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Host Cell Requirements for Efficient Infection of Quiescent Primary B Lymphocytes by Epstein-Barr Virus

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Quiescent primary B lymphocytes are efficiently immortalized by Epstein-Barr virus (EBV). This process requires both the delivery and expression of the viral genome and results in activation of the cell division cycle. Infection of B lymphocytes depends on a direct interaction between the viral glycoprotein gp340/220 and CD21, the C3dg complement receptor. This interaction is required for the adsorption of EBV. In addition, several lines of evidence suggest that the interaction of EBV with CD21 modulates the phenotype of cells. CD21 forms part of a multimeric signal transduction complex with CD19, TAPA-1, and Leu-13. In normal B lymphocytes, CD19 becomes tyrosine phosphorylated following stimulation of the antigen receptor and recruits the signal-transducing enzyme phosphatidylinositol 3-kinase kinase. Here, we investigated the involvement of signal transduction pathways in efficient infection. Protein synthesis is not required for events leading to the transcription of the viral genome, suggesting that the early stages of infection do not depend on the expression of novel cell genes and consistent with the Wp promoter being the first viral promoter used upon infection. Since the stimulation of cells with gp340/220 leads to an increase in the level of CD19 tyrosine phosphorylation, we investigated the potential contribution of both tyrosine and phosphatidylinositol 3-kinase kinases to efficient infection. Both kinases contribute to the posttranscriptional control of viral gene expression following infection, but neither is required for the entry or initial transcription of the virus. Thus, it appears that EBV exploits a host signal transduction pathway to efficiently infect primary cells.

The ability of Epstein-Barr virus (EBV) to immortalize primary B lymphocytes was first discovered nearly 30 years ago (13, 27), yet many of the strategies used by EBV to overcome normal cell growth controls remain elusive. In vivo, the majority of B lymphocytes exist in a quiescent state characterized by low levels of cellular RNA and protein synthesis. This does not provide an optimal environment for establishing viral gene expression. Nevertheless, up to 50% of a population of B lymphocytes express EBV genes following infection, suggesting that the virus has developed specific strategies to exploit this large pool of quiescent target cells.

When EBV enters quiescent primary B lymphocytes, 11 viral genes that promote cell proliferation and presumably prevent terminal differentiation are expressed, resulting in the outgrowth of immortal lymphoblastoid cell lines (LCLs). This process is dependent on both the acquisition and subsequent expression of the viral genome (16, 37). EBV binds to B lymphocytes through a direct interaction of a short region of the EBV glycoprotein gp340/220 with the complement receptor CD21 (16, 19, 22, 24, 25, 35). EBV and CD21 colocalize in patches on the cell surface, followed by polar capping and endocytosis into smooth vesicles (23). Subsequently the viral DNA is transported to the nucleus. Over the next 6 days, at least 11 viral genes are expressed, and the cells start to proliferate (16, 37).

Stimulation of CD21 with its natural ligand, C3dg, or with anti-CD21 antibodies alters the phenotype of B lymphocytes (reviewed in references 9, 22, and 36). In addition, several lines of evidence suggest that the interaction of EBV with CD21 causes subtle changes in the surface phenotype of primary B lymphocytes. Infection with either transformation-defective strains of EBV or UV-inactivated virus results in increased expression of CD23 at the cell surface (11). Interaction with polymeric gp340/220 results in homotypic cell adhesion (35) and an increase in cell size (35). We have recently shown that exposure of primary B lymphocytes to purified gp340/220 will also permit expression of transfected DNA (31) but is not sufficient to stimulate the cells to progress into the G_1 or S phase of the cell division cycle (31, 35). The phenotypic changes resulting from stimulation of CD21 during virus entry into B lymphocytes may facilitate the subsequent expression of viral genes, allowing EBV to exploit the large target population of quiescent B lymphocytes found in vivo.

CD21 is part of a multimeric signal transduction complex comprising CD21, CD19, TAPA-1, and Leu-13 (5, 20, 34). Following stimulation of quiescent B lymphocytes with either anti-immunoglobulin M (IgM) or anti-CD19 antibodies, tyrosine phosphorylation of CD19 is increased (6, 39). CD19 contains two copies of the YXXM motif, which, following tyrosine phosphorylation, act as binding sites for the SH2 domains of the signal-transducing enzyme phosphatidylinositol 3-kinase (PI3 kinase) (39) (reviewed in references 9 and 36).

This led us to consider whether the efficient immortalization of quiescent B lymphocytes by EBV might involve stimulation of the multimeric CD21 complex, generating a cellular environment capable of expressing viral genes. In this paper, we show that events leading to the initial transcription of the viral genome are independent of protein synthesis, demonstrating that no novel cell or viral gene expression is required to mediate these events. However, we show that signal transduction through both tyrosine and PI3 kinases is required for the efficient expression of viral genes following infection and furthermore that this is regulated at a posttranscriptional level. Since stimulation of cells with gp340/220 results in increased tyrosine phosphorylation of CD19, this suggests that the stimulation of the viral receptor results in the activation of a signal transduc-

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tion pathway involving tyrosine and PI3 kinases that is required for the efficient expression of the incoming viral genome.

MATERIALS AND METHODS

Cell culture. Primary B lymphocytes were purified from adult peripheral blood as described previously (31). Briefly, B cells were isolated by positive selection on Pan-B Dyna-beads (Dynal) and removed from the beads by competition with Detachabead (Dynal). The cells were at least 95% pure and were cultured at a density of 10^6/ml for 24 h before use. IB4 (15) and B95-8 (21) are lymphoblastoid cell lines. Cells were cultured in RPMI medium supplemented with penicillin, streptomycin, and 15% fetal calf serum for primary cells and 10% fetal calf serum for the LCLs.

Inhibitors. Wortmannin (Sigma), genistein (Calbiochem), and tyrphostin A25 (Calbiochem) were dissolved in dimethyl sulfoxide (DMSO). Anisomycin (Sigma) was dissolved in H_2O. In each experiment, the concentration of DMSO was maintained at 0.1% of the total volume.

Preparation of virus stocks. EBV was prepared from the B95-8 cell line (21) as described previously (31). Briefly, B95-8 cells were treated with tetradecanoyl phorbol acetate (TPA) at 40 ng/ml for 6 days. Cell debris was removed by centrifugation at 1,300 rpm for 5 min, the supernatant was filtered through a 0.8-μm unit, and then the virus was pelleted by centrifugation at 25,000 rpm for 60 min. The TPA was removed by washing and repelleting the virus. The pellet was resuspended in 1/50 the original volume and stored in liquid nitrogen.

gp340/220 stimulation experiments. Purified gp340/220 (17) (a kind gift from J. Arrand and M. Mackett) was used to coat plastic tissue culture plates as described previously (31). Purified primary B lymphocytes were added to the plates at a density of 10^6/ml in normal culture medium. At 0, 30, 60, 120, and 240 min, 10^7 cells were removed, washed with phosphate-buffered saline (PBS), and stored in liquid N_2. The cells were lysed in 1 ml of Nonidet P-40 lysis buffer (39) supplemented with protease inhibitors and precleared by incubation with protein A-Sepharose beads. Each extract was divided in two and incubated with 5 μl of either normal rabbit serum or 5 μl of anti-CD19 rabbit serum (39). Immune complexes were collected on protein A-Sepharose beads, denatured in protein sample buffer, and fractionated on 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels. After transfer to nitrocellulose and blocking with 3% skim milk in PBS, the tyrosine-phosphorylated proteins were identified by Western immunoblotting with a 1/100 dilution of antiphosphotyrosine antibody (Oncogene Sciences), followed by a second layer of 1:1,000 dilution of rabbit anti-mouse Ig (Z259; Dako), followed by ^{125}I-labeled protein A. The signals were detected by autoradiography.

Analysis of proteins in whole-cell lysates. Total-cell lysates were prepared by resuspending the cells in protein sample buffer at a density of 5×10^4 cells per μl, sonicating, and heating at 95°C for 5 min. Total cell lysates from 5×10^6 cells were fractionated on 10% polyacrylamide–SDS gels and transferred to nitrocellulose filters. After blocking with 3% skim milk powder in PBS, the filters were incubated with one of the following antisera: 1:500 dilution of EBNA-2 monoclonal antibody PE-2 (Dako), 1:10 dilution of EBNA-LP monoclonal antibody JF186 (10), 1:100 dilution of anti-human Ig (Sera-lab), or 1:200 dilution of bcl-2 monoclonal antibody (Dako). A second layer of 1:1,000 dilution of rabbit anti-mouse Ig (Z259; Dako) was used for the monoclonal antibodies, and a final layer of 1:5,000 horseradish peroxidase-conjugated goat anti-rabbit antibody (Dako) was used for all filters. The specific signals were detected by enhanced chemiluminescence (Amersham).

Total protein synthesis was measured by [^{35}S]methionine incorporation assays. Aliquots of 10^6 cells were incubated with 5 μCi of [^{35}S]methionine for 3 or 12 h at 37°C. Cells were lysed in 10% trichloroacetic acid, and the resulting precipitates were harvested on GF/C filters. Each experiment was performed in duplicate, and the average [^{35}S]methionine incorporation was determined.

Plasmid constructs. The plasmid (pBSKS-29BAY) used to generate the riboprobe to map EBNA-2-specific transcripts contained B95-8 EBV sequences from positions 48273 to 48467 (3), originally subcloned into the *Sma*I site of mp8 and subsequently transferred as a *Bam*HI-*Eco*RI fragment into Bluescript II KS (Stratagene). To prepare a riboprobe, the plasmid was linearized with *Bam*HI and transcribed with T3 RNA polymerase.

The plasmid 5′W0-W1, used to generate the riboprobe to map specific transcripts from the EBV *Bam*HI W region, was constructed in a four-step procedure. A fragment spanning W0 to W1 spliced RNA was amplified from a partial cDNA clone from the IB4 cell line (32) by PCR with primers 482M and 526M. The remainder of W0 was added in a further round of amplification with primers 482M and 481M, and the resulting product was subcloned into pGEM3Z. A further 18 nucleotides were added to the 5′ end by amplification with oligonucleotides 482M and G1144, and the resulting fragment was subcloned into Bluescript. A 901-bp *Pst*I fragment from the *Bam*HI region of B95-8 EBV was then inserted into the *Pst*I site. The resulting plasmid contains contiguous EBV DNA from the *Pst*I site at position 13465 to the end of W0 at 14410, immediately adjacent to sequence from 14554 to 14619, which includes the W1 exon. To prepare a riboprobe, the plasmid was linearized with *Sna*BI (at position 14296) and transcribed with T7 RNA polymerase. The oligonucleotides used were G1144 (5′ GGGAAATTCCTGCAGCTATTTCTGGTCGTCGCATCAGAGC GCCAGGAGT), 418M (5′ ATGAATTCTCAGAGCGCCAGGAGTCCACAC AAATCC), 526M (5′ GGAGTCCACACAAATCCTAGGGGAGACCGAAGT GAAG), and 482M (5′ ATTCTAGACTCTGGCCCGATACCGGG).

Analysis of RNA. Cytoplasmic RNA was prepared from the B95-8 and IB4 cell lines as previously described (8). Total-cell RNA was prepared from 5×10^7 purified primary B lymphocytes for each point (RNAgents; Promega). Any residual DNA was removed by treatment with DNase I, and the resulting RNA was subjected to a quantitative RNase protection assay (Ambion) with the various EBV riboprobes. The 18S rRNA riboprobe (Ambion) was used essentially as described previously (31).

RESULTS

Novel cellular gene expression is not required for events leading to transcription of the viral genome. During the first 3 days postinfection, 11 viral genes are expressed in a sequential manner (1, 2, 29), and the infected cells display many features of activated B lymphocytes (11, 38). The initial transcription of the EBNA genes is directed from the Wp promoter (28, 30, 32, 40). Later, this promoter is downregulated, and an upstream promoter, Cp, is activated (4, 40). The multiple complex structures of the mRNAs from this region (reviewed in reference 33) make mapping difficult. All of the EBNA mRNAs initiated at Wp contain exon W0 followed by a variable number of copies of a repeat unit formed by two exons from the *Bam*HI W region (W1 of 66 bp and W2 of 132 bp) and a single copy of two exons, Y1 and Y2 (Fig. 1A to C). This forms a leader sequence, which is then spliced onto one of the unique coding regions for EBNA-1, -2, -3A, -3B, and -3C. A minority of the transcripts contain an alternative second exon; a splice acceptor 5 nucleotides into exon W1 is used, generating exon W1′.

In order to establish a sensitive assay to detect viral transcripts early after infection, we devised an RNase protection probe to detect transcription from this region (Fig. 1D). Mapping of the 5′ region of these transcripts is hindered by the small size of exon W0 (27 bp), so the RNase protection probe was constructed with exon W1 adjacent to exon W0. Sequences from the 5′-flanking region from the B95-8 strain of EBV were also included. A riboprobe made from this clone was used to map the RNA species found in two well-characterized cell lines (Fig. 2). IB4 is a lymphoblastoid cell line derived from human cord B lymphocytes (15) which uses the Wp promoter (30, 32). B95-8 is a lymphoblastoid cell line (21) in which the Wp promoter is silent and the upstream Cp promoter is used (4). For each cell line, the strongest protected species of 66 nucleotides corresponds to the W1 exon (Fig. 2A). This was anticipated, since multiple copies of W1 are contained in the leader sequence of the RNA transcripts initiating from both Wp and Cp. The signal resulting from protection of exon W1′ was not detected, since it is difficult to distinguish from the predominant W1 species. As expected, a fragment of 93 nucleotides, corresponding to the W0W1 species characteristic of transcription initiation at Wp followed by splicing to exon W1, was detected only with the IB4 RNA. Two additional fragments were detected in RNA from both cell lines. One of about 180 nucleotides corresponds to RNA containing the W0W1 splice but initiating upstream from the W0 start site (labeled 5′W0W1). The second fragment of about 115 nucleotides (labeled 5′W0) also appears to initiate upstream from W0 but is not spliced to W1. Together, these species constitute a significant proportion of the RNA from this region. Since their sizes correspond to protection of EBV sequences up to the *Sna*BI site, this does not define the 5′ end of the transcript, and further investigation into the structure is required.

This assay was then used to monitor EBV transcripts 12 h after infection of primary B lymphocytes with EBV (Fig. 2B). All four protected fragments were found. Pretreatment of the cells with anisomycin had little effect on the levels of the viral transcripts (Fig. 2B) even though it inhibited [^{35}S]methionine

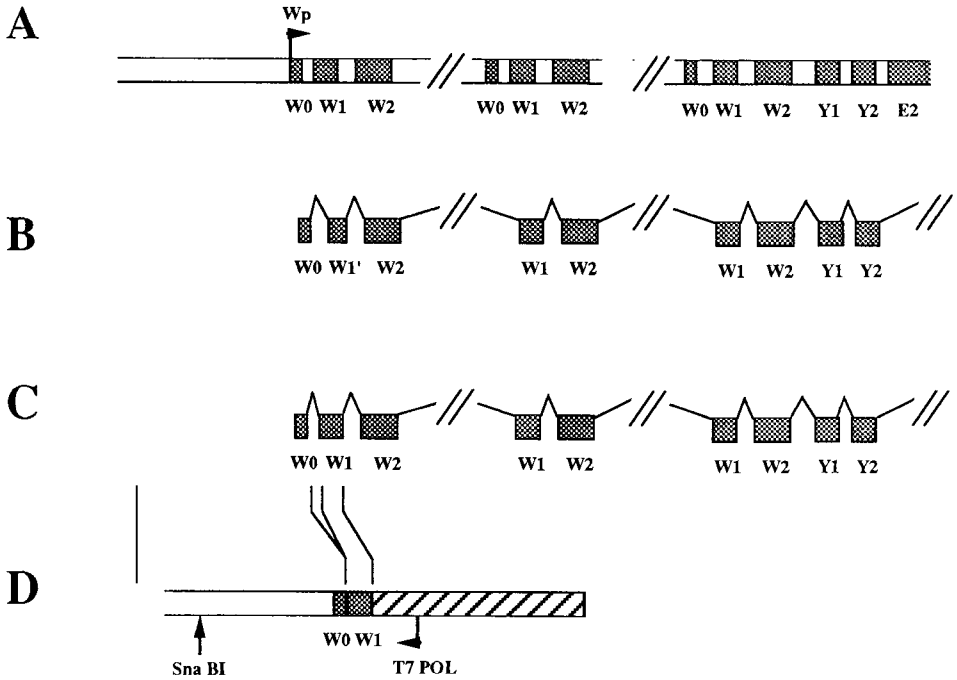

FIG. 1. Transcription from the *Bam*HI W region of the genome. (A) The positions of the EBNA exons are shown relative to the transcription start site at Wp. Transcription can initiate from each copy of the internal repeat at Wp, resulting in transcripts containing different numbers of the W1-W2 repeat unit. (B) Structures of the transcripts encoding the EBNA genes. RNA initiating at Wp contains exon W0. An alternative splice acceptor within exon W1 is used, creating exon W1′. This juxtaposes an adenosine nucleotide from W0 next to a thymidine and a guanosine dinucleotide from exon W1′, generating an initiation codon. Note that this transcript usually contains one or more copies of exon W1 but only one copy of exon W1′. (C) The structure of the more abundant transcripts encoding the other EBNA genes is shown for comparison. Exon W0 is spliced to exon W1. (D) Region of EBV DNA used to construct the riboprobe to map the initial transcripts from Wp. This region contains exon W0 adjacent to exon W1 and includes sequence 5′ to exon W0. Antisense RNA transcripts are synthesized with T7 RNA polymerase and terminate at the *Sna*BI site indicated.

incorporation by 93% and prevented the expression of EBNA-LP protein (data not shown). The level of the 18S rRNA used as a control in the RNase protection assays was unchanged.

Interaction of quiescent B lymphocytes with viral gp340/220 induces tyrosine phosphorylation of CD19. Since CD19 is a target for tyrosine phosphorylation during the physiological activation of B lymphocytes (9, 22), we investigated whether an increase in tyrosine phosphorylation of CD19 could occur following the interaction of gp340/220 with these cells. Primary B lymphocytes were incubated with purified gp340/220, and the resulting cell lysates were immunoprecipitated with antiserum specific for CD19 (39). The level of tyrosine phosphorylation in CD19 was determined by immunoblotting with a phosphotyrosine-specific antibody (Fig. 3). No phosphotyrosine was observed in unstimulated cells either at the start or at the end of the experiment; however, there was a dramatic increase in the level of phosphotyrosine on CD19 between 60 and 120 min poststimulation (Fig. 3). These data suggested that the interaction of EBV gp340/220 with its receptor is capable of activating signal transduction through tyrosine kinases. However, the kinetics of the tyrosine phosphorylation of CD19 are slower following gp340/220 stimulation than following IgM stimulation (39), suggesting that this may not be a direct result of the interaction with gp340/220. This experiment did not establish whether the observed increase in tyrosine phosphorylation was necessary for efficient viral infection.

Tyrosine and PI3 kinase activities are required for the expression of EBV proteins following infection. The potential role of tyrosine kinases in controlling the expression of viral proteins was assessed by investigating the effects of two specific tyrosine kinase inhibitors, genistein and tyrphostin A25, on virus-dependent events: the expression of the viral proteins EBNA-LP and EBNA-2 and the appearance of homotypic cell adhesion. Multiple forms of the EBNA-LP protein are readily detectable in whole-cell extracts by Western blotting with an EBNA-LP-specific monoclonal antibody by 10 h postinfection (Fig. 4A). Addition of either genistein or tyrphostin A25 prior to infection with EBV inhibited the expression of EBNA-LP protein in a dose-dependent manner (Fig. 4A) and also reduced the level of EBNA-2 protein detected (data not shown). This inhibition appeared to be specific, since it was not reflected by a change in the level of a cell protein, bcl-2 (Fig. 4A), or by the small change in total cellular protein synthesis determined by [^{35}S]methionine incorporation assayed after 3 h (Fig. 4B) or 12 h (data not shown). In addition, both inhibitors prevented homotypic cell adhesion (data not shown).

During the physiological activation of normal B lymphocytes, one of the proteins known to be recruited by tyrosine-phosphorylated CD19 is the signal-transducing enzyme PI3 kinase (39); reviewed in references 9 and 36). Having shown that the levels of tyrosine phosphorylation on CD19 increase following stimulation with the EBV glycoprotein gp340/220 and that tyrosine kinase activity is required for two early events associated with virus-mediated immortalization, we next investigated the effect of the PI3 kinase inhibitor wortmannin on the expression of EBV proteins. Following the infection of primary B lymphocytes, both EBNA-LP and EBNA-2 proteins are detected in whole-cell extracts by Western blotting with monoclonal antibodies by 12 h (Fig. 5A). Addition of wortmannin inhibited the appearance of both proteins in a dose-dependent manner. This was not a reflection of a general change in protein stability or synthesis, since the expression of Ig was

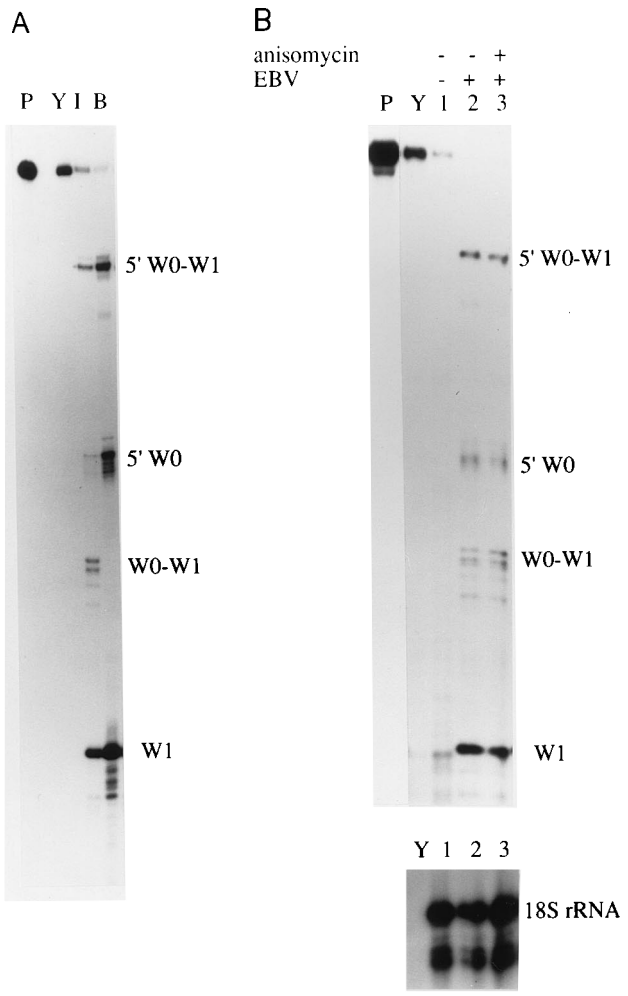

FIG. 2. Transcription from the *Bam*HI W region of EBV occurs in the absence of novel protein synthesis following infection. Quantitative RNase protection assays for transcripts from the *Bam*HI W region and for 18S rRNA were performed, and the products were separated on a 6% polyacrylamide–urea denaturing gel. The positions of the undigested probes are shown in lane P, and the result of protection with yeast RNA is shown in lane Y. The identities of the protected bands are shown on the right. (A) Cytoplasmic RNA was prepared from the B95-8 (lane B) and IB4 (lane I) cell lines. (B) Purified primary B lymphocytes were incubated with 100 μM anisomycin where indicated. After 30 min, B95-8 EBV was added where indicated. Total-cell RNA was prepared 12 h postinfection.

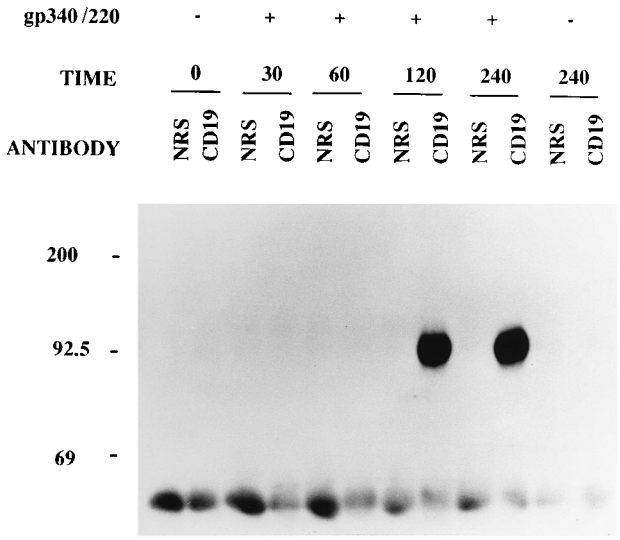

FIG. 3. Induction of tyrosine-phosphorylated CD19 following gp340/220 stimulation of primary B cells. Purified primary B cells were incubated with gp340/220 plates for the time indicated (in minutes). Extracts were prepared and precipitated with either normal rabbit serum (NRS) or CD19 antiserum (CD19). Tyrosine-phosphorylated proteins were then detected by Western blotting with a tyrosine-specific monoclonal antibody. The positions of molecular size markers are indicated on the left-hand side (in kilodaltons).

scarcely altered (Fig. 5A) and there was no change in total protein synthesis, as determined by [^{35}S]methionine incorporation assayed after 3 h (Fig. 5B) or 12 h (data not shown).

Neither tyrosine nor PI3 kinase activity is required for events leading to transcription of the viral genome. Having established that events occurring within the first 10 to 12 h of viral infection require both tyrosine and PI3 kinases, we were interested to determine which stage in the process was sensitive to the kinase inhibitors. Using the riboprobe specific for W0-W1 (Fig. 1), we investigated the effects of wortmannin (Fig. 6A) and genistein (Fig. 6B) on the transcription of the viral genome following infection. Neither wortmannin nor genistein inhibited the expression of the RNA transcripts 5′W0-W1, 5′W0, or W0-W1. Interestingly, there was a modest reduction in the W1 signal in response to genistein (Fig. 6B) but not in response to wortmannin (Fig. 6B). The level of the 18S rRNA used as a control in the RNase protection assays remained unchanged.

To extend this analysis, we devised an RNase protection probe to detect EBNA-2-specific transcripts, using the splice junction at the start of the EBNA-2 coding exon (Fig. 7A). Using this probe, we detected a series of protected fragments by 10 to 12 h postinfection (Fig. 7B and C). The longest band corresponds to full-length protection of the EBV content of the probe and probably represents unprocessed RNA. The shorter bands are the expected size for protection of RNA with the EBNA-2 splice acceptor; the signal is split into several bands that probably result from overdigestion of the probe due to the high AT content at the end of this exon. The integrity of each RNA sample was verified by determining the level of 18S rRNA (see Fig. 6). Interestingly, the level of the unspliced EBNA-2-specific RNA was affected by both inhibitors; wortmannin treatment led to a modest increase, and genistein led to a decrease. This suggests that the signal transduction may affect RNA processing or stability. Importantly, the level of processed RNA found with the EBNA-2-specific splice acceptor probe is not altered in response to treatment of cells with either wortmannin (Fig. 7B) or genistein (Fig. 7C) prior to and during infection.

DISCUSSION

The transcription of the EBV genome following infection in the presence of protein synthesis inhibitors has important implications for understanding early events during infection. It shows that EBV does not require the induction of cellular genes for events leading up to the transcription of the genome (Fig. 8). The EBV genome is delivered to the cells as a linear double-stranded DNA molecule (16). Circularization of about 1% of the viral genome can be detected soon after infection (1, 14), and it appears to be required for the outgrowth of immortal cell lines (14). It was not previously clear whether the initial transcription of the viral genome was from the linear or circu-

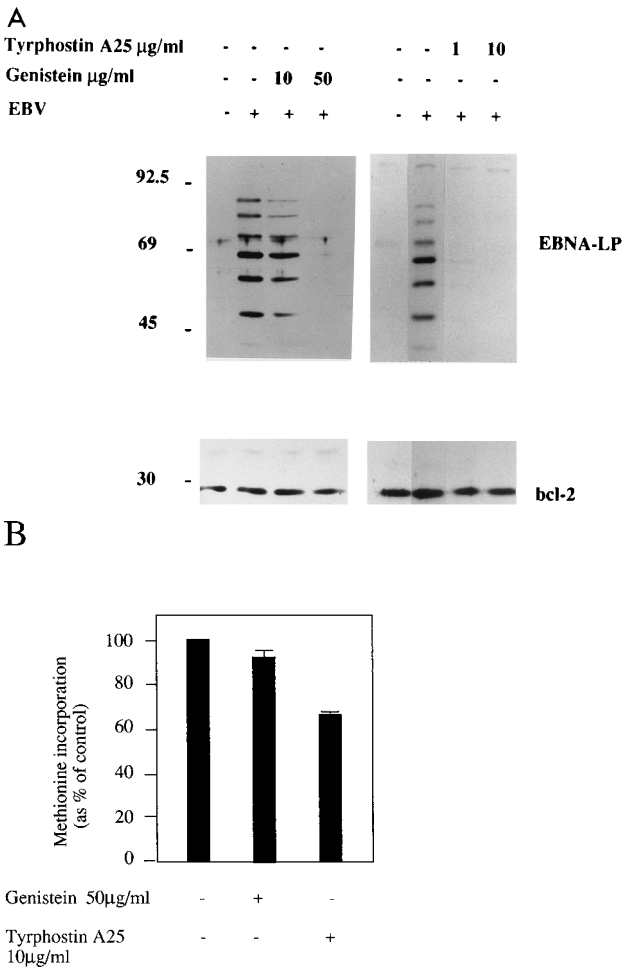

FIG. 4. Effect of tyrosine kinase inhibitors genistein and tyrphostin A25 on expression of EBV proteins following infection. Primary B lymphocytes were purified and incubated with the indicated concentration of genistein or tyrphostin A25. (A) After 30 min of incubation, B95-8 EBV was added to the cultures where indicated. The cells were harvested at 10 h postinfection, and total protein extracts were prepared and fractionated on 10% polyacrylamide–SDS gels. Western blot analysis was carried out with antisera specific for EBNA-LP and bcl-2 as indicated. (B) The levels of total protein synthesis were determined by measuring [^{35}S]methionine incorporation over a 3-h period in control cells (untreated) and cells treated with the maximum dose of genistein or tyrphostin A25 used in panel A.

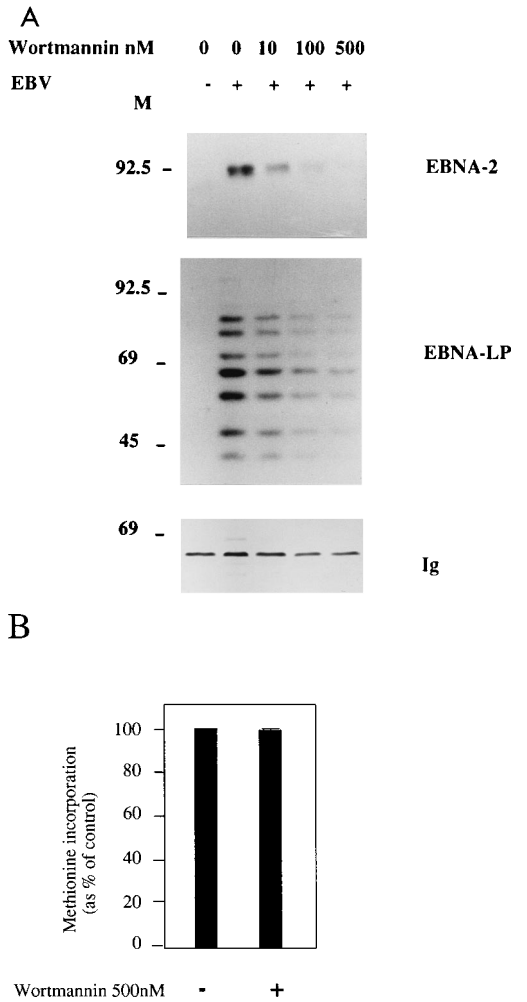

FIG. 5. Effect of PI3 kinase inhibitor wortmannin on the expression of EBV genes following infection. Primary B lymphocytes were incubated with the indicated concentration of wortmannin, which was supplemented with an equal dose at two hourly intervals. (A) After 30 min of incubation, B95-8 EBV was added to the culture where indicated. The cells were harvested at 12 h postinfection, and total protein extracts were prepared and fractionated on 10% protein gels. Western blot analysis was carried out with antisera specific for EBNA-2, EBNA-LP, and Ig, as indicated. The positions of the molecular size markers are shown on the left (in kilodaltons). (B) The levels of total protein synthesis were determined by measuring [^{35}S]methionine incorporation over a 3-h period in control cells (untreated) and those treated with the maximum dose of wortmannin used in panel A.

lar DNA. Since we have now established that the initial transcription of the viral genome occurs in the absence of novel protein synthesis, and since circularization requires novel protein synthesis (14), we can conclude that the initial transcription is from the linear genome.

The structure of the processed RNA from the *Bam*HI W region of the viral genome appears to be more complex than had previously been described (7). We identified a novel form of processed RNA which represents about 50% of the RNA after infection of primary B cells and is also present in established LCLs (Fig. 2 and 6). This signal shows that sequences upstream from the characterized start are included in RNA that appears to be processed, since it contains an authentic W0 to W1 splice. There are two possible models to account for the origins of this RNA: it may originate from a novel transcription start upstream from Wp, or it may initiate at one of the characterized start sites at Cp or Wp but not use the W2 to W1 splicing pattern. We have not identified a novel start site.

The stimulation of CD21 with gp340/220 resulted in the tyrosine phosphorylation of CD19 (Fig. 3), suggesting a role for both tyrosine and PI3 kinases in the infection process. To investigate this further, it would be necessary either to attempt to disrupt the potential signal transduction pathway with dominant negative constructs or to use specific inhibitors. Since quiescent B lymphocytes are not amenable to gene transfer techniques (26, 31), it was necessary to use specific kinase inhibitors. We chose to assay the expression of EBNA-LP and EBNA-2 because they are the first viral genes known to be expressed following infection (1, 2, 29) and both genes have been shown to contribute to the ability of EBV to immortalize primary B lymphocytes; EBNA-2 is absolutely required for immortalization (12, 18), and mutation of EBNA-LP results in a low efficiency of immortalization that is initially feeder cell dependent (12, 18). From the experiments shown in Fig. 4 and 5, it is clear that both tyrosine and PI3 kinases are required for

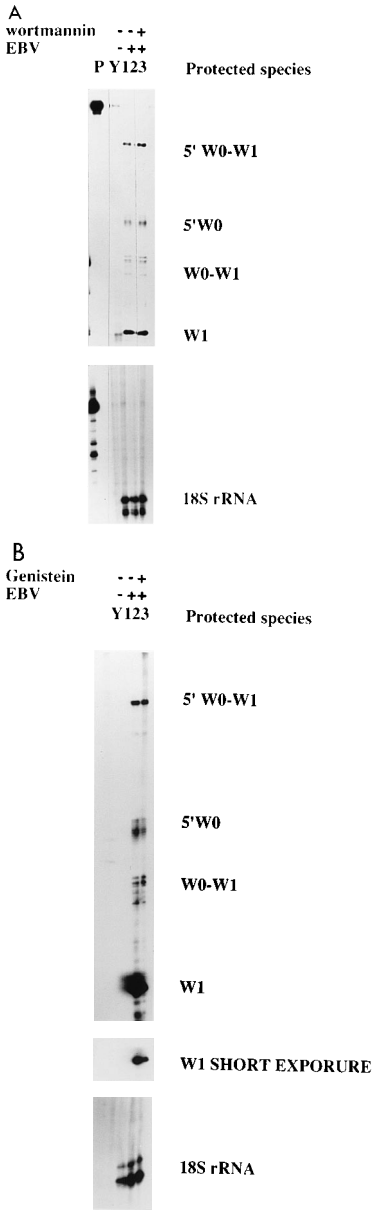

FIG. 6. Initial transcription of viral RNA is independent of tyrosine or PI3 kinase. Quantitative RNase protection assays for 5′W0-W1 and for 18S rRNA were performed, and the products were separated on a 6% polyacrylamide–urea denaturing gel. The positions of the undigested probes are shown in lane P, and the result of protection with yeast RNA is shown in lane Y. The identities of the protected bands are shown on the right. (A) Purified primary B lymphocytes were incubated with 500 nM wortmannin where indicated, and this was supplemented every 2 h. After 30 min, B95-8 EBV was added where indicated, and total-cell RNA was prepared at 12 h postinfection. (B) Purified primary B lymphocytes were incubated with 50 μg of genistein per ml where indicated. After 30 min, B95-8 EBV was added where indicated, and total-cell RNA was prepared at 10 h postinfection.

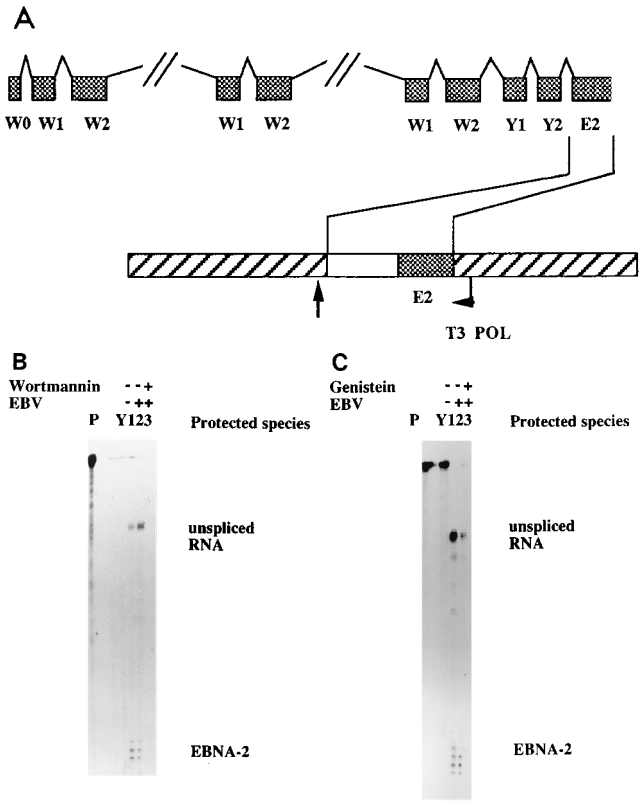

FIG. 7. Expression of EBNA-2 RNA is not inhibited by wortmannin or genistein. (A) Exon structure of EBNA-2-specific RNA initiating at the Wp promoter. The region of EBV DNA used to construct the EBNA-2-specific riboprobe is indicated below. The hatched box represents plasmid sequence, and the open box represents viral genomic DNA. Antisense RNA transcripts are synthesized with T3 RNA polymerase and terminate at the *Bam*HI site indicated. (B and C) Quantitative RNase protection assays for EBNA-2 and for 18S rRNA were performed, and the products were separated on a 6% polyacrylamide–urea denaturing gel. The positions of the undigested probes are shown in lane P, and the result of protection with yeast RNA is shown in lane Y. The identities of the protected bands are shown on the right. (B) Purified primary B lymphocytes were incubated with 500 nM wortmannin where indicated, and this was supplemented every 2 h. After 30 min, B95-8 EBV was added where indicated, and total-cell RNA was prepared at 12 h postinfection. (C) Purified primary B lymphocytes were incubated with 50 μg of genistein per ml where indicated. After 30 min, B95-8 EBV was added where indicated, and total-cell RNA was prepared at 10 h postinfection.

the efficient expression of these two viral proteins following infection. Although the data do not establish whether the kinase activities can be termed "essential" for virus infection and virus-mediated immortalization, they clearly show that both signalling pathways are quantitatively important for the efficient expression of at least two viral genes involved in the immortalization process. Since the initial transcription from the *Bam*HI W domain of the viral genome is not affected by the kinase inhibitors (Fig. 6 and 7), the tyrosine and PI3 kinase activities are not required for events prior to this, such as the adsorption, internalization, and uncoating of the virus or transport of the genome to the nucleus (Fig. 8).

It is difficult to establish precisely where these signalling pathways impinge on the control of viral gene expression. The difficulties in mapping EBNA-LP-specific RNA mean that we cannot exclude a potential contribution from changes in RNA levels. However, since neither the level of Wp-initiated RNA nor the level of EBNA-2-specific RNA alters, it seems most likely that the inhibition by genistein and wortmannin occurs at a posttranscriptional level. During this study, we identified two novel features of EBNA gene expression. We found that at least 50% of the initial RNA following infection contains sequences upstream from the previously characterized start at W0 (Fig. 2 and 7) and that the expression of RNA containing exon W1 can be regulated differentially from that of RNA containing the W0 to W1 splice (Fig. 7B). There are several possible explanations for the differential regulation of RNA

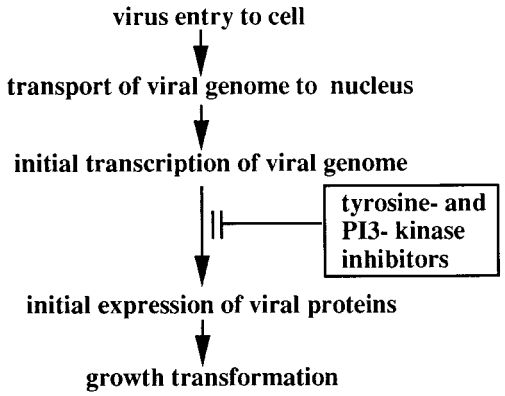

FIG. 8. Dependence on signal transduction for stages in the infection of primary B lymphocytes by EBV. The entry of EBV into quiescent B lymphocytes, transport to the nucleus, and initial transcription are independent of novel protein synthesis or tyrosine or PI3 kinase activity. However, signal transduction through both tyrosine and PI3 kinases is required for expression of EBNA proteins and thus for subsequent events, such as growth transformation.

containing these elements; it could result from premature termination of transcription within the *Bam*HI W region, it might reflect a change in the copy number of the repeat unit in the leader sequence generated by alternate splicing or promoter selection within the repeated copies, or it may reflect differential stability of the heterogeneous transcripts from this region.

In summary, we propose a working model in which the stimulation of CD21 by virus binding activates a cell signal transduction pathway involving both tyrosine and PI3 kinases. This is not required for events leading to the initial transcription of the viral genome, but it is required for the subsequent expression of the viral genes. This may represent a specific strategy to efficiently target quiescent cells.

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