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oriP Is Essential for EBNA Gene Promoter Activity in Epstein-Barr Virus-Immortalized Lymphoblastoid Cell Lines

MARYANN T. PUGLIELLI,[1,2] MAXIMILIAN WOISETSCHLAEGER,[3] AND SAMUEL H. SPECK[2*]

Committee on Virology of Harvard University at Harvard Medical School, Boston, Massachusetts 02115[1]; Sandoz Forschungsinstitut, Vienna, Austria[3]; and Departments of Pathology and Molecular Microbiology, Center for Immunology and Division of Molecular Oncology, Washington University School of Medicine, St. Louis, Missouri 63110[2]

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During Epstein-Barr virus latent infection of B lymphocytes in vitro, six viral nuclear antigens (EBNAs) are expressed from one of two promoters, Cp or 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 region upstream of Cp was analyzed for the presence of *cis* elements involved in regulating the activities of the EBNA gene promoters in established in vitro immortalized lymphoblastoid cell lines (LCLs). It was determined that *oriP*, the origin for episomal maintenance during latency, is essential for efficient transcription initiation from either Cp or Wp in LCLs, as well as in some Burkitt's lymphoma cell lines. Deletion of the EBNA2-dependent enhancer located upstream of Cp resulted in a ca. two- to fivefold reduction in Cp activity in the LCLs assayed. More extensive deletion of sequences upstream of Cp, including the EBNA2-dependent enhancer, resulted in nearly complete loss of Cp activity. This loss of activity was shown to correlate with deletion of two CCAAT boxes, a proximal CCAAT box located at bp −61 to −65 and a distal CCAAT box located at bp −253 to −257, upstream of Cp. Site-directed mutagenesis of these *cis* elements demonstrated that Cp activity is highly dependent on the presence of a properly positioned CCAAT box, with the dependence on the distal CCAAT box apparent only when the proximal CCAAT box was deleted or mutated. Deletion of the glucocorticoid response elements located at ca. bp −850 upstream of Cp did not result in a significant loss in activity. In general, deletions which diminished Cp activity resulted in induction of Wp activity, consistent with suppression of Wp activity by transcriptional interference from Cp. The identification of *oriP* and the EBNA2-dependent enhancer as the major positive *cis* elements involved in regulating Cp activity in LCL suggests that EBNA gene transcription is largely autoregulated by EBNA 1 and EBNA 2.

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. Two features of EBV infection of B lymphocytes in tissue culture have long been thought to be directly relevant to the issue of EBV-associated oncogenesis. (i) EBV establishes predominantly a latent infection in B lymphocytes with little or no virus production; thus, the virus persists for life in the infected individual. (ii) There is a concomitant growth transformation (immortalization) of the infected lymphocytes that gives rise to lymphoblastoid cell lines (LCL) that proliferate indefinitely in vitro (reviewed in references 12 and 16a).

In latently EBV-infected, growth-transformed B lymphocytes, six viral nuclear antigens (EBNAs) and three viral membrane proteins (latent membrane proteins [LMPs]) are expressed (reviewed in reference 12). EBNA 1 is required for replication from the latency origin of replication, *oriP* (32, 33), which also functions as an EBNA 1-dependent enhancer (19). EBNA 2 is essential for growth transformation of B lymphocytes (5, 8, 10, 11, 18) and has been shown to modulate the activity of several viral and cellular promoters (1, 6, 13, 23, 25–28, 34). EBNA 3c has recently been shown to modulate the function of EBNA 2, through interaction with the same cellular transcription factor that interacts with EBNA 2 (14a, 19a). LMP 1 oncogenically transforms some established rodent cell lines and appears to be expressed at high levels in nasopharyngeal carcinoma tumors (reviewed in reference 12). LMP 2a interacts with some Src tyrosine kinases family members, and this interaction appears to interfere with signalling via the B-cell antigen receptor (16). The functions of the other viral genes expressed during latency are not known. Of those viral antigens expressed in latently infected lymphocytes, only LMP 2 and EBNA 3b are not essential.

Although the viral genes which are expressed during EBV latency are not clustered in one region of the viral genome (reviewed in reference 21), the viral promoters involved in regulating the transcription of the latency-associated genes are clustered in a relatively small region of the genome (Fig. 1). The six EBNAs are all encoded on the r strand of the genome and are spread out over approximately 110 kb. Characterization of the EBNA mRNAs has revealed that they all have common 5′ exons which are spliced to unique 3′ coding exons. Two viral promoters that are involved in generating these viral mRNAs one of which maps to the unique region of the viral *Bam*HI C fragment (Cp) and the other of which maps to the *Bam*HI W repeats (Wp) (Fig. 1), have been identified. Several years ago, it was observed that in all clonal EBV cell lines examined, one or the other of these promoters was exclusively used (mutually exclusive promoter usage) (29). Early in infection of primary B cells Wp is exclusively employed to drive

* Corresponding author. Mailing address: Department of Pathology, Box 8118, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-0367. Fax: (314) 362-4096. Electronic mail address: speck@pathology.wustl.edu.

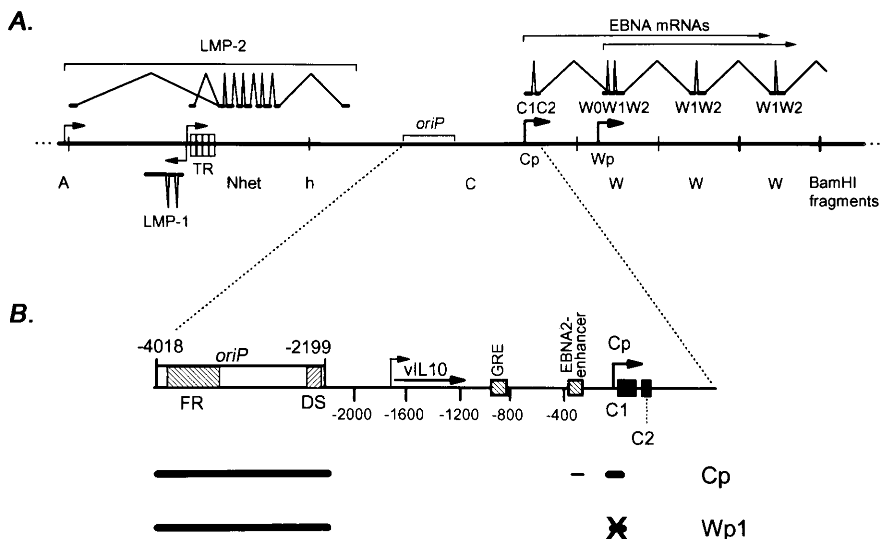

FIG. 1. Summary of genomic organization and sequences involved in regulating Cp and Wp activities. (A) Genomic map of the region near the fused terminal repeats in the EBV episome, illustrating the viral promoters active during latent infection in LCL. *oriP*, the latency origin of replication; TR, fused terminal repeats; Cp and Wp, EBNA gene promoters. (B) Detailed map of the region upstream of the Cp EBNA gene promoter and the regions that affect Cp and Wp activity. The positions of several previously identified *cis* elements are indicated, including the location of the viral IL10 gene, a glucocorticoid response element (GRE), the EBNA 2-dependent enhancer, and the latency origin of replication (*oriP*), which is composed of a family of repeats (FR) and a dyad symmetry element (DS). The regions that affect Cp and Wp activity are indicated below the map. The thickness of the lines is proportional to the effect of the indicated sequences on activity. For Wp activity, the bold X represents the requirement for inactivation of Cp.

transcription of the EBNA genes, followed by a switch to Cp usage and expression of all the EBNA genes (28, 30).

EBNA 2 plays a critical role during the early stages of B-cell transformation and is essential for immortalization of human B lymphocytes by EBV. Furthermore, continued expression of EBNA 2 is required for growth of established LCL (11). EBNA 2 is one of the first viral antigens expressed upon infection of resting B cells (20), and it is directly involved in activating the transcription of other viral genes expressed during latency, as well as activating the transcription of the cellular CD23 and c-*fgr* genes (13, 25, 26). It has recently been demonstrated that this activity is mediated through binding of EBNA 2 to a cellular DNA-binding protein, CBF1 (7a, 9, 10a, 14, 35). CBF1-binding sites are found upstream of Cp, the LMP 1, 2a, and 2b gene promoters, and a number of cellular genes. We have previously shown that $EBNA2^{-}/EBNA4^{mut}$ virus (clone-13 virus) fails to switch from Wp to Cp usage during the initial stages of infection of primary B cells (28) and that among the EBNA gene products, only EBNA 4 is expressed in these cells (20). These studies indicated that the distal EBNA 1, 3a, 3b, and 3c genes are not transcribed from Wp during the initial stage of infection of primary B cells. Our more recent work has demonstrated that the EBNA 1 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) (20b). Thus, it is still unclear whether EBNA 1, EBNA 2, or both are required for switching from Wp to Cp activity during the establishment of viral latency.

Although a number of *cis* elements have been implicated in the regulation of Cp and Wp activities, their contribution to the activities of the EBNA gene promoters in the context of the viral sequences surrounding Cp and Wp has not been carefully investigated. Here, we report the characterization of *cis* elements upstream of Cp which are involved in regulating transcription from Cp and Wp in EBV-immortalized LCL.

MATERIALS AND METHODS

Cell lines and transfections. X50-7 and JY are human LCL transformed with EBV, which have been previously described and characterized (29, 30). DG75 (3) is an EBV-negative BL cell line. Clone-13 is an EBV-positive BL cell line in which the endogenous viral genome has a deletion spanning the EBNA 2 gene and 3′ end of the EBNA 4 gene. Akata (24) is a group I BL cell line that by definition expresses only EBNA 1. 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 with Lipofectin (Bethesda Research Laboratories) as previously described (29) 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 under 5% CO_2 for 16 to 18 h in 15-ml conical tubes and then transferred to 20 ml of RPMI 1640 containing 10% fetal calf serum. Transfected cells were harvested for RNA preparation ca. 72 h posttransfection.

DG75 cells were transfected by electroporation with a Bio-Rad Gene Pulser set at 230 V and 960 mF capacitance. Prior to electroporation, ca. 10^7 cells were washed in RPMI 1640 and resuspended in 200 μl of cold RPMI 1640 per transfection sample. To an electroporation cuvette, 50 μl of cold 1× phosphate-buffered saline (PBS) was added along with 15 μg of CsCl-prepared plasmid DNA. To each cuvette, 200 μl of cell suspension was added, and the mixture was incubated on ice for 10 min before being shocked. Immediately following electroporation of the samples, 750 μl of cold 1× PBS was added to each sample, followed by vigorous shaking to separate the cells from cell debris. Transfected cells were then transferred to 20 ml of RPMI 1640 containing 10% fetal calf serum in T25 flasks. As above, transfected cells were harvested ca. 72 h posttransfection.

RNA preparation and S1 nuclease protection analysis. Total cellular RNA was prepared by the single-step method with either TriReagent (Molecular Research Center, Inc.) as specified by the manufacturer or guanidinium thiocyonate (GITC)-phenol by the method of Chomczynski and Sacchi (4). In the latter case, the reagent used consisted of three solutions, (i) 4 M GITC–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 (by vol) ratio.

S1 nuclease protection analyses were carried out as previously described (28–30), with the following modifications. In the hybridization buffer (29), 4 ng of ^{32}P 5′-end-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 (unless otherwise stated in the figure legend). The probe was hybridized to cellular RNA at 42°C for 16 to 18 h. S1 nuclease (30 U)

was added to each sample in S1 nuclease reaction buffer, and the mixtures were incubated at 37°C for 30 min and then extracted with an equal volume of phenol-chloroform (1:1) and precipitated with ethanol. Protected fragments were separated on a 10% denaturing polyacrylamide gel. S1 nuclease protection analysis to detect β-actin was carried out as described above, except that only 10 μg of total cellular RNA was used in the hybridization. The sequences of the oligonucleotide probes used in the S1 nuclease protection analyses were as follows: 5′-CATCTGGGCCACGCGTCTTGTCTCTATGCCATCTGATCTAA AATTTGCAGCAGAAC-3′ (Cp), 5′-GTCTCCCCTAGGATTTGTGTGGCC AGGACGGCCCATGGATGCGACCAGAAATAGCTGCAGG-3′ (Wp), and 5′-ACATAGGAATCCTTCTGACCCATGCCCACCATCACGCCCTGGGAA GGAAAGGACAAGA-3′ (β-actin).

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

Construction of reporter plasmids. In all cases, the reporter constructs used contained the rabbit β-globin gene fused to the W1 exon, as previously described (29), and in these constructs, the β-globin gene was fused to the second W1 exon (thus, these constructs contained a complete copy of the *Bam*HI W repeat). 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 with primers that incorporated these sites. In all cases when fragments of the viral genome were generated by PCR amplification (17) 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 (28), to allow transcripts arising from the transfected reporter constructs to be distinguished from those arising from transcription of the endogenous viral genome. The *Sac*I site at bp 11088 in the viral genome (within the viral *Bam*HI C fragment) was replaced with a unique *Xba*I site. Nested and internal deletions were generated by using PCR primers with relevant combinations of these unique restriction sites.

Mutations in the proximal and distal CCAAT boxes upstream of Cp were generated by PCR mutagenesis with primers which disrupted the CCAAT box motif and introduced a restriction endonuclease site. For mutation of the proximal CCAAT box, the native sequence was altered from 5′-ACCAAT-3′ to 5′-CTCGAGT-3′ to generate an *Xho*I site, while for the distal CCAAT box, the sequence was mutated from 5′-GCCAATA-3′ to 5′-AGATCTA-3′ to create a *Bgl*II site. The locations of the proximal and distal CCAAT boxes are indicated in Fig. 7.

RESULTS

Detection of specifically initiated transcripts employing reporter constructs transiently transfected into B-cell lines. To identify *cis* elements involved in regulating Cp and Wp, a relatively large region of the viral genome encompassing Cp and Wp was used to generate reporter constructs. We chose this approach because the mutually exclusive behavior of these promoters suggests that they are functionally linked. The parental plasmid, on which all the reporter constructs used in this study were based, contained EBV sequences extending from just upstream of *oriP* to the second W1 exon (bp 7315 to 17633 in the EBV genome). To facilitate generation of stable transcripts, the W1 exon was fused to the rabbit β-globin cDNA. The steady-state abundance of transcripts initiating from Cp and Wp was determined by S1 nuclease protection with total RNA prepared from cell lines transiently transfected with relevant reporter constructs. All reporter constructs contained sequence tags within the C1 exon and W0 exons, as previously described (28), such that transcripts initiating from the transfected plasmid could be distinguished from endogenous viral transcripts. It should be noted that the probes used were specific for the transfected reporter constructs and that they span the respective transcription initiation sites of these mRNAs,

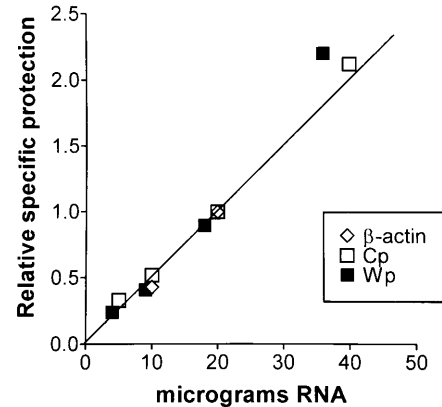

FIG. 2. Linear relationship between level of protection from S1 nuclease digestion and amount of input RNA. Titration of specific protection observed in S1 nuclease analyses with probes for Cp- or Wp-initiated transcripts, or exon 3 of the cellular β-actin gene, as a function of amount of total RNA added to the hybridization. For analysis of Cp activity, X50-7 cells were transfected with the wt reporter construct, and for analysis of Wp activity, X50-7 cells were transfected with the $d-1814/+961$ construct, as described in Materials and Methods. The structures of these reporter constructs are illustrated in Fig. 3A. Total RNA was prepared from the transfected cells, and the indicated amounts of RNA were hybridized with 4 ng of ^{32}P-labeled oligonucleotide probe, followed by digestion with S1 nuclease and fractionation of the digested fragments on a denaturing acrylamide gel as described in Materials and Methods. The protected fragments were quantitated with a PhosphorImager (Molecular Dynamics). The level of protection observed with 20 μg of total RNA was defined as 1.0.

making it possible to distinguish between protection of the probe by mRNA (which gives rise to appropriate size fragments) from protection by DNA contamination in the RNA preparations (which results in total protection of the probe). The tagged C1 and W0 exons were designed such that protection by endogenous viral RNA or DNA would give rise to very small fragments. A β-actin probe was routinely used to monitor RNA loading.

To demonstrate that the assay conditions were sensitive to changes in the level of transcript, an RNA titration was carried out with the probes used in this study (Fig. 2). As shown for the Cp and Wp probes, the assay was linear over a range of 5 to 40 μg of input RNA. Only 10 and 20 μg of RNA were analyzed with the β-actin probe, but the results of these protections also demonstrated the expected linear relationship between input RNA and amount of probe specifically protected. The S1 nuclease protection analyses described in this paper routinely involved 35 μg of total RNA for analysis of transcripts initiating from Cp and Wp and 10 μg of RNA of analysis of the abundance of β-actin transcripts.

***OriP* is required for efficient transcription initiation from either Cp or Wp in LCL and some BL cell lines.** While it has previously been determined that the presence of *oriP* can enhance transcription from Cp and Wp (22, 29), the dependence of these promoters on *oriP* in LCL has not been carefully examined. To assess the importance of *oriP* for Cp and Wp activity, a series of nested deletions was generated and the activities of these reporter constructs were assessed in several LCL and BL cell lines. The structures of the nested deletions are illustrated in Fig. 3A. In X50-7 and JY cells, two EBV-positive LCL, *oriP* was absolutely required for Cp and Wp activity (Fig. 3B and C). Only RNA prepared from cells transfected with the wild-type (wt) construct, which contains Cp and *oriP*, exhibited specific Cp protection. In contrast, Wp protection was detected only when *oriP* was present and Cp was deleted, as seen with the $d-1814/+961$ construct. S1 nuclease

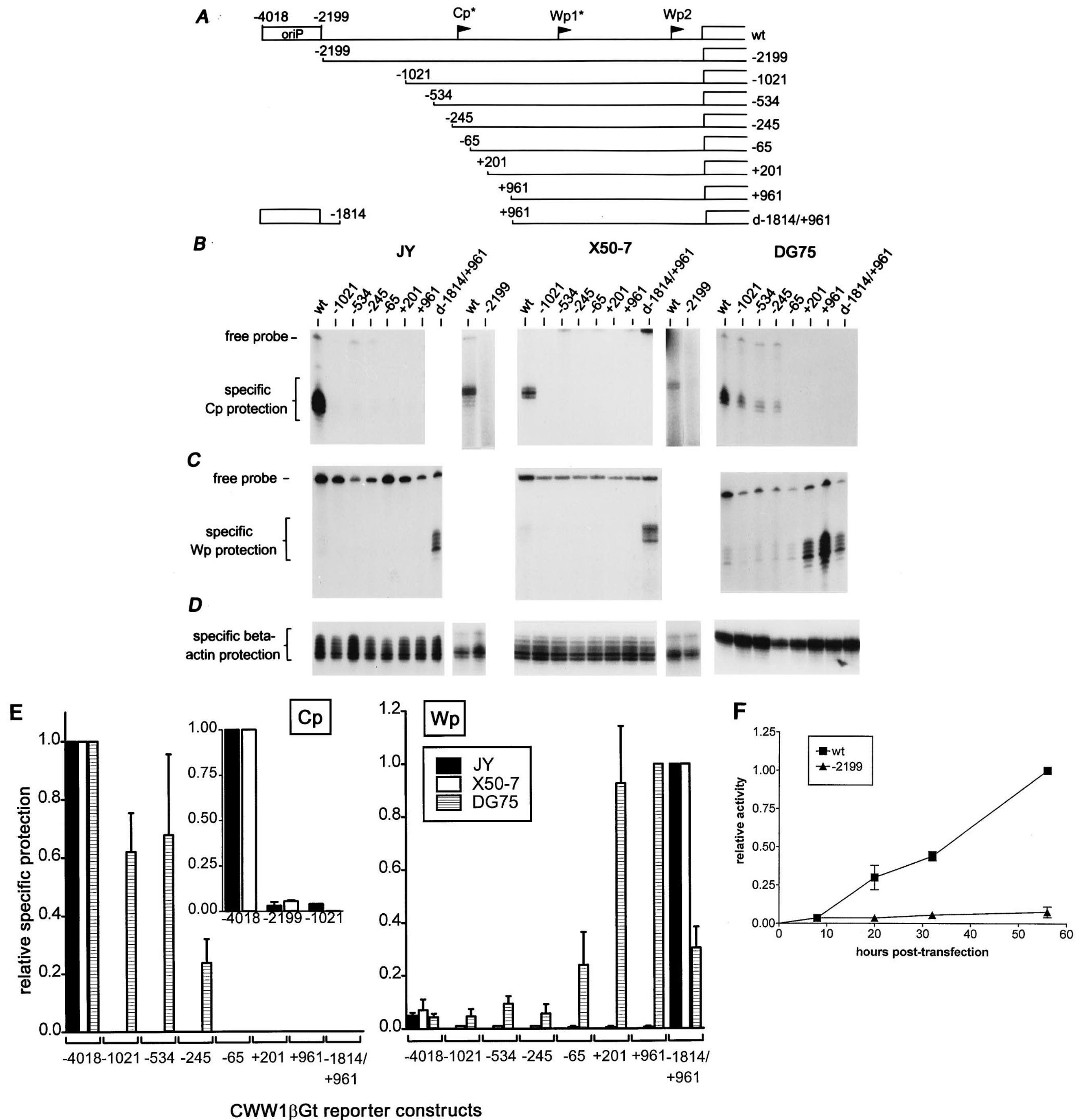

FIG. 3. Dependence of Cp and Wp on *oriP* for activity in LCL. (A) Schematic illustration of the reporter constructs used to assess the requirements for Cp and Wp activity. Asterisks indicate promoters for which a sequence tag has been introduced into the first exon such that transcription from that promoter can be distinguished from that from endogenous viral transcripts (see Materials and Methods). (B) Detection of Cp activity by S1 nuclease protection analysis as described in Materials and Methods. An oligonucleotide probe spanning the C1 exon transcription initiation site, which was specific for the transfected reporter construct, was used for this analysis. (C) S1 nuclease analysis of Wp-initiated transcripts. An oligonucleotide probe spanning the W0 exon transcription initiation site, which was specific for the transfected reporter construct, was used. (D) S1 nuclease analysis of β-actin initiated transcripts. An S1 probe to detect exon 3 of a cellular gene, β-actin, was used to demonstrate RNA integrity and loading consistency for samples lacking both Cp and Wp activity. Transfections, RNA preparations, and S1 nuclease protection assays were carried out as described in Materials and Methods. JY and X50-7 are EBV-immortalized LCL, and DG75 is an EBV-negative BL cell line. (E) Compiled data from independent transfections of the indicated reporter constructs (JY/Cp, $n = 3$; JY/Wp, $n = 2$; X50-7/Cp, $n = 2$; X50-7/Wp, $n = 3$; DG75/Cp, $n = 3$; DG75/Wp, $n = 3$). The inset in the left-hand panel for Cp activity summarizes the activity of a reporter construct containing sequences from bp −2199 upstream of Cp to the second W1 exon (−2199 reporter construct), and the activity of this reporter construct is shown in comparison with the activity observed with wt (−4018) and −1021 reporter constructs. The level of specific protection was quantitated by either densitometry (Molecular Dynamics) or direct analysis on a PhosphorImager (Molecular Dynamics). The activities of the reporter construct are given relative to that of the most active reporter construct, which was defined as 1.0. (F) Time course analysis of Cp activity in JY cells transfected with the full-length *oriP*-containing reporter construct (wt) or the −2199 reporter construct lacking *oriP*. JY cells were transfected as described in Materials and Methods and harvested at 8, 20, 32, or 56 h posttransfection. Total RNA prepared from the transfected cells was assessed for the presence of Cp-initiated transcripts as described in Materials and Methods. The level of specific protection was quantitated on a PhosphorImager (Molecular Dynamics). Cp activities are given relative to the level of Cp activity observed with the wt reporter construct at 56 h posttransfection (which was defined as 1.0).

protection analysis with a probe complementary to exon 3 of the β-actin gene, an abundant cellular transcript, was carried out to verify the integrity of the RNA used in these studies. The actin probe demonstrated that the lack of detectable Cp and Wp activity observed with reporter constructs lacking *oriP* was not due to variations in the amount or quality of RNA used (Fig. 3D). These results confirm the previously reported observation that the activities of Cp and Wp are mutually exclusive and that Cp is the dominant promoter when both promoters are present.

In contrast to the results obtained with LCL, neither Cp nor Wp activity was dependent on *oriP* in the EBV-negative BL cell line DG75 (Fig. 3). The presence of sequences from bp −4018 to −1021 appeared to increase Cp activity (Fig. 3; compare the activity of wt with that of −1021 or −534). This region includes *oriP*; however, *oriP* enhancer activity has previously been associated only with expression of EBNA 1 (19). It is perhaps more likely that positive *cis* elements, which serve to slightly enhance Cp activity in DG75 cells, lie between bp −1021 and *oriP*. However, in contrast to the results observed with Cp, the presence of *oriP* appeared to diminish Wp activity (Fig. 3; compare the activity of +961 with that of *d*−1814/+961). Notably, while Cp activity in the −65 construct was undetectable owing to disruption of the CCAAT box upstream of Cp, Wp activity failed to increase significantly until sequences to bp +201 were deleted (Fig. 3; compare the activities of −245, −65, and +201). This result suggests that we may have mapped a negative *cis* element(s) to the region from bp −65 to +201, which is involved in downregulating Wp activity in DG75 cells. However, as discussed below, this negative element does not appear to be important in regulating Wp activity in LCL, and its identification is therefore of questionable significance.

To further examine the dependence of Cp and Wp activities on *oriP* in other BL cell lines, we assessed their activities in the Akata and clone-13 cell lines (data not shown). In the Akata cell line, which is a group 1 BL cell line that expresses only EBNA 1, Cp activity was dependent on the presence of *oriP*, suggesting that EBNA 1 expression may play an important role in the dependence of Cp and Wp on *oriP* for activity. However, in the clone-13 cell line, *oriP* was completely dispensable for Cp activity. Clone-13 is a subclone of the P3HR-1 BL cell line and harbors a viral genome in which the entire EBNA 2 gene and the 3′ end of the EBNA 4 gene have been deleted. Clone-13 cells do express EBNA 1, but the presence of EBNA 1 and *oriP* did not result in enhancement of Cp activity, indicating that in some BL cell lines, other *cis* elements are involved in activating transcription from Cp and Wp. Importantly, the distinct behavior of Cp/Wp reporter constructs in clone-13 and DG75 cells compared with their behavior in LCL raises the concern that BL cell lines cannot be assumed to be good model systems for characterizing the requirements for these promoters. Thus, our further characterization of the regions of the viral genome involved in regulating Cp and Wp activity has been restricted to analyses in LCL.

To more finely map the sequence requirements for Cp activity in LCL, an intermediate deletion construct (−2199 [Fig. 3A]) which lacks only *oriP* sequences (from bp −4018 to −2199) was generated. When the −2199 reporter construct was assayed in the JY and X50-7 LCL, only a very low level of Cp-initiated transcripts was detected (Fig. 3B; quantitation is shown in the inset in Fig. 3E). This result underscores the dependence of Cp activity on the presence of *oriP*. It is possible that there are weakly positive *cis* elements in the region from bp −2199 to −1021. However, as shown below, deletion of these sequences in the context of a reporter construct containing *oriP* did not result in a loss in activity (see Fig. 6). Efficient transcription initiation from Wp was observed only in the LCL with the reporter construct containing a large deletion from bp −1814 to +961.

It is possible that the observed dependence on *oriP* for Cp and Wp activity in our assay system reflected the plasmid maintenance function of *oriP*, in conjunction with EBNA 1, rather than its enhancer activity. As such, the absence of activity with the reporter constructs lacking *oriP* might reflect loss of these plasmids during the 72 h between transfection and harvesting the cells. To directly address this possibility, JY cells were transfected with either the full-length *oriP*-containing reporter construct (CWW1βGlobin; referred to as wt) or the equivalent reporter construct lacking *oriP* (−2199CWW1βGlobin; referred to as −2199). Cells were harvested at various times posttransfection (ranging from 8 to 56 h), RNA was prepared, and the abundance of Cp-initiated transcripts was assessed by S1 nuclease protection. As shown in Fig. 3F, we could readily detect Cp transcripts from the *oriP*-containing plasmid by 20 h posttransfection, and the abundance of these transcripts increased over the time course. However, we failed to detect any significant level of Cp-initiated transcription from the −2199 reporter construct at any time posttransfection. Thus, even if the reporter constructs lacking *oriP* were lost by 72 h posttransfection, their abundance at 20 h posttransfection should not be significantly different from that of *oriP*-containing plasmids. However, the *oriP*-minus reporter construct exhibited activity that was less than 10% of the activity of the *oriP*-containing reporter construct (Fig. 3F). Thus, it appears likely that it is the EBNA 1-dependent enhancer function of *oriP* that is required for Cp activity and not the plasmid retention function of the element. Finally, consistent with this interpretation, as discussed above, *oriP* was dispensable for Cp and Wp activity in two BL cell lines. The latter results demonstrated that reporter constructs lacking *oriP* are functional in some cell lines and that their activities could be detected by the nuclease protection assay used in these studies.

Analysis of the region upstream of Cp identifies the EBNA 2-dependent enhancer and two CCAAT boxes that are important for Cp activity. Previously, we reported that a deletion (from bp −429 to −245 upstream of the Cp transcription initiation site) which encompassed the EBNA 2-dependent enhancer upstream of Cp ablated Cp activity (28). During subsequent identification of the *cis* elements involved in this phenomenon, we determined that the sequence tag introduced into the C1 exon had been lost during cloning (presumably as a result of contamination of the original site-directed mutated plasmid preparation with unmutated plasmid). Loss of this sequence tag resulted in an inability to detect by S1 nuclease protection Cp-initiated transcripts arising from the transfected reporter construct. After reintroduction of the tag into this construct, Cp activity was detected in both the X50-7 and JY LCL (Fig. 4B). In JY and X50-7 cells, deletion of the EBNA 2-dependent enhancer gave variable results ranging from ca. twofold to greater than fivefold decrease in Cp activity. Shown in Fig. 4B are the results of a typical assay in JY cells and an example of the maximum reduction in activity observed in X50-7 cells. The compiled results of several independent assays are shown in Fig. 4C. These results indicate that the EBNA 2 enhancer contributes to Cp activity in LCL but is not essential for activity. The variability in the impact of this deletion may reflect a complex regulation of this enhancer by both EBNA 2 and EBNA 3c (see Discussion).

To identify other *cis* elements involved in regulating Cp activity, nested deletions that encompassed the Cp EBNA

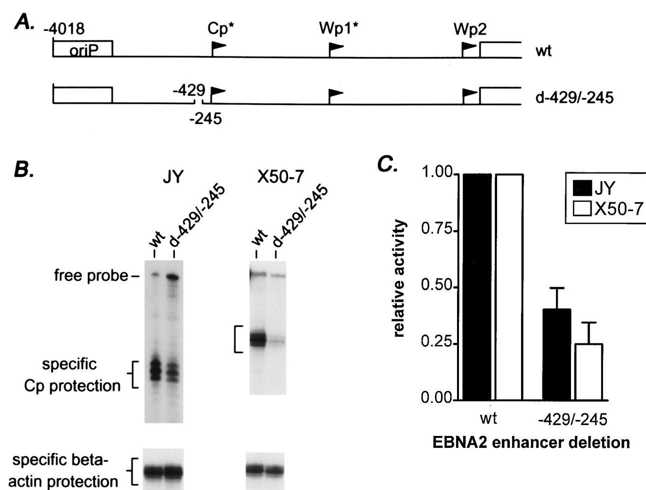

FIG. 4. Deletion of the EBNA 2-dependent enhancer upstream of Cp does not ablate Cp activity in LCL. (A) Structures of the reporter constructs used. Asterisks indicate that the first exon contains a sequence tag such that transcripts from the transfected reporter construct can be discerned from endogenous viral transcripts. (B) S1 nuclease protection analysis of Cp-initiated transcripts. JY and X50-7 LCL were transfected with the indicated reporter constructs. S1 nuclease protection analyses and structure of probes are as described in Materials and Methods. (C) Compiled data from independent transfections (JY, n = 3; X50-7, n = 3). Quantitation of the S1 nuclease protection analyses was carried out as described in the legend to Fig. 3.

2-dependent enhancer and extended upstream were generated (Fig. 5A). While deletion of the sequences from bp −245 to −45 upstream of Cp (these sequences lie downstream of the EBNA 2 enhancer) did not diminish Cp activity, extending this deletion to bp −1021 relative to the Cp transcription initiation site abolished Cp activity in JY and X50-7 cells (Fig. 5B). Thus, while a smaller deletion that removed the EBNA 2 enhancer (bp −429 to −245) only modestly decreased the Cp activity (Fig. 4B), a larger deletion which spans the EBNA 2 enhancer abolished it. The presence of the C1 exon tag in the deletion constructs which did not exhibit Cp activity was verified by diagnostic digests and sequencing. As observed above (Fig. 3C), the Wp activity was significantly upregulated when Cp activity was absent (Fig. 5C, compare the activities of *d*−2199/−45, *d*−1814/−45, and *d*−1021/−45 with those of *d*−245/−45 and wt). In addition, the activities of these deletion constructs indicated that there are no *cis* elements between bp −2199 and −1021 that are important for Wp activity (Fig. 5C and E).

Whether the loss of Cp activity with the bp −1021 to −45 deletion was the result of loss of a promoter element(s) or enhancer sequences, or both, was unclear. Interestingly, deletion of sequences from bp −245 to −45 upstream of Cp, which did not diminish Cp activity (Fig. 4B), removed the Cp CCAAT box. However, inspection of the sequences upstream of Cp revealed that this deletion juxtaposed an upstream putative CCAAT box (located at bp ca. −250) in nearly the same position relative to the Cp transcription initiation site (see Fig. 7). To determine whether the loss of Cp activity reflected the loss of positive *cis* elements upstream of bp −245, nested deletions were generated from bp −1021 to various downstream positions (Fig. 6A). Notably, deletions extending as far downstream as bp −245 did not result in any significant loss of Cp activity when assayed in either the X50-7 or JY cells (Fig. 6B). This supports the contention that the activity of the −245/−45 reporter construct was due to juxtaposing the CCATT box located at bp −257 in a position similar to the position occupied by the Cp CCATT box located at bp −65 (Fig. 7). In addition, it should be noted that deletion of the glucocorticoid elements located at bp ca. −850 upstream of Cp did not affect Cp activity (Fig. 6B; compare the activities of *d*−1021/−920 and *d*−1021/−815).

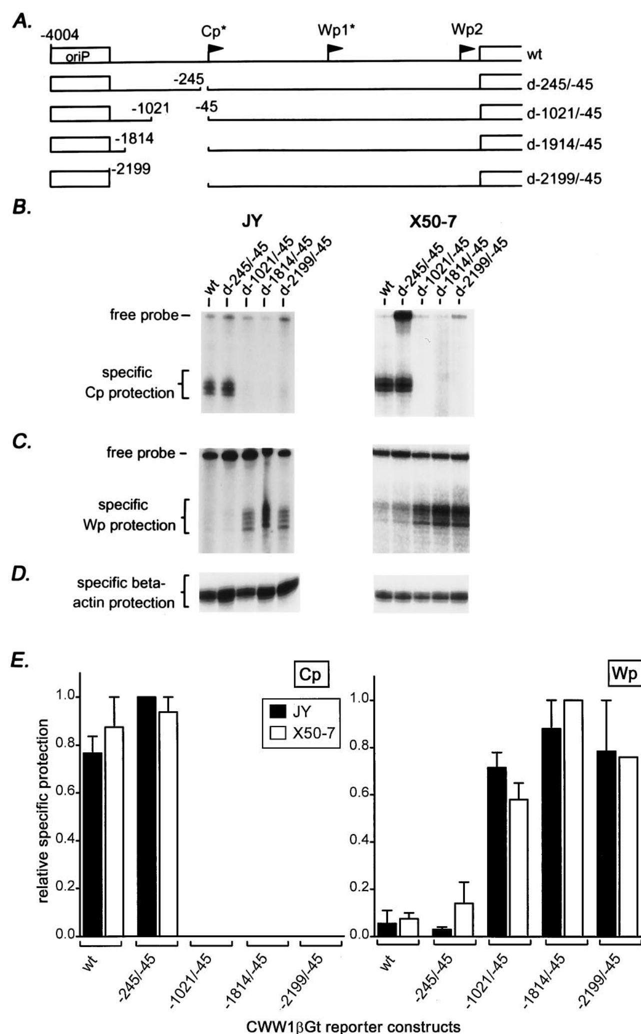

FIG. 5. Deletion of sequences from bp −45 to −1021 upstream of Cp abrogates Cp activity and induces Wp activity. (A) Structures of the reporter constructs used. Asterisks indicate that the first exon contains a sequence tag such that transcripts from the transfected reporter construct can be discerned from endogenous viral transcripts. (B) S1 nuclease protection analysis of Cp-initiated transcripts with RNA prepared from JY and X50-7 cells transfected with the indicated reporter constructs. (C) S1 nuclease protection analysis of Wp-initiated transcripts with the indicated reporter constructs. (D) S1 nuclease analysis of RNAs in panels B and C for levels of β-actin transcripts. (E) Compiled data from independent transfection (JY/Cp, n = 3; JY/Wp, n = 2; X50-7/Cp, n = 2; X50-7/Wp, n = 2). Activities are shown as relative specific protection, which was determined either by densitometry or by quantitation on a PhosphorImager (Molecular Dynamics). The activity of the most active construct was assigned a value of 1.0.

A summary of the results of several independent transfections with these reporter constructs is shown in Fig. 6c. In addition to analyzing deletions extending from bp −1021 to various positions upstream of Cp, we analyzed the sequences between *oriP* and bp −1021 for the presence of *cis* elements affecting Cp activity by generating a deletion that extended from bp −2199 to −1021 (*d*−2199/−1021) (Fig. 6A). Deletion of the sequences from bp −2199 to −1021 upstream of Cp (in

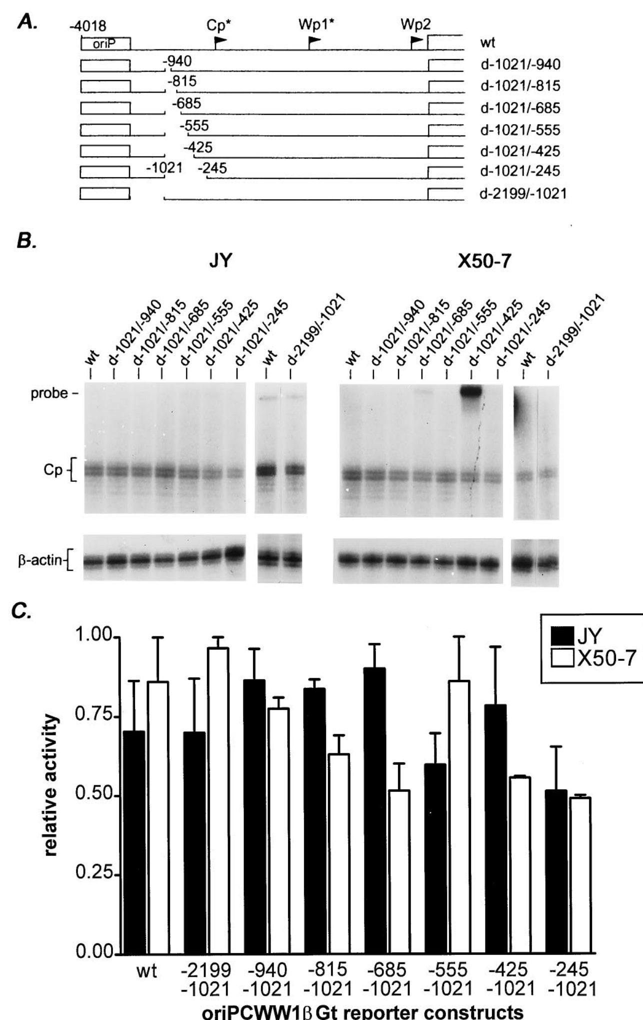

FIG. 6. Sequences from bp −245 to −1021 upstream of Cp are not essential for Cp activity. (A) Structures of the reporter constructs used. Asterisks indicate that the first exon contains a sequence tag such that transcripts from the transfected reporter construct can be distinguished from endogenous viral transcripts. (B) S1 nuclease protection of Cp-initiated transcripts with RNA prepared from JY and X50-7 LCL transfected with the indicated reporter constructs. Also shown are the same RNA preparations analyzed for levels of β-actin transcripts. (C) Compiled data from independent transfections, as described in the legend to Fig. 3 (JY, $n = 3$; X50-7, $n = 2$).

the context of sequences extending from bp −4018 to the second W1 exon) did not diminish Cp activity (Fig. 6B). Notably, this deletion removes the viral interleukin-10 gene which lies between *oriP* and Cp (Fig. 1). Taken together, these results indicate that the only significant regulatory element within the region from bp −2199 to −245 upstream of Cp is the EBNA 2-dependent enhancer.

Support for the hypothesis that the distal CCAAT box upstream of Cp is important for the activity of the $d-245/-45$ reporter construct was obtained by creating additional nested deletions (Fig. 8). The $d-150/-45$ deletes the proximal CCAAT box and moves the distal CCAAT box closer to Cp but further upstream than the proximal CCATT box normally is positioned. The $d-300/-45$ reporter construct extends the deletion through the distal CCAAT box, removing both CCAAT sequences. The activities of these mutants indicate that (i) the position of the distal CCAAT box relative to the Cp transcription initiation site has a significant effect on Cp activity in the presence of a deletion that removes the proximal CCAAT box (Fig. 8, compare the activities of $d-245/-45$ and $d-150/-45$) and (ii) deletion of both CCAAT boxes severely diminishes Cp activity (Fig. 8, see the activity of $d-300/-45$). In addition, we have repeatedly observed that a small amount of totally protected Cp probe is present with RNA samples prepared from cells transfected with the $d-150/-45$ reporter construct. One interpretation of this result is that by moving the CCATT box further upstream, we have moved the site of transcription initiation to a more upstream position.

To more definitively characterize the contribution of the proximal and distal CCAAT boxes to Cp activity, these *cis* elements were mutated as described in Materials and Methods. The activity of the construct with the proximal CCAAT box mutated ($m-63$) was ca. fourfold lower than that of the wt reporter construct (Fig. 8C). A reporter construct in which both the proximal and distal CCAAT boxes were mutated ($m-63,-255$) exhibited little detectable Cp activity. These results confirm the importance of a properly positioned CCAAT box for Cp activity.

Sequences from bp −2199 upstream of Cp to bp +1155 downstream of Cp are dispensable for Wp activity. To assess the presence of *cis* elements upstream of Cp, other than *oriP*, that contribute to Wp activity, several reporter constructs containing internal deletions were generated (Fig. 9A). Comparison of the activity of a reporter construct containing an internal deletion extending from bp −245 upstream of Cp to bp +1155 downstream of Cp ($d-245/+1155$) with that of the $d-1021/+1155$ reporter construct revealed a slight decrease in activity upon deletion of the sequences from bp −245 to −1021 (Fig. 9B). This may reflect deletion of the EBNA 2-dependent enhancer. Further deletion of upstream sequences to bp −2199 had little or no effect on the observed Wp activity in either JY or X50-7 cells, indicating that these sequences are dispensable for Wp activity. The activity of the $d-1814/+961$ construct, which has routinely been used as a positive control for Wp activity, is shown for comparison. Protection of the β-actin exon 3 probe demonstrated that the RNA preparations all appeared to contain equivalent levels of RNA (Fig. 9C). Compiled data from independent experiments are summarized in Fig. 9D.

DISCUSSION

In this study, we have examined the region upstream of Cp for *cis* elements involved in regulated EBNA gene transcription. These studies clearly demonstrate the dependence of the EBNA gene promoters on *oriP* for activity in LCL. In addition, the only other *cis* elements upstream of Cp which appear to be involved in regulating Cp activity are the EBNA 2-dependent enhancer and a properly positioned CCAAT box. Wp activity was observed only when *oriP* was present and Cp was inactive. The regions required for Cp and Wp activity are summarized in Fig. 1B.

The analyses presented here also indicate that the cellular context in which Cp and Wp are characterized can have a large effect on the results obtained. Notably, *oriP* is required for Cp and Wp activity in LCL and some BL cell lines but not in two BL cell lines which we examined (DG75 and clone-13). This difference in the behavior of these cell lines did not correlate with the expression of EBNA 1 or EBNA 2 but appears to reflect a more complex regulation in which specific cellular transcription factors in DG75 and clone-13 cells may obviate the requirement for *oriP*, perhaps by acting through positive *cis*

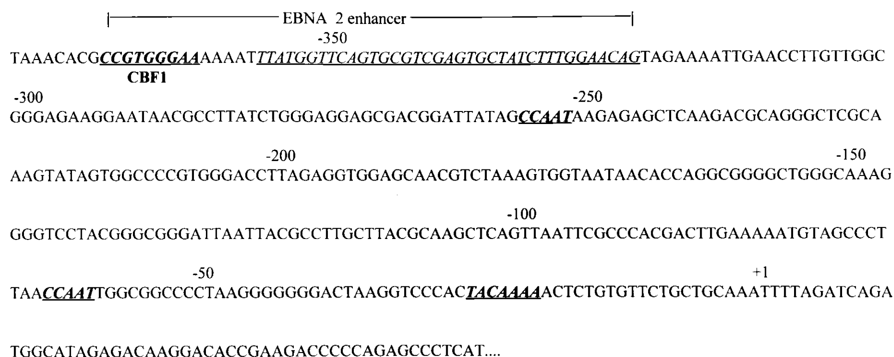

FIG. 7. Cp contains two putative CCAAT boxes. The nucleotide sequence of the EBV B95-8 genome spanning from bp 10949 to 11385 (2) is shown. The Cp-proximal CCAAT box and TATAA box are indicated. In addition, the EBNA 2 responsive enhancer, which contains a CBF1-binding site as well as binding sites for at least two other cellular transcription factors, is indicated. The proximal putative CCAAT box is also indicated. The sequence is numbered relative to the Cp transcription initiation site.

elements which are not functional in LCL. On the basis of the inconsistent behavior of Wp and Cp in BL cell lines, we have purposely focused our characterization on the behavior of reporter constructs in LCL, since these cells are derived from primary B lymphocytes that are EBV transformed in vitro. Thus, LCL are likely to provide a more accurate model for EBV transcriptional regulation than are BL cell lines.

For the identification of the regions upstream of Cp that are involved in regulating Cp and Wp activity, the JY and X50-7 LCL were used. We are aware of the possible limitations of using cell lines that have been passaged in culture for a long period. Indeed, significant effort was expended to identify newly established LCL which could be used in these analyses. However, we were unsuccessful in identifying any low-passage LCL that could be transfected efficiently enough for these studies. While many low-passage LCL could be transfected efficiently enough to detect sensitive reporter gene activity (e.g., chloramphenicol acetyltransferase or luciferase) driven from a strong promoter, the studies presented here required detection of transcription initiation by nuclease protection, which is significantly less sensitive than are most enzymatic reporter gene assays. Notwithstanding the reservations of using LCL which have been passaged in culture for a long time, it should be emphasized that the results obtained with the JY and X50-7 cell lines were nearly identical. Therefore, we think that it is very likely that their behavior with the Cp/Wp reporter constructs accurately reflects the regulation of the EBNA gene promoters in LCL.

Presumably, the essential role of *oriP* for Cp and Wp activity in LCL relates to the EBNA 1-dependent enhancer function associated with this replication origin (19). It has recently been shown that *oriP* may also play an important role in regulating transcription from the LMP promoters (7). In addition, analogous to the observations reported here, these authors noted that the *oriP* enhancement of LMP promoter activity did not require the EBNA 2-responsive element. Thus, *oriP* may be the central regulatory element controlling transcription from the viral promoters active in LCL. With respect to this issue, we have recently determined that transcription from Cp in non-B-cell lines requires only the presence of EBNA 1 (20a). This underscores the role of the *oriP* EBNA 1-dependent enhancer in regulating EBNA gene transcription. The absence of Cp activity in EBV-infected epithelial cell lines appears to correlate with methylation of the viral genome (20a).

With respect to the dependence of Cp and Wp activities on *oriP*, it is worth noting that the EBNA 2-dependent enhancer upstream of Cp is not sufficient to activate these promoters in LCL. Deletion of the Cp EBNA 2-dependent enhancer resulted in a modest reduction of Cp activity. It is possible that the contribution of the EBNA 2 enhancer to Cp activity is variable, depending on as yet unidentified requirements. The recent identification of an interaction between EBNA 3c and CBF1 (RBP-J_κ), which blocks EBNA 2 binding and abrogates DNA binding of CBF1 (14a, 19a), suggests that transcriptional activation through CBF1 may be tightly controlled. Furthermore, it has recently been shown that EBV lacking a functional Cp EBNA 2 enhancer is still able to transform primary B cells and to give rise to LCL which exhibit Cp activity (5a, 33a). It is clear, however, from recent studies that continued expression of EBNA 2 is required for proliferation of LCL (11). Whether this reflects EBNA 2 control of viral and/or cellular gene expression remains to be determined. Taking all these results into consideration, we propose that the EBNA 2-dependent enhancer may function as a rheostat to adjust the levels of transcription from Cp.

We have shown that Wp does not require the sequences from bp −2199 upstream of Cp to bp +1155 downstream of Cp in LCL. Wp activity is, however, dependent on (i) the presence of *oriP* and (ii) inactivation of Cp. The observation that Wp activity is closely linked to inactivation of Cp is consistent with a transcriptional interference model in which transcription from Cp blocks transcription initiation from Wp. Alternatively, it is possible that the presence of a functional Cp effectively competes for *oriP* enhancer activity. Since *oriP* is essential for either Cp or Wp activity, preferential interaction of *oriP* with Cp would be expected to effectively block Wp activity. Distinguishing between these possibilities will require further investigation.

Finally, in considering the requirement of *oriP* for Wp activity in established LCL, it is of interest to consider the requirements for initial Wp activity upon infection of primary B cells. On the basis of immunoblots from early time points after infection of primary B cells, it does not appear that EBNA 1 is packaged in the virion (20). It therefore seems likely that either the initial signal triggered by binding of virus to CD21 and/or a virion-associated factor (perhaps analogous to the herpes simplex virus tegument protein VP16) acts to stimulate transcription from Wp. In addition, it is possible that in primary resting B cells, there are transcription factors that direct transcription from Wp. The latter would be consistent with a model in which Wp is specifically designed to function in resting B cells to initiate viral transcription and Cp is designed to func-

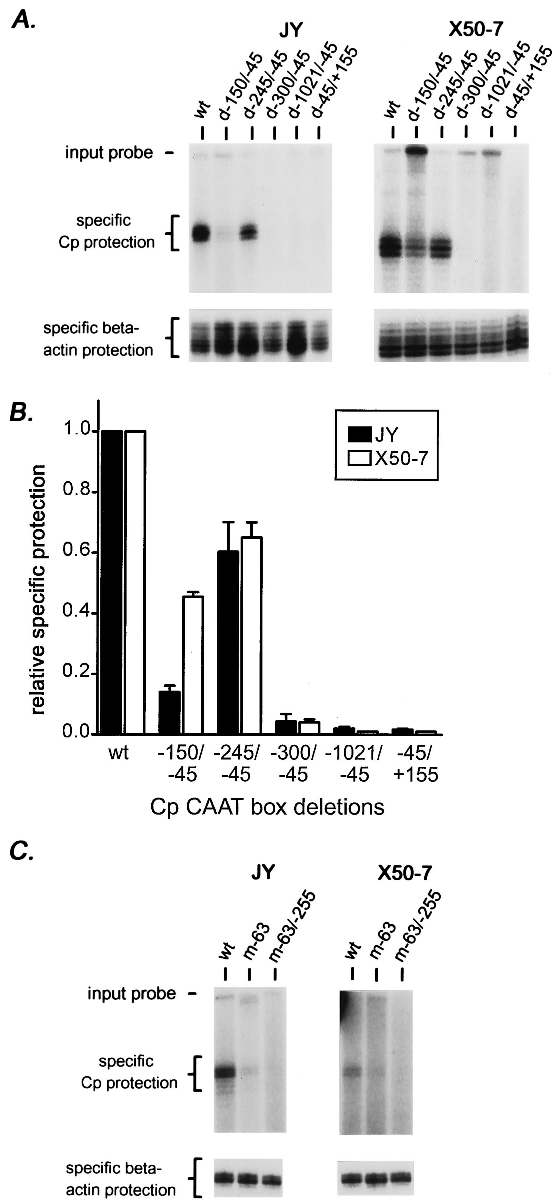

FIG. 8. Cp activity is dependent on the presence of an upstream CCATT box. (A) A series of constructs which either delete both proximal and distal CCATT boxes (Fig. 7) or delete the proximal CCAAT box and move the distal CCATT box were assessed for Cp activity. Cp activity was monitored by S1 analysis with RNAs prepared from either JY or X50-7 LCL transfected with the indicated reporter constructs as described in Materials and Methods. The sequences deleted are indicated in the name of reporter construct. Corresponding β-actin protections are shown and demonstrate that equivalent amounts of RNA were used for each analysis. (B) Compiled data from independent transfections of the indicated reporter constructs (JY, $n = 3$; X50-7, $n = 2$). Quantitation was carried out as described in the legend to Fig. 3. (C) Activities of reporter constructs containing Cp CCAAT box mutations. Reporter constructs were generated as described in Materials and Methods and contained site-directed mutations in either the proximal CCAAT box ($m-63$) or both the proximal and distal CCAAT boxes ($m-63,-255$). Both reporter constructs contained sequences from just upstream of *oriP* (bp −4018) to the second W1 exon. Cp activity was monitored by S1 analysis with RNAs prepared from either JY or X50-7 LCL transfected with the indicated reporter constructs as described in Materials and Methods.

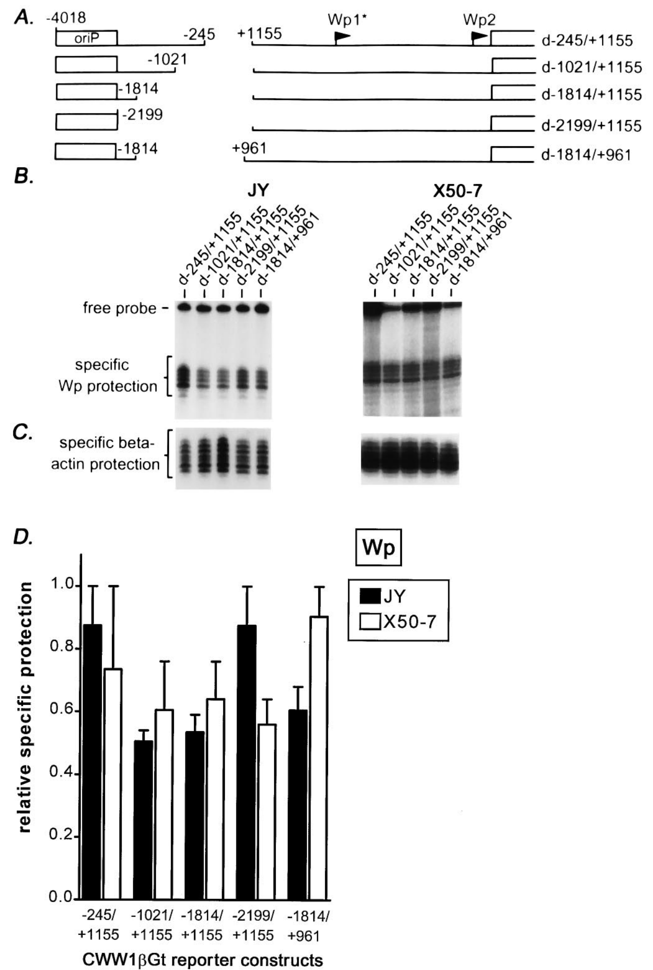

FIG. 9. Sequences from bp −2199 upstream of Cp to bp +1155 downstream of Cp are dispensable for Wp activity. (A) Structures of the reporter constructs used. The asterisk denotes a sequence tag introduced into the W0 exon of Wp1 to distinguish transcripts arising from the transfected reporter construct from endogenous viral transcripts. (B) S1 nuclease protection analysis of Wp-initiated transcripts with RNA prepared from JY or X50-7 LCL transfected with the indicated reporter constructs. (C) S1 nuclease protection analysis of a β-actin exon 3 probe with the same RNA preparations from transfected JY or X50-7 cells. (D) Compiled data from independent transfections of the indicated reporter constructs into the JY and X50-7 LCL (JY, $n = 2$; X50-7, $n = 2$). The data were analyzed as described in the legend to Fig. 3.

tion once EBNA gene expression is established. Investigation of these issues will require careful characterization of viral and cellular factors influencing Wp activity during the early stages of infection of primary B cells.

In addition to *oriP*, we have shown that Cp activity appears to be highly dependent on the presence of a CCAAT box. Deletion of the sequences from bp −245 to −45 upstream of Cp, which removed the proximal CCAAT box, juxtaposed a distal CCAAT box in nearly the same position and did not adversely affect activity. However, a slightly larger deletion from bp −300 to −45, which deleted both CCAAT boxes, nearly completely abrogated Cp activity. Further support for the role of the distal CCAAT box came from deleting the sequences from bp −150 to −45, which removed the proximal CCAAT box and moved the distal CCAAT box to a position ca. 100 bp upstream of the position of the normal CCAAT box. This reporter construct exhibited diminished activity compared

with the wt and bp −245 to −45 deletion constructs, suggesting that the distance of the CCAAT box from the site of transcription initiation is important. Furthermore, site-directed mutation of the proximal and distal CCAAT boxes conclusively demonstrated their importance in Cp activity. Overall, these results indicate that Cp activity is dependent on the presence of an appropriately positioned CCAAT box.

Our deletion analysis also indicated that the region upstream of Cp containing the glucocorticoid response elements is not necessary for transcription initiation from Cp or Wp (Fig. 1). This is consistent with the ability to generate EBV mutants lacking these elements which exhibit Cp activity and are still able to immortalize primary B cells (5a). It seems likely that this region plays an important regulatory role in the context of natural infection, which is not reproduced by in vitro infection of primary B cells.

In summary, we have identified the major regulatory *cis* elements in the viral genome upstream of Cp which modulate EBNA gene promoter activity (summarized in Fig. 1). In addition to the promoter elements, the only other sequences which appear to have a significant effect on Cp activity are *oriP* and the EBNA 2-dependent enhancer. For Wp activity, the presence of *oriP* and inactivation of Cp are essential for activity.

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