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Expression of a second Epstein–Barr virus-determined nuclear antigen in mouse cells after gene transfer with a cloned fragment of the viral genome

(simian virus 40 selection vector/anticomplement immunofluorescence/immunoblotting)

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ABSTRACT **Large Epstein–Barr virus (EBV) DNA restriction fragments corresponding to regions transcribed in transformed, proliferating cells were cloned in a cosmid derivative of the dominant-acting selection vector pSV2-*gpt*. Recombinant vectors carrying the *Eco*RI A fragment of EBV DNA were modified in the region corresponding to the deletion of the virion DNA in the non-transforming viral substrain P3HR-1, to create a series of recombinants lacking parts of this region. The recombinant vectors were introduced into 3T3 mouse fibroblasts under selective conditions, and resistant clones shown to contain EBV DNA sequences were analyzed for the expression of EBV-related antigens detectable by direct, indirect, and anticomplement immunofluorescence techniques. Cells that contained the *Bam*HI K fragment expressed the EBV-determined nuclear antigen (EBNA) as expected. Cells transfected with recombinant vectors containing the *Bam*HI W, Y, and H fragment part of the *Eco*RI A fragment also express a nuclear antigen detectable with certain anti-EBNA-positive human sera in anticomplement immunofluorescence tests. The *Bam*HI WYH-induced EBNA polypeptide is similar in size to the EBNA2 polypeptide in Raji cells, as shown by gel electrophoresis and immunoblotting. The antigen is not detected in cells transfected with *Eco*RI A-derived vectors in which the *Bam*HI H fragment has been deleted or in cells transformed with vectors carrying the *Bam*HI H fragment alone. Direct and indirect immunofluorescence did not reveal the presence of antigens associated with productive infection in any of the EBV DNA-transfected fibroblast clones.**

Immortalization of human B lymphocytes by Epstein–Barr virus (EBV) is regularly accompanied by the appearance of a nuclear antigen, EBNA, detectable by an anticomplement immunofluorescence (ACIF) test with the sera of EBV-seropositive donors (1). EBNA is present in all EBV-genome carrying cells, including Burkitt lymphoma and nasopharyngeal carcinoma biopsies (2, 3). Electrophoretic analysis and immunostaining showed that at least part of the ACIF-detected EBNA reactivity can be associated with a group of polypeptides that vary in size between 70 and 92 kDa in different EBV-transformed cells (4, 5). Recent studies with DNA-mediated gene transfer techniques (6, 7) and the use of a gene fusion between EBV DNA and the β-galactosidase gene in a bacterial expression vector (8) have established a relationship between EBNA and the internal repeat region contained within the *Bam*HI K fragment of EBV DNA. This was also shown directly by the EBNA-specific ACIF staining obtained with antibodies directed against a synthetic peptide deduced from the known nucleotide sequence of the *Bam*HI K internal repeat region (9).

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However, there is increasing evidence that more than one species of EBNA exist. Strnad *et al.* (4) and Hennessy and Kieff (8) have reported the existence of an EBV-associated 81-kDa intranuclear polypeptide in EBV-transformed cells. This polypeptide, designated EBNA2, was shown by Hennessy and Kieff (8) to be structurally different from the nuclear antigen induced by the *Bam*HI K fragment of EBV DNA. Further, Grogan *et al.* (10) recently demonstrated, by means of cotransformation of LTK^- cells, that the *Bam*HI M fragment of EBV DNA induces the expression of a nuclear antigen with antigenic properties different from those of the *Bam*HI K-encoded EBNA.

Studies on the expression of EBV-related RNA in transformed, nonproducer cell lines and Burkitt tumor biopsies have defined the regions of the EBV genome expressed in such cells (11–17). The major polyadenylylated transcripts are encoded by regions contained within the *Eco*RI A, the *Bam*HI K, and the *Eco*RI D_{het} fragments of B95-8 EBV DNA, respectively. Notably, the region corresponding to the deletion in virion DNA from the non-transforming P3HR-1 strain, which is in the *Eco*RI A fragment of B95-8 EBV DNA (Fig. 1), is always transcribed in transformed cells.

In this study, we address the question whether EBV genome regions transcribed in transformed, non-virus-producing cells, other than the *Bam*HI K fragment region, are also involved in the induction of EBV-associated nuclear antigens. EBV DNA fragments representing these regions were introduced into 3T3 mouse fibroblast by using transducing selectable vectors. Cells transformed stably with EBV DNA were selected and characterized with regard to the expression of EBV-related antigens as detected by immunofluorescence and immunoblotting techniques. We show here that cells transfected with recombinant vectors containing the *Bam*HI WYH-fragment part of the *Eco*RI A fragment of EBV DNA express a nuclear antigen that reacts with certain anti-EBNA-positive human sera in ACIF tests. The size of the *Bam*HI WYH-induced EBNA polypeptide is similar to that of the EBNA2 polypeptide extracted from Raji cells, as determined by gel electrophoresis and immunoblotting.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. NIH/3T3 mouse fibroblasts were obtained from R. Jaenisch (Hamburg, F.R.G.). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Cells from subconfluent monolayers were transfected in

Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-determined nuclear antigen; ACIF, anticomplement immunofluorescence.

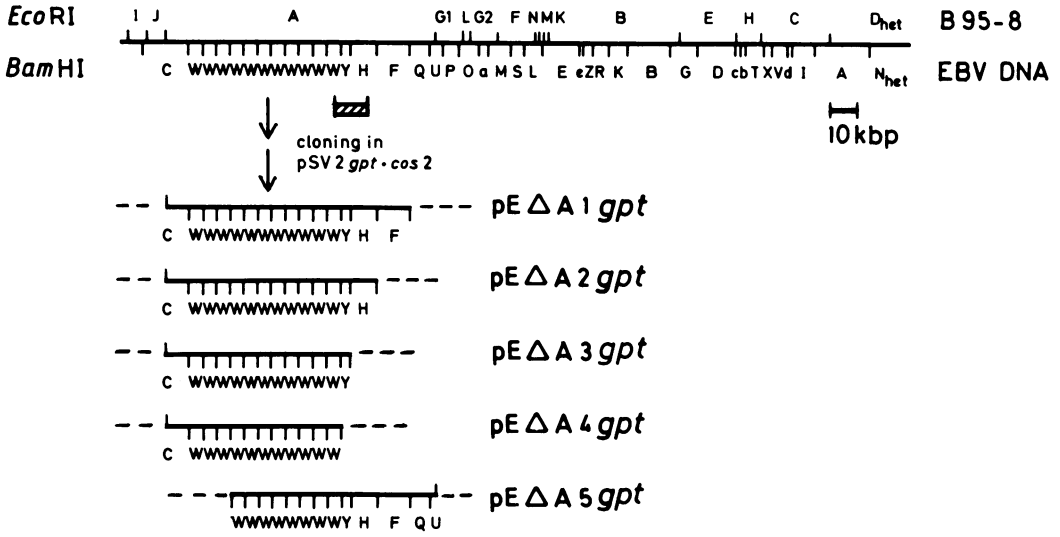

FIG. 1. *Eco*RI and *Bam*HI restriction fragment maps of linear B95-8 EBV DNA (18–20). Letters above and below the map designate *Eco*RI and *Bam*HI cleavage fragments, respectively. The region deleted in virion DNA from the nontransforming strain P3HR-1 is indicated by the hatched bar. The structures of the EBV DNA part of *Eco*RI fragment A-derived recombinant vectors used for transfection are illustrated schematically. The broken lines represent pSV2-*gpt·cos* sequences (see Fig. 2). kbp, Kilobase pairs.

suspension with the appropriate recombinant vector DNA (10^6 cells and 10 μg of DNA per ml) by the calcium phosphate-DNA precipitation technique of Graham and van der Eb (21) as modified by Shen *et al.* (22). After plating and polyethylene glycol "shocking" (22) the transfected cells were maintained in DMEM containing 10% fetal calf serum for 2–3 days. The cells then were seeded at a density of 2×10^5 cells per 9-cm tissue culture dish, and the medium was replaced by DMEM containing 10% fetal calf serum, xanthine (250 μg/ml), mycophenolic acid (8 μg/ml), hypoxanthine (15 μg/ml), aminopterin (2 μg/ml), and thymidine (10 μg/ml). The medium containing these supplements was changed the next day and thereafter every 3–4 days. Mycophenolic acid-resistant colonies were isolated with cloning cylinders after 14–21 days. The cloned cells were maintained in Iscove's modification of Dulbecco's medium (GIBCO) with the same supplements.

Recombinant Plasmids. A library of cloned restriction enzyme fragments of EBV DNA covering the whole genome was established earlier (20). The plasmids pSV2-*gpt* and pSV2-*gpt*/*Bgl* IIdel were obtained from P. Berg (Stanford University, CA) (23, 24). A cosmid derivative of pSV2-*gpt*, designated pSV2-*gpt·cos*2, was constructed as outlined in Fig. 2.

Immunoblotting, Preparation of Cellular Nucleic Acids, and Hybridization Procedures. $NaDodSO_4$/PAGE, electrophoretic transfer to nitrocellulose, and immunostaining were performed essentially as described (26). The procedures for the preparation of cellular nucleic acids and ^{32}P-labeled probes and the conditions for the hybridization to DNA immobilized on nitrocellulose membranes have been described (17).

RESULTS

Construction of Transfection Vectors Containing EBV DNA Sequences. Plasmids containing the *Eco*RI B and *Bam*HI H, K, and M fragments of B95-8 EBV DNA and the *Eco*RI D_{end} fragment of circular Raji EBV DNA, respectively, were digested with the appropriate restriction endonucleases, and the excised fragments were purified in agarose gels and recloned in the dominant-acting selection vectors pSV2-*gpt* or pSV2-*gpt·cos*2 by standard techniques. The vectors containing the *Eco*RI fragments B and D_{end} were designated pEB-*gpt* and pED$_{end}$-*gpt*(Raji), respectively, and those containing the *Bam*HI fragments H, K, and M were designated pBH-*gpt*, pBK-*gpt*, and pBM-*gpt*, respectively.

We wanted to construct a series of deletions in the *Eco*RI A fragment involving sequences corresponding to the region that is deleted in virion DNA from the non-transforming P3HR-1 strain. Thus, a plasmid carrying the *Eco*RI A fragment was partially digested with *Bam*HI under conditions that resulted in cleavage of one *Bam*HI site per molecule, on the average. The plasmid DNA then was digested to completion with *Eco*RI and the resulting *Bam*HI–*Eco*RI fragments were directionally cloned in the vector pSV2-*gpt·cos*2 between the *Eco*RI and the *Bam*HI sites. Recombinant clones that hybridized to the appropriate *Bam*HI fragments of EBV DNA and to simian virus 40 DNA were characterized further by small-scale isolation of plasmid DNA and restriction enzyme analysis. Five clones designated pEΔA1-*gpt*, pEΔA2-*gpt*, pEΔA3-*gpt*, pEΔA4-*gpt*, and pEΔA5-*gpt* were selected on the basis of their EBV DNA *Bam*HI-fragment composition and used for transfection experiments (Fig. 1).

Transformation of NIH/3T3 Mouse Fibroblasts with EBV DNA-Containing Selection Vectors. Rymo (27) has argued that human lymphoid cells would be the recipient of choice for transformation studies with EBV DNA. So far, it has not been possible, however, to transform lymphoid cells stably with our large EBV DNA-carrying vectors by using the calcium phosphate procedure. It has been shown that EBV genes might be expressed after transfer to murine cells (6, 28). NIH/3T3 mouse fibroblasts appeared, therefore, to be potentially useful recipient cells for investigation of the expression of EBV genes carried in the selection vectors.

The recombinant vectors listed in Table 1 were introduced into the mouse cells by the calcium phosphate technique, transformants were selected for growth in mycophenolic acid-containing medium, and clones were isolated with cloning cylinders. Small-scale preparations of cellular DNA from a large number of clones were analyzed with dot hybridization (30) for the presence of EBV DNA sequences. Five representative EBV DNA-containing clones for each recombinant selection vector were chosen for analysis of the expression of EBV-related cellular antigens, except for pEΔA3-*gpt*- and pEΔA5-*gpt*-transfected cells, where only two and three positive clones had been obtained, respectively. In most cases at least 50% of the mycophenolic acid-re-

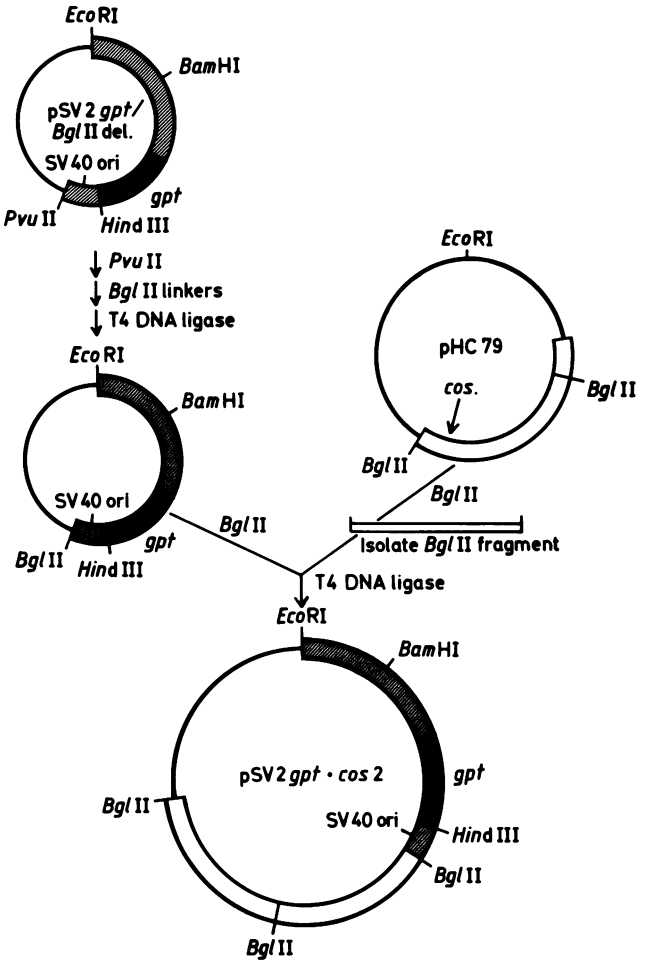

FIG. 2. Construction of a cosmid derivative of the selective vector pSV2-*gpt*. The pSV2-*gpt*/*Bgl* IIdel plasmid is a variant of pSV2-*gpt* in which the first 121 nucleotides of the *gpt* segment, including the *Bgl* II site, have been deleted (24). Plasmid pSV2-*gpt*/*Bgl* IIdel was linearized with *Pvu* II and ligated with DNA ligase to *Bgl* II linkers (C-A-G-A-T-C-T-G; New England Biolabs) present in a 200-fold excess. Following digestion with *Bgl* II, purification on a Aca22 (LKB) column, and recircularization with T4 DNA ligase, the plasmid molecules were used to transform *E. coli* HB101. *Bgl* II cleavage of small-scale plasmid preparations identified pSV2-*gpt*/*Bgl* II-del variants in which the *Pvu* II site had been exchanged for a *Bgl* II site. This plasmid is referred to as pSV2-*gpt*/*Bgl* II. Cosmid pHC79 (25) was cleaved with *Bgl* II and the 1.8-kilobase-pair fragment containing the *cos* sequences was isolated by agarose gel electrophoresis and purified. This fragment was ligated into the *Bgl* II site of pSV2-*gpt*/*Bgl* II. Recombinant plasmids were identified by colony hybridization and characterized by restriction endonuclease mapping. The plasmid chosen for further work contains a dimer of the *cos*-carrying *Bgl* II fragment. Single line represents pBR322 DNA sequences. The unshaded area between double lines is the λ fragment containing the *cos* sequences, the shaded area is the *E. coli* fragment containing the *gpt* gene, and hatched regions are simian virus 40 (SV40) DNA.

sistant clones contained EBV DNA sequences, although the amount of EBV DNA varied from 1 to about 5 genome equivalents per cell among different clones, as judged from the dot hybridizations.

Recombinant vector-containing 3T3 fibroblast clones were examined for the expression of EBV-related antigens with human sera containing anti-EBV antibodies. Table 1 summarizes the results. Cells transfected with the *Bam*HI K fragment expressed a nuclear antigen, as expected (6). We also found that cells stably transformed with the recombinant vectors pEΔA1-*gpt*, pEΔA2-*gpt*, and pEΔA5-*gpt* express a nuclear antigen detectable by certain anti-EBNA-positive human antisera in ACIF tests (Fig. 3). These vectors contain the major part of the *Eco*RI A fragment, as illustrated schematically in Fig. 1. The nuclear staining pattern of the transformed cells was diffuse and finely granular, similar to the EBNA pattern of conventionally stained EBV-transformed lymphoblastoid cell lines and mouse fibroblasts stably transfected with the *Bam*HI K fragment. Between 10 and 70% of the cells expressed the nuclear antigen in the different *Eco*RI A-transfected clones. The intensity of the nuclear fluorescence varied considerably between different cells within a certain clone. The nuclear antigen could not be detected when an antiserum against a chemically synthesized 14-residue copolymer of glycine and alanine, the structure of which was deduced from the internal repeat sequence of the *Bam*HI K fragment (9), was used in the ACIF test. This antiserum identifies the *Bam*HI K-encoded nuclear antigen and gave a positive staining reaction with our *Bam*HI K-transfected clones. Cells transfected with recombinant vectors in which EBV DNA sequences corresponding to the *Bam*HI H fragment (pEΔA3-*gpt*) or the *Bam*HI H and Y fragments (pEΔA4-*gpt*) had been deleted did not contain antigen in the nucleus, nor did cells transfected with vectors carrying the *Bam*HI H fragment alone.

Table 1. EBV-associated antigens in cells transformed with recombinant selection vectors containing EBV DNA fragments

Recombinant vector	Antigen-containing clones, number positive/number tested		
	EA	VCA	EBNA
pEΔA1-*gpt* (CWYHF)*	0/5	0/5	4/5
pEΔA2-*gpt* (CWYH)*	0/5	0/5	5/5
pEΔA3-*gpt* (CWY)*	0/2	0/2	0/2
pEΔA4-*gpt* (CW)*	0/5	0/5	0/5
pEΔA5-*gpt* (WYHFQU)*	0/3	0/3	1/3
pBH-*gpt*	NT	NT	0/5
pEB-*gpt*	0/5	0/5	0/5
pBK-*gpt*	0/5	0/5	4/5
pBM-*gpt*	NT	NT	0/5
pED_{end}-*gpt* (Raji)	0/5	0/5	0/5

Early antigen (EA), viral capsid antigen (VCA), and EBNA were detected on fixed cell smears as described (1, 29). At least two EBNA-antibody-positive and two EBV-antibody-negative sera were tested against each clone. Positive reactions were seen with EBNA-antibody-positive sera but not with EBV-antibody-negative sera. Each clone was tested between two and five times in independent repeat tests. NT, not tested.

*Letters within parentheses denote the *Bam*HI-fragment sequences of EBV DNA present in the recombinant. The number of *Bam*HI W repeats in the different vectors has not been determined.

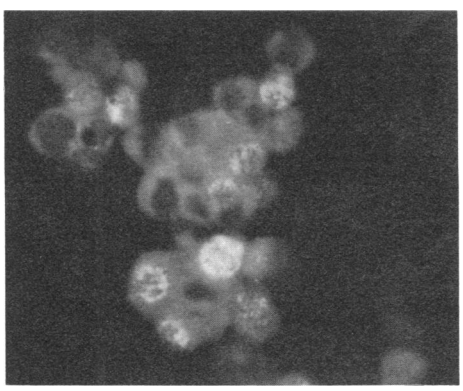

FIG. 3. Expression of nuclear antigens in mouse fibroblasts stably transfected with the recombinant vector pEΔA2-*gpt* (see Fig. 1). Cells were stained by using the ACIF technique and a human serum that contained antibodies against EBNA1 and EBNA2. (×400.)

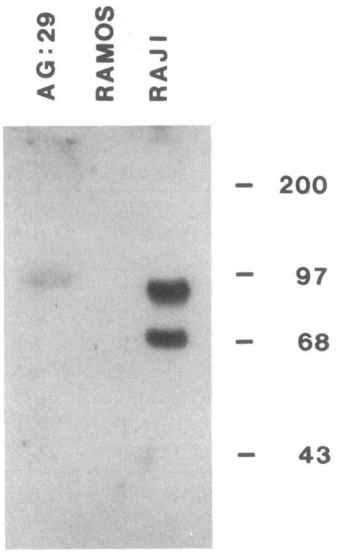

FIG. 4. NaDodSO$_4$/PAGE/immunoblotting analysis of the *Bam*HI WYH-induced EBNA polypeptide. Nuclei prepared from AG:29, Ramos, and Raji cells were suspended in sample buffer (26), sonicated, and centrifuged. The supernatants were analyzed as described (26), using an EBNA antibody-positive serum diluted 1:10 and ^{125}I-labeled protein A. AG:29 is a mouse fibroblast cell line stably transfected with pEΔA2-*gpt* DNA. Ramos is an EBV-negative, and Raji is an EBV-positive B-lymphoid cell line. Protein size standards were myosin heavy chain (200 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa).

Nuclear extracts prepared from pEΔA2-*gpt*-transfected cells expressing the nuclear antigen were analyzed by NaDodSO$_4$/PAGE and immunoblotting (Fig. 4). A 90-kDa polypeptide band, of similar mobility as the EBNA2 polypeptide in Raji cells, was identified using anti-EBNA-positive human sera. Only those sera (4 out of 9 tested) that reacted with the EBNA2 polypeptide in Raji cell extracts visualized the 90-kDa band from pEΔA2-*gpt*-transfected cells on the immunoblots. No bands were seen with EBV antibody-negative sera.

Neither direct immunofluorescence with an anti-EA (early antigen) and anti-VCA (viral capsid antigen) antibody-positive, fluorescein isothiocyanate-conjugated human IgG nor indirect immunofluorescence with an anti-VCA-positive anti-EA-negative human serum revealed the presence of antigens associated with productive infection in any of the EBV DNA-transfected fibroblast clones.

To demonstrate the presence of EBV DNA sequences in cells transfected with *Eco*RI A-carrying recombinant vectors that expressed the nuclear antigen, high molecular weight cellular DNA was prepared and cleaved with restriction endonucleases *Eco*RI and *Bam*HI. The resulting DNA fragments were separated by electrophoresis in agarose gels, transferred to nitrocellulose, and analyzed by hybridization (Fig. 5). The results show that fragments corresponding to the *Bam*HI W, Y, and H fragments of B95-8 EBV DNA were present in the cells.

DISCUSSION

We have used DNA-mediated gene transfer to assign EBV-specific antigens detected by direct, indirect, or anticomplement immunofluorescence techniques to defined segments of the EBV genome. In this investigation we used NIH/3T3 mouse fibroblasts as recipient cells for EBV DNA fragments representing regions of the EBV genome known to be transcribed in EBV-transformed, nonproducing lymphoid cells.

Our results confirm the findings of Summers *et al.* (6) showing that the *Bam*HI K fragment induces the expression of a nuclear antigen in mouse cells. It has now been firmly established that this antigen corresponds to the EBNA polypeptides that vary in size between 70 and 92 kDa. These polypeptides are in part encoded by the internal repeat sequences within the *Bam*HI K fragment and their size variation actually corresponds to differences in the length of the repeat region that characterize the viruses carried by different cell lines (5, 7–9).

The major new finding of our study concerns the EBV-specific nuclear fluorescence detected by the ACIF reaction in cells that have received the major part of the *Eco*RI A fragment EBV DNA. The nuclear fluorescence was not induced when the *Bam*HI H fragment was deleted from the transducing vector. The *Bam*HI C, F, Q, and U fragment regions of the *Eco*RI A fragment were not necessary for the expression of the nuclear antigen and the *Bam*HI W and Y region did not seem to be sufficient. Furthermore, the *Bam*HI H fragment could not induce the nuclear antigen by itself. This implies that the coding sequence for the antigen is within the *Bam*HI W, Y, and H fragment region.

The results are in line with the fact that a major transcript is generated from this area of the genome in EBV-transformed cells. A model for the generation of a cytoplasmic polyadenylylated 3-kilobase mRNA has been provided by van Santen *et al.* (31) from RNA hybridization data. The primary transcript is synthesized in a left-to-right direction on a standard physical map. It is spliced from a large primary transcript and consists of small segments from the *Bam*HI W repeat and larger, possibly continuous, exons encoded by the *Bam*HI Y and H fragments plus a polyadenylate tail.

The transcription model is supported by DNA sequence data identifying strong promoter sequences (-C-C-A-A-T- and -T-A-T-A-A-A-) and potential splice sites in *Bam*HI W;

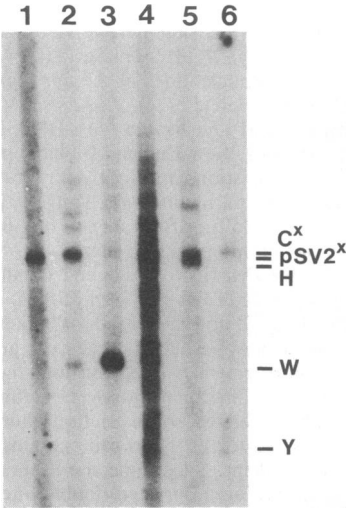

FIG. 5. Hybridization of EBV DNA to *Eco*RI/*Bam*HI cleavage fragments of cellular DNA from a mouse fibroblast clone transformed with the vector pEΔA2-*gpt*. High molecular weight cell DNA was doubly cleaved with *Eco*RI and *Bam*HI and the fragments were separated in a 0.5% agarose gel and transferred to a nitrocellulose membrane. Strips were cut from the membrane and hybridized with the following ^{32}P-labeled DNA probes: plasmid pSV2-*gpt* (lane 1); plasmid pBR322 containing EBV *Bam*HI C (lane 2); purified EBV *Bam*HI W fragment (lane 3); plasmid pBR322 containing EBV *Bam*HI Y (lane 4); plasmid pBR322 containing EBV *Bam*HI H (lane 5); and plasmid pBR322 containing EBV *Bam*HI F (lane 6). Vector pEΔA2-*gpt* DNA was cleaved in the same way and run in parallel on the same gel. The positions of these fragments are indicated at right: C^x is the *Eco*RI A fragment part of the *Bam*HI C fragment of EBV DNA, pSV2^x is the pSV2-*gpt* fragment; H, W, and Y indicate the corresponding *Bam*HI fragments of EBV DNA.

long open reading frames in *Bam*HI W, Y, and H; and an -A-A-T-A-A-A polyadenylylation signal in *Bam*HI H (32–34).

Strnad *et al.* (4) used immunostaining techniques to identify EBV-associated nuclear antigens that had been electrophoretically separated and transferred to nitrocellulose membranes. B95-8, P3HR-1, and Raji cells were analyzed with four different anti-EBNA antibody-containing human sera. All sera detected an antigen band in the different cell extracts corresponding to EBNA polypeptides with a molecular mass of 72, 70, and 65 kDa, respectively. Three of the sera also detected a second antigen in B95-8 cells, with a mobility corresponding to 81 kDa, and two of these sera identified a similar polypeptide in Raji cells. Notably, the 81-kDa polypeptide was not detected in P3HR-1 cells by any of the sera. The 81-kDa antigen was shown to be a DNA-binding protein with clearly distinctive chromatographic properties compared to the 65-kDa antigen in Raji cells. These results have been confirmed by Hennessy and Kieff (8), who identified an 82-kDa intranuclear antigen, designated EBNA2, in all EBV-transformed cell lines tested but not in EBV-negative cells. The EBNA2 polypeptide did not react with an anti-EBNA1-β-galactosidase rabbit antiserum specific for the glycine-alanine polymer domain of EBNA1, showing that EBNA1 and EBNA2 are unrelated proteins encoded by different genes.

Recently, Grogan *et al.* (10) suggested that the *Bam*HI M fragment of EBV DNA codes for an EBV-determined nuclear antigen. This region of the viral genome is not transcribed in EBV-transformed, non-virus-producing cells, however, nor have we been able to induce any nuclear antigen in 3T3 cells by transfection experiments involving the *Bam*HI M fragment (Table 1). Since antibodies to the protein encoded by the *Bam*HI M fragment region appear early during acute mononucleosis, similarly to antibodies to various members of the early antigen (EA) complex, and since at least five proteins within this complex are DNA-binding nuclear proteins, it appears likely that the M fragment codes for an early antigen associated with the viral cycle rather than a nuclear antigen expressed in a proliferating, transformed cell.

It is clear from the present study that the nuclear antigen induced by the *Eco*RI A fragment in mouse cells is different from the *Bam*HI K-encoded EBNA, since the A-induced antigen does not react with an antiserum raised against a chemically synthesized glycine-alanine peptide that has been shown to identify EBNA1 (9). The *Eco*RI A-induced antigen might be related to the other previously described EBNA subtype, EBNA2, or it might represent still another EBNA species. It should be noted that Hennessy and Kieff (8) suggested that EBNA2 is encoded by the *Eco*RI D_{het} region of EBV DNA, because transfection of this fragment into monkey cells induced an intranuclear antigen detectable with a serum containing a high titer of anti-EBNA2 antibodies. However, in the present investigation mouse cells stably transfected with a corresponding fragment (*Eco*RI D_{end}) of circular Raji EBV DNA did not express EBV-associated nuclear antigens detectable with the ACIF technique (Table 1). Furthermore, the absence of a detectable 81-kDa antigen in P3HR-1 cells (4), together with the present observation of a similarity between the *Eco*RI A-induced antigen and EBNA2 with regard to size and reactivity against different anti-EBNA antibody-containing sera, implies that the coding sequence for EBNA2 is within the *Bam*HI W, Y, and H fragment region of the EBV genome.

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