain that directly contacts a cell factor. If so, then it may represent a different class of activation domains because it lacks the hydrophobic motif present in domain 2. Other activators also may have combinations of different types of activation domains. The activation domain of E1A has been extensively mutagenized, and the results indicate it to be a zinc finger domain (46). However, immediately preceding this domain is the sequence shown in Fig. 6A. Deletion of one or more of the boxed hydrophobic residues abolished transactivation activity by GAL4-EIA. (Deletion of sequences adjacent to the other side of the zinc domain did not affect activation). Furthermore, the boxed hydrophobic residues of

E1A in Fig. 6A are highly conserved among adenovirus strains.

The activation domains of EBV Rta and VP16 were compared in dose-response and competition assays. Because the GAL4 antibody did not function in a Western analysis, we could not quantitate protein for a direct comparison of activation levels per microgram of activator. However, quantitation of protein from transfected cells is likely not to reflect the effective concentration of an activator because immunofluorescence assays indicated that some fusion proteins were transported to the nucleus more efficiently than others. Despite these limitations, we were able to make two observations. (i) Transactivation activity by GAL4-VP16 was saturable while that of GAL4-Rta2 was not (even though both produced increasing amounts of protein with increasing amounts of transfected plasmid). These results suggest the possibility that a cell factor required by VP16 is present in limiting quantities. (ii) A reciprocal competition assay indicated that native VP16 and Rta acted as competitive inhibitors of both VP16 and Rta2 activation domains linked to GAL4. However, native VP16 and Rta were more efficient at inhibiting their own Rta activation domains than at inhibiting each other's domains. This suggested that each activator can bind up some but not all the cell factors required by the other. It has been suggested that members of different classes of activation domains—acidic, glutamine rich (e.g., SP1) and proline rich (e.g., CTF; Fig. 6A)—interact with different cellular transcription factors (11, 48, 55, 65). Evidence presented here suggests that different acidic activators, even those with conserved hydrophobic motifs, may interact with distinct cell factors.

Computer analysis of Rta predicted that domains 2a and 2b form alpha helices connected by a turn. The positionally conserved hydrophobic residues are all on one side of the helix, and the alignment of leucines on the helices resembles short leucine zippers (leucines in every seventh position). When 2c is drawn as a helix (although not predicted to be one), a similar pattern is observed. Since the glycine substitutions would destabilize a helix, we cannot determine whether these substitutions disrupted protein conformation, hydrophobicity, or amphipathy or blocked specific interactions with cell factors. Although these structures were predicted for the activation domain of VP16 and others, mutagenesis has indicated that neither the amphipathy nor the helicity is important for activation (13, 21). Furthermore, nuclear magnetic resonance analysis of the purified C-terminal 79 amino acids of VP16 indicated that it had little secondary structure and no alpha helix formation (51). These results are consistent with circular dichroism spectroscopic analyses of the VP16 and GCN4 activation domains (14, 67). However, these results do not preclude the possibility that these activation domains form secondary structures upon interaction with their targets.

There is yet another level of activation in which Rta can participate; native Rta can synergize with another EBV-encoded transactivator, Zta. The Rta-responsive enhancer in EBV also contains binding sites for Zta, and when both are cotransfected into cells they transactivate with an activity approximately five times greater than the sum of their individual activities (12). Possibly one or more Rta subdomains can mediate this response.

Although activation by GAL4-Rta2 was significantly enhanced by the presence of a GAL4 binding site, we were surprised by the efficiency of activation in the absence of a binding site. Comparison of several activation domains from different proteins suggested that the ability to activate in the absence of a binding site was proportional to activation in the presence of a binding site. This implies that a stronger activator is more likely to activate without a specific binding site. Perhaps low-affinity interactions with DNA are sufficient to mediate activation at a lower level. It is possible that such activation is still biologically important. We have tested native Rta on a variety of EBV targets, and found Rta to specifically activate the promoters for BHRF1 and a third EBV transactivator, Mta (25). Both of these promoters have Rta-responsive enhancers upstream (5, 12, 30, 60). Most other EBV promoters tested were activated by Rta to at least some low level, perhaps mediated through nonspecific interactions with DNA.

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