Epstein-Barr Virus Gene Expression in P3HR1-Superinfected Raji Cells

MARK BIGGIN,^{1†} MYRIAM BODESCOT,² MICHEL PERRICAUDET,² AND PAUL FARRELL^{3*}

Ludwig Institute for Cancer Research, Medical Research Council Centre, Cambridge CB2 2QH, England¹; ER 272 Centre National de la Recherche Scientifique, Institut de Recherches Scientifiques sur le Cancer, 94800 Villejuif, France²; and Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, London W2 1PG, England³

Received 1 April 1987/Accepted 7 July 1987

The pattern of Epstein-Barr virus (EBV) RNAs expressed in Raji cells superinfected with P3HR1 EBV was examined. RNAs whose expression was of an immediate-early type (resistant to treatment of the cells with anisomycin) were identified. These RNAs, encoding the EBV reading frames BZLF1 and BRLF1, were probably expressed from defective virus within the P3HR1 preparation, and some of them were responsible for the induction of the EBV productive cycle in the Raji cells. The structures of the B95-8 RNAs equivalent to the anisomycin-resistant RNAs were determined. The RNA encoding the BZLF1 reading frame contained two splices which extended and modified the reading frame from that previously described.

Epstein-Barr virus (EBV) is a human herpesvirus that infects B lymphocytes and certain epithelial cells (P. J. Farrell and B. G. Barrell, in B. Roizman, ed., *The Herpesviruses*, in press). Lymphocytes infected by the virus give rise to immortalized lymphoblastoid cell lines. Depending on the source of the lymphocytes, these may be latently infected (making no virus) or spontaneously productive, in which case a small fraction of the cells spontaneously enter the productive cycle. In most such cell lines, the fraction of cells producing virus can be dramatically increased by treating the cells with various chemicals, such as butyrate or phorbol esters (for example, TPA [32]). Two basic types of EBV gene expression can be distinguished in these various cell lines: a latent type, in which a small number of particular genes are expressed (including EBV nuclear antigens 1 to 4, the leader protein, the latent membrane protein, and the EBER RNAs), and a productive type, in which most of the 80 to 100 genes are expressed (11, Farrell and Barrell, in press). The purpose of the work described in this paper was to study genes involved in the switch from latent to productive gene expression.

Many viruses cause latent infections. In some viruses, latency and subsequent reactivation have important clinical and pathological consequences. Other members of the herpesvirus group, such as herpes simplex virus (HSV) and varicella-zoster virus, are good examples of such viruses. It is generally difficult to mimic these latent infections in tissue cultures, but EBV readily displays a latent state in tissue cultures. It is thus an attractive virus for studying latency. However, a fundamental problem with studying the switch from the latent to the productive cycle in EBV is that we do not understand the natural stimulus which causes the induction of the productive cycle. It might be a particular stage of host cell differentiation or some chemical or biological signal, or induction might be a purely stochastic process.

Apart from chemical induction, an alternative way to obtain a productive cycle in cells latently infected by EBV is by superinfection with an appropriate strain of EBV. Superinfection of Raji cells with the P3HR1 strain of EBV results

in virus production (13), and this is the system we have studied. It has been established that the P3HR1 virus contains a population of rearranged, defective viral genomes which actually trigger the induction (8, 24, 26). One example of these defective viral genomes, called het (het DNA), has been functionally characterized in detail (23, 24). It is presently unknown whether the defective viral genomes represent a physiologically relevant mechanism of induction in vivo.

Productive gene expression in HSV, the most studied herpesvirus in this respect, has been divided into three broad categories: alpha, beta, and gamma (14). These categories are also called immediate-early, delayed-early, and late, respectively (9). EBV productive gene expression has been sorted into early and late classes relative to DNA synthesis (16, 30; Farrell and Barrell, in press), and these may correspond loosely to the beta and gamma classes of HSV. We hope to identify immediate-early genes of EBV by studying which genes are expressed during P3HR1 superinfection of Raji cells. Alpha genes of HSV and immediate-early genes of other viruses, e.g., adenovirus, can be distinguished by the fact that they are transcribed in the productive cycle even in the presence of inhibitors of protein synthesis (17). We have accordingly analyzed the genes which are expressed during superinfection even in the presence of inhibitors of protein synthesis. While the P3HR1 superinfection system is not strictly analogous to simple infection with a lytic virus, the experiments yield results strikingly consistent with those of other, quite different approaches to identifying EBV immediate-early genes and reveal several new related phenomena.

MATERIALS AND METHODS

Cell and virus preparation. Cell lines Raji, B95-8, and P3HR1 were grown in RPMI 1640 media supplemented with 10% fetal calf serum. Some cultures were treated with TPA at 30 ng/ml, and PAA was used at 125 µg/ml.

P3HR1 virus was prepared from P3HR1 cell cultures which, after reaching 10⁶ cells per ml, had been treated for 3 days with TPA. The cells were then centrifuged and discarded, and the supernatant was centrifuged at 13,000 rpm for 2 h at 4°C in a Sorvall GSA rotor (average relative centrifugal force, 22,500). The virus pellet was suspended in a 1/200 volume of medium and stored frozen at -70°C.

* Corresponding author.

† Present address: Department of Biochemistry, University of California at Los Angeles, Los Angeles, CA 90024.

FIG. 1. Map of *EcoRI* and *BamHI* restriction sites in the B95-8 EBV genome shown next to a scale in kilobases. Most of the probes used in Northern blotting experiments (see Fig. 2) were cloned *EcoRI* or *BamHI* fragments. Probes DH1 to DH4 are also shown.

Superinfection of Raji cells. Raji cells (2×10^7) were spun down from cultures which had grown to about 5×10^5 cells per ml. The cells were suspended in either 2 ml of P3HR1 virus suspension or, for mock infections, in 2 ml of medium. The cells were gently shaken at 37°C for 1 h, diluted to 5×10^5 cells per ml, and incubated at 37°C.

Cells blocked for protein synthesis were treated similarly by suspension in 2 ml of either medium or P3HR1 virus suspension with 100 μ M anisomycin (17), and after 1 h at 37°C they were diluted to 5×10^5 cells per ml in medium with 100 μ M anisomycin. As judged by trichloroacetic acid-precipitable radioactivity from [35 S]methionine-labeled cells, protein synthesis was reduced to less than 3% of normal after 1 h of 100 μ M anisomycin treatment. After 12 h of treatment with 100 μ M anisomycin followed by washing of cells to remove anisomycin, the protein synthesis rate immediately returned to 26% of normal.

Northern blot (RNA blot) analysis. Cytoplasmic RNA was prepared from Raji cells but was not poly(A) selected. Samples (2 μ g) were glyoxylated, electrophoresed through 1% agarose, and blotted onto nitrocellulose (29). RNA blots were prehybridized in $5 \times$ Denhardt solution– $5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate–100 μ g of denatured salmon sperm DNA per ml at 65°C for 2 h. They were hybridized overnight in a fresh sample of the above-described solution at 65°C to either nick-translated DNA probes or single-stranded DNA “prime-cut” probes (12). Nick-translated probes typically contained 5×10^7 to 5×10^8 dpm/ μ g of DNA, and prime-cut probes contained 1×10^6 to 3×10^6 dpm/ μ g of DNA. The blots were washed repeatedly in $0.1 \times$ SSC– $1 \times$ Denhardt solution–0.1% sodium dodecyl sulfate at 65°C for 2 to 4 h. Marker DNAs were bacteriophage lambda DNAs cut with *HindIII* and radioactively labeled with Klenow polymerase.

Mapping of RNA termini. Riboprobes were prepared as described by Melton et al. (22) from EBV DNA restriction fragments cloned into pSP64. Probes (50,000 dpm) were annealed overnight with 5 μ g of RNA and digested with RNases A and T₁ (22). S1 nuclease analysis (2) was performed with single-stranded prime-cut probes as described previously (12). The products of RNase and S1 nuclease digestions were denatured and then electrophoresed on 6% denaturing acrylamide gels. Radioactively labeled *MspI*-cut pBR322 DNAs were used as markers. Primer extension experiments (4) were carried out as described earlier (3), except that 60 μ g of actinomycin D per ml was included in the reverse transcription reaction.

In vitro transcription analysis. Restriction fragments used in transcription runoff assays were isolated in low-gelling-temperature agarose from digests of B95-8 *BamHI*-Z. In vitro transcription was done with HeLa whole-cell extracts (20), and the resultant RNAs were fractionated on denaturing polyacrylamide gels or agarose gels after glyoxylation (21).

RESULTS

Superinfection of Raji cells with P3HR1 EBV. Cytoplasmic RNA was prepared from Raji cells 4, 8, and 12 h and 3 days after superinfection with P3HR1 EBV and from mock-infected cells after 12 h. Cytoplasmic RNA was also prepared after 12 h from mock-infected or superinfected Raji cells which had been treated with 100 μ M anisomycin. The anisomycin was added at the same time as the superinfecting virus. Replicate Northern blots (29) of these various RNA samples were probed with a series of nick-translated restriction fragment probes representing the B95-8 EBV genome (Fig. 1 and 2) and with a B/8 mouse actin probe. The blots were arranged to correspond to the linear order of probes on the EBV genome. RNAs were sized on the gels by comparison with DNA markers. RNA and DNA migrated differently under the conditions used here. A correction curve was obtained by coelectrophoresing RNAs and DNAs of known sizes (Fig. 3), and the sizes of all the RNAs quoted were corrected to their true values. The sizes of the RNAs detected by the various probes are summarized in Table 1.

The actin probe detected the 1.9-kilobase (kb) cytoplasmic beta and gamma actin RNAs (Fig. 2 and Table 1). This probe was used as an independent check on the integrity of the various RNA samples and as a marker for host gene transcription. This RNA was present at the same level in most samples, but there was a reduction in its level in mock-infected cells treated with anisomycin for 12 h and a more marked reduction after 72 h of superinfection. These effects could have been due to a general reduction in cell metabolic activity or, late in infection, to the lysis of some cells. In anisomycin-treated cells, a slightly longer transcript was visible, possibly indicating a change in mRNA processing in these cells.

Latent RNAs. A few RNAs were detectable in uninfected Raji cells (Fig. 2, lanes U, and Table 1). The most abundant was the 2.5-kb RNA encoded by the *BamHI*-N het region (11). This RNA was transcribed from the latent promoter ED-L1 in B95-8 cells, and an RNA of the same size encoded

FIG. 2. Northern blotting of EBV RNAs in P3HR1-superinfected Raji cells. Cytoplasmic RNA was prepared from uninfected Raji cells (lanes U) and Raji cells 4, 8, and 12 h and 3 days (lanes 4, 8, 12, and 3, respectively) after superinfection with P3HR1 virus. RNA was also prepared from Raji cells treated with anisomycin for 12 h (lanes AU) or superinfected in the presence of anisomycin for 12 h (lanes A12). The

RNA samples were electrophoresed and blotted onto nitrocellulose filters, which were hybridized with the indicated radioactive probes, together covering the whole B95-8 genome. Panels 1 to 27 cover the B95-8 genome from left to right on the standard map. In Panel 15, the BamHI-e3 probe is an M13 clone (LTB 32, rightward strand) covering most of e3. The BamHI-e1 and BamHI-e2 probes detected nothing (data

not shown). Panels 24 to 27 cover the *EcoRI* Dhet region from left to right. DH1 is the leftmost *EcoRI*-*Bgl*II fragment, DH2 is the adjacent *Bgl*II-*Hind*III fragment, DH3 is the next *Hind*III-*Bgl*II fragment, and DH4 is the terminal *Bgl*II-*EcoRI* fragment. Panels 29 to 32 represent the results with probes derived from various M13 clones of parts of the *Bam*HI-R or *Bam*HI-Z regions (R122, Z31, and Z44) or from a highly spliced cDNA (P) (5). The prime-cut probes used were RAM122-*Bgl*II, BAZ31-*Ban*II, BAZ44-*Sph*I, and P-*Eco*RI. Radioactive DNA size markers (lambda DNAs cut with *Hind*III) were coelectrophoresed on each gel, and their sizes are indicated at the left side of each panel in kilobases. The actin probe (pRT.d3) detects beta and gamma actin mRNAs and is a 1.7-kb *Pst*I subfragment of a mouse beta actin gene (or pseudogene) in the *Pst*I site of pBR322. It was the kind gift of J. Rogers.

by sequences overlapping the latent RNA was induced by TPA from a separate promoter, ED-L1A (15). The level of 2.5-kb RNA increased in the presence of anisomycin in mock-infected cells. The level of 2.5-kb latent RNA detected by probe DH3 (Fig. 2) dropped dramatically after 4 h of superinfection, and then 2.5-kb RNA gradually reappeared. This result may represent a switching off of transcription from the latent promoter ED-L1 and an increase in transcription from the late-lytic-cycle promoter ED-L1A during superinfection.

Of the other RNAs previously found in cells latently infected with EBV, the 3.2-kb EBV nuclear antigen 1 RNA was weakly detected by the *Bam*HI-K probe (Fig. 2). The level of this RNA increased after 4 h of superinfection but

returned to uninfected levels at later times. We failed to detect the latent transcripts encoded by the *Bam*HI-W, -Y, and -H regions (5, 11) with nick-translated probes.

Using a prime-cut probe (probe P-*Eco*RI) derived from a cDNA clone (5), we detected 3.3- and 3.0-kb transcripts. The levels of these RNAs increased at 4 h after superinfection but decreased thereafter. These RNAs accumulated in cells treated with anisomycin. A 2.0-kb RNA was also induced upon superinfection, and this RNA was also present in cells superinfected in the presence of anisomycin. The low level of this RNA and the presence of latent transcripts of about this size in uninfected Raji cells (5) made us uncertain about classifying this as a true productive-cycle, anisomycin-resistant RNA. In some cases anisomycin treatment of Raji

FIG. 3. (A) Comparison of mobilities of glyoxylated DNA and RNA in neutral agarose gel electrophoresis. Various radioactively labeled RNAs and DNAs were glyoxylated and electrophoresed on 1% agarose gels. Lanes contained end-labeled influenza virus RNAs (FLU), an end-labeled mixture of *Hind*III-digested simian virus 40 and bacteriophage lambda DNAs (SV40/λH3), internally labeled *Escherichia coli* rRNA, human rRNA, and mouse rRNA. Numbers represent kilobases. (B) Plot of mobility of RNAs and DNAs in panel A against RNAs and DNAs of known sizes (in kilobases) revealing the slower mobility of RNA than of DNA on these gels. This graph was used to correct the sizes of the RNAs shown in Table 1.

TABLE 1. Summary of the sizes (corrected for the mobility differences between RNA and DNA) of EBV RNAs detected in P3HR1-superinfected Raji cells^a

Probe	RNA size (kb)
Actin.....	2.2 and 1.9
<i>Eco</i> RI-I.....	3.9 and 1.8
<i>Bam</i> HI-C.....	6.0 and 3.7
<i>Bam</i> HI-W.....	
<i>Bam</i> HI-Y.....	
<i>Bam</i> HI-H.....	2.4, 1.3, and 0.9
P.....	3.6, 3.3, 2.8, and 2.0
<i>Bam</i> HI-F.....	5.5, 4.8, 3.7, 2.4, 1.4, and 1.3
<i>Bam</i> HI-Q.....	20, 4.7, 3.4, and 2.3
<i>Bam</i> HI-U.....	20 and 15
<i>Eco</i> RI-G1.....	20 and 15
<i>Bam</i> HI-O.....	20, 15, 5.6, 3.7, and 2.9
<i>Eco</i> RI-G2.....	20, 15, 5.2, 3.1, 2.5, 2.1, and 1.3
<i>Eco</i> RI-F.....	5.2, 3.7, 3.2, 1.6, and 1.1
<i>Bam</i> HI-L.....	4.6, 2.8, 2.2, 1.0, and 0.7
<i>Bam</i> HI-E.....	3.7
<i>Bam</i> HI-e3.....	4.2
<i>Bam</i> HI-Z.....	6.0, 3.5, 2.8, 1.0, and 0.5
<i>Bam</i> HI-R.....	6.0 and 2.8
<i>Bam</i> HI-K.....	4.8, 3.2, 2.4, 1.6, 1.2, and 0.9
<i>Bam</i> HI-B.....	6.2, 3.9, 3.4, 3.2, 2.5, 2.2, 1.8, 1.3, 1.0, and 0.7
<i>Bam</i> HI-G.....	4.0, 3.3, 3.0, 2.0, 1.7, and 1.3
<i>Eco</i> RI-E.....	3.8, 3.0, 2.2, and 1.4
<i>Eco</i> RI-H.....	5.2, 3.9, 3.5, 3.1, 2.2, 1.7, and 1.6
<i>Eco</i> RI-C.....	6.4, 5.0, 3.9, 2.8, 1.6, and 1.2
DH1.....	6.6, 3.8, and 3.3
DH2.....	3.8
DH3.....	2.5 and 0.8
DH4.....	

^a Data were deduced from the Northern blots in Fig. 2.

cells appeared to superinduce latent EBV RNAs; for example, compare lanes U and AU in Fig. 2, panel 26, or lanes U and AU in Fig. 2, panel 32.

A previously uncharacterized 1.6-kb RNA was detected in uninfected Raji cells by *Eco*RI-F (Fig. 2).

Lytic-cycle RNAs. All RNAs which were not detected in mock-infected cells but whose levels increased during superinfection were designated as viral lytic-cycle RNAs. Only a few such RNAs were detected at 4 h after superinfection (Fig. 2). These were the 2.8- and 1.0-kb RNAs hybridizing to *Bam*HI-Z, the 2.8-kb RNA detected by *Bam*HI-R, the 2.2-kb RNA detected by *Bam*HI-B, and the 3.8-kb RNA detected by probe DH1. At 8 and 12 h after superinfection many lytic-cycle RNAs were detected (Fig. 2 and Table 1). There were some variations between the relative levels of the same RNAs at those two times, possibly relating to their time of maximal expression in the lytic cycle. After 72 h most RNAs were found at lower levels; taken with the reduced level of the actin RNA, this result may indicate a general reduction in transcription in these cells or the beginning of cell death. Given that nick-translated probes failed to detect the latent RNAs encoded by *Bam*HI-W, -Y, and -H and given the low level of some of the detected RNAs, it is probable that some other low-level RNAs were not detected in these experiments.

The effect of cotreatment with 100 μM anisomycin upon superinfection was the prevention of the expression of almost all lytic-cycle RNAs. Apart from the 2.0-kb RNA weakly detected by a cDNA clone (discussed earlier) among

FIG. 4. Summary of structures of the 1.0- and 2.8-kb RNAs and the probes used for mapping.

the latent RNAs encoded by *BamHI*-W, -Y, and -H, the only lytic-cycle RNAs which were detected in anisomycin-treated cells were the 1.0-kb RNA detected by *BamHI*-Z and the 2.8-kb RNA detected by *BamHI*-Z and *BamHI*-R. In superinfected cells blocked for protein synthesis, the 2.8-kb

RNA was more heterogeneous in size, reminiscent of the effect seen with the actin probe; again, this may reflect some mRNA processing effect in these cells. Given the dramatic increase in the levels of these RNAs, their insensitivity to anisomycin treatment, and their appearance as some of the

FIG. 5. Mapping the 5' end of the 1.0-kb RNA. (Lanes 1 to 5) S1 nuclease mapping with probe LTB138-AhaIII (B95-8 EBV bases 103083 to 103228). Lane 1, Untreated probe. The probe was hybridized to tRNA (lane 2) as a negative control, RNA from control B95-8 cells (lane 3), RNA from B95-8 cells treated with TPA (lane 4), and RNA from B95-8 cells treated with TPA and PAA (lane 5). Size markers (lane M) were pBR322 DNAs cut with *MspI*. (Lanes 6 to 9) Primer extension with LTB138-AvaII (B95-8 EBV bases 103083 to 103145). Lane 6, Untreated primer. The primer was hybridized to tRNA (lane 7) as a negative control, RNA from control B95-8 cells (lane 8), and RNA from TPA-treated B95-8 cells (lane 9). Size markers (lane M) were pBR322 DNAs cut with *MspI*. (Lanes 10 to 18) In vitro transcription from the 1.0-kb promoter. In vitro transcription extracts were supplied with various template DNAs, and the resulting radioactive transcripts were analyzed on agarose (lanes 10 to 14) or acrylamide (lanes 15 to 18) gels. Lane 12, Extract with no added DNA. Templates were *BamHI*-Z DNA (lane 14), *BamHI*-Z DNA cut with *NcoI* (lane 13), an *SmaI*-*BamHI* fragment (bases 102756 to 103743) containing the 1.0-kb RNA promoter (lane 15), and a lower concentration of the *SmaI*-*BamHI* template (lane 16). Markers were a *HindIII* digest of simian virus 40 DNA (lane 10), human rRNA (lane 11), an *MspI* digest of pBR322 DNA (lane 17), and an *AluI* digest of pAT153 (lane 18). The relevant protected fragments are indicated by arrows. Numbers beside panels represent kilobases.

S1 nuclease mapping of the RNAs from the P3HR1-superinfected Raji cells would be very complicated because

We are confident that the 1.0-kb RNA has a promoter separate from that of the 2.8-kb RNA. The S1 nuclease mapping and primer extension results for the 1.0-kb RNA agree on the start point, there is no consensus splice acceptor within the error limits of the mapping, and in vitro

FIG. 7. Mapping of splice junctions near the 3' end of the 2.8- and 1.0-kb RNAs. (Lanes 1 to 11) S1 nuclease mapping with probe BAZ31-*Ban*II (containing B95-8 EBV bases 102393 to 102615). Lane 1, Untreated probe. The probe was hybridized to tRNA (lane 2) as a negative control, RNA from untreated Raji cells (lane 3), RNA from Raji cells 4, 8, and 12 h after superinfection with P3HR1 EBV (lanes 4, 5, and 6, respectively), RNA from Raji cells 12 h after superinfection in the presence of anisomycin (lane 7), RNA from Raji cells treated for 12 h with anisomycin (lane 8), RNA from Raji cells 3 days after superinfection (lane 9), and RNA from TPA-treated B95-8 cells (lane 10). Size markers (lane 11) were from an *Alu*I digest of pAT153 DNA. (Lanes 12 to 17) S1 nuclease mapping with probe BAZ13-*Fok*I (containing B95-8 EBV bases 102363 to 102576). Lane 13, Untreated probe. The probe was hybridized to tRNA (lane 14) as a negative control or to RNA from B95-8 cells untreated (lane 15), treated with TPA (lane 16), or treated with TPA and PAA (lane 17). Size markers (lane 12) were from an *Msp*I digest of pBR322 DNA. (Lanes 18 to 22) S1 nuclease mapping with probe 58BAZ-*Bgl*II (containing B95-8 EBV bases 102210 to 102400). Lane 18, Untreated probe. The probe was hybridized to tRNA (lane 19) as a negative control or to RNA from B95-8 cells untreated (lane 20), treated with TPA (lane 21), or treated with TPA and PAA (lane 22). (Lanes 23 to 26) S1 nuclease mapping with probe 191LTB-*Sma*I (containing B95-8 EBV bases 102565 to 102756). Lane 23, Untreated probe. The probe was hybridized to RNA from untreated B95-8 cells (lane 24) or to RNA from B95-8 cells treated with TPA (lane 25). Size markers (lane 26) were from an *Msp*I digest of pBR322 DNA. The relevant protected fragments are indicated by arrows. Numbers beside panels represent kilobases.

transcription experiments have shown that there is a promoter with a transcription start site at base 103195.

In vitro transcription of the isolated *Bam*HI-Z DNA yielded a specific transcript 1,245 bases long together with a template full-length artifact transcript (Fig. 5). The 1,245-base band was shortened to 1,075 bases when the template was cleaved with *Nco*I, indicating that transcription was leftward, starting at 1,075 bases from the *Nco*I site, i.e., at about base 103195. The in vitro transcript was sized more accurately by transcribing the *Sma*I-*Bam*HI (bases 102756 to 103743) piece of DNA, yielding the predicted major runoff 440 bases long (Fig. 5). The 5' end of the 1.0-kb RNA was also mapped with riboprobe SP64-BAZ44 (data not shown). In this experiment, RNA from P3HR1-superinfected Raji cells was compared with B95-8 cell RNA. The same-sized protected fragment was observed, indicating that the transcription start site in the superinfection system was the same as that in B95-8 cells.

Sequence of a partial cDNA copy of 2.8-kb RNA. Because

of the complexity of mapping overlapping RNAs by S1 nuclease protection, we attempted to isolate cDNA clones corresponding to the RNAs.

A cDNA library made from B95-8 cytoplasmic poly(A)⁺ RNA was screened with the probe BAZ44-*Sph*I (bases 102984 to 103419). Positive lambda clones were subcloned into pUC 11, and their structures were analyzed. The clone pUC 33-25 was found to contain the 3' poly(A), confirming that it arose by reverse transcription of RNA. The insert in this clone was 1,787 base pairs long, including 12 bases of poly(A). The clone evidently did not correspond to the 1.0-kb RNA, since it was too long, and was apparently a partial cDNA copy of the 2.8-kb RNA. In fact, no cDNAs small enough to be the 1.0-kb RNA were obtained, perhaps because a size selection step in the preparation of the library excluded them. The DNA sequence of the pUC 33-25 clone was determined by subcloning restriction fragments of pUC 33-25 to appropriate M13 vectors and dideoxynucleotide sequencing. The pUC 33-25 sequence had two splices rela-

FIG. 8. Mapping of the 5' boundary of the coding exon of the 2.8-kb RNA. (Lanes 1 to 6) Riboprobe mapping with probe RAM122-*Bgl*II (containing B95-8 EBV bases 104959 to 105415). Lane 1, Untreated probe. The probe was hybridized to tRNA (lane 2) as a negative control, RNA from uninfected Raji cells (lane 3), RNA from Raji cells 12 h after superinfection with P3HR1 EBV (lane 4), RNA from Raji cells 12 h after superinfection in the presence of anisomycin (lane 5), and Raji cells treated for 12 h with anisomycin (lane 6). The resulting hybrids were treated with RNase, and the protected fragments were electrophoresed on acrylamide gels. (Lanes 7 to 11) S1 nuclease mapping with probe RAM128-*Bgl*II (containing B95-8 EBV bases 105060 to 105415). Lane 7, Untreated probe. The probe was hybridized to tRNA (lane 8) as a negative control, RNA from untreated B95-8 cells (lane 9), RNA from TPA-treated B95-8 cells (lane 10), and RNA from B95-8 cells treated with TPA in the presence of PAA (lane 11). The hybrids were digested with S1 nuclease, and the protected fragments were electrophoresed on acrylamide gels. (Lanes 12 to 16) Primer extension with probe RAM128-*Fok*I (containing B95-8 EBV bases 105060 to 105151). Lane 12, Primer alone. The primer was hybridized to RNA from untreated B95-8 cells (lane 13), RNA from TPA-treated B95-8 cells (lane 14), and RNA from B95-8 cells treated with TPA and PAA (lane 15). The hybrids were extended with reverse transcriptase, and the products were electrophoresed on acrylamide gels. Size markers (lanes M) were from an end-labeled *Msp*I digest of pBR322 DNA. In each panel, the relevant protected fragment or primer extension product is indicated by an arrow. Numbers beside panels represent kilobases.

tive to the B95-8 genomic sequence but was otherwise identical to the B95-8 sequence (Fig. 6). The 5' end of the cDNA was at base 104102. The sequence was like the leftward strand of B95-8 DNA but lacked bases 102654 to 102531 and 102425 to 102342, terminating at base 102126 and containing a short tract of poly(A). Because the EBV sequence contains two adenine residues at the point at which poly(A) begins, the precise 3'-end cleavage point for poly(A) addition could not be deduced. This cDNA sequence confirmed our notion from S1 nuclease mapping experiments (described below) that the RNAs might be spliced near the 3'

FIG. 9. (Lanes 1 to 4) Northern blot of RNA from B95-8 cells with probe 23FAL-*Bst*XI (B95-8 EBV bases 106074 to 106223). Lanes: 1, RNA from untreated B95-8 cells; 2, RNA from B95-8 cells treated with TPA; 3, RNA from B95-8 cells treated with TPA and PAA; and 4, end-labeled *Hind*III digest of bacteriophage lambda DNAs as size markers. The 2.8-kb RNA is indicated by an arrow. (Lanes 5 to 9) Northern blot of RNA from B95-8 cells treated with TPA and PAA (lanes 6 and 8) and RNA from P3HR1-superinfected Raji cells (lanes 7 and 9). The probes were 23FAL-*Bst*XI (lanes 6 and 7) and BAZ44-*Sph*I (lanes 8 and 9). Size markers (lane 5) were from a *Hind*III digest of bacteriophage lambda DNA. The 2.8-kb RNA is indicated by an arrow. Numbers beside panels represent kilobases.

end. These splices have important consequences for the BZLF1 reading frame (see Discussion).

S1 nuclease mapping of splice junctions. To substantiate the cDNA clone sequence and to show that the structure of this clone was representative of the structure of the great majority of the RNA population, we S1 nuclease mapped the region upstream of the 3' end of the 2.8- and 1.0-kb RNAs.

S1 nuclease mapping with probe BAZ31-*Ban*II (bases 102393 to 102615) of RNA from P3HR1-superinfected Raji cells and B95-8 cells and S1 nuclease mapping with probe BAZ13-*Fok*I (bases 102363 to 102576) of B95-8 cell RNA confirmed the structure of the cDNA clone and showed that this splicing pattern was the major structure in both cell systems. It also confirmed that the AAUAAA site at base 102500 was not used for 3'-end generation to any measurable extent. Both mapping experiments revealed a protected fragment of 108 to 110 bases in length (Fig. 7). This fragment was larger than the 105-base exon deduced from the cDNA sequence because the sequences brought together by the splicing happened to extend the region complementary to the probes.

We attempted to confirm the presence of the splice acceptor at base 102341 by using probe 58BAZ-*Bgl*II (bases 102210 to 102400), which should reveal a protected fragment of 132 bases. In fact, a shorter protected fragment of about 115 bases long was seen (Fig. 7). One end of the protected region (bases 102210 to 102222) was exceedingly rich in adenine plus thymine, and we believe that this shorter fragment was the result of S1 nuclease digestion of the transiently melting end of the RNA-DNA duplex down to the point at which a more stable guanine-plus-cytosine-rich duplex was encountered. The unique nature of the protected fragment indicated that there was only one dominant structure in the RNA population in this region.

S1 nuclease mapping with probe 191LTB-*Sma*I (bases

102565 to 102756) revealed a protected fragment of 102 bases (Fig. 7), again consistent with the splice donor found in the cDNA sequence at base 102655.

The absence of any full-length protection of the EBV content of the probes in these S1 nuclease mapping experiments and the unique protected fragments indicated that both the 2.8- and 1.0-kb RNAs were spliced in the same way.

5'-end mapping of 2.8-kb RNA. S1 nuclease protection mapping with riboprobe RAM122-*Bg*II (containing bases 104959 to 105415) revealed a protected fragment of 226 bases (Fig. 8). S1 nuclease mapping with probe RAM128-*Bg*II (containing bases 105060 to 105415) revealed a shorter protected fragment of 126 bases (Fig. 8). The reduction in size of the protected fragment with these overlapping probes by the same amount as the difference in the probe boundaries indicated that the RNA was colinear with the 5' end of EBV DNA in both probes. Therefore, the mapped 5' end of this exon was 126 bases into RAM128-*Bg*II from base 105060, i.e., at base 105185. To determine if this was the 5' end of the RNA, we performed primer extension analysis with probe RAM128-*Fok*I. This probe contains 92 bases of EBV (bases 105060 to 105151) and, with its M13 leader, is 140 bases long. If base 105185 were the 5' end of the RNA, the probe should have been extended 40 bases to base 180. In fact, the primer extension product was 235 bases long (Fig. 8), indicating a further 5' exon(s) totalling 55 bases upstream of the boundary at base 105185. Indeed, the DNA sequence at base 105185 corresponded to a consensus splice acceptor sequence.

We have not properly identified the 5' end of the 2.8-kb RNA but have obtained some evidence as to its likely location in B95-8 virus. The next reading frame to the right of BRLF1 is in the opposite (rightward) direction and exactly abuts the N terminus of BRLF1. We searched further up the genome with short Northern blotting probes to find the exon(s) which corresponded to the remaining 55 bases of the leftward 2.8-kb RNA. Probe 23FAL-*Bst*XI (bases 106074 to 106223) was the only probe tested which detected an early RNA of exactly the same size as the 2.8-kb anisomycin-resistant RNA (Fig. 9). This result was particularly interesting, because 23FAL-*Bst*XI overlaps a gap between two rightward reading frames (1); this gap could contain the leftward leader and its promoter. The downstream end of this gap has an excellent consensus splice donor at base 106126, and within this gap there is a TATA box appropriately placed for the promoter. 23FAL-*Bst*XI also detected another, larger leftward RNA, possibly indicating that this putative leader exon (bases 106180 to 106126) contributes to another unknown leftward gene. Our attempts to S1 nuclease map this exon have, however, yielded poor and variable results.

Comparison of 2.8- and 1.0-kb RNA structures in B95-8 cells and P3HR1-superinfected Raji cells. We have not analyzed both RNAs exhaustively, but all the information we have indicates that the 1.0-kb RNA has the same overall structure in B95-8 cells and P3HR1-superinfected Raji cells. The 2.8-kb RNA, however, has a structural difference which probably indicates that most of the transcription of the 2.8-kb RNA in the superinfected cells is coming from the defective genomes.

Two groups have analyzed the structures of the P3HR1 defective DNAs. The first does not contain an intact BRLF1 reading frame (24), but the defective genomes characterized by Cho et al. (7) contain most of *Bam*HI-R and -Z, *Bam*HI-R being fused to *Bam*HI-W. The predominant defective genomes in the P3HR1 cells used here seem to be similar to

those characterized by Cho et al. (7), as judged by Southern blotting with probes in the *Bam*HI-R region (data not shown).

The putative 5' leader exon detected in B95-8 RNA with probe 23FAL-*Bst*XI was absent in anisomycin-blocked, P3HR1-superinfected Raji cells. This is evident from the Northern blots shown in Fig. 9. Although probe BAZ44-*Sph*I detected both the 2.8- and 1.0-kb RNAs in B95-8 cells, probe 23FAL-*Bst*XI detected the 2.8-kb RNA only in B95-8 cells. Since there is no reason to believe that P3HR1 lacks this piece of DNA sequence (its *Bam*HI-R fragment is the conventional size), we conclude that most of the expression is coming from the defective genomes (these lack the leader exon DNA). It must follow that the 2.8-kb gene is being expressed by an alternative mechanism in the superinfected, anisomycin-blocked cells, perhaps from another spliced leader which then utilizes the standard acceptor in front of the BRLF1 reading frame.

DISCUSSION

We have studied the expression of EBV RNAs in P3HR1 virus-superinfected Raji cells. Over 60 productive-cycle RNAs have been identified in this system, and their approximate times of peak expression have been determined. By also examining RNA levels in superinfected Raji cells treated with anisomycin, a powerful inhibitor of protein synthesis, we have identified a novel small class of RNAs, of which the predominant members are 2.8- and 1.0-kb RNAs from the *Bam*HI-R-*Bam*HI-Z region of the genome and a 2.0-kb RNA detected by the cDNA clone P. These RNAs, whose transcription is independent of cellular protein synthesis, behave as immediate-early RNAs in this system, but the complexity of EBV genome structures in the superinfection system lead us to question this conclusion. Other evidence described below argues strongly that the 1.0-kb RNA is a true immediate-early RNA, but the case for the 2.8- and 2.0-kb RNAs is much less clear.

We used probes corresponding only to the B95-8 genome, which has a region deleted relative to other EBV strains. We therefore cannot exclude the possibility that there may be other anisomycin-resistant RNAs hybridizing to the B95-8 deletion region.

A combination of S1 nuclease protection mapping, Northern blotting, and cDNA clone sequencing was used to determine the structures of the 2.8- and 1.0-kb RNAs in B95-8 cells and P3HR1-superinfected Raji cells. These experiments showed that the structures are generally similar in the two systems, with the difference that a spliced leader on the 2.8-kb RNA 5' end is different in the P3HR1-superinfected Raji cells. Several splices were identified in both RNAs. These splices have no effect on the integrity of reading frame BRLF1, which is encoded by the 2.8-kb RNA. The structure of BZLF1 is altered by the splices, with the result that a repetitive region is spliced out and the reading frame is extended beyond the simple reading frame assumed from the DNA sequence (1).

Our conclusions on identification of RNAs in superinfected Raji cells which are resistant to inhibitors of protein synthesis differ substantially from those of Sample et al. (27). They found a predominant cycloheximide-resistant RNA hybridizing to *Bam*HI-M (encoding BMRF1). We consider that this difference in results arises from their use of only intermediate cycloheximide concentrations, which do not completely inhibit protein synthesis, and the detection of RNAs by dot blotting in their experiments. This technique is

less sensitive and specific than Northern blotting, and we believe that their experiments detected only those very abundant delayed early RNAs which leaked through the partial cycloheximide block.

Recent experiments directly examining *trans*-activating gene products in EBV show that the BMLF1 reading frame encodes a protein important in the activation of productive-cycle gene expression (6, 18). However, the RNA for this protein does not behave as an anisomycin-resistant RNA.

Our experiments were performed originally with the intention of identifying immediate-early genes of EBV which might be involved in the latent cycle-productive cycle switch. A central question now is whether the genes we have identified as having anisomycin-resistant RNAs are immediate-early genes. Although the procedure of superinfecting with virus and inhibiting the resulting productive cycle with anisomycin is superficially similar to a conventional immediate-early analysis, in this case we are probably studying those genes set up for constitutive expression in the defective P3HR1 particles. Some of these are probably equivalent to true immediate-early genes of EBV, since the defective genomes are the active agents in the induction. The identification of immediate-early transcription of the *Bam*HI-Z region, notably the 1.0-kb mRNA traversing BZLF1, is in striking correspondence with the results of transfection experiments of other groups (10, 28). They showed by transfection of fragments of DNA from the defective genomes that this same region (WZhet) is capable of inducing a productive cycle in cells latently infected with EBV. There can be little doubt, therefore, that the expression of BZLF1 (presumably the spliced version of the reading frame that we have identified here) is critical for the switch to the productive cycle.

Why then do we also observe transcription of the 2.8-kb anisomycin-resistant RNA encoding BRLF1? Either this observation is a clue that BZLF1 is not the only gene in the defective genomes relevant to activation or it might just be chance that this gene, adjacent to BZLF1, is also expressed. The trivial explanation that BRLF1 is being expressed by chance through its splice acceptor being accessed by the highly spliced latent RNA leaders coming from *Bam*HI-W (*Bam*HI-W is next to *Bam*HI-R in the defective genomes) is not supported by our failure to detect the anisomycin-resistant 2.8-kb RNA with the clone P probe (Fig. 2). However, if a very short piece of the leaders were on the front of the 2.8-kb RNA, the clone P probe might not have detected it. We have not yet established the structure of the anisomycin-resistant 2.0-kb RNA which is detected by clone P.

In HSV infections, the productive cycle is initiated by expression of the immediate-early group of genes. Activation of transcription of these genes requires the consensus sequence TAATGARAATTC, which is found upstream of all the immediate-early genes of HSV (19, 31). The upstream promoter regions of the immediate-early genes are also characterized by guanine-plus-cytosine-rich sequences which are binding sites for the Sp1 transcription factor. We examined the B95-8 EBV sequences upstream of the transcription start sites of the 1.0- and 2.8-kb RNAs for homologies to the TAATGARAATTC motif. The only substantial homology was TAATGAAATCT on the leftward strand at bases 103462 to 103452. This is about 270 base pairs upstream of the 1.0-kb RNA transcription start site. The sequence is also preceded by a block of 14 to 18 guanine or cytosine residues. This might be an indication that TAATGARAATTC is an important immediate-early se-

quence element for activation of expression of the 1.0-kb RNA. However, several points must be weighed against this. Homologies (90%) to TAATGAAAATC can be found in four other places in the EBV genome with no obvious significance. Also, the guanine-plus-cytosine-rich sequences near EBV base 103452 TAATGARAAT do not match the Sp1-binding consensus sequence.

In conclusion, we have identified and determined the structures of two RNAs in B95-8 cells and P3HR1-superinfected Raji cells. These RNAs may be in a special regulatory class. The protein product of at least one of them is likely to be important in the switch from the latent cycle to the productive cycle. The RNAs are both spliced, and the splicing modifies the structure of the BZLF1 reading frame, extending it and removing a repetitive region from the simple reading frame previously surmised from DNA sequence analysis. We are using the cDNA clone spanning BZLF1 to try to obtain large amounts of this protein to directly study its effects on EBV gene expression.

LITERATURE CITED

1. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, et al. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* (London) **310**:207-211.
2. Berk, A. J., and P. A. Sharp. 1978. Spliced early mRNAs of SV40. *Proc. Natl. Acad. Sci. USA* **75**:1274-1278.
3. Biggin, M., P. J. Farrell, and B. G. Barrell. 1984. Transcription and DNA sequence of the Bam HI L fragment of B95-8 Epstein-Barr virus. *EMBO J.* **3**:1083-1090.
4. Bina-Stein, M., M. Thoren, N. Salzman, and J. A. Thompson. 1979. Rapid sequence determination of late SV40 16s mRNA leader by using inhibitors of reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **76**:731-736.
5. Bodescot, M., B. Chambraud, P. Farrell, and M. Perricaudet. 1984. Spliced RNA from the IRI-U2 region of Epstein-Barr virus: presence of an opening reading frame for a repetitive polypeptide. *EMBO J.* **3**:1913-1917.
6. Chevallier-Greco, A., E. Manet, P. Chavrier, C. Mosnier, J. Daillie, and A. Sergeant. 1986. Both Epstein-Barr virus (EBV)-encoded transacting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. *EMBO J.* **5**:3243-3250.
7. Cho, M.-S., G. W. Bornkamm, and H. zur Hausen. 1984. Structure of defective DNA molecules in Epstein-Barr virus preparations from P3HR-1 cells. *J. Virol.* **51**:199-207.
8. Cho, M.-S., K.-O. Fresen, and H. zur Hausen. 1980. Multiplicity dependent biological and biochemical properties of Epstein-Barr virus (EBV) rescued from non-producer lines after superinfection with P3HR1 EBV. *Int. J. Cancer* **26**:357-363.
9. Clements, J. B., R. J. Watson, and N. M. Wilkie. 1977. Temporal regulation of HSV1 transcription; location of transcripts on the viral genome. *Cell* **12**:275-285.
10. Countryman, J., and G. Miller. 1985. Activation of expression of latent Epstein-Barr herpes virus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proc. Natl. Acad. Sci. USA* **82**:4085-4089.
11. Dambaugh, T., K. Hennessy, S. Fennwald, and E. Kieff. 1986. The virus genome and its expression in latent infection, p. 13-46. In M. A. Epstein and B. G. Achong (ed.), *The Epstein-Barr virus; recent advances*. William Heinemann Medical Books Ltd., London.
12. Farrell, P. J., A. Bankier, C. Seguin, P. Deininger, and B. G. Barrell. 1983. Latent and lytic cycle promoters of the Epstein-Barr virus. *EMBO J.* **2**:1331-1338.
13. Henle, W., G. Henle, B. Zajac, G. Pearson, R. Waubke, and M. Scriba. 1970. Differential reactivity of human serums with early antigens induced by Epstein-Barr virus. *Science* **169**:188-190.
14. Honess, R., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* **14**:8-19.

15. Hudson, G. S., P. J. Farrell, and B. G. Barrell. 1985. Two related but differentially expressed potential membrane proteins encoded by the *Eco*RI Dhet region of Epstein-Barr virus B95-8. *J. Virol.* **53**:528–535.
16. Hummel, M., and E. Kieff. 1982. Epstein-Barr virus RNA. VIII. Viral RNA in permissively infected B95-8 cells. *J. Virol.* **43**:262–272.
17. Lewis, J. B., and M. B. Mathews. 1980. Control of adenovirus early gene expression: a class of immediate early products. *Cell* **21**:303–313.
18. Lieberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward. 1986. Promiscuous *trans* activation of gene expression by an Epstein-Barr virus-encoded early nuclear protein. *J. Virol.* **60**:140–148.
19. Mackem, S., and B. Roizman. 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. *J. Virol.* **44**:939–949.
20. Manley, J. L., A. Fire, A. Cano, P. Sharp, and M. Gelfer. 1980. DNA dependent transcription of adenovirus genes in a soluble whole cell extract. *Proc. Natl. Acad. Sci. USA* **77**:3855–3859.
21. McMaster, G. K., and G. C. Carmichael. 1977. Analysis of single stranded and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**:4835–4838.
22. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. *Nucleic Acids Res.* **12**:7035–7056.
23. Miller, G., L. Heston, and J. Countryman. 1985. P3HR-1 Epstein-Barr virus with heterogeneous DNA is an independent replicon maintained by cell-to-cell spread. *J. Virol.* **54**:45–52.
24. Miller, G., M. Rabson, and L. Heston. 1984. Epstein-Barr virus with heterogeneous DNA disrupts latency. *J. Virol.* **50**:174–182.
25. Polack, A., H. Delius, U. Zimmer, and G. W. Bornkamm. 1984. Two deletions in the Epstein-Barr virus genome of the Burkitt lymphoma nonproducer line, Raji. *Virology* **133**:146–157.
26. Rabson, M., L. Heston, and G. Miller. 1983. Identification of a rare Epstein-Barr virus variant that enhances early antigen expression in Raji cells. *Proc. Natl. Acad. Sci. USA* **80**:2762–2766.
27. Sample, J., A. Tanaka, G. Lanez, and M. Nonoyama. 1984. Identification of Epstein-Barr virus genes expressed during the early phase of virus replication and during lymphocyte immortalisation. *Virology* **139**:1–10.
28. Takada, K., N. Shimizu, S. Sakuma, and Y. Ono. 1986. *trans* activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. *J. Virol.* **57**:1016–1022.
29. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77**:5201–5205.
30. Weigel, R., and G. Miller. 1983. Major EB virus-specific cytoplasmic transcripts in a cellular clone of the HR-1 Burkitt lymphoma line during latency and after induction of viral replication by phorbol esters. *Virology* **125**:287–298.
31. Whitton, J. L., F. L. Rixon, A. J. Easton, and J. B. Clements. 1983. Immediate early mRNA-2 of HSV1 and 2 is unspliced: conserved sequences around the 5' and 3' termini correspond to transcription regulatory signals. *Nucleic Acids Res.* **11**:6271–6287.
32. zur Hausen, H., F. O'Neill, and U. Freese. 1978. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. *Nature (London)* **272**:373–375.