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Epstein–Barr virus small nuclear RNAs are not expressed in permissively infected cells in AIDS-associated leukoplakia

(latency/Epstein–Barr virus replication/permissive infection/Epstein–Barr virus transcription)

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ABSTRACT **Epstein–Barr virus (EBV) DNA structure and gene expression were analyzed in tissue specimens from oral hairy leukoplakia (HLP), a mucocutaneous lesion that develops in patients infected with human immunodeficiency virus (HIV). The structure of the terminal restriction enzyme fragments of EBV revealed that HLP is a permissive infection without a predominant, detectable population of EBV episomal DNA. In RNA preparations from this uniquely permissive infection, EBV replicative mRNAs could be identified by Northern analysis; however, the virally encoded small nuclear RNAs, the EBERs, were not detected in most HLP RNA preparations. *In situ* hybridization detected EBER expression in very rare cells. These data indicate that unlike other viral small nuclear RNAs, the EBERs are not expressed during viral replication and must participate in the complex maintenance of latent EBV infection.**

Oral hairy leukoplakia (HLP) is a newly recognized lesion, most often located on the tongue, that occurs almost exclusively in patients with human immunodeficiency virus (HIV) infection (1). Epstein–Barr virus (EBV), usually thought of as a lymphotrophic herpesvirus, is the etiologic agent of this mucocutaneous epithelial cell disorder. HLP is the only EBV-associated disease in which acyclovir, an antiviral drug that inhibits the replication of EBV, induces clinical remission (2). This response and the detection of masses of viral particles in the infected cells make these lesions unique in EBV biology and indicate that the virus is actively replicating (1, 2).

Replication is relatively unusual in EBV-infected cells. Although the virus replicates in oropharyngeal epithelial cells, the transforming infection of B lymphocytes, which is the hallmark of EBV infection, is largely nonpermissive (3–6). In the latently infected B-cell lines, the viral DNA is maintained as an episome (7, 8). Permissive infection develops in a small subset of cells with the expression of multiple replicative RNAs and the generation of the linear, virion form of DNA. The structure of the EBV termini permits discrimination between the linear and episomal DNA forms and therefore between permissive and latent states (9, 10).

In EBV-infected cells, the most abundantly expressed viral transcripts are the nonpolyadenylylated polymerase III transcripts, EBER1 and EBER2 (EBV-encoded small nuclear RNA), which are found in ribonucleoprotein particles (RNP) (11, 12). Despite their abundance, the function of these RNAs is unknown. The EBERs can partially substitute for the similarly sized polymerase III virus-associated (VA) transcripts encoded by adenovirus that function during viral replication (13, 14). This complementation suggested that perhaps, like the VA RNAs, the EBERs also function during lytic replication. This observation was supported by studies that revealed that the EBERs are more abundant in the partially permissive virus-producer cell line B95-8 than in a latently infected lymphoid line, IB4, established by infection with B95-8 virus (15, 16). However, in contrast to the cytoplasmic VA RNAs, which are expressed late after infection, the EBERs are expressed early after infection and detected predominantly in the nucleus (17, 18).

In this study, the state of EBV infection in HLP was further characterized through analysis of the terminal restriction-enzyme fragments of EBV. Analysis of the EBV termini revealed that HLP is an unusually permissive infection with abundant ladder arrays representing virion DNA. EBV replicative mRNAs and the latent membrane protein (LMP) mRNA were expressed in the infected tissues; however, the EBER RNAs could not be detected in most specimens. EBER expression was detected in a few cells in *in situ* hybridizations. These data indicate that the EBER RNAs, which are abundantly expressed in latently infected cells, are not expressed in permissively infected cells and are not required for viral replication.

MATERIALS AND METHODS

Cell Cultures and Tissue Specimens. The lymphoblastoid B-cell lines B95-8, AG876, Raji, Louckes, and CB4 were grown at 37°C in RPMI 1640 medium with 10% (vol/vol) fetal calf serum and antibiotics. The B95-8 and AG876 cell lines were treated with 40 ng of phorbol 12-myristate 13-acetate to induce viral replication. The nasopharyngeal carcinoma (NPC) cell line NPC-KT was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (19). The NPC tumor C15 is an undifferentiated, nonkeratinizing NPC, which has been serially propagated in nude mice (20).

Nucleic Acid Extraction and Blotting. Tissue specimens of HLP and NPC were obtained at biopsy, frozen, pulverized in a microdismembrator (Braun Instruments/Fisher Scientific), and dissolved in 4 M guanidine thiocyanate (21, 22). Total cellular RNA was obtained from the cell lines by resuspending the pelleted cells in 4 M guanidine thiocyanate (23). RNA and DNA were separated by centrifugation through a cesium chloride step gradient. The DNA fraction was dialyzed, treated with proteinase K, and extracted with phenol and chloroform prior to digestion with restriction enzymes and electrophoresis through a 0.6% agarose gel. Polyadenylylated mRNA was enriched by oligo(dT)-cellulose chromatog-

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Abbreviations: EBV, Epstein–Barr virus; EBER, Epstein–Barr virus-encoded small nuclear RNA; HLP, hairy leukoplakia; LMP, latent membrane protein; NPC, nasopharyngeal carcinoma; TR, terminal repeat; RNP, ribonucleoprotein particles.

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raphy prior to electrophoresis through a 0.8% agarose/formaldehyde gel or through a 2% NuSieve agarose gel (24).

Preparation of Radiolabeled Probes. Single-stranded RNA probes were synthesized from the EBV fragments cloned into the pGEM vector (Promega) by using either SP6 or T7 RNA polymerase in a 50-μl reaction mixture consisting of 40 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 100 μg of bovine serum albumin per ml, 10 mM dithiothreitol, 0.5 mM unlabeled nucleotides, and 120 μCi of [^{32}P]UTP. The EBV fragments used to identify the terminal fragments were the *Eco*RII portion of *Bam*HI NJhet, adjacent to the left terminal repeats (TRs) and the *Xho* I 1.9-kilobase (kb) fragment representing unique DNA adjacent to the right TRs (9, 25). RNA probes were synthesized from the *Bam*HI Z, H, and L fragments to identify EBV replicative mRNAs, from the *Xho* I 1.9-kb fragments to identify LMP mRNA, and from the *Eco*RI J fragment to identify the EBER RNAs. The EBER1-specific probe is a 1.45-kb *Sau*3A subfragment of *Eco*RI J fragment (bases 5418–6794).

Hybridization Conditions *in Situ*. Frozen biopsies of HLP were fixed in 4% paraformaldehyde/0.1% deoxycholate/0.1% Triton X-100 in phosphate-buffered saline. The HLP tissues were divided in pieces and hybridized at 50°C [in 50% formamide/600 mM sodium chloride/1 mM EDTA/10 mM dithiothreitol/10% SDS/10% PEG 8000/0.25 mg of *Escherichia coli* tRNA per ml/10 mM Tris·HCl, pH 7.5/0.02% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone] to antisense and sense RNA probes representing LMP and EBER RNAs. The probes were hydrolyzed in alkali to ≈100–200 base pairs (bp). After hybridization the tissues were treated with ribonuclease A (20 μg/ml) in 500 mM NaCl/10 mM Tris, pH 8.0, at 37°C for 30 min. The tissue was washed, dehydrated, fixed, and sectioned. Slides were coated with Kodak NTB2 emulsion and exposed for 1–3 weeks.

RESULTS

The Structure of the EBV Termini in HLP. The terminal restriction enzyme fragments of linear virion DNA are heterogeneous in size and vary by increments of 500 bp reflecting differing numbers of copies of direct tandem repeats of ≈500 bp at each terminus (TR) (26, 27). Fused terminal fragments are formed after infection through the joining of the ends of the linear DNA to form the intracellular, episomal form of EBV (25, 27). The fused terminal fragments can be distinguished from the terminal fragments of the linear genomes because they will hybridize to DNA probes of unique DNA adjacent to the TR from both ends of the linear genome (9). This type of analysis revealed a single band representing the fused EBV termini in NPC, an EBV-associated epithelial malignancy, as well as in monoclonal lymphomas (9, 22). The detection of homogeneous, clonal EBV genomes indicated, by extension, cellular clonality. Similar analyses of cell lines cloned *in vitro* and EBV-infected lymphoproliferations *in vivo* identified predominant clonal or oligoclonal fused terminal fragments and faint ladder arrays representing linear DNAs (10, 28).

In HLP tissue, EBV virions are detected. However, in contrast to lytic infections with other herpesviruses, in HLP the infected cells continue to proliferate and produce keratin filaments, an observation that inspired the appellation "hairy" leukoplakia. Total intracellular DNA was extracted from tissue biopsy specimens representing active HLP. Duplicate Southern blots prepared with DNA from five specimens of HLP were hybridized to probes representing unique DNA from the left or right ends of the linear EBV genome, adjacent to the TRs (Fig. 1). In contrast to the pattern of terminal fragments detected in NPC, lymphomas, lymphoproliferations, and B-cell lines, in HLP abundant ladder

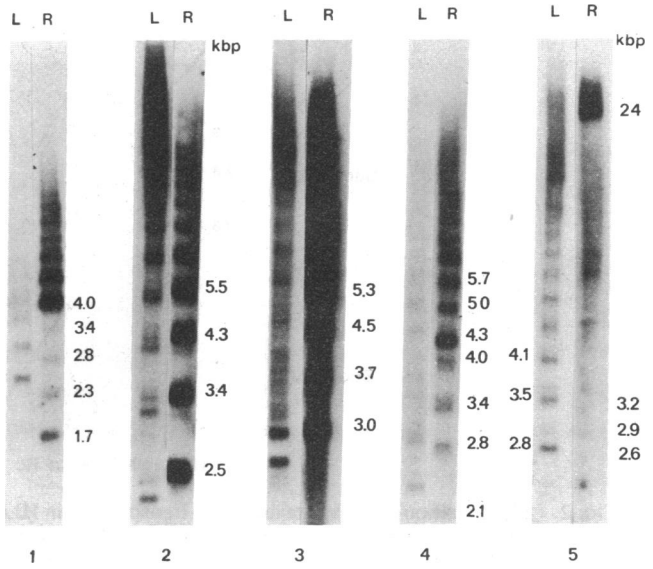

FIG. 1. The structure of the EBV termini in HLP. Duplicate blots prepared with DNA extracted from five specimens of HLP digested with *Bam*HI were hybridized to SP6-generated RNA probes representing the *Eco*RII portion of *Bam*HI NJhet, adjacent to the left TRs (lanes L) and to the *Xho* I 1.9-kb fragment representing unique DNA adjacent to the right TRs (lanes R) (6).

arrays representing the left (L) or right (R) termini were detected without evidence of predominant fused fragments representing episomal DNA. Although it is likely that the multiple larger fragments are heterogeneous fused terminal fragments, which would include replicative intermediates, the lack of predominant fused fragments indicates that HLP is not a reactivation of EBV with clonal or oligoclonal cellular proliferation and viral replication in some of the cells. Rather, the unusual pattern of the termini in HLP indicates that HLP is primarily a permissive EBV infection. Interestingly, the periodicity of the EBV terminal fragments varies between specimens. This suggests that perhaps the size of the TR is distinct in these wild-type strains.

Analysis of EBV Transcription in HLP. All studies of EBV expression during replication have analyzed mixed populations of cells where a large portion of the cells is latently infected. Therefore, HLP provides a unique opportunity to analyze expression of replicative functions. However, in most cases, the total RNA obtained from a 2- to 3-mm HLP biopsy specimen was sufficient for one or two Northern blots. Therefore, the top portion of the blot was hybridized to an EBV probe to identify specific EBV replicative RNAs, and the bottom portion was hybridized to probes specific for the EBERs. Transcription in HLP was compared with transcription in latently and permissively infected lymphoid lines, an EBV-infected epithelial cell line, and a latently infected NPC passaged in nude mice (29, 30). The two strain variants of EBV, A and B, which have considerable sequence divergence in the Epstein–Barr nuclear antigen *EBNA2* gene, were also included (31). The EBER genes are 99% homologous between the two strains; however, a point mutation results in a restriction enzyme polymorphism that can distinguish A and B types (32).

The late mRNAs that encode the glycoproteins gp350 and gp220, the two major envelope proteins of EBV, are transcribed from the *Bam*HI L fragment (33, 34). A single-stranded RNA probe transcribed rightward identified the 3.3- and 2.8-kb mRNAs that encode the envelope proteins and a late 1.8-kb mRNA also transcribed from these sequences in two specimens of HLP (Fig. 2*A*). These late mRNAs were not detected in the RNA obtained from the latently infected

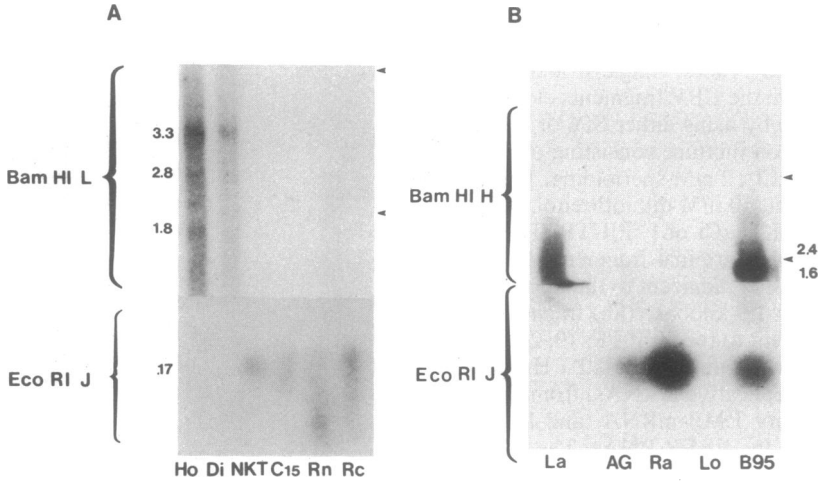

FIG. 2. Identification of EBV late and early transcription in HLP. (*A*) Northern blot prepared from a 2% NuSieve gel containing total RNA extracted from two HLP biopsies (lanes Ho and Di) and 5 μg each of nonpolyadenylylated RNA from NPC-KT, a cell line formed by fusion of an NPC with an established adenocarcinoma cell line (lane NKT) (30); from C15, an NPC passaged in nude mice (lane C15) (20); and nuclear and cytoplasmic RNA from the Raji cell line (lanes Rn and Rc). The blot was cut in half to separate the high and low molecular weight RNAs. The top half of the blot was hybridized to a single-stranded RNA probe homologous to leftward *Bam*HI L fragment mRNAs encoding the viral envelope glycoproteins gp350 and gp220 (3.3 and 2.8 kb). The bottom half was hybridized to an RNA probe that was transcribed leftward from the *Eco*RI J fragment and is homologous to the EBER RNAs (0.17 kb). The 5.2- and 2.0-kb ribosomal RNAs are denoted by arrows on the right. The approximate sizes of detected bands are indicated in kilobases. (*B*) A second Northern blot was prepared from a 1% agarose gel containing total RNA extracted from one HLP specimen (lane La) and 5 μg each of polyadenylylated RNA from phorbol 12-myristate 13-acetate (PMA)-induced AG876 (lane AG), Raji (lane Ra), Louckes, an EBV-negative American Burkitt lymphoma (lane Lo), and PMA-induced B95-8 (lane B95). This blot was cut in half and the top half was hybridized to a single-stranded RNA probe homologous to rightward transcripts of *Bam*HI H fragment. The bottom half was hybridized to *Eco*RI J fragment.

NPC-KT and C15 NPC epithelial cells or cytoplasmic and nuclear RNA from the Raji cell line. Hybridization of the bottom panel to *Eco*RI J fragment identified the EBERs in NPC-KT, C15, and Raji cells but did not detect EBER expression in the HLP RNAs.

Several early replicative mRNAs are transcribed from the *Bam*HI H fragment of EBV (15, 35). The multiple RNAs, transcribed from left to right in *Bam*HI H fragment, are 3′ coterminal and encode a 17-kDa early replicative protein (36). A Northern blot was prepared with total RNA extracted from HLP and with RNA obtained from the EBV producer cell lines B95-8 and AG876 (which represent the prototype A and B strains), the latently infected Raji cell lines, and the EBV-negative lymphoid line Louckes. Hybridization with a leftward RNA probe transcribed from *Bam*HI H fragment identified the 2.4- and 1.6-kb mRNAs in B95-8 RNA and the 1.6-kb mRNA in AG876. Strong hybridization was detected to the HLP RNA sample La. Hybridization of the bottom panel to *Eco*RI J fragment detected the EBERs in AG876, B95-8, and Raji cells but did not detect EBER expression in the La RNA. These data confirmed that HLP specimens contain replicative EBV transcripts in the absence of detectable EBER transcription.

The EBV replication activator protein ZEBRA is an immediate-early function that can activate expression of replicative antigens (37, 38). Hybridization with a single-stranded RNA probe synthesized rightward in map orientation from the *Bam*HI Z fragment identified the 4.6-kb, 3.4-kb, and 1.2-kb early mRNAs transcribed from *Bam*HI Z fragment in the polyadenylylated RNA from B95-8 and the 1.2-kb mRNA in AG876 RNA (39). A low level of transcription was also detected in Raji RNA. Abundant hybridization was also detected to RNA extracted from the HLP specimen La (Fig. 3*A*). Hybridization with the *Bam*HI Z probe identified multiple discrete mRNAs in two additional specimens, Hu and Ma (Fig. 3*B*). In RNA from B95-8, large mRNA forms are also detected in long exposures of the autoradiogram, indicating that transcription into *Bam*HI Z is complex (Fig. 3*B*).

Hybridization of the bottom portion of the blot to the *Eco*RI J fragment (Fig. 3*A*) or to a probe specific for EBER1 (Fig. 3*B*) detected expression of the EBERs in RNA fractions from the B95-8, AG876, and Raji cell lines but did not detect the EBERs in RNA from the HLP specimens. Long exposures of the heavily loaded Ma channel detected trace levels of hybridization to the EBER1 probe. These results indicated that although EBV replicative RNAs were readily detected in the RNA preparation from HLP specimens, the EBERs were not detected or were expressed at trace levels.

The EBV LMP is expressed in all latently infected lymphoid cells; however, expression of LMP or a related truncated protein transcribed from the same sequences increases after induction of replication (40–42). Expression of LMP can transform rat fibroblasts *in vitro* and induces in transgenic mice epithelial proliferation and hyperkeratin production (43, 44). Hybridization of an RNA probe homologous to LMP detected the 2.8-kb LMP mRNA in B95-8 and 3.7-kb and 2.8-kb mRNAs in HLP RNA (Fig. 3*C*). A 3.7-kb mRNA homologous to LMP also has been detected in NPC, an epithelial malignancy associated with EBV (9, 45). Transcription of the 3.7-kb LMP mRNA in both HLP and NPC may indicate that this form of LMP mRNA is transcribed more frequently in epithelial cells.

The EBER RNAs are generally resistant to degradation, perhaps because they are found in RNP particles and can readily be detected in most RNA preparations even when mRNAs are not detectable. The absence of detectable EBERs in HLP RNA preparations where replicative transcripts were detected indicates that the EBERs are not transcribed in permissive infection and are not required for EBV replication. Previous studies had revealed that EBER abundance did not detectably change when a predominantly latent cell line was induced to a high percentage of lytically infected cells (46). However, these studies could not determine whether EBERs, which were present in the latently infected cells prior to induction, also functioned in the permissively infected cells.

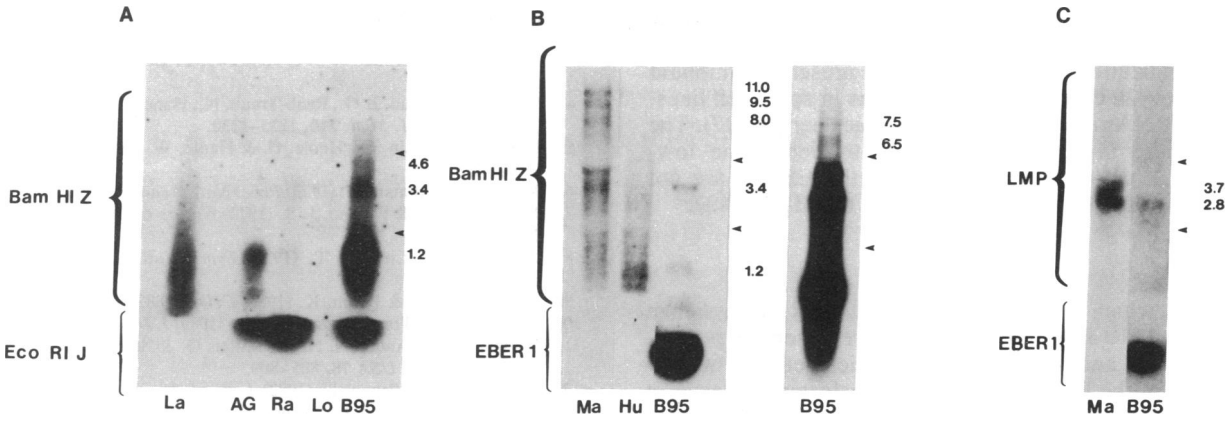

FIG. 3. Identification of EBV immediate-early and latent transcription in HLP. (*A*) An identical Northern blot of Fig. 2*B* was prepared and cut in half to separate high and low molecular weight RNAs. The top portion of the blot was hybridized to a single-stranded RNA probe homologous to leftward transcripts of *Bam*HI Z fragment. The bottom portion was hybridized to an *Eco*RI J fragment RNA probe. The approximate sizes of the detected RNAs are indicated in kilobases. (*B*) A Northern blot was prepared with total RNA from two HLP specimens (lanes Ma and Hu), and 10 μg of B95-8 (lane B95). The blot was cut in half, the top was hybridized to the *Bam*HI Z fragment probe, and the bottom was hybridized to a probe homologous to EBER1. The lane on the right contains 5 μg of polyadenylylated B95-8 RNA hybridized to the *Bam*HI Z probe, identifying additional transcripts after lengthy exposure to x-ray film. (*C*) A duplicate Northern blot of Fig. 3*B* containing HLP (Ma) and B95-8 (B95) total cellular RNA was hybridized to an *Xho* I 1.9-kb probe homologous to LMP (*Upper*) or to the EBER1 probe (*Lower*).

Analysis of EBV Transcription by *in Situ* Hybridization. Activation of EBV replication is believed to occur as oropharyngeal epithelial cells differentiate (4). It is possible that the EBERs and perhaps other functions that are expressed in latently infected cells may be localized to the more undifferentiated cells and not be detected in RNA prepared from the whole tissue homogenate. Therefore, specimens of HLP were analyzed by *in situ* hybridization with ^{35}S-labeled single-stranded RNA probes specific for EBER1 and the EBV LMP in antisense and sense orientation. Hybridization with the LMP antisense probe detected expression in all layers of the infected tissue, with particularly strong hybridization in the differentiated squamous cell layer (Fig. 4*A*). Hybridization was not detected with the LMP sense probe (Fig. 4*B*), indicating that the hybridization was specific for the EBV RNAs and was not detecting EBV DNA. Hybridization with the LMP antisense probe to EBV-negative normal glandular tissue from the nasopharynx was also negative (Fig. 4*C*).

Hybridization with the EBER1 antisense probe did not detect EBER expression in the majority of cells in the superficial or basal layers of the HLP tissue (Fig. 4*D*). In some specimens, grains were detected on an extremely rare cell within the basal layer, denoted with an arrow. Such cells perhaps represent an occasional cell that remains latently infected and account for the trace levels of hybridization detected on some Northern blots. In contrast, in NPC tissue, which contains clonal EBV episomal DNA and is predominantly latently infected, EBER1 is abundantly transcribed and readily detected by *in situ* hybridization (Fig. 4*E*). Hybridization was not detected with EBER sense probe in the NPC tissue (Fig. 4*F*). Although LMP RNA could be

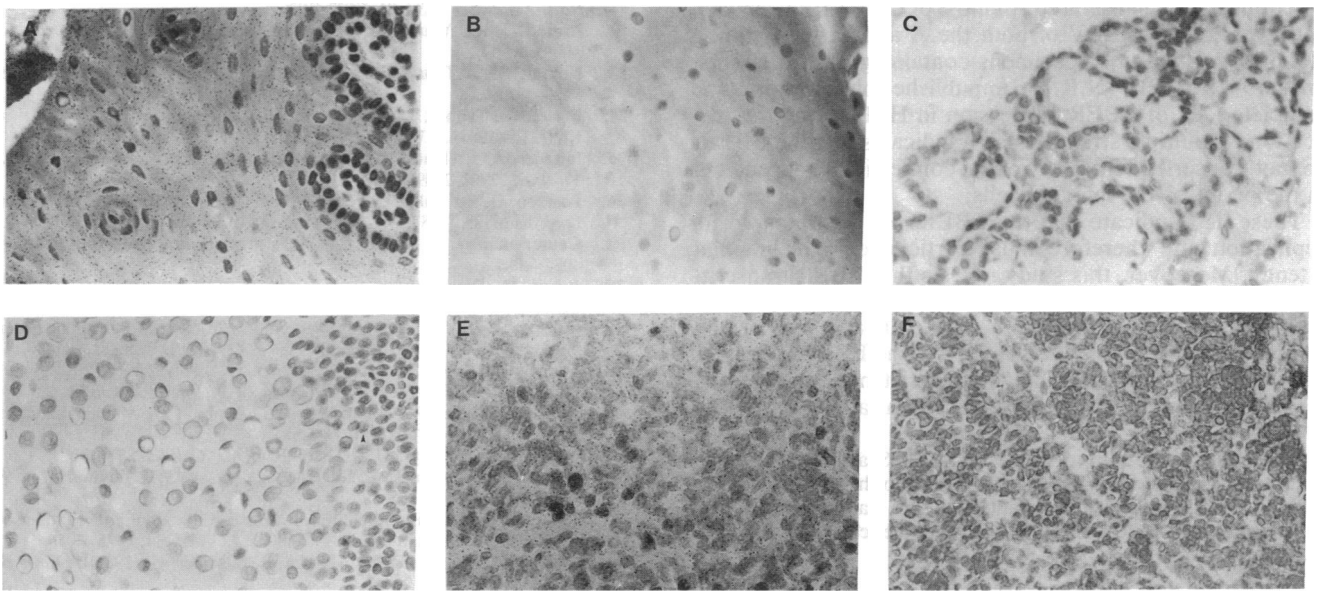

FIG. 4. Identification of LMP and EBER RNA by *in situ* hybridization. (*A*) An SP6-generated ^{35}S-labeled antisense RNA probe synthesized from an *Xho* I 1.9-kb fragment homologous to LMP RNA was hybridized to a portion of a shave biopsy of HLP. (*B*) A ^{35}S-labeled LMP sense RNA probe was hybridized to a second portion of the HLP biopsy. (*C*) Hybridization of the LMP antisense probe to normal nasopharyngeal glandular tissue. (*D*) Hybridization of the ^{35}S-labeled EBER1-specific antisense RNA probe to an HLP specimen. (*E*) Hybridization of the ^{35}S-labeled EBER antisense probe to C15 NPC tissue. (*F*) Hybridization of a ^{35}S-labeled EBER1 sense probe to C15 NPC tissue. (×140.)

consistently detected in the HLP tissue sections, EBER expression was not detected in 10 HLP specimens tested by *in situ* hybridization. The LMP mRNA is present in lymphoid cell lines at ≈20 copies per cell, whereas in some cell lines, the EBER RNAs are detected at 10^7 copies per cell (17). The ability of the *in situ* hybridizations to detect the low-abundance LMP mRNA suggests that the EBERs are not expressed at low levels in most cells in the HLP tissue.

DISCUSSION

These data, which reveal the absence of EBER expression in most of the EBV-infected cells in HLP representing permissive infections, and previous studies, which detected nuclear expression of the EBERs early after transformation, suggest that the EBERs function during latent infection where they may participate in RNA processing, stabilization, or transport (17, 18). Structural and sequence analysis of the EBER genes has revealed a seven-nucleotide sequence homologous to the region of the cellular U6 small nuclear RNA, which is involved in base-pairing with the U4 RNA in the RNP spliceosome complex (47). This region of EBER2 is also single-stranded in RNP particles, suggesting that EBER2 may hybridize with another RNA and potentially could function during the intricate RNA splicing that is characteristic of the latent mRNAs of EBV (48). Another key element in the regulation of EBV expression during latent infection is the selective stabilization and transport of transcripts (16). The EBERs may participate in this process. This explanation would be consistent with the predominant nuclear localization with some detection in the cytoplasm.

In contrast to the lack of EBER expression in permissive infection, LMP expression is maintained in the permissively infected cells. Interestingly, HLP is characterized by abundant keratin production and continued proliferation of the epithelium, a phenotype exhibited by transgenic mice expressing LMP (44). The continued expression of LMP in HLP may provoke these histopathologic characteristics.

The lack of a permissive cell system and the resulting inability to generate specific mutants have impeded the identification of the specific biologic functions of EBV genes. Therefore, a more indirect approach to ascertain potential functions has been required. In most of the HLP specimens, multiple strains of EBV of both the A and B types can be identified, with some specimens containing four to six distinct strains of EBV (N.R.-T., unpublished observation). The complete lack of EBER expression in HLP during the replication of multiple strains of EBV indicates that the EBERs are not transcribed from any of the coinfecting viral genomes in the permissively infected cells.

These data indicate that the EBERs are not required for replication and therefore must participate in maintaining latency. Moreover, this study reveals that EBER transcription can be considered a marker of latent EBV infection, whereas LMP is expressed in both latently and permissively infected cells. The relative stability of the EBERs and their considerable abundance allow for consistent detection by *in situ* hybridization which may be useful as a diagnostic tool.

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