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Identification of a Short Amino Acid Sequence Essential for Efficient Nuclear Targeting of the Epstein-Barr Virus Nuclear Antigen 3A

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The Epstein-Barr virus nuclear antigen 3A is expressed in the nuclei of cells latently infected by the Epstein-Barr virus. We have previously shown that a fragment of 265 amino acids was essential for the proper subcellular localization of the Epstein-Barr virus nuclear antigen 3A. As described in this paper, we have used deletion analysis to identify a decapeptide, RDRRRNPASR, which is essential for nuclear localization of this protein. Furthermore, this decapeptide is a functional nuclear localization signal as demonstrated by its ability to target expression of β-galactosidase in the nuclei of transfected cells.

Epstein-Barr virus (EBV) is a human herpesvirus associated with nasopharyngeal carcinoma, Burkitt's lymphoma, and other lymphoproliferative disorders. Infection of human primary B lymphocytes by EBV, in vitro, confers upon the cells the ability to grow permanently in culture (12). These growth-transformed B cells express nine viral proteins, including six nuclear antigens (LP and EBNAs 1, 2, 3A, 3B, and 3C) and three membrane proteins (LMP, TP1, and TP2) (reviewed in references 17 and 21). These proteins are likely to be required for the immortalization of B cells, the maintenance and regulation of expression from the viral genome, or the control of latency.

EBNA1 is required for autonomous maintenance of the circularized EBV genome (20) and activates a transcriptional enhancer located in the oriP region (24). EBNA2 is essential for EBV-induced B-lymphocyte transformation (4, 10) and transactivates viral and cellular gene expression (5, 18, 31–33). LMP also contributes to the induction of cell activation markers, alters the growth properties of rodent fibroblast cell lines, and upregulates the expression of the bcl2 proto-oncogene which is able to protect the cells from apoptosis (11, 30, 32). The genes encoding the EBNA3A, EBNA3B, and EBNA3C proteins exhibit homologies in their sequences, structures, and sizes. They are tandemly located in the *Bam*HI E region of the EBV genome. The EBNA3 proteins are high-molecular-weight, high-proline-content polypeptides which are found in the nucleus. Their C-terminal regions contain different repeating polypeptide domains (13, 14, 16, 22, 23, 26, 28). Since EBNA3C induces CD21 expression, the EBNA3 proteins may be transactivators of B-lymphocyte gene expression (32).

To investigate EBNA3A functional domains, we constructed a recombinant plasmid able to direct the expression of the full-length EBNA3A antigen. A large deletion in the N-terminal end of this protein modified its subcellular localization (14). We present here the identification of the amino acid sequence required for nuclear targeting of the EBNA3A protein. Nuclear localization signals (NLSs) have already been found in several nuclear proteins; however, no universal sequence for nuclear localization has been described (reviewed in references 7 and 29).

Recombinant plasmids containing deletions in the EBNA3A open reading frame. The cDNA sequence encoding the EBNA3A protein downstream from the adenovirus 2 major late promoter (19) was inserted in the *Eco*RI site of the pUC13 vector, generating pUCE3 recombinant DNA. The Δ795 mutant was constructed by cutting pUCE3 with *Bgl*II and ligating the ends together. Internal in-frame deletions between these *Bgl*II sites were performed as follows. The *Xho*I-*Stu*I restriction fragment of pUCE3 was partially cleaved by *Sau*3A (Fig. 1). The resulting *Xho*I-*Sau*3A fragments were then inserted between the *Xho*I and *Bgl*II (second site) sites of pUCE3. In this way, three mutants (Δ654, Δ624, and Δ417) were generated that corresponded to the deletion of 218, 208, and 139 amino acids, respectively, from the EBNA3A open reading frame. The Δ216 mutant was constructed by cleavage with *Bgl*II and *Bst*EII restriction enzymes, filling in the ends, and ligation.

Transient expression of EBNA3A-deleted proteins. The deletion mutants described above were transfected into cells from cell line 293 by the standard CaPO4 precipitation method (8), and the protein extracts were assayed in a Western blot (immunoblot) experiment (2). The results show that deletions within the coding sequence led to the expression of shorter polypeptides that were still detected by an EBV-immune human serum which was previously shown to recognize EBNA3A (Fig. 2).

To study specifically the requirements for nuclear localization, we first investigated whether a transient expression-immunofluorescence system using the deleted variants of EBNA3A would reproduce the nuclear localization seen after transfection of pUCE3 into human 293 and HeLa cells. The cells were assayed for EBNA3A expression by indirect immunofluorescence 48 h after transfection. The anti-EBNA serum described above was used for these experiments (14). No more than 10% of cells expressed EBNA3A, reflecting the level of transfection. Mock-transfected cells showed a very low level of background fluorescence (data not shown). The deleted EBNA3A proteins were found to be nuclear when constructs Δ417 and Δ624 (Fig. 3A) were used in transfection experiments. Thus, we concluded that amino

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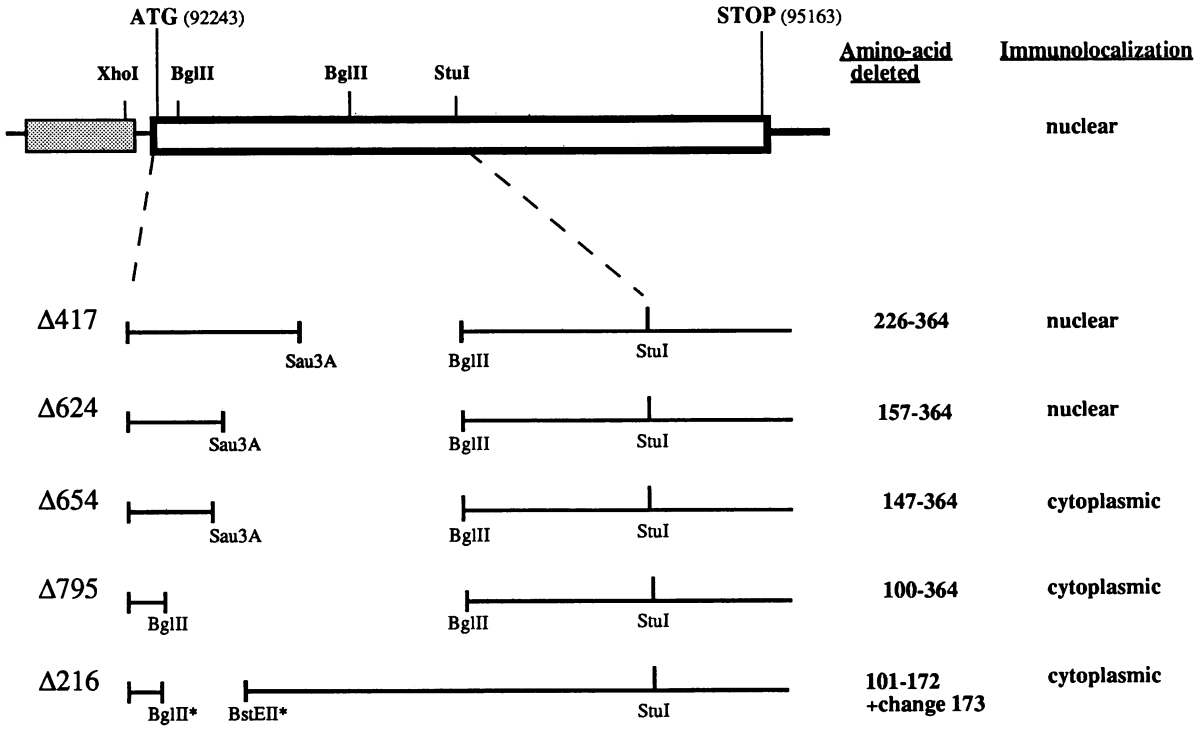

FIG. 1. Schematic representation of deletions in the EBNA3A open reading frame and subcellular localization of the corresponding protein. The top line represents the entire EBNA3A open reading frame (open box) taken from a cDNA placed downstream of the adenovirus 2 major late promoter (shaded box) (12). The coordinates in the EBV genome of the initiation and stop codons are indicated, as well as the positions of the restriction sites used. Below are EBNA3A deletions: the solid lines represent the sequences present on the recombinant plasmid. The numbers of the first and last amino acids lacking in the deleted protein are indicated, as well as the subcellular localization of the corresponding protein. In the Δ216 mutant amino acid 173 is changed from G to R.

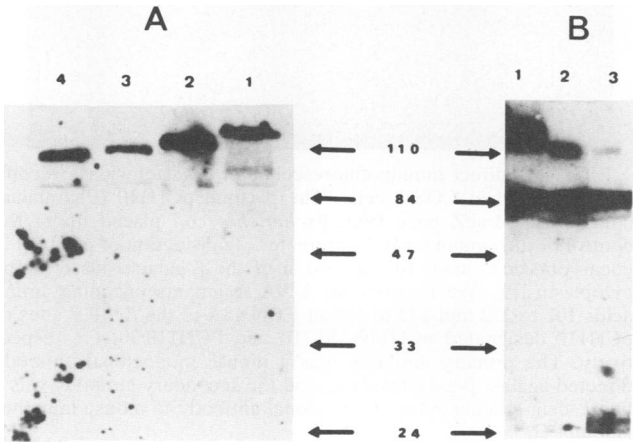

FIG. 2. Immunological detection of EBNA3A in cells from cell line 293 transfected with pUCE3 and various constructs by using an anti-EBNA-human serum (dilution, 1/100). The antigen-antibody complexes were visualized after incubation with 1 μCi of ^{125}I-labeled protein A. (A) Lanes: 1, pUCE3; 2, Δ417; 3, Δ624; 4, Δ795. (B) Lanes: 1, pUCE3; 2, Δ624; 3, Δ654. Molecular mass estimates in kilodaltons are shown in the center of the figure and were determined with prestained standard proteins.

acids 157 to 364 were probably not essential for the nuclear localization of EBNA3A. In contrast, the Δ654 and the Δ795 expressed proteins were excluded from the nucleus (Fig. 3B and C, respectively); immunostaining was observed throughout the cytoplasm. The Δ624 plasmid harbors a fragment of the EBNA3A open reading frame which is 30 bp longer than the one included in the Δ654 recombinant DNA. All or part of the amino acid sequence encoded by these 30 bp would thus seem to be essential for nuclear localization. Another possibility is that this deletion disrupted the protein folding and the nuclear localization signal could thus be inefficient. We constructed another recombinant plasmid, the Δ216 mutant (Fig. 1), whose deleted sequence included also the fragment coding for the 10-amino-acid sequence. It was transferred into HeLa cells, and the intracellular localization of the protein was determined. The fact that the EBNA3A encoded by this mutant is not found in the nucleus either (data not shown) is further evidence of the importance of the decapeptide sequence in nuclear localization.

In summary, amino acids 147 to 172 were deleted in the three truncated EBNA3A proteins which were found in the cytoplasm and thus all or some of these amino acids are implicated in the nuclear targeting of EBNA3A. The comparison of the cellular localization of Δ624 and Δ654 proteins indicates that the domain(s) responsible for EBNA3A nuclear localization is composed of at least one essential sequence which is contained in the 10 amino acids: RDRRRNPASR.

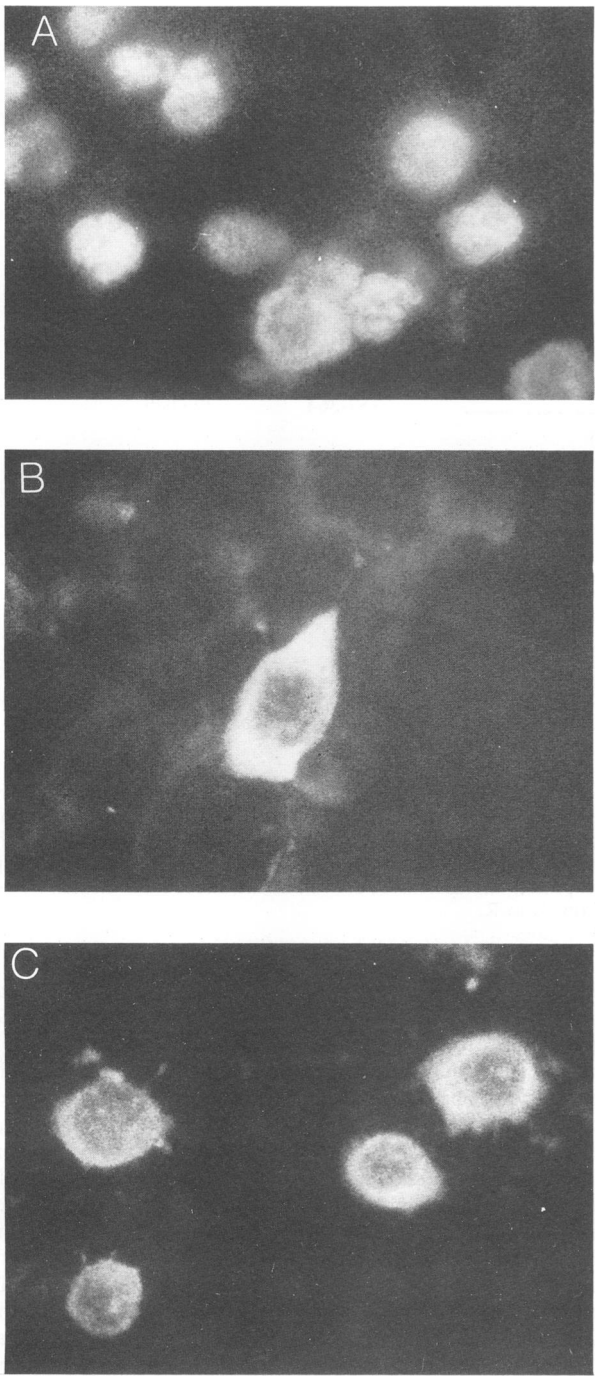

FIG. 3. Indirect immunofluorescence of HeLa cells transfected with EBNA 3A mutants Δ624 (A), Δ654 (B), and Δ795 (C). Primary antibody was polyclonal EBV-positive human serum (dilution, 1/10) which detects EBNA3A protein (control); the secondary antibody was fluorescein-conjugated goat anti-human immunoglobulin G.

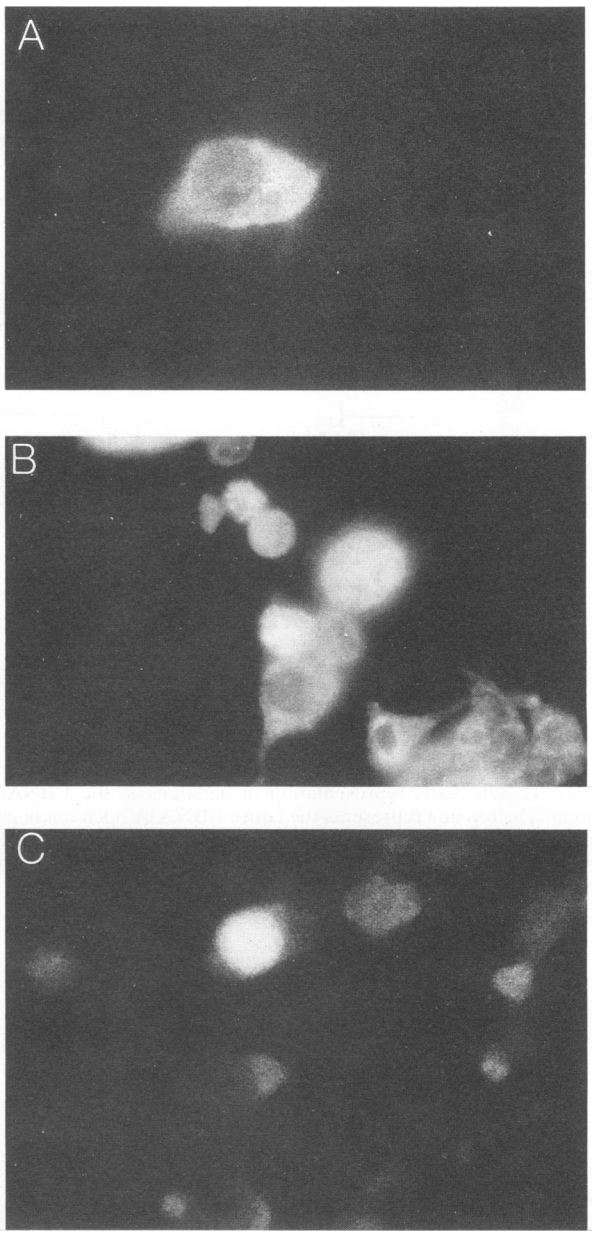

FIG. 4. Indirect immunofluorescence of β-galactosidase recombinant protein in COS-1 cells. The plasmid pCH110 (Pharmacia) contains the LacZ gene from *Escherichia coli* placed under the control of the simian virus 40 promoter. Transfection of pCH110 in eucaryotic cells leads to expression of the β-galactosidase in the cytoplasm (A). We inserted the DNA fragments encoding amino acids 101 to 172 and 147 to 157 of EBNA3A in the *Eco*RV sites of pCH110 designated pCH110/220 (B) and PCH110/30 (C), respectively. The primary antibody was a mouse monoclonal antibody directed against β-galactosidase, and the secondary antibody was a fluorescein-conjugated goat polyclonal antibody to mouse immunoglobulin G.

Insertion of EBNA3A sequences into a heterologous protein. β-Galactosidase was used as a recipient of the putative nuclear localization signal of EBNA3A. To determine whether the sequence (amino acids 101 to 172) absent in the Δ216 mutant is sufficient to induce nuclear localization, it was fused in frame to the coding sequence of the β-galactosidase gene (designated pCH110/216). This insertion not only allows the expression of β-galactosidase, as shown by immunofluorescence staining with a mouse monoclonal antibody to β-galactosidase (Zymed Laboratories), but also leads to a nuclear localization of this protein (Fig. 4B). This

experiment shows that the 216-bp sequence of EBNA3A contains information necessary for the nuclear localization of a heterologous protein.

To determine whether the decapeptide sequence which is necessary for the proper EBNA3A subcellular localization can also direct nuclear targeting of β-galactosidase, we inserted an oligonucleotide encoding these amino acids (RDRRRNPASR) in the LacZ gene (designated pCH110/30). This plasmid encodes a nuclear β-galactosidase as shown by immunofluorescence staining (Fig. 4C). Thus, we have demonstrated that these 10 amino acids not only are necessary for the nuclear localization of EBNA3A but also can induce the nuclear targeting of a heterologous protein.

Some proteins contain more than one functionally redundant NLS (27). For example, Dang and Lee (6) identified two regions of the human c-myc protein necessary for nuclear targeting of the protein. One region of the human c-myc protein functions as an NLS and the other induces only partial nuclear targeting. In contrast, the simian virus 40 large T antigen contains only one NLS (15). The progesterone receptor also has an NLS similar to the NLS of simian virus 40 large T antigen. This NLS is constitutive, and when it is deleted, the ligand-free receptor becomes cytoplasmic. However, this receptor has a second NLS which requires the binding of the hormone to be effective (9). In the polyomavirus large T antigen, the two nuclear targeting sequences are functional. However, only one (VSRKRPRP) is able to direct a reporter protein to the cell nucleus (25). Interestingly, the smallest known targeting signal, that of the adenovirus E1A protein, KRPR, is present in three EBNAs (EBNA1, EBNA2, and EBNA3C). Recently, Ambinder and al. (1) reported the identification of the EBNA1 NLS which, in fact, contains this peptide (LKRPRSPSS). The region of EBNA2 containing this amino acid sequence is also implicated in nuclear transport. However, it is likely that another sequence in this protein is able to function as an NLS (3).

Thus, even though many NLSs have been identified, no single consensus sequence has emerged (reviewed in reference 7). Examination of the different NLSs, however, reveals two common features: they are usually short (less than 12 amino acids), and they contain a high proportion of positively charged amino acids. In this report we have shown that the NLS of EBNA3A antigen (RDRRRNPASR) contains at most 10 amino acids, including five arginine residues (R). Although we identified a minimal EBNA3A sequence that directs a β-galactosidase fusion protein to the nucleus, we cannot totally exclude the possibility that another sequence will be implicated in the nuclear targeting of EBNA3A. If such a sequence exists, we have demonstrated that it is not sufficient for directing the EBNA3A protein to the nucleus. Our results (see also reference 14) strongly suggest that the EBNA3A protein contains a single NLS whose role must be considered in studies of functional domains of the EBNA3A antigen.

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REFERENCES

1. **Ambinder, R. F., M. Mullen, Y. Chang, G. S. Hayward, and S. D. Hayward.** 1991. Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. J. Virol. **65**:1466–1478.
2. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibodies and radioiodinated protein A. Anal. Biochem. **112**:195–203.
3. **Cohen, J., F. Wang, and E. Kieff.** 1991. Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation. J. Virol. **65**:2545–2554.
4. **Cohen, J., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86**:9558–9562.
5. **Cordier, M., A. Calender, M. Billaud, U. Zimber, G. Rousselet, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm, and G. Lenoir.** 1990. Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. J. Virol. **64**:1002–1013.
6. **Dang, C. V., and W. M. F. Lee.** 1988. Identification of the human *c-myc* protein nuclear translocation signal. Mol. Cell. Biol. **8**:4048–4054.
7. **Garcia-Bustos, J., J. Heitman, and M. N. Hall.** 1991. Nuclear protein localization. Biochem. Biophys. Acta **1071**:83–101.
8. **Graham, F. L., and A. J. van der Ed.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
9. **Guichon-Mantel, A., H. Loosfelt, P. Lescop, S. Sar, M. Atger, M. Perrot-Applanat, and E. Milgrom.** 1989. Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. Cell **57**:1147–1154.
10. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340**:393–397.
11. **Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. Rickinson.** 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65**:1107–1115.
12. **Henle, W., V. Diehl, G. Kohn, H. Zur Hausen, and G. Henle.** 1967. Herpes-type virus and chromosome marker in normal leucocytes after growth with irradiated Burkitt cells. Science **157**:1064–1065.
13. **Hennessy, K., F. Wang, E. Woodland-Bushman, and E. Kieff.** 1986. Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family. Proc. Natl. Acad. Sci. USA **83**:5693–5697.
14. **Joab, I., D. T. Rowe, M. Bodescot, J. C. Nicolas, P. J. Farrell, and M. Perricaudet.** 1987. Mapping of the gene coding for Epstein-Barr-virus-determined nuclear antigen EBNA3A and its transient overexpression in a human cell line by using an adenovirus expression vector. J. Virol. **61**:3340–3344.
15. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
16. **Kerdiles, B., D. Walls, H. Triki, M. Perricaudet, and I. Joab.** 1990. cDNA cloning and transient expression of Epstein-Barr virus-determined nuclear antigen EBNA3B in human cells and identification of novel transcripts from its coding region. J. Virol. **64**:1812–1816.
17. **Kieff, E., and D. Leibowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1990. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. M. Monath, and B. Roizman (ed.), Virology. Raven Press, Ltd., New York.
18. **Knutson, J. C.** 1990. Level of *c-fgr* RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization. J. Virol. **64**:2530–2536.
19. **Levrero, M., V. Barban, S. Manteca, A. Ballay, C. Balsamo, M. L. Avantaggiati, G. Natoli, H. Skellekens, P. Tiollais, and M. Perricaudet.** 1991. Defective and nondefective adenovirus vectors for expressing foreign genes in vitro and in vivo. Gene **101**:195–202.

20. **Middleton, T., and B. Sugden.** 1992. EBNA1 can bind the enhancer element to the initiator element of Epstein-Barr virus plasmid origin of DNA replication. J. Virol. **66:**489–495.
21. **Miller, G.** 1990. Epstein-Barr virus biology, pathogenesis, and medical aspects, p. 1921–1957. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. M. Monath, and B. Roizman (ed.), Virology. Raven Press, Ltd., New York.
22. **Petti, L., and E. Kieff.** 1988. A sixth Epstein-Barr virus nuclear protein (EBNA3B) is expressed in latently infected growth-transformed lymphocytes. J. Virol. **62:**2173–2178.
23. **Petti, L., J. Sample, F. Wang, and E. Kieff.** 1988. A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. J. Virol. **62:**1330–1338.
24. **Reisman, D., and B. Sugden.** 1986. *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6:**3838–3846.
25. **Richardson, W. D., B. L. Roberts, and A. E. Smith.** 1986. Nuclear location signals in polyoma virus large T. Cell **44:**77–85.
26. **Ricksten, A., B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Rymo.** 1988. BamH1 E region of the Epstein-Barr virus genome encodes three transformation associated nuclear proteins. Proc. Natl. Acad. Sci. USA **85:**995–999.
27. **Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64:**615–623.
28. **Shimizu, N., M. Yamaki, S. Sakuma, Y. Ono, and K. Takada.** 1988. Three Epstein-Barr virus (EBV)-determined nuclear antigens induced by the BamH1 E region of EBV DNA. Int. J. Cancer **41:**744–751.
29. **Silver, P. A.** 1991. How proteins enter the nucleus. Cell **64:**489–497.
30. **Wang, D., D. Leibowitz, and E. Kieff.** 1985. An EBV membrane protein expressed in immortalized lymphocytes transform established rodent cells. Cell **43:**831–840.
31. **Wang, F., C. Gregory, M. Rowe, A. Rickinson, D. Wang, M. Birkenbach, H. Kikutami, T. Kishimoto, and E. Kieff.** 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc. Natl. Acad. Sci. USA **84:**3452–3456.
32. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Leibowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
33. **Zimber-Strobl, U., K. Suentzenich, G. Laux, D. Eick, M. Cordier, A. Calender, M. Billaud, G. Lenoir, and G. Bornkamm.** 1991. Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene. J. Virol. **65:**415–423.