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The Cellular YY1 Transcription Factor Binds a *cis*-Acting, Negatively Regulating Element in the Epstein-Barr Virus BRLF1 Promoter

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Disruption of Epstein-Barr virus latency is induced by expression of either the BZLF1 (in B cells and epithelial cells) or BRLF1 (in epithelial cells only) immediate-early protein. Regulation of BZLF1 and BRLF1 transcription may therefore modulate the stringency of viral latency. The cellular transcription factor YY1 negatively regulates BZLF1 transcription. Here we show that the BRLF1 promoter (Rp) sequences from −206 to −227 (relative to the mRNA start site) and from −7 to +6 are directly bound by YY1. Mutation of the upstream YY1 binding site increases constitutive Rp activity in epithelial cells and B cells, while mutation of the downstream YY1 binding site does not significantly affect Rp activity. Negative regulation of BZLF1 and BRLF1 transcription by YY1 may act to maintain viral latency.

Epstein-Barr virus (EBV) is a human herpesvirus which is associated with the development of B-cell lymphomas and nasopharyngeal carcinoma (30, 57). Infection in B cells results in primarily latent infection, whereas infection of epithelial cells is more frequently permissive (20, 25, 30, 46). Expression of the EBV immediate-early (IE) gene product BZLF1 (Z) disrupts viral latency in B cells (4, 5, 7, 18, 38, 39, 47). In epithelial cells, expression of either BZLF1 or another EBV IE gene product, BRLF1 (R), disrupts viral latency (15, 52). Therefore, the transcriptional regulation of both the BZLF1 and BRLF1 gene products by cellular factors is critical in determining the stringency of viral latency.

Disruption of viral latency may require not only the activation of cellular transcription factors which positively regulate BZLF1 and/or BRLF1 transcription but also the inactivation of repressor factors. The BZLF1 gene product is primarily derived from the Zp promoter, and the BRLF1 gene product is primarily derived from the Rp promoter (27). In addition, small amounts of BZLF1 may be derived from Rp, since the messages derived from this promoter have the potential to translate the BZLF1 gene product bicistronically (27).

The BZLF1 promoter (Zp) has been extensively studied and shown to contain a number of positive, as well as negative, regulatory elements. Important positively regulating elements in Zp include the ZII motif (a CREB-like motif which helps mediate 12-*O*-tetradecanoylphorbol-13-acetate [TPA] induction) and the ZI motifs (which act as positive regulators in the presence of TPA but negative regulators in the absence of TPA) (10, 11, 40). Zp is bound by the cellular YY1 transcription factor and is negatively regulated by this protein (33, 34). A series of HI motifs (some of which overlap the YY1 binding sites) have also been shown to negatively regulate BZLF1 transcription (41).

Chemical and biological agents which disrupt EBV latency activate Zp and Rp transcription essentially simultaneously (9, 21, 35, 48), suggesting that BZLF1 and BRLF1 expression is regulated through common cellular transcription factors. The BRLF1 promoter contains Sp1 binding sites, which are important for constitutive activity (53), and Zif268 (Egr-1) binding sites, which are required for TPA-induced stimulation (54). However, specific cellular factors which regulate both Zp and Rp transcription have not been identified (19).

In this study, we show that Rp, like Zp, contains binding sites for YY1 and is negatively regulated by this cellular transcription factor. The YY1 (yin-yang 1) protein is a Kruppel-related zinc finger protein which functions as either a negative or positive regulator, depending upon the position in the promoter and the cellular environment (8, 16, 36, 44). Our results suggest that YY1 promotes EBV latency by negatively regulating the two viral IE proteins which induce lytic infection.

Binding of cellular proteins to Rp was assessed in electromobility shift assays (EMSAs) (12). A ^{32}P-labelled DNA probe spanning the Rp sequences from −188 to +6 was incubated with a nuclear cell extract derived from Raji cells (a latently infected EBV-positive Burkitt's lymphoma cell line). Binding reaction mixtures consisted of 20,000 cpm of the ^{32}P-end-labelled probe incubated with 4 μg of nuclear extract in a buffer consisting of 50 mM Tris (pH 7.9), 250 mM NaCl, 2.5 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, and 50% glycerol. Poly(dI-dC) · poly(dI-dC) (4 μg) was added as a nonspecific competitor DNA, and the final volume was adjusted to 20 μl.

As shown in Fig. 1, the BRLF1 promoter is bound by several different proteins in Raji cell extracts. One of these complexes is the previously reported Sp1 complex (53). Another complex, previously designated complex B (53), was specifically competed by the murine leukemia virus YY1 binding site (8) but not by a series of other competitor DNAs. Thus, complex B, which we have previously shown is present in both B cells and epithelial cells (53), may in fact be the YY1 transcription factor.

To confirm that YY1 binds to Rp, partially purified bacterial YY1 fusion protein (produced from a construct containing the YY1 cDNA cloned into the pDS56-6X His vector) (a gift from Tom Shenk) was incubated with 20,000 cpm of radioactively labelled BRLF1 probe (containing Rp sequences from −188 to +6) in a buffer consisting of 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM KCl, 0.25 μM $ZnSO_4$, 10 ng of bovine serum albumin per μl, and 7% glycerol. The reaction mixture was loaded on a 4% polyacrylamide–Tris-glycine gel

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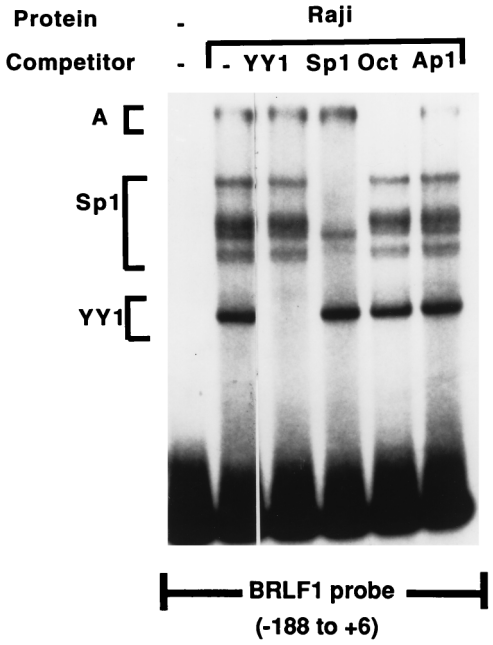

FIG. 1. A YY1-like factor binds to Rp. A ^{32}P-labelled probe containing Rp sequences (from −188 to +6) was incubated with Raji cell nuclear extract in an EMSA. Raji cell extracts contain a complex binding to the Rp probe which is specifically competed with unlabelled oligonucleotide competitor DNA containing the YY1 binding site from the murine leukemia virus LTR (YY1) but is not competed by cold competitor DNA containing the Ap1 or Sp1 binding motif. The potential YY1 complex, the previously identified Sp1 complex (53), and the as yet unidentified A complex are indicated in brackets.

FIG. 2. Bacterially synthesized YY1 binds to Rp. Protein extracts from HB101 bacteria expressing the YY1 protein linked to a His tag (a gift from Tom Shenk) versus extracts from HB101 bacteria containing the pDS56-6X His vector were incubated with radioactively labelled BRLF1 probe (containing Rp sequences from −188 to +6). A labelled BRLF1 probe containing Rp sequences from −179 to −575 also bound bacterially synthesized YY1 (data not shown).

and run in 1× Tris-glycine buffer (0.025 mM Tris, 0.19 M glycine) at 20 mA at room temperature. The bacterial extracts containing the vector alone (pDS56-6X His) did not bind to Rp, whereas the YY1-expressing bacterial extracts produced a complex binding to Rp which was specifically competed by the murine leukemia virus YY1 binding site but not competed by competitor DNA containing the Ap1 motif (Fig. 2). A probe containing the BRLF1 sequences from −179 to −575 produced similar results (data not shown). Thus, there appear to be at least two YY1 binding sites in the BRLF1 promoter.

To determine the exact sites of YY1 binding in the BRLF1 promoter, we performed methylation protection studies as previously described (53). The ^{32}P-end-labelled BRLF1 probes (100,000 cpm) were incubated with 2 volumes of the reaction mix used in the EMSAs (containing partially purified bacterial YY1 protein) and treated with dimethylsulfate immediately before loading the reaction mixture on the gel. The YY1-competed bands and free probes were cut separately from the gel, transferred to DEAE membrane paper, incubated with 1 M piperidine at 90°C for 15 min, and then eluted with high-salt buffer. Equal amounts of radioactive bound and free probes were then loaded on a 6% polyacrylamide–7 M urea gel and subjected to autoradiography.

As shown in Fig. 3A and B, two YY1 binding sites in Rp were defined in these experiments. The first (downstream) YY1 site is located over the BRLF1 mRNA initiation start site. The second (upstream) YY1 binding site is located between −206 and −227 (relative to the BRLF1 mRNA start site). To confirm that the sites defined in Fig. 3A and B bind YY1, labelled oligonucleotide probes spanning each of the two YY1 binding sites were incubated with bacterially synthesized YY1 in an EMSA (Fig. 3C). The two Rp oligonucleotide probes bound YY1 with similar efficiency, whereas a control oligonucleotide probe (spanning the CCAAT motif in the early EBV BHLF1 promoter) did not bind YY1. Likewise, unlabelled oligonucleotides spanning either of the two BRLF1 YY1 sites efficiently competed against YY1 binding (from HeLa cell nuclear extracts) to a labelled probe containing the murine leukemia virus YY1 site (8), whereas oligonucleotides containing specific mutations in the BRLF1 YY1 sites did not compete (Fig. 3D). Finally, a labelled BRLF1 probe (containing the BRLF1 promoter sequences from −254 to +6) in which the two YY1 sites were both specifically mutated no longer bound the YY1-competed complex in HeLa cell nuclear extracts (Fig. 3E).

The functional significance of the YY1 sites in Rp was examined in transient reporter gene assays. Site-directed mutants altering each of the YY1 sites in the RpBSCAT construct (which contains the Rp sequences from −962 to +5 linked to the chloramphenical acetyltransferase [CAT] gene) (54) were made with the Bio-Rad in vitro mutagenesis kit as shown in Fig. 4a. The wild-type and mutant RpBSCAT plasmids were transfected into HeLa epithelial cells or EBV-positive Raji Burkitt cells as previously described (54), and the level of CAT activity induced by each construct was measured (14).

As shown in Fig. 4b and c, mutation of the upstream YY1 site alone in Rp increased the constitutive activity of the RpBSCAT plasmid in both HeLa and Raji cells. Thus, the upstream YY1 binding site may function as a negative regulator of Rp. In contrast, mutation of the downstream YY1 site alone, which is positioned over the mRNA start site, slightly reduced Rp activity in HeLa cells and had little effect in Raji cells. Simultaneous mutation of both YY1 sites reduced Rp activity in HeLa cells but increased Rp activity in Raji cells.

Disruption of EBV latency can be induced by a variety of agents, including the phorbol ester TPA, and anti-immuno-

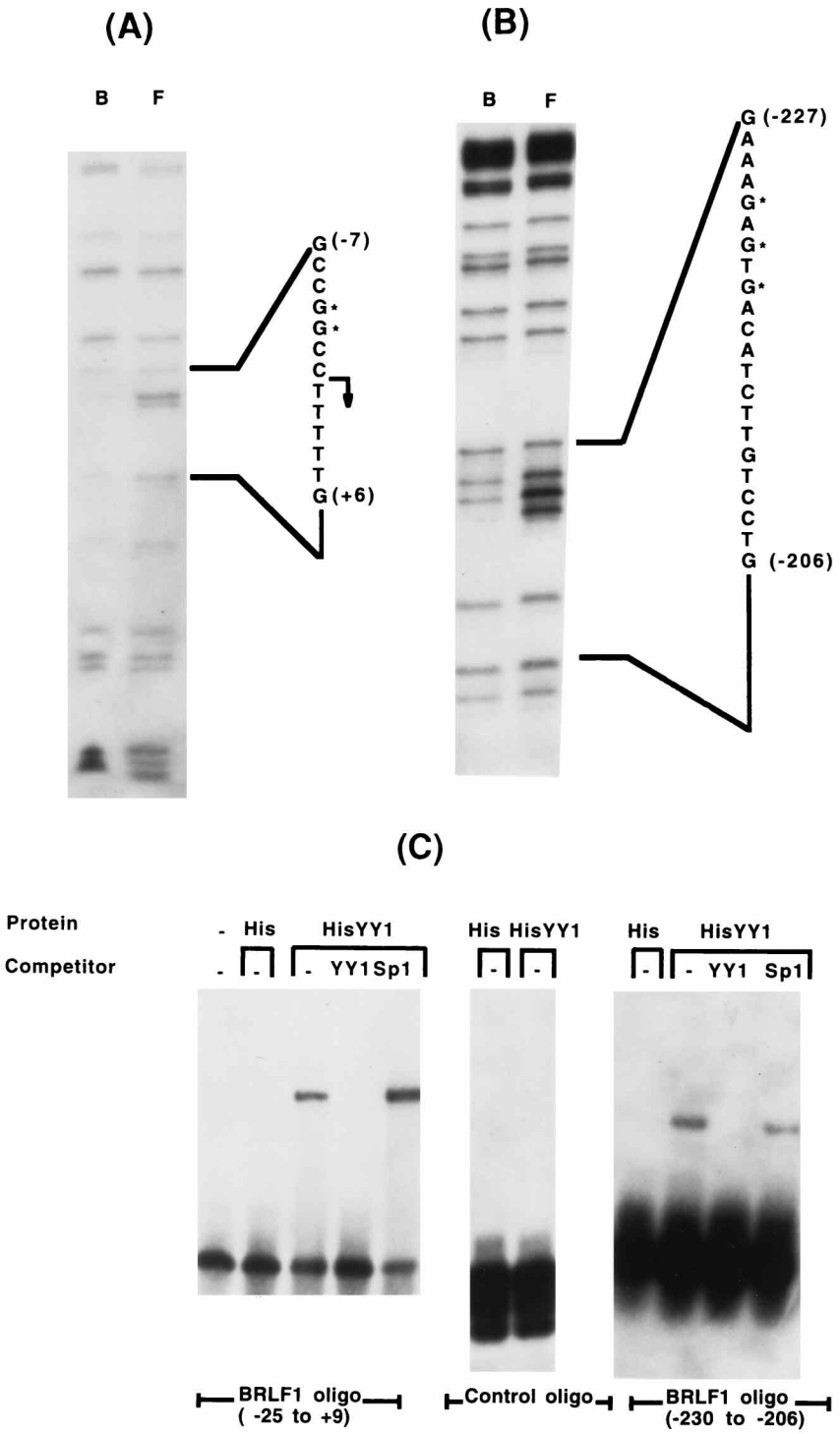

FIG. 3. Mapping of YY1 sites in Rp. ^{32}P-labelled probes containing Rp sequences from −188 to +6 (A) or −179 to −575 (B) were incubated with partially purified bacterial YY1 protein, and a methylation protection assay was performed. The bands representing the YY1-bound (lanes B) and free probe (lanes F) are shown. Areas of partial YY1-induced protection are indicated. The BRLF1 promoter sequence is shown in the regions of protection, numbered relative to the BRLF1 mRNA initiation site. (C) Labelled oligonucleotides containing the Rp promoter sequences indicated (spanning each of the YY1-protected regions shown in panels A and B) were incubated with extracts from HB101 bacteria expressing the YY1 protein or extracts from HB101 bacteria containing the pDS56-6X His vector. Each of the Rp probes bound to the bacterially synthesized YY1 protein, whereas the control probe (containing the CCAAT motif from the EBV BHLF1 promoter) did not. (D) A labelled oligonucleotide probe containing the YY1 binding motif from the murine leukemia virus LTR (8) was incubated with HeLa cell nuclear extract and competed with cold oligonucleotides containing either the Sp1 binding motif, the NF-κB binding motif, sequences spanning the upstream BRLF1 promoter YY1 binding site (lane YY1-2), sequences spanning the downstream BRLF1 YY1 binding site (lane YY1-1), or oligonucleotides which had been mutated within the upstream (lane ΔYY1-2) and downstream (lane ΔYY1-1) YY1 binding sites as shown in Fig. 4a. (E) A ^{32}P-labelled probe containing the wild-type BRLF1 promoter sequences from −254 to +6 or a mutant BRLF1 probe spanning the same sequences but mutated over the two YY1 sites (as shown in Fig. 4a) was incubated with HeLa cell nuclear extract and competed with cold oligonucleotide competitor DNA containing either the Sp1 binding motif or the YY1 binding motif (from the murine leukemia virus LTR) (8).

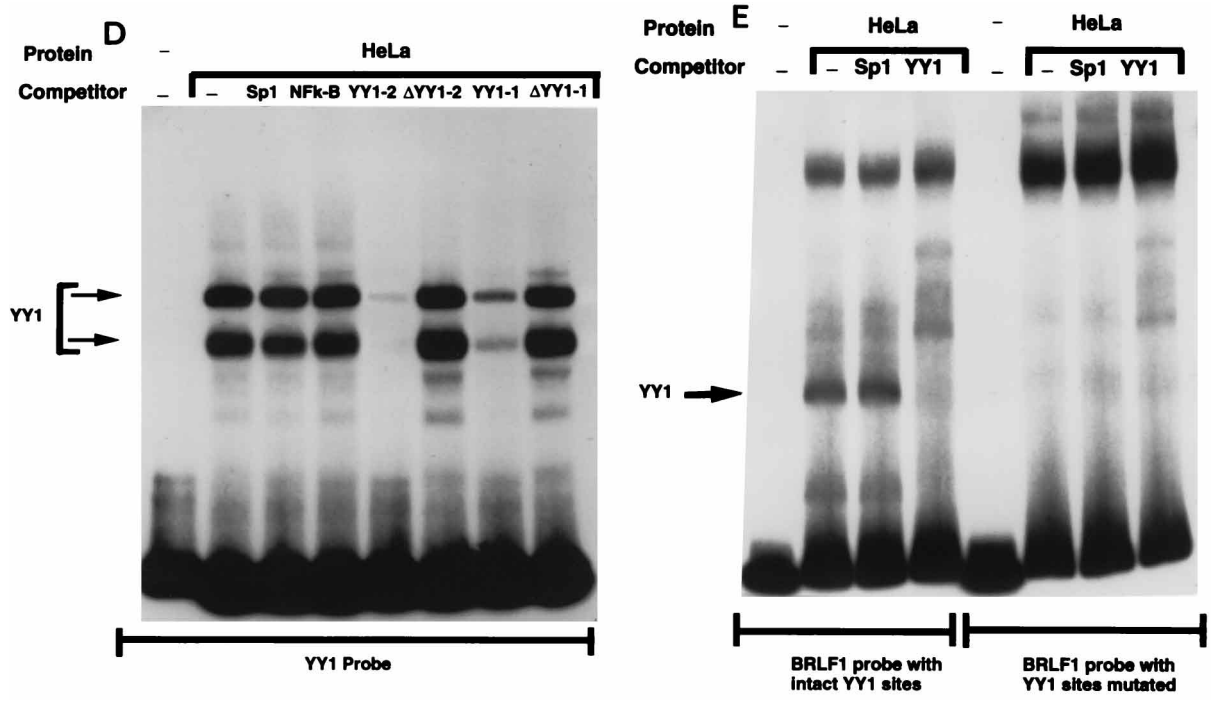

FIG. 3—*Continued.*

globulin cross-linking of surface immunoglobulin (21, 48). Agents which disrupt EBV latency could mediate their effects by modulating YY1 binding to the BRLF1 and/or BZLF1 promoters. Montalvo et al. have reported that TPA treatment of Raji cells is associated with loss of YY1 binding to the BZLF1 promoter (although the intracellular level of YY1 is not affected), whereas cross-linking of surface immunoglobulin does not affect YY1 binding to Zp (34). We examined the effect of TPA and cross-linking of surface immunoglobulin on YY1 binding to the BRLF1 promoter (Fig. 5). Nuclear extracts were prepared from uninduced Akata cells (48) or cells treated for various times (from 0.5 to 4 h as indicated) with anti-human immunoglobulin G (100 μg/ml) and used in EMSAs with labelled probes spanning the two Rp YY1 binding sites. The level of YY1 binding to Rp was not altered by cross-linking of surface immunoglobulin in Akata cells, even though BZLF1 binding to Rp was clearly induced. Induction of lytic viral infection in the B95-8 cell line with TPA (20 ng/ml) likewise had no effect on YY1 binding to either of the YY1 sites in Rp (data not shown). Therefore, disruption of EBV latency by cross-linking of surface immunoglobulin or TPA does not appear to require loss of YY1 binding to the BRLF1 promoter.

YY1 regulates the expression of numerous different viruses. The adeno-associated virus P5 promoter (44), the human immunodeficiency virus type 1 long terminal repeat (LTR) (28), the human cytomegalovirus major IE gene (26), and the LTR of murine leukemia virus (8) are transcriptionally repressed by YY1. In contrast, YY1 activates expression of the P6 promoter of B19 parvovirus (32) and the major capsid protein (VP5) gene of herpes simplex virus type 1 (3, 31). YY1 can either positively or negatively regulate the long control region of human papillomavirus, depending upon the cell type and the presence or absence of a switch region (1, 2).

YY1 function is modulated by viral and cellular proteins. The adenovirus E1A protein converts YY1 from a negative to a positive regulator of AAV transcription, without affecting DNA binding of YY1 (44). Relief of YY1-induced transcriptional repression by E1A may involve direct interactions between E1A and YY1 (24), as well as interactions between the E1A-associated protein, p300, and YY1 (23). In the case of the c-*fos* promoter, YY1 represses transcription by physically interacting with ATF/CREB proteins bound to the −67 cyclic AMP response element, and E1A reverses this effect by disrupting the ATF/CREB-YY1 complex (13, 55, 56). YY1 also directly interacts with Sp1 and modulates Sp1-dependent transcription (22, 43). c-Myc (45), cyclophilin A, and FK506-binding protein 12 (50) have likewise also been recently shown to directly interact with and regulate YY1.

In this report, we have identified two YY1 binding sites in the BRLF1 promoter. The downstream site (CGGCCTTTT) is located over the BRLF1 transcription initiation site, and the upstream site (GAGTGACATCTT) is located between −206 and −227 relative to the RNA start site. Both sites are similar (but not identical) to the preferred YY1 binding motif ([C/g/a][G/t][G/t/a]CATN[T/a][T/g/c], where capital letters indicate the preferred sequence and lowercase letters indicate possible substitutions) (17, 51). Under appropriate conditions, YY1 can function as an initiator element, binding to the core promoter and recruiting the polymerase to the initiation complex (42, 49). The downstream Rp YY1 site, located over the transcriptional initiation site, could thus function as an initiator element. In HeLa cells, mutation of the downstream YY1 site alone had little effect on Rp activity, suggesting that YY1 binding at this site is not necessarily required for transcription initiation. Nevertheless, in the context of the upstream YY1 binding site mutation (a mutation which increases constitutive Rp activity), mutation of the downstream YY1 site significantly reduced Rp activity in HeLa cells. Thus, it remains possible that under certain conditions the downstream YY1 binding site is required for efficient transcriptional initiation.

Deletion of the upstream YY1 site increased Rp activity in both HeLa cells and Raji cells. Thus, the upstream YY1 site

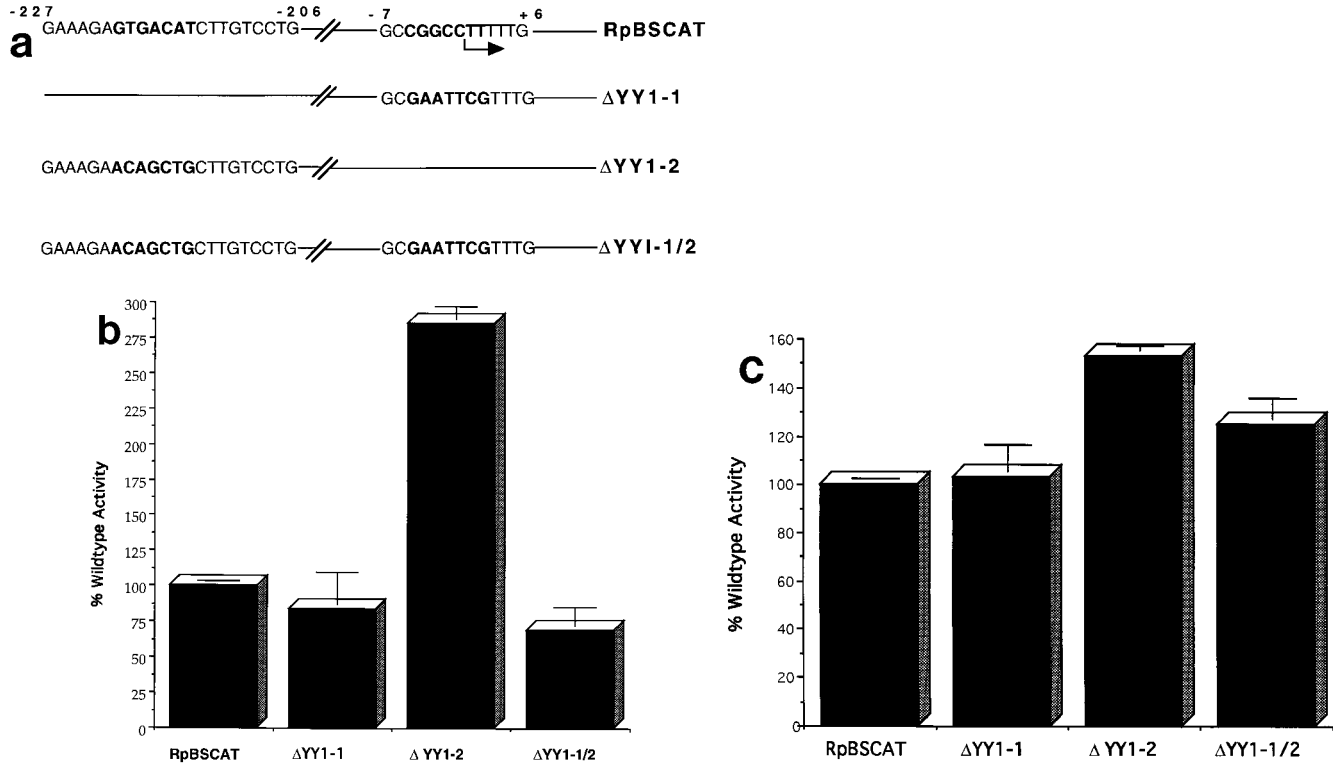

FIG. 4. Functional significance of YY1 binding sites in Rp. (a) Site-directed mutations of the RpBSCAT vector were constructed as shown, altering each of the two YY1 binding sites. (b) Five micrograms of the wild-type or mutant RpBSCAT plasmids was transfected into HeLa cells, and CAT assays were performed 2 days after transfection as described previously (14, 53). Results are normalized such that the activity of wild-type RpBSCAT plasmid in each experiment is set at 100%. (c) Experiments were performed as described for panel b, except in the EBV-positive Burkitt lymphoma line, Raji.

may function as a negative regulator of BRLF1 transcription. We have previously shown that the constitutive activity of Rp is considerably greater in epithelial cells than in B cells (53). Deletion of the upstream YY1 binding site increased Rp activity considerably more in HeLa cells than in Raji cells, suggesting that additional factors repress Rp activity in Raji cells even in the absence of YY1 binding. Although we have defined the upstream BRLF1 YY1 site as a negatively regulating element in this report, YY1 effects are notoriously cell type dependent and influenced by a myriad of different factors (1, 2, 44, 45, 50). Thus, it would not be surprising if the upstream YY1 site in Rp could function as a positive regulator under certain conditions.

Montalvo et al. have likewise reported that YY1 binding sites in the BZLF1 promoter function as *cis*-acting negative elements (33, 34). Disruption of EBV latency could therefore require inhibition of YY1 binding or modulation of YY1 function. We did not find that either TPA treatment of cells or cross-linking of surface immunoglobulin reduced YY1 binding to the BRLF1 promoter, although Montalvo et al. have reported that TPA reduces YY1 binding to the BZLF1 promoter (34). However, as is the case with the E1A protein, TPA treatment of cells or cross-linking of surface immunoglobulin could regulate YY1 function without affecting its DNA binding capacity.

Suppression of BZLF1 and BRLF1 transcription is likely required for the maintenance of viral latency in epithelial cells (52). Lytic viral infection may be selected against during the evolution of nasopharyngeal carcinoma, since this EBV-associated epithelial malignancy involves predominently latent infection (37). Cervical carcinomas induced by extrachromo-

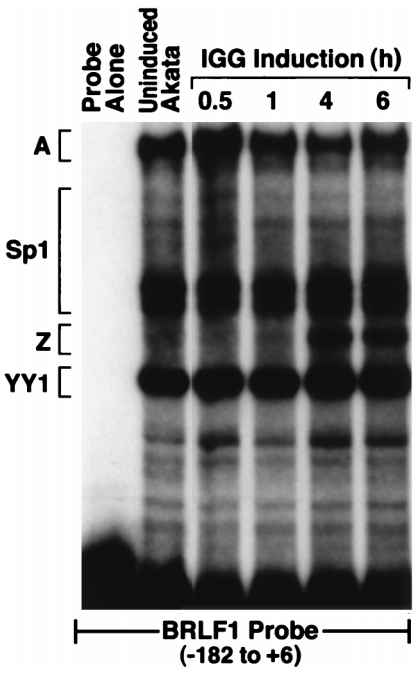

FIG. 5. Anti-immunoglobulin treatment of Akata cells does not affect YY1 binding to Rp. Nuclear extracts were prepared from uninduced Akata cells or cells treated for various times (from 0.5 to 4 h as indicated) with anti-human immunoglobulin G (IgG) and used in EMSAs with labelled probes spanning the two BRLF1 YY1 binding sites. The positions of the Sp1, BZLF1 (Z), YY1, and A binding complexes are indicated in brackets. Similar results were obtained with a probe spanning the upstream YY1 binding site in Rp (data not shown).

somal human papillomavirus type 16 often contain mutations which alter YY1 sites within the long control region (6, 29). Modulation of YY1 regulation in EBV-associated tumors could likewise affect the stringency of EBV latency.

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