JOURNAL OF VIROLOGY, May 1995, p. 3108–3116 Vol. 69, No. 5
0022-538X/95/$04.00+0


# Epstein-Barr Virus Nuclear Protein 3C Modulates Transcription through Interaction with the Sequence-Specific DNA-Binding Protein Jκ

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Received 29 November 1994/Accepted 9 February 1995

**The Epstein-Barr virus (EBV) nuclear protein 3C (EBNA 3C) is essential for EBV-mediated transformation of primary B lymphocytes, is turned on by EBNA 2, and regulates transcription of some of the viral and cellular genes which are regulated by EBNA 2. EBNA 2 is targeted to response elements by binding to the DNA sequence-specific, transcriptional repressor protein Jκ. We now show that EBNA 3C also binds to Jκ. EBNA 3C causes Jκ to not bind DNA or EBNA 2. Jκ DNA binding activity in EBV-transformed lymphoblastoid cells is consequently reduced. More than 10% of the EBNA 3C coimmunoprecipitated with Jκ from extracts of non-EBV-infected B lymphoblasts that had been stably converted to EBNA 3C expression. EBNA 3C in nuclear extracts from these cells (or in vitro-translated EBNA 3C) prevented Jκ from interacting with a high-affinity DNA binding site. Under conditions of transient overexpression in B lymphoblasts, EBNA 2 and EBNA 3C associated with Jκ and less EBNA 2 associated with Jκ when EBNA 3C was coexpressed in the same cell. EBNA 3C had no effect on the activity of a −512/+40 LMP1 promoter-CAT reporter construct that has two upstream Jκ sites, but it did inhibit EBNA 2 transactivation of this promoter. These data are compatible with a role for EBNA 3C as a "feedback" down modulator of EBNA 2-mediated transactivation. EBNA 3C could, in theory, also activate transcription by inhibiting the interaction of the Jκ repressor with its cognate DNA. The interaction of two viral transcriptional regulators with the same cell protein may reflect an unusually high level of complexity or stringency in target gene regulation.**

Epstein-Barr virus (EBV) establishes a latent infection in human B lymphocytes and induces their proliferation, in vitro or in vivo (for a review see reference 41). These growth-transformed B lymphocytes express a characteristic repertoire of viral nuclear proteins (EBNAs) and integral membrane proteins (LMPs) which maintain latent infection and cause the infected lymphocyte to perpetually proliferate (for a review see reference 26). EBV recombinant-based genetic analyses indicate that EBNA LP, 2, 3A, and 3C and LMP1 are important in primary B-lymphocyte growth transformation in vitro (9, 10, 20, 22, 25, 34, 51, 53; for a review see reference 26). EBNA 1 is also important for transformation, since it binds to a site in the EBV genome, thereby enhancing transcription and enabling the site to serve as an origin for S-phase replication by cellular DNA polymerase (47, 48, 63). Most of the rest of the EBV genome, including genes that in latent infection encode EBNA 3B, LMP 2A and 2B, the BARFO RNA, and the EBERs, is dispensable for B-lymphocyte immortalization (30–32, 42, 43, 49, 50).

This report focuses on EBNA 3C (40), which appears to partially overlap in function with EBNA 2, a gene whose role in EBV infection is considerably better understood than that of EBNA 3C. In the transformation of primary B lymphocytes to lymphoblastoid cell lines (LCLs), EBNA 2 is one of the first two viral genes expressed (2, 3, 44) and is a key regulator of viral and cellular gene transcription (1, 13, 14, 28, 59–61). EBNA 2 up regulates transcription of the EBNA 3s, of EBNA 1, and of the LMPs, and it also up regulates the transcription of cellular genes, including CD21, CD23, and c-*fgr* (1, 7, 13, 28, 54, 59–61). EBNA 2 is an acidic-type transactivator (8, 52) which recruits cellular transcription factors to specific promoters through its interaction with sequence-specific DNA-binding proteins, including Jκ (18, 21, 24, 36, 65, 66) and PU.1 (24). EBNA 2 interacts with Jκ through a 27-amino-acid domain which includes two tryptophans (18, 62). Mutation of these two tryptophans abrogates the binding of Jκ to EBNA 2 and leads to a loss of EBV transforming activity, indicating the importance of EBNA 2-Jκ interaction in the transformation of primary B lymphocytes (62).

EBNA 3C can up regulate the expression of some of the genes that are up regulated by EBNA 2. EBNA 3C up regulates CD21 expression in non-EBV-infected Burkitt lymphoma (BL) cells (60) and LMP1 expression in Raji cells (4). Raji cells are infected with an EBV that lacks EBNA 3C, and LMP1 expression is down modulated in $G_1$-arrested Raji cells (6). Expression of EBNA 3C in Raji cells up regulates LMP1 expression during $G_1$ arrest (4). The mechanism by which EBNA 3C up regulates CD21 in BL cells and LMP1 expression in $G_1$-arrested Raji cells is obscure. Although the EBNA 3C amino acid sequence includes basic residues and a coiled coil domain reminiscent of cFos- and cJun-type transcription factors, EBNA 3C does not bind DNA specifically (37, 45, 56).

The experiments presented here begin with the surprising observation that stable EBNA 3C expression in BL cell lines results in markedly diminished Jκ activity, as measured by the ability of nuclear extracts to gel shift a probe containing a Jκ cognate sequence. Since Jκ is a key mediator of EBNA 2

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transactivation in EBV-infected cells (18, 21, 29, 36, 62, 65, 66) and is a repressor of transcription in the absence of EBNA 2 (11) an effect of EBNA 3C on Jκ could be an important aspect of EBNA 3C's effects in EBV infection. Further, Jκ is a ubiquitously expressed and evolutionarily conserved gene, essential in *Drosophila melanogaster* embryonic development (15, 16, 19, 46, 55). We have therefore investigated the basis for this surprising effect of EBNA 3C expression on interaction of Jκ with its cognate sequence.

## MATERIALS AND METHODS

**Cell lines, culture conditions, and plasmids.** Raji is a BL cell line infected with an EBV strain in which EBNA 3C is deleted. P3HR-1 is a BL cell line infected with an EBV strain in which EBNA LP and EBNA 2 are deleted, which renders the virus nontransforming (10, 20). P3LCL and B95LCL are LCLs infected with a P3HR-1 virus marker rescued for transformation with EBNA LP and EBNA 2 and with B95-8 virus, respectively. BJAB is an EBV-negative BL cell line (60). Stable cell lines were prepared by transfection of pZipneo EBNA 3A, EBNA 3B, or EBNA 3C or vector into BJAB cells followed by neomycin selection (60). Cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (50 U/ml), and neomycin (200 μg/ml) when necessary. Cultures were incubated at 37°C in 5% $CO_2$ and expanded as appropriate.

Plasmids GST-Jκ[1-48] and GST-Jκ are constructs containing the first 48 amino acids (*Nco*I-to-*Sca*I fragment) and the complete cDNA (*Nco*I-to-*Nco*I fragment) of the Jκ gene isoforms I and III, respectively (18), cloned into the pGex vector. pSG5Flag-Jκ is an *Sma*I-*Bgl*II fragment containing the Jκ isoform III open reading frame cloned into a pSG5Flag vector (unpublished data) at the *Pml*I and *Bgl*II sites. The pSG5-EBNA 2, pSG5Flag-EBNA 2, and pSG5Flag-CAM-Kinase plasmids have been described previously (38, 54, 62). pSG5-EBNA 3C is a construct containing entire EBNA 3C open reading frame cloned into the pSG5 vector.

**Northern (RNA) blot analysis.** A total of $10^8$ cells from each culture were harvested by centrifugation, and total RNA was isolated by using RNAzol B (Tel-Test, Inc.). The cell pellet was resuspended in 20 ml of RNAzol B. Total RNA was harvested by standard procedures (38). Total RNA (15 μg) was electrophoresed on a 1% formaldehyde denaturing gel at 50 V for 12 h, and the RNA was transferred to GeneScreen Plus (NEN-Dupont) (38). The membrane was incubated with [α-$^{32}$P]dCTP-labeled Jκ and GAPDH probes (38).

**Nuclear extracts and EMSAs.** Probes or competitors for use in electrophoretic mobility shift assays (EMSAs) were prepared by using complementary synthetic oligonucleotides and were labeled with the Klenow fragment of DNA polymerase I and [$^{32}$P]dGTP (18, 24). Crude nuclear extract (5 μg), S-Sepharose or DNA affinity column fractions (1 to 5 μl), or programmed reticulocyte lysate (7.5 μl) was used in the EMSA experiments (24).

**Electrophoresis of proteins, immunoblotting, and immunoprecipitations.** Proteins were analyzed on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels, transferred to 0.4-μm-pore-size nitrocellulose filters, and incubated with human sera recognizing the EBV EBNAs (42, 43). Jκ antibody is a rabbit polyclonal antibody against glutathione *S*-transferase GST-Jκ residues 1 to 48 from Jκ isoform I (18, 19). Immunoprecipitations were done by using Flag monoclonal antibody M2 linked to agarose beads (IBI/Kodak) or polyclonal rabbit antibody followed by protein A-Sepharose. Immunoprecipitates were washed six times with radioimmunoprecipitation assay (RIPA) buffer (52) prior to performance of SDS-polyacrylamide gel electrophoresis (PAGE).

**Protein-protein interaction assays.** EBNA 3A, 3B, and 3C and Jκ were translated in vitro (TNT; Promega) from T7 expression plasmids (18, 24, 62) with or without $^{35}$S-Express (NEN-Dupont) (18, 24, 62). The GST-Jκ and GST-EBNA 2 (positions 243 to 337) proteins were purified on glutathione-Sepharose beads (18, 62). Labeled EBNA 3s or Jκ was incubated with glutathione-Sepharose-bound GST-Jκ or GST-EBNA 2 in binding buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 150 mM NaCl, 0.5% Nonidet P-40 [NP-40], 250 mg of bovine serum albumin [BSA] per ml, 2 mM dithiothreitol, 10 μg of aprotinin per ml, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin) at 4°C for 30 min and centrifuged briefly. After the pellet was washed three times in binding buffer, proteins were eluted in SDS-PAGE lysis buffer and electrophoresed on SDS–8% PAGE gels (18, 42, 43). For competition experiments, unlabeled in vitro-translated EBNA 3A or EBNA 3C was added as a lysate to labeled in vitro-translated Jκ for 15 min on ice prior to addition of the mixture to GST-EBNA 2 (positions 243 to 337) beads.

**Transfections.** BJAB cells ($10^6$) were transfected with 50 μg of pSG5Flag-Jκ, pSG5Flag-EBNA 2, pSG5-EBNA 2, or pSG5-EBNA 3C in 400 μl of RPMI 1640 containing 10% fetal bovine serum by using a Bio-Rad electroporation apparatus at 220 V and 960 μF (18, 42, 43). After electroporation, cells were resuspended in 10 ml of complete medium as described above and incubated at 37°C for 20 h. Cell pellets were resuspended in phosphate-buffered saline, extracted in RIPA buffer on ice for 1 h, and clarified by centrifugation at 4°C for 6 min. The lysate was transferred to a fresh tube, the specific antibody was added, and the mixture was vortexed briefly and incubated at 4°C for 1 h. The protein A-Sepharose or M2 agarose beads (IBI/Kodak) were collected by centrifugation, washed six times in RIPA buffer, resuspended in lysis buffer, and then heated at 100°C for 5 min and fractionated by SDS–8% PAGE followed by Western blotting (immunoblotting).

## RESULTS

**Stable EBNA 3C expression leads to decreased Jκ gel shift activity.** Multiple clones of BJAB non-EBV-infected BL cells that had been converted to neomycin resistance after transfection with the pZipneoSV(X) expression vector or with the expression vector and an EBNA 3A, 3B, or 3C cDNA insert were established. The clones were screened by immunoblotting for EBNA 3A, 3B, or 3C expression by using EBV-immune human serum, and at least four clones expressing each EBNA 3 were studied. There was no apparent difference in the ease of isolating clones that had vector only or those that were expressing EBNA 3A, 3B, or 3C. The growth characteristics of these stable cell lines were also similar.

Jκ-specific EMSA activity was assayed with a Jκ cognate sequence probe and nuclear extracts from various cells lines, including at least four of the BJAB clones expressing EBNA 3A, 3B, or 3C, Raji, and EBV-transformed LCLs (Fig. 1 and data not shown). As expected, nuclear extracts from EBV-infected BL cells (Raji and P3HR-1) or from BJAB, BJAB vector control, BJAB EBNA 3A-converted, or BJAB EBNA 3B-converted cells yielded two major complexes with a labeled LMP2 promoter Jκ site probe (Fig. 1A); the faster EMSA complex (indicated by an arrow) comigrates with a complex produced by in vitro-translated Jκ cDNA (18). This complex was inhibited with a minimal Jκ binding site (lane O34) but not with an oligonucleotide that was mutated for the core binding site (O910) (18, 24). The more slowly migrating complex in all lanes just above the Jκ complex was due to a different protein that binds to an adjacent site in this probe (18). Surprisingly, nuclear extracts from all clones of BJAB cells which had been converted to stable EBNA 3C expression had significantly less or no Jκ EMSA activity (Fig. 1, lanes E3C.1 to E3C.7). No novel EMSA supershift appeared with BJAB EBNA 3C nuclear extracts which would have been indicative of a complex of EBNA 3C, Jκ, and probe. This is in contrast to the case with EBNA 2, which can supershift a Jκ-probe complex (18, 21, 29, 62, 66). The level of EBNA 3C expression in the converted BJAB clones was two to threefold higher than that in a typical EBV-transformed LCL as determined by immunoblotting with EBV-immune human serum (Fig. 1B; compare lanes E3C.1 to E3C.7 with lane B95LCL). Importantly, the level of Jκ EMSA activity in LCLs was intermediate between those in BJAB and BJAB EBNA 3C-converted cells, consistent with the intermediate level of EBNA 3C in the LCLs (P3LCL and B95LCL in Fig. 1). Thus, expression of EBNA 3C in BJAB cells or LCLs is associated with less Jκ-specific EMSA activity. The ease of selecting clones with such high-level EBNA 3C expression and the normal growth of these cells with substantially diminished Jκ gel shift activity are indications that normal levels of Jκ activity are not critical to BL cell growth.

**EBNA 3C does not affect Jκ RNA levels.** To determine whether EBNA 3C alters Jκ EMSA activity by decreasing Jκ transcription, the level of Jκ mRNA in EBNA 3C-converted BJAB cells was compared with those in controls. Jκ mRNA was detected in all BL cell lines and LCLs by Northern blotting of total RNA (Fig. 2). While the ratio of Jκ mRNAs to GAPDH mRNAs varied as much as sixfold among the cell lines, EBNA 3C-converted BJAB cell lines (Fig. 2, lanes 9 to

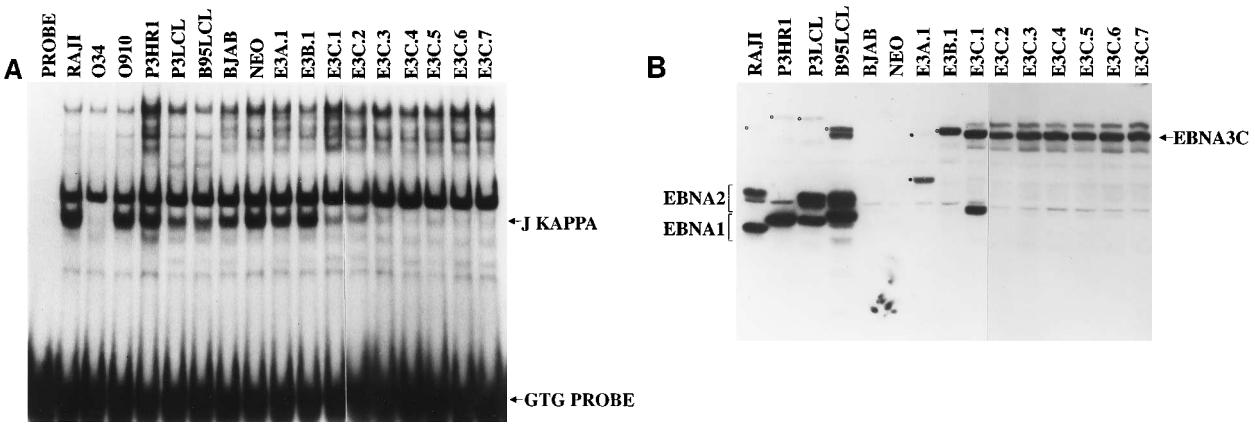


FIG. 1. (A) EMSA for Jκ activity in nuclear extracts of cell lines which express EBNA 3C or controls. Nuclear extracts (5 μg) from each cell line were used for EMSA (18, 24, 62). The LMP2 probe (CTCGCGACTC**GTGGGAA**AATGGGCGGAAGGGCA [5, 65]) contained a Jκ binding site (boldface). The specific competitor O34 (GACTC**GTGGGAA**AATGGGC) also contained a Jκ binding site, while the nonspecific competitor O910 was mutated at the Jκ site (GACTC**TCTGGAA**AAT GGGC). The positions of the Jκ EMSA complex and the unbound probe are indicated. The lanes indicate the extract used in each reaction. Raji is a BL line infected with a type 1 EBV in which EBNA 3C is deleted. The O34 and O910 lanes contain Raji extract incubated with probe and a 100-fold excess of specific (O34) or nonspecific (O910) competitor. P3HR-1 is a BL line infected with a type 2 EBV in which EBNA LP and EBNA 2 are deleted. P3LCL is a recently derived LCL infected with a P3HR-1 recombinant with wild-type EBNALP and EBNA 2 (26). B95-8 LCL (B95LCL) is a recently derived LCL infected with the B95-8 EBV type 1 strain. BJAB is an EBV-negative BL cell line. NEO is pZipneo-converted BJAB, while E3A.1, E3B.1, and E3C.1 to E3C.7 are BJAB cell lines converted to EBNA 3A, 3B, or 3C expression. (B) Immunoblot with EBV-immune human serum which detects all of the EBNAs in EBV type 1 strains (42, 43). Nuclear extracts (50 μg) were resolved on an SDS–8% PAGE gel, and EBNA proteins were detected (42, 43). The open circles indicate the position of EBNA 3B, and the closed circles indicate that of EBNA 3A.

18) and other cell lines (Fig. 2, lanes 1 to 8) exhibited the same range of variability and did not differ overall. Thus, the decrease in Jκ gel shift activity observed with EBNA 3C-converted BJAB cells was not due to an effect of EBNA 3C on Jκ mRNA abundance. The variability observed in the relative levels of Jκ mRNA abundance was probably due to the large size of Jκ mRNA and to variation in the quality of the RNA preparations.

**EBNA 3C directly inhibits Jκ EMSA activity in vitro.** To investigate whether EBNA 3C directly affects the Jκ EMSA shift, in vitro-translated EBNA 3C or in vitro-translated luciferase (as a control) was mixed with Jκ-enriched S-Sepharose column fraction (CF) prepared from a Louckes nuclear extract or with BJAB nuclear extract and the Jκ EMSA activity was assayed (Fig. 3A). The Louckes CF contained a slightly higher concentration of Jκ activity than the BJAB extract. Despite the high concentration of Jκ in the Louckes CF, 10 μl of in vitro-translated EBNA 3C almost completely inhibited the Louckes CF Jκ probe shift (lanes E3C10), while the same amount of in vitro-translated luciferase had no effect (Fig. 3A, lane LUC10). No new supershifted band appeared, as might be anticipated for a Jκ-probe-EBNA 3C complex. Instead, the amount of free probe appeared to be increased in the lane to which 10 μl of EBNA 3C was added, which is compatible with the possibility that EBNA 3C directly affects the ability of Jκ to interact with its cognate probe. Similar results were obtained with in vitro-translated EBNA 3C and the BJAB nuclear extract. Curiously, the slightly more slowly migrating complex evident in nuclear extract gel shifts appeared to be affected by 10 μl of luciferase or EBNA 3C in vitro translation product, while the Jκ shift was specifically affected only by the EBNA 3C in vitro translation product.

The impact of EBNA 3C on the Jκ shift was also readily apparent when in vitro-translated Jκ was used instead of Jκ-enriched CF (Fig. 3A, right panel). In this experiment, 10 μl of in vitro-translated luciferase or 1 μl of in vitro-translated EBNA 3C had no effect on the Jκ gel shift, while 10 μl of in vitro-translated EBNA 3C nearly completely blocked the Jκ gel shift. The amount of free probe also appeared to increase in the lane containing 10 μl of EBNA.

To more easily observe the effect of EBNA 3C in disrupting the ability of Jκ to shift probe, a 1/10 amount of probe was incubated with an amount of Jκ-enriched CF that resulted in the specific shift of most of the probe (Fig. 3B). The amounts of free probe and of Jκ-shifted probe in each lane were quantitated with a PhosphorImager. Increasing amounts of EBNA 3C in vitro translation product (Fig. 3B, lanes 5 through 8) resulted in less Jκ-shifted probe (80, 70, 40, and 15 counts) and more free probe (50, 90, 120, and 140 counts), while similar additions of luciferase in vitro translation product had no effect on the amount of Jκ-shifted probe. This experiment demonstrates that EBNA 3C directly interferes with the ability of Jκ to interact with its cognate sequence.

**EBNA 3C and Jκ form a stable complex in vitro.** To evaluate whether EBNA 3C and Jκ directly interact, a bacterial GST-Jκ

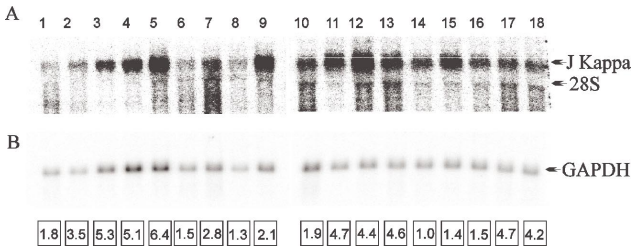


FIG. 2. Northern blots with Jκ or GAPDH cDNA probes and 12 μg of total RNA from EBNA 3C-converted or control cell lines. The numbers in the boxes indicate the ratios of Jκ to GAPDH as determined by using Imagequant Software (PhosphorImager; Molecular Dynamics Corp.). Lanes 1 to 8 contain RNA from P3HR-1 c16, BJAB, B95-8 LCL, P3LCL, Raji, and BJAB pZipneo vector control cells and BJAB cells expressing EBNA 3A or 3B, respectively. Lanes 9 to 18 contain BJAB clones expressing EBNA 3C (E3C.1 to E3C.10). The position of the Jκ transcripts is indicated by the arrow above the 28S rRNA.

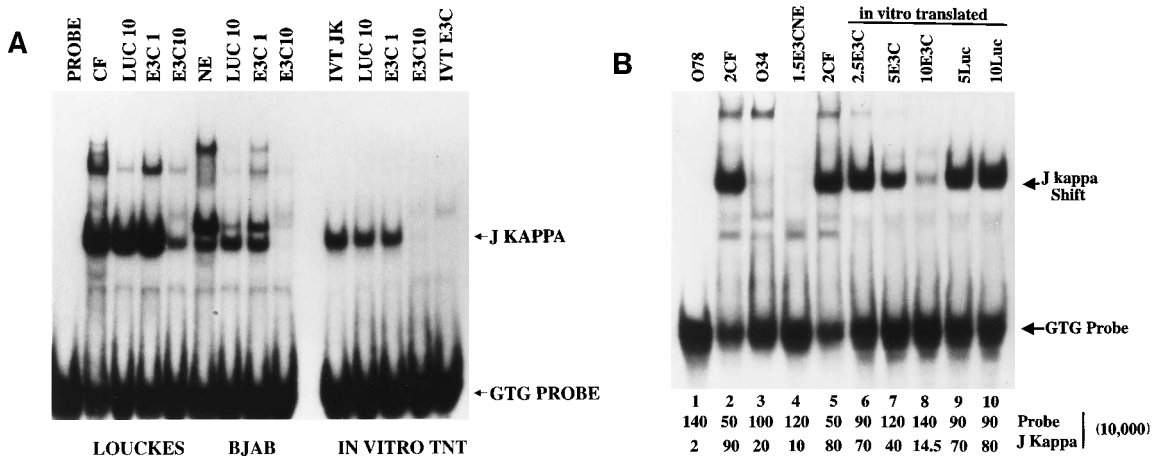


FIG. 3. EBNA 3C inhibits Jκ DNA binding activity. (A) EMSA with an LMP2 promoter-derived probe and Jκ-enriched CF from EBV-negative Louckes BL cells, nuclear extract from BJAB BL cells (5 μg per lane [18]), or in vitro-translated Jκ (right panel [18]). The lanes, from left to right, contain probe, probe plus CF, probe plus CF plus 10 μl of in vitro-translated luciferase (LUC10), and probe plus CF plus 1 and 10 μl of in vitro-translated EBNA 3C, respectively (E3C1 and E3C10). The set is repeated with BJAB nuclear extracts (NE) and in the right panel with 10 μl of in vitro-translated Jκ (IVT Jκ) in each lane. Lane IVTE3C in the right panel contains in vitro-translated EBNA 3C added to the probe without any Jκ. (B) LMP2 probe (lane 1) was incubated with CF (lane 2); with CF plus a specific competitor (O34; lane 3); with 20% of the usual amount of nuclear extract from BJAB clone E3C 1.10 (lane 4); with CF (lane 5); with CF plus 2.5, 5, or 10 μl of in vitro-translated EBNA 3C (lanes 6 to 8); or with CF plus 5 or 10 μl of in vitro-translated luciferase (lanes 9 and 10). Arrows on the right include free LMP2 probe and the Jκ EMSA complex. The amounts of free and Jκ-bound probe were quantitated with a PhosphorImager (Molecular Dynamics Corp.), and the estimates (in counts) are displayed below the gel.

fusion protein was adsorbed to glutathione-Sepharose and then incubated with in vitro-translated EBNA 3A, 3B, or 3C. The fractions of in vitro-translated proteins that adsorbed to the GST-Jκ fusion were analyzed by SDS-PAGE (Fig. 4A). More than 25% of the in vitro-translated EBNA 3C bound to Jκ, while no binding to GST control protein was observed. In vitro-translated EBNA 3A did not bind to GST-Jκ or to GST. Surprisingly, in vitro-translated EBNA 3B significantly (more than 20%) bound to GST-Jκ, and the binding to GST-Jκ was much greater than that to the GST control. These data indicate that EBNA 3C binds efficiently, specifically, and directly to bacterially expressed and presumably unmodified Jκ in vitro and that EBNA 3B can also interact with Jκ in vitro.

**EBNA 3C associates with Jκ in BL cells and can inhibit the association of Jκ with EBNA 2.** Coimmunoprecipitation experiments were performed with BL cells transiently or stably expressing EBNA 3C to evaluate the association of EBNA 3C and Jκ in vivo. In the first series of experiments, BJAB cells were transiently cotransfected with an EBNA 3C expression vector and amino-terminally epitope-tagged (Flag; IBI/Kodak) Jκ, Flag-EBNA 2, or Flag-CAM kinase IV control (38) expression vectors. Monoclonal antibody to the Flag epitope was used to immunoprecipitate the Flag-tagged fusion proteins from nonionic detergent-containing cell extracts, and the presence of EBNA 3C and EBNA 2 in the immunoprecipitates was detected by immunoblotting with monoclonal antibodies (EBNA 3C in Fig. 4B and EBNA 2 in Fig. 4C; EBNA 2 is indicated by dots and is the band just under the monomeric immunoglobulin G from the anti-FLAG epitope monoclonal antibody). Flag-Jκ associated with EBNA 3C, as detected on immunoblots with A10 monoclonal antibody to EBNA 3C (lanes 1, 4, and 5), while Flag-CAM kinase did not associate with EBNA 3C. Surprisingly, cotransfection of Flag-EBNA 2 and EBNA 3C constructs followed by Flag immunoprecipitation and immunoblotting for EBNA 2 and 3C resulted in coimmunoprecipitation of EBNA 3C with EBNA 2 (lane 2), although the amount of EBNA 3C brought down by Flag-EBNA 2 was slightly less than that brought down by Flag-Jκ (lanes 1, 4, and 5). Cotransfection of Flag-Jκ and EBNA 2 into

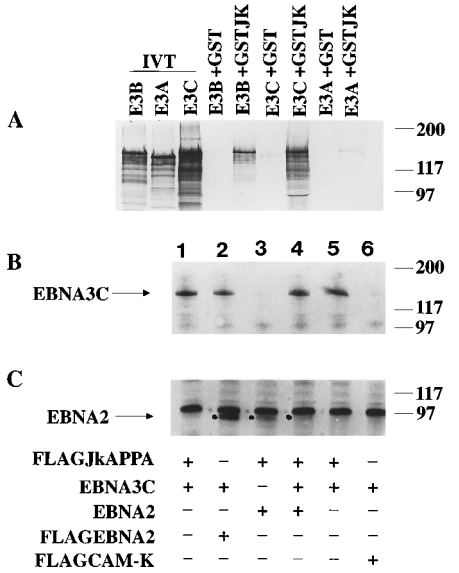


FIG. 4. EBNA 3C interacts with Jκ in vitro and in vivo. (A) EBNA 3B (E3B), EBNA 3A (E3A), and EBNA 3C (E3C) were translated in vitro with [$^{35}$S]methionine, and 7.5 μl of in vitro-translated (IVT) protein was incubated with 7.5 μl of GST or GST-Jκ fusion protein bound to beads. After 30 min of incubation at 4°C followed by three washes, bound protein was eluted in SDS sample buffer and analyzed on an SDS–8% PAGE gel in comparison with 7.5 μl of in vitro translation product. (B and C) In vivo association of Jκ with EBNA 3C. A Flag epitope was cloned at the amino-terminal end of the Jκ (FLAGJκ), EBNA 2 (FLAGEBNA2 [62]), or CAM kinase IV (FLAGCAM-K [38]) open reading frame in the pSG5 expression vector. BJAB cells were transfected with FLAGJκ and EBNA 3C expression vectors (lanes 1 and 5); with FLAGEBNA2 and EBNA 3C expression vectors (lane 2); with FLAGJκ and EBNA 2 expression vectors (lane 3); with FLAGJκ, EBNA 2, and EBNA 3C expression vectors (lane 4); or with FLAGCAM-K and EBNA 3C expression vectors (lane 6). After 24 h, the cells were lysed in 1% NP-40 buffer and adsorbed with 50 μl of anti-Flag M2 affinity matrix (IBI/Kodak chemicals) at 4°C for 12 h. The beads were washed six times in 1% NP-40, and half of the sample was electrophoresed on an SDS–8% PAGE gel. The gel was transferred to a nitrocellulose membrane and blotted for EBNA 3C (B) or EBNA 2 (C) by using A10 or PE2 monoclonal antibodies (35, 64) and alkaline phosphatase-conjugated goat anti-mouse antibody. The dark band in panel C above the position of EBNA 2 is M2 monomeric immunoglobulin G and the closed circles indicate the position of EBNA 2 in the lighter band below.

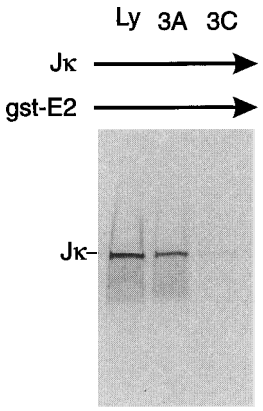


FIG. 5. In vitro-translated EBNA 3C specifically blocks the binding of in vitro-translated $^{35}$S-labeled Jκ to GST-EBNA 2 (gst-E2). $^{35}$S-labeled Jκ was incubated with in vitro-translated EBNA 3A (3A) or EBNA 3C (3C) or with control reticulocyte lysate (Ly) and with a GST-EBNA 2 (positions 243 to 337) fusion protein. The beads were washed with 1% NP-40 buffer, and bound protein was analyzed on an SDS–8% PAGE gel.

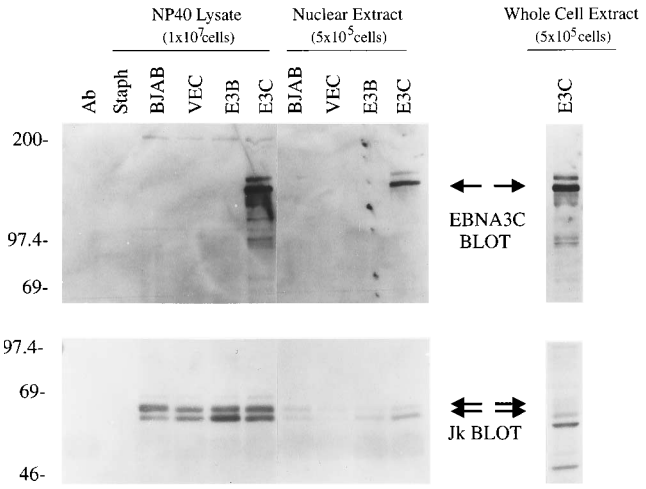


FIG. 6. EBNA 3C coimmunoprecipitates with Jκ from BJAB E3C.7 cells. Jκ was immunoprecipitated from an isotonic NaCl–1% NP-40 cell lysate of $10^7$ cells or from a 0.4 M NaCl nuclear extract by using a rabbit anti-Jκ antibody. Immunoprecipitates were resolved by SDS–8% PAGE and transferred to nitrocellulose. A whole-cell SDS lysate ($5 \times 10^5$ cells) was included as a control for total cellular EBNA 3C or Jκ. The lanes marked Ab and staph contain NP-40 lysate incubated with rabbit antibody and staph protein A alone, respectively. Lanes BJAB, Vec, and E3B are precipitations from BJAB cells, vector-transfected BJAB cells, and EBNA 3B-converted BJAB cells, respectively, while the lanes marked E3C are immunoprecipitations from BJAB E3C.7 cells. Membranes were blocked in 5% BSA, probed with a 1:100 dilution of rabbit anti-human Jκ antibody, and subjected to $^{125}$I-protein A detection and autoradiography. Subsequently, membranes were reblocked, probed with A10 antibody to EBNA 3C followed by a 1:5,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse antibody, and subjected to ECL detection. The positions of Jκ, EBNA 3C, and molecular size standards (in kilodaltons) are indicated.

BJAB cells followed by immunoprecipitation with Flag antibody resulted in coimmunoprecipitation of EBNA 2 with Flag-Jκ (lane 3), as expected from previous in vitro data (18, 29, 62, 66). When EBNA 3C was expressed in the same cells as EBNA 2, less EBNA 2 was coimmunoprecipitated with Flag-Jκ (compare lanes 3 and 4 in panel C), although the amount of EBNA 3C coimmunoprecipitated with Flag-Jκ was not decreased by EBNA 2 coexpression (compare lanes 4 and 5 in panel B). This indicates that EBNA 3C can inhibit the interaction of Jκ with EBNA 2 as well as the interaction of Jκ with probe. These experiments indicate that Jκ can associate with EBNA 3C or EBNA 2. Furthermore, when both EBNA 3C and EBNA 2 are expressed in the same cell, Jκ can associate with either protein, although EBNA 3C can also compete with EBNA 2 for Jκ interaction. Moreover, EBNA 2, overexpressed in a cell, can associate with EBNA 3C, possibly in a large complex with two or more Jκ molecules.

The ability of EBNA 3C to compete with EBNA 2 for Jκ association was further investigated by incubating GST-EBNA 2 with in vitro-translated labeled Jκ and in vitro-translated EBNA 3A or 3C (Fig. 5). Very little labeled Jκ bound to GST-EBNA 2 in the presence of in vitro-translated EBNA 3C, while in vitro-translated EBNA 3A had no effect on the binding of the labeled Jκ to GST-EBNA 2.

The association of EBNA 3C with Jκ in vivo in the EBNA 3C-stably converted BJAB cells was further substantiated by using a polyclonal antibody developed against residues 1 to 48 of Jκ. The antiserum precipitated 10 to 20% of the Jκ from NP-40-lysed cell extracts or from 0.4 M NaCl nuclear extracts (compare the Jκ immunoblot of a whole-cell extract with the Jκ immunoblot of a Jκ antibody immunoprecipitation from the nuclear extract of the same number of cells or the immunoprecipitation of the NP-40 lysate of 20 times as many cells in Fig. 6). As expected from the earlier Northern blotting data, the abundance of Jκ in the immunoprecipitates from EBNA 3C-expressing BJAB cells was not different from that in immunoprecipitates from BJAB cells, from vector-only BJAB cells, or from EBNA 3B-expressing BJAB cells (Fig. 6). The Jκ antiserum immunoprecipitations from BJAB cells expressing EBNA 3C brought down a fraction of the total cellular EBNA 3C that was similar to the immunoprecipitated fraction of total cellular Jκ (Fig. 6). In the experiment with $10^7$ cells, antibody was limiting and about 50% of the Jκ remained in the supernatant after immunoprecipitation (data not shown). Since the immunoprecipitation was not in antibody excess and only 50% of the Jκ in the NP-40 lysate was precipitated, at least 20% of the cellular EBNA 3C was associated with Jκ in this lysate. These data clearly demonstrate that a significant fraction of the EBNA 3C is stably associated with Jκ in lymphocytes.

**EBNA 3C modulates EBNA 2 transactivation of the LMP1 promoter in BL cells.** Given the physical association of EBNA 3C with the Jκ protein and its effect on Jκ DNA binding and EBNA 2 interaction, EBNA 3C could effect EBNA 2 transactivation of target promoters. This was evaluated by using a −512/+40 LMP1 promoter-CAT construct in BJAB cells (54) (Fig. 7). Expression of EBNA 3C had no effect on basal chloramphenicol acetyltransferase (CAT) expression when cotransfected with a −512/+40 LMP1 promoter-CAT construct. As expected, cotransfection of EBNA 2 with the −512/+40 LMP1 promoter-CAT construct resulted in approximately ninefold transactivation. EBNA 3C competitively inhibited the EBNA 2 transactivation in a dose-dependent fashion when the two proteins were coexpressed in BJAB cells along with −512/+40 LMP1 promoter-CAT (Fig. 7). The highest levels of cotransfected EBNA 3C expression vector resulted in complete inhibition of EBNA 2-mediated transactivation. A β-galactosidase expression plasmid was included in all transfections to evaluate potential differences in transfection efficiency or toxicity. No substantial differences in β-galactosidase expression were observed in any of the transfections, indicating an absence of significant toxicity. Thus, EBNA 3C can interfere with EBNA 2-specific transactivation without having generalized effects on transcription.

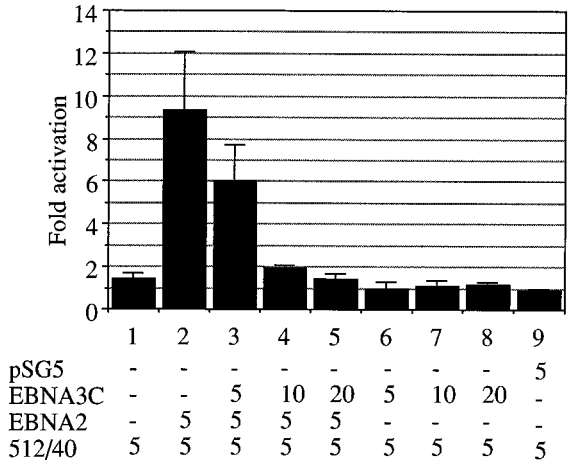


FIG. 7. EBNA 3C down regulates EBNA 2 transactivation of the LMP1 promoter. EBNA 2 transactivates a −512/+40 LMP1 promoter-CAT reporter construct (33, 54). EBNA 3C does not transactivate this construct, but it down regulates EBNA 2 transactivation. BJAB cells were transfected with the −512/+40 LMP1 promoter-CAT reporter alone (lane 1) or with the −512/+40 LMP1 promoter-CAT reporter and an EBNA 2 expression vector (lane 2); the EBNA 2 expression vector and 5, 10, or 20 μg of an EBNA 3C expression vector (lanes 3 to 5); 5, 10, or 20 μg of an EBNA 3C expression vector (lanes 6 to 8); or pSG5 vector (10). Cells were harvested and CAT assays were done at 24 h posttransfection (18, 24, 32). The results shown are the averages of those from three different experiments.

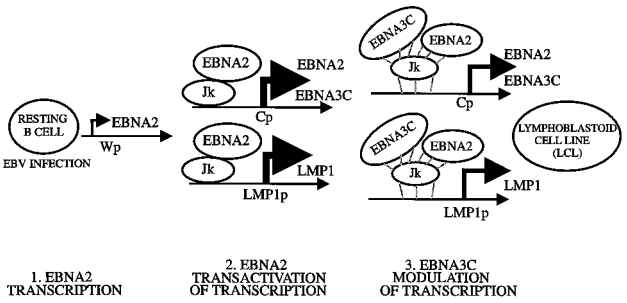


FIG. 8. The role of EBNA 3C modulation of Jκ activity in EBV infection. EBV infects resting B lymphocytes. The Wp EBNA promoter is constitutively active, causing transcription of EBNA LP and EBNA 2. EBNA 2 activates the Cp EBNA promoter further upstream and the LMP promoters. The EBNA transcripts now extend past the EBNA 2 polyadenylation site and terminate after the EBNA 1 polyadenylation site. EBNA LP, EBNA 2, EBNA 3A, EBNA 3B, EBNA 3C, and EBNA 1 mRNAs are now processed from the longer transcript. EBNA 1 binds to ori p, which is 3 kbp upstream of the Cp promoter and 4 kbp upstream of the LMP1 promoter, further increasing transcription. EBNA 3C binds to Jκ and modulates the activity of the Cp and LMP1 promoters (as well as cellular promoters having Jκ sites) to prevent the production of toxic levels of LMP1 and EBNAs.

## DISCUSSION

These experiments demonstrate that EBNA 3C and Jκ interact in vitro and in vivo and that EBNA 3C binding to Jκ can inhibit the interactions of Jκ with its cognate sequence or with EBNA 2. These biochemical interactions are likely to be fundamentally important in our ultimate understanding of the mechanisms by which EBNA 3C and EBNA 2 cooperate in the regulation of viral and cellular gene expression and participate in the establishment and maintenance of primary B-lymphocyte growth transformation. In opening this new aspect of the role of EBNA 3C and Jκ in EBV-mediated growth transformation, these experiments enable the formulation of testable models. However, a full understanding of the precise biochemical and physiological interactions among EBNA 3C, Jκ, and EBNA 2 in regulating EBV and cellular gene expression will require substantial further investigation of the interactions among these proteins in LCLs.

One simple model that is consistent with the available data is that EBNA 3C biochemically interacts with Jκ to down modulate the up regulatory effects of EBNA 2 and to up regulate the repressive effects that Jκ exerts when it is bound to DNA and not associated with EBNA 2. EBNA 3C down modulation of the activating effects of EBNA 2 is likely to be important in the establishment of growth transforming latent B-lymphocyte infection (Fig. 8). In the establishment of latent infection, EBNA 2 and EBNA LP are first expressed under the control of the Wp promoter (2, 3, 44). EBNA 2 then transactivates the Cp, LMP1, and LMP2 promoters as well as cellular promoters that have Jκ sites (18, 21, 23, 24, 59–61, 65, 66). Activation of the Cp promoter is associated with its turning on of EBNA 3A, 3B, and 3C and EBNA 1 mRNA expression, and EBNA 2 and EBNA LP mRNAs also continue to be expressed. EBNA 1 further enhances Cp activity (48). On the basis of the data presented here, we would expect that as EBNA 3C accumulates it would down modulate EBNA 2-mediated transactivation of Cp and LMP1 promoters by blocking interaction of Jκ with its cognate DNA and with EBNA 2. EBNA 3C would thereby prevent runaway transcription from the Cp promoter and modulate the activating effects of EBNA 2 on other promoters, most notably the LMP1 promoter. With appropriate levels of expression of all of the EBNAs and LMPs, the infected cell is able to progress through the first S phase and to continue to proliferate as long as growth conditions are maintained. Precise regulation of LMP1 expression in EBV-transformed cells is critical, since LMP1's physiologic effects require expression near the ordinary LCL level and expression above that level is toxic to cells (57, 58, 60). Thus, an effect of EBNA 3C in down modulating LMP1 up regulation in response to EBNA 2 and EBNA 1 is likely to be important to growth of infected cells.

In Raji cells and possibly in other EBV-infected cells, LMP1 expression wanes in $G_1$ arrest and may be cell cycle regulated (4, 6). Raji is an EBV-infected BL cell line and is presumably not dependent on the endogenous EBV genome for its growth in vitro. The Raji EBV genome lacks EBNA 3C, and growth arrest inhibition of LMP1 expression in Raji cells can be corrected by EBNA 3C expression (4, 6). Our experiments now link EBNA 3C to Jκ, to Jκ-mediated repression, and to Jκ-mediated EBNA 2 transactivation of the LMP1 promoter. The cell cycle dependence of LMP1 expression in Raji cells has not been traced to EBNA 2, but EBNA 2 is a prime candidate, since it undergoes extensive posttranslational modification, including phosphorylation (39). If EBNA 2's activating effects are lost in $G_1$ arrest, Jκ without EBNA 2 would exert a repressive effect (11) on promoters with Jκ sites. On the basis of the data presented here, EBNA 3C is expected to have the net effect of increasing basal expression from promoters with upstream Jκ sites because dislodgement of Jκ from its cognate DNA sites would reverse its repressive effects. Consistent with this model, stable EBNA 3C expression in BJAB cells results in up regulation of the CD21 promoter (60).

Several aspects of the model are incompletely investigated. First, the failure of transiently transfected EBNA 3C expression plasmid to activate the cotransfected LMP1 promoter in BJAB cells appears to be somewhat inconsistent with the expected EBNA 3C reversal of Jκ-mediated repression. However, this result was most likely due to the high copy number of

promoter templates that were taken up by transfected cells and the relatively small number of free Jκ molecules in cells. There may have been an inadequate amount of free Jκ to repress the transfected LMP1 promoter templates and no repression for EBNA 3C cotransfection to reverse. In fact, previous data demonstrating Jκ-mediated repression of the adenovirus pIX promoter in transient transfection assays relied on a Jκ expression vector which was cotransfected with a Jκ binding site-promoter-reporter construct (see Fig. 10 in reference 11). The poor repressive effect of endogenous Jκ in that experiment is consistent with the lack of an effect of EBNA 3C on basal (unstimulated by EBNA 2) LMP1 promoter activity in the experiments reported here.

Second, the effects of EBNA 3C on interactions of Jκ with DNA and EBNA 2 are impressive, but the stoichiometry of the in vivo interactions in EBV-transformed LCLs requires fuller delineation. EBNA 3C markedly reduced Jκ EMSA activity in BJAB cells, in which there is two- to threefold excess EBNA 3C expression, while in LCLs, EBNA 3C moderately reduced Jκ activity. The extent of stable interaction of Jκ with EBNA 2, cognate DNA, and EBNA 3C in LCLs remains to be determined. Other factors may affect the stability of the interaction of Jκ with DNA, EBNA 2, or EBNA 3C. PU.1, a hematopoietic lineage-restricted sequence-specific DNA-binding protein (17, 24, 27, 39), also has a key role in conveying EBNA 2 responsiveness to the LMP1 promoter and is likely to be an important component of the EBNA 2 responsiveness of B-lymphocyte-specific genes (24, 27, 39). The potential effect of PU.1 on the relative stability of EBNA 3C-Jκ-DNA-EBNA 2 interactions requires investigation.

Third, very little is as yet known about the effect of cell cycle or growth arrest on EBV regulation of viral and cellular gene expression. Multiple isoforms of each EBNA are present in cells (39). In vitro assays of function have not been established, and appropriate genetic analyses to evaluate the roles of specific phosphorylation sites in EBNA activity have not been done. The data reported here lay the groundwork for examining the effects of cell cycle and growth arrest on the interactions of Jκ with cognate DNA, EBNA 2, and EBNA 3C.

Fourth, Jκ is an important mediator not only of EBV and adenovirus transcription (11), but also of *Drosophila* development. In *D. melanogaster* Jκ is genetically linked to the Hairless, Notch, and Wingless pathways (15, 46). The Notch protein interacts directly with Jκ through its carboxy-terminal cytoplasmic domain, and ligation of the Notch extracellular domain effects Jκ nuclear localization (15). Human Notch (Tan-1) has been implicated in T-lymphocyte malignancy (12). Furthermore, a search of the human genome database reveals one or more high-affinity Jκ sites upstream of many genes that are important in cell growth and differentiation (reference 18 and unpublished data). Moreover, a homozygous null mutation in Jκ in *D. melanogaster* is lethal in embryonic development (15, 16). Thus, Jκ-mediated transcriptional repression, EBNA 2 activation, and EBNA 3C modulation of Jκ and of EBNA 2 are all likely to be important in the complex regulation of EBV promoters and of a broad repertoire of cellular promoters. Given the large number of cellular promoters with upstream Jκ sites and the extent to which EBNA 3C overexpression in BJAB cells eradicates Jκ gel shift activity, the lack of significant changes in BJAB cell growth is quite surprising, and it probably reflects the complexity of regulation of many of these promoters. In fact, the CD23 promoter is not significantly up regulated by EBNA 3C expression in BJAB cells even though it has Jκ sites and can be up regulated by EBNA 2 (60).

The interaction of EBNA 3C and EBNA 2 with Jκ is an unusual example in which two viral transcriptional regulators interact with the same cellular protein. As described above, regulation through Jκ may even be more complex because of the potential involvement of cellular regulators of Jκ activity. Further, GST-Jκ also interacts with in vitro-translated EBNA 3B. Although EBNA 3B does not appear to have an effect on the interaction of Jκ with its cognate DNA, it could have a role in modulating the interaction of Jκ with EBNA 2 or EBNA 3C. This overlap with EBNA 3C may explain the nonessentiality of EBNA 3B for B-lymphocyte growth transformation in vitro (50). An ancillary role for EBNA 3B in Jκ modulation could be important to the survival, growth, or differentiation of EBV-infected B lymphocytes in vivo.

## ACKNOWLEDGMENTS

We thank Fred Wang for the pZipneo EBNA 3A, 3B, and 3C expression constructs; George Mosialos, Ramana Yalamanchili, and Kenneth Izumi for their helpful discussions; Martin Rowe and Alan Rickinson for the A10 and PE2 monoclonal antibodies to EBNA 3C and EBNA 2; and George Miller for the P3HR-1 cell line.

This work was supported by grant CA47006 from the National Cancer Institute of the USPHS. E.S.R. and B.T. were supported by postdoctoral fellowships from the Cancer Research Institute. S.G. was supported by a fellowship from the National Foundation for Infectious Disease. E.J. was supported by a fellowship from the Howard Hughes Medical Institute.

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