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A Second Epstein-Barr Virus Early Antigen Gene in *Bam*HI Fragment M Encodes a 48- to 50-Kilodalton Nuclear Protein

MYUNG-SAM CHO,[1]† GREGORY MILMAN,[2] AND S. DIANE HAYWARD[1]*

Department of Pharmacology and Experimental Therapeutics, School of Medicine,[1] and Department of Biochemistry, School of Hygiene and Public Health,[2] The Johns Hopkins University, Baltimore, Maryland 21205

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We used antiserum raised against the bacterially synthesized product of one of the open reading frames in Epstein-Barr virus (EBV) *Bam*HI fragment M to demonstrate that this reading frame (BMRF1) codes for a nuclear protein of the diffuse early antigen (EA) class. In indirect immunofluorescence assays, the rabbit anti-BMRF1 antiserum gave nuclear staining in approximately 5% of Raji cells which had been treated with sodium butyrate, and positive fluorescence was observed in both acetone- and methanol-fixed cells. Uninduced Raji cultures contained less than 0.1% positive cells regardless of whether indirect immunofluorescence or anti-complement immunofluorescence was used. In immunoblot analyses, the rabbit serum identified a family of polypeptides of 46 to 55 kilodaltons (kDa) in total protein extracts from B95-8 cells or from butyrate-induced Raji cells. In both cell types, the dominant polypeptides were the 48- and 50-kDa species. This same family of polypeptides was identified when the immunoblots were reacted with the R3 monoclonal antibody, and we concluded that this antibody also recognized the product of the BMRF1 open reading frame. Fibroblast cell lines containing EBV *Bam*HI fragment M were established by cotransfection of baby hamster kidney cells with *Bam*HI-M and the gene for neomycin resistance. Aminoglycoside G418-resistant colonies which showed evidence for EBV antigen expression in immunofluorescence assays were selected, and clonal cell lines were established. After 3 to 4 months of passaging, constitutive synthesis of EA was no longer detectable in these cell lines either by immunofluorescence or by immunoblot analysis. However, in the one cell line examined, synthesis of the 48- to 50-kDa EA was induced by treatment of the culture with sodium butyrate. Thus, the regulation of expression of this EA in transfected fibroblasts is analogous to that seen in Raji lymphoblasts. We showed previously that *Bam*HI fragment M also contains the coding sequences for a 60-kDa nuclear EA, and hence *Bam*HI-M encodes two separate components of the diffuse EA complex.

Epstein-Barr virus (EBV) is a member of the human herpesvirus group and establishes latent infection in B lymphocytes. EBV-specified polypeptides are classified as nuclear antigens (EBNAs), early antigens (EAs), viral capsid antigens, or membrane antigens. By definition, EBNAs consist of those nuclear proteins which are synthesized during latent infection and are present in all cells carrying the EBV genome. EAs, can be demonstrated either in producer cell lines, such as P3HR-1 or B95-8, in which their synthesis is unaffected by inhibitors of virus DNA synthesis, or in latently infected cell lines, such as Raji, after superinfection or after induction with phorbol esters or sodium butyrate (21, 29). The EA class is divided into diffuse (D) and restricted (R) subsets (15, 16), which are distinguished by their cellular localization (predominantly nuclear [D] versus cytoplasmic [R]) and their differential susceptibility to methanol fixation. The viral capsid antigens include the late polypeptides of the viral lytic cycle, while the membrane antigen class contains both latency and lytic cycle viral membrane-associated polypeptides.

A large number of virus-specific polypeptides have been recognized, and many of these have been assigned to antigen classes on the basis of their reaction with characterized human sera. Additionally, in vitro translation of hybrid-selected mRNA has led to the association of polypeptides of specific sizes with templates in particular DNA restriction fragments (6, 19). However, relatively few of the in vivo-synthesized antigens have been mapped. Among those to be correlated with specific open reading frames are two EBNAs (7, 17, 27), a diffuse EA[EA(D)] (5a), a viral capsid antigen (5), and both a latency and a lytic membrane antigen (2, 11, 20). More general mapping, to the restriction fragment level, has been reported for a restricted EA component (12), for components of the EA(D) complex (5, 23, 28), and for another EBNA (14). Curiously, each of the last four reports describes mapping of antigens to the *Bam*HI-M region of the genome. Pearson et al. (23) used the R3 monoclonal antibody to immunoprecipitate the in vitro translation products of hybrid-selected mRNA and to map a 50- to 52-kilodalton (kDa) EA(D) polypeptide to *Bam*HI-M. Takaki et al. (28) used DNA transfection to associate a nuclear EA with *Hin*dIII fragment G (*Hin*dIII-G overlaps with *Bam*HI-M), whereas Grogan et al. (14) reported an EBNA synthesized in a *Bam*HI-M-transfected fibroblast cell line. We have described an EA(D) coded by P3HR-1-defective DNA which was mapped by DNA transfection experiments to the *Bam*HI-M–*Bam*HI-S region of the standard genome (5). Subsequently, we determined that the open reading frame coding for this antigen was BMLF1, and we identified a 60-kDa polypeptide product (5a).

In this report, we used the bacterially synthesized product of one of the other open reading frames in *Bam*HI-M (BMRF1) to generate a specific antiserum which enabled us to characterize the product of this reading frame. We determined that BMRF1 codes for a 48- to 50-kDa EA(D) polypeptide and hence that *Bam*HI fragment M codes for two separate components of the EA(D) complex. In addition, a *Bam*HI-M cotransfected cell line was established in which expression of the 48- to 50-kDa EA(D) polypeptide was

* Corresponding author.
† Present address: Cutter Laboratories, Berkeley, CA 94710.

induced by butyrate treatment. No data were obtained to support the existence of a *Bam*HI-M-encoded EBNA.

MATERIALS AND METHODS

Cell lines. Raji and B95-8 lymphoblastoid cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Baby hamster kidney (BHK) cells and the BEM cell lines derived by DNA transfection were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. To construct cell lines containing EA coding regions, 3 μg of the plasmid pSL74, which contains EBV (B95-8) *Bam*HI fragment M cloned in pBR322, was mixed with 300 ng of pSV2neo DNA (26), and calcium phosphate precipitates were formed as previously described (5, 13). BHK cells were transfected with the precipitated DNAs, and 48 h later the cells were seeded into 100-mm-diameter dishes and subjected to selection by growth in medium containing 300 μg of the aminoglycoside G418 per ml (5a). Individual colonies were isolated and transferred to 24-well dishes. Cells from each colony were tested for EBV antigen expression by immunofluorescence with human serum.

DNA hybridization. Cell DNA (10 μg) from Namalwa lymphocytes and from the *Bam*HI-M-containing BEM-4722 fibroblast cell line was cleaved with *Bam*HI and electrophoresed at 40 V for 15 h through an 0.8% agarose gel. The DNA fragments were transferred to a nitrocellulose filter as previously described (3). After an overnight preincubation at 42°C, the nitrocellulose blot was hybridized in 50% formamide at 42°C for 2 days with a nick-translated, ^{32}P-labeled probe of the P3HR-1-defective DNA fragment *Bam*HI-W'C' cloned in bacteriophage lambda L-47 (5).

Rabbit anti-BMRF1 antiserum. The BMRF1 open reading frame is completely contained within a 4,340-base-pair (bp) *Bcl*I-*Bam*HI subfragment of *Bam*HI-M. This subfragment (mapping between bases 79,895 and 84,233 on the EBV [B95-8] genome [1]) was inserted, in phase, into the *Bam*HI site of the *Escherichia coli* expression plasmid pHE6 (21a). The resulting construct synthesized the BMRF1 product as a fusion polypeptide with the normal initiator methionine residue of the BMRF1 protein linked to the amino-terminal 33 amino acids of the bacteriophage lambda N protein. The resulting 47-kDa fusion polypeptide was identified in extracts of induced bacteria by reaction with EBV-positive human serum and was purified by DEAE-cellulose and phosphocellulose column chromatography (J. Halprin, A. L. Scott, L. Jacobson, P. H. Levine, J. H. C. Ho, J. C. Niederman, S. D. Hayward, and G. Milman, submitted for publication). The peak fractions from the phosphocellulose column were used to generate anti-BMRF1-specific antiserum. New Zealand White rabbits were injected subcutaneously with 250 μg of the purified BMRF1 fusion polypeptide in Freund complete adjuvant, followed by a series of four biweekly booster injections of 250 μg of BMRF1 protein in incomplete adjuvant. Serum was collected 1 week after the final inoculation. For immunofluorescence assays, the rabbit serum was diluted 1:200, and for immunoblot analyses a 1:50 dilution was used.

Immunofluorescence. Assays were performed as previously described (4). Cells were stained by indirect immunofluorescence (16) with human serum, rabbit monospecific antiserum, or the R3 monoclonal antibody, and by anticomplement immunofluorescence (24) with human serum and the rabbit antiserum. To induce EA synthesis, cell cultures were grown in medium supplemented with 3 mM sodium butyrate for 3 days before harvesting. Lymphoblast cells were spotted onto glass slides and air dried, and the *Bam*HI-M-containing BEM fibroblast cells were grown directly on glass slides. Cells were washed in phosphate-buffered saline and fixed at −20°C for 4 min in acetone or methanol. Human serum (W.C.) was kindly provided by W. Henle (The Children's Hospital, Philadelphia, Pa.) and J. Strominger (Dana-Farber Cancer Institute, Boston, Mass.), and the R3 monoclonal antibody was provided by G. Pearson (Georgetown University, Washington, D.C.).

Immunoblotting. Immunoblot analyses were performed as previously described (5a). Cells that had been washed in phosphate-buffered saline were solubilized by sonication in a buffer containing 2.5% sodium dodecyl sulfate, 4% mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.01% bromophenol blue, and 52.5 mM Tris hydrochloride (pH 6.8), followed by heat treatment (5 min at 100°C). The protein extracted from 5×10^4 cells was electrophoresed at 200 V for 3 h through a 12.5% sodium dodecyl sulfate-polyacrylamide gel. The separated polypeptides were transferred to a nitrocellulose membrane by electrophoresis at 40 mA for 2 h in buffer containing 200 mM glycine, 20% methanol, and 25 mM Tris hydrochloride (pH 8.3). The nitrocellulose filter was preincubated in 0.05% Tween 20–150 mM NaCl–10 mM Tris hydrochloride (pH 7.4) for 60 min at room temperature, followed by incubation with the rabbit antiserum or the R3 monoclonal antibody for 90 min at room temperature. The filter blot was washed extensively in the same buffer and incubated with a 1:100 dilution of alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.). Bands reacting with the alkaline-phosphatase conjugate were visualized with indicator substrate (0.2% naphthol AS-B1, 0.1% fast blue BB salt, 0.1% fast violet B salt in 1 mM $MgCl_2$ and 100 mM Tris hydrochloride [pH 9.5]).

RESULTS

BMRF1 reading frame codes for an EA(D) antigen. Baer et al. (1) identified within the EBV (B95-8) DNA sequence open reading frames which they named according to their restriction fragment location and directionality. For example, the BMRF1 open reading frame is the first rightward open reading frame in *Bam*HI fragment M. A monospecific antiserum raised against the bacterially synthesized product of the BMRF1 reading frame was used to determine whether BMRF1 coded for an EBNA, an EA, or a viral capsid antigen. A *Bcl*I-*Bam*HI subfragment of *Bam*HI-M containing the complete BMRF1 reading frame was inserted, in phase, into the *Bam*HI site of the *E. coli* expression plasmid pHE6 (21a). The resulting construct synthesized the BMRF1 polypeptide as a run-on product, fused to the amino terminus of the bacteriophage lambda N protein. The BMRF1 reading frame theoretically codes for a polypeptide of approximately 45 kDa, and an appropriately sized 47-kDa fusion polypeptide was identified with EBV-positive human serum, purified by column chromatography from the bacterial cultures, and inoculated into rabbits to generate antisera which would be specific for the product of BMRF1 reading frame. When the anti-BMRF1 rabbit serum was incubated with Raji cells, either in an anti-complement immunofluorescence assay or in an indirect immunofluorescence assay, 0.05 to 0.1% of cells were stained (Fig. 1a). However, after butyrate treatment, approximately 5% of the Raji cells contained brilliantly stained nuclei by indirect immunofluorescence (Fig. 1b). The same staining pattern was observed whether the cells were fixed in acetone or in methanol and was also observed in 4 to 5% of cells from the virus-producing B95-8

line (data not shown). This immunofluorescence pattern is characteristic of the EA(D) class of antigens (15, 16).

Identification of the polypeptide products of the BMRF1 reading frame. Total protein extracts were made from the producer lymphoblastoid cell line B95-8, latently infected Raji cells, and Raji cells induced for EA expression by treatment with sodium butyrate. The solubilized polypeptides were separated electrophoretically, transferred to nitrocellulose, and incubated with rabbit antiserum. The rabbit serum reacted with a family of polypeptides (46, 48, 50, 51, 52, 53, and 55 kDa) in extracts from B95-8 cells and butyrate-treated Raji cells (Fig. 2A). The two major bands contained the 48- and 50-kDa polypeptides. None of these bands were present in extracts from untreated Raji cells. This multiband pattern was routinely obtained with freshly prepared protein extracts. However, in samples which had been stored for some weeks at 4°C or which had been subjected to multiple cycles of freezing and thawing, only the 48- to 50-kDa bands remained, and the other bands were no longer detectable.

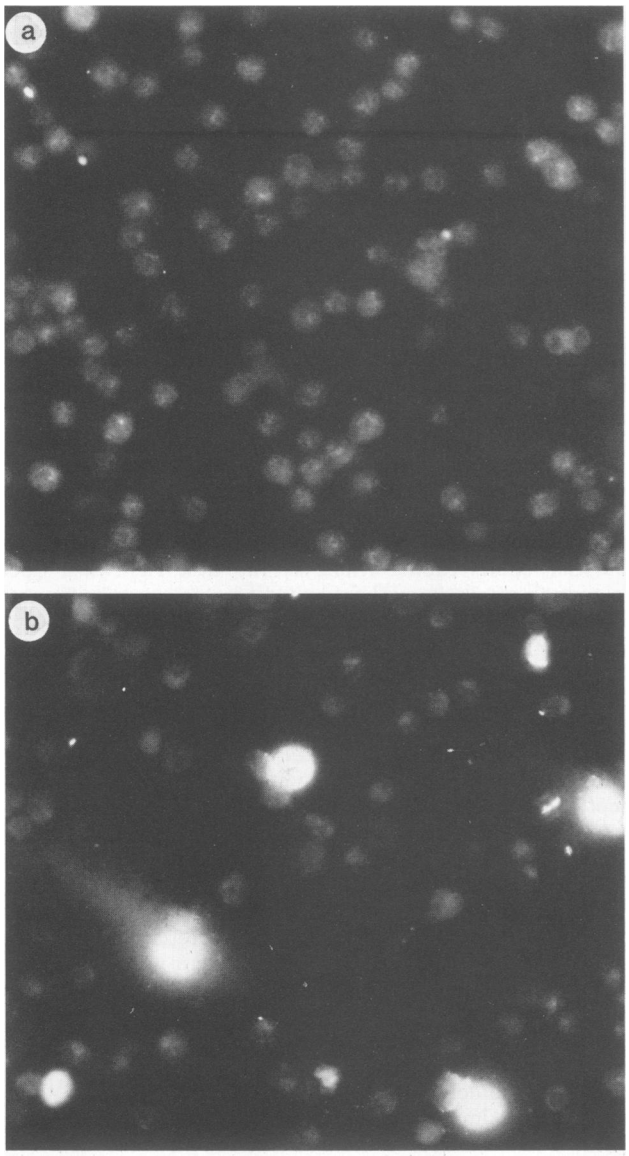

FIG. 1. Raji lymphoblastoid cells stained with rabbit anti-BMRF1 antiserum in an indirect immunofluorescence assay. (a) Untreated culture; (b) 72 h after induction with sodium butyrate.

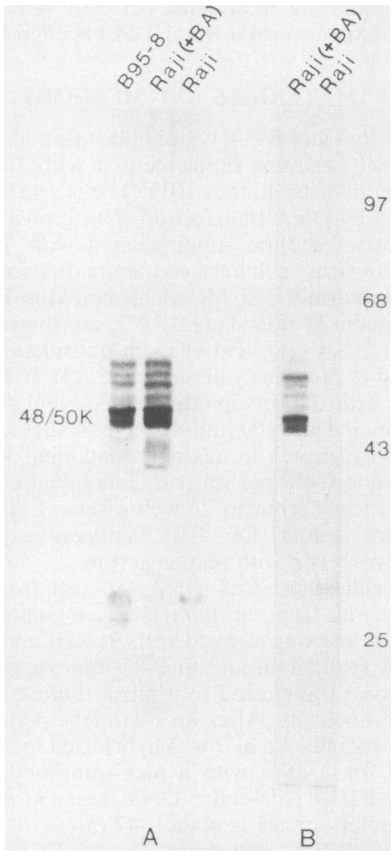

FIG. 2. Reactivity of rabbit anti-BMRF1 antiserum and R3 monoclonal antibody in immunoblot analyses. Solubilized proteins from B95-8, Raji lymphoblasts, or Raji cultures treated with sodium butyrate (BA) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was incubated with monospecific BMRF1 rabbit antiserum (A) or R3 monoclonal antibody (B), followed by reaction with the appropriate alkaline phosphatase-conjugated antibody. Molecular sizes are given in kilodaltons.

R3 monoclonal antibody recognizes the same family of polypeptides. Pearson et al. (23) described a monoclonal antibody, R3, which immunoprecipitated the in vitro translation product of RNA hybrid selected by *Bam*HI fragment M and which recognized 50- and 52-kDa polypeptides of the EA complex. When immunoblots of Raji cell extracts were probed with the R3 monoclonal antibody, a multiband pattern was obtained with the butyrate-treated cells (Fig. 2B) which was identical to that seen with induced Raji cells and our rabbit monospecific antiserum (Fig. 2A). Again, these polypeptides were absent from extracts of untreated Raji cells. A similar multiband pattern was also obtained when the R3 monoclonal antibody was reacted with extracts of B95-8 cells (data not shown).

Expression of the M-EA(D) polypeptides in a fibroblast cell line. To enable us to study M-EA(D) in isolation from the rest of the EBV polypeptides, *Bam*HI fragment M, cloned in pBR322, was cotransfected with pSV2neo DNA into BHK fibroblasts. Colonies which grew in G418-containing medium

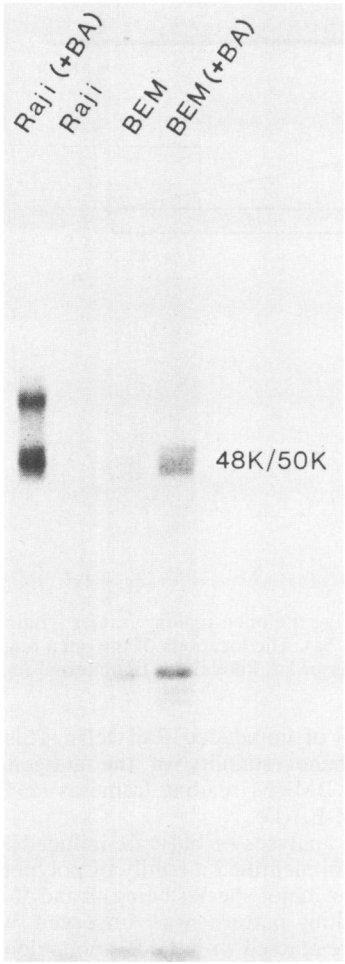

FIG. 3. The 48- to 50-kDa M-EA(D) polypeptide (48K/50K) is inducible with sodium butyrate in a permanent DNA-transfected BHK cell line. Raji lymphoblasts and the *Bam*HI-M-containing BEM-4722 fibroblast cell line were grown with or without the addition of 3 mM sodium butyrate (BA) to the culture medium. Filter blots of the separated polypeptides from these cells were incubated with the R3 monoclonal antibody, followed by reaction with alkaline phosphatase-conjugated anti-mouse immunoglobulin G and color substrate.

were tested for EA expression by indirect immunofluorescence with EA(D)-positive human serum. Immediately after selection, colonies were obtained which contained between 1 and 10% EA-positive cells. The staining was nuclear and was present in cells fixed with either acetone or methanol. Clonal cell lines were established from the positive colonies, but after a few months of passaging, EA expression in these cell lines became essentially undetectable by immunofluorescence. A small proportion of the cells showed spotty nuclear fluorescence, but the staining was weak and fell into the plus-minus category. This result was not altered by the use of anti-complement immunofluorescence as opposed to indirect immunofluorescence. We next looked for EA expression by immunoblot analysis. One of the cell lines, BEM-4722, was grown in the presence or absence of sodium butyrate, the culture was extracted, and a filter blot of the separated polypeptides was incubated with the R3 monoclonal antibody. The R3 antibody recognized bands of 48 and 50 kDa in extracts from the butyrate-treated BEM-4722 fibroblasts (Fig. 3). These bands were not present in extracts from the untreated cells.

BEM-4722 fibroblast cell line contains EBV DNA. The BEM cell lines were established by unlinked cotransfection of EBV *Bam*HI fragment M and pSV2neo, with the primary selection being for the retention and expression of the neomycin gene. To demonstrate that EBV DNA sequences were also retained, DNA from BEM-4722 cells and from Namalwa lymphocytes was restricted with *Bam*HI, electrophoresed through an agarose gel, and transferred to nitrocellulose paper. The Southern blot was incubated with a probe of the P3HR-1-defective DNA fragment *Bam*HI-W'C' cloned in a bacteriophage lambda vector. *Bam*HI-W'C' contains portions of standard *Bam*HI fragments S, M, and B1 and identified these three fragments in the *Bam*HI digest of Namalwa cell DNA (Fig. 4). (The less intense fourth band in the Namalwa digest is *Bam*HI fragment H which contains the *Not*I repeat cluster and therefore cross-hybridizes with the homologous *Pst*I repeat sequences present in the probe [8, 18].) In the *Bam*HI digest of BEM-4722 DNA, the probe detected two DNA species, both of which were of a larger size than input *Bam*HI fragment M (Fig. 4). This result is compatible with the integration of transfected EBV *Bam*HI fragment M into the cellular genome and with integration at more than one site. (The lambda DNA sequences present in the probe did not cross-react with pBR322 DNA under the hybridization conditions used, and thus the transfected

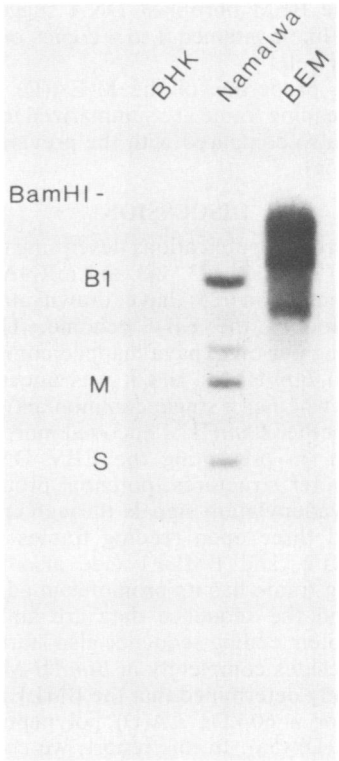

FIG. 4. Autoradiograph showing the presence of EBV *Bam*HI-M DNA in the BEM-4722 cell line. A Southern blot of *Bam*HI-cleaved DNA from the parent BHK cells, Namalwa lymphoblasts, and the *Bam*HI-M-cotransfected BEM-4722 fibroblast cell line was incubated with a ^{32}P-labeled probe of the P3HR-1-defective DNA fragment *Bam*HI-W'C' cloned in a lambda vector (*Bam*HI-W'C' contains portions of standard *Bam*HI fragments B1, M, and S).

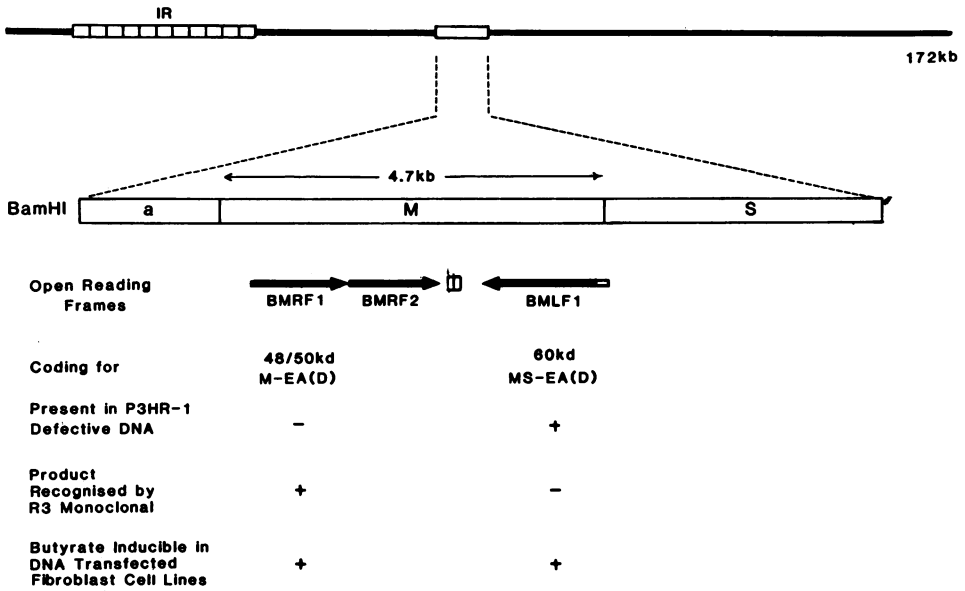

FIG. 5. Schematic representation of the *Bam*HI-M region of the EBV genome showing the open reading frames within *Bam*HI-M and comparing the properties of the EA(D)s coded by two of these, BMRF1 and BMLF1 (5, 5a). The locations of the open reading frames and of the 75-base-pair repeats (▥) were taken from Baer et al. (1). Abbreviations: kb, kilobase; kd, kilodalton; IR, internal repeat.

vector sequences would not have been detected.) A comparison of the intensity of hybridization of the probe to the Namalwa DNA, which has one copy of the EBV genome per cell, and to the BEM fibroblast DNA suggested that the BEM-4722 cell line contained 4 to 6 copies of EBV *Bam*HI fragment M per cell.

Some of the properties of the M-EA(D) coded by the BMRF1 open reading frame are summarized in Fig. 5, where this antigen is also compared with the previously described MS-EA(D) (5, 5a).

DISCUSSION

A number of recent publications describing the mapping of EBV antigens (5, 5a, 14, 23, 28) and mRNA transcription early in lytic infection (25) have drawn attention to the *Bam*HI-M region of the EBV genome. Three different groups, including our own, have mapped components of the EA complex to *Bam*HI-M, and it was uncertain from the data presented whether a single common antigen was being observed or whether *Bam*HI-M encoded more than one EA. Baer et al. (1), in presenting the EBV DNA sequence, identified promoter structures, potential protein coding regions, and polyadenylation signals throughout the genome. They described three open reading frames in *Bam*HI-M: BMRF1, BMRF2, and BMLF1 (see also Fig. 5). (The BMLF1 reading frame has its promoter in adjacent *Bam*HI fragment S, and the sequence data are ambiguous as to whether the protein coding sequence also starts in *Bam*HI-S or whether it resides completely in *Bam*HI-M.)

We had already determined that the BMLF1 open reading frame coded for a 60-kDa EA(D) polypeptide which we termed MS-EA(D) (5a). In this report, we concentrated on the BMRF1 open reading frame. By synthesizing the product of the BMRF1 open reading frame in bacteria by using an expression plasmid, we were able to obtain sufficient purified material to immunize rabbits and to generate anti-BMRF1-specific antiserum. In indirect immunofluorescence assays, the rabbit antiserum recognized a nuclear antigen in approximately 5% of butyrate-induced Raji cells and reacted with less than 0.1% of uninduced Raji cells. This result, along with the methanol stability of the antigen, defined the product of the BMRF1 reading frame as an EA, which we have termed M-EA(D).

Immunoblot analyses of butyrate-induced Raji cells with the rabbit serum identified a family of polypeptides of 46 to 55 kDa, with the major species being 48 and 50 kDa. Since an identical banding pattern was observed when the immunoblots were reacted with the R3 monoclonal antibody of Pearson et al. (23), it seems reasonable to conclude that the R3 monoclonal antibody also recognized the product of the BMRF1 open reading frame. On the other hand, the MS-EA(D) (5a) corresponds to the EA observed by Takaki et al. (28) in *Hin*dIII-G-transfected cells. The coding region for the MS-EA(D) is contained within EBV *Hin*dIII fragment G, whereas that for the M-EA(D) is not. Similarly, it is the MS-EA(D) which is encoded by P3HR-1-defective DNA (5). The coding sequences for the M-EA(D) are absent from the predominant defective species. Thus, each of the previous reports of *Bam*HI-M-encoded EAs (5, 5a, 23, 28) can be correlated with one of these two proteins. It remains unclear, however, where a *Bam*HI-M EBNA (14) would be encoded. The third open reading frame in *Bam*HI-M, BMRF2, is described by Baer et al. (1) as being transcribed into a late, lytic cycle mRNA, and we found no evidence for EBNA expression in *Bam*HI-M-cotransfected cell lines, even when anti-complement immunofluorescence was done with a human serum (W.C.) which has been previously characterized as having activity against the second EBNA (14).

Fibroblast cell lines containing *Bam*HI-M were established by cotransfection, and although initially EA synthesis could be detected by immunofluorescence in a small percentage of the cells, spontaneous EA expression was lost on passaging. However, even at late passages, the BEM-4722 cell line showed butyrate-inducible synthesis of the 48- to 50-kDa EA(D) polypeptides. Latently EBV-infected lymphoblastoid cell lines, such as Raji, do not express EAs unless they are superinfected or chemically induced. We

have now observed that both the 60-kDa MS-EA(D) (5a) and the 48- to 50-kDa M-EA(D)s were responsive to butyrate induction in DNA fragment-transfected fibroblast cell lines. It is presently unclear whether butyrate inducibility is an inherent property of all EA genes or whether the 48- to 50-kDa and 60-kDa proteins represent a special subclass of EAs.

Feighny et al. (10) described a subset of three early EBV polypeptides which they classed as immediate-early based on their time of synthesis in superinfection experiments. Two of these polypeptides were 48- and 63-kDa species, and they presumably corresponded to the 48- to 50-kDa M- and 60-kDa MS-EA(D)s. This assumption is strengthened by the observations of Sample et al. (25), who used superinfection in the presence of cycloheximide to identify an immediate-early EBV mRNA class. One of the two DNA fragments most actively transcribed under these conditions was *Bam*HI-M. The interest in the immediate-early proteins lies in the hypothesis that conversion from the latent state to the lytic cycle may be mediated via a switch protein or proteins whose synthesis would then irreversibly trigger the onset of the viral replicative cycle and the death of that particular cell. Such a protein would, of necessity, be synthesized ahead of other early polypeptides, and by this criterion the MS- and M-EA(D)s are both candidates for such a role.

Another shared property of these two antigens is that they each appear in immunoblots as a multiband family of polypeptides. This electrophoretic behavior may well be a reflection of phosphorylation modification since the known EBV phosphoproteins include both a 48- and a 60-kDa species (9, 22).

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