Epstein-Barr Virus RNA

VIII. Viral RNA in Permissively Infected B95-8 Cells

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More than 50 RNAs expressed by Epstein-Barr virus late in productive infection have been identified. B95-8-infected cells were induced to a relatively high level of permissive infection with the tumor promotor 12-O-tetradecanoyl-phorbol-13-acetate. Polyadenylated RNAs were extracted from the cell cytoplasm, separated by size on formaldehyde gels, transferred to nitrocellulose, and hybridized to labeled recombinant Epstein-Barr virus DNA fragments. Comparison of RNAs from induced cultures with RNAs from induced cultures also treated with phosphonoacetic acid to inhibit viral DNA synthesis identifies two RNA classes: a persistent early class of RNAs whose abundance is relatively resistant to viral DNA synthesis inhibition and a late class of RNAs whose abundance is relatively sensitive to viral DNA synthesis inhibition. The persistent early and late RNAs are not clustered but are intermixed and scattered through most of segments UL and US. The cytoplasmic polyadenylated RNAs expressed during latent infection were not detected in productively infected cells, indicating that different classes of viral RNA are associated with latent and productive infection. Non-polyadenylated small RNAs originally identified in cells latently infected with Epstein-Barr virus are expressed in greater abundance in productively infected cells and are part of the early RNA class.

The Epstein-Barr virus (EBV) genome is a linear double-stranded molecule of approximately 170×10^3 base pairs (bp) (6, 7, 14, 18, 21, 39). There are variable numbers of tandem direct repeats of a 500-bp sequence, TR, at both ends of the molecule (14, 16, 30). A variable number of tandem direct repeats of a 3,000-bp sequence, IR, separates the largely unique DNA into a segment of 14×10^3 bp, US, and a segment of 135×10^3 bp, UL (6, 7, 14, 15, 20). The DNA of the B95-8 isolate differs from most EBV DNAs in a deletion from UL of 15×10^3 bp (5, 7, 21, 40, 41). Despite this deletion, the B95-8 virus, like other EBV isolates, can infect and growth transform normal B lymphocytes and replicate in some of the transformed cell lines (33, 34).

In latently infected cells growth transformed by the B95-8 virus or in Burkitt tumor cells grown in culture, stable RNAs are transcribed from at least 30% of the double-stranded viral DNA (19, 28, 29, 36, 50). RNA encoded by only 10% of the DNA accumulates on polyribosomes (19, 28, 29, 38, 50). There are three relatively more abundant viral polyadenylated cytoplasmic RNAs in latently infected cells. These RNAs appear as a few copies per cell and are encoded by DNA mapping at the juncture of IR and UL, by DNA near the center of UL at $63 \times$

10^6 to 66×10^6 daltons, and by the right end of UL (28, 29, 51). Two less abundant cytoplasmic polyadenylated RNAs (51) and two "VA-like" RNAs are encoded by US (32, 43, 51).

RNAs encoded by the EBV genome in abortive or productive infection are less well characterized. Raji, a latently infected Burkitt tumor cell line, can be induced to an abortive state of virus infection in which early antigen, a complex of several viral polypeptides not found in latently infected cells, can be detected (13, 17, 23, 25, 26, 36, 37). In association with early antigen expression, the complexity of viral cytoplasmic polyadenylated RNA increases and is similar to that of nuclear RNA in latently infected cells (29, 38). The additional cytoplasmic polyadenylated RNAs are encoded largely by regions of UL which map at 39×10^6 to 49×10^6, 51×10^6 to 59×10^6, 66×10^6 to 77×10^6, and 102×10^6 to 105×10^6 daltons (29).

In permissively infected cells, the complexity of polyadenylated RNA is similar to the full single-strand complexity of the EBV genome (38). More than 30 new polypeptides are synthesized in productively infected cells (10, 11, 25, 26, 35–37). Only a few of these polypeptides are synthesized in Raji cells after induction (37). Treatment of permissively infected cells with

inhibitors of viral DNA synthesis results in inhibition of expression of many polypeptides, which are thereby defined as being late viral polypeptides, and continued synthesis of early viral polypeptides, including early antigen (11, 12, 26, 37). There have been no previous descriptions of the early and late viral RNAs expressed in the course of EBV replication.

In this report, the viral DNAs encoding abundant cytoplasmic polyadenylated RNAs of permissively infected cells are identified by hybridizing cDNA made from the RNAs to Southern blots of restriction endonuclease fragments of EBV DNA. The DNA mapping is confirmed and the size of the RNAs is determined by hybridization of labeled recombinant EBV DNA fragments to RNA blots (49, 51). For these experiments, B95-8-infected cells were induced to a relatively high level of permissive infection by treatment with a tumor-promoting phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (53). These productively infected cells also continue to express some early viral polypeptides (11, 12, 25, 26, 37). To distinguish persistent early and late RNAs, some cultures were induced by TPA and treated with phosphonoacetic acid (PAA) to inhibit viral DNA synthesis and late gene expression (47).

MATERIALS AND METHODS

Cell culture. B95-8-infected cells (34) were maintained by dilution twice weekly in an equal volume of RPMI 1640 medium supplemented with 10% fetal calf serum. Permissive infection was induced by adding TPA to a concentration of 20 ng/ml (53) and harvesting the cultures 3 days later. At this time, approximately 25% of the cells were positive for viral capsid antigen (VCA) and 30% were positive for membrane antigen (MA) (22, 31, 46). For the preparation of early RNA, PAA was added to the cultures at a concentration of 125 µg/ml 3 h after the addition of TPA (52). Three days later, at harvest, 15% of the cells were positive for early antigen. The PAA-treated cultures were negative for MA and VCA.

Preparation of RNA. Initial steps in RNA preparation were at 0 to 4°C. RNA was prepared (4, 51) by pelleting the cells at 3,000 rpm for 5 min in a GS3 rotor and suspending the cells in a lysing buffer containing 20 mM Tris-hydrochloride (pH 7.4), 10 mM NaCl, 0.25% Triton X-100, 1.25% sucrose, and 10 mM vanadyl ribonucleoside complex (3). The cell suspension was homogenized 10 times with a Dounce homogenizer, and the nuclei were removed by centrifugation for 5 min at 5,000 rpm in the SS34 rotor. An equal volume of a solution containing 7 M urea, 0.35 M NaCl, 50 mM Tris-hydrochloride (pH 7.4), and 1% sodium dodecyl sulfate (SDS) was added to the cytoplasmic extract. Proteins and lipids were extracted with a mixture of phenol and chloroform, and the RNA was precipitated with ethanol.

Nuclei were suspended in a solution containing 8 M guanidine hydrochloride, 25 mM potassium phosphate (pH 5.0), and 20 mM vanadyl ribonucleoside complex. DNA was sheared by homogenizing for 90 s at full speed with a Tekmar Tissumizer. High-molecular-weight RNA was precipitated overnight at −20°C by adding 0.6 volumes of 95% ethanol and collected by centrifugation at 12,500 rpm for 20 min in an SW27 rotor. Nuclear RNA was suspended in 3.5 M urea–0.175 M NaCl–25 mM Tris (pH 7.4)–0.5% SDS, extracted with phenol-chloroform, and precipitated with ethanol. Polyadenylated and non-polyadenylated RNAs from the nucleus or the cytoplasm were separated by two cycles of oligodeoxythymidylate-cellulose chromatography (1, 38).

cDNA synthesis, DNA blots, and hybridization. DNA complementary to polyadenylated cytoplasmic RNA was synthesized with avian myeloblastosis virus reverse transcriptase (obtained through the resources program of the National Cancer Institute) and a random oligodeoxynucleotide primer derived by limit digestion of calf thymus DNA (48). The reaction mixture, consisting of 20 µg of RNA, 250 µg of primer per ml; 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 200 µCi of [^{32}P]dCTP (500 Ci/mmol), 80 mM Tris-hydrochloride (pH 8.3), 0.2% β-mercaptoethanol, 0.01% Nonidet P-40, 10 mM MgCl$_2$, 5 µg of actinomycin D per ml, and 50 U of avian myeloblastosis virus reverse transcriptase in 100 µl, was incubated at 37°C for 2 h. The reaction was terminated by adding SDS to a concentration of 0.2%, and the mixture was diluted to 1 ml with 50 mM Tris-hydrochloride, pH 7.4. Salmon sperm DNA was added to a concentration of 1 mg/ml, and the nucleic acid was precipitated two times with 0.3 M sodium acetate–ethanol to remove unincorporated [^{32}P]dCTP. The nucleic acid was suspended in 2 ml of water, made 0.3 M in NaOH, and incubated at 110°C for 10 min to hydrolyze the RNA. The mixture was neutralized and hybridized to blots of cloned EBV DNA fragments (6, 40) for 18 h at 72°C in 6× SSC (SSC, 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS–50 mM Tris-hydrochloride (pH 7.4)–0.02% polyvinylpyrrolidone–0.02% Ficoll–100 µg of denatured salmon sperm DNA per ml (8). Blots were washed for 60 min at 72°C in 0.2% SDS and decreasing concentrations of SSC to 0.1× SSC.

DNA blots were prepared by digesting recombinant phage or plasmid DNAs (gifts of T. Dambaugh and C. Beisel) with BamHI or BamHI and EcoRI, electrophoretically separating the fragments on 0.8% agarose gels, and transferring the fragments to nitrocellulose paper (45).

RNA gels, RNA blots, and hybridization to labeled EBV DNA fragments. RNA was denatured for 5 min at 60°C in 50% (vol/vol) formamide and 2.2 M formaldehyde in electrophoresis buffer (20 mM morpholinopropanesulfonic acid [pH 7.4; Sigma Chemical Co.], 5 mM sodium acetate, 1 mM EDTA) and electrophoretically separated in 0.8% agarose gels containing 2.2 M formaldehyde and 1 µg of ethidium bromide per ml in electrophoresis buffer (51). The RNA was transferred to nitrocellulose paper (Millipore Corp.) with 10× SSC (49), and the positions of the 5.2- and 2.0-kilobase (kb) (49), and the positions of the 5.2- and 2.0-kilobase (kb) ribosomal RNAs were determined by UV illumination.

Recombinant plasmid DNAs were labeled in vitro with Escherichia coli DNA polymerase I (Boehringer Mannheim Corp.) and [^{32}P]dCTP (500 Ci/mmol; Amersham Corp.) (27, 42). The specific activity of the labeled DNA was 5 × 10^7 to 10 × 10^7 cpm/µg. A total
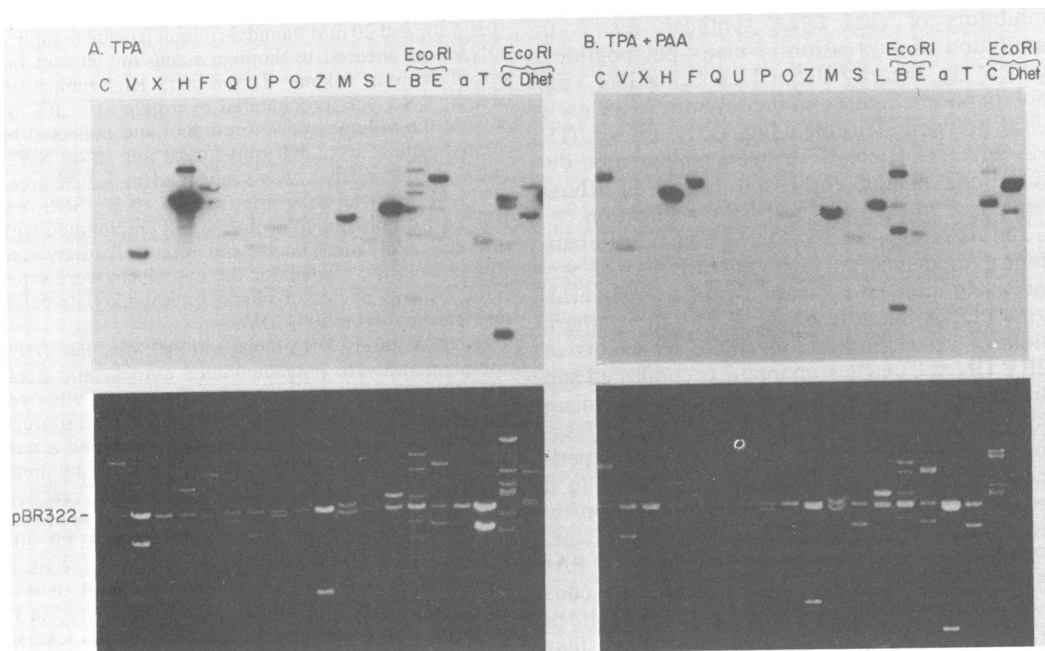
FIG. 1. Fluorogram of Southern blots (upper) and ethidium bromide fluorescence (lower) of gels of recombinant EBV DNAs. The fluorograms were made after hybridizing the blots to ^{32}P-labeled cDNA made from polyadenylated cytoplasmic RNA of TPA-induced productively infected cells (panel A) or of TPA-induced cells blocked in viral DNA synthesis by PAA (panel B). Lanes C, V, X, H, F, Q, U, P, O, Z, M, S, L, a, and T are *Bam*HI digests of recombinant plasmids containing the indicated *Bam*HI fragments of B95-8 DNA (6). The lanes indicated by brackets are *Bam*HI/*Eco*RI digests of EBV *Eco*RI fragments cloned in cosmid MUA-3 (*Eco*RI fragments B, E, and Dhet-I Jhet) (40) or Charon 4A (*Eco*RI-C) (6). The fragments of MUA-3/*Eco*RI-B visible in the ethidium bromide fluorescence are, in order of decreasing size, *Bam*HI-B, *Bam*HI-E, *Bam*HI-K, MUA-3, *Bam*HI-R, a portion of *Bam*HI-G, and *Bam*HI-Y. MUA-3/*Eco*RI-E contains *Bam*HI-D, MUA-3, and the other portion of *Bam*HI-G. A *Bam*HI/*Eco*RI digest of *Eco*RI-C cloned in Charon 4A generates, in order of decreasing size, two Charon 4A fragments, *Bam*HI-I, a portion of *Bam*HI-A which comigrates with a Charon 4A fragment, another 4A fragment, *Bam*HI-V$_1$, *Bam*HI-W, and *Bam*HI-b. A *Bam*HI/*Eco*RI digest of MUA-3/*Eco*RI-Dhet-I Jhet generates a portion of *Bam*HI-A, the fused ends, and MUA-3.

of 20×10^6 to 60×10^6 cpm were hybridized to the RNA blots in 50% formamide–5× SSC–0.5% SDS–0.02% polyvinylpyrrolidone–0.02% Ficoll–40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES; pH 6.5)–100 μg of denatured salmon sperm DNA per ml at 50°C for 18 h. The blots were washed twice at room temperature in 2× SSC–0.2% SDS and then at 72°C in 0.2% SDS and decreasing concentrations of SSC.

RESULTS

Viral DNA encoding persistent early and late RNA in permissively infected cells. The regions of the viral genome which encode early and late RNAs are identified by hybridizing labeled cDNA made from cytoplasmic polyadenylated RNA to Southern blots of separated restriction endonuclease fragments of EBV DNA. cDNA made from RNA of productively infected cells (persistent early and late RNA) hybridizes, in order of decreasing intensity, to *Bam*HI fragments H, L, D, M, b, K, Nhet, I, A, F, and V (Fig. 1A). With longer exposure of the fluorograms, hybridization to all of the viral DNA

fragments is evident. In contrast, cDNA made from RNA of cultures blocked in viral DNA synthesis by PAA (early RNA) hybridizes mostly to *Bam*HI fragments H, A, M, L, F, B, R, C, and Y (Fig. 1B). In this instance also, with longer exposure, hybridization to many other fragments is evident.

The intensity of hybridization of early RNA cDNA to *Bam*HI-H is less with a fivefold-longer fluorography exposure (Fig. 1B) than that observed with early and late RNA cDNA (Fig. 1A), indicating that late RNA is enriched for RNA encoded by *Bam*HI-H. Relative to the amount of *Bam*HI-H-encoded RNA, early RNA is differentially enriched for RNA encoded by *Bam*HI fragments A, C, B, R, Y, M, and F and deficient in RNA encoded by *Bam*HI fragments D, I, and b (cf. Fig. 1A and B).

Size and relative abundance of RNAs encoded by defined regions of EBV DNA. The size of the cytoplasmic polyadenylated RNAs encoded by fragments of EBV DNA and the relative abun-

dance of each of the RNAs encoded by a fragment were determined by hybridization of labeled recombinant EBV DNA restriction endonuclease fragments to blots of RNA which had been separated by size in agarose gels (49, 51). In general, there was a correspondence between the extent of cDNA hybridization to a fragment (Fig. 1A) and the extent of hybridization of that fragment to specific RNAs.

Using the cDNA data to estimate the relative abundance of RNAs encoded by the various fragments and the RNA blot results to determine the relative abundance of each of the RNAs encoded by a fragment, we determined that the most abundant RNAs are: a 2.5-kb RNA encoded by BamHI-H; RNAs of 3.4, 1.2, and 0.9 kb encoded by BamHI-L; RNAs of 3.4, 2.4, 5.2, and 1.05 kb encoded by BamHI-D; 2.65- and 1.9-kb RNAs encoded by BamHI-M; a 1.4-kb RNA encoded by BamHI fragments b and I; RNAs of 1.5, 2.2, and 1.9 kb encoded by BamHI-K; a 1.1-kb RNA encoded by BamHI-I; 4.2- and 3.0-kb RNAs encoded by BamHI-A; and RNAs of 4.0, 3.6, 1.4, 1.6, 0.96, and 2.8 kb encoded by
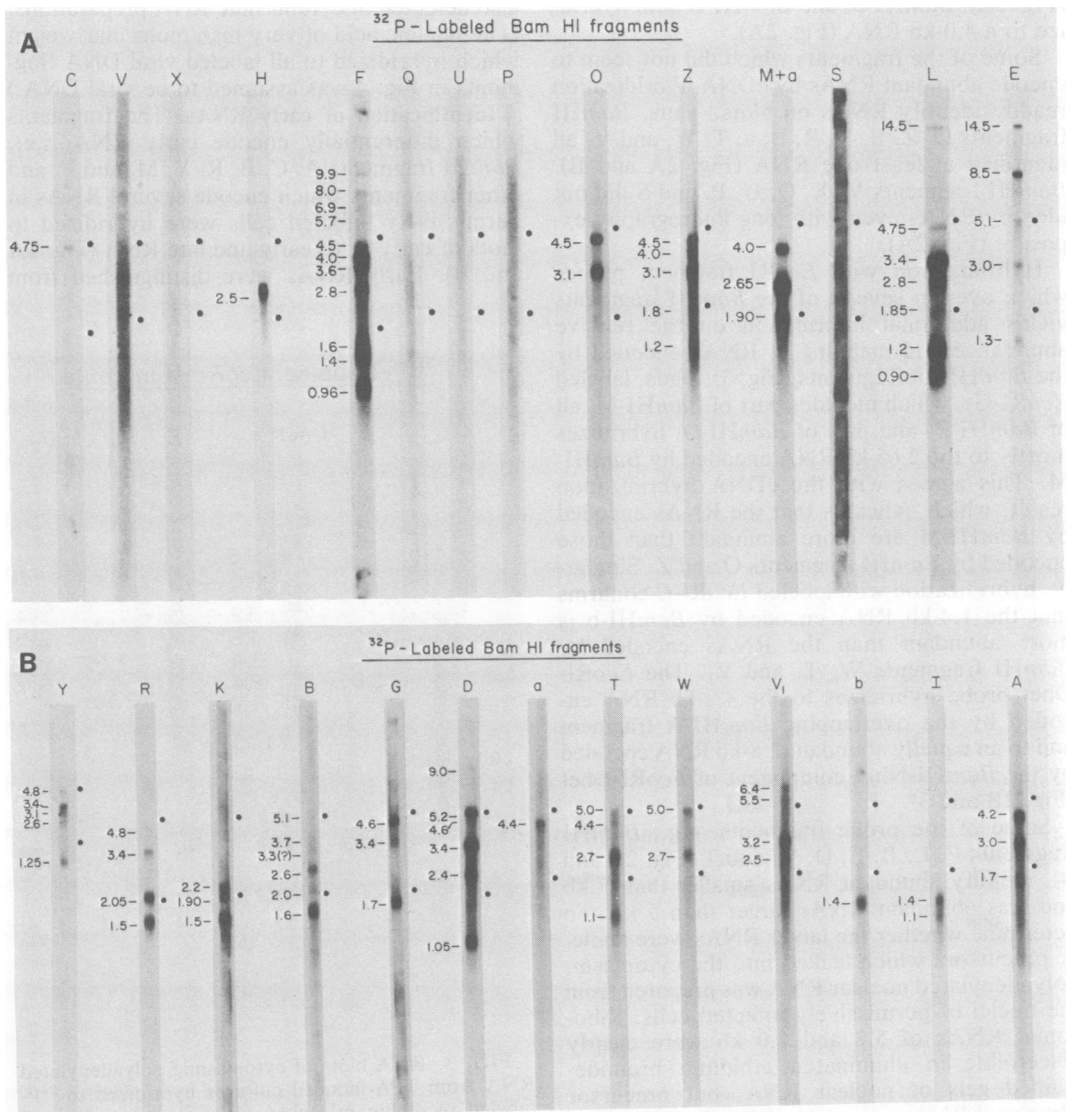

FIG. 2. RNA blots of cytoplasmic polyadenylated RNA from TPA-induced cultures hybridized to denatured
^{32}P-labeled recombinant plasmid DNAs containing the indicated BamHI fragments of B95-8 DNA (6). The fluorography exposure of each blot differs to obtain the clearest representation of the RNAs identified by each probe DNA. The dots indicate the positions of 2.0- and 5.2-kb ribosomal RNAs on each blot.

*Bam*HI-F (Fig. 2A and B). In some instances these fragments also identified RNAs with less intensity. For example, *Bam*HI-F also identifies RNAs of 9.9, 8.0, 6.9, and 5.7 kb, and *Bam*HI-L identifies RNAs of 14.5, 4.75, 2.8, and 1.85 kb (Fig. 2A). Some of these less abundant RNAs may be precursors of the more abundant RNAs. Other RNAs are identified weakly by one fragment and intensely by an adjacent fragment by which they are presumed to be largely encoded. For example, the 3.4-kb RNA is presumed to be primarily encoded by *Bam*HI-D and to a lesser extent by *Bam*HI-G (Fig. 2B). Both *Bam*HI-O and *Bam*HI-Z hybridize to 4.5- and 3.1-kb RNAs (Fig. 2A). *Bam*HI-M and *Bam*HI-Z both hybridize to a 4.0-kb RNA (Fig. 2A).

Some of the fragments which did not seem to encode abundant RNAs by cDNA hybridization readily identify RNAs on blots. Thus, *Bam*HI fragments O, Z, E, Y, R, B, a, T, W, and V_1 all identified at least one RNA (Fig. 2A and B). *Bam*HI fragments V, X, Q, U, P, and S did not identify RNAs even with long fluorography exposure (Fig. 2A).

Hybridization with *Eco*RI fragment probes which overlap several of the *Bam*HI fragments yields additional information on the relative abundance and mapping of RNAs encoded by the *Bam*HI subfragments (Fig. 3). Thus, labeled *Eco*RI-G_2, which includes part of *Bam*HI-M, all of *Bam*HI-Z, and part of *Bam*HI-O, hybridizes mostly to the 2.65-kb RNA encoded by *Bam*HI-M. This agrees with the cDNA hybridization result, which indicates that the RNAs encoded by *Bam*HI-M are more abundant than those encoded by *Bam*HI fragments O and Z. Similarly, hybridization with labeled *Eco*RI-C confirms that the 1.4-kb RNA encoded by *Bam*HI-b is more abundant than the RNAs encoded by *Bam*HI fragments W, T, and V_1. The *Eco*RI-Dhet probe hybridizes to the 4.2-kb RNA encoded by the overlapping *Bam*HI-A fragment and to an equally abundant 2.8-kb RNA encoded by the *Bam*HI-Nhet component of *Eco*RI-Dhet (Fig. 2B and 3).

Some of the probe fragments, e.g., *Bam*HI fragments F, L, B, E, D, V_1 and T (Fig. 2A and B), identify abundant RNAs smaller than 5 kb and less abundant RNAs larger than 5 kb. To determine whether the larger RNAs were nuclear precursors which leaked into the cytoplasm, polyadenylated nuclear RNA was prepared from the nuclei of permissively infected cells. Ribosomal RNAs of 5.2 and 2.0 kb were clearly discernible in illuminated ethidium bromide-stained gels of nuclear RNA, but precursor ribosomal RNAs were not, indicating that there was degradation or inhibition of synthesis of the precursor ribosomal RNAs. Degradation could be a consequence of extraction, whereas inhibi-

tion of synthesis could be a consequence of the permissive state of viral infection. Identification of specific polyadenylated viral RNAs was also more difficult with blots of nuclear RNA preparations than with blots of cytoplasmic RNA preparations (cf. Fig. 2 and 4). Higher-molecular-weight RNAs encoded by *Bam*HI fragments F, Z, and E could be identified in the fluorograms of nuclear RNA blots (Fig. 4). However, only with *Bam*HI-Z was there definite enrichment for the higher-molecular-weight putative nuclear precursor polyadenylated RNAs over the lower-molecular-weight, more abundant, "mature" polyadenylated RNAs which were also detected in cytoplasmic RNA preparations. (The nucleic acid of very high molecular weight which hybridized to all labeled viral DNA fragments in Fig. 4 was assumed to be viral DNA.)

Identification of early RNAs. The fragments which differentially encode early RNA, i.e., *Bam*HI fragments A, C, B, R, Y, M, and F, and other fragments which encode several RNAs in permissively infected cells were hybridized to blots of early or of early and late RNA (Fig. 5A and B). Early RNAs were distinguished from
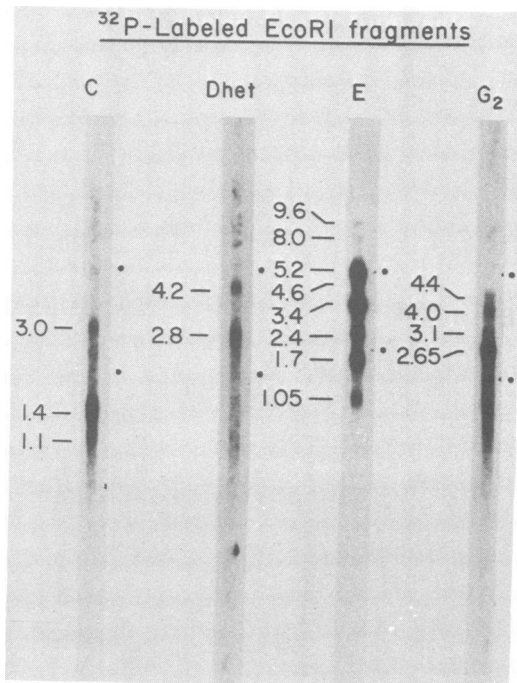

FIG. 3. RNA blots of cytoplasmic polyadenylated RNA from TPA-induced cultures hybridized to ^{32}P-labeled recombinant DNAs containing the indicated B95-8 *Eco*RI DNA fragment (6, 40). The fluorography exposure intervals differ as in Fig. 2. The dots indicate the position of 2.0- and 5.2-kb ribosomal RNAs on each blot.
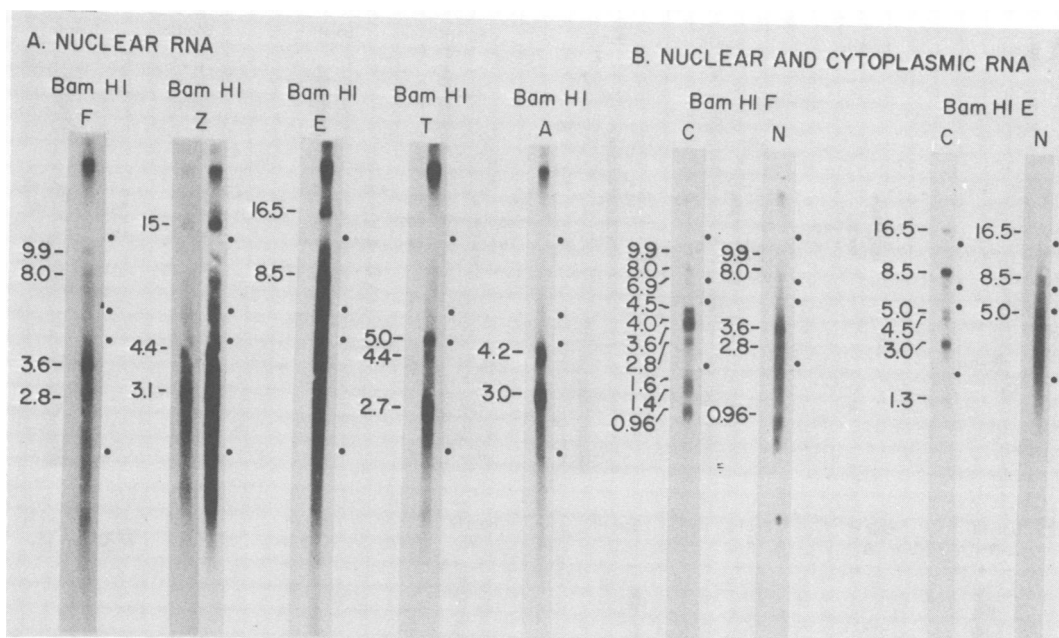
FIG. 4. Polyadenylated nuclear RNAs from TPA-induced B95-8-infected cells. Blots of polyadenylated nuclear (panel A) or of polyadenylated nuclear or cytoplasmic RNAs (panel B) were hybridized to denatured ^{32}P-labeled recombinant EBV plasmid DNAs (6). The dots indicate the positions of 2.0- and 5.2-kb ribosomal RNAs on the blots and the position of lymphocyte ribosomal precursor RNA in parallel lanes of the same gel. The molecular weight of the large RNA encoded by BamHI-E was estimated to be 16.5 kb when ribosomal precursor RNAs were used as markers.

late RNAs by their relative persistence in the face of inhibition of viral DNA synthesis by PAA. The 4.75- and 1.9-kb RNAs and the less abundant 2.5-kb RNA encoded by BamHI-C are inhibited by PAA, whereas the 0.17-kb RNA is not. Thus, the 0.17-kb RNA is an early RNA and the 4.75-, 1.9-, and 2.5-kb RNAs are late RNAs (Fig. 5A).

Hybridization with the BamHI-H probe shows that the 2.5-kb RNA and a second RNA of 1.9 kb are early RNAs (Fig. 5A). BamHI-F encodes early RNAs of 4.0 and 1.6 kb and late RNAs of 4.5, 3.6, 2.8, 1.4, and 0.96 kb (Fig. 5A). The BamHI-Z probe hybridizes to early RNAs of 4.0 and 3.1 kb and to a late RNA of 4.5 kb (Fig. 5A). The RNAs of 4.0, 2.65, and 1.9 kb identified by a probe containing BamHI-M and BamHI-a are all early RNAs encoded by BamHI-M (Fig. 5A and BamHI-M hybridization data not shown). Of the RNAs encoded by BamHI-L, only the 1.2-kb RNA is detectable in early RNA (Fig. 5A). BamHI-E does not encode any early RNAs, since early RNA cDNA does not hybridize to BamHI-E (Fig. 1B) and early RNA blots do not hybridize to the BamHI-E probe (Fig. 5A). Early RNAs of 4.8, 3.4, and 1.25 kb and late RNAs of 3.1 and 2.6 kb are

identified by the BamHI-Y probe (Fig. 5A). BamHI-R encodes an early RNA of 1.5 kb. Hybridization with BamHI-B shows that the 2.6-kb RNA encoded by that fragment is an early RNA, whereas the 1.6-kb BamHI-B RNA is late (Fig. 5B). The RNAs of 5.2, 3.4, 2.4, 1.7, and 1.05 kb encoded by EcoRI-E are late RNAs, whereas the 4.6-kb EcoRI-E RNA is an early RNA (Fig. 5B). Hybridization of RNA blots with BamHI-T shows that the 5.0-kb RNA is an early RNA and the 4.4- and 2.7-kb RNAs are late RNAs (Fig. 5B). The BamHI-W probe also identifies the 5.0-kb RNA as early and the 2.7-kb RNA as late (Fig. 5B). The BamHI-I probe does not hybridize to any early RNA, showing that the 1.4- and 1.1-kb RNAs encoded by that fragment are late RNAs (Fig. 5B). BamHI-A encodes an early 4.2-kb RNA and late RNAs of 3.0 and 1.7 kb (Fig. 5B). Hybridization with the labeled EcoRI-Dhet probe confirms that the 4.2-kb RNA is an early RNA and indicates that the 2.8-kb RNA encoded by the BamHI-Nhet component of EcoRI-Dhet is a late RNA (Fig. 5B).

Comparison of RNAs of productively and latently infected cells. BamHI-C and EcoRI-Dhet identify RNAs similar in size to the RNAs previously identified in latently infected IB4
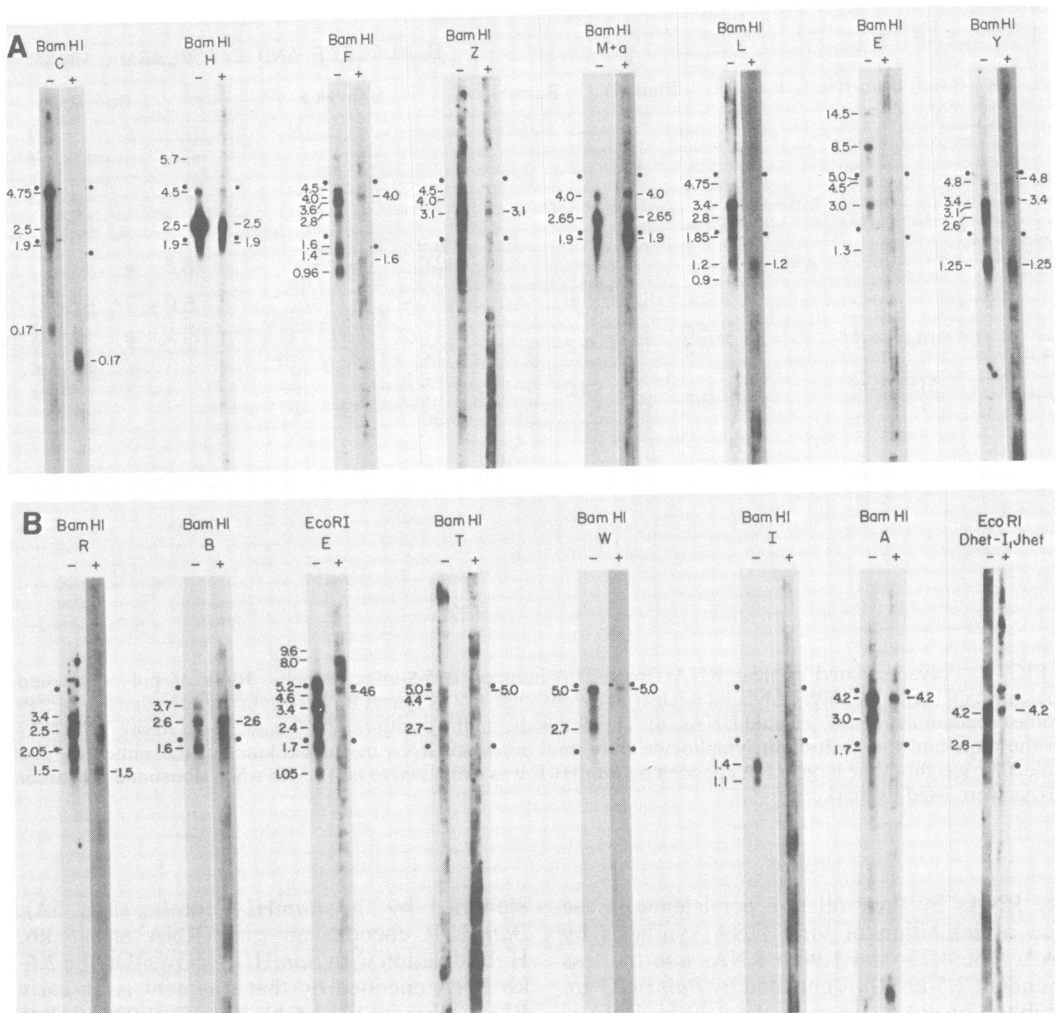
FIG. 5. Comparison of early and late RNAs. RNAs from induced B95-8-infected cultures which were incubated with PAA(+) or, as a control, without PAA(−) were separated on formaldehyde gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled recombinant plasmids containing the indicated B95-8 DNA fragments (6). Early RNAs are those identified by probe DNA in blots made from cells incubated with PAA. The PAA(+) blots required a two- to fivefold-longer fluorography exposure than the PAA(−) blots. The positions of 2.0- and 5.2-kb ribosomal RNAs on each blot are indicated by dots alongside each strip.

cells (51). The 2.5-kb RNA encoded by BamHI-C is not an abundant RNA in productively infected cells and appears to be inhibited by PAA (Fig. 5A). This suggests that it is a late RNA and, therefore, probably different from the 2.3- and 2.0-kb RNAs previously identified in latently infected cells. Hybridization with the labeled EcoRI-J probe indicates that all three RNAs are encoded in part by the EcoRI-J region of BamHI-C and that the 2.5-kb productively infected cell RNA is larger than the 2.3-kb RNA of latently infected cells (Fig. 6A). The 0.17-kb RNA also identified by EcoRI-J in productively

infected cells (Fig. 6A) is an early RNA (Fig. 5A) and is identical in size to the non-polyadenylated small RNA of latently infected cells (Fig. 6B). Approximately 90% of the non-polyadenylated RNA is separated from the polyadenylated RNA by a single passage through an oligodeoxythymidylate column (29, 51; cf. Fig. 6A and B). The 0.17-kb RNA was not detectable on blots of the polyadenylated RNA fraction of latently infected cells (Fig. 6A). The amount of residual 0.17-kb RNA in the polyadenylated RNA fraction of induced B95-8-infected cells was 10-fold more than is in the non-polyadenylated RNA fraction
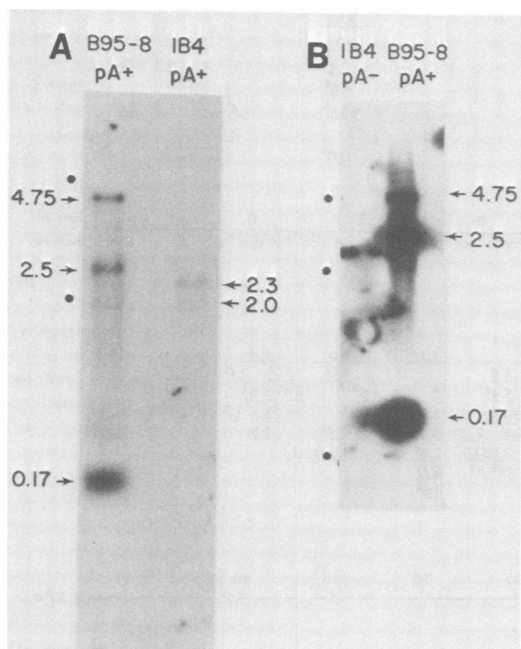
FIG. 6. Fluorograms of RNA blots. Panel A, Fluorograms of RNA blot of B95-8 and IB4 polyadenylated RNAs hybridized to denatured ^{32}P-labeled purified B95-8 EcoRI-J insert (51); panel B, fluorograms of RNA blot of IB4 non-polyadenylated RNA (pA−) and B95-8 polyadenylated RNA (pA+) hybridized to the same probe as in panel A. The dots indicate the positions of 5.2- and 2.0-kb ribosomal RNAs and tRNA.

of latently infected IB4 cells (Fig. 6B), suggesting that the concentration of the 0.17-kb RNA is approximately 100-fold higher in productively infected cells.

The cytoplasmic polyadenylated (2.9- and 2.8-kb) RNAs encoded by the BamHI-Nhet component of EcoRI-Dhet in latently (51) and productively infected cells (Fig. 3 and 5B) could not be distinguished from each other in blots of agarose gels in which the RNAs were in adjacent lanes. The 2.8-kb RNA of productively infected cells is inhibited by PAA (Fig. 5B), indicating that it is a late RNA, and is therefore probably different from the 2.9-kb RNA encoded by this fragment in latently infected cells (51).

DISCUSSION

The biochemistry of EBV replication can be studied by inducing productive infection in partially permissive B95-8- or P3HR-1-infected cultures or by superinfecting Raji cells with P3HR-1 virus (2, 10–12, 25, 26, 35–37, 52, 53). In the experiments described in this report, virus replication was induced in cultures of B95-8-infected cells. Before induction, 99% of the cells are

latently infected and only 1% of the cells contain VCA or MA. After induction, the number of productively infected cells increases for 3 days, at which time 25% of the cells are VCA positive and 30% are MA positive (46). One reason for using this system is that B95-8 virus has been the prototype for previous studies of EBV DNA and RNA (5–7, 14–16, 18–21, 28–30, 32, 38–41, 50, 51). Although the DNA of the B95-8 virus differs from other EBV DNAs in the deletion of 15,000 bp (21, 40), the B95-8 virus is similar to other EBV isolates in its biological properties. These include cellular growth transformation and virus replication (33). A difference has been described in the size of the outer membrane glycoprotein of the B95-8 virus (9). It is not known if this difference is a consequence of altered glycosylation, of an altered polypeptide, or of altered regulation (9). Most recent studies of the polypeptides synthesized in productive virus infection have used Raji cells superinfected with P3HR-1 virus (2, 10–12, 26, 35, 36). Productive infection following superinfection can be more rapid and more uniform. However, P3HR-1 virus and Raji cells have substantial populations of defective EBV DNAs (21). Thus, a second reason for using induced B95-8-infected cultures for studies of viral RNA is to avoid the possibility that the defective EBV DNAs in P3HR-1 virus and Raji cells might encode altered transcripts.

The data reported in this manuscript are summarized in Fig. 7. Induction of productive infection leads to synthesis of RNAs encoded by almost every region of the EBV genome. Inhibition of viral DNA synthesis results in reduction in abundance of all RNAs. Some RNAs encoded by BamHI fragments A, B, C, D, F, G, H, K, L, M, Nhet, O, R, T, W, Y, and Z are less affected by PAA treatment than other RNAs and are therefore designated as early RNAs in Fig. 7. Other RNAs encoded by these fragments and by BamHI fragments E, I, V_1, and b are not present in cells treated with PAA. These RNAs are therefore designated as late RNAs. It is apparent from Fig. 7 that early and late RNAs are not clustered but are intermixed and scattered throughout segments UL and US.

Three points should be made about the assignment of RNAs to the early or late class based on the differential effect of PAA on RNA abundance. First, although the effects of PAA on the synthesis of viral DNA and on the synthesis of individual viral polypeptides is similar to that of acycloguanosine, another inhibitor of viral DNA synthesis (12), the possibility exists that PAA could inhibit the synthesis of some viral RNA(s) through mechanisms other than inhibition of viral DNA synthesis. Second, there is not a consistent correlation between the time of maximal synthesis of some viral proteins and the
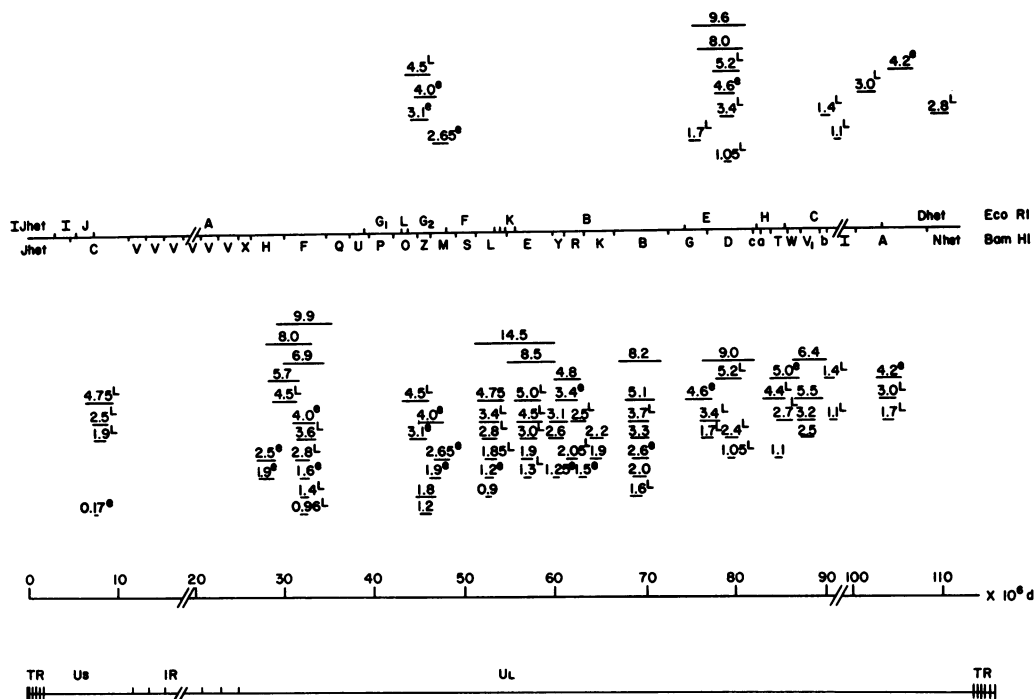
FIG. 7. Summary of locations and sizes of RNAs encoded by B95-8 DNA (6, 7, 14) in productive infection. Superscript e indicates early RNAs which are present in cells treated with PAA. Superscript L indicates late RNAs whose synthesis is differentially inhibited by PAA. The 8.2-kb RNA encoded by BamHI-B and the 1.9-kb RNA encoded by BamHI-E are visible only after long exposure of the fluorograms.

dependence of the protein synthesis on prior viral DNA replication (12, 26, 37). Both DNA synthesis-dependent and DNA synthesis-independent proteins are synthesized at late times after superinfection or induction. Similarly, the data reported here indicate that both PAA-sensitive and PAA-resistant RNAs persist at late times after induction. Third, other herpesvirus genomes have more elaborate regulatory schemes involving three tiers of sequential protein synthesis in productive infection (24). EBV probably also has at least three classes of sequential protein synthesis (10–12). Some EBV polypeptides are expressed for transient intervals after induction or superinfection (10–12). The RNAs encoding these polypeptides would not have been detected in this study. No RNAs have been detected from BamHI fragment Q, U, P, or S, either in this study or in previous studies of viral RNA in latent infection (28, 29, 51). These regions probably do encode RNA and are therefore likely candidates for sequences encoding transiently expressed polypeptides.

Several restriction endonuclease fragments of UL, BamHI fragments F, E, D, and L, encode abundant polyadenylated RNA whose cumulative size is larger than that of the fragment. In the case of BamHI-L, the cumulative size of the RNA is larger than both DNA strands. The data therefore indicate that some of these RNAs must overlap in nucleotide sequence and differ in coding strand, splicing, initiation, or termination. However, in no instance did three adjacent or distant DNA fragments hybridize to the same cytoplasmic polyadenylated RNA as might occur if these RNAs had exons encoded by separated regions of the genome as has been described for adenovirus late RNAs (44).

Very little can be concluded as to the function of individual RNAs which have been identified. The 150×10^3-dalton major capsid protein, the 145×10^3-dalton core membrane protein, and the 135×10^3-dalton outer envelope protein precursor are abundant late polypeptides (25, 26, 35–37). These polypeptides are likely to be encoded by abundant late cytoplasmic polyadenylated RNAs of at least 3.4 kb. Late RNAs larger than 3.4 kb are encoded by BamHI fragments C, D, and L. Hybrid selection and in vitro translation of these RNAs have shown that they encode proteins of 130×10^3 to 150×10^3 daltons (manuscript in preparation). A 135×10^3-dalton abundant early polypeptide continues to be made after induction and would also re-

quire an RNA of 3.4 kb (25, 26, 35–37). BamHI-A encodes an early RNA of 4.2 kb which is translated in vitro into a polypeptide of 135 × 10^3 daltons (manuscript in preparation).

Although only 25% of the induced cells contain VCA and 30% of them contain MA, the remaining VCA- and MA-negative cells could be transcribing, processing, and even translating some early or late viral RNAs. With the possible exception of the 2.8-kb RNA encoded by EcoRI-Dhet, which seems to be a late RNA, viral cytoplasmic polyadenylated RNAs previously identified in cells latently infected with B95-8 virus (51) were not detected in the TPA-induced cultures. The latently infected cell RNAs may be less abundant in TPA-induced cultures or much less evident because of the large quantity of early and late viral RNAs from the cells which are productively infected. In either case, the identification of many new viral RNAs in the induced cultures and the failure to detect those polyadenylated cytoplasmic RNAs previously identified in growth-transformed latently infected cells support the hypothesis that different classes of viral RNAs are specifically associated with latent versus productive viral infection.

The 170-base non-polyadenylated RNA which was originally identified in cells latently infected by EBV has been shown to be two distinct RNAs of 166 and 173 nucleotides (32, 43, 51). The genes encoding these RNAs are separated by 161 bp and are transcribed from left to right in the EBV map orientation shown in Fig. 7 (43). Like adenovirus VAI and VAII RNAs, both EBV small RNAs are polymerase III products and have extensive intramolecular homology (43). The EBV RNAs also have some homology to adenovirus VAI and VAII RNAs (43). The adenovirus VA RNAs are expressed in productive, but not latent, infection. The results reported here indicate that, unlike the EBV cytoplasmic polyadenylated RNAs which are detected in latently infected cells, the small RNA is made in much larger amounts in productive infection. Thus, the function of one or both of these EBV small RNAs is more analogous to that of the adenovirus VA RNAs than was previously appreciated.

LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69:**1408–1412.
2. **Bayliss, G., and M. Nonoyama.** 1978. Mechanisms of infection with Epstein-Barr virus. III: The synthesis of proteins in superinfected Raji cells. Virology **87:**201–207.
3. **Berger, S., and C. Birkenmeier.** 1979. Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes isolation of RNA from resting lymphocytes. Biochemistry **18:**5143–5149.
4. **Berk, A., F. Lee, T. Harrison, J. Williams, and P. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17:**935–944.
5. **Bornkamm, G. W., H. Delius, U. Zimber, J. Hudewentz, and M. A. Epstein.** 1980. Comparison of Epstein-Barr virus strains of different origin by analysis of the viral DNAs. J. Virol. **35:**603–618.
6. **Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub, and E. Kieff.** 1980. Epstein-Barr virus DNA. VII. Molecular cloning and detailed mapping of EBV (B95-8) DNA. Proc. Natl. Acad. Sci. U.S.A. **77:**2999–3003.
7. **Dambaugh, T., N. Raab-Traub, M. Heller, C. Beisel, M. Hummel, A. Cheung, S. Fennewald, W. King, and E. Kieff.** 1980. Variations among isolates of Epstein-Barr Virus. Ann. N.Y. Acad. Sci. **602:**711–719.
8. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23:**641–646.
9. **Edson, C. M., and D. A. Thorley-Lawson.** 1981. Epstein-Barr virus membrane antigens: characterization, distribution, and strain differences. J. Virol. **39:**172–184.
10. **Feighny, R. J., M. P. Farrell, and J. S. Pagano.** 1980. Polypeptide synthesis and phosphorylation in Epstein-Barr virus-infected cells. J. Virol. **34:**455–463.
11. **Feighny, R. J., B. E. Henry II, and J. S. Pagano.** 1981. Epstein-Barr virus polypeptides: identification of early proteins and their synthesis and glycosylation. J. Virol. **39:**651–655.
12. **Feighny, R. J., B. E. Henry II, and J. S. Pagano.** 1981. Epstein-Barr virus polypeptides: effect of inhibition of viral DNA replication on their synthesis. J. Virol. **37:**61–71.
13. **Gerber, P.** 1972. Activation of Epstein-Barr virus by 5'-bromodeoxyuridine in virus free human cells. Proc. Natl. Acad. Sci. U.S.A. **69:**83–85.
14. **Given, D., and E. Kieff.** 1978. DNA of Epstein-Barr virus. IV. Linkage map of restriction enzyme fragments of the B95-8 and W91 strains of Epstein-Barr virus. J. Virol. **28:**524–542.
15. **Given, D., and E. Kieff.** 1979. DNA of Epstein-Barr virus. VI. Mapping of the internal tandem reiteration. J. Virol. **31:**315–324.
16. **Given, D., D. Yee, K. Griem, and E. Kieff.** 1979. DNA of Epstein-Barr virus. V. Direct repeats of the ends of Epstein-Barr virus DNA. J. Virol. **30:**852–862.
17. **Hampar, B., J. G. Derge, L. M. Martos, and J. L. Walker.** 1972. Synthesis of Epstein-Barr virus after activation of the viral genome in a "virus-negative" human lymphoblastoid cell (Raji) made resistant to 5-bromodeoxyuridine. Proc. Natl. Acad. Sci. U.S.A. **69:**78–82.
18. **Hayward, S. D., and E. Kieff.** 1977. DNA of Epstein-Barr virus. II. Comparison of the molecular weights of restriction endonuclease fragments of the DNA of Epstein-Barr virus strains and identification of end fragments of the B95-8 strain. J. Virol. **23:**421–429.
19. **Hayward, S. D., and E. D. Kieff.** 1976. Epstein-Barr virus-

specific RNA. I. Analysis of viral RNA in cellular extracts and in the polyribosomal fraction of permissive and nonpermissive lymphoblastoid cell lines. J. Virol. 18:518–525.

20. Hayward, S. D., L. Nogee, and G. S. Hayward. 1980. Organization of repeated regions within the Epstein-Barr virus DNA molecule. J. Virol. 33:507–521.

21. Heller, M., T. Dambaugh, and E. Kieff. 1981. Epstein-Barr virus DNA. IX. Variation among viral DNAs from producer and nonproducer infected cells. J. Virol. 38:632–648.

22. Henle, G., and W. Henle. 1966. Immunofluorescence in cells derived from Burkitt's lymphoma. J. Bacteriol. 91:1248–1256.

23. 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.

24. Honess, R. W., 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.

25. Kallin, B., J. Luka, and G. Klein. 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in n-butyrate-treated P3HR-1 cells. J. Virol. 32:710–716.

26. Kawanishi, M., K. Sugawara, and Y. Ito. 1981. Epstein-Barr virus induced polypeptides: a comparative study of superinfected Raji, IudR treated and n-butyrate treated P3HR-1 cells. Virology 109:77–81.

27. Kelley, R. B., N. R. Cozzarelli, M. P. Deutscher, I. R. Lehman, and A. Kornberg. 1970. Enzymatic synthesis of deoxyribonucleic acid. J. Biol. Chem. 245:39–45.

28. King, W., A. L. Thomas-Powell, N. Raab-Traub, M. Hawke, and E. Kieff. 1980. Epstein-Barr virus RNA. V. Viral RNA in a restringently infected, growth-transformed cell line. J. Virol. 36:506–518.

29. King, W., V. van Santen, and E. Kieff. 1981. Epstein-Barr virus RNA. VI. Viral RNA in restringently and abortively infected Raji cells. J. Virol. 38:649–660.

30. Kintner, C., and B. Sugden. 1979. The structure of the termini of the DNA of Epstein-Barr virus. Cell 17:661–671.

31. Klein, G., G. Pearson, J. S. Nadkarni, J. J. Nadkarni, E. Klein, G. Henle, W. Henle, and P. Clifford. 1968. Relation between Epstein-Barr viral and cell membrane immunofluorescence of Burkitt tumor cells. I. Dependence of cell membrane immunofluorescence on presence of EB virus. J. Exp. Med. 128:1011–1020.

32. Lerner, M., N. Andrews, G. Miller, and J. Steitz. 1981. Two small RNAs encoded by EBV and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosis. Proc. Natl. Acad. Sci. U.S.A. 78:805–809.

33. Miller, G., D. Coope, J. Niederman, and J. Pagano. 1976. Biological properties and viral surface antigens of Burkitt lymphoma- and mononucleosis-derived strains of Epstein-Barr virus released from transformed marmoset cells. J. Virol. 18:1071–1080.

34. Miller, G., and M. Lipman. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. 70:190–194.

35. Mueller-Lantzsch, N., B. Georg, N. Yamamoto, and H. zur Hausen. 1980. Epstein-Barr virus-induced proteins. II. Analysis of surface polypeptides from EBV-producing and superinfected cells by immunoprecipitation. Virology 102:401–411.

36. Mueller-Lantzsch, N., B. Georg, N. Yamamoto, and H. zur Hausen. 1980. Epstein-Barr virus-induced proteins. III. Analysis of polypeptides from P3HR-1 EBV superinfected NC37 cells by immunoprecipitation. Virology 102:231–233.

37. Mueller-Lantzsch, N., N. Yamamoto, and H. zur Hausen. 1979. Analysis of early and late Epstein-Barr virus associated polypeptides by immunoprecipitation. Virology 97:378–387.

38. Orellana, T., and E. Kieff. 1977. Epstein-Barr virus-specific RNA. II. Analysis of polyadenylated viral RNA in restringent, abortive, and productive infections. J. Virol. 22:321–330.

39. Pritchett, R. F., S. D. Hayward, and E. D. Kieff. 1975. DNA of Epstein-Barr virus. I. Comparative studies of the DNA of Epstein-Barr virus from HR-1 and B95-8 cells: size, structure, and relatedness. J. Virol. 15:556–569.

40. Raab-Traub, N., T. Dambaugh, and E. Kieff. 1980. DNA of Epstein-Barr virus. VIII. B95-8, the previous prototype, is an unusual deletion derivative. Cell 22:257–267.

41. Raab-Traub, N., R. Pritchett, and E. Kieff. 1978. DNA of Epstein-Barr virus. III. Identification of restriction enzyme fragments that contain DNA sequences which differ among strains of Epstein-Barr virus. J. Virol. 27:388–398.

42. Rigby, P., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling DNA to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–252.

43. Rosa, M. D., E. Gottlieb, M. R. Lerner, and J. A. Steitz. 1981. Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and the adenovirus-associated ribonucleic acids VAI and VAII. Mol. Cell. Biol. 1:785–796.

44. Sharp, P., J. Manley, A. Fire, and M. Gefter. 1980. Regulation of adenovirus mRNA synthesis. Ann. N.Y. Acad. Sci. 354:1–15.

45. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

46. Strnad, B. C., R. H. Neubauer, H. Rabin, and R. A. Mazur. 1979. Correlation between Epstein-Barr virus membrane antigen and three large cell surface glycoproteins. J. Virol. 32:885–894.

47. Summers, W. C., and G. Klein. 1976. Inhibition of Epstein-Barr virus DNA synthesis and late gene expression by phosphonoacetic acid. J. Virol. 18:151–155.

48. Taylor, J. M., R. I. Imensee, and J. Summer. 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta 442:324–330.

49. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77:5201–5205.

50. Thomas-Powell, A. L., W. King, and E. Kieff. 1979. Epstein-Barr virus-specific RNA. III. Mapping of DNA encoding viral RNA in restringent infection. J. Virol. 29:261–274.

51. van Santen, V., A. Cheung, and E. Kieff. 1981. Epstein-Barr virus (EBV) RNA. VII: Viral cytoplasmic RNA in a restringently infected cell line transformed in vitro by EBV. Proc. Natl. Acad. Sci. U.S.A. 78:1930–1934.

52. Yamamoto, N., and H. zur Hausen. 1980. Effect of inhibition of DNA synthesis on Epstein-Barr virus induction by tumor promotors. Virology 101:104–110.

53. zur Hausen, H., E. Hecker, F. J. O'Neill, and U. K. Freese. 1978. Persisting oncogenic herpesvirus induced by tumor promotor TPA. Nature (London) 272:373–375.