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Identification of Transcribed Regions of Epstein-Barr Virus DNA in Burkitt Lymphoma-Derived Cells

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RNA was extracted from the Burkitt lymphoma-derived cell line Raji and from Burkitt lymphoma tumor biopsies, isotope labeled in vitro by iodination with ^{125}I, and hybridized to electrophoretically separated restriction endonuclease fragments of Epstein-Barr virus DNA on nitrocellulose membranes. The results indicated that only certain parts of the Epstein-Barr virus genome are represented as polyribosomal RNA in Raji cells, with a pronounced dominance of RNA sequences complementary to a 2.0×10^6-dalton segment of Epstein-Barr virus DNA located close to the left end of the viral genome. A map of virus-specific polyribosomal RNA sequences was constructed, which indicated that a minimum of three regions of the Epstein-Barr virus genome are expressed in Raji cells. Total-cell RNA preparations from five Burkitt lymphoma biopsies contained RNA sequences homologous to the same regions of Epstein-Barr virus DNA as polyribosomal RNA from Raji cells, albeit at different relative proportions.

Since the discovery of a herpes-like virus in a cultured line of Burkitt lymphoma cells by Epstein et al. (8), this virus, commonly called the Epstein-Barr virus (EBV), has attracted attention as a major candidate for a human tumor virus. Thus, EBV is able to transform differentiated, nondividing, human B lymphocytes in vitro into continuously dividing immortalized cells with a new set of stable properties that resemble those of tumor cells (25), and in vivo the virus induces malignant lymphomas experimentally in some New World monkeys (32). The relationship between EBV and human disease is, however, not fully unraveled. It is generally accepted that EBV causes the heterophile antibody-positive form of infectious mononucleosis. The evidence for an etiological role of EBV in Burkitt's lymphoma and nasopharyngeal carcinoma is persuasive but as yet not conclusive (7, 8, 15, 22, and 34). If EBV is necessary for the development of these two human neoplasias, the virus alone is not sufficient to cause the disease.

The concept that the virus-transformed cell phenotype results from the continuous expression of persistent viral genes is supported by data obtained with both DNA and RNA tumor viruses. Studies on the expression of the EBV genome in cells that contain EBV DNA but do not produce virus, i.e., in transformed, nonproductive cell lines and in tumor biopsies, therefore might yield information about gene products responsible for initiation and maintenance of the transformed state. Because of the inherent difficulties of the EBV system, progress of our knowledge about this virus at the molecular level has been slow, and little is known about the transcription of EBV DNA in transformed cells. Kieff and collaborators have analyzed viral RNA extracted from permissive and nonpermissive EBV-infected lymphoblastoid cell lines (13, 23). They reported that polyribosomal RNA fractions of two Burkitt lymphoma-derived EBV DNA-containing cell lines, Raji and Namalwa, which do not contain progeny virus or antigens associated with productive infection, are enriched for a class of EBV RNA encoded by approximately 5% of EBV DNA. The same EBV DNA sequences encode the polyribosomal RNA of both Raji and Namalwa cells. The fraction of virus-specific RNA in the polyribosomal RNA preparations is approximately 5×10^{-5}. In recent studies Kieff and co-workers have used restriction endonuclease fragments of EBV DNA to determine the location of transcribed DNA sequences within the EBV genome. The results indicated that Raji and Namalwa polyadenylated virus-specific RNA is encoded primarily by 0.6×10^6 daltons of a 2×10^6-dalton segment of DNA, which is tandemly reiterated approximately 10 times in the *Eco*RI A fragment of the virus genome, and to a lesser extent by DNA in certain other fragments (26). RNA extracted from Burkitt lymphoma biopsies was found to contain viral sequences homologous mainly to

the regions of the EBV genome contained in the *Eco*RI A and *Hsu*I D fragments of B95-8 EBV DNA and also an abundant RNA species encoded by the additional DNA present in the W91 isolate of EBV (5).

I have analyzed the EBV-specific RNA sequences present in the cytoplasm of Raji cells by using hybridization between in vitro iodinated [^{125}I]RNA and separated restriction endonuclease fragments of EBV DNA adsorbed to nitrocellulose membranes. The results indicate that only certain parts of the EBV genome are represented as virus-specific polyribosomal RNA, with a pronounced dominance of RNA sequences complementary to a 2.0×10^6-dalton segment of EBV DNA, the *Eco*RI J fragment, located close to the left end of the viral DNA molecule. When the techniques were applied to total cell RNA preparations of Burkitt lymphoma biopsies, a similar pattern was obtained. All five biopsies contained detectable amounts of RNA sequences complementary to the *Eco*RI J fragment.

MATERIALS AND METHODS

Cell lines and tumor biopsies. P3HR-1, B95-8, Raji, and Ramos cells were obtained from Alice Adams, University of Gothenburg, Gothenburg, Sweden. The cell lines were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Flow Laboratories) and were diluted to a concentration of 0.2×10^6 cells per ml twice a week. For virus production cells were diluted to 0.2×10^6 cells per ml in medium containing 2% fetal bovine serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; and mycostatin, 25 U/ml) and incubated for 3 days at 37°C followed by 8 to 10 days at 34°C. They were then stored at 4°C until the supernatant medium containing the virus was harvested. The five Burkitt lymphoma tumor biopsies (D.Om., D.Or., T.M., K.K., and I.M.) investigated were from Kenyan patients treated by surgery. This material was a gift from George Klein, Karolinska Institute, Stockholm, Sweden.

Virus and viral DNA. Virus was concentrated from cell supernatants by precipitation with polyethylene glycol and viral DNA purified as described by Adams (1), with care taken to minimize shearing of the DNA. All DNA samples were purified through two equilibrium centrifugation steps in CsCl, dialyzed, and concentrated by ethanol precipitation. DNA concentrations were determined with the ethidium fluorescence technique of Le Pecq and Paoletti (18).

Restriction endonuclease cleavage and separation of fragments. Restriction endonucleases *Eco*RI, *Hin*dIII, and *Bam*HI were purchased from Boehringer. Adenovirus type 2 DNA (a gift from Ulf Pettersson, University of Uppsala, Uppsala, Sweden) was used as a substrate to determine the amounts of restriction endonucleases required to achieve complete digestion under the specified conditions. Digestions were usually performed for 2 h at 37°C and virus DNA concentrations of 20 μg/ml. The reaction mixutre contained the following components: for *Eco*RI, 50 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol; and for *Hin*dIII and *Bam*HI, 20 mM Tris-hydrochloride (pH 7.5), 20 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. I routinely used five times the quantity of restriction endonucleases needed for complete hydrolysis of an equivalent amount of adenovirus DNA. Reaction was stopped by the addition of 0.2 volume of 50% sucrose–0.25 M EDTA–0.05% bromophenol blue. DNA samples of 10 μl were loaded into slots (5 by 2 by 4 mm) in horizontal 0.35% agarose slab gels (26 by 16 by 0.5 cm; Agarose HSC; Litex, Denmark). The gels were run at 6°C for 18 h at a potential of 1.5 V/cm by using a buffer system containing 50 mM Tris-acetate (pH 7.9), 20 mM sodium acetate, 1 mM EDTA, and 0.5 μg of ethidium bromide per ml. They were photographed through a Kodak 23A filter onto Polaroid type film by using an MP4 camera and short-wave UV illumination.

Blotting and hybridization. Slab gels containing DNA were immersed in 0.25 M NaOH–1.5 M NaCl for 14 h at 6°C. They were then submerged in 0.5 M Tris-hydrochloride (pH 7)–3 M NaCl for 2 h at room temperature. The DNA was transferred to a sheet of nitrocellulose (BA85; Schleicher and Schüll) by a flow of 20× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0) through the gel, using essentially the technique described by Southern (33). After blotting, the nitrocellulose membranes were washed in 2× SSC, allowed to dry at room temperature, and heated at 80°C for 4 to 6 h. Usually the baked filters were cut into strips (2 by 17 cm) and inserted into polyethylene bags which were formed to appropriate sizes by heat-sealing. Hybridization was carried out at 67°C for 40 h in a total volume of 2 ml which contained 4× SSC, 0.1% sodium dodecyl sulfate, 0.1 mg of yeast RNA per ml, 2 mM KI, and about 10×10^6 cpm of ^{125}I-labeled RNA (specific activity, 1×10^7 to 5×10^7 cpm/μg). After hybridization the nitrocellulose sheets were washed in 2× SSC at room temperature for several hours, treated with 20 μg of pancreatic RNase A (Boehringer) per ml in 2× SSC at 37°C for 1 h, and washed with 2× SSC–0.1% sodium dodecyl sulfate at 67°C for 1 h and in 1× SSC at room temperature for 2 h. They were then air-dried, mounted on a sheet of paper, and subjected to fluorography at −70°C for 4 to 14 days with Kodak Xomat R film and Agfa-Gevaert M.R. 600 intensifying screens. Membranes to be hybridized with nick-translated EBV DNA were preincubated with Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.02% bovine serum albumin) in 4× SSC–0.1% sodium dodecyl sulfate–50 μg of sonicated calf thymus DNA per ml at 67°C for 6 h. Hybridization was carried out at 67°C for 40 h in 1 ml of Denhardt solution containing 4× SSC, 0.1% sodium dodecyl sulfate, 50 μg of sonicated DNA per ml, 2 mM KI, and approximately 0.01 μg of ^{125}I-labeled nick-translated B95-8 EBV DNA. After hybridization the membranes were rinsed in 2× SSC and washed at

67°C in the hybridization buffer for 3 h, in 2× SSC–0.1% sodium dodecyl sulfate for 30 min, in 1× SSC for 30 min, in 0.5× SSC for 30 min, and in 0.25× SSC for 30 min. They were then dried and fluorographed as described above.

In vitro synthesis of ^{125}I-labeled nucleic acid homologous to EBV DNA. Carrier-free [^{125}I]CTP and [^{125}I]dCTP were prepared by a modification of the procedure described by Scherberg and Refetoff (31). The standard reaction mixture contained 0.1 M sodium acetate buffer (pH 4.2), 0.25 mM CTP or dCTP, 0.5 mM thallic trichloride (Merck), and 10 mCi of Na^{125}I (carrier-free ^{125}I; pH 8 to 10; 100 mCi/ml; The Radiochemical Centre, Amersham, England) in a final volume of 0.4 ml. The mixture was incubated at 60°C for 30 min. Unstable reaction components were dissociated by the addition of 0.4 ml of 0.5 M Tris-hydrochloride (pH 8.0) containing 10 mM 2-mercaptoethanol, and incubation was continued at 60°C for 20 min. [^{125}I]CTP and [^{125}I]dCTP were separated from unlabeled nucleoside triphosphates by chromatography on a column (0.9 by 20 cm) of DEAE-Sephadex A-25 equilibrated with 0.5 M ammonium formate (pH 3.8). Nucleotides were eluted by 40 ml of a linear gradient of 0.5 to 5 M ammonium formate (pH 3.8). The ^{125}I-labeled triphosphate peak was pooled, diluted with 9 volumes of purified water, and added to a column (0.5 by 1 cm) of AG 1-X8 (200 to 400 mesh; Bio-Rad Laboratories) in formate form. The column was washed with 10 ml of water, followed by 25 ml of 0.1 M triethylammonium bicarbonate. The triphosphate was eluted with a 30-ml 0.5 to 2 M triethylammonium bicarbonate linear gradient, and peak fractions were pooled and desalted by repeated evaporation from water. The purified product, which was more than 90% ^{125}I-labeled nucleotide triphosphate as judged from polyethyleneimine cellulose thin-layer chromatography in 0.75 M LiCl, was stored in 10 mM triethylammonium bicarbonate–50% ethanol at −20°C. It could be used for in vitro labeling for at least 1 month without decreasing incorporation rates. It might be noted that desalting of ^{125}I-labeled CTP or dCTP on Sephadex G-10 was not used, as the iodinated triphosphates did not separate efficiently from low-molecular-weight substances and were recovered from the columns at low yields. Apparently the carrier-free [^{125}I]CTP and [^{125}I]dCTP interacted with the gel matrix at low ionic strengths, eluting from the Sephadex columns at higher elution volumes than expected. Purification procedures involving evaporation from hydrochloric acid solutions were also avoided, as this resulted in degradation of the iodinated triphosphates, especially [^{125}I]CTP.

EBV DNA was labeled with [^{125}I]dCTP to specific activities of 1×10^8 to 2×10^8 cpm/μg by the nick translation reaction of *Escherichia coli* DNA polymerase I, using a procedure based on those of Rigby et al. (27) and Maniatis et al. (21). Approximately 10^9 cpm of carrier-free [^{125}I]dCTP dissolved in 50% ethanol was vacuum dried and redissolved in 100 μl of a solution containing 50 mM Tris-hydrochloride (pH 7.5), 7 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM dCTP, 20 μM dATP, 20 μM dGTP, and 20 μM TTP; 1 μg of EBV DNA, 10 pg of pancreatic DNase I, and 30 U of *E. coli* DNA polymerase I (Boehringer) were added, and the reaction mixture was incubated at 14°C for 3 h. About 40% of the ^{125}I radioactivity was incorporated into trichloroacetic acid-precipitable material. The reaction was stopped by the addition of 100 μl of 0.1 M EDTA, and the solution was extracted with equal volumes of phenol–cresol mixture (phenol–*m*-cresol–water, 100:14:11, containing 1.8 g of 8-hydroxyquinoline per liter) and chloroform–isoamylalcohol (100:1). The aqueous phase was chromatographed on a Sephadex G-50 column (0.5 by 10 cm) equilibrated with 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. The excluded fractions were pooled, heated to 100°C for 5 min, divided into portions, and stored frozen at −20°C.

EBV DNA complementary RNA (cRNA) was synthesized in vitro by using [^{125}I]CTP and *E. coli* RNA polymerase containing sigma factor (a generous gift of Karin Carlsson, University of Tromsø, Tromsø, Norway) and EBV DNA as a template. Reaction conditions were essentially those given by Lindahl et al. (19). The purification of the [^{125}I]cRNA has been described previously (28).

Preparation of cytoplasmic RNA. Cells were suspended at 20×10^6 cells per ml in cold 10 mM Tris-hydrochloride (pH 7.5)–1 mM $MgCl_2$–0.5 mM $CaCl_2$–0.25 M sucrose–0.5% Nonidet P-40. After 10 min on ice more than 90% of the cells were ruptured, as judged from phase-contrast microscopy of the suspension. Nuclei were deposited by centrifugation for 5 min at $1{,}300 \times g$. The cytoplasmic fraction was adjusted to 10 mM EDTA–0.5% sodium dodecyl sulfate and deproteinized by repeated extractions (three times) with equal volumes of phenol–cresol mixture and chloroform. After addition of NaCl to 1.4 M, the aqueous phase was precipitated with 2 volumes of ethanol at −20°C. The precipitate was dissolved in 2 ml of a solution containing 50 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 50 mM $MgCl_2$, and 2 mM $CaCl_2$ and treated with 50 μg of DNase I (type DPFF, electrophoretically purified; Worthington Biochemicals) per ml at 37°C for 15 min. The solution was adjusted to 0.5% sodium dodecyl sulfate–25 mM EDTA–1 mg of self-digested pronase (2 h, 37°C) per ml and incubated at 37°C for 1 h. The mixture was then extracted with equal volumes of phenol–cresol mixture and chloroform, and the aqueous phase was precipitated with ethanol. The precipitate was dissolved in 0.3 ml of 50 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl and passed through a column (0.9 by 10 cm) of G-100 Sephadex in the same buffer. Material eluting in the void volume of the column was collected and adjusted to 50 mM $MgCl_2$, and DNase digestion, phenol–chloroform extraction, and Sephadex G-100 chromatography were repeated once. RNA was finally collected by ethanol precipitation of excluded fractions, dissolved in 10 mM Tris-hydrochloride (pH 7.5), and stored at −20°C until used.

Preparation of RNA from frozen tumor biopsies. A 0.5-g amount of frozen tumor tissue was minced by grinding with dry ice, suspended in 5 ml of a solution containing cold 25 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 1 mg of heparin per ml, and 2% Triton X-100, and homogenized in a Dounce homogenizer with loose- and tight-fitting glass pestles, following essentially the method of Palmiter (24). After the addition of 1 volume of 0.1 M sodium

acetate (pH 5.0) and sodium dodecyl sulfate to a concentration of 0.5%, the mixture was again homogenized with the tight-fitting pestle and then extracted four times with equal volumes of phenol–cresol mixture and chloroform. The aqueous phase was precipitated with 2 volumes of ethanol at −20°C. The precipitate was further purified by two cycles of DNase digestion, deproteinization, Sephadex G-100 chromatography, and precipitation, as described above for the preparation of cytoplasmic RNA. The yield of RNA was 100 to 400 μg/g of frozen tumor tissue.

Preparation of polyribosomal RNA and separation of polyadenylated and non-polyadenylated RNA. The magnesium chloride precipitation technique of Palmiter was used to prepare polyribosome-associated material (24). About 10^9 cells were suspended in 10 ml of a solution containing ice-cold 25 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 2% Triton X-100, and 1 mg of heparin per ml, and homogenized by 30 strokes in a Dounce homogenizer with a tight-fitting pestle. The suspension was clarified by centrifugation at 20,000 × *g* for 5 min at 4°C. The supernatant was diluted with an equal volume of a solution containing 25 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, 0.2 M $MgCl_2$, 2% Triton X-100, and 1 mg of heparin per ml. After 90 min on ice, the precipitated polyribosome-associated material was deposited by centrifugation of 8-ml samples through 4-ml pads of 0.2 M sucrose in 25 mM Tris-hydrochloride (pH 7.5)–25 mM NaCl–0.1 M $MgCl_2$ at 4°C and 15,000 rpm for 10 min in an MSE SW6× 14-ml rotor. The precipitated material was resuspended in 2 ml of a solution containing 25 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 2% Triton X-100, and 1 mg of heparin per ml, diluted with an equal volume of 0.1 M sodium acetate (pH 5.0), adjusted to 0.5% sodium dodecyl sulfate, and extracted three times with equal volumes of phenol–cresol mixture and chloroform. After precipitation with ethanol, the RNA was further purified by two cycles of DNase digestion, deproteinization, Sephadex G-100 chromatography, and precipitation as described above for the preparation of cytoplasmic RNA.

Polyadenylated and non-polyadenylated polyribosomal RNAs were separated by chromatography on polyuridylic acid-Sepharose; 1-ml columns of polyuridylic acid-Sepharose 4B (Pharmacia Fine Chemicals) were prepared in disposable syringes and equilibrated with 10 mM Tris-hydrochloride (pH 7.5)–0.5 M NaCl–0.5% sodium dodecyl sulfate. Polyribosomal RNA from 2×10^7 to 5×10^7 cells in the equilibration buffer was passed through the column two times, and the column was then washed with 10 ml of the same buffer. RNA not binding to the column was collected by ethanol precipitation. Polyadenylated RNA was eluted with 3 ml of a buffer containing 90% (vol/vol) formamide, 10 mM Tris-hydrochloride (pH 7.5), and 0.5% sodium dodecyl sulfate. The formamide was removed by dialysis against 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA at 4°C overnight. After the addition of NaCl to a final concentration of 1.4 M and 30 μg of polyadenylic acid (Boehringer) as carrier, the polyadenylated RNA was collected by ethanol precipitation.

Iodination of RNA. The iodination technique was adapted from that described by Commerford (4). The standard reaction mixture contained 25 mM sodium acetate buffer (pH 4.2), 1 mCi of $Na^{125}I$, 1 mM thallic trichloride (Merck), and 5 to 10 μg of RNA in a final volume of 20 μl. The reaction was carried out for 20 min at 68°C, after which 200 μl of 0.5 M Tris-hydrochloride (pH 8.0) containing 10 mM 2-mercaptoethanol was added, and the incubation continued for 30 min at 68°C. RNA was separated from low-molecular-weight material by chromatography on a column (0.9 by 10 cm) of Sephadex G-50 equilibrated with 50 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–10 mM EDTA. The material eluting with the void volume was adjusted to 30% (vol/vol) in ethanol and applied to a 1-ml column of Whatman CF11 cellulose (9) equilibrated with 50 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–10 mM EDTA diluted with ethanol to 30% (vol/vol). The column was washed with 15 ml of the same buffer and the RNA was then eluted with Tris–NaCl–EDTA buffer adjusted to 13% (vol/vol) in ethanol. In some cases the cellulose chromatography step was repeated once. RNA was precipitated with ethanol, dissolved in 0.5 ml of 10 mM Tris-hydrochloride (pH 7.5), and used for hybridization, usually within 1 week. The specific activity of the labeled product was calculated from the amount of radioactivity eluting with the void volume of the Sephadex G-50 column and the known input of RNA. On this basis a specific activity of 1×10^7 to 5×10^7 cpm/μg was generally obtained.

Purification of RNA by potassium iodide equilibrium density gradient centrifugation. The procedure was based on those of De Kloet and Andrean (6) and Gonzales et al. (11). ^{125}I-labeled cytoplasmic RNA in 2 ml of 10 mM Tris-hydrochloride (pH 7.5) containing 1 mM EDTA and 10 mM sodium bisulfite was mixed with 5 ml of 5.78 M KI and layered over 5 ml of 5.78 M KI in a 14-ml polycarbonate tube. Gradients were centrifuged for 60 h in an MSE SW6× 14-ml rotor at 20°C and 25,000 rpm. Fractions were collected from the bottom of the tube, and the refractive index was determined with an Abbe refractometer. The density (*d*) of the fractions was calculated from the refractive index (*n*) by using the following formula: $n = 0.1731 \times d + 1.1617$, as given by Blin et al. (3). Fractions were pooled (see Fig. 3) and dialyzed overnight at 4°C against 50 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl. RNA was collected by ethanol precipitation.

RESULTS

EBV DNA cRNA sequences in Raji cells. The detection and characterization of virus-specific RNA sequences in EBV-infected cells has been seriously hampered by the fact that the infected cells contain only small amounts of virus-specific RNA (13, 23). It has also been difficult to label viral nucleic acids to high specific activities in vivo. I used iodination with ^{125}I to introduce isotope label to high specific activities directly in vitro into small amounts of RNA from different EBV-infected cells. The ^{125}I-labeled RNA was purified by Sephadex gel filtration followed by chromatography on cellulose (9). Virus-specific RNA was identified by hy-

bridization to restriction endonuclease fragments of EBV DNA adsorbed to nitrocellulose membranes (33). Without purification of the ^{125}I-labeled RNA on cellulose columns, the hybridization backgrounds were high and prevented identification of specific hybridization on the Southern blots.

When ^{125}I-labeled cytoplasmic RNA from Raji cells was incubated under hybridization conditions with nitrocellulose membranes containing the separated fragments of EBV DNA from virus strain B95-8, obtained after cleavage with endonuclease *Eco*RI, fragment J was identified as the major hybridizing species (Fig. 1). Long exposure times revealed hybridization to fragments A, B, E (and/or D), and G_1 (and/or G_2), but, judging from the radiofluorograms, at least 10 times more RNA was associated with the small fragment J than with any of the larger fragments.

There is no a priori reason to suspect that RNA extracted from the cytoplasm of Raji cells should not be representative of actively translated, ribosome-associated mRNA. Nevertheless, polyribosomal RNA was prepared, separated into polyadenylated and non-polyadenylated RNA, iodinated, and characterized with regard to its content of RNA sequences complementary to EBV DNA by hybridization to blots of *Eco*RI-cleaved EBV DNA (Fig. 1). The patterns obtained with polyribosomal RNA preparations were qualitatively the same as those obtained with cytoplasmic RNA. Sequences complementary to fragments A, B, E (and/or D), G, and J were present, albeit at different proportions as compared with cytoplasmic RNA, the sequences homologous to the J fragment not being quite as dominant. Polyadenylated and non-polyadenylated RNA preparations both primarily contained sequences which hybridized to fragment J. Long exposure times also revealed some hybridization over background to fragments E (and/or D) and A in both cases. There was no obvious qualitative difference between polyadenylated and non-polyadenylated RNA.

Incubation of ^{125}I-labeled cytoplasmic RNA from Raji cells with blots containing the *Eco*RI fragments of EBV DNA from a different virus strain, P3HR-1, showed that fragment M, which is similar in size to the *Eco*RI J fragment of the B95-8 strain, hybridized extensively to the labeled RNA (Fig. 2). With long exposure times hybridization to some other fragments was evident.

To characterize the hybridizing material in the ^{125}I-labeled RNA preparations as RNA, one preparation of ^{125}I-labeled cytoplasmic RNA from Raji cells was further purified by buoyant density equilibrium centrifugation in potassium

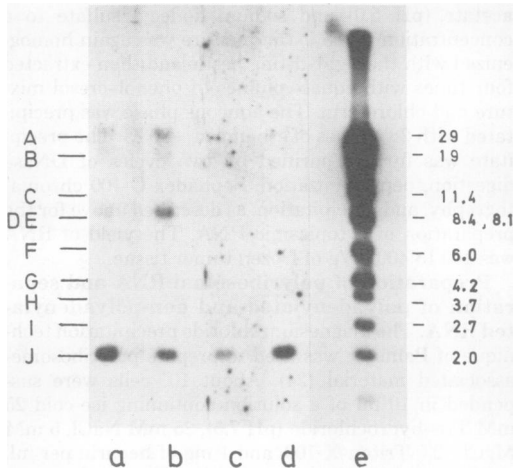

FIG. 1. *Detection of RNA sequences complementary to EcoRI fragments of B95-8 EBV DNA in Raji cells. Samples (0.2 μg) of B95-8 EBV DNA cleaved by EcoRI were applied to a 0.35% agarose slab gel, fractionated by electrophoresis, and transferred to a nitrocellulose sheet. The sheet was cut into strips which were incubated under hybridization conditions with cytoplasmic [^{125}I]RNA (strip a), polyribosomal [^{125}I]RNA (strip b), polyadenylated polyribosomal [^{125}I]RNA (strip c), non-polyadenylated polyribosomal [^{125}I]RNA (strip d), and nick-translated ^{125}I-labeled B95-8 EBV DNA (strip e). The molecular weights (×10^6) of the EBV DNA fragments (28) are indicated in the right margin. For further details, see text.*

iodide. In vitro iodinated denatured EBV [^{125}I]DNA and double-stranded *Bacillus subtilis* [^{3}H]DNA were run in parallel gradients as density markers. Their peak positions in the gradient corresponded to densities of about 1.52 and 1.48 g/cm^3, respectively. Fractions with densities between 1.57 and 1.62 g/cm^3 were pooled (Fig. 3) and dialyzed, and the RNA was recovered by ethanol precipitation. When the purified [^{125}I]RNA preparation was incubated under hybridization conditions with blots containing *Eco*RI fragments of B95-8 EBV DNA, extensive hybridization to fragment J could still be seen (Fig. 4). Long exposure times revealed hybridization also to fragments A and E (and/or D).

Further evidence for the RNA nature of the hybridizing material was obtained from an experiment where samples of ^{125}I-labeled cytoplasmic RNA were incubated with RNase T1 or DNase I and subsequently used as hybridization probes. Incubation with RNase T1 completely destroyed the ability of the RNA preparation to hybridize to the EBV DNA fragments on the nitrocellulose blots, whereas incubation with DNase did not affect the hybridization proper-

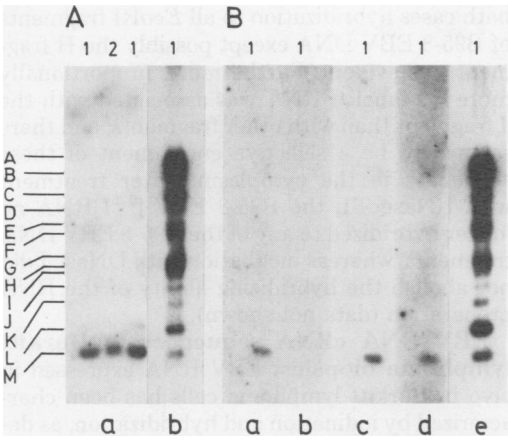

FIG. 2. *Detection of RNA sequences complementary to EcoRI fragments of P3HR-1 EBV DNA in Raji cells and a Burkitt lymphoma biopsy. Samples (0.2 μg) of P3HR-1 EBV DNA (lanes 1) and B95-8 EBV DNA (lane 2) cleaved by EcoRI were applied to 0.35% agarose slab gels, electrophoresed, and transferred to nitrocellulose sheets, which were cut into strips. (A) The strips were hybridized with Raji cytoplasmic [^{125}I]RNA (strip a) or B95-8 EBV DNA homologous [^{125}I]cRNA (strip b). (B) The strips were hybridized to DNase-treated Raji cytoplasmic [^{125}I]RNA (strip a), RNase T1-treated Raji cytoplasmic [^{125}I]RNA (strip b), Raji cytoplasmic [^{125}I]RNA (strip c), total cell [^{125}I]RNA from Burkitt lymphoma biopsy D.Or. (strip d), or B95-8 EBV DNA homologous [^{125}I]cRNA (strip e). For treatment with DNase or RNase approximately 2 × 10^7 cpm of cytoplasmic [^{125}I]RNA was incubated in a final volume of 50 μl with 0.1 mg of DNase I per ml or 1,000 U of RNase T1 per ml, in 50 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–10 mM MgCl$_2$ for 30 min at 37°C. Reactions were stopped by the addition of 10 μl of 0.25 M EDTA–5% sodium dodecyl sulfate and heating at 90°C for 5 min.*

ties of the RNA (Fig. 2 and 4).

It might be possible to explain the results so far by assuming that the EBV DNA preparations contain trace amounts of a cellular DNA species which happens to produce *Eco*RI fragments of the same size as some of the B95-8 and P3HR-1 EBV DNA fragments, and that the homologous RNA makes up a substantial proportion of the cytoplasmic RNA preparations. It would seem highly improbable, however, that cleavage of EBV DNA and the putative contaminating DNA with other endonucleases like *Hin*dIII and *Bam*HI would also produce fragments of overlapping sizes. Moreover, knowing the *Eco*RI and *Hin*dIII cleavage maps of B95-8 EBV DNA (10), it is possible to predict which *Hin*dIII fragments should hybridize to the ^{125}I-labeled cytoplasmic RNA preparations. *Eco*RI fragment J, which is the major hybridizing fragment, is contained in the *Hin*dIII A fragment. Consequently, this fragment would be expected to be a major hybridizing species. When blots containing the separated fragments of B95-8 EBV DNA obtained after cleavage with *Hin*dIII and *Bam*HI were incubated under hybridization conditions with ^{125}I-labeled cytoplasmic RNA from Raji cells, specific hybridization was found

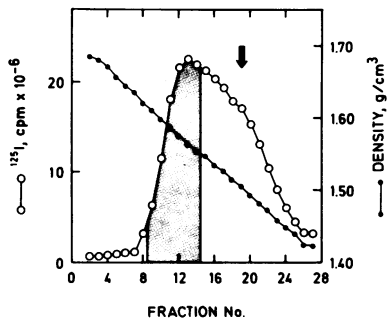

FIG. 3. *Equilibrium density gradient centrifugation in potassium iodide of ^{125}I-labeled cytoplasmic RNA from Raji cells. A 10-μg amount of cytoplasmic RNA was iodinated with ^{125}I, purified on cellulose columns supplemented with potassium iodide, and centrifuged in an MSE SW6 × 14-ml rotor for 60 h at 25,000 rpm and 20°C. Details on each step are given in the text. The position of single-stranded ^{125}I-labeled EBV DNA, which was run in a parallel gradient as a density marker, is indicated by the arrow.*

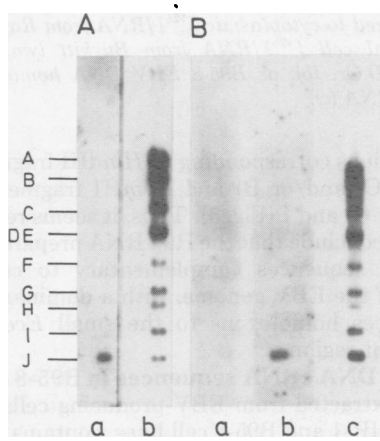

FIG. 4. *Detection of RNA sequences complementary to EcoRI fragments of B95-8 EBV DNA in Raji cells. Samples (0.2 μg) of B95-8 EBV DNA cleaved by EcoRI were applied to 0.35% agarose slab gels, electrophoresed, and transferred to nitrocellulose sheets which were cut into strips. (A) The strips were hybridized to cytoplasmic [^{125}I]RNA purified by potassium iodide density gradient centrifugation (strip a) or B95-8 EBV DNA homologous [^{125}I]cRNA (strip b). (B) The strips were hybridized to RNase T1-treated cytoplasmic [^{125}I]RNA (strip a), DNase-treated cytoplasmic [^{125}I]RNA (strip b), or B95-8 EBV DNA homologous [^{125}I]cRNA (strip c). For details see legend to Fig. 2 and text.*

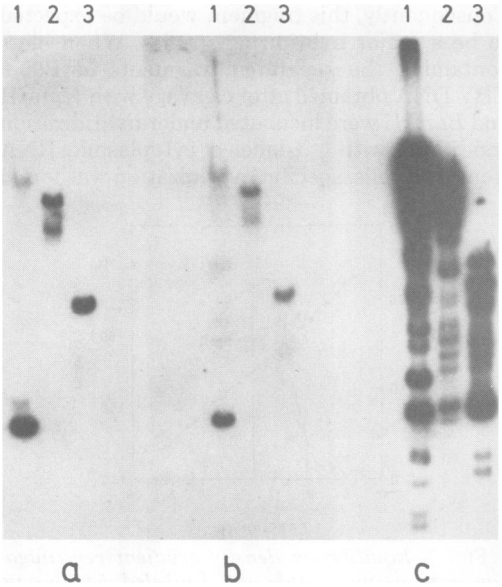

FIG. 5. *Identification in Raji cells and a Burkitt lymphoma biopsy of RNA sequences complementary to fragments of B95-8 EBV DNA obtained after cleavage with EcoRI, HindIII, or BamHI. Samples (0.2 μg) of B95-8 EBV DNA cleaved by EcoRI (lanes 1), HindIII (lanes 2), and BamHI (lanes 3) were applied to a 0.35% agarose slab gel, electrophoresed, and transferred to a nitrocellulose membrane. The membrane was cut into three similar parts which were hybridized to cytoplasmic [^{125}I]RNA from Raji cells (a), total cell [^{125}I]RNA from Burkitt lymphoma biopsy D.Or. (b), or B95-8 EBV DNA homologous [^{125}I]cRNA (c).*

in positions corresponding to *Hin*dIII fragments A and C (and/or B) and *Bam*HI fragments B (and/or C) and I (Fig. 5). Thus, it seems reasonable to conclude that the Raji RNA preparations contain sequences complementary to certain parts of the EBV genome, with a dominance of sequences homologous to the small *Eco*RI J fragment region.

EBV DNA cRNA sequences in B95-8 cells. RNA extracted from EBV-producing cells, like the P3HR-1 and B95-8 cell lines, contains RNA sequences encoded by at least 45% of the viral DNA, and most or all sequences are present in the polyadenylated, polyribosomal RNA fraction (13). Thus, iodination and hybridization of RNA extracted from B95-8 cells to blots containing the separated *Eco*RI fragments of B95-8 EBV DNA, as described above for RNA from Raji cells, would be expected to show hybridization in positions corresponding to most or all EBV DNA fragments. The result of such a positive control experiment is shown in Fig. 6. Both cytoplasmic and nuclear RNA were analyzed. In both cases hybridization to all *Eco*RI fragments of B95-8 EBV DNA except possibly the H fragment was evident. Furthermore, proportionally more ^{125}I-labeled RNA was associated with the J fragment than with other fragments, and there seemed to be a selective enrichment of these sequences in the cytoplasm. After treatment with RNase T1, the B95-8 EBV [^{125}I]RNA no longer hybridized to any of the B95-8 EBV DNA fragments, whereas incubation with DNase I did not abolish the hybridizing ability of the RNA preparation (data not shown).

EBV DNA cRNA sequences in Burkitt lymphoma biopsies. EBV RNA expressed in vivo in Burkitt lymphoma cells has been characterized by iodination and hybridization, as described above for RNA in Raji cells.

Total RNA extracted from four frozen Burkitt lymphoma biopsies was iodinated and incubated under hybridization conditions, with blots containing the separated fragments of *Eco*RI-cleaved B95-8 EBV DNA (Fig. 7). The results showed a pattern qualitatively very much like

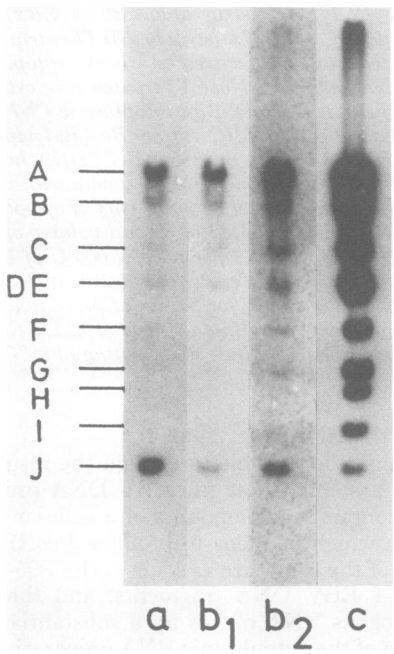

FIG. 6. *Identification of RNA sequences complementary to EcoRI fragments of B95-8 EBV DNA in B95-8 cells. Samples (0.2 μg) of B95-8 EBV DNA cleaved by EcoRI were applied to a 0.35% agarose slab gel, electrophoresed, and transferred to a nitrocellulose sheet. The sheet was cut into strips which were hybridized to B95-8 cytoplasmic [^{125}I]RNA (strip a), B95-8 nuclear [^{125}I]RNA (strips b_1 and b_2), or nick-translated ^{125}I-labeled B95-8 EBV DNA (strip c). Strips b_1 and b_2 represent fluorograms of the same strip exposed for different times.*

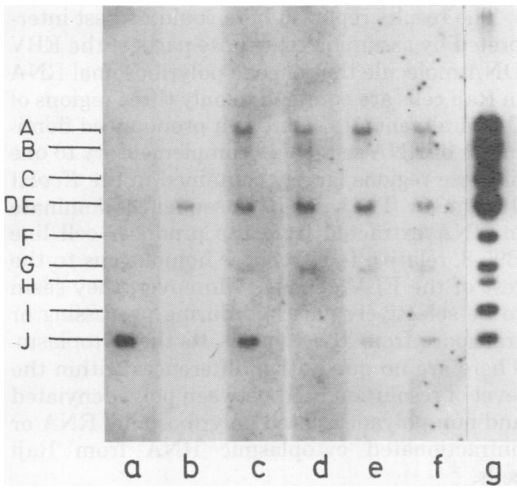

FIG. 7. *Identification of RNA sequences complementary to EcoRI fragments of B95-8 EBV DNA in Burkitt lymphoma biopsies. Samples (0.2 μg) of B95-8 EBV DNA cleaved by EcoRI were applied to a 0.35% agarose slab gel, electrophoresed, and transferred to a nitrocellulose sheet. The sheet was cut into strips which were hybridized to Raji cytoplasmic [^{125}I]RNA (strip a), polyribosomal [^{125}I]RNA from Burkitt lymphoma biopsy D.Om. (strip b), total cell [^{125}I]RNA from Burkitt lymphoma biopsies D.Or. (strip c), T.M. (strip d), K.K. (strip e), and I.M. (strip f), or B95-8 EBV DNA homologous [^{125}I]cRNA (strip g).*

that obtained with polysomal Raji RNA. All four total RNA preparations hybridized to *Eco*RI fragments A, B, E (and/or D), and G, and all four preparations could also be demonstrated to contain sequences complementary to the *Eco*RI J fragment, albeit at varying and lower relative ratios than Raji RNA. Incubation of one of these RNA preparations with blots containing the *Hin*dIII and *Bam*HI fragments of B95-8 EBV DNA revealed hybridization in positions corresponding to *Hin*dIII fragments A and C (and/or B) and *Bam*HI fragments B (and/or C) and I (Fig. 5).

Polyribosomal RNA prepared from a fifth frozen Burkitt lymphoma biopsy was iodinated and incubated under hybridization conditions with blots containing the *Eco*RI fragments of B95-8 EBV DNA (Fig. 7). Hybridization to fragments A, E (and/or D), G, and J could be demonstrated after long exposure times.

DISCUSSION

Lymphoid cells from Burkitt tumor biopsies, cell lines established from such tumors, and lymphoid cells transformed in vitro by EBV characteristically contain multiple copies of the complete EBV genome present as covalently closed circular DNA, as well as integrated viral DNA sequences (14). So far, EBV-transformed cells that carry only a fraction of the viral genome have not been identified with certainty, in contrast to the situation for papovavirus- and adenovirus-transformed cells. In these cases certain parts of the viral genomes could be defined as responsible for the transformed cell phenotype by analyzing which viral DNA sequences were present in different lines of virus-transformed cells (12, 29). Obviously this approach is not presently applicable to EBV-transformed cells. Instead, identification of the gene(s) coding for functions necessary for the maintenance of the transformed state of such cells might be derived from a characterization of viral mRNA sequences expressed in different EBV-transformed cell lines and tumor cells. There are, however, two complicating factors to this endeavor. First, no efficient producer system for EBV is available, making EBV DNA a limiting reagent. Second, the minute amounts of RNA sequences complementary to EBV DNA present in the transformed cells make their identification difficult. In the present study these problems were dealt with by using the Southern technique of transferring DNA from agarose gels to nitrocellulose filters and by hybridizing filters with cell RNA preparations isotope-labeled in vitro to high specific activities. This was achieved by the iodination of RNA with ^{125}I to obtain preparations of 1×10^7 to 5×10^7 cpm/μg. It is known that ^{125}I-labeled RNA tends to give high background levels in hybridization reactions (30). All [^{125}I]RNA preparations used in this investigation were therefore purified by chromatography on cellulose columns, which decreased hybridization backgrounds to levels where EBV-specific sequences in cell RNA could be detected on Southern blots.

I feel confident that the radioactive material binding to the DNA fragments on the nitrocellulose membranes represents virus-specific RNA, although I have not been able to reisolate enough hybrid material for a further characterization. However, the material is RNase sensitive and DNase resistant and has a buoyant density characteristic of RNA. It also binds to filters containing the separated fragments of EBV DNA obtained after cleavage with *Eco*RI and *Hin*dIII in a way consistent with the physical map of EBV DNA, showing that the hybridizing material is specific for defined parts of the virus genome. If EBV DNA was a significant contaminant of the RNA preparations, it would also be labeled by the iodination procedure and hybridize to the EBV DNA fragments on the blots. It is hard to rationalize, however, a pref-

erential purification and labeling of only parts of the putative contaminating EBV DNA, resulting in the dominant hybridization to the *Eco*RI J fragment observed here.

The hybridization data obtained with the *Eco*RI, *Hin*dIII, and *Bam*HI fragments of B95-8 EBV DNA allowed the construction of a map of viral RNA sequences present in the cytoplasm of Raji cells consistent with all results, assuming that RNA complementary to adjacent restriction endonuclease fragments belong to the same transcription unit (Fig. 8). Obviously this map is rather speculative and needs confirmation on many points, but it might serve as a schematical representation of the findings presented in this paper. First, the most abundant viral RNA sequences present in Raji cells are homologous to *Eco*RI fragment J. This fragment is adjacent to the *Eco*RI A fragment, which also contains sequences homologous to viral RNA. Since I cannot detect RNA sequences hybridizing to the 10-fold-redundant *Bam*HI S fragment, which is contained within the left part of *Eco*RI fragment A, I assume that only a small part of the very left end of fragment A is transcribed. Second, *Eco*RI fragments B and E are adjacent to each other, and their intervening cleavage site is contained within the *Hin*dIII C fragment. Since I find RNA hybridizing to these three fragments but not to *Hin*dIII fragments I and L on either side of the C fragment or to the *Hin*dIII D fragment, which contains most of the *Eco*RI D fragment, I define the second transcribed region as corresponding to *Hin*dIII fragment C. Third, a small amount of RNA hybridizes to *Eco*RI fragment G_1 or G_2. Since I failed to detect RNA sequences homologous to *Hin*dIII fragments G and J and my Southern blots are consistent with the possibility that some hybridization to the *Hin*dIII B fragment is obscured by a more prominent hybridization to the C fragment (Fig. 5), I position the third transcribed region as corresponding to *Eco*RI fragment G_1. The right end of the adjacent *Eco*RI A fragment might be included in this region.

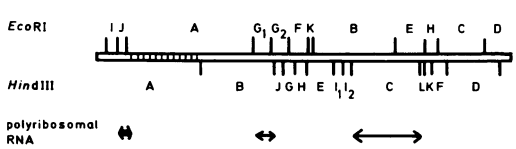

FIG. 8. *Map of B95-8 EBV DNA sequences expressed in Raji cells. The linkage map of the EcoRI and HindIII cleavage fragments is that of Given and Kieff (10). The position of repetitious DNA corresponding to the BamHI S fragment is indicated by the vertical lines within the open bar. The arrows represent the tentative map positions of the B95-8 EBV DNA transcripts found in Raji cells.*

The results reported here could be best interpreted by assuming that those parts of the EBV DNA molecule that encode polyribosomal RNA in Raji cells are confined to only three regions of the viral genome. There is a pronounced dominance of RNA sequences complementary to one of these regions largely contained in the *Eco*RI J fragment. The same RNA sequences dominate in RNA extracted from the producer cell line B95-8, relative to sequences homologous to the rest of the EBV genome. Moreover, they seem to be selectively enriched during processing or transport from the nucleus to the cytoplasm. There are no qualitative differences within the level of resolution used between polyadenylated and non-polyadenylated polyribosomal RNA or unfractionated cytoplasmic RNA from Raji cells.

A striking aspect of this work is the finding that total cell RNA extracted from Burkitt lymphoma biopsies contains viral RNA sequences homologous to the same fragments of EBV DNA as polyribosomal RNA from Raji cells. RNA sequences complementary to the *Eco*RI J fragment were detected in all five biopsies investigated, although at varying and lower relative ratios than in Raji cells. The map of virus-specific polyribosomal RNA in Raji cells shown in Fig. 8 might thus also be valid for RNA extracted from Burkitt lymphoma tumors, suggesting that a minimum of three regions of the EBV genome are expressed in transformed cells. These regions comprise, from left to right, approximately 3, 4, and 18% of the viral genome, respectively, corresponding to the information content of 4,500, 6,000, and 27,000 DNA base pairs. The results presented do not allow us to single out any of these regions as the location for a putative "transformation gene(s)." It is of interest to note, however, that only 50% of papovavirus DNA (molecular weight, 3.6×10^6), 5 to 10% of adenovirus DNA (molecular weight, 22.9×10^6), and 1 to 2% of herpes simplex virus DNA (molecular weight, 100×10^6) are at most required to maintain a transformed state (12, 17, 29).

Since the *Eco*RI J fragment of B95-8 EBV DNA encodes RNA sequences abundant in transformed cell lines and also present in tumor cells in vivo, one might ask if this part of the genome contains the gene for the EBV-associated nuclear antigen (EBNA), a virus-related antigen with properties similar to those of the large T-antigens of smaller DNA tumor viruses. Certainly the information content of the J fragment is high enough for a protein the size of EBNA, which is comprised of presumably identical subunits having molecular weights of 53,000 (20). Two further pieces of evidence provide support for the assumption that the *Eco*RI J

fragment might contain sequences which code for EBNA. (i) When intracellular EBV DNA was partly purified from 23 EBNA-positive cell lines or biopsies of Burkitt lymphoma, nasopharyngeal carcinoma, infectious mononucleosis, or healthy carrier origin and analyzed by *Eco*RI endonuclease cleavage, considerable sequence variability between different EBV DNA isolates was found, but the *Eco*RI J fragment region was always conserved in apparently unaltered form (L. Rymo, T. Lindahl, and A. Adams, Proc. Natl. Acad. Sci. U.S.A., in press). (ii) A few human lymphoid cell lines of B-cell type have been established which are EBNA negative and do not contain EBV DNA in amounts high enough to be detected by the standard hybridization techniques employed (2, 16). Fragments of EBV DNA comprising 10% or less of the genome would, however, have escaped detection. We have analyzed RNA extracted from such an EBNA-negative cell line (Ramos) with the techniques described above. Although there seemed to be small amounts of RNA sequences homologous to certain limited parts of the EBV genome present in these cells, no RNA species homologous to the *Eco*RI J fragment could be detected (Rymo, unpublished data).

In a recently published paper Powell et al. (26) have reported results indicating that EBV-specific polyadenylated, polyribosomal RNA in Namalwa cells is encoded primarily by 0.6×10^6 daltons of a 2.0×10^6-dalton segment of B95-8 EBV DNA, the *Bam*I S fragment, which is tandemly reiterated approximately 10 times in the *Eco*RI A fragment of the genome, and to a lesser extent by DNA in the *Eco*RI B and *Hsu*I D fragments. Polyadenylated, polyribosomal RNA in Raji cells was found to contain sequences homologous to a similar fraction of the *Eco*RI A fragment. Dambaugh et al. (5) reported that RNA extracted from Burkitt lymphoma biopsies contains viral RNA sequences homologous primarily to the *Hsu*I A, B, and D fragments of B95-8 EBV DNA and also an abundant RNA species encoded by the additional DNA present in the W91 isolate of EBV. Both studies failed, however, to detect RNA sequences complementary to the *Eco*RI J fragment region of B95-8 EBV DNA. Obviously, these results are at variance with the results reported in this paper. The iodination procedure used by us for in vitro labeling of RNA should give a uniform distribution of isotope in all RNA species present in the mixture. Consequently, the relative amount of radioactivity bound to each DNA band on the Southern blots after hybridization, measured here by fluorography, should reflect the product of abundance and size of each transcript, assuming an approximately equal efficiency of transfer of all EBV DNA fragments to the blots. The conclusion of our results thus must be that the *Eco*RI J fragment of B95-8 EBV DNA encodes an RNA species present in high abundance in Raji cells and also present in Burkitt tumor cells in vivo. It is conceivable that the more indirect method to identify virus-specific RNA used by Kieff and collaborators, namely to select for the fraction of ^{32}P-labeled nick-translated EBV DNA homologous to different cell RNA preparations and to use that material as a hybridization probe on Southern blots containing separated EBV DNA fragments, might have distorted the quantitative aspects of the transcription patterns. Isolated restriction endonuclease fragments of EBV DNA were also used in those studies to show by hybridization in solution that Namalwa and Raji polyadenylated, polyribosomal RNAs are homologous to approximately 25% of the *Eco*RI and *Hsu*I A fragments, mostly contained in the *Bam*I S fragment, and that RNA in Burkitt tumor tissue is homologous to a sizeable fraction of the *Hsu*I D fragment. These results are compatible with my data only if so few copies of RNA sequences complementary to the *Bam*I S and *Hsu*I D fragments are present in Raji and Burkitt lymphoma cells that my method of detection fails to detect them, in which case they cannot be major transcripts. Further work is needed to investigate these observed differences and to give a more detailed map of the parts of the EBV genome that are expressed in transformed cells. Such studies would be greatly facilitated if larger quantities of EBV DNA were made available, e.g., by cloning of EBV DNA fragments in a bacterial plasmid.

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