JOURNAL OF VIROLOGY, Oct. 1990, p. 4948–4956
0022-538X/90/104948-09$02.00/0

Vol. 64, No. 10

Novel Transcription from the Epstein-Barr Virus Terminal *Eco*RI Fragment, DIJhet, in a Nasopharyngeal Carcinoma

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Received 6 April 1990/Accepted 2 July 1990

Transcription of Epstein-Barr virus (EBV) genes in epithelial tissue, one of the two principal cell types infected by EBV, is not well characterized. EBV transcription in a nasopharyngeal carcinoma established in nude mice, C15, has been analyzed by using strand-specific RNA probes and sequence analysis of a C15 cDNA library. In C15, two equally abundant mRNAs of 3.7 and 2.8 kilobases (kb) are encoded by the sequences that encode latent membrane protein (LMP). Hybridization with probes specific for the 3′ end of the LMP mRNA to Northern (RNA) blots and sequence analysis of cDNAs representing the messages indicated that the 3.7- and 2.8-kb mRNAs are 3′ coterminal. Sequence analysis of additional cDNAs revealed an mRNA that is spliced identically to the LMP mRNA but is initiated 5′ to the promoter for LMP. A probe representing the sequences contained within the cDNA which are 5′ to the LMP promoter identified the 3.7-kb mRNA in C15 and a low-abundance 3.7-kb mRNA in B95-8 RNA. These data indicate that transcription of the LMP-encoding sequences is complex and that LMP can be expressed from an additional RNA in both nasopharyngeal carcinoma and lymphoid cells. Hybridization with *Bam*HI-A identified a predominant 4.8-kb mRNA and two less abundant larger-molecular-weight mRNAs transcribed in C15. These mRNAs are consistently expressed in all passages in nude mice of the C15 tumor. Hybridization with strand-specific probes and sequence analysis of three cDNAs revealed that these mRNAs are transcribed from left to right. Sequence analysis of cDNAs representing the 3′ end of the mRNAs identified an open reading frame that could potentially encode a protein of 174 amino acids. In situ hybridization of a ^{35}S-labeled RNA probe homologous to the *Bam*HI-A cDNA to tissue sections revealed that the *Bam*HI-A mRNA is not focally expressed and is transcribed in all cells within the C15 tumor. Linear forms of EBV DNA were not detected in any of the C15 tumors, and replicative viral antigens have not been detected. These data suggest that the C15 tumor represents a latently infected tumor and that the transcription from *Bam*HI-A, which is expressed in all cells, is not associated with virus replication.

Epstein-Barr virus (EBV), like other herpesviruses, exhibits a dual cellular tropism, infecting B lymphocytes and certain epithelial cells in vivo (4, 30). The ability of the virus to infect these cell types is thought to be a contributing factor in the occurrence of two human malignancies, African Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) (7, 8, 23).

In vitro, the infection of B lymphocytes with EBV induces continuous proliferation and establishment of B-cell lines (20, 21). EBV gene expression has been primarily studied in these lymphoid cell lines. These studies have revealed that viral transcription is complex, with multiple intricately and differentially spliced RNAs (3, 27, 31). Transcripts encoding each of the six EBV nuclear antigens in B-lymphoid cell lines all appear to be initiated either within or immediately 5′ to the large internal repeat sequence IR1, whereas the latent membrane protein (LMP) mRNA is transcribed in the opposite direction from the Dhet fragment at the far right end of the genome (9, 26, 27).

In comparison, relatively few details are known concerning expression in epithelial cells. It has been difficult to identify the viral genes expressed in EBV-infected epithelial cells because cell lines cannot be established from NPC tumors, nor can epithelial cell lines be generated by infection in vitro (24, 30). In addition, NPC tissue does not readily transplant into nude mice (5). However, analyses of RNA obtained from NPC tissue taken at biopsy indicated that in some tumors the same sequences are transcribed as are found in latently infected lymphocytes (24). In other tumors, transcription was detected only from the terminal fragment, DIJhet, or the large internally repeated sequence, IR1. Two NPC that had been passaged extensively in nude mice showed transcription from most of the genome, in agreement with the detection of virus in some of the NPC when passaged in nude mice, indicative of complete viral replication in a small proportion of the tumor cells in some nude mouse-propagated NPC (24).

Another NPC tumor, C15, which has been successfully heterotransplanted into nude mice, is of particular interest because protein analyses indicated a restricted pattern of viral antigen expression similar to that seen in NPC biopsy specimens (5, 35). EBV transcription has been analyzed in C15 to identify the viral mRNAs expressed in malignant epithelial tissues and to characterize transcription from the DIJhet fragment.

The study presented here revealed that in contrast to previously analyzed nude mouse passaged NPC, gene expression in C15 is largely confined to regions coding for latent gene functions (24, 32). Analysis of the EBV DNA structure revealed the presence of episomal DNA without the detection of the linear, virion form of EBV DNA.

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Transcription has not been detected from sequences that encode immediate-early or early replicative mRNAs, suggesting that EBV expression in this tumor is indeed restricted to latent genes.

In the C15 tumor, two distinct mRNAs, 3.7 and 2.8 kilobases (kb), are transcribed from the sequences that encode LMP within the *Eco*RI DIJhet fragment. Sequence analysis of the C15 LMP cDNAs revealed that the 3.7-kb mRNA is spliced identically to LMP mRNA but initiates 5′ to the established promoter for LMP. A related mRNA was also detected in B95-8 cells. These data indicate that LMP can be encoded by another mRNA which must utilize a distinct promoter.

The C15 cDNA library also contained cDNAs homologous to the *Bam*HI A fragment. Hybridization with strand-specific probes revealed rightward transcription from *Bam*HI-A in all passages of the C15 tumor. Sequence analysis of the *Bam*HI-A cDNAs revealed an open reading frame (ORF) at the 3′ end of the mRNAs which could encode a protein of 18 to 20 kilodaltons.

Hybridization in situ detected the *Bam*HI-A and LMP transcripts in all cells within the C15 tumor. These data indicate that the *Bam*HI-A transcript does not represent a replicative transcript which is sporadically expressed in a few abortively infected cells in the C15 tumor. The consistent detection of transcription from *Bam*HI-A in all cells within the C15 tumor suggests that in NPC or perhaps in epithelial cells these sequences encode a function associated with latent infection.

MATERIALS AND METHODS

Cell cultures and tumor material. The lymphoblastoid B-cell lines B95-8 (20), Raji (15), Louckes, and CB4 were grown at 37°C in RPMI 1640 medium with 10% fetal calf serum and antibiotics. The NPC tumor, C15, is an undifferentiated, nonkeratinizing NPC that has been serially propagated in nude mice (5).

RNA preparation and nucleic acid blotting. Total cellular RNA was obtained from the cell lines by suspending the pelleted cells in 4 M guanidine thiocyanate (6). RNA and DNA were separated by centrifugation through a cesium chloride step gradient. The DNA fraction was dialyzed to remove the cesium chloride and guanidine thiocyanate, treated with proteinase K, and extracted with phenol and chloroform (29). To obtain RNA from frozen specimens, the specimens were pulverized in a microdismembrator (Braun Instruments, Fisher Scientific, Lexington, Mass.) and dissolved in 4 M guanidine thiocyanate for CsCl step-gradient separation (24). poly $(A)^+$ RNA was separated from the total RNA by oligo(dT)-cellulose chromatography (1). The molecular weights of individual mRNAs were determined by Northern (RNA) blot hybridization after electrophoresis through a 0.8% agarose-formaldehyde gel and transfer onto nitrocellulose paper (1, 18).

Preparation of radiolabeled probes and hybridization. Riboprobes were prepared under the following conditions: 1 μg of linearized template in a 50-μl reaction consisting of 40 mM Tris hydrochloride (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 (19). The 6.8-kb *Eco*RI-*Bam*HI portion of *Bam*HI-A was purified by electroelution and was labeled with ^{32}P by nick translation (25). Hybridization solution consisted of 5× SSC (SSC is 0.1 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution, 0.2% sodium dodecyl sulfate, 100 μg of calf thymus DNA per ml, and 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4) in 50% formamide. Radiolabeled RNA probes were hybridized at 52°C.

cDNA synthesis. A cDNA library was prepared from poly$(A)^+$ C15 RNA by the method of Gubler and Hoffman (11). Reagents for this synthesis were obtained in kit form from Amersham Corp. The double-stranded cDNA was treated with *Eco*RI methylase before ligation to *Eco*RI linkers, digested with *Eco*RI, and size fractionated over a Sepharose CL-4B column. The cDNA was cloned into *Eco*RI-digested λgt11, pretreated with calf intestinal alkaline phosphatase, and plated onto the *Escherichia coli* Y1090 (36). A second cDNA library was prepared by the same method, with the modification of using as a primer a synthetic oligomer from bases 168542 to 168562. The cDNAs were cloned into λgt10 and plated on *E. coli* C600 *hfl*.

Screening of the cDNA library. Approximately 3×10^6 bacteriophage λgt11 recombinants were plated onto a lawn of *E. coli* Y1090 on 150-mm petri dishes, and the phage were allowed to grow for approximately 8 h. Plaques were transferred to nitrocellulose filters and hybridized overnight to EBV virion DNA labeled with ^{32}P by nick translation (25). The filters were washed for 2.5 h at 68°C in 0.1× SSC–2% sodium dodecyl sulfate–1% sodium pyrophosphate. Positive plaques were picked and purified. Inserts were excised and subcloned into $pGem_2$ vector for amplification and sequencing. The λgt10 library was screened with a 1.9-kb *Xho*I riboprobe (Fig. 1).

cDNA sequencing. Sequencing of cDNAs was accomplished by the GemSeq/RT system (Promega Corp., Madison, Wis.). The cDNAs were ligated into the $pGem_2$ vector, and an RNA template of the cDNA insert was synthesized in vitro by using the SP6 or T7 promoters and annealed to primer complementary to the T7 promoter or vice versa. The sequencing reaction was accomplished by the dideoxy-chain termination method of Sanger et al. (28), using reverse transcriptase in the presence of [^{35}S]dATP. SP6 or T7 promoter primers were used to initiate reverse transcription.

Hybridization conditions in situ. Frozen pieces of the C15 tumor were fixed in 4% paraformaldehyde–0.1% deoxycholate–0.1% Triton X-100 in phosphate-buffered saline. The NPC tissue was divided in pieces and hybridized at 50°C in 50% formamide–600 mM sodium chloride–1 mM EDTA–10 mM dithiothreitol–10% sodium dodecyl sulfate–1× Denhardt solution–10% polyethylene glycol 8000–*E. coli* tRNA (0.25 mg/ml)–10 mM Tris hydrochloride (pH 7.5) to antisense and sense RNA probes representing LMP and *Bam*HI-A RNAs. The probes were hydrolyzed in alkali to approximately 100 to 200 base pairs (bp). After hybridization, the tissues were treated with RNase A (20 μg/ml) in 500 mM NaCl–10mM 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 to 3 weeks.

RESULTS

The state of viral infection in C15. Earlier studies of EBV expression in NPC cultivated in nude mice indicated that viral expression was not tightly restricted and that virus replication or abortive infection occurred in some cells (24). To determine whether foci of cells in the C15 tumor were permissive for viral replication and produced linear forms of DNA, the structure of the terminal fragments was analyzed in each passage of C15 in nude mice.

EBV virion DNA has multiple copies of 500-bp direct

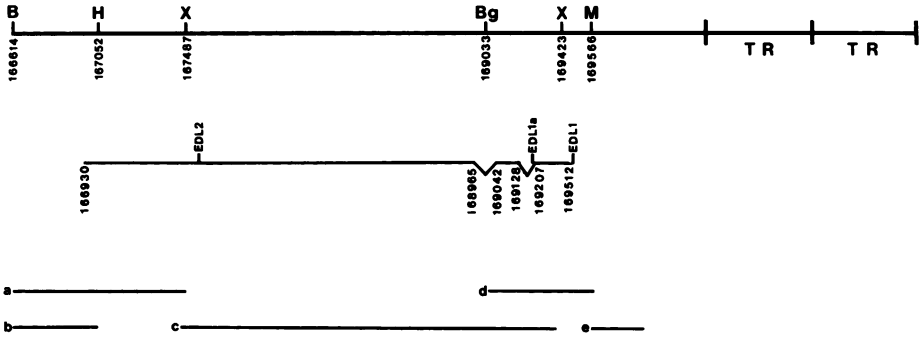

FIG. 1. Diagram of the coding region for LMP. The top line shows a partial restriction map, with cut sites for *Bam*HI (B), *Hin*cII (H), *Xho*I (X), *Bgl*II (Bg), and *Mlu*I (M) indicated. The second line is a schematic of the mRNA coding for LMP, indicating known promoters. The transcription start site and splice donor and acceptor sites are numbered according to genomic base pair coordinates from the B95-8 sequence (2). Probes made from subfragments of this region for Northern blot analysis are as follows: a, *Bam*HI to *Xho*I; b, *Bam*HI to *Hin*cII; c, *Xho*I to *Xho*I; d, *Bgl*II to *Mlu*I; e, *Mlu*I to bp 169787. These subfragments were transferred to $pGem_2$ vector for the synthesis of strand-specific riboprobes.

tandem repeats at each terminus (10). After digestion with restriction enzymes, the terminal fragments are heterogeneous in size, varying by increments of 500 bp, and form a ladder on gels or Southern blots. The linear termini are joined intracellularly to form covalently closed episomal DNA (16). The fused terminal fragments can be distinguished from the linear forms by their larger size and will hybridize to probes from either end of the genome. In cell lines that produce virus, both fused termini and ladder arrays of smaller fragments are detected (22).

A restriction enzyme map of the *Bam*HI portion of the Dhet fragment, including two terminal repeats and the structure of the LMP gene, is shown in Fig. 1; the kilobase coordinates with respect to the B95-8 genome are designated (2). Hybridization with a ^{32}P-labeled 1.9-kb *Xho*I fragment (Fig. 1, probe c), representing unique DNA adjacent to the terminal repeats, to a Southern blot prepared with *Bam*HI-digested DNA from two different passages of C15 and DNA from the Raji cell line identified a single fused terminal fragment of 8.0 kb in C15 and a 23-kb fragment in Raji DNA (Fig. 2A). The fused terminal *Bam*HI fragment in Raji DNA is exceptionally large because of the loss of the rightmost *Bam*HI site characteristic of most EBV strains, which is retained in C15 DNA. Linear forms of the viral DNA were not detected in any of the nude mouse passages of the C15 tumor. These data suggest that there were no foci of lytically infected cells in any of the passages of the C15 tumor.

Transcription from the sequences that encode LMP. Earlier studies of EBV expression in NPC biopsy specimens or NPC passaged in nude mice detected abundant transcription from the *Eco*RI Dhet fragment (24). To identify the specific mRNAs encoded by *Eco*RI DIJhet, the 1.9-kb *Xho*I fragment within the LMP-coding sequences (Fig. 1, probe c) was used to compare transcription in C15 with that in lymphoid cell lines. The *Xho*I 1.9-kb probe identified a highly abundant 2.8-kb transcript in poly(A)$^+$ RNA from B95-8 cells and a 2.8-kb mRNA in the CB4 cell line, which was established by infection of neonatal lymphocytes with B95-8. In C15, two

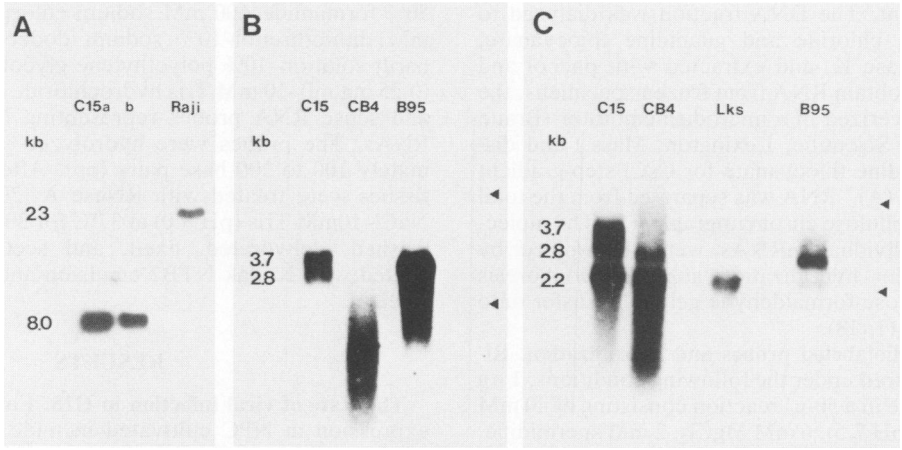

FIG. 2. (A) Identification of the EBV terminal fragments. Shown is hybridization of a ^{32}P-labeled *Xho*I 1.9-kb fragment (Fig. 1, probe c) to DNA digested with *Bam*HI from two separate passages of C15 and the lymphoid cell line Raji. Sizes of the fragments are indicated on the left. (B) Identification of mRNAs homologous to LMP-encoding sequences. Shown is hybridization to a Northern blot prepared with poly(A)$^+$ RNA from C15 and the EBV-infected lymphoid cell lines CB4 and B95-8 to a strand-specific riboprobe complementary to LMP (Fig. 1, probe c). Arrowheads indicate locations of the 5.0- and 2.0-kb rRNAs. (C) Relative abundance of the LMP mRNA in C15, CB4, and B95-8 cells. A Northern blot prepared with poly(A)$^+$ RNA from C15, CB4, B95-8, and the EBV-negative lymphoid cell line Loucke (Lks) was hybridized to 175×10^6 cpm of a probe representing the 5′ end of the LMP mRNA (probe d) and 2×10^6 cpm of a probe for actin mRNA.

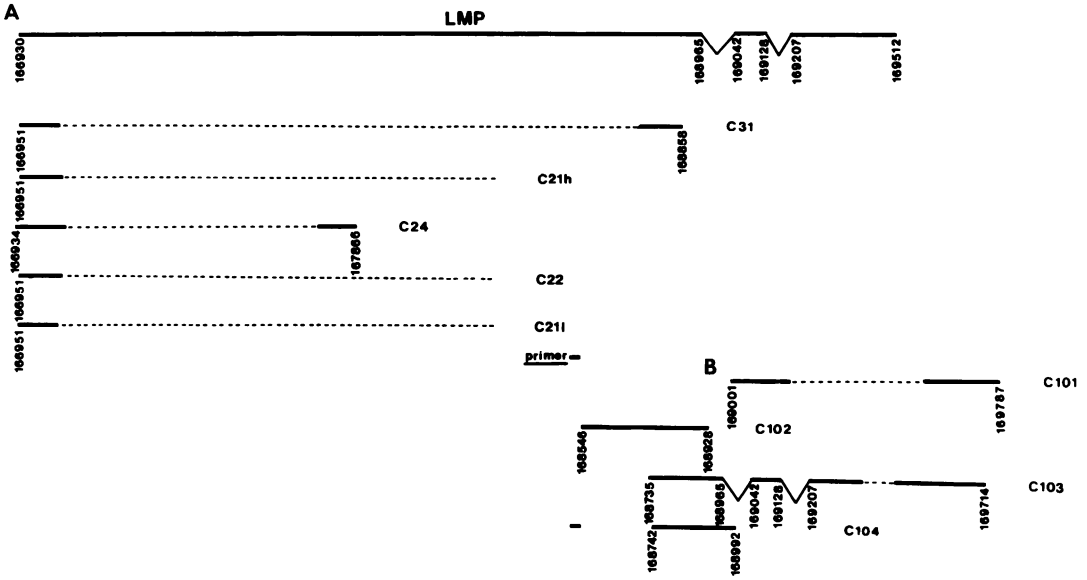

FIG. 3. Sequence of LMP-specific cDNAs in C15. Symbols: ■, sequenced region; - - -, inferred sequence. The primer used for cDNA synthesis of C101, C102, C103, and C104 from bp 168542 through 168562 is indicated. C104 contained the primer sequence but did not contain the intervening sequence to base 168742.

mRNAs of approximately equal abundance of 2.8 and 3.7 kb were detected (Fig. 2B).

In B95-8, the LMP mRNA is initiated from the EDL1 promoter at bp 169512 (9, 14). A related abundant 2.8-kb mRNA is transcribed during replication from the EDL1a promoter (Fig. 1) (14). To determine the relative abundance of the 2.8- and 3.7-kb mRNAs in C15 in comparison with the LMP mRNA in B95-8 and CB4, a Northern blot was hybridized to a probe that spans the sequences from the *Bgl*II site at bp 169033 to the *Mlu*I site at bp 169566 (Fig. 1, probe d). This probe is relatively specific for the LMP RNA initiated from the EDL1 promoter, since only 135 of the 533 bp are included in the replicative mRNA transcribed from the EDL1a promoter. A small amount of probe for actin mRNA was included in the hybridization to determine the relative levels of RNA per lane. Because the LMP mRNA is abundantly transcribed in B95-8, to permit alignment with the C15 mRNA, the blot was prepared with approximately one-fourth as much B95-8 RNA. The actin probe identified the 2.2-kb actin mRNA at equivalent levels in C15 and in CB4 and at an expected lower level in B95-8. The 5′-end LMP probe identified the 3.7- and 2.8-kb mRNAs at equivalent levels in C15, an equally abundant 2.8-kb mRNA in CB4, and an abundant 2.8-kb mRNA in the B95-8 lane, which contained much less total RNA (Fig. 2C). In the EBV-negative lymphoid line, Louckes, only the 2.2-kb actin mRNA was identified. These data revealed that the two mRNAs in C15 contained sequences specific for the 5′ end of LMP and were transcribed at approximately the same level as the LMP mRNA in the CB4 cell line.

To further characterize the 2.8- and 3.7-kb mRNAs, probes were constructed which extended from the *Bam*HI site at bp 166614 to the *Xho*I site at bp 167487 (Fig. 1, probe a) and to the *Hin*cII site at bp 167052 (Fig. 1, probe b). In B95-8, the LMP mRNA utilizes the polyadenylation signal at bp 166950 and terminates approximately at bp 166930. These probes overlap the LMP mRNA coding sequence at the 3′ end by approximately 550 and 120 bp, respectively, and therefore should hybridize more strongly to the 3.7-kb message if it extends past the LMP termination signal. The probes identified the 3.7- and 2.8-kb messages in C15 with approximately equal intensity (data not shown), suggesting that both RNAs terminate at the polyadenylation signal at bp 166950.

Analysis of C15 cDNAs. To analyze EBV expression in NPC in greater detail, a cDNA library was constructed in λgt11 from polyadenylated RNA after priming first-strand synthesis with oligo(dT). Alternative polyadenylation and splicing are key elements in the regulation of EBV expression; therefore, priming cDNA synthesis with oligo(dT) should produce a cDNA library which is representative of the processed mRNA population (20). EBV-positive clones that were homologous to the coding region of LMP were identified. Restriction enzyme analysis of the longest cDNA, C31, indicated that this 1.8-kb cDNA was colinear with the 3′ end of LMP (Fig. 3).

The LMP cDNAs were subcloned into the $pGem_2$ vector (Promega) and were sequenced by the GemSeq method. The sequence of the left end of the cDNAs (C31, C21h, C22, and C211) revealed that the cDNAs extended to bp 166951, apparently utilizing the polyadenylation signal at bp 166950, the same signal utilized by the LMP transcript in B95-8 and in IB-4, a cell line established by infection of neonatal lymphocytes with B95-8 (Fig. 3) (9, 14). The cDNAs retained multiple restriction sites common to the 3′ exon of LMP (data not shown). The rightward end of C31 and C24 were also sequenced. These data, together with the size estimation of the cDNAs, indicated they were not spliced. Base pair polymorphisms were not detected in the sequenced regions of these five cDNAs.

To further characterize the 2.8- and 3.7-kb transcripts, a synthetic oligomer, representing bp 168542 to 168562, complementary to the LMP mRNA at a region 3′ to the second splice acceptor site was used as a primer for the synthesis of second cDNA library that would extend further 5′ (Fig. 4). Four cDNAs were identified. The C101 (0.8 kb), C102 (0.4 kb), C103 (0.9 kb), and C104 (0.3 kb) cDNAs were subcloned into $pGem_2$ and sequenced. The sequenced regions are

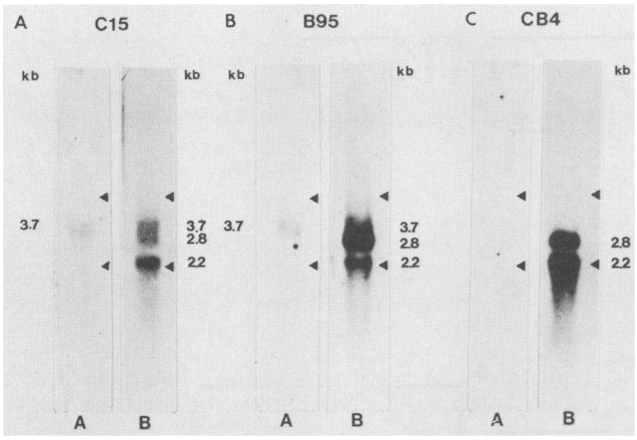

FIG. 4. Identification of the 3.7-kb mRNA. Duplicate blots were prepared with poly(A)$^+$ RNA from C15, CB4, and B95-8 cells. Lanes A were hybridized to 165×10^6 cpm of a strand-specific riboprobe prepared from the C101 cDNA representing sequences 5′ to the start of the LMP mRNA (Fig. 1, probe e); lanes B were hybridized to 175×10^6 cpm of the probe representing the 5′ end of the LMP mRNA and to 2×10^6 cpm of actin probe. The individual lanes for C15, CB4, and B95-8 were aligned by using the internal rRNAs (arrowheads).

presented in Fig. 3B. One of these cDNAs, C103, revealed two splices that are identical to the splices of the latent form of LMP (14). The 5′ end of this cDNA extended to bp 169714, which is 200 bp 5′ to the transcription initiation site for LMP in IB-4 and B95-8. This indicated that a transcript that could encode LMP can be initiated from a different promoter.

Both C101 and C104 contain sequences that represent intron sequences in B95-8 and in the C103 cDNA. The size of the C101 cDNA and the presence of characteristic restriction enzyme cut sites suggested that there are no splices in the unsequenced region of the C101 cDNA. It is likely that the C101 and C104 cDNAs represent unspliced precursor RNAs, since these cDNAs were synthesized by using a specific oligonucleotide. A cDNA library synthesized from C15, which was primed with random oligonucleotides, also contained spliced and unspliced cDNAs (13).

To identify the mRNAs represented by these cDNAs, the 5′ end of the C101 cDNA was subcloned from the *Mlu*I site at bp 169566 to the end of the cDNA at bp 169787. This probe only contains sequences which are 5′ to the published LMP mRNA start site and which were part of the unspliced C101 cDNA and the spliced C103 cDNAs (Fig. 1, probe e). Duplicate Northern blots were prepared with RNA from C15, CB4, and B95-8. One panel was hybridized to the cDNA 5′ end subclone (Fig. 1, probe e). The second panel was hybridized to the 5′ end LMP probe (Fig. 1, probe d) and to a small amount of actin probe. After hybridization, the individual lanes were aligned, using the rRNAs as internal size markers. The 5′ end LMP probe identified both the 2.8- and 3.7-kb mRNAs in C15, which were expressed at equal levels (Fig. 4A, lane B). The probe also identified a discrete 2.8-kb mRNA in CB4 and an abundant 2.8-kb mRNA in B95-8 which trailed into the 3.7-kb size range. The 5′ end cDNA probe identified the 3.7-kb mRNA in C15 and a 3.7-kb mRNA in B95-8 but did not identify the 2.8-kb mRNA in C15, B95-8, or CB4 (Fig. 4, lanes A). These data indicate that in C15, the 3.7-kb mRNA but not the 2.8-kb mRNA contains sequences 5′ to the LMP promoter. A related 3.7-kb mRNA is transcribed in B95-8 at much lower levels than the 2.8-kb LMP mRNA. The presence of the 3.7-kb mRNA in B95-8 RNA is obscured by the abundant 2.8-kb LMP mRNA; therefore, it can only be detected by a probe that does not hybridize to the 2.8-kb LMP mRNA. These hybridizations and the sequence data suggest that the C103 cDNA, which would encode LMP, represents the 3.7-kb mRNA.

Polymorphisms of LMP sequence. A total of seven base pair polymorphisms were observed in the sequence of the C103 cDNA as compared with B95-8 (Fig. 5) (2). One of these polymorphisms, a conversion of a T to an A, is present in the untranslated region 5′ to the translation start site of LMP at bp 169474. The other six are present in the coding region for LMP, and all theoretically cause changes in the amino acid sequence when compared with the EBV strain B95-8 (Fig. 5). The amino acid conversions were all conservative and would alter neither the net charge nor the probable conformation of the protein. Five of the six changes are located in the amino-terminal transmembrane region of the protein. The other substitution was detected in the C102 cDNA and occurred in the negatively charged carboxy terminus, resulting in conversion of a serine to a threonine.

Transcription from the *Bam*HI-A region. The C15 cDNA library primed with oligo(dT) contained several clones homologous to *Bam*HI-A. To identify the mRNAs represented by these cDNAs, a nick-translated probe consisting of the *Eco*RI Dhet portion of *Bam*HI-A was hybridized to a Northern blot of C15 and B95-8 RNA (Fig. 6A). This probe identified a 4.8-kb mRNA in the C15 sample and a 4.2-kb message in both uninduced and 12-*O*-tetra decanoylphorbol-13-acetate-induced B95-8 samples. Related mRNAs were not detected in either Raji or CB4, although other early mRNAs expressed from other regions of the viral genome could be detected in these cell lines.

One of the cDNAs homologous to *Bam*HI-A, C25a, was estimated to be 1.3 kb long and contained a single *Eco*RI site. The C25a cDNA and the *Eco*RI subfragments were transferred to the $pGem_2$ vector for sequence analysis and to generate strand-specific riboprobes for Northern blot analysis. A strand-specific riboprobe synthesized from the 1.3-kb C25a cDNA was hybridized to a Northern blot that contained lanes of C15 and CB4 duplicate to those shown in Fig. 2C. The C25a cDNA probe identified in C15 RNA an abundant 4.8-kb mRNA transcribed rightward and two larger mRNAs of 6.4 and 7.6 kb (Fig. 6B). This finding was surprising since the cDNAs were within a major leftward ORF, BALF3, and a rightward ORF was not described (2). Although both the LMP mRNA and actin mRNA were readily detected in the duplicate blot of CB4 RNA (Fig. 2C), transcription from *Bam*HI-A was not detected in CB4 RNA.

Sequence analysis of three of the *Bam*HI-A cDNAs revealed that all three terminated at an AATAAA polyadenylation signal at bp 160986 (Fig. 6C). The longest of the cDNAs, C25a, terminated at the left end at bp 159541. The sequence of this cDNA revealed a single splice which eliminated an intron of 171 bp. A search for rightward ORFs in C25a identified a methionine codon 752 bases downstream of the 5′ end of the cDNA at bp 160293, within an uninterrupted reading frame extending nearly the entire length of the cDNA. An identical ORF is present in the B95-8 sequence and extends from bp 159579 through 160991 (2). Although a stop codon is not present in the cDNA, a TAA stop codon is 6 bp 3′ to the end of the cDNA and is presumably present in the analogous polyadenylated parent molecule. This ORF would produce a peptide of 18 to 20

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-240  CCGCTTTTCTAACTCAAACACACGCTTTCTACTTCCCTTTCTACGCTTACATG
-186  CACACACACACCGCCGCTTTCGGGTCTGTACCCGTACTGCCTCCGGCAGACCC
-133  CGCAAATCCCCCGGGCCTACATCCCAAGAAACACGCGTTACTCTGACGTAGCC
 - 80  GCCCTACAATAAGCCTCTCACACTGCTCTGCCCCCTTCTTTCCTCAACTGCCT
 - 27  TGCTCCTGACACACTGCCCTGAGGATGGAACACGACCTTGAGAGGGGCCCACC
B95                          MetGluHisAspLeuGluArgGlyProPro
C15                          MetGluHisAspLeuGluArgGlyProPro

  26  GGGCCCGCGACGGCCCCCTCGAGGACCCCCCCTCTCCTCTTCCCTAGGCCTTG
       GlyProArgArgProProArgGlyProProLeuSerSerSerLeuGlyLeu
       GlyProArgArgProProArgGlyProProLeuSerSerSerLeuGlyLeu

  79  CTCTCCTTCTCCTCCTCTTGGCGCTACTGTTTTGGCTGTACATCGTTATGAGT
      AlaLeuLeuLeuLeuLeuLeuAlaLeuLeuPheTrpLeuTyrIleValMetSer
      AlaLeuLeuLeuLeuLeuLeuAlaLeuLeuPheTrpLeuTyrIleValMetSer

 132  GACTGGACTGGAGGAGCCCTCCTTGTCCTCTATTCCTTTGCTCTCATGCTTAT
       AspTrpThrGlyGlyAlaLeuLeuValLeuTyrSerPheAlaLeuMetLeuIle
       AspTrpThrGlyGlyAlaLeuLeuValLeuTyrSerPheAlaLeuMetLeuIle

 185  AATTATAATTTTGATCATCTTTATCTTCAGAAGAGACCTTCTCTGTCCACTTG
       IleIleIleLeuIleIlePheIlePheArgArgAspLeuLeuCysProLeu
       IleIleIleLeuIleIlePheIlePheArgArgAspLeuLeuCysProLeu

 238  GAGCCCTTTGTCTACTCCTACTGATGATCACCCTCCTGCTCATCGCTCTCTGG
      GlyAlaLeuCysIleLeuLeuLeuMetIleThrLeuLeuLeuIleAlaLeuTrp
      GlyAlaLeuCysLeuLeuLeuLeuMetIleThrLeuLeuLeuIleAlaLeuTrp

 291  AATTTGCACGGACAGGCATTGTACCTTGGAATTGTGCTGTTCATCTTCGGGTG
       AsnLeuHisGlyGlnAlaLeuPheLeuGlyIleValLeuPheIlePheGlyCys
       AsnLeuHisGlyGlnAlaLeuTyrLeuGlyIleValLeuPheIlePheGlyCys

 344  CTTACTTGTCTTAGGTGTCTGGATCTACTTATTGGACATTCTCTGGCGACTTG
       LeuLeuValLeuGlyIleTrpIleTyrLeuLeuGluMetLeuTrpArgLeu
       LeuLeuValLeuGlyLeuTrpIleTyrLeuLeuAspIleLeuTrpArgLeu

 397  GTGCCACCATCTGGCAGCTTTTGGCCTTCTTCCTAGCCTTCTTCCTAGACCTC
      GlyAlaThrIleTrpGlnLeuLeuAlaPhePheLeuAlaPhePheLeuAspLeu
      GlyAlaThrIleTrpGlnLeuLeuAlaPhePheLeuAlaPhePheLeuAspLeu

 450  ATCCTGCTCATTATTGCTCTCTATCTACAACAAAACTGGTGGACTCTATTGGT
       IleLeuLeuIleIleAlaLeuTyrLeuGlnGlnAsnTrpTrpThrLeuLeuVal
       IleLeuLeuIleIleAlaLeuTyrLeuGlnGlnAsnTrpTrpThrLeuLeuVal

 503  TGATCTCCTTTGGCTCCTCCTGTTTCTGGCGATTTTAATCTGGATGTATTACC
       AspLeuLeuTrpLeuLeuLeuPheLeuAlaIleLeuIleTrpMetTyrTyr
       AspLeuLeuTrpLeuLeuLeuPheLeuAlaIleLeuIleTrpMetTyrTyr

 556  ATGGACAACGACACAGTGATGAACACCACCACGATGACTCCCTCCCGCACCCT
      HisGlyGlnArgHisSerAspGluHisHisHisAspAspSerLeuProHisPro
      HisGlyGlnArgHisSerAspGluHisHisHisAspAspSerLeuProHisPro

 609  CAACAAGCTACCGATGATTCTGGCCATGAATCTGACTCTAACTCCAACGAGGG
       GlnGlnAlaThrAspAspSerGlyHisGluSerAspSerAsnSerAsnGluGly
       GlnGlnAlaThrAspAspSerGlyHisGluSerAspSerAsnSerAsnGluGly

 662  CAGACACCACCTGCTCGTGACTGGAGCCGGCGACGGACCCCCACTCTGCTCTC
       ArgHisHisLeuLeuValSerGlyAlaGlyAspGlyProProLeuCysSer
       ArgHisHisLeuLeuValThrGlyAlaGlyAspGlyProProLeuCysSer

 715  AAAACCTAGGCGCACCTGGAGGTGGTCCTGACAATGGCCCACAGGACCCTGAC
      GlnAsnLeuGlyAlaProGlyGlyGlyProAspAsnGlyProGlnAspProAsp
      GlnAsnLeuGlyAlaProGlyGlyGlyProAspAsnGlyProGlnAspProAsp

 768  AACACTGATGACAATGGCCCACAGGACCCTGACAACACTGATGACAATGGCC
       AsnThrAspAspAsnGlyProGlnAspProAspAsnThrAspAspAsnGly
       AsnThr----------

 821  CACATGACCCGCTGCCTCAGGACCCTGACAACACTGATGACAATGGCCCACAG
      ProHisAspProLeuProGlnAspProAspAsnThrAspAspAsnGlyProGln
       ----------

 874  GACCCTGACAACACTGATGACAATGGCCCACATGACCCGCTGCCTCATAGCCC
       AspProAspAsnThrAspAspAsnGlyProHisAspProLeuProHisSerPro
       ----------

 927  TAGCGACTCTGCTGGAAATGATGGAGGCCCTCCACAATTGACGGAAGAGGTTG
       SerAspSerAlaGlyAsnAspGlyGlyProProGlnLeuThrGluGluVal
       ----------

 980  AAAACAAAGGAGGTGACCAGGGCCCGCCTTTGATGACAGACGGAGGCGGCGGT
      GluAsnLysGlyGlyAspGlnGlyProProLeuMetThrAspGlyGlyGlyGly
       ----------

1033  CATAGTCATGATTCCGGCCATGGCGGCGGTGATCCACACCTTCCTACGCTGCT
       HisSerHisAspSerGlyHisGlyGlyGlyAspProHisLeuProThrLeuLeu
       ----------

1086  TTTGGGTTCTTCTGCTTCCGGTGGAGATGATGACGACCCCCACGGCCCAGTTC
       LeuGlySerSerGlySerGlyGlyAspAspAspAspProHisGlyProVal
       ----------

1139  AGCTAAGCTACTATGACTAA
      GlnLeuSerTyrTyrAsp---
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kilodaltons. A C15 cDNA library prepared by using random hexamers as primer for cDNA synthesis has been previously described. This library contained multiple spliced and unspliced cDNAs homologous to *Bam*HI-A; however, the cDNAs did not encompass the 3′ end of the mRNAs and did not contain this ORF.

Hybridization in situ. To identify the functions expressed in latent lymphoid infection, it was important to determine that the cell lines did not express RNAs associated with virus replication and to demonstrate that the function is expressed in every latently infected cell. Although transcription from the *Bam*HI Z, H, and M fragments, which encode functions involved in replication, was not detected (data not shown), it is possible that replicative mRNAs could be abundantly expressed in a few abortively infected cells the majority of which are latently infected. To investigate the extent of expression of the *Bam*HI-A gene in comparison with LMP, pieces of the C15 tumor were hybridized in situ to ^{35}S-labeled RNA probes synthesized from the *Bam*HI cDNA and the *Xho*I 1.9-kb fragment in both sense and antisense orientations. Hybridization with the single-stranded RNA probe antisense to the *Bam*HI-A mRNA revealed that the mRNA was expressed in all cells within the C15 tumor (Fig. 7A). Hybridization was specific to the tumor cells and was not detected in the normal mouse epithelium at the bottom left of the tissue section. Hybridization with an RNA probe antisense to the LMP mRNA (shown at higher magnification in Fig. 7B) detected LMP transcription specifically in all of the tumor cells. Hybridization with the sense probes for both *Bam*HI-A and LMP was negative, indicating that the opposite strands were not transcribed and that hybridization was specific for the EBV RNAs and was not detecting EBV DNA (Fig. 7C and D).

DISCUSSION

This study reveals several differences in virus expression in an NPC, C15, in comparison with the prototype lymphoid strain, B95-8. In the C15 tumor, two mRNAs are transcribed from the sequences that encode LMP. Interestingly, sequence analysis of the C15 LMP cDNAs suggests that another promoter can be utilized for LMP expression. The 5′ end of the C103 cDNA, which is spliced identically to the B95-8 LMP mRNA and could encode LMP, is at bp 169714, 200 bp 5′ to the LMP promoter identified in B95-8-transformed lymphocytes. A probe representing these sequences identified the 3.7-kb mRNA in C15 and in B95-8. Since the 5′ end of the C103 cDNA is only 200 bp away from the start of the terminal repeats (TR), the 3.7-kb mRNA may be a transcript that originates at the left end of the EBV genome and is transcribed leftward across the TR from the episomal DNA or may initiate within the TR. Two other mRNAs that originate in *Bam*HI-A and transverse the TR rightward have been described in B95-8 (17). The detection of the 3.7-kb mRNA in B95-8 and C15 suggests that a putative promoter for LMP at the left end of the genome may be utilized in both lymphoid and epithelial cells. Two potential promoters have been described for expression of the EBNAs (3, 27). The data presented here suggest that the regulation of LMP may be equally complex and that different promoters may be used

FIG. 5. Sequence of LMP in C15, showing a comparison of the C103 cDNA sequence data with the sequence of LMP in B95-8 cells. Differences in nucleotides and amino acids are in boldface. - - -, Inferred sequence of LMP in C15.

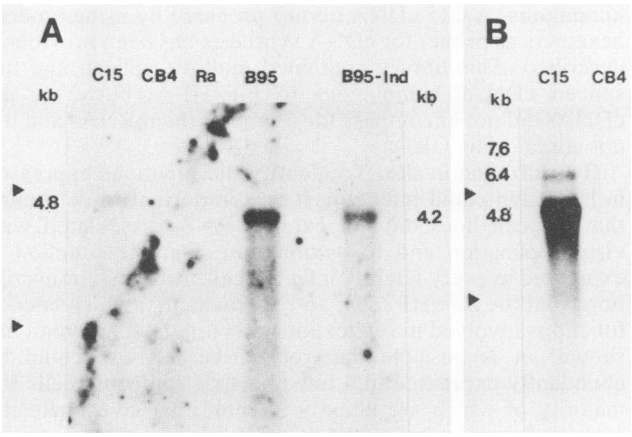
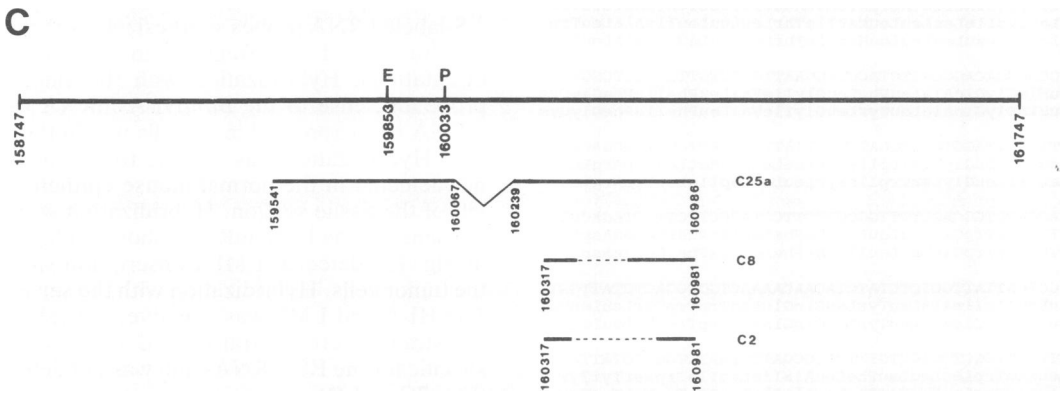


FIG. 6. Identification of mRNAs encoded by *Bam*HI-A. (A) The 6.7-kb *Eco*RI-*Bam*HI subfragment of *Bam*HI-A was labeled with ^{32}P by nick translation and hybridized to a Northern blot prepared with poly(A)$^+$ RNA from C15, Raji, CB4, and uninduced (B95) and 12-*O*-tetradecanoylphorbol-13-acetate-induced (B95-Ind) B95-8 cells. Positions of rRNAs are indicated with arrowheads. Sizes of identified mRNAs are indicated. (B) Identification of the mRNAs represented by the C15 cDNAs homologous to *Bam*HI-A. A strand-specific riboprobe synthesized from right to left was prepared from the C25a cDNA, representing bases 160986 to 159541. The probe was hybridized to a Northern blot containing poly(A)$^+$ RNA from C15 and CB4 prepared in duplicate with that shown in Fig. 2C. rRNAs are indicated with arrowheads. (C) Structure of the *Bam*HI A cDNAs. The schematic at the top is a partial restriction map of *Bam*HI-A from bp 158747 to 161747. Symbols: ■, sequenced region; - - -, inferred sequence.



in different situations. The 3.7-kb mRNA but not the 2.8-kb mRNA has also been identified in mRNA from an NPC biopsy; therefore, it is possible that the promoter for this mRNA is more frequently utilized in NPC or perhaps in epithelial cells (22).

Sequence analysis of the C15 LMP cDNAs reveals several changes in comparison with the LMP sequence in B95-8 cells. Seven base pair changes were detected in the sequence of 800 bp of the 5′ end of the LMP cDNA. Of these, six result in conservative amino acid changes that would not alter the net charge or folding pattern of the protein. This may indicate structural and functional constraints on variation in LMP. Sequence analysis of the LMP-encoding sequence in the Raji EBV strain revealed 30 base pair substitutions in 1,300 nucleotides, resulting in 21 amino acid substitutions (12). Although the LMP-encoding sequence in C15 is considerably less divergent than in the Raji strain, there may be selection favoring antigenic variation in LMP while conserving important structural components.

LMP is apparently an important component in EBV-induced transformation in that it has profound effects on the phenotype of lymphoid cells and can transform Rat-1 cells in vitro, conferring the ability to grow in nude mice (33, 34). As it is a rare NPC that can be established in nude mice, the successful transplantation of the C15 tumor may reflect the continued expression of LMP, which may not be expressed in all tumors in vivo (5). Analysis of EBV proteins expressed in NPC biopsy samples detected LMP in approximately half of the tumors and detected strong expression of the 60-kilodalton full-length form of LMP in C15 on immunoblots probing with the CS1-4 pool of monoclonal antibodies. However, another anti-LMP monoclonal antibody, S12, did not react with C15 LMP (36). It is possible that divergence in the LMP expressed in NPC biopsy specimens may have prevented recognition by the monoclonal antibodies.

The detection of transcription from the *Bam*HI A fragment is intriguing in that *Bam*HI-A is not transcribed in latently infected lymphocytes. The lack of detectable linear DNA or expression of early functions in C15 indicates that the *Bam*HI-A transcripts are not associated with replication (36). A recent study of EBV transcription in C15 also identified abundant cDNAs from the *Bam*HI A and adjacent *Bam*HI I fragments in a library synthesized by using random oligonucleotides as primer (18). This library represented total RNA and contained both spliced and unspliced cDNAs. The absence of a major ORF within the previously described cDNAs prompted the suggestion that the RNAs may be negative regulators for expression of replication functions encoded by the opposite strand. However, the cDNAs analyzed here were synthesized by priming with oligo(dT) and encompassed the 3′ end of the mRNAs. The sequence of these cDNAs revealed an ORF with an internal methionine codon that could potentially encode an 18- to 20-kilodalton protein. The consistent expression of rightward transcription from this ORF in all of the C15 tumor cells suggests that this ORF encodes an essential function in epithelial cells.

The regulation of herpesvirus expression is complex and involves both cellular and virus-encoded transcription factors. Therefore, it is likely that epithelial- or lymphoid-specific cellular transcription factors would influence the expression of viral genes. The expression of distinct viral genes could, in turn, alter the growth regulation of epithelial or lymphoid cells. It is possible that the 3.7- and 4.8-kb mRNAs are preferentially expressed in epithelial cells. The

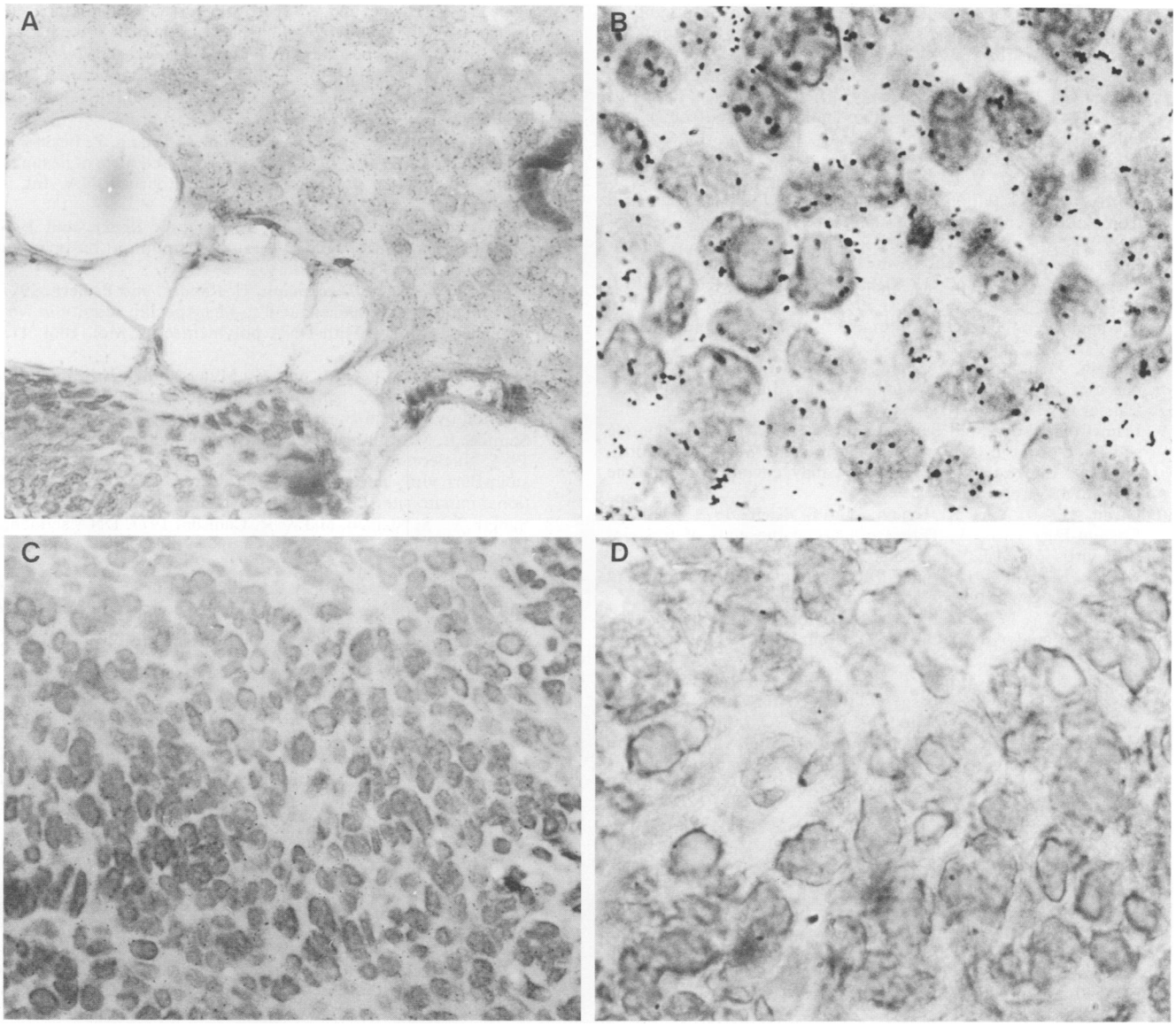


FIG. 7. Detection of the *Bam*HI-A RNA and LMP RNA in situ. (A) A ^{35}S-labeled RNA probe synthesized to the *Bam*HI-A cDNA, homologous to the mRNA, was hybridized to a piece of C15 tissue, exposed to emulsion for 1 week, and photographed. Magnification, ×400. (B) A ^{35}S-labeled RNA probe homologous to the LMP mRNA was hybridized to C15 tissue, exposed to emulsion for 1 week, and photographed. Magnification, ×1,000. (C) A ^{35}S-labeled RNA probe synthesized in the sense orientation to the *Bam*HI A cDNA was hybridized to C15 tissue, exposed to emulsion for 1 week, and photographed. Magnification, ×400. (D) A ^{35}S-labeled RNA probe synthesized in the sense orientation to the LMP mRNA was hybridized to C15 tissue, exposed to emulsion for 1 week, and photographed. Magnification, ×1,000.

consistent expression of the *Bam*HI-A transcript in all of the EBV-infected epithelial cells suggests that the mRNA encodes a critical function that is involved in maintenance of latency in epithelial cells or affects cellular proliferation.

ACKNOWLEDGMENTS

We are grateful for the excellent assistance of Kathryn Flynn and Stephanie Oberhaus.

This work was supported by Public Health Service grant CA 19014 from the National Cancer Institute.

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