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The Conserved Domain CR2 of Epstein–Barr Virus Nuclear Antigen Leader Protein Is Responsible Not Only for Nuclear Matrix Association but Also for Nuclear Localization

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There is a growing body of evidence for the importance of the nuclear matrix in various nuclear events including gene expression and DNA replication. Epstein–Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) is a nuclear matrix-associated protein that has been suggested to play an important role in EBV-induced transformation. To define the biological significance of the association of EBNA-LP with the nuclear matrix, we mapped the domain of EBNA-LP responsible for nuclear matrix association and investigated the functions of the EBNA-LP mutant mutagenized by substitution of alanines for the cluster of arginine residues in the mapped region. The results of the present study were as follows. (i) Transiently expressed EBNA-LP in COS-7 or BOSC23 cells was associated with the nuclear matrix, similarly to that in EBV-infected B cells. (ii) Mutational analysis of EBNA-LP revealed that a 10-amino acid segment of EBNA-LP is critical for nuclear matrix association of the protein. Interestingly, the identified region overlapped with the region CR2 of EBNA-LP conserved among a subset of primate gammaherpesviruses. The identified segment is referred to as EBNA-LP NMTS (nuclear matrix targeting signal). (iii) The EBNA-LP mutant with the arginine to alanine substitutions in NMTS was no longer localized not only to the nuclear matrix but also to the nucleus. (iv) The EBNA-LP mutant lacked its ability to coactivate EBNA-2-dependent transactivation. These results indicated that EBNA-LP needs to be localized in the nucleus and/or associated with the nuclear matrix through CR2 to elicit its function such as the coactivation of the EBNA-2-dependent transcriptional activation. © 2001 Academic Press

INTRODUCTION

Epstein–Barr virus (EBV) is frequently associated with a variety of neoplastic diseases including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, several lymphomas, and gastric carcinoma (Kieff, 1996; Rickinson and Kieff, 1996). *In vitro*, EBV can efficiently immortalize human B cells (Kieff, 1996; Rickinson and Kieff, 1996). The resultant cell line, lymphoblastoid cell line (LCL), carries EBV genome in an episomal form (Kieff, 1996; Rickinson and Kieff, 1996) and constitutively expresses several viral proteins including six EBV nuclear antigen (EBNA) proteins [EBNA-1, -2, -3A, -3B, -3C, and leader protein (LP)] and three latent membrane proteins (LMP) (LMP-1, -2A, and -2B; Kieff, 1996; Rickinson and Kieff, 1996). Extensive studies have been performed to determine the mechanisms of this immortalization process, and LMP-1, EBNA-2, EBNA-3A, and EBNA-3C have been shown to be essential for the establishment of LCLs (Cohen *et al.,* 1989; Hammerschmidt and Sugden, 1989; Kaye *et al.,* 1993; Tomkinson *et al.,* 1993).

Epstein–Barr virus nuclear antigen leader protein (EBNA-LP), the subject of this report, is the first viral gene product to be expressed together with EBNA-2 after EBV infection of B cells (Alfieri *et al.,* 1991). A genetic analysis with recombinant viruses showed that EBNA-LP is not essential for EBV-induced B-cell immortalization (Hammerschmidt and Sugden, 1989; Mannick *et al.,* 1991). However, the efficiency of B-cell immortalization by the recombinant EBV lacking the C-terminal Y1Y2 domain of EBNA-LP is severely reduced and LCLs established by the recombinant virus require fibroblast-feeder cells for proliferation (Mannick *et al.,* 1991). These results support the hypothesis that EBNA-LP is a critical regulator of EBV-induced B-cell transformation. Although the mechanisms by which EBNA-LP acts in EBV-induced B-cell immortalization remain to be elucidated, several lines of evidence listed below suggest roles of EBNA-LP in the immortalization process. First, EBNA-LP has been demonstrated to activate cellular and viral gene expression in cooperation with EBNA-2. EBNA-LP and EBNA-2 cooperatively stimulate expression of cyclin D2 in resting B cells and progression of these cells from G_0 to G_1 (Sinclair *et al.,* 1994). These proteins also cooperate in up-

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regulating viral gene expression of LMP1 in B cells (Harada and Kieff, 1997; Nitsche *et al.,* 1997). Second, EBNA-LP has been shown to interact with various cellular proteins. EBNA-LP was shown to bind to p53 and pRb in *in vitro* binding assays (Szekely *et al.,* 1993) and to be colocalized with an antigenically distinct form of pRb in nuclear domains named ND10 (Jiang *et al.,* 1991, Szekely *et al.,* 1996). EBNA-LP and the 70-kDa family of heat-shock proteins (hsp70s) are associated *in vivo*, colocalized in ND10, and translocated to the nucleolus under cellular stress conditions such as heat shock and high-cell density (Mannick *et al.,* 1995; Kitay and Rowe, 1996; Szekely *et al.,* 1995). Recently, we showed that EBNA-LP is also localized in the cytoplasm and interacts with a cellular cytoplasmic protein, HAX-1, that was suggested to be involved in B-cell signal transduction and apoptosis (Kawaguchi *et al.,* 2000). These results suggested that EBNA-LP modulates the function of the interactive cellular proteins and EBNA-2-mediated activation of cellular and viral gene expression during EBV-induced immortalization.

EBNA-LP associates with the nuclear matrix like other EBNAs such as EBNA-2, -3A, and -3C do (Petti *et al.,* 1990). The nuclear matrix (also known as nuclear scaffold and nuclear skeleton) is a filamentous ribonucleoprotein complex composed of lamine polymers, core filaments, and associated proteins (Berenzy and Coffey, 1975; Fey *et al.,* 1984). A number of studies indicated that the organization of this structure is linked to various nuclear events including transcription, splicing, and DNA replication (Mortillaro *et al.,* 1996; Jackson and Cook, 1995). For instance, the cellular transcription factor, AML1 (PEBP2αB/Cbfa2) (Miyoshi *et al.,* 1991; Bae *et al.,* 1993), controls transcription of its target genes as well as polyomavirus DNA replication through association with the nuclear matrix (Zeng *et al.,* 1998; Kanno *et al.,* 1998; Chen *et al.,* 1998). These results suggested the importance of the nuclear matrix in regulation of nuclear events.

To demonstrate the significance of the interaction of EBNA-LP with the nuclear matrix, we mapped and mutagenized the nuclear matrix targeting signal (NMTS) of EBNA-LP. Here, we report that amino acid substitutions in the NMTS of EBNA-LP abolished not only its nuclear matrix association but also nuclear localization of the protein. We also showed that mutation of EBNA-LP abrogated its cooperative transcriptional activation with EBNA-2.

RESULTS

Transiently expressed EBNA-LP in COS-7 or BOSC23 cells is associated with nuclear matrix

Petti *et al.* (1990) previously reported that EBNA-LP is associated with nuclear matrix in B cells latently infected with EBV). One of the objectives of this report was to identify the domain responsible for the association of EBNA-LP with nuclear matrix. For this purpose, we used COS-7 or BOSC23 cells and carried out transient transfection assay in the cells using expression vectors for various EBNA-LP mutants. First of all, we performed two sets of experiments to confirm that transiently expressed EBNA-LP is in fact localized at nuclear matrix in these cells. The results were as follows.

(i) In the first series of experiments, FLAG epitope-tagged EBNA-LP was transiently expressed in COS-7 cells and its association with nuclear matrix was investigated by *in situ* nuclear matrix isolation and subsequent indirect immunofluorescence assay using a monoclonal antibody to FLAG epitope (M2). As shown in Fig. 1A, EBNA-LP was clearly detected after nuclease digestion (Fig. 1A–c), while DNA staining by DAPI was barely detectable (Fig. 1A–d), indicating that EBNA-LP is retained at nuclear matrix *in situ*.

(ii) In the second series of experiments, BOSC23 cells were transfected with indicated expression plasmids, fractionated as described under Materials and Methods, subjected to electrophoresis in denaturing gels, transferred to nitrocellulose sheets, and reacted to the anti-FLAG antibody (M2). As shown in Fig. 1B, the major portion of EBNA-LP transiently expressed in BOSC23 cells was detected in the nuclear matrix fraction (Fig. 1B, lane 10) and the small protein was also found in other subnuclear fractions (Fig. 1B, lanes 7 to 9). These results were consistent with those of a previous study using EBV-infected B cells (Petti *et al.,* 1990). Figure 1C shows that AML1b, which is known to be sequestered into the nuclear matrix (Zeng *et al.,* 1997), was detected in the nuclear matrix fraction, while the alternatively spliced form of AML1b, AML1a, that lacks ability to localize in the nuclear matrix (Miyoshi *et al.,* 1995; Chen *et al.,* 1998), was not detectable in this fraction. This confirmed that cells were appropriately fractionated in these experiments. These results indicated that transiently expressed EBNA-LP in COS-7 or BOSC23 cells was associated with the nuclear matrix.

W2 repeat domain of EBNA-LP is responsible for association of the protein with the nuclear matrix

To map the domain of EBNA-LP that is involved in association of the protein with the nuclear matrix, we constructed a series of deletion mutants of EBNA-LP (Fig. 2A) and tested whether these mutants transiently expressed in BOSC23 cells were localized in the nuclear matrix. Preliminary experiments indicated that molecules with only one W repeat domain were difficult to express in BOSC23 cells, presumably due to their instability (data not shown). We, therefore, constructed plasmids expressing FLAG epitope-tagged EBNA-LP mutants fused to the GAL4 DNA binding domain to increase the stability of the proteins. Whole-cell extract and nuclear matrix fraction were prepared as described under Materials and Methods, separated in a denaturing gel, and sub-

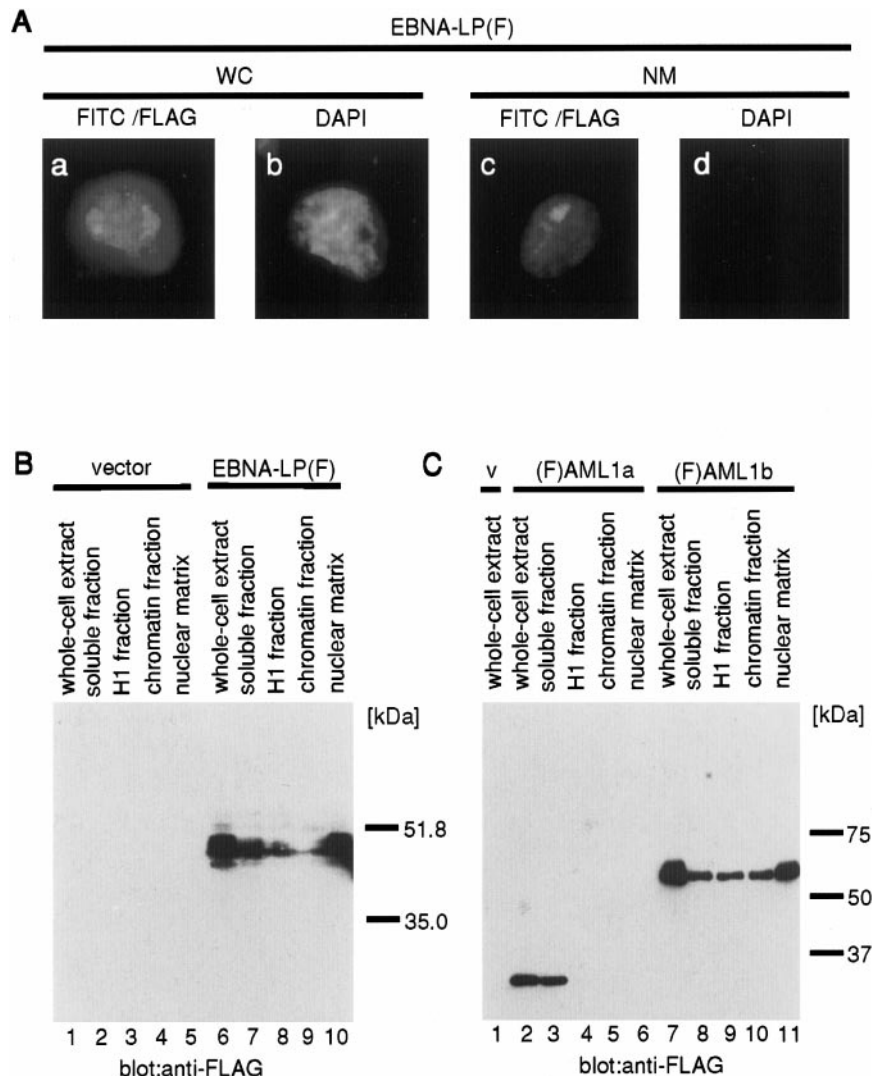
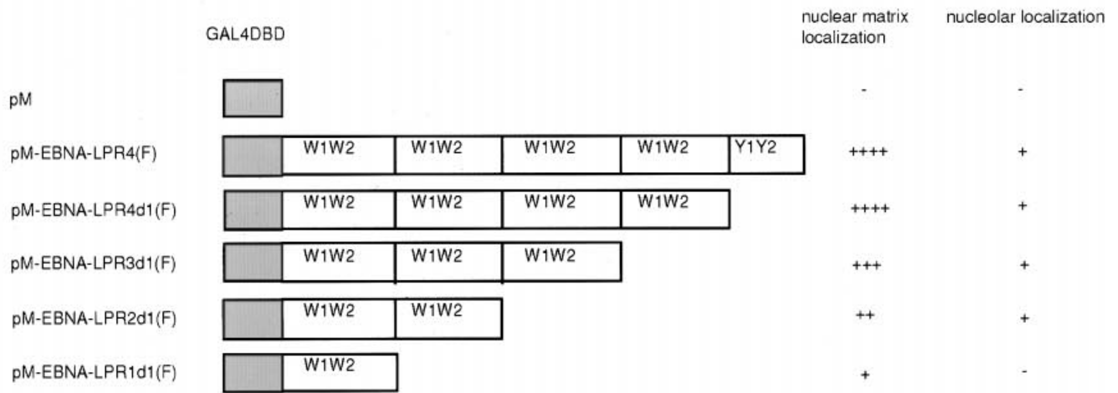
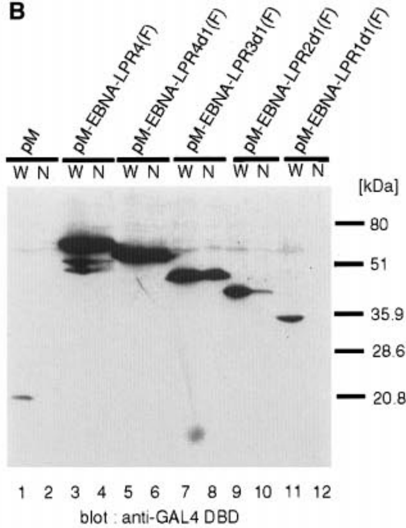


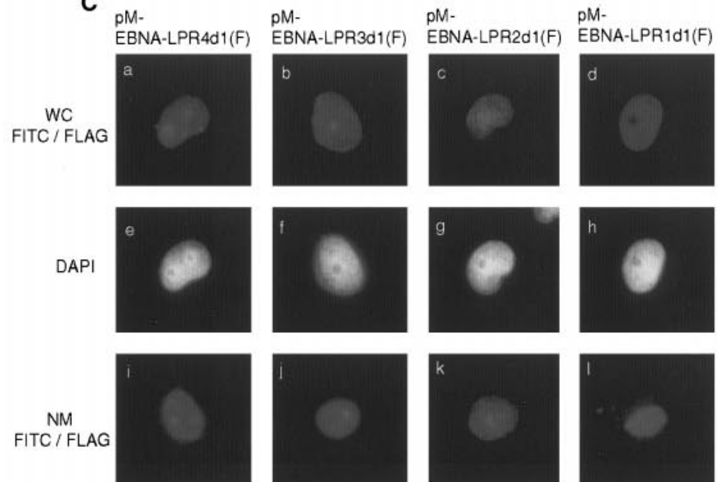
FIG. 1. Transiently expressed EBNA-LP in COS-7 or BOSC23 cells was associated with the nuclear matrix. (A) Photographic images of COS-7 cells transfected with pcDNA4/HisMax-EBNA-LP(F) on immunofluorescence assay. Whole cells (WC) *and in situ* nuclear matrix (NM) preparations of the transfected cells were analyzed for the presence of EBNA-LP by immunofluorescence assay with the anti-FLAG antibody (M2), and anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (a, c). DNA content was visualized by DAPI staining (b, d). (B) Photographic image of an immunoblot of electrophoretically separated subcellular fractions of BOSC23 cells transfected with the indicated vectors. BOSC23 cells were transfected with the empty expression vector (vector) or pEBVHis-EBNA-LP(F) (EBNA-LP(F)) and harvested 48 h after transfection. The cells were divided into two pools equally; one was solubilized as whole-cell extract, and the other was fractionated as described under Materials and Methods. The cell lysates were then subjected to electrophoresis in a denaturing gel, transferred onto a nitrocellulose sheet, and subjected to immunoblotting with anti-FLAG antibody (M2). Molecular weights (kDa) are shown on the right. The soluble fractions in CSK, extraction, and digestion buffers are referred to as soluble, histone H1, and chromatin fractions, respectively. The nuclear matrix fraction was the residual insoluble fraction after treatment with digestion buffer. (C) Photographic image of an immunoblot of electrophoretically separated subcellular fractions of BOSC23 cells transfected with the empty expression vector (v), pLNSX-FLAG-AML1a ((F)AML1a), or pLNSX-FLAG-AML1b ((F)AML1b). The transfected cells were fractionated as described in (B) and subjected to immunoblotting with the anti-FLAG antibody (M2). Molecular weights (kDa) are shown on the right.

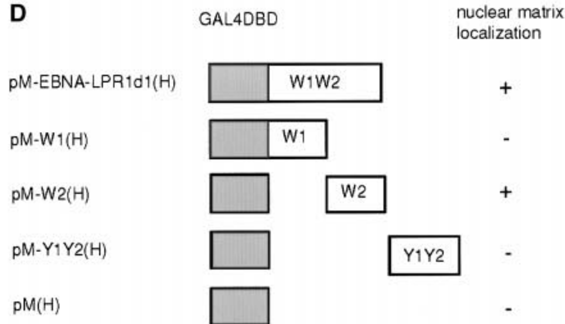
jected to immunoblotting with an antibody to the GAL4 DNA binding domain (RK5C1). As shown in Fig. 2A, Y1Y2-deleted EBNA-LP associated with the nuclear matrix as efficiently as wild-type EBNA-LP (Fig. 2B, lanes 3 to 6), indicating that the domain of the EBNA-LP protein responsible for the nuclear matrix association is the W repeat domain. These results were confirmed by *in situ* nuclear matrix isolation and subsequent immunofluorescence assays using an antibody to FLAG epitope (M2) (Fig. 2C).

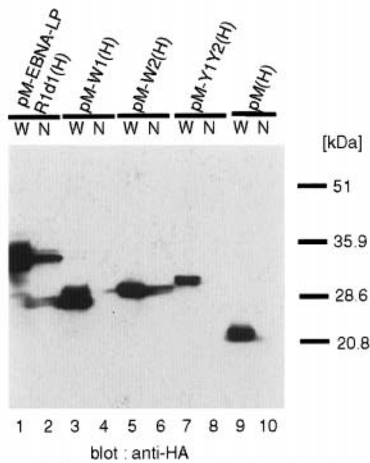
Three further EBNA-LP mutants were generated to determine whether the copy number of W repeats of EBNA-LP influences the association with the nuclear matrix (Fig. 2A). The results shown in Fig. 2B indicated that the ratio of EBNA-LP mutants detected in the nuclear matrix fraction to that in the whole-cell extract was reduced as the copy number of W repeat decreased. These results suggested that association of EBNA-LP with the nuclear matrix depends on the number of W repeats of the protein and it is likely that the affinity to the nuclear matrix indeed increases as the copy number of W repeat

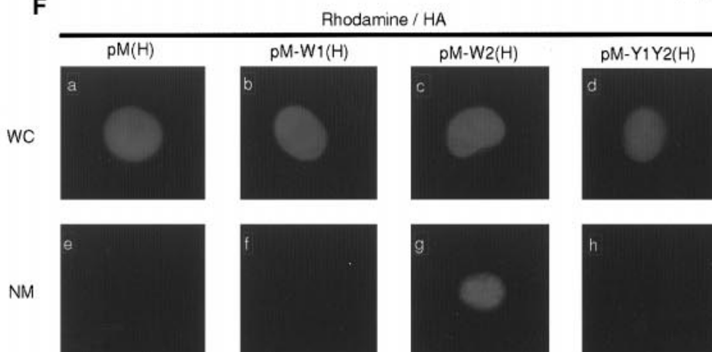

A
GAL4DBD
nuclear matrix localization
nucleolar localization
pM
pM-EBNA-LPR4(F)
pM-EBNA-LPR4d1(F)
pM-EBNA-LPR3d1(F)
pM-EBNA-LPR2d1(F)
pM-EBNA-LPR1d1(F)
W1W2
Y1Y2
++++
+++
++
+
-


B
pM
pM-EBNA-LPR4(F)
pM-EBNA-LPR4d1(F)
pM-EBNA-LPR3d1(F)
pM-EBNA-LPR2d1(F)
pM-EBNA-LPR1d1(F)
W N
[kDa]
80
51
35.9
28.6
20.8
1 2 3 4 5 6 7 8 9 10 11 12
blot : anti-GAL4 DBD


C
pM-EBNA-LPR4d1(F)
pM-EBNA-LPR3d1(F)
pM-EBNA-LPR2d1(F)
pM-EBNA-LPR1d1(F)
WC FITC / FLAG
DAPI
NM FITC / FLAG


D
GAL4DBD
nuclear matrix localization
pM-EBNA-LPR1d1(H)
pM-W1(H)
pM-W2(H)
pM-Y1Y2(H)
pM(H)
W1W2
W1
W2
Y1Y2


E
pM-EBNA-LPR1d1(H)
pM-W1(H)
pM-W2(H)
pM-Y1Y2(H)
pM(H)
W N
[kDa]
51
35.9
28.6
20.8
1 2 3 4 5 6 7 8 9 10
blot : anti-HA


F
Rhodamine / HA
pM(H)
pM-W1(H)
pM-W2(H)
pM-Y1Y2(H)
WC
NM

increases. The experiments using *in situ* nuclear matrix isolation and subsequent immunofluorescence assays with GAL4-EBNA-LP mutants with different copy numbers of W repeats revealed that approximately 30% of cells expressing EBNA-LP derivatives containing more than two W repeats clearly showed their nucleolar localization (Fig. 2C, a–c and i–k) as reported previously (Szekely *et al.,* 1995), while EBNA-LP with only a single W repeat was no longer localized in the nucleolus (Fig. 2C, d and l).

The W repeat of EBNA-LP consists of W1 and W2 domains. Next, we attempted to determine which domain of the W repeat is responsible for the nuclear matrix localization of EBNA-LP. We constructed various HA-tagged EBNA-LP mutants fused to the GAL4 DNA binding domain in expression plasmids (Fig. 2D) examined whether their products were detected in the nuclear matrix fraction of COS-7 cells. Although GAL4-EBNA-LPR1d1 was not detectable in the nuclear matrix fraction as shown in Fig. 2B, it was detected when 10-fold concentrated cell lysate was subjected to immunoblotting (data not shown). Further, we used an antibody to the HA epitope (3F10) to detect the HA-tagged GAL4-EBNA-LP fusion proteins because it can detect the fusion protein more sensitively than the antibody to the GAL4 DNA binding domain (RK5C1) (Fig. 2E, lanes 1 and 2). The results (Fig. 2F) showed that all GAL4 and GAL4-EBNA-LP proteins were expressed in the nucleus, while only the GAL4-W2 fusion protein was detectable in the nuclear matrix. These results were confirmed by cell fractionation analysis as shown in Fig. 2E. Although the expression level of GAL4-Y1Y2 is lower than that of other GAL4 fusion proteins, it could not be detected in the nuclear matrix fraction even with the much longer exposure. These results indicated that the W2 domain of EBNA-LP is involved in its nuclear matrix localization.

CR2 region of the W2 domain of EBNA-LP is essential for its nuclear matrix localization

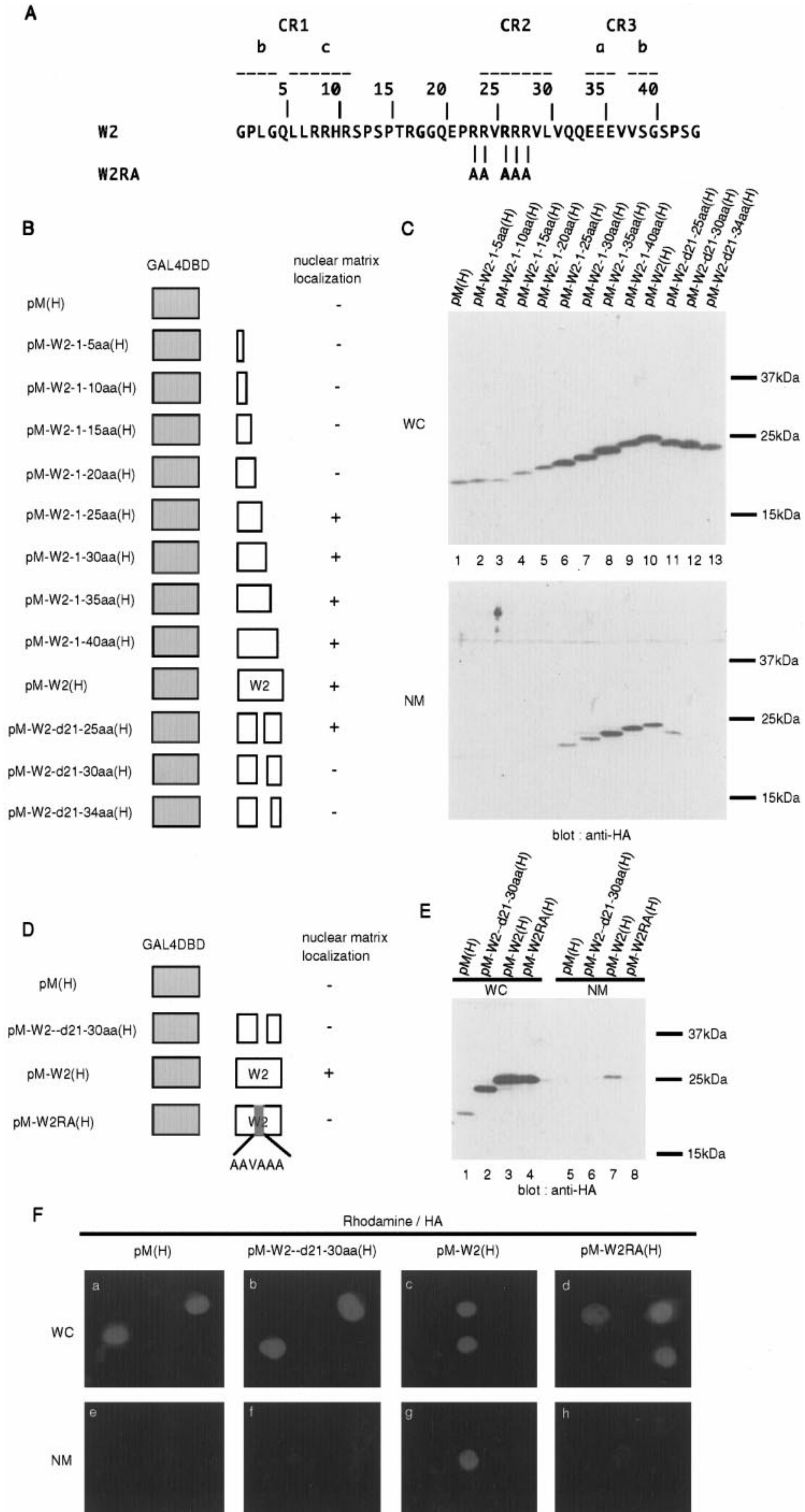
To further map the site of EBNA-LP interacting with the nuclear matrix, we constructed a series of 3′ sequential and internal deletion mutants of the W2 domain tagged with HA at the C-terminal ends and fused to the GAL4 DNA binding domain in expression plasmids as shown in Fig. 3B and tested them for their association with the nuclear matrix by cell fractionation and immunoblotting probed with the antibody to the HA epitope (3F10). As shown in Fig. 3C, the site in the W2 domain of EBNA-LP that interacts with the nuclear matrix mapped the amino acids EPRRVRRRVL between codon 21 to 30 (relative to the first amino acid residue of the W2 domain; Fig. 3A). Interestingly, the mapped region was completely overlapped with the conserved region, named CR2 (Peng *et al.,* 2000), among a subset of primate gammaherpesviruses (Fig. 3A). Next, we mutagenized the GAL4-W2 construct by replacing clustered arginine residues in the region between codons 21 to 30 to alanines as described under Materials and Methods (Figs. 3A and 3D). The experiments with the mutant (GAL4-W2RA) showed that the amino acid substitutions resulted in abolishment of nuclear matrix association of the protein (Figs. 3E and 3F), although the mutant protein was expressed in the nucleus (Fig. 3F, d).

From these results, we concluded that the 10-amino acid segment from codons 21 to 30 in the W2 domain of EBNA-LP is the major determinant of its interaction with the nuclear matrix. We referred to this segment as the nuclear matrix targeting signal (NMTS) of EBNA-LP.

NMTS of EBNA-LP is critical for its nuclear localization as well as its nuclear matrix association

Nitsche *et al.* (1997) previously reported that more than two copies of the W repeat are required for EBNA-LP to express one of its biological phenotypes, the up-regulation of LMP1 expression in combination with EBNA-2. To investigate the importance of nuclear matrix localization for the function of EBNA-LP in more detail, we mutagenized the EBNA-LP cDNA containing four repeats of W domains in the expression vector by introducing the same alanine substitutions for the clustered arginine in NMTS of each W repeat domain (Fig. 4A). BOSC23 cells

FIG. 2. Mapping of the domain of EBNA-LP responsible for nuclear matrix association. (A) Schematic representation of various deletion mutants of EBNA-LP fused to the GAL4 DNA binding domain (GAL4 DBD) tagged with a FLAG epitope. The levels of nuclear matrix association and nucleolar localization of the mutants are also shown. (B) Photographic image of an immunoblot of electrophoretically separated subcellular fractions of BOSC23 cells transfected with a series of expression vectors for EBNA-LP mutants shown in (A). At 48 h after transfection, whole-cell extracts (W) and nuclear matrix fractions (N) were prepared as described under Materials and Methods, separated by electrophoresis in a denaturing gel, transferred onto a nitrocellulose sheet, and subjected to immunoblotting with anti-GAL4 DNA binding domain antibody (RK5C1). Molecular weights (kDa) are shown on the right. (C) Photographic images of COS-7 cells transfected with the indicated expression vectors for GAL4-EBNA-LP containing various numbers of W repeats on immunofluorescence assay. At 48 h after transfection, whole cells (WC) (a to d) or nuclear matrix (NM) (i to l) isolated *in situ* as described under Materials and Methods were fixed, subjected to immunofluorescence with the anti-FLAG antibody (M2), and anti-mouse IgG conjugated to FITC. DNA was detected by DAPI staining (e to h). (D) Schematic representation of each EBNA-LP mutant expressing W1W2, W1, W2, or Y1Y2 domain of EBNA-LP fused to the GAL4 DNA binding domain (GAL4 DBD) tagged with an epitope of influenza hemagglutinin (HA). The levels of nuclear matrix association of the mutants are also shown. (E) Photographic image of an immunoblot of electrophoretically separated subcellular fractions of BOSC23 cells transfected with the expression vectors for EBNA-LP mutants shown in (D). Whole-cell extracts (W) and nuclear matrix fractions (N) of the transfected cells were analyzed as described in (B) using the anti-HA antibody (3F10). Molecular weights (kDa) are shown on the right. (F) Photographic images of BOSC23 cells were transfected with the expression vectors for EBNA-LP mutants shown in (D) on immunofluorescence assay. Localizations of EBNA-LP mutants in whole cells (WC) (a to d) and nuclear matrix (NM) (e to h) were analyzed as described in (C) using the anti-HA antibody (3F10), and anti-rat IgG conjugated to rhodamine.


A
CR1
CR2
CR3
b c a b
5 10 15 20 25 30 35 40
W2 GPLGQLLRRHRSPSPTRGGQEPRRVRRRVLVQQEEEVVSGSPSG
W2RA AA AAA
B
GAL4DBD
nuclear matrix localization
pM(H) -
pM-W2-1-5aa(H) -
pM-W2-1-10aa(H) -
pM-W2-1-15aa(H) -
pM-W2-1-20aa(H) -
pM-W2-1-25aa(H) +
pM-W2-1-30aa(H) +
pM-W2-1-35aa(H) +
pM-W2-1-40aa(H) +
pM-W2(H) W2 +
pM-W2-d21-25aa(H) +
pM-W2-d21-30aa(H) -
pM-W2-d21-34aa(H) -
C
pM(H)
pM-W2-1-5aa(H)
pM-W2-1-10aa(H)
pM-W2-1-15aa(H)
pM-W2-1-20aa(H)
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pM-W2-d21-30aa(H)
pM-W2-d21-34aa(H)
WC
37kDa
25kDa
15kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
NM
37kDa
25kDa
15kDa
blot : anti-HA
D
GAL4DBD
nuclear matrix localization
pM(H) -
pM-W2--d21-30aa(H) -
pM-W2(H) W2 +
pM-W2RA(H) W2 -
AAVAAA
E
pM(H)
pM-W2--d21-30aa(H)
pM-W2(H)
pM-W2RA(H)
WC
pM(H)
pM-W2--d21-30aa(H)
pM-W2(H)
pM-W2RA(H)
NM
37kDa
25kDa
15kDa
1 2 3 4 5 6 7 8
blot : anti-HA
F
Rhodamine / HA
pM(H)
pM-W2--d21-30aa(H)
pM-W2(H)
pM-W2RA(H)
WC
a b c d
NM
e f g h

were transfected with the indicated expression plasmids, solubilized, and subjected to immunoblotting with the monoclonal antibody to the FLAG epitope (M2) or EBNA-LP (JF186). As shown in Fig. 4B, EBNA-LP with the mutation was expressed in BOSC23 cells. The EBNA-LP mutant (EBNA-LPRA(F)) migrated more slowly than wild-type EBNA-LP in denaturing gels (Fig. 4B) possibly because the amino acid substitutions resulted in a change in electronic charge of the mutant protein. Surprisingly, when localization of transiently expressed EBNA-LPRA(F) was examined in COS-7 cells by immunofluorescence assay with anti-FLAG antibody, the mutant protein was detected only in the cytoplasm of the cells (Fig. 4C). To confirm these results, BOSC23 cells transiently transfected with wild-type EBNA-LP or the mutant EBNA-LP expression vector were fractionated as described under Materials and Methods and each fraction was subjected to electrophoresis in denaturing gels and immunoblotting with anti-FLAG antibody. The results (Fig. 4D) showed that wild-type EBNA-LP was detectable mainly in the nuclear fractions (Fig. 4D, lanes 4 to 7) as well as in the cytoplasmic fraction (Fig. 4D, lane 3), while the mutant EBNA-LP was detected only in the cytoplasm (Fig. 4D, lane 9). These results indicated that NMTS of EBNA-LP is responsible not only for its nuclear matrix association but also for its nuclear localization.

EBNA-LPRA fused to GAL4 DNA binding domain is retained in cytoplasm of COS-7 cells

To further investigate the mechanism by which the EBNA-LP mutant (EBNA-LPRA) is localized in the cytoplasm, we constructed EBNA-LPRA mutant fused to GAL4 DNA binding domain in an expression vector (Fig. 5A). In BOSC23 cells transfected with the expression plasmids [pM-EBNA-LP(F) and pM-EBNA-LPRA(F)], the expected proteins were expressed (Fig. 5B). Earlier studies indicated that the GAL4 DNA binding domain possesses a nuclear localization signal (NLS) within the domain from codons 1 to 74 that is sufficient to translocate heterologous cytoplasmic proteins into the nucleus (Silver *et al.,* 1984). COS-7 cells were transfected with the indicated plasmids, fixed 2 days after transfection, reacted with the anti-GAL4 antibody (RK5C1), and examined with a Nikon fluorescence microscope. As shown in Fig. 5C, GAL4 by itself and wild-type EBNA-LP fused to GAL4 DNA binding domain were detected as nuclear staining. In contrast, EBNA-LPRA was still localized in the cytoplasm of the cells even when the mutant was expressed as a fusion protein with the GAL4 DNA binding domain (Fig. 5C). These results raised two possibilities that (i) EBNA-LP possesses nuclear export signal to be excluded from the nucleus, or (ii) the protein has a domain that is involved in its retention in the cytoplasm.

EBNA-LPRA lacks the ability to stimulate EBNA-2-mediated transcriptional activation

To examine the biological significance of nuclear and nuclear matrix localization of EBNA-LP, we performed two series of experiments.

Harada and Kieff (1997) reported that EBNA-LP is a coactivator for EBNA-2-dependent transcriptional activation. In the first series of experiments, the expression vector for EBNA-2 fused to the GAL4 DNA binding domain was constructed and tested for coactivation with EBNA-LP or EBNA-LPRA in COS-7 or BOSC23 cells cotransfected with a reporter construct (pFR-luc) containing five tandem repeats of the 17bp GAL4 binding element upstream of a basic promoter element (TATA box). At 48 h after transfection, the cells were harvested and subjected to luciferase assay as described under Materials and Methods. As shown in Figs. 6A and 6B, wild-type EBNA-LP significantly stimulated EBNA-2-mediated transcriptional activation by approximately 20-fold, while EBNA-LPRA did not.

Nitsche *et al.* (1997) also reported that transiently expressed EBNA-LP along with EBNA-2 induces endogenous LMP-1 expression in EBV-positive cell lines. In the second series of experiments, wild-type EBNA-LP or EBNA-LPRA mutant along with EBNA-2 was introduced into Akata cells. We examined the LMP-1 expression by

FIG. 3. CR2 of EBNA-LP is the region responsible for nuclear matrix association. (A) The amino acid sequence of W2 exon of EBNA-LP. Conserved regions defined by Peng *et al.* (2000) are indicated as broken lines with their designation given above. Arginine to alanine substitutions (W2RA) in the segment mapped by truncation of the W2 sequence are also indicated. (B) Schematic representation of 3′ sequential, and internal deletion mutants of W2 domain fused to the GAL4 DNA binding domain tagged with the HA epitope. The level of nuclear matrix association of the mutants is also shown. (C) Photographic images of immunoblots of electrophoretically separated whole-cell extract (WC) and nuclear matrix fraction (NM) of BOSC23 cells transfected with the indicated expression vectors for EBNA-LP mutants are shown in (B). Cell extracts were prepared as described under Materials and Methods, separated by electrophoresis in denaturing gels, transferred onto nitrocellulose sheets, and subjected to immunoblotting with the anti-HA antibody (3F10). Molecular weights (kDa) are shown on the right of each panel. (D) Schematic representations of various mutants of EBNA-LP W2 domain fused to the GAL4 DNA binding domain tagged with the HA epitope, including the internal deletion mutant lacking the amino acids between codons 21 to 30 (relative to the first amino acid residue of W2 domain), and arginine–alanine substitution mutant in this region. (E) Photographic image of an immunoblot of electrophoretically separated whole-cell extract (WC) (lanes 1 to 4) and nuclear matrix fraction (NM) (lanes 5 to 8) of BOSC23 cells transfected with the indicated expression vectors for EBNA-LP mutants shown in (D). Whole-cell extracts (WC) and nuclear matrix fractions (NM) of the transfected cells were analyzed as described in (C) using the anti-HA antibody (3F10). Molecular weights (kDa) are shown on the right. (F) Photographic images of BOSC23 cells were transfected with the expression vectors for EBNA-LP mutants shown in (D) on immunofluorescence assay. Localizations of EBNA-LP mutants in whole cells (WC) (a to d) and nuclear matrix (NM) (e to h) were analyzed as described in Fig. 2F using the anti-HA antibody (3F10), and anti-rat IgG conjugated to rhodamine.

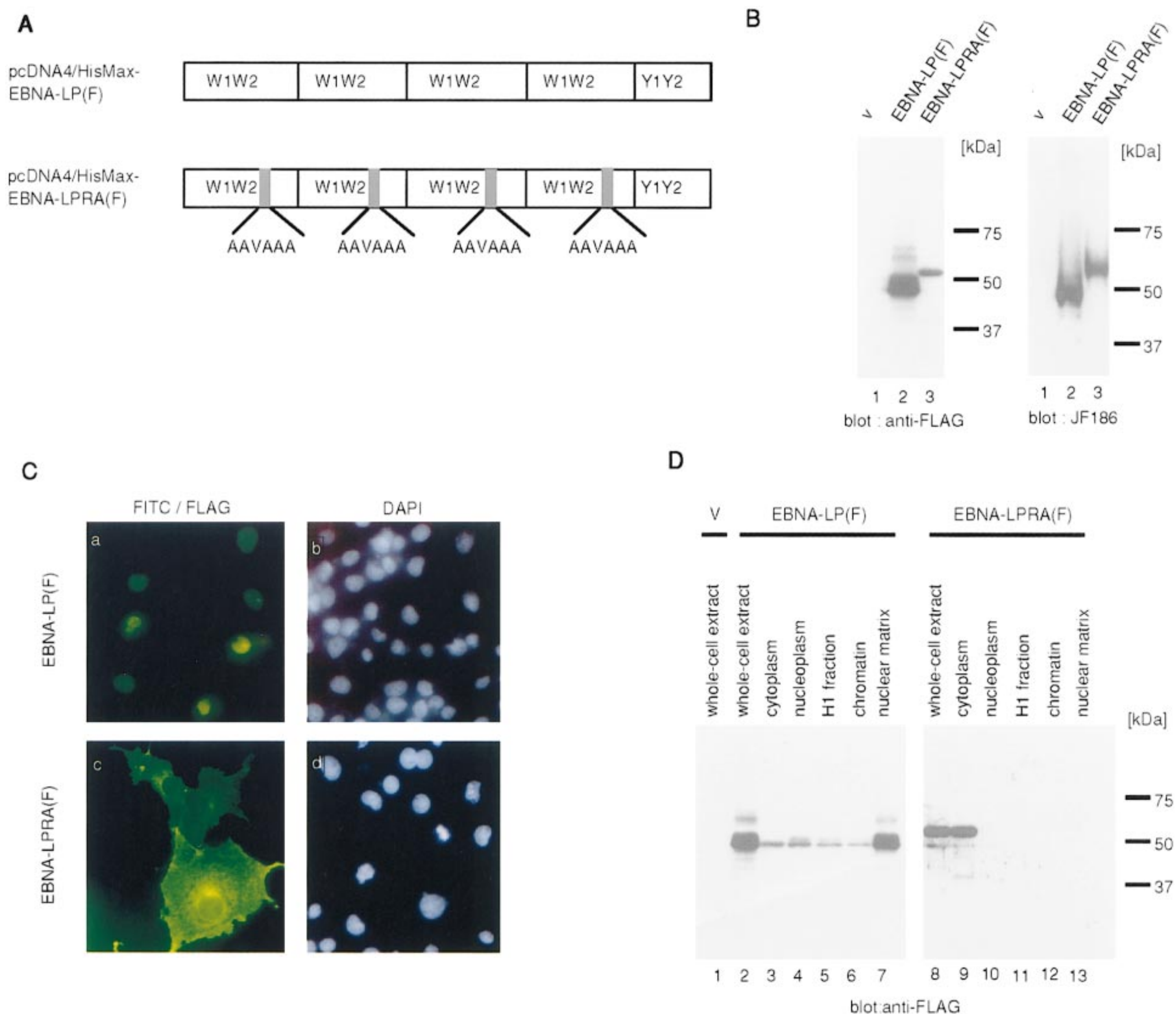

FIG. 4. CR2 is responsible for not only nuclear matrix localization but also nuclear localization. (A) Schematic representation of FLAG epitope-tagged wild-type EBNA-LP and EBNA-LPRA mutant with arginine to alanine substitutions in nuclear matrix targeting signal (NMTS) of EBNA-LP. (B) Photographic images of immunoblots of electrophoretically separated lysates of BOSC23 cells transfected with pcDNA4/HisMax vector (v), pcDNA4/HisMax-EBNA-LP(F) (EBNA-LP(F)), or pcDNA4/HisMax-EBNA-LPRA(F) (EBNA-LPRA(F)). The blots were probed with the anti-FLAG antibody (M2) (left panel) or the mouse monoclonal antibody to EBNA-LP (JF186) (right panel). Molecular weights (kDa) are shown on the right of each panel. (C) Photographic images of COS-7 cells transfected with pcDNA4/HisMax-EBNA-LP(F) (EBNA-LP(F)) or pcDNA4/HisMax-EBNA-LPRA(F) (EBNA-LPRA(F)) on immunofluorescence assay. The EBNA-LP proteins were visualized with the anti-FLAG antibody (M2) and FITC-conjugated anti-mouse IgG antibody (left panels). DNA was stained with DAPI (right panels). (D) Photographic images of immunoblots of electrophoretically separated subcellular fractions of BOSC23 cells transfected with pcDNA4/HisMax vector (v), pcDNA4/HisMax-EBNA-LP(F) (EBNA-LP(F)), or pcDNA4/HisMax-EBNA-LPRA(F) (EBNA-LPRA(F)). The transfected cells were fractionated as described under Materials and Methods, separated by electrophoresis in denaturing gels, transferred onto nitrocellulose sheets, and reacted with anti-FLAG antibody. Each subnuclear fraction except the nucleoplasm fraction was obtained as described in Fig. 1B. The nucleoplasm was the soluble fraction obtained after treatment of the whole nuclear fraction with CSK buffer. Molecular weights (kDa) are shown on the right of each panel.

immunoblotting with anti-LMP-1 monoclonal antibody (S-12). As shown in Fig 6C, EBNA-LPRA failed to induce LMP-1 expression, while EBNA-LP efficiently induces the expression of LMP-1 as reported previously (Nitsche *et al.,* 1997). The expressions of EBNA-LPs and EBNA-2 were also examined with anti-EBNA-LP monoclonal antibody (JF186) and anti-EBNA-2 monoclonal antibody (PE2), respectively.

These results indicated that nuclear and/or nuclear matrix localization of EBNA-LP are essential for cooperative transcriptional activation with EBNA-2.

DISCUSSION

The salient features of this study can be summarized as follows.

(i) We defined a 10-amino acid segment as NMTS that mediates association of EBNA-LP with the nuclear matrix and also showed that the NMTS is essential for nuclear localization of EBNA-LP. We referred to this segment as EBNA-LP NMTS/NLS. Three lines of evidence listed below supported our conclusion. First, EBNA-LP possesses five evolutionarily well-conserved regions (CR1 to CR5)

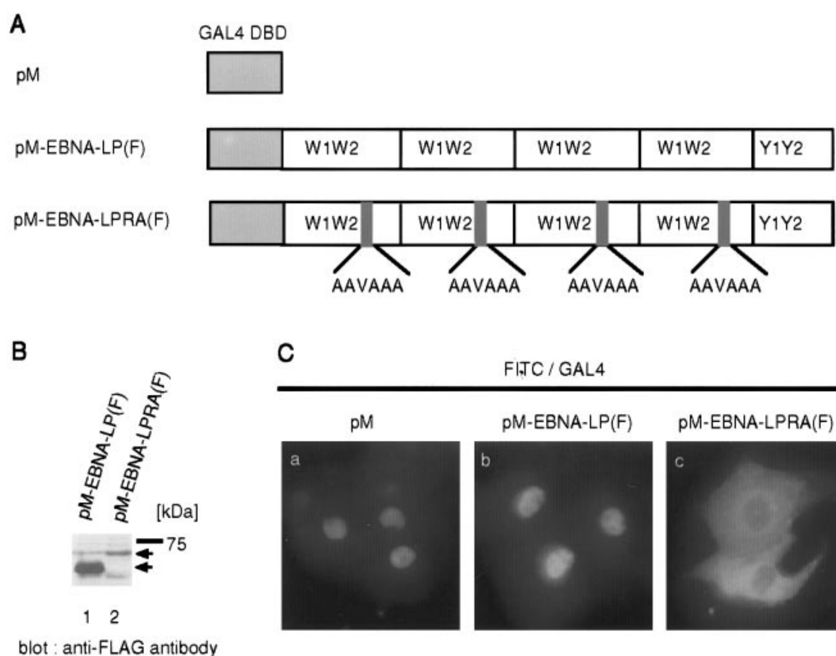

FIG. 5. EBNA-LP RA mutant fused to the GAL4 DNA binding domain is retained in the cytoplasm. (A) Schematic representation of wild-type EBNA-LP or EBNA-LP RA mutant fused to GAL4 DNA binding protein tagged with the FLAG epitope. (B) Photographic image of an immunoblot of electrophoretically separated lysates of BOSC23 cells transfected with pM-EBNA-LP(F) or pM-EBNA-LPRA(F). The blot was probed with the anti-FLAG antibody (M2). Arrows indicate each band of GAL4-EBNA-LP or GAL4-EBNA-LPRA. (C) Photographic images of COS-7 cells transfected with pM, pM-EBNA-LP(F), or pM-EBNA-LPRA(F), fixed and reacted with anti-GAL4 DNA binding domain (RK5C1), and anti-mouse IgG conjugated to FITC.

among gammaherpesvirus EBNA-LP homologues (Peng *et al.,* 2000), and these regions are suggested to be important functional domains of the protein. As predicted, our mutational analyses of EBNA-LP revealed that one of the conserved regions, CR2, plays essential roles in both nuclear matrix association and nuclear localization of EBNA-LP. Second, EBNA-LP NMTS/NLS shares similar features with typical NLSs. NLSs fall into two categories: a single-type signal consisting of a stretch of basic amino acids such as NLS of simian virus 40 T antigen (Kalderon *et al.,* 1984), and a bipartite signal consisting of two basic amino acid stretches separated by a 10-amino acid spacer such as that of nucleoplasmin (Dingwall *et al.,* 1988; Robbins *et al.,* 1991). As shown in Fig. 7, EBNA-LP NMTS/NLS is composed of a cluster of basic amino acid and shows significant similarity to the single-type NLS of simian virus 40 T antigen. Furthermore, the EBNA-LP NMTS/NLS together with another conserved region, CR1c, showed similarities to a nucleoplasmin-like bipartite NLS (Fig. 7). CR1c consists of a cluster of basic amino acids positioned approximately 10-amino acids upstream of the EBNA-LP NMTS/NLS. Third, Zou *et al.* (2000) recently reported that a cluster of basic amino acids residues of E2 protein of human papillomavirus type 11 (HPV-11) is required for both nuclear localization and nuclear matrix association. As shown in Fig. 7, EBNA-LP NMTS/NLS showed consistent homology with the cluster of basic amino acids in HPV-11 E2 protein. Taken together, these observations support our conclusion that EBNA-LP NMTS/NLS identified in this study is critical for both nuclear localization and nuclear matrix association.

(ii) EBNA-LPRA mutant fused to the GAL4 DNA binding domain is retained in the cytoplasm although the GAL4 DNA binding domain is known to have the ability to confer nuclear localization on heterologous cytoplasmic proteins (Silver *et al.,* 1984). These results suggested that EBNA-LP contains a functional domain responsible for cytoplasmic retention as well as NMTS/NLS for import into the nucleus. This may account for our recent observation that EBNA-LP containing a single W repeat transiently expressed in COS-7 cells was localized predominantly in the cytoplasm, while EBNA-LPs with more than two W repeats were mainly localized in the nucleus (Kawaguchi *et al.,* 2000). It appears that EBNA-LPs with more than two W repeats have sufficient ability to be transported into the nucleus, while the ability of EBNA-LP with only one W repeat to retain in the cytoplasm is stronger than the ability to be translocated to the nucleus. Taken together, these results suggest that more than two NMTS/NLS are required for efficient nuclear localization of the protein.

(iii) EBNA-LP with a single W repeat shows biological activities different from those of EBNA-LPs with more than two W repeats. First, Nitsche *et al.,* (1997) reported that the stimulation of EBNA-2-dependent transactivation requires more than two copies of the W repeat of EBNA-LP. Second, we previously reported that two copies of the W repeat of EBNA-LP are necessary for its nuclear localization as described above (Kawaguchi *et al.,* 2000).

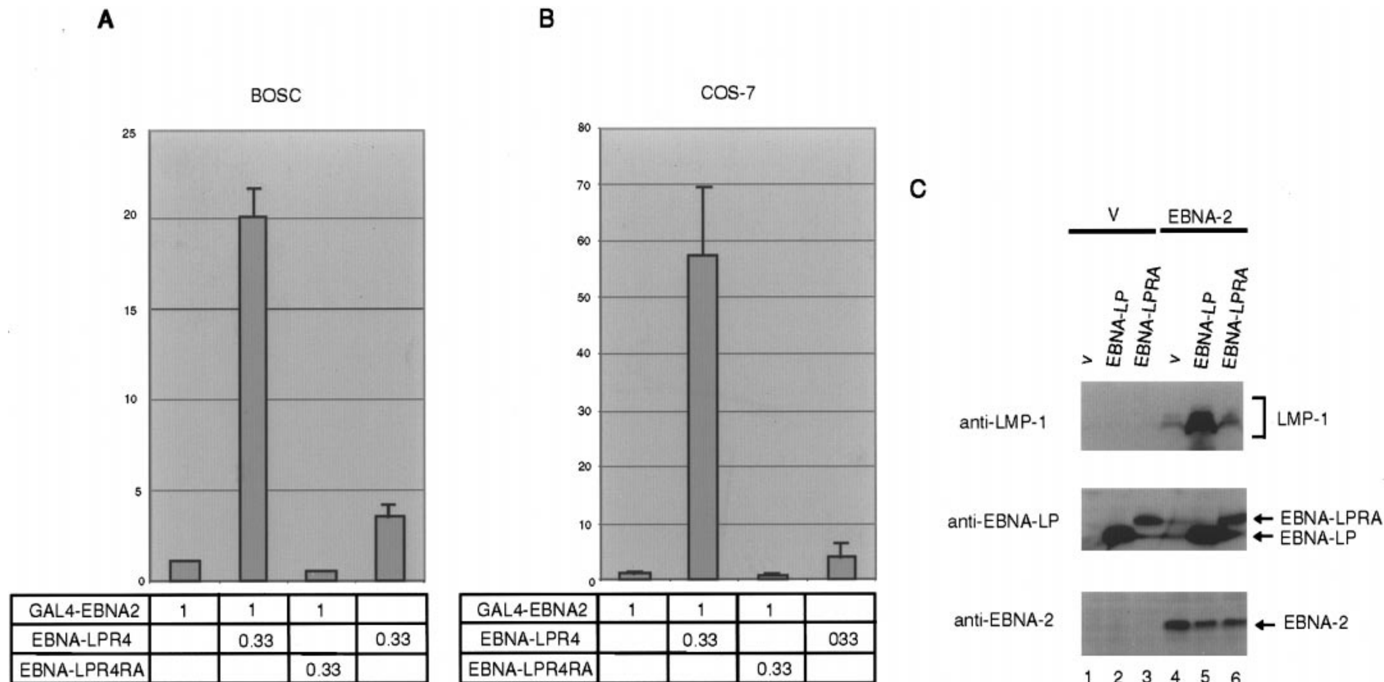

FIG. 6. The EBNA-LPRA mutant failed to stimulate EBNA-2-dependent transactivation. 0.33 μg of pcDNA4/HisMax vector, pcDNA4/HisMax-EBNA-LP(F) (EBNA-LPR4), or pcDNA4/HisMax-EBNA-LPRA(F) (EBNA-LPR4RA) was cotransfected into BOSC23 cells (A) or COS-7 cells (B) cultured in 24-well plates with 1 μg of pM-EBNA-2, 0.16 μg of pFR-luc, and 0.016 μg of pRL-tk. At 48 h after transfection, cells were lysed and assayed for luciferase activity as described under Materials and Methods. Fold activation relative to that with the pcDNA4/HisMax vector is shown. The results in (A) represent averages of two independent experiments, each of which was performed in quadruplicate. The results in (B) represent averages of two independent experiments, each of which was performed in duplicate. (C) Akata cells were transfected with 5 μg of pcDNA4/HisMax vector (v), pcDNA4/HisMax-EBNA-LP(F) (EBNA-LP), or pcDNA4/HisMax-EBNA-LPRA(F) (EBNA-LPRA) in combination with 20 μg of pME18S vector (V) or pME-EBNA-2 (EBNA-2) by electroporation. At 48 h after transfection, cells were lysed and subjected to Western blotting with anti-EBNA-LP mouse monoclonal antibody (JF186), anti-EBNA-2 mouse monoclonal antibody (PE2), anti-LMP-1 mouse monoclonal antibody (S-12).

Third, we showed here that the nucleolar localization of EBNA-LP requires more than two copies of the W repeat. Although the biological significance of nucleolar localization of EBNA-LP is unknown at present, there might be a possibility that the nucleolar localization of the protein is linked to its biological activity.

basic NLS

single type

SV40 largeT antigen	126	PKK-KRKV
EBNA-LP CR2	43	PRRVRRRV

bipartite type

nucleoplasmin	155	KRPAATKKAGQAKKK-K
EBNA-LP CR1c to CR2	31	HRSPSPTRGGQEPRRVRRR

signal for both nuclear localization and nuclear matrix targeting

E2 of HPV11	236	PPRKRAR
EBNA-LP CR2	43	PRRVRRRV

FIG. 7. Comparison of amino acid sequence of EBNA-LP CR2 with typical nuclear localization signals, and a signal for both nuclear localization, and nuclear matrix localization identified in human papillomavirus type 11 (HPV-11) E2 protein. The numbers in front of the amino acid sequences refer to the positions relative to the first amino acid of each protein.

(iv) There is accumulating evidence suggesting the importance of the nuclear matrix in regulation of gene expression. It has been reported that the nuclear matrix associated protein EBNA-LP is a coactivator of EBNA-2-mediated transcriptional activation (Harada and Kieff, 1997). Although it is unknown whether nuclear matrix association of EBNA-LP is important for its function as a coactivator, we show that mutations in NMTS/NLS of EBNA-LP abrogated its cooperative transcriptional activation with EBNA-2. As mutant EBNA-LP that was unable to associate with the nuclear matrix was not localized in the nucleus, we could not directly address whether nuclear matrix association of EBNA-LP is critical for its regulatory function. However, this is probably the case, based on the following observations. First, it has been reported that NMTS of the transcriptional regulator AML1/CBFα can act as a transcriptional activation domain (Zeng *et al.,* 1998). Second, the nuclear matrix has been suggested to be scaffold for various nuclear events including gene expression (Jackson and Cook, 1995) and it would therefore be important to target EBNA-LP to the right place.

In conclusion, we identified a target sequence of

EBNA-LP involved in both nuclear matrix association and nuclear localization. The identified sequence of EBNA-LP showed similarity to NMTS/NLS of HPV-11 E2, raising the possibility that a new type of specific signal for both nuclear matrix association and nuclear localization exists in a subset of proteins.

MATERIALS AND METHODS

Cells

BOSC23 cells are derived from 293T cells (Pear *et al.,* 1993). BOSC23 and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Akata cells are derived from a sporadic Burkitt's lymphoma (Takada and Ono, 1989). Akata cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS).

Plasmids

pEBVHis-EBNA-LP(F) was generated by cloning the EBNA-LP cDNA (a generous gift from Dr. Kieff) tagged with the FLAG sequence at its C-terminal end into the pEBVHis vector (Invitrogen). pME-EBNA-2 was constructed by cloning the *Eco*RI fragment of pSG-E2 (a generous gift from Dr. Fujiwara) into the *Eco*RI site of pME18S (a generous gift from Dr. Maruyama). pM-EBNA-2 was generated by inserting the *Eco*RI fragment of pSG-E2 into the *Eco*RI site of pM (Clontech) in frame with the GAL4 DNA binding domain. pMn was identical to pM except that a *Not*I site was introduced in front of the *Xba*I site of pM. pM-EBNA-LP(F) was generated by cloning the *Bam*HI-*Not*I fragment of pEBVHis-EBNA-LP(F) containing the entire coding region of EBNA-LP tagged with the FLAG epitope sequence into *Bam*HI-*Not*I sites of pMn in frame with the GAL4 DNA binding domain. Deletion mutants of pM-EBNA-LP(F) (Fig. 2A) were constructed by cloning into pMn the DNA fragments amplified by polymerase chain reaction (PCR) with appropriate primer pairs. The resultant plasmids expressed various FLAG-tagged EBNA-LP mutants fused to the GAL4 DNA binding domain. Plasmids (Figs. 2D and 4B) expressing various HA-tagged EBNA-LP domains fused to the GAL4 DNA binding domain were constructed by cloning DNA fragments amplified by PCR using appropriate primer pairs into pM. To generate pM-W2-RA(H) (Fig. 3D) and pM-EBNA-LPRA(F) (Fig. 5A), the arginine codons in the region mapped by deletion analysis were replaced with alanine codons using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) with the oligonucleotide 5′-CCAGTCCTACCAGAGGGGGCCA-AGAACCCGCAGCAGTCGCGGCCGCGGTCCTCGTCCAG-CAAGAAGAGGAGGTGG-3′ according to the manufacturer's instructions. pcDNA4/HisMax-EBNA-LP(F) and pcDNA4/HisMax-EBNA-LPRA(F) were generated by cloning the *Eco*RI-*Xba*I fragments of pM-EBNA-LP(F) and pM-EBNA-LPRA(F), respectively, into the pcDNA4/HisMax C vector (Invitrogen). pLNSX-(F)AML1a and pLNSX-(F)AML1b were constructed by cloning of the *Stu*I/*Hin*dIII fragment of the PCR product amplified from the cDNA of AML1a or AML1b with appropriate primer pairs, one of which contained the FLAG epitope sequence, into pLNSX (Miller *et al.,* 1993).

Transfection

COS-7 or BOSC23 cells were seeded 1 day before transfection and 60–80% confluent cells were transfected with various expression plasmids by calcium phosphate precipitation using the Profection mammalian transfection system (Promega). Twelve hours after transfection, the cells were washed and placed in fresh DMEM growth medium. At 48 h after transfection, the cells were harvested and used for further experiments. Akata cells growing in log phase were washed twice with RPMI supplemented with 10% FCS. 10^7 cells were suspended in 0.5 ml of RPMI 10% FCS medium and mixed with 25 μg of DNA. Cells were electroporated at 0.327 kV and 960 μF and then suspended in 10 ml of RPMI 10% FCS medium and cultured at 37°C in humidified 5% CO_2. Cells were harvested 48 h after transfection by pelleting down and washed with phosphate buffered saline (PBS) and lysed with SDS-PAGE sample buffer at the concentration of 5×10^7 cells/ml.

Cellular fractionation

Nuclear and cytoplasmic fractions were obtained by the method described previously (Kawaguchi *et al.,* 1997a). Briefly, BOSC23 cells transiently transfected with various EBNA-LP expression plasmids were harvested, washed with PBS, and resuspended in buffer A [10 mM HEPES [pH 7.4], 1.5 mM $MgCl_2$, 10 mM NaCl, 1 mM AEBSF (4-(2-aminoethyl) benzene sulfonyl fluoride) (Boeringer Mannheim)]. The cells were then lysed by addition of Nonidet-P40 to 0.1% and the nuclei were pelleted by centrifugation at top speed in a microcentrifuge. The supernatant (cytoplasmic fraction) was transferred to a new tube. The pellet was washed twice with buffer A, resuspended with CSK buffer [10 mM PIPES [pH 6.8], 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM VRC (vanadyl riboside complex) (5 Prime–3 Prime), 1 mM AEBSF], and subjected to nuclear matrix fractionation. Subnuclear fractionation was performed as described elsewhere (Fey *et al.,* 1984; Spector *et al.,* 1998) by sequential extraction with CSK buffer and extraction buffer (10 mM PIPES [pH 6.8], 250 mM ammonium sulfate, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM VRC, 1 mM AEBSF) each for 4 min at 4°C, and then incubated with digestion buffer [10 mM PIPES [pH 6.8], 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM VRC, 1 mM AEBSF, 300 units/ml of RNase-free DNaseI (Roche)] for 50 min at 32°C. Nucleoplasm, histone H1, and chromatin fractions were soluble materials obtained after

extraction with CSK, extraction, and digestion buffers, respectively. The residual insoluble fractions after extraction with digestion buffer were used as the nuclear matrix fractions.

In situ nuclear matrix isolation and indirect immunofluorescence

To isolate nuclear matrix *in situ* (Fey *et al.,* 1984, Spector *et al.,* 1998), cells on coverslips were washed with PBS and extracted in CSK buffer and extraction buffer for 4 min at 4°C. DNA digestion was performed in digestion buffer at 32°C for 50 min followed by treatment with extraction buffer twice. The cells were then fixed in 4% formaldehyde in PBS. Whole-cell samples were directly fixed in 4% formaldehyde in PBS, followed by permeabilization with 0.25% Triton X-100 in PBS.

For immunofluorescence assays, the fixed cells were blocked for 1 h at 37°C in PBS containing 10% FCS, reacted for 1.5 h at 37°C with appropriate primary antibody, rinsed three times with PBS, reacted for 1 h at 37°C with appropriate secondary antibody, rinsed three times with PBS, and mounted in PBS containing 90% glycerol. Goat anti-mouse and anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) and goat anti-mouse and anti-rat IgG conjugated to rhodamine (Santa Cruz Biotechnology) were used as secondary antibodies. For DAPI staining, the fixed cells were reacted in 2 μg/ml DAPI in PBS for 3 min at room temperature, then washed four times with PBS and mounted in PBS containing 90% glycerol. The coverslips were examined with a Nikon fluorescence microscope.

Immunoblotting

The electrophoretically separated proteins transferred onto nitrocellulose sheets were reacted with appropriate antibodies as described elsewhere (Kawaguchi *et al.,* 1997b). Briefly, the nitrocellulose sheets were blocked with 5% skim milk in T-PBS (PBS containing 0.1% Tween 20) for 1 h or overnight, rinsed twice, washed once for 15 min and twice for 5 min, and reacted for 2 h with primary antibodies. The blots were then washed as before, reacted for 1 h with peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology), rinsed twice, washed once for 15 min and four times for 5 min each time in T-PBS, and developed using the ECL chemiluminescence reagent (Amersham-Pharmacia).

Antibodies

Anti-FLAG mouse monoclonal antibody (M2; Sigma), anti-HA rat monoclonal antibody (3F10; Boehringer Mannheim), anti-GAL4 DNA binding domain mouse monoclonal antibody (RK5C1; Santa Cruz Biotechnology), anti-EBNA2 mouse monoclonal antibody (PE2; DAKO), anti-EBMA-LP mouse monoclonal antibody (JF186; a generous gift from Dr. G. Klein), and anti-LMP-1 mouse monoclonal antibody (S-12; a generous gift from Dr. E. Kieff) were used as the primary antibodies for immunoblotting and/or immunofluorescence.

Reporter gene assay

BOSC23 or COS-7 cells in 24-well culture plates were transfected with 1 μg of pM-EBNA-2 and 0.16 μg of pFR-luc (Stratagene) together with 0.33 μg of pcDNA4/HisMaxCvector,pcDNA4/HisMax-EBNA-LP(F)orpcDNA4/HisMax-EBNA-LPRA(F) as described above. pRL-tk (0.016 μg; Promega) was also cotransfected, being used as an internal control for transfection efficiency as described by the manufacturer. At 48 h after transfection, cells were harvested and assayed for luciferase activity using the Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). Fold activation activity was relative to that of the control expression vector pcDNA4/HisMaxC.

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