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EBNA-2 and EBNA-3C Extensively and Mutually Exclusively Associate with RBPJκ in Epstein-Barr Virus-Transformed B Lymphocytes

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Although genetic and biochemical data indicate that the cell protein RBPJκ is a mediator of EBNA-2 and EBNA-3C effects on transcriptional regulatory elements, the extent of association of these Epstein-Barr virus nuclear proteins with RBPJκ in transformed B lymphocytes has not been determined. We now report that most of the EBNA-2 and at least 20% of the EBNA-3C coimmunoprecipitated with RBPJκ from extracts of transformed B lymphocytes that contained most of the cellular EBNA-2 and EBNA3C. Both proteins are associated preferentially with the smaller of the two RBPJκ isoforms. EBNA-2–RBPJκ complexes do not contain EBNA-3C, and EBNA-3C–RBPJκ complexes do not contain EBNA-2. Although EBNA-2 and EBNA-3C are extensively associated with RBPJκ, a fraction of RBPJκ appears to be free of EBNAs after repeated immunoprecipitations with anti-EBNA, Epstein-Barr virus-immune, human antibody. Promoters with RBPJκ sites in their regulatory elements are likely to be differentially regulated by these RBPJκ–EBNA-2 and RBPJκ–EBNA-3 complexes.

The experiments reported here evaluate the extent of association in Epstein-Barr virus (EBV)-transformed B lymphocytes (lymphoblastoid cell lines [LCLs]) between virus-encoded nuclear proteins (EBNAs) and a cellular protein, RBPJκ. RBPJκ was identified and erroneously named as a potential recombinase that appeared to bind to the immunoglobulin κ J region (30). Molecular genetic and biochemical data now indicate that RBPJκ is a sequence-specific DNA-binding protein that is a key mediator of the transcriptional regulatory effects of EBNA-2 (1, 6, 16, 19, 21, 23, 25, 28, 42, 43, 45, 47) and of EBNA-3A and -3C (2, 27, 29, 33, 34, 42) in EBV transformation of primary B lymphocytes (7, 8, 18, 24, 37, 38, 44, 45), of the pIX promoter in adenovirus infection (10), and of signaling from activated Notch receptors in neural and muscular development (4, 5, 9, 13–15, 22, 26, 35). Since constitutive Notch activation is an important etiologic factor in human T-cell leukemia (11), RBPJκ is also implicated in leukemogenesis. Thus, the level of steady-state association of EBNAs with RBPJκ is likely to be important in EBV-driven B-lymphocyte proliferation.

The findings that the overall level of RBPJκ in LCLs is similar to those in other cells and that free RBPJκ is reduced as measured by gel shift assays (23, 34) are consistent with the notion that RBPJκ could be extensively complexed with EBNA proteins in LCLs. However, complexes of RBPJκ, probe DNA, and EBNAs have been difficult to detect (23, 25, 45, 47), leaving substantial uncertainty as to the steady-state level of EBNA association with RBPJκ.

The extent of stable association of RBPJκ with EBNAs in isotonic salt and nonionic detergent lysates from recently established EBV-transformed LCLs (LCL3 and LCL10) was determined by immunoprecipitation of putative RBPJκ-EBNA complexes with RBPJκ- or EBNA-specific antibody followed by immunoblot detection of RBPJκ and EBNAs. The cell lysates used for these precipitations contained most of the EBNA-1, EBNA-2, EBNA-3C, or total EBNA-3 reactivity in LCLs. When blotted with the appropriate antibodies, these EBNAs were readily detected in 5% of the soluble cell lysate (Fig. 1 and 2, lys lanes). There was no significant background in immunoblots of 5% of the sodium dodecyl sulfate (SDS)-solubilized residual cell debris, and these EBNAs were substantially less evident in the SDS-solubilized residual cell debris than in the soluble cell lysate when these samples were directly compared on the same blot (data not shown). In contrast, about half of the total EBNA-LP was in the soluble lysate and half was in the residual cell debris (data not shown).

Multiple immunoprecipitation experiments of each type were done, and the results from representative experiments are presented. Immunoprecipitations from two different LCLs with polyclonal RBPJκ antibody (34), followed by immunoblotting with the same antibody, resulted in detection of two RBPJκ isoforms in LCLs (Fig. 1), consistent with previous Northern (RNA) blot and immunoblot analyses (17, 34, 47). A background band migrating slightly faster than both isoforms was present in the input lysate but not in RBPJκ or EBNA immunoprecipitates (Fig. 1A). Comparisons of the abundance of RBPJκ in the RBPJκ antibody immunoprecipitate and that in the lysate indicate that the antibody brought down about 50% of both isoforms and that the upper isoform was at least as abundant as the lower isoform (Fig. 1 and data [for an experiment in which the background band in the lysate lane was separated from both isoforms by running the gels for longer times] not shown). The abundance of proteins in the immunoprecipitate lanes was estimated by band intensity relative to the lysate lane. In instances such as the RBPJκ immunoprecipitates, where the RBPJkappa bands in the immunoprecipitate lane had a stronger signal than the lysate lane, estimates were also based on the difference in the film exposure time necessary for the immunoprecipitate band intensity to approximate the lysate band intensity. Only a small fraction (less than 5%) of the RBPJκ coprecipitated with EBNA-2- or EBNA-3C-specific antibodies, and the lower isoform was consistently over-

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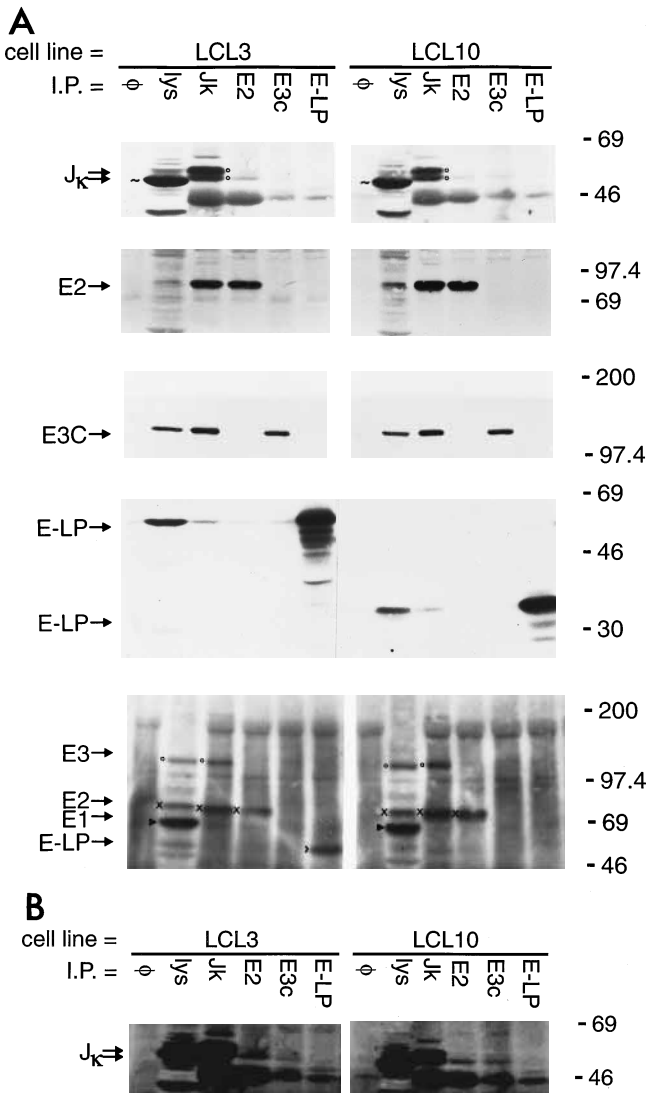

FIG. 1. Immunoblot analysis of EBNA-RBPJκ complexes in LCLs. EBV-transformed LCLs (40 ml of LCL3 or LCL10 culture) in log-phase growth at a cell density of 5×10^5/ml with viability of >95% as determined by trypan blue exclusion were lysed by vortexing in 1 ml of lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10 mg each of aprotinin and leupeptin per ml, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate) for 30 min at 4C. After clarification by low-speed centrifugation, lysates were immunoprecipitated with 3 μl of rabbit RBPJκ (Jk) antibody, 2 μl of rabbit EBNA-2 (E2) antibody, 50 μl of monoclonal D8 (EBNA-3C [E3c]) antibody, 10 μl of monoclonal JF186 (EBNA-LP [E-LP]) antibody, or no antibody (ϕ) (12, 16, 20, 31). Each immunoprecipitation mixture was allowed to incubate overnight at 4°C. To capture immunoprecipitates (I.P.), Pansorbin (Calbiochem) or rabbit anti-mouse (Cappel) coupled to Pansorbin was rotated with the lysates for 30 min at 4°C and the lysates were pelleted and washed three times in lysis buffer. Immunoprecipitates were then resuspended in 50 μl 2× gel loading buffer, boiled for 5 min, and separated by 7 to 12% gradient polyacrylamide gel electrophoresis. A 50-μl portion of cleared lysate was reserved and run out with the other samples (lys). After transfer to nitrocellulose, blots were probed with antibody as indicated on the left. EBNA-2 was detected with an EBNA-2 monoclonal antibody (46); otherwise, blotting primary antibodies were the same as those used for immunoprecipitation. (Protein A-horseradish peroxidase conjugate or sheep anti-mouse–horseradish peroxidase conjugate was used as a secondary detection reagent in conjunction with an ECL kit [Amersham].) Detected bands are indicated by arrows. (A) The top panel demonstrates two RBPJκ isoforms (◦) and a background band seen only in the lysate (~). In the experiment for the bottom panel an EBV-immune human serum (32) was used to detect all of the EBNA proteins: unseparated EBNA-3s, EBNA-2, EBNA-1 (E1), and EBNA-LP. Note that EBNA-LP proteins differ in size in the two LCLs and that the EBNA-LP from LCL10 is not demonstrated in the IHS blot. (B) Darker exposure of the RBPJκ blot.

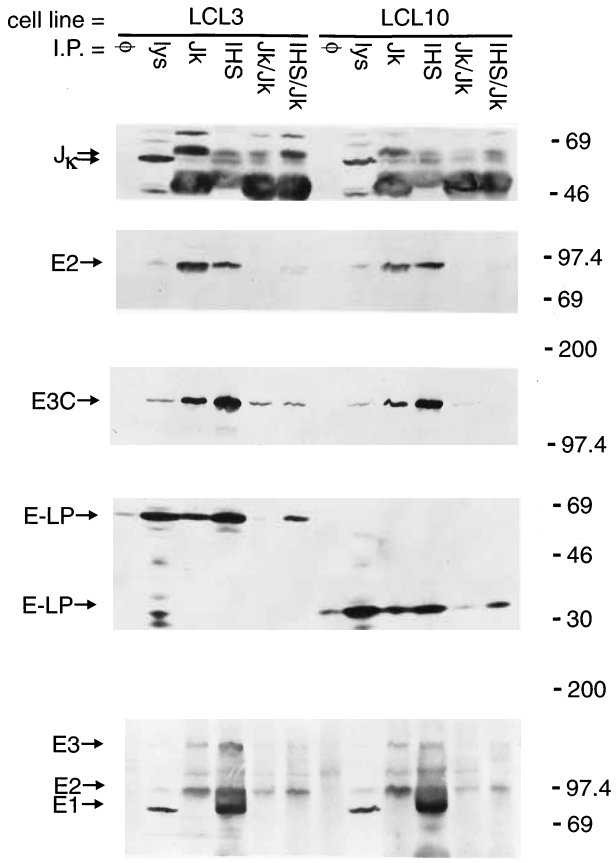

FIG. 2. LCLs contain RBPJκ uncomplexed to EBNA proteins. Immunoprecipitations were performed as described in the legend to Fig. 1. Supernatants from an initial RBPJκ antibody or EBV-immune human serum (IHS) polyvalent anti-EBNA antibody immunoprecipitation were reserved and subsequently re-immunoprecipitated with RBPJκ antibody (Jk/Jk and IHS/Jk, respectively) or human anti-EBNA serum (Jk/IHS and IHS/IHS, respectively). The Jk/IHS and IHS/IHS data are not shown. The precipitates were then solubilized, electrophoresed, and immunoblotted as for Fig. 1. Abbreviations are as defined in the legend to Fig. 1.

represented (Fig. 1 and data not shown). This indicates that EBNA-2 and EBNA-3C associate somewhat preferentially with the smaller isoform. Little or no RBPJκ immunoprecipitated with an EBNA-LP-specific monoclonal antibody (Fig. 1A).

Probing of the same blot with an EBNA-2-specific monoclonal antibody (46) revealed as much EBNA-2 in the RBPJκ immunoprecipitate as it did in the EBNA-2 immunoprecipitate (Fig. 1A). Comparisons of shorter exposures of this and similar immunoblots with the longer exposure of the immunoblot of input lysate indicate that the RBPJκ and EBNA-2 antibodies precipitated about 50% of the soluble EBNA-2 (data not shown). Since the RBPJκ antibody precipitated about 50% of the RBPJκ and about 50% of the EBNA-2, most of the soluble EBNA-2 appears to be in a complex with RBPJκ. However, despite the stability of the RBPJκ complex in immunoprecipitation, only a small fraction of the RBPJκ coimmunoprecipitated with EBNA-2 in the EBNA-2 antibody lane, indicating that RBPJκ is in substantial excess of EBNA-2 (Fig. 1).

Reprobing with an EBNA-3C-specific monoclonal antibody indicated that only 10 to 20% of the EBNA-3C had immunoprecipitated with EBNA-3C-specific monoclonal antibody (Fig. 1A; compare lysate with EBNA-3C immunoprecipitation). Accordingly, the very small fraction of RBPJκ in the EBNA-

3C immunoprecipitate lane is an underestimate of the amount of RBPJκ associated with EBNA-3C. Immunoprecipitation with RBPJκ-specific antibody resulted in retrieval of as much EBNA-3C as was obtained with the EBNA-3C-specific antibody or more. At least 10 to 20% of the soluble EBNA-3C precipitated as a complex with RBPJκ. Since only about 50% of the RBPJκ was precipitated, more than 20% of the EBNA-3C is probably associated with RBPJκ. Interestingly, EBNA-2 was not detected in EBNA-3C-specific immunoprecipitates and EBNA-3C was not detected in EBNA-2 immunoprecipitates (Fig. 1A), indicating that higher-order complexes of EBNA-2 and EBNA-3C with RBPJκ are not detectable in LCLs by this approach.

Only a trace of EBNA-LP was detected in EBNA-2- or EBNA-3C-specific immunoprecipitates, and this trace was not above levels found in the negative control lane (Fig. 1A; the negative control lane is the leftmost lane and is an immunoprecipitation without primary antibody). Slightly more EBNA-LP was consistently found in the RBPJκ immunoprecipitates, but the amount of EBNA-LP was much smaller than that in the lane containing 5% of the lysate, suggesting that a very small fraction of EBNA-LP, probably less than 1%, may be associated with RBPJκ (Fig. 1A). However, little or no RBPJκ was found in the EBNA-LP immunoprecipitates, and these were efficient for EBNA-LP retrieval (Fig. 1A).

Immunoblotting with an EBV immune human serum (IHS) known to recognize all the EBNA proteins (32) revealed an absence of EBNA-1 in the RBPJκ immunoprecipitate (Fig. 1A). Absence of EBNA-1 was a consistent result with immunoblots of every RBPJκ immunoprecipitation. Despite the faint detection of EBNA-3C by IHS in the EBNA-3C immunoprecipitate lane and the precipitation of a similar amount of EBNA-3C in the RBPJκ immunoprecipitate lane (see the EBNA-3C blot in Fig. 1A mentioned above), IHS detected more proteins similar in size to EBNA-3C in the RBPJκ immunoprecipitate lane than in the EBNA-3C lane. These other proteins are likely to be EBNA-3A or EBNA-3B that also coimmunoprecipitated with RBPJκ-specific antibody, since EBNA-3A and -3B also interact with RBPJκ in vitro (33, 34).

To further assess the extent of association between RBPJκ and EBNAs, two-step immunoprecipitations from LCL extracts were done with RBPJκ antibody or IHS. As previously, antibody to RBPJκ immunoprecipitated about half of the RBPJκ. A smaller amount was brought down by a repeat RBPJκ immunoprecipitation (Fig. 2, Jk/Jk lane). Immunoblots for EBNA-2 and EBNA-3C with the first RBPJκ-specific immunoprecipitation confirm the association of RBPJκ with most of the EBNA-2 and with about 20% of the EBNA-3C (Fig. 2 and data not shown). Blotting for EBNA-LP again suggested a slight association with RBPJκ (Fig. 2; compare the Jk lane with the negative control lane and with the lane containing 5% of the cell lysate). Substantially less EBNA-2, EBNA-3C, or total EBNA-3s were brought down by the repeat RBPJκ antibody immunoprecipitation, as expected from the smaller amount of RBPJκ in the repeat as opposed to the first immunoprecipitation (Fig. 2, Jk/Jk lane). IHS immunoprecipitated about half of the EBNA-2 and EBNA-3C (Fig. 2; compare the respective immunoprecipitations with 5% input lysate). Reimmunoprecipitation of the IHS supernatant with IHS and blotting for EBNA-2 and EBNA-3C confirmed that most of these EBNAs had been removed by the first IHS immunoprecipitation (data not shown). IHS did not immunoprecipitate RBPJκ from an EBV-negative B-cell line (data not shown). A significant fraction, estimated to be close to 30%, of the total RBPJκ was immunoprecipitated by IHS from the LCL extracts (Fig. 2 and data not shown). The RBPJκ antibody immunoblots of the IHS immunoprecipitates shown in Fig. 2 were atypical among several similar experiments in showing equal amounts of the two RBPJκ isoforms in the IHS immunoprecipitate. Still, there was relatively more of the lower isoform in the IHS precipitate lane than in the RBPJκ immunoprecipitate lane (compare the Jk and IHS lanes in the Jk antibody immunoblot in Fig. 2). Further, RBPJκ-specific antibody immunoprecipitated more of the upper isoform from the supernatant after the IHS precipitation (Fig. 2, IHS/Jk lane). The RBPJκ antibody reimmunoprecipitation brought down as much RBPJκ as the initial IHS immunoprecipitation (Fig. 2; compare the IHS and IHS/Jk lanes); this is compatible with the notion that about half of the RBPJκ is associated with EBNAs.

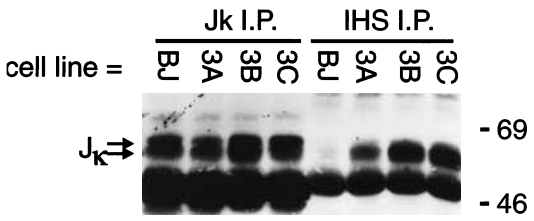

FIG. 3. RBPJκ associates with EBNA-3A, -3B, and -3C in stably transfected B lymphocytes. BJAB (BJ) is an EBV-negative BL cell line. BJAB/EBNA-3A.1 (3A), BJAB/EBNA-3B.7 (3B), and BJAB/EBNA-3C.7 (3C) are BJAB cell lines stably expressing EBNA-3A, EBNA-3B, and EBNA-3C, respectively (34). Lysates from the indicated cell lines were immunoprecipitated with IHS or RBPJκ (Jk) antibody and then immunoblotted with RBPJκ antibody. I.P., immunoprecipitate.

In another series of experiments, immunoprecipitations were done twice sequentially with IHS- or RBPJκ-specific antibodies and the residual supernatants were split and reimmunoprecipitated with either IHS or RBPJκ-specific antibody. The results were consistent with the previous results. More than 20% of the EBNA-3C or of the total EBNA-3 reactivity was still in the supernatant after two RBPJκ specific immunoprecipitations, confirming that at least 20% of each of the EBNA-3s is not stably associated with RBPJκ (data not shown). In contrast, almost all of the EBNA-2 had been precipitated by two sequential RBPJκ immunoprecipitations and 5% or less of the EBNA-2 was in the supernatant (data not shown). Thus, EBNA-2 is more extensively complexed with RBPJκ than are the EBNA-3s.

Immunoprecipitation with IHS or EBNA-3A, EBNA-3B, or EBNA-3C from non-EBV-infected Burkitt lymphoma (BL) cells stably overexpressing EBNA-3A, EBNA-3B, or EBNA-3C demonstrated that each of the EBNA-3s preferentially associated with the smaller RBPJκ isoform in BL cells, while precipitation with RBPJκ antibody brought down comparable amounts of both isoforms in these cell lines (Fig. 3; the upper RBPJκ isoform is not visible in the EBNA-3A, EBNA-3B, or EBNA-3C immunoprecipitates with IHS [right panel], even though the blot is overexposed so that the upper and lower bands have begun to merge in the RBPJκ immunoprecipitates [left panel]). The apparent smaller amount of RBPJκ in the IHS immunoprecipitation from the EBNA-3A BL cell line probably reflects a lower concentration of protein in this lysate, since the RBPJκ level in the RBPJκ immunoprecipitation from the EBNA-3A-expressing cells was also reduced (Fig. 3). Reblotting of the RBPJκ immunoprecipitates and input lysates with IHS demonstrated that similar fractions of EBNA-3A, EBNA-3B, and EBNA-3C had immunoprecipitated from the respective cell lines (data not shown). Thus, the extent of association of EBNA-3A and EBNA-3B with RBPJκ is probably similar to that of EBNA-3C. Taken together, this suggests

that the EBNA-3s bind preferentially in vivo to the lower RBPJκ isoform.

These data are important for understanding the role of EBNA-RBPJκ interactions in mediating gene regulation in EBV-transformed B lymphocytes. Most of the EBNA-2 and at least 20% of the EBNA-3C in these LCLs is associated with RBPJκ, pointing to a key role for RBPJκ in mediating effects of these proteins and to the importance of genes with RBPJκ regulatory sites as targets for EBNA-2, EBNA-3A, and EBNA3C effects in primary B-lymphocyte growth transformation. Although EBNA-3A and EBNA-3B are not specifically identified in the gels shown here, data obtained with EBV-immune human serum and RBPJκ immunoprecipitations from LCL (Fig. 2 and data not shown) and from EBNA-3A-, EBNA-3B-, and EBNA-3C-converted BL cell extracts (Fig. 3) (33) indicate that EBNA-3A and EBNA-3B associate with RBPJκ to the same extent as EBNA-3C does.

RBPJκ appears to be in excess of EBNA-2 and EBNA-3s, and perhaps as much as half of the total soluble RBPJκ may be free of EBNA-2 and EBNA-3s. This estimate is compatible with a similar reduction in free RBPJκ gel shift activity in EBV-transformed B lymphocytes (23, 34). The excess of free RBPJκ makes it less likely that EBNA-2 and EBNA-3s compete for soluble RBPJκ interaction.

However, the apparent excess of "free" RBPJκ may be deceptive. In adenovirus-infected epithelial cell lines there is barely sufficient free RBPJκ to repress the adenovirus pIX promoter. Adenovirus DNA replication leads to cognate sequence excess and escape from RBPJκ-mediated repression (10). Further, there may be differential competition among the EBNAs for RBPJκ at specific promoter sites, dependent on the interactions of the EBNA-RBPJκ complexes with other cell proteins that bind near promoters (23, 25). Moreover, "free" RBPJκ may, in fact, be complexed with other nuclear proteins or sequestered in the cytoplasm. RBPJκ can interact not only with EBNAs but also with cellular proteins, including Notch and the vertebrate homolog of *Drosophila melanogaster* Hairless (4, 5, 13, 22, 26), although the extent of association of RBPJκ with these proteins in B lymphocytes remains to be determined.

Recombinant EBV-based molecular genetic evidence indicates that EBNA-2 is essential for lymphocyte transformation because of its role as a transactivator of viral and cellular gene expression (7, 8, 44, 45). Molecular genetic and biochemical evidence indicates that EBNA-2 interaction with RBPJκ is important in localizing EBNA-2 to specific promoters so that the EBNA-2 acidic domain can recruit basal and activated transcription factors to increase transcription (7, 16, 19, 23, 25, 28, 39–45). The coimmunoprecipitation data shown here reinforce the link between RBPJκ and EBNA-2. These results are in contrast to the difficulty in demonstrating RBPJκ–EBNA-2 complexes by DNA probe shift. This difficulty may be related to a negative effect of EBNA-2 and EBNA-3s on RBPJκ affinity for DNA in vitro (16, 33, 34). The EBNA-2–RBPJκ coimmunoprecipitations also contrast with an inability to coimmunoprecipitate EBNA-2 with PU.1 (reference 25 and our unpublished observations) despite the importance of PU.1 in EBNA-2 responsiveness of the LMP1 and LMP2 promoters and the demonstration that some EBNA-2-derived fusion proteins can deplete PU.1 from nuclear extracts (23). EBNA-2 interaction with PU.1 may be dependent on PU.1 interaction with its cognate DNA or with another protein. The stable association of most of the soluble EBNA-2 with RBPJκ favors a primary role for RBPJκ in efficiently conveying EBNA-2 to response elements, where EBNA-2 can exhibit its strong activating effects.

While the extent of association of EBNA-3C and of other EBNA-3s with RBPJκ demonstrated here is evidence of an important role for RBPJκ in transcriptional effects of the EBNA-3s, the precise role of EBNA-3A, EBNA-3B, and EBNA-3C in EBV-induced primary B-lymphocyte growth transformation is less certain than the role of EBNA-2. Promoters with RBPJκ sites are frequently activated by EBNA-2, and such promoters are variably affected by EBNA-3A, EBNA-3B, and EBNA-3C. In BL cells, EBNA-3C can up-regulate CD21 expression (42) or LMP1 expression when cells reach saturation (2) and EBNA-3B can up-regulate CD40 (36). By using recombinant EBV molecular genetics, only one mutation each has been studied for EBNA-3B (37) and EBNA-3C (38) and only two have been studied for EBNA-3A (24, 38). The EBNA-3A and EBNA-3C mutations substantially affected transformation but left intact the N-terminal domains that interact with RBPJκ and destabilize the interaction of RBPJκ with DNA in vitro (33). Thus, this interaction is not sufficient for efficient transformation even though destabilization of the repressive effects of RBPJκ (10, 21) could be activating. EBNA-3A, EBNA-3B, and EBNA-3C also have glutamine- and proline-rich domains. The EBNA-3C domain can activate a nearby promoter (29) and in the context of full-length EBNA-3C is presumed to localize to promoters at least in part through interaction with RBPJκ. These biochemical properties of the EBNA-3s are dependent on relatively small parts of these antigens, while the EBNA-3s are each nearly 1,000 amino acids long. Thus, other, as yet unidentified EBNA-3 domains are likely to be important in the differential activity of EBNA-3-RBPJκ complexes at specific promoters.

The smaller isoform of RBPJκ appears to preferentially associate with the EBNAs. Treatment of RBPJκ immunoprecipitates with phosphatase did not alter the relative amount of either RBPJκ isoform (data not shown). RBPJκ is encoded by several transcripts that differ in their first two exons (3). Splice variants 1 and 3 have been detected in B lymphocytes and likely encode the two isoforms (16). Since these variants differ only in the first 20 amino acids, the preference of EBNAs for the smaller isoform is somewhat surprising, especially since residues 179 to 361 of RBPJκ appear to mediate EBNA-2 interaction (21).

The finding of separate in vivo EBNA-2–RBPJκ and EBNA-3–RBPJκ complexes points to a finely balanced viral mechanism for regulating transcription through RBPJκ. This mechanism is similar to that proposed for *D. melanogaster*, whereby the RBPJκ isolog Su(H) is regulated by a balance of Hairless/Su(H) versus Notch(intracellular domain)/Su(H) complexes (4, 5). *Drosophila* genetic analyses also point to an important dosage dependency of Su(H) activity consistent with regulation based on the quantitative amounts of various Su(H) complexes (35). The observations that overexpression of Notch in T lymphocytes is associated with leukemia, that Notch positively regulates transcription through RBPJκ, that EBNA-2 positively regulates transcription through RBPJκ, and that EBNA-2 extensively associates with RBPJκ in EBV-transformed B lymphocytes are consistent with EBNA-2 mimicking Notch and activating a cellular gene(s) important in growth regulation. Conversely, the high-level interaction of EBNA-2 and EBNA-3s with RBPJκ in EBV transformation of primary B lymphocytes suggests the possibility that signaling through Notch or another regulator of RBPJκ may be important in normal B-lymphocyte growth or differentiation. The ability of the EBNA-3s to destabilize the interaction of RBPJκ with DNA in vitro and to antagonize activating effects through RBPJκ suggests that the EBNA-3s may be similar to Hairless (5).

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