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SHORT COMMUNICATION

Both A type and B type Epstein–Barr virus nuclear antigen 6 interact with RBP-2N

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Using the yeast two-hybrid system, Epstein–Barr virus nuclear antigen 6A (EBNA6A) was found to interact with the RBP-2N isoform of RBP-Jκ. The interaction of EBNA6A and EBNA6B with RBP-2N was compared and the results indicated that EBNA6B was less efficient at interacting with RBP-2N than was EBNA6A. Deletion mutation analysis of EBNA6A identified a region involved in the interaction with RBP-2N, while analysis of RBP-2N identified a domain which interacts with EBNA6A. The region of RBP-2N to which EBNA6A binds has previously been shown to interact with EBNA2.

Epstein–Barr virus (EBV) is the aetiological agent of infectious mononucleosis and is associated with Burkitt's lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disease in immunocompromised individuals (reviewed in Rickinson & Kieff, 1996). Transformation of B lymphocytes by EBV results in the expression of a family of latent antigens which includes six Epstein–Barr nuclear antigens (EBNAs 1–6). EBV strains can be categorized into one of two types, A type or B type (also known as type I or II), which show sequence divergence in the EBNA2, -3 (3A), -4 (3B) and -6 (3C) gene products. Differences in these proteins, expressed during EBV-induced transformation of B lymphocytes, are thought to be reflected in biological differences between A type and B type EBV (Rickinson *et al*., 1987). Expression of the full array of latent gene products induces uncontrolled proliferation of B lymphocytes into lymphoblastoid cell lines (LCLs). Of the nuclear antigens, expression of EBNA2 (Hammerschmidt & Sudgen, 1989), EBNA3, EBNA6 (Tomkinson *et al*., 1993) and EBNA5 (Mannick *et al*., 1991) is essential for the transformation of B lymphocytes.

Little is known about the functions of EBNA proteins and the role they play in the transformation process. EBNA2 transactivates both cellular and viral genes through its interaction with the DNA-binding protein RBP-Jκ (also known as CBF1) (Ling *et al*., 1993) and other proteins (Johannsen *et al*., 1995). Several alternatively spliced cDNAs of RBP-Jκ which differ in their first exon but share common exons 2–11 have been identified (Amakawa *et al*., 1993; Brou *et al*., 1994).

Recently, interaction of EBNA6 with RBP-Jκ was identified both *in vitro* and *in vivo* (Robertson *et al*., 1995; Marshall & Sample, 1995; Zhao *et al*., 1996). EBNA6 was shown to form stable complexes with RBP-Jκ, inhibiting RBP-Jκ binding to an oligonucleotide sequence containing an RBP-Jκ binding site, and to disrupt the interaction of RBP-Jκ with EBNA2 (Robertson *et al*., 1995). Co-expression of EBNA6 and EBNA2 demonstrated the inhibition of EBNA2-mediated transactivation (Robertson *et al*., 1995).

To identify an interaction between EBNA6A and other viral or cellular proteins, EBNA6A was expressed in the yeast two-hybrid system (CLONTECH). EBNA6A cDNA was prepared by the digestion of plasmid T36 (Petti *et al*., 1988) (containing the EBV strain B95.8 EBNA6 coding sequence) with restriction enzymes, and subsequent ligation into the shuttle expression vector pGBT9. The construct gave a fusion of the GAL4-binding domain (bd) and amino acids 1–992 of the EBNA6A protein sequence. Correct insertion of the EBNA6A coding sequence into pGBT9 was confirmed by digestion with restriction enzymes and DNA sequencing of the insertion site at the 5′ end of the EBNA6A insert. Expression of the fusion protein in yeast strains HF7c and SFY526 was confirmed by Western blot analysis using a human serum (MCr) known to react with the EBNA6A protein (results not shown). To ensure that the fusion protein did not cryptically transactivate the reporter *lacZ*, a series of control transformations were performed according to the manufacturer's recommendations (CLONTECH).

Yeast strain HF7c was sequentially co-transformed with the EBNA6–GAL4 bd vector then with a GAL4 activation domain (ad) cDNA fusion library (CLONTECH). This library is constructed from an EBV-transformed human B cell population in the shuttle expression vector pACT. Interaction between the GAL4(bd)–EBNA6A fusion protein and a GAL4(ad)–library fusion protein reconstitutes GAL4 function and transactivates two reporters, HIS3 and *lacZ*. Transformants were plated on synthetic minimal medium without the amino acids leucine, tryptophan and histidine to select for growth of co-transformants in which the two hybrid proteins interact. The

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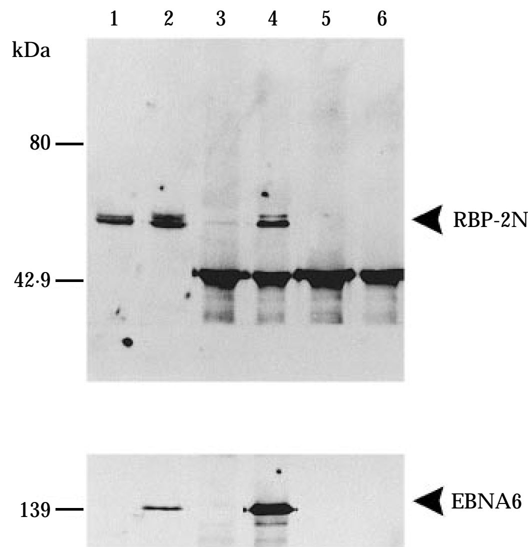

Fig. 1. Co-immunoprecipitation of RBP-2N with EBNA6. Lane 1, Western blot of DG75 showing expression of RBP-Jκ and RBP-2N; lane 2, Western blot of LCL (DJM, B95.8) showing expression of RBP-Jκ, RBP-2N and EBNA6 (stained with E3cA10 anti-EBNA6 monoclonal antibody); lane 3, immunoprecipitation with DG75 lysate showing that no co-immunoprecipitation of RBP-2N occurs in the absence of EBNA6; lane 4, co-immunoprecipitation of RBP-Jκ and RBP-2N with EBNA6; lanes 5 and 6, immunoprecipitation with DG75 and LCL lysate, respectively, lacking anti-EBNA6 antibody and showing that co-immunoprecipitations are antibody-specific.

resulting colonies were assayed for *β*-galactosidase activity using a filter assay, as recommended by the manufacturer. Of the 510 colonies assayed, only five clones were positive for *β*-galactosidase activity.

β-Galactosidase-positive colonies were checked for cryptic activation, as recommended by the manufacturer. The resultant library plasmid DNA was rescued and used to transform SURE *Escherichia coli* (Stratagene) by electroporation, from which purified activation domain plasmid DNA was isolated and sequenced. Sequencing of the cDNA insert was performed using primers (5′ TACCACTACAATGGATG 3′ and 5′ AGTTGAAGTGAACTTGCG 3′) either side of the multiple cloning site of pACT. The PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) was used and the products analysed on an Applied Biosystems Model 373A DNA sequencing system. The resulting DNA sequences were all found to have identity with the RBP-2N isoform of RBP-Jκ. Four of the clones were full-length RBP-2N, while the fifth clone commenced from amino acid 159 of RBP-2N.

To confirm that EBNA6 interacted with RBP-2N *in vivo*, co-immunoprecipitations were performed using an anti-EBNA6 monoclonal antibody (E3cD8). An LCL (DJM, B95.8) was lysed by the addition of NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris–HCl pH 8). The cell lysate was incubated with the E3cD8 antibody for 1 h at 4 °C. The immune complex was immunoprecipitated with rabbit anti-mouse IgG antibody and Sepharose A beads. The immune complex was washed and then subjected to SDS–PAGE and Western blot analysis using an anti-RBP-Jκ monoclonal antibody (T6719). Two forms of RBP-Jκ were co-immunoprecipitated with EBNA6; the smaller isoform predominated (Fig. 1). Since RBP-2N has a lower molecular mass than RBP-Jκ, and given that we demonstrated that RBP-2N was the major isoform of RBP-Jκ in LCLs and BLs (Krauer *et al*., 1996), it is likely that the smaller isoform detected on the Western blot represents RBP-2N. A recent report by Johannsen *et al*. (1996) also showed that EBNA6 co-immunoprecipitated with a smaller isoform of RBP-Jκ.

Isoform-specific amino-terminal regions of RBP-Jκ may modulate the affinity or specificity of RBP-Jκ to bind DNA (Brou *et al*., 1994). RBP-2N binds to a specific recognition sequence 5′ TGGGAAAGAA (Dou *et al*., 1994). This recognition sequence is similar to that described for RBP-Jκ-binding by Jin & Speck (1992), with a core DNA binding domain of GTGGGAA (Tun *et al*., 1994). Considering the interest in understanding the immortalization of B lymphocytes by EBV and the difference in binding affinity or specificity of different isoforms of RBP-Jκ, it is important that further studies on the interaction of the EBNAs with RBP-Jκ should utilize the RBP-2N isoform.

To examine the interaction between EBNA6A and RBP-2N more closely, deletion mutants of EBNA6A were prepared in the shuttle expression vector pGBT9 (Fig. 2*a*). Correct insertion of the coding sequences into pGBT9 was confirmed by DNA sequencing. All constructs were tested to ensure they did not cryptically transactivate the *lacZ* reporter gene. Yeast co-transformed with pACT-RBP-2N and the deletion mutants of EBNA6A was then assayed for *β*-galactosidase activity using a modified form of the quantitative liquid *o*-nitrophenylgalactoside (ONPG) assay according to the manufacturer's recommendations (CLONTECH). Assays were performed in triplicate for five different clones of each deletion mutant. Protein–protein interaction with RBP-2N was detected in yeast transformed with clones expressing full-length EBNA6A and amino acids 365–992 and 182–440 of EBNA6A (Fig. 2*a*). These data suggest that a region from amino acids 182–440 is involved in interaction with RBP-2N. This region overlaps the one identified by Zhao *et al*. (1996), when amino acids 181–257 of EBNA6 were found to interact with RBP-Jκ. However, there is virtually no overlap with the region of EBNA6 (amino acids 1–183) identified by Robertson *et al*. (1996) as being sufficient for interaction with RBP-Jκ in co-immunoprecipitation experiments.

To compare the interaction of EBNA6A and EBNA6B with RBP-2N, the second exons of EBNA6B (EBV strain AG876) and EBNA6A (EBV strain B95.8) were cloned into the shuttle expression vector pGBT9 (Fig. 2*b*, *c*). Correct insertion of EBNA6A and EBNA6B into pGBT9 in-frame with GAL4(bd) was confirmed by DNA sequencing. All constructs were tested to ensure they did not cryptically transactivate the *lacZ* reporter gene. Yeast co-transformed with pACT-RBP-2N and

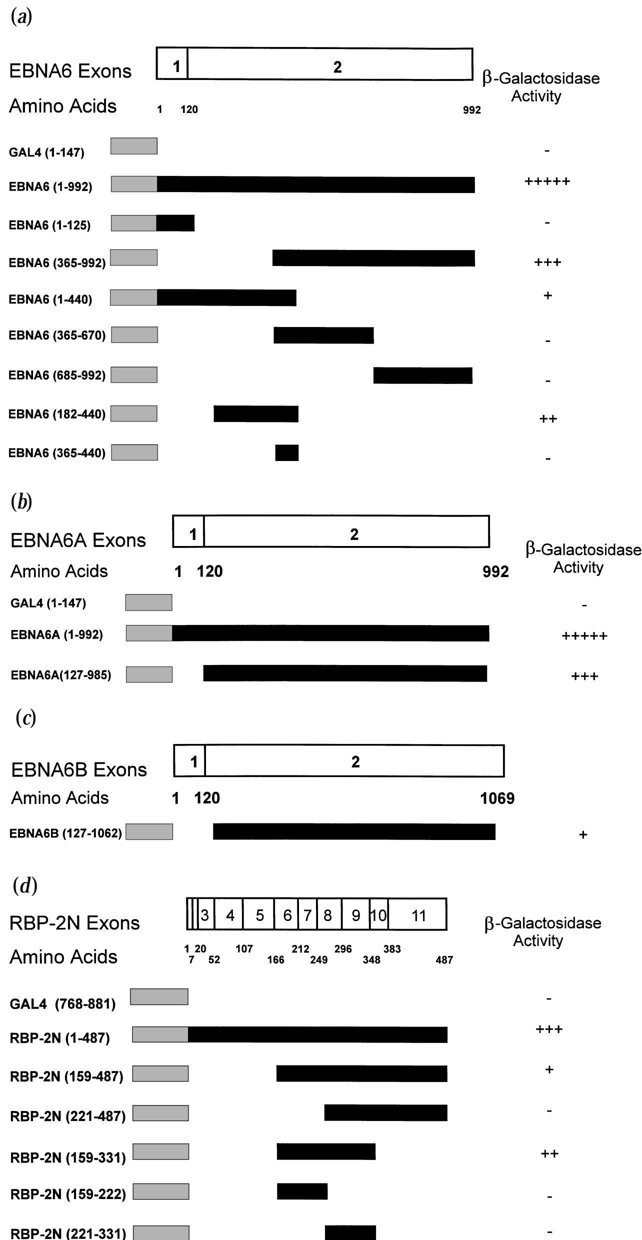

Fig. 2. (*a*) Interaction of EBNA6A deletion mutants with RBP-2N. The GAL4 DNA bd protein (grey boxes) was expressed in fusion with EBNA6A deletion mutants (black boxes). Yeast expressing EBNA6 deletion mutants did not transactivate the β-galactosidase reporter in the absence of RBP-2N. (*b*, *c*) Interaction of A type (*b*) and B type (*c*) EBNA6 proteins with RBP-2N. The GAL4 DNA bd protein (grey boxes) was expressed in fusion with EBNA6 (black boxes). (*d*) Interaction of deletion mutants of RBP-2N with EBNA6A. The GAL4 ad protein (grey boxes) was expressed in fusion with RBP-2N deletion mutants (black boxes). Relative levels of expression of the reporter gene, β-galactosidase, are presented to the right of each fusion protein. The numbers in brackets are the amino acids expressed from each construct.

the second exon of EBNA6A or EBNA6B in pGBT9 was then assayed for β-galactosidase activity using the quantitative liquid ONPG assay. Assays were performed in triplicate for five different clones of each co-transformant. EBNA6B was shown to interact with RBP-2N. However, our data suggest that EBNA6A interacts more strongly with RBP-2N than does EBNA6B. The apparent differences in affinity of EBNA6A and -B for RBP-2N may be a contributing factor in the reduced transforming activity of B type EBV.

To define the region of RBP-2N which interacted with EBNA6A, deletion mutants of RBP-2N were cloned into the shuttle expression vector pGAD424 in-frame with the GAL4 ad (Fig. 2*d*). Correct insertion of the deletion mutants was confirmed by DNA sequencing. All deletion mutants were tested to ensure they did not cryptically transactivate the *lacZ* reporter gene. Yeast was co-transformed with pGBT9-EBNA6A and the deletion mutants of RBP-2N, and β-galactosidase activity was measured using the liquid ONPG assay. Assays were performed in triplicate for five different clones of each deletion mutant. Protein–protein interaction with EBNA6A was detected in yeast expressing full-length RBP-2N, RBP-2N (159–487) and deletion mutant RBP-2N (159–331) (Fig. 2*d*). RBP-2N (159–331) includes the region recently identified to interact with EBNA6 (Zhao *et al*., 1996), EBNA3 and EBNA4 (Krauer *et al*., 1996) and is almost identical to the domain of RBP-Jκ amino acids 179–361, which interacts with EBNA2 (Hsieh & Hayward, 1995).

Due to the low level of expression of hybrid proteins from the vectors used it was not possible to measure expression levels by Western blot (CLONTECHniques, 1995). To attempt to compensate for possible variations in expression level, assays were performed in triplicate for five different clones.

EBNA2 appears to be the first latent EBV protein expressed after infection of B lymphocytes (Alfieri *et al*., 1991). EBNA2 then apparently interacts with RBP-Jκ-binding to the C promoter of EBV, initiating transcription of EBNA6 and other latent viral proteins. Since EBNA6 interacts with the same region of RBP-Jκ as EBNA2, it is likely that competition between EBNA2 and -6 for RBP-Jκ may contribute to regulation of the transactivating function of EBNA2. This would explain the observation that EBNA6 inhibits activation of viral gene LMP1 by EBNA2 (Robertson *et al*., 1995; Marshall & Sample, 1995) and that EBNAs 3, 4 and 6 repress EBNA2-mediated transactivation of a viral terminal protein 1 gene promoter (Roux *et al*., 1994).

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