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Transcription of the Epstein-Barr Virus Genome during Latency in Growth-Transformed Lymphocytes

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Nuclear run-on assays revealed extensive transcription of the Epstein-Barr virus genome during latent infection in in vitro-infected human fetal lymphoblastoid cells (IB-4). The EBER genes were the most heavily transcribed viral genes in these cells. Their transcription was partially inhibited in the presence of 1 μg of α-amanitin per ml and fully inhibited at 100 μg/ml, consistent with RNA polymerase III transcription. All other transcription was inhibited at 1 μg of α-amanitin per ml, consistent with RNA polymerase II sensitivity to α-amanitin. Other than EBER transcription, almost no transcription occurred from the U1 region. Specifically, no transcription was detected from the U1 latent promoter. RNA polymerase II transcription was highest in IR1, extending rightward through U2 and IR2 into the U3 domain and gradually decreased, but was measurable throughout the rest of the genome. This is consistent with EBNA gene transcription initiation within IR1. The higher level of transcription of the IR1 and U2 domains, which encode EBNA-LP and EBNA-2, as opposed to the domains which encode EBNA-3A, EBNA-3B, or EBNA-3C or EBNA-1, correlated with a higher level of EBNA-LP/EBNA-2 mRNA. Transcription extended through U4 into U5, even though no known latent-gene mRNAs are expressed from U4 downstream of the EBNA-1 open reading frame. This may result from inefficient termination of EBNA gene transcription. Leftward transcription from the latent membrane protein promoter was lower than EBNA transcription, although the latent membrane protein mRNA was the most abundant of the latent-gene mRNAs, indicating that this mRNA is more efficiently processed or has a longer half-life. Although transcription was detected from the D_L strong early promoters and to a lesser extent from other early promoters, early mRNAs were less abundant than EBNA mRNAs or undetectable, suggesting that there may be posttranscriptional as well as transcriptional control over early mRNA expression in these latently infected cells.

Epstein-Barr virus (EBV) is a human herpesvirus that establishes latent infection in human B lymphocytes and growth-transforms these cells so that they can proliferate indefinitely in vitro. Latently infected cell lines, however, frequently contain a small population of cells which are spontaneously permissive for virus replication. Within latently infected lymphocytes the viral genome is maintained primarily as an episome, but may integrate into cell DNA (28, 31, 33). Viral gene expression during latent infection is limited to six nuclear proteins (Epstein Barr nuclear antigen [EBNA]-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP) and one integral membrane protein, latent membrane protein (LMP) (8, 14–18, 24, 32, 36, 37, 40, 46, 47, 49). Two polyadenylated RNAs, which by sequence and in vitro translation are predicted to encode membrane proteins (LMP-2A and LMP-2B), are also detected in latently infected cells, as are two abundant small nuclear RNA-like EBV RNAs (EBERS) which are transcribed by RNA polymerase III (22, 26, 42).

The structures of the latent-gene mRNAs have been determined from S1 nuclease protection or cDNA analyses (with the exception of EBNA-3B, which is predicted by analogy to EBNA-3A and EBNA-3C) and are illustrated in Fig. 1 (3–5, 9, 26, 37, 41, 42, 44, 47, 48). The EBNA and LMP-2 mRNAs are unusual among herpesvirus mRNAs in that they are extensively spliced from long primary transcripts. The EBNA transcripts initiate under a promoter in IR1 or a promoter in U1 5′ to the IR1 promoter, depending on cellular or EBV-specific factors which are not as yet characterized. The EBNA mRNAs are produced by alternative splicing and 3′ processing of a putative common precursor RNA that is encoded by at least 100 kilobases (kb) of the EBV genome (3, 5, 37, 41, 44, 47). Thus, the EBNA proteins appear to be products of a single transcription-regulatory domain. Alternative selection of the splice acceptor site between exons 1 and 2 in IR1-initiated transcripts, or between exons 2 and 3 in U1-initiated transcripts can create the AUG initiator codon for the translation of EBNA-LP from the leader sequence of the EBNA mRNAs (41, 47, 49; M. Birkenbach, unpublished observations).

The LMP-2A and LMP-2B mRNAs are initiated within the U5 domain of the viral genome and transverse the fused terminal repeats of the EBV episome. Although cDNA sequence analyses indicate that these are overlapping mRNAs which share eight of nine exons and encode nearly identical proteins, the mRNAs initiate at different points and their transcription is therefore assumed to be regulated by different promoters (26, 42). The LMP gene (hereafter referred to as LMP-1) encodes the most abundant EBV mRNA in latently infected cells and is the only latent-infection gene that is transcribed in a leftward direction (9).

Although EBV mRNAs and proteins expressed during latent infection have been well characterized, the extent and rate of transcription have only been inferred from analyses of the structure and abundance of EBV mRNAs and from liquid hybridization studies with whole-cell and polysomal RNA (7, 13, 34, 38). On the basis of mRNA abundance, the LMP-1 promoter would appear to be the strongest of all the latent-gene promoters. However, differences in latent-gene mRNA abundance could also be due to differential mRNA

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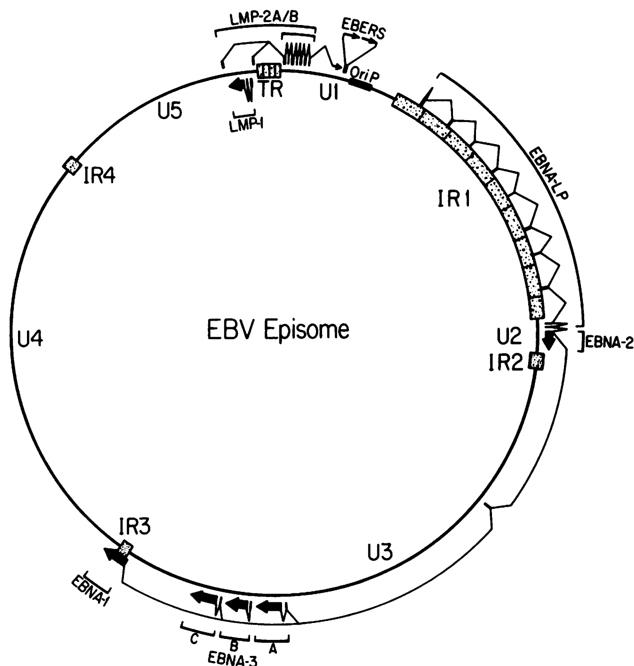

FIG. 1. The EBV genome and its expression during latent infection. The major DNA repeat (TR, IR1 to IR4) and largely unique domains (U1 to U5) of the genome are indicated, as are the latency-associated mRNAs and the EBERs. The exons which make up the ORFs encoding the latent-infection proteins are bracketed. The EBNA-3B mRNA sequence has not been reported and is depicted here on the basis of analogy to the EBNA-3A and EBNA-3C genes. The EBNA mRNAs are shown initiating within IR1, although they may also initiate in U1 (6). OriP is the latent-infection episomal origin of DNA replication (39, 50).

processing, transport, or stability rather than transcription. To directly determine the role of transcription in latent-infection gene regulation, we undertook an analysis of EBV genome transcription by nuclear run-on assay.

MATERIALS AND METHODS

Cells. The EBV-infected lymphoblastoid cell lines IB-4 and B95-8 were maintained in medium consisting of RPMI 1640 with 10% fetal bovine serum. The IB-4 and B95-8 cells used in this study exhibited levels of EBV replication cycle antigen expression that were undetectable and 5%, respectively, as determined by indirect immunofluorescence staining with human sera reactive with EBV early and viral capsid proteins.

Isolation of nuclei. Nuclei were isolated by the method of Hewish and Burgoyne (19) as modified by Schibler et al. (45). Cells (2×10^9 to 3×10^9) were collected by centrifugation at 4,000 rpm in a Beckman JA-10 rotor for 5 min at 4°C, washed in ice-cold 150 mM NaCl, repelleted, and suspended on ice in 0.3 M sucrose in buffer A [60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 140 mM β-mercaptoethanol, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 2 mM EDTA, 15 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES; pH 7.5)]. All subsequent manipulations were done on ice or at 4°C. Cell membranes were ruptured in a Dounce homogenizer by repeated strokes with a tight-fitting pestle. The homogenate was layered onto an 18.5-ml cushion of 30% (wt/vol) sucrose in buffer A and was spun at 2,400 rpm in a Beckman SW28 rotor for 10 min. Nuclei were suspended in 28 ml of 2 M sucrose in buffer B (as buffer A, but 0.1 mM EGTA and 0.1 mM EDTA), layered onto an 8-ml cushion of 2 M sucrose in buffer B, and spun at 26,900 rpm in an SW28 rotor for 2 h. The nuclei were suspended in 0.5 ml of nucleus storage buffer (20 mM Tris hydrochloride [pH 7.9], 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride, 50% glycerol), spun for 2 min in a microcentrifuge, and suspended in the same solution at approximately 2×10^8 nuclei per ml. Aliquots sufficient for one run-on assay (2×10^7 nuclei) were frozen immediately in a dry ice-ethanol bath and stored at −80°C. No loss in the efficiency of [^{32}P]UTP incorporation by nuclei was noted for nuclei stored in this manner for at least 9 months. Some preparations of nuclei were treated with pancreatic RNase A as described previously (45) prior to sedimentation through 2 M sucrose. After this treatment, nuclei were twice sedimented through 30% sucrose cushions (as above) and then sedimented through 2 M sucrose.

Nuclear transcription and RNA isolation. Conditions for the nuclear run-on transcription assay were similar to those described by Gariglio et al. (10). The assays were carried out at 26°C for 10 or 15 min in a 200-μl reaction volume of 2×10^7 nuclei in 100 mM Tris hydrochloride (pH 7.9)–50 mM NaCl–4 mM $MnCl_2$–1.2 mM dithiothreitol–0.1 mM phenylmethylsulfonyl fluoride–0.4 mM EDTA–1 mM each GTP, ATP, and CTP–5 μM UTP–200 μCi of [α-^{32}P]UTP (800 Ci/mmol; Du Pont, NEN Research Products)–29% glycerol–10 mM creatine phosphate–150 U of RNasin (Promega Biotec) per ml. Reactions done in the presence of α-amanitin were incubated on ice for 5 min prior to incubation at 26°C. The reactions were terminated by the addition of DNase I to 95 μg/ml and further incubation at 26°C for 5 min. RNA was isolated as described by Groudine et al. (11). Briefly, the reaction mixture was deproteinized by digestion with proteinase K, extracted with phenol, and precipitated in 5% trichloroacetic acid, and the acid-insoluble material was collected onto nitrocellulose disks. The bound material was then digested with DNase I, eluted from the disk, digested again with proteinase K, extracted twice with phenol and then chloroform, and finally precipitated in ethanol. The level of ^{32}P incorporation, determined from the purified RNA, was typically about 10 cpm per nucleus and did not vary noticeably among reactions or different preparations of nuclei. The RNA pellets were dissolved in 0.5 ml of 10 mM Tris hydrochloride (pH 7.4)–5 mM EDTA–1% sodium dodecyl sulfate (SDS) and added directly to filter hybridization buffer for hybridization to Southern or slot blots containing a molar excess of individual EBV DNA fragments.

Southern blot hybridization. Plasmids containing EBV DNA were digested with an appropriate restriction endonuclease(s), and the resulting fragments were fractionated by electrophoresis in 1% agarose gels. The DNA was blotted to GeneScreen Plus nylon membranes (Du Pont, NEN), which were then prehybridized and hybridized at 42°C in a 0.2-ml/cm^2 mixture of 50% formamide, 1 M NaCl, 10% dextran sulfate, and 1% SDS. Filters were prehybridized for 1 to 4 h, [^{32}P]RNA from two run-on reactions (4×10^7 nuclei) was then added with yeast tRNA (100 μg/ml), and incubation was continued for 48 h. Filters were rinsed briefly in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]) and then washed for 1 h each at 65°C in 1× SSC–1% SDS, 0.5× SSC–1% SDS, and 0.2× SSC–1% SDS. Washed filters were incubated for 15 min at room temperature in 2× SSC containing 10 μg of RNase A per ml, rinsed, dried, and

exposed at −80°C to preflashed Kodak XAR-5 film with an intensifying screen. Slot blots of single-stranded EBV DNA on nylon membranes were hybridized and washed under the same conditions as were Southern blots. Relative levels of transcription were determined by densitometric scanning of autoradiograms representing various lengths of exposure.

Northern (RNA) blot hybridization. IB-4 cell cytoplasmic RNA was isolated as described previously (21), and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography (1). RNA was fractionated in a 1.2% agarose–2.2 M formaldehyde gel (29) and blotted onto nylon membranes for hybridization to ^{32}P-labeled (by nick translation) EBV DNA fragments. Conditions for hybridization and washing were as described previously (21) and above, respectively.

RESULTS

RNA synthesis across the EBV genome. Relative transcription rates across the EBV genome during latency were determined by nuclear run-on assays with nuclei isolated from IB-4 cells. IB-4 cells were chosen for these assays of transcription in latently infected cells because (i) this cell line was established by infection and growth transformation in vitro; (ii) the genome of the EBV isolate that was used to establish the IB-4 cell line is completely cloned and sequenced; (iii) the IB-4 cell line is a latently infected lymphoblastoid cell line in which lytic-cycle protein expression is restricted to less than 0.01% of the cell population; and (iv) EBV mRNAs from IB-4 cells have been well characterized. Nascent [^{32}P]RNA was hybridized to Southern blots of approximately 1- to 5-kilobase-pair (kbp) restriction endonuclease fragments of cloned EBV DNA, and RNA synthesis rates were inferred from densitometric scans of the resultant autoradiograms. When possible, EBV DNA fragments on Southern blots were cut to be gene specific. The cloned EBV DNAs used in these experiments are described in Table 1. Data representative of several experiments with IB-4 nuclei are shown in Fig. 2A. Because the DNA fragments were unequal in size and the thymidine content over the length of the EBV genome is not constant, values achieved by densitometric scanning of autoradiograms were normalized to the thymidine content and size of each DNA fragment and are summarized in Fig. 3. The highest transcription rate occurred within a 3-kbp *Bgl*I fragment spanning the EBER 1 and 2 genes located within the *Bam*HI C fragment (Fig. 2A, lane 2). Transcription of EBER 1 and 2 is considerably higher than is indicated by the level of hybridization shown in lane 2, since all of this transcription originates from a small segment of the 3-kbp *Bgl*I fragment. The rate of EBER transcription in the summary diagram (Fig. 3) has been corrected for the small size of the EBER DNA template. To confirm that this was largely due to RNA polymerase III transcription of the EBER genes, we performed run-on assays in the presence of α-amanitin at concentrations sufficient to inhibit transcription by RNA polymerase II (1 μg/ml) and both RNA polymerases II and III, although not RNA polymerase I (100 μg/ml [30]). All detectable transcription was abolished at 100 μg of α-amanitin per ml (data not shown), whereas under conditions which completely inhibit RNA polymerase II only, EBER transcription was only modestly diminished (Fig. 2B). These data do not exclude the possibility, however, of an accessory role for RNA polymerase II or its associated factors in EBER transcription. In fact, recent data indicate that RNA polymerase II-related regulatory elements exist upstream of the EBER genes and are required for maximum expression (20).

TABLE 1. EBV DNA fragments in Fig. 2

Lane	DNA	Digest[a]	Genomic coordinates[b]
1	*Eco*RI-I		2–4163
2	*Bam*HI-C	*Bgl*I	3994–13215
3	*Bam*HI-W, *Bam*HI-Y		13215–48848
4	*Bam*HI-H	*Acc*I	48848–54853
5	*Bam*HI-F	*Stu*I	54853–62249
6	*Bam*HI-Q	*Bgl*II-*Oxa*NI	62249–66121
7	*Bam*HI-U		66121–69410
8	*Bam*HI-P	*Pvu*II	69410–73468
9	*Bam*HI-O	*Pvu*II	73468–77835
10	*Eco*RI-G_2	*Bam*HI	76596–82920
11	*Eco*RI-F	*Pvu*II	82920–91421
12	*Bam*HI-L	*Ava*III	87650–92703
13	*Bam*HI-E	*Acc*I	92703–100613
14	*Bam*HI-Z, *Bam*HI-R		101947–103741 103816–107457
15	*Bam*HI-K	*Hin*dIII	107565–112620
16	*Bam*HI-B	*Bgl*II	112620–122313
17	*Bam*HI-G	*Eco*RI	122313–128848
18	*Eco*RI-E	*Bam*HI	125316–137221
19	*Bam*HI-T, *Bam*HI-X		139352–144862
20	*Bam*HI-V, *Bam*HI-I		144862–148007 149115–154747
21	*Bam*HI-A	*Eco*RI	154747–166614
22	*Eco*RI-Dhet	*Bam*HI	159853–172281

[a] In addition to digestion with *Bam*HI or *Eco*RI to release the EBV DNA fragment from the vector DNA.
[b] According to Baer et al. (2).

The next-highest transcription rate, at least 10-fold lower than that of the EBERs, was of the 3.1-kbp IR1 *Bam*HI W fragment (Fig. 2A, lane 3), a 3.9-kbp *Acc*I fragment (genomic coordinates 50478 to 54413) of *Bam*HI-H (lane 4), and two *Stu*I fragments spanning coordinates 54853 to 56824 and 57154 to 59417 of *Bam*HI-F (lane 5; a 330-bp fragment separates these two *Stu*I fragments and is likely to be transcribed at the same rate but was not detected because of its small size). No transcription was detected downstream of the alternate U1 EBNA promoter. This is consistent with S1 nuclease mapping data, which suggest that in IB-4 cells, EBNA transcription does not originate in *Bam*HI-C (6), but, instead, uses the IR1 promoter (Fig. 1). The high rate of transcription of IR1, U2, IR2, and the left part of U3 is probably due to synthesis of EBNA transcripts initiated downstream of an IR1 promoter. The higher rate of transcription of the IR2 and neighboring U3 DNA segments (Fig. 3) than of U2, which encodes EBNA-2, may be due to the strong D_L leftward and rightward early replicative-cycle promoters located at the left end of U3 (12, 23, 25, 27) which may be active in a small population of the cells. The higher apparent rate of IR1 transcription relative to U2 is probably due to the multiple IR1 repeats in the EBNA primary transcripts.

Transcription occurred at a reduced rate from the right of map coordinate 61 in U3 through much of the remainder of the genome (Fig. 2A and 3). Slightly higher levels of transcription at some loci, such as that observed for the *Bam*HI M region (Fig. 3, map coordinates 79.5 to 84.2), may be due, as with D_L, to the spontaneous activation of the EBV replication cycle early promoters in a very few of the cells. Minor differences in apparent transcription rates (≤0.5), however, are not necessarily significant. The data summarized in Fig. 3 also suggested that synthesis of the EBNA primary transcript was not constant over its entirety. An analysis of latent-gene transcription involving strand-specific

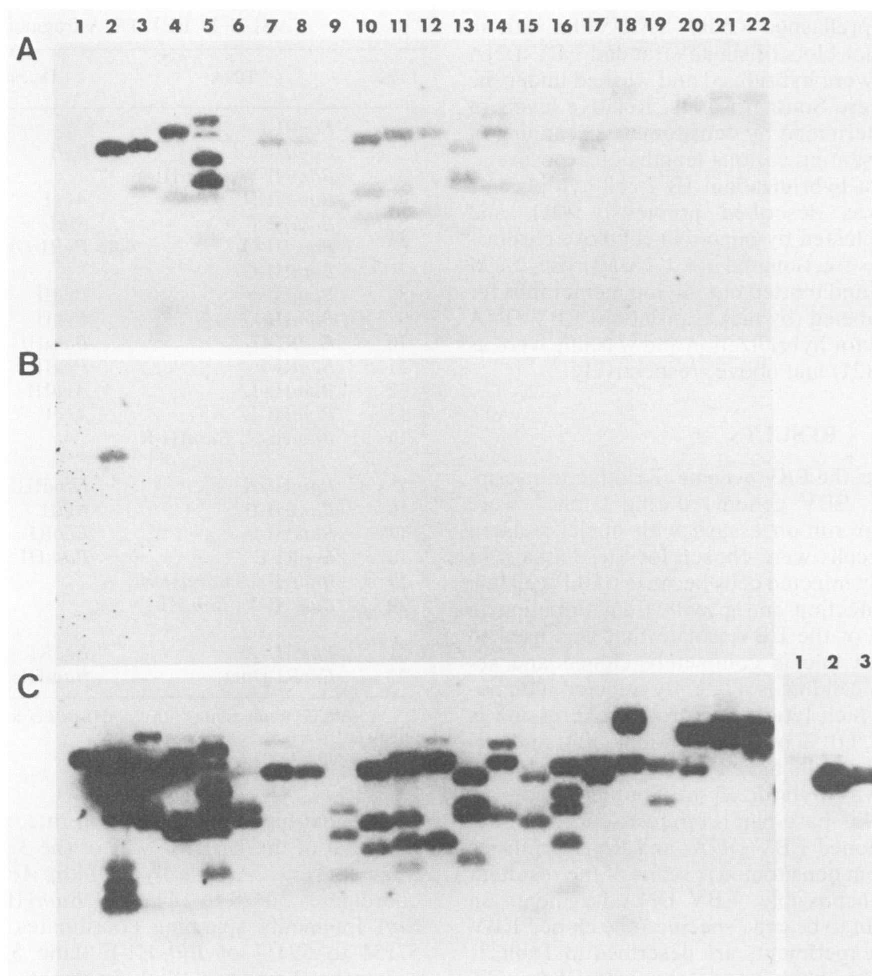

FIG. 2. Transcriptional activity across the EBV genome. Southern blots of cloned B95-8 EBV DNA fragments, representing essentially the entire B95-8 EBV genome (left to right), were hybridized to ^{32}P-labeled nuclear RNA from 4×10^7 (A) IB-4 nuclei, (B) IB-4 nuclei in the presence of 1 μg of α-amanitin per ml, or (C) B95-8 nuclei. Autoradiograms of blots A and C represent identical exposure lengths; a lighter exposure of lanes 1 to 3 (B95-8 blot) is presented to the right of blot C. See Table 1 for a description of the DNA in each lane.

DNA confirmed this, indicating that the relative rates of transcription through the EBNA open reading frames (ORF) were EBNA-2 > EBNA-1 > EBNA-3A and EBNA-3C > EBNA-3B (Fig. 4, − strand). The apparent rate of RNA synthesis across the IR1 repeat in these experiments was determined to be six to nine times greater (depending on the length of exposure of the blot to X-ray film) than that which occurred across the adjacent and downstream EBNA-2 ORF. As stated above, this may be artificially high due to the multiple IR1 repeats in the transcripts. In addition to transcription that would be specific for the latent gene RNAs, at least some transcription of the opposite strand (Fig. 4, + strand) was detected. This is unlikely to be due to nonspecific hybridization, as no hybridization to the negative control DNA (M13mp18) was detected (Fig. 4). Opposite-strand transcription through the EBNA-2 coding region is consistent with some leftward transcription from the D_L promoter continuing into U2 and terminating largely before IR1.

Transcription through the LMP-1 and LMP-2 genes (Fig. 2A, lanes 22 and 1, respectively; Fig. 4) was notably low, even though these encode relatively abundant mRNAs (see below). Opposite-strand transcription through the LMP-1 coding region would have been due in part to transcription of the LMP-2A gene (Fig. 1).

Relative transcription rates across the same EBV genome in B95-8 cell nuclei were determined in parallel to evaluate the effect of spontaneous activation of the replication cycle in 5% of otherwise latently infected cells. A representative run-on assay of the EBV genome transcription in B95-8 nuclei is presented in Fig. 2C. The most notable difference relative to IB-4 was a much higher overall level of RNA synthesis in B95-8 nuclei, possibly due to a higher copy number of the EBV genome in B95-8 cells. In contrast to IB-4 nuclei, transcription downstream of the U1 EBNA promoter was detected (Fig. 2C, lane 2, lowest band), consistent with S1 nuclease protection and cDNA analyses, which indicate that this is the dominate EBNA promoter in B95-8 cells (6). Surprisingly, the relative rates of transcription across the EBV genome in B95-8 and IB-4 cells were very similar, even though early-gene mRNAs are often quite abundant in B95-8 cells. Slightly higher rates of transcription, relative to IB-4 cells, were detected for the regions containing the *Bam*HI H and *Eco*RI G_2 and F (*Bam*HI M region) restriction fragments, although this is not readily apparent in the autoradiogram in Fig. 2C, owing to the length of exposure. These differences probably reflect increased transcription of early lytic cycle genes.

Levels of the latent-infection mRNAs. The relative latent-

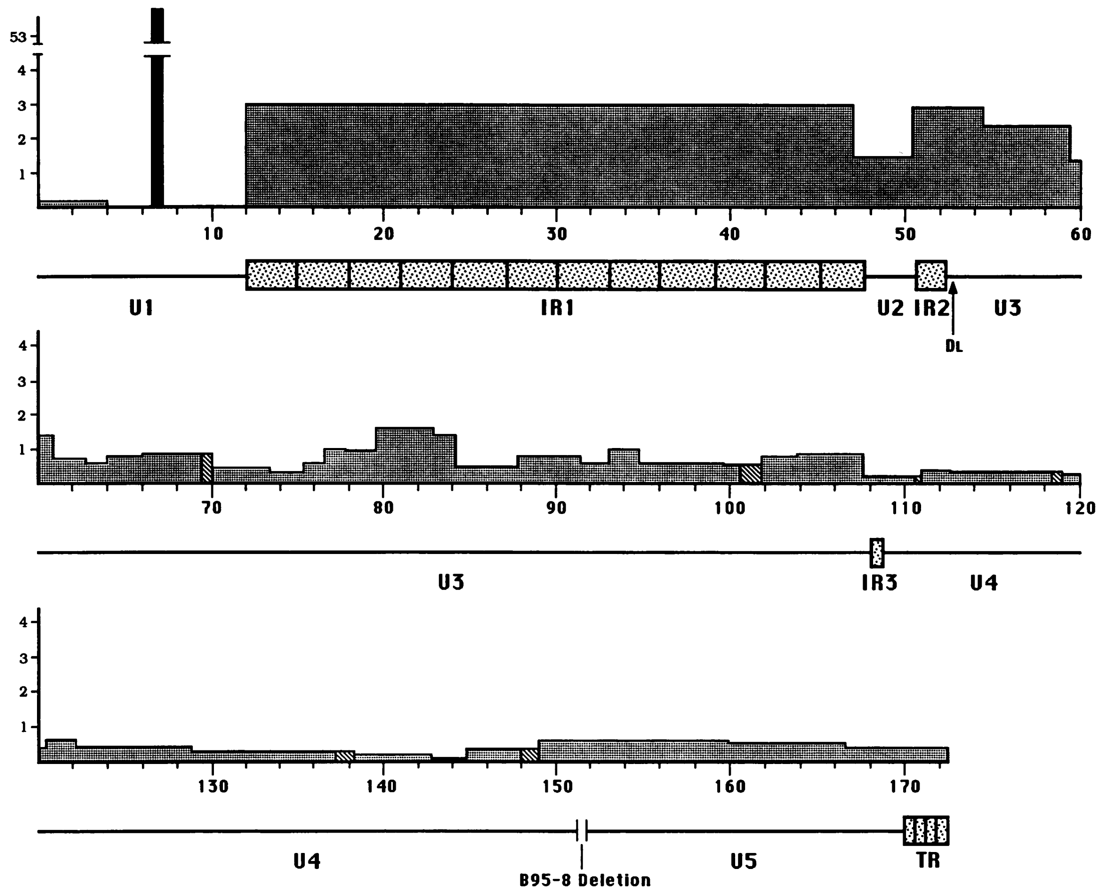

FIG. 3. Summary of transcription across the EBV genome in IB-4 nuclei. The relative levels of transcription across the different DNA fragments were determined by densitometric scanning of autoradiograms (such as Fig. 2A) and were normalized for the thymidine content of each respective fragment; the scale is arbitrary and is presented to the left of each segment of the linear genome. The EBV genome scale is in kilobase pairs (2). EBER gene transcription by RNA polymerase III is represented by the solid bar between map units of 6 and 7 in U1. DNA fragments that were not included in Fig. 2 and for which the level of transcription was not determined are indicated (▧).

infection gene mRNA levels in IB-4 cells were determined by Northern blot analysis of poly(A)$^+$ RNA (Fig. 5). All blots, which contained equal amounts of RNA and which were from the same gel, were hybridized to a ^{32}P-labeled DNA fragment (2×10^8 to 3×10^8 cpm/μg) specific for one of the latent-infection genes. The probes were the same EBV DNA fragments as those cloned into M13 phage (Table 2) for use in the transcription run-on assays described above, except that cDNA probes were used to detect the LMP-2A/B and EBNA-LP or IR1 mRNAs. In order of decreasing abundance were the mRNAs for LMP-1 (2.6 kb), EBNA-LP/EBNA-2 (2.8 kb), and EBNA-1 (3.3 kb); the LMP-2A (2.2 kb), EBNA-3A (4.5 kb), and EBNA-3C (4.7 kb) mRNAs were approximately the same, and the LMP-2B (1.9 kb) and EBNA-3B (6.8 kb) mRNAs were the least abundant. Several larger, faint bands were detected in the LMP-2 blot, which may be transcripts derived from integrated copies of the EBV genome in IB-4 cells (31, 41). A 1.4-kb transcript, detected in the EBNA-LP (IR1) blot (Fig. 5), may be an mRNA encoding EBNA-LP only, since partial cDNAs of this type have been obtained (4; J. Sample and M. Birkenbach, unpublished observations). We have also characterized several cDNAs that represent transcripts of this size but would not encode EBNA-LP. These are identical to the leader of the EBNA mRNAs in their IR1 splicing pattern, except that an alternate splice between exons 1 and 2 does

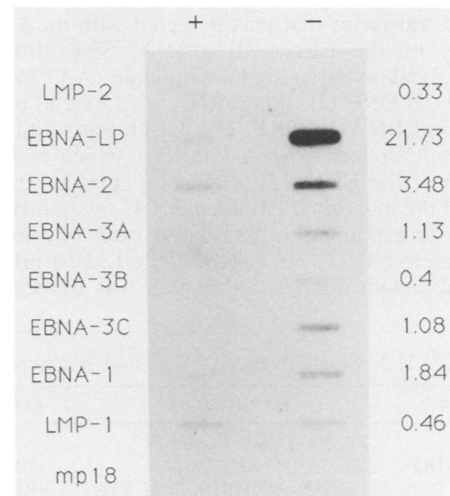

FIG. 4. Strand-specific latent-infection gene transcription. The + and − (coding) DNA strands of the individual latent-infection genes (cloned in M13 phage) were fixed to a nylon membrane and hybridized to ^{32}P-labeled nuclear RNA from IB-4 nuclei. The relative levels of latent gene-specific transcription are presented next to the autoradiogram. The EBV DNA fragments that were cloned into M13 for this experiment are described in Table 2.

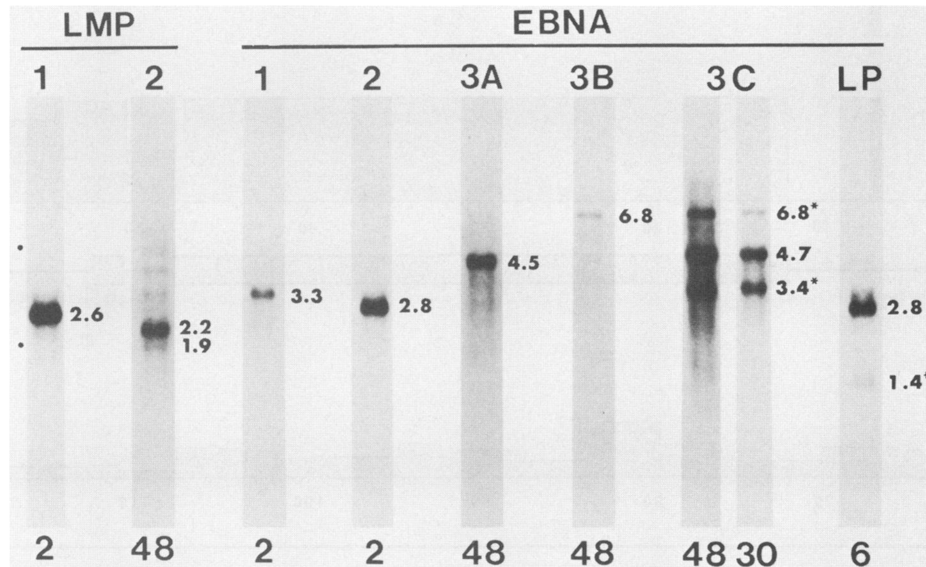

FIG. 5. Northern blot analysis of the latent-infection gene mRNAs. Each lane of the gel blotted contained 5 μg of IB-4 cell poly(A)$^+$ RNA. The probes used were the same EBV DNA fragments that were cloned into M13 phage (see Table 2), except those for the LMP-2 and EBNA-LP (IR1) mRNAs; these were a full-length LMP-2A cDNA (42) and the IR1-derived portion of the full-length T65 cDNA (41), respectively. The length of exposure (in hours) required to provide each autoradiogram is indicated at the bottom of the lanes. The positions of the 28S (5.0-kb) and 18S (2.0-kb) rRNAs are indicated to the left of the blots (dots), whereas the sizes (in kilobases) of the known or putative latent-gene mRNAs are shown to the right. The sizes of less well characterized transcripts are denoted by asterisks.

not create the EBNA-LP initiator codon. These transcripts have polyadenylated variable 3′ termini just upstream of the EBNA-2 ORF, although the canonical polyadenylation signal sequence AATAAA is absent (Sample and Birkenbach, unpublished observations). Two additional transcripts of 6.8 and 3.4 kb were detected with the EBNA-3C probe (Fig. 5). We did not observe these in blots of IB-4 poly(A)$^+$ RNA that was isolated several years earlier (37). However, Sawada et al. (44) have recently reported similar-sized transcripts with the same relative abundance in lymphoblastoid cell lines as well as the Burkitt lymphoma-derived cell line Namalwa. The 6.8-kb transcript that was detected with the EBNA-3C probe may be the EBNA-3B mRNA. Since there is no obvious polyadenylation signal sequence (AATAAA) after the EBNA-3B ORF (2), this mRNA may extend across the downstream EBNA-3C ORF. The 3.4-kb transcript could be an alternately spliced EBNA-1 mRNA which includes the small exon of the EBNA-3C ORF (48) (Fig. 1). In general, the level of the individual EBNA mRNAs in latently infected IB-4 cells corresponded to the relative rates of transcription of the respective ORFs. In contrast, the LMP-1 mRNA was the most abundant mRNA, although it was transcribed at a substantially lower rate than the most abundant EBNA mRNA (EBNA-2). In part, this discrepancy could occur because an EBNA-2 primary transcript may also mature into EBNA-1, EBNA-3A, EBNA-3B, or EBNA-3C mRNAs.

TABLE 2. Single-stranded EBV DNA fragments

Latent gene	DNA fragment	Coordinates[a]
LMP-2	*Eco*RI-*Xho*II (*Eco*RI-I)	2–1071
EBNA-LP (IR1)	*Sau*3A (*Bam*HI-W)	13945–15200
EBNA-2	*Fnu*DII-*Aha*III (*Bam*HI-YH)	48472–50303
EBNA-3A	*Eco*RI-K	93162–95239
EBNA-3B	Random (*Bam*HI-E [36])	95963–97906
EBNA-3C	T36 cDNA (37)	98389–101788
EBNA-1	*Sau*3A-*Pvu*II (*Bam*HI-K)	107930–110174
LMP-1	cDNA	166950–169474

[a] Coordinates for cDNAs are inclusive of introns, but intron sequences were omitted in the calculation of transcription values.

DISCUSSION

We have shown that the EBV genome is extensively transcribed during latent infection, although only a small fraction of this RNA is processed to the mRNAs encoding the latent-infection proteins. A significant amount of the transcription beginning in IR1 and continuing through at least the EBNA-1 ORF in U4 was undoubtedly due to the continuous synthesis of the long EBNA primary transcript. The apparent rate of synthesis of the EBNA transcript was not constant, however, but decreased substantially approximately 10 kb downstream of the EBNA-2 ORF. Precise analysis of the point(s) of transcription fall-off was obscured by early lytic-infection gene transcription from this same region. The observation that transcription decreases 3′ of the EBNA-2 ORF suggests the possibility that RNA polymerase II fall-off is of some significance in regulating the relatively greater abundance of EBNA-LP and EBNA-2 mRNAs than that of the other EBNA mRNAs. After this region, EBNA transcription appeared to continue at a similar rate through the EBV U4 domain. Thus, alternative splicing of the same primary transcript may regulate the relative amounts of the EBNA-1, EBNA-3A, EBNA-3B, and EBNA-3C mRNAs. Differential posttranscriptional processing or stability may also be important, since the LMP-1 mRNA is the most abundant mRNA, even though transcription through the EBNA-2 coding region was substantially higher. Because one of the estimated five copies of the EBV genome in IB-4 cells is integrated (31), it is uncertain what effect, if any, cellular control elements may have on transcription of the integrated genome. The most direct effect would be on the

expression of the LMP-2 genes, which are inactivated as a result of integration into chromosome 4 in IB-4 cells.

It was surprising to find a high rate of transcription by RNA polymerase II from the U2-IR2-U3 domain (*Bam*HI H and F restriction fragments) in latently infected cells. This transcription is probably of prominent early lytic-cycle genes, namely the BHLF1 and BHRF1 genes. This could be due to spontaneous activation of the lytic cycle in a very small percentage of the cells, since transcription was detected from other regions of the genome encoding prominent early proteins, e.g., the *Bam*HI M region (43). However, fewer than 0.01% of our IB-4 cells express detectable BHRF1 protein, as determined by immunofluorescence staining with an anti-BHRF1 monoclonal antibody (C. Alfieri, personal communication). Furthermore, although BHRF1 cDNAs have been isolated from latently infected cell cDNA libraries (5, 35), we were unable to detect the BHLF1 and BHRF1 mRNAs by Northern blot analysis of IB-4 poly(A)$^+$ RNA (data not shown), even though they are abundant within permissively infected cells (21, 23). Since these mRNAs and proteins are not detectably expressed in these latently infected cells despite transcription, there is probably restriction of these genes by posttranscriptional mechanisms.

Processes other than transcriptional initiation must also be crucial in expression of the latent-gene mRNAs themselves, particularly the EBNA transcripts. Transcription termination and 3'-processing signals of the many interspersed lytic-cycle genes must be ignored or suppressed during synthesis of the EBNA primary transcript, yet be actively recognized during lytic infection.

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