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Striking Similarities Are Exhibited by Two Small Epstein-Barr Virus-Encoded Ribonucleic Acids and the Adenovirus-Associated Ribonucleic Acids VAI and VAII

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The nucleotide sequence of the region of the Epstein-Barr virus genome that specifies two small ribonucleic acids (RNAs), EBER 1 and EBER 2, has been determined. Both of these RNAs are encoded by the right-hand 1,000 base pairs of the *Eco*RI J fragment of EBV deoxyribonucleic acid. EBER 1 is 166 (167) nucleotides long and EBER 2 is 172 ± 1 nucleotides long; the heterogeneity resides at the 3' termini. The EBER genes are separated by 161 base pairs and are transcribed from the same deoxyribonucleic acid strand. In vitro, both EBER genes can be transcribed by RNA polymerase III; sequences homologous to previously identified RNA polymerase III intragenic transcription control regions are present. Striking similarities are therefore apparent both between the EBERs and the two adenovirus-associated RNAs, VAI and VAII, and between the regions of the two viral genomes that specify these small RNAs. We have shown that VAII RNA as well as VAI RNA and the EBERs exist in ribonucleoprotein complexes which are precipitable by anti-La antibodies associated with systemic lupus erythematosus. Finally, we have demonstrated that the binding of protein(s) from uninfected cells confers antigenicity on each of the four virus-encoded small RNAs.

Patients suffering from the rheumatic disease systemic lupus erythematosus produce antibodies against a variety of cellular antigens (24). It has recently been established that the antigens recognized by four of these autoantibody classes are small ribonucleoprotein (RNP) complexes (19). The molecular identities of the ribonucleic acid (RNA) components of the particles bound by anti-Sm have been shown to be the highly abundant small nuclear RNA species U1, U2, U4, U5, and U6 (20). The U1-containing particle is also immunoprecipitated by anti-RNP antibodies (20). Antibodies to the La and Ro antigens precipitate distinctly different spectra of RNP particles (19). The La antigen is found not only on particles in uninfected mammalian cells but also on protein-nucleic acid complexes containing the adenovirus type 2-encoded VAI RNA and the two small Epstein-Barr virus (EBV)-encoded RNAs, EBER 1 and EBER 2 (18).

Mapping of the viral transcripts synthesized in the EBV-transformed human nonproducer cell line Raji (27) has identified the most heavily transcribed region of the EBV genome as a 3,000-base pair (bp) *Eco*RI restriction fragment (*Eco*RI-J), located close to the left end of the viral deoxyribonucleic acid (DNA) molecule (6, 28). A preliminary characterization (18) of the two EBERs determined that their coding regions are also located within the *Eco*RI J fragment. The two RNAs have different T_1 ribonuclease (RNase) fingerprints and are thus distinct species; neither of the approximately 175-nucleotide-long RNAs is capped or polyadenylated.

Here, we have determined the nucleotide sequence of the region of the EBV genome that specifies the two EBERs and shown that these RNAs can be transcribed in vitro by RNA polymerase III. The parallels that we were able to draw between the EBERs and the two small adenovirus-associated RNAs, VAI and VAII, prompted us to ascertain that VAII RNA, as well as VAI RNA, can exist in an anti-La immunoprecipitable RNP complex. Finally, we have demonstrated that normal cellular protein(s) is sufficient for recognition by the anti-La antibody of RNP particles containing either the EBERs or VA RNAs.

MATERIALS AND METHODS

Cell line maintenance. Raji, B95-8, and BJAB-B1 cells were obtained from G. Miller (Yale University). HeLa cells were derived from standard laboratory stocks. Cell lines were maintained at 37°C, 5% CO_2, at approximately 2×10^5 cells per ml in RPMI 1640

medium supplemented with 5% heat-inactivated bobby calf serum (GIBCO Laboratories) and 60 μg of penicillin and 100 μg of streptomycin per ml.

Viral infection. Actively growing HeLa cells were seeded at a concentration of 5×10^7 cells per ml in medium containing the normal level of phosphate. Cells were incubated in the presence of 100 plaque-forming units of adenovirus type 2 per cell. After infection, cells were washed twice and subsequently diluted 100-fold with phosphate minus-minimal essential medium (GIBCO) supplemented with 5% dialyzed heat-inactivated bobby calf serum and 60 μg of penicillin and 100 μg of streptomycin per ml. Cells were labeled with $^{32}PO_4$ 1 h postinfection and harvested approximately 17 h later. EBER-producing cells are chronically infected with EBV and hence do not require infection before labeling.

Preparation of ^{32}P-labeled RNA. Cell sonicates were prepared from approximately 10^8 cells by the method of Lerner et al. (18). RNAs were prepared by phenol extraction and ethanol precipitation of the antibody-precipitated RNP complexes. RNA from EBV-transformed cells was fractionated on 5% polyacrylamide (20:1, acrylamide-bisacrylamide) gels in 8.3 M urea–100 mM tris(hydroxymethyl)aminomethane (Tris)-borate (pH 8.3)–2 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA). RNA from adenovirus-infected cells was fractionated on a 12% polyacrylamide (29:1, acrylamide-bisacrylamide)–7 M urea gel in 100 mM Tris-borate (pH 8.3)–2 mM Na_2EDTA after denaturation of the RNA at 65°C for 3 min in the presence of 90% formamide. EBER 1, EBER 2, VAI, and VAII RNAs were extracted from gel slices by the crush and soak method (22).

Recombinant DNA. A molecular clone which we have designated pEBV HIC contains the *Bam*HI C restriction fragment of the EBV strain B95-8 in pBR322 and was obtained from J. L. Strominger (Harvard Medical School). The plasmid pEBV HIC was used to transform *Escherichia coli* LE392. Ampicillin-resistant tetracycline-sensitive colonies were picked and grown at 37°C before isolation and purification of plasmid DNA. The *Eco*RI J fragment of the EBV genome is contained within the *Bam*HI C fragment (6, 28). A molecular clone, pEBV RIJ, was generated by ligation of the *Eco*RI J fragment into pBR325. A 0.5-μg amount of *Eco*RI-cleaved pBR325 (dephosphorylated) and 1 μg of *Eco*RI-cleaved pEBV HIC were incubated with 10 U of T4 DNA ligase (P-L Biochemicals) in 20 μl of 50 mM Tris-hydrochloride (pH 7.5)–1 mM Na_2EDTA–10 mM $MgCl_2$–10 mM dithiothreitol–30 μM adenosine 5′-triphosphate (ATP) overnight at 15°C. After transformation of *E. coli* LE392, chloramphenicol-sensitive, tetracycline-resistant colonies were selected and analyzed by the minilysate protocol of Birnboim and Doly (4).

Restriction endonuclease mapping of the EBV *Eco*RI J fragment. A 10-μg amount of pEBV RIJ DNA was cleaved by *Eco*RI, treated with bacterial alkaline phosphatase, and 5′ ^{32}P end labeled by T4 polynucleotide kinase by the method of Maxam and Gilbert (22). The two 5′ ^{32}P-end-labeled termini of the EBV *Eco*RI J fragment were separated after incubation with *Sst*I. Restriction endonuclease maps of these two EBV *Eco*RI J/*Sst*I fragments were generated by the method of Smith and Birnstiel (29).

Hybridization procedures. Restriction fragments of the EBV *Eco*RI J fragment were electrophoresed on 1.4% agarose gels in 40 mM Tris-acetate (pH 7.9)–5 mM disodium acetate–1 mM Na_2EDTA and were transferred to diazobenzyloxymethyl-paper by the method of Alwine et al. (2). Hybridizations of in vivo ^{32}P-labeled EBER 1 and EBER 2 to the DNA covalently attached to diazobenzyloxymethyl-paper were performed as described by Alwine et al. (2). 5′ ^{32}P-end-labeled *Hae*III fragments of φX174 were used as markers.

DNA sequencing. DNA sequencing was performed by the Maxam and Gilbert (22) protocol.

Fingerprint analysis of RNAs. T_1 and pancreatic RNase digests were fingerprinted as described by Barrell (3), using thin-layer homochromatography on Cel PEI 300 (Brinkmann Instruments, Inc.) for the second dimension. Oligonucleotides were subsequently eluted and analyzed by digestion of T_1 spots with pancreatic RNase and digestion of pancreatic spots with T_1 RNase.

Identification of the 3′ terminal sequences of EBER 1 and EBER 2. RNA was extracted from an anti-La immunoprecipitate prepared from unlabeled B95-8 cells. The 3′ termini of the RNAs were ^{32}P-labeled with ^{32}pCp and T4 RNA ligase by the method of England and Uhlenbeck (10). EBER 1 and EBER 2 were fractionated by electrophoresis through 5% polyacrylamide–8.3 M urea gels and eluted by the crush and soak method (22). Some aliquots were subjected to total cleavage under alkaline conditions and the 3′ ^{32}P-labeled terminal nucleotides were identified after electrophoresis on Whatman 3MM paper at pH 3.5 by the method of Barrell (3). Other aliquots were analyzed by the rapid RNA sequencing method described by Donis-Keller et al. (8).

In vitro assembly of anti-La precipitable RNPs. Anti-La immunoprecipitated ^{32}P-labeled RNA (from EBV-transformed or adenovirus-infected cells) and unlabeled cell sonicates (from HeLa, EBV-transformed, or adenovirus-infected cells) were incubated together and analyzed as follows. All cell extracts were obtained from approximately 10^8 cells and fractionated over 2-ml DE-52 columns; the fraction eluting between 0.2 and 0.5 M NaCl in 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$ were used in the subsequent steps. In vivo ^{32}P-labeled RNAs were prepared by phenol extracting a DE-52 fractionated cell extract, followed by ethanol precipitation and suspension in 10 M urea. Unlabeled DE-52 fractionated cell extracts were adjusted to 2 M LiCl–5 M urea and mixed with the ^{32}P-labeled RNA in an approximately 1:1 ratio of original cell volumes. After 40 min at room temperature, the reassorted mixture was dialyzed against 40 mM Tris-hydrochloride (pH 7.5)–130 mM NaCl for 3 h at room temperature.

To make certain that the RNA molecules were not degraded during incubation, half of each reassorted mixture was treated with 100 μg of proteinase K for 1 h at 37°C before phenol extraction and ethanol precipitation of the RNA. The remaining half of the mixture was subjected to the standard immunoprecipitation treatment to assess the formation of labeled RNA-containing RNPs. Aliquots were analyzed by gel elec-

trophoresis through 5% polyacrylamide in 8.3 M urea-100 mM Tris-borate (pH 8.3)-2 mM Na_2EDTA.

Enzymes. Restriction endonucleases were purchased from New England Biolabs, Miles Laboratories, or Bethesda Research Laboratories and were used in buffers as directed by the supplier. T4 DNA ligase and T4 RNA ligase were from P-L Biochemicals. T4 polynucleotide kinase was from Boehringer Mannheim. Pancreatic and T_1 RNases were from Calbiochem. Bacterial alkaline phosphatase was a gift from J. E. Coleman (Yale University).

RESULTS

Location of the EBER genes within the EBV genome. Previous hybridization experiments had determined that the EBV-associated RNAs, EBER 1 and EBER 2, are complementary to the *Eco*RI J fragment of the 170-kilobase EBV genome (18). This 3-kilobase fragment lies within the *Bam*HI C fragment which is located near the left-hand end of the standard map of EBV DNA (Fig. 1). Before DNA sequence analysis of the EBER genes, we determined their genomic locations more precisely. A molecular clone, pEBV RIJ, containing the EBV *Eco*RI J fragment inserted into pBR325 was constructed as described above. The plasmid pEBV RIJ DNA was cleaved by *Eco*RI and *Sst*I, generating one 2,000-bp and one 1,000-bp EBV-specific fragment. Unfractionated in vivo ^{32}P-labeled EBERs hybridized to the 1,000-bp *Eco*RI/*Sst*I fragments (data not shown).

To ascertain the location of the *Eco*RI/*Sst*I 1,000-bp fragment within the EBV genome, we analyzed the plasmid pEBV HIC (see above), which contains the EBV *Bam*HI C fragment inserted into pBR322. By analysis of restriction enzyme cleavage products of pEBV HIC, we determined that the EBV *Eco*RI J/*Sst*I 1,000-bp fragment is located at the right-hand end of EBV *Eco*RI-J (Fig. 1).

Nucleotide sequence of the EBER coding region. A detailed restriction endonuclease map for the EBV *Eco*RI/*Sst*I 1,000-bp fragment was first obtained to design a strategy for determining the nucleotide sequence of the EBER genes. Figure 1 shows the sites that were utilized in sequence analysis. The DNA sequence of the 707-bp *Hae*III/*Hae*III fragment, which is contained within the EBV *Eco*RI J/*Sst*I 1,000-bp fragment, is presented in Fig. 2.

Correlation of RNA fingerprint data for EBER 1 and EBER 2 (Fig. 3) with the DNA sequence (Fig. 2) revealed that both EBER genes are contained entirely within the 707-bp

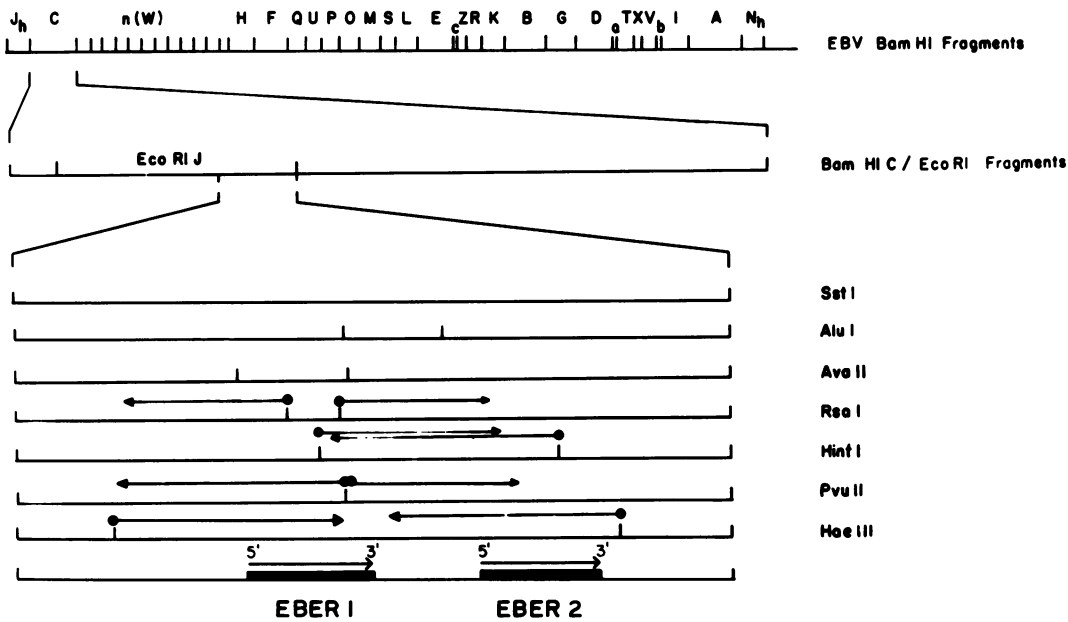

FIG. 1. *Restriction map of the coding region for EBERs. The BamHI restriction map of EBV DNA is from Skare and Strominger (28). The EBV BamHI C fragment is cleaved by EcoRI into three fragments, one which includes EcoRI-J. The cleavage map of the 1,000-bp EcoRI J/SstI fragment was determined as described in the text. The closed circles represent the sites of 5' terminal labeling by* γ-^{32}P*-labeled adenosine 5'-triphosphate; the arrows indicate the direction and the extent of the nucleotide sequence determinations. The locations of the coding regions for EBER 1 and EBER 2 are indicated by the solid boxes, together with the 5' and 3' termini of the RNAs.*

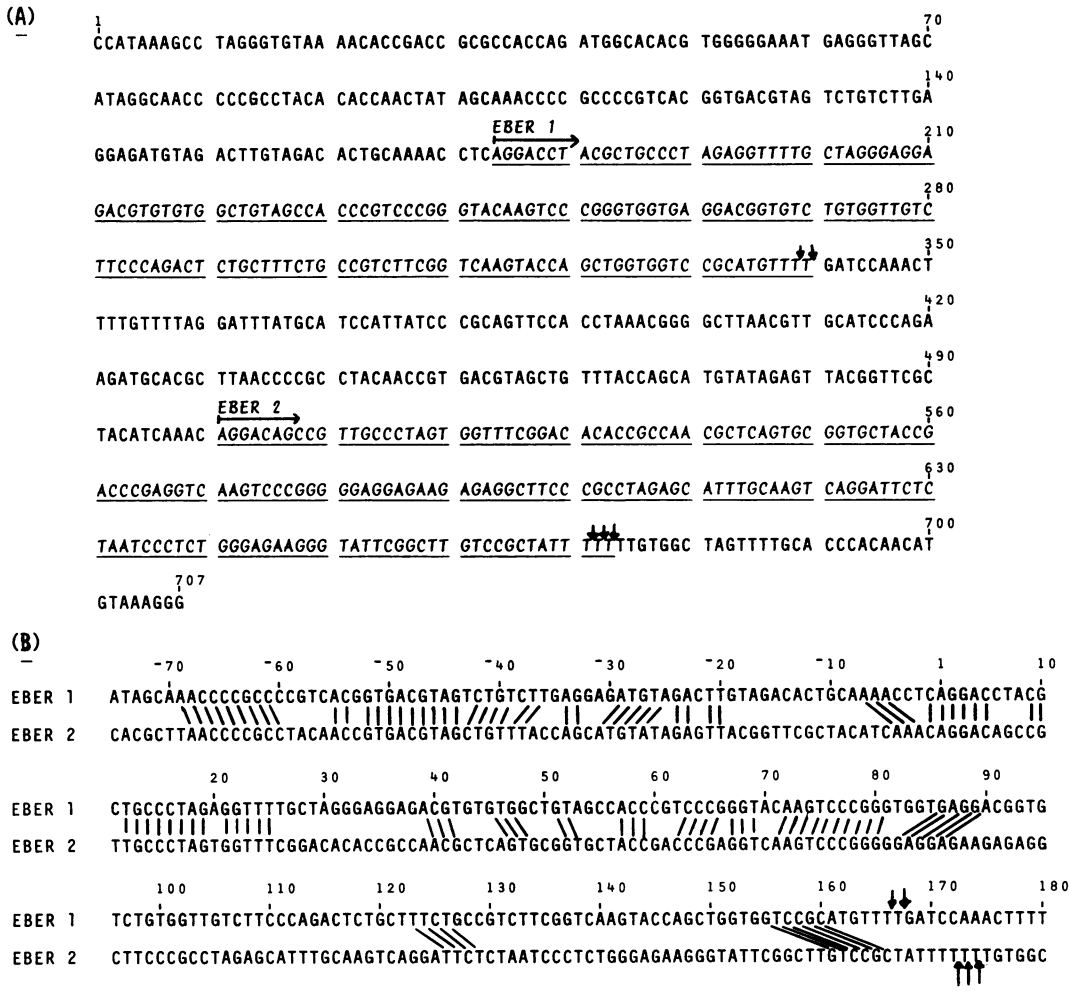

FIG. 2. *(A) Nucleotide sequence of the region on the EBV genome that encodes EBER 1 and EBER 2. The genes for the EBERs are shown underlined. The 3′ termini of the EBERs are indicated by the arrows. (B) Comparison of the primary sequences of EBER 1 and EBER 2 and of their flanking sequences, showing homologous regions where at least two consecutive bases can be matched.*

*Hae*III/*Hae*III fragment and that transcription proceeds as indicated in Fig. 1. The 5′ terminus and 2nd nucleotide of each EBER had been previously identified as pppA and G, respectively (18). Analysis of the pppA-containing spots from the pancreatic RNase fingerprints (indicated in Fig. 4B as spot 15 for EBER 1 and in Fig. 4D as spot 20 for EBER 2) revealed that the 5′ terminal sequence of both EBER 1 and EBER 2 was pppAGGAC. The EBER 1 gene is located between nucleotide 174 and approximately 340, and the EBER 2 gene has its 5′ terminus at nucleotide 501 and its 3′ terminus at approximately 672 (Fig. 2). The DNA sequence of EBER 2 predicted the presence of two long pancreatic RNase oligonucleotides, between 578 and 596 and between 641 and 651, neither of which appeared to be present in the 2nd dimension fingerprint. Since it is known that long G-rich oligonucleotides transfer in low yield from the first to the second dimension of the fingerprint fractionation, aliquots of pancreatic RNase digests of both EBER 1 and EBER 2 were subjected directly to homochromatography (the 2nd dimension). Figure 4D clearly demonstrates the presence of long pancreatic RNase-generated oligonucleotides in EBER 2; subsequent RNase T_1 digestion of these long oligonucleotides showed that a, b, and f are derived from positions 578 to 596, c corresponds to nucleotides 641 to 651, and d and e arise from the 5′ end.

The 3′ terminal nucleotide sequences of EBER 1 and EBER 2 were ascertained by analysis of the species labeled at the 3′ termini with

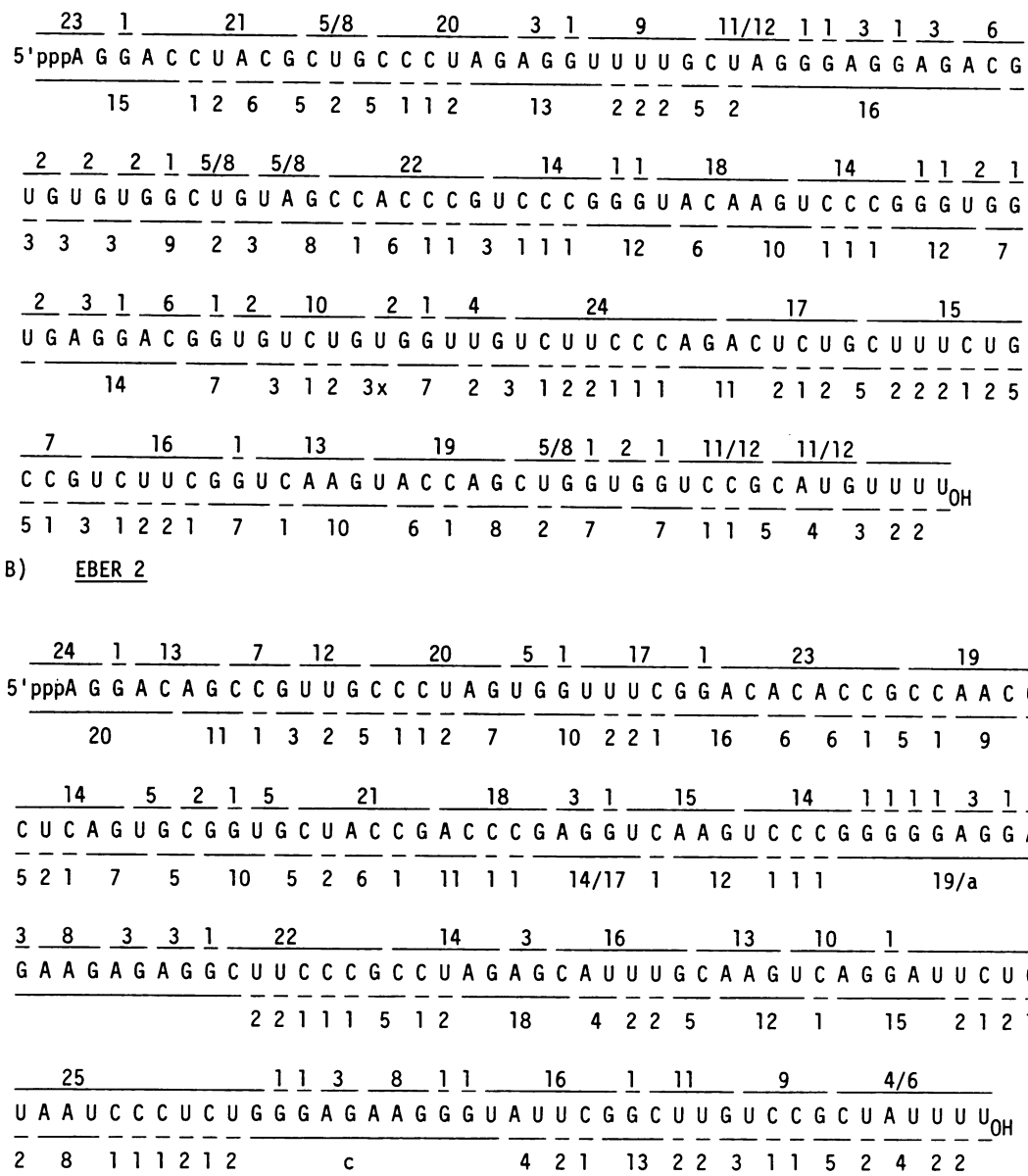

FIG. 3. *Primary structure of EBERs. The bars above and below the sequences represent fragments obtained by total T_1 RNase and total pancreatic RNase digestions, respectively. The numbers refer to the numbered oligonucleotides shown in Fig. 4. (A) EBER 1; (B) EBER 2.*

^{32}pCp, using T4 RNA ligase (data not shown). Analysis of total alkaline hydrolysates of EBER 1 and EBER 2 indicated that both RNA species terminate with a uridine residue. Yet the results of partial T_1 RNase digestions of the 3′ ^{32}P-end-labeled EBERs were consistent with both RNA species having heterogeneous termini. The partial T_1 RNase pattern for EBER 1 agreed with the pattern that would be predicted if approximately 50% of the ligated molecules have the sequence 5′-GCAUGUUU^{32}pCp, and the remaining molecules have the sequence 5′-GCAUGUUUU^{32}pCp. Thus, EBER 1 apparently terminates at both nucleotides 339 and 340

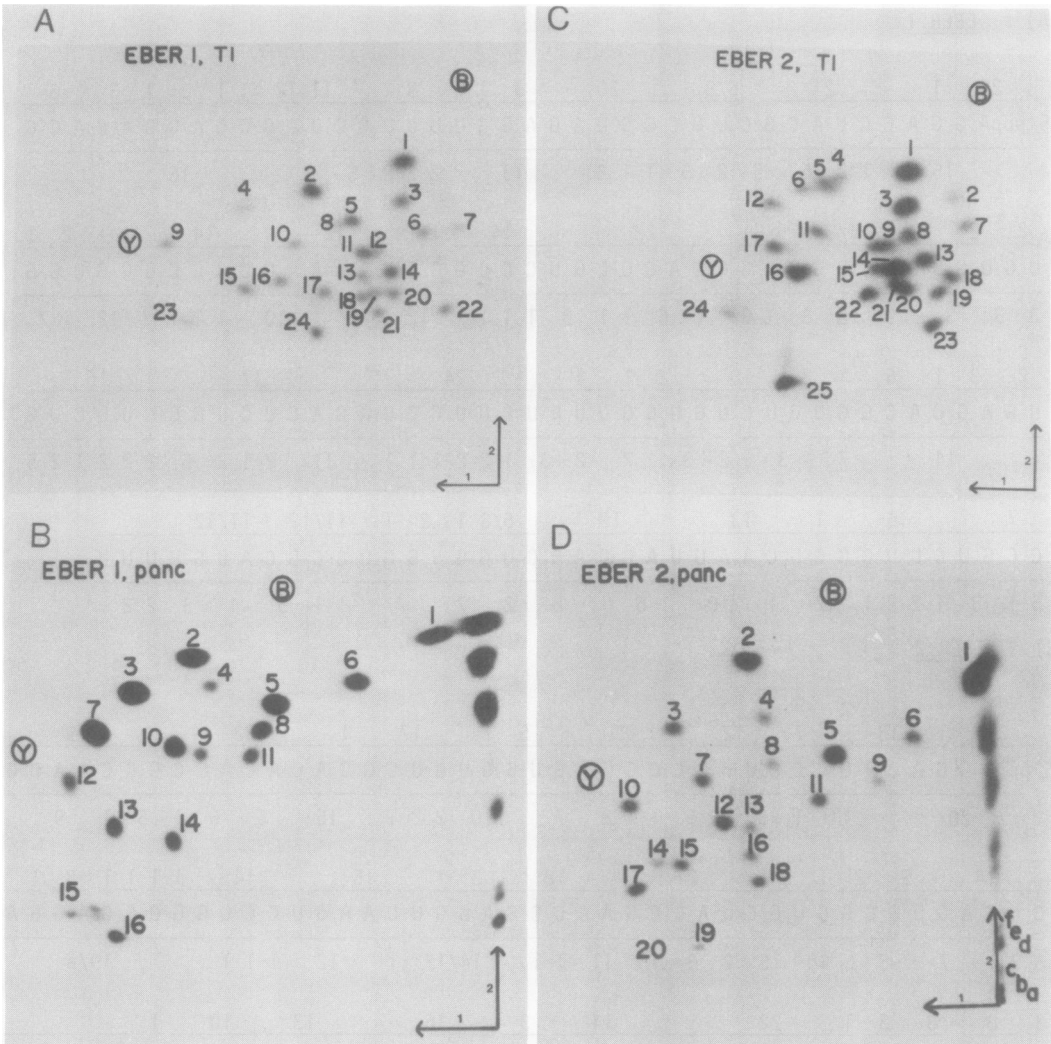

FIG. 4. *RNase fingerprints of EBER 1 and EBER 2. The RNase T_1 fingerprints (A and C) and the pancreatic RNase fingerprints (B and D) were obtained by electrophoresis from right to left and by homochromatography on PEI thin-layer plates from bottom to top, as described in the text. B and Y indicate the positions of the blue and yellow dyes in the 1st and 2nd dimensions, respectively.*

within the DNA sequence presented (Fig. 2). Similarly, the partial T_1 RNase cleavage pattern for EBER 2 would be expected if a fraction of the molecules terminates with the sequence 5'-GCUAUUU^{32}pCp, another fraction is extended by an additional uridine, and a small portion has a stretch of five uridines. Hence, EBER 2 appears to terminate at residues 671, 672, and 673 (Fig. 2). The lengths of EBER 1 and EBER 2 are therefore 166 (167) and 172 ± 1 nucleotides, respectively.

In vitro transcription of EBER 1 and EBER 2. To ascertain whether the EBERs are synthesized in vitro by RNA polymerase III, the plasmid pEBV RIJ DNA was tested for its ability to stimulate RNA synthesis in the system described by Wu (31). Figure 5 shows the specific transcription of two discrete RNA species that are insensitive to low (0.5 μg/ml) but inhibited by high concentrations (200 μg/ml) of α-amanitin. The two in vitro-synthesized RNAs comigrate with EBER 1 and EBER 2 isolated from EBV-transformed cells (data not shown).

Anti-La precipitates an RNP complex containing VAII RNA. Immunoprecipitation of adenovirus-infected cell sonicates have previously revealed that the highly abundant adenovirus-associated VAI RNA is present in anti-

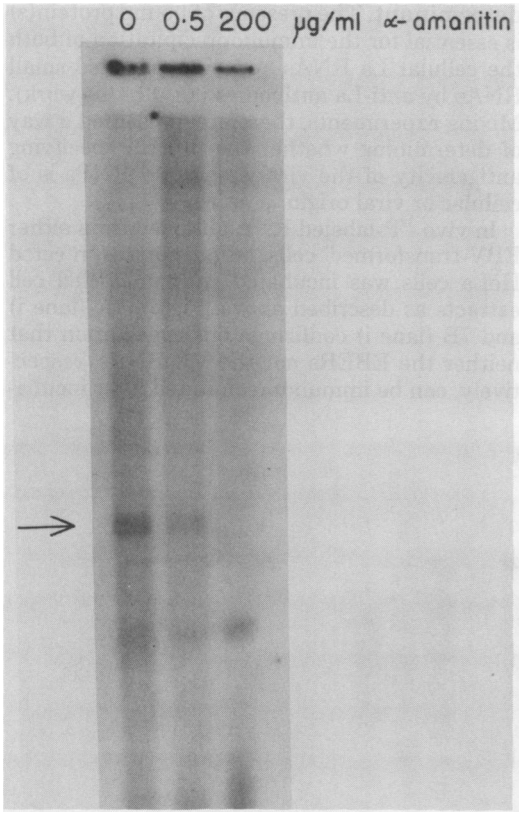

FIG. 5. *Polyacrylamide gel electrophoresis of ^{32}P-labeled RNA transcribed by RNA polymerase III in vitro. ^{32}P-labeled RNA was transcribed from pEBV-RIJ DNA using an in vitro cell-free system (31) in the presence of varying concentrations of α-amanitin and fractionated by electrophoresis through a 5% polyacrylamide gel containing 8.3 M urea. An autoradiograph of the gel is shown. The arrow marks the RNA polymerase III specific transcripts.*

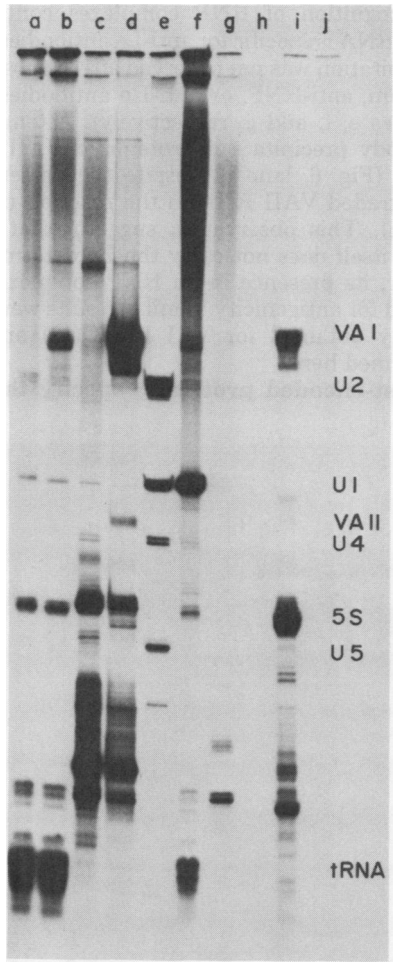

FIG. 6. *Immune precipitates of ^{32}P-labeled cell sonicates from adenovirus-infected and uninfected HeLa cells. Total small RNAs present in extracts of uninfected and adenovirus-infected HeLa cells are shown in lanes a and b, respectively. Lanes c and d show RNAs precipitated by anti-La antibody from uninfected and infected cells sonicates, respectively. In several trials, the immune precipitability of VAI and VAII RNAs varied between 20 and nearly 100%. The reasons for this variability are unclear. RNAs from virus-infected cell sonicates precipitated by anti-Sm, anti-RNP, and anti-Ro are presented in lanes e, f, and g, respectively. La-precipitable RNA found in adenovirus-infected HeLa cells was deproteinized, and reprecipitation by the anti-La serum was attempted. Precipitated RNAs are exhibited in lane j, and the RNAs remaining in the supernatant are shown in lane i. No sample was loaded in lane h.*

genic RNP complexes carrying the La determinant (19). The similarities exhibited by the two EBERs and the two VA RNAs prompted us to ascertain whether the far less abundant VAII RNA is also immunoprecipitated by anti-La antibodies.

Gel fractionation of immune precipitates of ^{32}P-labeled cell sonicates from adenovirus-infected and uninfected HeLa cells is shown in Fig. 6. In addition to the heterogeneous spectrum of RNAs precipitated by anti-La from uninfected HeLa cells (Fig. 6, lane c), two new species appeared in the lane showing RNAs from adenovirus-infected cells (Fig. 6, lane d). Subsequently, anti-La-precipitated RNA from infected cells was fractionated on a preparative gel, and the two virus-specific bands were eluted and subjected to fingerprint analysis (data not shown). Comparison of the resulting oligonucleotides with those predicted from their known nucleotide sequences (1) positively identified the two virus-specific RNA bands as VAI and VAII (see labeling in Fig. 6).

Recognition of RNP complexes containing VAII RNA is specific for anti-La antibodies since precipitation was not observed after exposure to anti-Sm, anti-RNP, or anti-Ro antibodies (Fig. 6, lanes e, f, and g, respectively). Attempts to antibody precipitate deproteinized VAII RNA failed (Fig. 6, lane j), despite the presence of undegraded VAII RNA in the reaction (Fig. 6, lane i). This observation suggests that VAII RNA itself does not carry the La determinant; rather, its presence in an RNP complex is required for antigenicity. Similar results were previously obtained for VAI RNA (19) and are confirmed here.

Host-encoded protein(s) specify the La-determinant. The presence of bound protein(s) is essential for the immunoprecipitation of both the cellular La RNAs and virus-encoded small RNAs by anti-La antibodies (18, 19; this work). Mixing experiments, therefore, presented a way of determining whether the protein specifying antigenicity of the viral associated RNPs is of cellular or viral origin.

In vivo ^{32}P-labeled RNA obtained from either EBV-transformed cells or adenovirus-infected HeLa cells was incubated with unlabeled cell extracts as described above. Figure 7A (lane i) and 7B (lane i) confirmed our expectation that neither the EBERs nor the VA RNAs, respectively, can be immunoprecipitated after incuba-

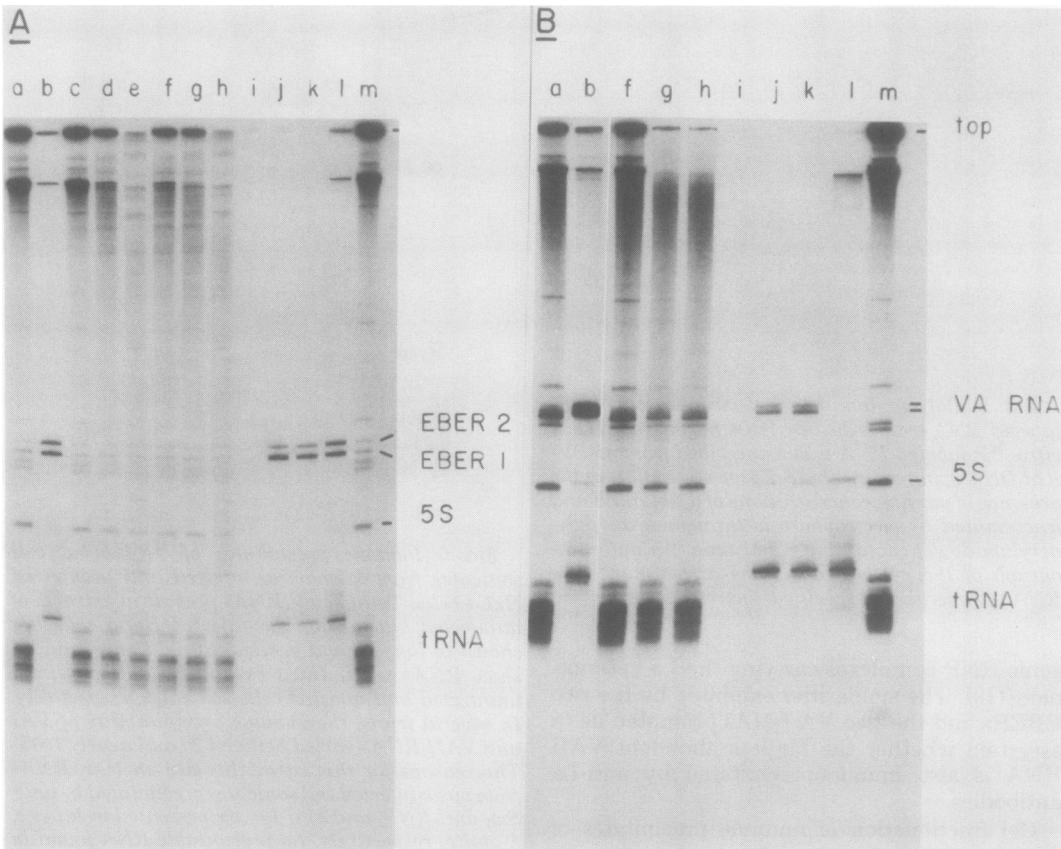

FIG. 7. *In vitro assembly of anti-La precipitable RNPs. Anti-La precipitable RNPs were assembled in vitro by mixing ^{32}P-labeled RNA isolated from (A) the EBV-transformed cell line BJAB-B1 or (B) adenovirus-infected cells and unlabeled cell sonicates as described in the text. Lanes (A) a and m and (B) a show total small RNAs from extracts of virus-infected cells. Lanes (A) b and l and (B) b show small RNAs precipitated by anti-La antibodies from extracts of virus-infected cells. Lanes (B) l and m show small RNAs precipitated by anti-La antibodies and total small RNAs, respectively, from extracts of uninfected HeLa cells. Fractions after in vitro reassembly with no (c, f, and i), virus-infected (d, g, and j), or uninfected HeLa (e, h, and k) cell sonicates are shown in the remaining lanes. Lanes c, d, and e show RNAs present in the supernatants after anti-La precipitation of the reassembled mixtures. Lanes f, g, and h show total RNAs present in the reassembled mixtures. Lanes i, j, and k show the RNA present in anti-La precipitable complexes after reassembly.*

tion in the absence of protein. However, protein required for antigenicity was present in uninfected HeLa cells, as well as in the EBV-transformed or adenovirus-infected cells (Fig. 7A, lanes k and j, and 7B, lanes k and j). Both VAI and VAII RNAs were shown, by fingerprint analysis, to be included in the adenovirus-specific RNA band labeled as VA RNA in Fig. 7B (data not shown). Conservation of the antigenic protein(s) across species is indicated by the finding that mouse (Ehrlich ascites) cell extracts can also be used to form antibody-precipitable EBER particles (data not shown). Finally, we can conclude that the EBERs and VA RNAs interact specifically with the La antigen, since the regenerated RNPs could not be precipitated by anti-Sm antibodies (data not shown).

DISCUSSION

Nucleotide sequence of the EBER coding region of the EBV genome. The nucleotide sequence of the region of the EBV genome encoding two small RNA species has been determined. Correlation with RNA fingerprint data demonstrates that the DNA sequences corresponding to EBER 1 and EBER 2 are completely contained within a 707-bp *Hae*III/*Hae*III fragment located near the right-hand end of the *Eco*RI J fragment. The 5′ terminal oligonucleotide for both EBERs is pppAGGAC. RNA sequence analyses of 3′ ^{32}P-end-labeled EBERs established that the 3′ termini of both RNAs exhibit some heterogeneity. EBER 1 and EBER 2 are 166 (167) and 172 ± 1 nucleotides long, respectively. The 3′ terminus of EBER 1 is separated by 161 bp from the 5′ terminus of EBER 2. Both RNAs are transcribed from the same DNA strand, and transcription proceeds from left to right on the standard map of the EBV genome.

Comparison between EBER 1 and EBER 2. Inspection of the primary structures of the EBER 1 and EBER 2 genes and flanking sequences (Fig. 2B) reveals several noteworthy features. The region preceding EBER 1 contains two 9-bp sequences that are present at corresponding positions before EBER 2. Explanations for this apparent homology include the possibility that the genes for EBER 1 and EBER 2 arose by duplication of a single ancestral gene. This does not, however, preclude an alternate explanation which invokes the importance of these regions in the efficiency of transcription of the two genes. Modulating effects of the 5′ flanking region have been observed in other RNA polymerase III systems (7, 30; see below). The 3′ flanking regions show little homology. Within the coding regions of EBER 1 and EBER 2, there are four regions of greatest homology: at the 5′ termini, centered around nucleotide 20, centered around nucleotide 65, and near the 3′ termini (Fig. 2B). It is probably no coincidence that two of these regions are in locations analogous to the two intragenic RNA polymerase III transcription control regions recently identified in adenovirus type 2 VAI RNA (13; see below). Using the in vitro transcription system devised by Wu (31), we have ascertained that the genes for both EBERs are transcribed by RNA polymerase III.

Parallels between the adenovirus-associated RNAs, VAI and VAII, and the EBV-encoded RNAs, EBER 1 and EBER 2. Striking similarities exist between the two small RNAs transcribed from the adenovirus genome and the two EBV-encoded RNAs. All of these RNAs are similar in size, 160 to 170 nucleotides. Secondary structures may be drawn for all four RNAs in which the 5′ termini are hydrogen bonded to regions adjacent to the 3′ termini (1; Fig. 8). The EBER genes are separated by 161 bp, whereas the VA RNA genes are separated by 98 bp. The four genes are all transcribed in vitro by RNA polymerase III. The two VA RNAs and the two EBERs are transcribed in the same direction from the same strand of the adenovirus and EBV genomes, respectively. Comparison of the nucleotide sequences of the coding regions for the four RNAs indicates that the two intragenic transcription control regions in VAI RNA (13) have homologs in the other three RNAs. The nucleotide sequences for these regions are shown in Fig. 9, along with sequences from two murine cell-encoded RNAs that also become involved in small RNPs precipitable by anti-La. It is not yet understood how these two intragenic transcription control regions function.

Despite the parallels that can be drawn between the EBV- and adenovirus-derived RNAs, the viruses that encode them exhibit only a few known similarities at the molecular level. The genomes of both EBV and adenovirus are linear double-stranded DNA molecules whose replication takes place in the cell nucleus. Epidemiologically, it is known that both viruses cause latent infections and that the majority of adult North Americans harbor antibodies to each virus.

EBV has assumed an importance in studies in viral oncology because of its association with the two human neoplasias, Burkitt's lymphoma and nasopharyngeal carcinoma (11). Although the etiological role of EBV in these two diseases remains obscure, it has been demonstrated that EBV is the cause of infectious mononucleosis. In vitro, the virus is capable of transforming human

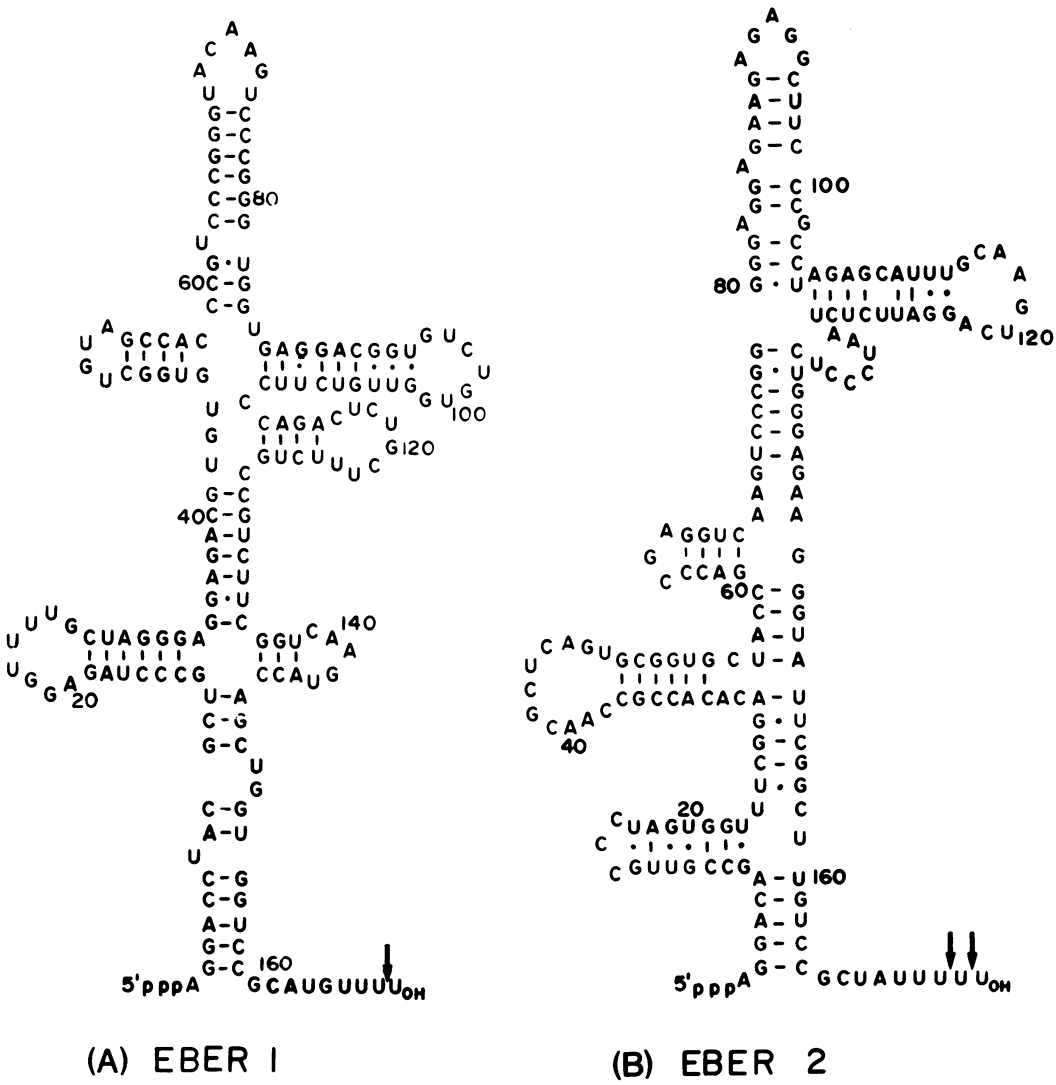

FIG. 8. *Potential secondary structures of EBERs. The arrows indicate alternate 3' termini. (A) EBER 1; (B) EBER 2.*

B lymphocytes into continuously dividing cells, thus presenting a model system for studies of latent viruses. Adenovirus, on the other hand, has not been associated with any human neoplasia. It is capable, however, of producing tumors in rodents. Infections of human fibroblasts by adenovirus are always lytic (12).

That two viruses of such diverse nature should similarly encode small RNA components that associate with the same host protein(s) poses a perplexing puzzle in viral evolution. Perhaps further similarities between the two viruses will emerge as our knowledge of the EBV system approaches that of the extensively characterized adenovirus.

Potential functions of the La RNPs. Functions of the RNP complexes containing the EBER or VA RNAs are currently a matter of speculation. The many shared features of the RNA molecules enumerated above and the fact that they bind a common antigenic host protein supports the supposition that these RNPs play similar roles in virus-infected cells. However, in neither case is it known whether additional host or viral proteins or both are present in the RNP particles, nor can it even be asserted that the RNP complexes are the functional forms of the viral-specified small RNAs.

If we assume a common function of VA RNAs and EBERs, it seems unlikely that the EBER

	EBER 1	5'pppAGGACCTACGCTGCCCTAGAGGTTT	-	37nuc	-	CCCGGGTACAAGTCCCGG...
	EBER 2	5'pppAGGACAGCCGTTGCCCTAGTGGTTT	-	36nuc	-	CCCGAGGTCAAGTCCCGG...
AD-2	VAI	5'pppAGCGGGCACTCTTCCGTGGTCTGGTGGATA	-	27nuc	-	CCGGGGTTCGAACCCCGG...
AD-2	VAII	5'ppp GGCTCGCTCCCTGTAGCCGGAGGGTTA	-	25nuc	-	CCCCGGTTCGAGTCTCGG...
MOUSE	4.5S	5'pppGCCGGTAGTGGTGGCGCACGCCGGT	-	33nuc	-	CACGAGTTCGAGGCCAGC...
RAT	4.5S	5'pppGGTCGAGAGGATGGCTCAGCCGTTA	-	25nuc	-	TAAGAGTTCGGTTCCCAG...
CONSENSUS SEQUENCE		...GTGGYNNPGTGG ...	25 - 35nuc			...GGGTTCGAANCC...

FIG. 9. *Nucleotide sequence comparison of EBERs, VA, and 4.5S RNAs. The VA RNA sequences are from Akusjärvi et al. (1), the mouse 4.5S RNA sequence is from Harada and Kato (15), and the rat hepatoma 4.5S RNA sequence is from Ro-choi et al. (25). The consensus sequence for the RNA polymerase III transcription control region is from Fowlkes and Shenk (13); their consensus sequence was derived from 5S RNA and a number of transfer RNA sequences in addition to the VAI, VAII, and mouse 4.5S RNA sequences shown here. Ad-2, Adenovirus type 2; nuc, nucleotides.*

RNPs can be directly responsible for the induction or maintenance of EBV-induced transformation of B lymphocytes since DNA encoding VA RNAs is not required for adenovirus type 2-induced transformation (14). A specific role in the splicing of adenovirus messenger RNAs has been proposed for the VA RNAs (23). The demonstration of a direct physical association between the VA RNAs and certain adenovirus late messenger RNAs supports this proposal (21). However, as pointed out by Mathews (21), the ability of VA RNAs to associate only with spliced messenger RNAs, and not their precursors, may indicate that the role of VA RNAs is in the transport of the spliced message from the nucleus to the cytoplasm. EBERs could well perform comparable functions in either splicing or transport of EBV messenger RNAs.

Alternatively, the finding that the EBER and VA RNA-containing particles are members of the La family of small RNPs suggests other potential functions. The heterogeneous spectrum of mouse host cell-specified La RNAs (19) includes two sequenced 4.5S species (J. Hendrick, personal communication). These, like the VA RNAs and EBERs, are uncapped and exhibit sequence homology to internal RNA polymerase III transcription control sites (Fig. 9), hinting that all La RNAs may be RNA polymerase III transcripts. One of these 4.5S RNAs, first described by Jelinek and Leinwand (16), also shares sequence homology (15) with a highly repetitive dispersed family of DNA sequences in mammalian genomes known as the Alu family (17, 26). This sequence homology, together with the fact that Alu family elements are known to be templates for RNA polymerase III transcription (9), suggests that the functions of Alu family sequences and the La family of RNPs might be considered together. The location of Alu sequences adjacent to RNA polymerase II transcription units has led to the postulate of their involvement in the control of RNA synthesis (23a). Finally, participation of Alu sequences or their transcripts in DNA replication has been suggested. However, the unusual virus-specific mechanism established for the initiation of adenovirus DNA synthesis (5) renders it unlikely that the VA RNAs function in this particular process.

Presumably, further insights into the functions of La RNPs containing either host- or viral-specified RNAs will be provided by additional studies of their localization in mammalian cells, their appearance in specific tissues or developmental states, and their protein components.

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LITERATURE CITED

1. **Akusjärvi, G., M. B. Mathews, P. Andersson, B. Vennström, and U. Pettersson.** 1980. Structure of genes for virus-associated RNA_I and RNA_{II} of adenovirus type 2. Proc. Natl. Acad. Sci. U.S.A. **77**:2424-2428.
2. **Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl.** 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods Enzymol. **68**:220-242.
3. **Barrell, B. G.** 1971. Fractionation and sequence analysis of radioactive nucleotides. Proc. Nucleic Acid Res. **2**: 751-779.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513-1523.
5. **Challberg, M. D., and T. J. Kelly.** 1979. Adenovirus DNA replication *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **76**:655-659.
6. **Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub,**

and E. Kieff. 1980. Epstein-Barr virus (B95-8) DNA VII: molecular cloning and detailed mapping. Proc. Natl. Acad. Sci. U.S.A. **77**:2999–3003.

7. **DeFranco, D., O. Schmidt, and D. Söll.** 1980. The control regions for eukaryotic tRNA gene expression. Proc. Natl. Acad. Sci. U.S.A. **77**:3365–3368.
8. **Donis-Keller, H., A. M. Maxam, and W. Gilbert.** 1977. Mapping adenines, guanines and pyrimidines in RNA. Nucleic Acids Res. **4**:2527–2538.
9. **Duncan, C., P. A. Biro, P. V. Choudary, J. T. Elder, R. R. C. Wang, B. G. Forget, J. K. De Riel, and S. M. Weissman.** 1979. RNA polymerase III transcriptional units are interspersed among human non-α-globin genes. Proc. Natl. Acad. Sci. U.S.A. **76**:5095–5099.
10. **England, T. E., and O. C. Uhlenbeck.** 1978. 3′-Terminal labelling of RNA with T4 RNA ligase. Nature (London) **275**:560–561.
11. **Epstein, M. A., and B. G. Achong (ed.).** 1979. The Epstein-Barr virus. Springer-Verlag, Berlin.
12. **Flint, J.** 1977. The topography and transcription of the adenovirus genome. Cell **10**:155–166.
13. **Fowlkes, D. M., and T. Shenk.** 1980. Transcription control regions of the adenovirus VAI RNA gene. Cell **22**:405–413.
14. **Graham, F. G., P. J. Abrahams, C. Mulder, H. L. Heijneker, S. O. Warnaar, F. A. J. de Vries, W. Fiers, and A. J. van der Eb.** 1974. Studies on *in vitro* transformation by DNA and DNA fragments of human adenovirus and SV40. Cold Spring Harbor Symp. Quant. Biol. **39**:637–650.
15. **Harada, S., and N. Kato.** 1980. Nucleotide sequences of 4.5S RNAs associated with poly(A)-containing RNAs of mouse and hamster cells. Nucleic Acids Res. **8**:1273–1285.
16. **Jelinek, W., and L. Leinwand.** 1978. Low molecular weight RNAs hydrogen-bonded to nuclear and cytoplasmic poly(A)-terminated RNA from cultured Chinese hamster ovary cells. Cell **15**:205–214.
17. **Jelinek, W. R., T. P. Toomey, L. Leinwand, C. H. Duncan, P. A. Biro, P. V. Choudary, S. M. Weissman, C. M. Rubin, C. M. Houck, P. L. Deininger, and C. W. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. U.S.A. **77**:1398–1402.
18. **Lerner, M. R., N. C. Andrews, G. Miller, and J. A. Steitz.** 1981. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. U.S.A. **78**:805–809.
19. **Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz.** 1981. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Science **211**:400–402.
20. **Lerner, M. R. and J. A. Steitz.** 1979. Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. U.S.A. **76**:5495–5499.
21. **Mathews, M. B.** 1980. Binding of adenovirus VA RNA to mRNA: a possible role in splicing? Nature (London) **285**:575–577.
22. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
23. **Murray, V., and R. Holliday.** 1979. Mechanism for RNA splicing of gene transcripts. FEBS Lett. **106**:5–7.

23a. **Pan, J., J-T. Elder, C. H. Duncan, and S. M. Weissman.** 1981. Structural and transcription analysis of interspersed repetitive polymerase III transcription units in human DNA. Nucleic Acids Res. **9**:1151–1170.

24. **Provost, T. T.** 1979. Subsets in systemic lupus erythematosus. J. Invest. Dermatol. **72**:110–113.
25. **Ro-Choi, T. S., R. Reddy, D. Henning, T. Takano, C. W. Taylor, and H. Busch.** 1972. Nucleotide sequence of 4.5S ribonucleic acid of Novikoff hepatoma cell nuclei. J. Biol. Chem. **247**:3205–3222.
26. **Rubin, C. M., C. M. Houck, P. L. Deininger, T. Friedman, and C. W. Schmid.** 1980. Partial nucleotide sequence of the 300-nucleotide interspersed repeated human DNA sequences. Nature (London) **284**:372–374.
27. **Rymo, J.** 1979. Identification of transcribed regions of Epstein-Barr virus DNA in Burkitt lymphoma-derived cells. J. Virol. **32**:8–18.
28. **Skare, J., and J. L. Strominger.** 1980. Cloning and mapping of BamHI endonuclease fragments of DNA from the transforming B95-8 strain of Epstein-Barr virus. Proc. Natl. Acad. Sci. U.S.A. **77**:3860–3864.
29. **Smith, H. O., and M. L. Birnstiel.** 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. **3**:2387–2398.
30. **Sprague, K. U., D. Larson, and D. Morton.** 1980. 5′-Flanking sequence signals are required for activity of silkworm alanine tRNA genes in homologous *in vitro* transcription systems. Cell **22**:171–178.
31. **Wu, G.-J.** 1978. Adenovirus DNA-directed transcription of 5.5S RNA *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **75**: 2175–2179.