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Epstein-Barr Virus-Specific RNA

III. Mapping of DNA Encoding Viral RNA in Restringent Infection

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Namalwa and Raji cells, originally obtained from a Burkitt tumor biopsy, grow as continuous cell lines in vitro and contain the Epstein-Barr virus (EBV)-related nuclear antigen EBNA (B. M. Reedman and G. Klein, Int. J. Cancer **11**:499-520, 1973) and RNA homologous to at least 17 and 30% of the EBV genome, respectively (S. D. Hayward and E. Kieff, J. Virol. **18**:518-525, 1976; T. Orellana and E. Kieff, J. Virol. **22**:321-330, 1977). The polyribosomal and polyadenylated [poly(A)+] RNA fractions of Namalwa and Raji cells are enriched for a class of viral RNA homologous to 5 to 7% of EBV DNA (Hayward and Kieff, J. Virol. **18**: 518-525, 1976; Orellana and Kieff, J. Virol. **22**:321-330, 1977). The objective of the experiments described in this communication was to determine the location within the map of the EBV genome (D. Given and E. Kieff, J. Virol. **28**:524-542, 1978) of the DNA which encodes the viral RNA in the poly(A)+ and non-polyadenylated [poly(A)−] RNA fractions of Namalwa cells. Hybridization of labeled DNA homologous to Namalwa poly(A)+ or poly(A)− RNA to blots containing *Eco*RI, *Hsu* I, or *Hsu* I/*Eco*RI double-cut fragments of EBV (B95-8) or (W91) DNA indicated that these RNAs are encoded by DNA contained primarily in the *Hsu* I A/*Eco*RI A and *Hsu* I B/*Eco*RI A fragments and, to a lesser extent, in other fragments of the EBV genome. Hybridizations of Namalwa poly(A)+ and poly(A)− RNA in solution to denatured labeled *Eco*RI A or B fragments, *Hsu* I A, B, or D fragments, and *Hsu* I A/*Eco*RI A or *Bam* I S fragments and of Raji polyribosomal poly(A)+ RNA to the *Eco*RI A fragment indicated that (i) Namalwa poly(A)+ RNA is encoded primarily by 6×10^5 daltons of a 2×10^6-dalton segment of DNA, *Bam* I S, which is tandemly reiterated, approximately 10 times, in the *Hsu* I A/*Eco*RI A fragment and is encoded to a lesser extent by DNA in the *Hsu* I B, *Eco*RI B, and *Hsu* I D fragments. Raji polyribosomal poly(A)+ RNA is encoded by a similar fraction of the *Eco*RI A fragment as that which encodes Namalwa poly(A)+ RNA. (ii) The fraction of the *Bam* I S fragment homologous to Namalwa poly(A)− RNA is similar to the fraction homologous to Namalwa poly(A)+ RNA. However, Namalwa poly(A)− RNA is homologous to a larger fraction of the DNA in the *Hsu* I B, *Hsu* I D, and *Eco*RI B fragments.

Lymphocytes infected with Epstein-Barr virus (EBV) in vivo or in vitro can be grown indefinitely as continuous cell lines in vitro (11, 14, 19, 35, 40-42). Many EBV-infected continuous lymphoblastoid cell lines are nonpermissive for virus replication (12, 21-24, 31, 32, 35). These cells contain a new intranuclear antigen, EBNA (26, 46, 47), probably encoded by the viral genome, but do not contain other antigens associated with abortive or productive virus infection. This state of partial but restricted expression of EBV has been termed "restringent infection" (39, 48). Many restringently infected cell lines, including the Raji cell line, contain multiple copies (55, 56) of more than 90% of the EBV genome (20, 37, 44) in a circular form (1, 2, 25) not linked to cell DNA (3, 36). Some viral DNA may be covalently linked to cell DNA (3, 4). Kinetic hybridization experiments suggest that one cell line, Namalwa, may contain one or two copies of less than the complete viral genome (44). The Raji and Namalwa cell lines were established by growing Burkitt tumor biopsy cells in vitro. Raji and Namalwa cells contain RNA encoded by at least 30 and 17%, respectively, of the DNA of the HR-1 strain of EBV (17, 39). RNA complementary to only 5% of EBV (HR-1) DNA is found on the polyribo-

somes (17, 39) and in the polyadenylated [poly(A)+] RNA fraction (39) of Raji and Namalwa cells. Summation hybridization experiments with poly(A)+ and polyribosomal RNAs from Raji and Namalwa cells suggest that these RNAs are largely encoded by the same DNA sequences (39). That the RNAs are encoded by the same DNA sequences suggests that these RNAs may mediate viral functions essential to the maintenance of restringent infection or to EBV-induced enhancement of cell growth. The objective of this report is to identify and map the DNA sequences which encode these RNAs.

Current knowledge of the structure and sequence arrangement of EBV DNA can be summarized (E. Kieff, D. Given, A. Powell, W. King, T. Dambaugh, and N. Raab-Traub, Biochim. Biophys. Acta, Rev. Cancer, in press) as follows. (i) Because of the relatively large amount of EBV produced by B95-8 cultures, an infectious mononucleosis isolate which has been passaged in marmoset lymphocytes (34), the DNA of this isolate is the best characterized. The DNA is 105×10^6 daltons (43) and has a buoyant density in cesium chloride of 1.718 g/ml (43), suggesting an average guanine-plus-cytosine content of 57 to 58 mol%. The order of the *Hsu* I, *Eco*RI, and *Sal* I restriction endonuclease fragments (18) within the DNA has been determined (15; E. Kieff, N. Raab-Traub, D. Given, W. King, A. T. Powell, R. Pritchett, and T. Dambaugh, *in* F. Rapp and G. de Thé, ed., *Oncogenesis and Herpesviruses III,* in press; Fig. 9). Ninety-five to 100×10^6 daltons of DNA is invariant in sequence arrangement (15). This DNA has homologous termini which consist of between 1 and 10 copies of a 3×10^5-dalton sequence (15). Digestion of EBV (B95-8) DNA with *Bam* I yields an overabundant 2×10^6-dalton fragment (18), *Bam* I S, approximately 10 copies of which are tandemly reiterated in the DNA common to the *Hsu* I A and *Eco*RI A fragments (15, 49; D. Given and E. Kieff, manuscript in preparation). Although EBV (B95-8) is similar to other isolates of EBV in its ability to induce EBNA and to transform lymphocytes into lymphoblasts capable of continuous growth in culture (30, 33), it is known to be missing DNA contained in two Burkitt tumor isolates of EBV, W91 (45) and Jijoye/HR-1 (43, 45). (ii) The W91 Burkitt tumor isolate has been passaged in marmoset cells (31). The size and arrangement of the *Hsu* I, *Eco*RI, and *Sal* I restriction endonuclease fragments of this DNA have also been determined (15; Fig. 9). The DNA is similar in structure and sequence arrangement to EBV (B95-8) DNA but contains an additional 7×10^6 to 8×10^6 daltons of DNA inserted at a single site approximately 15×10^6 daltons from one end of the DNA (15, 45). This additional DNA is viral and not cellular, since it is present in a second Burkitt tumor isolate, Jijoye/HR-1 (15, 45). (iii) Jijoye cells are largely nonpermissive of EBV replication, and much less is known about the arrangement of sequences in the Jijoye isolate. The fragments produced by cleavage of EBV (Jijoye) or (W91) DNAs with *Eco*RI restriction endonuclease are similar in molecular weight (15, 18). EBV (Jijoye) DNA is therefore similar to EBV (W91) DNA. The DNA of the more permissive HR-1 clone of Jijoye differs from EBV (Jijoye) DNA (18) and is known to have a deletion (45). Furthermore, the arrangement of restriction endonuclease fragments in EBV (HR-1) DNA is not fully known.

Because of the relatively larger amount of virus produced by cultures of B95-8 cells, the similarity in the biological properties of this virus to other isolates of EBV, and the similarity in the sequence arrangement of EBV (B95-8) and (W91) DNAs, the location of DNA sequences homologous to viral RNA in Namalwa cells was first determined with EBV (B95-8) DNA. However, since the Namalwa cell line was derived from a Burkitt tumor, insofar as was feasible the results were compared to those obtained with DNA from the Burkitt tumor isolate of EBV, W91.

MATERIALS AND METHODS

Cell culture and virus purification. Cultures of Namalwa and Raji cells (obtained from G. Klein, Karolinska Institute, Stockholm, Sweden) were maintained at 35°C at a concentration of 1×10^5 to 5×10^5 viable cells per ml and fed every third day with 2 volumes of a medium consisting of RPMI 1640 supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). B95-8 and W91 cultures (obtained from G. Miller, Yale University, New Haven, Conn.) were maintained at 35°C and fed with one-third volume of medium every third day. Cells were maintained for 3-month cycles in media containing tylocine (60 μg/ml; Grand Island Biological Co.) and spectinomycin (200 μg/ml; Upjohn Co., Kalamazoo, Mich.) or in medium without antibiotics.

The procedures used to purify virus from B95-8 and W91 culture supernatants have been described in detail previously (8). Briefly, culture supernatants were centrifuged at 15,000 × *g* for 90 min to obtain a crude extracellular fluid pellet. The pellet containing virus was homogenized in 5×10^{-3} M sodium phosphate buffer, pH 7.4, clarified by centrifugation at 4,000 × *g* for 15 min, and sedimented through a 5 to 30% (wt/vol) gradient of dextran T10 (Pharmacia Corp., Uppsala, Sweden) for 1 h at 76,000 × *g*. The single light-scattering band which forms midway in the gradient was aspirated, diluted in 5×10^3 M phosphate buffer (pH 7.4), and concentrated to a pellet by centrifugation at $1.2 \times 10^5 \times g$ for 90 min.

Preparation of RNA. The supernatant medium

was decanted from 30 liters of Namalwa cell cultures. The cells were further concentrated by centrifugation for 5 min at 100 × *g* and 20°C. The loose cell pellet was resuspended at 4°C in 50 ml of a solution consisting of 10^{-3} M EDTA and 0.01 M Tris-hydrochloride, pH 7.5. An equal volume of 8 M guanidine hydrochloride (Bethesda Research Laboratories, Bethesda, Md.) was added at 4°C (6). The mixture was vigorously agitated. Three hundred micrograms of proteinase K (EM Laboratories, Inc., Elmsford, N.Y., obtained through Scientific Products, McGaw Park, Ill.) per ml and 1/20 volume of 20% (wt/vol) sodium dodecyl sulfate (SDS; British Drug House Laboratories, Poole, England) were then added, and the solution was rapidly brought to 65°C with vigorous shaking. One-tenth volume of 20% Sarkosy NL97 (ICN Pharmaceuticals, Inc., Plainview, N.Y.) and 0.5 g of CsCl (specially pure grade, EM Laboratories) per ml (50) was then added, and the solution was homogenized with three strokes of tight-fitting Dounce apparatus at 65°C. Thirty milliliters of the solution was layered over a 7-ml cushion of CsCl at a density of 1.735 g/ml in 0.01 M EDTA and 0.01 M Tris-hydrochloride, pH 7.4. The lysate was then centrifuged for 8 h at 44,000 × *g* and 20°C in six 37-ml nitrocellulose tubes in a 50.2 rotor (Beckman Corp., Palo Alto, Calif.). The lipid and proteinaceous debris at the top of the tube was removed by aspiration. The remainder of the viscous, translucent, upper phase was aspirated down to the 7-ml cushion. The cushion of dense CsCl was then aspirated, leaving the RNA in a hard pellet. The RNA pellets were resuspended in 100 ml of a solution consisting of 5×10^{-3} M EDTA, 0.2% (wt/vol) SDS, and 0.01 M Tris-hydrochloride, pH 7.5. The yield from 30 liters of cells was 4,000 to 5,000 absorbancy units at 260 nm. The ratio of absorbancy at 260 nm to that at 280 nm of the RNA solution was 1.8 to 2.0, and the DNA content was less than 1%. Polyribosomal RNA was prepared as described previously (33).

Poly(A)+ RNA which contains at least 35 adenylic acid residues (9, 16) was separated from the remainder of the RNA by chromatography on columns of oligodeoxythymidylate-cellulose (T3, Collaborative Research, Waltham, Mass.) as previously described (5, 7, 39). Approximately 96% of the starting RNA did not bind to the column and was the poly(A)− RNA fraction. The RNA which was retained on the column was eluted with a buffer consisting of 0.05% (wt/vol) SDS, 0.005 M EDTA, and 0.01 M Tris-hydrochloride, pH 7.4. This RNA was heated at 60°C for 2 min, cooled quickly in ice, adjusted to 0.4 M NaCl, and reapplied to an oligodeoxythymidylate-cellulose column. The column was washed, and the poly(A)+ RNA (approximately 2% of the starting RNA) was eluted as described before (39). The poly(A)+ and poly(A)− RNAs were adjusted to 0.1 M NaCl, extracted twice with phenol and chloroform-isoamyl alcohol, and precipitated overnight with 2 volumes of ethanol. The RNAs were dissolved at a concentration of 2 mg/ml in a solution consisting of 0.01 M NaCl, 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, and 0.01 M Tris-hydrochloride, pH 7.4. Iodoacetate-treated DNase (DNase I, RNase-free; Worthington Biochemicals Corp., Freehold, N.J.) was added to a concentration of 50 μg/ml, and the solution was incubated for 20 min at 37°C. The digestion was terminated by the addition of SDS to 0.5% (wt/vol). The solution was extracted twice with phenol and chloroform-isoamyl alcohol, and the RNA was reprecipitated at −20°C, overnight, after the addition of sodium chloride to 0.1 M and 2 volumes of ethanol.

Preparation of EBV and *Klebsiella pneumoniae* DNAs. The procedures used in extracting DNA from purified EBV have been described previously (43). *K. pneumoniae* DNA was used as a control labeled DNA of similar guanine-plus-cytosine content (3). *K. pneumoniae* (ATCC 13883) was grown in Trypticase soy broth and inoculated into 1 liter of Müller-Hinton broth (Baltimore Biological Laboratory, Cockeysville, Md.). After 4 h of incubation at 37°C, 1 mCi of [^{3}H]thymidine (50 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to the culture, which contained 10^6 bacteria per ml. Incubation was continued for 4 h, at which time the culture contained approximately 10^8 bacteria per ml. The cells were separated from the media by centrifugation for 10 min at 10,000 × *g* and were resuspended in 100 ml of a solution consisting of 0.002 M EDTA and 0.020 M Tris-hydrochloride, pH 7.5. SDS, 0.5 g/ml, and proteinase K, 500 μg/ml, were added, and the lysate was rolled for 4 h at 37°C. Sarkosyl, 1 g/ml, and CsCl (EM Laboratories, Inc.), to a final density of 1.720 g/ml, were added, and the solution was centrifuged for 2 days at 20°C and 150,000 × *g* in a 50.2 rotor (Beckman Corp.). Fractions from the gradient between 1.710 and 1.720 g/ml were combined, dialyzed against 0.1 M NaCl-0.01 M EDTA-0.05 M Tris-hydrochloride (pH 7.4), sonically treated for 1 min (Artek 300 Sonifier, Artek Corp., Farmingdale, N.Y.), extracted with phenol and chloroform-isoamyl alcohol, and precipitated after the addition of 2 volumes of ethanol. The specific activity of the DNA was 10^4 cpm/μg. The size of denatured ^{3}H-labeled *Klebsiella* DNA was determined to be 400 to 800 bases by sedimentation in alkaline sucrose gradients (43).

Restriction enzymes and separation of fragments of EBV DNA. The procedures used in treating EBV DNA with *Eco*RI, *Hsu* I, or *Bam* I restriction endonucleases and in separating the fragments of EBV DNA in 0.4% agarose gels (15, 18) have been described previously. *Eco*RI and *Bam* I were obtained from Bethesda Research Laboratories. *Hsu* I was prepared as described previously (18). Double-cut fragments (*Hsu* I/*Eco*RI) were produced by incubating 2 μg of EBV DNA with 10 U of *Hsu* I restriction endonuclease in 100 μl of *Hsu* I buffer (15) for 3.5 h at 37°C. The solution was adjusted to 0.1 M sodium chloride and 0.05 M Tris-hydrochloride. Ten units of *Eco*RI restriction endonuclease was added, and the incubation was continued for 2.5 h at 37°C. To prepare fragments for labeling in vitro, the *Hsu* I, *Eco*RI, and *Hsu* I/*Eco*RI fragments were initially separated in cylindrical 0.3% agarose gels (1 by 20 cm) containing 0.5 μg of ethidium bromide per ml. Individual fragments were cut from the gel under UV illumination. The gel slice containing separated fragments was placed on the top of a cylindrical 0.4% agarose gel (1 by 20 cm) containing ethidium bromide, sealed in place with 0.35% agarose, and subjected to electrophoresis a second time to further purify the fragments. The single band which appeared in the second gel was cut from the gel in a 1- to 2-mm

slice under UV illumination. The *Bam* I S fragment (49) was similarly twice separated by electrophoresis in 0.8% agarose gels.

Preparation of [α-^{32}P]dCTP-labeled EBV DNAs (^{32}P-labeled EBV DNA). The procedures used in extracting fragments of EBV DNA from agarose gels and in labeling EBV DNA and fragments of EBV DNA in vitro using *Escherichia coli* DNA polymerase I (Boehringer-Mannheim Corp., Indianapolis, Ind.) and [α-^{32}P]dCTP (300 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) have been described (15). The specific activity of the ^{32}P-labeled EBV DNA was 1×10^8 to 2×10^8 cpm/μg. The specific activity of the ^{32}P-labeled fragments of EBV DNA was 0.5×10^8 cpm/μg. The size of the DNA strands after labeling in vitro was 4×10^2 to 6×10^2 nucleotides. The extent of reassociation of ^{32}P-labeled EBV DNA in the presence of excess unlabeled EBV DNA varied from 0.75 to 0.85, and for ^{32}P-labeled fragments of EBV DNA it varied from 0.65 to 0.75. Each ^{32}P-labeled EBV DNA restriction endonuclease fragment was shown to be free of sequences contained in other fragments by hybridization of the labeled fragment to blots made from agarose gels containing all of the fragments produced by cleavage of EBV DNA with the same enzyme.

Identification of restriction endonuclease fragments of EBV DNA homologous to viral RNA in Namalwa cells. The strategy used to identify the fragments of EBV DNA which encode RNA in Namalwa cells is outlined in Fig. 1. Briefly, 2×10^7 cpm of ^{32}P-labeled EBV DNA was mixed with 5 mg of poly(A)− or 0.5 mg of poly(A)+ RNA in 0.5 ml of a solution consisting of 0.3 M NaCl, 0.01 M EDTA, 5% (vol/vol) formamide, and 0.02 M Tris-hydrochloride, pH 7.4. The mixture was incubated at 108°C for 5 min and at 68°C for 18 h, dialyzed against 0.005 M Tris-hydrochloride (pH 7.4) for 3 h at 4°C, and diluted to 8 ml in S1 buffer, which consists of 25 μg of denatured calf thymus DNA per ml, 50 μg of native calf thymus DNA (Worthington Biochemicals Corp.) per ml, 0.1 M NaCl, 10^{-3} M $ZnSO_4$, and 0.025 M potassium acetate, pH 4.5. The mixture was then incubated at 42°C with twice the concentration of S1 nuclease required to digest 95% of denatured ^{32}P-labeled EBV DNA (39). Denatured [^{3}H]thymidine-labeled *K. pneumoniae* DNA was added so that the digestion of single-strand DNA could be followed. By 30 min of incubation 80 to 90% of the ^{3}H-labeled *K. pneumoniae* DNA was acid soluble. After 45 min of incubation an equal amount of S1 nuclease was added, and the incubation was continued for an additional 45 min. More than 95% of

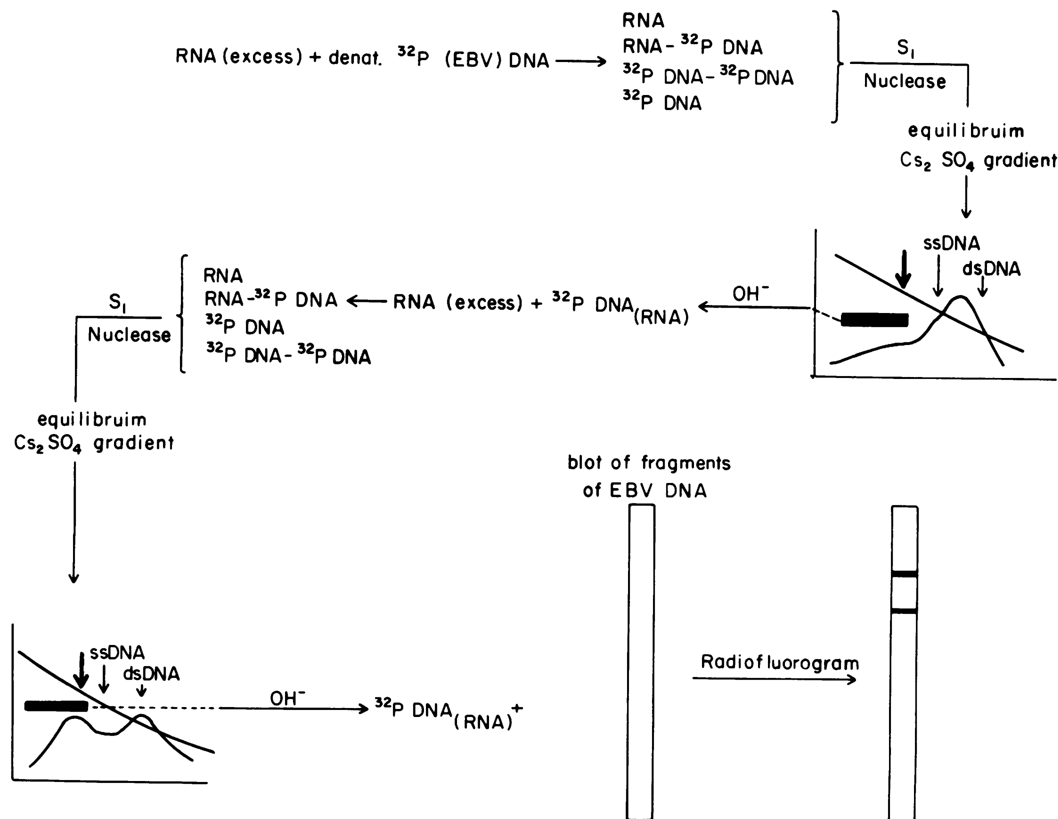

FIG. 1. *Strategy used to identify fragments of EBV DNA which encode RNA of Namalwa cells. The heavy arrow indicates the fraction containing cesium sulfate at a density of 1.49 g/ml, the density of a calf thymus DNA-RNA synthetic hybrid (52).*

the ^{3}H-labeled *Klebsiella* DNA was acid soluble at the end of the second 45-min incubation period. One-twentieth volume of 20% (wt/vol) SDS was added to the mixture, and the pH was adjusted to 7.4 with 1 M Tris-hydrochloride, pH 8.5. The nucleic acid mixture was extracted twice with phenol and chloroform-isoamyl alcohol and stored overnight at −20°C after the addition of 2 volumes of ethanol. The nucleic acid precipitate was collected by centrifugation for 20 min at 8,000 × *g* and 4°C and was resuspended in 1 ml of 0.1% Sarkosyl, 0.01 M EDTA, and 0.02 M Tris-hydrochloride, pH 7.4. Two micrograms of native and 2 μg of denatured ^{3}H-labeled *K. pneumoniae* DNA were added as markers for DNA similar in density to native and denatured EBV DNA (3). The solution was adjusted to a final density of 1.5 g/ml with cesium sulfate (EM Laboratories). The solution was then centrifuged for 36 to 48 h at $1.4 \times 10^{5} \times g$ and 20°C in a type 65 rotor (Beckman Corp.) (51). The gradient was collected in 40 fractions of equal volume after bottom puncture of the tube. The refractive index, ^{32}P, and tritium content of each fraction were determined. The fractions with density between 1.50 and 1.62 g/ml which contain putative DNA-RNA hybrid (52) were combined with 50 μg of native calf thymus DNA per ml and incubated with 0.3 M NaOH for 4 h at 37°C to partially digest the RNA. The mixture of labeled DNA and oligo ribonucleotides was adjusted to pH 7.4, dialyzed three times against 1 liter of 0.15 M NaCl-0.01 M EDTA-0.05 M Tris-hydrochloride (pH 7.4), and precipitated overnight at −20°C after addition of 2 volumes of ethanol. An aliquot of the labeled DNA preparations which had been hybridized to poly(A)− or poly(A)+ RNA was tested for specificity by hybridization to poly(A)− or poly(A)+ RNA in excess, as described below. Between 50 and 75% of the labeled DNA from the cesium sulfate gradient was driven into hybrid by the poly(A)− or poly(A)+ RNA.

The labeled DNA which had been hybridized to poly(A)− or poly(A)+ RNA was resuspended in 0.4 ml of hybridization buffer containing 4 mg of poly(A)− RNA or 0.4 mg of poly(A)+ RNA, respectively, and incubated at 68°C for 18 h. The hybridization mixture was digested with S1 nuclease and centrifuged in isopycnic cesium sulfate gradients as described above. The labeled DNA was then incubated in 5 ml of a solution consisting of 1 M NaCl, 0.01 M EDTA, and 0.05 M Tris-hydrochloride, pH 7.5, for 20 h at 68°C with one-half of a nitrocellulose filter to which restriction enzyme fragments of 1 to 2 μg of EBV (B95-8) DNA or 0.5 to 1 μg of EBV (W91) DNA had been transferred from an agarose gel, as previously described (15, 51). The filters were washed for 4 h at 55°C in 0.6 M NaCl-0.06 M sodium citrate (pH 7.4), dried, and exposed at −70°C to SB5 film (Eastman Kodak Corp., Rochester, N.Y.), using a Cronex Lightning Plus screen (Dupont Corp., Wilmington, Del.). The nitrocellulose filters were then incubated with ^{32}P-labeled EBV DNA to determine the location and relative amount of each of the DNA fragments present on the filter.

Hybridization of RNA to ^{32}P-labeled EBV DNA and to ^{32}P-labeled fragments of EBV DNA in solution. A 10^{-3}-μg amount of ^{32}P-labeled EBV DNA or fragments of EBV DNA were mixed with 1 mg of poly(A)− RNA, 0.1 mg of poly(A)+ RNA, 1 mg of yeast RNA (Sigma Chemical Co., St. Louis, Mo.), or 5 μg of EBV DNA in 0.1 ml of hybridization buffer, which consisted of 5% (vol/vol) formamide, 0.3 M NaCl, 0.005 M EDTA, and 0.05 M Tris-hydrochloride, pH 7.4. The mixture was sealed in 5-μl micropipettes, heated at 110°C for 5 min, and incubated at 70°C for variable intervals of up to 18 h. The samples were frozen at −20°C and processed simultaneously. The procedures used to determine the extent of self-hybridization of the ^{32}P-labeled EBV DNA and of DNA-RNA hybridization and for determining the configuration of the curves drawn in the figures have been described previously (10, 17, 39). The data plotted in the figures indicate the extent of DNA-RNA hybridization after correction of the raw data for the extent of renaturation of the ^{32}P-labeled EBV DNA. The extent of renaturation of the ^{32}P-labeled EBV DNA was 0.05 at 18 h with ^{32}P-labeled EBV DNA and varied from 0.15 with ^{32}P-labeled EBV (B95-8) DNA *Hsu* I D or *Eco*RI B fragments to 0.3 with ^{32}P-labeled EBV (B95-8) DNA *Hsu* I A, *Eco*RI A, or *Hsu* I A/*Eco*RI A fragments. An aliquot of each RNA preparation was treated with 0.3 N KOH for 18 h, neutralized, and incubated with ^{32}P-labeled EBV DNA in a parallel control to demonstrate that the nucleic acid which hybridized to ^{32}P-labeled EBV DNA was RNA and not DNA (39).

RESULTS

Hybridization of poly(A)+ and poly(A)− RNA from Namalwa cells to ^{32}P-labeled EBV (B95-8) or (W91) DNAs. To determine the fraction of EBV (B95-8) or (W91) DNAs homologous to poly(A)+ and poly(A)− RNA from Namalwa cells, RNAs were prepared and hybridized in excess in solution to in vitro labeled EBV (B95-8) or (W91) DNA. The Namalwa poly(A)− RNA is homologous to at least 18% of EBV (B95-8) DNA (Fig. 2A) and 16% of EBV (W91) DNA (Fig. 2C). From the kinetics of hybridization of the RNA to labeled DNA (10, 17, 39), the RNA is estimated to be 0.5×10^{-3} to 1×10^{-3}% virus specific. The poly(A)+ fraction of Namalwa cells contains RNA which is enriched (0.5×10^{-2} to 1×10^{-2}% virus specific) for RNA homologous to 7% of EBV (B95-8) and (W91) DNA (Fig. 2B and D, respectively).

Identification of EBV (B95-8) or (W91) DNA restriction endonuclease fragments containing sequences homologous to Namalwa poly(A)+ and poly(A)− RNAs. The strategy used to identify the fragments of EBV (B95-8) or (W91) DNAs which contain sequences homologous to Namalwa poly(A)+ or poly(A)− RNA involves two cycles of hybridization of denatured ^{32}P-labeled EBV DNA to excess RNA in solution and isolation of the ^{32}P-labeled EBV DNA-RNA hybrid in neutral cesium sulfate gradients. The scheme is outlined in Fig. 1. After an initial hybridization of ^{32}P-

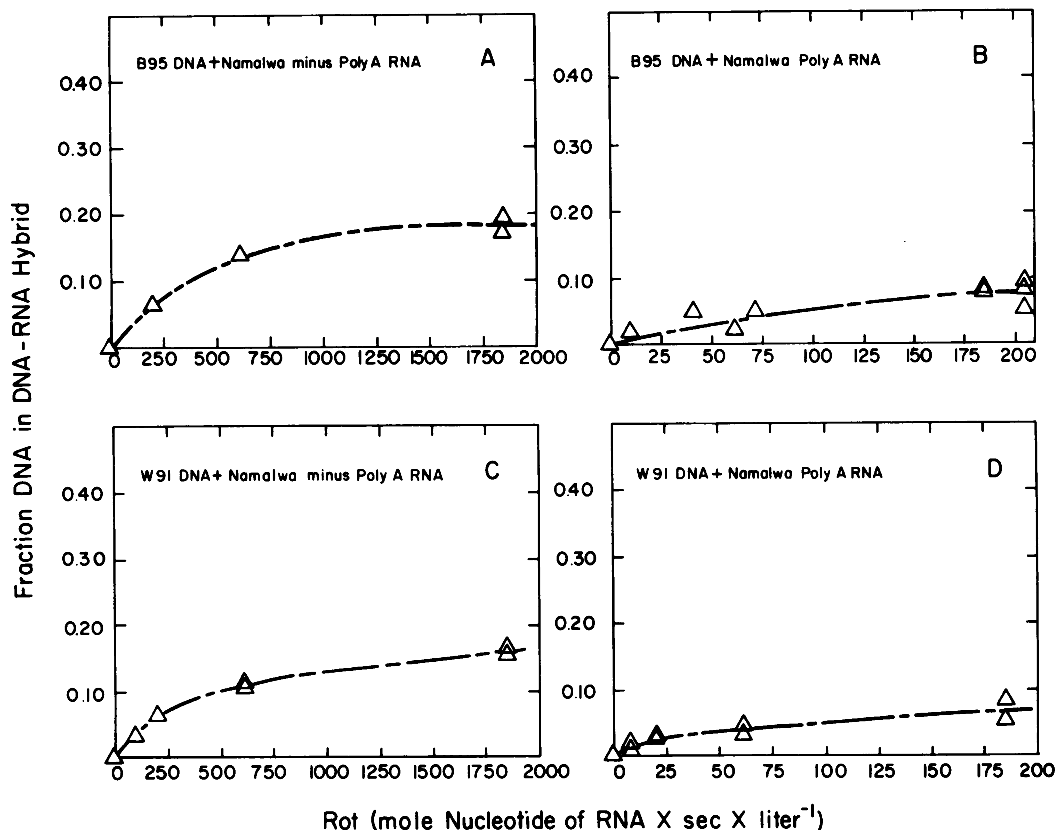

FIG. 2. *Hybridization of the poly(A)− (A and C) and poly(A)+ (B and D) RNA from Namalwa cells with denatured ^{32}P-labeled EBV (B95-8) DNA (A and B) or with denatured ^{32}P-labeled EBV (W91) DNA (C and D).*

labeled EBV DNA with Namalwa RNA, S1 nuclease digestion, and isopycnic banding in neutral cesium sulfate (Fig. 3A), most of the ^{32}P-labeled EBV DNA was in a broad region of the gradient which extended to include fractions that contained native (density, 1.43 g/ml) as well as denatured (density, 1.46 g/ml) ^{3}H-labeled *K. pneumoniae* DNA, indicating that there was extensive reassociation of the ^{32}P-labeled EBV DNA and incomplete digestion of the single-stranded ^{32}P-labeled EBV DNA. As is apparent from a comparison of the amount of ^{32}P-labeled EBV DNA in fractions 20 and 18 with that in fractions 36 and 38, there was some enrichment for ^{32}P-labeled EBV DNA in the region of the gradient where a DNA-RNA hybrid would be expected to band. As indicated in Materials and Methods, at least 50% of the ^{32}P-labeled EBV DNA which banded at a density in excess of that expected for the calf thymus DNA-RNA hybrid (53) was homologous to Namalwa RNA. The putative ^{32}P-labeled EBV DNA-RNA hybrid was treated with alkali and subjected to a second cycle of hybridization with Namalwa RNA in excess, S1 nuclease digestion, and isopycnic centrifugation in cesium sulfate. In the second cesium sulfate gradient most of the ^{32}P-labeled EBV DNA banded either at the density of the DNA-RNA hybrid or at the density between native and denatured ^{3}H-labeled *K. pneumoniae* DNA (Fig. 3B). ^{32}P-labeled EBV DNA which banded at the density of the DNA-RNA hybrid in the second cesium sulfate gradient was treated with alkali, neutralized, and incubated with blots of agarose gels which contained *Hsu* I, *Eco*RI, or *Hsu* I/*Eco*RI double-cut restriction endonuclease fragments of EBV DNA. After radiofluorography to determine which fragments contain DNA homologous to poly(A)− or poly(A)+ RNA, the blots were rehybridized to denatured ^{32}P-labeled EBV DNA to demonstrate that the blots contained all fragments of the viral genome.

The results (Fig. 4) indicate the following: (i) EBV (B95-8) DNA homologous to Namalwa poly(A)+ RNA hybridizes most extensively to the *Hsu* I A, *Eco*RI A, or *Hsu* I A/*Eco*RI A fragments and, to a lesser extent, to *Hsu* I B and

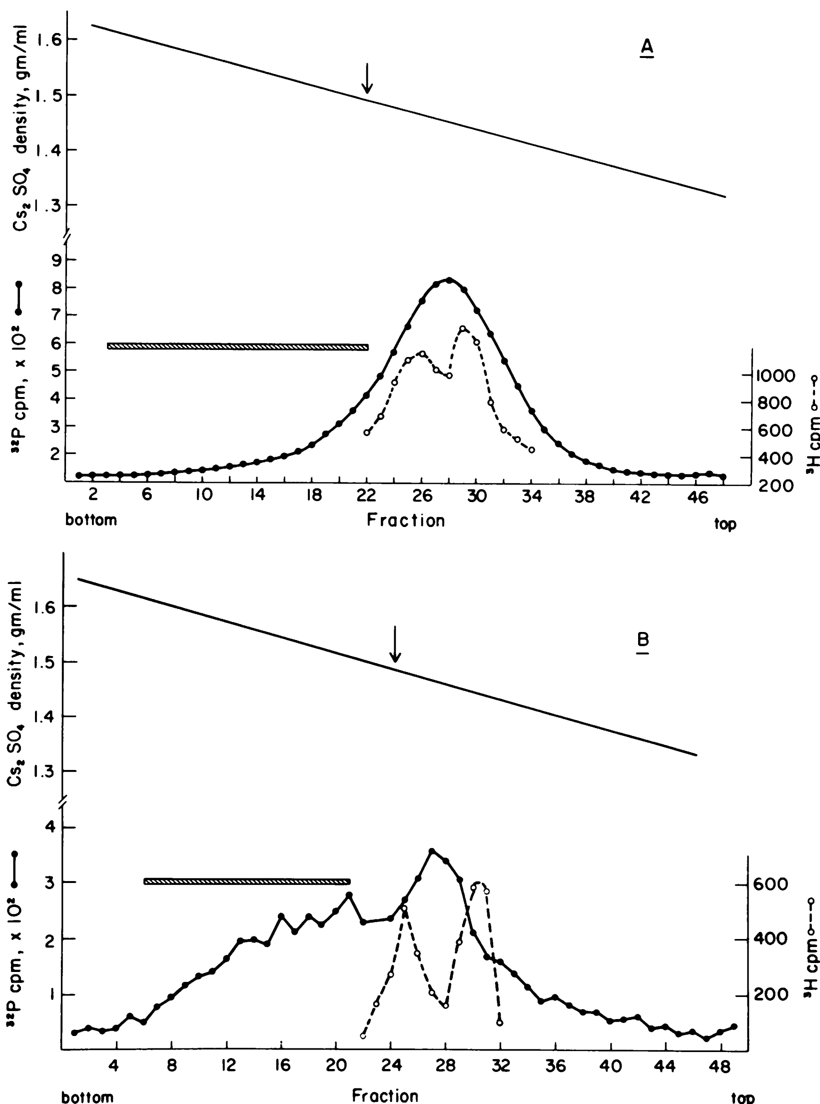

FIG. 3. *Results of first (A) and second (B) cycle of isopycnic centrifugation of denatured ^{32}P-labeled EBV (B95-8) DNA which had been incubated with Namalwa poly(A)+ RNA and treated with S1 nuclease before banding in cesium sulfate. Native and denatured [^3H]thymidine-labeled K. pneumoniae DNA was added before centrifugation. The total amount of ^{32}P in each fraction was determined by Cerenkov counting. Background of 10 cpm was not subtracted from the data plotted in the figure. The hatched bar indicates fractions which were combined for subsequent analyses. The arrow indicates the reported density for a calf thymus synthetic RNA:DNA hybrid (53).*

Hsu I B/*Eco*RI A fragments of EBV (B95-8) DNA. With much longer intervals of radiofluorography, trace hybridization to other fragments of EBV (B95-8) DNA was evident. (ii) Radiofluorograms of blots of *Hsu* I, *Eco*RI, or *Hsu* I/*Eco*RI fragments of EBV (B95-8) DNA hybridized to ^{32}P-labeled EBV (B95-8) DNA, homologous to poly(A)− RNA, were similar to those obtained by hybridization with ^{32}P-labeled EBV DNA homologous to poly(A)+ RNA. ^{32}P-labeled EBV (B95-8) DNA, homologous to poly(A)− RNA, hybridized primarily to the *Hsu* I A, *Eco*RI A, or *Hsu* I A/*Eco*RI A fragments and, to a lesser extent, to the *Hsu* I B or *Hsu* I B/*Eco*RI A fragments. With longer intervals of radiofluorography some hybridization of the ^{32}P-labeled EBV (B95-8) DNA, homologous to Namalwa poly(A)− RNA, to other fragments was apparent.

To determine whether the ^{32}P-labeled EBV

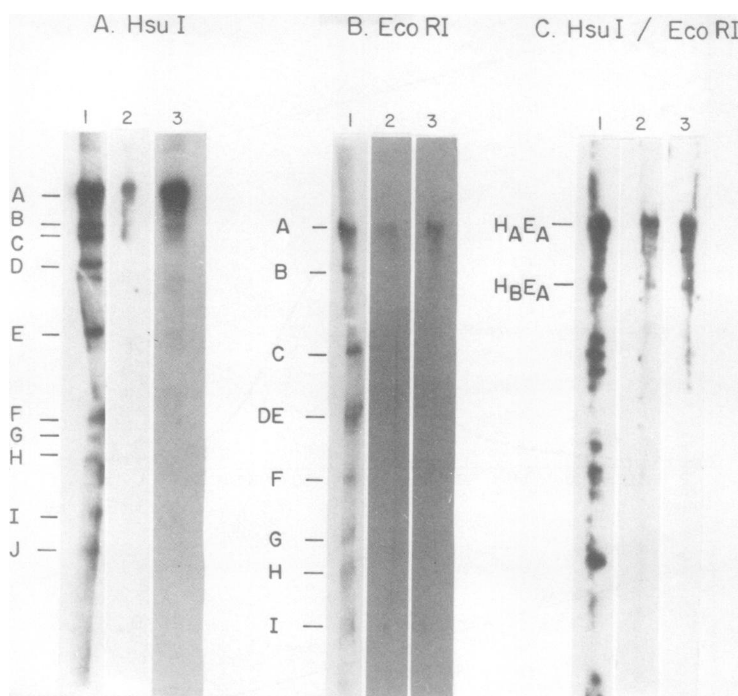

FIG. 4. *Radiofluorogram of halves of blots of agarose gels containing separated Hsu I (A), EcoRI (B), or Hsu I/EcoRI (C) restriction endonuclease-cleaved fragments of EBV (B95-8) DNA which were hybridized to denatured ^{32}P-labeled EBV (B95-8) DNA homologous to Namalwa poly(A)+ RNA (fluorogram labeled 2 in each panel) or Namalwa poly(A)− RNA (fluorogram labeled 3 in each panel) and then rehybridized to ^{32}P-labeled EBV (B95-8) DNA (fluorogram labeled 1 in each panel).*

DNA selected for homology to Namalwa RNA hybridized preferentially to the *Hsu* I A, *Hsu* I B, *Eco*RI A, *Hsu* I A/*Eco*RI A, and *Hsu* I B/*Eco*RI A fragments, blots of agarose gels containing *Hsu* I, *Eco*RI, or *Hsu* I/*Eco*RI fragments were hybridized to an equivalent amount of ^{32}P-labeled EBV DNA. The extent of hybridization of the ^{32}P-labeled EBV DNA to each fragment was consistent with its molecular weight, although the heterogeneous fragments which contain the terminal repetitions and the *Hsu* I A, *Eco*RI A, and *Hsu* I A/*Eco*RI A fragments which contain the internal tandem repetition (15) were slightly over-represented. The extent of hybridization of the ^{32}P-labeled EBV DNA, selected for homology to Namalwa RNA, to the *Hsu* I A, *Hsu* I B, *Eco*RI A, *Hsu* I A/*Eco*RI A, and *Hsu* I B/*Eco*RI A fragments relative to other fragments of EBV DNA was consistently greater than that observed with ^{32}P-labeled EBV DNA which was not selected for homology to Namalwa RNAs. To directly demonstrate that the blots which had been hybridized to the ^{32}P-labeled EBV DNA, selected for homology to Namalwa poly(A)− RNA, contained all of the fragments of EBV DNA and that the extent of hybridization to the *Hsu* I A, *Hsu* I B, *Eco*RI A, *Hsu* I A/*Eco*RI A, and *Hsu* I B/*Eco*RI A fragments was in excess of that obtainable with the same blot and ^{32}P-labeled EBV DNA, these blots were rehybridized with ^{32}P-labeled EBV DNA (Fig. 4).

To determine whether the RNA in Namalwa cells was homologous to the additional DNA present in the W91 isolate and missing in EBV (B95-8) DNA, ^{32}P-labeled EBV (W91) DNA homologous to Namalwa poly(A)+ or poly(A)− RNA was similarly selected by two cycles of hybridization to RNA in excess, S1 digestion, and isopycnic banding in cesium sulfate. The ^{32}P-labeled EBV (W91) DNA which was homologous to Namalwa poly(A)+ or poly(A)− RNA was hybridized to blots of EBV (B95-8) or (W91) DNA (Fig. 5). DNA homologous to poly(A)+ RNA hybridized most extensively to EBV (W91) *Hsu* I A and *Eco*RI A fragments and to EBV (B95-8) *Hsu* I A, *Eco*RI A, and *Hsu* I A/*Eco*RI A fragments. Hybridization was also evident to EBV (W91) *Hsu* I B and *Eco*RI B and to EBV (B95-8) *Hsu* I B and *Eco*RI B fragments and the

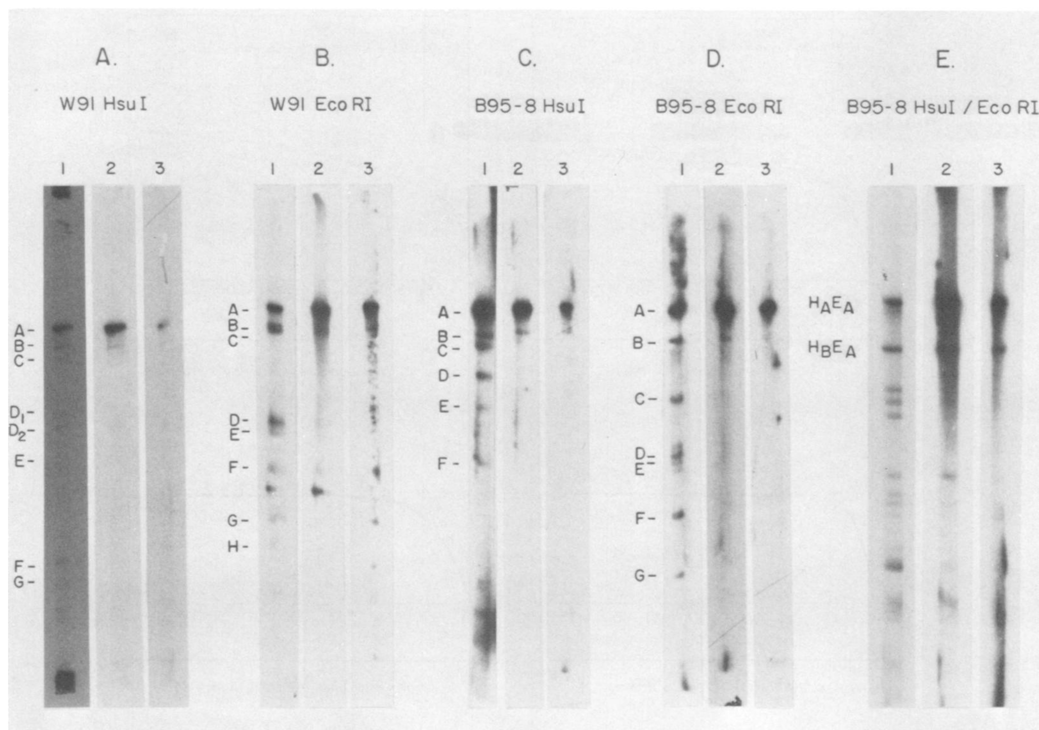

FIG. 5. *Radiofluorograms of halves of blots of agarose gels containing separated Hsu I (A) or EcoRI (B) of restriction endonuclease-cleaved fragments of EBV (W91) DNA or Hsu I (C), EcoRI (D), or Hsu I/EcoRI (E) fragments of EBV (B95-8) DNA which were hybridized to denatured ^{32}P-labeled EBV (W91) DNA homologous to Namalwa poly(A)+ RNA (fluorogram labeled 2 in each panel) or Namalwa poly(A)− RNA (fluorogram labeled 3 in each panel) and then rehybridized to ^{32}P-labeled EBV (W91) DNA (fluorogram labeled 1 in each panel).*

Hsu I B/*Eco*RI A double-cut fragment. With longer exposure of the radiofluorograms, hybridization could be seen to other restriction endonuclease fragments. Hybridization of the blots with ^{32}P-labeled EBV (W91) DNA demonstrated that each of the blots contained the restriction endonuclease fragments.

Determination of the extent of homology between fragments of EBV (B95-8) DNA and Namalwa poly(A)+ or poly(A)− RNA or Raji polysomal poly(A)+ RNA. The extent of homology between Namalwa poly(A)+ or poly(A)− RNA and the *Eco*RI A, *Hsu* I A, and *Eco*RI A/*Hsu* I A and *Bam* I S fragments was determined by hybridizing ^{32}P-labeled EBV (B95-8) DNA fragments to excess RNA in solution as described in Materials and Methods. Namalwa poly(A)+ RNA is homologous to at least 25% of the *Eco*RI A fragment (Fig. 6A), 21% of the *Hsu* I A fragment (Fig. 6C), 23% of the *Hsu* I A/*Eco*RI A fragment (data not shown), and 31% of the *Bam* I S fragment (Fig. 6E). Namalwa poly(A)− RNA is homologous to at least 30% of the *Eco*RI A fragment (Fig. 6B), 21% of the *Hsu* I A fragment (Fig. 6C), and 28% of the *Bam* I S fragment (Fig. 6F). From the rapid rate of hybridization of Namalwa poly(A)+ and poly(A)− RNA to *Eco*RI A, *Hsu* I A, or *Bam* I S DNA, the percentage of RNA homologous to these DNAs was estimated (10, 39) to be approximately 10^{-2} and 10^{-3}%, respectively.

To evaluate the significance of the hybridization observed to EBV (B95-8) *Hsu* I B and *Eco*RI B fragments and the trace hybridization to other fragments, including the *Hsu* I D fragment, these fragments were labeled in vitro and hybridized in solution to Namalwa poly(A)+ and Namalwa poly(A)− RNA.The results indicated that poly(A)+ RNA is homologous to at least 6% of the *Hsu* I B fragment (Fig. 7A), 3% of the *Eco*RI B fragment (Fig. 7C), and 2% of the *Hsu* I D fragment (Fig. 7E). Poly(A)− RNA is homologous to at least 18% of the *Hsu* I B fragment (Fig. 7B), 15% of the *Eco*RI B fragment (Fig. 7D), and 6% of the *Hsu* I D fragment (Fig. 7F).

To confirm previous summation hybridization results (39) indicating that poly(A)+ and polyribosomal RNAs of Raji cells are largely encoded

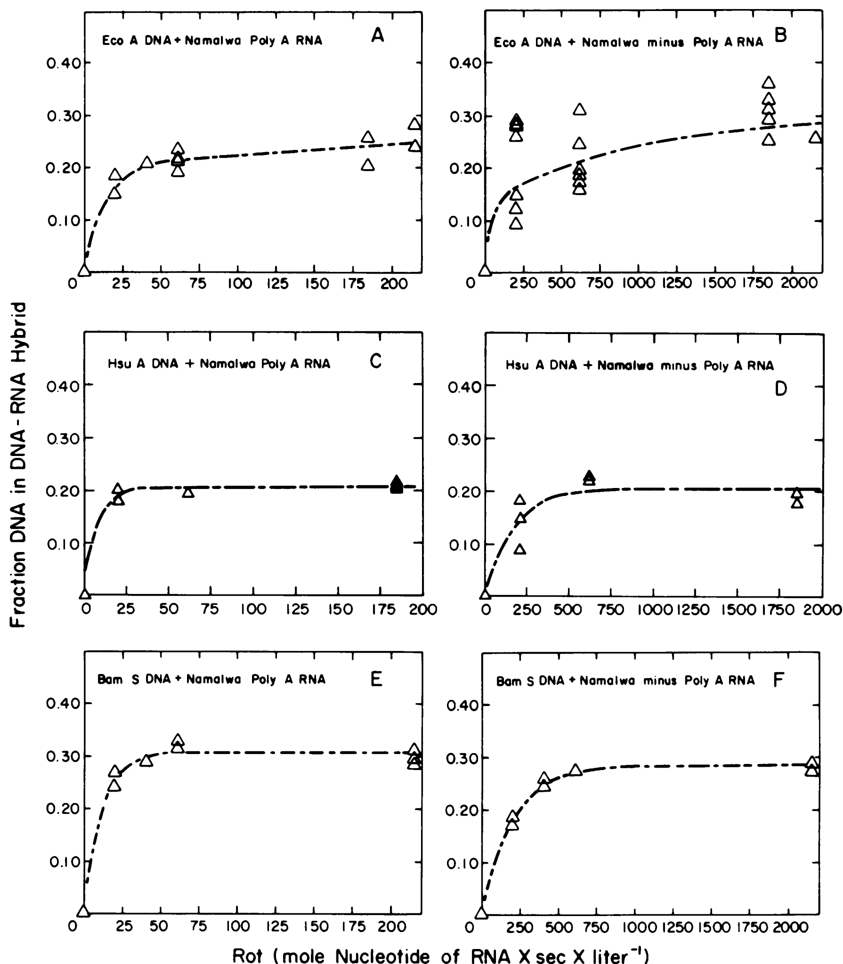

FIG. 6. *Hybridization of unlabeled Namalwa poly(A)+ (A, C, E) or poly(A)− (B, D, F) RNAs with denatured ^{32}P-labeled restriction endonuclease fragments of EBV (B95-8) DNA: EcoRI fragment A (A and B); Hsu I fragment A (C and D); and Bam I fragment S (E and F).*

by the same DNA sequences which encode poly(A)+ and polyribosomal RNA from Namalwa cells, Raji poly(A)+ polyribosomal RNA was hybridized in excess to ^{32}P-labeled EBV (B95-8) DNA and ^{32}P-labeled EBV (B95-8) DNA *Eco*RI A fragment. The results indicate that Raji poly(A)+ polyribosomal RNA is homologous to at least 8% of EBV (B95-8) DNA (Fig. 8A) and 19% of the *Eco*RI A fragment (Fig. 8B).

DISCUSSION

Several aspects of the RNA mapping data reported here and summarized in Fig. 9 require discussion. (i) The preparation of Namalwa poly(A)+ and poly(A)− RNA for this study used techniques which are more rapid and efficient than those used previously (39). Nevertheless, the results of hybridization of Namalwa poly(A)+ and poly(A)− RNAs to ^{32}P-labeled EBV (B95-8) or (W91) DNAs are similar to those reported previously (39) and indicate that Namalwa poly(A)− RNA is homologous to at least 17% of EBV DNA, whereas the poly(A)+ RNA is enriched for sequences homologous to only 7% of EBV (B95-8) and (W91) DNA.

(ii) The map of *Hsu* I and *Eco*RI fragments of EBV (B95-8) and (W91) DNAs (15) is shown in Fig. 9. Namalwa poly(A)+ and Raji poly(A)+ polyribosomal RNAs are homologous to at least 25 and 20% of the *Eco*RI A fragment, respectively. The *Eco*RI A fragment is approximately 38×10^6 daltons (15, 18). Therefore, approximately 9×10^6 daltons of DNA in the *Eco*RI A fragment is homologous to Namalwa poly(A)+ RNA. The *Eco*RI A fragment contains approx-

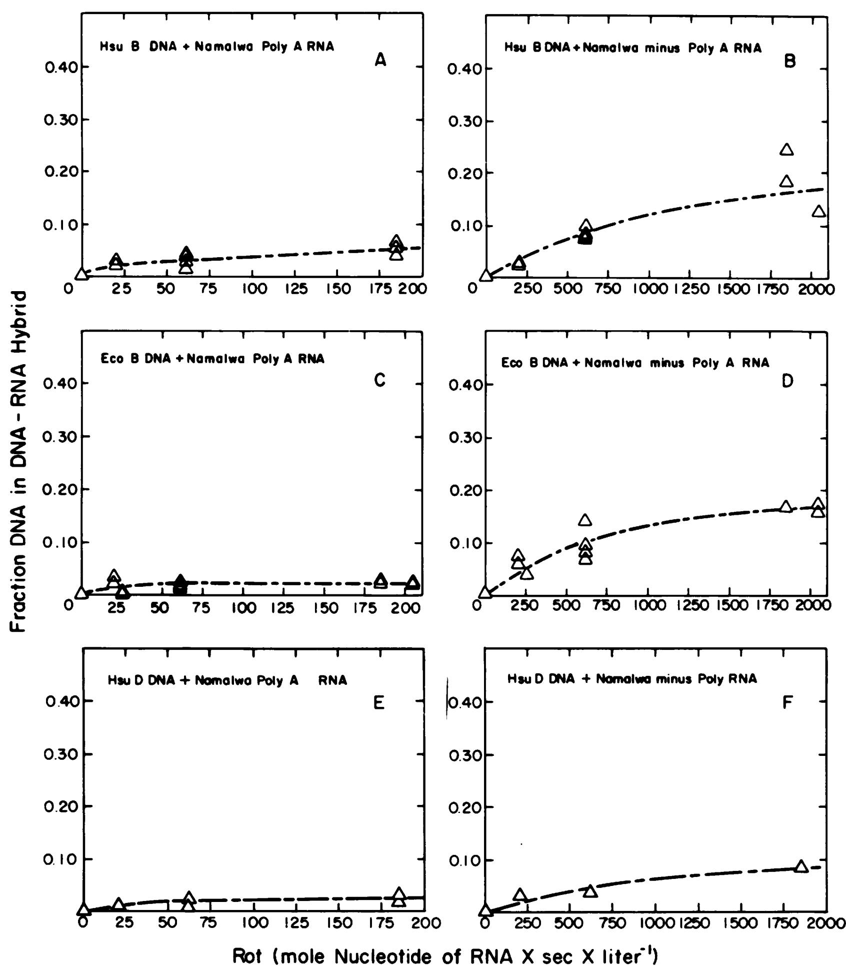

FIG. 7. *Hybridization of unlabeled Namalwa poly(A)+ (A, C, E) or poly(A)− (B, D, F) RNAs with denatured ^{32}P-labeled restriction endonuclease fragments of EBV (B95-8) DNA: Hsu I fragment B (A and B); EcoRI fragment B (C and D); and Hsu I fragment D (E and F).*

imately 26 × 10^6 daltons of DNA in common with the *Hsu* I A fragment and 12 × 10^6 daltons in common with the *Hsu* I B fragment (15; Fig. 9). Hybridizations of ^{32}P-labeled EBV DNA selected for homology to Namalwa RNAs to blots containing fragments of *Hsu* I/*Eco*RI double digests of EBV DNA indicated that the *Hsu* I A/*Eco*RI A and *Hsu* I B/*Eco*RI A fragments contain regions of homology to the Namalwa RNAs. The molecular weight of the *Hsu* I A fragment is approximately 32 × 10^6 (15, 18). Approximately 21%, or 6.7 × 10^6 daltons, of the *Hsu* I A fragment is homologous to Namalwa poly(A)+ RNA. The molecular weight of the *Hsu* I B fragment is 20 × 10^6 (18). Namalwa poly(A)+ RNA is homologous to approximately 6% of the *Hsu* I B fragment. This could account for an additional 1.2 × 10^6 daltons of the DNA in the *Eco*RI A fragment which is homologous to Namalwa RNA.

The molecular weight of the *Hsu* I A/*Eco*RI A EBV (B95-8) DNA fragment is approximately 26 × 10^6 (15; Given and Kieff, manuscript in preparation) and contains nine to 10 (15, 49) tandem reiterations of the 2 × 10^6-dalton fragment, *Bam* I S. The reiterations of the *Bam* I S fragment are at the right end of the *Hsu* I A/*Eco*RI A fragment (Given and Kieff, manuscript in preparation). Namalwa poly(A)+ RNA is homologous to 30% of the *Bam* I S fragment. The 6 × 10^5 daltons of DNA present in each of the 9 to 10 copies of the *Bam* I S fragment accounts for 6 × 10^6 daltons of the DNA in the *Hsu* I A fragment which is homologous to Namalwa poly(A)+ RNA. Thus, the sequence complexity of the Namalwa poly(A)+ RNA homol-

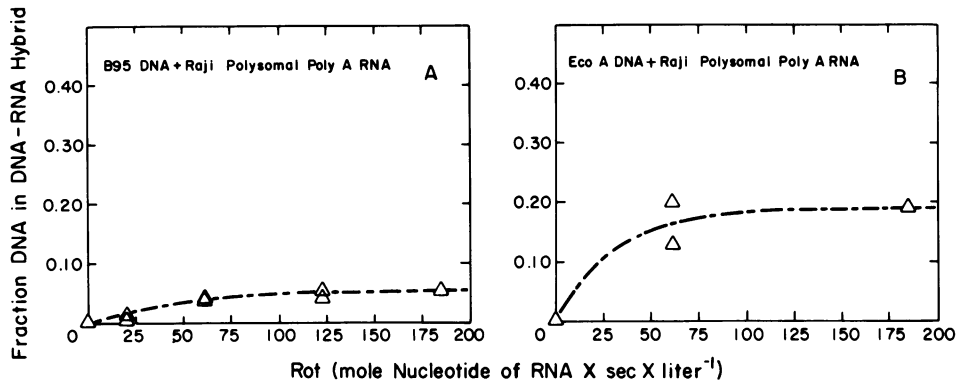

FIG. 8. *Hybridization of unlabeled Raji polyribosomal poly(A)+ RNA with denatured ^{32}P-labeled EBV (B95-8) DNA (A) or denatured ^{32}P-labeled EcoRI A fragment of EBV (B95-8) DNA (B).*

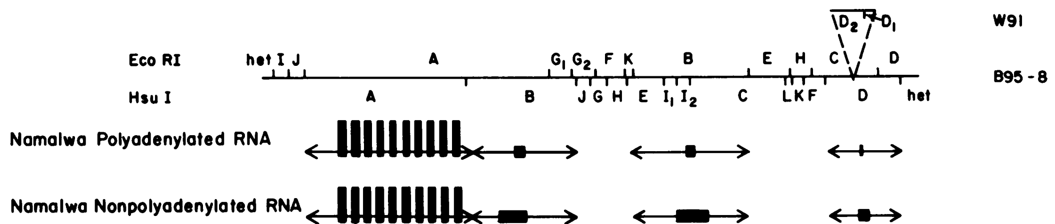

FIG. 9. *Map of Hsu I and EcoRI restriction endonuclease fragments of EBV (B95-8) and (W91) DNA and diagrammatic representation of the location of DNA sequences which encode stable RNAs in Namalwa cells. The width of the bar indicates the length of DNA within the fragments which is homologous to stable RNA. The length is twice the product of the fraction of the fragment homologous to RNA and the known molecular weight of the fragment. The rationale for doubling the product is that preliminary data indicate that most of the RNA in these cells is an asymmetric transcript. The height of the bars represents the relative abundance of stable RNA.*

ogous to *Hsu* I A and *Eco*RI A fragments is approximately 5×10^6 daltons less than is estimated from the hybridization of Namalwa poly(A)+ RNA to ^{32}P-labeled *Hsu* I A or *Eco*RI A DNA fragments. The rapid rate of hybridization of Namalwa poly(A)+ RNA to the labeled *Bam* I S fragment and other fragments which contain this reiterated DNA indicates that this DNA is homologous to a relatively abundant class of viral RNA.

The *Hsu* I D fragment is 14×10^6 daltons (18). Namalwa poly(A)+ RNA is homologous to 2%, or approximately 3×10^5 daltons, of the *Hsu* I D fragment. The molecular weight of the *Eco*RI B fragment is 21×10^6 (18), and Namalwa poly(A)+ RNA is homologous to 2%, or approximately 4×10^5 daltons, of the *Eco*RI B fragment.

(iii) Comparison of the data obtained with Namalwa poly(A)− RNA to those obtained with poly(A)+ RNA indicates that poly(A)− RNA also consists largely of RNA homologous to the reiterated DNA in the *Hsu* I A/*Eco*RI A fragment and to DNA in the *Hsu* I B fragment. The similarity in the mapping of the poly(A)− and poly(A)+ RNAs indicates that poly(A)− RNA contains many of the RNA species present in poly(A)+ RNA. This could arise if those RNA species in the poly(A)+ fraction also accumulate in the poly(A)− fraction before adenylation or after partial degradation. The data also indicate that most of the additional DNA homologous to poly(A)− RNA but not to poly(A)+ RNA is contained in the *Hsu* I B, *Eco*RI B, *Hsu* I D, and other fragments of the viral genome and is not contained in the *Eco*RI A or *Hsu* I A fragments.

The functions of these viral RNAs in Namalwa cells are not known. The viral poly(A)+ and polyribosomal RNAs in Namalwa cells are largely encoded by the same EBV DNA sequences (39), suggesting that most of the poly(A)+ RNAs function as mRNA. Only one virus-related antigen, EBNA, has been demonstrated in Namalwa cells (46). Several lines of evidence indicate that EBNA is specified by the viral genome. Thus, EBNA has been found only in cells and tissues which contain EBV DNA (26, 46, 47). Furthermore, EBNA appears in cells after EBV infection (29). Moreover, human or

chimpanzee lymphocytes infected with the chimpanzee EBV contain an antigenically distinct EBNA, Ch EBNA, whereas human or chimpanzee lymphocytes infected with human EBV contain EBNA (13). The soluble component of EBNA has a molecular weight of 174×10^3 and dissociates in high salt into two components of 98×10^3 daltons each (28, 38), which may consist predominantly of a single polypeptide of 48×10^3 daltons (27). EBNA could therefore be encoded by 1.3×10^3 to 1.5×10^3 bases or approximately 5×10^5 daltons of DNA. The most abundant RNA in Namalwa cells is homologous to 30% of the 2×10^6-dalton internal reiterated sequence. If this RNA is homologous to only one strand of viral DNA, the 6×10^5 daltons of DNA would be adequate to encode EBNA. Other functions of the viral RNAs in Namalwa cells may include functions necessary for the maintenance of restringent infection or continuous cell growth in vitro. The HR-1 clone of EBV, which lacks the ability to transform lymphocytes into lymphoblasts capable of long-term growth in vitro, is missing DNA from the *Hsu* I B fragment (45) and the *Hsu* I cleavage site between the *Hsu* I A and B fragments (D. Given, N. Raab-Traub, and E. Kieff, manuscript in preparation).

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