Mapping of Genes in *Bam*HI Fragment M of Epstein-Barr Virus DNA That May Determine the Fate of Viral Infection

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We used nuclease digestion to map RNA transcripts encoded in the *Bam*HI M fragment of the Epstein-Barr virus (EBV) genome (strain B95-8). Of the five RNAs, three are rightwardly transcribed, have different cap sites but common 3' termini, and are unspliced. The two remaining RNAs are leftwardly transcribed and are 5' and 3' coterminal. One of these transcripts is spliced, resulting in the removal of a small intron from the 5' region of this RNA. We have previously published data which indicated that the *Bam*HI M region is the first actively transcribed region of the viral genome during the replicative cycle, suggesting that one or more genes in this region is important in the initiation of EBV replication. We have now mapped two large *Eco*RI restriction fragments which span approximately 75% of the P3HR-1 defective genome and which contain DNA from the *Bam*HI M region of the standard genome. The data indicate that only the coding and 5' flanking sequences for the leftwardly transcribed RNAs are intact within the defective genome. Fewer than 500 bases coding for the 3'-most regions of the rightwardly transcribed RNAs are intact, and it is unlikely that these encode functional native polypeptides. Therefore, it seems that transcriptional activation of the *Bam*HI M-region genes is not mediated directly by the rearrangement of M genes in defective P3HR-1 EBV.

The infection of primate B lymphocytes with Epstein-Barr virus (EBV) leads to the establishment of latent infection and immortalization of the cells. In cultures of immortalized lymphoblastoid cells which are predominately latently infected but spontaneously produce EBV in 1 to 5% of the cells (such as the B95-8 and P3HR-1 cell lines), the virus replication cycle can be induced in a higher percentage of cells (20 to 30%) by treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (35). EBV produced in P3HR-1 cells has two unique biological properties. First, the P3HR-1 EBV strain is nontransforming, presumably due to a deletion in a gene necessary for growth transformation of B lymphocytes (6, 24, 32, 33). Second, this EBV strain can superinfect latently infected lymphoblastoid cells to develop lytic replication of the superinfecting virus and also disrupt latency by activating the latently infected viral genomes (16, 22, 26, 30, 34).

It has been reported that the superinfecting ability of P3HR-1 EBV may require the presence of virus particles containing defective genomes in the superinfecting virus population (23, 27). Analyses of the DNAs in the defective genome revealed that they contain specific noncontiguous regions of the standard EBV genome and are similar in structure to defective genomes of other herpesviruses (5, 7, 12, 14, 23). After transfection of fragments of the defective P3HR-1 genome into EBV-negative fibroblast cells, there is expression of both EBV early antigen and viral capsid antigen, suggesting that some of the functional genes associated with virion replication are present in the defective genome (8). However, the mechanism(s) by which the defective virus may help superinfection is unknown.

We have previously identified a region of the standard EBV genome, composed primarily of the *Bam*HI M restriction fragment, which is the first actively transcribed region of

the genome after superinfection of latently infected Raji cells. Furthermore, this region of the genome is not transcriptionally active during growth-transforming EBV infection of fresh lymphocytes (29). These findings are consistent with a model in which expression of a gene(s) within the *Bam*HI M region is necessary for initiation of the EBV replication cycle and may determine the fate of viral infection, immortalization or lytic replication.

Analyses of the defective P3HR-1 genome by Southern blot hybridization indicate that portions of the *Bam*HI M and adjacent S fragments are present within the defective molecules (7, 14, 23). Rearrangement of this region of the genome could result in alternative organizational forms of the transcriptional units that control the expression of the genes that are important for the initiation of the replication cycle. To evaluate this possibility, as well as other mechanisms of activation of the EBV replication cycle, knowledge of the precise organization of the genes in the *Bam*HI M region of the standard and defective genomes is required.

We characterized and mapped the RNA transcripts encoded by the *Bam*HI M region of the EBV genome. Of the five transcripts, three were transcribed in a rightward direction. They had different cap sites but had coterminal 3' ends and were unspliced. The two remaining transcripts were leftwardly transcribed and had coterminal 5' and 3' ends, and one of these transcripts was spliced. Two large *Eco*RI restriction fragments that made up approximately 75% of the defective P3HR-1 EBV genome were cloned and mapped. The structure of these fragments was nearly identical to that previously reported by Cho et al. (7). The entire coding region only for the two leftwardly transcribed RNAs was present in the defective genome DNA. Furthermore, approximately 3 kilobases (kb) of the 5' flanking region of this gene was intact, and therefore *cis*-mediated activation of this gene by rearrangement seems unlikely. Less than 500 bases encoding the 3' regions of the three rightwardly transcribed RNAs were present in the defective genome and do not likely encode functional polypeptides.

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FIG. 1. Identification of the RNA species transcribed from the *Bam*HI M region of the EBV genome. Cytoplasmic poly(A)⁺ RNA from productively infected cells was denatured, electrophoresed in a 1% agarose gel, and blot transferred to a nitrocellulose membrane. The RNA blot was hybridized to ³²P-labeled plasmid DNA containing the *Bam*HI M restriction fragment. Each lane (left to right) contained 5 µg of RNA from: Raji cells superinfected with P3HR-1 EBV in the absence (S.I.-, lane 1) or presence (S.I.+, lane 2) of cycloheximide (5 µg/ml), TPA-treated B95-8 cells (lane 3), or TPA-treated P3HR-1 cells (lane 4). Sizes are indicated in kilobases.

MATERIALS AND METHODS

Cells. The EBV-harboring lymphoblastoid cell lines B95-8, P3HR-1, and Raji were maintained at 37°C in RPMI 1640 medium containing 50 U of penicillin per ml, 50 µg of streptomycin per ml, and 5% heat-inactivated (56°C, 30 min) newborn calf serum. Fresh medium was added to the cultures every 3 to 4 days. Induction of the EBV replicative cycle in B95-8 and P3HR-1 cells was achieved by adding TPA (20 ng/ml) to the cell cultures. These cells were harvested 3 to 4 days after the addition of TPA, at which time 20 to 30% of the cells were positive for early antigen expression as determined by indirect immunofluorescence staining (15).

Isolation of cytoplasmic RNA. Cells were washed with and resuspended (10^7 to 10^8 cells per ml) in an ice-cold solution containing 10 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, and 3 mM CaCl₂. The plasmid membranes were disrupted by the addition of 0.1 volume of 5% Nonidet P-40 followed by incubation on ice for 7 min. Nuclei were pelleted by centrifugation at $8,000 \times g$ (Beckman JA-20 rotor) for 5 min at 4°C. The supernatant (cytoplasmic fraction) was removed and mixed with an equal volume of a solution containing 50 mM Tris hydrochloride (pH 7.4), 7 M urea, 0.35 M NaCl, and 1% sodium dodecyl sulfate (4). This mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and then once with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). The RNA was precipitated at -20°C in ethanol and collected by centrifugation. Poly(A)⁺ RNA was isolated by three cycles of oligo(dT)-cellulose (type 3; Collaborative Research, Inc., Waltham, Mass.) chromatography (1). All RNA was stored at -80°C in 70% ethanol at 1 mg/ml.

Nuclease mapping of EBV RNA. S1 and exonuclease VII mapping were performed as described by Berk and Sharp (4) except that end-labeled DNA fragments were used instead of uniformly labeled DNA. Restriction fragments of EBV DNA were labeled at their protruding 5' termini with [γ - 32 P]ATP (3,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and T4 polynucleotide kinase after removal of terminal phosphate with bacterial alkaline phosphatase as described previously (19). Recessed 3' termini were labeled with [α - 32 P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase I as described previously (18). Asymmetrically labeled fragments were generated by cleavage with a second restriction endonuclease. End-labeled DNA fragments were purified by polyacrylamide gel electrophoresis and isolated from the gel as described by Maxam and Gilbert (19). For each hybridization reaction, 2×10^4 cpm of an end-labeled DNA fragment was denatured at 72°C for 15 min in 50 μ l of 80% formamide–400 mM NaCl–40 mM PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]) (pH 6.4)–1 mM EDTA containing 150 μ g of yeast tRNA (control) or 150 μ g of cytoplasmic RNA from TPA-treated B95-8 cells. Hybridization was then carried out for 4 h at 50°C. After hybridization, half of the mixture was rapidly diluted into 10 volumes of ice-cold 250 mM NaCl–30 mM sodium acetate (pH 4.5)–1 mM ZnSO₄–20 μ g of thermally denatured salmon sperm DNA per ml–5% glycerol–1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. This was incubated at 45°C for 30 min. The reaction was terminated by adding 10 μ g of tRNA and ethanol precipitation. The remaining half of the hybridization mixture was rapidly diluted into 10 volumes of ice-cold 20 mM Tris hydrochloride (pH 7.4)–30 mM KCl–10 mM EDTA–12 U of *E. coli* exonuclease VII (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) per ml. Digestion with exonuclease VII was carried out at 45°C for 1 h and was terminated by ethanol precipitation. The products of nuclease digestion were subjected to electrophoresis in 1.4% alkaline or neutral agarose gels as described previously (20). After electrophoresis the gels were dried and exposed to X-ray film.

Isolation of P3HR-1 EBV DNA. P3HR-1 virions were recovered by centrifugation from the cell-free medium obtained from 10- to 20-liter cultures of TPA-treated P3HR-1 cells as described previously (11). The virus pellet was suspended in 0.4 ml of 50 mM Tris hydrochloride (pH 9.0)–400 mM NaCl–1 mM EDTA per 10 liters of original cell culture. Proteinase K and sodium dodecyl sulfate were added to concentrations of 200 μ g/ml and 1%, respectively, and the preparation was incubated at 37°C overnight. This was then layered on a linear 10 to 30% glycerol gradient and centrifuged at $178,000 \times g$ (Beckman SW41 rotor) for 4 h at 18°C. Fractions of 0.5 ml were collected from the top of the gradient and monitored for UV absorbance at 260 nm. Peak fractions from the center of the gradient were pooled, and the DNA was precipitated with ethanol.

Gel electrophoresis and blot hybridizations. Agarose gels for RNA or DNA blots were cast and run in either 10 mM

FIG. 3. Nuclease mapping of the 3' termini of the rightwardly transcribed *Bam*HI M RNAs. The DNA fragment (I) used in this experiment contained one radiolabeled 3' terminus. The organization of this figure is as described in the legend to Fig. 2. b, Bases.

sodium phosphate (pH 7.0) or 40 mM Tris hydrochloride (pH 8.0)–2 mM EDTA, respectively. For RNA gels the buffer was recirculated during electrophoresis to maintain pH. Before electrophoresis the RNA was denatured in 1 M glyoxal–50% dimethyl sulfoxide–10 mM sodium phosphate (pH 7.0) (21). The conditions for blotting, prehybridization, and washing of nitrocellulose blots were as described previously (18, 29). 32 P-labeled DNA probes were prepared by nick translation (28), using [α - 32 P]dCTP (3,000 Ci/mmol; New England Nuclear Corp.). Although not shown in the figures, all RNA and DNA gels contained phage lambda or pBR322 DNA fragments as standard size markers. DNA marker fragments used for RNA gels were denatured in an identical manner to the RNA before electrophoresis.

RESULTS

Identification of the EBV RNAs encoded by the *Bam*HI M region. Cytoplasmic poly(A)⁺ RNA isolated from productively infected lymphoblastoid cells was subjected to Northern blot hybridization to identify the RNAs transcribed from the *Bam*HI M region of the EBV genome (Fig. 1). Transcripts of 4.0, 2.65, 2.0, and 1.9 kb were detected in each of

FIG. 2. Nuclease mapping of the 5' termini of the rightwardly transcribed *Bam*HI M RNAs. Restriction fragments of EBV DNA labeled with 32 P (asterisks) at both (I) or at one (II and III) 5' terminus were thermally denatured and allowed to hybridize to yeast RNA (Y) as a control or cytoplasmic RNA from TPA-treated B95-8 cells (B). The products of the hybridization reaction were digested with either endonuclease S1 (S1) or exonuclease VII (Exo). Nuclease-resistant DNA fragments were separated by electrophoresis in 1.4% alkaline agarose gels and were detected by autoradiography of the dried gel (upper portion of the figure). The full-length DNA fragment is shown in the leftmost lane of each autoradiograph. The numbers to the left of each autoradiograph indicate the position of DNA fragments used as size markers; all numbers indicate size in kilobases. In the lower portion of the figure, the map positions of the RNAs (horizontal arrows) are indicated above a restriction map of the *Bam*HI α -M-S region of the EBV genome (B95-8). The map positions of the end-labeled DNA fragments are shown below the restriction map.

FIG. 4. Nuclease mapping of the 5' and 3' termini of the leftwardly transcribed *Bam*HI M RNAs. DNA fragment I contained one radiolabeled 3' terminus whereas fragments II and III each contained one labeled 5' terminus. The rightmost panel (top of figure) is an autoradiograph of a neutral agarose gel; all others are of alkaline agarose gels. The organization of this figure is as described in the legend to Fig. 2. The caret symbol within one RNA depicts the intervening sequence that is removed during the splicing process.

the different EBV-infected cells. Additionally, a 5.5-kb transcript was detected in the cytoplasmic fraction of Raji cells that had been superinfected with P3HR-1 EBV (lane 1) and in TPA-treated P3HR-1 cells (lane 4) but not in B95-8 cells (lane 3). This RNA is most likely transcribed from the defective P3HR-1 genome which contains a portion of the standard *Bam*HI M DNA (see below) and which is present in the P3HR-1 but not the B95-8 virus population. Production of the 4.0-, 2.65-, 2.0-, and 1.9-kb transcripts occurred in superinfected Raji cells held in the presence of cyclohexi-

imide. Thus, under conditions of superinfection these are immediate-early or early RNAs as suggested previously (29).

Nuclease mapping of the *Bam*HI M RNAs. The relative map positions of the *Bam*HI M RNAs were determined by Northern blot hybridization employing subfragments of the *Bam*HI M fragment and the adjacent entire *Bam*HI a and S fragments as probes. The data from these experiments (not shown) indicated that the 4.0-, 2.65-, and 1.9-kb poly(A)⁺ RNAs are encoded by the left half of the *Bam*HI M fragment and that the 4.0-kb transcript is partially encoded by the

FIG. 5. Summary of nuclease mapping of the *Bam*HI M RNAs. The map positions of the RNAs (horizontal arrows) are indicated above a *Bam*HI and *Hind*III restriction map of the *Bam*HI M region of the EBV genome. All numbers indicate length in kilobases. The length of each transcript as determined by nuclease analysis of RNA-DNA hybrids is shown above each arrow. The size of each transcript after polyadenylation of 3' termini is indicated by the numbers to the right of the arrows.

FIG. 6. Analysis of P3HR-1 viral DNA by Southern blot hybridization. Southern blots of *EcoRI*- or *BamHI*-digested P3HR-1 viral DNA were hybridized to ^{32}P -labeled plasmids containing the *BamHI* *a*, *M*, or *S* DNA fragment of the EBV genome. The *BamHI* and *EcoRI* restriction maps for this region of the standard P3HR-1 EBV genome are illustrated at the bottom of the figure. Letters indicate the expected standard size fragments, whereas the arrowheads indicate nonstandard fragments. Sizes are given in kilobase pairs.

adjacent *BamHI a* fragment. The 2.0-kb poly(A)⁺ RNA was encoded by the right half of the *BamHI M* fragment.

The transcription initiation sites for the 4.0-, 2.65-, and 1.9-kb RNAs were located by using 5'-end-labeled DNA fragments in the S1 and exonuclease VII mapping experiments that are illustrated in Fig. 2. In an alkaline agarose gel, two nuclease-resistant DNA fragments of 1.67 (full length) and 0.75 kb were detected when mapping the 5' terminus of the 4.0-kb RNA (Fig. 2, panel I). The 0.75-kb band represents the DNA fragment protected by hybridization to the 4.0-kb RNA, indicating that its 5' terminus is approximately 750 bases to the left of the *BamHI* restriction site separating the *BamHI a* and *M* fragments. The full-length DNA band may result from a nuclease-resistant hybrid of two RNAs to the same end-labeled DNA fragment. The second RNA, which is encoded within *BamHI-a* and the adjacent *BamHI O* fragment, overlaps the 5' terminus of the 4.0-kb RNA by approximately 70 bases (13). The DNA fragment protected by the 3' portion of this RNA alone would be 1.1 kb. This fragment would not be observed because the 3' ends of the RNA would not extend beyond and protect the labeled 5' end of the complementary DNA strand used in this experiment. Detection of nuclease-resistant fragments by Southern blot hybridization did reveal the presence of full-length 0.75- and 1.1-kb fragments as predicted (data not shown).

The nuclease-resistant bands in Fig. 2, panels II and III, indicate that the 5' termini of the 2.65- and 1.9-kb poly(A)⁺ RNAs were approximately 300 and 1,200 bases, respec-

tively, to the right of the *BamHI* restriction site separating *BamHI-a* and *-M*. The data also indicated that the 4.0-, 2.65-, and 1.9-kb RNAs share a common transcription termination site approximately 100 bases to the right of the *HindIII* restriction site within the *BamHI M* fragment (Fig. 3). In each alkaline gel represented in Fig. 2 and 3, S1- and exonuclease VII-resistant DNA bands of equivalent size were detected, indicating that the 4.0-, 2.65-, and 1.9-kb RNAs are not spliced.

The results of nuclease mapping experiments from which the structure of the 2.0-kb poly(A)⁺ RNA was determined are shown in Fig. 4. Although this transcript appeared as a single band on Northern blots, nuclease mapping indicated that there are actually two transcripts, one of which is spliced. The S1- and exonuclease VII-resistant DNA band that was produced as a result of hybridization to the unspliced RNA was approximately 700 bases in an alkaline gel (Fig. 4, panel III). A 500-base S1 nuclease-resistant DNA band was also detected which had no corresponding exonuclease VII-resistant band of equivalent size. It is likely that the 950-base (full-length) DNA band represents undigested rather than hybrid-protected DNA, even though it was not prevalent in the controls, since a transcript that would be large enough to completely protect this DNA fragment has not been detected in Northern blots after hybridization to a *BamHI S* probe (data not shown) (17, 31). Thus, the corresponding exonuclease VII-resistant DNA band for the 500-base band must also be a 700-base band, indicating that the

FIG. 7. Organization of the 18- and 21-kbp *Eco*RI fragments within the defective genome of P3HR-1 EBV. (A) Organization of the 18-kbp (left) and 21-kbp (right) *Eco*RI fragments. Letters indicate the standard *Bam*HI fragments (B95-8) that hybridized to the *Bam*HI fragments of the P3HR-1 defective DNA. The origins of hybridization probes used in this experiment are indicated by the numbered boxes. Subcomponents of the 18-kbp fragment added up to 17.8 kbp and those the 21-kbp fragment added up to 21.2 kbp. (B) Autoradiographs of Southern blots of *Bam*HI-digested P3HR-1 viral DNA that were hybridized to the indicated ³²P-labeled probes (numbers). Standard size fragments are indicated by a letter, and nonstandard fragments are indicated by an arrowhead. a indicates fragments that hybridized due to partial homology between *Bam*HI-C and -W (probe 1) and -B' and -WH (probe 3). (C) Restriction fragments of the defective genome that were detected by Southern blot hybridization are positioned above the *Bam*HI restriction map of the adjacent 18- and 21-kbp *Eco*RI fragments (D). Dashed lines represent DNA adjacent to these *Eco*RI fragments that is heterogeneous.

transcription initiation site is the same for these RNAs. Because these RNAs are both 2.0 kb, the intron must be short, and therefore polyadenylation of the spliced RNA results in a transcript that is indistinguishable from the unspliced RNA on Northern blots. In a nondenaturing gel, a single S1 nuclease-resistant DNA band of 680 to 700 bases was detected (Fig. 4, panel IV), indicating that the neutral gel did not distinguish the two species of spliced and unspliced DNA-RNA hybrid after S1 treatment. Thus, the intron is relatively small, i.e., 100 base pairs (bp) or less.

The transcription initiation site of the 2.0-kb RNAs was determined to be approximately 100 bases to the right of the *Bam*HI restriction site separating the *Bam*HI M and S fragments. The transcription termination site was approximately 200 bases to the left of the *Eco*RI restriction site within the *Bam*HI M fragment. The structure and the map positions of all five of the *Bam*HI M RNAs are illustrated in Fig. 5.

Characterization of the defective P3HR-1 genome for *Bam*HI-a-M-S region DNA. The defective genome of P3HR-1 EBV is known to contain DNA from the *Bam*HI M fragment region of the standard genome (7, 14, 24). If rearrangement of this region occurs during the generation of the defective genome, loss of the DNA sequences required for transcriptional regulation, e.g., the 5' flanking region, may occur.

This could lead to the expression of early replicative genes within the *Bam*HI M region and initiation of the replicative cycle after superinfection.

To identify DNA sequences present in the defective genome which would contain coding sequences for the *Bam*HI M RNAs, Southern blots of *Eco*RI- or *Bam*HI-digested P3HR-1 virion DNA were hybridized to ³²P-labeled cloned *Bam*HI a, M, or S DNA fragments (B95-8 strain). In addition to the expected standard restriction fragments that span the *Bam*HI a-M-S region, nonstandard DNA fragments partially homologous to *Bam*HI-M and -S, but not -a, were also detected (Fig. 6, arrowheads). These data are comparable to those of previous reports (7, 14, 24), suggesting that the structure of this specific region of the defective genome DNA is the same in all defective P3HR-1 genomes. Further, these data indicate that sequences of a-M-S region DNA found in the defective genome have undergone rearrangement.

Characterization of the defective-DNA-containing sequences from the *Bam*HI M region. The structure of the restriction fragments of the defective P3HR-1 genome that may contain genes in the *Bam*HI M region was determined. Two large *Eco*RI restriction fragments containing defective genome DNA were cloned and subsequently characterized by restriction analysis and Southern blot hybridization using

FIG. 8. Location of the junction site within the *Bam*HI M fragment. Plasmid DNA (pBR-M) containing the *Bam*HI M fragment (B95-8 strain) was digested with *Bam*HI, *Hind*III, and *Ava*I (lane 1) or *Bam*HI, *Hind*III, and *Pst*I (lane 2) and electrophoresed in a 1.2% agarose gel, and a Southern blot of this was hybridized to 32 P-labeled pJS184 DNA. This plasmid contains the 21-kbp *Eco*RI fragment from the defective genome that spans the *Bam*HI M-B' junction. Letters and open boxes indicate subfragments of *Bam*HI-M which hybridized; other bands which hybridized are vector DNA. A restriction map of the standard *Bam*HI M fragment is shown below the autoradiograph. Shaded boxes represent subfragments of *Bam*HI-M which did not hybridize. The junction site (X) as determined from these data is located within subfragment B, between the *Hind*III and the left adjacent *Ava*I restriction site. The position of the *Eco*RI restriction site (vertical arrow) that is the left terminus of the 21-kbp *Eco*RI fragment is indicated. The horizontal arrow represents the 800-bp *Eco*RI-*Hind*III fragment that is in the opposite orientation relative to the defective genome.

cloned fragments of the *Bam*HI library of B95-8 EBV DNA as probes. These were 18- and 21-kbp fragments which appeared as a single band in Southern blots after hybridization to *Bam*HI M and S probes (Fig. 6). The standard *Bam*HI restriction fragments of EBV DNA (B95-8 strain) that hybridized to the *Bam*HI subfragments of the 18- and 21-kbp fragments are indicated in Fig. 7A.

Three lines of evidence indicated that these two *Eco*RI fragments are contiguous within the defective genome. First, the right terminal region of the 18-kbp fragment and the left terminal region of the 21-kbp fragment both hybridized to *Bam*HI-M. Second, the positions of *Bam*HI, *Eco*RI, and *Hind*III (not shown) restriction sites are as would be predicted if the *Eco*RI fragments are contiguous. Third, the 9.4-kbp nonstandard *Bam*HI fragment that would span the termini of the *Eco*RI fragments was detected in Southern blots of P3HR-1 viral DNA after hybridization to 32 P-labeled *Bam*HI-M (Fig. 6) or to a labeled terminal subfragment from either of the *Eco*RI fragments (Fig. 7B, panels 2 and 3).

In addition to the region with homology to the *Bam*HI M-S

region, the organization of the remaining defective genome contained within these two *Eco*RI fragments was also determined (Fig. 7A through D). It was determined by one or more of the following criteria that incomplete copies of the *Bam*HI W, R, d', S, M, and B' fragments were present within the defective P3HR-1 genome. (i) The *Bam*HI restriction fragment within the defective genome was smaller than the corresponding standard *Bam*HI fragment to which it hybridized; (ii) two standard *Bam*HI restriction fragments with no known homology hybridized to the same *Bam*HI subfragment of the defective genome; or (iii) the absence of an expected restriction site(s) within a subfragment of the defective genome. It was also found that the regions of the defective genome derived from the *Bam*HI d'-d-Z-R and the *Bam*HI M-S fragment regions were in the opposite orientation relative to the standard genome.

Location of the junction site between the *Bam*HI M and B' fragments. The data indicated that the *Bam*HI M fragment was joined to an incomplete copy of the *Bam*HI B' fragment within the defective genome. To locate the approximate site

FIG. 9. Structure of the defective P3HR-1 EBV genome. (A) *EcoRI* and *BamHI* restriction map of the defective genome characterized in this study. Letters represent the standard *EcoRI* and *BamHI* fragments that are contained within each respective subfragment of the defective genome. Letters preceded by a triangle indicate that the entire standard fragment is not present within the defective molecule. The dashed lines represent the heterogeneity between termini of defective genomes. Vertical arrows indicate the approximate junction site between noncontiguous segments of the standard genome that have been joined during generation of the defective genome. Horizontal arrows indicate the orientation of these segments relative to the standard genome. (B) *EcoRI* and *BamHI* restriction map of the standard P3HR-1 EBV genome (24). The regions of the standard genome that are present in the defective genome are indicated by the lines above the map.

within *BamHI*-M that is joined to the B' fragment, Southern blots of plasmid DNA containing the *BamHI* M fragment were hybridized to ³²P-labeled plasmid containing the 21-kbp *EcoRI* fragment (Fig. 8). The junction site was determined to be within a 400-bp *AvaI*-*HindIII* subfragment of *BamHI*-M (Fig. 8, bottom). Since the transcription termination site for the 4.0-, 2.65-, and 1.9-kb *BamHI* M RNAs is approximately 100 bases to the right of the *HindIII* restriction site, 500 bases or less of the coding sequence for these RNAs are present in the defective genome. Thus, it is unlikely that they encode native functional polypeptides.

Approximately 3 kbp of the *BamHI* S fragment that is adjacent to *BamHI*-M and that contains the 5' flanking region and cap site of the 2-kb *BamHI* M RNAs was found to be intact. Since the transcription termination site for these RNAs is to the right of the *HindIII* restriction site, the entire coding region for these RNAs is present within the defective genome. The structure of the P3HR-1 defective DNA that we characterized relative to the standard P3HR-1 genome is illustrated in Fig. 9.

DISCUSSION

We have previously reported data which suggest that expression of the *BamHI* M region of the EBV genome likely results in the initiation of virus replication (29). This conclusion is based on the observations that (i) the *BamHI* M region is the first actively transcribed region of the genome after superinfection of Raji cells with P3HR-1 EBV; (ii) the *BamHI* M region is transcriptionally active under immediate-early conditions in superinfected Raji cells; and (iii) the *BamHI* M region is not transcriptionally active during growth-transforming infection of human lymphocytes.

We have now characterized the RNA transcripts encoded within the *BamHI* M region of the EBV genome by nuclease mapping. Mapping of the 5'-terminal region of the 4.0-kb poly(A)⁺ RNA encoded within the *BamHI* a and M frag-

ments has been reported by Gibson et al. (13) and is confirmed here. The DNA sequence data of Baer et al. (2) for the *BamHI* a-M-S region, deposited in the GenBank database, indicate that there are homologs of the TATA box sequence near each RNA cap site and that the polyadenylation consensus sequence AATAAA or ATTAAA (25) is present near each transcription termination site. Furthermore, splice donor and acceptor consensus sequences are present at the splice points shown in Fig. 4. Thus, our mapping data are consistent with transcriptional information derived from the deoxynucleotide sequence of this region. In addition, open translational reading frames occur within each RNA, suggesting that these transcripts do function as mRNAs.

The ability of the P3HR-1 strain of EBV to initiate the virus replication cycle after superinfection seems dependent upon the presence of virus particles that contain a defective genome in the superinfecting virus population (23). Subtle differences within the P3HR-1 standard genome relative to the genome of prototype EBV strains, moreover, may also contribute to this unique property of P3HR-1 EBV. These differences are evidenced by multiple restriction site polymorphisms in P3HR-1 DNA (23; unpublished observations). The significance of these polymorphisms has not been evaluated.

Although the mechanism by which the P3HR-1 defective virus induces superinfection is unclear, the initiation of the replicative cycle appears to be mediated, at least in part, by activation of one or more of the genes within the *BamHI* M region (29). It is apparent from data presented here, with the possible exception of the gene encoding the 2-kb *BamHI* M RNAs, that production of the standard *BamHI* M RNAs after superinfection is not derived from the defective genome of EBV. Because approximately 3 kb of the 5' flanking region of the DNA encoding the 2-kb RNAs is present within the defective genome, it is unlikely that there has been a

change in the 5' upstream region immediately adjacent to *Bam*HI-M. It is, however, possible that a *cis*-acting element for regulating transcription is present beyond 3 kbp of the 5' flanking region. Notwithstanding, the transcriptional activity of the *Bam*HI M region genes after superinfection is not likely a direct consequence of their incorporation into or their rearrangement within the P3HR-1 defective genome.

The structure of the P3HR-1 defective genome characterized in this paper is essentially identical to that reported by Cho et al. (7), who have derived the complete structure of the defective genome; we found only one complete copy of the standard *Bam*HI W fragment within the defective genome instead of two as reported by these authors. The data of Cho and co-workers and earlier data from the same laboratory (10) indicate that the defective genome forms concatemers of three to four genome units. It is possible that this may result in a gene dosage effect in which expression of early-replicative-cycle genes, such as that for the 2-kb *Bam*HI M RNAs, is favored on a stoichiometric basis. Concatenation could also cause the reiteration of DNA sequences to which putative proteins capable of repressing transcription of early replicative cycle genes could bind. In either case the result could be initiation of the replicative cycle of the EBV genome after superinfection with defective virus. It remains to be determined which, if any, of the *Bam*HI M-region gene products including one that is a DNA-binding protein (9) induces viral replication.

Biggin et al. recently reported (M. Biggin, P. J. Dyson, and P. Farrel, Herpesvirus Workshop, Ann Arbor, Mich., 1985) that by using anisomycin *Bam*HI-R and -Z were the regions coding for the immediate-early transcription and what we had seen previously was due to the insufficient inhibition of protein synthesis by cycloheximide. However, the transcriptional patterns that we have observed (29) were specific and different from those that occur during early transcription (31); particularly some of the abundant transcriptions seen in the early phase were not observed. (For example, compare Fig. 1b in reference 31 and Fig. 2 in reference 29.) It is possible that the difference in the systems, even the difference in Raji cells carried in the separate laboratories, may have caused this discrepancy, as we have frequently observed different types of responses to EBV infection in separately cloned Raji cells.

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