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Recombinant Epstein–Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates *in vitro*

(recombination/transfection/interferon)

SANKAR SWAMINATHAN, BLAKE TOMKINSON, AND ELLIOTT KIEFF

Departments of Medicine and Microbiology and Molecular Genetics, Harvard Medical School, 75 Francis Street, Boston, MA 02115

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ABSTRACT **Strains of Epstein–Barr virus (EBV) with deletions of the small RNA (EBER) genes were made by homologous recombination using the EBV P3HR-1 strain, which has undergone deletion of the essential transforming gene that encodes the EBV nuclear antigen, EBNA-2, and a DNA fragment that was wild type at the EBNA-2 locus but from which the EBER genes had been deleted. Even though the EBER and EBNA-2 genes are separated by 40 kilobases, selection for transforming P3HR-1 recombinants that required a restored EBNA-2 gene resulted in 20% cotransfer of the EBER deletion. EBER-deleted recombinants transformed primary B lymphocytes into lymphoblastoid cell lines (LCLs), which were indistinguishable from LCLs transformed by wild-type EBV in their proliferation, in latency-associated EBV gene expression, and in their permissiveness for EBV replication cycle gene expression. EBER-deleted virus from infected LCL clones could infect and growth-transform primary B lymphocytes. These procedures should be applicable to the construction of other EBV recombinants within 40 kilobases of the EBNA-2 gene. The EBER-deleted EBV recombinants should be useful in further evaluating the role of EBERs in EBV infection.**

Epstein–Barr virus (EBV) causes infectious mononucleosis and lymphoproliferative syndromes, and it is associated with Burkitt lymphoma and nasopharyngeal carcinoma (for reviews see refs. 1 and 2). EBV immortalizes and growth-transforms B lymphocytes, resulting in lymphoblastoid cell lines (LCLs) that proliferate indefinitely *in vitro*. In latently infected proliferating lymphocytes, EBV does not usually undergo lytic replication but does express a limited gene repertoire of six nuclear and two membrane proteins and two small RNAs: EBER 1 and EBER 2 (for review see refs. 1 and 3). These genes are of interest because they are likely to mediate EBV's persistence or its effects on lymphocyte growth.

The EBERs are of particular interest because they are by far the most abundant virus RNA in EBV-infected LCLs. The EBERs are not polyadenylylated and are transcribed by RNA polymerase III (4). The EBERs and the other EBV genes expressed in latently infected lymphoblastoid cells have no homology to genes of other herpes viruses, while most EBV replicative cycle genes have homologous counterparts in other herpes viruses. Curiously, herpes virus saimiri, which is unusual among herpes viruses in being oncogenic in heterologous primate species, also expresses five abundant, small, nonpolyadenylylated RNAs in transformed cells (5).

Little is known about the function of the EBERs. They are largely complexed to the nuclear La antigen (6). The EBERs are detectable early in infection of primary lymphocytes, and they persist in abundance throughout latent infection (7). Because the EBERs are predominantly located in the nucleus and are similar in primary and secondary structure to cell U6 small nuclear RNA, which is involved in RNA processing, the EBERs could also be involved in nuclear RNA processing (8, 9). The EBERs are also similar to adenovirus virus-associated (VA) RNAs in size and potential double-stranded structure (9). In fact, EBER RNAs are able to functionally substitute for VA RNAs in rescuing infected cells from virus-induced translational arrest (10).

To directly address the role of EBERs in EBV infection, we devised a genetic approach to produce recombinant EBV from which the EBER genes have been deleted. Recombinant, EBER-deleted, virus was tested for its ability to transform primary B lymphocytes *in vitro* or to express viral replicative cycle proteins in the small subpopulation of latently infected cells that characteristically become permissive for virus replication.

MATERIALS AND METHODS

Cell Lines, Virus, and Plasmid DNA. The P3HR-1 clone 16 (11) and B95-8 (12) cell lines were kindly provided by G. Miller (Yale University, New Haven, CT). IB4 is a human cord blood leukocyte line transformed by EBV from B95-8 cells (13). Louckes is an EBV-negative B-lymphoma cell line (14). The plasmid pUC EcoJ was constructed by inserting the EBV B95-8 DNA *Eco*RI J fragment into pUC 19 (New England Biolabs) from which the *Acc* I site had previously been removed. pUC EcoJ(−)EBER was constructed by an *Acc* I digestion and religation removing base pairs 6612–7263 of the EBV genome (15), which includes the EBER 1 and 2 genes. The SalA cosmid was cloned from EBV DNA purified from the B95-8 cell line as previously described (16). *Sal* I-digested DNA was cloned in the cosmid pDVcosA2 (17), packaged with Gigapack packaging extract (Stratagene), and used to infect *Escherichia coli* PLK-A (Stratagene). The cosmid SalA(−)EBER was constructed by digestion of the parent SalA cosmid with *Eco*RI, removal of the EcoJ fragment by gel purification, and ligation of the rest of SalA with the EBER-deleted *Eco*RI J fragment from pUC EcoJ(−) EBER. pSVNaeZ was constructed by blunt-end ligation of the *Nae* I–*Bam*HI subclone of the EBV DNA fragment, BamZ, in the *Bam*HI cloning site of the pSG5 expression vector (Stratagene).

Transfection, Induction, and Infection. EBV insert DNA was excised from 20 μg of circular cosmid DNA, mixed with 40 μg of pSVNaeZ, precipitated with ethanol, and electroporated into 5×10^6 P3HR-1 cells with a Bio-Rad Gene Pulser, with a pulse of 220 V, at 960 μF. After 48 hr, the transfected cells were lysed by three cycles of freezing and thawing. The crude virus was filtered through a 0.45-μm-pore

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; EBNA-LP, EBNA leader protein; EBER, EBV-encoded RNA; LCL, lymphoblastoid cell line; VA, virus-associated; TPA, 12-*O*-tetradecanoylphorbol 13-acetate (phorbol 12-tetradecanoate 13-acetate).

filter and pelleted at 8000 × *g* for 2 hr. The pellet was resuspended and incubated with 5×10^7 human peripheral blood mononuclear cells from an EBV-seronegative donor for 1 hr and plated in 96-well microtiter plates at 1.5×10^5 cells per well. The cells were fed weekly with RPMI 1640 medium with 10% fetal calf serum.

Virus Passage. Three million EBV-transformed lymphoblastoid cells were treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; phorbol 12-tetradecanoate 13-acetate) at 20 ng/ml for 3 days to induce viral replication, washed in Dulbecco's phosphate-buffered saline (PBS), irradiated with 10,000 rads (1 rad = 0.01 gray), and cocultivated with 2.5×10^7 peripheral blood mononuclear cells from an EBV-seronegative donor in 96-well plates at 1.5×10^5 mononuclear cells and 3×10^4 transformed cells per well, in the presence of cyclosporin A. Irradiated cells were also plated without mononuclear cells as a control to ensure that outgrowth of transformed cell lines was due to virus transfer and not to persistence of viable irradiated cells.

PCR Analysis. Oligonucleotides corresponding to nucleotides 6444 to 6463 and 7304 to 7324 (15) were synthesized and purified (18). Total cellular DNA from 1×10^6 transformed lymphocytes was prepared by lysis in 0.2× PBS, boiling, digestion with protease K, and reboiling. DNA from 1.25×10^5 cells was used in each 35-cycle amplification reaction with oligonucleotides at 1 μM, dNTPS at 200 μM, and Taq DNA polymerase (United States Biochemical) at 0.25 unit per reaction.

Miscellaneous Techniques. Indirect immunofluorescence analysis of live and fixed cells and Southern, Northern, and immunoblot analyses were performed as previously described (19).

RESULTS

Construction of EBER-Negative Mutants. The strategy for constructing recombinant EBV with EBERs deleted exploits the existence of an EBV strain, P3HR-1, which can be induced to replicate in infected lymphocytes but which is incapable of establishing growth transformation of normal B lymphocytes because it has undergone deletion of an essential transforming gene that encodes nuclear antigen EBNA-2 (19, 20). Since herpes virus genomes undergo homologous recombination with transfected homologous DNA (for review see ref. 21), recombinant transforming P3HR-1 viruses can be generated by transfection of P3HR-1-infected cells with wild-type EBV DNA spanning the P3HR-1 deletion, and can be selected by their ability to transform human B lymphocytes (19, 20). These recombinant viruses will acquire variable amounts of DNA flanking the EBNA-2 coding region. The EBV DNA *Sal* I A fragment includes the P3HR-1 deletion which maps from 48.5 to 50.7 kilobases (kb) and the EBER genes at 6.6–7.3 kb. A fraction of the recombinant virus derived from transfection of the P3HR-1 cell line with SalA might thus acquire EBER genes from the transfected DNA. Since mutations or deletions of the EBER genes could potentially be introduced into recombinant virus, we constructed an EBER-deleted cosmid, SalA(−)EBER, by removing the *Acc* I fragments from base pairs 6612 to 7263, which contain both EBER sequences and 313 bases of flanking and intergenic sequence (Fig. 1). No other known EBV open reading frames are affected by this deletion.

Recombinant transforming viruses were obtained by transfecting P3HR-1 cells with EBV SalA or SalA(−)EBER, inducing EBV replication by cotransfecting pSVNaeZ, which encodes the EBV replication cycle transactivator BZLF1 protein, and infecting peripheral blood lymphocytes from an EBV-negative donor with the resultant virus. The infected B lymphocytes were plated in limiting dilution in microtiter wells under conditions where one in six wells yielded a cell line infected with a transforming recombinant virus. In this way, independent clones of cells transformed by EBNA-2-positive recombinant virus could be obtained and scored. The number of transformed cell lines obtained was approximately 40 per experiment with SalA or SalA(−)EBER DNA. Obtaining similar numbers of transformants with either DNA was not surprising, since we expected the majority of recombinant virus to be EBER- and EBNA-2-positive, regardless of the DNA used for transfection. Further, our method was devised such that any transformation-defective EBER-negative viruses could be complemented in trans by parental, EBER-positive, P3HR-1 virus, which might initially coinfect the target cells.

Transformed cell clones were screened for EBER-negative EBV DNA by PCR analysis. Oligonucleotide primers flanking the deleted *Acc* I fragment were used in the amplification reaction, resulting in an 871-base-pair (bp) product with intact SalA DNA or a 220-bp fragment with SalA(−)EBER DNA as a template (Fig. 2, lanes 8 and 9). A representative PCR analysis of seven transformed LCLs is shown in Fig. 2 *Upper*. PCR analysis demonstrates EBER-deleted DNA in LCLs 15, 26, and 58 (lanes 3, 4, and 6) and the presence of wild-type EBER DNA in LCLs 8, 22, and 65 (lanes 1, 2, and 7), and both types of DNA in LCL 25 (lane 5), probably indicating coinfection with an EBNA-2-positive EBER-negative recombinant virus and a parental, EBER-positive, P3HR-1 virus. Southern analysis of total cellular DNA (Fig. 2 *Lower*) confirms the PCR data; LCLs 15, 26, and 58 contain

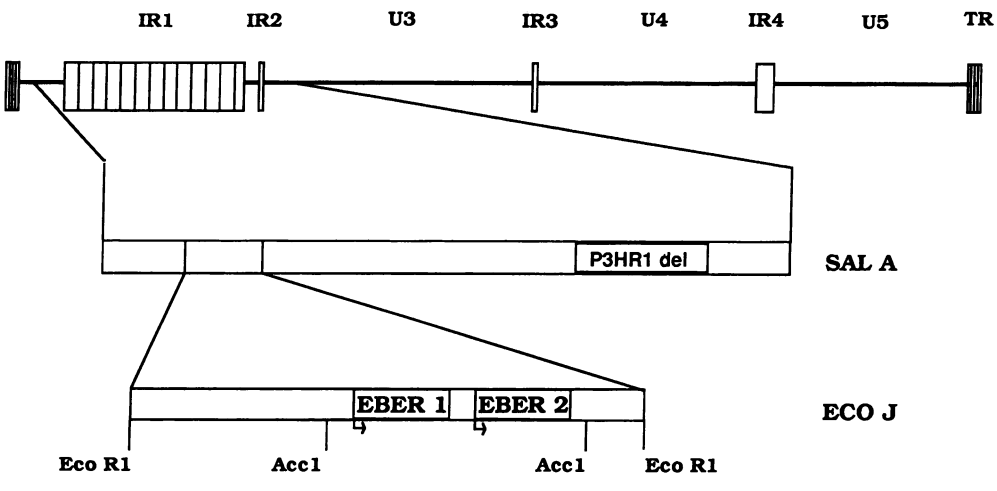

FIG. 1. Schematic representation of the EBV genome and cosmid constructs. The first line depicts the 173-kilobase pair EBV genome, internal repeats (IR), unique regions (U), and terminal repeats (TR). The second line depicts the EBV *Sal* I A fragment (base pairs 644 to 56,081). The relative positions within SalA of the *Eco*RI J fragment (base pairs 4163 to 7315) and of the DNA missing from the P3HR-1 genome (P3HR-1 del) are indicated. The third line depicts the *Eco*RI J fragment, which contains the EBER genes. The *Acc* I sites at base pairs 6612 and 7263 which were used to delete the EBER genes to construct pUC EcoJ(−)EBER and SalA(−)EBER are also shown.

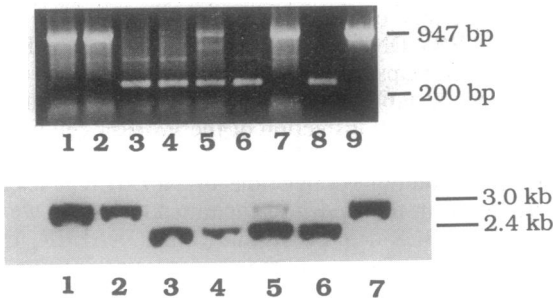

FIG. 2. EBER gene PCR and Southern blot analyses from LCL DNA. (*Upper*) Photograph of an ethidium bromide-stained agarose gel of PCR products from EBER-positive LCLs 8, 22, and 65 (lanes 1, 2, and 7), EBER-negative LCLs 15, 26, and 58 (lanes 3, 4, and 6), or dually infected LCL 25 (lane 5). SalA(−)EBER and SalA cosmid DNA were used as controls (lanes 8 and 9, respectively). Positions of 947- and 200-bp DNA markers are indicated. (*Lower*) Corresponding autoradiogram of a Southern blot of the *Eco*RI-digested cell DNA from the same LCLs that had been hybridized with EcoJ probe. EBER-positive LCLs (lanes 1, 2, and 7), EBER-negative LCLs (lanes 3, 4, and 6), or a dually infected cell line (lane 5), contain EcoJ fragments corresponding in size to those expected from the PCR shown above.

the expected EBER-deleted EBV EcoJ fragment of 2.4 kbp; LCLs 8, 22, and 65, a wild-type 3.0-kbp fragment; and LCL 25, both fragments. The sensitivity of the Southern analysis was assessed by hybridization to serial dilutions of EcoJ(−)EBER plasmid DNA. We could easily detect 10 pg of plasmid DNA (corresponding to one copy of EBER DNA per cell). By comparison the EBV copy number was estimated to be 25–50 per cell (data not shown). Of 70 transformed cell clones derived from transfection with SalA(−)-EBER DNA and screened by PCR, 9 contained only EBER-deleted DNA, 5 contained both EBER-deleted and parental, P3HR-1, EBER-positive DNA, and the remainder contained only parental, P3HR-1, EBER-positive DNA. Thus, the EBERs are nonessential for maintaining B lymphocyte growth transformation. Furthermore, the frequency of cotransfer of the $EBER^-$ and EBNA-2 markers was surprisingly high (14/70), given the 40-kb distance between the EBER and EBNA-2 genes. These results indicate that this genetic strategy is useful in generating mutations outside the P3HR-1 deletion. Further, the high frequency of EBER-negative recombinants suggests that there is not a significant requirement for EBERs in the initiation of lymphocyte transformation *in vitro*.

RNA Analysis. To confirm the absence of EBER RNAs in the LCLs infected with EBER-negative virus, 10 μg of cell RNA from each of two EBER-negative cell lines was analyzed by Northern blot (Fig. 3). Whereas LCLs infected with EBER-positive recombinant virus (8 and 22) contain abundant EBERs, LCLs infected with EBER-negative recombinant virus (15 and 26) are devoid of EBER RNA (Fig. 3 *Upper Left*). EBER RNA was readily detectable in 100 ng of cell RNA from LCL 8 (EBER-positive), indicating that 1 EBER-positive cell would have been detected among 100 EBER-negative cells. The same blot probed with labeled B-actin cDNA demonstrates the presence of undegraded RNA in all four RNA preparations (Fig. 3 *Upper Right*).

Growth of LCLs Infected with EBER-Negative Virus. LCLs infected with EBER-deleted virus were not different from EBER-positive LCLs in their time to initial outgrowth, morphology, growth rate, or saturation density. At early passage, EBER-negative or -positive LCLs grow in clumps, with a doubling time of 48–72 hr. EBNA-1, -2, and -LP gene expression in EBER-negative and -positive cell lines as measured by immunoblotting were also not different (Fig. 4 *Upper*). As expected, LCLs transformed by recombinant

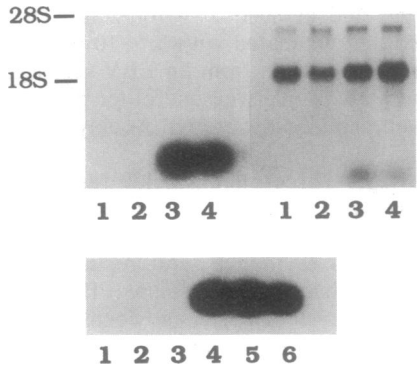

FIG. 3. EBER RNA blot hybridization. RNAs (10 μg per lane) from EBER-negative LCLs 15 and 26 (lanes 1 and 2) or EBER-positive LCLs 8 and 22 (lanes 3 and 4), were hybridized with either EcoJ (*Upper Left*) or B-actin probes (*Upper Right*). The positions of 28S and 18S ribosomal RNA bands are indicated. RNAs (10 μg per lane) from three EBER-negative secondary LCLs (lanes 1–3) or three wild-type LCLs (lanes 4–6) were hybridized with EcoJ probe (*Lower*).

virus express EBNA-2 identical in size to that of B95-8, and they express a slightly larger EBNA-1, consistent with its derivation from the P3HR-1 parent. In addition, both EBER-positive and EBER-negative LCLs express leader proteins of various sizes. This size variation is thought to be due to variable splicing across the IR1 repeats, which make up most of EBNA-LP. Heterogeneity in the size of EBNA-LP is typical of newly transformed lymphocytes. In addition, EBER-positive and EBER-negative cell lines express similar latent membrane protein levels (Fig. 4 *Lower*).

EBV Replication in LCLs Infected with EBER-Negative Virus. Although primary human lymphocytes transformed by

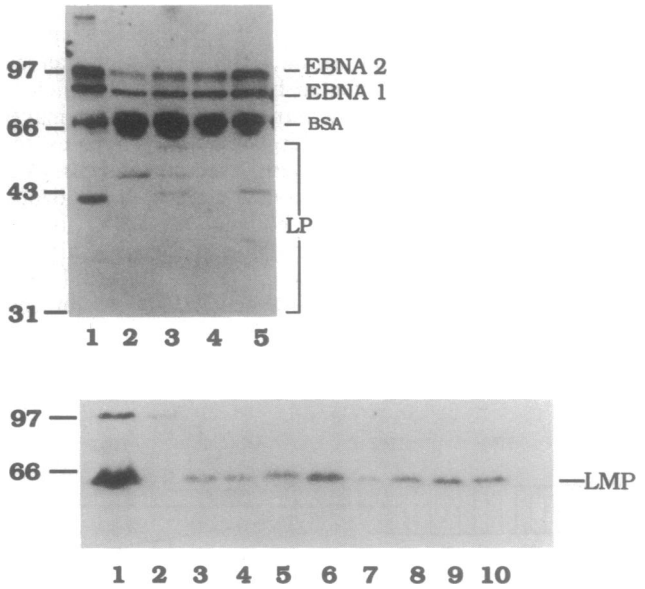

FIG. 4. Immunoblot analyses for EBNA (*Upper*) or latent membrane protein (LMP) (*Lower*) in LCLs infected with EBER-deleted or -nondeleted EBV. (*Upper*) Protein lysates from IB4, which is a tightly latent EBV-transformed cord blood LCL (lane 1), EBER-negative LCLs (lanes 2–4), or an EBER-positive LCL (lane 5) were allowed to react with human sera specific for EBNA-1, EBNA-2, and EBNA leader protein (EBNA-LP). BSA, bovine serum albumin. Molecular masses in kDa are shown on the left. (*Lower*) Lysates from IB4 (lane 1), an EBV-negative B-lymphoma cell line, Louckes (lane 2), four EBER-negative LCLs (lanes 3–6), or four EBER-positive LCLs (lanes 7–10) were allowed to react with the anti-LMP monoclonal antibody S12.

EBV are largely nonpermissive for EBV replication, most clonal cell lines have a small number of cells in which virus replicates spontaneously. Wild-type and EBER-negative cell lines were examined for spontaneous EBV replicative antigen expression by fixed cell immunofluorescence. Staining with the 5B11V monoclonal antibody, which is specific for the 17-kDa BHRF1 protein (22), a member of the restricted subset of early replicative antigens (EA), revealed a similar percentage of EA-positive cells among EBER-negative or -positive cell lines. Five EBER-negative LCLs were 1–2% EA-positive, and five EBER-positive LCLs were 0–4% EA-positive. None of the LCLs spontaneously expressed the late replicative cycle antigen gp110, as measured by indirect immunofluorescence with the L2 monoclonal antibody (23). Inducibility of both early and late lytic antigens was also similar in both EBER-positive and -negative cell lines. BHRF1 expression increased approximately 2-fold after induction of EBV replication with TPA treatment (1.2–3.6% in EBER-deleted cell lines and 1.6–6.8% in EBER-expressing LCLs). Similar gp110 expression was induced by TPA treatment in both types of LCLs, 0.1–3.6% among EBER-negative LCLs and 0–4.5% among EBER-positive LCLs. Plasma membrane expression of the gp350/220 EBV late glycoprotein detected with the 2L10 monoclonal antibody (24) was less than 1% on both EBER-positive and -negative cell lines, after induction with TPA.

Passage of EBER-Deleted Virus. Although the frequent initial clonal outgrowth of LCLs infected with EBER-negative virus demonstrates that the EBERs are not significant for the maintenance of lymphocyte transformation, the EBER-negative cell lines could have been initially coinfected with P3HR-1, which would express EBERs. The putative coinfecting P3HR-1 could have been subsequently lost if the EBERs were necessary only for initiation of cell growth transformation. Alternatively, EBER expression in adjacent P3HR-1-infected cells could have induced the elaboration of a diffusible factor required for the initial establishment of transformation. To prove that parental EBER-positive P3HR-1 was not required for the establishment of lymphocyte transformation by EBER-negative virus, it was necessary to demonstrate that EBV from an EBER-negative LCL could transform primary lymphocytes. EBV-transformed LCLs usually produce little virus, so that cocultivation of irradiated infected LCLs with primary lymphocytes is frequently necessary to passage transforming virus. Accordingly, virus replication was induced with TPA treatment of clonal EBER-negative or -positive LCLs, the LCLs were lethally irradiated, and the irradiated cells were cocultivated with primary blood lymphocytes. Secondary transformants were obtained from all eight EBER-negative cell lines tested. Both EBER-positive and EBER-negative LCLs yielded approximately 15 secondary transformants per 10^5 irradiated lymphoblastoid cells. As expected, secondary transformant LCLs infected with EBER-deleted virus contained only EBER-deleted EBV DNA by PCR analysis (data not shown). More sensitive Northern blot analysis confirmed that these LCLs were devoid of EBER RNA (Fig. 3 *Lower*). Secondary LCLs transformed by EBER-negative virus were not different from EBER-positive LCLs in their time to outgrowth (3 weeks), growth rate (doubling time of 48–72 hr), sensitivity to dilution in fresh medium, or morphology. Furthermore, EBER-negative secondary LCLs were indistinguishable from EBER-positive LCLs in their expression of latent or early replicative cycle proteins as measured by immunoblot analysis (Fig. 5). The variation between LCLs in spontaneous early antigen expression is typical of EBV-transformed cell lines, and it occurs equally among EBER-negative and EBER-positive LCLs (Fig. 5).

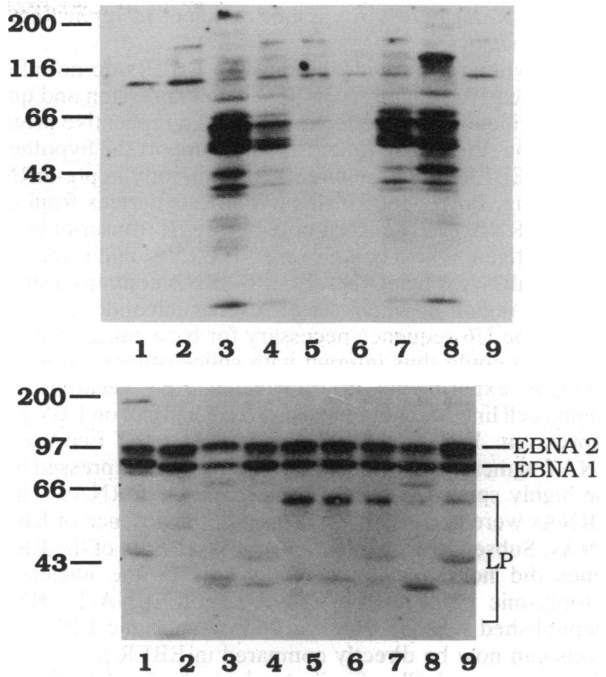

FIG. 5. Immunoblot analyses of early replicative cycle (*Upper*) or latent (*Lower*) EBV proteins in secondary LCLs. Protein lysates from four EBER-negative LCLs derived by infection with virus from EBER-negative LCLs (lanes 2–5), from four EBER-positive LCLs (lanes 6–9), or from IB4 cells (lane 1) were allowed to react with serum specific for early replicative proteins (*Upper*) or EBNA (*Lower*). In *Upper*, the 50- to 60-kDa proteins are the diffuse early antigen complex (EA-D) of EBV replicative proteins (25).

DISCUSSION

Given the abundance of the EBERs and their evolutionary conservation, the finding that they are not essential for EBV latent infection, growth transformation, or replication in B lymphocytes *in vitro* is surprising. At 10^7 copies per cell (8), the EBERs are orders of magnitude more abundant than EBV mRNAs, and they are expressed in all LCLs, Burkitt lymphoma cells, and nasopharyngeal carcinoma biopsy samples, regardless of the infecting EBV type (26). The baboon EBV homologue, herpes virus papio, also expresses large amounts of two small RNAs with similar primary sequence and predicted secondary structure (27). Thus, the EBERs are likely to be important in EBV infection. Still, the high frequency of EBER-negative LCLs derived from the initial transformation with putative recombinant virus, the ease of obtaining secondary transformants, and the similarity in growth and EBNA or latent membrane protein expression in EBER-negative and -positive LCLs indicate that the EBERs do not play an important role in latent B-lymphocyte infection or growth transformation *in vitro*. Further, the immunoblot analyses provide quantitative evidence that there is similar early EBV replicative protein expression in EBER-negative and -positive LCLs. Late protein expression has so far been demonstrated to be equal only by immunofluorescence microscopy, and virus production has been shown to be similar only by cocultivation of EBER-negative or -positive LCLs with primary lymphocytes. These experiments do not exclude the possibility that the EBERs could have a quantitative effect on late protein synthesis or on virus maturation in LCLs *in vitro*. Nevertheless, the similarity in early or late viral replication cycle protein expression and in the amount of transforming virus derived from induced EBER-negative or -positive LCLs supports the hypothesis

that EBER-negative virus is not deficient in replication in LCLs *in vitro*.

In providing firm evidence that the EBERs do not affect latent or early replicative EBV protein expression and qualitative evidence for no effect on late EBV replicative protein expression, these experiments do not support the hypothesis that the EBERs function directly or indirectly in pre-mRNA processing. Precedent for this hypothesis derives from cellular U6 RNA, also an RNA polymerase III transcript bound by La antigen, which complexes to U4 RNA, and is required for pre-mRNA splicing (28). EBER-2 RNA contains a single-stranded region in which six of seven nucleotides are identical to the U6 sequence necessary for base pairing with U4 (9), and it could thus interact with spliceosomes. However, previous experiments in transfected EBV-negative lymphoma cell lines did not reveal any EBER effect on EBV gene expression. For example, transfection of the EcoA EBV DNA fragment yielded cell lines which stably expressed both the highly spliced EBNA-LP and EBNA-2 mRNAs. These mRNAs were accurately processed in the absence of EBER RNAs. Subsequent transfection and expression of the EBER genes did not alter the overall levels or the nuclear-to-cytoplasmic ratios of the EBNA-LP or EBNA-2 mRNAs (unpublished results). Nuclear and cytoplasmic EBV RNA levels can now be directly compared in EBER-positive and EBER-negative LCLs. Similarly, the lack of an EBER effect on latent or early replicative protein expression is against the alternative hypothesis that the EBERs indirectly affect gene expression through binding La antigen, thereby altering the processing of other RNA polymerase III transcripts, including transfer RNAs (29).

Given that deletion of the EBERs does not affect latent or early replicative infection in LCLs *in vitro*, the significant role of the EBERs is perhaps more likely to derive from cell interactions which are important *in vivo*, such as might be mediated by an antiviral or antiproliferative effect of interferon. The EBERs are similar to the adenovirus VA RNAs, which antagonize interferon's antiviral effects. Interferon induces a kinase which, when activated by viral infection, phosphorylates eukaryotic initiation factor 2 (eIF-2), preventing initiation of translation. VA RNAs inhibit eIF-2 kinase activation, permitting virus growth (30). The binding of VA RNA to the kinase is dependent on the presence of an apical stem-loop structure, and inhibition of kinase activation requires the central domain of the VA RNA molecule (31). Experimental data from nuclease and chemical protection experiments suggest that EBER-1 has a similar structure (9). Moreover, EBER RNAs can partially substitute for VA RNA (10). The EBERs could be necessary for maximal viral protein synthesis during primary infection or reactivation *in vivo*, where high interferon levels may be induced by virus replication. Such a protective role might be superfluous *in vitro*, in the absence of interferon. The predominantly nuclear localization of the EBERs has been adduced as evidence against their involvement in cytoplasmic processes such as protein translation, and the addition of purified EBER RNA to interferon-treated cell extracts did not affect eIF-2 kinase activity (8). However, given their abundance, a small fraction of EBER RNAs could act in regulating cytoplasmic events such as protein translation. The otherwise isogenic EBER-negative and -positive cell lines will permit testing these hypotheses by measuring the effect of interferon treatment on virus replication and eIF-2 kinase in EBER-negative and positive LCLs. They will also be useful in examining the potential role of EBERs in modulating an antiproliferative effect of interferon. Precedent for an interferon-modulatory role for the EBERs derives not only from their similarity to the VA RNAs but also from the recent identification of another EBV gene, *BCRF1*, which encodes a close homologue of interleukin-10, an inhibitor of T-cell γ-interferon secretion (32).

These experiments significantly extend the potential for recombinant EBV molecular genetic analyses. Previous mutations were confined to the EBNA-2 or immediately adjacent EBNA-LP locus, which map in the DNA segment deleted from nontransforming EBV strain P3HR-1. Our results indicate that a mutation in a locus 40 kb distant from the wild-type EBNA-2 gene can be cotransferred into the recombinants with a 20% efficiency. The dual recombinants can be readily identified by PCR. We have not determined whether physical, albeit distant, linkage to the EBNA-2 gene enhances the efficient transfer of the EBER mutation. Further investigation of the role of physical linkage is necessary to reveal whether this procedure is limited to the 40 kb flanking the EBNA-2 locus or can be extended to the entire 170-kb EBV genome.

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