JOURNAL OF VIROLOGY, Jan. 1997, p. 120–128 Vol. 71, No. 1
0022-538X/97/$04.00+0

Regulation of EBNA Gene Transcription in Lymphoblastoid Cell Lines: Characterization of Sequences Downstream of BCR2 (Cp)

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Received 21 June 1996/Accepted 11 September 1996

During Epstein-Barr virus (EBV) latent infection of B lymphocytes in vitro, six EBV nuclear antigens (EBNAs) are expressed from one of two promoters, Cp and Wp, whose activities are mutually exclusive. Upon infection, Wp is initially active, followed by a switch to Cp for the duration of latency. In this study, the impact on Cp and Wp activity of sequences downstream of the distal EBNA gene promoter, Cp, was assessed in two lymphoblastoid cell lines. Cp activity was detected in constructs extending from just upstream of *oriP* to the first W1 exon. In contrast, Wp activity required the presence of the next downstream exon, W2. Viral sequences from −2199 to +2680 bp, relative to the Cp transcription start site, were dispensable for Wp activity. Sequences from +155 to +2680 bp, relative to the Cp transcription start site, were dispensable for Cp activity. Deletion of a 200-bp region from +2680 to +2880 bp downstream of Cp decreased both Cp and Wp activity two- to fivefold. Wp activity was also significantly diminished by deletion of the sequences from +2880 to +3000 bp downstream of the Cp transcription initiation site, which encompassed the Wp CCATT box. Based on deletion analyses of 10.3 kb of viral genomic sequence extending from just upstream of *oriP* to the first Wp, the only deletions which significantly upregulated Wp activity were those which abrogated Cp activity. However, reporter constructs in which the orientation of Cp was reversed displayed Wp activity comparable to that of reporter constructs in which Cp was deleted, even though the steady-state level of Cp-initiated transcripts from the inverted promoter was indistinguishable from that observed with Cp in normal orientation. This is the first direct evidence to support transcriptional interference as the mechanism for the mutually exclusive behavior of Cp and Wp.

Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus which is the etiologic agent of infectious mononucleosis, a self-limiting lymphoproliferative disorder. In addition, EBV is closely associated with two human cancers, African Burkitt's lymphoma (BL) and nasopharyngeal carcinoma, and also appears to be associated with a significant percentage of Hodgkin's lymphoma as well as the non-Hodgkin's lymphomas that arise in immunosuppressed patients. During group III latency, the form of latency observed in peripheral B lymphocytes EBV immortalized in vitro (LCLs), six viral nuclear antigens (EBNAs) and three viral membrane proteins (latent membrane proteins [LMPs]) are expressed (reviewed in reference 9). EBNA1 is required for replication from the latency origin of replication, *oriP* (32, 33), which also functions as an EBNA1-dependent enhancer (16, 22). EBNA2 is essential for growth transformation of B lymphocytes (3, 6–8, 15) and has been shown to modulate the activity of several viral and cellular promoters (1, 5, 10, 23, 25–27, 30, 34). EBNA3c has recently been shown to modulate the function of EBNA2, through interaction with the same cellular transcription factor that has been shown to interact with EBNA2 (11, 18). LMP1 oncogenically transforms some established rodent cell lines and appears to be expressed at high levels in nasopharyngeal carcinoma tumors (reviewed in reference 9). LMP2a interacts with some Src tyrosine kinases family members, and this interaction appears to interfere with signalling via the B-cell antigen receptor (12). The functions of the other viral genes expressed during latency are not known. Of those viral antigens expressed in latently infected lymphocytes, only LMP2 and EBNA3b are not essential.

Transcripts encoding the EBNA family of proteins are expressed from two promoters, one of which maps to the unique region of the viral *Bam*HI C fragment (Cp), while the other maps to the *Bam*HI-W repeats (Wp) (reviewed in reference 1). Several years ago, it was observed that in all clonal EBV cell lines examined, one or the other of these promoters was exclusively employed (mutually exclusive promoter usage) (28). Early in infection of primary B cells, Wp is exclusively employed to drive transcription of the EBNA genes, followed by a switch to Cp usage and expression of all the EBNA genes (29, 30). In addition, EBNA2 appears to be required for the Wp-to-Cp switch, based on the observation that clone-13 virus (which lacks EBNA2 and has a mutant EBNA4) fails to switch from Wp to Cp usage during the initial stages of infection of primary B cells (30). These studies indicated that the distal EBNA1, -3a, -3b, and -3c genes are not transcribed from Wp during the initial stage of infection of primary B cells (19, 30). However, more recent data have demonstrated that the EBNA1 gene is transcribed from Wp during the early stages of infection of peripheral B cells with an immortalizing strain of EBV (B95.8 virus) but not with EBNA2/EBNA4^{mut} virus (clone-13 virus) (20). Thus, it is still unclear whether EBNA1, EBNA2, or both are required for switching from Wp to Cp activity during the establishment of viral latency.

In an effort to elucidate sequences involved in regulating EBNA gene transcription, an extensive deletion analysis of 10.3 kb of viral sequence was carried out. The region analyzed extended from just upstream of *oriP* to the second W1 exon. Previously we demonstrated the presence of three critical *cis* elements upstream of Cp (14). In LCLs, *oriP* was absolutely

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essential for both Cp and Wp activity. This element normally serves as the latency-associated origin of episomal replication and has been demonstrated to act as an EBNA1-dependent enhancer (16, 22). Deletion of the EBNA2-responsive enhancer upstream of Cp resulted in a two- to fivefold decrease in Cp activity. The third *cis* element identified was the CCAAT box of Cp. There are two CCAAT box sequences upstream of Cp, and a deletion which removed the proximal CCAAT box and moved the distal CCAAT box to nearly the same position relative to the transcription start site did not affect Cp activity. However, a site-directed mutation which ablated the proximal CCAAT box, leaving the distal CCAAT box in its normal position, dramatically reduced Cp activity. Thus, Cp appears to require the presence of a properly positioned CCAAT box for optimal activity in LCLs.

In this report, the previous deletion analyses are extended to analyze the region between Cp and Wp. Sequences downstream of Cp that extend to the second W1 exon are characterized for the presence of regulatory sequences. In addition, we present data which address the mechanism for the mutually exclusive activities of Cp and Wp.

MATERIALS AND METHODS

Cell lines and transfections. X50-7 and JY are human LCLs transformed with EBV which have been previously described and characterized (28, 29, 31). All cell lines were grown in RPMI 1640 medium with 10% fetal calf serum supplement. X50-7 and JY cells were transfected by liposome-mediated fusion using Lipofectin (Bethesda Research Laboratories) as previously described (28), with some modifications. Approximately 10^7 cells were washed in RPMI 1640 and resuspended in 1 ml of Opti-MEM I (GIBCO) per transfection. In parallel, 4 μg of CsCl-purified plasmid DNA was added to a mixture of 10 μl of Lipofectin and 1 ml of Opti-MEM I and was incubated at room temperature for 20 min before addition of 1 ml of washed cells in Opti-MEM I. Cultures were incubated at 37°C and 5% CO_2 for 16 to 18 h in 15-ml conical tubes, followed by transfer to 20 ml of RPMI 1640 containing 10% fetal calf serum. Transfected cells were harvested for RNA preparation ca. 72 h posttransfection.

RNA preparation and S1 nuclease protection analysis. Total cellular RNA was prepared by the single-step method using guanidinium isothiocyanate-phenol according to the method of Chomczynski and Sacchi (2). The reagent used consisted of three solutions ([i] 4 M guanidinium isothiocyanate–0.1 M β-mercaptoethanol–0.5% sarcosyl, [ii] 2 M sodium acetate [pH 4.0] and [iii] water-saturated molecular biology grade phenol [Bethesda Research Laboratories]) which were mixed in a 1:0.1:1 (vol/vol) ratio.

S1 nuclease protection analyses were carried out as previously described (28–30), with the following modifications. In the hybridization buffer (28), 4 ng of ^{32}P-labeled oligonucleotide probe, homologous to the region of the viral genome spanning the Cp or Wp transcription initiation site, was annealed to 35 μg of total cellular RNA. Hybridization of the probe to total RNA was carried out at 42°C for 16 to 18 h. In most cases, 30 or 60 U of S1 nuclease was added to each sample in S1 nuclease reaction buffer and incubated at 37°C for 30 min, followed by extraction with an equal volume of phenol-chloroform (1:1) and ethanol precipitation. Protected fragments were separated on a 10% denaturing polyacrylamide gel. S1 nuclease protection analysis to detect β-actin was carried out as described above with the exception that only 10 μg of total cellular RNA was used in the hybridization. Sequences of the oligonucleotide probes used in the S1 nuclease protection analyses were as follows: Cp, 5′-CATCTGGGCCACGCG TCTTGTCTCTATGCCATCTGATCTAAAATTTGCAGCAGAAC-3′; Wp, 5′-GTCTCCCCTAGGATTTGTGTGGCCAGGACGGCCCATGGATGCGA CCAGAAATAGCTGCAGG-3′; and β-actin, 5′-ACATAGGAATCCTTCTGA CCCATGCCCACCATCACGCCCTGGGAAGGAAAGGACAAGA-3′.

The results of S1 nuclease protection analyses were quantitated either on a PhosphorImager (Molecular Dynamics) or by densitometry (Molecular Dynamics). For those assays in which a reporter construct(s) exhibited activity which was significantly less than that of the wild-type (or positive control) reporter construct, the nuclease protection assay was repeated, using the same RNA preparations. This ensured that the observed differences in level of protection was not due to loss of protected fragments during the assay. In addition, the integrity of these RNAs was assessed by using the β-actin probe described above.

Construction of reporter plasmids. In all cases, the reporter constructs used contained the rabbit β-globin gene fused with either the first W1 exon, the first W2 exon, or the second W1 exon. The region of the viral genome which was analyzed for impact on Cp and Wp activities spanned from the *Eco*RI site at bp 7315 to the W1 exon at bp 17633 in the viral genome.

To facilitate cloning, the *Bam*HI sites at the *Bam*HI-C/*Bam*HI-W border and the border of the first and second *Bam*HI-W repeats were replaced with unique *Sal*I and *Cla*I sites, respectively, which were generated by PCR amplification using primers that incorporated these sites. In all cases where fragments of the viral genome were generated by PCR amplification from an appropriate template, a large amount (500 ng) of input plasmid was used and a minimum of number of amplification cycles (10 to 15 cycles) were carried out to avoid introduction of mutations. In all cases, the reporter constructs contained tagged sequences in the C1 exon and the first W0 exon, as previously described (30), to allow transcripts arising from the transfected reporter constructs to be distinguished from transcription of the endogenous viral genome. The *Sac*I site at 11088 bp in the viral genome (within the viral *Bam*HI C fragment) was replaced with a unique *Xba*I site.

Plasmids depicted in Fig. 1 were constructed by PCR amplification of viral sequence between each 3′ end of the deletion and the unique *Sal*I site. These fragments were cloned into an oriPCWW1d−2199/+1155 background as *Xba*I/*Sal*I fragments. These nested deletions were subsequently converted to 200-bp internal deletions by generating PCR products extending from the *Kpn*I site in *Bam*HI-C to the 5′ border of the deletion. A three-part ligation was done to connect the two PCR fragments (*Xba*I/*Sal*I and *Kpn*I/*Xba*I) with the oriPCWW1 background (*Kpn*I/*Sal*I).

The d−245/−45 plasmid in Fig. 3 was generated by cloning a *Xba*I/*Sal*I PCR fragment extending from −45 to +1880 into an oriPCWW1 background (containing the unique *Xba*I site at −245, as described above). A PCR fragment including +1155 to +1880 of viral sequence was cloned into a oriPCW1 background as an *Xba*I/*Sal*I fragment. This construct was then digested with *Kpn*I/*Xba*I, *Sac*II/*Xba*I, or *Hpa*I/*Xba*I and religated by use of a cloning adaptor or by blunt-end ligation. These constructs were converted to CWW1 constructs by cloning in the *Bam*HI-W repeat as a *Sal*I/*Hin*dIII fragment and are presented in Fig. 4. In Fig. 5, deletions in *Bam*HI-W were generated by digestion of this sequence with *Acc*I/*Cla*I (+3543 to +4951) and *Pst*I (+5205 to +6105). These basic deletions were then assembled in different combinations. The plasmid in which the β-globin gene is fused to the W2 exon was made by inserting a PCR product into an oriPCW1 background that spans the *Xho*I site in *Bam*-W to the end of the W2 exon (*Xho*I/*Hin*dIII).

The series of 200-bp nested deletions from the *Bam*HI-C/*Bam*HI-W junction to just upstream of Wp1 was made with three-part ligations. Two PCR products were generated for each construct; one spans from the *Sal*I site to the 5′ border of the deletion, and the other spans from the 3′ border to the *Hin*dIII site at the beginning of the β-globin gene. These fragments were mixed with a *Sal*I/*Hin*dIII-digested oriPCW1 background vector. To transfer these deletions to a CW2 background, the above constructs were digested with *Sal*I/*Nco*I and the deletion-containing fragments were cloned into a *Bam*HI W fragment extending to the W2 exon. These intermediates were then digested with *Sal*I/*Hin*dIII and transferred to a oriPCd−2199/+1155 background via a two-part ligation. Results of experiments done with these plasmids are presented in Fig. 7 and 8.

Constructs in which the orientation of Cp was reversed were generated and analyzed in Fig. 11. A cassette containing EBV sequences from genomic coordinate 10888 to the terminus of the C2 exon at 11657 was fused to either the chloramphenicol acetyltransferase (CAT) gene or the β-globin gene and was inserted in reverse orientation into the CWW1 reporter construct. Using PCR cloning techniques as described above, a unique *Xba*I site was generated to create a junction at 10888 and 12288 in the EBV *Bam* C fragment, while an endogenous *Kpn*I site at 10312 provided a cloning site for the 3′ terminus of the reporter gene. As a result, Cp is 560 bp closer to Wp1 than it normally would be in the genomic context.

RESULTS

The region from bp +155 to +1880 downstream of the Cp transcription initiation site is dispensable for Cp and Wp activity. To determine whether sequences downstream of Cp are required for Wp activity, a series of 200-bp nested deletions spanning from bp 9134 to 13213 in the viral genome, in the context of viral sequences extending from just upstream of *oriP* to the 5′ end of the second W1 exon (CWW1 background), was generated (Fig. 1A). These reporter constructs were introduced into two LCLs, JY and X50-7, and Wp activity was assessed as previously described (14) (note that data are not shown for deletion extending to +1880 bp). Notably, although some small fluctuations in Wp activity were observed, none of these deletions had a significant impact on Wp1 activity (Fig. 1B and D).

To assess the impact of deletions in this region on Cp activity, this set of nested deletions was converted to a series of 200-bp internal deletions spanning the viral genome from bp 10888 to 12888 (Fig. 2A). A modest reduction in Cp activity was observed with the d−445/−245 construct (Fig. 2B and D). This result is consistent with our previous observation that

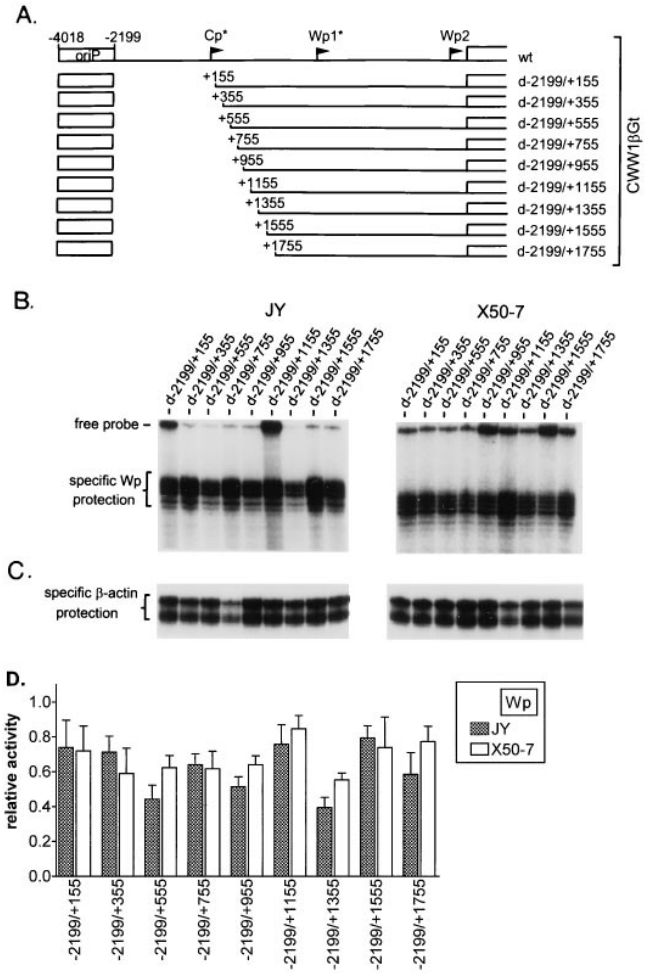

FIG. 1. Sequences between −2199 and +1755 bp are dispensable for Wp1 activity. (A) Schematic illustration of reporter constructs transfected into X50-7 and JY cells. An asterisk indicates those promoters for which a sequence tag was introduced into the first exon such that transcription from that promoter could be distinguished from endogenous viral transcripts as described in Materials and Methods. wt, wild type. (B) Detection of Wp1-initiated transcripts by S1 nuclease protection as described in Materials and Methods. An oligonucleotide probe spanning the W0 exon transcription initiation site, which was specific for the transfected reporter construct, was used for this analysis. (C) β-Actin exon 3 S1 nuclease protection to control for loading consistency and RNA integrity in the assay. Transfections, RNA preparation, and S1 nuclease protection assays were carried out as described in Materials and Methods. JY and X50-7 are EBV-immortalized LCLs. (D) Compiled data from independent transfections of the indicated reporter constructs (JY, $n = 4$; X50-7, $n = 3$). The level of specific protection was quantitated by either densitometry (Molecular Dynamics) or direct analysis on a PhosphorImager (Molecular Dynamics). Activities of reporter constructs are given relative to that of the most active reporter construct for a particular assay, which was defined as 1.0.

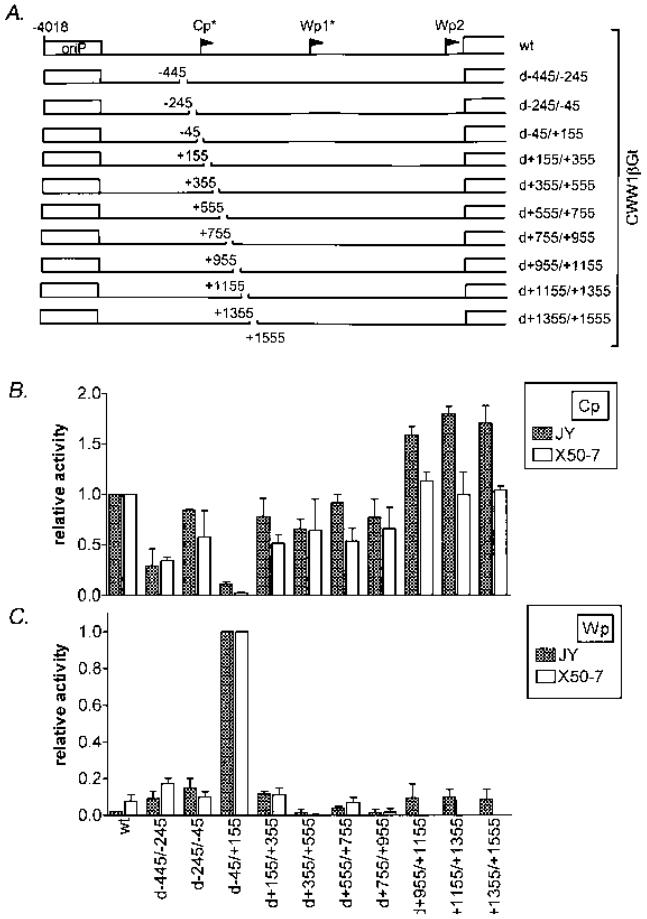

FIG. 2. Sequences between +155 and +1555 bp downstream of the Cp transcription initiation site are dispensable for Cp activity. (A) Schematic illustration of reporter constructs. An asterisk indicates promoters which were specifically tagged, as described in the legend to Fig. 1 and in Materials and Methods. wt, wild type. (B) Analysis of Cp-initiated transcripts with the reporter constructs shown in panel A. Shown are compiled results from independent transfections (JY, $n = 2$; X50-7, $n = 2$). Quantitation of specific protection was determined as described in the legend to Fig. 1. Activities of reporter constructs are given relative to the activity of the intact parent reporter construct (wt), which was defined as 1.0. (C) Analysis of Wp1-initiated transcripts with the reporter constructs illustrated in panel A. Shown are compiled results from independent transfections (JY, $n = 2$; X50-7, $n = 4$). Activities of reporter constructs are given relative to that of the most active construct (d−45/+155), which was defined as 1.0.

deletion of the EBNA2-dependent enhancer (ca. −330 to −380 bp) resulted in a two- to fivefold reduction of Cp activity (14). In addition, we previously demonstrated that the CCATT box just upstream of Cp is also essential for its activity and is removed in the −245/−45 deletion. However, as previously reported, this deletion brings another CCATT box into nearly the identical position relative to Cp (14). Thus, the activity of the d−245/−45 reporter construct did not vary significantly (less than twofold) from that of the wild-type control. As expected, Cp activity was not detected with the d−45/+155 construct since Cp and the C1 exon are deleted in this reporter construct. It should be noted that the low-level activity detected with the d−45/+155 reporter construct represents background noise in the assay and underscores the point that the activities of reporter constructs which are less than 10% of the positive control reporter construct are frequently difficult to accurately assess.

Deletions across the region from +155 to +1555 bp had little impact on Cp activity (Fig. 2). In X50-7 cells, and to a lesser extent in the JY cell line, deletion of sequences from +155 to +955 modestly reduced activity (less than twofold reduction in Cp activity). In JY cells, deletion of sequence from +955 to +1555 bp appeared to have slightly greater activity than the parent reporter construct. However, this increase in activity was less than twofold and was not observed in the X50-7 cell line.

In contrast to the pattern of activity observed for Cp in this deletion series, Wp activity was strongest with the reporter construct in which Cp was deleted (d-−45/+155). Compared

to the wild-type construct, and any other deletion construct in which Cp was intact, Wp activity was 5- to >50-fold higher with the Cp-deleted construct in both JY and X50-7 cells. Variable, low-level protection was observed for nearly all of the deletion constructs in both JY and X50-7 cells. Whether this represents bona fide Wp1 activity or is the result of cross-hybridization of the probe to partially spliced endogenous viral transcripts is unclear. As noted above, this is a difficult issue to resolve. However, it is clear that deletion of Cp results in a strong upregulation of Wp1 activity.

The results for Cp activity are in contrast to the findings of Walls and Perricaudet (24), who reported the presence of a strong positive *cis* element in the region from +417 to +868 bp downstream of the Cp transcription initiation site. This element was shown to upregulate Cp activity ca. 10-fold in P3HR-1-converted BL41 cells. We previously reported that the behavior of Cp/Wp reporter constructs in some BL cell lines was distinctly different than that observed in LCL (14). BL41/P3HR-1 is an EBV-negative BL cell line which has been infected with a mutant form of EBV lacking the EBNA2 gene and a portion of the EBNA4 coding sequences. As such, it has been transformed by events independent of EBV infection and is lacking viral gene products known to be involved in regulating viral transcription. Therefore, this cell background may not represent a relevant setting in which to address the impact of viral sequences on Cp and Wp activity. Alternatively, it is possible that since the IR1 repeat begins at +665 bp downstream of the Cp transcription initiation site, our failure to detect a positive *cis* element in the region from +417 to +868 bp was due to the fact that some of these sequences are repeated downstream of Wp1 in the CWW1 reporter construct. Thus, we assessed the impact of a panel of 200-bp deletions spanning the region from +555 to +1880 bp downstream of Cp in the context of a CW1 reporter construct in which the rabbit β-globin gene was fused to the first W1 exon. Consistent with the results obtained with the CWW1 reporter constructs, no evidence of a positive *cis* element in this region was observed when the CW1 deletion constructs were assayed in the JY and X50-7 cell lines (data not shown).

Dependence of Wp1, but not Cp, on sequences downstream of Wp1. Our previous analysis of sequences upstream of Cp involved in regulating Cp and Wp activities were carried out in the context of sequences extending through an entire *Bam*HI-W repeat (14). A significant amount of the sequences upstream of the first Wp (Wp1) are repeated upstream of the second Wp (Wp2) (i.e., downstream of Wp1) and are present in the CWW1 reporter constructs. Since it has previously been reported that there are positive regulatory sequences in the region immediately upstream of Wp1 (17, 24), having these sequences repeated downstream of Wp1 might lead to difficulties in analyzing their affect on Cp and Wp1 activity. Thus, in order to eliminate this complication, we investigated the impact of deleting the downstream repeated sequences in the reporter constructs used. We initially sought to determine what sequences downstream of the first W promoter (Wp1) were required for Cp and Wp activity. To assess the requirement of these sequences for Cp activity, four constructs were generated (Fig. 3A). The parent reporter constructs both start just upstream of *oriP* and extend either to the first W1 exon (CW1) or to the second W1 exon (CWW1). The latter construct contains a complete *Bam*HI-W repeat. In addition to the parent constructs, two reporter constructs containing a small deletion upstream of Cp (CW1d−245/−45 and CWW1d−245/−45) were also assayed. Deletion of the sequences from −245 to −45 bp was previously shown not to diminish Cp activity (14), and as such the deletion constructs simply provided an independent assessment of the impact of downstream *Bam*HI-W sequences on Cp activity. Both CWW1 reporter constructs were slightly less active than their CW1 counterparts (Fig. 3B and D), possibly due to higher transfection efficiency of the smaller plasmids. S1 nuclease protection analysis of the RNA prepared from the transfected cells by using a probe for exon 3 of the cellular β-actin gene transcript demonstrated that quantitation and integrity of the RNA were comparable between samples (Fig. 3C).

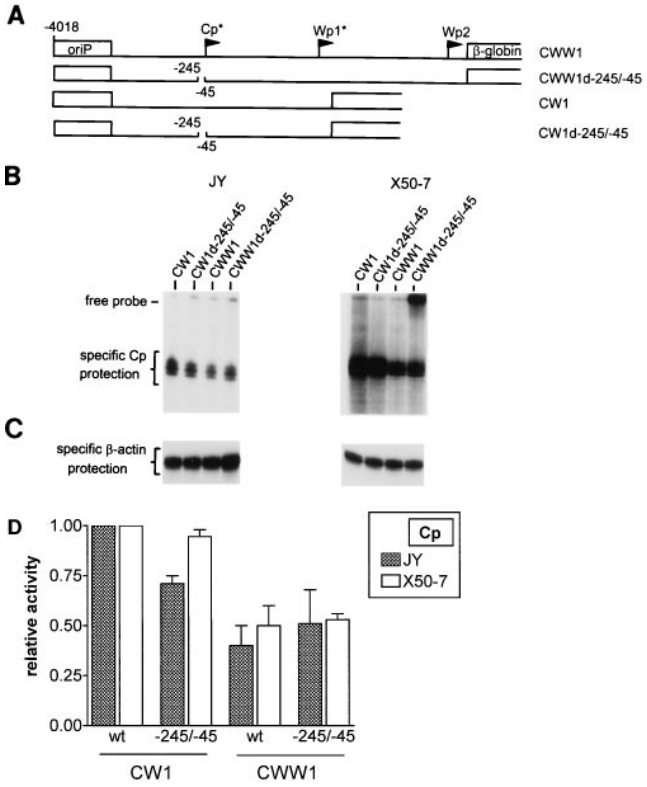

FIG. 3. Cp is not dependent on sequences downstream of the first W1 exon for activity. (A) Schematic illustrations of the reporter constructs transfected into X50-7 and JY cells. An asterisk indicates promoters which were specifically tagged in the C1 or W0 exon. (B) S1 nuclease protection analysis to detect Cp-initiated transcripts from the reporter construct depicted in panel A. Quantitation was carried out as described in the legend to Fig. 1. (C) S1 nuclease protection analysis for β-actin transcripts, as described in Materials and Methods. (D) Compiled results from independent transfections (JY, $n = 2$; X50-7, $n = 2$). wt, wild type.

As previously demonstrated, Wp activity in LCLs is dependent on the presence of *oriP* and the absence of Cp (14). Thus, to assess the impact of downstream *Bam*HI-W sequences on Wp1 activity, we initially generated two reporter constructs which span the region from *oriP* to the first or second W1 exon and contain a deletion from −2199 to +1155 bp relative to the Cp transcription initiation site (Fig. 4A). When these reporter constructs were transfected in JY and X50-7 cells, Wp activity could readily be detected from the CWW1d−2199/+1155 reporter construct but not from the CW1d−2199/+1155 reporter construct. This result implied that sequences downstream of the first W1 exon are required for Wp activity. It should be emphasized that only activity from Wp1 (and not Wp2) can be detected with the oligonucleotide probe used, since we have introduced a sequence tag into the first W0 exon which is not present in the second W0 exon. To determine whether the lack of Wp activity with the shorter reporter construct lacking downstream *Bam*HI-W sequences could be restored by includ-

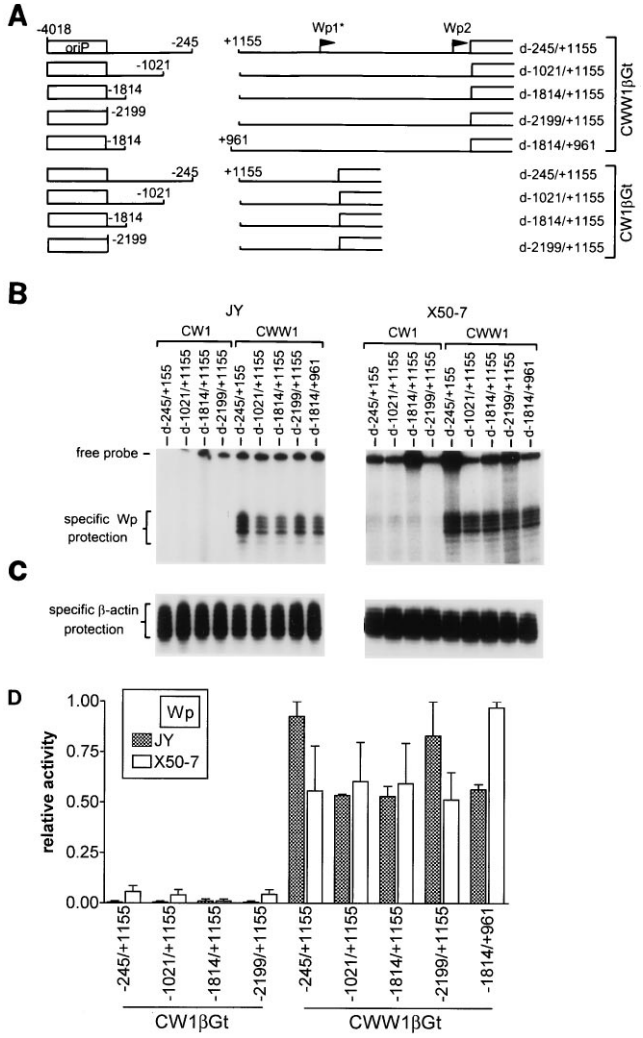

FIG. 4. Wp activity is dependent on sequences downstream of the first W1 exon. (A) Schematic illustration of the CWW1- and CW1-based deletions reporter constructs transfected into X50-7 and JY cells. An asterisk indicates the presence of a sequence tag introduced into the first exon, as described in the legend to Fig. 1 and in Materials and Methods. (B) S1 nuclease detection of Wp1-initiated transcripts in JY and X50-7 cells, using the reporter constructs diagrammed in panel A. (C) S1 nuclease protection analysis of cellular β-actin transcripts. Protection assays were carried out with total RNA as described in Materials and Methods. (D) Compiled results from independent transfections (JY, $n = 2$; X50-7, $n = 3$). The level of specific protection was assessed as described in the legend to Fig. 1 and in Materials and Methods. Activities are expressed relative to the activity of the most active reporter construct in a given assay, which was defined as 1.0.

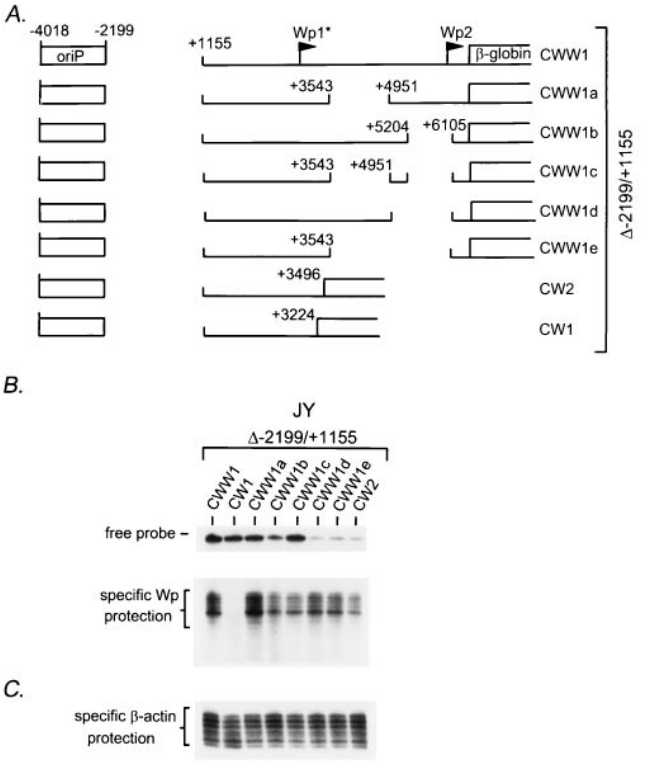

FIG. 5. Identification of sequences downstream of the first W1 exon required for Wp1 activity. (A) Schematic illustration of the reporter constructs used. All deletion coordinates in the diagram are given relative to the Cp transcriptional start site and correspond to the Wp1 transcriptional start site in the following manner: Cp +3543 to +4951 = Wp1 +495 to +1903; Cp +5204 to +6105 = Wp1 +2156 to +3057; Cp +4951 to +6105 = Wp1 +1903 to +3057; and Cp +3543 to +6105 = Wp1 +495 to +3057. (B) S1 nuclease protection analysis of Wp1-initiated transcripts in JY cells. (C) S1 nuclease protection analysis of cellular β-actin transcripts. Protections were carried out as described in Materials and Methods.

ing sequences upstream of Cp, a series of nested deletions was generated (Fig. 4A). Inclusion of the sequences from −2199 to −245 bp upstream of the Cp transcription initiation site did not alter the dependence of Wp1 activity on the sequences downstream of the first W1 exon (Fig. 4B and D). In addition, this series of CWW1 reporter constructs did not exhibit significantly different levels of Wp1 activity. The latter indicates that these sequences upstream of Cp, which include the EBNA2-dependent enhancer, are not able to modulate Wp1 activity in established LCL under the assay conditions used.

The dependence of Wp1 activity on sequences downstream of the transcription initiation site suggested that the additional sequences are involved in stabilizing the resulting transcript and/or that there is a positive regulatory element(s) downstream of the first W1 exon. In an effort to distinguish between these two possibilities, we generated a group of reporter constructs with identical Cp deletions plus deletions downstream of Wp1 from +3543 to +6105 relative to Cp transcription initiation site (the positions of these deletions relative to the Wp1 transcription initiation site are given in the legend to Fig. 5) (Fig. 5A). These reporter constructs were transfected into the JY cell line, and Wp1 activity was assessed (Fig. 5B). As shown in Fig. 4, a reporter construct containing sequences to the first W1 exon did not exhibit detectable levels of Wp1 activity (Fig. 5B; compare activities of CWW1 and CW1). A construct with a deletion downstream of Wp1 spanning the sequences from +3543 to +4951 bp was as active as the positive control (Fig. 5B; compare CWW1 to CWW1a). In contrast, the other deletion constructs exhibited a two- to threefold decrease in Wp1 activity (Fig. 5B; compare activities of CWW1b, -c, -d, and -e). A common feature of these reporter constructs is the deletion of intronic sequences spanning from +5204 to +6105 bp. As shown below, this region contains positive *cis* elements that affect both Cp and Wp activities (see Fig. 7 and 8). Notably however, fusion of the reporter gene to the 3′ end of the W2 exon (CW2) gave rise to a steady-state level of Wp1-initiated transcripts that was comparable to the levels observed with the CWW1b, -c, -d, and -e deletion constructs.

From the results presented here, it is reasonable to conclude that the 3 kb of viral sequence between the first W1 and second W1 exons likely contains both a positive intronic *cis* element(s)

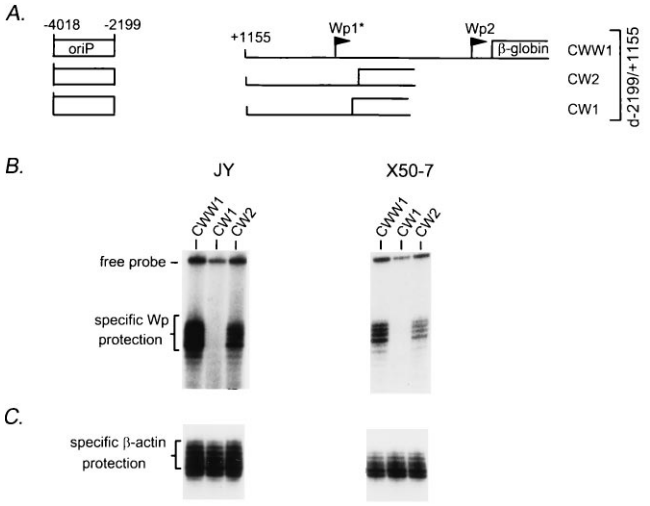

FIG. 6. The presence of the W1 and W2 exons allows detection of Wp1-initiated transcripts in JY and X50-7 cells. (A) Schematic illustrations of the reporter constructs transfected into X50-7 and JY cells. An asterisk indicates promoters which contain sequence tags introduced into either the C1 or W0 exon, as described in Materials and Methods. (B) S1 nuclease protection analysis of Wp1-initiated transcripts, carried out as described in Materials and Methods. (C) S1 nuclease protection analysis of the level of cellular β-actin transcripts.

and a necessary exon (W2 exon) which may serve to stabilize Wp1-generated transcripts. The dramatic increase in Wp activity observed when sequences were extended to the W2 exon was verified in the X50-7 cell line as well as the JY cell line (Fig. 6A and B). S1 protection analysis of these RNAs with a probe that detects the cellular β-actin gene was carried out to ensure equal loading of samples and to assess RNA integrity in samples lacking detectable Wp1 activity (Fig. 5C and 6C).

Identification of a positive *cis* element 2680 to 2880 bp downstream of Cp. To characterize the region upstream of Wp1, a series of 200-bp internal deletions spanning from +1880 to +3080 bp relative to the Cp transcription start site was generated in the context of the CW1 background for Cp analysis (Fig. 7A). A corresponding set of deletions extending to +3000 was inserted in the CW2 background for Wp analysis (Fig. 8A) since, as discussed above, the CW2 background represents the minimal context in which Wp1 activity could be assessed (Fig. 5 and 6). By using the minimal context in which Cp or Wp1 activity can be assessed (CW1 or CW2 background, respectively), the presence of naturally duplicated sequences, spanning the beginning of the second *Bam*-W repeat to the second W1 exon, was eliminated. When sequences between +2680 and +2880 bp were removed, a modest reduction in Cp activity ranging from three- to fourfold in JY and X50-7 cells, was observed (Fig. 7B). No other deletion in this series had a significant impact on Cp activity in either cell line. Walls and Perricaudet (24) localized a positive *cis* element to the region from +2700 to +3066 bp downstream of the Cp cap site. However, they reported a 10-fold effect on Cp activity, whereas we observed a less dramatic impact on Cp activity. As discussed above, the analysis by Walls and Perricaudet was carried out only in the BL41/P3HR-1 cell line.

Wp1 activity was also affected when the sequences between +2680 and +2880 bp were deleted (Fig. 8B). In addition, when the sequences from +2880 to +3000 bp were deleted, Wp1 activity was significantly diminished, while Cp activity was not affected by this deletion (Fig. 7B). The latter deletion removes the CCAAT box upstream of Wp1. We have previously shown that a site-directed mutation which ablates the CCAAT box

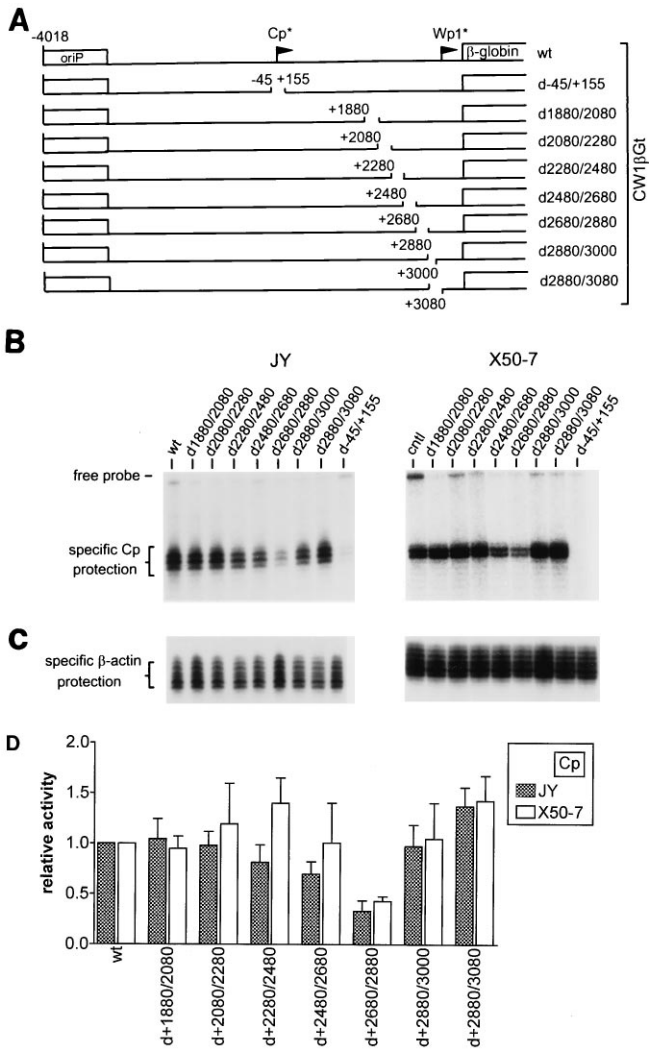

FIG. 7. Identification of a region from +2680 to +2880 bp downstream of the Cp transcription initiation site which enhances Cp activity. (A) Schematic illustration of the CW1-based reporter constructs used in the analysis of sequences from +1880 to +3080 bp downstream of the Cp transcription initiation site. wt, wild type. (B) S1 nuclease protection analysis of the level of Cp-initiated transcripts. (C) S1 nuclease protection analysis of the level of cellular β-actin transcripts. Protection analyses were carried out as described in Materials and Methods. (D) Compiled results from independent transfections (JY, $n = 4$; X50-7, $n = 3$). Activities of reporter constructs were determined as described in the legend to Fig. 1 and in Materials and Methods and are expressed relative to the activity observed with the intact parent reporter construct (wt), which was defined as 1.0.

upstream of Wp1 dramatically reduces promoter activity (29). In addition, we have recently demonstrated that Cp is a CCAAT box-dependent promoter (14). Thus, like Cp activity, Wp1 activity may also be highly dependent on the presence of a functional CCAAT box. Whether other positive Wp1-specific *cis* elements map to this region remains to be determined.

Inhibition of Wp1 activity by transcriptional interference from Cp. Figure 9 schematically illustrates the regions of the viral genome that contain *cis* elements which regulate either Cp or Wp1 activity. In the course of this deletion analysis, no major negative *cis* elements that could explain the mutually exclusive behavior of Cp and Wp have been identified. The only reporter constructs in which Wp activity was high were those in which Cp was crippled or deleted. Thus, the presence

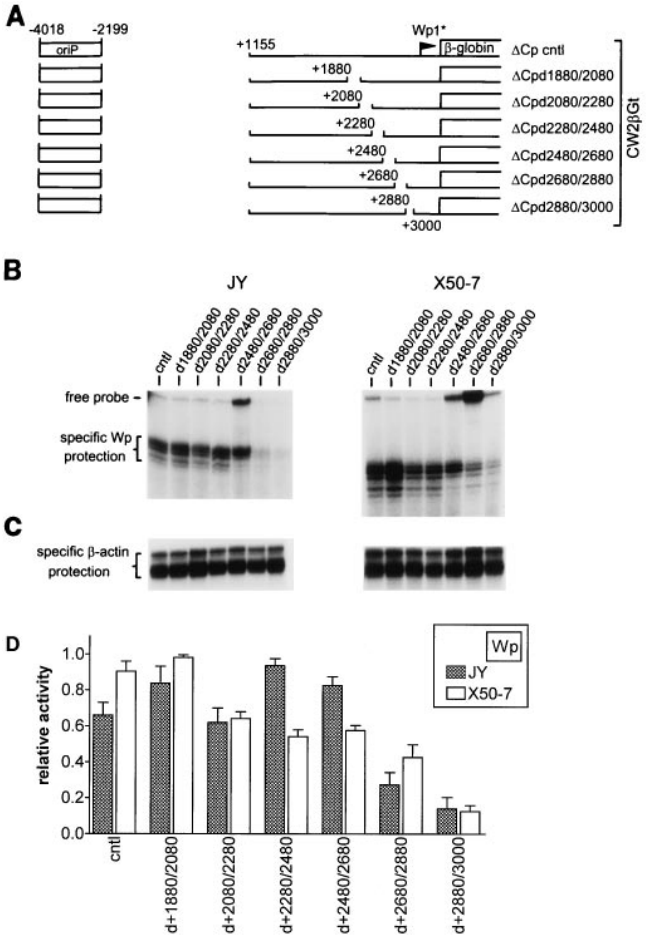

FIG. 8. Deletion of sequences from +2680 to +2880 bp and +2880 to +3000 bp downstream of the Cp transcription initiation site diminish Wp1 activity. (A) Schematic illustration of the CW2d−2199/+1155-based reporter constructs used in this analysis. cntl, control. (B) S1 nuclease protection analysis of the level of Wp1-initiated transcripts in cells transfected with the indicated reporter constructs. (C) S1 nuclease protection analysis of the level of cellular β-actin transcripts. Protection analyses were carried out as described in Materials and Methods. (D) Compiled results from independent transfections (JY, $n = 4$; X50-7, $n = 5$).

of a functional Cp appears sufficient to lead to the downregulation of transcription initiation from Wp. Two distinct mechanisms could account for this phenomenon: (i) since *oriP* is essential for the activity of both Cp and Wp in established LCL (14), presumably through its EBNA1-dependent enhancer function (16), competition for enhancer function could lead to downregulation of Wp and upregulation of Cp if Cp competed much more efficiently for enhancer activity; or (ii) transcription interference from Cp could squelch transcription initiation from Wp, since Cp lies upstream of Wp (Fig. 10). To distinguish between these possibilities, we generated two reporter constructs in which the orientation of Cp was inverted. Sequences from just upstream of the EBNA2-dependent enhancer through Cp to the C2 exon were inverted in the CWW1 reporter construct (Fig. 11A). In addition, either the CAT or β-globin reporter gene was linked to the C2 exon in order to stabilize the resulting Cp-initiated transcripts (Fig. 11A). The rationale for using two different reporter genes in the inverted constructs was to avoid the possible complication that one of the reporter genes could contain cryptic positive *cis* elements that might influence Cp or Wp activity. When compared to the CWW1 wild-type positive control with Cp in its normal orientation, Cp in the reverse orientation appeared to be just as active (compare oriPCWW1 to oriPCinvCAT and oriPCinvBg in Fig. 11B). As expected, the CWW1d−1021/+1155 construct (in which Cp was deleted) had no detectable Cp activity and a strong Wp signal. Furthermore, the Cp-inverted reporter constructs exhibited comparable levels of Wp1-initiated transcripts as the Cp-deleted reporter construct (Fig. 11C). The CWW1 control construct exhibited only very low levels of Wp1-initiated transcripts. These results are most consistent with the transcription interference model since Cp activity with the inverted constructs appeared very similar to that observed with the wild-type reporter construct, while Wp activity was dramatically upregulated by inverting Cp.

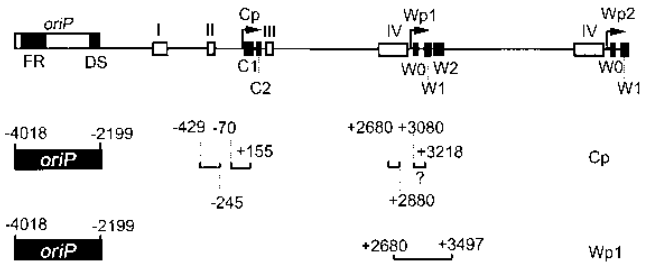

FIG. 9. Schematic diagram of the sequences identified as important for Cp and/or Wp activity. The top panel depicts the 10.3-kb region characterized in this paper and in previous work (14). Several known *cis* elements are displayed: I is a known glucocorticoid response element; II is the EBNA2-responsive enhancer; III and IV are *cis* elements identified by Perricaudet and Walls (24). Small black boxes represent exons present in either Cp- or Wp-derived transcripts. FR, family of repeats; DS, dyad symmetry element. Illustrated below the genomic map are the regions which we have identified as important for either Cp or Wp1 activity in LCL. Regions denoted by a question mark have not been addressed by the deletion analysis presented here.

DISCUSSION

In this paper, the sequences between Cp and Wp were analyzed for the presence of *cis* elements that might be involved

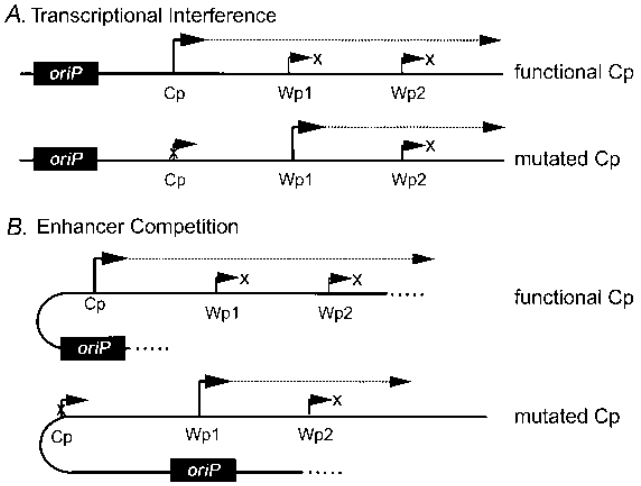

FIG. 10. Models to account for the observed mutually exclusive behavior of Cp and Wp. (A) Transcriptional interference, in which transcription from the distal promoter promoter (Cp) interferes with the ability to initiate transcription from the proximal promoter (Wp1). When Cp is deleted or inactivated, transcription initiation from Wp1 is possible. (B) Enhancer competition, in which the essential *oriP*/EBNA1 enhancer preferentially interacts with Cp, thereby preferentially upregulating transcription from Cp. When Cp is deleted or mutated, *oriP* becomes available to interact with Wp1, thereby activating transcription from this promoter.

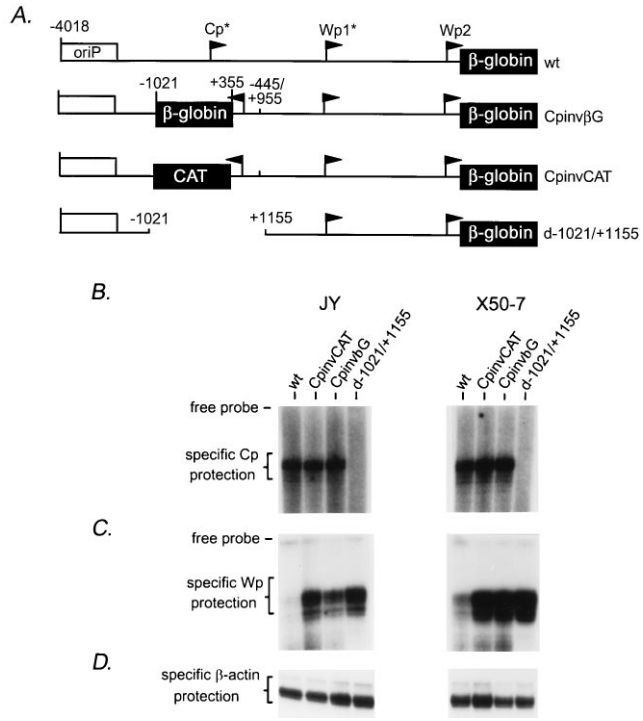

FIG. 11. Inversion of Cp leads to an induction of Wp1 activity. (A) Schematic illustration of the reporter constructs used, which are described in detail in Materials and Methods. Both Cp and Wp1 are tagged, as indicated by asterisks in the diagram. wt, wild type. (B) S1 nuclease protection analysis of the level of Cp-initiated transcripts driven from the indicated reporter constructs in X50-7 and JY cells. (C) S1 nuclease protection analysis of the level of Wp1-initiated transcripts driven from the indicated reporter constructs in X50-7 and JY cells. (D) S1 nuclease protection analysis of the level of cellular β-actin transcripts. Protection analyses were carried out as described in Materials and Methods.

in regulating EBNA gene expression in established LCL. It was shown that Cp and Wp require different sequences downstream of Wp1. Cp-initiated transcripts were readily detectable by using reporter constructs extending to the first W1 exon, while Wp1-initiated transcripts could be detected only when Cp was deleted and the sequences downstream of Wp1 were extended past the first W1 exon. Further analysis revealed that both the addition of W1 and W2 exon sequences and the presence of sequences just upstream of Wp2 served to greatly enhance the steady-state level of Wp1-initiated transcripts. It seems likely that the addition of W1 and W2 exon sequences serve to stabilize the resulting reporter gene transcripts. However, the enhancement observed with the sequences upstream of Wp2 most likely correlates with the presence of a positive *cis* element(s) in this region since the analysis of the homologous region upstream of Wp1 indicates the presence of an enhancer sequence that upregulates both Cp and Wp1. With respect to exon sequences stabilizing reporter gene transcripts, we have observed that for Cp reporter constructs lacking extensive downstream sequences, fusion of a reporter construct to the C1 exon leads to much lower reporter gene activity than fusion of the reporter gene to the C2 exon (30a). However, as shown here, the deletion analysis did not indicate the presence of any strong positive *cis* elements in the region between the C1 and C2 exons. Thus, at least in the case of Cp-initiated reporter gene transcripts, the 5′ transcript structure appears to plays a significant role in dictating mRNA stability. Whether this is the case with Wp activity remains to be determined.

Extensive deletion analyses of the viral genome, spanning from −2199 to +1880 bp relative to the Cp cap site, revealed that none of these sequences had a significant impact on Wp activity (although deletion of Cp was required for efficient transcription initiation from Wp). Similarly, sequences from +155 to +1880 bp were dispensable for Cp activity. Walls and Perricaudet (24) reported a positive *cis* element mapping within the region from +417 to +868 bp downstream of the Cp cap site that increased Cp activity 10-fold as assessed by RNase protection. We failed to observe a significant impact of these sequences on Cp or Wp activity. As discussed in Results, Walls and Perricaudet assayed their reporter constructs in only one cell line (BL41/P3HR-1). The BL41/P3HR-1 cell line is an EBV-negative BL that has been infected with a nonimmortalizing strain of EBV. In contrast, we used two LCLs which, although they have been in culture for a number of years, exhibited nearly identical behavior with the panel of deletion constructs assayed. Previously, we observed that the dependence of Cp and Wp activities on *oriP* in LCL was not observed in some BL cell lines (14). Taken together, these observations indicate that the behavior of Cp/Wp reporter constructs in some BL cell lines may not accurately reflect their behavior in LCL. In addition, it should be noted that the study by Walls and Perricaudet (24) used reporter constructs that in many cases fused the CAT reporter gene to C2/W1 intron sequences. This could lead to aberrant processing of the resulting primary transcripts, which in turn may alter message stability. All of the reporter constructs used here have the β-globin gene fused to either the W1 or W2 exon, allowing normal processing of the resulting primary transcripts and thus minimizing the chance of variable mRNA stability.

Further deletion analysis of the region upstream of Wp1 identified a positive *cis* element within the sequences from +2680 to +2880 bp downstream of the Cp cap site, whose deletion modestly reduced the activities of both Cp and Wp. In addition, deletion of the sequences from +2880 to +3000 significantly diminished Wp1 activity but had little effect on Cp activity. The latter region contains the Wp1 CCAAT box, and given the differential effect of this deletion on Cp and Wp1 activities, it is likely that the loss of this *cis* element accounts for the impact of this deletion. Consistent with this interpretation, we have previously shown that a site-directed mutation of the Wp1 CCAAT box strongly diminished Wp activity (29). With respect to the positive element within the region from +2680 to +2880 bp, two other groups have reported similar observations (17, 24). Walls and Perricaudet (24), characterizing Cp activity, mapped a region from +2700 to +3066 bp whose deletion resulted in an ca. 10-fold decrease in activity. Ricksten et al. (17), characterizing Wp activity by using reporter constructs with very limited upstream sequences, identified a positive element within the region from +2751 to +2912 bp. All three regions share an extensive overlap from +2751 to +2880 bp, and it seems likely that the positive regulatory sequences map within this region. As discussed above, the major drawback of the other studies is that they used BL cell lines to characterize Cp or Wp activities, which may not accurately mimic the regulation of these promoters in LCL.

Nilsson et al. (13) previously reported the characterization of several Cp- and Wp-driven reporter constructs in a panel of EBV-negative and EBV-positive BL cell lines. In these studies, the Cp reporter constructs used contained various amounts of upstream sequence but did not contain sequences downstream of the C1 exon. Conversely, the Wp-driven reporter constructs used contained very limited sequences upstream of Wp. They demonstrated that in general, group I BL cell lines exhibited higher activity with the Wp-driven reporter construct than with the Cp-driven reporter construct, while in group III BL cell

lines, the *oriP*-containing Cp reporter construct was more active than the most active Wp-driven reporter construct. The significance of this observation is unclear, since no reporter construct containing both promoters in their normal physiological context was examined. However, the observation that minimal Wp-driven reporter constructs are active in the group I BL cells may indicate that these cells express cellular transcription factors that are required for initial Wp activity during infection of resting B cells whereas group III BL cell lines and LCL lack expression of critical factors for Wp activity (in the absence of the *oriP*/EBNA1 enhancer). Whether this is indeed true awaits future studies identifying the critical *cis* elements involved in regulating Wp activity during the establishment of viral latency.

Overall, few *cis* elements appear to be involved in modulating Cp and/or Wp activity in established LCL. As previously shown (14), the dominant *cis* element is *oriP* whose presence (presumably in conjunction with EBNA1) is essential for Cp and Wp activity in LCL. The EBNA2-dependent enhancer has a modest effect on Cp activity and does not appear to affect Wp activity. In addition, the sequences upstream of Wp1 (+2660 to +2880 bp downstream of Cp cap site) also modestly enhance both Cp and Wp activity. Finally, those elements which define Cp and Wp (CCAAT and TATA boxes) are required for efficient transcription initiation from each promoter. The major conclusions from the analysis of Cp and Wp activity in LCL, based on the data present here and in our previous characterization of the region upstream of Cp (14), are that (i) EBNA gene transcription in LCL is largely autoregulated by EBNA gene products through *oriP* and the EBNA2-dependent enhancer upstream of Cp and (ii) transcription from Cp interferes with transcription initiation at the downstream Wp promoters.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA43143 to S.H.S. S.H.S. is a Scholar of the Leukemia Society of America.

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