Identification of Epstein-Barr virus sequences that encode a latent antigen expressed in latently infected lymphocytes

(polyoma virus expression vector/radioimmunoelectrophoresis)

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ABSTRACT The Epstein-Barr virus (EBV) BamHI 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 Mr 88,000 polypeptide in 3T3 cells transfected with the BamHI fragment K-containing polyoma virus plasmid by radioimmunoelectrophoresis. A Mr 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 BamHI fragment K directed the synthesis of truncated forms of the Mr 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 BamHI 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 Mr 65,000 and Mr 73,000 in EBV-positive lymphoblastoid cell lines by radioimmunoelectrophoresis (RIE); these species were not detected in EBV-negative lines (8). It has been suggested that the BamHI 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-laclZ 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 Mr of 88,000. The coding sequences for this antigen were mapped by deletion and insertion mutagenesis within the EBV (B95-8 strain) BamHI 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 × 10⁶ 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 NaHPO₄/5 mM KCl and incubated for 8 min at room temperature with 0.5 ml of TS buffer containing 2.0 μg of plasmid DNA and DEAE-dextran (0.5 mg/ml; M₂, 2 × 10⁶, Pharmacia). Cells were washed once with TS buffer and incubated in growth medium supplemented with 100 μM 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) HindIII–BamHI fragment of polyoma virus A2 strain (nucleotides 3918–4632; ref. 20) between the BamHI and HindIII sites of pML2 (21), thus deleting a 346-bp BamHI–HindIII fragment from pML2. The EBV (B95-8 strain) BamHI fragment K was isolated from

Abbreviations: EBV, Epstein-Barr virus; EBNA, Epstein-Barr virus nuclear antigen; ACIF, anti-complement immunofluorescence; RIE, radioimmunoelectrophoresis; kb, kilobase(s); IR, internal repeat; bp, base pair(s).
pDF225 (22) and inserted in both possible orientations in the unique BamHI site of pMLPyA2 to yield the plasmids pMLPyA2K and pMLPyA2K2 (see Fig. 1). The insertion mutants H1 and H2 (Fig. 5) were constructed by partially digesting pMLPyA2K2 with HindIII, 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 pMLPyA2K2 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 pMLPyA2K2 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.

**Fig. 1.** Restriction endonuclease map of the plasmids containing EBV BamHI 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 BamHI site. EBV BamHI fragment K (B95-8 strain) was inserted into the vector at the unique BamHI 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).

**RESULTS**

Expression of an EBV-Associated Nuclear Antigen. A polyoma virus vector was constructed to permit the transient expression of EBV poly peptides 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-
lished results). Approximately 1% of the cells expressed polyoma tumor antigen(s) as determined by indirect immunofluorescence (unpublished observations). The 5.0-kilobase (kb) BamHI fragment K from the B95-8 strain of EBV was inserted into the unique BamHI site of this vector in both possible orientations (Fig. 1). This EBV DNA is transcribed into stable polyadenylated 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 BamHI 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 BamHI 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-BamHI fragment K antigen titer on pMLPyA2K'-transfected 3T3 cells (Fig. 3). A good correlation was observed between the anti-EBNA titer and the anti-BamHI K antigen titer, indicating that the polypeptide(s) induced by EBV BamHI 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 Mr 65,000 (Raji) to Mr 90,000 (Namalwa). These proteins were not detected in the EBV-negative Burkitt tumor cell lines BJAB, Lourcs, 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 Mr 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 Mr 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 Mr 82,000 was also observed in cells transfected with pMLPyA2K'. The Mr 88,000 protein was present in approximately 10 times greater amounts in cells transfected with pMLPyA2K' compared with pMLPyA2K (Fig. 4, lanes f and k).

**M, 88,000 Protein Is EBV-Encoded.** It was of interest that the Mr 88,000 protein induced in pMLPyA2K'-transfected 3T3 cells comigrated with the major protein detected in IB4 cells and B95-8 cells. The BamHI 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 BamHI 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 Mr 88,000 protein are contained in BamHI 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...
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 BamHI 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-BamHI K antigen antibody titer on 3T3 cells transfected with pMLPyA2K' (Fig. 3). This survey identified the BamHI 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 BamHI 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, 88,000 protein coding sequences lie to the left of the HindIII site at 2.9 kb (Fig. 5). The nucleotide sequence of an 1153-bp HindIII 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, 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 BamHI 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, 88,000 polypeptide detected in 3T3 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, 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, 88,000 and M, 82,000 polypeptides detected in 3T3 cells transfected with pMLPyA2K' as well as the relationship between the M, 92,000 and M, 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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