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The Amino-Terminal Domains of Epstein-Barr Virus Nuclear Proteins 3A, 3B, and 3C Interact with RBPJκ

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The ability of Epstein-Barr virus (EBV) latent infection nuclear protein EBNA3C to activate transcription of two EBNA2-responsive genes and to inhibit EBNA2 activation of transcription in transient-transfection assays appears to be due to its ability to interact with RBPJκ, a cell protein that links EBNA2 to its response elements. We now show that EBNA3A and EBNA3B expressed in non-EBV-infected Burkitt tumor lymphoblasts are similar to EBNA3C in binding to glutathione *S*-transferase–RBPJκ in vitro and in coimmunoprecipitating from cell lysates with antibody to RBPJκ. EBNA3A and EBNA3B can also inhibit the interaction of RBPJκ with cognate DNA in vitro. Although EBNA3 open reading frames are each close to 1,000 codons long, EBNA3A amino acids 1 to 138, EBNA3B amino acids 1 to 311, and EBNA3C amino acids 1 to 183 are sufficient for RBPJκ interaction, while EBNA3B amino acids 1 to 109 have less or no binding. The RBPJκ interacting domains overlap with the most highly conserved domain (amino acids 90 to 320) among the EBNA3 proteins. Thus, the EBNA3 gene family appears to have evolved to differentially regulate promoters with RBPJκ binding sites. EBNA2, EBNA3A, and EBNA3C are important in EBV transformation of primary human B lymphocytes. Their interaction with RBPJκ links EBV transformation to the notch signaling pathway and the effects of activated notch in T-cell leukemogenesis.

This report focuses on three nuclear proteins, EBNA3A, EBNA3B, and EBNA3C, encoded by the Epstein-Barr virus (EBV) genome in transformed human B lymphocytes (for a review, see reference 26), and their interaction with RBPJκ, a cellular sequence-specific DNA-binding protein. RBPJκ had initially been mistakenly identified in a search for a protein involved in kappa chain joining (23) and is now known to mediate transcriptional activation by EBNA2 in EBV-transformed B lymphocytes (11, 12, 18, 21, 32, 33) and repression of the pIX promoter in adenovirus-infected cells (6). RBPJκ also mediates transcriptional activation by the notch mutation in sensory and muscle cell development (2, 3, 5, 8, 9, 14, 19, 27). Since activated notch is implicated in T-cell leukemia and notch regulates transcription through interaction with RBPJκ (2, 7, 14), promoters with nearby RBPJκ sites are likely to effect transcription of genes important to T-cell leukemogenesis.

Several lines of evidence are consistent with the possibility that EBNA3A, EBNA3B, and EBNA3C also regulate the transcription of genes with nearby RBPJκ sites. EBNA3C can transactivate some EBNA2-regulated genes (1, 31), can bind to RBPJκ in vitro and in yeast cells (22, 25), can associate with RBPJκ in human lymphoblasts (25), and can block EBNA2 transactivation of the LMP1 and LMP2 promoters in transient-transfection assays (20, 22, 25). EBNA3C has a glutamine- and proline-rich domain that can functionally substitute for the EBNA2 acidic domain in transcriptional activation (4, 22). EBNA3A and EBNA3B are distantly homologous to EBNA3C, and the genes encoding the three proteins are in tandem in the EBV genome, indicating that they have evolved from the same gene (26). EBNA3A and EBNA3B, like EBNA3C, can also specifically block EBNA2 activation of the LMP2 promoter in transient-transfection assays (20), consistent with an interaction with a protein in the EBNA2 transactivation pathway. In fact, in vitro-translated EBNA3B can bind to glutathione *S*-transferase (GST)-RBPJκ as well as in vitro-translated EBNA3C binds to GST-RBPJκ (25). However, in vitro-translated EBNA3A had an affinity for GST-RBPJκ which was only slightly above background (25). This discrepancy and recombinant EBV genetic analyses that indicate that EBNA3A and EBNA3C are critical to EBV-mediated primary B-lymphocyte growth transformation (16, 29, 30) prompted us to further evaluate the interactions of these proteins with RBPJκ in vitro and in vivo.

MATERIALS AND METHODS

Cell lines, culture conditions, and plasmids. BJAB is an EBV-negative cell line (25). BJAB EBNA3A, EBNA3B, EBNA3C, EBNA2, or EBNALP or control converted cell lines were derived by transfection of pZipneo EBNA or vector control into BJAB cells followed by neomycin selection (25, 31). LCLs are lymphoblastoid cell lines infected and transformed by EBV. All 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.

Nuclear extracts and EMSAs. Competitors and probes for electrophoretic mobility shift assays (EMSAs) were prepared by using synthetic oligonucleotides (Applied Biosystems). Probes were labeled with Klenow fragment of DNA polymerase I and [^{32}P]dGTP (25). Extracts and EMSAs were previously described (11, 15, 25).

Electrophoresis of polypeptides, immunoblotting, and immunoprecipitations. Proteins were denatured in sodium dodecyl sulfate (SDS)-β-mercaptoethanol lysis buffer and heated for 5 min at 95°C. They were then analyzed on SDS-polyacrylamide gels, transferred to 0.4-μm-pore-size nitrocellulose filters, and incubated with polyclonal rabbit anti-RBPJκ antibody or polyclonal human EBV immune sera (25). Immunoprecipitations were done by lysing the cells in radioimmunoprecipitation assay (RIPA) buffer for 1 h on ice, preclearing by incubation with 2 μl of normal rabbit serum for 0.5 h with rotation at 4°C, and collecting the bound molecules with protein A-Sepharose. The cleared supernatant was then used for immunoprecipitation using the RBPJκ rabbit antiserum (25) followed by protein A-Sepharose. Immunoprecipitates were washed six times in RIPA buffer, boiled in SDS lysis buffer, and then electrophoresed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein-protein interaction assays. Plasmids containing the EBNA3A, EBNA3B, and EBNA3C genes under control of the T7 promoter were digested with restriction enzymes (New England Biolabs) to facilitate transcription of the

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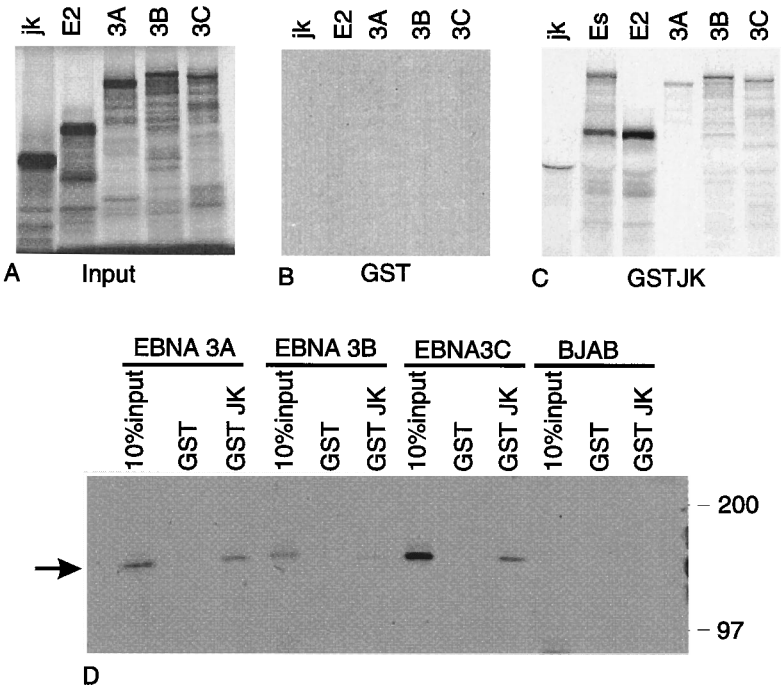

FIG. 1. EBNA3A, EBNA3B, and EBNA3C bind to GST-RBPJκ. In vitro-translated [^{35}S]methionine-cysteine-labeled EBNA3A, EBNA3B, EBNA3C, EBNA2, or RBPJκ (the input amount for each binding assay is shown in panel A) was precleared with GST beads, and the supernatant was then adsorbed to GST-RBPJκ beads or to beads bound with a fivefold excess of GST. Labeled proteins were eluted from the beads in sample buffer and were run on an SDS–8% polyacrylamide gel. The dried gels were developed on phosphorimager plates (B and C). Lane Es in panel C contains a mixture of equal amounts of each EBNA protein. Nonionic detergent lysates of BJAB cells stably converted by transfection with an EBNA3A, EBNA3B, or EBNA3C expression vector were incubated with GST- or GST-RBPJκ coated beads (D). Lysate (lanes 10% input) or proteins eluted from 10 times as much lysate incubated with beads coated with GST (lanes GST) or GST-RBPJκ (lanes GST JK) were subjected to SDS–8% PAGE and immunoblotted with an EBV immune human serum. Molecular masses (in kilodaltons) are indicated on the right.

full open reading frame or of 3′ truncations of the open reading frame. The nucleic acids were phenol-chloroform extracted before translation in vitro using ^{35}S-Express or a ^{14}C-amino acid mixture (NEN-Dupont) and wheat germ T7-TNT (Promega). Binding assays using GST-RBPJκ fusion protein were done as described previously (25). In vitro translation products were adsorbed to GST-coated beads before adsorption to GST- or GST-RBPJκ-coated beads.

RESULTS

In vitro-translated EBNA3A binds less efficiently than EBNA3B or EBNA3C to GST-RBPJκ but in vivo-expressed EBNA3A, EBNA3B, and EBNA3C bind similarly to GST-RBPJκ. In vitro-translated EBNA3A, EBNA3B, EBNA3C, or EBNA2 bound to GST-RBPJκ and did not bind to fivefold more GST (Fig. 1). Approximately 12% of input EBNA2, 3% of EBNA3C or EBNA3B, and 1% of EBNA3A adsorbed to RBPJκ in the experiment whose results are shown. The adsorption of in vitro-translated RBPJκ to GST-RBPJκ was <0.3%. Although the latter was intended to be a negative control, 0.3% was still above-background adsorption to GST, leaving open the possibility that RBPJκ may have some ability to self-associate (Fig. 1). While the absolute GST-RBPJκ adsorption efficiencies varied slightly among experiments, the relative efficiencies were constant in three repetitions of the experiment. Similar results were obtained when the EBNA proteins were mixed and incubated with GST-RBPJκ: EBNA2 was most efficient in RBPJκ binding, and the binding of EBNA3B and EBNA3C, which migrate together, and of EBNA3A, which is slightly smaller, is not affected by the presence of other EBNA proteins (lane Es in Fig. 1C). These results confirm the previous finding that in vitro-translated EBNA3A is less efficient than EBNA3B or EBNA3C in binding to GST-RBPJκ but also demonstrate that EBNA3A can bind specifically to GST-RBPJκ. Furthermore, given excess GST-RBPJκ, EBNA2, EBNA3B and EBNA3C, and EBNA3A do not appear to interact so as to alter their binding to RBPJκ.

To evaluate whether in vivo-expressed EBNA3A also binds more weakly to GST-RBPJκ, nuclear extracts from BJAB cell lines expressing EBNA3A, EBNA3B, or EBNA3C were incubated with GST or GST-RBPJκ. Surprisingly, EBNA3A, EBNA3B, and EBNA3C bound similarly to GST-RBPJκ and did not bind to GST (Fig. 1D). Approximately 5% of EBNA3A, EBNA3B, and EBNA3C bound to GST-RBPJκ in the experiment whose results are shown. Thus, EBNA3A expressed in human lymphoblasts is similar to EBNA3B and EBNA3C in its binding to GST-RBPJκ; only in vitro-translated EBNA3A is deficient in RBPJκ binding.

EBNA3A, EBNA3B, and EBNA3C associate with RBPJκ in human B lymphoblasts. RBPJκ was specifically immunoprecipitated from nuclear extracts of BJAB cells that had been converted to stable expression of EBNA3A, EBNA3B, EBNA3C, EBNALP, or EBNA2. As is apparent from an immunoblot of the cell extracts, the level of EBNA3A, EBNA3B, and EBNA3C expression in the BJAB cells was in excess of that in an EBV-transformed lymphoblastoid cell line (LCL4), with EBNA3C expression being in greatest excess (Fig. 2, compare lane 7 with lanes 2 through 4). The high level of overexpression of EBNA3C in this BJAB cell line probably accounts for the decreased levels of free RBPJκ previously observed in gel shifts with nuclear extracts from this cell line (25). At least 30% of the RBPJκ in cells was specifically immunoprecipitated by RBPJκ immune rabbit serum, and at least 5 to 10% of EBNA3A, EBNA3B, or EBNA3C coimmunoprecipitated with RBPJκ (Fig. 2, compare 2% of the lysate with immunoprecipi-

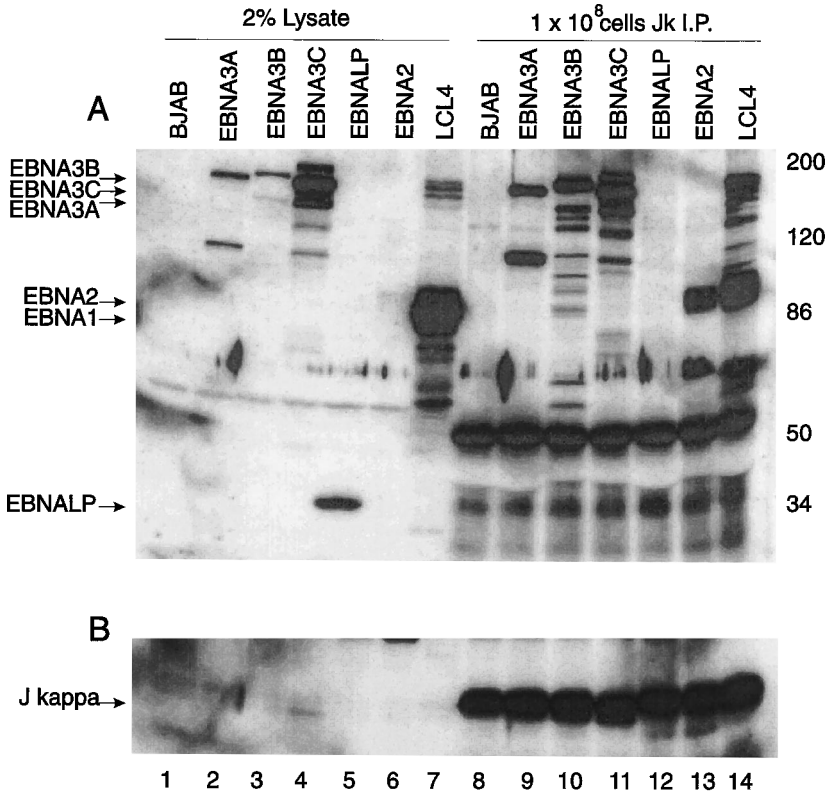

FIG. 2. Coimmunoprecipitation of EBNA3A, EBNA3B, EBNA3C, or EBNA2 with RBPJκ from cell extracts. Lysates from BJAB cells stably converted to EBNA3A, EBNA3B, EBNA3C, EBNA2, or EBNALP expression were incubated with rabbit antibody to RBPJκ. BJAB and EBV-transformed primary B-lymphocyte (LCL4) lysates were treated similarly to the controls. Antibody and antibody-antigen complexes were collected on protein A beads, solubilized in SDS sample buffer, run on the rightward seven lanes of an SDS–8% polyacrylamide gel, and immunoblotted with an EBV immune human serum (A) or with rabbit antibody to RBPJκ (B). The first seven lanes are immunoblots of 2% of the starting lysates. The exposure shown in the LCL4 lanes is intended to be more illustrative of EBNA3A, EBNA3B, and EBNA3C, while the EBNA2 and the EBNA1 bands are overexposed. EBNA2 is the upper component of the complex of EBNA2 and EBNA1. EBNA2 coprecipitated with RBPJκ but EBNA1 did not, so a lower EBNA1 component is missing from lane 14. The blot was sequentially reprobed with antisera specific for EBNA1, EBNA2, and EBNALP to confirm the data for these proteins. Molecular masses (in kilodaltons) are indicated on the right. I.P., immunoprecipitate; Jk, RBPJκ.

tation lanes). In comparison, RBPJκ immune precipitation coimmunoprecipitated at least 30% of the EBNA2 from an EBNA2-expressing BJAB cell line and very little, if any, EBNALP from an EBNALP-expressing BJAB cell line and very little, if any, EBNALP from an EBNALP-expressing BJAB cell line (Fig. 2, lanes 12 and 14). Similar results were obtained with the immunoprecipitation of RBPJκ from LCL4, as determined by an immunoblot with EBV immune serum detecting all the EBNA proteins (Fig. 2, compare lanes 7 and 14). Reblotting with EBNA1-, EBNA2-, or EBNALP-specific antibody distinguished the EBNA1 (lower) and EBNA2 (upper) components of the EBNA1-EBNA2 complex (Fig. 2 and data not shown). The more efficient precipitation of at least 30% of EBNA2 as opposed to 5 to 10% of the EBNA3 protein from Burkitt's lymphoma (BL) cell lysates with RBPJκ immune serum is consistent with the fourfold-greater binding of in vitro-translated EBNA2 to GST-RBPJκ. However, we cannot exclude the possibility that overexpression of EBNA3C, EBNA3A, and EBNA3B in the BL cells might contribute to a smaller fraction associating with RBPJκ.

The amino-terminal domains of EBNA3A, EBNA3B, and EBNA3C bind similarly to RBPJκ. A series of carboxy-terminal truncations were made in the EBNA3A, EBNA3B, and EBNA3C open reading frames, and the truncated fragments were transcribed and translated in vitro (Fig. 3A). Equivalent amounts of products were precleared with GST and incubated with GST (Fig. 3B) or GST-RBPJκ (Fig. 3C). (The gels in Fig. 3B and C were exposed for 5 days, while that in Fig. 3A was exposed for 1 day.) As before, full-length in vitro-translated EBNA3A (Fig. 3, lane 8) bound to GST-RBPJκ with lower efficiency than EBNA3B or EBNA3C (Fig. 3, lanes 5 and 3, respectively). However, C-terminally truncated EBNA3A *Spe*I (amino acids 1 to 302) or *Nco*I (1 to 138) polypeptides (EBNA3A 1–302 and 1–138, respectively) bound specifically to GST-RBPJκ with efficiencies similar to those of C-terminally truncated EBNA3B or EBNA3C polypeptides (compare lanes 6 and 7, lane 4, and lanes 1 and 2 of panel A with the same lanes of panel C in Fig. 3). The EBNA3A 1–138 and 1–302 polypeptides, the EBNA3B *Nco*I 1–311 polypeptide, and the EBNA3C *Spe*I 1–365 and *Eco*RV 1–183 polypeptides all bound to GST-RBPJκ with similar efficiencies. Thus, the lower efficiency of binding of full-length in vitro-translated EBNA3A appears to be due to an inhibitory effect of the C terminus when the protein is translated in vitro. Further, the EBNA3A 1–138, EBNA3B 1–311, and EBNA3C 1–183 polypeptides have nearly full GST-RBPJκ binding activity. The EBNA3B 1–109 polypeptide could be labeled only with ^{14}C-amino acids. Binding to GST-RBPJκ was $<1\%$ of input and was not detectable above background (Fig. 3, lane 9). Although the sensitivity of detection of EBNA3B 1–109 polypeptide binding to RBPJκ is only adequate to exclude binding at nearly the same level as the other C-terminally truncated polypeptides, the data indicate that EBNA3B residues between 109 and 311 are important for RBPJκ interaction, while EBNA3A 1–138 and

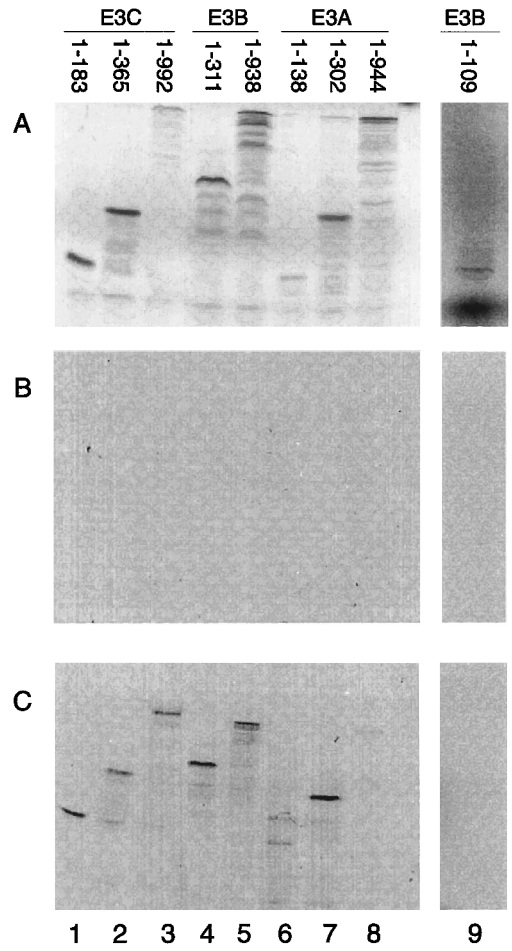

FIG. 3. The amino-terminal domains of EBNA3A, EBNA3B, and EBNA3C bind to RBPJκ. EBNA3A C-terminally truncated at the *Spe*I site (after codon 302) or at the *Nco*I site (after codon 138), EBNA3B C-terminally truncated at the *Nco*I site (after codon 311) or at the *Spe*I site (after codon 109), and EBNA3C C-terminally truncated at the *Spe*I site (after codon 365) or at the *Eco*RV site (after codon 183) were translated in vitro, precleared with GST beads, and incubated with GST- or GST-RBPJκ-coated beads. Input (A), GST-adsorbed (B), and GST-RBPJκ-adsorbed (C) proteins were eluted from the beads in SDS sample buffer and subjected to SDS–12% (left panels) or SDS–15% (right panels) PAGE. EBNA3B truncated at the *Spe*I site (after codon 109) and labeled with a ^{14}C-amino acid mixture (NEN-Dupont) is shown (A, right panel). The left panel A was exposed to film for 1 day at −70°C, while panels B and C were exposed for 5 days at −70°C. The panels on the right are prints from a phosphorimager scan after 7 days.

EBNA3C 1–183 polypeptides are sufficient for RBPJκ interaction.

EBNA3A, EBNA3B, or EBNA3C inhibits RBPJκ interaction with cognate DNA probes; the EBNA3C N-terminal domain has most of the inhibitory activity. In vitro-translated EBNA3C can prevent RBPJκ from interacting with cognate DNA in vitro, resulting in a decrease in the RBPJκ probe gel shift and an increase in free probe (probe 25, Fig. 4). As in the GST-RBPJκ binding assay, in vitro-translated EBNA3A had less of an effect than EBNA3B or EBNA3C on RBPJκ gel shift activity, whereas in vitro-translated EBNA3B had as much of an effect as or more of an effect than EBNA3C (Fig. 4). Although similar amounts of the proteins were translated in vitro, 2 μl of in vitro-translated EBNA3B or 5 μl of EBNA3C almost completely blocked RBPJκ gel shift activity, while 10 μl of EBNA3A only partially blocked RBPJκ gel shift activity (Fig. 4).

To evaluate whether probe release was dependent on interactions with parts of EBNA3 proteins beyond the N-terminal interaction domain, progressive carboxy-terminal truncations of EBNA3C were assayed for their effects on RBPJκ gel shift inhibitory activity (Fig. 5). The EBNA3C *Spe*I 1–365 polypeptide blocked RBPJκ activity as well as full-length EBNA3C, while the EBNA3C *Eco*RV 1–183 polypeptide had nearly as much activity (Fig. 5). Thus, EBNA3C 1–183 is sufficient for most of the RBPJκ binding activity and gel shift-disruptive activity. The finding that in vitro-translated EBNA3A is as deficient relative to EBNA3B and EBNA3C in the probe release assay as in the RBPJκ binding assay suggests that probe release simply reflects the extent of EBNA3A binding to RBPJκ.

DISCUSSION

EBNA3A, EBNA3B, and EBNA3C consist of 944, 938, and 992 amino acids, respectively (for a sequence comparison, see reference 26) and encompass most of the protein-coding capacity of the EBV genome in latently infected cells. EBNA3A and EBNA3C are critical for primary B-lymphocyte growth transformation in vitro (16, 30), while EBNA3B is not (29). All three EBNA3 proteins include epitopes that are frequently recognized by EBV immune cytotoxic T lymphocytes (10, 17, 24). Given the importance of EBNA3A, EBNA3B, and EBNA3C epitopes in cytotoxic T-cell recognition of EBV-infected lymphocytes, strains with deletions of all or part of each of the EBNA3 proteins would have been expected to have arisen and been identified if these proteins were not important for infection in vivo.

The data presented here indicate that EBNA3A, EBNA3B, and EBNA3C can independently bind to RBPJκ can alter its ability to recognize its cognate DNA sequence, and can associate with RBPJκ in B lymphoblasts. These observations are consistent with the ability of EBNA3A, EBNA3B, or EBNA3C to down modulate EBNA2-mediated transactivation of the LMP2 promoter in transient-transfection experiments (20), since that transactivation is substantially dependent on EBNA2 interaction with RBPJκ and on RBPJκ binding to specific cognate DNA (11, 12, 18, 32).

EBNA3C can up regulate expression of LMP1 in growth-arrested cells (1) and can up regulate CD21 expression in non-EBV-infected BL cells (31), while EBNA3B appears to be able to up regulate CD40 and vimentin expression in non-EBV-infected BL cells (28). RBPJκ is likely to be a mediator of these transactivating effects. The most likely model is that the EBNA3 proteins are functionally analogous to EBNA2. EBNA3C has a glutamine-proline-rich domain which can substitute for the acidic transactivating domain of EBNA2 (4, 22); EBNA3B and EBNA3C have similar glutamine-proline-rich domains (for a sequence comparison, see reference 26). RBPJκ is a critical component of EBNA2 interaction with response elements (11–13, 15, 18, 21, 32, 33), and the binding of EBNA3A, EBNA3B, and EBNA3C to RBPJκ likely indicates that RBPJκ is a critical mediator of EBNA3 interactions with response elements. While EBNA3B shares with EBNA3C and EBNA3A the ability to down regulate EBNA2 transactivation of the LMP1 promoter (20), the effects of EBNA3B and EBNA3C on cell promoters differ and are distinct from those of EBNA2 (28, 31). The different effects of EBNA3C, EBNA3B, and EBNA2 in non-EBV-infected BL cells likely indicate a role for other protein-protein interactions as determinants of EBNA3A, EBNA3B, and EBNA3C transcriptional effects on specific cell genes. EBNA2 transactivation of the LMP1 promoter is dependent not only on interaction with

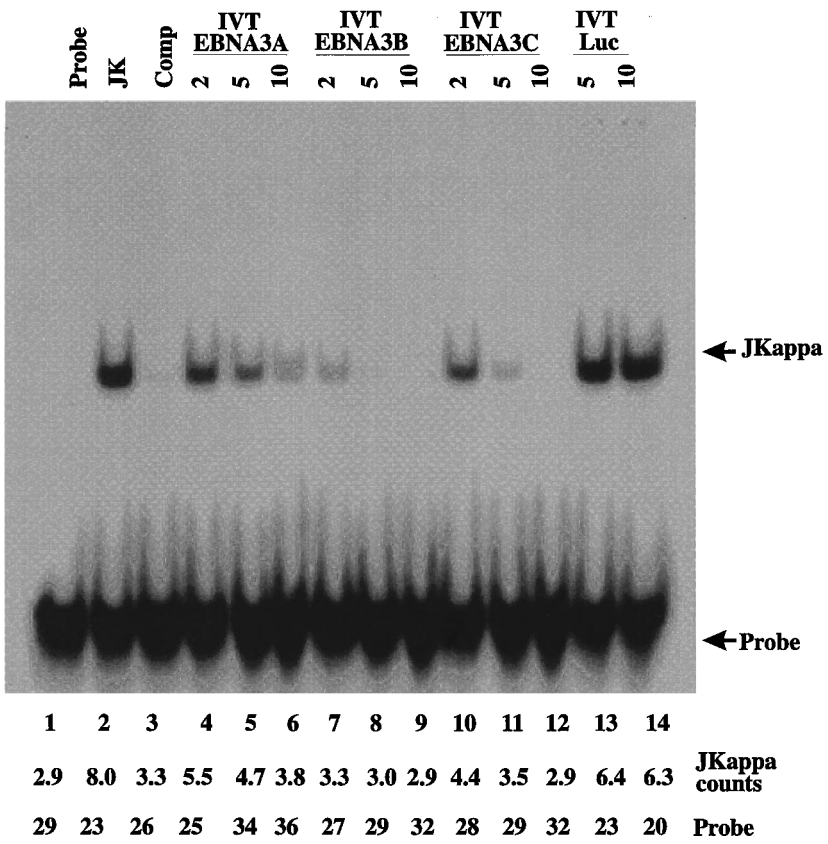

FIG. 4. In vitro-translated EBNA3B, EBNA3C, or (to a lesser extent) EBNA3A inhibits RBPJκ binding to cognate DNA. An LMP2 promoter probe (25) was incubated with 2 μl of in vitro-translated RBPJκ or with RBPJκ and 2, 5, or 10 μl of an in vitro-translated EBNA3 product (as indicated above the lanes) in a total reaction volume of 50 μl. The efficiencies of transcription and translation of EBNA3A, EBNA3B, and EBNA3C were similar, as detected by electrophoresis and autoradiography of the [^{35}S]methionine-cysteine-labeled polypeptides (data not shown). Lane 1, probe alone; lane 2, probe and 2 μl of RBPJκ; lane 3, probe, RBPJκ, and cold probe oligonucleotide competitor; lanes 5 to 7, probe, RBPJκ, and EBNA3A; lanes 7 to 9, probe, RBPJκ, and EBNA3B; lanes 10 to 12, probe, RBPJκ, and EBNA3C; lanes 13 and 14, probe, RBPJκ, and 5 or 10 μl of luciferase (Luc) used as a control. The total counts for the RBPJκ gel shift (as quantitated with a Molecular Dynamics phosphorimager) and the probes are indicated below the gel. IVT, in vitro translated.

RBPJκ but also on interaction with PU.1 (15), and the EBNA3 proteins may be similarly dependent on interactions with factors other than RBPJκ. Such putative factors are difficult to define because of problems in achieving EBNA3-mediated transactivation in transient-transfection assays (22, 31; data not shown).

An alternative model consistent with the in vitro effects of EBNA3 proteins in inhibiting RBPJκ binding to cognate DNA is that the EBNA3 proteins transactivate transcription by regulating the interaction of RBPJκ with DNA. RBPJκ has transcriptional repressive effects on the adenovirus pIX promoter (6), and GAL4-RBPJκ fusions have repressive effects on promoters with multiple GAL4 binding sites (13). Thus, dissociation of RBPJκ from DNA could activate promoters with RBPJκ sites. A weakness in this model is the lack of an apparent need for the size and complexity of the EBNA3 gene family, since the N-terminal domain of one EBNA3 protein would suffice for this effect.

The data reported here enable a better understanding of the effects of previous recombinant EBV-based molecular genetic analyses of the EBNA3 proteins. The EBNA3B analysis used a nonsense codon insertion after codon 109 (29). On the basis of the data presented here, this mutant EBNA3B would be deficient in RBPJκ binding and would be expected to be a null mutant. Studies of EBNA3A and EBNA3C placed nonsense codons after codons 302 and 365, respectively (30). Both would result in expression of cross-reactive proteins that include the RBPJκ interactive domains (Fig. 6). Assuming that both cross-reactive proteins were stable, the data indicate that the need for EBNA3 proteins extends beyond their cumulative ability to interact with RBPJκ and includes an important role for the rest of EBNA3A and EBNA3C in primary B-lymphocyte growth transformation. The EBNA3A mutation appeared to be a weak dominant negative (30), perhaps in part because of its ability to interact with RBPJκ in the absence of the rest of EBNA3A.

Consistent with their common RBPJκ binding activity, the N-terminal EBNA3 domains overlap with the most highly conserved domain among the EBNA3 proteins (Fig. 6). The most highly conserved domain is from amino acids 90 to 320, and there is little homology in the first 90 amino acids. Since EBNA3B 1–311, EBNA3A 1–138, and EBNA3C 1–183 are sufficient for RBPJκ interaction, the core RBPJκ interacting domain is likely to be in the partially conserved sequence between amino acids 90 and 138. EBNA3A, EBNA3B, and EBNA3C lack the two consecutive tryptophans that are the core of the EBNA2 domain that interacts with RBPJκ (11, 32).

The largeness of the EBNA3 proteins, the critical requirement for EBNA3A and EBNA3C in the transformation of primary B lymphocytes in vitro (16, 30), the apparent need for EBNA3A, EBNA3B, and EBNA3C in vivo, and the diversity in effects of EBNA3B and EBNA3C are most compatible with

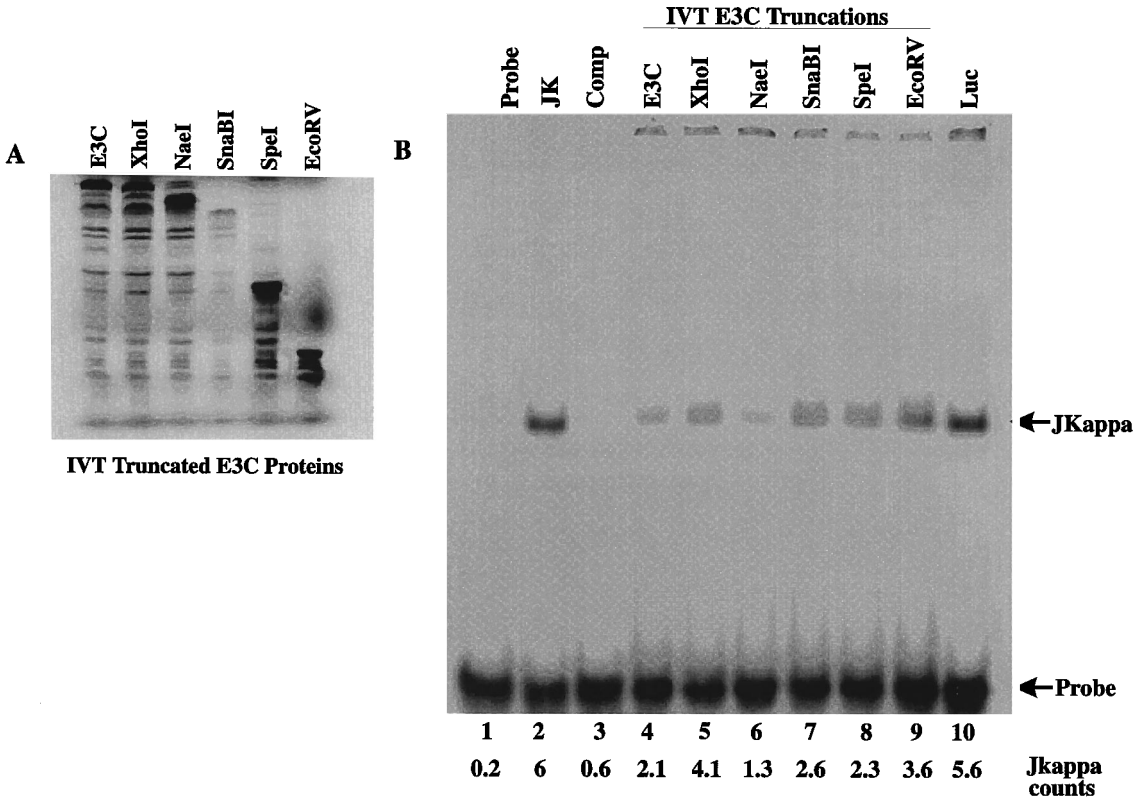

FIG. 5. The EBNA3C amino terminus can inhibit RBPJκ binding to its cognate DNA sequence. An LMP2 promoter probe (25) containing an RBPJκ binding site was incubated with in vitro-translated RBPJκ and with equal amounts of in vitro-translated EBNA3C or carboxy-terminally truncated EBNA3C. (A) Relative amounts of the ^{35}S-labeled EBNA3C in vitro translation (IVT) products. (B) Protein-DNA complexes resolved by EMSA on a 5% native polyacrylamide gel. The RBPJκ EMSA shift (JKappa) and free probe are indicated on the right. Lane 1, probe alone; lane 2, probe with RBPJκ; lane 3, probe with RBPJκ and excess unlabeled competitor (Comp) oligonucleotide; lanes 4 to 10, probe with RBPJκ and in vitro-translated EBNA3C polypeptides or in vitro-translated luciferase (Luc) control. The total counts for the RBPJκ gel shift were quantitated with a Molecular Dynamics phosphorimager and are indicated at the bottom. The C-terminal truncations of EBNA3C polypeptides produced as a consequence of digestion of the DNA with restriction endonuclease are *Xho*I after codon 942, *Nae*I after codon 754, *Sna*BI after codon 630, *Spe*I after codon 365, and *Eco*RV after codon 183. These are shown in Fig. 6.

these proteins differentially regulating transcription through RBPJκ. RBPJκ is now recognized to be an important mediator of transcriptional regulation in developing tissues, in which it is directly activated by the notch mutation (2, 3, 8, 9, 14, 19, 27). Overexpression of human notch is associated with T-cell malignancies (7), consistent with the notion that transcriptional activation of genes with appropriately placed RBPJκ binding sites can be important in cell growth transformation. Thus,

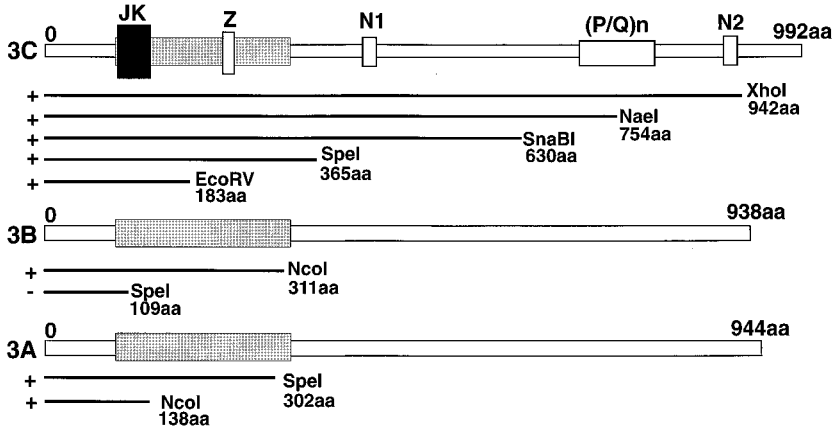

FIG. 6. Schematic diagram of EBNA3 proteins. The region having 22 to 27% identity among the EBNA3 proteins (26) (gray box), a putative leucine zipper region (Z), the potential nuclear localization signals (N1 and N2), and the activation domain [(P/Q)n] (27) are indicated. The C-terminally truncated polypeptides used in these assays are indicated along with the enzymes used, the codons translated, and the results of binding assays (positive or negative [+ or −, respectively]). The most likely RBPJκ (JK) interacting domain (black box) is indicated on the basis of binding of EBNA3A 1–138 and of EBNA3C 1–183, the absence of significant homology in the first 90 amino acids, and the partial homology among the EBNA3 proteins in amino acids 90 to 320. aa, amino acids.

EBNA2, EBNA3A, EBNA3B, and EBNA3C effects on such genes could well be important in EBV-mediated cell growth transformation. Conversely, the apparent central importance of RBPJκ in EBV-mediated primary B-lymphocyte growth transformation, as evidenced by interaction with EBNA2, EBNA3A, EBNA3B, and EBNA3C, may indicate an important role for notch homologs and ligands in B-lymphocyte growth or differentiation.

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