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In Vitro Selection of RNA Ligands for the Ribosomal L22 Protein Associated with Epstein-Barr Virus-Expressed RNA by Using Randomized and cDNA-Derived RNA Libraries

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The Epstein-Barr virus (EBV)-expressed RNA 1 (EBER1) associates tightly with the ribosomal protein L22. We determined the general requirements for an RNA to bind L22 in a SELEX experiment, selecting RNA ligands for L22 from a randomized pool of RNA sequences by using an L22–glutathione *S*-transferase fusion protein. The selected sequences all contained a stem-loop motif similar to that of the region of EBER1 previously shown to interact with L22. The nucleotides were highly conserved at three positions within the stem-loop and identical to the corresponding nucleotides in EBER1. Two independent binding sites for L22 could be identified in EBER1, and mobility shift assays indicated that two L22 molecules can interact with EBER1 simultaneously. To search for a cellular L22 ligand, we constructed a SELEX library from cDNA fragments derived from RNA that was coimmunoprecipitated with L22 from an EBV-negative whole-cell lysate. After four rounds of selection and amplification, most of the clones that were obtained overlapped a sequence corresponding to the stem-loop between nucleotides 302 and 317 in human 28S ribosomal RNA. This stem-loop fulfills the criteria for optimal binding to L22 that were defined by SELEX, suggesting that human 28S ribosomal RNA is likely to be a cellular L22 ligand. Additional L22 binding sites were found in 28S ribosomal RNA, as well as within 18S ribosomal RNA and in RNA segments not present in sequence databases. The methodology described for the conversion of a preselected cellular RNA pool into a SELEX library might be generally applicable to other proteins for the identification of cellular RNA ligands.

The lymphotrophic herpes virus, Epstein-Barr virus (EBV), can efficiently transform primary human B lymphocytes, and EBV is associated with a variety of human malignancies (reviewed in references 13 and 16). A small subset of the genes carried on the 172-kbp EBV genome is expressed in transformed B lymphocytes. These include six nuclear proteins termed EBNAs, two integral membrane proteins termed latent membrane proteins (LMPs), and two small RNAs termed EBERs for EBV-expressed RNA.

EBER molecules accumulate to high levels in cells latently infected or transformed by EBV (15, 30). EBER1 and EBER2, two short, polymerase III-transcribed transcripts, associate with the La antigen (12, 15). Additionally, EBER1 interacts with the ribosomal protein L22 (21, 23). When L22 is in a complex with EBER1, it does not associate with the ribosome but is relocalized to the nucleus (25). By mutational analysis, a domain of EBER1 involved in complex formation with L22 has been mapped to a region called stem-loop 3 (27).

Since L22 is an RNA-binding protein and a constituent of the large ribosomal subunit, one would anticipate that it interacts directly with 28S ribosomal RNA. It is also possible that it interacts with additional cellular RNAs. Since the identification of cellular RNA ligands for L22 could help to elucidate the function of both L22 and EBER1 (25–27), we first employed the SELEX (systematic evolution of ligands by exponential enrichment) protocol (4, 28) to identify a set of relatively optimal L22 RNA-binding sites, and then we used a modification of the procedure that we term cDNA-SELEX to search for natural cellular L22 ligands.

The application of the SELEX procedure to L22 produced a consensus RNA stem-loop sequence that includes three conserved nucleotides. Two sequences corresponding to this motif were evident in EBER1. One comprised stem-loop 3, the EBER1 domain that was shown previously to interact with L22 (27), and one corresponded to stem-loop 4 of EBER1. Both EBER1 domains interact with L22, although the protein binds to the stem-loop 3 sequence more efficiently than to the stem-loop 4 sequence. Mobility shift assays indicate that the viral RNA can simultaneously interact with two molecules of the protein.

The cDNA-SELEX procedure consisted of immunoprecipitation of RNA-protein complexes with an antibody to L22, followed by a SELEX amplification of L22-binding sequences present in the immunoprecipitate. This analysis revealed three sites on 28S ribosomal RNA that have the potential to interact with L22, one site on 18S ribosomal RNA, and two RNA segments that are not in current sequence databases.

MATERIALS AND METHODS

PCR, in vitro transcription, reverse transcription, sequencing. A SELEX library was constructed with primers and PCR conditions as described previously (20), except that *Escherichia coli* single-stranded DNA-binding protein (Perfect Match; Stratagene) was added to the PCR mixture as recommended by the supplier. In brief, a 77-nucleotide oligonucleotide composed of a randomized 30-nucleotide region flanked by two primer-binding sites was amplified in a PCR with two primers corresponding to these defined flanking sequences. One of the primers also contained a sequence corresponding to a T7 promoter appended to its 5′ end. RNA was transcribed from this initial pool with T7 RNA polymerase as previously described (18), in the presence of trace amounts of [α-^{32}P]UTP for quantitation. After digestion of the DNA template with DNase I, the RNA was purified by gel filtration (Sephadex G50; Pharmacia) and diluted into binding buffer (20 mM Tris Cl [pH 7.5], 250 mM NaCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM dithiothreitol). After selection and purification of the RNA ligands (see below), reverse transcription was carried out with avian myeloblastosis virus reverse transcriptase and the cDNA was amplified in 15 PCR cycles as described previously (28). A total of nine rounds of selection and

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amplification was performed. The PCR products of the final round were cloned into pUC19 with the restriction endonucleases *Bam*HI and *Hin*dIII. Single clones were isolated and sequenced with Sequenase 1.0 (United States Biochemical Corp.). The predicted secondary structure of the RNA corresponding to these sequences was determined as described previously (35).

Selection of RNA molecules binding L22 in vitro. A glutathione *S*-transferase (GST)–L22 fusion protein (27) was expressed in *E. coli* DH5α. Fifty milliliters of bacterial culture (with an optical density at 600 nm of 0.7) was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 30°C, resuspended in 0.5 ml of lysis buffer (20 mM Tris Cl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5 μg of pepstatin per ml, 0.5 μg of leupeptin per ml, 0.5 μg of aprotinin per ml, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% β-mercaptoethanol), and lysed by sonication. The NaCl and aprotinin concentrations were increased to 500 mM and 0.5 mg/ml, respectively, and glycerol was added to 20% (vol/vol). The lysate was incubated for 30 min with 30 μl (packed volume) of glutathione-Sepharose beads (Pharmacia) at 4°C and washed four times with wash buffer (lysis buffer containing 500 mM NaCl and 0.035% β-mercaptoethanol) for 1 min. The RNA transcript (20 μg of RNA in 0.5 ml of binding buffer) was incubated with the beads containing bound GST-L22 for 30 min at room temperature, after which it was washed in five 1-min washes and one 30-min wash in binding buffer. The protein was eluted together with its RNA ligands with 50 mM glutathione in binding buffer (with the pH adjusted to 7.5 with NaOH) for 30 min, and then the RNA was extracted with phenol and chloroform and precipitated with ethanol.

EMSA. GST-L22 was prepared as described above, eluted from a glutathione matrix, and dialyzed into phosphate-buffered saline. The PCR product from each round was transcribed and radiolabeled with [α-^{32}P]UTP with T7 RNA polymerase (Maxiscript; Ambion) according to the manufacturer's instructions. The RNA was purified by gel filtration (Chromaspin 30; Clontech). RNA (1 fmol) was incubated with 1 pmol of GST-L22 in electrophoretic mobility shift assay (EMSA) buffer (20 mM Tris Cl [pH 7.5], 300 mM NaCl, 5 mM $MgCl_2$, 10% glycerol; final volume, 20 μl) for 10 min at room temperature and then subjected to electrophoresis on a 4% polyacrylamide gel in 1× TBE (50 mM Tris base, 50 mM boric acid, 0.5 mM EDTA), followed by autoradiography. For supershift assays, 5 μl of rabbit serum was added to the reaction mixture prior to the addition of RNA. For competition experiments, EBER1 and other RNAs were transcribed in vitro with constructs described by Toczyski and Steitz (27), with the proportion of [α^{32}P]UTP versus unlabeled UTP being 5,000-fold less than that used for the transcription from the final SELEX round PCR product. One-tenth or one picomole of these competitor RNA molecules (representing 10-fold and 100-fold excesses, respectively) was added to 100 fmol of GST-L22 10 min prior to the addition of the RNA transcribed from the PCR product. All other EMSA experiments were carried out under the same conditions with the concentrations of ligands indicated in the figure legends. The location of radioactively labeled RNA in the gels was visualized with a PhosphorImager (Molecular Dynamics) and processed for presentation with the software Adobe Photoshop 3.0.

In vitro synthesis of RNA. Oligonucleotides that contained the same primer binding sites as those of the PCR template used for SELEX were synthesized, but instead of the randomized region, they contained sequences complementary to stem-loop 3 of EBER1 (GCCGGATCCGGGCCTCATGTCGAACACCACC CGGGACTTGTACCCGGGACGGGTTTGAGCGTTTATTCTGAGCTCCC), stem-loop 4 of EBER1 (GCCGGATCCGGGCCTCATGTCGAACAGAGTCT GGGAAGACAACCACAGACACCGTTGAGCGTTTATTCTGAGCTCCC), nucleotides 295 to 324 of 28S ribosomal RNA (GCCGGATCCGGGCCTCAT GTCGAAAGCCTTAGATGGAGTTTACCACCCGCTTTGTTGAGCGTTT ATTCTGAGCTCCC), nucleotides 3859 to 3888 of 28S ribosomal RNA (GCC GGATCCGGGCCTCATGTCGAAGCTCAACAGGGTCTTCTTTCCCCGC TGATTTTGAGCGTTTATTCTGAGCTCCC), and nucleotides 1355 to 1384 of 18S ribosomal RNA (GCCGGATCCGGGCCTCATGTCGAAGTTCGTTATC GGAATTAACCAGACAAATCGTTGAGCGTTTATTCTGAGCTCCC). PCR amplification and in vitro transcription were performed as described above for the SELEX library.

Construction of a cDNA SELEX library. BJAB cells (21), derived from human EBV-negative Burkitt's lymphoma, were cultivated in RPMI 1640 containing 10% fetal bovine serum (Gibco). L22 was immunoprecipitated from a BJAB cell lysate with a polyclonal rabbit antibody (25), and RNA was purified with a commercial kit (RNeasy; Qiagen). Two micrograms of RNA was annealed to 100 pmol of primer 1 (GCCGGATCCGGGCCTCATGTCGAANNNNNNNN; N represents the incorporation with equal likelihood of any of the four nucleotides during synthesis) in a total volume of 10 μl, after which reverse transcription was carried out with reverse transcriptase (Superscript II; Gibco) according to the manufacturer's instructions. The strands were separated by boiling for 5 min and then treated with RNase I and RNase H. The cDNA was separated from unincorporated primer by gel filtration (Chromaspin 400; Clontech). The RNase was inactivated by the addition of sodium dodecyl sulfate (final concentration, 0.3%) and by boiling for 2 min, after which it was extracted with phenol and chloroform and precipitated with ethanol. The first strand was annealed to 25 pmol of primer 2 (GGGAGCTCAGAATAAACGCTCAANNNNNN) in a total volume of 20 μl and then elongated with DNA polymerase I (Sequenase 1.0; United States Biochemical Corp.). The newly synthesized DNA was purified by gel filtration as described above and amplified by PCR over 15 cycles under the same conditions as those employed with the conventional SELEX library (20), except that the extension times (at 72°C) were set to 3 min. The selection and amplification steps were done as described above, except that the size selection was continued. That is, before PCR, the reverse-transcribed RNA was purified on Chromaspin 400 (Clontech), and after each round of PCR amplification, the PCR product was size selected on Chromaspin 200 before transcription. This was necessary to avoid the competitive effect of very short PCR products during the amplification. After four rounds of selection and amplification, the PCR product was cloned. The number of rounds was kept lower than that performed during the conventional SELEX procedure in order to avoid skewing the original composition of L22 ligands in the precipitated material. DNA was prepared from individual clones, and the size of the insert derived from the PCR product was determined by restriction analysis. Clones with an insert size greater than ~120 bp were sequenced.

RESULTS

Definition of an RNA motif with which L22 interacts. In an effort to determine the sequence and structural characteristics of an RNA molecule that can form a specific complex with L22, a SELEX experiment was carried out according to the protocol described previously (20). A library was created by PCR amplification from a pool of chemically synthesized oligonucleotides that contained a 30-nucleotide randomized region flanked by primer-binding sites. One of the primers contained a T7 promoter sequence that had been appended by PCR and was subsequently used to direct in vitro transcription. The resulting RNA library was incubated with an immobilized GST-L22 fusion protein (27). The RNA that bound to the matrix was eluted with free glutathione and amplified by sequential reverse transcription and PCR, and a new RNA pool was then prepared by transcription of the amplified DNA with T7 RNA polymerase. Nine sequential rounds of RNA capture and amplification were performed.

To monitor the progress of the selection process, EMSAs were carried out with the RNA pools from each round. As shown in Fig. 1A, the amount of RNA that could be shifted in its mobility by an excess of GST-L22 increased during the progression of SELEX rounds, indicating that the average level of affinity of the RNA pools for the protein was enhanced from round to round. The complex of GST-L22 and the final RNA pool from round 9 could be supershifted with a polyclonal antibody against L22 but not with preimmune serum (Fig. 1B, compare lanes 3 and 4), confirming that the RNA was forming a complex with GST-L22 and not with a contaminating bacterial protein. The supershifted complex migrated near the well at the top of the gel, presumably because multiple antibody molecules interacted at different locations on each L22 molecule, producing a very large complex. Both immune and preimmune antibodies also bound directly to RNA, as evidenced by the shifted complexes produced by antibodies plus RNA in the absence of GST-L22 protein (Fig. 1B, lanes 5 and 6). This nonspecific interaction is not responsible for the complex we have identified as a GST-L22 plus RNA plus antibody complex (Fig. 1B, lane 3), because the complex is not formed by the preimmune serum, which also interacts directly with the RNA probe (Fig. 1B, lane 4). To determine whether the interaction of the selected RNA from round 9 with L22 is similar to the interaction of EBER1 with L22, a competition experiment was performed. An excess of EBER1 could efficiently inhibit the selected RNA from binding to L22 (Fig. 1C, lanes 3 and 4), whereas an unrelated RNA, termed HSUR 3, a herpesvirus saimiri-encoded small RNA (19, 27, 33), could not (Fig. 1C, lanes 5 and 6). These results indicate that the selection procedure yielded specific RNA ligands that interact with the target protein in a way similar to that of EBER1.

After nine rounds of SELEX, individual molecules from the final PCR pool were cloned and sequenced. As shown in Fig.

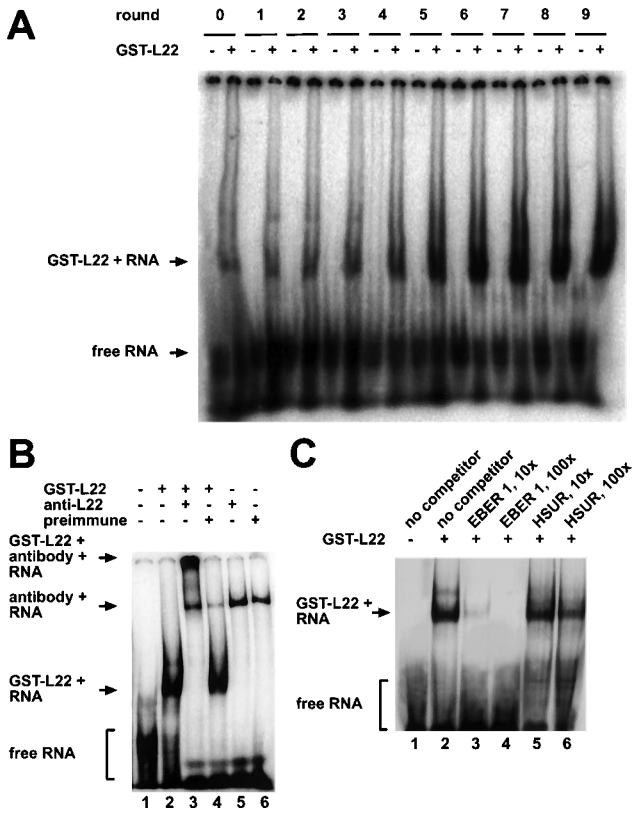

FIG. 1. EMSAs to monitor the progression of SELEX. (A) Radiolabeled RNA pools from the different SELEX rounds (round 0 refers to the original SELEX library) were transcribed and incubated with (+) or without (−) GST-L22. The increase in the amount of shifted RNA reflects the expected increased average level of affinity of the RNA pools during subsequent rounds. (B) Supershift of L22-RNA complex formed with RNA from the final SELEX pool. The RNA associated with L22 could be supershifted with a polyclonal anti-L22 antibody but not with a nonspecific control antibody. Note that both antibodies display a similar amount of nonspecific interaction with RNA. (C) Competition analysis of the complex formation of L22 with the final RNA pool. RNA from the final pool was radiolabeled, incubated with GST-L22 in the presence (+) or absence (−) of various unlabeled competitors in 10-fold or 100-fold molar excesses, and then subjected to EMSA. EBER1 competed efficiently, whereas an unrelated RNA of similar length (HSUR [33]) did not compete.

2A, a region that was predicted to fold into a stem-loop structure was present in all of the RNA sequences obtained. This folding pattern was confirmed by calculations (not shown) made according to the method described by Zuker (35). The sequences forming this structure are shown in Fig. 2B, and the consensus structure is diagrammed in Fig. 2C. The stem consisted of 3 to 6 bp, and in all but one case, a GC base pair formed the apical end of the stem (G on the 5′ side, C on the 3′ side). The loop consisted of 6 to 9 nucleotides, and in all cases, the most 3′ nucleotide within the loop was a U. These consensus criteria are exactly met by the stem-loop 3 structure on EBER1 (Fig. 2B), which has previously been shown to be involved in the complex formation with L22 (27). Interestingly, a mutation changing the conserved U residue at the 3′ end of the loop in the stem-loop 3 sequence has been shown to reduce the level of affinity of EBER1 for L22 by more than a factor of 5 (27). Thus, changing the base that is most conserved in the RNAs derived from the SELEX experiment substantially decreases the binding affinity of the naturally occurring EBER1, again confirming that in vitro selection (SELEX) and natural evolution yielded corresponding results.

EBER1 has the potential to bind two molecules of L22. An inspection of the EBER1 sequence revealed that not only stem-loop 3 but also stem-loop 4 is compatible with the consensus motif derived by SELEX, although there is a 1-base bulge in the stem. This raised the possibility that two molecules of L22 might be able to associate with one molecule of EBER1. To test this, we compared wild-type EBER1 with a mutant lacking stem-loop 3 but still containing stem-loop 4 (EBER Δstlp; cf. reference 27) and with an unrelated RNA (HSUR) for their abilities to associate with GST-L22. Two RNA-protein complexes were evident in an EMSA with wild-type EBER1 as the probe and high concentrations of GST-L22 (Fig. 3A, lanes 1 to 4), while only one complex was generated with EBERΔstlp as the probe (Fig. 3A, lanes 6 to 9) and no shifted complex was evident in the control experiment using the nonspecific HSUR probe (Fig. 3A, lanes 11 to 14). This is in agreement with the interpretation that a mutant EBER1 lacking stem-loop 3 can still associate with one molecule of L22, while wild-type EBER1 can bind either one or two molecules of L22. The fact that only one shifted complex was produced on the EBERΔstlp probe argues that the second complex formed on the wild-type EBER1 probe does not result from dimerization of GST-L22 molecules through a protein-protein interaction.

To directly demonstrate the interaction of GST-L22 with stem-loop 3 and stem-loop 4 and to compare the relative affinities of GST-L22 for the two structures, we synthesized the fragments as independent molecules. The sequences corresponding to these fragments were embedded into a surrounding sequence that was the same as that used for the random pools of SELEX by synthesizing a corresponding oligonucleotide, after which the sequences were amplified by PCR and transcribed in vitro. Both stem-loop sequences were shown to associate with GST-L22 independently (Fig. 3B, lanes 2 and 8). Stem-loop 4 RNA could be efficiently inhibited from interacting with L22 by an excess of stem-loop 3 RNA (Fig. 3B, lanes 9 and 10), whereas competition was far less efficient when assayed in the reverse combination (Fig. 3B, lanes 5 and 6). This indicates that stem-loop 4 can associate with GST-L22, albeit with a relatively lower level of affinity than that of stem-loop 3.

Identification of potential cellular ligands for L22. The specificity of the L22 RNA-binding site suggested that a cellular RNA that forms a specific complex with L22 in the absence of EBER1 might exist. In fact, the 28S ribosomal RNA is one candidate for an interaction since L22 is named for its presence in the large ribosomal subunit. In an effort to identify such cellular RNA molecules, we used a variation of SELEX that we call cDNA-SELEX. To create a cDNA-SELEX library, L22 was immunoprecipitated with a polyclonal antibody (22) from a lysate of BJAB cells, a cell line derived from an EBV-negative human B-cell lymphoma. RNA was purified from the precipitated material, yielding a pool that was enriched for RNA species present in complexes with L22 in vivo. This RNA pool was transformed into a SELEX library as outlined in Fig. 4. A primer with a randomized octamer at its 3′ end was annealed to the RNA and then reverse transcribed, yielding the first DNA strand of the library (cf. references 5 and 6). After enzymatic removal of the RNA, a second primer, which was randomized over 6 bases at its 3′ end, was used to initiate the second-strand synthesis; the second strand extended to the first-strand primer and could be used as a PCR template with the same primers used for the first SELEX experiment, which employed an artificial, randomized sequence library. The primer comprising the T7 promoter had the nonrandomized sequence of the second-strand primer at its 3′ end. Therefore,

A

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Eap11  gggagctcagaataaacgctcaaTCTCTAACTGTAGAGCAACGAGCCGATAAttcgacatgaggcccggatccggc
Eap15  gggagctcagaataaacgctcaaCACCCCGAGTTATCTTCGGGAGTGCAGAttcgacatgaggcccggatccggc
Eap13  gggagctcagaataaacgctcaaGACTGCAGCGGCAACCTCGCTGTATCCTTCttcgacatgaggcccggatccggc
 Eap3  gggagctcagaataaacgctcaaTCTGCGGCAACCTCGCAGAACAACAACACAAttcgacatgaggcccggatccggc
Eap17  gggagctcagaataaacgctcaaCTCGCGGCCACATTCCGCGATATTTAACACttcgacatgaggcccggatccggc
Eap14  gggagctcagaataaacgctcaaCCGCGGTAACCTTCCGCGCAATTGCAAttcgacatgaggcccggatccggc
Eap18  gggagctcagaataaacgctcaaTGCGGTTACCTTCCGCAACACAGTGCTACAttcgacatgaggcccggatccggc
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 Eap5  gggagctcagaataaacgctcaaCAGCAGCGAACCTTCTGCTAGGGCGCTTCGttcgacatgaggcccggatccggc
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 Eap2  gggagctcagaataaacgctcaaTAGAATAAACGCTTCAACCCGAAGTAACAATCttcgacatgaggcccggatccggc
 Eap8  gggagctcagaataaacgctcaaTAAGCATGACTGAGGAAACCATAACTCGCCttcgacatgaggcccggatccggc
Eap20  gggagctcagaataaacgctcaaACTTGTCCAGTAAGTAGGCCCCTTCTACTAGttcgacatgaggcccggatccggc
Eap22  gggagctcagaataaacgctcaaACTGGGTTACATTTCCAGTACAGCACTGTAttcgacatgaggcccggatccggc
Eap16  gggagctcagaataaacgctcaaACAACACATAGGGTACCTTCCCTATTCCAttcgacatgaggcccggatccggc
 Eap7  gggagctcagaataaacgctcaaAGGGACACCTTCCCTTGAAAATTCCACGTCttcgacatgaggcccggatccggc
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B

clone	5' stem	loop	3'stem
15	cccg	agttatctt	cggg
13	cagcg	gcaacct	cgctg
3	tctgcg	gcaacct	cgcaga
17	tcgcgg	ccacatt	ccgcga
14	cgcgg	taacctt	ccgcg
18	tgcgg	ttacctt	ccgca
19	acagag	cccatt	ctctgt
5	agcag	cgaacctt	ctgct
9	ccgcag	accccatt	ctgcgg
20	agtag	gcccctt	ctact
22	actgg	gttacattt	ccagt
16	ataggg	tacctt	ccctat
7	aaggg	acacctt	ccctt
11	ctct	aactgt	agag
EBER1			
stem-loop 3	cccggg	tacaagt	cccggg
stem-loop 4	gtctgtgg	ttgtctt	cccagac

C

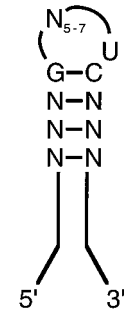

FIG. 2. L22-binding RNA sequences. (A) Sequences obtained after cloning the final PCR product generated by the SELEX procedure. The sequences shown correspond to the RNA that would have been synthesized from the PCR product by T7 RNA polymerase. Sequences derived from the PCR primer are shown in lowercase type, whereas sequences that originated from the randomized region of the library are shown in uppercase type. Nucleotides that are predicted to form stems are underlined. (B) Alignment of the predicted stem-loop structures observed in the sequences shown in panel A. Only those sequences that are entirely derived from the randomized region of the library but that do not partially consist of the primer binding sites are shown. All but one of these structures shows a GC base pair at the apical end of the stem with G on the 5′ side and C on the 3′ side. The base at the 3′ extreme of the loop invariably is U. Included is an alignment with the stem-loop 3 of EBER1, which has been previously shown to interact with L22 and which also displays this configuration. Previous experiments have shown that a base exchange at the conserved U residue reduces the affinity level of EBER1 to L22 from 100% to 17% (27). Included is an alignment with stem-loop 4 of EBER 1. Note that there is a 1-base bulge in the stem structure of stem-loop 4. (C) Model derived from the data shown in panel B for a generalized RNA motif that mediates the interaction of RNA with L22: a stem with a GC base pair at the apical end loops out 6 to 9 bases, the most 3′ of which is a U residue.

a directional cDNA pool that would yield only fragments of naturally occurring RNA molecules upon transcription with T7 RNA polymerase was created. In preliminary experiments, such a cDNA library was created out of a total RNA preparation from human fibroblasts. From this library, fragments of mRNAs of low abundance, such as p53 or NF-κB mRNAs, could be detected by PCR amplification (data not shown), indicating that the procedure can generate a highly representative library.

The cDNA-SELEX library derived from RNA coimmunoprecipitated with L22 was further enriched with RNA fragments binding GST-L22 by the same selection procedures outlined for the SELEX experiment using a randomized sequence library. After four selection rounds, individual cDNA fragments were cloned and sequenced (Fig. 5). A database search showed that 11 of the 15 selected cDNA fragments are derived from the human 28S ribosomal RNA (10), and of those, 6 include the stem-loop structure between nucleotides 300 and 320. Indeed, this structure on 28S ribosomal RNA matches the consensus motif previously defined by the SELEX experiment using a randomized sequence library. The fact that the cDNA clones obtained overlap in the stem-loop region but are not identical indicates that they were synthesized and selected independently from each other. A database search revealed that the sequence in this region of 28S ribosomal RNA is highly conserved among all eukaryotes (it is named stem-loop 7 according to the nomenclature of reference 17), suggesting that if L22 does indeed interact with this sequence in vivo, then its interaction is well conserved through evolution.

Two additional domains of the 28S ribosomal RNA and one segment from the 18S ribosomal RNA were isolated less frequently than the 28S domain between nucleotides 300 and 320 by the cDNA-SELEX protocol as potential ligands for L22. Also, in two cases, clones that did not have corresponding sequences in the database were identified (Fig. 5). In all of the sequences which were isolated less frequently, a motif similar to that of the consensus (Fig. 2C) could be identified. However, in some cases, the consensus criteria are only partially fulfilled: a sequence derived from 28S rRNA (nucleotides 3859 to 3888) is predicted to fold into a stem-loop structure with an apical GC base pair, but it does not contain a U at the 3′ end of the loop. Other motifs contain mismatches in the stem region (e.g., 18S rRNA, nucleotides 1355 to 1384).

The relative binding efficiencies of some of these different motifs for L22 were assessed by an EMSA. To this end, RNA molecules corresponding to these fragments were produced in the same way as described above for RNA corresponding to stem-loop 3 and stem-loop 4 of EBER1. Equal amounts of each RNA species were then mixed with different concentrations of purified GST-L22, and the resulting complexes were analyzed. The RNA pool from round 9 of the SELEX procedure and RNA corresponding to stem-loop 3 of EBER1 formed complexes with GST-L22 at similar efficiencies (Fig. 6, compare lanes 1 and 2 with lanes 4 and 5). Stem-loop 4 of EBER1 also associated with GST-L22, albeit with a slightly reduced level of efficiency (Fig. 6, lanes 7 and 8), in agreement with the competition experiments whose results are displayed in Fig. 3B. Among the sequences derived from cDNA-SELEX, the sequence motif identified most frequently (nucleotides 295 to 324 of 28S rRNA) interacted with GST-L22 most efficiently (Fig. 6, lanes 10 and 11), whereas two other sequences (nucleotides 3859 to 3888 of 28S rRNA and nucleotides 1355 to 1384 of 18S RNA) mediated relatively weak interactions with the protein (Fig. 6, lanes 13 to 18).

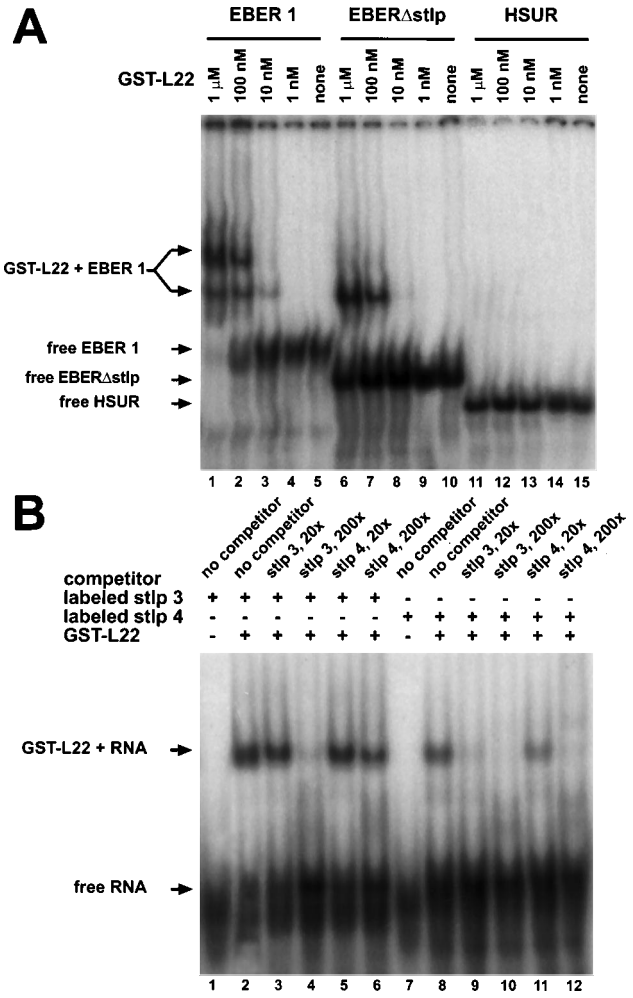

FIG. 3. EMSAs demonstrating the association of EBER1 with L22. (A) In vitro-transcribed EBER1, EBERΔstlp, and HSUR RNAs at a concentration of 1 nM were incubated with purified GST-L22 at the indicated concentrations (total volume, 10 μl) and subjected to electrophoresis. In the case of wild-type EBER1, two different shifted bands can be identified, the upper band possibly corresponding to a complex of two L22 molecules with one EBER1 molecule. Shifted complexes and free RNAs are labeled. (B) Mutual competition of stem-loop 3 and stem-loop 4 derived from EBER1 for binding GST-L22. α-^{32}P-labeled RNA at a concentration of 1 nM was incubated in the presence of 100 nM GST-L22 and the indicated amounts of nonlabeled competitor RNAs and then electrophoresed. Shifted complexes and free RNAs are labeled.

DISCUSSION

Using the SELEX procedure, we have obtained RNA pools that form complexes with L22 protein (Fig. 1A and B). Competition analysis showed that an excess of EBER1 can compete with the final RNA pool to interact with L22 (Fig. 1C). This suggests that both types of RNA are interacting with L22 in a similar way, probably involving similar structures on the RNA and on the protein. Analysis of individual L22-binding RNA sequences derived from the SELEX procedure (Fig. 2A and B) revealed that L22 specifically binds to RNA that is structured as a stem-loop and has conserved nucleotides in three positions (Fig. 2C).

The EBV RNA that can bind L22, EBER1, contains a sequence (stem-loop 3) that corresponds precisely to the consensus motif identified by SELEX (Fig. 2B), and this sequence has been shown previously to interact with L22 (27). The EBER-like herpesvirus papio type 1 RNA (12) encoded by the baboon lymphotropic virus, also binds to L22 (27), and an inspection of its sequence also reveals a precise match to the L22-binding motif: 5′ TCCCGGGTACAAGTCCCGGGA 3′ (nucleotides 119 to 139). The efficiencies with which the EBER1 stem-loop 3 and the final SELEX pool interact with L22 are similar (Fig. 6), and this suggests that the affinities of the two interactions are similar. This is in contrast to the results obtained in earlier SELEX experiments using other proteins that bind to viral RNAs. Specifically, the RNA sequences that were found to confer optimal in vitro binding to the Tat or Rev proteins from human immunodeficiency virus did not correspond to the TAR or RRE motif, respectively (29). In the case of the interaction of L22 with EBER1, our results suggest that natural selection during the evolution of EBV yielded a solution similar to that of the in vitro selection. This strengthens the suspicion (26) that EBER1 evolved at least in part to interact with L22. Further, since the SELEX procedure ultimately selects for RNA ligands that bind to a protein with a high level of affinity, it appears that the EBER1 sequence has evolved to contribute to the formation of an optimally stable complex with L22 protein.

Both stem-loop 3 and stem-loop 4 of EBER 1 separately interacted with L22 when synthesized as fragments, although the apparent level of affinity of stem-loop 4 for L22 is lower when compared with that of stem-loop 3 (Fig. 3B). This raises the possibility that EBER1 can form a bridge between two L22 molecules. This model is further supported by the observation that EBER1 and GST-L22 can form complexes with different electrophoretic mobilities, which are produced in different proportions, depending on the concentration of GST-L22 (Fig. 3A). We suspect that the band with the slower mobility corresponds to a complex of two GST-L22 molecules with one EBER1 molecule and that the faster-migrating band reflects the complex of one molecule of each. The ability of EBER1 to associate with two L22 molecules might contribute to its biological function in vivo.

To find cellular RNA molecules that can interact with L22, we devised a modified SELEX procedure that we termed cDNA-SELEX (Fig. 4). This protocol combines the SELEX procedure with the preselection of RNA species that interact with the protein of interest by immunoprecipitation. This method should be generally useful to screen for cellular interacting partners of proteins that specifically bind RNA. A similar methodology has been described by others (7) and was used to amplify sequences close to the 3′ ends of messenger RNA from an unselected polyadenylated RNA preparation, after which the sequences were subjected to SELEX. In contrast, our method allows the amplification of cDNA fragments from all locations within a cellular RNA and it focuses the screen on RNA molecules that are coimmunoprecipitated with the target protein.

Several novel RNAs that interacted with L22 in the cDNA SELEX procedure were isolated (Fig. 5). These RNA sequences were not found in the GenBank and EMBL nucleotide sequence databases, and work is in progress to ascertain their identities and to determine whether their putative interactions with L22 are physiologically relevant. However, the RNA that we isolated most often by the cDNA-SELEX procedure as a potential interacting partner of L22 was the 28S ribosomal RNA. Importantly, the fragments that were amplified from this RNA were not randomly distributed over the more than 5,000 nucleotides of this molecule but rather were tightly clustered around two regions that displayed motifs identical or similar to that defined from a random RNA sequence library by the conventional SELEX procedure. Several lines of evidence suggest that the stem-loop sequence between nucle-

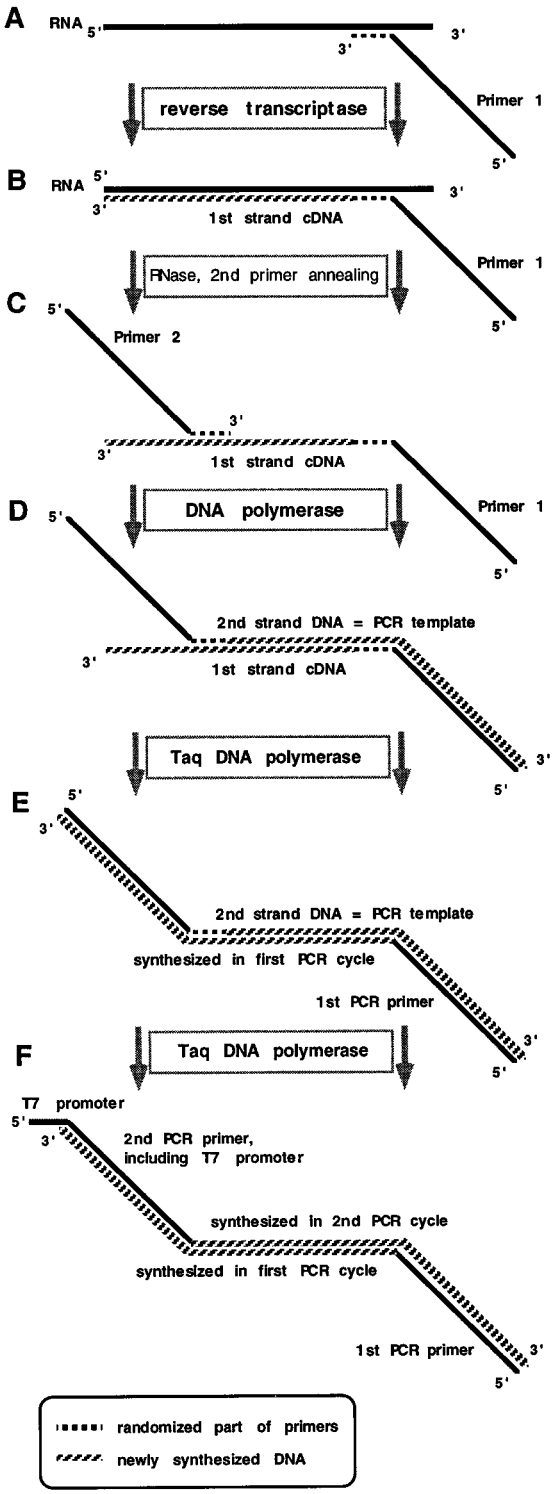

FIG. 4. Construction of a cDNA-SELEX library. (A) RNA was preselected by coimmunoprecipitation with an L22-specific polyclonal antibody from (EBV-negative) BJAB cell lysate and then subjected to reverse transcription. A primer (primer 1′) that contains a region corresponding to the first PCR primer (solid line) but additionally contains a randomized region of eight nucleotides at its 3′ end (dotted line) was used to allow random annealing to the RNA. (B) DNA (hatched line) was synthesized with reverse transcriptase. (C) After the enzymatic removal of RNA and free primer 1, the second-strand primer (primer 2) was annealed. Similar to primer 1, primer 2 had a randomized region of six nucleotides (dotted line) at its 3′ end. The 5′ region (solid line) corresponds to the second primer used in the PCR step. (D) The second DNA strand was synthesized with DNA polymerase. Note that the strand complementary to primer 1 is synthesized at the 3′ end of the second cDNA strand. (E) First PCR cycle. The first PCR primer is bound to the 3′ end of every DNA that was synthesized during the second-strand cDNA synthesis and then elongated. Note that the complementary strand to primer 2 is synthesized at the 3′ end of the DNA corresponding to the first strand cDNA. (F) Second PCR cycle. The second PCR primer contains the promoter for T7 RNA polymerase. The T7 promoter region will become double stranded during all subsequent PCR cycles. During subsequent in vitro transcription, RNA that corresponds to fragments of cellular RNA but that is flanked by primer binding sites will be synthesized. Thus, the cDNA-SELEX library can be used like a conventional SELEX library for the iterative selection and amplification of L22 ligands.

otides 300 and 320 is at least one important binding partner of L22 in vivo. First, independent isolates of RNA fragments comprising this region were obtained most often, and the relative levels of affinity of these RNA fragments appear to be higher than those of other RNA sequences isolated by cDNA-SELEX (Fig. 6). Second, this region is highly conserved among various eukaryotic species and includes the three residues apparently required for tight binding to L22, and its folding into a stem-loop structure in vitro and in vivo has been previously predicted (2, 34). The sequences of ribosomal RNA within this region are 100% identical for humans, *Mus musculus*, *Rattus norvegicus*, *Xenopus laevis*, and *Drosophila melanogaster*; the *Saccharomyces cerevisiae* large subunit RNA has a homologous region that is identical in 20 of 21 residues and in which the predicted stem-loop structure is not affected and the motif defined by SELEX is conserved. This suggests that an important function is mediated by this region. Since L22 is also well conserved through evolution (1, 3), this function might be the interaction of the 28S ribosomal RNA with L22 protein. Third, this region perfectly matches the criteria that were shown to allow tight binding to L22 in the conventional SELEX experiment. Fourth, L22 is found in the large subunit of ribosomes, consistent with the prediction that it might interact directly with 28S ribosomal RNA in vivo. Finally, the associations of L22 with EBER1 or the ribosome are mutually exclusive (25). This suggests that at least one important contact between L22 and the rest of the ribosome may be formed through an RNA structure that has features similar to those of EBER1, resulting in the competition of EBER1 with a ribosomal RNA to bind L22.

The isolation of RNA fragments containing additional sites for L22 binding in both 28S and 18S ribosomal RNA by the cDNA-SELEX procedure (Fig. 4) raises the possibility that several molecules of the protein could be present in the ribosome. However, it is difficult to be certain whether sites that interact in the cDNA-SELEX protocol are available to bind L22 in vivo. The accessibility of these sites could be influenced by RNA folding interactions and by other proteins interacting with the ribosomal RNAs.

We cannot be certain that all of the cellular RNAs with the potential to bind to L22 were identified in the cDNA-SELEX screen. RNAs could have been missed if their association with L22 is relatively weak or transient and failed to survive the immunoprecipitation step. Further, RNAs containing sequences that are difficult to copy with reverse transcriptase or amplify by PCR could have been missed. Finally, in our experiment we examined a limited number of clones, isolating several clones representing interactive RNAs only once. Thus, we could have failed to identify the full range of RNAs that might interact with L22 in our immunoprecipitated RNA population.

In the absence of EBER1, L22 presumably functions at least in part as a constituent of the ribosome. Since partially reconstituted ribosomes lacking detectable amounts of L22 were found to be active for translation in vitro (14), it is possible that

FIG. 5. Cellular L22-binding RNA sequences. Aligned sequences obtained after cloning the final PCR pool generated by four rounds of cDNA-SELEX are shown. Sequences derived from the primer-binding sites are not shown. Sequences that originated from the region of the library derived from cellular RNA are shown in uppercase type. Sequences derived from the randomized parts of the primers used in the construction of the cDNA-SELEX-library are shown in lowercase type. Nucleotides that are predicted to form base pairs in stem-loop structures are underlined. Nucleotides corresponding to the conserved GC base pair and U residue are shown in boldface type. Most often, sequences corresponding to 28S ribosomal RNA were obtained. Among those, sequences that overlapped the stem-loop region between nucleotides 300 and 320 of 28S ribosomal RNA were the most frequent. Sequences that originated from 18S ribosomal RNA or from sequences not found in the database were also isolated.

L22 is not essential for basal ribosomal function and plays a regulatory rather than a constitutive role. Since a considerable amount of L22 can be found in the nucleolus of uninfected cells (our unpublished observations and Toczyski et al. [25]), it might also regulate the assembly of parts of the ribosome before nucleocytoplasmic export occurs.

What is the purpose of the L22-EBER1 interaction? Perhaps EBER1 antagonizes one or more of the natural functions of L22 by binding and sequestering the protein. This model is attractive since, as discussed above, the EBER1 stem-loop 3 sequence appears to bind L22 with a high level of affinity, suggesting that it might compete efficiently with cellular RNAs for interaction with the protein. However, it is difficult to be certain of the affinity of EBER1 compared with that of cellular ligands for L22, since protein-protein interactions could play a major role in the stability of L22-containing complexes. In this regard, the ability of EBER1 to compete for L22 might be enhanced by its apparent ability to interact with two molecules of the protein. If the L22 proteins interact with each other as well as with EBER1, then they might enter the putative viral

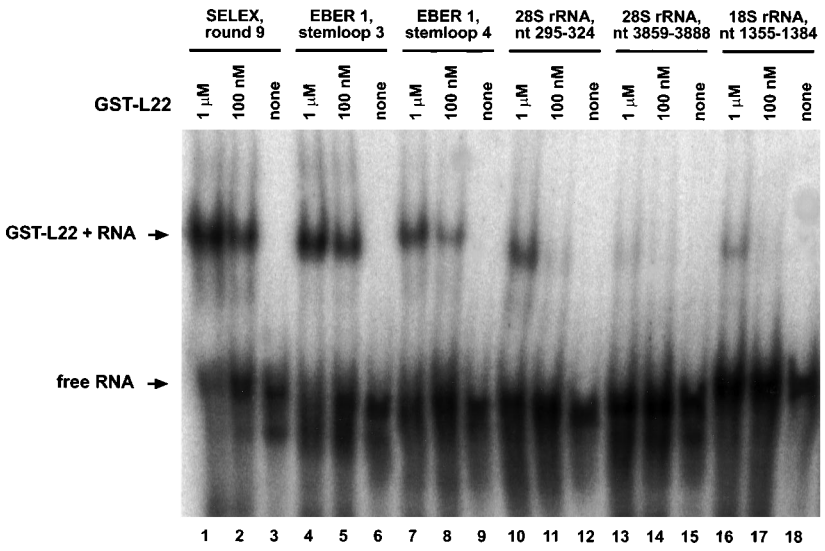

FIG. 6. Comparison of the relative efficiencies with which RNA fragments bind L22 protein in an EMSA. In vitro-transcribed RNA derived from the final round of the conventional SELEX procedure, EBER1, or ribosomal RNAs at a concentration of 1 nM was incubated with purified GST-L22 at the indicated concentrations (total volume, 10 μl) and subjected to electrophoresis.

sequestration complex in a cooperative fashion. Our initial studies suggest that two L22 molecules might interact with EBER1 in a cooperative fashion, since mobility shift assays indicate that two molecules of L22 bind to one molecule of EBER1 in the presence of an excess of free EBER1 (Fig. 5A, lanes 2 and 3). However, these assays have employed a GST-L22 fusion protein and it will be necessary to perform more extensive studies with native L22 protein to determine with confidence that the L22-EBER1 interaction is cooperative.

It is also possible that the L22-EBER1 interaction does not function merely by sequestering L22. Perhaps the complex carries out an active function in EBV-infected cells. This function might also involve the La antigen, since this protein is known to interact with EBER1 (9, 12).

Since neither of the two EBER genes is essential for EBV replication or immortalization when assayed in cultured cells (23, 24), EBERs might function primarily within an infected host, perhaps by antagonizing a host antivirus defense. This view is consistent with the observation that EBERs are almost always expressed in EBV-associated neoplasms (13, 16). In contrast, the lesions found in AIDS-associated and non-human immunodeficiency virus-associated oral hairy leukoplakia contain a variety of EBV gene products but no EBERs (8, 11, 31, 32). Since these lesions arise in individuals with partially compromised immune functions, one might speculate that the EBERs are normally selected to be expressed because they somehow protect cells from a host immune defense. Alternatively, the difference in expression may be due to the difference in the DNA template, which is linear in hairy leukoplakia and episomal in latency. Perhaps the identification of the nonribosomal cellular RNAs that interact with L22 (Fig. 5) will ultimately provide a clue to the function of the EBER ribonucleoprotein complex.

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