Epstein–Barr virus nuclear antigen 1 is a DNA-binding protein with strong RNA-binding activity

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Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA-1) plays key roles in both the regulation of gene expression and the replication of the EBV genome in latently infected cells. To characterize the RNA-binding activity of EBNA-1, it was demonstrated that EBNA-1 binds efficiently to RNA homopolymers that are composed of poly(G) and weakly to those composed of poly(U). All three RGG boxes of EBNA-1 contributed additively to poly(G)-binding activity and could mediate RNA binding when attached to a heterologous protein in an RNA gel mobility-shift assay. In vitro-transcribed EBV and non-EBV RNA probes revealed that EBNA-1 bound to most RNAs examined and the affinity increased as the content of G and U increased, as demonstrated in competition assays. Among these probes, the 5′ non-coding region (NCR) (nt 131–278) of hepatitis C virus RNA appeared to be the strongest competitor for EBNA-1 binding to the EBV-encoded small nuclear RNA 1 (EBER1) probe, whereas a mutant 5′ NCR RNA with partially disrupted secondary structure was a weak competitor. Furthermore, the interaction of endogenous EBNA-1 and EBER1 in EBV-infected cells was demonstrated by a ribonucleoprotein immunoprecipitation assay. These results revealed that EBNA-1 is a DNA-binding protein with strong binding activity to a relatively broad spectrum of RNA and suggested an additional biological impact of EBNA-1 through its ability to bind to RNA.

INTRODUCTION

Epstein–Barr virus (EBV), a ubiquitous human gamma-herpesvirus, infects most of the world’s population. EBV infections usually result in latency; Epstein–Barr virus nuclear antigen 1 (EBNA-1) is the only viral protein that is detectable in all EBV-associated malignancies (Kieff, 1996). EBNA-1 initiates DNA replication at the latent replication origin, oriP, and maintains EBV DNA molecules in cells by binding to a specific DNA sequence in oriP (Middleton & Sugden, 1992). In addition to modulation of EBV latent promoters, transient expression of EBNA-1 is sufficient to induce the recombinese-activating genes RAG1, RAG2 (Srinivas & Sixbey, 1995) and CD25 (interleukin 2 receptor z-chain; Kube et al., 1999) in cell lines. However, the mechanism whereby EBNA-1 regulates the expression of these cellular genes remains unknown.

EBNA-1 is a 641 aa DNA-binding protein (Fig. 1) that binds to multiple sites within the family of repeat (FR) and dyad symmetry (DS) elements that comprise the 1.8 kbp boundary of oriP (Rawlins et al., 1985). The DNA-binding and dimerization domains of EBNA-1 have been mapped to the C-terminal 459–607 aa (Chen et al., 1993) and the crystal structure of EBNA-1 reveals a glycine-rich region that is responsible for direct DNA recognition (Bochkarev et al., 1996). The N-terminal 50 aa region of EBNA-1 shares 50% sequence similarity with the N-terminus of the human ribosomal protein S2, but the functional significance remains obscure (Yates & Camiolo, 1988). EBNA-1 also contains three RGG-like motifs, repeated at aa 33–56, 330–350 and 354–377, that have been hypothesized to be responsible for RNA binding (Snudden et al., 1994).

Investigations seeking proteins that interact with EBNA-1 have identified cellular proteins involved in RNA processing, including p32 (Wang et al., 1997; Chen et al., 1998) and EBNA-1-binding protein 2 (EBP2; Shire et al., 1999). The cellular protein p32, which is also known as SF2-associated protein or TAP (HIV Tat-associated protein), was first isolated from HeLa cells as a protein that was associated tightly with the essential splicing factor ASF/SF2 (Krainer et al., 1990). ASF/SF2 is a member of the SR family of splicing factors and can stimulate constitutive splicing and regulate alternative RNA splicing factors positively or negatively, depending on where they bind to the pre-mRNA (Fu, 1995; Manley & Tacke, 1996). p32 was shown to inhibit the phosphorylation of ASF/SF2 and thereby prevent a stable interaction between ASF/SF2 and
RNA (Petersen-Mahrt et al., 1999). Mutational studies of EBNA-1 revealed that the p32-binding regions of EBNA-1, which overlap RGG motifs, are important for transcriptional activation of a reporter gene that is linked to FR elements and for maintenance of oriP-containing plasmids (Van Scoy et al., 2000). The other EBNA-1-interacting protein relating to RNA, EBP2, is a 35 kDa protein and the region of EBNA-1 that interacts with EBP2 is important for maintenance of plasmids containing oriP in human cells (Shire et al., 1999). The study of EBP2 homologues in the yeast system revealed that it encodes a protein that is essential for pre-rRNA processing (Huber et al., 2000). More interestingly, it was reported by Sugawara et al. (1999a) that infection with EBV successfully converted several cell lines to permissiveness for hepatitis C virus (HCV) RNA replication. EBNA-1 was demonstrated to support HCV RNA replication by transfecting individual plasmids into MT-2 cells (Sugawara et al., 1999b).

We were prompted to further characterize the RNA-binding abilities of EBNA-1, which may be mediated through multiple RGG motifs. RGG motifs were first identified in heterogeneous nuclear ribonucleoprotein (hnRNP) U, which is a member of a family of polypeptides that bind hnRNP particles and control the post-transcriptional pathways of expression of individual genes (Swanson & Dreyfuss, 1988; Kiledjian & Dreyfuss, 1992). RGG motifs have been found in a wide variety of RNA-binding proteins that are involved in RNA processing or transport, such as herpes simplex virus (HSV) ICP27 and splicing factor TLS (Sandri-Goldin, 1998; Lerga et al., 2001), whereas fragile X mental retardation protein recognizes GGGG GU-rich sequences or with high degrees of secondary structure. Observation of the in vivo interaction of EBNA-1 with EBER1 further confirmed EBNA-1 as a DNA sequence-specific transactivator with strong RNA-binding activity.

**METHODS**

**Plasmids.** For in vitro translation of EBNA-1, plasmids pMRC72 [E1(1–102, 325–641)] (Chen et al., 1994), pRA362 [E1(408–641)] (Ambinder et al., 1991), pWS61 [E1(459–641)] (Shah et al., 1992) and pMRC77 [E1(462–641)] (Chen et al., 1994) were used. The following EBNA-1-coding fragments were cloned into a pGEM2 derivative.

![Diagram of EBNA-1 structure and functional domains](image-url)
pCW21, which encoded E1 (1–102, 325–641), was generated by using LMRC19 (5’-CTAGAGATCTATGCTGAGGAGGAGCA-3’) (restriction site underlined) as the forward primer and LMRC14 (5’-AACTGAGATCTCCTGTGAGGAGGAGCA-3’) as the reverse primer, with p367 (Yates & Camiolo, 1988) as the template. pCW22 [E1[ARGGG]], which contained deletions of aa 40–60 and aa 103–324 of EBNA-1, was generated using LMRC14 and LMRC19 with p378dG2 (Yates & Camiolo, 1988) as the template. The PCR product was digested with BsPI and SacI and used to replace the BsPI/SacI fragment of pMRC72. pCW20 [E1[ARGGG,2,3]], which contained a deletion between aa 93 and 381, was generated by using LMRC14 and LMRC19 as primers and p385dG2 (Yates & Camiolo, 1988) as the template. pCW24 [E1[ARGGG,2,3]], which contained a deletion of aa 40–60 and 93–381 of EBNA-1, was generated by replacing the PfuII/BspEI DNA fragment of pCW20 with the PfuII/BspEI DNA fragment from pCW18.

Three constructs were generated for glutathione S-transferase (GST)—EBNA-1 RGG fusion proteins. The primer sets that were used for pCW12 [GST-E1[32–58]] were 5’-GAAGATCTCAAGAAGAGGGGTGTA-3’ and 5’-CGGGATCCCGCCCGGCTCTCGTCG-3’ (GST-RGG1); for pCW13 [GST-E1[32–360]] were 5’-GAAGATCTCGAGGAGGTTTGGCC-3’ and 5’-GATCGGATCCCTCTTACGTCTCT-3’ (GST-RGG2); and for pCW14 [GST-E1[351–382]] were 5’-GAAGATCTCGAGGAGGTTTGGCC-3’ and 5’-GCGGA-TCCCTGGGGCTCTTTCCTC-3’ (GST-RGG3). PCRs were performed by using pRA17 (Ambinder et al., 1990) as the template and the amplified products were digested with BamHI and BgIII and cloned into a derivative of pGEX-3 (Pharmacia).

To construct a recombinant EBNA-1-expressing baculovirus, the PCR product that was amplified by using primers 5’-CTAGAGATCTCATATGGCTGAGGAGGAGCA-3’ and 5’-GAAGATCTCAAGAAGAGGGGTGTA-3’ with p367 as the template was digested with BamHI and BgIII and cloned into the BgIII site of pACHLT-B (Pharmingen) to generate pTY1. To express EBNA-1 in transfected cells, a HindIII/BamHI fragment from pRA17 was cloned into pSG5.

To generate RNA probes, pCW16 (pGEM3-EBER1, encoding 167 nt of EBER1) and pCW17 (pGEM3-ZEBER2, encoding 172 nt of EBER2) were generated by using a B95-8 cell lysate as the template and primers 5’-GAAGATCTTACATGGCTGAGGAGGAGCA-3’ and 5’-GAAGATCTCCTGGGGCTCTTTCCTC-3’ for EBER1 and 5’-GAAGATCTCGAGGAGGTTTGGCC-3’ and 5’-GCGGATCCCTCGGACACGGCCGCA-3’ for EBER2. PCR products were digested with BamHI and BgIII and cloned into the pGEM-3Z. pCL3 and pCL4 were linearized with SmaI and transcribed by T7 RNA polymerase. The mixture was incubated at 37°C until the OD600 reached 0.6 and added to 20 ml of T7 or SP6 RNA polymerase. The mixture was incubated at 37°C for 1 h, 1 U RQ1 DNase was added and the mixture was incubated for another 15 min. The RNA product was purified on a Chroma-spin column (Clontech) and quantified by using a Beckman scintillation counter.

**Interaction of EBNA-1 and RNA**

Expression of in vitro-translated EBNA-1 mutants and RNA homopolymer-binding assays. In vitro translation was performed by using a TNT reticulocyte lysate (Promega) in the presence of [35S]methionine. To assess binding of in vitro-translated protein to ribonucleotide homopolymers (Kiledjian & Dreyfuss, 1992), 10⁵ c.p.m. trichloroacetic acid-precipitable translated protein was made up to a final volume of 0.5 ml with binding buffer (10 mM Tris-HCl, pH 7.6; 2.5 mM MgCl₂; 0.5% Triton X-100; appropriate concentration of NaCl). Washed agrose beads (50 μg) containing the attached ribonucleotide homopolymer (Pharmacia or Sigma) or ssDNA (Pharmacia) were added to the mixture and incubated for 60 min at 4°C on a rocker platform. The beads were pelleted in a microfuge and washed five times with binding buffer. Bound protein was eluted in SDS sample buffer [50 mM Tris/HCl, pH 6.8; 4% SDS; 20% glycerol; 0.04% bromophenol blue; 200 mM diethiothreitol (DTT)] and analysed by 10 or 12% SDS-PAGE. Radioactive bands were detected with a phosphorimag (Storm 840; Molecular Dynamics).

Expression and purification of recombinant EBNA-1 (rEBNA-1). Recombinant baculovirus expressing EBNA-1 was generated by co-transfection of pTY1 and baculovirus DNA with a BaculoGold Transfection kit (Pharmingen). For preparation of rEBNA-1, virus-infected Sf9 cells were incubated for 46 h at 27°C and the cells were scraped into cold PBS and harvested by low-speed centrifugation. The cells were lysed in 10 mM Tris/HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF and protease inhibitor cocktail and incubated on ice for 45 min. After sonication and clarification at 30000 r.p.m. (Beckman L-80, SW41 rotor) for 45 min, the supernatant was applied to a 5 ml Econo-heparin column (Bio-Rad) and eluted with eluting buffer (20 mM HEPES, pH 7.5; 0.5 mM EDTA; 1 mM PMSF; 2 mM DTT; 20% glycerol) that contained 250, 500, 750 or 1000 mM NaCl. The fractions that contained EBNA-1 were pooled, the NaCl concentration was adjusted to 100 mM and the fractions were applied to a MonoQ column (Bio-Rad). The protein was eluted with eluting buffer that contained 100, 250, 500, 750 or 1000 mM NaCl. The purified protein produced from recombinant baculovirus will be referred to as rEBNA-1.

Expression and purification of GST–EBNA-1 fusion proteins. To express GST or GST–EBNA-1 fusion proteins, bacterial culture at exponential phase was diluted with 10 vols fresh medium, cultured at 37°C until the OD₆₀₀ reached 0.6 and induced with 0.1 mM IPTG for 3 h. The GST–EBNA-1 fusion protein or GST was expressed in JM109 cells and purified by using a glutathione column (Chen et al., 2001).

**RNA probes.** In a typical 20 ml reaction, 1 μg linearized plasmid DNA was added to 4 ml 5 x transcription buffer, 1 ml 10 mM ATP, CTP and GTP, 2–5 μl 100 μM UTP, 2 μl 5 x-PRLP (3000 Ci mmol⁻¹=111 TBq mmol⁻¹), 1 μl RNasin (40 U μl⁻¹) and 1 ml T7 RNA polymerase. The mixture was incubated at 37°C for 1 h, 1 U RQ1 DNase was added and the mixture was incubated for another 15 min. The RNA product was purified on a Chroma-spin column (Clontech) and quantified by using a Beckman scintillation counter.

In vitro synthesis of RNA. RNA was prepared by using an in vitro transcription kit (Promega). For a 100 ml reaction, 4 μl linearized plasmid was dissolved in an appropriate amount of deionized water and added to 20 μl 5 x transcription buffer, 10 μl each 10 mM ATP, CTP and GTP, 2–5 μl 100 μM UTP, 2 μl 5 x-PRLP (3000 Ci mmol⁻¹=111 TBq mmol⁻¹), 1 μl RNasin (40 U μl⁻¹) and 1 ml T7 RNA polymerase. The mixture was incubated at 37°C for 1 h, 1 U RQ1 DNase was added and the reaction mixture was incubated for a
further 1 h. RQ1 DNase (4 U) was added and the mixture was incubated for 15 min at 37 °C to remove the DNA template. As the plasmid pSP64c191(A)M307 was not transcribed efficiently, PCR was performed with a forward primer containing a T7 promoter sequence (shown in bold) (5’-TAATACGACTCACTATAGATGCCTCGGGGAGAGCCATTGGGACACCAGCTGGTAC-3’) and the reverse primer (5’-AAGCTTTCGGGACCACTCTCGG-3’). All RNA products were purified by using Chroma-spin columns (Clontech). For quantification, an aliquot of RNA was denatured at 65 °C, electrophoresed on a 2 % TBE/agarose gel, stained with SYBR green II RNA stain (Molecular Probes) and analysed by using a Storm 840 phosphorimager (Molecular Dynamics) and QuantImage by comparison with RNA standards.

RNA-binding GMSA. RNA-binding conditions were modified from those described by Snudden et al. (1994). Briefly, 2 x 10^5 c.p.m. [α-32P]UTP-labelled RNA probe was incubated with purified EBNA-1 in a 20 μl reaction mixture that contained binding buffer (25 mM Tris/HCl, pH 8-0; 1 mM MgCl2; 0-4 M NaCl; 5 mM spermidine; 1 mM DTT; 5 mM EDTA; 5 % glycerol), 40 U Rnasin, 0-4 μg yeast tRNA and 1 μg poly(I).poly(C) (Pharmacia) and incubated at 30 °C for 30 min. The binding complex was analysed by gel shift using non-denaturing 19:1 PAGE containing 50 mM Tris/glycine (pH 8-8) and 5 % glycerol. After electrophoresis, the gel was dried and analysed by using a phosphorimager. In the supershift assay, 2 μl purified EBNA.OT1x (Chen et al., 1993) or anti-His tag antibody (Qiagen) was added to the reaction and incubated for another 10 min. In competition assays, different amounts of non-radiolabelled RNA were included in the reaction.

Ribonucleoprotein immunoprecipitation (RIP) assay. In vivo cross-linking of the protein–RNA complex was described as modified by Niranjankumari et al. (2002). To increase the intracellular amount of EBNA-1 protein, 2 x 10^7 Akata cells were activated into the lytic cycle with 0-5 % anti-human IgG (Zetterberg et al., 2002) and harvested at 18 h post-induction. In the epithelial system, 15 μg EBNA-1 expression plasmid pTY2 or vector was transfected into 2 x 10^6 EBV-positive NA cells (Chang et al., 1999) and harvested at 48 h post-transfection. The cell pellets were resuspended in 10 ml PBS that contained 1 % (v/v) formaldehyde and incubated at room temperature for 10 min with slow mixing. The cross-linking reaction was quenched by the addition of glycine (pH 7-0) to a final concentration of 0-25 M and incubated at room temperature for 10 min. Cells were then harvested by centrifugation, washed twice with ice-cold PBS and resuspended in 1 ml RIPA buffer (50 mM Tris/HCl, pH 7-5; 1 % NP-40; 0-5 % sodium deoxycholate; 0-5 % SDS; 1 mM EDTA; 150 mM NaCl) in the presence of protease inhibitors. After sonication, the cell lysate was clarified by centrifugation at 14 000 r.p.m. in a microfuge for 10 min at 4 °C and the supernatant was pre-cleared with 50 μl protein A–Sepharose beads. The lysate was then diluted with 1 vol. RIPA buffer, mixed with EBNA.OT1x-coated beads and 40 U RNaseOUT (Invitrogen) and incubated with rotation at room temperature for 90 min. The immunocomplexes were pelleted, washed five times with high-stringency RIPA buffer (50 mM Tris/HCl, pH 7-5; 1 % NP-40; 1 % sodium deoxycholate; 0-1 % SDS; 1 mM EDTA; 1 M NaCl; 1 M urea; 0-2 mM PMSE) and suspended in 50 mM Tris/HCl (pH 7.0), 5 mM EDTA, 10 mM DTT and 1 % SDS. To reverse the cross-linking, the resuspended beads were incubated at 70 °C for 45 min. Bound RNA was extracted from these samples by using Trizol (Invitrogen) followed by digestion with 2 U DNases 1 (Ambion) for 30 min at 37 °C, and the DNase activity was inactivated by adding 0-1 vol. 25 mM EDTA. The RNA was reverse-transcribed with random hexamers and PCR-amplified with EBER1-specific primers (5’-CTACGCTGGCC- TAGAGGTTTTGCTA-3’ and 5’-ATGCAGGACCACGCTGGTACTTG-3’).

**RESULTS**

**EBNA-1 binds to poly(G) RNA homopolymers.** To characterize the RNA-binding potential of EBNA-1, in vitro-translated EBNA-1 with aa 102–325 deleted [E1(Δ102–325)], with an apparent molecular mass of 52 kDa, was chosen to avoid the possible non-specific binding effects of the Gly-Gly-Ala repeats. In vitro-translated [35S]methionine-labelled EBNA-1 protein was incubated with RNA homopolymer agarose beads. Binding reactions and washing steps were performed in buffer that contained 0-125, 0-25 or 0-5 M NaCl. The bound protein was eluted and analysed by SDS-PAGE and phosphorimaging. Significant amounts of EBNA-1 bound to poly(G) ribopolymers in buffer that contained various concentrations of NaCl and EBNA-1 also bound weakly to poly(U) ribopolymers, but not to poly(A), poly(C) or ssDNA (Fig. 2).

![Fig. 2. Binding of in vitro-translated EBNA-1 to RNA homopolymers.](https://microbiologyresearch.org)
RGG boxes contribute additively to poly(G) binding of EBNA-1

To identify the sequences in EBNA-1 responsible for poly(G) binding, EBNA-1 deletion mutants were translated in vitro and tested in the binding assay at various NaCl concentrations (Fig. 3). By using mutant E1(Δ102–325), approximately 48% of the input protein bound to poly(G), whereas the binding of E1(ΔRGG1) and E1(ΔRGG2,3) was markedly reduced to approximately 26 and 5%, respectively, in 0.75 M NaCl buffer. The C-terminal DNA-recognition domain (aa 462–477) of EBNA-1 did not show significant binding to poly(G) beads in the presence of 0.75 M NaCl, but may have contributed to the residual binding in 250 mM NaCl. These results indicated that RGG1 and RGG2,3 are functional and additive in their RNA-binding abilities. Interestingly, the RNA-binding ability of E1(Δ102–325) appeared to be higher in the presence of 0.5 or 0.75 M NaCl in comparison with buffer that contained 0.125 (not shown) or 0.25 M NaCl, whereas the binding affinities of the other EBNA-1 constructs decreased in buffers that contained higher concentrations of NaCl.

RNA-binding ability of EBNA-1 can be transferred to a heterologous protein

To verify that the individual RGG boxes of EBNA-1 mediate RNA binding, GST–RGG1, GST–RGG2 and GST–RGG3 were expressed, purified and examined for RNA binding in GMSAs according to the protocol of Snudden et al. (1994), which included 0.4 M NaCl in the binding buffer. Various amounts of purified GST or GST–RGG fusion proteins were incubated with [α-32P]UTP-labelled EBER1 RNA and the RNA/protein mixtures were fractionated by 4% native PAGE. The RNA-binding ability of all three RGG boxes within EBNA-1 could be transferred individually to the GST protein, whereas GST alone or GST–E1(390–641), which contained the DNA-binding domain, did not show significant binding (Fig. 4). A weak smearing pattern, which could be attributed to a non-specific interaction of the DNA-binding domain, was observed in the reaction that contained 2 μg GST–E1(390–641).

Characterization of EBNA-1 RNA-binding specificity by using a recombinant EBNA-1 protein

To further characterize the RNA-binding preference of EBNA-1, a recombinant baculovirus expressing rE1(Δ102–325) was generated. Although rE1(Δ102–325) contained a 6-His tag at the N-terminus for purification, it was unable to bind to a nickel column, possibly due to internalization of the tag sequence during protein folding. Therefore, rEBNA-1 was purified by using a heparin column and a monoQ column to approximately 90% homogeneity, as determined by Coomassie brilliant blue staining (Fig. 5a). The identity of rEBNA-1 was confirmed by immunoblotting using EBNA-1 mAb 5C11 (Chen et al., 1999) and an anti-His tag antibody (data not shown).

As increasing amounts of rEBNA-1 were incubated with the [α-32P]UTP-labelled EBER1 RNA probe, a stable, shifted RNA–protein complex (Fig. 5b, complex I) and larger
complex(es) trapped in the well (Fig. 5b, complex II) were visible. As all three RGG boxes in EBNA-1 are functional, we suggest that a large complex(es) may form among EBNA-1 and RNA molecules. To confirm the identity of EBNA-1 in these complexes, the mAb EBNA.OT1x, which recognizes an epitope between aa 420 and 432 of EBNA-1, or a His tag-specific mAb was added to the binding reaction. Complex I was supershifted with EBNA.OT1x, but...
not with the anti-His tag antibody, whereas complex II was partially disrupted in the presence of EBNA.OT1x or anti-His-tag mAb (Fig. 5c). Thus, it was confirmed that EBNA-1 is responsible for RNA binding in both complexes. The specific RNA-binding activities were calculated as the sum of the radioactivities in complexes I and II in this study. The molecular structures of complexes I and II are unknown, but the differential competition of these complexes when two different competitors were used (Fig. 6c) suggested some structural specificity for the binding of RNA to EBNA-1.

**EBNA-1 binding to various RNA probes in GMSAs**

In order to examine the RNA target preference of EBNA-1, we used several types of *in vitro*-transcribed RNA in GMSAs. The first group of RNAs contained a high degree of secondary structure: EBER1, EBER2, HCV 5’NCR(1–130) and HCV 5’NCR(131–278). The 5’ region of the EBV early gene DNase, obtained from a cDNA clone (−83 to +72), was chosen to represent a lytic transcript. In addition, as several RGG proteins have been shown to be involved in alternative RNA splicing, the intron and intron–exon junction sequences of EBNA-3A, which can be spliced differentially (Kienzle et al., 1999), were also generated as probes. The polylinker region of pGEM-3Z was included as an irrelevant RNA. The GMSA results showed that EBNA-1 bound to all RNA probes tested to varying degrees, except for HCV 5’NCR(1–130) (data not shown).

To compare the binding affinities of EBNA-1 for the different RNA molecules, an RNA competition assay was performed by using EBER1 as the probe in the presence of 0.2 μg EBNA-1 and various cold RNA competitors (Fig. 6a and data not shown). As demonstrated in Fig. 6b, HCV 5’NCR(131–278) appeared to be the strongest competitor, followed by pGEM-3Z, EBER2, EBER1, EBNA-3A intron, EBNA-3A junction, DNase RNA and HCV 5’NCR(1–130). The G, U and G+U contents of individual probes were calculated. The stronger competitors, including HCV 5’NCR (131–278), pGEM-3Z and EBER1 and EBER2, had >30% G or 50% G+U contents, whereas the weaker competitors had lower G or G+U contents. The stronger competitors, including HCV 5’NCR(131–278), EBER1 and EBER2, are also known to contain a high degree of secondary structure.

**Secondary structure of the HCV IRES RNA affects the RNA-binding ability of EBNA-1**

To examine the possible effect of RNA structure on EBNA-1 binding, a mutant RNA (M307) of HCV 5’NCR(131–278) was tested in the competition assay (Fig. 6c, d). This RNA contained mutations at nt 241–245 (GGGAG→CGGTC), which affect the stem–loop III region of the secondary structure, resulting in a predicted long hairpin instead of a multistem structure. It has a lower affinity for a cellular transcription factor (Yen et al., 1995; Chang et al., unpublished data). In comparison with NCR(131–278) of M307, wild-type HCV 5’NCR(131–278) was a 10-fold stronger competitor. The predicted secondary structures of wild-type and mutant HCV 5’NCR(131–278) are shown in Fig. 6(e, f).

**Detection of intracellular EBNA-1–EBER complexes by RIP**

To test whether binding of RNA to EBNA-1 occurred in EBV-positive lymphocyte and epithelial cells, Akata cells were induced into the lytic cycle to increase the expression of endogenous EBNA-1 (Zetterberg et al., 2002). EBNA-1–RNA complexes were cross-linked *in vivo* at 18 h post-induction and immunoprecipitated with EBNA.OT1x. RNA isolated from the immunoprecipitated complex was reverse-transcribed with random hexamers and detected with EBER1-specific primers by PCR. In the presence of reverse transcriptase, the EBER1-specific 157 bp product was detected in the immunocomplex pellet from the reaction by using anti-EBNA-1 antibody, but not anti-GST antibody (Fig. 7a). The supernatant of each reaction was also examined for EBER1 to ensure the integrity of RNA molecules. In addition, EBV-positive NA cells were transfected with a plasmid expressing EBNA-1 for a similar RIP assay. The results of a 25-cycle PCR revealed that the EBER1 PCR product was only detected in the presence of transcribed EBNA-1 (Fig. 7b), whereas the EBER1 RNA bound by endogenous EBNA-1 could also be detected in a 40-cycle PCR (data not shown). Each fraction was also subjected to Western blot analysis to confirm the simultaneous presence of EBNA-1 (Fig. 7c).

**DISCUSSION**

EBNA-1 is known to be crucial for EBV oriP-dependent DNA replication and modulation of viral latent promoters, mainly through its ability to bind DNA and interact with cellular proteins. However, domain-mapping studies of EBNA-1 indicated that the RGG motifs located at aa 33–56, 330–354 and 354–377 overlapped with the p32- and EBP2-interacting domains and also with the chromosome-binding region of EBNA-1 (Fig. 1). To dissect RNA-binding ability from these different biological activities, *in vitro*-translated EBNA-1 was used to demonstrate its binding preference to RNA homopolymers that were composed of poly(G) and poly(U). The binding preference of EBNA-1 is similar to that of HSV ICP27 (Mears & Rice, 1996), but different from some RGG proteins that prefer poly(U), such as TBRGG1 of *T. brucei* (Vanhamme et al., 1998). Compared with other RGG proteins, such as hnRNP U protein (Kiledjian & Dreyfuss, 1992) and HSV ICP27 (Mears & Rice, 1996), EBNA-1 appears to be a RNA-binding protein with very high affinity, as about 50% of input [35S]-labelled protein bound to poly(G) beads in the presence of 0.75 M NaCl (Fig. 3).

It was interesting to note that EBNA-1 bound to poly(G) more efficiently in buffer that contained 0.5 or 0.75 M NaCl than in buffer that contained 0.25 M NaCl. Perhaps the
folding of EBNA-1 determines the exposure of the RGG boxes and therefore regulates its RNA-binding ability. A negative regulatory domain for RNA-binding activity was observed in the C-terminus of HSV ICP27, but whether these domains are biologically relevant remains to be elucidated. We showed that all three RGG boxes of EBNA-1 could contribute additively to RNA-binding activity (Fig. 3) and that each individual box could mediate RNA binding.
in GMSAs (Fig. 4). Furthermore, as the three RGG motifs can function independently of each other, large RNA–rEBNA-1 complexes could be formed, as observed in the GMSAs (Figs 5 and 6).

We tested eight different RNA probes for rEBNA-1 binding in GMSAs. The results indicated that EBNA-1 bound to a broad spectrum of RNA, except for HCV 5’NCR(1–130), and that the binding affinity correlated with the G or G+U contents of the different probes (Fig. 6). The stronger competitors had 30–35 % G or 50–60 % G+U contents, whereas the weaker competitors had 23–25 % G or 43–48 % G+U. Interestingly, the probe with highest affinity, HCV 5’NCR(131–278), contained a region that is bound by several unidentified cellular factors (Yen et al., 1995) and the La antigen (Ali & Siddiqui, 1997). The possible contribution of the secondary structure of the RNA probe to EBNA-1 binding was analysed by comparing wild-type HCV 5’NCR(131–278), which was predicted to form a multistem structure, with HCV 5’ NCR-M307, which harbours a long hairpin-like structure and showed much weaker binding (Fig. 6). How the altered RNA structure affects the binding of EBNA-1 remains to be determined.

The RNA-binding abilities of EBNA-1 reported here support the idea that many transactivators, including p53 and STAT1, are able to bind specific DNA sequences and RNA molecules [reviewed by Cassiday & Maher (2002)]. Although lacking sequence homology, we found that the arrangement of RNA-binding motifs and the RNA-binding characteristics of EBNA-1 share some similarities with the La antigen, which is also composed of three separate RNA-binding domains with a hinge region in between [reviewed by Maraia & Intine (2001)]. Similar to EBNA-1, the La antigen binds to most RNAs and the affinity increases as the number of terminal Us increases. The La antigen was found to bind many pol III transcripts, including EBER1 and -2, VAI and VAII of adenovirus and the NCR of HCV (Maraia & Intine, 2001). Functionally, the La antigen is required for the processing and maturation of precursor tRNA (Van Horn et al., 1997; Pannone et al., 1998). It also binds to some IRESs in viral and cellular mRNAs and has been shown to stimulate IRES-mediated translation (Belsham et al., 1995; Holcik & Korneluk, 2000). A stimulatory effect of rEBNA-1 on HCV IRES–luciferase has also been observed in our laboratory (data not shown).

The *in vivo* EBNA-1–EBER1 interaction detected by the RIP assay indicated that EBNA-1 can form ribonucleoprotein complexes in EBV-infected cells. The biological impact of EBNA-1 RNA binding should be considered in relation to previously mapped functional domains that overlap the RGG motifs. For instance, it is possible that the EBNA-1-interacting protein EBP2, which is involved in rRNA processing and EBNA-1 chromosome binding (Kapoor & Frappier, 2003), associates with metaphase chromosomes through an RNA-mediated mechanism. This might be similar to the scaffold attachment factor A (SAF-A), which binds to a specific DNA sequence and a non-coding RNA, XIST. The RGG box of SAF-A is required for its binding to the inactive X chromosome (Helbig & Fackelmayer, 2003). In addition, the transactivation function of EBNA-1 could also involve RNA binding. This suggestion is based
on the similarity of EBNA-1 to the human papilloma virus E2 protein, which also contains an RGG motif and interacts with p32 and was found to turn on gene expression by functioning as a transcription transactivator and as a pre-mRNA processing modulator (Li et al., 1998).

The RNA-binding properties of EBNA-1 characterized here seem to be affected by the secondary structure of the RNA (Fig. 6). Therefore, we searched for sequence similarity with the dsRNA-binding domain (dsRBD), a 65–70 aa sequence/structure motif that mediates dsRNA interaction, in EBNA-1 by using the PIR Domain Similarity Search and Pairwise Alignment tool (http://pir.georgetown.edu). However, no dsRBD consensus sequence was observed in EBNA-1. Insight into the significance and structural specificity of the EBNA-1–RNA interaction is most likely to come from identification of physiological RNA ligands of EBNA-1 in EBV-infected cells. The RIP protocol described in this report could be used to search for the most abundant RNA species bound by EBNA-1 in EBV-positive cells, as has been described previously for identifying the substrates of HSV US11 (Attrill et al., 2002).

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REFERENCES


