

Identification of Epstein–Barr virus sequences that encode a nuclear antigen expressed in latently infected lymphocytes

(polyoma virus expression vector/radioimmuno-electrophoresis)

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ABSTRACT The Epstein–Barr virus (EBV) *Bam*HI restriction endonuclease fragment K (B95-8 strain) was introduced into a polyoma virus expression vector and used to transfect murine NIH 3T3 cells. An EBV-associated nuclear antigen was detected in these cells in an indirect immunofluorescence test using anti-EBV nuclear antigen-positive human sera. These sera recognized a M_r 88,000 polypeptide in 3T3 cells transfected with the *Bam*HI fragment K-containing polyoma virus plasmid by radioimmuno-electrophoresis. A M_r 88,000 polypeptide also was detected in a B-cell line latently infected with the B95-8 strain of EBV. Plasmids containing insertion and deletion mutations in *Bam*HI fragment K directed the synthesis of truncated forms of the M_r 88,000 polypeptide in 3T3 cells. These data directly demonstrate that the polypeptide identified in EBV-infected lymphocyte lines by anti-EBV nuclear antigen-positive human sera is encoded by the viral genome.

Infection of human B lymphocytes with Epstein–Barr virus (EBV) results in the establishment of permanent cell lines (1). These immortalized cells contain most, if not all, of this herpesvirus genome as a multicopy episome (2). B-cell lines that are EBV genome-positive may also be established from the blood of individuals who have had a prior EBV infection (infectious mononucleosis or inapparent infection; ref. 3) and from tumor tissue in 90% of the cases of Burkitt's lymphoma in Africa (4). EBV remains in a latent state in most of these lymphoblastoid cell lines with 10% or less of the viral DNA expressed as cytoplasmic polyadenylated RNA (5); only rare cell lines express late viral functions and produce infectious virus. Very little is known about the proteins encoded by the EBV genome in latently infected lymphocytes, but potential functions include immortalization of B lymphocytes, control of EBV gene expression, and maintenance of the viral genome as an episome.

All EBV-infected cells contain a nuclear antigen (EBNA) that may be detected in an anti-complement immunofluorescence (ACIF) test using serum from individuals who have had a prior EBV infection (6). This antigen is not detected in EBV-negative lymphoblastoid cell lines using the same sera (6). DNA transfer experiments have demonstrated that the *Bam*HI restriction endonuclease fragment K of EBV DNA is able to induce the expression of a new nuclear antigen in mouse fibroblasts that may be detected with EBNA antibody-positive but not EBNA antibody-negative human sera (7). In addition, anti-EBNA sera identified polypeptides varying between M_r 65,000 and M_r 73,000 in EBV-positive lymphoblastoid cell lines by radioimmuno-electrophoresis (RIE); these species were not detected in EBV-negative lines (8). It has been suggested that the *Bam*HI fragment K

contains at least part of the coding sequences for the antigen detected by RIE and that variation in the size of this protein between different EBV-infected cell lines is due to variation in the size of a repeated DNA sequence (internal repeat 3, IR3) contained within that fragment (9). Recent experiments demonstrated that rabbit antisera to an IR3-*lacZ* gene fusion product recognized similar-sized polypeptides by RIE as did EBNA antibody-positive human sera (10). The experiments described here identify an EBV-encoded nuclear polypeptide, detected with EBNA antibody-positive human sera, with an apparent M_r of 88,000. The coding sequences for this antigen were mapped by deletion and insertion mutagenesis within the EBV (B95-8 strain) *Bam*HI restriction endonuclease fragment K.

MATERIALS AND METHODS

Cell Lines and Transfections. Lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Flow Laboratories). BJAB, Loucks, and Ramos are EBV-negative Burkitt lymphoma cell lines (11–13). Raji and Namalwa are Burkitt lymphoma cell lines that are latently infected with EBV (14, 15). IB4 is a latently infected B-cell line established by infection of human umbilical cord lymphocytes with EBV (16). B95-8 and Jijoye are EBV-producing lymphoblastoid cell lines (17, 18). NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Mouse 3T3 cells were transfected with plasmid DNA by a modification of a published procedure (19). These cells were seeded at 0.7×10^6 cells per 10-cm dish and incubated overnight at 37°C. The monolayers were washed twice with Tris-buffered saline (pH 7.4) (TS buffer; 25 mM Tris-HCl/137 mM NaCl/0.7 mM Na₂HPO₄/5 mM KCl) and incubated for 8 min at room temperature with 0.5 ml of TS buffer containing 2.0 μ g of plasmid DNA and DEAE-dextran (0.5 mg/ml; M_r 2×10^6 , Pharmacia). Cells were washed once with TS buffer and incubated in growth medium supplemented with 100 μ M chloroquine diphosphate (Sigma) for 2.5 hr at 37°C. Chloroquine diphosphate was removed by washing the cells twice with TS buffer, and the cells were then fed with growth medium and incubated at 37°C.

Plasmids and Construction of Mutations. All plasmids used in these experiments were propagated in *Escherichia coli* HB101 (rec A⁻). The vector pMLPyA2 was constructed by inserting a 4576-base-pair (bp) *Hind*III–*Bam*HI fragment of polyoma virus A2 strain (nucleotides 3918–4632; ref. 20) between the *Bam*HI and *Hind*III sites of pML2 (21), thus deleting a 346-bp *Bam*HI–*Hind*III fragment from pML2. The EBV (B95-8 strain) *Bam*HI fragment K was isolated from

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Abbreviations: EBV, Epstein–Barr virus; EBNA, Epstein–Barr virus nuclear antigen; ACIF, anti-complement immunofluorescence; RIE, radioimmuno-electrophoresis; kb, kilobase(s); IR, internal repeat; bp, base pair(s).

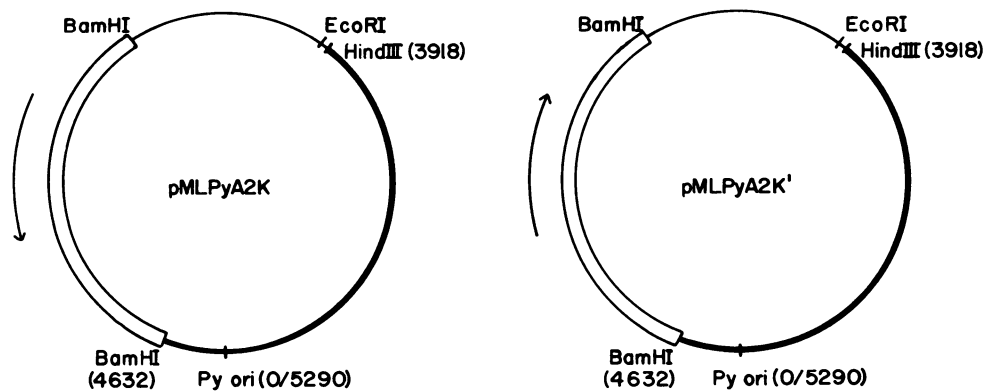


FIG. 1. Restriction endonuclease map of the plasmids containing EBV *Bam*HI fragment K. The numbers in parentheses indicate nucleotide positions in the polyoma virus A2 genome (20). This fragment of polyoma virus contains the entire early region and origin of replication. The cap sites and reiterated leader sequence for polyoma late mRNAs are also present in this plasmid between the polyoma virus origin of replication and the *Bam*HI site. EBV *Bam*HI fragment K (B95-8 strain) was inserted into the vector at the unique *Bam*HI site in both possible orientations. The direction of transcription of this fragment in an EBV growth-transformed lymphoblastoid cell line is indicated by the arrows (12).

pDF225 (22) and inserted in both possible orientations in the unique *Bam*HI site of pMLPyA2 to yield the plasmids pMLPyA2K and pMLPyA2K' (see Fig. 1). The insertion mutants H1 and H2 (Fig. 5) were constructed by partially digesting pMLPyA2K' with *Hind*III, repairing the ends with *E. coli* DNA polymerase I Klenow fragment (23), and ligating gel-purified full-length linear molecules with T4 DNA ligase. The insertion mutant X1 (see Fig. 5) was constructed in a similar fashion using the restriction endonuclease *Xba* I. The mutant S2 (see Fig. 5) was constructed by partially digesting pMLPyA2K' with *Sma* I and introducing 8-bp *Bgl* II linkers (Collaborative Research, Waltham, MA). Excess linkers were removed by digestion with *Bgl* II, and gel-purified full-length linear molecules were ligated with T4 DNA ligase. Several deletion mutants were also isolated by partially digesting pMLPyA2K' with *Sma* I and ligating less than full-length molecules (see Fig. 5; mutants S1, S3-1, S4, S6, and S7). Mutants S3-1 and S7 contain a *Bgl* II restriction site at the positions of the deletions.

RIE. Cell extracts were prepared by sonicating washed cell pellets in a small volume of 2% (wt/vol) NaDodSO₄/1% (vol/vol) 2-mercaptoethanol/80 mM Tris·HCl, pH 6.8. Samples were heated at 100°C for 5 min and centrifuged at 12,800 × g for 5 min. Supernatants were stored at -20°C. The protein concentration was determined by a Bio-Rad protein assay. Samples were subjected to NaDodSO₄/PAGE by a modification of the procedure of Laemmli (24). The separating gel was composed of 12.5% acrylamide and 0.1% methylenebisacrylamide. Buffers were as described (24). Conditions for the electrophoretic transfer of proteins to nitrocellulose and subsequent probing with antibody and radioiodinated *Staphylococcus aureus* protein A were as described by Burnette (25). ¹²⁵I-labeled protein A was purchased from New England Nuclear. Human sera were diluted 1:100.

Immunofluorescence. Twenty-four hours after transfection of NIH 3T3 cells with plasmid DNA, the cells were trypsinized and seeded onto glass slides in growth medium. Twenty-four hours later, the cells were fixed with methanol/acetone, 1:2 (vol/vol), for 15 min at -20°C and allowed to air dry. The cells were then incubated with a 1:10 dilution of EBNA antibody-positive or -negative human sera for 45 min at 37°C. Cells were washed well with phosphate-buffered saline and then incubated with a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-human IgG, IgA, and IgM (Cappel Laboratories, Cochranville, PA) for 45 min at 37°C. After extensive washing, the cells were visualized by fluorescence microscopy. ACIF staining of lymphocytes was performed as described (6).

RESULTS

Expression of a EBV-Associated Nuclear Antigen. A polyoma virus vector was constructed to permit the transient expression of EBV polypeptides in mouse cells. This vector, pMLPyA2, contains the early region, origin of replication, and the late transcriptional control region of polyoma virus and is able to replicate to high copy number when introduced into NIH 3T3 cells by DEAE-dextran transfection (unpub-

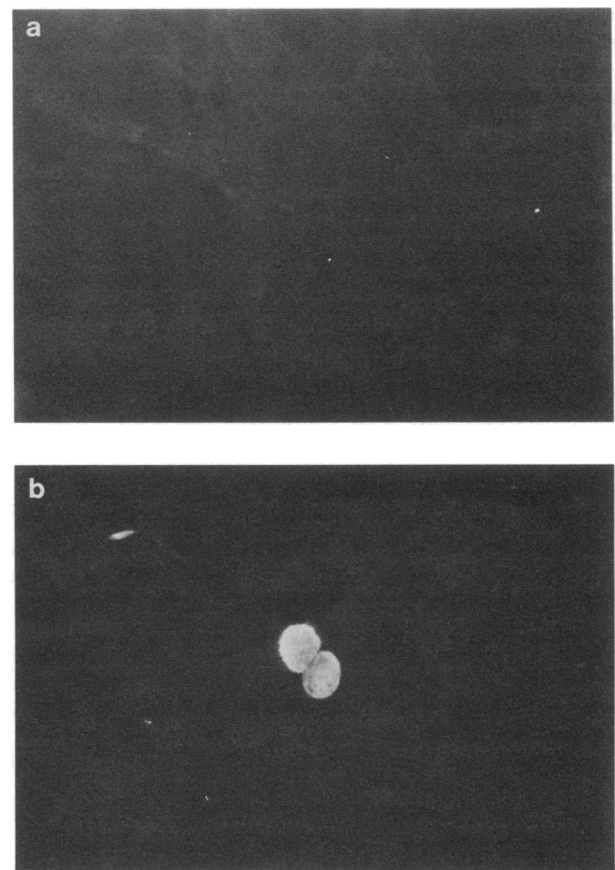


FIG. 2. Indirect immunofluorescence of NIH 3T3 cells transfected with pMLPyA2K'. 3T3 cells were transfected with pMLPyA2K' as described and examined by indirect immunofluorescence 48 hr after transfection. (a) EBNA-negative human serum (ACIF anti-EBNA titer < 1:2). (b) EBNA-positive human serum (ACIF anti-EBNA titer > 1:80).

lished results). Approximately 1% of the cells expressed polyoma tumor antigen(s) as determined by indirect immunofluorescence (unpublished observations). The 5.0-kilobase (kb) *Bam*HI fragment K from the B95-8 strain of EBV was inserted into the unique *Bam*HI site of this vector in both possible orientations (Fig. 1). This EBV DNA is transcribed into stable polyadenylylated RNA in latently infected lymphocytes (12, 15), and the direction of transcription of a 3.7-kb mRNA that is encoded at least in part by this restriction fragment in IB4 cells (12) is indicated by the arrow.

Plasmids containing EBV *Bam*HI fragment K and the parent vector were individually transfected into NIH 3T3 cells, and the expression of EBV-associated antigens was investigated by indirect immunofluorescence. EBNA-positive human sera identified a nuclear antigen in approximately 1% of the cells transfected with pMLPyA2K' (Fig. 2b) and pMLPyA2K (data not shown). Nuclear fluorescence was not observed when cells transfected with pMLPyA2 (lacking *Bam*HI fragment K) were stained with EBNA antibody-positive human sera (data not shown) nor when 3T3 cells transfected with pMLPyA2K' were stained with EBNA antibody-negative human sera (Fig. 2a).

A series of 38 human sera were characterized for their anti-EBNA titer on Raji cells (EBV-positive Burkitt lymphoma cell line) and anti-*Bam*HI fragment K antigen titer on pMLPyA2K'-transfected 3T3 cells (Fig. 3). A good correlation was observed between the anti-EBNA titer and the anti-*Bam*HI K antigen titer, indicating that the polypeptide(s) induced by EBV *Bam*HI fragment K constitute the major antigenic determinant(s) recognized by EBNA-positive human sera in latently infected lymphocytes.

EBV-Associated Polypeptides in Transfected 3T3 Cells. The EBV-associated proteins synthesized in 3T3 cells transfected with pMLPyA2K and pMLPyA2K' were compared to those present in latently infected and producer lymphoblastoid cells by RIE (Fig. 4). Total cellular protein was extracted from 3T3 cells 48 hr after DEAE-dextran transfection. A single polypeptide was detected in each of the EBV-positive Burkitt lymphoma cell lines Raji, Namalwa, and Jijoye, ranging in size from M_r 65,000 (Raji) to M_r 90,000 (Na-

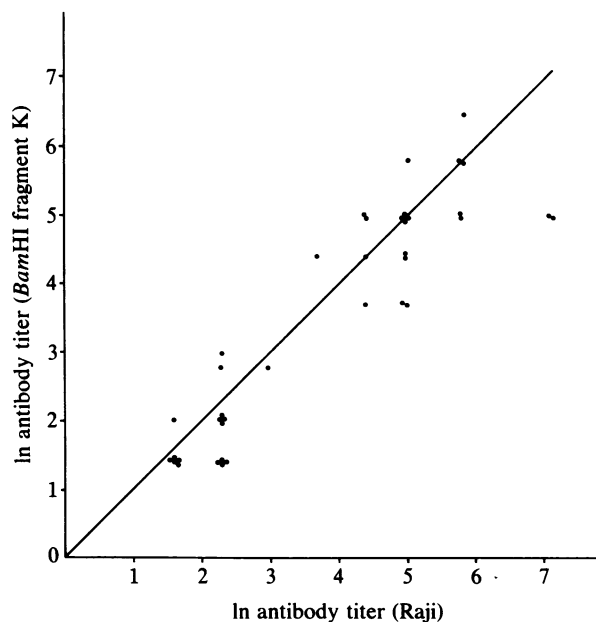


FIG. 3. Relationship between the anti-EBNA titer and anti-*Bam*HI K antigen titer of human sera. The anti-*Bam*HI K antigen antibody titer of 38 human sera was determined and is shown as a function of the anti-EBNA titer of the same sera as determined by ACIF staining of Raji cells.

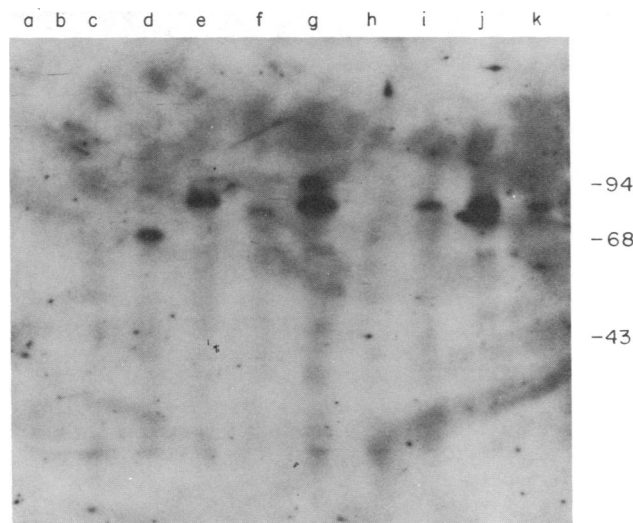


FIG. 4. EBV-associated proteins detected in lymphoblastoid cell lines and transfected NIH 3T3 cells. NIH 3T3 cells were transfected with the plasmids indicated by a modification of the DEAE-dextran-chloroquine diphosphate method (19). One hundred fifty micrograms of protein (all lanes except k) were electrophoresed on a Na-DodSO₄/12.5% polyacrylamide gel. The proteins were transferred electrophoretically to nitrocellulose and probed with a 1:100 dilution of EBNA-positive human serum (ACIF anti-EBNA titer > 1:80), and the bound antibodies were detected with radioiodinated *S. aureus* protein A. An autoradiogram of the nitrocellulose filter is shown. Lanes: a, BJAB; b, Loucks; c, Ramos; d, Raji; e, Namalwa; f, Jijoye; g, IB4; h, 3T3 cells transfected with pMLPyA2; i, 3T3 cells transfected with pMLPyA2K; j, 3T3 cells transfected with pMLPyA2K'; k, 15 μ g of the extract used in lane j. Antibody-reactive bands of lower intensity and lower molecular weights may be proteolytic breakdown products, cross-reactive antigens, or cellular antigens that react with extraneous antibodies in human sera. M_r s are shown $\times 10^{-3}$.

malwa). These proteins were not detected in the EBV-negative Burkitt tumor cell lines BJAB, Loucks, and Ramos (Fig. 4) and also were not detected when EBNA antibody-negative human sera were used (data not shown). These data are in good agreement with observations made by Strnad *et al.* (8). Two polypeptides of approximate M_r s 92,000 and 88,000 were detected in IB4 cells, a cell line established by the immortalization of human umbilical cord lymphocytes with the B95-8 strain of EBV (Fig. 4). A polypeptide of M_r 88,000 was also detected in the EBV-producer cell line B95-8 (data not shown) and in 3T3 cells transfected with the plasmids pMLPyA2K and pMLPyA2K' but not pMLPyA2 (Fig. 4). An additional protein of M_r 82,000 was also observed in cells transfected with pMLPyA2K'. The M_r 88,000 protein was present in approximately 10 times greater amounts in cells transfected with pMLPyA2K' compared with pMLPyA2K (Fig. 4, lanes i and k).

M_r 88,000 Protein Is EBV-Encoded. It was of interest that the M_r 88,000 protein induced in pMLPyA2K'-transfected 3T3 cells comigrated with the major protein detected in IB4 cells and B95-8 cells. The *Bam*HI fragment K used in construction of the plasmids was derived from the same strain of EBV as is present in these two lymphoid cell lines. These data, together with the correlation between the size of the IR3 component of *Bam*HI fragment K and the size of the polypeptide detected with EBNA antibody-positive human sera by RIE (9), suggested that the coding sequences for the M_r 88,000 protein are contained in *Bam*HI fragment K. This hypothesis was tested by introducing a series of insertion and deletion mutations into the EBV sequences present in the plasmid pMLPyA2K' (Fig. 5 Lower). The EBV-associated polypeptides induced in 3T3 cells transfected with these

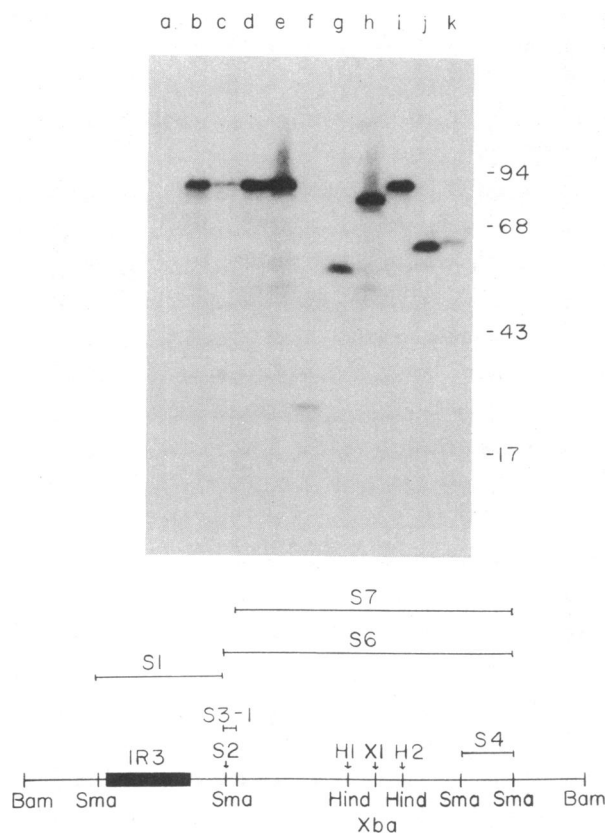


FIG. 5. EBV-associated polypeptides synthesized in 3T3 cells transfected with plasmids containing insertion and deletion mutations in EBV *Bam*HI fragment K. The locations of the mutations are indicated on the map at the bottom of the figure. The EBV-associated proteins synthesized in cells transfected with these mutant plasmids were examined by RIE as described. An autoradiogram of the filter is presented. Lanes: a, pMLPyA2; b, pMLPyA2K'; c, H1; d, H2; e, X1; f, S1; g, S2; h, S3-1; i, S4; j, S6; k, S7. M_r s are shown $\times 10^{-3}$.

mutants were examined by RIE (Fig. 5 *Upper*). Mutations to the right of the *Hind*III site at 2.9 kb (Fig. 5; mutants H1, H2, X1, and S4) had no effect upon the size of the EBV-associated polypeptides synthesized in transfected 3T3 cells. Deletion and insertion mutations to the left of this *Hind*III site, however, resulted in the synthesis of truncated forms of the M_r 88,000 protein (mutants S1, S2, S3-1, S6, and S7; Fig. 5).

DISCUSSION

These data directly (*i*) demonstrate that the major antigenic polypeptide identified in latently infected lymphocytes by EBNA antibody-positive human sera is encoded by the EBV genome and (*ii*) map the gene for this protein to the *Bam*HI restriction fragment K. A panel of human sera were characterized for their anti-EBNA antibody titer on a Burkitt lymphoma cell line and their anti-*Bam*HI K antigen antibody titer on 3T3 cells transfected with pMLPyA2K' (Fig. 3). This survey identified the *Bam*HI K antigen as containing the major antigenic determinant of the protein referred to as EBNA (6).

It was previously determined that the direction of transcription of *Bam*HI fragment K in a latently infected lymphocyte line is from left to right as drawn in Fig. 5 (12). The sizes of the polypeptides obtained by introducing insertion and deletion mutations in this gene are consistent with the transcription data and demonstrate that the majority, if not all, of the M_r 88,000 protein coding sequences lie to the left of the *Hind*III site at 2.9 kb (Fig. 5). The nucleotide sequence

of an 1153-bp *Hinf*I fragment that spans the internal repeat IR3 has been determined (26). One of the three possible reading frames in this fragment is completely open; in this translational frame, the IR3 sequence would be translated to yield a glycine and alanine repeat. Antibodies raised against this glycine and alanine copolymer were able to recognize polypeptides of various molecular weights in EBV-infected lymphocytes by RIE (10). Deletion of IR3 (mutant S1) resulted in the truncation of the M_r 88,000 protein, confirming that this repeated sequence encodes part of this EBV protein. In addition, these data indicate that translation of this EBV protein initiates between the leftward *Bam*HI site and the *Sma*I site at 0.6 kb (Fig. 5).

The level of expression of this EBV gene from the polyoma virus expression vector described in this report is high compared to the level observed in EBV-infected lymphocytes. This is evident by the ability of EBNA antibody-positive human sera to detect this antigen in transfected mouse fibroblasts by an indirect immunofluorescence assay. In order to detect EBNA in EBV-positive lymphocytes, it is necessary to use the more sensitive ACIF test (6). Furthermore, although the amount of the M_r 88,000 polypeptide detected in IB4 cells by RIE is similar to that observed in 3T3 cells transfected with pMLPyA2K' (Fig. 4), this represents the total amount of this protein synthesized by 100% of the cells in a latently infected lymphocyte line compared to that synthesized by only 1% of the 3T3 cells. The orientation of this gene in the polyoma vector had an effect upon the level of its expression. Transfection of pMLPyA2K' into 3T3 cells resulted in the synthesis of approximately 10 times more M_r 88,000 polypeptide than 3T3 cells transfected with pMLPyA2K (Fig. 4). Although the reason for this difference is not known, it is interesting that in pMLPyA2K', the DNA strand encoding this polypeptide is the same as that containing the polyoma late transcriptional control sequences (Fig. 1).

The relationship of the M_r 88,000 and M_r 82,000 polypeptides detected in 3T3 cells transfected with pMLPyA2K' as well as the relationship between the M_r 92,000 and M_r 88,000 proteins observed in IB4 cells (Fig. 4) is not yet clear. Development of monospecific antibodies to these proteins will permit a more detailed analysis.

Results similar to those presented here were recently reported by Fischer *et al.* (27) using an SV40-based vector.

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