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An Enhancer within the Divergent Promoter of Epstein-Barr Virus Responds Synergistically to the R and Z Transactivators

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The EA-R and *Not*I repeat genes of Epstein-Barr virus (EBV) are oriented head to head and separated by a 1,000-base-pair (bp) divergent promoter region. We have identified functional domains within this divergent promoter which are important for regulation of the rightward EA-R gene. Both the R transactivator (Rta) and the Z transactivator (Zta) increase the abundance of correctly initiated EA-R transcripts. A 258-bp fragment (−114 to −372 from the EA-R cap site) contained the primary Rta and Zta response elements and was capable of transferring Rta and Zta activity to a heterologous promoter in an orientation- and position-independent manner. Rta activated this 258-bp enhancer region in both EBV-positive and EBV-negative cells. However, Zta activity appeared to be dependent on another EBV gene product, since Zta activated the enhancer efficiently (500- to 2,000-fold) in EBV-positive cells but had little or no activity in EBV-negative cells. The combination of Rta and Zta produced a striking synergistic effect on the enhancer in the absence of any additional EBV components, suggesting that the interaction between Zta and Rta accounts for the Zta response observed in EBV-positive cells. An Rta response element was mapped to a domain located 60 bp away from a Zta-binding site within the enhancer. Although Rta activated the enhancer and other early promoters without additional EBV- or B-cell-specific factors, it did not activate the lytic cycle of EBV, in contrast to Zta. Immunofluorescence patterns of Rta and Zta with antipeptide antisera indicated that they have overlapping but different subcellular localizations. Both transactivators were found in the nucleus, but Rta was also found in the cytoplasm.

Epstein-Barr virus (EBV) infects and replicates in oropharyngeal and cervical epithelium (39–41), and progeny viruses subsequently infect B lymphocytes. In B lymphocytes and perhaps epithelial cells as well, EBV establishes a latent infection which persists for life (33). In the latent state, the EBV genome is maintained as a plasmid episome, and only a specific subset of genes is expressed (12, 23, 29, 32, 35). These latent cycle genes are presumed to be responsible for the ability of EBV to immortalize B lymphocytes and contribute to the malignancies associated with EBV (34). Periodic reactivation to produce lytic virus may be required to infect new cells and maintain a population of latently infected cells. Reactivation of the lytic cycle can be achieved in tissue culture by treatment of latently infected B cells with various chemicals, including tetradecanoylphorbol acetate (TPA), a phorbol ester (47). Although the events leading to reactivation following TPA treatment have not been delineated, several lytic cycle promoters, including the promoter for the Z transactivator (Zta) can be stimulated by TPA (16). The Zta promoter is normally silent or suppressed, a requirement for maintaining latency since Zta expression leads to reactivation (8, 14, 43). A small percentage of latently infected cells in culture undergo spontaneous reactivation. This may occur on the infrequent occasion when the Zta promoter is activated by cellular factors. Also, defective EBVs can induce the lytic cycle, presumably because their rearranged genomes place Zta behind a strong latency promoter (36).

It is not clear how Zta expression leads to activation of the lytic cycle. We have identified EBV promoters which appear to be targets for Zta, the most responsive being the divergent promoter (16). This 1,000-base-pair (bp) promoter region regulates the expression of its flanking early genes, the leftward *Not*I repeat gene and the rightward EA-R gene, and contains the lytic origin of replication (15). Most EBV strains contain a second copy of this promoter-origin domain which regulates the expression of the leftward *Pst*I repeat gene but lacks a flanking rightward promoter and gene (2, 22). This *Pst*I repeat promoter has also been identified as a target for Zta (8).

Zta is a 36- to 39-kilodalton protein encoded by three exons (3, 17, 38, 45). Recently, it was found that the second exon of Zta contains a basic amino acid sequence motif which is shared with the cellular transcription factors Jun, Fos, GCN4, and C/EBP (13). This basic motif is believed to directly contact DNA (46). Like Jun and Fos, Zta recognizes and binds to AP1 sites (13) and other related sites (P. M. Lieberman, J. M. Hardwick, J. Sample, G. S. Hayward, and S. D. Hayward, submitted for publication). Although Zta alone can activate the *Not*I repeat gene through the proximal promoter (6, 25), we found that Zta activation via upstream elements requires the R transactivator (Rta) and that together Zta and Rta induce a synergistic response.

The gene encoding Rta is immediately upstream of the gene encoding Zta, and their mRNAs are 3′ coterminal (3, 16). The unspliced Rta open reading frame is sufficient to encode a 66.6-kilodalton protein. However, Rta-specific antibodies detect a 94- to 98-kilodalton protein in infected cells, indicating that Rta may be posttranslationally modified (28, 38). Several lines of evidence suggest that Rta is likely to play an important role in reactivation of latent virus. (i) Rta can activate the promoters for several early EBV genes, including the divergent promoter and the promoter for the MS transactivator (Mta) (16). Although Mta fails to activate the divergent promoter, it increases expression from a wide range of target promoters, perhaps by a posttranscriptional mechanism (19, 26), and also is likely to play an important role in reactivation of the lytic cycle. (ii) Like the genes for

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Zta and Mta, the gene for Rta is preserved in defective viral genomes (P3HR1), in which it appears to be regulated by a latent cycle promoter like Zta (3, 10, 37). (iii) Studies in highly inducible, latently infected Akata cells suggest that Rta may be an immediate-early gene because its expression does not depend on prior protein synthesis (42). In this study we show that Rta does not require Zta to activate gene expression from an EBV enhancer located within the divergent promoter. By deletion analysis of the enhancer we identified sequences important for transactivation by Rta.

MATERIALS AND METHODS

Plasmid construction. The divergent promoter-chloramphenicol acetyltransferase (CAT) construction, pMH49, contains the *Stu*I-*Bgl*II subfragment of *Bam*HI-H of the B958 strain of EBV cloned upstream of the CAT gene as described previously (16). pMH32 (H9), pMH55 (H1), pMH54 (H10), pMH56 (H2), pMH61 (H3), pMH64 (H4), pMH65 (H6), pMH69 (H5), pMH104 (H7), and pMH109 (H8) were generated by partial or complete restriction enzyme digestions of pMH49 (see Fig. 3). pMH96 (H13), pMH97 (H11), pMH99 (H14), and pMH102 (H12) were generated by partial restriction enzyme digestions of pMH69 (see Fig. 4). pJL47 (L1) and pJL48 (L2) were generated by unidirectional Bal31 digestion of pMH49 from the *Tth*111I site (see Fig. 8). The 258-bp *Xma*I subfragment of pMH49 was ligated into the *Xma*I site of pGH56 (16) to generate pMH78. *Bgl*II linkers were added at the *Eco*RI site of pMH78 to generate pMH91, and the *Bam*HI-*Bgl*II fragment of pMH91, containing the *Xma*I fragment, was inserted at the *Bgl*II site of A10CAT (G. Khoury, National Institutes of Health, Bethesda, Md.) to generate pMH103. pMH105 contains the same EBV sequences as pMH103 but in the opposite orientation as for the *Not*I repeat gene. The *Bgl*II fragment of pMH91, containing the EBV *Xma*I subfragment, was ligated into the *Bam*HI site downstream of the CAT gene in A10CAT to generate pMH110 (forward orientation) and pMH111 (reverse orientation). pMH53 contains the rightward divergent promoter driving EA-R and was constructed as described previously (16). pPL17 (simian virus 40 [SV40]-driven *Bam*HI-Z) and pMH48 (SV40-driven *Hin*dIII-12) have been described previously (16, 44). An *Mst*II-*Nae*I deletion in pPL17 (pMH82) was generated to remove the sequences which encode the carboxy-terminal 85 amino acids of Rta which could potentially contribute to the effect of Zta.

Cells, transfections, immunofluorescence, and CAT assays. Vero, BHK, and NIH 3T3 cells were maintained in Dulbecco modified Eagle minimal essential medium with 10% fetal calf serum and seeded into two-well slides (Lab-Tek Products) at 2×10^5 cells per well the day before the experiment. Cells were transfected with calcium phosphate as described previously (16). At 44 h posttransfection, cells were fixed in acetone and stored at −20°C or used immediately for an immunofluorescence assay as described previously (16). Rabbit sera were diluted 1:50, monoclonal antibody R3.3 (31) was diluted 1:5,000, and monoclonal antibody 5B11 (30) was diluted 1:500.

IB4 cells, obtained from J. Sample (20), were maintained in Opt medium with 2.5% fetal calf serum. Raji cells and BJAB 107 cells (21, 24) were maintained in RPMI 1640 with 10% fetal calf serum. Cells (5×10^6 per sample) were transfected with DEAE-dextran as described previously (16). Transfected cells were either harvested for CAT assays or washed and dried onto slides for immunofluorescence assays as described previously (16). For CAT assays, 100 μl of lysate was prepared from 5×10^6 cells. Lysate samples of 5 to 100 μl were used in CAT assays, and only those results obtained within the linear range of the experiment were used to calculate relative activities.

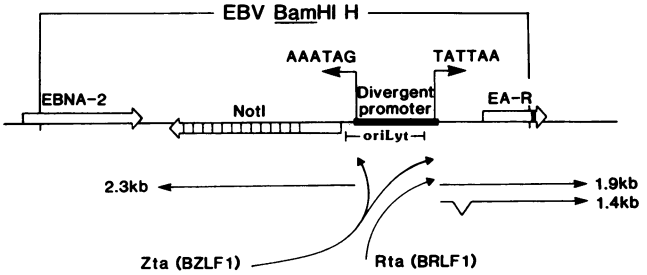

FIG. 1. Map of the *Bam*HI H fragment of EBV. Open reading frames (open arrows), initiation sites of transcription (bent arrows), mRNAs (straight arrows), and specificities of EBV transactivators (curved arrows) are indicated.

Isolation and analysis of RNA. IB4 cells were cotransfected with pMH53 (divergent promoter plus the EA-R gene) and Zta (pPL17), Rta (pMH48), or a control plasmid (pBR322). From approximately 6×10^7 transfected cells, RNA was isolated (9) and poly(A) was selected as described previously (16). Approximately 1 μg of RNA was hybridized to 20 ng of denatured probe at 56°C for 3 h. Samples were treated with 800 U of S1 nuclease (Bethesda Research Laboratories, Inc.) at 37°C for 45 min (27). Samples were precipitated twice and run on a DNA sequencing gel along with a chemically sequenced probe. The 827-bp *Sfa*NI-*Bam*HI fragment of pMH65 labeled at the *Sfa*NI site was used as a probe.

Antibody preparation. Synthetic peptides used for immunization corresponded to amino acids 335 to 349 of Rta and 106 to 120 of Zta. Ser-Cys was added to the carboxy terminus to provide a spacer and a sulfhydryl group for linkage. D-Tyr was added to the amino terminus to help prevent degradation and for quantitation. The peptides were linked to keyhole limpet hemocyanin with *N*-maleimidobutyryloxy-succinimide (GMBS). GMBS (1.4 mg) in 25 μl of dimethylformamide was added dropwise to 10 mg of keyhole limpet hemocyanin in 1.5 ml of 0.2 M sodium phosphate (pH 7.0)–1 mM EDTA–0.02% sodium azide. The synthetic peptide (1 to 10 mg) in 1 ml of 0.01 M sodium phosphate (pH 6.0)–1 mM EDTA–0.02% sodium azide was added to 1 ml of a keyhole limpet hemocyanin-GMBS mixture and incubated at 37°C for 2 h with frequent agitation. The sample was prepared for injection by mixing 1 ml of cross-linked peptide with 1 ml of Freund complete adjuvant and sonicated to generate a paste. Rabbits were immunized at 10 intradermal sites and boosted 6 weeks later with 0.5 ml of cross-linked peptide which had been stored at −20°C plus 0.5 ml of incomplete adjuvant. The mixture was sonicated as before and injected subcutaneously. A second boost 2 weeks later was prepared in the same manner wth 0.25 ml of cross-linked peptide.

RESULTS

Identification of EA-R transcripts induced by Rta and Zta. The divergent promoter in *Bam*HI-H regulates the expression of the leftward *Not*I gene and the rightward EA-R gene (Fig. 1). Northern analyses of transcripts from TPA-induced B958 cells identified a leftward 2.3-kilobase (*Not*I) RNA and two rightward (EA-R) RNAs of 1.9 and 1.4 kilobases (16,

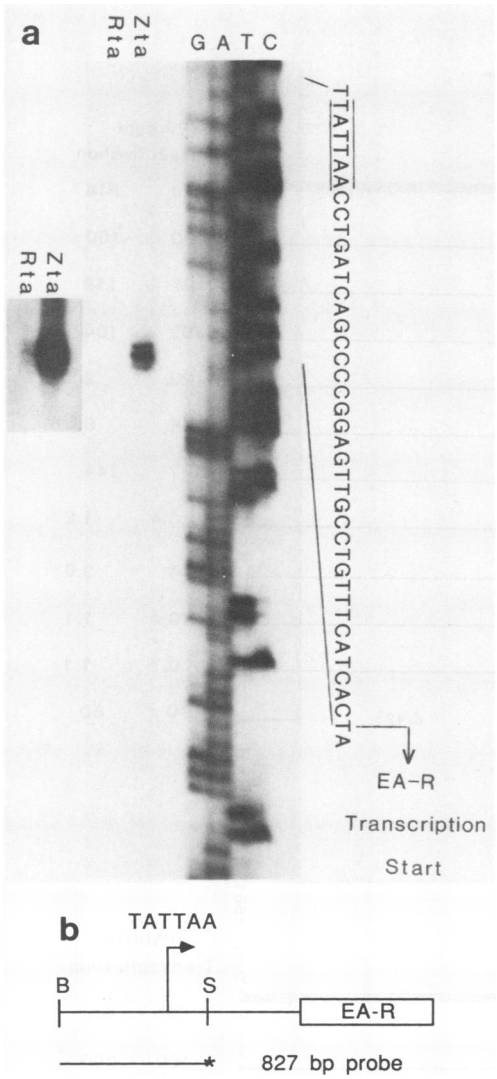

FIG. 2. (A) S1 analysis of Rta- and Zta-induced transcripts form the rightward divergent promoter. Polyadenylated RNA was isolated from IB4 cells transfected with Rta (pMH48) or Zta (pPL17) and the divergent promoter linked to the EA-R gene (pMH53). A chemically sequenced probe was run alongside as a marker. A sevenfold-longer exposure is shown on the left to reveal the Rta-induced product. We estimated that Zta is approximately 20- to 30-fold more active than Rta is. (B) Diagram of the probe used for S1 analysis. B, *Bam*HI site in the vector; S, *Sfa*NI site in the EA-R leader (^{32}P-labeled end is marked with an asterisk).

30). These transcripts were initiated just downstream from their putative TATA boxes, GATAAA (*Not*I) and TATTAA (EA-R) (25, 30). To determine where EA-R transcripts were initiated in response to transactivation by Zta or Rta, S1 analysis was done on polyadenylated RNA isolated from transfected IB4 cells, a latently infected B-cell line. For this assay, SV40-driven transactivators were cotransfected with a plasmid containing the divergent promoter and EA-R gene. The only detectable initiation site observed in cells transfected with Zta was located 34 bp downstream from the TATA box (Fig. 2). A longer exposure revealed a transcript that was initiated at the same position in cells transfected with Rta. Since basal levels of expression were not detectable, the fold increase in Rta- and Zta-induced RNA could not be determined. However, the ratio of Rta- to Zta-induced RNA approximated the levels of transactivation observed in CAT assays (see Fig. 4). We conclude that Rta and Zta increase the abundance of EA-R transcripts in our transient assay system and that these EA-R transcripts appear to be the same as the lytic cycle transcripts induced by TPA in B958 cells (30).

Mapping sequences within the divergent promoter that are required for Zta and Rta transactivation. The divergent promoter and the leader sequences for both the left and right transcripts are contained within a 1,794-bp *Stu*I-*Bgl*II subfragment of *Bam*HI-H. This subfragment was cloned into a CAT vector so that the rightward EA-R promoter was driving the CAT gene. The rightward orientation was utilized in these experiments because Rta transactivates primarily in the rightward direction, although Zta works in both directions. The effects of promoter mutations on transactivation by Rta and Zta were measured in a CAT assay and expressed as a percentage of wild-type activity (Fig. 3A).

Results obtained with mutants H5 and H9 suggested that the Rta and Zta response elements were located between −114 and −372 (hatched box). The deletion in mutant H3 (−237 to −671) significantly reduced Zta transactivation and nearly abolished Rta transactivation, indicating that sequences from −237 to −372 were important for Zta and required for Rta. Extending the deletion in H3 to −858 (H4) increased the total Rta and Zta activity. When these mutants were analyzed in BHK and Vero cells, in which basal levels were high, we found that deletion of sequences −591 to −858 (black box) increased the basal levels of activity (data not shown). Thus, the increase in activity of H4 relative to H3 and of H5 relative to the wild type is probably due to an increase in basal activity and not to alterations in Zta and Rta responsiveness. Deletion of the 258 bp between −114 and −372 reduced Rta and Zta activity by 97 to 99% (compare H6 with H1 and H2). Therefore, this 258-bp domain is likely to contain the predominant Rta and Zta response elements. The reduced Zta response of H6 could be partially reconstituted by moving the Zta response elements for the proximal leftward *Not*I repeat promoter (open box) close to the EA-R promoter (compare H7 and H8). A large deletion in the EA-R leader reduced Zta and Rta transactivation to 70 and 50%, respectively (H10). The role of the EA-R leader sequence in gene regulation has not been determined.

To further delineate the Rta and Zta response elements, we made additional deletions within the 258-bp responsive domain from −114 to −372 (Fig. 3B). Deletion of the left half of this domain (H11 and H12) had a greater effect on Rta transactivation than on Zta transactivation and suggested that sequences around the *Tth*111I site may be important for Rta. Deletion of the right half (H13 and H14) reduced both Zta and Rta activity by approximately 85%. Therefore, sequences in the right half are required for full Rta and Zta activity. The basal levels of expression by these mutants did not vary more than twofold. Thus, the strong basal element (deleted in H11) which is active in HeLa cells (6) and BHK cells (J. M. Hardwick, unpublished data) was not apparent in IB4 cells.

Enhancer properties of the Rta and Zta response element. To confirm that the 258-bp *Xma*I fragment (−114 to −372) was sufficient for Rta and Zta transactivation, we inserted this fragment into the *Bgl*II site of A10CAT, approximately 100 bp upstream from the enhancerless SV40 promoter. A10CAT containing the EBV sequences (pMH103) responded to both Zta and Rta at levels comparable to those of the enhancer upstream of its native promoter (H5) (Fig. 4A;

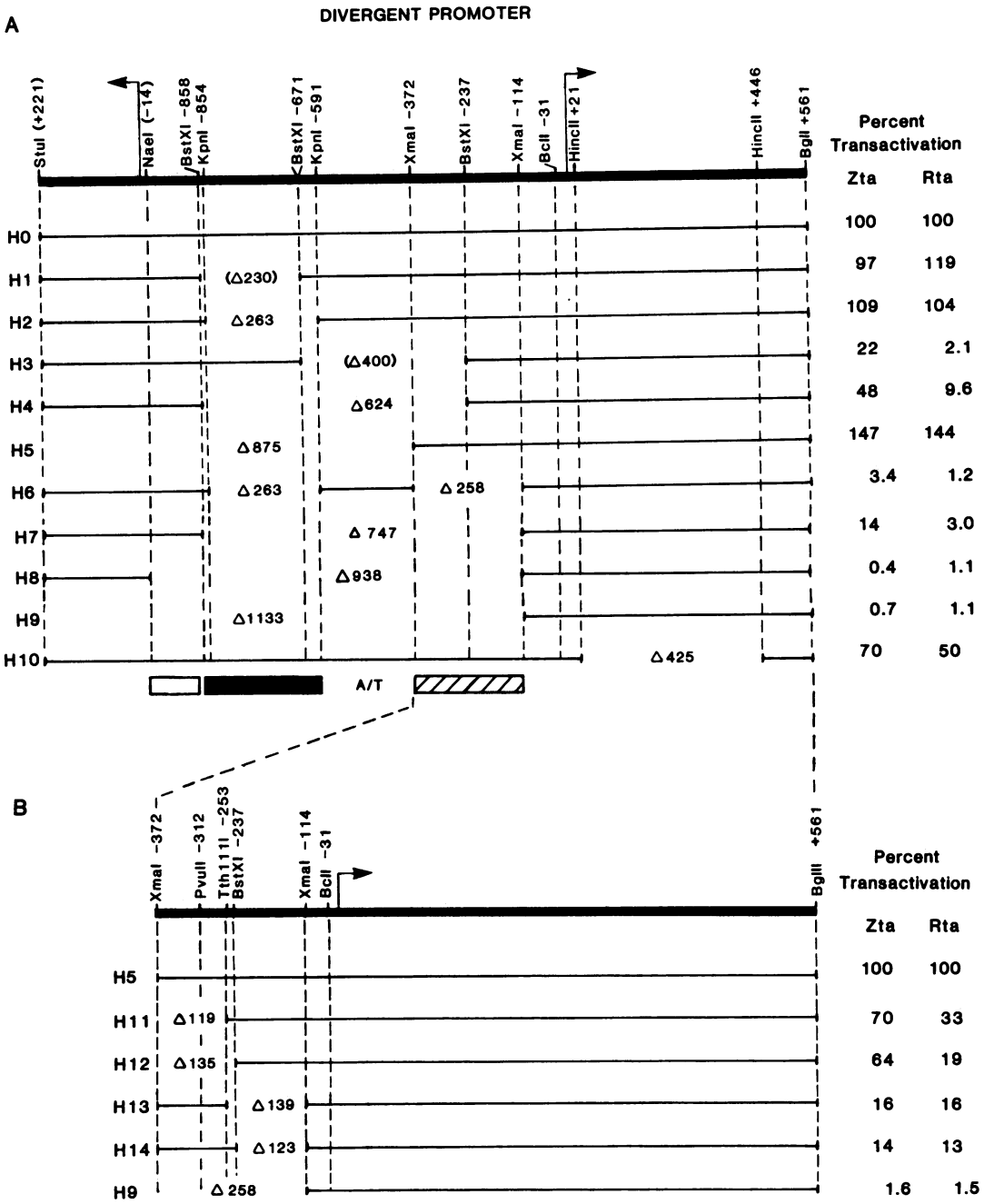

FIG. 3. Deletion analysis of the divergent promoter. Deletion mutants of the divergent promoter linked to the CAT gene in the rightward direction were cotransfected into IB4 cells with Zta (pPL17) or Rta (pMH48). The average of several CAT assays is shown and expressed as a percentage of wild-type activity. The transcription start site is indicated by the rightward arrow. The Rta- and Zta-responsive domain (hatched box), negative basal domain (solid box), leftward Zta-responsive domain (open box), and two A+T-rich palindromes (A/T) are indicated. Position numbers in parentheses are for the leftward direction.

see Fig. 3 for a map of H5). A10CAT alone was transactivated only two- to threefold by Rta and Zta. When the EBV insert was placed in the opposite orientation, as it is for the *Not*I repeat gene (pMH105), it also responded to Rta and Zta. Virtually 100% of the Zta response and approximately 50% of the Rta response were transferred to A10CAT by the 258-bp *Xma*I fragment. In a separate experiment, we tested the ability of EBV sequences to confer Zta and Rta inducibility when placed 3′ of the CAT gene in either the forward (pMH110) or the reverse (pMH111) orientation (Fig. 4B). In this downstream position, Zta and Rta maintained 15 to 20% of their activities relative to the forward, upstream position (pMH103). Thus, it appears that EBV encodes two transactivators which are involved in the regulation of gene expression via an enhancer within the divergent promoter.

Synergistic effect of Zta plus Rta on the enhancer. The experiments described thus far were done in EBV-positive IB4 cells. Because Zta is known to activate endogenous virus, we wanted to know if the Zta response on the divergent promoter was due to Zta alone or to other lytic

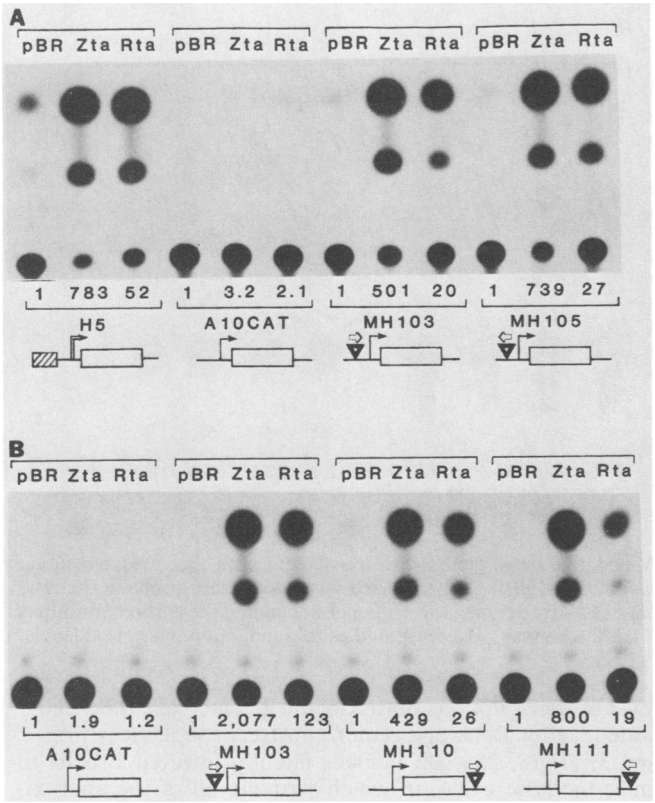

FIG. 4. Enhancer properties of the Rta and Zta responsive domain. pBR322, Zta (pPL17), or Rta (pMH48) was cotransfected into IB4 cells with target promoters containing the EBV enhancer (hatched box) with its native promoter (see Fig. 3 for a map of H5) or inserted into A10CAT (triangle) either upstream or downstream of the CAT gene (open box) in the orientations indicated by the open arrows. Results of the CAT assays are presented as fold induction over basal levels. For data to remain in the linear range of the assay, 5 μl of cell lysates with Zta and 50 μl of cell lysates with pBR322 or Rta were assayed for CAT activity. Two separate representative experiments are shown in panels A and B.

cycle gene products induced by Zta. The EBV enhancer in A10CAT (pMH103) was tested for Rta and Zta responsiveness in EBV-negative BJAB 107 cells. Although Zta activated this construction 500 to 2,000-fold in EBV-positive cells, there was virtually no Zta response in EBV-negative cells (Fig. 5A). However, the enhancer linked to its native EA-R promoter (H5) responded to Zta 12-fold. This response may have been due to sequences in the proximal EA-R promoter and not to enhancer sequences. We conclude that Zta alone has little or no ability to activate expression via the enhancer. In contrast, Rta was capable of activating the enhancer in the absence of other EBV components (Fig. 5A). The striking observation was that the combination of Rta plus Zta yielded a substantial increase in activity. Thus, it appears that Rta and Zta together have a synergistic effect that is approximately five times the sum of their individual activities. The combined effect of these transactivators on H5 approaches the effect observed with Zta alone in IB4 cells. Therefore, the effect of transfected Zta in EBV-positive cells may represent the combined effect of Rta and Zta.

This synergistic response appears to be specific for several reasons. (i) The combined effect of Zta plus Rta was not due to stimulation of the SV40 promoters driving the transactivators, since Zta stimulated SV2CAT by only twofold and Rta had no effect (Fig. 5B). (ii) A synergistic response was not observed with Rta plus Zta on the nonspecific target promoter in SV2CAT (Fig. 5B). (iii) When Rta and Zta were cotransfected into EBV-positive cells, no synergistic effect was observed (Fig. 5C) (16). Because Zta activates lytic cycle gene expression and presumably endogenous Rta as well, no synergistic effect would be expected by adding more Rta to EBV-positive cells. Therefore, transfected Rta does not produce a nonspecific increase in activity. These results suggest that in EBV-infected cells, Rta and Zta work in concert.

Mapping sequences important for the Rta response. The results presented in Fig. 3 suggested that Rta and Zta had different response elements and that sequences near the central *Tth*111I site may be important for Rta (compare mutant H3, H11, and H12). A deletion of 8 bp at this restriction site (mutant L1) diminished Rta activity by more than 80% as compared with the parent (Fig. 6). Although these deleted sequences appear to be critical for Rta responsiveness, additional sequences may also be important, since the Rta response on mutant L1 was not abolished. A larger deletion of 54 bp (mutant L2) did not further decrease Rta activity. The combined effect of Rta plus Zta in cotransfected BJAB 107 cells was diminished commensurately by the deletions in L1 and L2. These deletions also reduced the synergistic repsonse in Zta-transfected Raji cells (which presumably contain Rta). Reduction of the synergistic effect indicates that Rta response elements are important for synergism.

Rta does not activate the lytic cycle. The Zta transactivator was previously shown to stimulate latent virus to enter the lytic cycle (8, 14, 43). Because Rta can activate several EBV early promoters (linked to CAT) (16) as well as an EBV enhancer, we tested for the ability of Rta to activate the lytic cycle. SV40-driven Rta was transfected into latently infected Raji cells or IB4 cells. To monitor the induction of lytic cycle gene expression, we stained transfected cells in an immunofluorescence assay with antibodies to early antigens (Table 1). Cells transfected with Zta but not those transfected with Rta stained with the R3.3 monoclonal antibody specific for EA-D (BMRF1). Rta also failed to activate the expression of EA-R in Raji and IB4 cells. This result was puzzling in view of the results shown in Fig. 3 and 4, in which Rta activated the EA-R (rightward divergent) promoter when this promoter was present on transfected CAT plasmids. This result suggests that the endogenous viral promoter is less responsive to Rta than the same promoter present on transfected plasmids or that the immunofluorescence assay is not sufficiently sensitive. As in the CAT assays, Rta was capable of inducing EA-R in an immunofluorescence assay in cotransfected BHK and NIH 3T3 cells (Table 1). Not reflected by the numbers of positive cells was the observation that EA-R immunofluorescence induced by Rta in transfected BHK and NIH 3T3 cells was much more intense than Zta-induced immunofluorescence. Zta-induced EA-R was detected with the monoclonal antibody, but it could not be detected with lower-titer human nasopharyngeal carcinoma sera. Although it remains possible that Rta could activate lytic cycle gene expression in latently infected B cells at levels not detected by immunofluorescence, clearly Rta does not have the same activity as Zta.

Detection of Rta and Zta in transfected cells by antibodies to synthetic peptides. Synthetic peptides corresponding to amino acids 356 to 369 of Rta and amino acids 106 to 120 of

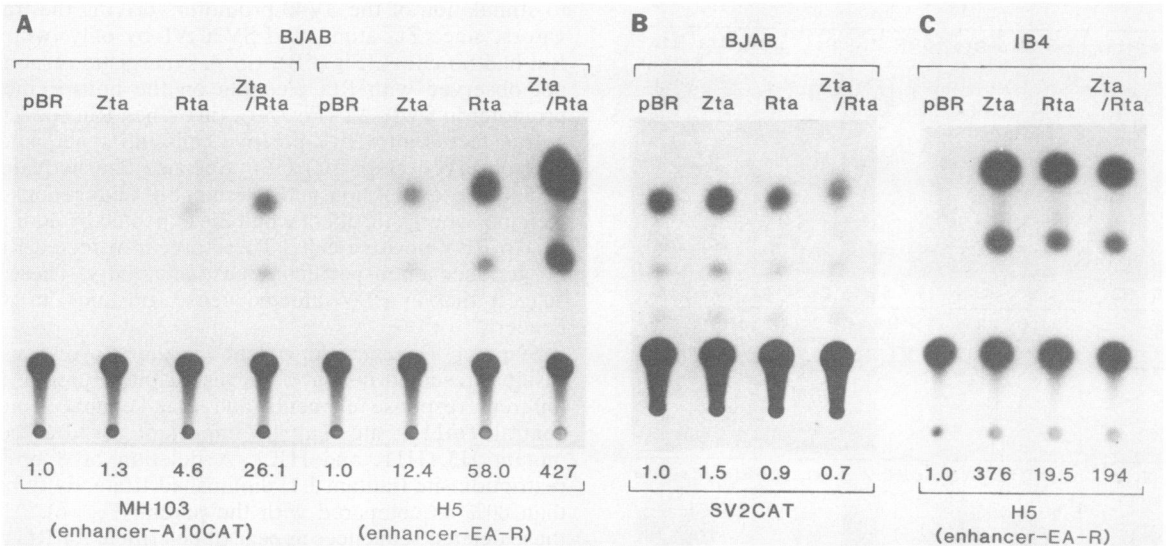

FIG. 5. CAT assay demonstrating synergism by Rta plus Zta in EBV-negative cells. Cells were transfected with the indicated target promoters and 2 μg each of pBR322, Zta (pMH82), and/or Rta (pMH48). Additional pBR322 was added when necessary to make the total DNA concentration of all the samples the same. pMH82 was generated from pPL17 by deleting the sequences encoding the carboxy terminus of Rta to ensure that Rta did not contribute to the effect of Zta. Results of the CAT assays are presented as fold induction over basal levels. Three representative experiments are shown.

Zta were prepared and used to generate specific rabbit antisera. To verify that our plasmid constructions produced Rta and Zta proteins, we transfected these plasmids into NIH 3T3 cells and stained them with antisynthetic peptide antisera. When stained with Zta-specific serum, cells transfected with Zta produced a diffuse nuclear immunofluorescence pattern which spared nucleoli (Fig. 7A). The immunofluorescence pattern observed when cells were transfected with Rta and stained with Rta-specific serum was complex (Fig. 7B). Some cells had nuclear staining only, some had cytoplasmic staining only, and others had both cytoplasmic and nuclear fluorescence. The presence of Rta in the cytoplasm may indicate that it is inefficiently transported to the nucleus or that its transport requires a second limiting factor. A similar disparate immunofluorescence pattern had been observed for the DNA-binding protein encoded by *erbA* (5). No Rta or Zta immunofluorescence was observed when cells were transfected with pBR322 (data not shown).

DISCUSSION

EBV encodes two transactivators, Rta (BRLF1) and Zta (BZLF1), which appear to regulate gene expression at the level of transcription. Both Rta and Zta increase the steady-state level of RNA and require upstream sequences (Fig. 2 and 3) (7, 18, 25). Zta induces latently infected B cells to enter the lytic cycle, in which virtually all of the approximately 100 genes are expressed. The molecular events leading to reactivation are not understood. Evidence presented here suggests that Rta works in concert with Zta to facilitate reactivation.

To delineate molecular events leading to reactivation of the lytic cycle, we have identified target promoters for Zta and Rta. The most responsive of these is the divergent promoter region located in the *Bam*HI H fragment of EBV (16). The divergent promoter region contains a leftward TATA box for the *Not*I repeat gene and a rightward TATA box for the EA-R gene separated by 1,000 bp of complex sequence and regulatory elements. By deletion analysis of the divergent promoter, we have identified domains which influence rightward transcription. The primary response elements for both Rta and Zta were contained within a 258-bp fragment (−114 to −372). This same domain has also been shown to confer Zta responsiveness in the leftward direction (25). When this 258-bp fragment was inserted upstream of a heterologous promoter, it conferred Rta and

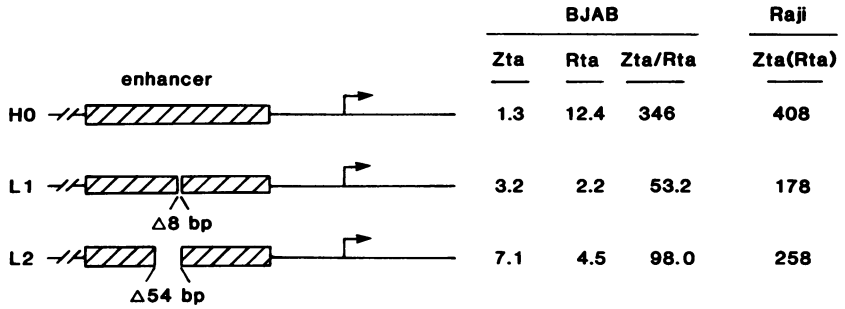

FIG. 6. Mapping the Rta-responsive region within the enhancer. Bal31 deletions were generated leftward from the *Tth*111I site (see Fig. 3 for a map of wild-type H0). Results of three CAT assays were averaged and are presented as fold induction over basal levels for the indicated transactivator(s). Raji cells were transfected only with Zta (pMH82) but are likely to express Rta from the endogenous virus, as indicated in parentheses.

TABLE 1. Immunofluorescence assay for induction of early antigens by EBV transactivators

Target	Trans-activator	No. of positive cells/10^5 transfected cells					
		EA-R[a]		EA-D[b]			
		Raji	IB4	Raji	IB4	BHK	3T3
Endogenous virus	pBR322	1	7	2	0		
	Zta	226	400	224	179		
	Rta	3	6	10	0		
Divergent promoter driving EA-R (pMH53)	pBR322					71	7
	Zta					189[c]	156[c]
	Rta					344	264

[a] Monoclonal antibody 5B11 (30).
[b] Monoclonal antibody R3.3 (31).
[c] Weak fluorescence intensity (not detectable with human NPC sera).

Zta responsiveness in an orientation- and position-independent manner. However, Zta activation of this enhancer domain occurred only in EBV-positive cells, indicating the requirement for a second EBV gene product. In contrast, Rta activated this enhancer in the absence of any other EBV components. Because Zta fully activates the lytic cycle leading to the production of progeny viruses (14), it is likely to induce the expression of endogenous Rta in EBV-positive cells. The Rta promoter predicted by Biggin et al. (3) and its upstream 1,300 bp responded weakly to Zta in a CAT assay (J. M. Hardwick, unpublished data). Other than induction of Rta, Zta appears to have a more direct role in enhancer activation. When Zta and Rta were transfected together into EBV-negative cells, a synergistic effect on the enhancer was observed. Further evidence for a direct role for Zta comes from our recent finding that Zta binds to four sites within the enhancer (Fig. 8) (Lieberman et al., submitted). Zta binding to the enhancer, which apparently is not sufficient to activate transcription, also may have a role in replication, since this enhancer is located within a large domain that overlaps the divergent promoter and functions as the lytic origin of replication (Fig. 1). The enhancer region is required for replication and can be functionally replaced by the cytomegalovirus enhancer (15).

Even though Zta alone had little effect on the enhancer, Zta apparently can still activate transcription without Rta via sequences close to the TATA box (6, 25). Activation of these proximal promoters in EBV-negative cells apparently does not involve the enhancer and, therefore, does not require Rta. Zta activation of proximal promoters, presumably by interaction with cellular TATA box factors, is much weaker than activation mediated by the enhancer. Zta also activates the expression of other EBV early promoters (16, 18) in EBV-negative cells, perhaps by a related mechanism.

Rta alone does not appear to be sufficient to induce the expression of lytic antigens from the endogenous genome or activate the lytic cycle. However, Rta may be an important facilitator of reactivation by activating EBV enhancers. To study the mechanism of transactivation by Rta, we sought to identify an Rta response element within the enhancer. An 8-bp deletion near the center of the enhancer domain reduced Rta responsiveness by 80%. This deletion interrupts two overlapping palindromes, either of which potentially could mediate Rta activity. This deletion did not completely abolish Rta activity, suggesting that additional sequences also are important. To the left of this 8-bp domain are two related palindromes which differ from each other and those

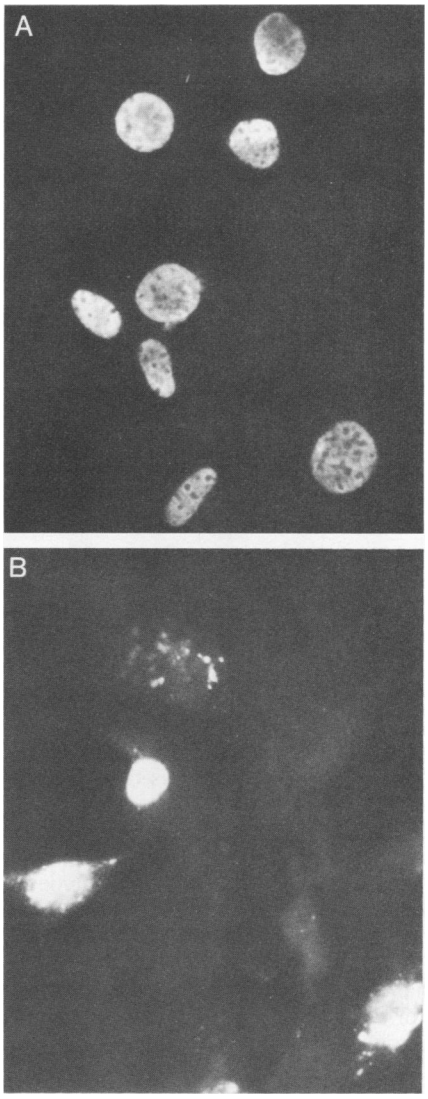

FIG. 7. Immunofluorescence assay showing subcellular localization of Zta and Rta. NIH 3T3 cells were transfected with Zta (pMH82) (A) or Rta (pMH48) (B) and stained in an indirect immunofluorescence assay with the corresponding rabbit antiserum directed against a synthetic peptide.

deleted in L1 by the number of base pairs at their axes of symmetry (Fig. 8). A 54-bp deletion (mutant L2) which deletes or interrupts all of these palindromes did not further decrease Rta activity. Thus, additional sequences, presumably to the right of the 8-bp deletion, may form part of the Rta response signal. In fact, sequences deleted in L1 are duplicated just to the right. Mutants with deletions of both of these copies (H3 and H4, Fig. 3) have Rta responses further reduced by 90 to 98%. A 28-bp oligonucleotide encompassing the L1 deletion from the analogous *Pst*I repeat promoter was sufficient to transfer the Rta response to a heterologous promoter (7). Thus, it appears that the 8-bp domain deleted in mutant L1 contains or interrupts an Rta response element. The Rta response element appears to be required for Zta-induced activation of the enhancer, consistent with the observation that Zta, without Rta, does not activate the enhancer.

Another domain within the divergent promoter which

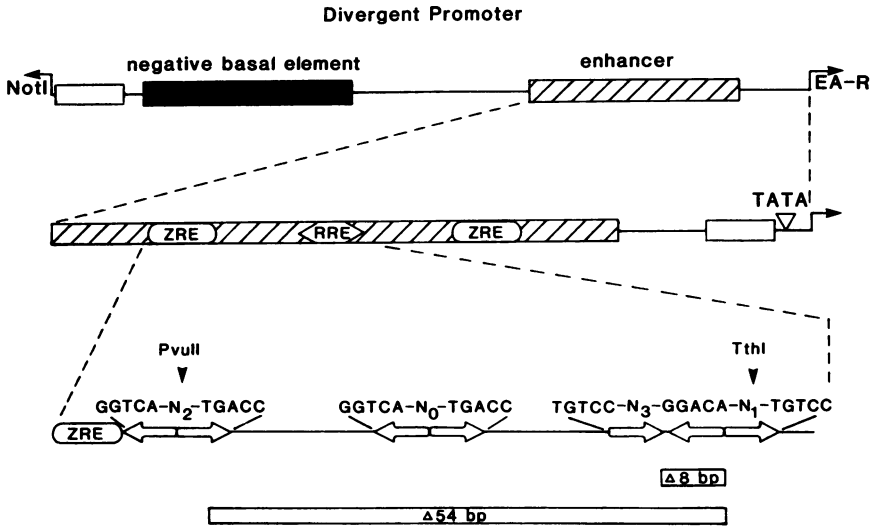

FIG. 8. Transcription elements of the divergent promoter. The Zta-responsive proximal promoter (open boxes), Zta response element (ZRE), Rta response element (RRE), palindromes (open arrows), and deletion mutations described in Fig. 6 are indicated.

appears to influence gene expression is located between the leftward promoter and the enhancer (−600 to −850). This domain suppresses basal levels of activity in both the leftward (25) and rightward directions. This region contains a 75-bp direct repeat which may contribute to its negative transcriptional effect. However, this negative domain probably does not contribute to the unidirectional effect of Rta on the divergent promoter. Because Rta can activate the enhancer alone in an orientation-independent manner, presumably other sequences outside the enhancer determine why Rta (without Zta) activates the divergent promoter less efficiently in the leftward direction than in the rightward direction.

This enhancer is strategically located to activate the expression of two flanking genes. The leftward *Not*I repeat gene encodes a protein which appears to be transported to nucleoli (25). The rightward EA-R gene encodes a protein which appears to be integrated into cytoplasmic membranes (16) and shares amino acid sequence homology with the *bcl*-2 oncogene (11). EA-R and *bcl*-2 have similar overall structures and intracellular localizations. Highly spliced latent cycle EA-R transcripts have also been reported (1, 4, 30), suggesting that EA-R expression may be regulated by a distant latent-cycle promoter as well as the lytic cycle divergent promoter. The *Not*I and EA-R gene products are likely to have important functions in EBV-infected cells.

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