JOURNAL OF VIROLOGY, June 1996, p. 3561–3570 Vol. 70, No. 6
0022-538X/96/$04.00+0

Transcription of the Epstein-Barr Virus Nuclear Antigen 1 (EBNA1) Gene Occurs before Induction of the BCR2 (Cp) EBNA Gene Promoter during the Initial Stages of Infection in B Cells

SABINE SCHLAGER,[1,2] SAMUEL H. SPECK,[3]* AND MAXIMILIAN WOISETSCHLÄGER[1]

Sandoz Forschungsinstitut, A-1235 Vienna,[1] and Institute of Microbiology, Karl-Franzens Universität, A-8010 Graz,[2] Austria, and Departments of Pathology and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110[3]

Received 22 January 1996/Accepted 6 March 1996

The purpose of this study was to gain insights into the regulation of Epstein-Barr virus (EBV) gene transcription during the establishment of viral latency in B cells. During the early stages of EBV infection in B lymphocytes, transcription of six viral nuclear antigens (EBNAs) is initiated from an early promoter (Wp). This is followed by a switch of promoter usage to an upstream promoter, Cp, whose activity is autoregulated by both EBNA1 and EBNA2. Previously it was demonstrated that infection of primary B cells with EBNA2-negative (EBNA2$^-$) EBNA4-mutant (EBNA4^{mut}) virus resulted only in the expression of mutant EBNA4 protein and failure to express the other EBNA gene products (C. Rooney, H. G. Howe, S. H. Speck, and G. Miller, J. Virol. 63:1531–1539, 1989). We extended this research to demonstrate that Wp-to-Cp switching did not occur upon infection of primary B cells with an EBNA2$^-$ EBNA4^{mut} virus (M. Woisetschlaeger, X. W. Jin, C. N. Yandara, L. A. Furmanski, J. L. Strominger, and S. H. Speck, Proc. Natl. Acad. Sci. USA 88:3942–3946, 1991). Further characterization of this phenomenon led to the identification of an EBNA2-dependent enhancer upstream of Cp. On the basis of these data, a model was proposed in which initial transcription from Wp gives rise to the expression of EBNA2 and EBNA4, and then transcription is upregulated from Cp via the EBNA2-dependent enhancer (Woisetschlaeger et al., as noted above). Implicit in this model is that transcription of the EBNA1 and EBNA3a to -3c genes is dependent on the switch from Wp to Cp, since primary cells infected with EBNA2$^-$ EBNA4^{mut} virus fail to switch and also fail to express these viral antigens. Here we critically evaluate this model and demonstrate, in contrast to the predictions of the model, that transcription of both the EBNA1 and EBNA2 genes precedes activation of Cp. Furthermore, the level of EBNA1 gene transcription was strongly reduced in primary B cells infected with EBNA2$^-$ EBNA4^{mut} virus compared with that of cells infected with wild-type virus. Switching to Cp, as well as EBNA1 gene transcription, was observed upon infection of EBV-negative Burkitt's lymphoma (BL) cell lines with EBNA2$^-$ EBNA4^{mut} virus, thus establishing a correlation between early EBNA1 gene transcription and upregulation of transcription initiation from Cp. However, in EBV-negative BL cell lines infected with EBNA2$^-$ EBNA4^{mut} virus, transcription of the EBNA1 gene at early time points postinfection initiated from Qp, the EBNA1 gene promoter active in group I BL cells (B. C. Schaefer, J. L. Strominger, and S. H. Speck, Proc. Natl. Acad. Sci. USA 92:10565–10569, 1995), rather than from Wp. The data support a model in which EBNA1 plays an important role in the cascade of events leading to successful switching from Wp to Cp and subsequent immortalization of the infected B cell.

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that infects two major cell types in vivo, epithelial cells in the oropharynx and B lymphocytes. EBV is the etiologic agent of infectious mononucleosis, a self-limiting lymphoproliferative disease. In addition, EBV is closely associated with a number of human cancers, including African Burkitt's lymphoma (BL) and nasopharyngeal carcinoma. In B lymphocytes the virus establishes a latent infection, which is the basis of viral persistence throughout the life of the infected individual. EBV latency can be partially mimicked by infection of B cells in vitro. The infected cells establish a latent phenotype with very limited viral gene expression and little or no virus production. There is a concomitant immortalization of the cells which gives rise to lymphoblastoid cell lines that proliferate indefinitely in culture. In such cells the virus expresses six nuclear antigens (EBNAs) and three latent membrane proteins (LMP). This pattern of viral gene expression is referred to as group III latency (reviewed in reference 11).

A different EBNA expression pattern is found in fresh EBV-positive BL biopsies and certain BL cell lines (group I cell lines). These cells express EBNA1 but not the other EBNAs or LMP genes (reviewed in reference 20). Downregulation of EBNA and LMP gene expression in BL cells may be an important mechanism of evading cytotoxic T-lymphocyte immune surveillance. Two groups mapped a candidate EBNA1 gene promoter (Fp) thought to be active in group I BL cell lines (21, 25). The resulting transcripts contained two noncoding exons, FQ and U, at their 5′ ends which then splice into the EBNA1-encoding K exon. Recent studies, however, demonstrated that Fp activity correlated with lytic viral gene expression (12, 23) and that the vast majority of EBNA1 transcripts in group I cell lines initiated from a promoter (Qp) ca. 200 nucleotides downstream of Fp (24). As a consequence, Qp-initiated mRNAs have a Q-U-K exon structure, while further analysis has revealed that most Fp-derived transcripts do not splice from the U exon to the K exon (23, 24). The struc-

* Corresponding author. Mailing address: Departments of Pathology and Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-0367. Fax: (314) 362-0089. Electronic mail address: speck@pathology.wustl.edu.

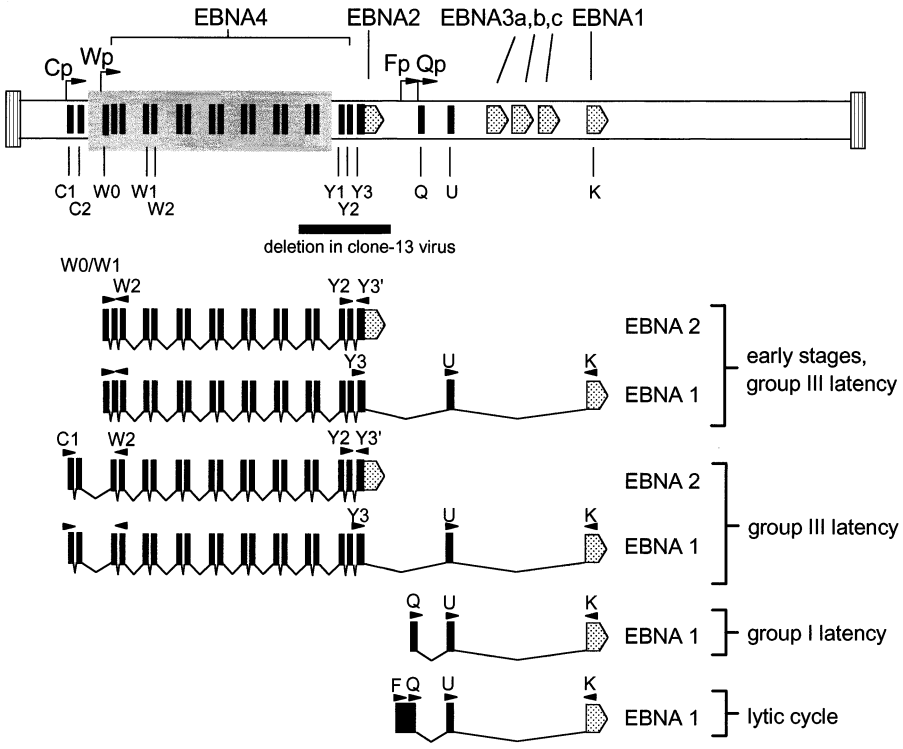

FIG. 1. Simplified schematic representation of EBNA1 and EBNA2 gene transcripts generated during the various EBV latency and lytic programs in B cells. The names and locations of the various PCR primers employed in these studies are noted. Regions encoding the various EBNAs are indicated on the viral genome, and specific exons present in some of the transcripts are labeled. The large shaded rectangle on the viral genome represents the major internal repeat, IR1. All EBNA transcripts present in group III latently infected B cells have common 5′ exons (the W1 and W2 repeat exons encoded within IR1 and the Y1, Y2, and Y3 exons), which are alternatively spliced to downstream coding exons. EBNA gene transcription in group III latently infected cells initiate from either Wp or Cp, while EBNA 1 gene transcripts in group I latently infected cells initiate from Qp. As illustrated, many of the group III EBNA transcripts contain the U exon, which is noncoding. The exons encoding EBNA3a, -3b, and -3c are actually each composed of two coding exons, i.e., a short exon which is spliced to a closely spaced large exon. In group III latently infected B cells, many alternatively spliced variants have been detected, but their relative abundance is unknown at this time. Evidence for the existence of Wp-initiated EBNA1 gene transcripts is presented in this paper.

tures of Fp- and Qp-initiated EBNA1 gene transcripts are shown in Fig. 1.

During group III latency, all six EBNA transcripts have common 5′ exons which are alternatively spliced to unique 3′ exons encoding individual EBNAs, with the exception of EBNA4, which is encoded by the 5′ common exons (Fig. 1) (reviewed in reference 27). Transcription initiation starts from one of two promoters, Cp or Wp, which are located near the left end of the viral genome. Cp activity appears to be regulated by EBNA1 and EBNA2 via two *cis* elements: (i) an enhancer in *oriP* (17), which also serves as the origin of replication for the maintenance of the viral genome during latency (both functions require EBNA1 binding to *oriP*) (37), and (ii) an EBNA2-dependent enhancer located in the proximal region upstream of Cp (29, 34). EBNA2 transactivation is mediated via an interaction with a DNA-binding protein called CBF1 or RBP-Jκ, which serves to target EBNA2 to this enhancer, thereby stimulating transcription from Cp, as well as to target EBNA2 to several other viral and cellular promoters (13, 32, 39).

It has been shown that the activities of Cp and Wp are mutually exclusive in all clonal cell lines which have been examined (35). Infection of peripheral B lymphocytes with EBV leads initially to exclusive Wp usage and then to upregulation of Cp, which appears to be the EBNA promoter of choice during established group III latency (34, 36). EBNA2 may be required for the upregulation of transcription from Cp during the establishment of latency since (i) infection with EBNA2-negative (EBNA2$^-$) EBNA4-mutant (EBNA4^{mut}) virus results in a failure to switch from Wp to Cp (34), (ii) an EBNA2-dependent enhancer exists upstream of Cp, and (iii) primary B cells infected with EBNA2$^-$ EBNA4^{mut} virus express only mutant EBNA4 and fail to express EBNA1 or EBNA3a to -3c (19). These determinations led to the proposal that the downstream of EBNA genes can be expressed only from Cp-derived transcripts.

In this paper we investigate the activities of the three latency-associated EBNA gene promoters (Cp, Qp, and Wp) with respect to the appearance of EBNA1 and EBNA2 transcripts during the establishment of viral latency. The data demonstrate (i) that Wp initiates the transcription of EBNA1 and EBNA2 during the initial phase of viral infection in peripheral blood mononuclear cells (PBMC) with wild-type virus before transcription initiation from Cp is detected; (ii) a failure to efficiently transcribe the EBNA1 gene and to activate transcription from Cp in PBMC infected with EBNA2$^-$ EBNA4^{mut} virus; (iii) efficient transcription of the EBNA1 gene and induction of Cp activity in an EBV-negative BL cell line with EBNA2$^-$ EBNA4^{mut} virus; and (iv) early activation of Qp in EBV-negative BL cells infected with EBNA2$^-$ EBNA4^{mut} virus. With respect to the latter point, Qp activity could be correlated with both the onset and the level of transcription of the EBNA1 gene. In all cases, a direct correlation between transcription of the EBNA1 gene during the initial stages of infection and efficient induction of transcription from Cp was observed, implicating EBNA1 in the upregulation of Cp activity.

TABLE 1. PCR primer pairs and probes to measure promoter activities and EBNA transcripts

Promoter activity type or transcript	Upstream primer	Downstream primer	Annealing temp (°C)	Southern blot probe
Wp	W0W1-W0W1′	W2	68	W1h
Cp	C1	W2	63	C1h
Qp	Q	K	58	Uh
Fp	F	K	58	Uh
EBNA2 mRNA	Y2	Y3a	70	Y3h
EBNA1 mRNA (total)	U	K	58	Uh
EBNA1 mRNA (from Wp or Cp)	Y3	K	58	Uh

MATERIALS AND METHODS

Cell lines. B95.8 is a spontaneously productive marmoset lymphoblastoid cell line containing EBV recovered from a human patient (14). Jijoye (3) and clone-13, a subclone of the EBNA2$^-$ cell line P3HR1 (15), are EBV-positive BL cell lines. Akata (31) is a group I BL cell line. BL2 and BL41 are EBV-negative BL cell lines (4). B95.8, Jijoye, and clone-13 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO, Grand Island, N.Y.). Akata, BL2, and BL41 cells were kept in enriched IMDM medium (38) supplemented with 10% heat-inactivated fetal calf serum at 37°C with 5% CO_2.

In vitro infection of peripheral blood lymphocytes and EBV-negative BL cells. Immortalizing virus was induced in B95.8 cells by growing the cells for 12 to 14 days without changing the culture medium. Nonimmortalizing EBNA2$^-$ EBNA4^{mut} virus was induced from the clone-13 BL cell line as previously described (34). Viral particles were concentrated 50-fold from supernatants by centrifugation at 13,000 × *g* for 2 h. Peripheral blood lymphocytes were enriched by centrifugation onto a Lymphoprep cushion (Nycomed Pharma, Oslo, Norway). B cells were infected at a concentration of 2×10^7 to 5×10^7 cells per ml of 50× virus stock for 2 h at 37°C, after which the cells were washed and cultured in enriched IMDM medium. Aliquots were taken at various time points for preparation of RNA.

S1 nuclease protection analyses. Total cellular RNA was prepared by the method (procedure C) of Auffray and Rougeon (1a). Fifty micrograms of RNA was used in S1 nuclease protection analyses with specific oligonucleotides as previously described (35) with the C1-58mer and the W0W1-60mer as radiolabeled probes (36).

RT-PCR and Southern hybridization analyses of viral transcripts. Total RNA was extracted with TRIzol reagent (GIBCO BRL Life Technologies, Renfrewshire, Scotland) in accordance with the manufacturer's instructions. RNA (15 μg) was treated with 10 U of RQ1 RNase-free DNase (Promega Corporation, Madison, Wis.) for 20 min at 37°C. After a phenol extraction, RNA was precipitated with ethanol, dissolved in distilled water, and denatured for 4 min at 68°C. Reverse transcription (RT) was initiated with 0.4 μg of oligo(dT) (Pharmacia LKB, Uppsala, Sweden), 0.4 μg of random primer (GIBCO BRL), and avian myoblastosis virus reverse transcriptase (Pharmacia) in a total volume of 50 μl at 42°C for 50 min. cDNA was denatured at 95°C for 5 min, and its quality was tested with dihydrofolate reductase-specific PCR primers.

PCR was performed in a final volume of 100 μl with 1 μl of cDNA per reaction mixture in a Perkin-Elmer Cetus DNA thermal cycler. The DNA was denatured for 30 s at 94°C, annealed between 58 and 70°C (depending on the primer pair) (Table 1) for 1.5 min, and extended at 72°C for 1 min. PCR products were fractionated on 0.8% agarose gels, transferred onto Hybond-N nylon membranes (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom) according to the method of Sambrook et al. (20a), and fixed by UV irradiation. Southern hybridization was carried out according to the method of Chujor et al. (5) with specific oligonucleotides (Table 1) labeled at their 3′ ends (with a terminal transferase kit; Boehringer, Mannheim, Germany). The nucleotide sequences of oligonucleotides used in this study are listed in Table 2.

To test for the relative sensitivities and specificities of the oligonucleotide primer-probe combinations, RNA from B95.8, clone-13, or Akata cells was serially diluted with RNA prepared from the EBV-negative BL2 cell line, resulting in dilutions ranging from 10^{-2} down to 10^{-6}. The combined RNA (15 μg) was reverse transcribed, and 1 μl of cDNA was amplified for 35 cycles, after which it was blotted to nylon membranes and detected with the appropriate internal probe as described above. Southern blots were exposed to film (X-Omat AR films; Kodak Company, Rochester, N.Y.) for 2 h.

RESULTS

Wp-to-Cp promoter switching in the absence of EBNA2 gene expression in EBV-negative BL cells but not in primary B cells. Our previous studies have shown that infection of primary B cells with EBNA2$^-$ EBNA4^{mut} EBV (recovered from induced clone-13 cells) led to Wp activity at early times postinfection, with a level of Wp-initiated transcripts comparable to that seen with wild-type virus (34). However, no switching to Cp was observed. As discussed above, earlier studies have shown that infection of primary B cells with EBNA2$^-$ EBNA4^{mut} virus resulted in the expression of EBNA4 only and not the other EBNA gene products (19). However, infection of EBV-negative BL cell lines with EBNA2$^-$ EBNA4^{mut} virus resulted in the expression of all the EBNA gene products except EBNA2 (19). On the basis of these data, we have proposed that EBNA1 and EBNA3a to -3c expression requires switching to Cp. To investigate this issue, we carried out infections of primary B cells and EBV-negative BL cell lines with wild-type and EBNA2$^-$ EBNA4^{mut} viruses.

As reflected in Fig. 2 (left panel), the activities of Cp and Wp in PBMC infected with wild-type virus (B95.8 virus) or EBNA2$^-$ EBNA4^{mut} virus (clone-13 virus) were monitored by S1 nuclease protection analysis. In agreement with our previous work, Wp activity was detected by 10 h postinfection with wild-type virus and by 6 h postinfection with the EBNA2$^-$ EBNA4^{mut} virus. At these early time points no Cp activity was observed. No Cp or Wp activity was detected in RNA samples prepared from uninfected PBMC (data not shown). Consistent with our previous results, induction of Cp activity was only observed in PBMC infected with wild-type virus and was detected by 45 to 115 h postinfection (Fig. 2). RNA from the clone-13 cell line (in which the endogenous viral genomes exhibit constitutive Wp activity) and from the Jijoye BL cell line (in which the endogenous viral genomes drive EBNA gene transcription from Cp) were used as positive controls for Cp and Wp activities. We have previously shown (18) that the clone-13 cell line exhibits very high steady-state levels of Wp-initiated transcripts while the Jijoye cell line contains fairly low steady-state levels of Cp-derived transcripts. Thus, the observed differences in the levels of protection of the Cp and Wp probes by these RNAs reflect the differences in the abundance of Cp- and Wp-initiated transcripts in these cell lines and not differences in the sensitivities of these probes (Fig. 2).

To address the issue of EBNA gene expression in EBV-negative BL cell lines infected with EBNA2$^-$ EBNA4^{mut} virus, infection time courses were carried out with two EBV-negative BL cell lines (BL2 and BL41). These cell lines were selected on the basis of cell surface expression of CD21, the EBV receptor

TABLE 2. Sequences of oligonucleotides used in this study

Name	Sequence (5′→3′)	EBV genome coordinates[a]
C1	TGTAGATCTGATGGCATAGAGAC	11342–11355
C1h	AAGGACACCGAAGACCCCCAGAG	11356–11378
K	GATCGAATTCCATTTCCAGGTCCTGTACCT	107987–107968
Q	ATATGAGCTCGGGTGACCACTGAGGGA	62333–62349
F	ATATGAGCTCGTGCGCTACCGGATGGCG	62441–62458
U	GCATAAGCTTAGAGAGTAGTCTCAGGGCATC	67545–67564
Uh	GGTGAATCTGCTCCCAGGTC	67629–67610
W0W1	CAGGAGATCTGGAGTCCACACAAATCCT	14396–14556
W0W1′	GAGGAGATCTGGAGTCCACACAAATGGG	14396–14561
W1h	GAGACCGAAGTGAAGGCCCTGGACCAACCC	14562–14590
W2	ACTGAAGCTTGACCGGTGCCTTCTTAGGAG	14735–14716
Y2	TGACAAGCTTGCGCCAATCTGTCTACATAG	47941–47960
Y3	TGGCGTGTGACGTGGTGTAA	48398–48418
Y3a	ACTGGAATTCCCCCCATGTAACGCAAGATAG	48536–48516
Y3h	GGGTGCTTAGAAGGTTGTTG	48474–48454

[a] See reference 6.

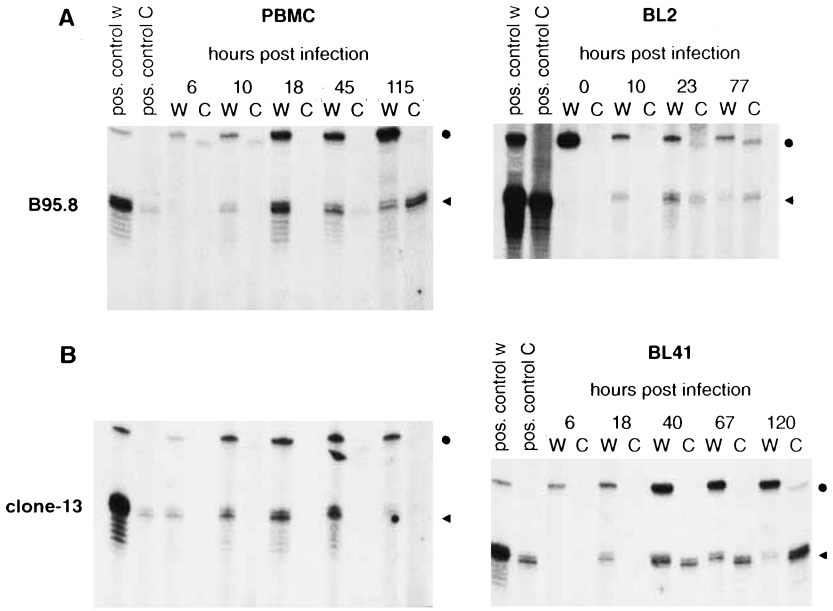

FIG. 2. Infection time courses of adult PBMC and EBV-negative BL cell lines (BL2 and BL41) infected with either wild-type virus (B95.8) (A) or EBNA2$^-$ EBNA4^{mut} virus (clone-13) (B). Total cellular RNA was prepared from cells harvested at the indicated hours postinfection, hybridized to Cp (lanes C)-specific (C1 exon) or Wp (lanes W)-specific (W0-W1 splice junction) oligonucleotide probes, and digested with S1 nuclease. The filled circles indicate the length of input probes, and the arrowheads indicate the S1-nuclease-resistant fragments. Ten micrograms of total cellular RNA from clone-13 and Jijoye BL cell lines was employed as a positive control for Wp and Cp activity, respectively. It should be noted that the level of Wp-initiated transcripts in clone-13 cells is much greater than the level of Cp-initiated transcripts in Jijoye cells.

(data not shown), and because they had been used in the studies described above (19). Infection of BL2 cells with wild-type virus (B95.8) resulted in detectable Wp activity by 10 h postinfection, similar to the results obtained upon infection of primary B cells (Fig. 2A, right panel). No EBNA gene transcription from Cp was detected at this time point; however, by 23 h postinfection both Wp and Cp were active and by 77 h the abundance of Cp-initiated transcripts was greater than that of Wp-derived mRNA. This result demonstrated that Wp-to-Cp promoter switching also occurs upon infection of EBV-negative BL cell lines (infection of BL41 cells gave similar results; data not shown).

Infection of BL41 cells with EBNA2$^-$ EBNA4^{mut} virus (clone-13 virus) also led to initial Wp usage without any detectable transcription initiation from Cp at 18 h postinfection. Wp activity was maximal by 40 h postinfection and decreased thereafter (Fig. 2B, right panel). In contrast to the results obtained with primary cells, Cp-initiated transcripts were detectable by 40 h, and their levels increased with time. The relative activities of Cp and Wp were very similar to those observed with wild-type virus infection of PBMC. This demonstrated that promoter switching can occur in the absence of EBNA2 expression in BL cell lines, in contrast to the results obtained with primary B cells. Furthermore, since previous studies had demonstrated expression of the downstream EBNA gene products in EBV-negative BL infected with EBNA2$^-$ EBNA4^{mut} virus (19), these results are consistent with the hypothesis that transcription of the EBNA1 and EBNA3a to -3c genes requires switching from Wp to Cp.

Establishment of an RT-PCR assay to detect transcription of the EBNA1 and EBNA2 genes. The observation that switching from Wp to Cp does not require EBNA2 in EBV-negative BL cell lines raises the question of the mechanism involved. In addition to EBNA2, EBNA1 is known to activate transcription from Cp via the EBNA1-dependent enhancer in *oriP* (28, 35). Thus, to extend the analysis of EBNA gene transcription, we attempted to employ S1 nuclease protection analysis to directly measure the production of EBNA1 and EBNA2 transcripts as well as to monitor the activities of Cp, Wp, and Qp. Initial attempts to detect EBNA1 and EBNA2 transcripts by S1 nuclease protection indicated that this approach was not sufficiently sensitive for the amounts of RNA which could be isolated from the aliquots of cells harvested during the infection time courses. To circumvent this problem, a sensitive RT-PCR technique was established.

To carry out a comparison of the appearance of transcripts initiating from the EBNA gene promoters and transcription of the EBNA1- and EBNA2-coding exons, the relative sensitivities of the PCR primer pairs (designed to monitor the abundance of various RNA species) were determined for three different established cell lines (Fig. 3) (a summary of the primers and probes employed in this study are given in Tables 1 and 2 and are illustrated in Fig. 1). B95.8 and clone-13 cells were chosen as examples of Cp and Wp active cell lines, respectively. In addition, because virus derived from these lines was used for the time course infection studies, this analysis verified that the RT-PCR primers were functional with these viral genomes. RNA prepared from the group I BL cell line Akata allowed assessment of primers specific for transcription initiation from Qp (24).

Wp signals with clone-13 RNA and Cp activity with B95.8 RNA were detectable down to a 10^{-4} dilution of RNA. Since previous S1 nuclease protection analyses indicated that the steady-state abundance of Wp-initiated transcripts in the clone-13 cell line is greater than the steady-state level of Cp-initiated transcripts in the B95.8 cell line (18), it appears that detection of Cp activity under the assay conditions employed is slightly more sensitive than detection of Wp activity. The ladder of bands detected with both Wp- and Cp-specific oligonucleotide pairs is consistent with the amplification of fragments

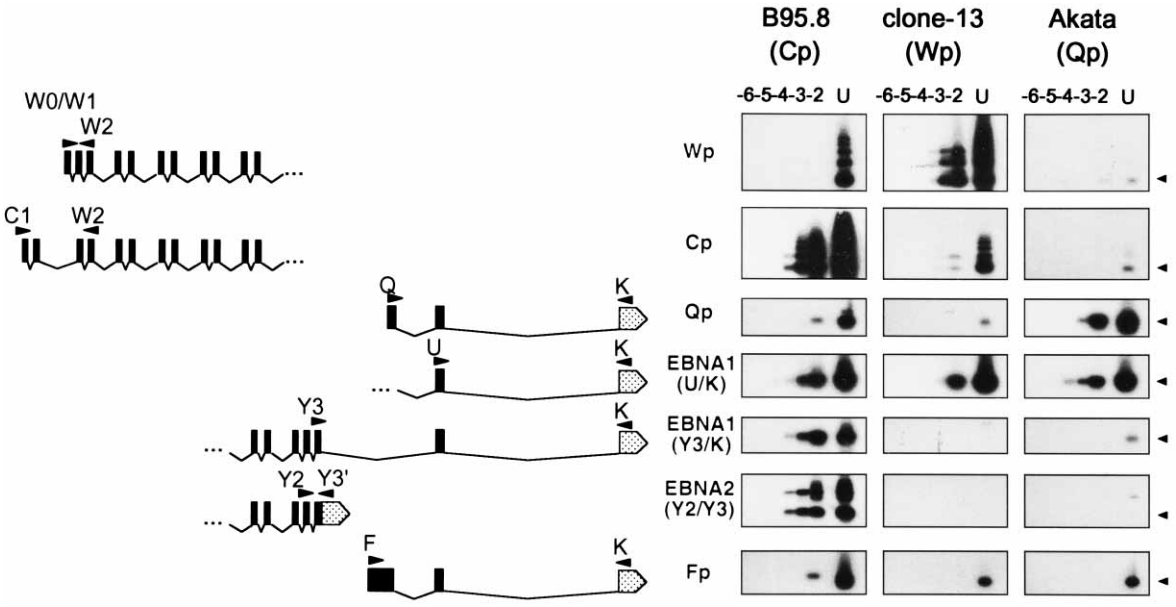

FIG. 3. Sensitivities of oligonucleotide primer pair combinations used to measure the levels of Cp, Wp, Qp, and Fp transcripts and EBNA1 and EBNA2 transcripts by RT-PCR. RNAs from three EBV-positive cell lines were serially diluted with RNA from the EBV-negative BL2 cell line, resulting in dilutions ranging from 10^{-2} down to 10^{-6}. After first-strand cDNA synthesis, RT-PCR followed by Southern blot hybridization was performed with the primer pairs and probes, as described in Materials and Methods. U, undiluted RNA from 2×10^4 EBV-positive cells. To the right, the predicted sizes of fully spliced PCR products are shown in nucleotides (nt). The ladder of bands observed with Wp and Cp primer pairs reflects hybridization of the downstream PCR oligonucleotide to one of several W2 exons present in the EBNA transcripts initiated from these promoters (Fig. 1). Data from an experiment representative of no less than three independent analyses are shown. The sizes of the PCR products indicated by the arrowheads are given in Fig. 4 and 5.

containing multiple copies of the W1W2 repeat exons (i.e., since the downstream primer employed in both cases lies within the W2 exon, products of various sizes can be obtained, depending upon the W2 repeat exon to which the primer hybridizes) (Fig. 1).

The increased sensitivity of the RT-PCR assay compared with that of S1 nuclease protection analysis is exemplified by the detection of Wp transcripts with RNA prepared from the B95.8 cell line and of Cp-initiated transcripts with RNA prepared from clone-13 cells, demonstrating that the activities of Cp and Wp are not absolutely mutually exclusive. In both cases, however, transcripts from the dominant promoter were 100- to 1,000-fold more abundant than mRNA initiated from the alternative promoter. In addition, only a very weak band for Cp- or Wp-initiated transcripts was observed with undiluted Akata RNA, in agreement with earlier observations that these two promoters are inactive in group I BL cell lines. Overall, these results demonstrated that the primer pairs for detecting Cp and Wp activity were specific and that the previously observed preferential promoter usage could be reproduced by RT-PCR.

EBNA1-specific transcripts were monitored with several different primer combinations. Primers within the *Bam*HI U-encoded exon and the EBNA1-encoding exon (U-K primer pair) should detect virtually all EBNA1 transcripts, whether they arise from Cp, Wp, or Qp, while primers within the *Bam*HI Y3 exon and the EBNA1 exon (Y3-K primer pair) should account for most of the EBNA1 transcripts which arise from either Cp or Wp (reviewed in reference 27) (Fig. 1). The Qp primer pair (Q-K) specifically detects EBNA1 transcripts arising from either Qp or Fp (24), while the Fp primer pair (F-K) detects only EBNA1 transcripts originating from Fp (23, 24) (Fig. 1 and 3). The latter transcripts appear to be produced at low levels during induction of the viral lytic cycle (23, 24). Subtracting the Fp signal from the Qp signal thereby gives an accurate assessment of the abundance of EBNA1 transcripts arising from Qp. This is valid since we have demonstrated by S1 nuclease protection analysis that the B95.8 cell line contains Fp-initiated transcripts (23) but not Qp-initiated transcripts. The near equivalence of the signals obtained with B95.8 RNA employing the Fp and Qp primer pairs indicates that they amplify Fp-initiated cDNA with the same level of efficiency.

The U-K primer pair, which was designed to detect EBNA1 mRNA regardless of its transcription origin, yielded a detectable signal at the 10^{-4} dilution with cDNA prepared with RNA isolated from either B95.8 or Akata cells and at the 10^{-3} dilution with clone-13-derived cDNA (Fig. 3). The Y3-K primer pair gave results nearly identical to those of the U-K primer pair with B95.8 RNA, consistent with Cp being the sole promoter used for the expression of EBNA1 transcripts in this cell line (Fig. 3). Because of the deletion in the clone-13 genome (Fig. 1), the Y3-K primer pair did not amplify anything with clone-13 RNA. Furthermore, the U-K primer pair was able to detect EBNA1 transcripts with a level of sensitivity similar to that of the Qp primer pair with RNA from the group I BL cell line Akata, consistent with the deduced structures of these transcripts (Fig. 3). In addition, the Y3-K amplification was nearly undetectable in this cell line. This finding is in line with the hypothesis that all EBNA1 transcripts in group I BL cell lines arise from Qp (24). These results demonstrate that the U-K and Y3-K primer pairs used in this study are well suited to distinguish Cp- and Wp-initiated EBNA1 transcripts from Qp-derived EBNA1 mRNA. It should be emphasized that the sensitivities of these amplifications were carefully titrated to allow a direct assessment of the kinetics of EBNA1 gene transcription relative to the onset of Wp, Cp, and Qp activities.

The Y2-Y3a-EBNA2-specific primer pair combination detected its target down to a 10^{-4} dilution of B95.8 cDNA but did not detect EBNA2 transcripts with Akata cDNA, in agreement with the group I latency phenotype of this cell line (Fig. 3). The

larger band observed, on the basis of its size, most likely represents a low level of either unspliced EBNA2 RNA or viral DNA in the RNA samples. With respect to the latter point, since all the PCR amplifications employed in this analysis cross one or more splice junctions, potential contamination of RNA samples by viral DNA should not affect the interpretation of the results since we rely on detection of the spliced product. The Q-K (Qp) amplification of Akata cDNA was at least 1,000-fold more sensitive than the F-K (Fp) primer pair combination. This result is in agreement with recent data demonstrating that only a small fraction of Fp-initiated transcripts splice to the EBNA1-coding exon and that these transcripts are expressed only during the lytic life cycle of EBV (23, 24). These determinations most likely explain the higher levels of Fp activity in B95.8 cells, of which ca. 1 to 5% are lytically infected. The F-K (Fp) primer combination therefore should be viewed as an indicator of a subfraction of Fp-initiated lytic transcripts which splice to the EBNA1-coding exon (Fig. 3). In addition, as discussed above, the studies of Schaefer et al. (24) demonstrated utilization of an alternative promoter, Qp, for EBNA1 transcripts in group I BL cells (located ca. 200 bp downstream of Fp). As a consequence, the Qp-specific Q-K primer combination efficiently amplified Qp-initiated transcripts down to the 10^{-3} dilution in Akata cells, while detecting Fp-initiated transcripts only in the undiluted RNA sample.

Transcription of the EBNA1 gene precedes the switch to Cp upon infection of either primary B cells or EBV-negative BL cell lines. The sensitivity and specificity of the RT-PCR assay allowed us to assess the appearance of EBNA1 and EBNA2 transcripts relative to the onset of transcription from Wp, Cp, and Qp during the initial stages of infection of BL2 cells and PBMC with both wild-type and EBNA2$^-$ EBNA4^{mut} mutant viruses. RT-PCR analyses of Wp and Cp activities during infection of PBMC gave results nearly identical to those obtained by S1 nuclease protection analyses (Fig. 4). Wp-initiated transcripts could be detected by 6 h postinfection with both viruses, although a stronger signal was observed in the wild-type-virus-infected cells. Upregulation of Cp activity was not observed until 69 h, at which time a strong signal was observed with cells infected with wild-type virus and only a very weak signal could be detected with EBNA2$^-$ EBNA4^{mut}-virus-infected cells.

EBNA2-specific transcripts were detectable as early as 6 h, and thus their presence correlated with the onset of Wp activity. This correlation is in line with published data in which EBNA2 and EBNA4 were shown to be the first viral nuclear antigens being expressed during in vitro infection with EBV (19). As anticipated, no EBNA2 mRNA was observed in cells infected with EBNA2$^-$ EBNA4^{mut} virus (clone-13 virus), demonstrating that the PBMC employed in this experiment were not detectably contaminated with endogenous EBV from the donor.

EBNA1 gene transcription was apparent by 15 h postinfection, well after EBNA2 transcripts were detectable. The latter data are in line with the delayed expression of EBNA1, compared with that of EBNA2, as determined by the immunoblotting of infection time courses (19). Since the signal intensities of the U-K and the Y3-K amplifications were very similar and since Qp-initiated transcripts were hardly detectable, it is likely that the EBNA1 transcripts detected at 15 and 25 h postinfection were derived from Wp-initiated transcripts. The level of EBNA1 transcripts was very low in EBNA2$^-$ EBNA4^{mut}-virus-infected cells compared with that of wild-type-virus-infected cells and appeared to correlate with the inefficient switch to Cp. In wild-type-virus-infected cells, Qp activation was consistently observed at 69 h, as with Cp activation, whereas

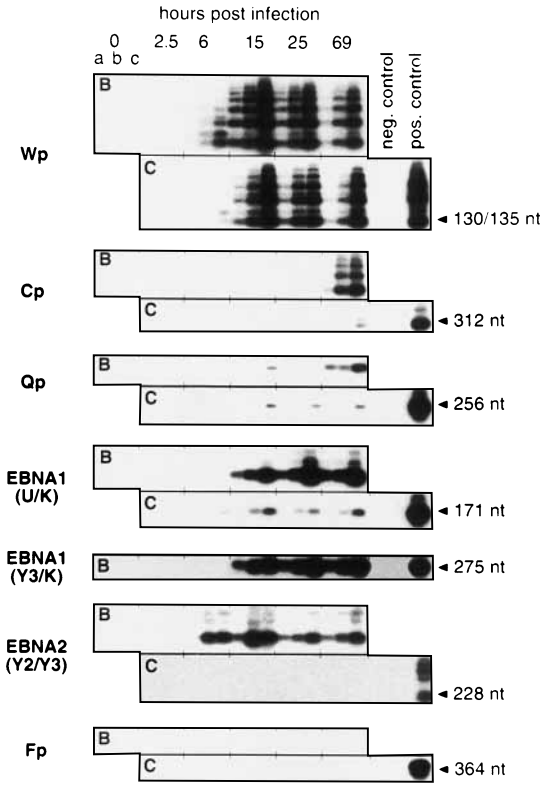

FIG. 4. Infection time courses of adult PBMC infected with either wild-type virus from the B95.8 cell line (B) or EBNA2$^-$ EBNA4^{mut} virus from induced clone-13 cells (C). RNA was prepared from cells harvested at the indicated hours postinfection, and RT-PCR and Southern hybridization were done as described in Materials and Methods. For each time point, different numbers of amplification cycles were performed (labeled a b c in the first lefthand panel) and were as follows: 28, 30, and 33 cycles were run for the detection of Cp and EBNA2 transcripts; 30, 33, and 35 cycles were run for the detection of Wp, Qp, EBNA1 (U-K and Y3-K), and Fp transcripts. PCRs without cDNA were included as negative controls. B95.8 cDNA was used as a positive control for Cp, EBNA1 (U-K and Y3-K), and EBNA2; clone-13 cDNA was used as a positive control for Wp; and Akata cDNA was used as a positive control for Qp and Fp. To the right the sizes of fully spliced PCR products are shown. The data show the results of an experiment representative of at least three independent analyses. nt, nucleotides; neg., negative; pos., positive.

EBNA2$^-$ EBNA4^{mut} infection resulted in an only marginally amplifiable template. Finally, FQ-U-K-spliced transcripts initiated from Fp were not detected upon infection of PBMC with either virus (i.e., there was no evidence of lytic cycle activity).

The same type of analysis was performed with BL2 cells infected with either wild-type (B95.8 virus) or EBNA2$^-$ EBNA4^{mut} virus (clone-13 virus) (Fig. 5). Wp activity was detectable as early as 4 h postinfection in wild-type-virus-infected cells and was stronger in cells infected with EBNA2$^-$ EBNA4^{mut} virus. Switching to Cp was seen at 19 h and occurred with similar efficiency in cells infected with either wild-type or mutant virus, consistent with the S1 nuclease protection analyses. As with the infection of primary B cells, EBNA2 gene transcription in wild-type-virus-infected cells correlated with the appearance of Wp-initiated transcripts at 6 and 10 h postinfection and remained relatively constant throughout the time course. In addition, in wild-type-virus-infected cells EBNA1 gene transcription was visible by 6 h postinfection when assessed by amplification with the U-K primer pair and by 10 h postinfection with the Y3-K primer pair. In EBNA2$^-$-EBNA4^{mut}-virus-infected BL2 cells EBNA1 transcripts were

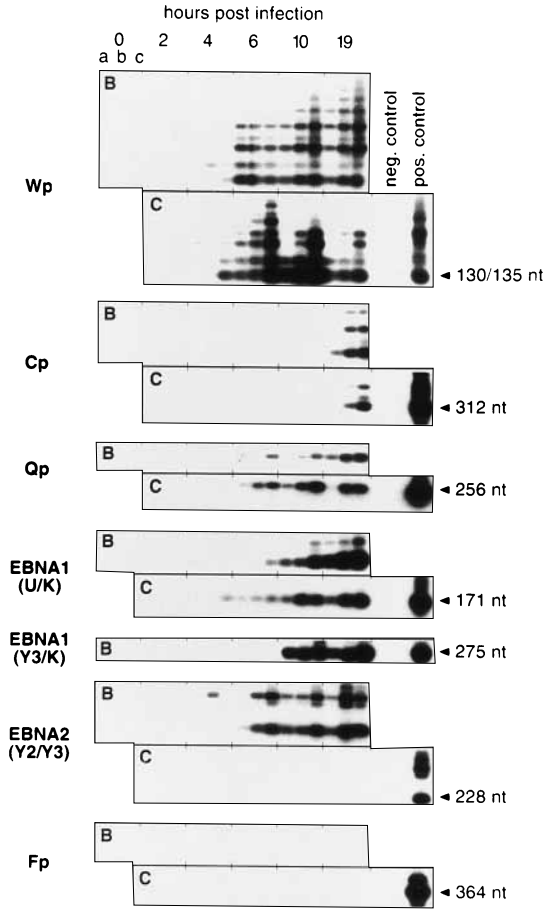

FIG. 5. Infection time courses of BL2 cells infected with either B95.8 virus (B) or clone-13 virus (C). RNA was prepared from cells harvested at the indicated hours postinfection, and RT-PCR and Southern hybridization were performed as described in Materials and Methods. The number of amplification cycles (a b c) and the positive controls employed are described in the legend to Fig. 4. To the right the sizes of fully spliced PCR products are shown. The data presented reflect the results of an experiment representative of at least three independent analyses. nt, nucleotides; neg., negative; pos., positive.

detected even earlier than in wild-type-virus-infected BL2 cells, at 4 h. Interestingly, the EBNA1 gene transcription level was only slightly lower than in wild-type-virus-infected cells, which is in sharp contrast to the situation with primary cells. The results from EBNA2$^-$ EBNA4^{mut} virus infection of primary B cells and BL2 cells suggest a relationship between the level of EBNA1 gene transcription and the efficiency of the switch from Wp to Cp.

In contrast to the results obtained upon infection of primary B cells, infection of BL2 cells led to the generation of Qp-specific transcripts early in the infection time course. Indeed, strong transcription from Qp in EBNA2$^-$-EBNA4^{mut}-virus-infected BL2 cells was observed as early as 6 h postinfection, and the intensity of this signal correlated with the abundance of U-K-spliced transcripts (Fig. 5). This indicated that EBNA1-specific transcripts originated largely from Qp at these early time points. Again, no Fp-derived transcripts were amplified in these experiments. However, in wild-type-virus-infected BL2 cells, the Qp activity level was lower and did not account for the majority of EBNA1 gene transcription. Thus, it appears likely that the bulk of EBNA1 transcripts at early time points in wild-type-virus-infected BL2 cells arose predominantly from Wp-initiated transcription. The latter point is substantiated by the comparable levels of EBNA1 transcripts containing the Y3 exon (detected with the Y3-K primer pair) and the abundance of U-K-spliced transcripts. The expression of EBNA1 from Wp is likely to inhibit transcription initiation from Qp via the low-level-affinity EBNA1 sites downstream of the Qp transcription initiation site (22) and thus account for the lower level of Qp-initiated transcripts present in wild-type-virus-infected BL cells compared with that of EBNA2$^-$-EBNA4^{mut}-virus-infected cells.

DISCUSSION

In this paper we have presented data on the temporal sequence of events leading to activation of the Cp EBNA gene promoter. The data demonstrate that EBNA1 gene transcription precedes the switch from Wp to Cp in primary B cells and EBV-negative BL cell lines infected with wild-type virus (B95.8 virus), as well as in EBV-negative BL cells infected with EBNA2$^-$ EBNA4^{mut} virus. However, infection of primary B cells with EBNA2$^-$ EBNA4^{mut} virus resulted in a failure to efficiently transcribe the EBNA1 gene and to upregulate Cp. Thus, there is a direct correlation in each case with early transcription of the EBNA1 gene and switching to Cp. A logical interpretation of these results is that EBNA1 transactivation of Cp via the *oriP* EBNA1-dependent enhancer is required for efficient switching. This interpretation leaves unresolved the importance of EBNA2 transactivation of Cp in switching in primary B cells. However, EBNA2 is clearly dispensable for activation of Cp in EBV-negative BL cell lines.

The failure to efficiently transcribe the EBNA1 gene from the Wp transcripts present at early times postinfection of primary B cells infected with EBNA2$^-$ EBNA4^{mut} virus suggests that either (i) EBNA2 and/or EBNA4 play a role in regulating transcription of the downstream EBNA coding exons or (ii) the deletion in clone-13 virus impairs transcription of the downstream EBNA genes. It appears that in EBV-negative BL cells infected with EBNA2$^-$ EBNA4^{mut} virus there is also a failure to transcribe the EBNA1 gene from Wp-initiated transcripts. The EBNA1 transcripts present at early time points in these cells arise from Qp-initiated transcripts. Thus, in the EBV-negative BL cells infected with EBNA2$^-$ EBNA4^{mut} virus, upregulation of Cp appears to be dependent on transcription initiation from Qp (Qp-to-Cp switch).

It is formally possible that Qp-driven transcription of the EBNA1 gene does not occur in the same cells that ultimately exhibit Cp activity upon acute infection of the BL2 cell line. This appears to be unlikely since (i) transient transfection of this cell line with Cp- or Wp-driven reporter constructs does not lead to detectable levels of Cp activity (14a), indicating that the necessary transcriptional milieu is not present in uninfected BL2 cells; (ii) the long lag time between initial infection and the detection of Cp-initiated transcripts also suggests the requirement for production of a viral and/or cellular factor(s) to upregulate transcription from Cp; and (iii) the infection of a cell line (e.g., BL2) would be anticipated to behave in a homogeneous fashion. On the basis of the available data, in the absence of EBNA2 gene expression (i.e., infection of BL2 cells with EBNA2$^-$ EBNA4^{mut} virus), EBNA1 is the best candidate for providing the necessary transactivation signal to activate transcription initiation from Cp.

The concerns about heterogeneity in the infection of cell populations with EBV can be extended to the results obtained upon infection of PBMC. As discussed above for Qp and Cp activities in infected BL2 cells, it is formally possible that Wp and Cp activities are active in different cell populations during

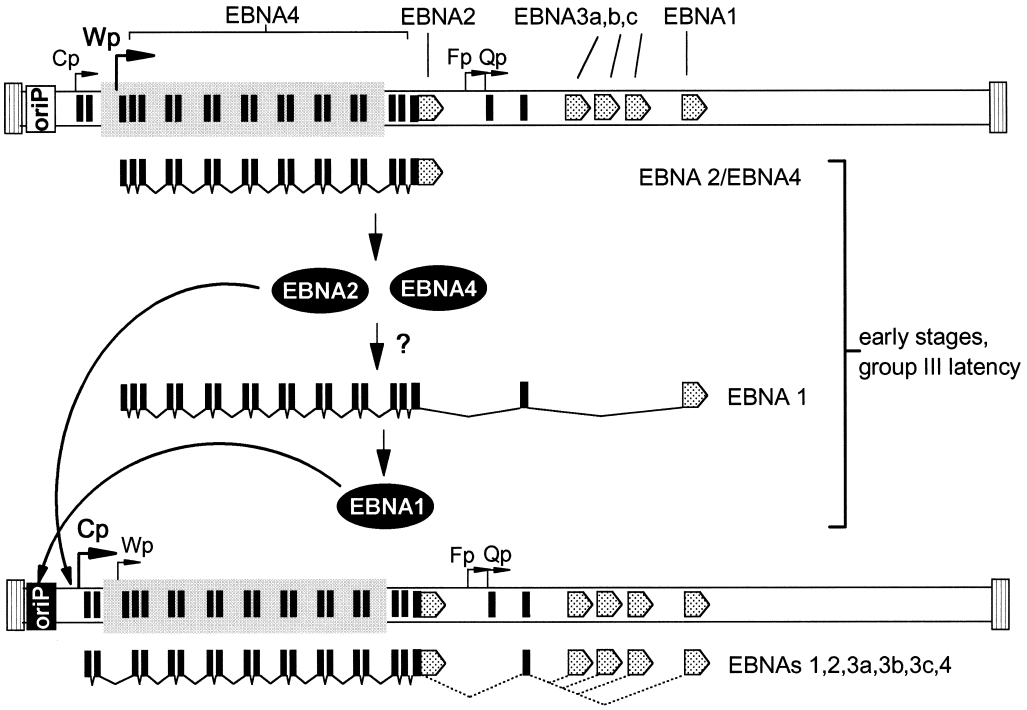

FIG. 6. Model for early events upon infection of peripheral B cells with wild-type EBV. Transcription from Wp leads to the production of EBNA2 and EBNA4. The synthesis of EBNA2 and/or EBNA4, by an unknown mechanism, leads to transcription of the downstream EBNA1 coding exon. The actions of EBNA1 and EBNA2, through *oriP* and the Cp-associated EBNA2-dependent enhancer, induce Cp activity. Transcription from Cp leads to a downregulation of transcription initiation from Wp.

acute infection of PBMC. This seems very unlikely, however, since analyses of Cp and Wp activities during infection time courses have demonstrated a significant decline in Wp activity levels with the appearance of Cp-initiated transcripts (reference 34 and Fig. 2), indicating that the activities of these promoters are linked. Furthermore, support for a role of EBNA1 in the induction of transcription initiation from Cp is provided by our recent observation that *oriP* is an essential element for Cp activity in established lymphoblastoid cell lines (14b). In addition, it has also recently been demonstrated that the presence of *oriP* strongly enhances LMP1 promoter activity in EBV-positive cell lines (7). Thus, it appears that the *oriP* EBNA1-dependent enhancer may play a central role in regulating viral transcription once viral latency is established.

In contrast to the results obtained with EBV-negative BL cells, Qp is inactive during the initial stages of infection of primary B cells with either wild-type or EBNA2$^-$ EBNA4^{mut} virus, becoming weakly active at late time points which roughly correspond to the time the infected cells enter S phase of the cell cycle. Thus, it is possible that Qp is a cell-cycle-regulated promoter. It should be noted that the detection of Qp activity during the establishment of viral latency in primary B cells is the first demonstration of Qp activity outside the setting of tumor cell populations. As such it provides support for the contention that Qp is a viral promoter which may play important roles at specific stages of viral infection. At this point it is unclear whether the Qp activity detected is present in the same cells which are also actively transcribing the EBNA genes from Wp and Cp or whether this reflects viral transcription in a discrete population of cells which are not destined to become immortalized by EBV. Finally, the failure to detect any transcription initiation from Fp provides further evidence that the functions of Qp and Fp are not linked.

Consistent with a possible role of EBNA2 and/or EBNA4 in regulating transcription of the downstream EBNA genes is the observation that the levels of EBNA2 transcripts which accumulate by 15 h postinfection in primary B cells infected with wild-type virus are circa threefold higher than the steady-state levels observed at later time points (Fig. 4). This higher level of EBNA2 transcripts suggests that there may be a transcription blockade early in infection which leads to the accumulation of EBNA2-EBNA4 transcripts and that this blockade is subsequently released upon production of sufficient levels of these EBNAs (Fig. 6). At this point nothing is known about the possible regulation of 3′ processing of the EBNA transcripts or any mechanisms which might function to regulate the levels of these antigens. Alternatively, the decrease in levels of EBNA2 transcripts could reflect the loss of infected cells expressing EBNA2, although this is not reflected by a concomitant decrease in EBNA1 gene transcription or Wp-initiated transcripts (Fig. 4).

The fact that Cp is not active during the initial stages of infection could reflect an active repression mechanism and/or the lack of necessary activation signals. If EBNA1 is required for switching to Cp, this would indicate that the absence of a necessary activation signal is responsible for the inactivity of Cp during the initial stages of infection. Support for an active repression of Cp comes from the observation of Hsieh and Hayward (9), who demonstrated that CBF1 (RBP-Jκ) binding repressed transcription initiation from a linked promoter. This repression was relieved by coexpression of EBNA2, which appeared to mask the repression domain in CBF1. A similar derepression mechanism may be operational for Cp and may set the stage for Cp *trans*-activation by EBNA1 once it is bound to its binding sites in *oriP* (16, 17, 28, 37).

The other major difference that was observed between the

infection of peripheral B-cell lines and EBV-negative BL cell lines was the kinetics of the upregulation of Cp activity. In BL cells, induction of Cp activity was observed much earlier than in primary B cells (19 h in BL cells compared with 69 h in B cells). This difference may reflect the fact that BL cells are actively proliferating, and thus early events involved in pushing primary infected cells from G_0 into the cell cycle are obviated. If this is the case, it would suggest that EBV-driven entry into the cell cycle is a prerequisite for switching from Wp to Cp activity, and would also indicate that these events are rate limiting.

Recent data in the literature describe EBNA2 as a general cell activator, as is reflected by its involvement in the regulation of cell cycle entry and the upregulation of B cell activation markers like CD21 and CD23 (8, 10, 26, 33). Sinclair et al. (26) showed that EBNA2 and EBNA4 cooperate to cause a G_0-to-G_1 transition during immortalization of resting B lymphocytes. These workers demonstrated that cyclin D2 and one of its dependent kinases (cdk4) were upregulated after cotransfection of EBNA2 and EBNA4. Cyclin D2 forms heterodimers with the tumor suppressor retinoblastoma gene product (pRB) and thus marks pRB for phosphorylation via cyclin-dependent kinases. Therefore, EBNA2-induced cyclin D2 expression results in higher levels of phosphorylated pRB, which acts as a key player for entry into the cell cycle (10).

In an attempt to assess whether the missing EBNA2 and EBNA4 functions in mutant-virus-infected primary B lymphocytes could be compensated for by alternative stimuli, infection time course experiments were carried out with clone-13 virus in the presence of interleukin-4 and anti-CD40 monoclonal antibody. These two stimuli have been shown not only to induce vigorous proliferation of human B cells but also to act via activation pathways similar to those of EBV (2, 10). However, in our hands, incubation of peripheral B cells with interleukin-4 and anti-CD40 antibody, either prior to or at the time of virus infection, resulted in significantly reduced viral gene expression from Wp and Cp, making interpretation of the results impossible (data not shown). It is possible that only resting primary B cells are susceptible to efficient EBV infection, as has been suggested from the results of other studies (1, 31a). We are currently investigating this issue to determine whether these experimental difficulties can be overcome.

In summary, the data presented in this paper indicate that EBNA1 is an important factor in regulating EBNA gene promoter switching during the initial stages of EBV infection. Further studies are required to determine the precise role of EBNA2 in Wp-to-Cp switching, as well as its possible role in regulating EBNA1 gene transcription from Wp during the establishment of latent infection. It should be noted that recombinant viruses lacking Cp have been shown to immortalize primary B cells, indicating that the switch from Wp to Cp is not obligatory under tissue culture conditions (30). It is likely, however, that the switch to Cp confers an advantage to lymphoblastoid cells in vivo, most likely during the acute stage of virus infection. Gaining insights into this issue, however, will require the development of appropriate animal model systems.

ACKNOWLEDGMENTS

This study was made possible by grant P08735 to M.W. by the Fonds zur Förderung der wissenschaftlichen Forschung, Vienna, Austria, and by grant R01 CA43143 to S.H.S. from the National Institutes of Health.

We thank David Leib and Skip Virgin for helpful comments on the manuscript and M. Grimling for skillful artwork.

REFERENCES

1. **Aman, P., J. Gordon, N. Lewin, M. Nordstrom, B. Ehlin-Henriksson, G. Klein, and A. Carstensson.** 1985. Surface marker characterization of EBV target cells in normal blood and tonsil B lymphocyte populations. J. Immunol. **135:**2362–2367.

1a. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. **107:**303–314.

2. **Banchereau, J., P. dePaoli, A. Valle, E. Garcia, and F. Rousset.** 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science **251:**70–72.

3. **Brandsma, J., and G. Miller.** 1980. Nucleic acid spot hybridization: rapid quantitative screening of lymphoid cell lines for Epstein-Barr viral DNA. Proc. Natl. Acad. Sci. USA **77:**6851–6855.

4. **Calender, A., M. Billaud, J. P. Aubry, J. Banchereau, M. Vuillaume, and G. M. Lenoir.** 1987. Epstein-Barr virus (EBV) induces expression of B cell activation markers on *in vitro* infection of EBV-negative B-lymphoma cells. Proc. Natl. Acad. Sci. USA **84:**8060–8064.

5. **Chujor, C. S. N., B. Kuhn, B. Schwerer, H. Bernheimer, W. R. Levis, and D. Bevec.** 1992. Specific inhibition of mRNA accumulation for lymphokines in human T cell line Jurkat by mycobacterial lipoarabinomannan antigen. Clin. Exp. Immunol. **87:**398–403.

6. **Farrell, P. J.** 1989. Epstein-Barr virus genome, p. 103–132. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, New York.

7. **Gahn, T. A., and B. Sugden.** 1995. An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. J. Virol. **69:**2633–2636.

8. **Gordon, J., L. Walker, G. Guy, G. Brown, M. Rowe, and A. Rickinson.** 1986. Control of human B-lymphocyte replication. II. Transforming Epstein-Barr virus exploits three distinct viral signals to undermine three separate control points in B cell growth. Immunology **58:**591–595.

9. **Hsieh, J. J.-D., and S. D. Hayward.** 1995. Masking of the CBF1/RBPJκ transcriptional repression domain by Epstein-Barr virus EBNA2. Science **268:**560–563.

10. **Kempkes, B., D. Spitkovsky, P. Jansen-Duerr, J. W. Ellwart, E. Kremmer, H. J. Delecluse, C. Rottenberger, G. W. Bornkamm, and W. Hammerschmidt.** 1995. BB cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2. EMBO J. **14:** 88–96.

11. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1920. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.

12. **Lear, A. L., M. Rowe, M. G. Kurilla, S. Lee, S. Henderson, E. Kieff, and A. B. Rickinson.** 1992. The Epstein-Barr virus (EBV) nuclear antigen 1 *Bam*HI F promoter is activated on entry of EBV-transformed B cells into the lytic cycle. J. Virol. **66:**7461–7468.

13. **Ling, P. D., J. J.-D. Hsieh, I. K. Ruf, D. R. Rawlins, and S. D. Hayward.** 1994. EBNA 2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1. J. Virol. **68:**5375–5383.

14. **Miller, G., and M. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. USA **70:**190–194.

14a. **Puglielli, M. T., and S. H. Speck.** Unpublished observation.

14b. **Puglielli, M. T., M. Woisetschläger, and S. H. Speck.** Unpublished data.

15. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Non-immortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **44:**834–844.

16. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42:**859–868.

17. **Reisman, D., and B. Sugden.** 1986. *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6:**3838–3846.

18. **Rogers, R. P., M. Woisetschlaeger, and S. H. Speck.** 1990. Alternative splicing dictates translational start in Epstein-Barr virus transcripts. EMBO J. **9:** 2273–2277.

19. **Rooney, C., J. G. Howe, S. H. Speck, and G. Miller.** 1989. Influences of Burkitt's lymphoma and primary B cells on latent gene expression by the nonimmortalizing P3J-HR-1 strain of Epstein-Barr virus. J. Virol. **63:**1531–1539.

20. **Rowe, M., and C. Gregory.** 1989. Epstein-Barr virus and Burkitt's lymphoma, p. 237–259. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, New York.

20a. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

21. **Sample, J., L. Brooks, C. Sample, L. Young, M. Rowe, C. Gregory, A. Rickinson, and E. Kieff.** 1991. Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. Proc. Natl. Acad. Sci. USA **88:**6343–6347.

22. **Sample, J., E. B. D. Henson, and C. Sample.** 1992. The Epstein-Barr virus

nuclear protein 1 promoter active in type I latency is autoregulated. J. Virol. **66:**4654–4661.
23. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** 1995. The Epstein-Barr virus *Bam*HI F promoter is an early lytic promoter: lack of correlation with EBNA 1 gene transcription in group 1 Burkitt's lymphoma cell lines. J. Virol. **69:**5039–5047.
24. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** 1995. Redefining the Epstein-Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription initiation site in group I Burkitt lymphoma cell lines. Proc. Natl. Acad. Sci. USA **92:**10565–10569.
25. **Schaefer, B. C., M. Woisetschlaeger, J. L. Strominger, and S. H. Speck.** 1991. Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **88:**6550–6554.
26. **Sinclair, A. J., I. Palmero, G. Peters, and P. J. Farrell.** 1994. EBNA 2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus. EMBO J. **13:**3321–3328.
27. **Speck, S. H., and J. L. Strominger.** 1989. Transcription of Epstein-Barr virus in latently infected, growth-transformed lymphocytes, p. 133–150. In G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, New York.
28. **Sugden, B., and N. Warren.** 1989. A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. J. Virol. **63:**2644–2649.
29. **Sung, N. S., S. Kenney, D. Gutsch, and J. S. Pagano.** 1991. EBNA-2 transactivates a lymphoid-specific enhancer in the *Bam*HI C promoter of Epstein-Barr virus. J. Virol. **65:**2164–2169.
30. **Swaminathan, S., R. Hesselton, J. Sullivan, and E. Kieff.** 1993. Epstein-Barr virus recombinants with specifically mutated BCFR1 genes. J. Virol. **67:** 7406–7413.
31. **Takada, K., and Y. Ono.** 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. J. Virol. **63:**445–449.
31a. **Thorley-Lawson, D., and K. Mann.** 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. J. Exp. Med. **162:**45–59.
32. **Waltzer, L., F. Logeat, C. Brou, A. Israel, A. Sergeant, and E. Manet.** 1994. The human Jκ recombination signal sequence binding protein (RBP-Jκ) targets the Epstein-Barr virus EBNA 2 protein to its DNA responsive elements. EMBO J. **13:**5633–5638.
33. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA 2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
34. **Woisetschlaeger, M., X. W. Jin, C. N. Yandava, L. A. Furmanski, J. L. Strominger, and S. H. Speck.** 1991. Role of the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. Proc. Natl. Acad. Sci. USA **88:**3942–3946.
35. **Woisetschlaeger, M., J. L. Strominger, and S. H. Speck.** 1989. Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **86:**6498–6502.
36. **Woisetschlaeger, M., C. N. Yandava, L. A. Furmanski, J. L. Strominger, and S. H. Speck.** 1990. Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proc. Natl. Acad. Sci. USA **87:**1725–1729.
37. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:** 3806–3810.
38. **Yssel, H., J. E. DeVries, M. Koken, W. VanBlitterswijk, and H. Spits.** 1984. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J. Immunol. Methods **72:**219–227.
39. **Zimber-Strobl, U., L. J. Strobl, C. Meitinger, R. Hinrichs, T. Sakai, T. Furukawa, T. Honjo, and G. W. Bornkamm.** 1994. Epstein-Barr virus nuclear antigen 2 exerts its *trans*activating function through interaction with recombination signal binding protein RBP-Jκ, the homologue of *Drosophila suppressor of hairless*. EMBO J. **13:**4973–4982.