Two small RNAs encoded by Epstein–Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus

(Epstein–Barr virus-encoded RNA/La antigen/small nuclear ribonucleoprotein)

MICHAEL R. LERNER*, NANCY C. ANDREWS*†, GEORGE MILLER‡, AND JOAN A. STEITZ*

Departments of *Molecular Biophysics and Biochemistry and ‡Pediatrics, Yale University, New Haven, Connecticut 06510

ABSTRACT   Primate cells harboring the Epstein–Barr virus (EBV) genome synthesize large amounts of two small RNAs: EBER 1 and EBER 2 (EBV-encoded RNA). These RNAs are approximately 180 nucleotides long, possess 5′ pppA termini, and lack poly(A). They have different T1 and pancreatic RNase digestion fingerprints. They are not found in normal B lymphocytes, in transformed B lymphocytes that lack EBV DNA, in T lymphocytes transformed by *Herpesvirus ateles*, or in a variety of other nonlymphoid mammalian cells. Hybridization analyses indicate that EBER 1 and EBER 2 are encoded by the *Eco*RI-J fragment of EBV (B95-8) DNA. *In vivo* both RNAs are associated with protein(s), allowing their specific precipitation by the systemic lupus erythematosus-associated antibody anti-La. The La antigen in uninfected mammalian cells consists of a heterogeneous class of small ribonucleoprotein particles, some of whose RNA components exhibit sequence homology with a highly repetitive, interspersed class of human DNA designated the Alu family. Possible functions for EBER 1 and EBER 2 in infection and cell transformation by EBV and their potential relationship to the pathogenesis of systemic lupus erythematosus are discussed.

Sera from patients with systemic lupus erythematosis (SLE) contain autoantibodies that react with components of normal cells. (See ref. 1 for a review.) Some of the antigens are composed of proteins complexed with small RNA molecules, about 80–200 nucleotides long (2–4). They may be localized either in the nucleus, in which case they are called small nuclear ribonucleoproteins (snRNPs) (2), or in the cytoplasm, in which case they are called small cytoplasmic ribonucleoproteins (scRNPs) (4).

Four different categories of small ribonucleoproteins (RNPs) can be distinguished on the basis of the RNA molecules that are included in immunoprecipitates formed by using specific SLE sera (4). Anti-Sm serum recognizes five different snRNPs, containing U1, U2, U4, U5, or U6 RNAs, whereas anti-RNP serum precipitates a subset of these particles, those containing only U1 RNA (2, 3). A third type of SLE serum, anti-Ro, precipitates a distinctly different set of RNA molecules, which are located in the cytoplasm of mammalian cells (4). A fourth type of SLE-associated antibody, designated anti-La (5) [also SS-B or Ha (1, 6)], precipitates a heterogeneous spectrum of nuclear RNAs of various sizes between 80 and 140 nucleotides (4). La antigen, originally reported to be cytoplasmic, is now generally believed to be nuclear. In our hands anti-La antisera give primarily nuclear staining by indirect immunofluorescence. Anti-La antibodies also recognize a RNP complex containing a small adenovirus-encoded RNA, virus-associated RNA (VA RNA), from infected HeLa cells (4). Whereas the U1-containing snRNP has been implicated in the splicing of heterogeneous nuclear RNA (3), functions for the other snRNPs and scRNPs remain obscure.

The studies reported here were prompted by the hypothesis that cells harboring the Epstein–Barr virus (EBV) genome might synthesize novel RNAs or proteins that would become incorporated into RNP complexes recognized by SLE antibodies. EBV has been implicated in several human diseases, including infectious mononucleosis, Burkitt lymphoma, and nasopharyngeal carcinoma (7–9). Its genome is a potent transforming agent that varies in copy number, base sequence, and gene expression in different infected and transformed primate cell lines (10). In a preliminary experiment we attempted to learn whether sera containing the SLE antibodies anti-Sm, anti-Ro, or anti-La would precipitate any new small RNPs from extracts of a continuous lymphoblastoid line that carries the EBV genome. We found that anti-La, but not the other sera tested, identified two new small RNAs (termed EBER, for EBV-encoded RNA), which we have characterized.

MATERIALS AND METHODS

Preparation of Cellular Supernatants and Antibody Precipitates. Raji, HR1K, B95-8, MCUV, Cory, BJAB-B1, or BJAB cells at $1–5 \times 10^5$ per ml in minimal essential medium with 5% bobby calf serum (GIBCO) containing 2% of the usual concentration of phosphate were labeled with [^{32}P]orthophosphate at 10 mCi/liter (1 Ci = 3.7×10^{10} becquerels) for 24 hr. Cellular supernatants were typically prepared from 10^7 cells by the following procedure, carried out at 0°C. Cells were harvested by centrifugation at $160 \times g$ for 5 min, washed in 20 ml of Tris-buffered saline (130 mM NaCl/40 mM Tris·HCl, pH 7.4), broken by sonication for 15 sec at setting 2 of a Branson sonifier, and clarified by centrifugation at $5300 \times g$ for 5 min. The cell supernatant was used immediately as a source of antigen.

Immunoprecipitations using previously characterized anti-La antibody (4) and Pansorbin (Calbiochem) were performed as described by Kessler (11). IgG was used at the same concentration as in the human serum from which it was derived. In a typical experiment, 10 μl of IgG was incubated with the supernatant from 10^7 cells at 0°C for 15 min; then 100 μl of Pansorbin was added and incubation was continued for 5 min. The complexes were pelleted by centrifugation and washed five times with NET2 buffer (11).

Purification and Analysis of RNAs. RNAs prepared by phenol extraction were fractionated on 10% polyacrylamide (27:1 acrylamide/bisacrylamide) gels in 7 M urea/45 mM Tris borate, pH 8.3/1.25 mM EDTA; bands were eluted electrophoretically. T1 and pancreatic RNase digests were finger-

printed by electrophoresis on Cellogel at pH 3.5 followed by homochromatography on thin layers of polyethyleneimine (Cel 300, Brinkmann) with homomix c (12). Oligonucleotides were subsequently eluted and analyzed by digestion of T1 spots with pancreatic RNase and of pancreatic spots with T1 RNase (12). Modified nucleotides were examined by two-dimensional chromatography (13) after digestion to completion with a mixture of T2, T1, and pancreatic RNases. Analysis for the presence or absence of capped 5′ ends was performed as described by Southern and Mitchell (14). The uncapped 5′ ends were identified by chromatography as described by Cashel *et al.* (15). Denaturing sodium dodecyl sulfate/urea gels (16) were used to determine the sizes of EBER 1 and EBER 2 by comparison with marker RNAs (U1, U2, U4, U5, U6) of known length (17).

Viral DNA Purification and Southern Blot Analysis. EBV was concentrated from B95-8 or HR1K cell supernatants by precipitation with polyethylene glycol (18). After the DNA was extracted by digestion with proteinase in the presence of Sarkosyl, samples were purified twice by centrifugation in CsCl, dialyzed, and concentrated by dialysis against solid polyethylene glycol. Restriction endonucleases BamHI, EcoRI, or both were incubated with viral DNA at 20 μg/ml for 2 hr at 37°C. Restriction enzyme buffers were: EcoRI in 100 mM Tris·HCl, pH 7.5/ 50 mM NaCl/5 mM MgCl$_2$; BamHI in 6 mM Tris·HCl, pH 7.5/ 50 mM NaCl/6 mM MgCl$_2$/6 mM 2-mercaptoethanol/10 μg of bovine serum albumin per ml.

To prepare blots, DNA samples were electrophoresed into 0.5% agarose horizontal slab gels in a buffer system containing 40 mM Tris·HCl at pH 7.7, 20 mM sodium acetate, 2 mM EDTA, and ethidium bromide at 1 μg/ml. Gels were then soaked in 0.5 M NaOH/1.5 M NaCl for 30 min at room temperature and neutralized in 1.0 M Tris·HCl, pH 6.0/0.6 M NaCl for 30 min at room temperature. The DNA was transferred to nitrocellulose by the procedure of Southern (19). The blot was washed in 2× NaCl/Cit (1× NaCl/Cit is 150 mM NaCl/15 mM sodium citrate, pH 6.35), dried, and heated at 80°C for 1.5 hr. Baked filters were cut into strips usually containing two lanes of EBV B95-8 DNA digested with EcoRI or BamHI. These were sealed in polyethylene bags for hybridization to EBER 1 or 2 labeled with ^{32}P.

Hybridization was carried out at 42°C for 72 hr in 5 ml of 50% (vol/vol) formamide, 5× NaCl/Cit containing tRNA carrier at 500 μg/ml and labeled EBER 1 or EBER 2 (isolated from Raji cells) at about 50,000 cpm. This solution was heated at 100°C for 4 min and chilled in ice before use. After hybridization, blots were rinsed once in 50% formamide, 5× NaCl/Cit and twice with 2× NaCl/Cit, each for 1 hr at 42°C. After brief drying, blots were wrapped in plastic and exposed to film on intensifying screens.

T1 RNase Hybridization-Protection Experiments. T1 hybridization-protection experiments were carried out by using 48,000 cpm of either EBER 1 or 2 and 1 μg of EBV B95-8 DNA as described by Weiner (20). Protected RNA fragments were electrophoresed into a Tris/borate/EDTA/urea/10% polyacrylamide gel as described above. The gel was dried and exposed to film on an intensifying screen.

RESULTS

EBER 1 and EBER 2 Are Encoded by EBV DNA. EBER 1 and 2 were first detected in polyacrylamide gels of RNAs obtained from immunoprecipitates between a ^{32}P-labeled cell extract of FF41 cells (a marmoset line containing EBV isolated from the saliva of a patient with mononucleosis) and anti-La serum from a patient with SLE. Other sera (anti-RNP, anti-Sm, and anti-Ro) known to recognize different classes of small RNPs failed to precipitate the EBERs.

To establish whether these small RNAs were related to the presence of the EBV genome, we performed similar immunoprecipitation analyses on a variety of other lymphoid cells (Fig. 1). These included three B cell lines derived from patients with Burkitt lymphoma, two with (HR1K and Raji) and one without (BJAB) the EBV genome; HR1K is a producer of virus, whereas Raji is a nonproducer. We examined the genome-negative BJAB line before and after its permanent conversion to carriage of EBV by infection with the HR1 virus (BJAB-B1). We also attempted to demonstrate the two EBERs in continuous marmo-
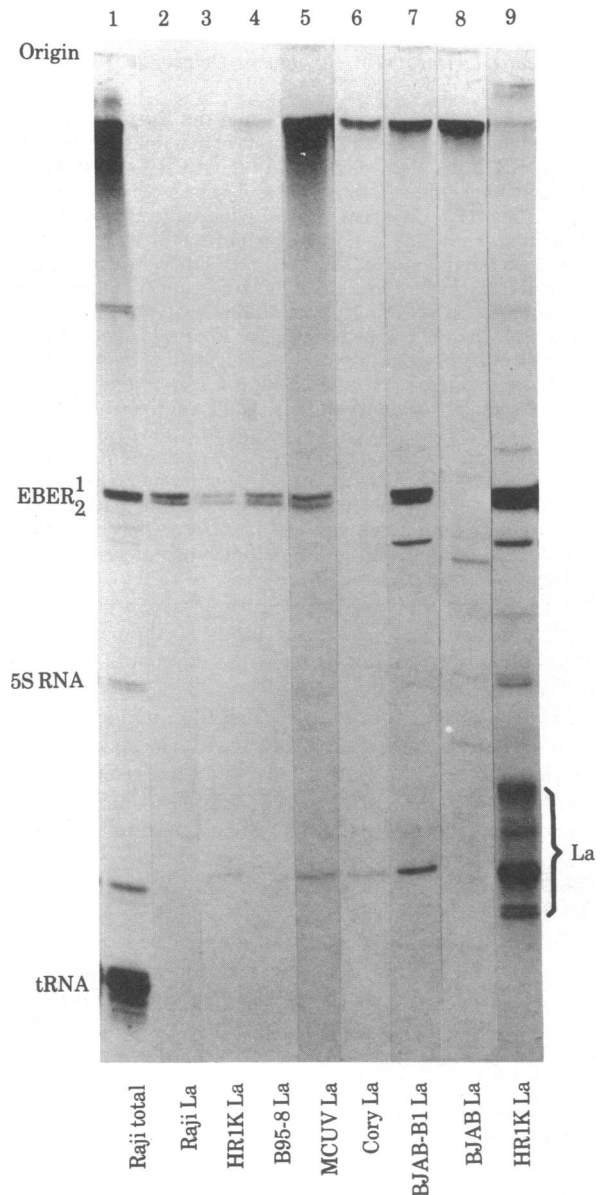

FIG. 1. Detection of EBER 1 and 2 in anti-La precipitates from EBV genome-positive cell lines. Cells were labeled in culture and RNAs were isolated from total (lane 1) or anti-La precipitated (lanes 2–9) extracts. Lane 1 shows the total RNA in Raji cells. Lane 2 shows those Raji cell RNAs specifically precipitated by anti-La. Lanes 3–5 show the La pattern obtained from the EBV genome-positive HR1K, B95-8, and MCUV cell lines, respectively. Lane 6 shows a parallel precipitation from EBV genome-negative Cory cells. Lanes 7 and 8 show anti-La precipitates from the *in vitro* EBV-infected BJAB-B1 and the uninfected BJAB parent cell lines. Lane 9 is a longer exposure of lane 3, showing the uninfected human cell La RNA spectrum. Comparable results were obtained with three different patient's sera, all of which have anti-La specificity (4).

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set B lymphoblastoid lines transformed *in vitro* by EBV (B95-8 and MCUV) and, as a control, a marmoset T lymphoid line immortalized by another lymphotropic herpes virus, *Herpesvirus ateles* (Cory). The data of Fig. 1 indicate that EBER 1 and EBER 2 are always present when the cells contain the EBV genome. EBER 1 and 2 are absent in the genome-negative BJAB and Cory cells, as well as in over 10 other nonlymphoid mammalian cell lines we have tested. From Fig. 1, lane 9, it is apparent that the La antiserum also precipitates from both EBV genome-positive and genome-negative cell lines a heterogeneous group of small RNA–protein complexes; these are comparable to those present in nonlymphoid human cells, such as HeLa cells (4).

All the EBV genome-positive lymphoid lines examined in the experiment of Fig. 1 contain multiple copies of EBV DNA. For example, our Raji line possesses about 100 copies per cell and the virus-producer lines HR1K, MCUV, and B95-8 have an average of several hundred copies per cell (21). An estimate that Raji cells contain about 10^7 EBER molecules can be made by comparing the relative intensity of labeling of EBER 1 and 2 with that of 5.8S ribosomal RNA (Fig. 1, lane 1), assuming comparable rates of synthesis and degradation. Fig. 1, lane 9, shows that EBER 1 and 2 are also more abundant in HR1K cells than are the other RNA components of the La snRNPs. We additionally tested a nonproducer EBV-transformed human neonatal B cell line (FF407-219) that has been found to contain only 1 copy of the EBV genome per cell (21); in this line EBER 1 and 2 were detected, but at lower levels, approximately comparable to those of the La RNAs (data not shown). Thus it appears that the copy number of EBERs is related to the copy number of EBV DNA molecules in each cell type.

The data at this point allowed two interpretations: EBERs are normal cellular RNA–protein complexes whose synthesis is induced by EBV, or EBERs are directly encoded by EBV DNA. To distinguish between these alternatives, ^{32}P-labeled EBER 1 and EBER 2 were eluted from acrylamide gels and hybridized to Southern blots of EBV (B95-8) DNA restriction fragments generated by *Eco*RI and *Bam*HI, singly and in combination. Both EBERs hybridized to the *Eco*RI-J and *Bam*HI-C fragments (Fig. 2), and in a double digest to a fragment identical in mobility to *Eco*RI-J (not shown). Published physical maps locate the 3000-base pair *Eco*RI-J fragment within *Bam*HI-C (22). The EBERs also hybridized to a fragment with mobility identical to that of the J fragment in a blot prepared from an *Eco*RI digest of the nontransforming HR1K viral DNA (not shown).

We further tested the hypothesis that hybridization of EBER 1 and EBER 2 might be due only to partial, rather than complete homology of the RNA sequences to EBV DNA. EBER 1 and 2 were individually hybridized to EBV DNA, the RNA·DNA hybrids were digested with T1 RNase, and the RNAs were eluted from the hybrids and electrophoresed in polyacrylamide gels. Full-sized molecules, indicating complete protection of the RNA sequences by EBV DNA, were observed (data not shown).

Properties of EBER 1 and EBER 2. EBER 1 and 2 are distinct, previously undescribed, small RNA molecules as demonstrated by T1 RNase fingerprint analysis (see Fig. 3). Pancreatic RNase fingerprints (not shown) are likewise different for the two RNAs. The fingerprints contain no spots consistent with poly(A) tails. Analysis of total T2, T1, plus pancreatic RNase digests (13) revealed few, if any modified bases. Capped 5′ termini were not detected by the method of Southern and Mitchell (14). Rather, the terminal nucleotide generated by RNase P1 digestion of both molecules ran with marker pppA in the system of Cashel *et al.* (15). Accordingly, all ^{32}P label from either this or the 5′ nucleotide generated by T2 digestion was released upon treatment with bacterial alkaline phosphatase (12). Analysis of the pppA-containing spot from the T1 RNase fingerprint (indicated by the

arrow in Fig. 3) revealed that the second residue of both EBER 1 and EBER 2 is G. Mobilities of the RNAs on sodium dodecyl sulfate/urea gels (16) suggested that EBER 1 and EBER 2 are approximately 175 and 180 nucleotides long, respectively.

EBER 1 and EBER 2 Are Incorporated into RNP Particles. EBER 1 and EBER 2, as recovered in cell extracts, are involved in specific RNA–protein complexes. Like the normal cellular La RNAs and VA RNA from adenovirus-infected HeLa cells (4), EBER 1 and 2 cannot be precipitated in detectable amounts after deproteinization by phenol extraction (data not shown). Moreover, anti-La precipitates from [^{35}S]methionine-labeled Raji or BJAB-B1 cells reveal two prominent proteins on sodium dodecyl sulfate/polyacrylamide gels (unpublished data). These observations agree with the original report that the La antigen requires a protein component (5) and indicate that normal host protein(s) are bound by EBER 1 and EBER 2.

DISCUSSION

Relationship of EBER 1 and EBER 2 to EBV. We have found that these two small RNAs are invariably synthesized in lymphoid cells transformed by EBV. They are direct products of the EBV genome. Moreover, the EBERs appear to exhibit a "gene dosage effect" in that their amount is correlated with the copy number of EBV DNA molecules per cell.

Southern hybridization analyses have localized the regions coding for both EBER 1 and 2 to the *Eco*RI-J fragment of EBV
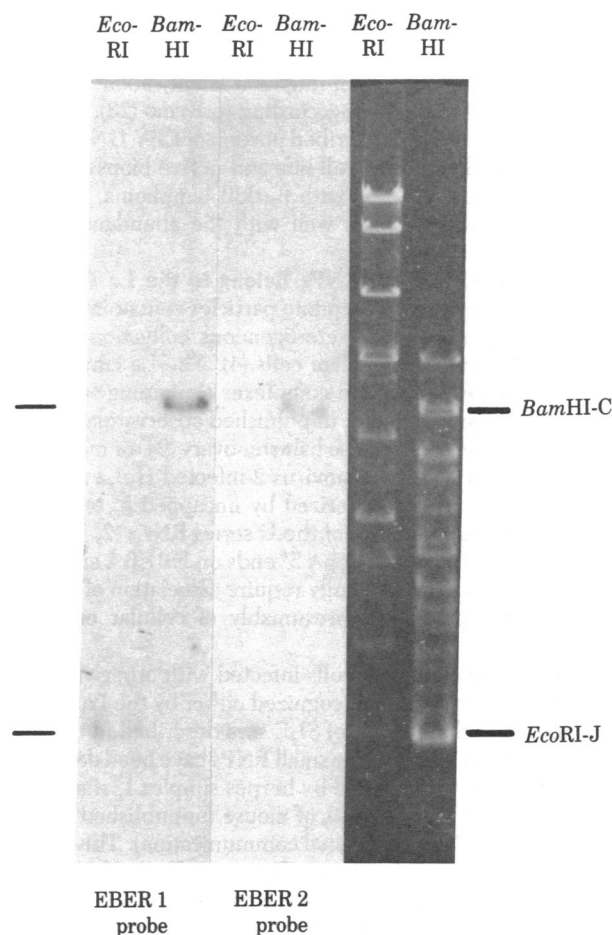

FIG. 2. **Southern hybridization of EBER 1 and EBER 2 to EBV DNA.** The third pair of lanes shows an ethidium bromide-stained agarose gel used to separate *Eco*RI and *Bam*HI restriction endonuclease fragments of the B95-8 EBV genome. The first and second pairs of lanes show Southern hybridizations to these fragments blotted onto nitrocellulose and probed with ^{32}P-labeled EBER 1 or EBER 2.
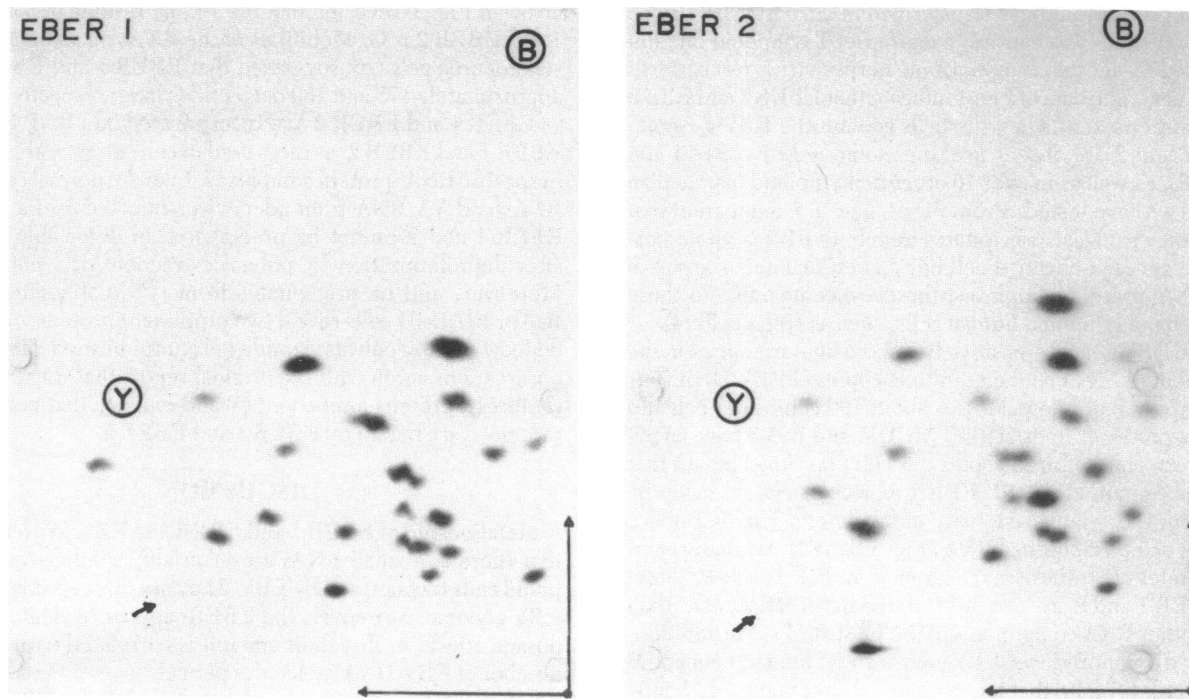
FIG. 3. T1 RNase fingerprints of EBER 1 and 2. B and Y indicate the positions of the blue and yellow dyes in the first (horizontal) and the second (vertical) dimensions. The streaked pppApGp 5′ end spot for each of the RNAs is identified with an arrow.

(B95-8) DNA. This restriction fragment is only about 3000 nucleotides long. Nonetheless, according to Rymo (23), it represents the most actively transcribed portion of EBV DNA both in the Raji Burkitt lymphoma cell line and in five biopsy samples taken directly from patients with Burkitt lymphoma. The high transcription rate correlates well with the abundance of the EBERs.

EBER 1 and EBER 2 RNPs Belong to the La Family of snRNPs. SLE sera that precipitate particles containing the two EBERs also precipitate a heterogeneous collection of small RNPs from various mammalian cells (4). The La family of snRNPs includes RNA–protein complexes containing such notable RNA components (ref. 4; unpublished observations) as 4.5S RNA from uninfected Chinese hamster ovary (24) or mouse cells (25) and VA$_I$ RNA from adenovirus 2-infected HeLa cells (26). All these RNAs are characterized by uncapped 5′ termini [in contrast to the capped ends of the U series RNAs (2, 17)], consistent with our finding of pppA 5′ ends on EBER 1 and EBER 2. All members of the La family require association of the RNA molecule with protein(s), presumably of cellular origin, for antigenicity.

We have also examined cells infected with other viruses for small RNPs that might be recognized either by the La antibody or by the other three types of SLE sera described in the Introduction. No new precipitable small RNPs have been detected in cells infected or transformed by herpes simplex I, simian virus 40, *H. ateles*, or minute virus of mouse (unpublished observations; Stephen Mount, personal communication). This suggests that not all viruses need carry information for producing these types of RNA–protein complexes. Nonetheless, it is tantalizing to note the parallels between the VA$_I$ and VA$_{II}$ RNAs of adenovirus and EBER 1 and 2. Like the EBERs, the two VA RNAs are similar in size (about 170 residues) but differ in sequence, and are encoded by nearby regions of the viral genome (26).

Studies on the easily accessible EBERs and VA RNA may be useful in further defining the function of the La family of snRNPs. It is possible that they all perform roles in heterogeneous

nuclear RNA processing. The U1-containing snRNP, precipitated by the SLE antibodies anti-Sm and anti-RNP (2), has been suggested to align splice junctions in heterogeneous nuclear RNA for removal of introns (3); antibody inhibition of the splicing of adenovirus early mRNAs in a nuclear system has recently provided strong support for this idea (27). Similarly, VA$_I$ RNA, a member of the La family, has been implicated in the splicing of late adenovirus mRNAs (28, 29), but no direct evidence has yet emerged.

Alternatively, other possible functions are suggested by overlap between the La family snRNPs and Alu family DNA sequences. The human Alu family consists of approximately 300,000 copies per genome of a highly conserved, widely interspersed 300-base pair DNA sequence (30, 31), many of which are transcribed into RNA (32). Also, 4.5S RNA (24, 25), which can be precipitated as a La snRNP (unpublished observations), shows strong similarity to the human Alu sequence (30, 31). Our preliminary rapid RNA sequence determination data on EBER 1 and EBER 2 also reveal significant homology with dominant oligonucleotides from the Alu family consensus sequence (30). We therefore anticipate a functional connection between the La family of cellular and viral RNAs and Alu family transcripts. It has been suggested (30, 31) that DNA replication or transcription by RNA polymerase II might exploit the widespread occurrence of the Alu sequences in human genomes. A viral version of the La antigen might mimic the normal cell system to the advantage of the virus, perhaps in the onset or maintenance of the transformed state. On the other hand, recent work with 7S cytoplasmic RNA demonstrates that it has considerable homology to Alu family sequences (20); 7S RNA has been suggested to be involved in the transport of mRNA from the nucleus to the cytoplasm (20). All the above aspects of cellular metabolism must be dealt with by a nonlytic virus such as EBV and are processes in which specific small RNA–protein complexes could fill pivotal roles.

EBER 1 and EBER 2 snRNPs as Antigens. All of the small RNAs precipitated by the four types of SLE antibodies de-

scribed in the Introduction must be complexed with protein(s) to be antigenic (2, 4). In the case of EBER 1 and 2, reconstitution data demonstrate that normal cellular protein(s) are indispensable for recognition by anti-La. Patients with either SLE or Sjogren syndrome develop autoantibodies with anti-La specificity (1) capable of precipitating the EBER-containing snRNPs. However, further seroepidemiologic studies using sera with and without anti-EBV antibodies from individuals with a variety of diseases are indicated. It will be of particular interest to learn whether sera from patients with Burkitt lymphoma or nasopharyngeal carcinoma recognize EBER snRNPs.

The fact that EBER 1 and EBER 2 are recognized by SLE antibodies only of the La specificity (and not by anti-RNP, -Sm or -Ro) suggests that EBV does not play a universal role in SLE. However, in certain patients with SLE, EBV might be involved in the induction of autoantibody formation. Synthesis of large amounts of viral EBERs and their assembly into RNPs might provide sufficient levels of the necessary antigen to provoke the autoimmune response. Additional work is necessary to correlate the presence of antibody that precipitates EBER with the presence or absence of anti-EBV antibody, with ongoing EBV infection, and with various autoimmune manifestations and clinical features in patients with SLE.

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