Human p32: A Coactivator for Epstein–Barr Virus Nuclear Antigen-1-Mediated Transcriptional Activation and Possible Role in Viral Latent Cycle DNA Replication

Sarah Van Sc oy, Ikuko Watakabe,* Adrian R. Krainer,* and Janet Hearing2

Department of Molecular Genetics and Microbiology, State University of New York, Stony Brook, New York 11794; and
*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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The Epstein–Barr virus (EBV) nuclear antigen-1 (EBNA-1) is required for the maintenance of the viral chromosome in latently infected, proliferating cells and plays a role in latent cycle DNA replication. EBNA-1 also functions as a positive and negative regulator of EBV gene expression. We have investigated the interaction of EBNA-1 with p32, a host mitochondrial protein that associates with EBNA-1 in EBV-positive Burkitt’s lymphoma cells. Using a chromatin immunoprecipitation assay, we found that a fraction of p32 localizes to the viral latent cycle origin of DNA replication oriP in vivo. p32 binds EBNA-1 independently of other proteins or DNA. EBNA-1 variants lacking one of two p32 binding elements did not interact stably with p32 in cultured cells and were defective for both transcriptional activation of a reporter gene linked to oriP FR and replication and/or maintenance of a plasmid bearing oriP. These results support a role for p32 in transcriptional activation by EBNA-1 and suggest that p32 plays a role in EBV latent cycle DNA replication. © 2000 Academic Press

INTRODUCTION

Epstein–Barr virus (EBV) has attracted much attention because of its ability to establish latency in resting B lymphocytes (Miyashita et al., 1997) and its association with a number of human cancers including nasopharyngeal carcinoma, endemic Burkitt’s lymphoma (BL), and immunoblastic B-cell lymphoma (Rickinson and Kieff, 1996). The viral genome is maintained as a plasmid in the nucleus of latently infected cells (Lindahl et al., 1976) and is replicated once per cell division cycle in parallel with replication of the cellular chromosomes (Adams, 1987). The EBV latent cycle origin of DNA replication, oriP, supports the efficient replication and maintenance of recombinant plasmids in EBV-positive cells (Yates et al., 1984). Notably, the replication of oriP-plasmids is also cell-cycle-regulated and an origin of DNA replication has been identified within oriP (Gahn and Schildkraut, 1989). Only one viral protein, the EBV nuclear antigen-1 (EBNA-1), is required for the replication and maintenance of plasmids bearing oriP (Yates et al., 1985).

oriP contains two clusters of EBNA-1 binding sites (the family of repeats, FR, and sites within the dyad symmetry element, DS) which are required for the stable maintenance of oriP-plasmids (Lupton and Levine, 1985; Reisman et al., 1985). EBNA-1 plays a crucial role in the maintenance of viral DNA in dividing cells through its interaction with FR (Aiyar et al., 1998). Derivatives of oriP-plasmids that lack FR are replicated but not retained in cells (Aiyar et al., 1998; Harrison et al., 1994; Shirakata and Hirai, 1998). The mechanism(s) by which EBV genomes are retained in cells and partitioned equally during cell division is not known but plasmid retention may involve the association of EBNA-1 and the EBV genome with cellular chromosomes during mitosis (Grogan et al., 1983; Harris et al., 1985). EBNA-1 self-associates through multiple “linking” domains (Laine and Frappier, 1995; Mackey et al., 1995). These interactions allow EBNA-1 to bring FR and DS together and loop the intervening DNA in vitro (Frappier and O’Donnell, 1991a; Su et al., 1991) and may underlie the association of oriP-plasmids with cellular chromosomes (Aiyar et al., 1998).

EBNA-1 is not required for the transient replication of plasmids bearing DS but the presence of EBNA-1 increases the likelihood that they will be replicated (Aiyar et al., 1998). The inability of EBNA-1 to unwind DNA in oriP (Frappier and O’Donnell, 1992; Hearing et al., 1992) distinguishes EBNA-1 from other eukaryotic viral origin recognition proteins which are DNA helicases (Bruckner et al., 1991; Stahl et al., 1986; Yang et al., 1993) and indicates that EBNA-1 must facilitate the synthesis of oriP-plasmids by a different mechanism. EBNA-1 interacts with replication protein A in vitro (Zhang et al., 1998) and this result suggests a direct role for EBNA-1 in the initiation of EBV latent cycle DNA replication.

An acidic cellular protein termed p32 was originally identified and cloned on the basis of its copurification with the human pre-mRNA splicing factor SF2/ASF (Krainer et al., 1991). p32 has been implicated in pre-
mRNA splicing (Petersen-Mahrt et al., 1999), transcriptional activation by HIV-1 Tat (Yu et al., 1995b), HIV-1 Rev function (Luo et al., 1994), and gC1q receptor activity (Ghebrehiwet et al., 1994), among other putative functions. p32 was also shown to interact with EBNA-1 in yeast and in human cells transiently expressing EBNA-1 (Wang et al., 1997). p32 is synthesized as a 282-amino-acid-long polypeptide and most p32 is posttranslationally imported into mitochondria by an amino-terminal targeting sequence which is later removed (Matthews and Russell, 1998; Muta et al., 1997; Seytter et al., 1998). The mature protein crystallizes as a doughnut-shape homotrimer with a striking asymmetric distribution of charged residues (Jiang et al., 1999). Although mostly mitochondrial, p32 has been detected in the nucleus of human A549 cells by confocal microscopy (Matthews and Russell, 1998). p32 binds two regions of EBNA-1 and derivatives lacking these interaction elements are defective for the transcriptional activation of a reporter gene linked to FR (Wang et al., 1997). Here we show that a fraction of p32 is localized to oriP in EBV-positive BL cells and demonstrate that EBNA-1 interacts with p32 independently of other proteins or DNA. EBNA-1 variants that lack one of the two p32 binding elements are defective for the stable interaction with p32 in vivo, transcriptional activation, and the replication and/or maintenance of a plasmid bearing oriP. These results provide further evidence supporting a role for p32 in the activation of transcription by EBNA-1 and suggest a role for p32 in EBV genome replication and/or maintenance.

RESULTS

EBNA-1 and p32 interact in EBV-positive Burkitt’s lymphoma cells

p32 was identified as an EBNA-1-interacting host protein in yeast two-hybrid screens (Aiyar et al., 1998; Wang et al., 1997) and in coimmunoprecipitation experiments performed with 293 cells transiently expressing EBNA-1 (Wang et al., 1997). To determine whether the interaction of endogenous EBNA-1 and p32 could be detected in cells latently infected with EBV, extracts prepared from EBV-positive (Raji and Akata) and -negative (Louckes) BL cells were immunoprecipitated with a rabbit anti-EBNA-1 serum and -negative (Louckes) BL cells were immunoprecipitated with a rabbit anti-EBNA-1 serum or preimmune serum and the precipitated proteins were examined by blotting with anti-p32 antibodies (Fig. 1A). p32 was present in the anti-EBNA-1 precipitates from Raji and Akata cell extracts and absent from the anti-EBNA-1 precipitate from the Louckes cell extract and precipitates formed with preimmune serum. p32 also coprecipitated with EBNA-1 from extracts prepared from 293 cells infected with an EBNA-1-expressing adenovirus, but not cells infected with a control virus, and from COS-7 cells expressing EBNA-1 transiently from a plasmid vector (Figs. 1B and 1C). Monkey p32 from COS-7 cells migrated more rapidly on SDS–gels than human p32 (data not shown) allowing the clear differentiation of a nonspecifically precipitated polypeptide (Figs. 1A and 1B) from p32. These results confirm the previously reported interaction of EBNA-1 and p32 in 293 cell extracts (Wang et al., 1997) and further show that an EBNA-1–p32 complex may be detected in extracts prepared from EBV-positive BL cells.

Specificity and mechanism of interaction

Because p32 binds two regions of EBNA-1 containing arginine- and glycine-rich repeats (Wang et al., 1997) and p32 is acidic (calculated pI of 4.57 and 4.15 for residues 1–282 and 74–282, respectively), we considered the pos-
Possibility that the interaction between EBNA-1 and p32 represented the nonspecific binding of proteins with opposite charges. To investigate the specificity of the interaction of p32 with EBNA-1, we tested whether p32 could interact with other arginine-rich proteins. The product of the herpes simplex virus type 1 Us11 gene binds 60S ribosomal RNA, and the RNA binding domain has been localized to the carboxy-terminal half of the protein (Roller et al., 1996). This region of Us11 (residues 79–161) is composed of a tripeptide repeat, Arg-X-Pro. GST/Us11(1–161)-beads did not, however, precipitate p32 from a HeLa cell extract (data not shown). Additionally, the carboxy-terminus of hepatitis B virus core antigen is arginine-rich (17/36 residues) and this protein did not interact detectably with p32 in a yeast two-hybrid assay (Bartel et al., 1993). This selectivity indicates that the interaction of p32 with EBNA-1 may not be explained as the irrelevant interaction of an acidic protein with arginine-rich proteins.

Although yeast two-hybrid assays (Aiyar et al., 1998; Wang et al., 1997) and coprecipitation experiments (Wang et al., 1997; Fig. 1) demonstrated an interaction between EBNA-1 and p32 independent of other EBV gene products, they could not distinguish between a direct or indirect interaction. To distinguish between these possibilities, an immunoprecipitation experiment was performed with purified proteins (Fig. 2). In agreement with the data in Fig. 1, p32 was precipitated by anti-EBNA-1 antibodies in the presence but not the absence of EBNA-1. Pretreatment of EBNA-1 and p32 with ethidium bromide to inhibit DNA-dependent protein interactions (Lai and Herr, 1992) or ribonuclease A (RNase A) to degrade any contaminating RNA did not prevent the coprecipitation of EBNA-1 and p32. Therefore, the interaction of EBNA-1 and p32 does not require additional proteins or DNA. Because RNase A cannot hydrolyze double-stranded RNA, it is possible that the interaction of EBNA-1 and p32 is mediated by binding of each protein to a piece of double-stranded RNA. However, p32 has not been observed to bind RNA (Petersen-Mahrt et al., 1999; Tange et al., 1996), making this explanation unlikely.

p32 associates with oriP in Raji cells

To determine whether p32 localizes to oriP in vivo, chromatin immunoprecipitation experiments were performed with Raji cells. Intact cells were treated with formaldehyde to crosslink proteins and nucleic acids and chromatin was isolated by isopycnic centrifugation in CsCl–Sarkosyl (Varshavsky et al., 1979). Purified chromatin was immunoprecipitated with antibodies to EBNA-1 or p32 and the precipitated DNA was analyzed by PCR to detect FR or DS. Each reaction contained a second set of primers that allowed detection of a region of EBV DNA located over 84 kbp from oriP (codons 1–89 of the EBNA-1 open reading frame [orf]) to monitor the nonspecific precipitation of chromatin. PCR analysis of twofold serial dilutions of the input DNA (Fig. 3C) showed that both DS and EBNAorf could be amplified from the chromatin preparations and these results were used to
quantify the amount of input DNA precipitated with various antibodies.

A monoclonal antibody (mAb) to EBNA-1 (mAb2B4) enriched DS 73-fold relative to EBNAorf (0.73% of DS and 0.01% of EBNAorf coprecipitated; averages of three experiments), whereas DS was not enriched in immunoprecipitates prepared with an mAb to an irrelevant antigen (LFA-1; Fig. 3A, left). Chromatin containing DS was also enriched compared to chromatin containing EBNAorf in immunoprecipitates prepared with a rabbit anti-EBNA-1 serum (1.28% of DS and 0.27% of EBNAorf coprecipitated) but not with the preimmune serum (less than 0.03% of DS and EBNAorf coprecipitated) (data not shown). Ascites fluid containing an anti-p32 mAb (mAb26) gave a 47-fold enrichment of DS relative to EBNAorf (average of two experiments; Fig. 3A, right) and similar results were obtained with three additional anti-p32 mAbs. All four anti-p32 mAbs which could precipitate oriP chromatin could precipitate DNA fragments bearing FR and DS provided that both EBNA-1 and p32 were present (representative data for mAb26 shown in Fig. 4). The precipitation of DS chromatin by the four anti-p32 mAbs was not due to cross reaction with EBNA-1, as they did not recognize EBNA-1 in immunoblot experiments and were unable to precipitate FR or DS when bound by purified EBNA-1 in vitro (Fig. 4 and data not shown). Furthermore, the precipitation of DS chromatin by mAb26 ascites fluid was specific to the anti-p32 antibody, as ascites fluid containing mAb to an irrelevant antigen (αHA) failed to precipitate specifically DS or EBNAorf (Fig. 3A, right) and adsorption of mAb26 ascites fluid with agarose beads coupled to a glutathione S-transferase (GST)–p32(74–282) fusion protein, but not GST-beads, eliminated its ability to precipitate DS chromatin (Fig. 3B). Finally, the rebanding of chromatin on a second CsCl–Sarkosyl gradient to eliminate any non-chromatin-bound p32 did not alter the ability of mAb26 to precipitate DS (data not shown). These results demonstrate that p32 is localized to oriP in Raji cells. Qualitatively similar results were obtained when immunoprecipitated chromatin was amplified with FR-specific primers. However, the fold enrichment of FR compared to EBNAorf could not be determined as all primer pairs and PCR conditions tested failed to yield a linear response to changes in input DNA.

Nuclear localization of p32(74–282) by EBNA-1

Our attempts to detect endogenous p32 in the nuclei of EBNA-1-expressing cells or control cells by immunofluorescence (IF) staining have not been successful. However, IF stains of HeLa cells expressing EBNA-1 and epitope-tagged p32 that lacks the presequence (M45-p32[74–282]) provided an in vivo assay for the interaction of EBNA-1, and EBNA-1 derivatives deleted for p32 binding sites, with p32. The expression of M45-p32(74–282) in HeLa cells was generally just visible over background staining. In one experiment, 31 of approximately 8000 cells present on a coverslip exhibited light cytoplasmic staining following transfection with an M45-p32(74–282) expression plasmid and, rarely (1 cell in this experiment), cells with faint nuclear fluorescence were observed (Figs. 5A and 5B). Coexpression of EBNA-1 gave both a dramatic increase in staining intensity and an apparent threefold increase in the number of cells expressing M45-p32(74–282). In two-thirds of these cells, M45-p32(74–282) was nuclear or predominantly nuclear, and the remaining cells exhibited a largely cytoplasmic stain (examples of the first two staining patterns are shown in Fig. 5C). Dual-labeling of cells with antibodies to M45-p32(74–282) and EBNA-1 showed that all cells with increased staining for M45-p32(74–282) expressed EBNA-1 (Fig. 5D).

p32 binds two regions of EBNA-1 containing arginine- and glycine-rich repeats (Wang et al., 1997). EBNA-1 variants lacking either or both p32 binding sites were created to investigate the function(s) of oriP-bound p32. For ease of discussion, the repeat located within amino acids 33–53 and the two adjacent repeats located within amino acids 329–382 are referred to as Box A and Box

![Fig. 4](https://example.com/image4.png)

**FIG. 4.** p32 coprecipitates with EBNA-1 and oriP FR and DS in vitro. EBNA-1 and p32 (lanes 4–7) were incubated together and then diluted into buffer containing end-labeled DNA fragments from pBS/oriP. Control reactions contained p32 alone (lane 2), EBNA-1 alone (lane 3), or neither protein (lane 1). Following incubation, the reactions were subjected to immunoprecipitation with ascites fluids containing anti-p32 mAb26 (lanes 1–7) and the precipitated DNA was analyzed by agarose gel electrophoresis and autoradiography. Lanes 4–7 contain increasing quantities (twofold increments) of the anti-p32 mAb. Input DNA fragments are displayed in the lane labeled M.
B/C. EBNA-1 variants lacking these repeats (deletions of residues 34–57 and/or 329–376) were derived from EBNA-1 lacking all but 12 residues of the Gly-Gly-Ala repeats (EBNA-1 \( ^{\text{Dgly-ala}} \)). The Gly-Gly-Ala repeats are not required for the transcriptional activation and DNA replication activities of EBNA-1 (Yates and Camiolo, 1988) nor for the interaction of EBNA-1 with p32 in yeast (Aiyar et al., 1998; Wang et al., 1997). The expression of these EBNA-1 derivatives was analyzed by IF staining (Fig. 5) and immunoblotting (Fig. 6). EBNA-1, EBNA-1 \( ^{\text{Dgly-ala}} \), and EBNA-1 \( ^{\text{Box A}} \) were predominantly nuclear while EBNA-1 \( ^{\text{Box B/C}} \) and EBNA-1 \( ^{\text{Box A/B/C}} \), although largely nuclear, could also be detected in the cytoplasm in some cells. Yeast two-hybrid experiments (Bartel et al., 1993) demonstrated that EBNA-1 \( ^{\text{A/B/C}} \) lacked the ability to interact with EBNA-1 (data not shown).

FIG. 5. EBNA-1 coexpression results in the increased nuclear accumulation of epitope-tagged p32(74–282). HeLa cells were cotransfected with pE1A-CMIX/p32(74–282) and pCMX (A and B), pCMX/EBNA-1 (C and D), pCMX/EBNA-1\(^{\text{Dgly-ala}} \) (E and F), pCMX/EBNA-1\(^{\text{Box A}} \) (G and H), pCMX/EBNA-1\(^{\text{Box B/C}} \) (I and J), or pCMX/EBNA-1\(^{\text{Box A/B/C}} \) (K and L) and IF stains were performed 48 h later with mAb M45 to detect epitope-tagged p32(74–282) and an anti-EBNA-1 serum. Primary antibodies were detected with FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG. A and B show labeling of M45-p32(74–282) in two different fields of cells. The distribution of M45-p32(74–282) (C, E, G, I, and K) and full-length or mutant EBNA-1 proteins (D, F, H, J, and L) in the same cells is shown. Identical results were obtained with a mouse mAb to EBNA-1 that recognizes an epitope in the DNA binding domain (Orlowski et al., 1990) and with NIH 3T3 cells.

FIG. 6. EBNA-1 and derivatives lacking the Gly-Gly-Ala repeats and Box A and/or Box B/C accumulate to similar levels in the nucleus. Nuclear (N) and postnuclear (C) extracts were prepared from HeLa cells transfected with pCMX (lanes 1 and 2), pCMX/EBNA-1 (lanes 3 and 4), pCMX/EBNA-1\(^{\text{Dgly-ala}} \) (lanes 5 and 6), pCMX/EBNA-1\(^{\text{Box A}} \) (lanes 7 and 8), pCMX/EBNA-1\(^{\text{Box B/C}} \) (lanes 9 and 10), or pCMX/EBNA-1\(^{\text{Box A/B/C}} \) (lanes 11 and 12) and analyzed by blotting with mAb2B4 to detect EBNA-1 and its derivatives (Grässer et al., 1994). The positions of molecular mass markers are shown on the right.
Coexpression of M45-p32(74–282) with EBNA-1Δgly-ala or EBNA-1ΔBox A resulted in the increased intracellular and intranuclear accumulation of M45-p32(74–282) (Figs. 5E and 5G). In contrast, EBNA-1ΔBox B/C and EBNA-1ΔBox A/B/C did not have this effect. Although both EBNA-1ΔBox B/C and EBNA-1ΔBox A/B/C were detected in the nucleus (Figs. 5J and 5L), M45-p32(74–282) was predominantly cytoplasmic when coexpressed with these EBNA-1 derivatives (Figs. 5I and 5K).

These results demonstrate that the nuclear accumulation of M45-p32(74–282) is mediated by its binding to EBNA-1 and suggest that the two p32 binding sites are not equivalent in this regard.

EBNA-1 derivatives that lack p32 binding site(s) are defective for the replication and/or maintenance of an oriP-plasmid

To determine whether EBNA-1 variants that failed to cause the nuclear accumulation of M45-p32(74–282) could support the replication and maintenance of an oriP-plasmid, HeLa cells were cotransfected with an oriP-plasmid (pHEBo-1.1) and pCMX/EBNA-1Δgly-ala (lane 3), pCMX/EBNA-1ΔBox A (lane 4), pCMX/EBNA-1ΔBox B/C (lane 5), or pCMX/EBNA-1ΔBox A/B/C (lane 6). Low-molecular-weight DNA was isolated 90 h post transfection, digested with DpnI and Clai, and examined by Southern blotting with a radiolabeled pHEBo-1.1 probe. A portion of the gel containing full-length linear plasmid DNA (L), supercoiled plasmid DNA (SC), and the largest DpnI restriction fragments (bottom) is shown.

FIG. 7. EBNA-1 derivatives lacking amino acids 329–376 are defective for the short-term replication and/or maintenance of an oriP-plasmid. HeLa cells were cotransfected with an oriP-plasmid (pHEBo-1.1) and pCMX/EBNA-1Δgly-ala (lane 3), pCMX/EBNA-1ΔBox A (lane 4), pCMX/EBNA-1ΔBox B/C (lane 5), or pCMX/EBNA-1ΔBox A/B/C (lane 6). Low-molecular-weight DNA was isolated 90 h post transfection, digested with DpnI and Clai, and examined by Southern blotting with a radiolabeled pHEBo-1.1 probe. A portion of the gel containing full-length linear plasmid DNA (L), supercoiled plasmid DNA (SC), and the largest DpnI restriction fragments (bottom) is shown.

Input plasmid DNA, isolated from a dam+ Escherichia coli strain, is digested by DpnI while replicated plasmid DNA is resistant to DpnI and linearized by Clai. The amount of linear DpnI-resistant pHEBo-1.1 DNA detected in this assay reflects the extent to which plasmids are both replicated and retained in the cell. Only a small amount of replicated pHEBo-1.1 was detected 90 h post-transfection of HeLa cells in the absence of EBNA-1 (not visible on the film exposure in Fig. 7) and EBNA-1Δgly-ala supported the stable maintenance of pHEBo-1.1 to a level similar to EBNA-1 (77 ± 19%). The amount of replicated pHEBo-1.1 in HeLa cells expressing EBNA-1ΔBox A, EBNA-1ΔBox B/C, and EBNA-1ΔBox A/B/C was 65 ± 5, 4 ± 1, and 3 ± 2%, respectively, the amount observed in the presence of EBNA-1 after four cell generations. If the amount of replicated plasmid DNA detected in these experiments was due to inefficient initiation of DNA replication and one assumes that plasmids that were not replicated in one S phase were present in the daughter cell nuclei in the subsequent S phase, the efficiency of pHEBo-1.1 replication supported by EBNA-1 derivatives lacking amino acids 34–57, 329–376, or 34–57 and 329–376 per cell generation was 80–85%, 6%, and 6%, respectively, compared to EBNA-1. If, however, replication of the plasmids occurred once per S phase and the reduced amount of replicated plasmid DNA detected in these experiments was the result of inefficient maintenance of the DNA, the efficiency of plasmid DNA retention supported by EBNA-1 derivatives lacking amino acids 34–57 or 329–376 per cell generation was 90 and 45%, respectively, compared to EBNA-1. These results show that regions of EBNA-1 that bind p32 are required for the efficient initiation of DNA replication of oriP plasmids and/or their retention in HeLa cells and that deletion of amino acids 329–376 had a greater effect than deletion of amino acids 34–57.

EBNA-1 derivatives that lack p32 binding sites are defective for transcriptional activation

It was previously reported that EBNA-1 deleted for amino acids 40–60 and all the Gly-Gly-Ala repeats did not activate expression of a reporter gene linked to FR (Wang et al., 1997). Because a similar deletion (amino acids 34–57) had only a small effect on oriP-plasmid replication and/or maintenance (Fig. 7) and previous genetic analyses of EBNA-1 have been unable to separate its transcription and replication functions (Yates and Camilo, 1988), the transcriptional activation function of EBNA-1 derivatives lacking p32 binding site(s) described above was analyzed (Table 1). EBNA-1 and EBNA-1Δgly-ala increased expression of a reporter gene in human 143 cells and mouse NIH 3T3 cells in an FR-dependent manner and to a similar extent (Table 1; data not shown). The level of transactivation for EBNA-1ΔBox A was 81% of the level observed with EBNA-1Δgly-ala in 143 cells.
and 58% of the level observed with EBNA-1 Δgly-ala in NIH 3T3 cells. In contrast, EBNA-1 derivatives lacking Box B/C were markedly defective for transcriptional activation. This defect was most apparent in NIH 3T3 cells where EBNA-1ΔBox B/C and EBNA-1ΔBox A/B/C only activated luciferase expression from FR-TK-luc 6 and 4%, respectively, of the level observed with EBNA-1Δgly-ala. The residual activity of EBNA-1ΔBox B/C and EBNA-1ΔBox A/B/C may be due to the acidic carboxy-terminal region of EBNA-1. This region was previously shown to be dispensable for transcriptional activation by EBNA-1, but largely responsible for activation by a truncated EBNA-1 consisting of amino acids 450–641 (Ambinder et al., 1991).

The inability of EBNA-1 derivatives lacking Box B/C to activate transcription to the same extent as EBNA-1, EBNA-1Δgly-ala, and EBNA-1ΔBox A could not be explained by their relative levels of expression (Fig. 6) nor by an inability of the cells expressing these EBNA-1 variants to support transcription from the TK promoter. Cotransfection of a plasmid bearing the lacZ gene under the control of the TK promoter with FR-TK-luc and expression plasmids for EBNA-1 and its derivatives yielded similar levels of β-galactosidase activity (Table 1). We also considered the possibility that the transcriptional activation defect of EBNA-1 derivatives lacking RGG Box B/C was due to a reduced ability of these proteins to bind DNA site-specifically. To address this possibility, we performed a DNA communoprecipitation assay with nuclear extracts prepared from HeLa cells transiently expressing EBNA-1, EBNA-1Δgly-ala, and the derivatives lacking Box A and/or Box B/C (Fig. 8). The 847-bp fragment bearing oriP FR was specifically precipitated from binding reactions containing EBNA-1, EBNA-1Δgly-ala, and derivatives lacking Box A and/or Box B/C and the other DNA fragments which lacked EBNA-1 binding sites were either not detected or present in relatively small amounts compared to the 847-bp fragment. We conclude that regions of EBNA-1 that bind p32 participate in the transcriptional activation function of EBNA-1 and, as was observed in the stable oriP-plasmid maintenance assay, deletion of amino acids 329–376 had a greater effect than deletion of amino acids 34–57.

**DISCUSSION**

The results of chromatin immunoprecipitation experiments shown here provide strong evidence for the localization of p32 to oriP in vivo and thereby implicate p32 in one or more functions of oriP. It is likely that p32 is recruited to oriP through a direct interaction with EBNA-1, as an EBNA-1–p32 complex can form in vitro in the absence of other proteins and DNA. Although both oriP FR and DS were present in immunoprecipitates prepared with anti-p32 antibodies, because of their proximity in the EBV genome and the ability of EBNA-1 to link FR and DS we do not know whether p32 is targeted to one or to both elements.

The finding that EBNA-1 recruits a mitochondrial protein to oriP was unexpected but may be explained by the binding of nascent p32 by EBNA-1 in the cytoplasm before it engages the mitochondrial protein import machinery and subsequent movement of the complex into the nucleus. This model is supported by the ability of EBNA-1 to cause the nuclear accumulation of p32 that either lacked the presequence or contained an amino-terminal extension that prevented mitochondrial targeting, and it predicts that the p32 localized to oriP lacks the presequence. Alternatively, it has been proposed that a fraction of p32 cycles between mitochondria and the nucleus (Matthews and Russell, 1998) and EBNA-1 may bind and retain p32 in the nucleus. A previous study reported that epitope (FLAG)-tagged p32(81–282) localized to the nucleus (Wang et al., 1997) whereas M45-p32(74–282) was only rarely observed in the nucleus of cells not expressing EBNA-1 (Fig. 5). It is likely that the different subcellular locations of the two proteins is due to differences in the epitope tags at their amino termini and the absence of the first seven amino acids of the mature form of p32 from p32(81–282). Perhaps truncation of the "A" alpha helix in p32(81–282) resulted in destabilization of p32 monomer interactions and an accumulation of monomers over dimers and trimers (Jiang et al., 1999). p32 monomers, but not trimeric p32, should be free to enter the nucleus by diffusion.

p32 is an evolutionarily conserved protein and, al-
though functional studies have been carried out in budding yeast, these studies have yielded conflicting data as to its function. One group found that disruption of the p32 gene resulted in cells that grew poorly in medium containing a nonfermentable carbon source, suggesting that p32 expression is important for mitochondrial oxidative phosphorylation (Muta et al., 1997), while another group did not observe impaired growth of a p32-null strain under the same conditions (Seytter et al., 1998). Although identification of the function of p32 in mitochondria may provide insight into the role it plays at oriP, it is possible that p32 exhibits a novel function when recruited to oriP.

p32 can interact independently with two regions of EBNA-1 containing related arginine- and glycine-rich repeat elements in a yeast two-hybrid assay (Wang et al., 1997); however, these elements are not functionally equivalent when examined in mammalian cells. The two repeats located between amino acids 329 and 382 (Box B/C), but not the single repeat located between residues 33 and 53 (Box A), were sufficient for the EBNA-1-mediated nuclear targeting of p32. A role for p32 in the transcriptional activation and stable plasmid maintenance functions of EBNA-1 is supported by our studies of EBNA-1 derivatives lacking this element. EBNA-1ΔBox B/C and EBNA-1ΔBox A/B/C were greatly reduced in their abilities to activate transcription of FR-TK-luc and support the replication and/or maintenance of an oriP-plasmid and these defects correlated with the inability of these EBNA-1 derivatives to cause the nuclear accumulation of M45-p32(7 4–282). The results of Wang et al. (1997) suggested that p32 plays a role in transcriptional activation by EBNA-1 but, in contrast to the results presented here, concluded that both p32 binding sites are required for EBNA-1 to increase transcription of a reporter gene linked to FR. This conclusion was based, in part, upon the inability of an EBNA-1 derivative lacking amino acids 40–60 (as well as most of the Gly-Gly-Ala repeats) to activate transcription. It is possible that the deletion of amino acids 34–57 (ΔBox A) did not remove amino acids that, in the presence of Box B/C, can give rise to a stable EBNA-1–p32 complex. A detailed analysis of the p32 binding sites is needed to determine whether Box B/C is sufficient for the formation of a stable complex with p32.

p32 contains a region that can function as a transcriptional activation domain (Wang et al., 1997; Yu et al., 1995b) and interacts with the general transcription factor TFIIB in vitro (Yu et al., 1995a) and these properties,
together with the acidic nature of p32, the localization of p32 to oriP, and the transcriptional and replication defects of EBNA-1 derivatives that fail to interact stably with p32 in vivo, strongly support a dual role for this cellular protein in EBNA-1-mediated transcriptional activation and DNA replication. There is widespread involvement of transcriptional elements in eukaryotic viral and cellular DNA replication (Li et al., 1998, and references therein) and information gathered from studies of these elements suggest two nonexclusive roles for EBNA-1 and p32 in the synthesis of oriP-plasmids. Nuclear factor 1 and the acidic activation domains of VP16 and p53 can prevent the inhibition of DNA replication by nucleosomes in vitro (Cheng and Kelly, 1989; Cheng et al., 1992; Li and Botchan, 1994) and EBNA-1-bound p32 may act similarly to allow recognition of the origin by the cellular replication machinery. Alternatively, EBNA-1 may direct the cellular replication machinery to oriP through protein–protein interactions. Both p53 and VP16 associate with the 70-kDa subunit of replication protein A through their acidic activation domains (He et al., 1993; Li and Botchan, 1993) and the ability of mutant derivatives of VP16 to bind RPA correlates with their ability to activate DNA replication (He et al., 1993). EBNA-1 interacts directly with the 70-kDa subunit of RPA (Zhang et al., 1998) and potential contacts between p32 and RPA may contribute to a functional interaction.

The interpretation of transcriptional activation and plasmid replication/maintenance experiments with EBNA-1 derivatives that fail to cause the nuclear accumulation of p32 is complicated by the apparent overlap or coincidence of amino acids involved in binding p32 (residues 40–60 and 325–376; Wang et al., 1997), the looping or linking of DNA containing EBNA-1 binding sites (residues 54–89, 331–361, and 372–391; Mackey et al., 1995), and elements with chromosome binding activity (residues 8–67, 72–84, and 328–375; Marechal et al., 1999). In addition, a host nucleolar protein has been reported to bind EBNA-1 through amino acids 330–386 (Shire et al., 1999). Further genetic analysis of these regions of EBNA-1 will be required to gain a better understanding of the contributions of these host proteins and activities to EBNA-1 function.

**MATERIALS AND METHODS**

**Cells**

BL cells were grown in RPMI 1640 medium plus 10% fetal bovine serum. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 5% fetal bovine serum. All other animal cells were grown as monolayer cultures in DMEM plus 10% bovine calf serum. HeLa S3 cells were grown in spinner culture in Eagle’s minimum essential medium with Earle’s salts and 5% bovine calf serum.

**Plasmid and virus constructions**

EBNA-1 was placed under control of the human cytomegalovirus (HCMV) immediate early gene enhancer and promoter by cloning the BglII fragment from pEBNA-1.2 containing the EBNA-1 orf (Hearing et al., 1992) into the BamHI site of pE1A-CMX (Obert et al., 1994) to create pE1A/CMX-EBNA1. Ad/CMV-EBNA1, an adenovirus EBNA-1-expression vector, was obtained by selecting recombinants between pE1A/CMX-EBNA1 and Ad5 dl309 (Schmidt and Hearing, 1999). pCMX/EBNA1 was created by inserting the BglII fragment from pE1N.2 containing the EBNA-1 orf into the BamHI site of pCMX (Umesono et al., 1991). The orf for EBNA-1 lacking all but 12 residues of the glycine-glycine-alanine repeat was amplified by PCR using p367 (Yates and Camilo, 1988) as template and cloned into the BamHI site of pCMX to create pCMX/EBNA1-gly-ala. A two-step PCR-based strategy (Freeman et al., 1996) using p367 as template was used to obtain expression plasmids for EBNA-1 derivatives lacking the glycine-glycine-alanine repeats and amino acids 34–57 (pCMX/EBNA1-Box A) or amino acids 329–376 (pCMX/EBNA1-Box B/C). The same strategy, employing pCMX/EBNA1-Box A as template, was used to construct pCMX/EBNA1-Box A/B/C. pE1A-CMX/p32 (74–282) encodes p32 amino acids 74–282 with an amino-terminal extension of 17 amino acids containing the adenovirus E4-6/7 M45 epitope (Obert et al., 1994). pGEX/p32, encoding a GST fusion protein with human p32 amino acids 74–282, was created by PCR-amplification and insertion of the fragment into the EcoRI site of pGEX-KG (Guan and Dixon, 1991).

**Protein purification**

HeLa S3 cells in spinner culture (5–6 x 10^6 cells/ml) were infected with Ad/CMV-EBNA1 at 200 particles/cell and harvested 24 h later. EBNA-1 was purified from 9 x 10^9 infected cells by heparin–agarose (Frappier and O’Donnell, 1991b) and DNA affinity (Hearing et al., 1992) chromatography. GST-p32 (74–282) was expressed in E. coli, purified from cell lysates, and released from GST with thrombin as described (Guan and Dixon, 1991).

**Antibodies**

An anti-EBNA-1 serum was obtained by immunization of rabbits with EBNA-1 purified from insect cells (Hearing et al., 1992). Bacterially produced p32(74–282) was further purified on SDS–gels prior to injection into chickens. Antibodies were purified from eggs from one seropositive hen as previously described (Jensenius et al., 1981). For preparation of anti-p32 mAbs, Balb-C mice were immunized with recombinant p32 (Krainer et al., 1991) purified by preparative SDS–PAGE. Standard procedures (Harlow and Lane, 1998) were used for immunization, generation of hybridomas with NS-1 myeloma cells, and...
mAb production in ascites fluid at the Cold Spring Harbor Laboratory Monoclonal Antibody Facility.

**Coimmunoprecipitation assays**

COS-7 cells (7 × 10^5) were transfected with 10 μg of salmon sperm DNA with or without 10 μg of the EBNA-1 expression plasmid p371 (Yates and Camiolo, 1988) by the calcium phosphate coprecipitation method and harvested 38 h after addition of the precipitates. Cells were lysed with 1 ml of 0.5% NP-40, 50 mM HEPES–NaOH (pH 7.5), 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, sonicated, and clarified by centrifugation at 10,000g, 15 min at 4°C. One-quarter of each lysate was incubated with 6 μl of rabbit anti-EBNA-1 serum for 1 h on ice. Subsequently, 50 μl of a 50-mg protein A-Sepharose/ml suspension was added and incubation was continued for 30 min at 4°C with mixing. Beads were washed four times with lysis buffer and boiled in SDS–gel sample buffer. EBV-positive and -negative BL cells (1.3 × 10^6) were lysed by the addition of 0.2 ml of 10 mM HEPES–NaOH (pH 7.4), 10 mM KCl, 0.75 mM spermidine, 0.75 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 0.1% NP-40, 0.5 mM PMSF, 2.5 mM benzamidine, 10 μg leupeptin/ml, and 10 μg pepstatin/ml. The nuclei were immediately pelleted in a microcentrifuge for 15 s, suspended in 0.02 ml of 20 mM HEPES–NaOH (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl_2_, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 2.5 mM benzamidine, 10 μg leupeptin/ml, and 10 μg pepstatin/ml, and incubated on ice for 30 min. The extracted nuclei were removed from the extracts by centrifugation.

**DNA coimmunoprecipitation assays**

Plasmids bearing FR and DS (pBS/oriP) or FR alone (pHEBo-2.1; M. Koons and J. Hearing, unpublished data) were digested with Ncol and Xhol (pBS/oriP) or SalI and Ncol (pHEBo-2.1) and the resulting DNA fragments were end-labeled with Klenow DNA polymerase and [α-^32P]dATP. End-labeled DNA fragments (5 ng total) were combined with purified proteins or nuclear extract in 20 mM HEPES–NaOH (pH 7.5), 5 mM MgCl_2_, 300 mM NaCl, 1 mM DTT, 0.1% NP-40, 5.3% glycerol, 1.5 mg single-stranded salmon sperm DNA/ml, 1.5 mg double-stranded salmon sperm DNA/ml, and 50 μg BSA/ml in a final volume of 20 μl. After 30 min of incubation at room temperature, the binding reactions were diluted 10-fold with 20 mM HEPES–NaOH (pH 7.5), 130 mM NaCl, 5 mM MgCl_2_, 0.1% NP-40, and incubated on ice for 30 min with hybridoma culture supernatant containing mAb to either the α subunit of LFA-1 (M17/4.4.11.9; American Type Culture Collection) or EBNA-1 (1H4; Grässer et al., 1994) or the ascites fluid containing mAb to p32. Immune complexes were precipitated with protein G–Sepharose, washed with 20 mM HEPES–NaOH (pH 7.5), 160 mM NaCl, 5 mM MgCl_2_, 0.1% NP-40, and released by heating to 60°C for 3 min in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% SDS, and electrophoresed on 1% agarose gels. Quantitation was performed with a Molecular Dynamics Storm 860 phosphorimager.

**Chromatin immunoprecipitations**

Raji cells at ~2.5 × 10^5 cells/ml were labeled with 0.5 μCi [³H]thymidine (60 Ci/mmol; ICN Radiochemicals)/ml for 24–30 h. One-tenth volume of 11% formaldehyde in 50 mM HEPES–NaOH (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, was added to the cultures and the cells were fixed for 14–16 h at 4°C. Chromatin was isolated by sonication and purified by centrifugation on CsCl–Sarkosyl gradients as described (Orlando et al., 1997). Aliquots of chromatin containing 60 μg DNA were subjected to immunoprecipitation and prepared for PCR as described (Meluh and Broach, 1999). PCR primers were 5’-GG-GAATTCTGACATCCCAATCCTTTAGC 3’ and 5’-GGGC-CAAGCCTTTCATCCTGAGG 3’ (DS; 792 bp), 5’-CGGAAT-TCATGTCTGACGAGG 3’ and 5’-GGGATCATTTGTTCC-

Immunoblots

Samples were electrophoresed on either SDS–10% polyacrylamide gels (Figs. 1C and 6) or SDS–5% to 20% gradient polyacrylamide gels (Figs. 1A and 1B), transferred to nitrocellulose, and blotted with antibodies using standard procedures. Anti-EBNA-1 serum and p32 antibodies were diluted 1:3200 and 1:5000, respectively. Appropriate proteins or buffer were combined and precipitated with anti-EBNA-1 serum. Ethidium bromide (50 μg/ml) was included in the first three washes of the sample pretreated with the dye.

Nuclear extracts

HeLa cells (1.3 × 10^5 cells/60-mm plate) were transfected with 5 μg of pCMX encoding wild-type or mutant derivatives of EBNA-1 using FuGENE6 (Roche Molecular Biochemicals) and harvested 48 h later. Washed cells were lysed by the addition of 0.2 ml of 10 mM HEPES–NaOH (pH 7.4), 10 mM KCl, 0.75 mM spermidine, 0.75 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 0.1% NP-40, 0.5 mM PMSF, 2.5 mM benzamidine, 10 μg leupeptin/ml, and 10 μg pepstatin/ml. The nuclei were immediately pelleted in a microcentrifuge for 15 s, suspended in 0.02 ml of 20 mM HEPES–NaOH (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl_2_, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 2.5 mM benzamidine, 10 μg leupeptin/ml, and 10 μg pepstatin/ml, and incubated on ice for 30 min. The extracted nuclei were removed from the extracts by centrifugation.

Substrate (Pierce).

Secondary antibodies were diluted 1:2500. Immunoblots were developed with SuperSignal Chemiluminescent Substrate (Pierce).

Immunoblots

Samples were electrophoresed on either SDS–10% polyacrylamide gels (Figs. 1C and 6) or SDS–5% to 20% gradient polyacrylamide gels (Figs. 1A and 1B), transferred to nitrocellulose, and blotted with antibodies using standard procedures. Anti-EBNA-1 serum and p32 antibodies were diluted 1:3200 and 1:5000, respectively. mAb2B4 hybridoma culture supernatant (Graß er et al., 1994) was diluted 1:20. Horseradish-peroxidase-coupled secondary antibodies were diluted 1:2500. Immunoblots were developed with SuperSignal Chemiluminescent Substrate (Pierce).
CACCCTGGGTCC 3' (EBNAorf; 287 bp). Serial dilutions of the input DNA and precipitated DNA (2- or 2.5-fold) were amplified to identify template concentrations within the linear range of the assay for quantitation. PCR products were resolved on agarose gels, stained with ethidium bromide, and photographed with Polaroid 665 film and scans of the negatives were quantified with NIH Image.

**Luciferase assays**

Cells (1.5 × 10^5 cells per 60-mm dish) were transfected with 1 μg reporter plasmid (TK-luc or FR-TK-luc; Middleton and Sugden, 1992) and 50 ng effector plasmid (pCMX or derivatives encoding wild-type or mutant EBNA-1) by calcium phosphate coprecipitation and analyzed after 40 h incubation using Promega's Luciferase Assay System. In two experiments using 143 cells, 1 μg of the reporter plasmid pTKβgal (Park et al., 1994), 1 μg of luciferase reporter plasmid, and 50 ng of effector plasmid were introduced into duplicate cultures using FuGENE6. One set of cultures was analyzed for luciferase activity following 48 h of incubation. Extracts from duplicate cultures were prepared by four freeze–thaw cycles in 100 μl of 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 25 mM sucrose, and 1 mM PMSF, clarified by centrifugation, and assayed for β-galactosidase activity as previously described (Bartel et al., 1993).

**IF staining**

Cells, transfected 24–48 h prior to staining, were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. The cells were washed with PBS, incubated for 20 min in PBS with 50 mM glycine, and permeabilized with 0.1% Triton X-100 in PBS. Primary antibodies were diluted 1:4 (mAb M45) or 1:200 (rabbit anti-EBNA-1 antibodies) in PBS. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG, and goat anti-rabbit IgG (Zymed Laboratories) were diluted 1:250 in PBS.

**DNA replication assay**

HeLa cells (2 × 10^5 cells/60-mm dish) were cotransfected with 2.5 μg pHEBo-1.1 (Hearing et al., 1992) and 2.5 μg pCMX or derivatives encoding wild-type or mutant EBNA-1 using FuGENE6. The cells were trypsinized 18 h after the addition of plasmid DNA and seeded in one 100-mm dish per original 60-mm dish for 72 h further incubation. The populations underwent approximately four cell divisions after the addition of plasmid DNA. Low-molecular-weight DNA was prepared and one-third of each sample was analyzed as previously described (Hearing et al., 1992). Replication products from three independent experiments were quantitated with a Molecular Dynamics Storm 860. The efficiencies with which EBNA-1 derivatives supported the replication of pHEBo-1.1 (e) or retained the plasmid in the cell (β) per cell generation compared to EBNA-1 were calculated using the formula

\[ \frac{N_i}{N_0} = \left( \frac{(1 + e)^{i} - (1 - e)^{i}}{2^{i}} \right) \beta^i, \]

where \( N_i \) is the amount of replicated plasmid DNA present after \( i \) cell generations in cells expressing an EBNA-1 derivative and \( N_0 \) is the amount of plasmid DNA present after 0 cell generations in cells expressing EBNA-1.

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