JOURNAL OF VIROLOGY, June 1993, p. 3182–3190
0022-538X/93/063182-09$02.00/0

Vol. 67, No. 6

Transcripts from the Epstein-Barr Virus *Bam*HI A Fragment Are Detectable in All Three Forms of Virus Latency

L. A. BROOKS, A. L. LEAR, L. S. YOUNG, AND A. B. RICKINSON*

Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham, Birmingham B15 2TJ, United Kingdom

Received 2 November 1992/Accepted 25 February 1993

An unexpected feature of the latency II form of Epstein-Barr virus (EBV) infection seen in the epithelial tumor nasopharyngeal carcinoma (NPC) is the presence of spliced polyadenylated RNAs encoded from the *Bam*HI A fragment of the viral genome and running in the opposite orientation to several *Bam*HI-A lytic cycle genes. The importance of these *Bam*HI-A transcripts and the specificity of their association with NPC remain to be determined. In this study, we examined the extent to which such RNAs are present in other transcriptionally distinct forms of EBV latency seen in B cells. Two independent assays of *Bam*HI-A transcription were employed: amplification across defined splice junctions in cDNAs, using the polymerase chain reaction, and in situ hybridization with a radiolabeled riboprobe specific for a putative open reading frame downstream of these splice junctions. Such methods, which easily detected *Bam*HI-A RNAs in fresh NPC biopsies and transplantable NPC lines, also revealed consistent expression of these transcripts in all EBV-positive Burkitt's lymphoma cell lines displaying the highly restricted latency I form of infection (*Bam*HI-F promoter usage) as well as in all EBV-transformed lymphoblastoid cell lines (LCLs) displaying the latency III form of infection (*Bam*HI-C/W promoter usage). Expression in established LCLs, occurring irrespective of virus producer status, was not a consequence of continued in vitro passage; thus, appropriately spliced *Bam*HI-A transcripts could be amplified from normal B cells within 1 day of their experimental infection in vitro, along with *Bam*HI-C/W promoter-initiated but not *Bam*HI-F promoter-initiated mRNAs. In situ hybridization both on Burkitt's lymphoma cell lines and on LCLs showed that essentially every cell contained *Bam*HI-A transcripts, although at levels apparently lower than those observed in NPC. We conclude that expression of the *Bam*HI-A RNAs is a consistent feature shared by all known forms of latent EBV infection.

Epstein-Barr virus (EBV), a herpesvirus now strongly linked to several human malignancies, can establish either fully productive (lytic) or nonproductive (latent) infections in target cells. The major target cell types for the virus in vivo are B lymphocytes and stratified squamous epithelium, and there is strong circumstantial evidence to suggest that both lytic and latent infections can be established in cells of either lineage, depending upon the precise stage of differentiation of the infected cell (5, 10, 17, 21). The association of the virus both with B-cell lymphomas and with nasopharyngeal carcinoma (NPC) emphasizes the importance of understanding the nature of virus latency both in lymphoid and in epithelial cell environments.

The analysis of EBV latent protein expression in cell culture model systems and in EBV-associated tumors themselves has highlighted the existence of three different forms of latency which we now refer to as I, II, and III. Latency I, seen in Burkitt's lymphoma (BL) biopsies and in derived BL cell lines which retain the tumor phenotype in vitro, is characterized by selective expression of the virus-coded nuclear antigen EBNA1 (11, 22). Latency II, seen in NPC and in some experimentally manipulated cells in vitro, is characterized by expression of EBNA1, of the latent membrane protein LMP1 (6, 30), and, from transcriptional analysis, probably also of LMP2 (2, 4, 26). Latency III, seen in immunoblastic B-cell lymphomas of the immunosuppressed and in EBV-transformed lymphoblastoid cell lines (LCLs) in vitro, is characterized by expression of all six EBNAs (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) in addition to LMP1 and LMP2 (15). Subsequent work at the RNA level has confirmed that these different forms of EBV latency are transcriptionally distinct. Thus, in latency I, EBNA1 is expressed from a 2.5-kb mRNA with a *Bam*HI-Q/U/K splice structure initiated from a novel promoter, Fp, located close to the *Bam*HI-FQ boundary; other latent promoters are silent (23, 25). In latency II, EBNA1 is again expressed from Fp and the LMPs are expressed from their individual promoters in *Bam*HI-N (2, 4, 14, 26, 27). In latency III, the LMP promoters are again active but Fp is silent (16) and the various EBNA mRNAs are all expressed from one of two upstream promoters, Cp or Wp, in the *Bam*HI-C or adjacent *Bam*HI-W region of the genome (28). One feature common to all three forms of latency, however, is abundant transcription of the small nonpolyadenylated EBER RNAs (8, 22), noncoding nuclear species whose function remains unknown.

In addition to the above-mentioned latent cycle transcripts, analysis of cDNA libraries established from the nude-mouse-passaged NPC line C15 has identified a series of multiply spliced rightward-running transcripts from the *Bam*HI-A region of the genome (9, 12). Northern (RNA) blotting with probes in this region detected a major 4.8-kb RNA and less abundant larger species in C15 and also in several other nude-mouse-passaged lines. In situ hybridization with similar probes confirmed that these transcripts were present in essentially every cell in the NPC lines and also detected specific labeling of the malignant cell population in several fresh NPC biopsies. These transcripts contain a number of potential open reading frames, in particular a region designated BARF0 or cBALF3 (complementary to the leftward-running lytic cycle open reading frame BALF3).

* Corresponding author.

The recent finding that in vitro transcription/translation of BARF0 yields polypeptides specifically recognized by NPC patient sera suggests that the *Bam*HI-A transcripts may indeed encode proteins in vivo (7).

To date, studies in this area have largely concentrated on NPC, and only recently has the question of *Bam*HI-A transcription in B-cell lines begun to be addressed (13). The present work uses well-characterized cell culture models to determine whether these *Bam*HI-A transcripts are a specific feature of epithelial cell infection, a specific feature of latency II, or a more general feature shared with other forms of EBV latency.

MATERIALS AND METHODS

Cells and biopsies. As reference material, these experiments used the transplantable EBV genome-positive NPC lines C15, C17, C18, and C19, originally established as heterotransplants in nude mice (3) and now serially propagated in severe combined immunodeficient (SCID) mice. The transplantable EBV genome-negative pharyngeal carcinoma cell line NOR (2) likewise maintained in SCID mice provided relevant control material. In addition, work was carried out on fresh NPC biopsies. Small punch biopsies taken from the postnasal space of Chinese NPC patients were immediately snap frozen; one half of the biopsy was used for histology and in situ hybridization analysis, and the other half was used for RNA analysis. In all cases, histological screening of an adjacent biopsy fragment had confirmed the presence of poorly differentiated NPC.

The panel of LCLs used in this study included C15 LCL, C18 LCL, and C19 LCL, established by transforming normal B lymphocytes from EBV-seronegative donors with infectious virus rescued from short-term cultures of the C15, C18, and C19 NPC cells, respectively (2, 12). The C15 LCL and C18 LCL were established and kindly supplied by K. Falk and I. Ernberg (Karolinska Institute, Stockholm, Sweden). Additional LCLs included lines established by B95.8 virus-induced transformation of adult B cells (designated SW/B95, etc.) or of fetal B cells (IB4 and FL389) and other lines of either adult or fetal origin carrying other EBV isolates (e.g., X50/7) (16). The panel of BL cell lines included Wan-BL, Chep-BL, Akata-BL, and WW2-BL, all EBV-positive lines retaining the group I BL cell phenotype in vitro (22). In addition, we used subclones of the Mutu-BL cell line displaying either the group I or group III phenotype (11). Throughout the work, the EBV-transformed marmoset cell line B95.8 and the EBV-negative B-cell lines BL40, BL41, and BJAB were used as positive and negative lymphoid cell controls, respectively.

RNA preparation and analysis. Total cellular RNA was extracted from cell lines or from tumor cells by using RNAzolB according to the protocol of the manufacturer (Cinna/Biotecx). Frozen tumor specimens were pulverized in a small glass homogenizer prior to extraction with RNAzolB.

For polymerase chain reaction (PCR) analysis, RNA samples (2 μg) were heated for 2 min at 90°C and then rapidly cooled on ice. Reverse transcription-PCR was carried out in a one-tube reaction consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.7 mM $MgCl_2$, 0.005% gelatin, 200 μM dATP, dTTP, and dCTP, 50 μM dGTP, 150 μM deaza-dGTP, and 2 μM each primer; 5 U of avian myeloblastosis virus reverse transcriptase (Pharmacia) and 10 U of RNAguard (Pharmacia) were added, and incubation was continued for 60 min at 42°C. After the addition of 2.5 U of *Taq* DNA polymerase, amplification was carried out for 40 cycles of denaturation for 30 s at 94°C, primer annealing for 90 s at 45°C, and extension for 240 s at 70°C. Amplified samples were subsequently analyzed by electrophoresis through 3% Nusieve agarose gels followed by Southern transfer onto Hybond N+ nylon membranes (Amersham).

For detection of *Bam*HI-A transcripts, the *Bam*HI-A oligonucleotide primer pairs A_1/A_2 and A_3/A_4 were designed across splice junctions in the 18.8 cDNA (originally reported by Hitt et al. [12]) as illustrated in Fig. 1. The sequences and genomic coordinates of these primers and of the relevant oligonucleotide probes are given in Table 1. Details of other primer/probe combinations used to detect specific EBV transcripts have been published as follows: 5′ Q and 3′ K primers plus U probe for Fp-initiated EBNA1 transcripts and 5′ Y3 and 3′ K primers plus U probe for Cp/Wp-initiated EBNA1 transcripts (2); 5′ W_0/W_1 and 3′ W_2 primers plus W_2 probe for Wp-initiated EBNA transcripts and 5′ C_1/C_2 and 3′ W_2 primers plus W_2 probe for Cp-initiated EBNA transcripts (14); and 5′ H_2 and 3′ H_3 primers and BHRF1 probe for lytic cycle BHRF1 transcripts (16).

In situ hybridization. To provide in situ hybridization riboprobes specific for *Bam*HI-A transcripts, a genomic fragment of B95.8 DNA which overlapped the putative BARF0 open reading frame and which was contained within the c25a cDNA (originally reported by Gilligan et al. [9]; Fig. 1) was subcloned into the *Bam*HI-*Eco*RI sites of pBluescript KS^+. The fragment (B95.8 genome coordinates 160212 to 160532 bp) was originally PCR amplified from the D214.HET M13Mp8 clone (kindly provided by P. Farrell, Ludwig Institute, London, England) by using primers in the M13 flanking sequences; the correct identity of the PCR-amplified fragment was confirmed by direct sequencing analysis (data not shown). For the preparation of in situ probes, sense and antisense runoff transcripts were generated from the linearized pBluescript KS^+ construct by using either T3 or T7 RNA polymerase in the presence of 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 5 mM each ATP, GTP, and CTP, 10 mM dithiothreitol, and 60 μCi of lyophilized [^{35}S]UTP (Amersham) in a total volume of 10 μl. After incubation for 90 min at room temperature, template DNA was removed with 1 U of DNase I (Pharmacia), and the length of RNA probes was adjusted to between 100 and 200 bases by controlled alkaline hydrolysis. Additional sense and antisense riboprobes were likewise prepared from the *Bam*HI-W internal repeat region of the genome cloned into pBluescript KS^+ (plasmid pBSW [18]); these riboprobes allowed specific detection of Cp- and Wp-initiated transcripts.

For in situ hybridization to RNA, frozen sections of tumor specimens or cytospin preparations of control cell lines were fixed in 4% (vol/vol) paraformaldehyde in phosphate-buffered saline and then dehydrated through graded ethanols. Hybridization was performed as previously described (18).

RESULTS

PCR analysis of *Bam*HI-A transcripts in NPC. The initial experiments used the nude-mouse-passaged NPC lines C15 to C19 as positive controls in order to validate *Bam*HI-A transcript detection by the A_1/A_2 and A_3/A_4 PCR primer pairs designed to amplify across splice junctions in the 18.8 cDNA (Fig. 1); this cDNA, representing the prototype *Bam*HI-A rightward-running transcript, was originally cloned from the C15 NPC line (12). Preliminary experiments confirmed the specificity of the amplified products obtained by using these primer pairs; thus, detection was absolutely

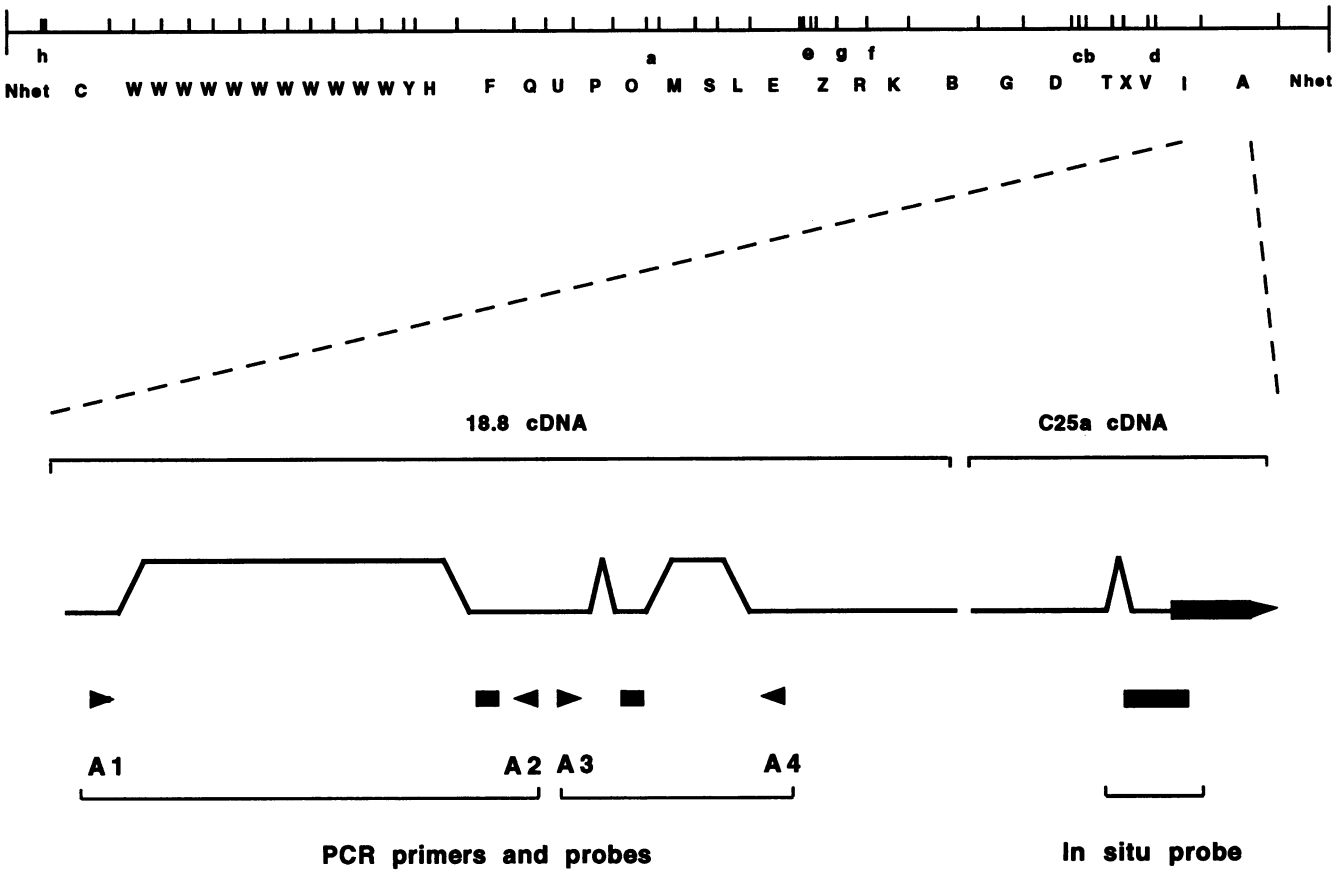

FIG. 1. Diagrammatic representation of the splice structure of *Bam*HI-A transcripts as revealed by the 18.8 and c25a cDNAs cloned from C15 NPC cells (9, 12). Transcripts are shown below a *Bam*HI restriction map of the genome displayed in its linear form. The positions of the two PCR primer combinations used in this work, A_1 (5′ ▶)/A_2 (3′ ◀) and A_3 (5′ ▶)/A_4 (3′ ◀), and of their internal oligonucleotide probes (■) are shown relative to the 18.8 cDNA structure; likewise, the position of the in situ hybridization riboprobe (▩) is shown relative to the c25a cDNA structure. The putative BARF0 open reading frame is identified within the C25a sequence (▬▶). Note that subsequent cDNA isolations have confirmed that the immediately adjacent 18.8- and C25a-derived sequences are indeed contiguous in *Bam*HI-A transcripts (13).

dependent upon the inclusion of a reverse transcription step in the reaction and also upon the use of the correct internal probe (data not shown). As illustrated in Fig. 2B and D, the predicted 163-bp A_1/A_2-primed product and 232-bp A_3/A_4-primed product were obtained upon amplification of RNA from all four EBV-positive SCID mouse-passaged NPCs but not from the EBV-negative SCID mouse-passaged pharyngeal carcinoma line NOR. We then extended the analysis to include a number of fresh snap-frozen NPC biopsy samples

TABLE 1. *Bam*HI-A oligonucleotide sequences

Designation	EBV genome coordinates[a]	Sequence
5′ A_1 primer	Raji insert 10604–10623	5′-ATGGCCGGAGCTCGTCGACG-3′
3′ A_2 primer	155866–155847	5′-CCTTCGATATCGAGTGTCTG-3′
A_1/A_2 probe	155777–155796	5′-ACCAGAGGACGCAGGATATC-3′
5′ A_3 primer	157154–157173	5′-AGAGACCAGGCTGCTAAACA-3′
3′ A_4 primer	159194–159175	5′-AACCAGCTTTCCTTTCCGAG-3′
A_3/A_4 probe	157359–157378	5′-AAGACGTTGGAGGCACGCTG-3′

[a] Coordinates refer to the B95.8 genomic sequence (1) with the exception of the *Bam*HI-A 5′ A_1 primer coordinates. This latter primer sequence lies within a 11,835-bp fragment present in the Raji (as well as most other) virus strains but deleted from B95.8 between nucleotides 152012 and 152013 of the B95.8 sequence; the *Bam*HI-A 5′ A_1 primer coordinates are therefore given relative to the 5′ end of the Raji insert sequence (19).

and found that these also gave specific amplification of the correctly sized cDNA products (Fig. 2A and C). Here the controls included the EBV-negative BL cell line BL41, which gave no signal with either primer combination, and the EBV-positive LCL B95.8, which showed specific amplification only with the A_3/A_4 primers; this was as expected since there is an 11.8-kb deletion within the *Bam*HI-I/A region of the B95.8 genome (1, 19) which removes the A_1 oligonucleotide primer sequence (Table 1).

These initial assays also included cDNA amplifications from three LCLs generated by in vitro transformation of normal B cells with infectious virus rescued from bromodeoxyuridine-induced C15, C18, and C19 NPC cells. The results are shown in Fig. 2B and D; it is clear that the *Bam*HI-A transcripts are also present in all three LCLs.

PCR analysis of *Bam*HI-A transcripts in LCL cells. In view of these findings, the next set of experiments examined a wider range of LCLs, including eight reference lines which recent analysis had carefully characterized for virus-producer status (16). Of these, four LCLs (IB4, X50/7, FL389, and SW/B95) were tightly latent by all criteria, whereas the other four (AR/B95, CM/B95, DH/B95, and B95.8) showed different degrees of spontaneous entry into lytic cycle (16). This finding was confirmed in the present analysis; all eight reference lines gave amplifiable *Bam*HI-Y3/U/K-spliced

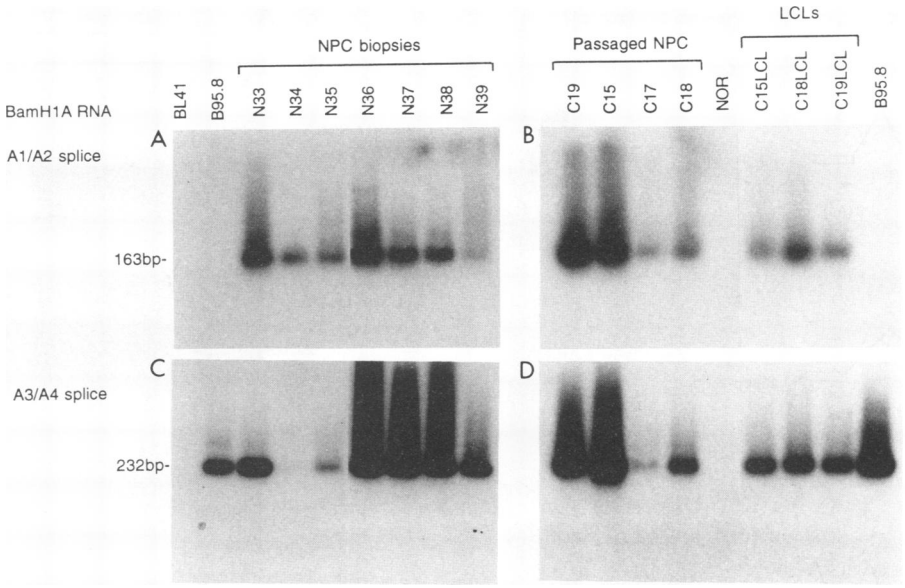

FIG. 2. PCR analysis of *Bam*HI-A transcription in fresh NPC biopsies N33 to N39 (A) and in SCID mouse-passaged NPCs C15 to C19 and in LCLs established by in vitro transformation of normal B cells with EBV rescued from C15 to C19 (B). Results are shown for assays using the A_1/A_2 primer combination (A and B) and the A_3/A_4 primer combination (C and D); in each case, the PCR products were screened by using the relevant internal oligonucleotide probe. Designated sizes of the amplified products are in accord with those predicted from the published structure of *Bam*HI-A transcripts (13). Control cells included in the analysis were the EBV-positive B95.8 LCL (in which the A_1 primer sequence is not present as a result of a deletion in the B95.8 EBV genome), the EBV-negative B-cell line BL41, and the EBV-negative SCID mouse-passaged carcinoma cell line NOR.

EBNA1 mRNA diagnostic of Cp/Wp-initiated latent transcripts (23), while only the four spontaneously productive LCLs contained amplifiable signals diagnostic of the spliced 1.7-kb BHRF1 early lytic cycle mRNA (20). Such reference amplifications are illustrated in Fig. 3B and C; we also confirmed the difference in virus-producer status between these two sets of lines by PCR amplification for the spliced BZLF1 immediate-early lytic cycle mRNA and by immunofluorescence staining for BZLF1 protein (16; unpublished observations). A parallel analysis of these same RNA preparations for *Bam*HI-A rightward transcripts is illustrated in Fig. 3A; we reproducibly detected the characteristic A_3/A_4 spliced product in all LCLs tested irrespective of virus-producer status, although in successive experiments we did note that the PCR signals tended to be slightly stronger from the spontaneously productive lines.

Since *Bam*HI-A transcripts appeared to be consistently present in established LCLs, we then examined whether such transcripts were detectable in a primary B-cell infection. Figure 4 presents the results of an experiment in which normal resting B cells isolated from tonsillar tissue were exposed to a B95.8 virus preparation and RNA was extracted from aliquots of the infected cells on days 1 to 4 postinfection. Using transcript-specific oligonucleotide primer/probe combinations, these RNA samples were then analyzed in parallel for the presence of *Bam*HI-A transcripts, of the Fp-initiated *Bam*HI-Q/U/K-spliced EBNA1 transcripts characteristic of latency I and II (2, 23), and of the Wp- and Cp-initiated EBNA transcripts characteristic of latency III (14, 29). As expected, the initial stages of infection were associated with the appearance of Wp- and Cp-initiated transcripts; interestingly, on day 1 postinfection, the Wp transcripts were more easily detectable, consistent with the reported sequential activation of Wp and

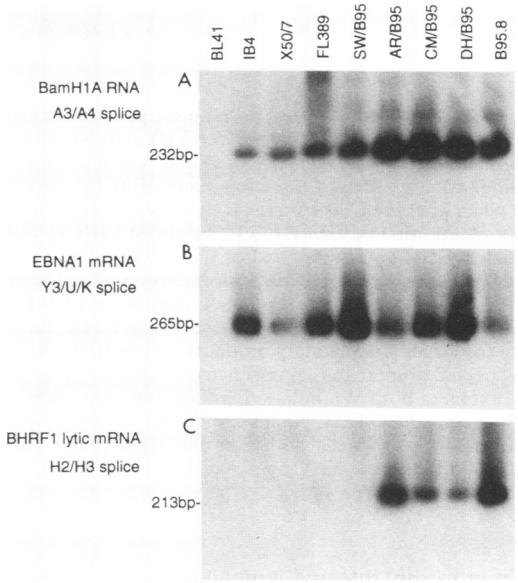

FIG. 3. PCR analysis of *Bam*HI-A transcription in a range of EBV-transformed LCLs previously characterized as tightly latent (IB4, X50/7, FL389, and SW/B95) or containing a minor subpopulation of lytically infected cells (AR/B95, CM/B95, DH/B95, and B95.8). (A) *Bam*HI-A transcript detection with the A_3/A_4 primer combination and relevant internal oligonucleotide probe; (B) EBNA1 mRNA detection in the same RNA samples with the Y3/U/K primer/probe combination. (C) BHRF1 lytic cycle mRNA (class IV BHRF1 cDNA structure [20]) detection in the same RNA samples with the H_2/H_3 primer combination and BHRF1-specific probe. Immunofluorescence staining for the immediate-early protein BZLF1 indicated that 2% AR/B95 cells, 1% CM/B95 cells, 0.2% DH/B95 cells, and 5% B95.8 cells were in lytic cycle at the time of RNA preparation.

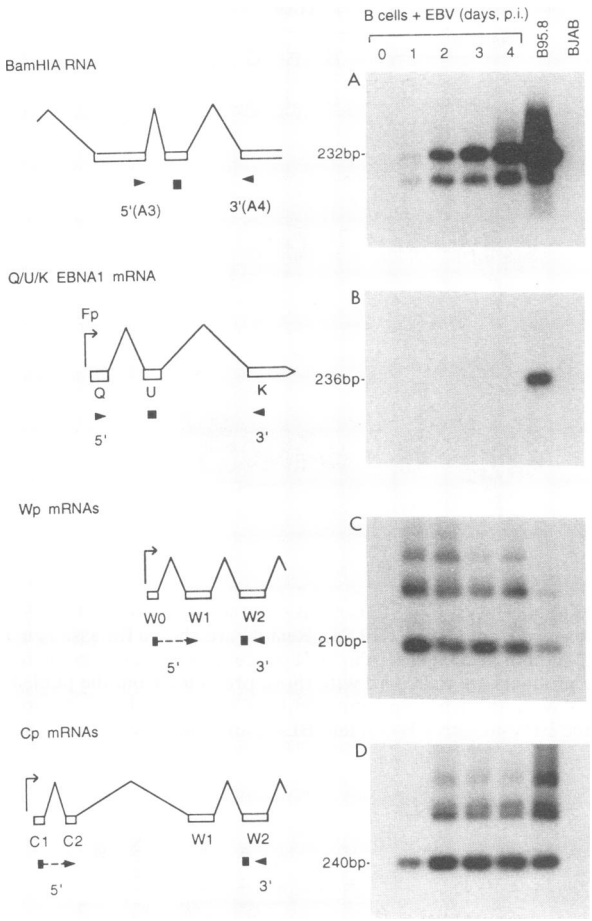

FIG. 4. PCR analysis of *Bam*HI-A transcription in normal B cells within 1 to 4 days of infection with B95.8 EBV. (A) Results obtained by using the A_3/A_4 primer combination and relevant internal oligonucleotide probe are shown in the top panel; (B to D) corresponding results when the same RNA preparations were analyzed for the presence of Fp-initiated *Bam*HI-Q/U/K-spliced EBNA1 mRNA, of Wp-initiated EBNA mRNAs, and of Cp-initiated EBNA mRNAs. The general designs of the various primer/probe combinations are illustrated with reference to the splice structures of the relevant mRNAs; full details have been published elsewhere (2, 14). We attribute the larger amplification products seen with the Wp-specific and Cp-specific primers to the presence of multiple copies of the W_1 and W_2 exons in both Wp-initiated and Cp-initiated mRNAs. Control cells included the EBV-positive B95.8 LCL (a virus-producing cell line known to express both Fp- and Cp/Wp-initiated EBNA transcripts [16]) and the EBV-negative B-cell line BJAB.

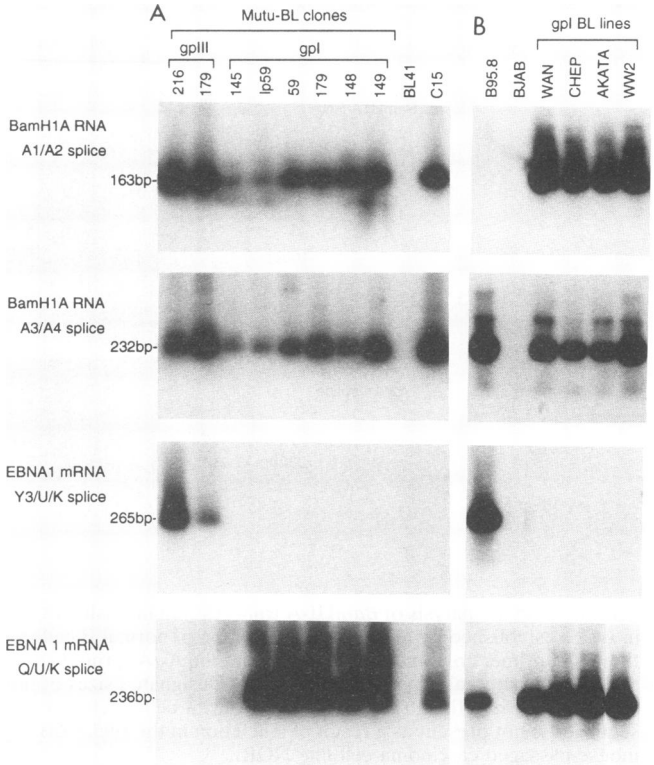

FIG. 5. PCR analysis of *Bam*HI-A transcription in EBV-positive BL cells. Cell lines analyzed included group I and group III Mutu-BL subclones, showing latency I and latency III forms of infection, respectively (A), and the latency I BL lines Wan, Chep, Akata, and WW2 (B). Results obtained by using the A_1/A_2 and A_3/A_4 primer combinations as in Fig. 2 are shown in the top two panels. Reference amplifications of the same RNA preparations were carried out for EBNA1 mRNAs by using the Y3/U/K (latency III) and Q/U/K (latency I) primer/probe combinations, respectively (bottom two panels). Control cells included the EBV-positive B95.8 LCL, the EBV-positive NPC line C15, and the EBV-negative B-cell lines BL41 and BJAB. Note that B95.8 cell populations contain both types of EBNA1 transcript because B95.8 is a spontaneously productive LCL (16).

then Cp in freshly infected lymphocytes (29). There was no evidence of Fp usage over the same period. However, amplification with the A_3/A_4 primer combination clearly showed that *Bam*HI-A transcription was initiated within 24 h of primary infection, with the strength of amplified signal increasing over subsequent days.

PCR analysis of *Bam*HI-A transcripts in BL cells. Further experiments examined *Bam*HI-A transcription in well-characterized BL cell lines which either had retained the original group I (i.e., biopsy-like) cell phenotype and latency I form of infection or had switched in vitro to the group III (i.e., LCL-like) cell phenotype and latency III form of infection. The data in Fig. 5A relate to phenotypically distinct subclones of the Mutu-BL cell line (11) and show that *Bam*HI-A transcripts are detectable with both A_1/A_2 and A_3/A_4 primer combinations not just in group III clones but also consistently in every group I clone. Also shown are reference amplifications from these same cells for Cp/Wp-initiated and for Fp-initiated EBNA1 mRNAs to confirm that these different clones did exhibit different patterns of latent infection; thus, group III clones expressed Cp/Wp-initiated Y3/U/K transcripts, whereas group I clones expressed Fp-initiated Q/U/K transcripts.

The analysis was extended to include a range of other BL cell lines, retaining the group I phenotype and latency I form of infection. Data from four such lines (Wan-BL, Chep-BL, Akata-BL, and WW2-BL; Fig. 5B) again show that A_1/A_2-spliced and A_3/A_4-spliced *Bam*HI-A transcripts are regularly detectable in BL cells with a pattern of EBNA1 mRNA transcription consistent with latency I. Controls in this series of experiments were the transplantable NPC line C15, the virus-producing B95.8 LCL, and the EBV-negative B-cell lines BL41 and BJAB.

In situ hybridization. A parallel series of experiments

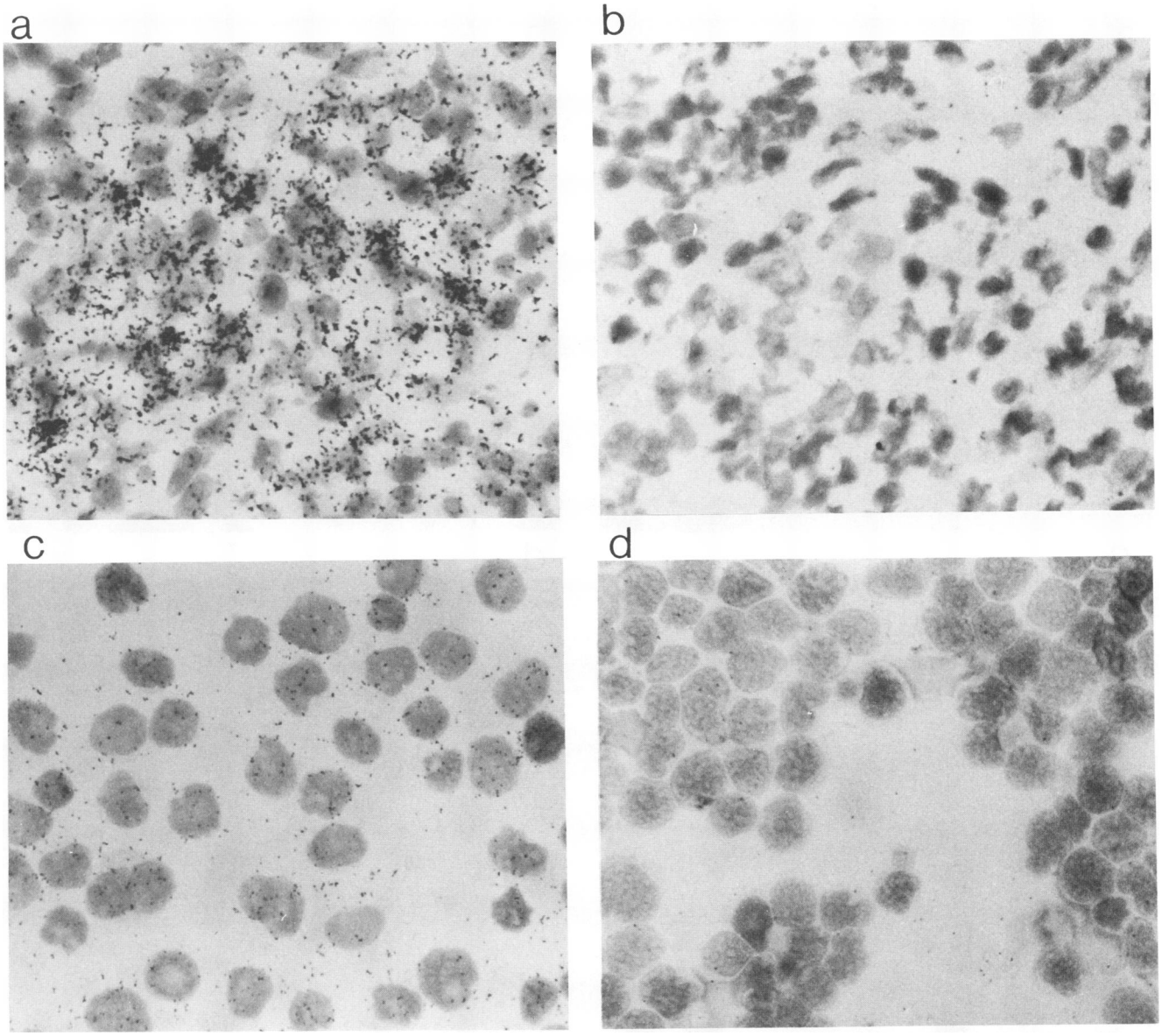

FIG. 6. In situ hybridization analysis of *Bam*HI-A transcription, using ^{35}S-labeled riboprobes from sequences overlapping the putative BARF0 open reading frame (Fig. 1). (a) Fresh NPC biopsy screened with the antisense riboprobe; (b) same NPC biopsy screened with the corresponding sense riboprobe as a control; (c) X50/7 LCL cells screened with the antisense riboprobe; (d) EBV-negative BL40 cells screened with the antisense riboprobe. Results in panels a and b are from 6-day exposures of the autoradiographs; results in panels c and d are from 14-day exposures.

sought to analyze *Bam*HI-A transcription at the single-cell level in these various cell populations, using in situ hybridization with RNA probes from a genomic region immediately downstream of the A_1/A_2 and A_3/A_4 splice sites and within the putative BARF0 open reading frame (Fig. 1). Frozen sections of NPC tumor material and cytocentrifuged preparations of B-cell lines were screened by using ^{35}S-labeled riboprobes in sense and antisense orientations with respect to rightward-running transcripts. Initial assays on SCID mouse-passaged material showed homogeneous labeling of essentially all cells in the NPC tumors C15 to C19 but not of the EBV-negative control tumor NOR (data not shown). Further assays of fresh snap-frozen NPC biopsy material likewise gave specific labeling of the malignant cell population with the antisense probe (Fig. 6a) but not with the control sense probe (Fig. 6b). We next used the same probes to screen a range of latently infected LCLs for *Bam*HI-A transcripts, using EBV-negative B-cell lines as controls. Exposure to the antisense riboprobe produced specific labeling, clearly distinct from background, across the whole population of LCL cells. A representative autoradiograph of X50/7 LCL cells after exposure to the antisense probe is shown in Fig. 6c alongside a parallel autoradiograph of EBV-negative BL40 cells as a control. It should be noted that 2-week exposures of the autoradiographs were required to produce LCL cell labeling of this intensity, whereas the stronger signals illustrated for NPC tissue were obtained after only 6 days. Thus, total levels of *Bam*HI-A transcripts

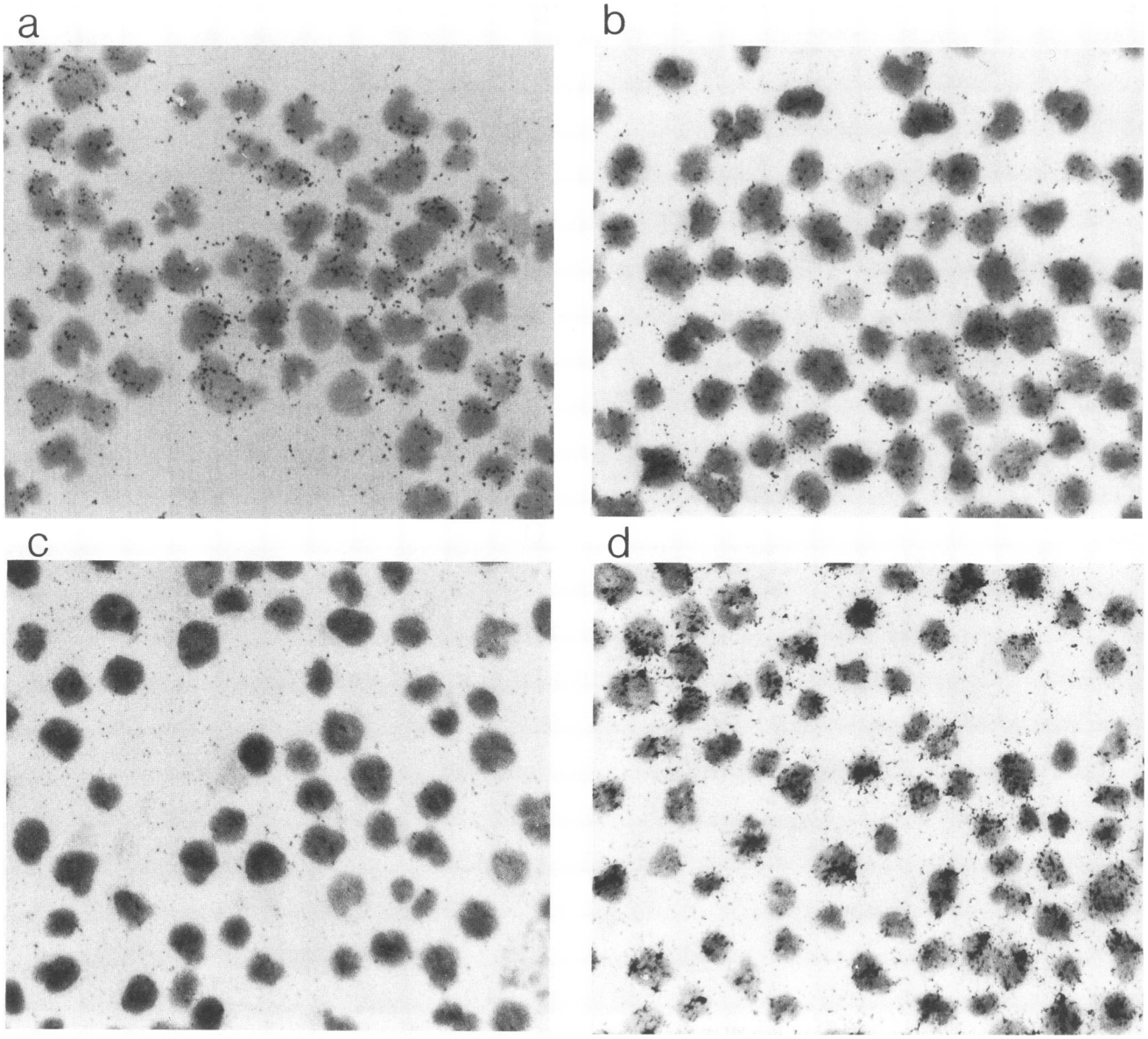

FIG. 7. In situ hybridization analysis of *Bam*HI-A and *Bam*HI-W transcription in phenotypically distinct subclones of the Mutu-BL cell line. (a) Group I subclone screened with the *Bam*HI-A antisense riboprobe; (b) group III subclone screened with the *Bam*HI-A antisense riboprobe; (c) group I subclone screened with the *Bam*HI-W antisense riboprobe; (d) group III subclone screened with *Bam*HI-W antisense riboprobe. Results in panels a and b are from 14-day exposures of the autoradiographs; results in panels c and d are from 3-day exposures.

appeared to be consistently lower in LCL cells compared with NPC.

The analysis was then extended to include EBV-positive group I and group III BL cell lines. Specific labeling was observed throughout both the group I and group III cell populations at intensities similar to those observed for LCLs. Results obtained with the *Bam*HI-A antisense riboprobe with representative group I and group III subclones of Mutu-BL are illustrated in Fig. 7a and b, respectively; in the same experiment, exposure to the *Bam*HI-A sense probe gave no labeling (data not shown). As a further control to check the pattern of EBV latency in these different subclones, we carried out parallel in situ hybridizations using a *Bam*HI-W antisense riboprobe specific for Wp/Cp-initiated transcripts. As expected, intense labeling of the group III cells (Fig. 7d) but not of the group I cells (Fig. 7c) was observed.

DISCUSSION

The demonstration that EBV *Bam*HI-A-encoded transcripts, first identified by cDNA cloning from the nude-mouse-passaged NPC line C15 (9, 12), are consistently present in fresh NPC biopsies (7, 13) highlights their potential importance. A key question which these studies left unresolved, however, was the extent to which such transcripts are expressed in other forms of EBV latent infection, particularly those involving B-lymphoid cells. Whereas

*Bam*HI-A transcripts of the appropriate size had been detected in some long-established B-cell lines by Northern blotting (13), other lines were apparently negative (9, 13), as were two EBV-positive B-cell lymphomas, when screened at the single cell level by in situ hybridization (7). We sought to examine this question more carefully, recognizing that two different forms of virus latency have been identified in B cells (latency I in BL cell lines; latency III in LCLs) which are transcriptionally distinct not just from one another (23, 25) but also from that seen in NPC (latency II [2]). We therefore screened representatives of these different forms of latency by using two independent assays specific for different regions of the *Bam*HI-A RNA (Fig. 1). The results clearly indicate that *Bam*HI-A transcription is a consistent feature not just of NPC but of all three forms of EBV latent infection recognized to date.

First, PCR amplification using two different primer/probe combinations across different splice junctions consistently detected appropriately spliced *Bam*HI-A RNAs in all four transplantable NPCs as well as in all seven fresh NPC biopsies tested (Fig. 2). Furthermore, in situ hybridization showed that the relevant transcripts were present in essentially every tumor cell within such biopsies (Fig. 6a and b). These results are consistent with earlier observations on NPC material (7, 13). Although not reported in detail here, we also screened a number of nonepithelial cell lines displaying the same latency II form of infection as observed in NPC (i.e., hybrid lines generated by fusing LCLs with EBV-negative cells [14]) and likewise found regular *Bam*HI-A transcription (2a).

Extending the analysis to B-cell lines with a latency III form of infection showed that *Bam*HI-A transcripts were detectable in all LCLs examined (Fig. 2 and 3). Importantly, the LCL panel included several lines which were tightly latent, indicating that the detectability of *Bam*HI-A transcripts was not a consequence of a few cells entering lytic cycle. Indeed, essentially all cells in a tightly latent LCL were *Bam*HI-A transcript positive by in situ hybridization (Fig. 6c), albeit with a lower intensity of signal than seen in NPC. This finding is consistent with the generally low level of transcripts detectable by Northern blotting in certain long-established B-cell lines (13). Our study clearly shows, however, that such transcription in LCLs is not an artifact of long-term maintenance in culture. Thus, we detected the appropriately spliced RNAs by PCR within 24 h of in vitro infection of resting B cells, essentially coincident with the detectability of the Wp/Cp-initiated EBNA mRNA transcripts (Fig. 4). Note that there was no accompanying transcription from Fp in these cultures, and in fact, separate amplifications confirmed that all of the detectable EBNA1 mRNA had the Y3/U/K splice structure (23) consistent with expression from Wp/Cp (data not shown); this was as expected since in vitro-transformed cells express Fp-initiated EBNA1 transcripts only if they switch from latency III into lytic cycle (16). The experiment therefore clearly shows that *Bam*HI-A transcription is not dependent upon Fp usage. In this context, recent work (27a) has localized the 5′ end of the *Bam*HI-A transcripts in C15 cells to a putative promoter region in the adjacent *Bam*HI-I fragment of the genome quite distinct from other known latent promoters.

The question of *Bam*HI-A transcription in BL cells required analysis of recently established BL cell lines which retain the latency I form of infection characteristic of the tumor itself (i.e., expression of EBNA1 mRNA from Fp in the absence of other EBNA and LMP mRNAs). The data clearly indicate that all latency I BL cell lines examined were positive for *Bam*HI-A transcripts by PCR amplification (Fig. 5). Furthermore, every cell in such lines gave positive signals by in situ hybridization (Fig. 7a); just as in LCLs, these signals were consistently weaker than in NPC, indicating a difference in the steady-state level of transcripts in lymphoid versus epithelial cell types. Such quantitative differences may well underlie the earlier inability to detect *Bam*HI-A transcripts by Northern blotting in certain EBV-positive B-cell lines (9, 13) and by in situ hybridization in two EBV-positive B-lymphoma biopsies (7).

The principal conclusion from these studies, namely, that *Bam*HI-A transcription is a characteristic of all three forms of EBV latency, requires some qualification. It must be remembered that the *Bam*HI-A RNAs found in NPC cells are a heterogeneous family of molecules with a variety of alternative splice structures. It remains possible, therefore, that subtle differences exist in the splicing patterns of *Bam*HI-A transcripts in different cellular environments. Nevertheless, it is clear that the expression of *Bam*HI-A RNAs is more widespread than first imagined and includes infections of the B-lymphoid system. In this context, published results of nuclear run-on assays are consistent with transcription through the *Bam*HI-A region in a prototype nonproducer LCL (24), while very recently the first *Bam*HI-A cDNA of B-cell origin has been cloned from an EBV-induced lymphoma of tamarins (31). As to the function of these transcripts, it has been postulated that they may act to maintain the latent state, perhaps as antisense RNAs interfering with the expression of *Bam*HI-A lytic cycle genes present on the complementary strand (13). In addition, the prototype *Bam*HI-A RNA sequence contains several potential open reading frames, the best characterized of which is BARF0, and may encode one or more novel latent proteins (7, 13). The present work suggests that, whatever their function, these RNAs are likely to be of general importance to virus latency in both epithelial and B-cell environments.

ACKNOWLEDGMENTS

This work was supported by the Cancer Research Campaign.

We are grateful to T. Tursz and P. Busson (Institut Gustave-Roussy, Paris) for access to the C15-C19 NPCs, to K. Falk and I. Ernberg (Karolinska Institute, Stockholm) for access to the C15 and C18 LCLs, to Rosemary Tierney and Liz Deacon for assistance with PCR, to Susan Williams for photography, and to Deborah Williams for excellent secretarial help.

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