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Structural Analyses of EBER1 and EBER2 Ribonucleoprotein Particles Present in Epstein-Barr Virus-Infected Cells

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The ribonucleoprotein (RNP) particles containing the Epstein-Barr virus-associated small RNAs EBER1 and EBER2 were analyzed to determine their RNA secondary structures and sites of RNA-protein interaction. The secondary structures were probed with nucleases and by chemical modification with single-strand-specific reagents, and the sites of modification or cleavage were mapped by primer extension. These data were used to develop secondary structures for the two RNAs, and likely sites of close RNA-protein contact were identified by comparing modification patterns for naked RNA and RNA in RNP particles. In addition, sites of interaction between each Epstein-Barr virus-encoded RNA (EBER) and the La antigen were identified by analyzing RNA fragments resistant to digestion by RNase A or T_1 after immunoprecipitation by an anti-La serum sample from a lupus patient. Our results confirm earlier findings that the La protein binds to the 3′ terminus of each molecule. Possible functions for the EBER RNPs are discussed.

The genome of Epstein-Barr virus (EBV), which infects and transforms primate B lymphocytes, encodes two small RNA molecules, EBER1 and EBER2, of 166 and 172 nucleotides, respectively (33). Both RNAs are transcribed by RNA polymerase III and are synthesized in large quantities in latently EBV-infected cells, in which they exist as ribonucleoprotein (RNP) particles complexed with the cellular La antigen (19, 33). Because of the relative abundance of EBER1 and EBER2 (approximately 10^7 copies per cell) and because they are two of only seven EBV gene products expressed in latently infected cells (15, 44), one or both EBV-encoded RNAs (EBERs) are potentially important in B-cell transformation. However, very little data on the biological functions of the EBERs are available at present, largely because the large 172-kilobase genome of EBV and the lack of a cell system for efficient virus growth make it relatively inaccessible to standard genetic approaches.

As a result, less direct approaches have been used to provide insight into the functions of the EBERs. One recent study has localized both EBERs to the nuclei of infected cells by in situ hybridization (12), suggesting that the EBERs may work at the level of replication, transcription, or RNA processing. In an effort to gather more basic information about the EBERs, we have determined the secondary structures of EBER1 and EBER2 as naked RNAs and as RNP particles by mapping the susceptibilities of various nucleotides to a series of chemical modifying agents and nucleases. We have also identified the binding site for the La protein on each EBER and confirmed and extended earlier indications that the primary recognition signal for La is the oligouridylate stretch at the 3′ ends of RNA polymerase III transcripts (10, 24, 30, 41).

MATERIALS AND METHODS

Materials. Raji cells (an EBV-immortalized Burkitt's lymphoma cell line) were grown in a suspension culture in RPMI 1640 medium (GIBCO Laboratories) containing 10% heat-inactivated fetal calf serum (GIBCO), penicillin (6 μg/ml), and streptomycin (10 μg/ml). La-specific antisera from patients have been described previously (19, 20); the serum used in this work was On, kindly provided by John Hardin (Department of Internal Medicine, Yale University). [γ-^{32}P]ATP (7,000 Ci/mmol) and $^{32}P_i$ (10 mCi/ml) were from New England Nuclear Corp., and [^{32}P]pCp (3,000 Ci/mmol) was from Amersham Corp. Cobra venom RNase (16.3 U/mg) and RNase T_1 (3,500 U/mg) were from Calbiochem-Behring, S1 nuclease (10^5 U/mg) and T4 RNA ligase were from Pharmacia, T4 polynucleotide kinase was from New England BioLabs, Inc., and avian myeloblastosis virus reverse transcriptase was from Promega Biotec. All ribo- and deoxyribonucleoside triphosphates were from Pharmacia. Dimethyl sulfate (DMS) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) were from Aldrich Chemical Co., and kethoxal was from United States Biochemical Corp.

Oligonucleotides. Oligodeoxynucleotides for primer extension analysis were synthesized by Aino Ruusala (in the laboratory of D. Crothers, Department of Chemistry, Yale University). The oligonucleotides included the following (sequences are 5′ to 3′, with complementary sequences given in parentheses): 1a, CCCTAGCAAAACCTCTAGGG (EBER1 14 through 33); 1b, GACAACCACAGACACCGTC (EBER1 89 through 107); 1c, AAAACATGCGGACCAC CAG (EBER1 149 through 167); 2a, ACCGCACTGAG CGTTGGCGGT (EBER2 33 through 53); 2b, GAATCCT GACTTGCAAATGC (EBER2 109 through 128); and 2c, AAAAATAGCGGACAAGCCGAA (EBER 2 153 through 173). Each oligonucleotide was purified on a 15% polyacrylamide–8 M urea gel containing TBE (89 mM Tris, 89 mM sodium borate, 1 mM EDTA) and concentrated on a DEAE-cellulose column (Whatman, Inc.). The eluate was desalted by passage over a Bio-Gel P4 column (Bio-Rad Laboratories).

Preparation of RNA and RNP. To prepare total-cell RNA, Raji cells at a concentration of 10^6 cells per ml were spun down for 10 min at 1,500 rpm in a Dynae II table-top centrifuge, washed once with ice-cold phosphate-buffered saline, suspended in 4 volumes (relative to the cell pellet) of the lysis buffer (0.15 M NaCl, 10 mM Tris hydrochloride [pH 7.9], 0.65% Nonidet P-40), and vortexed. The lysate was

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centrifuged for 2 min at 2,000 rpm, and the supernatant was mixed with an equal volume of urea buffer (7 M urea, 0.35 M NaCl, 10 mM Tris hydrochloride [pH 7.5], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]). This mixture was vortexed and PCA (phenol-chloroform-isoamyl alcohol [50:48:2]) extracted three times, and the RNA was precipitated with 2 volumes of ethanol. The precipitate was washed once with 100% ethanol, dried under a vacuum, and suspended in 1 ml of TES (10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 0.5% SDS). Proteinase K (Beckman Instruments, Inc.) was added to a final concentration of 0.2 mg/ml, and the sample was incubated for 30 min at 37°C. The digested sample was PCA extracted twice, chloroform extracted twice, and ethanol precipitated. The washed and dried precipitate was suspended in sterile distilled water to a concentration of 3 mg/ml (determined by A_{260}) and stored at −70°C.

EBER RNPs were contained in whole-cell sonicates of Raji cells prepared in the appropriate buffer as previously described (19).

Chemical modification. Modification reactions were based on those of Moazed et al. (25). Raji cell extract corresponding to roughly 10^7 cells (yielding about 50 μg of RNA upon isolation) or 50 μg of Raji cell RNA was used per reaction (final volume, 300 μl). RNA in the appropriate modification buffer was heated for 30 min at 37°C before modification; cell extracts in modification buffer were placed at room temperature for 10 min before modification. Mock-treated samples (no addition of modifying reagent) were run in parallel to all modification reactions. Samples were incubated for 15 min at room temperature after the addition of modifying reagent.

(i) DMS. A 0- to 2-μl quantity of DMS was added to 300 μl of RNP or RNA samples in CMK buffer (80 mM potassium cacodylate [pH 7.2], 0.3 M KCl, 20 mM magnesium acetate). RNP reactions were stopped by the addition of 150 μl of ice-cold DMS stop buffer (1.0 M Tris acetate [pH 7.5], 1 M β-mercaptoethanol, 1.5 M sodium acetate, 0.1 mM EDTA), 300 μl of cold CMK buffer, and 40 μl of 20% SDS and incubated on ice for 10 min. The samples were PCA extracted once and immediately ethanol precipitated. Precipitates were suspended in 300 μl of 0.3 M sodium acetate, PCA extracted twice, chloroform extracted twice, and precipitated with ethanol. Naked RNA reactions were stopped by the addition of 75 μl of DMS stop buffer, incubated on ice for 10 min, and then ethanol precipitated twice. All samples were suspended in 20 μl of sterile distilled water.

(ii) Kethoxal. A 0- to 15-μl quantity of a 37% kethoxal solution in 20% ethanol was added to 300-μl RNA or RNP samples in CMK buffer. Reactions were stopped by the addition of 25 μl of 0.5 M potassium borate (pH 7.0), 400 μl of cold CMK buffer, 80 μl of 3 M sodium acetate and for the RNP reaction, 40 μl of 20% SDS. The RNA and RNP samples were each treated exactly as described for the DMS modification, except that suspended samples were made 25 mM in potassium borate (pH 7.0) to stabilize the kethoxal adduct.

(iii) CMCT. A 0- to 150-μl quantity of CMCT (42 mg/ml) in BMK buffer (70 mM potassium borate [pH 8.1], 0.3 M KCl, 20 mM magnesium acetate) was added to 150 μl of RNA or RNP samples in BMK buffer. BMK was added to give a final volume of 300 μl. Reactions were stopped by the addition of 500 μl of cold 0.5 M potassium borate (pH 6.1) and 80 μl of 3 M sodium acetate, and RNA was isolated as described for the DMS modification.

To ensure that the respective stop treatments terminated modification and to assess the extent of modification occurring during the purification of RNA, a control reaction was run for each set of RNP reactions in which a maximum amount of modifying reagent was added to the sample after the addition of the appropriate stop reagent.

Enzymatic cleavage reactions. Digestions of RNA and RNP samples with cobra venom RNase and S1 nuclease were carried out essentially as described in Shelness and Williams (38). The amounts of RNA and cell extract used were as described for the chemical modification reactions. All reactions were stopped by the addition of SDS to a final concentration of 1% and immediate PCA extraction and ethanol precipitation. A stop control for the RNP reactions was run in which the sample was PCA extracted immediately after the addition of enzyme. Further isolation of RNA was exactly as described for DMS modification.

Primer extension. Primer extension analysis of modified RNA was performed as described by Moazed et al. (25), except that oligonucleotide primers were 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as previously described (22). For each reaction, 2.5 ng (0.4 pmol) of labeled primer and 7.5 μg of sample RNA were used, and extension reactions contained 0.3 mM of each deoxynucleoside triphosphate, 17.6 mM β-mercaptoethanol, and 17.6 μg of bovine serum albumin per ml (Bethesda Research Laboratories, Inc.), in addition to the previously described reaction buffer (22). Dideoxy sequencing reactions were performed with 7.5 μg of unmodified total RNA from Raji cells and a dideoxy/deoxy ratio of 1:5. After primer extension, all samples were ethanol precipitated, suspended in 80% formamide–10 mM EDTA–0.1% bromophenol blue–0.1% xylene cyanol, and run on 0.3-mm-thick 8% polyacrylamide–8 M urea gels in TBE. The gels were dried and autoradiographed at −70°C with an intensifying screen.

3′-End analysis. End labeling of Raji RNA with [^{32}P]pCp and T4 RNA ligase was carried out by the directions of the enzyme supplier. Bands corresponding to discrete isoforms of EBER1 and EBER2 were eluted after 5% polyacrylamide–8 M urea gel fractionation of the labeled products. Samples (4×10^{-3} cpm [Cerenkov]) of these purified RNAs were digested with various amounts of RNase T_1, S1 nuclease, and cobra venom RNase as previously described (38) in the presence of 10 μg of unlabeled yeast carrier RNA. Enzymatic sequencing reactions were performed on the labeled RNAs by using a kit and protocol from P-L Biochemicals, Inc., and run with the digested samples on 15% polyacrylamide–8 M urea gels in TBE. The gels were autoradiographed at −70°C with an intensifying screen.

Immunoprecipitation and analysis of protected fragments.
(i) In vivo labeling of RNA. Raji cells were grown for 18 h from a density of 2×10^5 cells per ml in phosphate-free modified Eagle medium (GIBCO) containing 10% fetal calf serum, 0.1 mCi of $^{32}P_i$ per ml, penicillin, and streptomycin. A whole-cell extract was prepared in NET-2 buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.05% Nonidet P-40) at a concentration of 10^7 cells per 0.5 ml. Portions (500 μl) of the extract were immunoprecipitated for 1 h at 4°C with 10 μl of anti-La serum or normal human serum prebound to 2.5 mg of protein A-Sepharose (PAS; Pharmacia). Undigested anti-La precipitable RNA was recovered after four NET-2 washes. To recover protected fragments, the PAS beads were washed four times with NET-2 and twice with NET-2 buffer lacking Nonidet P-40 and were digested at room temperature with RNase T_1 (150 U) or RNase A (50 μg) for various times. RNase-resistant fragments were recovered by PCA extraction and ethanol precipitation after addition of 1% SDS and 20 μg of carrier RNA.

(ii) In vitro labeling of RNA. Immunoprecipitation and

nuclease digestion were performed on an unlabeled Raji cell extract as described above for in vivo-labeled samples. RNA retained in the PAS-RNP complexes was end labeled with 50 μCi of [γ-^{32}P]ATP and 40 U of T4 polynucleotide kinase as previously described (22); the reaction mix (40 μl) was frequently agitated to ensure dispersal of the PAS beads. After multiple washes with NET-2 buffer containing 0.1 mg of carrier RNA per ml to inhibit nonspecific RNA binding, the labeled fragments were isolated as described for the in vivo-labeled fragments. Individual labeled fragments were purified from a 20% polyacrylamide–8 M urea gel of the labeled sample, and each was enzymatically sequenced by using the kit and protocol from P-L Biochemicals.

RESULTS

Secondary structure analyses of EBER. We examined the secondary structure of each EBER in both its native RNP form and as naked RNA by determining the sensitivities of various nucleotides to chemical and enzymatic probes. A crude Raji cell extract prepared by short bursts of sonication at 4°C in the appropriate reaction buffer was used as a source of EBER RNP. Extracts prepared in the various reaction buffers retained greater than 50% anti-La immunoprecipitability of EBERs compared with a control extract prepared in NET-2 buffer, from which EBERs are quantitatively immunoprecipitable (33; also results not shown). Therefore, we deemed these preparations a reasonable source of La-complexed EBER RNPs. We assume that any differences in modification between RNA and RNP preparations can probably be attributed to the binding of La protein, as no proteins other than La have been detected in stable association with either EBER by a variety of methods (see below).

Raji cell extracts or purified Raji RNAs were subjected to modification by DMS (reacts with N-1 of A residues and to a lesser extent with N-3 of C residues), kethoxal (reacts with N-1 and N-2 of G residues), or CMCT (reacts with N-3 of U residues), and the modified bases were mapped by primer extension on the modified RNA (13, 25) with primers complementary to each EBER. Each of these reagents attacks the base-pairing moieties of its target nucleotides and so modifies only single-stranded regions of RNA. As a result, reverse transcriptase stalls at the nucleotide immediately 3′ to a modified base, yielding a stopped extension product. This product is visible as a band on a denaturing polyacrylamide gel and can be sized precisely by running a parallel set of dideoxy sequencing reactions with the same primer. We performed similar experiments with the double-strand-specific cobra venom RNase and the single-strand-specific nuclease S1 (38). With these probes, reverse transciptase runs off the cut end of the RNA template and permits a similar analysis of the cleavage sites. For each reagent, the strong reproducible reactivity of a base may be taken as evidence that it is in a single-stranded (or for cobra venom RNase, a base-paired) state.

Previous chemical modification studies on RNP particles used rapid ethanol precipitation to terminate modification reactions (25). However, we observed that this treatment did not effectively stop modification in Raji cell extracts, possibly because reagent became trapped in the RNA-protein pellet and continued to modify the RNA during succeeding steps. To circumvent this problem, we used several alternative stop protocols, whereby cold stop buffer was added to quench the modification reaction (see Materials and Methods; also see reference 28). RNA was then collected by rapid PCA extraction and ethanol precipitation. For the DMS, CMCT, S1, and cobra venom RNase reactions, controls in which modifying reagent was added immediately before PCA extraction showed a level of modification no higher than the corresponding mock-treated sample (Fig. 1A, C, and D, lanes marked with an asterisk), indicating that the stop treatments were largely effective. The kethoxal stop control lane shows some reactivity above background (Fig. 1B, lane marked with an asterisk); accordingly, the extent of reactivity for this reagent was judged by comparison with this control.

Each set of modification or cleavage reactions was analyzed with several different oligonucleotide primers complementary to each EBER. Multiple primers were used because highly modified RNA templates yield principally short primer extension products, making the reactivity of bases far from the primer difficult to assess. Representative experiments for each reagent comparing EBERs and RNPs are shown in Fig. 1A to E. Base positions which show reactivity above background (the mock-treated 0 lane, which controls for endogenous reverse transcriptase stops) are indicated to the side of each gel. The complete modification and cleavage results are given in Fig. 2, which shows computer-derived secondary structures for each EBER (see Discussion). No significant changes in RNA modification and cleavage patterns were observed when the substrate RNA was heated at 80°C in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA for 5 min and slowly cooled to room temperature or when this renaturation was performed in the presence of 200 mM NaCl (results not shown). Changes in reactivity were determined by visually comparing band intensities in RNA and RNP lanes in which the overall extent of reaction seemed similar (for instance, Fig. 1A, RNA lane 1 and RNP lane 2). Bases which show significantly altered reactivity compared with the naked RNA, preferably with no reactivity in the RNP lanes, were designated as protected by an arrow at the side of the gel.

Primer extension analyses yielded data for all regions of both EBERs, except for the 20 nucleotides at the 3′ termini of the two molecules. To obtain more information on this region, we 3′-end labeled a sample of Raji total RNA with [^{32}P]pCp and T4 RNA ligase and fractionated the two EBERs on a 5% polyacrylamide–8 M urea gel. Samples of each labeled RNA were then digested with various amounts of T_1, S1, or cobra venom RNase. Polyacrylamide gel fractionation of the digested EBER1 is shown in Fig. 3, with enzymatic sequencing reactions of the same RNA sample run in parallel. Data on the 3′ termini of the EBER RNPs could not be obtained because the attempts to end label the RNA in this form were unsuccessful.

Identification of EBER fragments bound by La protein. Previous studies have demonstrated that the La protein selectively binds the oligouridylate stretch at the 3′ termini of RNA polymerase III transcripts (10, 24, 30, 41). Since such a sequence is present in the mature forms of both EBERs (33), it seemed likely to be the site of La binding. However, our inability to obtain chemical modification and enzymatic cleavage data for the 3′ ends of either EBER RNP made alternative approaches for analyzing RNA-protein interactions in this region necessary.

We localized the binding site(s) of La by identifying the EBER fragments which are protected from nuclease digestion in anti-La immunoprecipitates. First, we selected La-containing RNPs from a Raji cell extract with anti-La antibodies bound to PAS. The immunoprecipitated material was then treated with high concentrations of RNase T_1 or RNase A, and the PAS beads were extensively washed.

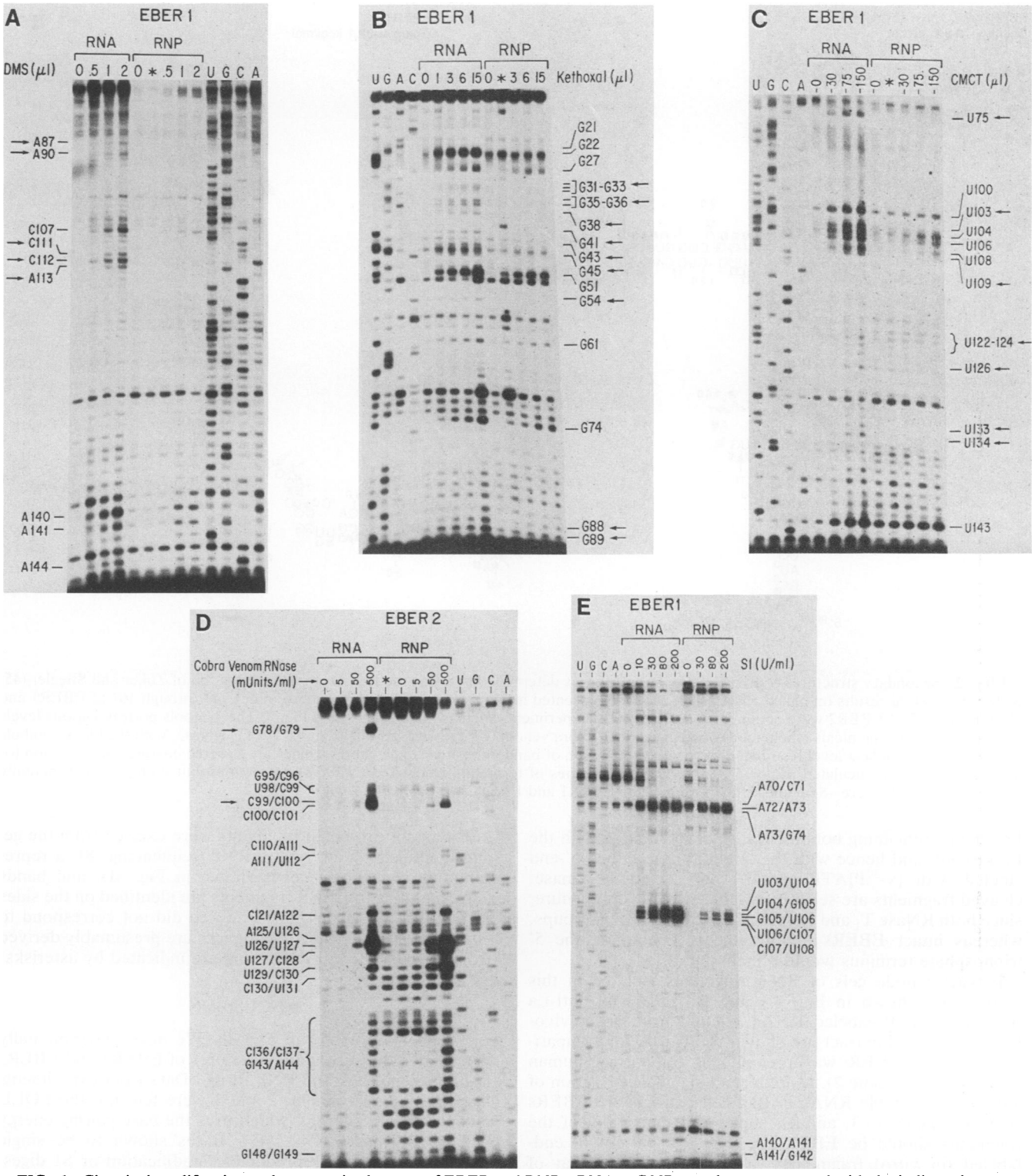

FIG. 1. Chemical modification and enzymatic cleavage of EBER and RNPs. RNA or RNP samples were treated with the indicated amount of reagent. RNA isolated from these reactions was subjected to primer extension with the oligonucleotide primer specified below, and the extension products were run on 8% polyacrylamide (acrylamide-bisacrylamide, 19:1)–8 M urea gels. The lanes with an RNP sample which received the maximum amount of reagent after the addition of the appropriate stop reagent are indicated with an asterisk. Dideoxynucleotide sequencing lanes are labeled according to the base they detect in the RNA (e.g., the U lane received ddATP). (A) DMS reaction, analyzed with primer 1c (complementary to EBER1 nucleotides 149 through 167); (B) kethoxal reaction, analyzed with primer 1b (complementary to EBER1 nucleotides 89 through 107); (C) CMCT reaction, analyzed with primer 1c (complementary to EBER1 nucleotides 149 through 167); (D) cobra venom RNase reaction, analyzed with primer 2c (complementary to EBER2 nucleotides 153 through 173); (E) S1 nuclease reaction, analyzed with primer 1c. Reactive bases or phosphodiester bonds are identified to the side of the modification lanes, and positions which show reduced reactivity in the RNP form are indicated with arrows.

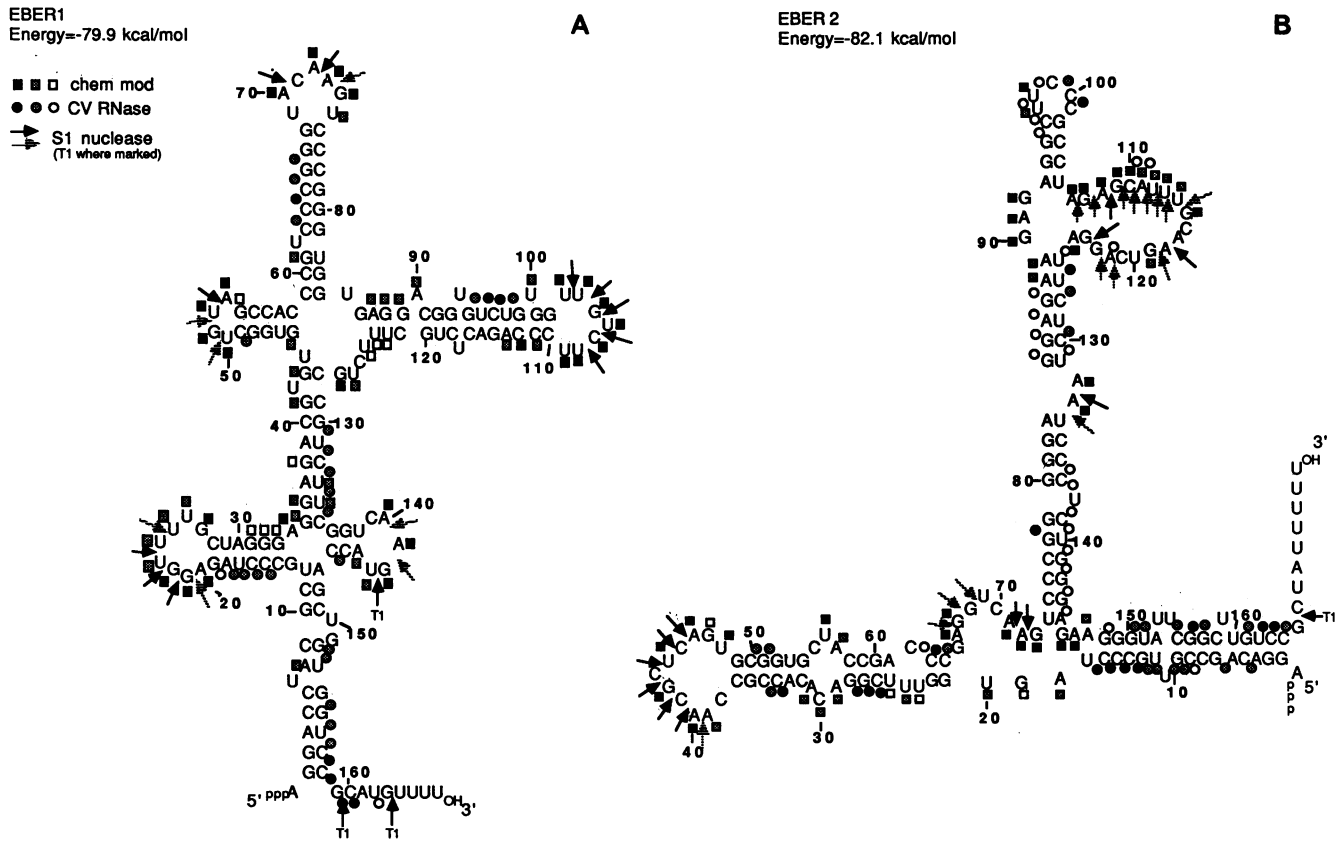

FIG. 2. Secondary structures of EBERs. The structure was determined by using the FOLD computer program of Zuker and Stiegler (45) with experimental results on naked RNAs such as those presented in Fig. 1 and 3. Data for the nucleotides 147 through 167 of EBER1 and 153 through 173 of EBER2 were obtained exclusively from experiments such as the one shown in Fig. 3. The symbols portray various levels of modification by chemicals (chem mod) or cleavage with cobra venom (CV) RNase, S1 nuclease, or T_1 (T1) RNase, with the black symbols representing the highest level (estimated by visual comparison of band intensities). The energy values are the free energies of formation for these structures as calculated by the FOLD program. Energies of formation calculated by the same program but without experimentally derived constraints were –84.6 and −90.0 kcal/mol for EBER1 and EBER2, respectively.

Fragments remaining bound because of interaction with the La protein (and hence with the antibody) were then 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase; cleaved fragments are selectively labeled by this procedure, since both RNase T_1 and RNase A leave 5′ hydroxyl groups, whereas intact EBERs and fragments containing the 5′ triphosphate terminus would remain unlabeled.

Polyacrylamide gels of RNA fragments isolated in this manner are shown in Fig. 4A and B. Total and anti-La precipitable [^{32}P]-labeled RNAs isolated from an in vivo-labeled Raji cell extract are shown in Fig. 4A for comparison. Neither EBER was precipitated by normal human serum (Fig. 4A, lane 2). Moreover, a significant fraction of anti-La-precipitable RNAs in Raji cell extracts are EBERs (Fig. 4A, lanes 1, 3, and 4), suggesting that many of the fragments should be EBER specific. The pattern of end-labeled (in vitro) fragments was very similar to that of fragments generated from in vivo-labeled RNA (Fig. 4C), demonstrating that all major protected fragments were accessible to the end-labeling protocol (compare Fig. 4A, lanes 5 and 6, with Fig. 4C). As a control, in vivo-labeled, anti-La-precipitated RNA was PCA extracted, then treated with corresponding concentrations of either RNase T_1 or RNase A; this yielded predominantly small RNA fragments (Fig. 4C, RNA lane), indicating that the fragments in Fig. 4A and B are generated by protein protection.

The major protected fragments were excised from the gel and identified by enzymatic RNA sequencing (8); a representative sequencing gel is shown in Fig. 4D, and bands corresponding to EBER fragments are identified on the sides of Fig. 4A and B. Fragments which did not correspond to either EBER1 or EBER2 sequences are presumably derived from other La-bound RNAs and are indicated by asterisks.

DISCUSSION

Secondary structure of EBERs. We have experimentally determined the secondary structures of EBER1 and EBER2 in both their RNA and RNP forms. Data from experiments like those shown in Fig. 1 and 3 were fed into the FOLD computer program (45), which uses the base-pairing energy values of Tinoco et al. (43). Bases shown to be single stranded by strong, reproducible modification or S1 digestion were constrained from base pairing. The secondary structures generated from these data are shown in Fig. 2, along with the complete results of the modification and cleavage experiments. We did observe some weak chemical modification of certain nucleotides which are shown to be base paired in the models (for instance, A8, A87, G88, and G89 of EBER1 and U25, A88, and A89 of EBER2). However, since most of these bases are located near the ends of helices and are often involved in A-U or G-U pairs, their

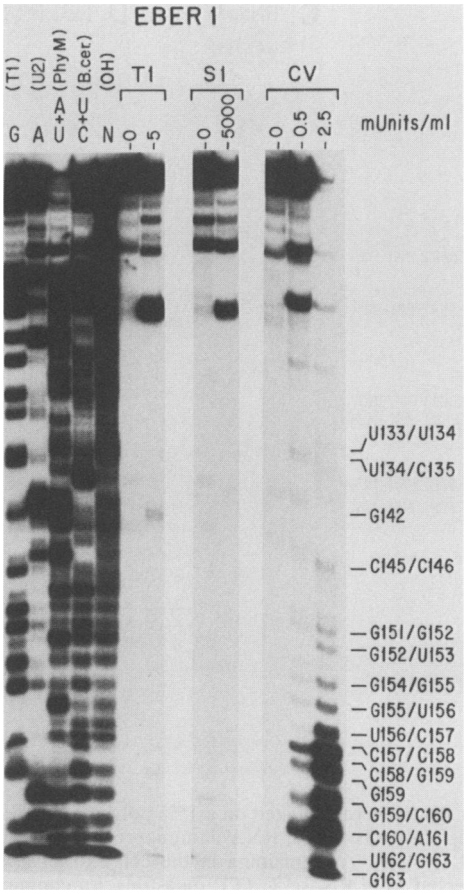

FIG. 3. Enzymatic cleavage of 3'-end-labeled EBER1 RNA. Gel-purified end-labeled EBER1 was digested for 15 min with the indicated concentrations (in milliunits [mUnits] per milliliter) of T_1 RNase (T1), S1 nuclease (S1), or cobra venom RNase (CV) under the same buffer conditions used for the experiments in Fig. 1. The digested RNA was ethanol precipitated and run on 20% polyacrylamide–8 M urea gels alongside enzymatic sequencing reactions of the same RNA (8). The sequence of the labeled RNA is indicated on the leftmost lanes (a small amount of impurities in the gel-purified RNA gave rise to some additional bands in the sequence ladder), and positions cut by T_1 RNase or S1 nuclease are indicated (these enzymes cleave the bond 3' to the identified base). Ribonucleases used to generate the sequencing ladder are indicated above their respective lanes, and an alkaline hydrolysis (OH) of the sample (N) is shown. Positions cut by cobra venom RNase are identified as two adjacent bases because this enzyme cuts either 5' or 3' to double-stranded bases. A parallel experiment was performed with end-labeled EBER2 (results not shown).

observed modification may indicate helical breathing, a phenomenon which has been observed in similar experimental systems (13, 25). The only major inconsistency between the data presented and the models occurs between U97 and C101 of EBER2, in which cobra venom RNase cuts are assigned to a loop (Fig. 2B). However, we also observed CMCT modification of U97 and U98, supporting the single-stranded character of these two nucleotides. Attempts to pair this region with its most obvious complement, G81 to G90 (as in the computer model of Rosa et al. [33]), in a manner consistent with the other data gave a structure considerably less stable than the one presented. The presence of weak cobra venom RNase cleavage sites is not necessarily inconsistent with the model of Fig. 2B, since this enzyme occasionally cuts at single-stranded positions in well-studied tRNA substrates (9, 21). Alternatively, one or more bases in the loop (perhaps C100 or C101, the positions of strongest cobra venom RNase cleavage) might be involved in tertiary interactions with distant nucleotides.

Both the EBER1 and EBER2 show extensively base-paired structures containing a number of short stem loops. The loops of these stems (for instance, A20 through G27, U50 through A53, U69 through U75, U103 through U109, and C139 through U143 of EBER1; C38 through U48 and U97 through C101 of EBER2) appear single stranded in our analysis and thus are potentially available for base-pairing interactions with other RNAs in vivo. In EBER2, there are several additional single-stranded regions (G65 through G73 and A106 through A125) which are longer than any in EBER1. Such differences imply that the EBERs could have different modes of action.

Structure of the EBER RNPs. The modification and cleavage patterns obtained for the two EBER RNPs were similar to those observed with the RNAs, although the RNPs were generally less reactive to chemical and enzymatic probes, and a number of weakly reactive nucleotides in the RNA reactions showed no reactivity in the RNP. Such observations have been interpreted to indicate that the RNP structure is generally tighter than the uncomplexed RNA (25). We did observe nucleotides which showed strongly reduced reactivity in the RNP (Fig. 5), attributable either to protection of the base by a bound protein or to a protein-induced RNA structural change. The modification and cleavage data alone cannot distinguish between these two possibilities.

The identification of RNA fragments which are protected by La protein from nuclease digestion gives a firmer idea of the regions which associate (either directly or indirectly) with this protein in the native RNPs and allows a more precise interpretation of the modification data. If a base is protected from modification or cleavage in the RNP and is contained in a La-protected fragment, then its decreased reactivity is most likely due directly to protein binding. The most abundant RNA fragments isolated in protection experiments include the 3' terminus of each EBER (Fig. 4 and 5), as predicted from earlier studies (10, 24, 30, 41); unfortunately, we could not obtain modification data for this region in the RNPs. The large size of the protected fragments relative to the oligouridylate stretch may reflect genuine protein contacts over this region, steric hindrance from the enzyme, or the absence of single-stranded cleavage sites for RNase T_1 or A within the base-paired stem. When RNAs purified from anti-La precipitates were treated with nuclease under the same conditions, low levels of fragments comparable in size to the major protected fragments were observed (Fig. 4C), suggesting that extended protection may be due in part to RNA secondary structure. Yet, the presence of cobra venom RNase cleavages in the opposite strands of the stems which are protected in the RNPs (Fig. 5) lends support to the hypothesis that La contacts these RNAs in the stem region; this is consistent with previous studies of La binding to adenovirus-associated (VA) RNAs (10) and to 4.5I RNA (30). Our failure to isolate protected fragments corresponding to the opposite strand, as might be expected given its proximity to the principal binding site, cannot be due to the presence of the 5' triphosphate terminus which precludes end labeling of such a fragment, as no major fragments from this region appear in preparations of in vivo-labeled RNA. The possibility does remain that a 5'-terminal fragment is a minor component of the in vivo-labeled fragment population.

Other EBER2 positions protected from cleavage in the RNP relative to the RNA (G78 or G79, C99 or C100 and the

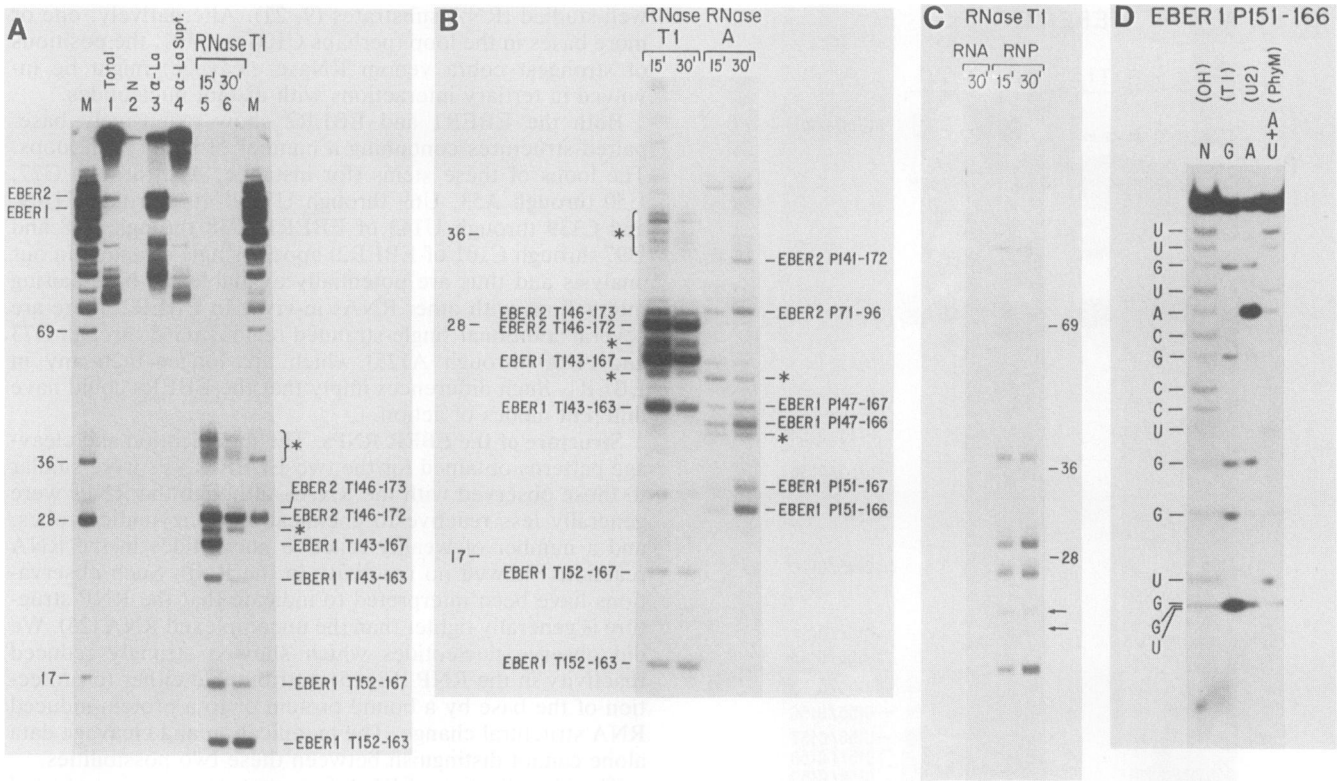

FIG. 4. EBER fragments protected from nuclease digestion by La protein. (A) RNAs were analyzed on a 15% polyacrylamide–8 M urea gel. Lanes 1 to 4 are samples of ^{32}P-Raji RNA labeled in vivo. Lanes: 1, total Raji RNA (Total); 2, RNA immunoprecipitated with normal human serum (N); 3, RNA immunoprecipitated with La-specific antiserum (α-La); 4, RNAs remaining in the cell extract after anti-La immunoprecipitation (α-La Supt.); 5 and 6, end-labeled (in vitro) fragments protected from RNase T_1 (T1) digestion. La-containing RNPs were immunoprecipitated, digested with 150 U of RNase T_1 at room temperature for the indicated time in minutes ('), and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Size markers are a filled-in *Hpa*II digest of pBR322 DNA. Sequenced bands are identified to the right of the gel, with the T or P referring to an RNase T_1 or RNase A fragment, respectively; bands with non-EBER sequences are indicated by asterisks. The positions (in nucleotides) of DNA size markers (M) are shown on the left. (B) End-labeled (in vitro) RNase T_1 (T1) and RNase A fragments from a separate experiment, resolved on a 20% polyacrylamide–8 M urea gel. Fragments were obtained as in panel A. RNase T_1 and A digestions were for the indicated time (in minutes ['']). Sequenced bands to either side of the gel are identified as in panel A, and the positions (in nucleotides) of DNA size markers are indicated on the left of the gel. (C) RNase T_1 fragments obtained from ^{32}P-labeled (in vivo) RNA fractionated on 15% gels as in panel A. Naked RNA, which was isolated from an anti-La precipitate from 10^7 Raji cells, was purified and treated with 150 U of RNase T_1 (T1) for 30 min ('). RNase T_1-resistant RNP fragments were obtained exactly as for panels A and B, except that the kinase step was omitted. Bands which do not appear among end-labeled (in vitro) fragments (compare panels A and B) are indicated by arrows. The positions of DNA size markers (in nucleotides) are indicated on the right of the gel. (D) A representative RNA sequencing gel of the RNase A fragment corresponding to EBER1 G151 through U167 labeled at its 5′ end. Base-specific enzymatic reactions (ribonucleases T1, U2, PhyM) are indicated above the appropriate lane, and an alkaline hydrolysis (OH) lane (N) is included. Bases are identified on the left. The three shortest partial products, pUp, pUpGp, and pUpGpGp, ran together at the salt front, giving a sequence consistent with the length of the protected fragment (Fig. 4B).

bond between A132 and A133; see legend to Fig. 3) are near or are contained in a less abundant fragment (P71-96) precipitated by the anti-La serum. These regions are probably areas more weakly associated with La than the oligouridylate stretch but in which RNA-protein interactions do occur. Regions of bases protected from modification in the RNP which are not near La-immunoprecipitable fragments (for instance, C14 through C16, G31 through G45, U50 through A53, A87 through A90, U109 through A113, and U122 through U126 of EBER1 and U11 through C14 of EBER2) could be sites of weak contact or occlusion by the protein or be due to structural changes which alter the conformation of the RNA. It is also possible that bound proteins other than La are involved in protection, although as yet no other specific polypeptides which copurify with EBER upon gel filtration, DEAE-column fractionation, or sucrose density gradient fractionation have been detected (E. Gottlieb and J. Stefano, unpublished results).

Possible functions of the EBER RNPs. Several results have suggested that either or both EBER RNPs may act along the same pathway as adenovirus VAI RNA to promote host cell survival and virus growth (4, 5). Viral mutants lacking the genes for one (VAI) or both VA RNAs grow to titers 60- to 80-fold lower than the wild-type virus (3, 5) and show a greatly reduced efficiency of translation of late viral mRNAs (36, 42). Recently, VAI RNA has been shown to prevent activation of an interferon-induced kinase which phosphorylates the initiation factor eIF-2α and arrests translation in the infected cell (16, 27, 35, 39). In the absence of VAI RNA, multiple kinase molecules bind to long stretches of double-stranded RNA produced by late viral transcription and become activated by autophosphorylation (14, 18, 34, 37). VAI RNA contains numerous shorter helical regions (26) which are proposed to bind single kinase molecules and thus prevent autophosphorylation (14). As a result, the host cell survives during the late phase of adenovirus infection.

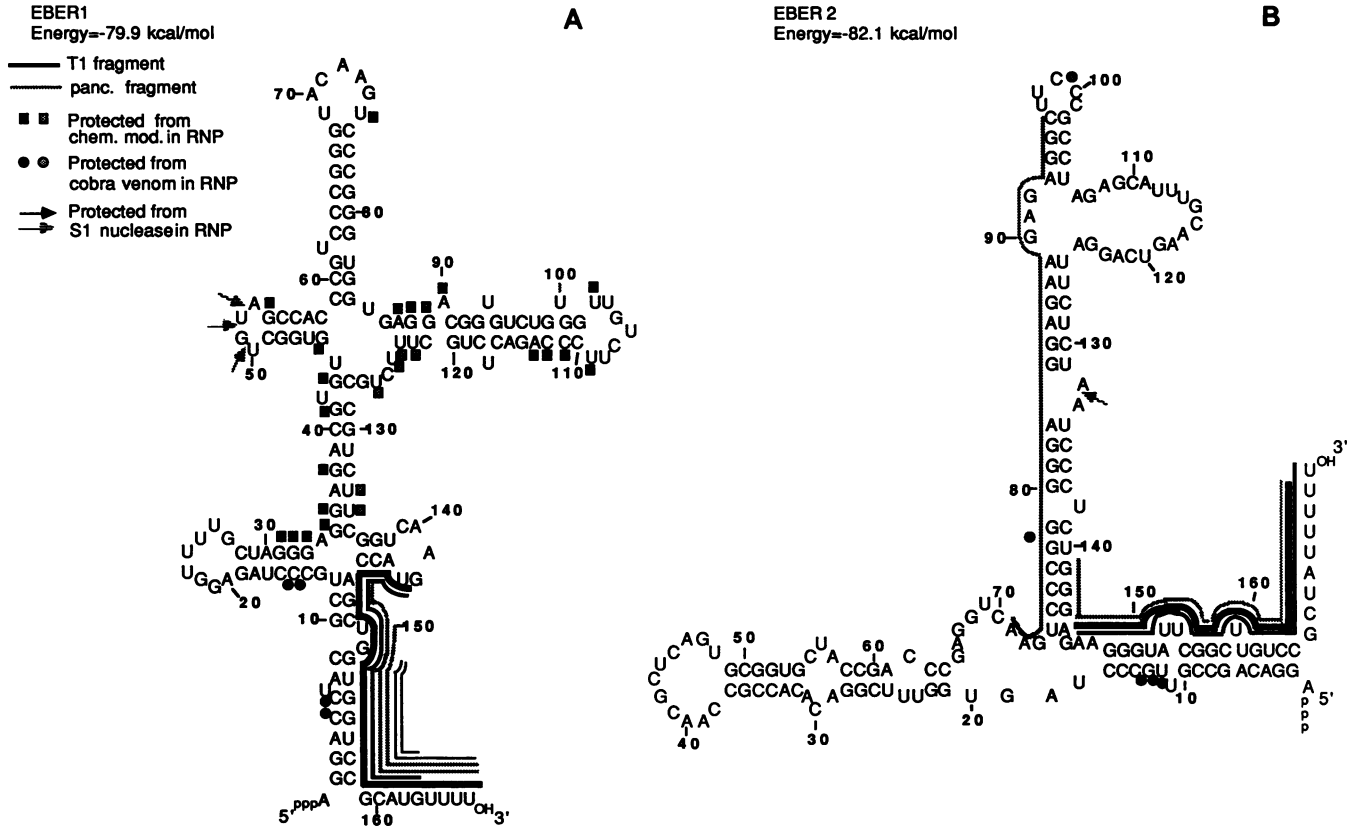

FIG. 5. Secondary structure of EBER RNPs and sites of RNA-protein interaction. Sites of differences in reactivity to chemical and enzymatic probes between the RNA and RNP (from Fig. 1 and similar experiments) are indicated. RNA fragments protected from RNase T_1 (T1) or RNase A (panc.) digestion and immunoprecipitated by anti-La antibodies (Fig. 4A and B) are shown as lines parallel to the corresponding sequence; the thickness of the lines roughly correlates with the relative abundance of the fragment. Black symbols indicate complete protection from modification or cleavage, and other symbols indicate partial protection. All protected fragments were reproducibly obtained in at least three experiments. Note that the non-3'-end fragment of EBER2 (P71-96) observed with RNase A contains only purines, except at its 5' end. Abbreviations: panc., pancreatic; chem. mod., chemical modification.

Our results show that both EBER1 and EBER2 contain analogous structural features (1, 25, 40) and could in principle mimic the action of VAI RNA in the adenovirus system. When cell line 293 was infected with an adenovirus substitution mutant lacking functional VAI and VAII genes but containing multiple EBER1 and EBER2 genes the EBERs were found to functionally substitute for adenovirus-associated RNAs to some extent (5). However, several findings argue against complete equivalence of the adenovirus-associated RNAs and EBERs. First, both EBERs have been definitively localized to the nuclei of Raji cells and EBV-infected human blood cord lymphocytes by in situ hybridization; by contrast, VAI RNA shows a nuclear and cytoplasmic distribution in adenovirus-infected HeLa cells by the same technique (12). A cytoplasmic location would be consistent with the role of VAI RNA in the modification of eIF-2α, which is involved in a cytoplasmic process. Second, VAI RNA accumulates markedly at late times after infection in a lytic pathway (39), whereas EBER transcription increases early after EBV transformation of B lymphocytes and remains high in latently infected B cells (G. Howe and C. Rooney, unpublished results; also see reference 44). Moreover, induction of EBV replication by phorbol esters does not significantly increase the already high steady-state levels of EBERs (44). Third, purified EBERs have no effect upon eIF-2α kinase activity when added to the same interferon-treated cell extracts in which VAI RNA has inhibitory activity (12).

The nuclear location of both EBER RNPs suggests that they may participate in some aspect of replication, transcription, or RNA processing in EBV-transformed cells. Since the EBERs are present in such large amounts, it is possible that their stable binding of the La protein could significantly reduce the pool of free La present in the nucleus. Since the La protein is intimately associated with RNA polymerase III transcripts in mammalian cells and may play a role in their biogenesis (32), this could differentially affect the metabolism of these important RNA molecules. However, at present it is unclear how such regulation would be related to EBV-dependent changes in cell growth.

Functions for cell-encoded small nuclear RNPs have been assigned in the splicing of pre-mRNAs (23), and the recent findings that U6 RNA is transcribed by RNA polymerase III and is complexed with the La antigen (17, 29) raise the possibility that other class III RNAs could be active in RNA processing. U6 RNA is found in the same small nuclear RNA particle as U4 RNA, to which it is apparently base paired (7, 11, 31); this particle is required for pre-mRNA splicing in vitro (2, 6). Interestingly, the eight-nucleotide region of U6 RNA involved in base pairing with U4 RNA (31) corresponds to a seven-nucleotide stretch of EBER2 (A111 through A117), with six out of seven nucleotides conserved

(U114 is an A in U6). We have found that this region of EBER2 is single stranded in both the RNA and RNP (Fig. 2B) and is therefore potentially accessible for base pairing with another RNA. If an EBER2 or U4 interaction exists, then a role for EBER2 in the regulation of RNA splicing in EBV-infected cells is conceivable. The implications of these observations are being pursued.

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