

Epstein-Barr Virus RNA

V. Viral RNA in a Restrictively Infected, Growth-Transformed Cell Line

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A continuous lymphoblastoid cell line, IB-4, was established by infection and growth transformation of normal neonatal B lymphocytes with the B95-8 isolate of Epstein-Barr virus (EBV). The IB-4 cells contained the intranuclear antigen, EBNA, but not early antigen, EA. The fragments produced by the digestion of intracellular episomal viral DNA (density, 1.700 to 1.720 g/cm³) with *EcoRI* restriction endonuclease were identical in size to the A, B, C, E, F, G, and H fragments of virion DNA. As expected from the previous observation that episomal intracellular DNA is circular, the fragment containing the rightward terminal sequences of EBV DNA in IB-4 cells was larger than the corresponding fragment of linear viral DNA, probably as a consequence of covalent linkage to the leftward terminal fragment. Also, two fragments, *EcoRI*-I and -J, which were adjacent to each other in the virion DNA, were absent from the intracellular DNA. The labeled *EcoRI*-J of viral DNA hybridized instead to a new fragment equal in size to *EcoRI*-I and -J combined. Analysis of viral RNA in IB-4 cells showed that RNAs encoded by more than 30% of the viral DNA comprised approximately 0.06% of the nuclear RNA, whereas RNAs encoded by 20% and 10% of the viral DNA comprised approximately 0.06% and 0.003% of the polyadenylated and polyribosomal RNAs, respectively. Viral mRNA (polyribosomal RNA) was encoded by DNA which mapped at 0.05×10^8 to 0.36×10^8 daltons and to a lesser extent by DNAs which mapped at 0.62×10^8 to 0.67×10^8 , 0.70×10^8 to 0.73×10^8 , and 1.13×10^8 to 1.15×10^8 daltons in the B95-8 genome. The most abundant nuclear viral RNAs were encoded primarily by DNA which mapped at the same loci; but RNAs encoded by many other fragments of viral DNA could also be detected among nuclear RNAs. Viral mRNA(s) (polyribosomal) was encoded by about 40% of the internal reiteration and by 25% of the *BamHI*-H fragments which mapped from 0.32×10^8 to 0.36×10^8 daltons; nuclear RNAs were encoded by at least 57% of the internal reiteration and 40% of *BamHI*-H. These data indicate that there is selective accumulation of some viral RNAs within the nucleus of IB-4 cells and that there is selective post-transcriptional processing of these RNAs. Finer mapping of the DNA which encodes mRNA (polyribosomal) in IB-4 cells indicated that some of this DNA is deleted in the DNA of the P3 HR-1 virus, the only isolate of EBV which cannot initiate growth transformation. These data, therefore, support the hypothesis that expression of this region of EBV genome is important for growth transformation or for the maintenance of restrictive infection.

Normal human lymphocytes grow poorly in vitro and usually cannot be maintained in culture beyond several months. Epstein-Barr virus (EBV) is able to infect B lymphocytes in vivo or in vitro (20, 21, 24). B lymphocytes infected with EBV can thereafter be grown in culture without finite life span (17). These continuous lymphoblastoid cell lines usually retain several EBV DNA molecules in an episomal, circular form (1, 2, 16). The cells also contain an intranuclear antigen, EBNA, found only in cells infected with

EBV (27). There is no abortive or productive virus infection in most EBV-infected lymphoblastoid cell lines. The state of virus infection in these cell lines in which expression of the virus is tightly restricted has been termed restrictive (22).

There have been several previous studies of viral RNA in restrictively infected continuous lymphoblastoid cell lines (10, 22, 25). Two cell lines, Namalwa and Raji, derived by growth in vitro of lymphocytes from human Burkitt tumor

biopsies, have been most extensively analyzed. Namalwa and Raji cells contain RNA encoded by a substantial fraction of the EBV genome, Raji cells containing RNA from at least 30% of EBV DNA and Namalwa cells containing RNA from at least 17% (10, 22, 25; W. King and E. Kieff, in preparation). There is evidence for selective processing of viral RNA, both before and after polyadenylation. Thus, Raji mRNA's are enriched for those viral RNAs encoded by approximately 10% of EBV DNA, whereas RNAs encoded by 20% of EBV DNA selectively accumulate in the polyadenylated RNAs of Raji cells (10, 22; King and Kieff, in preparation). Furthermore, the viral mRNA's of Raji and Namalwa cells are encoded largely by DNA sequences which map between 0.05×10^8 and 0.36×10^8 daltons and to a lesser extent by DNAs which map at 1.04×10^8 to 1.15×10^8 and 0.70×10^8 to 0.73×10^8 daltons, whereas RNA from many other regions of EBV DNA is detected only among nuclear RNAs (15, 25; King and Kieff, in preparation).

There are two limitations inherent in the study of viral RNA in cell lines derived by growth of Burkitt tumor cells in culture. First, there is variation among the DNAs of EBV isolates (8, 11, 14, 26). Since virus cannot be recovered from restringently infected cells, and it is difficult to separate viral DNA from infected cellular DNA, the sequence arrangement of the DNA of the virus which infected these cells is not known. Moreover, analysis of EBV DNA in Raji cells indicates that the viral DNA is heterogeneous in organization (M. Heller et al., in preparation). It is, therefore, not possible to align individual mRNA's with the DNA which encoded these RNAs. Second, malignant lymphocytes which are not EBV infected can be grown as continuous lymphoblastoid cell lines (7, 15). Some of these malignant cells can be infected with EBV *in vitro* (15), and such infection could presumably also occur *in vivo*. The possibility therefore exists that the growth of Burkitt tumor lymphocytes *in vitro* may not require expression of viral functions which promote the growth of normal cells.

For these reasons, we have infected normal human neonatal lymphocytes with an isolate of EBV, B95-8, whose DNA structure is well characterized (Fig. 9) (5, 8, 11, 14). Restringently infected, growth-transformed continuous lymphoblastoid cell lines have been derived. We report here the first analyses of viral RNA in one cell line, IB-4.

MATERIALS AND METHODS

Cell culture. Cultures of B95-8 cells were maintained as previously described (25) in complete me-

dium which consisted of RPMI 1640 medium supplemented with 10% fetal calf serum (both obtained from GIBCO Laboratories, Grand Island, N.Y.).

The IB-4 cell line was initiated by infection of cord blood lymphocytes with EBV produced by B95-8 cells. The lymphocyte fraction was first separated from heparinized cord blood by centrifugation on Ficoll-Hypaque gradients (3). The lymphocytes were freed from residual erythrocytes by incubation for 10 min at 4°C in 0.01 M potassium bicarbonate-0.1 M EDTA and were collected by centrifugation at 2,000 rpm for 5 min. The lymphocytes were resuspended at a concentration of 5×10^5 cells per ml of complete medium. Four cultures containing 0.5 ml of cells and 0.5 ml of a dilution of twice-filtered (0.22- μ m filter, Millipore Corp.) supernatant of B95-8 cultures were set up at each dilution of supernatant. The cultures were incubated in an atmosphere of 5% CO₂ at 37°C and were fed every 3 to 4 days by exchanging 0.5 ml of fresh complete medium for 0.5 ml of culture supernatant. By 6 to 10 weeks, the number of viable cells in one of four cultures infected with a 10^{-3} dilution and in one of four cultures infected with a 10^{-4} dilution of supernatant exceeded 2×10^5 cells per ml. These cultures were thereafter fed by dilution every 3 to 4 days in an equal volume of complete medium.

The culture which was initiated after infection with a 10^{-4} dilution of supernatant of B95-8 cultures, IB-4, was grown in large quantity for subsequent studies. The cultures contained more than 95% viable cells. The viral capsid antigen, VCA (12), and early antigen, EA (13), content of cells was monitored monthly and was consistently negative. In an attempt to induce early antigen, cultures were incubated in complete medium for 3 days in the presence of iododeoxyuridine, 30 μ g/ml, followed by 3 days in the absence of iododeoxyuridine. EA was not detected in IB-4 cells even after iododeoxyuridine treatment at the level of 1 positive cell per 1,000 negative cells.

Preparation of IB-4 DNA enriched for viral DNA. The pellet obtained by centrifuging 5×10^8 cells was suspended in 100 ml of 0.01 M EDTA-0.01 M Tris-hydrochloride, pH 7.5, at 4°C, and 0.05 volume of 20% (wt/vol) sodium dodecyl sulfate was added. After addition of 0.25 volume of 5.0 M NaCl, the mixture was stored at 4°C for 18 h. The precipitate was removed by centrifugation at 9,000 rpm for 15 min in a GSA rotor (Ivan Sorvall, Inc.). Proteinase K, 100 μ g/ml, and 0.025 volume of 20% (wt/vol) sodium dodecyl sulfate were added to the supernatant, which was then incubated at 60°C for 1 h and dialyzed at 20°C against 0.1% Sarkosyl, 0.01 M EDTA, and 0.02 M Tris-hydrochloride, pH 7.5. Cesium chloride was added to a density of 1.700 g/cm³, and the mixture was centrifuged for 20 h at 40,000 $\times g$ and 20°C in a VTI 50 rotor (Beckman Instruments, Inc.). The fractions of the gradients from 1.700 to 1.720 g/cm³ were combined and recentrifuged under the same conditions. The fractions of the gradient from 1.700 to 1.720 g/cm³ were combined, dialyzed against 0.1 M NaCl, 0.01 M EDTA, and 0.02 M Tris-hydrochloride, pH 7.5, and precipitated overnight at -20°C after the addition of 2 volumes of ethanol.

Purification of viral DNA, labeling of DNA *in vitro*, preparation of blots of DNA fragments,

and hybridization to blots. Virus was purified from the extracellular fluid of B95-8 cells (6), and the DNA was extracted as previously described (10). *Bam*HI fragments V, H, C, and X obtained from recombinant plasmids pDK14, pDK286, pDK10, and pDF322, respectively, were a kind gift of Timothy Dambaugh (5). The *Eco*RI Dhet-IJhet was obtained from a recombinant Charon 4A DNA preparation which was a gift of Chris Beisel and Nancy Raab-Traub. The procedures for labeling viral DNA, in vitro, by use of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Corp.) and 32 P-labeled dCTP (500 Ci/mmol, Amersham Corp.) were described previously as were the conditions for cutting DNA with *Eco*RI, *Hind*III, or *Bam*HI restriction endonucleases (Bethesda Research Laboratories), for separation of DNA fragments in agarose gels, for transfer of DNA fragments to nitrocellulose filters, and for hybridization, washing, and fluorography of blots (8, 25). The specific activity of the in vitro-labeled DNA was 0.5×10^8 to 2×10^8 cpm per μ g.

Preparation of IB-4 cellular RNAs and hybridization to labeled viral DNAs in solution. Ninety percent of the supernatant media was decanted from 40 liters of IB-4 culture. The concentrated cells were centrifuged at 2,000 rpm for 5 min at 4°C in a GS3 rotor (Ivan Sorvall, Inc.), and the cell pellet was resuspended in 50 ml of 0.01 M EDTA-0.02 M Tris-hydrochloride, pH 7.4, at 4°C. An equal volume of 8 M guanidine HCl (Bethesda Research Laboratories) was added at 4°C with vigorous shaking. Proteinase K, 300 μ g/ml, 0.1 volume of 10% sodium dodecyl sulfate, and 0.05 volume of 20% Sarkosyl NL97 were added, and the mixture was incubated at 60°C for 30 min. The RNA was separated from DNA by centrifugation through a cesium chloride cushion (density, 1.730 g/cm³; 25) and separated into polyadenylated and non-polyadenylated fractions by two cycles of chromatography on columns of oligodeoxythymidylate-cellulose (Collaborative Research Inc.) (22). Greater than 97% of the [³H]thymidine-labeled *Klebsiella pneumoniae* DNA which was added to the cellular DNA as a marker DNA similar in guanosine plus cytosine content to EBV DNA did not enter the dense CsCl cushion.

For the preparation of nuclear and polyribosomal RNAs, nuclear and polyribosomal fractions of cells were separated by differential centrifugation (10). Subsequent purification of nuclear and polyribosomal RNAs followed the procedures described above for cellular RNAs.

All RNA preparations were treated with DNase I under conditions in which 95% of added [³H]thymidine-labeled *K. pneumoniae* DNA was rendered soluble in 70% ethanol (25).

The procedures for hybridization of cellular RNAs to labeled viral DNA in solution, for S1 digestion of residual labeled single-strand DNA, and for analysis of the kinetics of hybridization have been described previously (10, 22). Alkali-treated portions of each RNA preparation had no effect on the rate of renaturation of labeled viral DNA (10, 22).

Identification of viral DNA fragments which encode RNA in IB-4 cells. Two approaches were employed to identify viral DNA fragments which en-

code RNAs in IB-4 cells. Both approaches involved hybridization of 32 P-labeled DNA complementary to IB-4 RNA to blots of fragments of viral DNA. For most experiments, labeled DNA complementary to IB-4 RNA was prepared by the hybridization of IB-4 RNA to labeled viral DNA, S1 digestion to degrade the unhybridized labeled DNA, isopycnic banding of the [32 P]DNA-RNA hybrid in cesium sulfate gradients, and alkali treatment of the [32 P]DNA-RNA hybrids to denature the hybrids and degrade the RNA. After S1 digestion, greater than 80% of added denatured [³H]thymidine-labeled *K. pneumoniae* DNA was rendered soluble in 70% ethanol. Native and denatured [³H]thymidine-labeled *K. pneumoniae* DNA was included in each of the cesium sulfate gradients as markers for DNA of guanosine and cytosine content similar to that of EBV DNA. The procedures which have been described in detail previously (25) were modified in that [32 P]DNA-RNA hybrid from the first cesium sulfate gradient was centrifuged to equilibrium in a second cesium sulfate gradient without denaturation, rehybridization to RNA in excess, or further S1 digestion. This modification avoided further loss and decrease in the size of the [32 P]DNA complementary to RNA. The second isopycnic banding resulted in enhanced separation of [32 P]DNA-RNA hybrids from 32 P-labeled denatured and renatured DNA (Fig. 3). There were 9×10^5 , 5×10^5 , and 3×10^5 cpm of 32 P-labeled DNA in the regions of native DNA, denatured DNA, and DNA-RNA hybrid, respectively, of the first cesium sulfate gradient, and there were 10×10^4 , 2×10^4 , and 1.5×10^5 cpm of 32 P-labeled DNA in the corresponding regions of the second cesium sulfate gradient, indicating that at least 98% of the renatured and 96% of the denatured labeled DNA were removed from the S1-resistant nucleic acid mixture by the first cycle through cesium sulfate. Nearly identical results were obtained in the cesium sulfate gradients of labeled DNA hybridized to polyribosomal RNA except that only 1.2×10^6 and 0.5×10^6 cpm of 32 P-labeled DNA were in the DNA-RNA hybrid region of the first and second cesium sulfate gradients. Assuming that the segregation of DNA-RNA hybrid from native and denatured labeled DNA is similar in the first and second cesium sulfate gradients, as the banding of native and denatured labeled *K. pneumoniae* DNA in both gradients suggests, then less than 0.6% of the labeled DNA in the DNA-RNA hybrid region of the second cesium sulfate gradient of DNA complementary to nuclear RNA and less than 2% of the labeled DNA in this region of the second cesium sulfate gradient of DNA complementary to polyribosomal RNA could be due to contamination with denatured labeled viral DNA. The level of contamination with native viral DNA is much less.

The second approach was to synthesize cDNA from polyadenylated polyribosomal RNA by using avian myeloblastosis virus reverse transcriptase (prepared by J. Beard and a kind gift of R. Gallo) and [32 P]dCTP (500 Ci/mmol, Amersham Corp.) in an oligodeoxynucleotide-primed reaction (29). The reaction was terminated after 4 h at 37°C with 0.5% sodium dodecyl sulfate and 0.01 M EDTA. Salmon sperm DNA was added to a final concentration of 50 μ g/ml. The solution was made to a final volume of 2 ml in 0.05 M Tris-

hydrochloride (pH 7.4)-0.15 M NaCl-0.01 M EDTA, extracted with phenol and chloroform, and precipitated by the addition of 2 volumes of ethanol. After overnight incubation at -20°C , the ethanol precipitate was resuspended in 2 ml of 0.05 M Tris-hydrochloride (pH 7.6)-0.15 M NaCl-0.01 M EDTA and ethanol precipitated. The DNA was resuspended in 1 ml of water and 0.3 M NaOH and incubated at 110°C for 10 min to hydrolyze the RNA. The mixture was neutralized and hybridized to blots of EBV DNA under conditions previously described (4). Approximately 0.75×10^6 cpm and 3.0×10^8 cpm of cDNA to nuclear and polyribosomal polyadenylated RNA, respectively, were hybridized to blots of EBV DNA.

RESULTS

EBV DNA in IB-4 cells. Hybridization of IB-4 DNA to in vitro-labeled viral DNA (26) indicated that IB-4 cells contain approximately five copies of viral DNA per diploid cell. To determine whether there were any changes in the organization of the B95-8 viral DNA in IB-4 cells, we compared the size of fragments produced by cleavage of intracellular and virion DNA with *EcoRI* restriction endonuclease. The *EcoRI* fragments were separated on 0.3% agarose gels and transferred to nitrocellulose filters (8). Labeled EBV (B95-8) DNA or labeled cloned fragments of EBV (B95-8) DNA identified fragments similar in size to the *EcoRI*-A, -B, -C, -E, -F, -G, and -H fragments of EBV (B95-8) DNA in digest of IB-4 cell DNA (Fig. 1). The labeled EBV (B95-8) DNA also hybridized to a fragment larger than *EcoRI*-E and a fragment smaller than *EcoRI*-F (Fig. 1). EBV DNA is known to circularize in infected cells by covalent joining of the ends (1, 2). To demonstrate that the fragment larger than *EcoRI*-E was the fragment anticipated from the joining of *EcoRI* Dhet and IJhet fragments, the component of *BamHI*-A which maps from 0.94×10^8 to 0.98×10^8 daltons in EBV B95-8 DNA (Fig. 9) was separated by electrophoresis from the other fragments of a *BamHI/EcoRI* digest of cloned *BamHI*-A, labeled in vitro, and hybridized to blots of *EcoRI* fragments of EBV (B95-8) or IB-4 cell DNAs. As expected, the labeled fragment hybridized extensively to the fragment larger than *EcoRI*-E in IB-4 cell DNA, which is, therefore, identified as *EcoRI*-Dhet-IJhet (Fig. 1).

The purified *EcoRI*-D component of *BamHI*-A was not completely free from the *EcoRI*-C component of *BamHI*-A, and the labeled probe hybridized to a lesser extent to the *EcoRI*-C fragment of EBV (B95-8) DNA and to a similar-size fragment of IB-4 cell DNA (Fig. 1). An unanticipated finding was the observation that this labeled DNA also hybridized to fragments slightly smaller than *EcoRI*-B and slightly larger

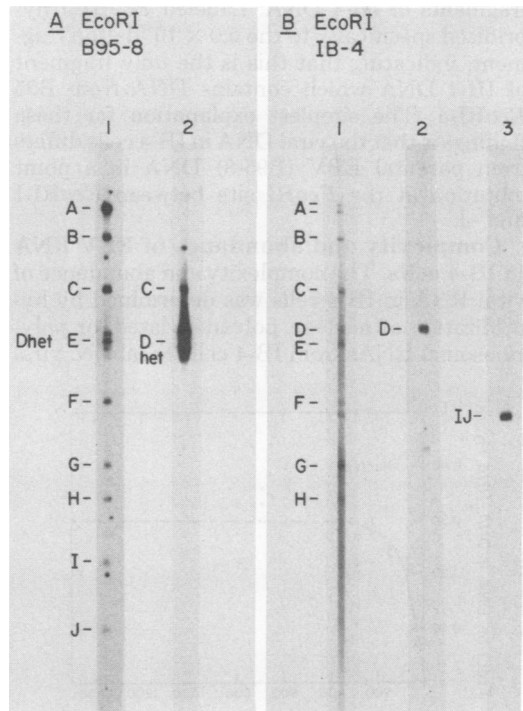


FIG. 1. *EcoRI* fragments of IB-4 and B95-8 DNA. ^{32}P -labeled EBV (B95-8) DNA (column 1), ^{32}P -labeled 4.6×10^6 dalton component of *BamHI*-A (column 2), and ^{32}P -labeled *EcoRI*-J component of *BamHI*-C (column 3) were hybridized to blots of *EcoRI* digests of EBV (B95-8) DNA (panel A) or high-density "viral" DNA from IB-4 cells (panel B).

than *EcoRI*-G in both EBV (B95-8) DNA and IB-4 cell DNA. These fragments may result from a minor population of defective molecules containing *BamHI*-A sequences. However, neither of these fragments was detected with labeled EBV (B95-8) DNA (Fig. 1).

Fragments the size of *EcoRI*-I and -J were not present in the autoradiograms of blots of *EcoRI* fragments of IB-4 DNA (Fig. 1). A new fragment was present, however, just below *EcoRI*-F (Fig. 1). The size of the new fragment, estimated from a log linear plot of the size and mobility of the other *EcoRI* fragments, was 5.0×10^6 daltons; estimates of the size of *EcoRI*-I and -J range from 2.8×10^6 to 2.6×10^6 and 2.2×10^6 to 2.0×10^6 daltons, respectively (5, 10). *EcoRI*-I and -J are adjacent to each other in EBV (B95-8) DNA (5, 8). The 5×10^6 dalton fragment could therefore be created by loss of the *EcoRI* site between *EcoRI*-I and -J. To test this hypothesis, we separated B95-8 *EcoRI*-J from a recombinant plasmid, pDK10, which contains a B95-8 *BamHI*-C insert. The *EcoRI*-J fragment was labeled in vitro and hybridized to blots of *EcoRI*

fragments of IB-4 DNA. Labeled *EcoRI*-J hybridized specifically to the 5.0×10^6 dalton fragment, indicating that this is the only fragment of IB-4 DNA which contains DNA from B95 *EcoRI*-J. The simplest explanation for these findings is that the viral DNA in IB-4 cells differs from parental EBV (B95-8) DNA in a point mutation at the *EcoRI* site between *EcoRI*-I and -J.

Complexity and abundance of EBV RNA in IB-4 cells. The complexity and abundance of viral RNA in IB-4 cells was determined by hybridization of nuclear, polyadenylated, or polyribosomal RNAs from IB-4 cells to labeled viral

DNA in solution. As shown in Fig. 2, nuclear RNA hybridized to 29 to 33% of the labeled EBV DNA; polyadenylated RNA, to 19 to 22% of the labeled EBV DNA; and polyribosomal RNA, to 8 to 10% of the labeled EBV DNA. From the kinetics of hybridization of the RNA to denatured labeled DNA, the viral RNA was estimated to be approximately 6×10^{-2} , 6×10^{-2} , and 3×10^{-3} % of the IB-4 cell nuclear, polyadenylated, and polyribosomal RNA, respectively (10, 22).

Mapping of IB-4 RNAs. Two series of experiments were done to identify on the restriction endonuclease map of EBV DNA (Fig. 9) the viral DNA sequences which encode IB-4 nuclear, polyadenylated, and polyribosomal RNAs. In the first series of experiments, labeled viral DNA which hybridized to IB-4 RNAs was then hybridized to blots of viral DNA fragments. As a consequence of the greater abundance of viral RNA in the nucleus than in the polyribosomal fraction of IB-4 cells, labeled DNA homologous to viral nuclear RNA could be more clearly separated from residual single-stranded DNA and small DNA fragments (Fig. 3). Despite the clear separation from residual single-stranded DNA, viral DNA homologous to nuclear or polyadenylated RNA hybridized to many of the *EcoRI* and *HindIII* fragments of EBV DNA (Fig. 4), and DNA homologous to polyribosomal RNA hybridized (Fig. 4) primarily to the *EcoRI*-A (map position, 0.07×10^8 to 0.46×10^8 daltons, Fig. 9), the *HindIII*-A and B fragments (map position, 0.00 to 0.52×10^8 daltons, Fig. 9), and to *BamHI*-V, -H, -C, and -X (map position, 0.05×10^8 to 0.37×10^8 daltons, Fig. 9). DNA selected for homology to polyribosomal RNA hybridized to a lesser extent (Fig. 4) to the *EcoRI*-B, -C, and -Dhet fragments (map positions, 0.63×10^8 to 0.82×10^8 , 0.93×10^8 to 1.15×10^8 daltons, Fig. 9), to the *HindIII*-D, -E, -FGHhet and possibly -I fragments (map positions, 0.61×10^8 to 0.72×10^8 and 0.96×10^8 to 1.15×10^8 daltons, Fig. 9) and to the *BamHI*-A, -E, -K, and -Jhet or -Nhet fragments (map positions, 0.61×10^8 to 0.66×10^8 , 0.70×10^8 to 0.73×10^8 , and 1.00×10^8 to 1.15×10^8 daltons, Fig. 9). The components of Jhet and Nhet overlap each other in the size range of 2×10^6 to 6×10^6 daltons, with Jhet and Nhet being the most prevalent components of the left and right end fragments, respectively (5).

In the second series of experiments, ^{32}P -labeled complementary DNA (cDNA) made from IB-4 nuclear RNA (cDNA-N) or polyadenylated polyribosomal RNA (cDNA-P) templates by use of oligodeoxynucleotide primers was hybridized to blots of *SalI*, *HindIII*, or *BamHI* fragments

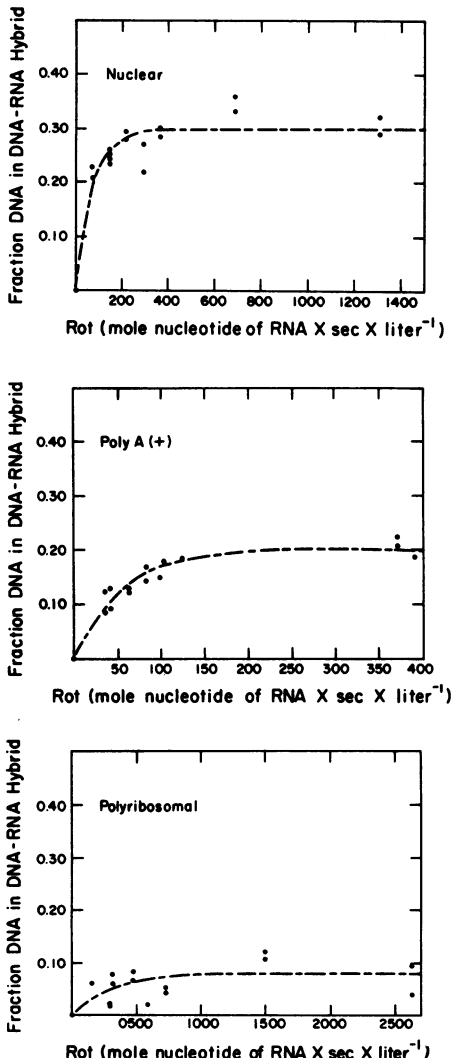


FIG. 2. Hybridization of nuclear, polyadenylated, or polyribosomal RNAs from IB-4 with denatured ^{32}P -labeled EBV (B95-8) DNA.

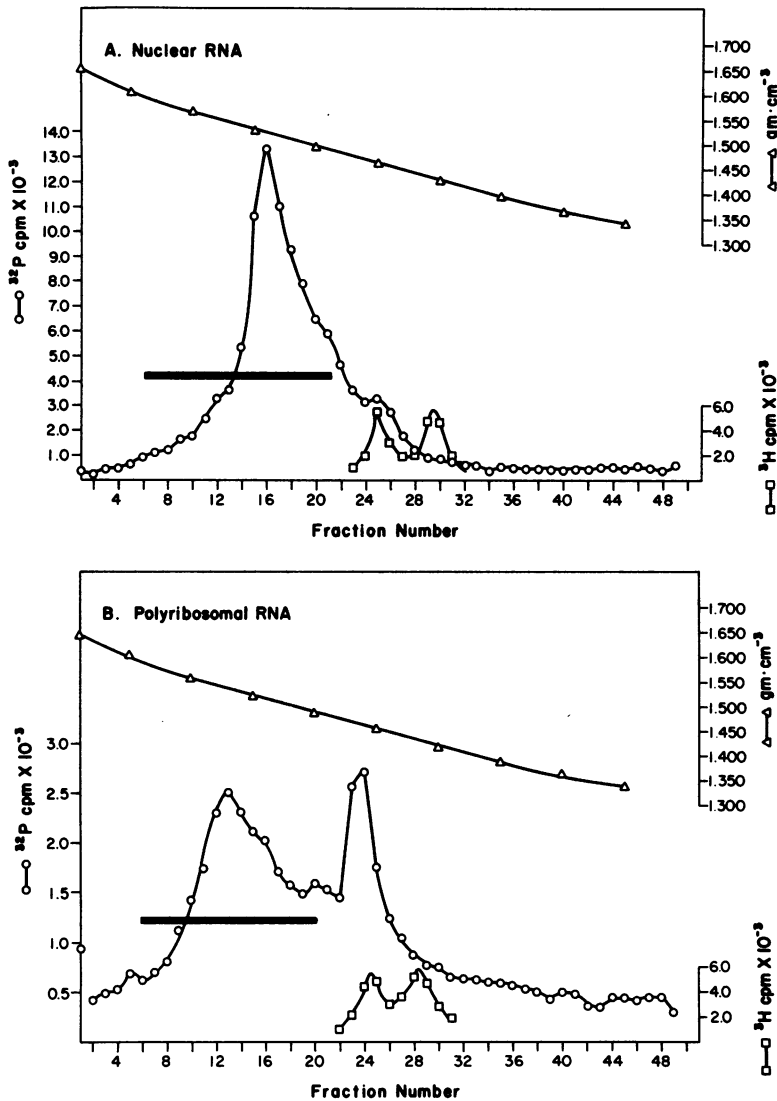


FIG. 3. Distribution of ^{32}P -labeled EBV (B95-8) DNA which had been hybridized to nuclear or polyribosomal RNA in the second equilibrium cesium sulfate gradient. The solid bar indicates fractions which were combined for hybridization to blots of EBV DNA. [^3H]thymidine-labeled native and denatured *K. pneumoniae* DNA was added prior to equilibrium banding.

of EBV DNA (Fig. 5). Labeled cDNA-N hybridized primarily to the *Bam*HI-C, -V, -X, -H, -E, -K, and -Nhet or -Jhet fragments (map positions, 0.05×10^8 to 0.36×10^8 , 0.61×10^8 to 0.66×10^8 , 0.70×10^8 to 0.73×10^8 , and 1.09×10^8 to 1.15×10^8 daltons, Fig. 9). With longer exposures, hybridization to many other fragments was evident. Labeled cDNA-P hybridized primarily to the *Sa*II-A, -C, -F, and -Dhet fragments (map positions, 0.04×10^8 to 0.38×10^8 , 0.50×10^8 to 0.74×10^8 , and 1.01×10^8 to 1.15×10^8 daltons, Fig. 9), to the *Hind*III-A, -B, -E, and -I fragments

(map positions, 0.00 to 0.52×10^8 and 0.62×10^8 to 0.73×10^8 daltons, Fig. 9), and to the *Bam*HI-C, -V, -X, -H, -E, -K, and -Jhet, or -Nhet fragments (map positions, 0.05×10^8 to 0.36×10^8 , 0.61×10^8 to 0.66×10^8 , 0.70×10^8 to 0.73×10^8 , and 1.09×10^8 to 1.15×10^8 daltons, Fig. 9). Several other dark areas are visible in the radiofluorogram of the *Bam*HI blot hybridized to cDNA-P (Fig. 5c). These are not indicated as positive results since they were not consistent with other experiments and, in many instances, did not coincide with the position of viral DNA

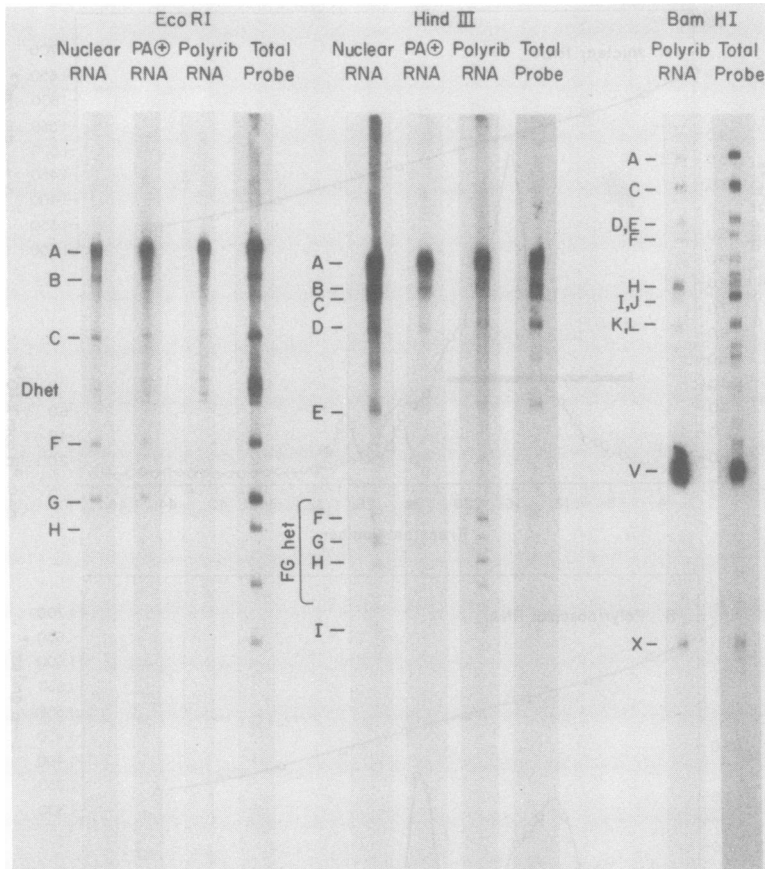


FIG. 4. Radiofluorograms of blots of agarose gels containing separated *EcoRI*, *HindIII*, or *BamHI* restriction endonuclease-cleaved fragments of EBV (B95-8) DNA which were hybridized to ^{32}P -labeled EBV (B95-8) DNA homologous to IB-4 nuclear, polyadenylated, or polyribosomal RNA.

fragments. They are, therefore, presumed to be an artifact of the large amount of radioactive probe to which the blot was hybridized.

The BamHI-C, -V, -X, and -H fragments are contiguous and define a continuous segment of EBV DNA (0.05×10^8 to 0.36×10^8 daltons, Fig. 9). To investigate whether IB-4 viral nuclear or polyribosomal RNAs are encoded by all or only part of these fragments, we undertook two further experiments. In the first type of experiment, cDNA to nuclear or polyadenylated polyribosomal RNA was hybridized to blots of *BamHI*, *EcoRI* fragments of plasmid pDK10, which contains *BamHI*-C (Fig. 6A), to *BamHI*, *BglII* fragments of pDK14, which contains *BamHI*-V (Fig. 6B), to *HindIII* fragments of pDF322, which contains *BamHI*-X (Fig. 6C) and to *BamHI*, *HinfI* fragments of pDK286, which contains *BamHI*-H (Fig. 6D). These cDNA's were also hybridized to blots of a *BamHI*, *EcoRI* digest of CB28, which contains *EcoRI*-Dhet-IJhet (Fig. 6E). The results were as follows. (i) Nuclear and polyribosomal cDNA's hybridized to the $3.9 \times$

10^6 dalton component of *BamHI*-C which contains the beginning of the internal reiteration (map position, 0.08×10^8 to 0.12×10^8 daltons) and hardly at all to the 2×10^6 dalton *EcoRI*-J component of *BamHI*-C. (ii) Both cDNA's hybridized to the 1.5×10^6 and 0.4×10^6 dalton *BamHI*, *BglII* fragments of *BamHI*-V. (iii) Both cDNA's hybridized to the components of *BamHI*-X which lie to the left (0.7×10^6 daltons) and the right (0.5×10^6 daltons) of the *HindIII* site at 0.32×10^8 daltons. (iv) Both cDNA's hybridized to a larger extent to the 0.4×10^6 dalton *HinfI* component of *BamHI*-H than to the 1.1×10^6 and 2.1×10^6 dalton components of *BamHI*-H. (v) cDNA to nuclear RNA hybridized to the 4.6×10^6 dalton *BamHI*-A component of *EcoRI*-D and only minimally to the *EcoRI*, *BamHI* joined ends, whereas cDNA to polyadenylated polyribosomal RNA hybridized more extensively to the *EcoRI*, *BamHI* ends, but relatively less to the *BamHI*-A component of *EcoRI*-D.

In the second type of experiment to determine

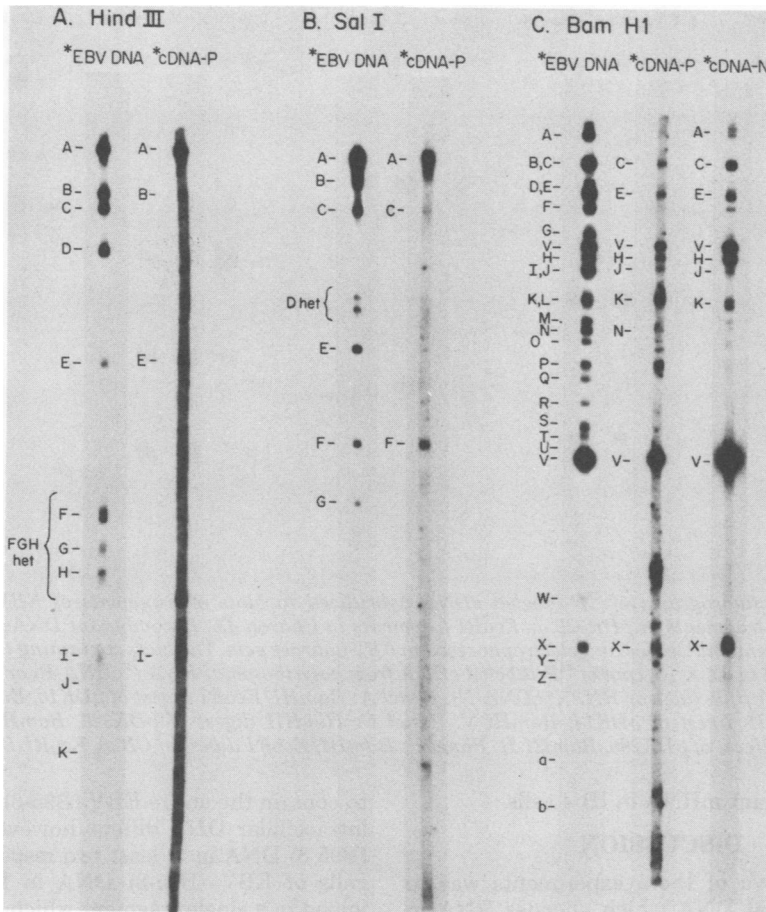


FIG. 5. Radiofluorograms of blots of agarose gels containing separated *HindIII*, *SalI*, or *BamHI* restriction endonuclease-cleaved fragments of EBV (B95-8) DNA which were hybridized to ³²P-labeled cDNA synthesized from polyribosomal polyadenylated (*cDNA-P) or nuclear (*cDNA-N) RNAs.

the extent to which individual fragments encode nuclear and polyribosomal RNA in IB-4 cells, nuclear or polyribosomal RNA from IB-4 cells was hybridized to denatured labeled *BamHI*-V or -H which had been separated from the respective recombinant plasmid DNAs. Nuclear RNA hybridized to at least 55% of *BamHI*-V and 40% of *BamHI*-H (Fig. 7). Polyribosomal RNA hybridized to at least 42% of *BamHI*-V and 25% of *BamHI*-H (Fig. 7). From the kinetics of hybridization of polyribosomal RNA to *BamHI*-V and -H, approximately 4×10^{-4} to 6×10^{-4} % of the IB-4 cellular polyribosomal RNA is estimated to be homologous to *BamHI*-H and *BamHI*-V. The abundance of viral nuclear RNA homologous to *BamHI*-H and -V is estimated to be approximately 10-fold greater.

EBV DNA in nontransforming P3HR-1 strain of EBV. Previous data indicate that the P3HR-1 strain of EBV differs from all other isolates in that it lacks the ability to growth

transform normal B lymphocytes (17, 19). Further, P3HR-1 DNA has been shown to have a deletion in the *HindIII*-B fragment (26). The deletion probably extends from 0.31×10^6 daltons rightward, as the map is drawn in Fig. 9, since P3HR-1 DNA has the internal reiteration but lacks the *HindIII* cleavage site between *HindIII*-A and -B (14). *BamHI*-X contains the *HindIII* site and *BamHI*-H is to the right of *BamHI*-X (5). To determine whether P3HR-1 DNA has the *BamHI*-X and -H sequences which encode mRNA in the EBV-infected, growth-transformed cell line IB-4, we hybridized ³²P-labeled P3HR-1 DNA to blots of fragments of a *HindIII* digest of pDF322 which contains *BamHI*-X and of a *BamHI/HinfI* digest of pDK286 which contains *BamHI*-H (Fig. 8). The results indicate that P3HR-1 DNA lacks the sequences to the right of the *HindIII* cut site in *BamHI*-X and the sequences of the 0.4×10^6 dalton fragment of *BamHI*-H, both of which

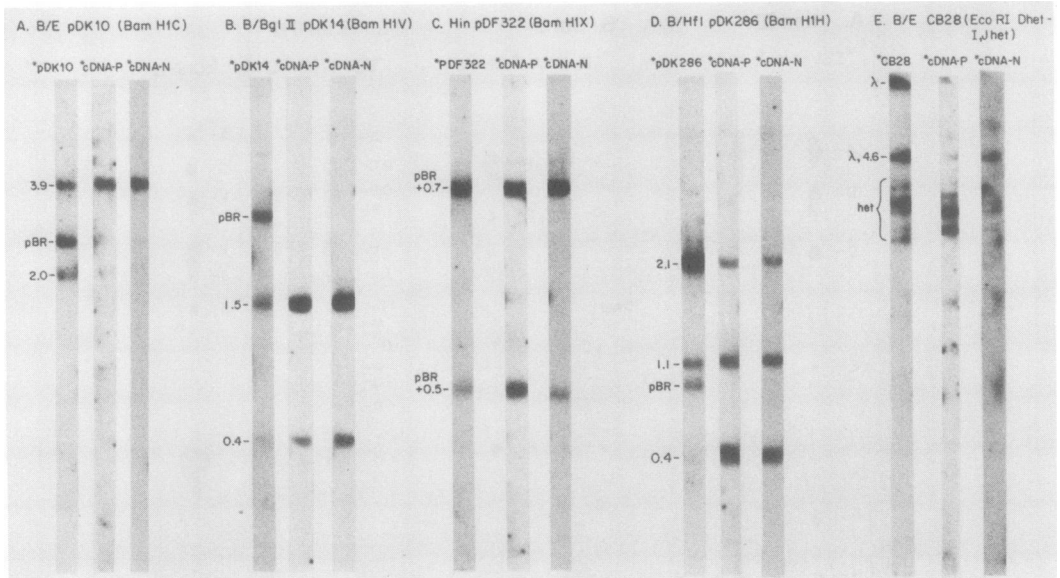


FIG. 6. Radiofluorograms of ^{32}P -labeled cDNA hybridized to blots of fragments of EBV recombinant DNAs: BamHI fragments in pBR322 or EcoRI fragments in Charon 4A. Recombinant DNAs were digested with restriction endonucleases and electrophoresed in 0.6% agarose gels. The blots containing these fragments were hybridized to 3.5×10^6 cpm of ^{32}P -labeled cDNA from polyribosomal RNA (*cDNA-P) or to 1×10^8 cpm of labeled cDNA from nuclear RNA (*cDNA-N). Panel A: BamHI/EcoRI digest of pDK10, BamHI-C. Panel B: BamHI/BglII digest of pDK14, BamHI-V. Panel C: HindIII digest of pDK322, BamHI-X. Panel D: BamHI/HinfI digest of pDK286, BamHI-H. Panel E: BamHI/EcoRI digest of CB28, EcoRI-Dhet-IJhet.

encode abundant mRNA in IB-4 cells.

DISCUSSION

The objective of these experiments was to define the viral DNA which encodes RNA in cells whose ability to grow in culture is unequivocally dependent on EBV infection. EBV is rarely, if ever, vertically transmitted (18). For these experiments, therefore, normal neonate lymphocytes, which could not establish long-term growth in culture, were infected with EBV. A continuous lymphoblastoid cell line established from a normal neonate lymphocyte culture infected with a dilution of the B95-8 isolate of EBV at which only one of four replica cultures were growth transformed was selected for these studies. Infection with a dilute suspension of virus makes it likely that the culture resulted from infection of a single cell. The resultant continuous lymphoblastoid cell line had a doubling time of approximately 24 h and has been passaged for over a year to obtain sufficient quantities of cells for the analyses described in this report. The viral infection remained stably stringent, with the cells expressing EBNA but not EA or VCA. EA and VCA cannot be induced in these cells with iododeoxyuridine.

From the similarity in size of the EcoRI fragments of the viral DNA in IB-4 cells to the fragments of EBV B95-8 DNA, IB-4 cells appear

to contain the entire EBV (B95-8) genome. The intracellular DNA differs, however, from EBV (B95-8) DNA in at least two respects. First, the ends of EBV (B95-8) DNA in IB-4 cells are joined in a single fragment which does not vary in the number of copies of the terminal reiteration. Second, the EcoRI-I and -J fragments are absent from the digest of the intracellular DNA and a new fragment is present which is equal in size to that of the I and J fragments together. This fragment contains the sequences of EcoRI-J, and since I and J are adjacent in EBV (B95-8) DNA it is likely that there has been an alteration in the EcoRI site in the DNA of the virus which infected IB-4 cells or an alteration in the site after infection.

Two lines of evidence suggest that there is selective processing of viral RNA in IB-4 cells as in restringently infected cell lines such as Raji (10, 22, 25; King and Kieff, in preparation). First, with IB-4 RNAs, as with Raji (10, 22), hybridization to labeled viral DNA, in solution, indicates that viral RNA encoded by at least 30% of EBV DNA accumulates in detectable quantity within the nucleus, whereas RNA encoded by 20% and 10% of EBV DNA selectively accumulates in the polyadenylated and polyribosomal RNAs, respectively. Second, mapping data suggest that the additional complexity of nuclear RNA is encoded, in part, by more of the DNA

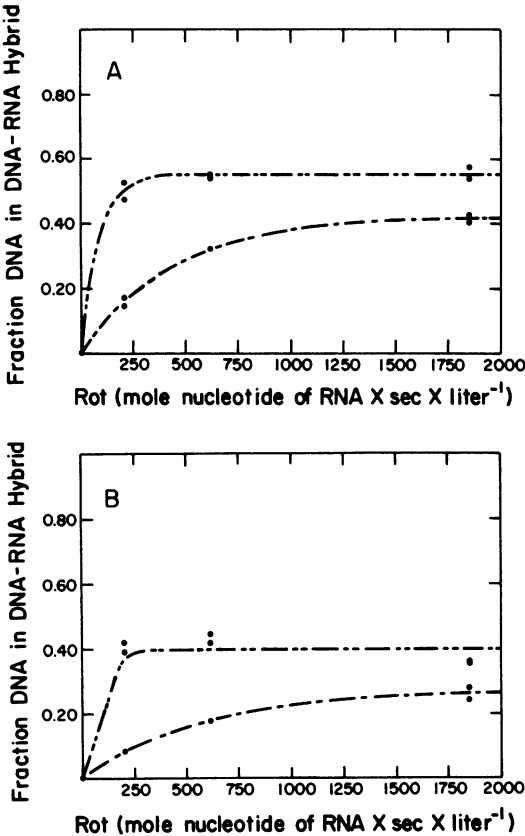


FIG. 7. Hybridization of ³²P-labeled BamHI-V (panel A) or BamHI-H (panel B) DNA to nuclear (●) or polyribosomal (○) RNAs.

from regions such as *Bam*HI-H and -V (Fig. 7) which encode both nuclear and polyribosomal RNAs and in part by other regions of EBV DNA which encode only nuclear RNAs. Comparison of the mapping of labeled DNA selected for homology to nuclear RNA with labeled DNA selected for homology to polyribosomal RNA indicated that nuclear RNA is encoded not only primarily by the *Eco*RI-A and the *Hind*III-A and -B fragments and to a lesser extent by the *Eco*RI-B, -C, and -Dhet and the *Hind*III-D, -E, -I, and -FGHhet fragments, but also by the *Eco*RI-F and -G and the *Hind*III-C, -F, -G, and -H fragments; labeled DNA homologous to polyribosomal RNA hybridized to the same extent to the former fragments, but hybridization to the latter fragments was not detected (Fig. 4). In both sets of experiments and particularly in those with nuclear RNA, residual labeled single-strand viral DNA should not have been detected since the level of contamination of labeled DNA selected by nuclear RNA was less than 0.4%; the level of contamination of labeled DNA selected by polyribosomal RNA was potentially greater but still less than 2%. Comparative assessment of the extent of hybridization of cDNA made from nuclear and polyribosomal RNAs by reverse transcriptase to these other regions of the viral DNA was more difficult since the large number of counts of ³²P-labeled cDNA-P needed to identify fragments encoding polyribosomal polyadenylated RNA resulted in high background scattered throughout the filter (Fig. 5).

Both sets of cDNA experiments indicate that

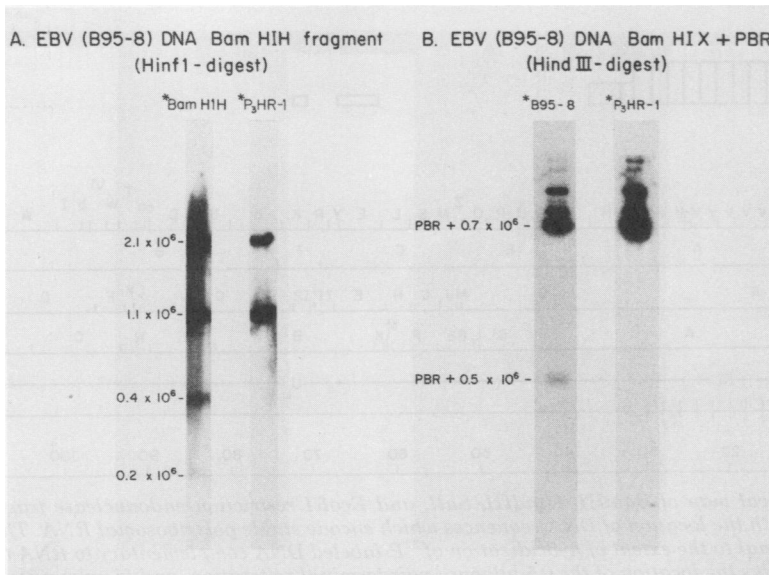


FIG. 8. Radiofluorograms of blots of BamHI/HinfI digest of (panel A) pDK286 (*Bam*HI-H) and (panel B) *Hind*III digest of pDF322 (*Bam*HI-X) to ³²P-labeled EBV B95-8 or P3HR-1 DNAs.

viral nuclear and polyribosomal RNAs in IB-4 cells are encoded largely by DNA in the *Bam*HI-C, -V, -H, -X, -E, -K, and -Nhet or -Jhet (or both) fragments. The *Bam*HI-C, -V, and -H fragments which encode polyribosomal RNA are contiguous in EBV DNA and stretch from 0.05×10^6 to 0.36×10^6 daltons (Fig. 9). The *Eco*RI-J fragment which maps from 0.05×10^6 to 0.07×10^6 daltons in *Bam*HI-C encodes less polyribosomal and nuclear RNA than the *Eco*RI-A fragment of *Bam*HI-C between 0.07×10^6 and 0.11×10^6 daltons, which contains the beginning of the internal reiteration. Whether the unique sequences or only the internal reiteration components of *Bam*HI-C encode IB-4 mRNA is unknown. Nuclear RNA from IB-4 cells is homologous to at least 57% of *Bam*HI-V and polyribosomal RNA is homologous to almost 50%. The hybridization of IB-4 nuclear RNAs to more than 50% of the internal reiteration probably indicates that there is some symmetric transcription of RNA in IB-4 cells. The internal reiteration contains sequences which have homology to each other (9). However, the extent of homology between components of the internal reiteration alone appears to be inadequate to account for the extent of hybridization beyond 50% (A. Cheung and E. Kieff, in preparation). *Bam*HI-X lies immediately to the right of *Bam*HI-V and contains at least 330 and less than 530 base pairs of the internal reiteration (5; Cheung and Kieff, in preparation). The compo-

nent of *Bam*HI-X which lies to the right of the *Hind*III cut site and has no homology to *Bam*HI-V also encodes messenger RNA in IB-4 cells. *Bam*HI-H maps to the right of *Bam*HI-X (5). The 0.4×10^6 dalton *Hinf*I fragment of *Bam*HI-H is the leftward terminus of *Bam*HI-H and the 2.1×10^6 and 1.1×10^6 dalton fragments are the principal internal fragments going from left to right (N. Raab-Traub, T. Dambaugh, and E. Kieff, submitted for publication). The extent of hybridization of polyribosomal cDNA to the 0.4×10^6 dalton *Hinf*I fragment of *Bam*HI-H indicates that this fragment encodes an abundant mRNA in IB-4 cells. Relative to the extent of hybridization to the 0.4×10^6 dalton *Hinf*I fragment, the hybridization of the labeled cDNA in decreasing amounts to the increasingly larger 1.1×10^6 and 2.1×10^6 dalton fragments suggests that the 1.1×10^6 and 2.1×10^6 dalton fragments encode progressively less abundant mRNA or progressively shorter and, necessarily, discontinuous regions of viral mRNA. Further, kinetic hybridization data of polyribosomal RNA to *Bam*HI-H indicate that only about 25% of *Bam*HI-H encodes polyribosomal RNA.

The data indicate that at least 50% of *Bam*HI-V, a substantial part of the unique component of *Bam*HI-X, and at least 25% of *Bam*HI-H encode polyribosomal RNA in IB-4 cells. These DNA sequences add up to at least 6×10^3 base pairs and are sufficient to encode polypeptides with a total molecular weight of 2×10^5 . This is

IB-4 Polyribosomal RNA

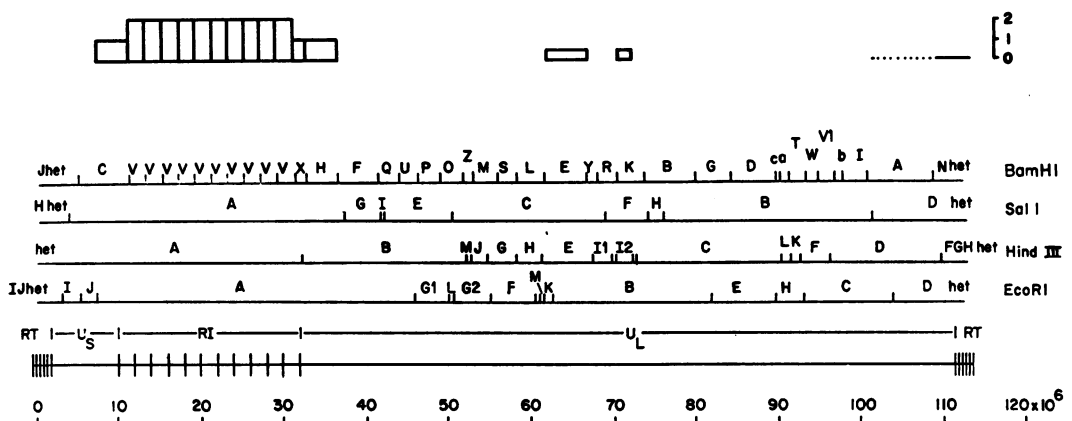


FIG. 9. Physical map of *Bam*HI, *Hind*III, *Sal*I, and *Eco*RI restriction endonuclease fragments of EBV (B95-8) DNA with the location of DNA sequences which encode stable polyribosomal RNA. The height of the bar is proportional to the extent of hybridization of ³²P-labeled DNA complementary to RNA to blots of viral DNA. Tr indicates the location of the 0.5-kilobase-pair terminal reiteration, and Ir represents the location of the 3-kilobase-pair internal reiteration.

a minimal estimate of the complexity of mRNA in IB-4 cells and assumes that the hybridization of IB-4 mRNA to *Bam*HI-C and the remainder of *Bam*HI-X is due to sequences in common with *Bam*HI-V and that the hybridization observed to other fragments is due to small DNA sequences which do not encode long stretches of mRNA. These other fragments such as *Bam*HI-E, -K, and -Nhet or -Jhet may, for example, encode leader sequences. Alternatively, *Bam*HI-E, -K, and -Nhet or -Jhet may encode significant portions of less abundant mRNA's.

The similarity between these results and those previously reported for Namalwa and Raji RNA (10, 22, 25; King and Kieff, in preparation) is striking. Although the possibility persists that these RNAs are a consequence of incidental similarity in the promoters for these regions of EBV DNA to cellular promoters, the concordance between these results with IB-4, a cell line whose growth in vitro is at least originally dependent on virus infection, and those obtained previously with Burkitt tumor cell lines suggests that these RNAs play a role in mediating growth transformation by EBV. Additional evidence in support of the importance of these viral RNAs in transformation comes from the previous finding, which is extended in these experiments, that the P3HR-1 isolate of EBV, which is alone among isolates and laboratory strains of EBV in its inability to induce growth transformation of normal human lymphocytes (17, 19), lacks the *Hind*III cut site in *Bam*HI-X (14), the sequences to the right of the *Hind*III cut site in *Bam*HI-X, and the 0.4×10^6 dalton *Hin*I fragment of *Bam*HI-H, which encode abundant mRNA in IB-4 cells. Further evidence for the role of these DNA sequences in cellular growth transformation should come from prospective studies of the ability of these fragments to transform cells in vitro. The studies will be facilitated by the availability of these fragments in large amounts from recombinant DNA grown in *Escherichia coli*.

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