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Epstein-Barr Virus-Specific RNA

II. Analysis of Polyadenylated Viral RNA in Restringent, Abortive, and Productive Infections

TESSA ORELLANA AND ELLIOTT KIEFF*

Section of Infectious Disease, Departments of Medicine and Microbiology, The Committee on Virology, University of Chicago, Chicago, Illinois 60637

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The complexity and abundance of Epstein-Barr virus (EBV)-specific RNA in cell cultures restringently, abortively, and productively infected with EBV has been analyzed by hybridization of the infected cell RNA with purified viral DNA. The data indicate the following. (i) Cultures containing productively infected cells contain viral RNA encoded by at least 45% of EBV DNA, and almost all of the species of viral RNA are present in the polyadenylated and polyribosomal RNA fractions. (ii) Restringently infected Namalwa and Raji cultures, which contain only intranuclear antigen, EBNA, and enhanced capacity for growth in vitro, contain EBV RNA encoded by at least 16 and 30% of the EBV DNA, respectively. The polyadenylated and polyribosomal RNA fractions of Raji and Namalwa cells are enriched for a class of EBV RNA encoded by approximately 5% of EBV DNA. The same EBV DNA sequences encode the polyadenylated and polyribosomal RNA of both Raji and Namalwa cells. (iii) After superinfection of Raji cultures with EBV (HR-1), the abortively infected cells contain RNA encoded by at least 41% of EBV DNA. The polyadenylated RNA of superinfected Raji cells is enriched for a class of EBV RNA encoded by approximately 20% of EBV HR-1 DNA. Summation hybridization experiments suggest that the polyadenylated RNA in superinfected Raji cells is encoded by the same DNA sequences as encode RNA present in Raji cells before superinfection, most of which is not polyadenylated. That the same EBV RNA sequences are present in the polyadenylated and polyribosomal fractions of two independently derived, restringently infected cell lines suggests that these RNAs may specify functions related to maintenance of the transformed state. The complexity of this class of RNA is adequate to specify a sequence of at least 5,000 amino acids. That only some RNA species are polyadenylated in restringent and abortive infection suggests that polyadenylation or whatever determines polyadenylation may play a role in the restricted expression of the EBV genome.

Lymphocytes infected with Epstein-Barr virus (EBV) acquire the capacity to be grown as continuous cell lines (3, 6, 7, 18, 37, 38) and contain an intranuclear antigen, EBNA (29, 42). Most EBV-infected cell lines contain multiple copies of more than 90% of the EBV genome (21, 23, 34, 35, 41). In one instance, i.e., the Namalwa cell line, analysis of the complexity of EBV DNA suggested the presence of one to two copies of 60% of the EBV genome (41). Most of the EBV-infected cell lines do not contain progeny virus or antigens associated with productive infection (24). Expression of EBV DNA in these cells must therefore be under tight control which permits the expression of a few viral functions, i.e., EBNA and cellular growth enhancement, while prohibiting the expression of many others. This state of EBV infection is referred to to as restringent. Some of these cell lines can be induced by treatment with chemical inducers (5, 13) or by superinfection (19) to enter an abortive state of virus replication characterized by the production of new membrane and cytoplasmic antigens (8) and by cytocidal effects (9). Incubation of superinfected cells in phosphate-free media (50) causes an otherwise abortive infection to become productive of progeny virus. Several of the EBV-infected cell lines, including the HR-1 (20) and B95-8 (33) lines, contain subpopulations, which produce progeny virus and express antigens associated with productive infection. Inhibition of viral DNA synthesis causes this productive infection to become abortive, and release from inhibition allows a return to the productive state (10). These data indicate that there are at

least three stages of expression of EBV in lymphoblastoid cell lines.

Studies of viral RNA have been undertaken to determine the biochemical mechanisms involved in the control of expression of the EBV genome (15, 16, 39). Analysis of the complexity and abundance of viral RNA in restringently and productively infected cultures (15) indicates that (i) HR-1 cultures that contain productively infected cells contain viral RNA encoded by at least 45% of EBV DNA (90% of the genome if the RNA is asymmetrically transcribed). Approximately 5×10^{-3}% of the RNA in HR-1 cultures is viral. Almost all of the viral RNA species are detectable in the polyribosomal fraction of HR-1 cells. (ii) In contrast, five independently derived, restringently infected cultures contain 0.6×10^{-3} to 1.5×10^{-3}% viral RNA encoded by as much as 26% of viral DNA. In these instances, the polyribosomal fraction contains RNA encoded by only 3 to 9% of EBV DNA. Therefore, in restringent infection there are two classes of viral RNA: those species that associate with polyribosomes and those species that are specifically excluded from polyribosomes. The latter class of RNA is either not used for translation or has a short functional half-life.

The purpose of the experiments reported in this paper is to deal with three issues raised by the previous data: (i) whether the RNA species selected for association with the polyribosomes in independently derived, restringently infected cultures are encoded by the same DNA sequences; (ii) whether there is a correlation between the class of RNA selected for association with polyribosomes and its polyadenylation; and (iii) whether there is a change in the transcription or polyadenylation of viral RNA after the transition of infection from restringent to abortive.

(The results of these studies were reported at the 76th Annual Meeting of the American Society for Microbiology, May 1976.)

MATERIALS AND METHODS

Cells. Continuous cultures of Raji, Namalwa, HR-1 (obtained from G. Klein, Karolinska Institute, Stockholm, Sweden), and B95-8 (obtained from G. Miller, Yale University, New Haven, Conn.) cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (both obtained from Grand Island Biological Corp., Grand Island, N.Y.). Between 5 and 10% of the cells in the HR-1 and B95-8 cultures contained viral capsid antigen (17). Cells were harvested 2 days after replenishment.

Raji superinfection. EBV was concentrated from 4 liters of clarified (by centrifugation at $600 \times g$ for 10 min) HR-1 culture supernatant by centrifugation at $15,000 \times g$ for 90 min. The virus pellet was suspended in RPMI 1640, homogenized in a 30-ml, tight-fitting glass Dounce homogenizer, and passed through a 0.45-μm nitrocellulose filter (HAWP, Millipore Corp., Bedford, Mass.). Raji cells (5×10^8) in log phase were gently pelleted, suspended to 5×10^6 cells/ml in the HR-1 virus suspension, and incubated with shaking at 37°C. After 2 h, cells were gently pelleted, resuspended to 5×10^5 cells/ml in fresh RPMI 1640 with 15% fetal calf serum, incubated 37°C, and harvested between 36 and 48 h after infection. Superinfected cultures contained between 7 and 18% early antigen (19)-positive cells at the time of harvest.

Preparation of total cellular RNA. Cell pellets were suspended at 4°C in a 30-fold volume of a solution consisting of 0.1 M NaCl, 0.01 M EDTA, and 0.02 M sodium acetate at pH 5.5 and were lysed by incubation in 0.5% sodium dodecyl sulfate at 60°C for 10 min. The lysate was extracted at 55°C twice with equal volumes of water-saturated redistilled phenol and chloroform containing 2% (vol/vol) isoamyl alcohol and three times with an equal volume of chloroform containing 2% (vol/vol) isoamyl alcohol. The combined phenol and chloroform-isoamyl alcohol phases, the interphases, and the first chloroform-isoamyl alcohol phase were washed with an equal volume of a solution containing 0.1 M NaCl, 0.01 M EDTA, and 0.05 Tris-hydrochloride, pH 9.0 (36). The nucleic acid was precipitated overnight with 2 volumes of ethanol at −20°C and resuspended to a concentration of 5 mg/ml in a solution consisting of 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.01 M NaCl, and 0.01 M Tris-hydrochloride, pH 7.4. DNA was digested by three cycles of incubation of the nucleic acid with 50 μg of iodoacetate-treated (51) DNase per ml (DNase I, Worthington Biochemical Corp., Freehold, N.J.) at 37°C for 45 min. After each incubation with DNase, the enzyme was inactivated by incubation in 0.5% sodium dodecyl sulfate at 60°C and extracted with phenol and chloroform-isoamyl alcohol as described previously. The nucleic acid solution was adjusted to 0.15 M NaCl and precipitated overnight with 2 volumes of ethanol at −20°C. Approximately 3 mg of RNA was obtained from 5×10^8 cells.

Preparation of polysomal RNA. The techniques used for isolation of polyribosomes from the cytoplasm of Raji and Namalwa cells have been described in an earlier publication (15). The purification of RNA from polyribosomes followed the scheme described for the preparation of total cellular RNA, except that the polyribosomal nucleic acid preparations were treated with only two 30-min cycles of incubation with iodoacetate-treated DNase.

Isolation of poly(A) and non-poly(A) RNA. Total cellular nucleic acid was prepared by sodium dodecyl sulfate, phenol, and chloroform-isoamyl alcohol extractions, precipitated, resuspended, and incubated with iodoacetate-treated DNase for 10 min under the conditions described above. Polyadenylated [poly(A)] RNA was isolated by oligodeoxythymidylate [oligo(dT)]-cellulose column chromatography (1, 43), a procedure that separates RNA with at least 35 adenylic acid residues at the 3′ terminus (2, 11). Briefly, 4 g of oligo(dT)-cellulose (T3, Collaborative

Research, Waltham, Mass.), suspended in loading buffer that consisted of 0.4 M NaCl, 0.005 M EDTA, 0.1% sodium dodecyl sulfate, and 0.01 M Tris-hydrochloride, pH 7.4, was poured into a 2.5-cm-diameter glass column (Bio-Rad Corp., Richmond, Calif.) and washed with loading buffer followed by 0.1 N NaOH. The column was stored between use at 4°C in 0.2% sodium azide and washed with 0.1 N NaOH and loading buffer prior to each usage. RNA at a concentration of 2 mg/ml in loading buffer was passed through the column four times. Unbound RNA, enriched for non-poly(A) RNA and RNA with short polyadenylate sequences [RNA minus poly(A) RNA], was removed by washing with 10 to 20 column volumes of loading buffer, adjusted to 0.15 M NaCl, and precipitated with 2 volumes of ethanol at −20°C. Poly(A)-enriched RNA was eluted in a buffer consisting of 0.05% sodium dodecyl sulfate and 0.01 M Tris, pH 7.4, adjusted to 0.4 M NaCl, and rechromatographed at approximately 50 μg/ml as described. After the second elution, the poly(A) RNA was adjusted to 0.15 M NaCl and precipitated with 2 volumes of ethanol at −20°C. At this stage the poly(A) RNA, which contained less than 0.1% DNA, was resuspended to a concentration of 0.5 mg/ml, treated for 20 min with DNase, phenol extracted, and reprecipitated as described previously. The crude RNA minus poly(A) RNA, which contained up to 20% DNA, was resuspended to a concentration of 5 mg/ml, and the DNA was digested by three 45-min cycles of incubation with DNase as described previously.

Preparation of ^{3}H-labeled EBV (HR-1) DNA. The procedures used in the preparation of ^{3}H-labeled EBV (HR-1) DNA have been described previously (40). Briefly, DNA was extracted from virus that was purified from the extracellular fluid of HR-1 cultures by velocity sedimentation in 10 to 50% sucrose gradients. The DNA was further purified by isopycnic centrifugation in neutral cesium chloride gradients. DNA preparations that contained a single UV-absorbing peak at 1.718 g/cm^3 in the analytical centrifuge (model E, Beckman Corp.) were labeled in vitro with [^{3}H]thymidine 5'-triphosphate (specific activity, 50 Ci/mmol; New England Nuclear Corp., Boston, Mass.) by nicked translation (22, 40) using *Escherichia coli* DNA polymerase I (obtained from Boehringer-Mannheim Corp.). The concentration of DNase used was adequate to yield denatured single-strand fragments approximately 350 bases long. Labeled DNA was dialyzed against a solution consisting of 0.28 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride, pH 7.2 (hybridization buffer), with 20% (wt/vol) Chelex (Bio-Rad Corp.) to remove heavy metals (49). The specific activity of the labeled DNA was approximately 4×10^6 cpm/μg. Less than 5% of denatured ^{3}H-labeled EBV (HR-1) DNA was resistant to digestion with S1 nuclease. Hybridization of denatured ^{3}H-labeled EBV (HR-1) DNA with unlabeled EBV (HR-1) DNA rendered the ^{3}H-labeled EBV (HR-1) DNA more than 90% resistant to S1 nuclease.

Preparation of S1 nuclease. All steps (46, 48) were carried out at 4°C. Thirty grams of alpha-amylase (type IV-A alpha-amylase, Sigma Corp., St. Louis, Mo.) was dissolved for 1 h in 200 ml of 0.01 M sodium phosphate buffer, pH 7.0, and then clarified by centrifugation at 8,000 × *g* for 15 min. The supernatant was stirred with ammonium sulfate (47.8 g/100 ml of enzyme solution) for 90 min, and the precipitate was removed by centrifugation at 8,000 × *g* for 10 min. The supernatant was exhaustively dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, and loaded onto a 100-g DEAE-cellulose column (DE52, Whatman). The column was serially washed with 0.5 liter of 5% (vol/vol) glycerol in 0.01 M sodium phosphate, pH 7.0, 0.5 liter of 5% glycerol in 0.05 M NaCl and 0.01 M potassium phosphate, pH 7.0, and 1 liter of 5% glycerol in 0.1 M NaCl and 0.02 M potassium phosphate, pH 7.0. Single-stranded nuclease activity was eluted with a solution consisting of 5% glycerol, 0.2 M NaCl, and 0.04 M potassium phosphate, pH 7.0. Fractions containing nuclease activity sufficient to digest more than 95% of labeled single-stranded DNA and less than 2% of labeled double-stranded DNA were pooled and stored at −20°C in 50% glycerol.

Hybridization conditions. Glassware and micropipettes were washed with nitric acid and Chelex-treated water and then autoclaved. Solutions were passed through a column of Chelex resin and autoclaved. RNAs [10 to 25 mg of total cellular RNA or RNA minus poly(A) RNA per ml, 5 to 20 mg of polyribosomal RNA per ml, or 1 to 2 mg of poly(A) RNA per ml] in hybridization buffer containing 5% formamide (vol/vol) and 0.01 to 0.02 μg of ^{3}H-labeled EBV (HR-1) DNA per ml were sealed into 10-μl micropipettes, heated to 105°C for 5 min, incubated at 65°C (30) for intervals of up to 24 h, frozen at −70°C, stored at −20°C, and assayed as a group for content of residual single-stranded DNA. Single-stranded DNA was differentiated from DNA-DNA and DNA-RNA hybrids by digestion of single-stranded DNA with S1 nuclease. Each 10-μl sample was diluted 1:170 in a solution containing 50 μg of native and 20 μg of denatured calf thymus DNA per ml, 0.0006 M zinc sulfate, 0.1 M NaCl, and 0.025 M potassium acetate, pH 4.5. Two-thirds of the sample was divided into two portions, which were each incubated with 10 μl of S1 nuclease for 2 h at 43°C to determine the amount of nuclease-resistant ^{3}H-labeled EBV (HR-1) DNA. The third portion was used to determine the total amount of ^{3}H-labeled EBV (HR-1) DNA. The three portions of each sample were individually precipitated at 4°C in 5% trichloroacetic acid and filtered onto 0.45-μm nitrocellulose filters, and the radioactivity was determined in a liquid scintillation spectrometer (LS230, Beckman Corp.). Each point on the hybridization curve represents an average of the two determinations of the nuclease-resistant fraction of ^{3}H-labeled EBV (HR-1) DNA.

Two types of control experiments were included with each hybridization. The extent of self-hybridization of ^{3}H-labeled EBV (HR-1) DNA, determined by hybridizations in which yeast RNA or RNA extracted from a lymphoblastoid cell line that does not contain EBV DNA (41) was substituted for sample RNA, varied from 4 to 6% at 24 h of incubation. The variation was less than 1% for experiments using the same preparation of ^{3}H-labeled EBV (HR-1) DNA. The extent of self-hybridization of probe RNA determined in this type of control (which was run

with each experiment) was subtracted from the observed values for DNA-RNA hybridizations as described in detail previously (15). The second type of control was to determine whether the EBV homologous nucleic acid in the sample was RNA rather than DNA. Samples of each RNA preparation were incubated at 37°C in 0.3 M KOH for 18 to 24 h prior to hybridization. The alkali-treated samples were hybridized to ^{3}H-labeled EBV (HR-1) DNA and processed simultaneously with the non-alkali-treated samples. In none of the alkali-treated preparations was the extent of hybridization greater than that observed for ^{3}H-labeled EBV (HR-1) DNA incubated in the presence of yeast RNA, thus indicating that there was no EBV homologous DNA in the RNA preparations. For summation hybridization experiments in which mixtures of RNAs were incubated with ^{3}H-labeled EBV (HR-1) DNA, controls included simultaneous testing of each of the component RNAs.

Analysis of hybridization kinetics. The complexity and abundance of viral RNA in total cellular, polyribosomal, and poly(A) RNA were determined from an analysis of the kinetics of hybridization of the RNAs to in vitro labeled viral DNA (4, 15). The data for the hybridization of viral RNA, in excess, to viral DNA should follow the kinetic curve for a second-order chemical reaction in which one of the reactants is present in excess. The data were analyzed by nonlinear least-squares regression to the equation (4)

$$D_t/D_0 = \sum_{I=1}^{n} A_I(1 - e^{-B_I t}) \quad (1)$$

which assumes for each series of calculations, the existence of n classes of viral-specific RNA in which the members of each class differ from members of the other classes in the number of copies. D_t/D_0 is the fraction of DNA hybridized at each time t. A_I is the fraction of DNA from which the class of RNA$_I$ is transcribed. B_I is a composite term, which is proportional to the number of copies of RNA$_I$ and equals the reaction rate constant, K_I, for DNA-RNA hybridization with nucleic acid of complexity A_I multiplied by the concentration, R_I, of RNA$_I$. The curves drawn in each figure represent the regression curves obtained by this analysis. In each instance, the data fit the model. The curve fit for $n = 2$ was not significantly different from that obtained when $n = 1$. The assumptions made in calculation of the concentrations of viral RNA have been described previously (4, 15).

Summation hybridization data were analyzed by comparison of the data obtained with mixtures of RNAs to that expected if the RNAs were encoded by the same or different DNA. If RNA$_a$ and RNA$_b$ were encoded by different DNA sequences, the reaction of the mixture of RNAs with EBV DNA would be expected to follow the curve

$$D_t/D_0 = \sum_{a=1}^{n} A_a(1 - e^{-K_a R_a t}) + \sum_{b=1}^{n} A_b(1 - e^{-K_b R_b t}) \quad (2)$$

in which a are the classes of RNA$_a$ and b are the classes of RNA$_b$. If the DNA sequences encoding RNA$_a$ were a subset of those encoding RNA$_b$, the reaction would be expected to follow a curve

$$D_t/D_0 = \sum_{b=1}^{n} A_b[1 - e^{-K_b(R_{ab} + R_b)t}] \quad (3)$$

in which b are the classes of RNA$_b$, R_{ab} and R_b are the concentrations in RNA$_a$ and RNA$_b$ of each class of RNA$_b$.

Since the proportion of viral specific RNA in total cellular RNA was small and the RNA concentration could not be increased above 25 mg/ml without a marked increase in viscosity, it was not possible to use sufficient concentrations of the individual RNAs to drive the reactions to completion in summation hybridizations using total cellular RNA. The data obtained from the summation hybridizations with total cellular RNA were therefore compared (44) to those obtained for each component RNA hybridized in parallel, using equations 2 and 3 to project the results anticipated, if the two RNAs arose from overlapping (Table 2, column X) or exclusive (Table 2, column Y) regions of the genome.

In those instances, i.e., the analyses of polyribosomal and poly(A) RNAs, in which the proportion of viral-specific RNAs was sufficiently high and the values of Bt correspondingly large for a 20-h interval of reaction, equation 2 reduces to

$$D_{20\,h}/D_0 = \sum_{a=1}^{n} A_a + \sum_{b=1}^{n} A_b$$

and equation 3 to

$$D_{20\,h}/D_0 = \sum_{b=1}^{n} A_b$$

In these instances, the data obtained for the mixtures was directly compared to that obtained in the same experiment for individual RNAs (44).

RESULTS

The complexity and abundance of viral RNA in productive, abortive, and restringent infection were determined by analysis of the hybridization of infected-cell RNA to labeled viral DNA.

EBV RNA in productive infection. Virus-producing B95-8 and HR-1 cultures in which 5 to 10% of the cells are productively infected contain RNA encoded by at least 43 (Fig. 1A) and 45% (15) of EBV (HR-1) DNA, respectively. The proportion of viral RNA in these cultures is between 4×10^{-3} and 9×10^{-3}% of the total cellular RNA (Table 1; 15). The poly(A) RNA fractions of B95-8 and HR-1 cultures contain viral RNA arising from at least 42% of EBV (HR-1) DNA and are three- to eightfold enriched for virus-specific sequences (Fig. 1C and 2B, Table 1). After removal of poly(A) RNAs by oligo(dT)-cellulose chromatography, the B95-8 and HR-1 poly(A[−]) RNAs contain a lower concentration of viral RNA but seem to contain all of the viral RNA species [RNA minus poly(A) RNA; Fig. 1B and 2A, Table 1].

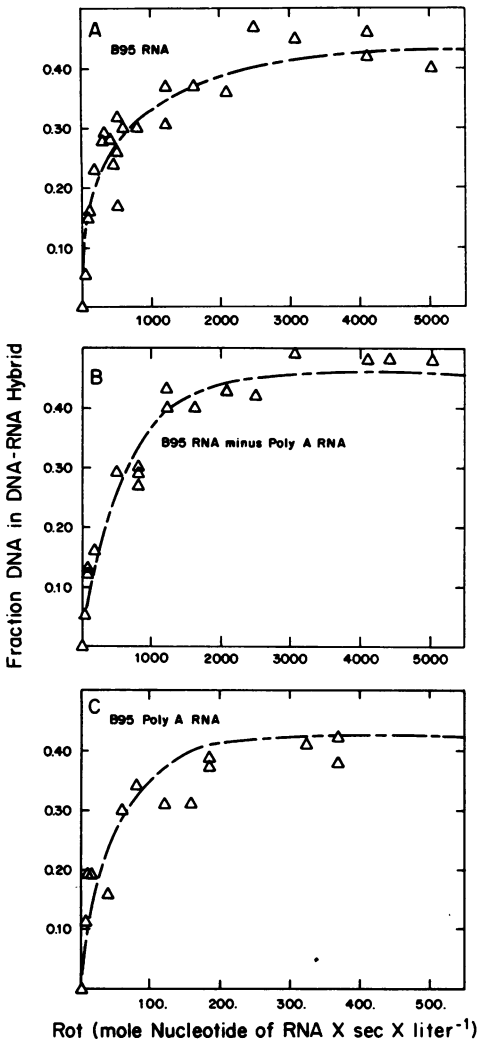

FIG. 1. *Hybridization of unlabeled RNAs extracted from B95-8 cells with denatured* ^{3}*H-labeled EBV (HR-1) DNA. (A) Total cellular RNA; (B) RNA minus poly(A) RNA; (C) poly(A) RNA.*

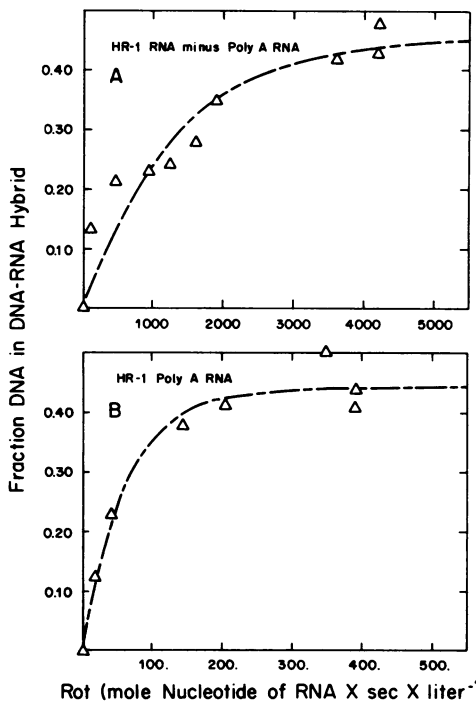

FIG. 2. *Hybridization of unlabeled RNAs extracted from HR-1 cells with denatured* ^{3}*H-labeled EBV (HR-1) DNA. (A) RNA minus poly(A) RNA; (B) poly(A) RNA.*

EBV RNA in restringent infection. Namalwa and Raji cells contain viral RNA encoded by at least 16% (Fig. 3A; 15) and 30% (Fig. 4A; 15) of EBV (HR-1) DNA. The proportion of virus-specific RNA in these cells is estimated to be approximately 10^{-3}% (Table 1; 15). Summation hybridization experiments with artificial mixtures of Raji and Namalwa RNAs suggest that the viral RNA in Namalwa cells is encoded by the same DNA sequences as many of the RNA species of Raji cells (Table 2). The poly(A) RNA preparations from Namalwa (Fig. 3D) and Raji (Fig. 4D) cells are selectively enriched in a class of viral RNA encoded by only 5 to 7% of EBV (HR-1) DNA. From the kinetics of hybridization of poly(A) RNA to ^{3}H-labeled EBV (HR-1) DNA, the proportion of viral specific RNA in the poly(A) RNA preparations is estimated to be 4×10^{-3} to 7×10^{-3}% (Table 1). RNAs encoded by 4 to 6% of EBV DNA are detected in the polyribosomal RNA preparations from Namalwa (Fig. 3B; 15) and Raji cells (Fig. 4B; 15).

Summation hybridzation tests in which artificial mixtures of poly(A) and polyribosomal RNAs of Raji and Namalwa cells were hybridized to in vitro labeled viral DNA were undertaken to determine if the viral RNA sequences found in these fractions of Raji and Namalwa cells were encoded by the same DNA sequences. The concentration of each of the RNA species was sufficiently high to ensure that the reaction with DNA would be near completion in the reaction interval tested. A mixture of the two RNAs, therefore, would be expected to drive the DNA encoding each into hybrid. As described in Materials and Methods, if the RNA species were encoded by different DNA sequences, the fraction of DNA driven into hybrid by the RNA mixtures would be the sum of the fractions of DNA driven into hybrid by each RNA. If the RNAs were encoded by the same DNA sequences, the observed rate of hybridiza-

TABLE 1. *EBV-specific antigens and viral RNA in lymphoblastoid cells*

Source of RNA	Viral antigen content of cultures[a]			% EBV (HR-1) DNA encoding RNA	% of RNA prepn estimated to be viral specific ($\times 10^{-3}$)
	EBNA	EA	VCA		
B95	+	+	+		
TC[b]				42	9
PA(−)[c]				46	3
PA(+)[d]				42	26
HR-1	+	+	+		
PA(−)				46	1.7
PA(+)				44	33
Namalwa	+	−	−		
TC				16	1
PA(−)				12	0.3
PA(+)				7	7
Polyribosomal				6	
Raji	+	−	−		
TC				32	0.7
PA(−)				22	0.6
PA(+)				5	4
Polyribosomal				4	
Raji superinfected	+	+	−		
PA(−)				41	1
PA(+)				20	22

[a] EBNA, intranuclear antigen; EA, early antigen; VCA, viral capsid antigen.
[b] TC, Total cellular RNA.
[c] PA(−), RNA minus poly(A) RNA.
[d] PA(+), Poly(A) RNA.

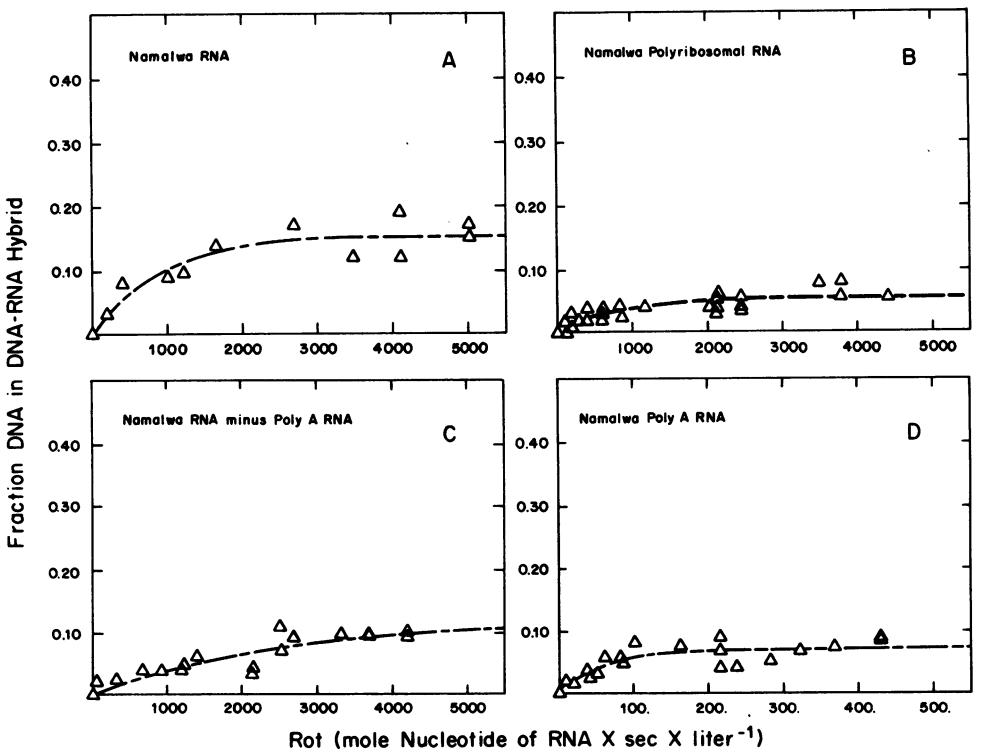

FIG. 3. *Hybridization of unlabeled RNAs extracted from restringently infected Namalwa cells with denatured ^{3}H-labeled EBV (HR-1) DNA. (A) Total cellular RNA; (B) polyribosomal RNA; (C) RNA minus poly(A) RNA; (D) poly(A) RNA.*

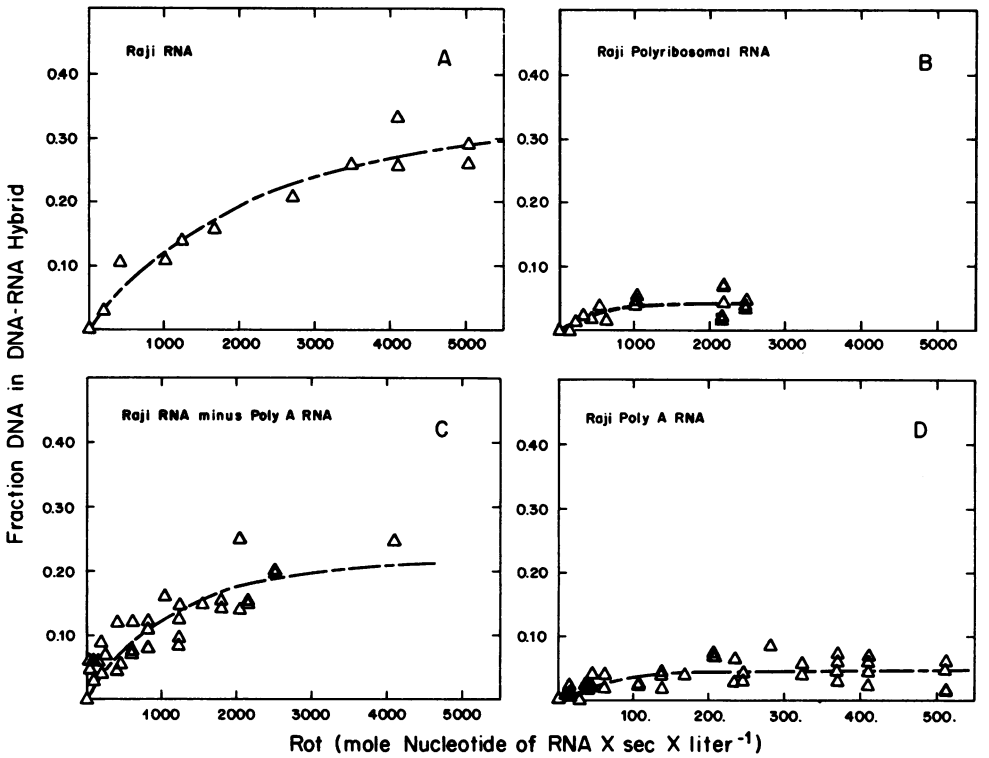

FIG. 4. *Hybridization of unlabeled RNAs extracted from restringently infected Raji cells with denatured ^{3}H-labeled EBV (HR-1) DNA. (A) Total cellular RNA; (B) polyribosomal RNA; (C) RNA minus poly(A) RNA; (D) poly(A) RNA.*

TABLE 2. *Summation hybridizations*

RNA prepn		% Labeled DNA in DNA-RNA hybrid[a]			Predicted value of % labeled DNA in DNA-RNA hybrid[b]		*P* value for rejection of Y[c]
A	B	A	B	Mixture A and B	X	Y	
Raji total cellular	Namalwa total cellular	16	13	19	23	29	0.9
Raji polysomal	Namalwa polysomal	4	4	4	5	8	0.999
Raji poly(A)	Namalwa poly(A)	6	6	5	6	12	0.999
Raji poly(A)	Raji polysomal	6	4	6	5	10	0.999
Raji total cellular	Superinfected Raji poly(A)	16	16	22	24	32	0.99

[a] The values of R_0t for each cellular, polyribosomal, and poly(A) RNA component of the reactions were 1,750, 1,200, and 200 mol nucleotide of RNA × s × liter^{-1}, respectively.

[b] The procedure used in projecting values for the two models is described in the text. Model X is based on the assumption that RNAs A and B are encoded by the same DNA sequences. Model Y is based on the assumption that RNAs A and B are encoded by different DNA sequences.

[c] Obtained by comparison of data observed for mixture with the sum of the observed values for A and B (44).

tion would be a function of the concentration of the common viral RNA species in each of the components of the mixture, and the fraction of DNA hybridized at high values of R_0t should be close to that of the component encoded by the larger fraction of the DNA. In each case, i.e., summation hybridizations with mixtures of Raji and Namalwa polyribosomal RNA, with Raji and Namalwa poly(A) RNA, and with Raji poly(A) and polyribosomal RNA, the observed data indicate that the RNA in the polyribosomal and poly(A) RNA fractions of Raji and

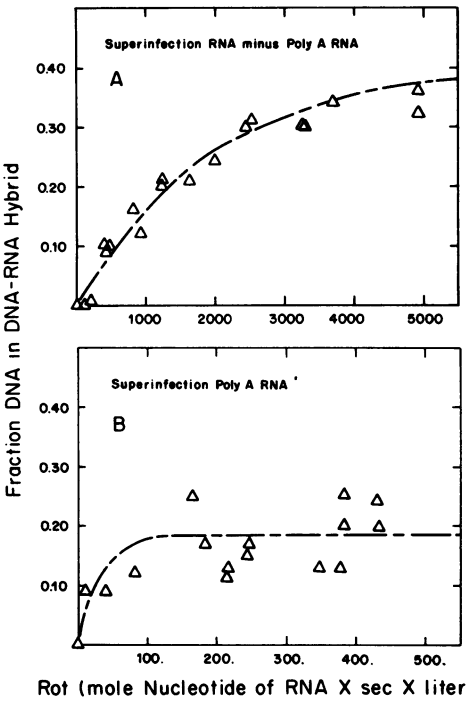

FIG. 5. *Hybridization of unlabeled RNAs extracted from superinfected (abortively infected) Raji cells with denatured ^{3}H-labeled EBV (HR-1) DNA. (A) RNA minus poly(A) RNA; (B) poly(A) RNA.*

Namalwa cells are encoded largely by the same sequences (Table 2). In each instance, the data were incompatible with the hypothesis that the RNAs are encoded by different viral DNA sequences ($P > 0.999$) (44) and fit closely the hypothesis that the RNAs are encoded by the same DNA sequences.

EBV-specific RNA in superinfected Raji cells. After superinfection, 7 to 18% of the Raji cells enter an abortive state of virus infection characterized by the production of early antigen. The complexity of viral RNA in Raji cells after superinfection is increased, and RNA species encoded by at least 41% (Fig. 5A) of EBV (HR-1) DNA are present. Poly(A) RNA from superinfected cells is markedly enriched for a class of RNA encoded by approximately 20% of EBV (HR-1) DNA (Fig. 5B, Table 1). Summation hybridization experiments in which poly(A) RNA from superinfected cells was mixed with total cellular RNA from Raji cells before superinfection indicate that most of the RNA species found in the poly(A) fraction after superinfection arise from the same DNA as RNA present in Raji cells prior to superinfection (Table 2).

DISCUSSION

Most nonhistone mRNA's of eukaryotic cells and many viral mRNA's have been shown to contain polyadenylic acid at their 3' terminus (28). The role of this post-transcriptional modification in the processing and function of mRNA is uncertain, although data suggest that it may be related to transport (32) or stability (27, 31) of the RNA. Previous analyses of viral RNA in cells transformed by EBV (15), adenovirus 12 (12), and simian virus 40 (26) demonstrated differential processing of viral RNAs such that species of viral RNA arising from a restricted region of the viral DNA selectively accumulate on polyribosomes, whereas other viral RNAs do not. In this paper, we report the results of analyses of poly(A) viral RNA in lymphocytes restringently, abortively, and productively infected with EBV. Several issues pertinent to these analyses require discussion.

(i) It is clear from these data and those reported previously that viral RNA is processed differently in restringent (15) infection than in productive infection. In restringent infection, Raji and Namalwa cells contain RNA encoded by a large (mostly common) segment of the EBV genome (16 to 30% of the DNA, or 32 to 60% of the genome if the RNA is an asymmetric copy of the double-stranded DNA), and a class of viral RNA encoded by 5 to 7% of the DNA is selectively polyadenylated. This same class of RNA selectively associates with polyribosomes. That only those species of viral RNA that associate with polyribosomes are polyadenylated suggests that polyadenylation (or whatever determines polyadenylation) may determine the fate of viral RNA in restringent infection. In contrast, productively infected cultures contain RNA encoded by at least 45% of EBV DNA (90% of the genome if the RNA is an asymmetric copy). Almost all of these RNA species are polyadenylated and can be identified on polyribosomes.

(ii) After superinfection, restringently infected Raji cells enter an abortive cycle characterized by the production of new membrane and cytoplasmic antigens, cytocidal effects, and viral RNA encoded by at least 41% of EBV DNA (82% of the genome, if transcription is asymmetric). As in restringent infection, in abortive infection only a class of viral RNA is polyadenylated. Summation hybridizations with mixtures of Raji total cellular RNA and superinfected Raji poly(A) RNA suggest that many of the RNA species that are not polyadenylated in Raji cells before superinfection are polyadenylated after superinfection and, conversely, that most of the sequences that are polyadenylated in superinfected cells are present in Raji cells before superinfection.

Studies by others (14) of the effect of actinomycin D on induction of abortive infection in cultures of Raji cells suggested that new RNA synthesis is required for the transition from restringent to abortive infection. The simplest interpretation of these data and those we report here is that either pre-existing RNA cannot be polyadenylated or that transcription of a viral regulatory gene may be necessary for the transition to abortive infection.

(iii) Three groups of summation hybridization data indicate that the RNA sequences that are polyadenylated and associated with polyribosomes of the independently derived, restringently infected Raji and Namalwa cell lines are encoded by the same EBV DNA sequences. Thus, artificial mixtures of the poly(A) RNAs of Raji and Namalwa cells, of polyribosomal RNAs of Raji and Namalwa cells, or of poly(A) and polyribosomal RNAs of Raji cells hybridize to the same fraction of EBV DNA as either of the RNAs alone, indicating that these RNAs are all encoded by the same DNA sequences. That the same sequences are present in the polyribosomal and poly(A) fraction of Raji and Namalwa adds weight to the suggestion (15) that the function of this RNA may be related to maintenance of the transformed state.

(iv) The complexity of viral poly(A) and polyribosomal RNAs from restringently infected cells is adequate to specify a sequence of at least 5,000 amino acids. The EBNA complex is the only antigen that has been identified to date in these cells. EBNA contains at least one soluble component estimated to be 180×10^3 daltons by sedimentation in sucrose velocity gradients (25). The relationship of EBNA to EBV-induced enhancement of cell growth or to the reduced serum dependence observed after infection of cell lines with EBV (45) is not clear. Although the presence of EBNA in all EBV-infected cell lines suggests that EBNA may be necessary for growth enhancement, indirect evidence suggests that it may not be sufficient (47).

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