JOURNAL OF VIROLOGY, Aug. 1995, p. 5039–5047 Vol. 69, No. 8
0022-538X/95/$04.00+0

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

BRIAN C. SCHAEFER,[1] JACK L. STROMINGER,[1] AND SAMUEL H. SPECK[2]*

Division of Tumor Virology, Dana-Farber Cancer Institute, Boston, Massachusetts 02216,[1] and Departments of Pathology and Molecular Biology, Center for Immunology and Division of Molecular Oncology, Washington University School of Medicine, St. Louis, Missouri 63110[2]

Received 11 January 1995/Accepted 5 May 1995

The Epstein-Barr virus *Bam*HI F promoter (Fp) was previously identified as the putative EBNA 1 gene promoter in group 1 Burkitt's lymphoma (BL) cell lines. Fp has also been shown to be activated in Epstein-Barr virus-positive B-cell lines following induction of the viral productive cycle (A. L. Lear, M. Rowe, M. G. Kurilla, S. Lee, S. Henderson, E. Kieff, and A. B. Rickinson, J. Virol. 66:7461–7468, 1992). Here we demonstrate that Fp is exclusively a lytic promoter which was incorrectly identified as the EBNA 1 gene promoter in group 1 BL cell lines. It is shown that while Fp activity was observed in two group 1 BL cell lines, it could not be detected in a third group 1 BL cell line. Furthermore, the level of Fp activity detected in both group 1 and group 3 cell lines appeared to correlate only with the level of spontaneous lytic activity. Induction of the lytic cycle in group 1 or group 3 BL cell lines resulted in a dramatic increase in Fp-initiated transcripts but no detectable increase in EBNA 1 transcripts. Anti-immunoglobulin induction of the lytic cycle in the Akata group 1 BL cell line revealed that induction of Fp activity was detectable by 2 to 4 h after induction of the lytic cycle and was dependent on de novo protein synthesis. In addition, Fp reporter constructs transiently transfected into group 1 BL cell lines exhibited activity which was independent of the Fp initiation site, TATAA box, or other upstream sequences. The sequences required for efficient reporter gene activity mapped to a region ca. 210 bp downstream of the Fp cap site. Furthermore, Northern (RNA) blot analyses indicated that there are two Fp-initiated lytic transcripts between 9 and 15 kb in size, neither of which correspond to the known EBNA 1 transcripts present in group 1 BL cell lines.

Epstein-Barr virus is a gammaherpesvirus which is capable of adopting at least three distinct programs of latency (latency types 1, 2, and 3) (10, 16) in which different combinations of latent viral genes are expressed. The factors which determine what form of latency the virus adopts in a given cell (or cell population) are not yet understood, but a great deal of information concerning the transcription patterns which characterize the various programs of latency has been gathered. The type 3 latency program is characteristic of in vitro-established lymphoblastoid cell lines (LCLs) and has been the most thoroughly studied. In type 3 latency, six nuclear antigens (EBNA 1, 2, 3a, 3b, 3c, and 4) are expressed from one of two viral promoters, Cp and Wp, located near the left-hand end of the viral genome (2, 20, 25, 26). In contrast, in type 1 and 2 latency, the expression of nuclear antigens is restricted to EBNA 1 (14, 15, 17), and neither Cp nor Wp is transcriptionally active (19, 22).

Previously, a putative promoter located within the viral *Bam*HI F fragment (Fp) was identified as a candidate EBNA 1 gene promoter active during type 1 (19, 22) and type 2 (24) latency. However, in our initial report identifying Fp (22), reservations were expressed concerning this assignment because we could not detect Fp activity by S1 nuclease protection analysis of RNA prepared from the group 1 Burkitt's lymphoma (BL) cell line Rael (group 1 BL cell lines exhibit type 1 latency, whereas group 3 BL cell lines have drifted to the type 3 latency phenotype). In a report by Lear et al. (12), Fp was shown to be activated during the lytic cycle, suggesting that Fp is a lytic promoter which may have been misidentified as the EBNA 1 gene promoter in group 1 BL cell lines. However, these investigators did not address the question of whether Fp is also involved in driving EBNA 1 gene transcription during type 1 or type 2 latency.

A previous characterization of elements regulating transcription from Fp reporter constructs reported that the major positive element influencing Fp activity is located in an unusual position, downstream of the Fp initiation site (between bp +25 and +280) (20). However, no evidence regarding the exact site of transcription initiation in these constructs was presented. Thus, the possibility that the downstream positive element acts as a promoter distinct from Fp was not addressed.

In this report, we present data which demonstrate that Fp activity does not correlate with transcription of the EBNA 1 coding exon but rather appears to correlate only with activation of the viral lytic cycle. It is also shown that Fp is activated in a protein synthesis-dependent fashion with the kinetics of an early lytic promoter. Additionally, transcriptional activity of transiently transfected reporter constructs does not require the Fp initiation site or immediately surrounding sequences but instead maps to a region 210 bp downstream of the Fp start site. As will be reported elsewhere, we have identified the bona fide EBNA 1 gene promoter in group 1 BL cell lines, which maps to this region at ca. +210 bp relative to Fp transcription initiation site. Finally, we present results of Northern (RNA) hybridization analyses which suggest that Fp is responsible for

* Corresponding author. Phone: (315) 362-0367. Fax: (315) 362-4096. Electronic mail address: speck@pathology.wustl.edu.

TABLE 1. Oligonucleotides used for S1 nuclease protection analyses

Name	Sequence
Cp	5′-TCTTCGGTGTCCTTGTCTCTATGCCATCTGATCTAAAATTTGCAGCAGAAC-3′
Fp	5′-CTAGTGGTCCCCTCCGGATCCCCCTCCTCTCTATCCACCGCCGCCCCGGAACAGGACCTG-3′
Zp	5′-AGCAAAGATAGCAAAGGTGGCCGGCAAGGTGCAATGTTTAGGAGTTACCTGTCT-3′
K exon	5′-CCAGGTCCTGTACCTGGCCCCTCGTCAGACATGATTCACACTAAAAGAGATCAATAGACA-3′
U exon	5′-GCTCATCCCAGGGAAGCTCAAGAAACGCTTCCTAAGTTACCTGCAGAAATTGAAGAATAA-3′
BHLF1p	5′-GCTGGGAGGTGTGCACCCCCCGAGCGTCTGGACGAGCTGGCGAGCCGGGCCGGCTCGCC-3′

the lytic cycle transcription of two messages ca. 9 to 15 kb in size which contain the previously described FQ and U exons (19, 22, 24) but not EBNA 1 coding-exon sequences.

MATERIALS AND METHODS

Cell lines and tissue culture. The group 3 BL cell lines Jijoye, clone-14, Raji, and Daudi, the marmoset cell line B95.8, and the LCLs X50-7, JY, JC5, IB4, and AK292 have been described and characterized previously (29, 30). DG75 and Ramos are EBV-negative BL cell lines. Rael (11) and Akata (27) are group 1 BL cell lines. Mutu group 1 and Mutu group 3 are group 1 and group 3, respectively, BL cell lines established from the same BL lymphoma tumor (9). All cell lines were propagated in RPMI 1740 supplemented with 10% fetal bovine serum.

Induction of the viral lytic cycle. Induction of the viral lytic cycle (asynchronous induction) in the clone-13 cell line was performed by the addition of TPA (12-*O*-tetradecanoylphorbol-13-acetate; Sigma) to a concentration of 21 ng/ml. Cells were harvested at the indicated hours after addition of TPA. Synchronous induction of the viral lytic cycle in the Akata cell line was performed by the addition of 1% anti-immunoglobulin G (Cappel) to the cell culture medium (6, 28). Cells were harvested at the indicated time points following anti-immunoglobulin G addition, immediately frozen on dry ice, and stored at −70°C until RNA was prepared. Protein synthesis was inhibited by the addition (at $t = 0$ h) of cycloheximide to a final concentration of 40 μM and anisomycin to a final concentration of 10 μM.

Generation of plasmids. The −715/+277, −129/+277, and +21/+277 Fp fragments were released from previously generated Bluescript-KS+ constructs by *Sac*I-*Hin*dIII digestion (−715, −129, and +21 correspond to *Bam*HI F coordinates 6664 [*Nae*I], 7260 [*Xho*I], and 7396 [*Bam*HI], respectively; +277 corresponds to *Bam*HI Q coordinate 259 [*Pvu*II]). These fragments were then ligated into the *Sac*I-*Hin*dIII sites of the pGL2-CAT vector (modified pGL2 basic vector [Promega] in which the luciferase reporter gene was replaced with the chloramphenicol acetyltransferase [CAT] reporter gene) (7). To generate the −129 Fp 5′ deletion mutants, the sequences from +21 to +277 were removed from the −129/+277 FpCAT construct by *Bam*HI-*Hin*dIII digestion and replaced by the +21/+237, +21/+218, or +21/+195 fragment. These fragments were generated by PCR amplification with sequence-specific primers which incorporated the natural +21 *Bam*HI site (5′ end) and appended a *Hin*dIII site (3′ end).

Electroporations and CAT assays. Cells were harvested, washed once in unsupplemented RPMI 1740 medium, and resuspended at a density of 4×10^6 cells per ml in unsupplemented RPMI 1740. Fifteen micrograms of reporter plasmid was added to 0.26 ml of cells in 0.4-cm-gap electroporation cuvettes. Cuvettes were then chilled on ice for 5 to 10 min. Cells were electroporated in a Bio-Rad Gene Pulser at 960 μF and 240 V. Cuvettes were returned to ice after electroporation, and 0.7 ml of ice-cold phosphate-buffered saline was added to each cuvette immediately after all electroporations were completed. Samples were then transferred to 26-cm^2 flasks containing 10 ml of the appropriate prewarmed media. Transfectants were then shaken vigorously several times to break up aggregates of cell debris and surviving cells and were placed in a 37°C, 5% CO_2 incubator for 40 to 48 h. CAT assays were performed as previously described (8).

Preparation of RNA and S1 nuclease analyses. Cytoplasmic RNA was prepared by the method of Favaloro et al. (5), and polyadenylated RNA was purified on an oligo(dT)-cellulose column as previously described (1). Total RNA was isolated from cells via the single-step method (3), using either Tri-Reagent (Molecular Research Center) or guanidium isothiocyanate-phenol prepared by the method of Chomczynski and Sacchi (3). RNA was then treated with RQ1 DNase (Promega) as instructed by the manufacturer.

Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP by using established protocols (18). The sequences of the oligonucleotide probes used are given in Table 1. Kinase-treated oligonucleotides were hybridized overnight to RNA samples, digested with S1 nuclease, and analyzed by electrophoresis on denaturing polyacrylamide gels as previously described (29).

Northern blot analysis and probe generation. Electrophoretic separation of RNA and Northern blotting were performed as previously described (22). The FQ exon probe was prepared by unidirectional PCR amplification of bases 1 to 193 of the FQ exon, using 21 pmol of the ^{32}P-end-labeled oligonucleotide 5′-GGCCAAGCTTTTTCGTTTTCGCAAAGCG-3′ and 0.26 pmol of the previously described FQ exon-containing RACE (rapid amplification of cDNA ends) PCR clone XPQF2 (22). PCR amplification was performed in the presence [α-^{32}P]dGTP, [α-^{32}P]dCTP, and unlabeled dATP and dTTP. The U exon probe was prepared by random-primed labeling ([^{32}P]dGTP and [^{32}P]dCTP) of bases 1 to 1831 (*Bam*HI to *Cla*I) of the EBV *Bam*HI U fragment, which includes the entire U exon (*Bam*HI-U bases 1454 to 1629). The probe for *Bam*HI U sequences downstream of the U exon was prepared by random-primed labeling of bases 1831 to 3299 (*Cla*I to *Bam*HI), which does not include any U exon sequences. Conditions for hybridization and washing were as previously described (22).

RESULTS

S1 nuclease protection analysis of Fp activity in a panel of cell lines reveals lack of correlation between group 1 phenotype and transcription initiation from Fp. In previously published work, we (22) and others (19) identified a putative promoter for the EBNA 1 transcript in group 1 BL cell lines near the *Bam*HI FQ junction of the EBV genome (Fig. 1). To further characterize the putative Fp and confirm that Fp has the properties expected of a group 1 BL EBNA 1 gene promoter, we performed an S1 nuclease protection analysis using total RNA from a panel of EBV-positive and EBV-negative cell lines and an oligonucleotide probe spanning the initiation site identified by RACE cloning (Fig. 2A). Surprisingly, we found that the pattern of cell lines positive for Fp usage did not segregate with the group 1 BL phenotype. Of the two group 1 BL cell lines in this panel, Rael and Akata, only Akata exhibited detectable Fp activity. In addition, the other cell lines in which Fp-initiated transcripts were detectable (clone-13, Daudi, JY, and B95.8) all exhibited spontaneous lytic cycle activity. In contrast, the cell lines which lacked detectable Fp activity (X50-7, Rael, Jijoye, IB4, and AK292) are known to produce negligible levels of virus. Ramos is an EBV-negative BL cell line which was included as a negative control. Thus, rather than segregating with the group 1 phenotype, Fp activity appeared to correlate with the phenotype of spontaneous lytic activity.

This observation is in agreement with the published findings of Lear et al. (12), who used reverse transcriptase-mediated PCR analysis to demonstrate that the putative splicing pattern for the group 1 BL EBNA 1 transcript (FQ/U/K) was detectable in cell lines which spontaneously produce virus, as well as group 1 BL cell lines, but was undetectable in tightly latent group 3 cell lines. These investigators also showed by reverse transcriptase-mediated PCR that the FQ/U/K splicing pattern could be induced in the tightly latent cell line X50-7 by ectopic expression of the EBV lytic transactivator Zta, which is known to be sufficient to trigger the EBV lytic cycle (4). Their conclusion based on these results was that Fp is not only a promoter for EBNA 1 gene transcription during the restricted latency program in group 1 BL cell lines but also a promoter for the EBNA 1 gene which is induced when EBV-infected cells become committed to the lytic cycle.

On the basis of the S1 analysis presented in Fig. 2A, the lack of detectable Fp activity in the Rael group 1 BL cell line raised concern over the assignment of Fp as the EBNA 1 gene promoter in this cell line. To more rigorously examine this ques-

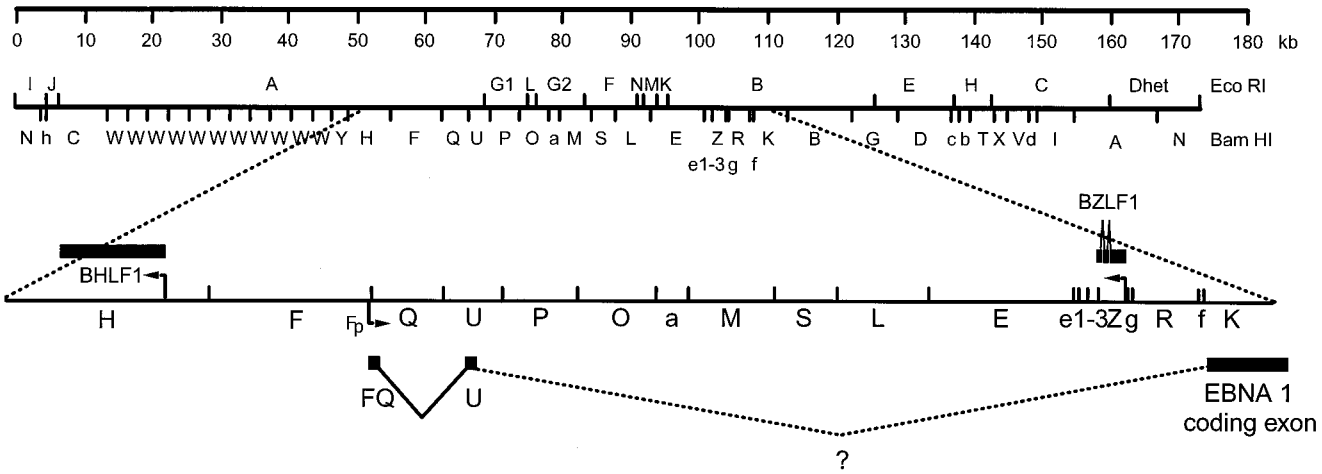

FIG. 1. Schematic illustration of the EBV genome, the putative structure of the type 1 latency EBNA 1 gene transcript, and the locations and exon structures of two viral genes associated with the lytic cycle, the immediate early BZLF1 gene and the early BHLF1 gene. Thick horizontal lines above and below the expanded portion of the viral genome map represent exons. In addition, Fp, BZLF1p, and BHLF1p are indicated. Splicing from the FQ exon to the U exon and from the U exon to the EBNA 1 coding exon (K exon) in the type 1 latency EBNA 1 gene transcript has previously been described (19, 22). The splice between the U exon and K exon in Fp-initiated transcripts is shown with a dotted line, since data presented in this report indicate that the vast majority of Fp-initiated transcripts do not splice to the EBNA 1 coding exon.

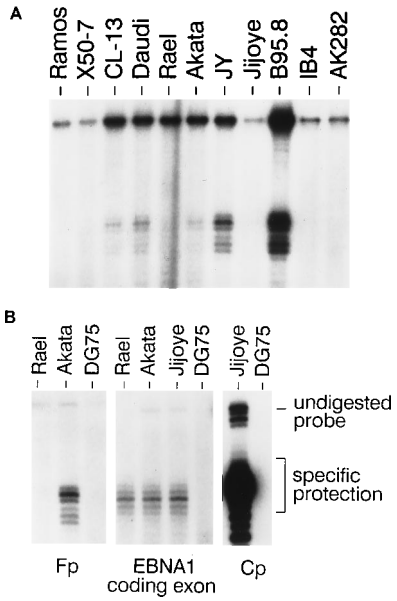

FIG. 2. Fp-initiated transcripts are present in EBV-infected cell lines exhibiting spontaneous lytic activity but absent in the tightly latent group 1 BL cell line Rael. (A) S1 nuclease protection analysis of Fp-initiated transcripts, using 26 μg of total RNA prepared from a panel of EBV-positive and -negative B-cell lines. Ramos is an EBV-negative BL cell line. Akata and Rael are group 1 BL cell lines. Clone-13, Daudi, and Jijoye are group 3 BL cell lines. AK292, IB4, JY, and X50-7 are in vitro-immortalized LCLs. The top band present in all lanes represents undigested probe, while the faster-migrating set of bands represent specific protection of the probe by Fp-initiated transcripts. The sequence of the oligonucleotide probe used in this analysis is given in Table 1. (B) S1 nuclease protection of oligonucleotide probes specific for transcription initiation from Fp or Cp or for the presence of the EBNA 1 coding exon. Polyadenylated RNA prepared from the group 1 BL cell lines Akata and Rael cell lines (10 μg) was compared with RNA prepared from the EBV-negative BL cell line DG75 and the group 3 BL cell line Jijoye. The Fp and Cp probes spanned the respective transcription initiation sites, while the EBNA 1 coding-exon probe spanned the BKRF1 splice acceptor site. The sequences of the probes used are given in Table 1.

tion, an S1 nuclease protection analysis using poly(A)-selected RNA from several cell lines was performed. In this analysis, the levels of Fp-initiated transcripts and EBNA 1 coding-exon-containing transcripts were examined in parallel, using equivalently labeled probes, which afforded a quantitative assessment of the relative abundances of Fp-initiated transcripts and EBNA 1 transcripts (Fig. 2B). The results confirmed that Fp activity could readily be detected in the group 1 cell line Akata but could not be detected in the group 1 cell line Rael and the EBV-negative control DG75. Analysis of the abundance of transcripts containing the EBNA 1 coding exon revealed that there were comparable levels of EBNA 1 transcripts in the group 1 Rael and Akata cell lines as well as in the group 3 BL cell line Jijoye. Therefore, from this limited analysis, it did not appear that Fp activity could account for EBNA 1 gene transcription in the Rael cell line, raising the possibility that the Akata cell line drives EBNA 1 gene transcription from Fp whereas the Rael cell line employs another promoter. However, given the similarities in the sizes of EBNA 1 transcripts produced in these cell lines (22), it seems more likely that they both employ the same promoter for driving expression of EBNA 1. If the latter is true, then the S1 nuclease protection data presented in Fig. 1 indicate that Fp is not the type 1 latency EBNA 1 gene promoter.

Fp activity, but not EBNA 1 gene transcription, is induced upon triggering of the viral lytic cycle. To examine the relationship, if any, between Fp-initiated transcription and transcription of the EBNA 1 coding exon, we stimulated clone-13 cells with TPA to trigger the lytic cycle. Samples were collected at 0, 25, and 48 h postinduction, and RNA was purified from these cells. S1 nuclease protection analyses of transcription initiation from the early lytic BHLF1 promoter (BHLFp; location shown in Fig. 1) demonstrated that the lytic cycle was efficiently activated by TPA in these cells (Fig. 3A). In parallel with BHLF1 activation, Fp-initiated transcription increased from undetectable levels at the 0-h time point to an easily detectable level at 25 and 48 h postinduction. In marked contrast to the substantial increase in Fp activity, the levels of transcripts containing the EBNA 1 coding exon remained unchanged throughout the time course. These findings demon-

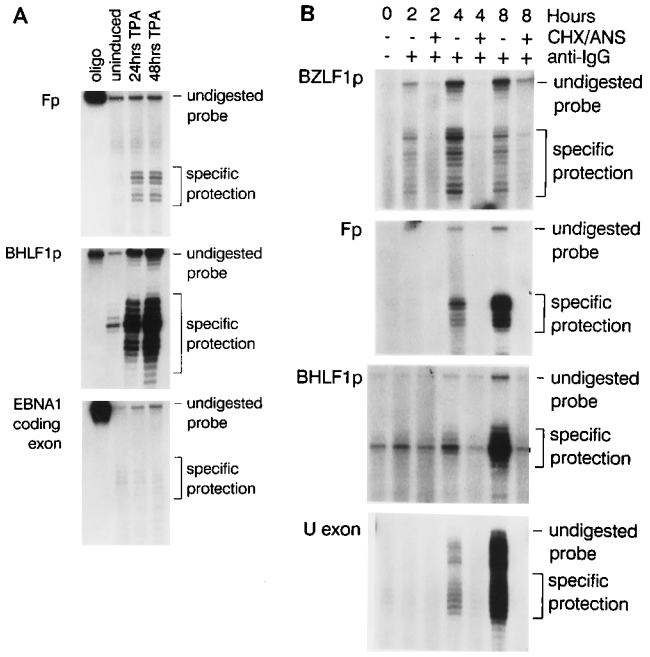

FIG. 3. Induction of the viral lytic cycle in group 1 or group 3 BL cell lines results in a dramatic increase in Fp-initiated transcripts but no detectable increase in EBNA 1 transcripts. (A) TPA induction of the viral lytic cycle in the clone-13 cell line. Total RNA isolated from uninduced and TPA-induced clone-13 cells was prepared at the indicated times after addition of TPA. The level of Fp-initiated transcripts was compared with the levels of EBNA 1 transcripts and the early lytic BHLF1 transcript. Twenty-six micrograms of total RNA was used for each protection assay, except that for the analysis of BHLF1 transcripts, only 10 μg of total RNA was used. (B) Anti-immunoglobulin induction of the viral lytic cycle in the group 1 BL cell line Akata in the presence and absence of the protein synthesis inhibitors cycloheximide (CHX) and anisomycin (ANS). Induction of Fp activity was compared with induction of the immediate-early BZLF1 gene and the early lytic BHLF1 gene. In addition, the levels of transcripts containing the U exon and the EBNA 1 coding exon were monitored. Oligonucleotide probes spanning the transcription initiation sites were used to monitor Fp, BHLF1p, and BZLF1p activities, while oligonucleotide probes spanning the splice acceptor site for the U exon and the EBNA 1 coding exon were used to assess the levels of the U exon and EBNA 1 coding-exon-containing transcripts. The sequences of the oligonucleotide probes used are given in Table 1. Twenty-six micrograms of total RNA was used for each protection assay, except that for the analysis of BHLF1 transcripts, only 10 μg of total RNA was used. IgG, immunoglobulin G.

strated that transcription from Fp increases markedly when the lytic cycle is triggered by TPA in clone-13 cells and that there is no correlation between the level of transcript initiated from Fp and the level of transcripts containing the EBNA 1 coding exon.

To confirm the findings for TPA-stimulated clone-13 cells and to better characterize the kinetics of Fp activation during the lytic cycle, the Akata group 1 BL cell line was synchronously induced into the lytic cycle by cross-linking surface immunoglobulin in the presence and absence of the protein synthesis inhibitors anisomycin and cycloheximide. Samples were collected at 0, 2, 4, and 8 h postinduction, and total RNA was purified from the harvested cells. Activation of the lytic cycle was monitored by measuring BZLF1 promoter (BZLF1p) and BHLF1p activities, using specific oligonucleotide probes in an S1 nuclease protection analysis (Fig. 3B). As has been previously described (6, 28), BZLF1 transcription was first apparent at 2 h, peaked at 4 h, and was less apparent by 8 h postinduction (the location of the BZLF1 gene is shown in Fig. 1). Induction of BHLF1 transcription was absent at 2 h, but a very strong signal was apparent at 4 h which became stronger by 8 h postinduction.

Induction of Fp-initiated transcription followed the same kinetics as induction of the early lytic BHLF1p, becoming activated to initially detectable levels at 4 h and increasing to much higher levels by 8 h postinduction (Fig. 3B). Concordant with the induction of transcription from Fp, transcripts containing the U exon were induced to similar levels, strongly suggesting that a large proportion of Fp-initiated transcripts contain the U exon (Fig. 3B). In contrast, a weak but constant level of transcripts containing the EBNA 1 coding exon was detected (data not shown), in agreement with data obtained from the clone-13 cell line. This experiment confirmed that there was no measurable correlation between Fp-initiated transcription and transcription of the EBNA 1 coding exon.

Transcription from Fp, as well as from Zp and BHLF1p, was sensitive to protein synthesis inhibitors, indicating that activation of Fp is dependent on synthesis of new proteins following initiation of the lytic cascade. The kinetics of Fp induction indicated that Fp has the characteristics of an early lytic promoter which is activated between 2 and 4 h following induction of the viral lytic cycle. In addition, although there was a correlation between transcription from Fp and transcription of the *Bam*HI U exon, the EBNA 1 coding exon was not transcribed from Fp at a detectable frequency.

The lack of correlation between the induction of Fp activity and transcription of the EBNA 1 coding exon was further underscored by analysis of Rael cells treated with 5-azacytidine, an agent that induces to various degrees demethylation of the EBV genome in group 1 BL cell lines and which we have previously shown will trigger the lytic cycle in the Rael cell line (22). In the experiment shown in Fig. 4, treatment of Rael cells with 5-azacytidine was not sufficient to trigger the shift from group 1 to group 3 latency but was sufficient to induce the viral lytic cycle. Northern analyses of RNA prepared from 5-azacytidine-treated Rael and Mutu group 3 cells, probed with the EBNA 1 coding exon, revealed the typical pattern of EBNA 1 transcripts for group 1 and group 3 cell lines, respectively (Fig. 4A) (22). In addition, this pattern did not change over the time course. Furthermore, as shown in the bottom panel of Fig. 4B, 5-azacytidine treatment of Rael cells in this case did not result in the induction of Cp activity. However, S1 nuclease probes specific for transcription initiation from Fp and BHLF1p indicated strong induction of the lytic cycle detectable by 28.5 h after addition of 5-azacyticine (Fig. 4B). In contrast to the induction of transcription from Fp, analysis of the abundance of transcripts containing the EBNA 1 coding exon failed to show any significant induction in the level of EBNA 1 transcripts. Thus, as was observed in both the Akata and clone-13 cell lines, induction of Fp activity did not result in a detectable increase in transcription of the EBNA 1 coding exon. Furthermore, the demonstration of inducible Fp activity in the Rael cell line indicates that the lack of detectable Fp activity in Rael cells in the absence of induction of the lytic cycle is unlikely to be due to deletion or mutation of this promoter. Thus, these results are consistent with the hypothesis that Fp is not active during group 1 latency.

To demonstrate that the foregoing findings regarding Fp activity in cell lines driven into the lytic cycle by exogenous stimuli could be generalized to untreated EBV-positive cell lines, poly(A)-selected mRNA from three group 1 BL cell lines, Akata, Rael, and Mutu group 1, as well as the marmoset line B95.8, was analyzed for Fp activity and early lytic gene expression. Oligonucleotide probes for Fp, BHLF1p, *Bam*HI U exon, and K (EBNA 1 coding) exon were used in an S1 nuclease analysis (Fig. 5). Akata, Mutu group 1, and B95.8 are

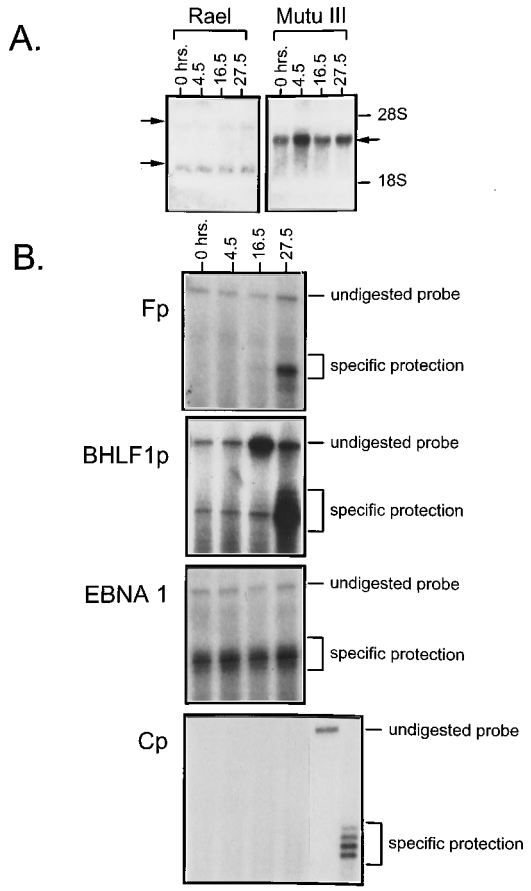

FIG. 4. Induction of the lytic cycle by 5-azacytidine in the Rael cell line results in an induction of Fp-initiated transcripts but does not always result in an increase in the abundance of transcripts containing the EBNA 1 coding exon. The experiment shown is an example of 5-azacytidine treatment which resulted in the induction of the viral lytic cycle in the Rael cell line but did not result in a shift from the group 1 to group 3 pattern of latent viral gene expression. (A) Northern analyses of RNA prepared from Rael and Mutu group 3 (Mutu III) cell lines at various times after addition of 5-azacytidine to the culture media. The blots were probed with a fragment of the EBV genome containing the EBNA 1 coding exon. The positions of migration of 18 and 28S rRNAs are indicated. Two transcripts were detected with RNA from the Rael cell line, consistent with previous observations of the sizes of the EBNA 1 transcripts in group 1 BL cell lines (22). In contrast, in the Mutu group 3 BL cell line, a single intensely hybridizing species of approximately 3.7 kb was observed. (B) S1 nuclease protection analyses of transcription initiation from Fp, BHLF1p and Cp and the abundance of transcripts containing the EBNA 1 coding exon. Protection analyses were carried out as described in Materials and Methods and in the legend to Fig. 3. In the Cp protection analysis, the two rightmost lanes represent undigested probe and positive control RNA from Cp, using the LCL JY.

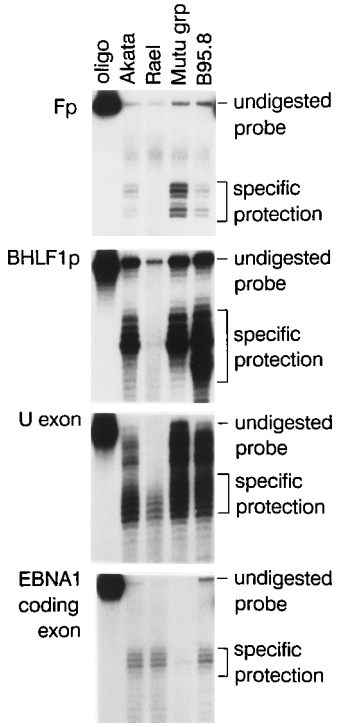

FIG. 5. The abundance of Fp-initiated transcripts correlates with the level of spontaneous lytic cycle activity and not with the level of transcripts containing the EBNA 1 coding exon in group 1 BL cell lines. The abundance of Fp-initiated transcripts was compared with the abundance of BHLF1 early lytic transcripts as well as with the abundance of U exon and EBNA 1 coding-exon-containing transcripts. The marmoset LCL B95.8, which is a producer cell line, is shown as a positive control. Ten micrograms of polyadenylated RNA was used in each protection assay.

cell lines which are known to exhibit significant spontaneous lytic cycle activity, whereas Rael is tightly latent. Analogous to the results obtained from exogenous triggering of lytic activity, the S1 nuclease protection analyses clearly demonstrated that Fp activity was seen only in producer cell lines (BHLF1p positive) and, most importantly, was not observed in the tightly latent group 1 BL cell line Rael. Increased levels of U exon protection observed in those cell lines exhibiting Fp activity suggested, as above (Fig. 3B), that a large proportion of Fp-initiated transcripts contain the *Bam*HI U exon. However, as shown in Fig. 3A and 4B, no correlation was observed between transcription from Fp and transcription of the EBNA 1 coding exon.

Northern blot analysis identifies two large transcripts containing FQ and U exon sequences which are candidates for lytic transcripts initiated from Fp. To obtain preliminary data regarding the structure of the transcript(s) initiated from Fp, a Northern blot of poly(A)-selected RNA from the Akata cell line was probed with a ^{32}P-labeled single-stranded DNA fragment antisense to the FQ exon. This probe was prepared by unidirectional (single-primer) PCR amplification of a double-stranded FQ exon DNA fragment in the presence of ^{32}P-labeled nucleotides. This Northern analysis detected two large transcripts with sizes in the range of 9 to 15 kb (Fig. 6A), as estimated by their migration relative to 19S and 29S rRNA species. Since S1 nuclease analysis suggested that Fp-initiated transcripts are frequently spliced from the FQ exon to the U exon (Fig. 3B and 5; illustrated in Fig. 1), a second Northern blot of poly(A)-selected RNA prepared from the cell lines Rael, Akata, and X50-7 was probed with a 1.7-kb ^{32}P-labeled DNA fragment containing the *Bam*HI U exon. A pattern of hybridization in the Akata lane identical to that observed with the FQ exon probe was seen, whereas no hybridizing species were detected in the Rael and X50-7 samples (Fig. 6A). Despite numerous attempts, we have never detected EBNA 1 transcripts in any EBV-positive cell line by using a *Bam*HI U exon probe, even though most or all EBNA 1 transcripts contain the U exon. Analysis of possible secondary structure at the 5′ end of the EBNA 1 transcript indicated that the U exon may be involved in a very stable secondary structure, which may substantially reduce hybridization to DNA probes spanning this region. In addition, it should be noted that latency-associated transcripts containing the U exon are much less abundant than Fp-initiated transcripts which contain the U exon (Fig. 3B and 5). Thus, among these three cell lines, Akata was

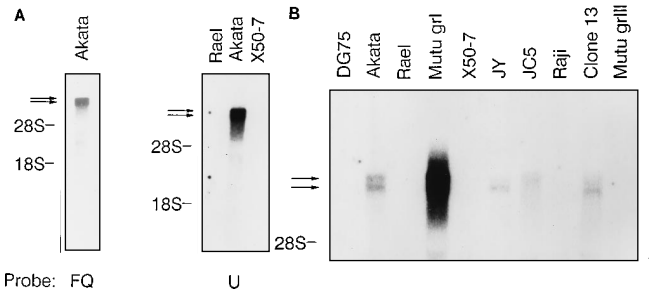

FIG. 6. Northern blot analysis of FQ exon-containing transcripts identifies two large transcripts ca. 9 to 15 kb in size which also appear to contain the U exon. (A) Ten micrograms of polyadenylated RNA from the Akata cell line was hybridized with single-stranded probes antisense to the FQ exon or the U exon. As a control for detection of latency-associated transcripts containing the U exon, polyadenylated RNA prepared from the tightly latent Rael group 1 BL cell line and the tightly latent LCL X50-7 were included. (B) Ten micrograms of polyadenylated RNA from a panel of cell lines was hybridized with a probe from the viral *Bam*HI U region of the genome downstream of the U exon. DG75 is an EBV-negative BL cell line; Akata, Rael, and Mutu group 1 (Mutu grI) are group 1 BL cell lines; clone-13, Mutu group 3 (Mutu grIII) and Raji are group 3 BL cell lines; X50-7, JY, and JC5 are in vitro-established LCLs.

positive for U exon hybridization and Fp usage, whereas the other two cell lines were negative for both (Fig. 2 and 6A).

To rule out the possibility that the transcripts detected originated from the oriLyt region of the genome, a Northern blot of Akata and Rael poly(A) RNA was hybridized with a ^{32}P-labeled DNA probe encompassing the entire *Bam*HI H fragment. A transcript corresponding in size to the BHLF1 mRNA was easily detected in the Akata sample, but no species near the size of the putative Fp-initiated messages was observed (data not shown). In addition, no hybridizing species were observed with RNA from the tightly latent group 1 Rael cell line, as anticipated (data not shown).

Both Northern hybridization with an EBNA 1 coding-exon probe (unpublished data) and S1 nuclease analyses indicated that Fp-initiated transcripts do not splice from the U exon to the K exon, which suggested that usage of the U exon splice donor might be suppressed during the lytic cycle when transcripts are initiated from Fp. To test this hypothesis, a Northern blot analysis of poly(A)-selected RNA was performed with RNA prepared from a panel of cell lines and a ^{32}P-labeled DNA probe derived from *Bam*HI U sequences downstream of the U exon. Again an identical hybridization pattern in the Akata RNA sample was observed, and this same pattern was also seen in the Mutu group 1, JY, and clone-13 samples (Fig. 6B). No hybridizing species were detected in the DG75, Rael, X50-7, Raji, and Mutu group 3 samples, and a weak and somewhat different pattern of hybridization was observed with RNA prepared from the LCL JC5 (Fig. 6B). The cell lines in which the hybridizing doublet was detected (Akata, Mutu group 1, JY, and clone-13) are also the only cell lines included in the Northern analysis which show evidence of Fp activity (Fig. 2), again suggesting that the two hybridizing transcripts are initiated from Fp. The data in Fig. 6B also indicate either that these two Fp transcripts do not splice at the normal U exon splice donor site or that the U exon is spliced to a previously undescribed exon downstream of the U exon in the *Bam*HI U fragment. We are currently screening cDNA libraries to further elucidate the structure of Fp-initiated transcripts.

Deletion analysis of Fp reporter constructs identifies downstream sequences as essential for reporter gene activity and does not show a clear dependence on Fp elements for reporter gene activity in EBV-negative and group 1 BL cell lines. The studies of Sample et al. (20) demonstrated that the activity of Fp reporter constructs was entirely dependent on elements located downstream of the Fp initiation site, between bp +25 and +280. Because the data presented above raised concerns about the assignment of Fp as the EBNA 1 gene promoter during type 1 latency, a series of deletion constructs was generated in an effort to begin to define the minimal elements required for FQ region reporter activity. The activities of these reporter constructs were assessed in two group 1 BL cell lines and the EBV-negative BL cell line DG75 (Fig. 7B). Deletions from the 3′ end confirmed the findings of Sample et al. (20), who observed that the EBNA 1 binding sites downstream of Fp exert a strong negative effect on reporter construct activity. As shown in Fig. 7B, deletion of sequences between bp +218 and +277, which removed both EBNA 1 binding sites (see Fig. 7A for structures of reporter constructs), resulted in a 21-fold or greater increase in reporter gene activity in the Mutu group 1 and Rael cell lines (compare activities of −129/+277 and −129/+218 reporter constructs). In our hands, this effect was always much smaller when activity was assessed in the Akata group 1 BL cell line (data not shown). However, this may have been due to loss of EBV genomes in Akata cells (and thus EBNA 1 gene expression), a phenomenon which has recently been reported to occur spontaneously during passage of this cell line (23). Notably, the −715/+277 and −129/+277 reporter constructs exhibit significant activity in the EBV-negative BL cell line DG75, consistent with the hypothesis that repression is mediated by EBNA 1 binding.

The majority of the repressive effect of the EBNA 1 binding sites was relieved when only the downstream EBNA 1 binding site was deleted (compare activities of −129/+237 and −129/+277), suggesting either that both binding sites are required to mediate repression or that the downstream binding site mediates the bulk of the repressive effect. It should be noted, however, the FQ exon splice donor site also maps to the region between +237 and +277; therefore, some of the increase in activity observed by this deletion may reflect alterations in RNA processing of the reporter gene transcript. This possibility is consistent with the observed increase in activity in the EBV-negative DG75 cell line when these sequences are deleted. In addition, it should be noted that deletion of the sequences from bp +237 to +218 resulted in a drop in activity in the DG75 cell line, suggesting that there may be a positive *cis* element in this region. Further deletion of sequences between bp +195 and +218 resulted in nearly complete abolishment of activity. Thus, it appears that there is an element absolutely essential for FQ region reporter activity which maps to the region between bp +195 and +218. The reporter constructs which contained only downstream sequences to +21 exhibited little activity above the basal activity of the parent reporter vector. The exception to this was the −715/+21 reporter construct in the Rael cell line, which could reflect a low level of transcription from Fp. However, we have been unable with an analogous β-globin reporter construct transiently transfected into the Rael cell line to demonstrate specific transcription initiation from Fp (data not shown).

Deletion of the Fp TATA box and transcription initiation site from the reporter constructs had no negative impact on the activities observed in the EBV-negative DG75 cell line (compare activities of −129/+237 and +21/+237 and activities of −129/+218 and +21/+218). In the group 1 BL cell lines, deletion of the sequences from −129 to +21 also had only a minimal impact on activity in the context of downstream sequences to +237. However, in the context of downstream sequences to +218, this same deletion had a pronounced effect. In the case of Rael, this deletion reduced activity about two-

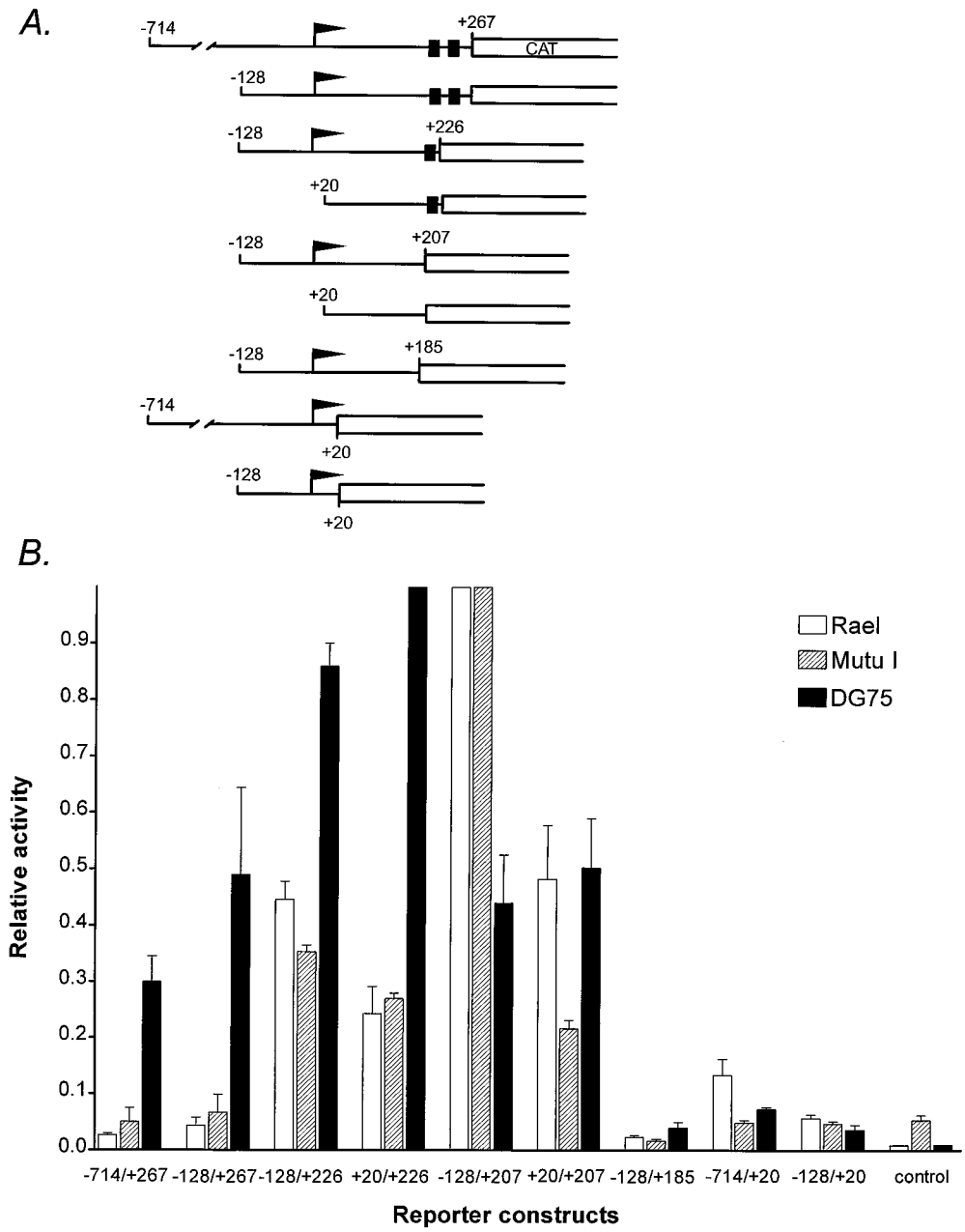

FIG. 7. Negative regulation of reporter gene activity by the low-affinity EBNA 1 binding sites and lack of dependence of activity on the presence of the Fp TATAA box, Fp transcription initiation site, or sequences upstream of Fp. (A) Structure of Fp-containing reporter constructs. The location of Fp is indicated, and the two low-affinity EBNA 1 binding sites are indicated by filled rectangles. The viral sequences included in the reporter constructs are indicated relative to the Fp transcription initiation site. (B) Analysis of Fp-CAT reporter constructs containing various amounts of upstream and downstream sequences in two group 1 BL cell lines and in the EBV-negative BL cell line DG75. Activities are expressed relative to that of the most active reporter construct. The activity of the parent reporter vector (pGLCAT) is also shown for reference (control).

fold, while in the Mutu group 1 cell line, this same deletion reduced activity about fourfold. We have repeatedly failed to demonstrate specific transcription initiation from Fp by nuclease protection using analogous β-globin reporter constructs transiently transfected in these cell lines (data not shown). Indeed, as will be reported elsewhere, it appears that transcription initiation maps not to the previously defined Fp initiation site but to the region identified by the 3′ deletions as being essential for reporter gene activity (+195 to +218 relative to the Fp transcription initiation site). This observation is also supported by data recently reported by Nonkwelo et al. (13). Thus, it appears likely that the region from bp −129 to +21 contains a positive *cis* element which has an effect that is most apparent in group 1 BL cell lines with reporter constructs in which both EBNA 1 binding sites have been deleted.

DISCUSSION

Previously, our group (22) and others (19) identified Fp as a candidate promoter for EBNA 1 gene transcription in group 1

BL cell lines. A rigorous evaluation of this hypothesis, presented here, demonstrates that Fp is a lytic promoter which initiates transcription of two messages which do not contain EBNA 1 coding-exon sequences. Notably, the inability to detect Fp-initiated transcripts in the tightly latent group 1 BL cell line Rael, as well as the direct correlation between the abundance of Fp-initiated transcripts and the level of early lytic transcripts, strongly indicates that Fp is strictly a lytic promoter. In addition, induction of Fp activity upon entry into the lytic cycle is not associated with a detectable increase in EBNA 1 transcripts. The latter result is in apparent contrast to those reported by Lear et al. (12), who provided evidence that FQ/U/K spliced transcripts were produced upon induction of the viral lytic cycle in group 1 and group 3 cell lines. However, these investigators only assessed the presence of FQ/U/K spliced transcripts by reverse transcriptase-mediated PCR, which is an extremely sensitive assay; the abundance of these transcripts relative to that of other Fp-initiated transcripts or EBNA 1 transcripts present during type 1 latency was not assessed. The lack of a requirement of reporter constructs containing the FQ region on Fp sequences for activity in group 1 BL cell lines further supports the interpretation that Fp is not active during type 1 latency. Finally, Northern blot analyses of RNA from group 1 and group 3 cell lines with probes containing the FQ exon or the region downstream of the U exon indicate that Fp-initiated transcripts fail to splice at the U exon splice donor site and continue downstream (or possibly splice to a downstream exon encoded with the *Bam*HI U (fragment). The FQ probe did not hybridize to any transcripts corresponding in size to those previously shown to hybridize to the EBNA 1 coding exon (19, 22).

In reassessing the initial identification of Fp as a candidate EBNA 1 gene promoter for group 1 BL cell lines, the source of the error which led to this assignment has become apparent. When cDNA for RACE cloning was primed from the K (EBNA 1 coding) exon, clones having the Q/U/K splicing pattern were recovered, and the 5′ ends of those clones mapped to the region near the 3′ end of the FQ exon. The original RACE clones also contained the U exon, which had previously been observed in a number of EBNA 1 and EBNA 3c cDNA clones recovered from in vitro-immortalized LCLs (type 3 latency) (reviewed in reference 26). Since there was no TATAA box immediately upstream of the RACE products, but there was a TATAA box ca. 221 bp further upstream, there was concern that these did not represent the bona fide 5′ end of the group 1 EBNA 1 gene transcript. Thus, to ensure that the RACE products indeed defined the 5′ end of the EBNA 1 transcript, cDNA was generated by using a primer in the U exon in hopes of generating full-length cDNA at the highest possible efficiency. RACE cloning of this cDNA, using a 3′ PCR primer in the Q exon (based on the previous RACE clones), resulted in very efficient amplification of RACE products whose 5′ ends mapped precisely to the region now defined as Fp (19, 22, 24). It is now apparent that Fp-initiated lytic transcripts have an FQ/U exon splice structure (Fig. 8). The existence of lytic transcripts containing the U exon severely complicated the analysis of the structure of EBNA 1 transcripts (i.e., by using U exon-primed cDNA, lytic transcripts rather than the type 1 latency EBNA 1 transcripts were identified by the second round of RACE cloning).

From the data obtained by Lear et al. (12), as well our data to be published elsewhere, it appears that a small percentage of Fp-initiated lytic transcripts splice from the U exon to the EBNA 1 coding exon (FQ/U/K splicing pattern). However, we have been able to detect the Fp-initiated transcripts containing the EBNA 1 coding exon only in cell lines exhibiting spontaneous lytic activity, and these transcripts are of much lower abundance than the EBNA 1 transcripts initiated at the bona fide EBNA 1 gene promoter active in group 1 BL cell lines (Fig. 8). Thus, as will be described elsewhere in detail, the results of the initial RACE analysis using a cDNA primer in the EBNA 1 coding exon correctly identified the region containing the EBNA 1 gene promoter active in group 1 BL cell lines.

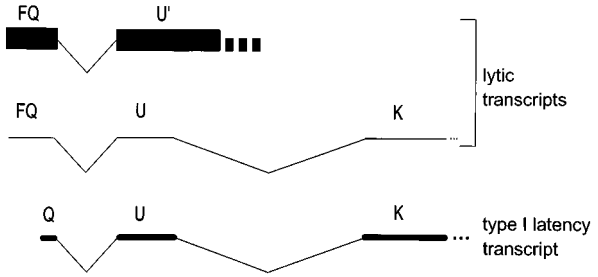

FIG. 8. Schematic illustration of proposed structures of lytic Fp-initiated transcripts and the type 1 latency EBNA 1 transcript. The relative abundances of the transcripts shown are indicated by the thicknesses of the lines representing exons. The distance between exons is not drawn to scale. The Fp and U exons have been previously described (19, 22, 24). The Q exon and the type 1 latency EBNA 1 gene transcript have been characterized in other studies (unpublished data). The U′ exon begins at the previously described U exon 5′ splice acceptor site (26) but extends past the U exon splice donor site for an undetermined distance.

The data presented here demonstrate that Fp is activated in a protein synthesis-dependent manner between 2 and 4 h after induction of the viral lytic cycle. As such, Fp is an early lytic promoter, and many, if not all, Fp-initiated transcripts splice from the FQ exon to the U exon. The precise structures of the Fp-initiated transcripts are uncertain at this point, but Northern blot analyses indicate that there are two transcripts of ca. 9 to 15 kb which either do not splice at the U exon splice donor sequence or splice to another exon within the *Bam*HI U fragment. Efforts are in progress to further define these transcripts.

Extensive reporter construct deletion analyses have shown that sequences necessary for FQ region reporter activity, in transiently transfected group 1 BL cell lines, map to a 23-bp region between bp +195 and +218 relative to the Fp transcription initiation site. The Fp TATAA box and initiation site do not contribute to reporter activity, and this observation was further confirmed by S1 nuclease protection analyses of RNA from prepared from transfected cells, which could demonstrate the presence of globin-containing transcripts but no evidence of transcription initiation from Fp (data not shown). Fp thus appears to be actively suppressed in the latent state and/or entirely dependent on lytic cycle transactivators for activity. Lear et al. (12) observed that BZLF1 gene expression (but not BRLF1 or BMLF1 gene expression) was sufficient to trigger Fp activity in the LCL X50-7, demonstrating that the BZLF1 gene product Zta, or a transactivator dependent on Zta for activity, is required for activation of Fp. Since the kinetics of Fp activation follow closely behind BZLF1 gene transcription, Zta or a factor immediately downstream of BZLF1 in the lytic cascade is most likely the critical transactivator initiating Fp activity. Fp may therefore be useful as a model system with which to study changes in the transcriptional milieu which occur early in the lytic cascade.

ACKNOWLEDGMENTS

This study was made possible by NIH grant CA47554 to J.L.S., NIH grant CA43153 to S.H.S., and an Office of Naval Research Graduate

Research fellowship to B.C.S. S.H.S. is a Scholar of the Leukemia Society of America.

REFERENCES

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligo-thymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69:**1408–1412.
2. **Bodescot, M., M. Perricaudet, and P. J. Farrell.** 1987. A promoter for the highly spliced EBNA family of RNAs of Epstein-Barr virus. J. Virol. **61:** 3424–3430.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogenous viral DNA. Proc. Natl. Acad. Sci. USA **82:**127–133.
5. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65:**718–749.
6. **Flemington, E. K., A. E. Goldfeld, and S. H. Speck.** 1992. Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis. J. Virol. **65:**7073–7077.
7. **Flemington, E. K., J. P. Lytle, C. Cayrol, A. Borras, and S. H. Speck.** 1994. DNA-binding-defective mutants of the Epstein-Barr virus lytic switch activator Zta transactivate with altered specificities. Mol. Cell. Biol. **14:**3041–3052.
8. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
9. **Gregory, C. D., M. Rowe, and A. B. Rickinson.** 1990. Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. J. Gen. Virol. **71:**1481–1495.
10. **Kerr, B. M., A. L. Lear, M. Rowe, D. Croom-Carter, L. S. Young, S. M. Rookes, P. H. Gallimore, and A. B. Rickinson.** 1992. Three transcriptionally distinct forms of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage. Virology **187:**189–201.
11. **Klein, G., L. Dombos, and B. Gothosokar.** 1972. Sensitivity of Epstein-Barr virus (EBV) producer and non-producer human lymphoblastoid cell lines to superinfection of with EB-virus. Int. J. Cancer **10:**44–57.
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. **Nonkwelo, C., E. B. D. Henson, and J. Sample.** 1995. Characterization of the Epstein-Barr virus Fp promoter. Virology **206:**183–195.
14. **Rowe, D. T., M. Rowe, G. I. Evan, L. Wallace, P. J. Farrell, and A. B. Rickinson.** 1986. Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. EMBO J. **5:**2599–2607.
15. **Rowe, M., and C. Gregory.** 1989. Epstein-Barr virus and Burkitt's lymphoma. Adv. Viral Oncol. **8:**237–259.
16. **Rowe, M., A. L. Lear, D. Croom-Carter, A. H. Davies, and A. B. Rickinson.** 1992. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J. Virol. **66:**122–131.
17. **Rowe, M., D. Rowe, C. Gregory, L. S. Young, P. Farrell, H. Rupani, and A. B. Rickinson.** 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. **6:**2743–2751.
18. **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.
19. **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.
20. **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.
21. **Sample, J., M. Hummel, D. Braun, M. Birkenbach, and E. Kieff.** 1986. Nucleotide sequences of mRNAs encoding Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site. Proc. Natl. Acad. Sci. USA **83:**5096–5100.
22. **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.
23. **Shimizu, N., A. Tanabe-Tochikura, Y. Kuroiwa, and K. Takada.** 1994. Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. J. Virol. **68:**6069–6073.
24. **Smith, P. R., and B. E. Griffin.** 1992. Transcription of the Epstein-Barr virus gene EBNA-1 from different promoters in nasopharyngeal carcinoma and B-lymphoblastoid cells. J. Virol. **66:**706–714.
25. **Speck, S. H., A. J. Pfitzner, and J. L. Strominger.** 1986. An Epstein-Barr virus transcript from a latently infected, growth-transformed B-cell line encodes a highly repetitive polypeptide. Proc. Natl. Acad. Sci. USA **83:**9298–9302.
26. **Speck, S. H., and J. L. Strominger.** 1989. Transcription of Epstein-Barr virus in latently infected, growth transformed lymphocytes. Adv. Viral Oncol. **8:**133–150.
27. **Takada, K., K. Horinouchi, Y. Ono, T. Aya, M. Osato, M. Takahashi, and S. Hayasaka.** 1991. An Epstein-Barr virus-producer line Akata: establishment of the cell line and analysis of viral DNA. Virus Genes **5:**147–156.
28. **Takada, K., and Y. Ono.** 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. J. Virol. **63:**445–449.
29. **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.
30. **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. Proc. Natl. Acad. Sci. USA **87:**1725–1729.