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Cell Cycle Stage-Specific Phosphorylation of the Epstein-Barr Virus Immortalization Protein EBNA-LP

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EBNA-LP is a viral nuclear oncoprotein implicated in the immortalization of B lymphocytes by Epstein-Barr virus. An analysis of EBNA-LP migration on polyacrylamide gels was performed with protein derived from the X50-7 lymphoblastoid cell line blocked by hydroxyurea or aphidicolin at the G_1/S phase of the cell cycle or by nocodazole at the G_2/M phase. More slowly migrating species of EBNA-LP were detected in G_2/M phase-arrested cell extracts. Release from nocodazole G_2/M block or treatment with phosphatase caused the more slowly migrating species of EBNA-LP to disappear. Analyses of $^{32}PO_4^{3-}$-labeled EBNA-LP protein immunoprecipitated from the drug-synchronized cells showed that phosphorylated EBNA-LP was present throughout the cell cycle but that phosphorylation increased in G_2 and was maximal at G_2/M. Phosphoamino acid analysis revealed that all phosphorylation was on serine residues only. The ability of EBNA-LP to be phosphorylated by $p34^{cdc2}$ kinase and casein kinase II exclusively on serines implicates these enzymes as being potentially involved in EBNA-LP phosphorylation.

The immortalization of B lymphocytes by Epstein-Barr virus (EBV) is an integral part of the virus life cycle (21). Molecular genetic analyses reveal that the viral genome encodes several proteins (latent membrane protein type 1 [LMP1], EBNA1, EBNA2, EBNA3A, EBNA3C, and EBNA-LP) expressed in immortalized cells that have roles in this process (15, 20, 21, 32). LMP1 is an integral membrane protein and may be a growth factor receptor, but it certainly plays some role in signal transduction since it interacts with tumor necrosis factor receptor-associated factor type 1 (TRAF1) and LMP-associated protein type 1 (LAP1), a RING finger motif-bearing protein with considerable homology to TRAF2 (37). EBNA2 and EBNA3C are transcriptional activators (34, 45). EBNA2, in particular, activates expression of the other EBNAs, including EBNA3C and LMP1, by activating EBNA2 response elements in the upstream regions of responsive promoters. EBNA2 can be found complexed with a cellular protein, RBP1-J_K, at the conserved core DNA sequence of the EBNA2 response elements (14, 16, 26, 29, 35, 46, 48, 49). Cellular genes for the B-cell activation markers CD21 and CD23 and the oncogene c-*fgr* are also activated in EBNA2-expressing cells (8). EBNA1 is a viral origin of replication (oriP)-binding protein which is essential for maintenance of the viral episome in proliferating cells (39).

The role of EBNA-LP is not understood. The open reading frame for EBNA-LP is derived from the repeating W1 and W2 leader exons of the major internal repeat (IR1) and the Y1 and Y2 exons which are located just beyond the 3′ end of IR1 in the standard EBV map. The length of the W1W2 reiteration varies in different cDNAs and leads to expression of a series of related protein species in virus-infected cells. The minimal EBNA-LP polypeptide with only one repeat would be 110 residues in length and rich in argenines (19 residues) and prolines (18 residues). Recombinant viruses lacking Y1 and Y2 exons are defective but not entirely deficient in the immortalization phenotype (15, 32). Immortalization efficiency is reduced 10-fold and requires the presence of fibroblast feeder layers. Immortalized cells grow slowly with an atypical small rounded appearance. With passage in culture, cells eventually achieve lymphoblastoid features and feeder layer independence; however, most colonies do not give rise to long-term lymphoblastoid cell lines. Previous work has focused attention on cell cycle-related effects of EBNA-LP expression (1). Along with EBNA2, it is the first viral gene product detected upon primary infection of resting lymphoblasts, and EBNA-LP mutant virus-immortalized cells show delayed transit through the G_1 phase of the cell cycle. Resting B cells, primed by CD21 cross-linking with the gp340 virus envelope protein to mimic virus infection and transfected with EBNA2 and EBNA-LP expression vectors, enter and progress through the cell cycle as evidenced by synthesis of cyclin D2 (42). The conclusion of these studies is that EBNA2 and EBNA-LP are key regulators of cyclin D2 expression and that these two viral proteins cooperate to induce a G_0-to-G_1 transition.

EBNA-LP has been found by immunofluorescence analysis to colocalize in the nucleus of infected cells with Rb (the retinoblastoma susceptibility gene product) (18) and also to bind the Rb and the tumor suppressor p53 in in vitro binding experiments (44). These findings have not been reproduced by other laboratories (23, 33), and studies designed to test the effect of EBNA-LP on Rb and p53 functions have failed to show any significant consequences of EBNA-LP expression (2, 17). Specific association with the 70-kDa family of heat shock proteins has been demonstrated (23, 33). In vitro interaction studies have shown that the COOH domain, which is required for immortalization, is also necessary for heat shock protein binding. Immunofluorescence studies suggest that under stress conditions, the HSP70 and EBNA-LP proteins coalesce in the nucleolar region of the nucleus (43). Biochemically, EBNA-LP is often detected in immortalized cells as a protein ladder with an iteration of 7 kDa, usually with one or two major bands surrounded by minor bands (10, 11). The size differences correspond to the number of W1W2 repeating exons incorporated into the final mRNA. The protein is phosphorylated (40), and

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each band may be separated by isoelectric focusing into several (as many as eight, in one case) isoforms with approximate pIs between 5.5 and 6.7 (38, 40). The isoforms have been suggested to arise by differential phosphorylation. Here, we report a detailed characterization of EBNA-LP phosphorylation. We show that the protein is phosphorylated on serine residues only. Phosphorylation occurs in a cell cycle stage-specific manner, with the protein being hyperphosphorylated in late G_2. In common with other proteins that are involved in cell cycle regulation and that have similar phosphorylation patterns, EBNA-LP contains a substrate motif recognized and phosphorylated by casein kinase II (CKII). The protein is also a substrate for the cyclin-dependent $p34^{cdc2}$ kinase which is active at the time EBNA-LP is hyperphosphorylated.

MATERIALS AND METHODS

Cell culture. X50-7 cells (a gift of G. Miller, Yale University, New Haven, Conn.) are human cord blood lymphocytes that are immortalized in vitro by B95-8 EBV and express only the genes for EBNAs and LMPs (47). IARC/BL36 (a gift of G. Lenoir, International Agency for Research on Cancer, Lyons, France) is an EBV-positive Burkitt's lymphoma cell line of the group III phenotype which also expresses the EBNAs and LMPs (27). Both cell lines were grown at 37°C in a 5% CO_2-containing humidified atmosphere in RPMI 1640 supplemented with Glutamax (Gibco BRL) and containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), penicillin (100 IU/ml), and streptomycin (100 μg/ml; Gibco BRL).

To obtain cells arrested at the G_1/S boundary, exponentially growing cells at 5×10^5/ml were incubated with either 1 mM hydroxyurea (HU; Sigma) or 5 μg of aphidicolin (Sigma) per ml for 16 h. To obtain cells arrested at the G_2/M boundary, 5×10^5 cells per ml were first synchronized at G_1/S by exposure to 1 mM HU for 16 h. The cells were then washed to remove trace amounts of HU and released in fresh media containing 50 ng of nocodazole [methyl-(5-[2-thienylcarbonyl]-1*H*-benzimidazol-2-yl) carbamate dissolved in dimethyl sulfoxide (Sigma) per ml for a further 12 h. For some experiments, cells were blocked at G_2/M by incubation in the presence of 50 ng of nocodazole per ml for 24 h without G_1/S synchronization. Cytofluorimetric analysis was used to confirm the cell cycle status.

For ^{32}P labeling, either exponentially growing cells or synchronized cells were washed twice in Tris-buffered saline (25 mM Tris-Cl [pH 7.4], 150 mM NaCl), resuspended in phosphate-free RPMI 1640 (ICN Biomedicals, Inc.) containing 2% dialyzed fetal bovine serum and 1 mM glutamine at 4×10^6 cells per ml, and labeled for 2 h with 1 mCi of $^{32}P_i$ (500 mCi/ml, carrier free; ICN Biomedicals). For arrested cells, the washing buffer and the labeling media contained drug at the concentration used for arrest. Following labeling, the cells were washed twice in ice-cold Tris-buffered saline containing 0.4 mM EDTA and 0.4 mM Na_3VO_4 and lysed as described below.

Propidium iodide staining and flow cytometry. Cells (2×10^6) from each fraction were collected by centrifugation, washed in Hanks balanced salt solution (Gibco BRL), and fixed with 70% ethanol for at least 1 h at 4°C. The fixed cells were washed twice with sodium citrate buffer (50 mM sodium citrate [pH 7.8], 150 mM NaCl), and the cell pellet was stained with 1 ml of propidium iodide staining solution (50 μg of propidium iodide and 50 mg of RNase A per ml of citrate buffer) at 4°C overnight. The fluorescence intensities of the samples were measured by quantitative flow cytometry with a Becton Dickinson FACSTAR scanner. Analysis was based on the accumulation of 5,000 cells, and all data were linearly scaled.

Immunoprecipitation and immunoblotting. Cells for immunoprecipitation were lysed in EBC buffer (50 mM Tris-Cl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) at 2×10^7 cells per ml for 1 to 2 h on ice. The DNA was sheared by sonication, and the lysates were cleared by centrifugation at 14,000 × *g* for 15 min. Extracts from ^{32}P-labeled cells were twice precleared for 1 h each with protein G-Sepharose beads (Sigma). EBNA-LP was immunoprecipitated from the supernatant with 10 μg of mouse monoclonal antibody JF186 per ml (kindly provided by M. Rowe, University of Birmingham, Birmingham, United Kingdom). Immunocomplexes were collected with 50 μl of protein G-Sepharose beads, washed extensively with EBC buffer, and eluted from the protein G-Sepharose with electrophoresis sample buffer (0.625 M Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.1% 2-mercaptoethanol, 10% glycerol, 0.02 mM bromophenol blue) by boiling for 3 min. Denatured immune complexes were separated by electrophoresis and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore). EBNA-LP was visualized by either exposing the membrane to XAR5 X-ray film (Kodak), as in the case for labeled samples, or immunoblotting, if the samples were unlabeled. EBNA-LP phosphorylation was quantitated on a PhosphorImager (Molecular Dynamics).

Whole-cell extracts were prepared by solubilization in electrophoresis sample buffer, sonication, and subsequent boiling for 3 min. Samples equivalent to 10^6 cells were electrophoresed by discontinuous SDS polyacrylamide gel electrophoresis (10% polyacrylamide). For immunoblotting, proteins were transferred to Immobilon PVDF membrane and blocked in Tris-saline (10 mM Tris-Cl [pH 8.0], 150 mM NaCl) containing 5% nonfat milk powder. The membrane was probed with JF186 at a total antibody concentration of 1 μg/ml at 4°C from 4 h to overnight. Goat anti-mouse ^{125}I-antibody (2 to 15 μCi/μg; ICN Biomedicals, Inc.) was used to visualize sites of antibody binding.

Phosphatase treatment. Immunocomplexes bound to protein G-Sepharose beads were washed with EBC buffer as described above. After the final wash, the beads were washed twice in AP buffer (100 mM Tris-Cl [pH 8.0], 50 mM $MgCl_2$, 1% aprotinin). Each sample was divided in half to give a control and an experimental sample. The control and experimental samples were resuspended in 45 μl of AP buffer to which 5 μl of alkaline phosphatase (1 U/μl; Boehringer Mannheim) was added and incubated at 37°C for 30 min. The reaction was stopped by adding 50 μl of electrophoresis sample buffer.

Phosphoamino acid analysis. ^{32}P-labeled protein was either immunoprecipitated with the JF186 antibody from labeled cells or phosphorylated in vitro with purified kinases. The protein was electrophoresed on an SDS–10% polyacrylamide gel, transferred to Immobilon PVDF membrane, and excised from the membrane with an autoradiogram as a template. The membrane was rehydrated with ethanol and water and washed extensively with water. The protein was hydrolyzed by boiling the membrane for 1 h in 6 N HCl. The acid was removed by Speed-Vac, and the hydrolysate was resuspended in 5 to 10 μl of pH 1.9 buffer (88% formic acid, glacial acetic acid, water; 25:78:897 [vol/vol/vol] to which 1.5 μg of unlabeled phosphoamino acid standards (phoshoserine, phosphotyrosine, and phosphothreonine [Sigma]) and 1 μl of xylene cyanol (1 mg/ml) were added. The samples were applied to a 0.250-mm thin-layer cellulose plate (Sigma-Aldrich) and separated by two-dimensional electrophoresis in a flatbed electrophoresis unit (Multiphor II System; Pharmacia LKB). Electrophoresis in the first dimension was performed in pH 1.9 buffer for 1.5 h at 900 V and in the second dimension in pH 3.5 buffer (pyridine, glacial acetic acid, water; 10:100:1,890 [vol/vol/vol]) for 45 min at 900 V. The position of unlabeled standards was determined by ninhydrin staining; labeled amino acids were detected by autoradiography and PhosphorImager analysis.

Preparation of bacterial fusion proteins. The plasmid pMAL-cRI, which encodes a maltose-binding protein (MBP)–β-galactosidase fusion protein (MBP-lacZ), was purchased from New England Biolabs. The plasmid pMBP was constructed by inserting a stop codon downstream of the *malE* gene on pMAL-cRI, thereby inhibiting the expression of *lacZ*. Plasmids encoding the MBP fused to a one-repeat EBNA-LP (pMBP-W1W2Y1Y2) or various fragments of EBNA-LP (pMBP-W1, pMBP-W1W2, pMBP-W2Y1Y2, PMBP-Y1Y2, and pMBP-Y2) were obtained by PCR amplification of a one-repeat EBNA-LP cDNA clone, pSp64-WY1 (derived from IB4WY and kindly provided by S. Speck, Washington University, St. Louis, Mo.). Each 5′ amplimer contained an *Eco*RI site, and each 3′ amplimer contained either a *Bam*HI site or an *Xba*I site such that the resulting PCR product, upon digestion with *Eco*RI and *Bam*HI or *Eco*RI and *Xba*I, could be ligated in frame downstream of the *malE* gene of pMAL-cRI. All clones were verified by restriction mapping and DNA sequencing with the Sequenase 2.0 kit (U.S. Biochemicals Corp.), using the protocol suggested by the manufacturer.

MBP fusion protein expression and purification were performed essentially as described by Smith and Johnson (42a). Overnight cultures of *Escherichia coli* (INVαF′; Invitrogen) transformed with plasmid DNA were diluted in Luria-Bertani broth (Difco Laboratories) containing ampicillin (100 μg/ml), and incubated at 37°C for 2 h. Fusion proteins were induced by addition of 0.2 mM isopropyl-β-D-galactoside (IPTG) to the culture for 3 h, after which cells were recovered by centrifugation at 5,000 × *g* for 15 min at 4°C and lysed on ice by sonication with two 30-s pulses in a 1/10 volume of NETN buffer (20 mM Tris-Cl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). The suspension was cleared by centrifugation at 12,000 × *g* for 15 min at 4°C. Cleared lysates were loaded onto amylose agarose beads (New England Biolabs), washed extensively with NTN (NETN buffer without EDTA), and eluted with 10 mM maltose. For analysis of the concentration and purity of the fusion proteins, samples were suspended in equal volumes of electrophoresis sample buffer and loaded onto SDS-polyacrylamide gels. The proteins were visualized by Coomassie blue staining in comparison with protein standards of known concentration. In some instances, the protein concentration was equilibrated as determined by the Lowry protein assay.

In vitro kinase assays. Purified CKII (15 U/μl; Promega) and purified $pp34^{cdc2}$ (7.5 U/μl; Promega) were used to phosphorylate EBNA-LP fusion proteins in vitro. Fusion proteins were expressed and purified on amylose agarose beads, washed twice with HB buffer [25 mM morpholinepropanesulfonic acid (MOPS; pH 7.2), 15 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 15 mM *p*-nitrophenylphosphate, 60 mM β-glycerophosphate, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1% Triton X-100, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml) and eluted from the beads with 50 μl HB–10 mM maltose. Equal amounts of protein (2 μg) were incubated with enzyme in HB buffer (total volume, 25 μl) with 5 μCi of [$\gamma^{32}P$]ATP (5,000 to 10,000 cpm/pmol; NEN) for 15 min at 30°C. The reaction was terminated by the addition of 25 μl of electrophoresis sample buffer.

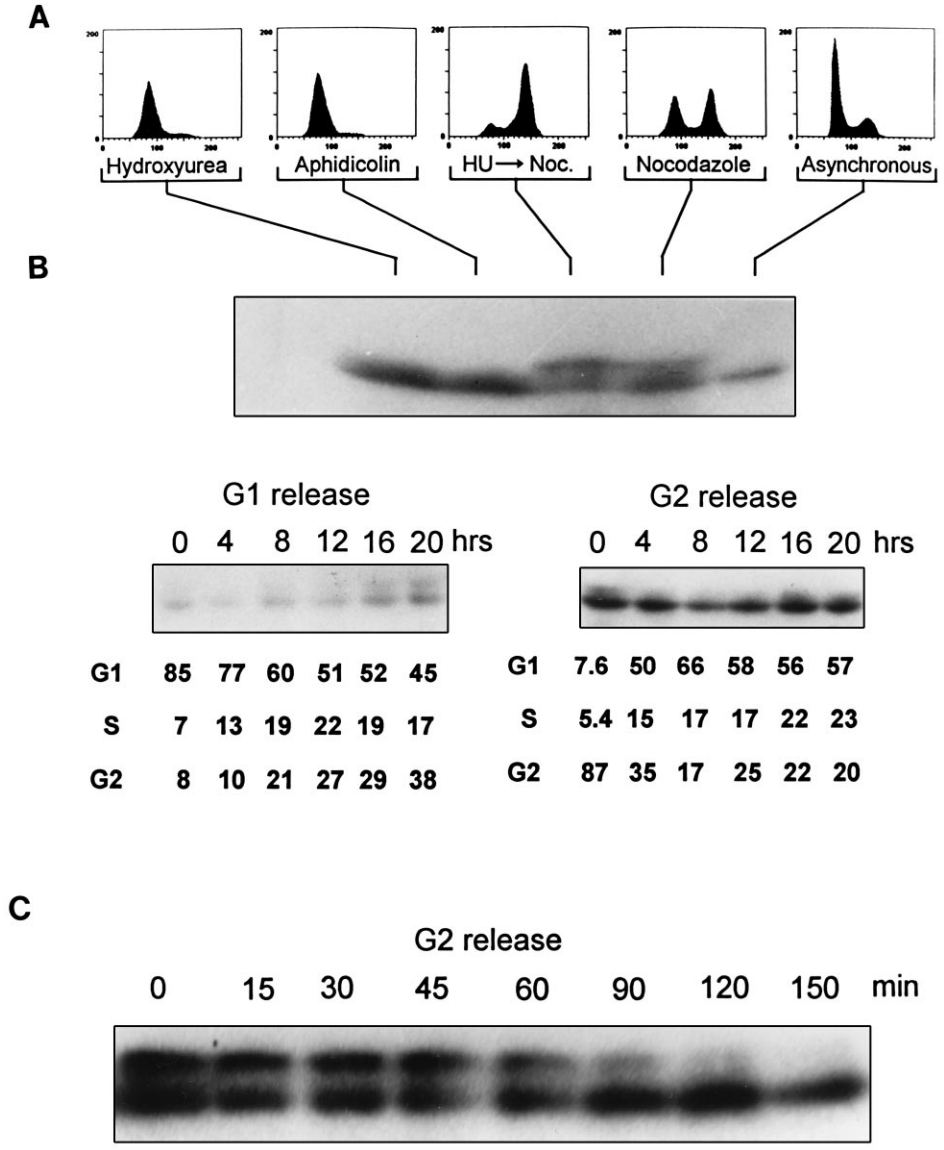

FIG. 1. Changes in EBNA-LP polyacrylamide gel electrophoresis migration related to cell cycle stage. (A) Flow-cytometric profiles of X50-7 cells treated with the indicated drugs to produce cell cycle arrest at G_1/S (HU, aphidicolin) and G_2/M (HU followed by nocodazole [Noc.] or nocodazole alone). (B) Western immunoblots of drug-arrested cells probed for EBNA-LP with JF186 monoclonal antibody. (C) Kinetics of EBNA-LP migration changes following release from the HU block (G_1 release) or HU-nocodazole block (G_2 release). Released cells were harvested in electrophoresis sample buffer at the times indicated and analyzed by Western immunoblotting with JF186 monoclonal antibody.

RESULTS

Cell cycle-specific changes in EBNA-LP migration in polyacrylamide gels. Previous studies had revealed that cells immortalized by recombinant EBV lacking the Y1 and Y2 exons (the COOH-terminal 45 amino acids of EBNA-LP) displayed phenotypic differences (delays) in cell cycle progression after release from starvation arrest (1). This preliminary work suggested that EBNA-LP functions might be dependent upon or perhaps even influence the stage of the cell cycle in which the cell was located. Therefore, cell populations synchronized to particular stages of the cell cycle should provide useful tools for analyzing EBNA4 function. As a technique for accomplishing synchronization the starvation arrest procedure coupled to [^{3}H]thymidine labeling had two serious drawbacks. First, the [^{3}H]thymidine-labeling procedure measured the entry of only some cells into S phase without revealing the status or proportions of cells in the other phases of the cell cycle. Second, starvation arrest caused a loss of viability in a large segment of the arrested population (in excess of 50% as determined by trypan blue exclusion).

We have used cytofluorimetric analysis of propidium iodide-stained EBV-immortalized lymphocytes to develop procedures for arresting the cells at the G_1/S or G_2/M boundary and rendering them viable and competent to proceed synchronously through the cell cycle. G_1 blocks are best accomplished with an 18-h exposure to 1 mM HU (Fig. 1). Although aphidicolin and other drugs are sufficient to establish the block, HU-blocked cells show better viability and synchronicity following release. G_2 blocks are best accomplished by first incubating the cells for 16 h in HU and then releasing them into

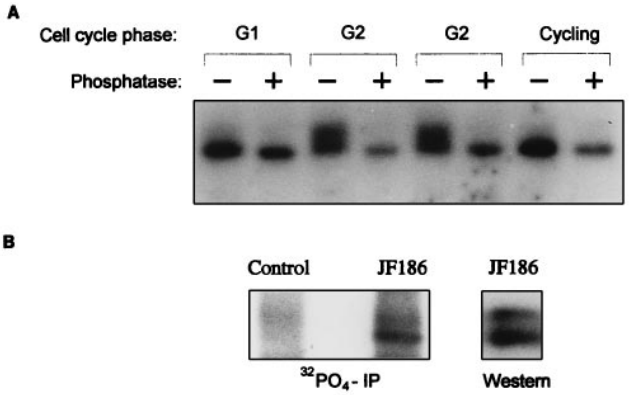

FIG. 2. Phosphatase treatment of EBNA-LP from cell cycle-arrested cells. (A) Immunoblot of EBNA-LP immunoprecipitated from X50-7 cells blocked at G_1 with HU or at G_2 with nocodazole alone or with HU and released into nocodazole. Untreated cycling cells were included as controls. Immunoprecipitated proteins were incubated with 10 U of alkaline phosphatase for 10 min. (B) Immunoprecipitates (IP) from $^{32}PO_4^{3-}$-labeled G_2-arrested BL36 cells with control or JF186 anti EBNA-LP antibodies are shown on the left. An immunoblot of whole-cell extracts of G_2-arrested BL36 is shown to the right.

medium containing 50 ng of nocodazole per ml for a further 12 h. When nocodazole is used alone, half the cells remain in G_1 regardless of the length of the incubation period with the inhibitor. This indicates that there is probably a nocodazole-sensitive step in the G_1 phase of the cell cycle of EBV-immortalized B cells. Cells arrested by HU (and aphidicolin) have apparently passed this critical step. Extracts of X50-7 cells arrested at the G_1/S boundary and the G_2/M boundary were subjected to polyacrylamide gel electrophoresis and probed by Western blotting (immunoblotting) for EBNA-LP expression (Fig. 1). EBNA-LP was present in the G_1 and G_2 phases of the cell cycle at the same levels as were detected in asynchronously growing cells. Therefore, EBNA-LP in X50-7 (and other lymphoblastoid cell lines and BL cells we have examined) did not show cell cycle-related changes in concentration. Cells arrested in G_2 had a component of the EBNA-LP band that migrated more slowly. From a comparison of the HU-released/nocodazole-arrested cells (a larger number of G_2 cells in the population) with the nocodazole-alone arrested cells (fewer G_2 cells in the population), it appeared that the amount of the more slowly migrating species correlated with the presence of cells in G_2.

To confirm that the more slowly migrating band was related to the G_2 status and was not an artifact of nocodazole treatment, cell populations arrested in G_1 with HU or in G_2 with nocodazole were released and sampled 4, 8, 12, 16, and 20 h post-release. The movement of cells arrested in G_1 or G_2 was monitored by flow cytometry. Fast-migrating EBNA-LP was converted to the slowly migrating form corresponding to the cell cycle transit. Cells arrested in G_2 by treatment with nocodazole alone rapidly shifted to G_1 DNA content when released, and most of the cells were in G_1 within 4 h. This transition was accompanied by the loss of the more slowly migrating species. By 12 to 16 h post-release, cells were reentering G_2 and the more slowly migrating species reappeared. Thus, the appearance of the more slowly migrating forms of EBNA-LP correlated with the cell cycle stage and was not due to either HU- or nocodazole-induced artifacts. The rapid loss of the slowly migrating species was analyzed in HU-released/nocodazole-arrested G_2-enriched populations. At most, only half of the EBNA-LP is present as the more slowly migrating form in the G_2-enriched populations. Cells released from the G_2 block were sampled at intervals of 15 min for the first 1 h and at intervals of 30 min thereafter for 2.5 h. The results showed that there was rapid loss of the more slowly migrating form of EBNA-LP following release (Fig. 1). More than 70% of the DNA content shift occurs within the first 4 h post-release, suggesting that the kinetics of the disappearance of the slowly migrating species corresponds to events related to the G_2-to-G_1 transition.

Slowly migrating species of EBNA-LP are modified by phosphorylation. The most likely explanation for the migration shift detected in the EBNA-LP protein during cell cycle transit is modification by phosphorylation. A rapid and simple test of this hypothesis involved treating the protein with alkaline phosphatase to remove the added phosphates. Treatment of EBNA-LP immunoprecipitates with alkaline phosphatase had little effect on the migration of the fast species of EBNA-LP (Fig. 2). Dephosphorylation caused complete loss of the slow species of EBNA-LP from the immunoprecipitate of G_2-arrested cells. Dephosphorylation always seemed to cause an overall reduction in the amount of EBNA-LP present. The untreated extracts were mock-incubated controls, suggesting that, to some extent, the slow species might also be phosphorylated and that phosphorylation may help to stabilize the protein.

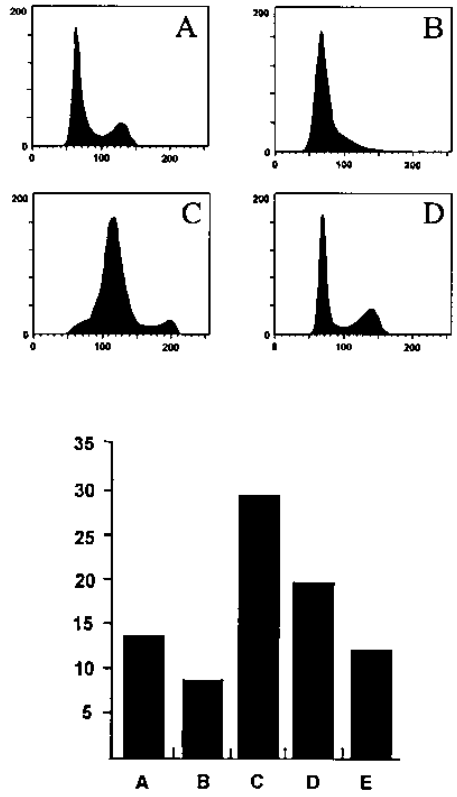

FIG. 3. Quantitation of EBNA-LP phosphorylation. EBNA-LP was immunoprecipitated from 2×10^7 IARC/BL36 cells labeled with $^{32}P_i$ at various positions in the cell cycle. All cells were verified for cell cycle position by propidium iodide staining and flow cytometry. (A) Cycling cells; (B) G_1/S-blocked cells; (C) G_2/M-blocked cells; (D) 2-h release from G_2/M block. The phosphorylated proteins were subjected to Western blotting, and the membranes were scanned in a Molecular Dynamics PhosphorImager and quantified by band volume integration. A histogram of the relative levels of phosphorylation was generated. A, Cycling cells; B, cells blocked at G_1/S with HU for 16 h; C, cells blocked at G_2/M with 16 h of HU treatment followed by 12 h in nocodazole; D, cells blocked at G_2/M and then released into fresh medium; E, cells blocked at G_2/M and then treated with alkaline phosphatase after immunoprecipitation.

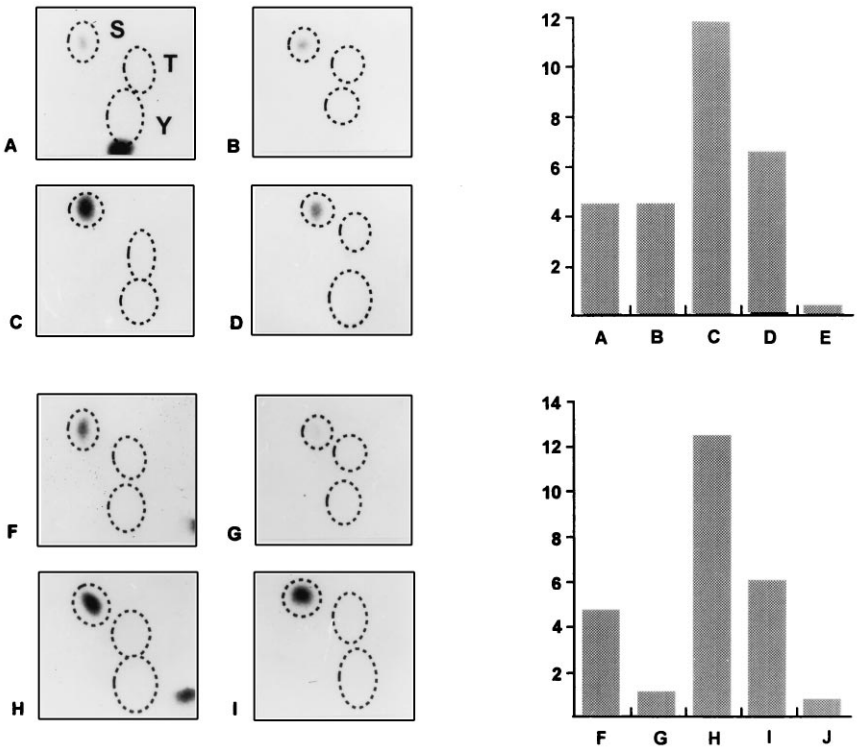

FIG. 4. Amino acid analysis of EBNA-LP phosphorylation. Immunoprecipitates of $^{32}PO_4^{3-}$-labeled EBNA-LP from different cell cycle stages were analyzed for phosphoramino acid content. Phosphorylated residues from the upper band (A to D) and the lower band (F to I) were analyzed. A and F) Cycling cells; (B and G) G_1/S-arrested cells; (C and H) G_2/M-arrested cells; (D and I) G_2/M-arrested cells released from the block for 2 h. Histograms show phosphorImager quantitation of the relative level of serine phosphorylation. E and J represent background signal. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

EBNA-LP phosphorylation level is related to cell cycle stage. Experiments with immunoprecipitation of $^{32}PO_4^{3-}$-labeled X50-7 cells were complicated by the presence of a major nonspecific species of 42 kDa (possibly actin) that comigrated with the major X50-7 EBNA4 band. BL36 EBNA-LP migrates as a 49-kDa protein whose cell cycle-related properties were identical to X50-7 EBNA-LP and whose further analysis following in vivo labeling was not hindered by backround contaminants. Immunoprecipitation of EBNA-LP from $^{32}PO_4^{3-}$-labeled nocodazole-arrested BL36 cells produced two bands on polyacrylamide gel electrophoresis that comigrated with the EBNA-LP doublet detected by Western immunoblotting (Fig. 2). Growing BL36 cells were switched into HU medium and incubated for a further 16 h. During the last 2 h of the block, the cells were resuspended in phosphate-free medium and labeled with $^{32}PO_4^{3-}$. A similar labeling procedure was used to $^{32}PO_4^{3-}$ label a population of cells released from the HU block into nocodazole-containing medium. Cytofluorometric profiles of similarly treated but unlabeled cells confirmed the enrichment of cells in G_1 or G_2 after the labeling procedure (Fig. 3). Immunoprecipitated EBNA-LP was run on a polyacrylamide gel, and the total amount of $^{32}PO_4^{3-}$ incorporated was estimated by PhosphorImager analysis. As expected, the smallest amount of $^{32}PO_4^{3-}$ was incorporated into EBNA-LP in G_1-arrested cells and the largest (threefold more) was incorporated into G_2-arrested cells. Some $^{32}PO_4^{3-}$ labeling was detected in all phases of the cell cycle; therefore, to some level, the protein appears to be constitutively phosphorylated but becomes hyperphosphorylated in the G_2 phase of the cell cycle. Cells released from the G_2 block showed a decrease in $^{32}PO_4^{3-}$ labeling. These results are consistent with the interpretation of the changing pattern of multiple species in Western blots as being due to altered phosphorylation.

Amino acid analysis of $^{32}PO_4^{3-}$ labeled EBNA-LP. The minimal unique sequence of EBNA-LP contains eight serines, five theonines, and one tyrosine. These amino acids are distributed fairly evenly along the length of the primary sequence. An analysis of the labeling pattern at different points around the cell cycle would provide valuable clues for determining which sites become phosphorylated. The $^{32}PO_4^{3-}$-labeled EBNA-LP was immunoprecipitated from asynchronous, G_1-arrested, G_2-arrested, and G_2-arrested-released BL36 cells. The slowly migrating and fast-migrating species were excised from polyacrylamide gels, hydrolyzed to their constituent amino acids, and analyzed by cellulose thin-layer electrophoresis to identify the amino acids which were phosphorylated. Only phosphoserine was detected (Fig. 4). Long exposures on the PhosphorImager plates failed to reveal even trace labeling of threonine or tyrosine at any stage of the cell cycle. The PhosphorImager analysis of the $^{32}PO_4^{3-}$ present in each serine spot indicated that cycling and G_1-arrested cells contained similar amounts of phosphoserine in the faster-migrating EBNA-LP species (Fig. 4A and B). This was approximately one-third the amount of phosphoserine from the same species in G_2-arrested cells (Fig. 4C). Release of cells from the G_2 block caused a drop in the phosphoserine content in this faster-migrating species (Fig. 4D). The more slowly migrating species showed a similar pattern of labeling (Fig. 4F to I). The main difference was in the very low levels of phosphorylated slowly migrating species recoverable at the G_1/S boundary (Fig. 4G). These changes in serine phosphorylation patterns which correlate with cell cycle

TABLE 1. Protein sequence motifs in nuclear oncoproteins phosphorylated by CKII

Protein	Species	Rb binding	LxCxE sequence[a]	P (S/T) (β turn) sequence	D/E (acidic domain) sequence
E1a	Adenovirus types 2 and 5	+	LTCHEAGF	-PPS-	DDEDEEGEE
1Tag	Simian virus 40	+	LFCSEEM-	PSS-	DDEATADS
E7	Human papillomavirus	+	LYCYEQLND-	SS-	EE
Fos	Human	−	VEQL-	SP-	EEEE
Myc	Human	−	HEE-	TPPTTSS-	DSEEEQEDEEE
EBNA-LP	EBV	−	HFE-	PPTVTTQRQSVYI	EEEEDED

[a] Underlines indicate residues of the LXCYE Rb-binding motif.

stage are reminiscent of the phosphorylation patterns observed in key cell cycle regulatory molecules (5, 6, 25, 31).

Phosphorylation of EBNA-LP by p34^{cdc2} kinase and casein kinase II. A long and growing list of cellular and viral nuclear proteins show cell stage-specific phosphorylation (4–6, 12, 36). Many of these proteins (e.g., Myc, Max, Myb, Fos, p53, Rb, Abl, E1a, human papillomavirus E7, and simian virus 40 Tag) are substrates for the same cellular kinases (3, 7, 31, 36). Inspection of the EBNA4-LP primary sequence revealed a motif at the COOH terminus of the protein that was similar to sequences in other proteins that were phosphorylated by CKII (Table 1). To determine if EBNA-LP was a potential phosphorylation substrate for CKII, a purified bacterial fusion protein containing MBP sequences linked to EBNA-LP sequences was incubated with CKII and [γ-^{32}P]ATP. Polyacrylamide gel analyses revealed that the MBP-LP fusion was a substrate for CKII which, as shown above, also autophosphorylates its own 45-kDa subunit in the in vitro reaction. MBP alone was not a substrate for CKII. In EBNA-LP, both threonine and serine residues are located in the CKII substrate motif. Amino acid analysis of the in vitro-labeled protein showed that CKII phosphorylates EBNA-LP on serine residues only, which is consistent with the lack of threonine phosphorylation in vivo.

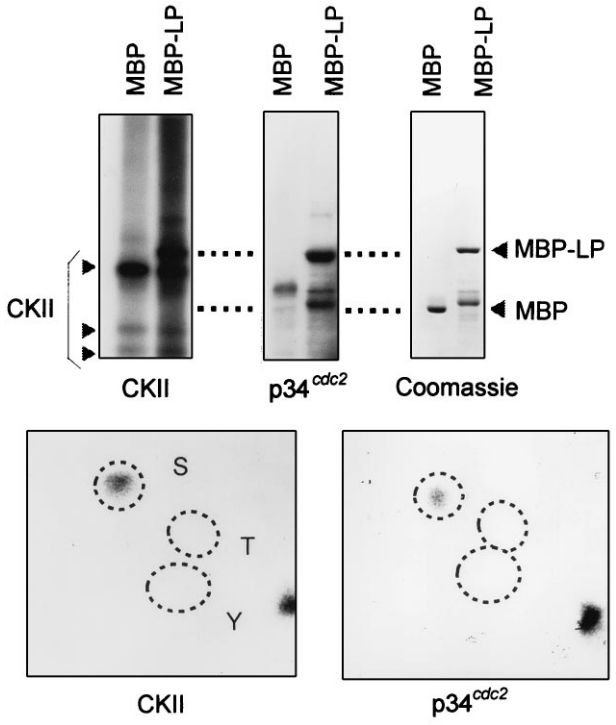

FIG. 5. In vitro phosphorylation of EBNA-LP. Purified CKII (15 U/μl; Promega) and p34^{cdc2} (7.5 U/μl; Promega) were used to phosphorylate purified bacterial fusion proteins MBP and MBP-LP. The proteins were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and either fixed and stained with Coomassie blue or transferred to Immobilon PVDF membrane before exposure to X-ray film. The Coomassie blue-stained gel shows the migration of the phosphorylated proteins. The CKII-phosphorylated MBP-LP band lies just above the autophosphorylated CKII 45-kDa subunit (arrows to the left of the lane). The phosphorylated proteins were excised from the PVDF membrane, hydrolyzed, and run in a two-dimensional thin-layer electrophoresis on cellulose plates. Both p34^{cdc2} and CKII phosphorylated the fusion protein on serine residues only.

Another enzyme which shares many of the same substrate proteins as CKII (p53, Rb, Myb, simian virus 40 Tag, RNA polymerase nucleolin, and CKII) is p34^{cdc2} kinase (9, 22, 25, 28, 30, 41). The activity of p34^{cdc2} is regulated and high in late G_2, when its action is considered to be critical to the onset of mitosis. Owing to the similarities in phosphorylation patterns between some of these cell cycle regulators and EBNA-LP, we tested the ability of EBNA-LP to serve as a substrate for p34^{cdc2} kinase in vitro (Fig. 5). MBP-LP was a substrate for p34^{cdc2} kinase, and thin-layer phosphoamino acid analysis of the in vitro-labeled protein showed that it was phosphorylated exclusively on serines.

Mapping in vitro sites of phosphorylation. As a preliminary step in the characterization of EBNA-LP phosphorylation in vivo, we attempted to determine the sites of phosphorylation by CKII and p34^{cdc2} in vitro. Fusion proteins that had deletions corresponding to the exon structure (W1W2Y1Y2) of the EBNA-LP gene were constructed. The mutants which were made had progressively fewer exons on the N or C terminus. Candidate phosphorylation sites for both enzymes were identified by comparison with consensus phosphorylation sequences. For the CKII enzyme, there was a single consensus site in Y2 which had the predicted β turn and acidic domain present in other nuclear oncogenes phosphorylated by CKII (Table 1). Mutants with progressive deletions of EBNA-LP sequences from the COOH terminus were not phosphorylated by the CKII, as would be predicted (Fig. 6). Unexpectedly, removal of the W2 exon, as in the Y1Y2 and Y2 constructs, also abolished phosphorylation. The alteration of conformation of the final fusion protein that we suspected to be primarily responsible for eliminating substrate specificity could not be relieved by heat denaturing the fusion proteins prior to incubation with the enzyme.

The same set of fusion proteins were used in in vitro kinase assays with purified p34^{cdc2} kinase. Potential phosphorylation sites (minimally a serine preceding a proline) are more difficult to predict, but two potential candidate residues reside in the W2 exon. Phosphorylation of EBNA-LP was dependent on the presence of the W2 exon in the sequence, indicating that one or both of the two serines (S-33 and S-62) in W2 may be phosphorylated by p34^{cdc2} kinase. Interestingly, removal of Y1Y2 sequences produced a fusion protein that was hyper-

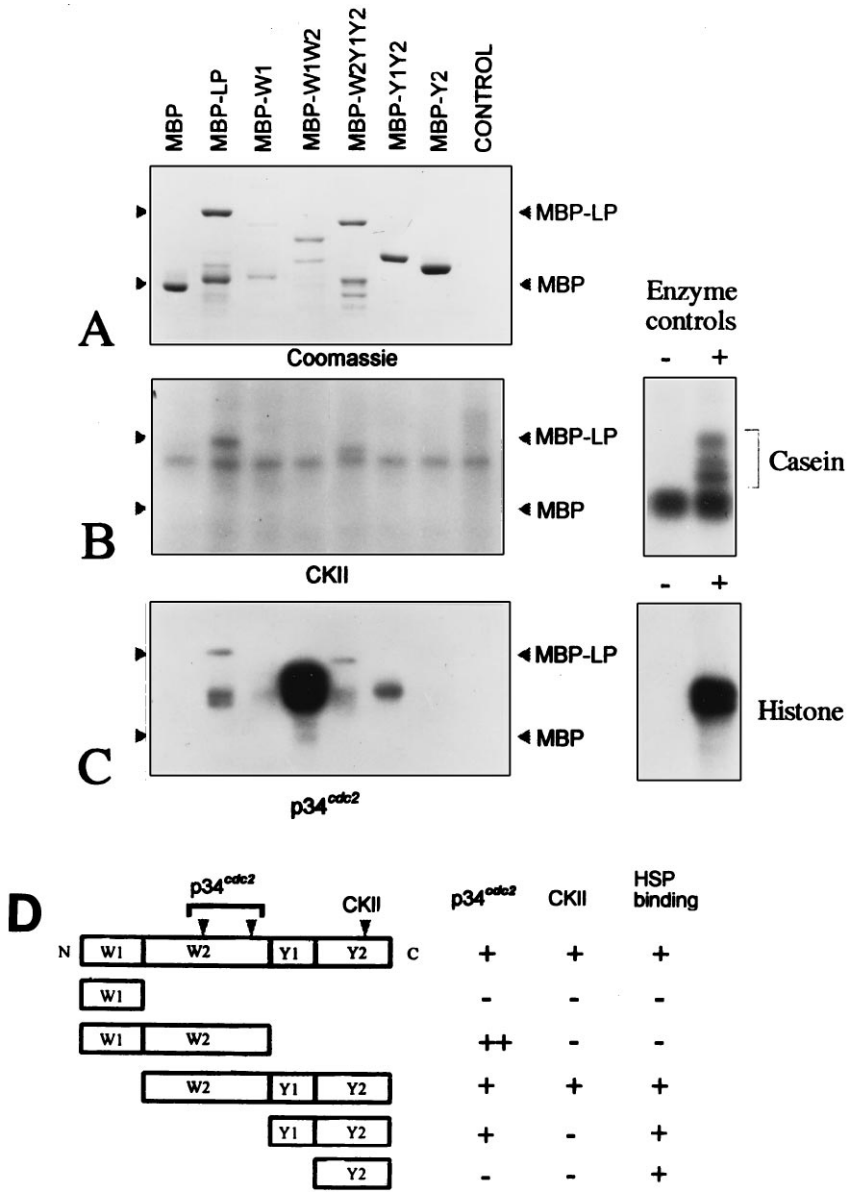

FIG. 6. In vitro phosphorylation of EBNA-LP fusion protein substrates with purified CKII and CDK1 ($p34^{cdc2}$) (A) Coomassie blue-stained gel of the purified fusion proteins with EBNA-LP exon deletions used in the kinase assays. (B) CKII phosphorylation of fusion protein MBP-LP and MBP-W2Y1Y1. (C) $p34^{cdc2}$ kinase phosphorylation pattern including hyperphosphorylation of MBP-W1W2. (D) Summary of phosphorylation patterns compared with the heat shock protein (HSP)-binding ability of the same proteins.

phosphorylatable relative to the wild-type EBNA-LP. The Y1Y2 fusion was also a substrate for $p34^{cdc2}$ kinase, but the Y1 sequence alone was not. Since Y1 contains no serines, its addition to the fusion protein must change the Y2 conformation and allow phosphorylation.

DISCUSSION

EBNA-LP is a viral nuclear protein that has been implicated in the immortalization of B cells by virus principally because of the phenotype of deletion mutants of the virus lacking the COOH-terminal Y1Y2 exons. Immortalization efficiency is reduced 10-fold and requires the presence of fibroblast feeder layers. Colonies of proliferating cells grow slowly, and most do not give rise to long-term lymphoblastoid cell lines. There is some evidence that early-passage mutant lines have G_1 delays after release from starvation arrest. By using transient transfection of resting B cells primed by CD21 cross-linking with vectors expressing EBNA-LP along with EBNA2, it has been shown that coexpression lifts the cells out of G_0 and into G_1 as evidenced by cyclin D2 gene expression.

All these studies suggest that EBNA-LP affects cell cycle regulation. Since EBNA-LP was a nuclear phosphoprotein and other nuclear phosphoproteins involved in cell cycle regulation have cell cycle-specific patterns of phosphorylation, it was reasonable to expect that if EBNA-LP were involved in cell cycle regulation, then it, too, might show cell-cycle-stage-specific phosphorylation. An analysis of stage-specific phosphorylation could also provide clues to function, which thus far remains unknown. Our first task was to identify conditions which could

be used to enrich cell populations in various stages of the cell cycle. While drug arrests with HU or aphidicolin were adequate for accumulating G_1-enriched cells, G_2 enrichment required releasing a G_1/S-blocked population into a G_2 block. Cells handled in this way tend to be fragile and may break up during subsequent processing for cytofluorometric analysis. Although this observation indicates that there are drug-related effects on these cells, block release experiments with HU and nocodazole alone suggest that the phosphorylation patterns of EBNA-LP are not artifactual. In addition, cell-cycle-stage-specific phosphorylation of the Rb protein, which has been studied in other systems (19), showed expected phosphorylation patterns in G_1- and G_2-arrested cells (data not shown).

Western blots of EBNA-LP from cells enriched for G_1 or G_2 populations or at various times after release from blocks showed that unlike some cell cycle regulatory molecules (e.g., cyclins), EBNA-LP was relatively stable and present at similar concentrations in all samples examined. There was a noticeable change in the migration of the protein in G_2, since the band seemed to broaden in some gels and resolve into two discernible species in high-resolution gels of cells arrested at the G_2/M boundary. Corresponding bands in $^{32}PO_4^{3-}$ immunoprecipitates contained phosphoserine. Phosphorimager analysis of the phosphoserine content indicated that EBNA-LP was hypophosphorylated in late G_1 and hyperphosphorylated in G_2. The evidence collected strongly indicated that hyperphosphorylation accounted for the slowly migrating species of the protein. Transition through the M phase corresponded to a disappearance of the hyperphosphorylated form of the protein. It is tempting to conclude from the biological evidence suggesting G_1-related effects for EBNA-LP expression and by analogy to the functional effects of the phosphorylation pattern of Rb (which EBNA-LP mimics) that hypophosphorylated EBNA-LP is active and hyperphosphorylated EBNA-LP is inactive.

Further elucidation of the functional significance of phosphorylation of EBNA-LP will require identification of the enzymes responsible for phosphorylation and the in vivo serine residues involved. The immunoprecipitates of EBNA-LP do not possess endogenous protein kinase activity (not shown). Nevertheless, potential phosphorylation sites for two candidate enzymes (CKII and $p34^{cdc2}$ kinase) are present in the EBNA-LP sequence, and the protein is a substrate for phosphorylation by these enzymes. CKII phosphorylates EBNA-LP on serine, and phosphorylation is dependent upon the presence of the COOH-terminal unique region where the consensus CKII site is located. This strongly implicates S-100 as a potential phosphorylation site in vivo. Two other EBV proteins, EBNA2 and ZEBRA, are also CKII phosphorylation targets (13, 24), although no EBV-specific consensus among these proteins is apparent. The capability of $p34^{cdc2}$ kinase to phosphorylate EBNA-LP on serines was also demonstrated. The behavior of fusions containing this site alone in the CKII kinase assay was unpredictable. The larger proteins with Y1Y2 termini were phosphorylated, but the shorter proteins were not. In a curious paradox, the Y1Y2 sequence also had an effect on phosphorylation by the $p34^{cdc2}$ kinase. After removal of the Y1Y2 COOH end, the W1W2 repeat sequence became hyperphosphorylated in the in vitro kinase assays. The Y1Y2 and Y2 fusion proteins might be considered to behave anamolously because they adopt unnatural configurations; however, in one other assay of their function (heat shock protein binding in vitro), these truncated products behave like the full-length fusion protein. It will be interesting to determine if mutant proteins have similar characteristics in vivo. In any event, there are two potential serines (S-33 and S-62) that match $p34^{cdc2}$ consensus sites within the 44 residues of the W2 exon to which the phosphorylation has been mapped. Either or both sites could be involved in vivo and could be responsible for the iteration pattern of spots previously described for isoelectric focused EBNA-LP (38, 40). Peptide mapping and mutational analyses will determine if these sites are also phosphorylated in vivo and if the posttranslated modifications are critical for function.

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