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Specific binding of a prokaryotic ribosomal protein to a eukaryotic ribosomal RNA: Implications for evolution and autoregulation

(protein–RNA interaction/RNA secondary structure/molecular evolution/autogenous regulation)

RICHARD L. GOURSE*, DAVID L. THURLOW†, SUSAN A. GERBI*, AND ROBERT A. ZIMMERMANN†

*Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912; and †Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01003

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ABSTRACT **Ribosomal protein L1 from the prokaryote *Escherichia coli* has been shown to form a specific complex with 26S ribosomal RNA from the eukaryote *Dictyostelium discoideum*. The segment of *Dictyostelium* rRNA protected from ribonuclease digestion by L1 and the corresponding region in *Dictyostelium* rDNA were investigated by nucleotide sequence analysis, and an analogous section in rDNA from *Xenopus laevis* was identified. When the L1-specific segments from eukaryotic rRNA were compared with those from prokaryotic rRNA, striking similarities in both primary and secondary structure were apparent. These conserved features suggest a common structural basis for protein recognition and indicate that such regions became fixed at a very early stage in rRNA evolution. In addition, certain structural elements of the L1 binding sites in rRNA are also found in the initial segment of the polycistronic L11-L1 mRNA, providing support for the hypothesis that L1 participates in the regulation of ribosomal protein synthesis by specific interaction with its own mRNA.**

Ribosomal particles are maintained in a compact, functionally active state by an intricate network of interactions among their protein and RNA constituents. Many ribosomal proteins exhibit high affinity for specific binding sites in rRNA, and stable complexes between purified components can be readily formed and studied *in vitro* (1). Protein L1 from the 50S ribosomal subunit of *Escherichia coli*, for example, associates strongly and independently with homologous 23S RNA at unit stoichiometry (2) and protects a sequence of approximately 115 bases from ribonuclease digestion at its presumed attachment site (3). *E. coli* L1 also has been found to form specific heterologous complexes with 23S RNAs from two species of *Bacillus* and four different archaebacteria (4, 5), which indicates that this interaction has been subject to strong evolutionary constraints within the prokaryotic domain. Although little is known about the function of L1 in the ribosome, recent investigations suggest that it may be capable of binding not only to rRNA but also to the mRNA from which it is translated, thus serving as a "feedback repressor" that regulates the synthesis of proteins in the L11–L1 operon (6, 7).

We report here the formation of a specific complex between protein L1 from a prokaryote, *E. coli*, and 26S RNA from the large ribosomal subunit of a eukaryote, the slime mold *Dictyostelium discoideum*. Sequence analyses of L1-associated fragments from *Dictyostelium* 26S RNA and the corresponding region of *Dictyostelium* rDNA and data on L1-specific segments from *E. coli* and *Bacillus stearothermophilus* 23S RNAs (3, 4) have allowed us to identify elements of primary and secondary structure common to all known L1 binding sites. In addition, we have found that a similar region occurs in the 28S rRNA of a vertebrate, *Xenopus laevis*. Therefore, the structural features required for interaction with protein L1 have been conserved in large-subunit rRNAs of both prokaryotes and eukaryotes despite the wide divergence between these two lines of descent. Moreover, we show that the initial portion of the L11–L1 operon possesses a number of features characteristic of the rRNA binding sites, which could provide the structural basis for regulatory interaction of L1 with the polycistronic L11–L1 mRNA.

MATERIALS AND METHODS

Isolation of Ribosomal Components. Protein L1 was purified from 50S ribosomal subunits of *E. coli* MRE600 by ion-exchange chromatography (8). Cells of *D. discoideum*, grown to a density of 10^7 cells per ml in HL/5 medium (9), were provided by B. S. Jacobson. The amoebae were lysed in 10 mM Tris·HCl, pH 7.6/100 mM LiCl/1 mM EDTA containing 0.5% sodium dodecyl sulfate and were extracted with phenol (10). RNA was then precipitated with ethanol, and the 26S component was isolated by centrifugation on 5–20% sucrose gradients in the same buffer as above.

Formation of Protein–rRNA Complexes. ^{3}H-Labeled protein L1 from *E. coli* was incubated with 26S RNA from *Dictyostelium* for 30 min at 40°C in buffer (50 mM Tris·HCl, pH 7.6/20 mM $MgCl_2$/350 mM KCl), and the mixture was chilled on ice (10). After the mixture was fractionated on 3–15% sucrose gradients, protein–RNA interaction was monitored by measuring the cosedimentation of ^{3}H-labeled protein L1 with the 26S RNA peak (11). Saturation curves for protein binding were constructed as described (10).

Preparation of L1-Specific rRNA Fragments. After complex formation, pancreatic RNase A (EC 3.1.4.22, Worthington) was added to the chilled reaction mixture at an enzyme/substrate ratio of 1:500 (wt/wt), and incubation was continued for 15 min at 0°C (10). When RNA fragments were to be used for nucleotide sequence analysis, bacterial alkaline phosphatase (EC 3.1.3.1, P-L Biochemicals) was also included at an enzyme/substrate ratio of 1:10 (wt/wt). The digests were loaded directly onto a 10% (wt/vol) polyacrylamide gel and fractionated by electrophoresis. Bands containing RNA and protein were visualized by successive staining with methylene blue and Coomassie brilliant blue, respectively (10). L1-specific RNA fragments were eluted from the minced gel and dissociated from protein by shaking overnight in 50 mM Tris·borate, pH 8.3/500 mM KCl/1 mM EDTA and were recovered by precipitation with 4 vol of absolute ethanol for 10 min at −80°C.

Preparation of Complementary rDNA Fragments. Plasmid pDd507 containing cloned *Dictyostelium* rDNA was obtained from R. A. Firtel; its properties are described in ref. 12. Propagation and isolation of the plasmid in accordance with National

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Abbreviation: kb, kilobase.

Institutes of Health guidelines and digestion of the rDNA with restriction endonucleases (New England BioLabs) were performed as reported (13). Restriction fragments were resolved by electrophoresis on 1.5% agarose or 3% polyacrylamide/0.5% agarose gels, transferred to nitrocellulose sheets (14), and hybridized with protein L1-associated fragments from *Dictyostelium* 26S RNA labeled with ^{32}P at their 5′ termini with [γ-^{32}P]ATP (New England Nuclear) and bacteriophage T4 polynucleotide kinase (EC 2.7.1.78, P-L Biochemicals).

Nucleic Acid Sequence Determinations. Fragments of *Dictyostelium* 26S RNA protected from RNase hydrolysis by *E. coli* L1 were labeled with [5′-^{32}P]pCp (New England Nuclear) at the 3′ end by incubation with bacteriophage T4 RNA ligase (EC 6.5.1.3, P-L Biochemicals) at a concentration of 400 units/ml (15). The labeled fragments were then fractionated by electrophoresis on 20% polyacrylamide gels in 90 mM Tris·borate, pH 8.3/2.5 mM EDTA/7 M urea, located by autoradiography, and eluted as described above in the presence of yeast tRNA carrier (25 μg/ml). Individual components were subjected to sequence analysis exactly as described (15). Restriction fragments of *Dictyostelium* rDNA containing sequences complementary to homologous RNA segments protected by protein L1 were sequenced as described (16) by using reactions as specified (17). Sequence comparisons were carried out with the computer program of Korn *et al.* (18).

RESULTS

Specificity and Stoichiometry of Protein L1–26S RNA Interaction. At the outset, we believed it was essential to establish rigorously the specificity of interaction between ribosomal protein L1 from *E. coli* and 26S ribosomal RNA from *Dictyostelium*, in view of the wide evolutionary distance separating the two donor organisms. Having observed the cosedimentation of radioactively labeled protein L1 with purified 26S RNA on sucrose gradients (5), we next determined that the protein associated only with this component upon incubation with a mixture of small- and large-subunit rRNAs from *Dictyostelium* and that it did not bind to poly(U) or to the RNA of bacteriophages Qβ, MS2, or R17 under our conditions (unpublished data). Furthermore, when increasing amounts of protein L1 were incubated with a fixed amount of 26S RNA, the molar protein/RNA binding ratios of the resulting complexes reached a saturation value of approximately 0.35:1 in the presence of a 5-fold molar excess of protein (Fig. 1). The relatively low level of the plateau in this case may have resulted either from the inaccessibility of protein binding sites in a portion of the RNA population or from the loss of L1 from the complex during fractionation (11). Together, the evidence suggests that the prokaryotic ribosomal protein interacts with a specific binding site in the eukaryotic rRNA, which should by inference possess the same distinctive structural features as its site of attachment in homologous large-subunit rRNA (1).

To isolate the segment(s) of *Dictyostelium* 26S RNA associated with protein L1 for structural analysis, we subjected the L1–26S RNA complex to partial hydrolysis with RNase A and separated the products by electrophoresis on nondenaturing polyacrylamide gels. A unique, slowly-migrating band containing both protein and RNA was present in the digest of the ribonucleoprotein but not in that of the 26S RNA alone (Fig. 2). The L1-specific RNA complex was extracted from the gel for use in the following experiments.

Location of the Protected Sequence in the *Dictyostelium* rRNA Gene. In order to ascertain its size and position within the *Dictyostelium* rDNA, the protected rRNA was first end-labeled with [$\gamma^{32}P$]ATP and polynucleotide kinase and then was purified electrophoretically on a nondenaturing gel. After subsequent electrophoresis on a 10% polyacrylamide gel in 8 M urea, the L1-specific RNA was found to comprise a number of fragments, the main components of which ranged from 40 to 60 nucleotides in length. When the protected RNAs were used to probe restriction digests of cloned *Dictyostelium* rDNA, they consistently hybridized to a single segment of the rRNA gene. Using hybridization techniques (14), we mapped the region complementary to the L1-protected RNA within a *Hin*fI fragment of about 400 base pairs (Fig. 3 *A* and *B*), which was then utilized for DNA sequence analysis. This fragment is located in the 3′ one-third of the 26S RNA gene (Fig. 3*C*) within a region previously shown to encompass sequences common to a variety of eukaryotic rDNAs (13).

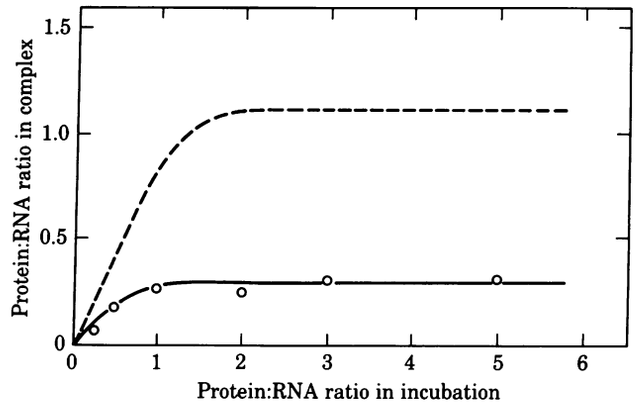

FIG. 1. Saturation curve for the interaction of protein L1 from *E. coli* with 26S RNA from *D. discoideum* (○). Molar quantities of protein and RNA in L1–26S RNA complexes isolated by sucrose gradient centrifugation were computed as described (10). Specific activity of ^{3}H-labeled protein L1 was 66 cpm/pmol; RNA concentration was determined assuming $A_{260\ nm}^{1\ mg/ml} = 24$. ——, Protein L1–26S RNA complex; ----, complex of protein L1 with *E. coli* 23S RNA (2) for comparison.

Nucleotide Sequence of Restriction Fragment from *Dictyostelium* rDNA. Both strands of the 400-base pair *Hin*fI restriction fragment were used for DNA sequence analysis as outlined in Fig. 3*C*. Through visual scanning and computer search, a unique segment of the *Dictyostelium* rDNA was identified that exhibited extensive homology with the sequence from *E. coli* 23S RNA most strongly protected by protein L1 (3). The relevant portion of the *Dictyostelium* rDNA sequence is shown in Fig. 4 with the corresponding sequence from *E. coli* 23S rDNA

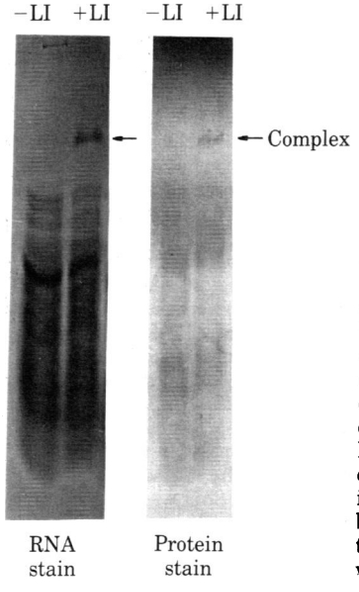

FIG. 2. Isolation of *Dictyostelium* rRNA fragments associated with *E. coli* protein L1. Fragments of *Dictyostelium* 26S RNA protected by L1 after partial hydrolysis of the L1–26S RNA complex with RNase A were prepared by electrophoresis of digestion mixtures on a 1.5-mm-thick, 10% polyacrylamide slab gel [acrylamide/bisacrylamide, 19:1 (wt/wt)] in 50 mM Tris·acetate, pH 7.6/1 mM $MgCl_2$ at 4°C. (*Left*) Digests of 26S RNA and L1–26S RNA complex, stained with methylene blue. (*Right*) After removal of methylene blue and restaining with Coomassie brilliant blue, the band (arrow) showed the color of the latter dye, revealing the presence of protein.

(21). In earlier hybridization studies, we identified restriction fragments at the same relative position in *Xenopus* rDNA that were homologous to *Dictyostelium* rDNA (13). The corresponding sequence from *Xenopus* rDNA (unpublished data) is included in Fig. 4 for comparison. These segments, which in all cases represent the RNA-like strand, have been aligned to emphasize homologies in primary structure. The rDNAs from *E. coli*, *Dictyostelium*, and *Xenopus* share other conserved sequences arranged in colinear fashion at relatively short distances upstream and downstream from the L1-specific region (unpublished data). Such analogies provide evidence that the L1-binding segment occurs at a comparable position in both *E. coli* 23S RNA and *Dictyostelium* 26S RNA and, more generally, suggest

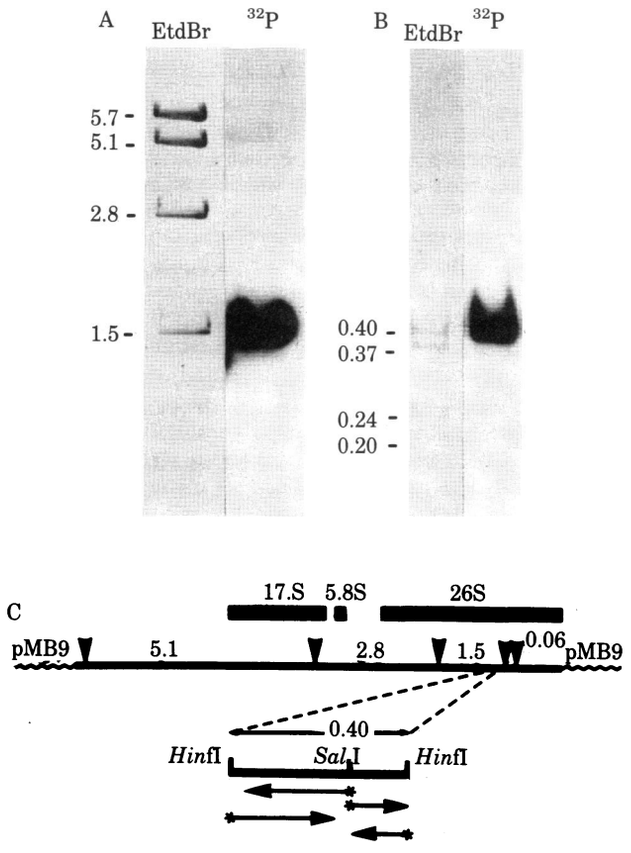

FIG. 3. Identification and location of rDNA fragments complementary to protein L1-specific rRNA from *Dictyostelium*. (*A*) DNA from clone pDd507 was digested with *Eco*RI, fractionated by electrophoresis on a 3-mm-thick, 1.5% agarose gel in 40 mM Tris·acetate, pH 8.0/20 mM Na acetate/18 mM NaCl/2 mM EDTA, transferred to a nitrocellulose sheet, and hybridized with ^{32}P-labeled *Dictyostelium* rRNA fragments protected from RNase digestion by L1. Lanes: EtdBr, gel stained with ethidium bromide; ^{32}P, autoradiograph of gel after hybridization. The rRNA fragments hybridized solely to the 1.5-kilobase (kb) band. (*B*) The 1.5-kb fragment was purified and redigested with *Hinf*I and *Alu* I, and the products were separated on a 1-mm-thick, 3% acrylamide/0.5% agarose gel in 90 mM Tris/90 mM boric acid/2.5 mM EDTA, pH 8.3. Transfer and hybridization were as in *A*. The rRNA fragments hybridized solely to the 0.4-kb band. (*C*) Map of *Dictyostelium* rDNA showing locations of the rRNA genes and of the fragments to which the L1-specific rRNA hybridized. Vertical arrows indicate sites of cleavage by *Eco*RI (19). Segments of the *Hinf*I fragment analyzed by DNA sequence determination are represented by horizontal lines at the bottom of diagram; arrowheads point in the direction of sequence determination, and asterisks show the positions of ^{32}P labels. Digestion of the 1.5-kb *Eco*RI fragment with *Sal* I was used to orient these fragments with respect to the *Eco*RI sites. The sizes of DNA bands were determined by comparison with sequences of restriction fragments of pBR322 (20).

that an overall similarity in the structural organization of rRNA has been preserved in prokaryotes and eukaryotes alike.

Sequence Analysis of Protein L1-Specific Segments from *Dictyostelium* 26S RNA. The precise base sequence(s) in *Dictyostelium* 26S RNA protected from nuclease digestion by *E. coli* protein L1 were determined directly by analysis of L1-associated fragments. RNA from the ribonucleoprotein band of the gel (Fig. 2) was extracted and the 3′ end was labeled with [5′-^{32}P]pCp using RNA ligase. Several discrete bands were then resolved by electrophoresis on polyacrylamide gels under denaturing conditions, and the three major components (39, 52, 56 bases in length) were sequenced by the chemical method of Peattie (15). Despite the recovery of multiple fragments, all shared a common 3′ terminus and were derived from the 56-base segment corresponding to residues 28 through 83 of *Dictyostelium* rDNA (Fig. 4). Thus, the RNA sequence confirms the structure of the L1-specific segment identified by a comparison of the rDNA sequences. In particular, this segment manifests strong similarities to residues 2115 through 2194 of the *E. coli* 23S RNA, which lie entirely within the region protected by L1 in the homologous complex (3).

DISCUSSION

The ability of *E. coli* ribosomal proteins to bind specifically to a wide variety of bacterial 5S, 16S, and 23S RNAs demonstrates that such associations have been highly conserved throughout prokaryotic evolution (4, 5, 11, 22, 23). In an effort to better understand the rRNA features recognized by these proteins, we have extended our studies to heterologous complexes between ribosomal components from prokaryotic and eukaryotic organisms. In this report, it has been shown that protein L1 from *E. coli* can bind to 26S RNA from *Dictyostelium* and that the association satisfies appropriate criteria of specificity (1). Moreover, the sequence to which protein L1 binds in *Dictyostelium* 26S RNA is very similar in primary structure to the homologous L1 binding site in *E. coli* 23S RNA. These observations establish the existence of a common functional region in rRNAs from prokaryotes and eukaryotes and indicate that such regions are of ancient origin in the evolutionary history of the protein synthetic apparatus.

We have examined alternative secondary structures for the sequences that encompass the L1 binding site and find that an extended stem and loop can be formed in analogous segments from *E. coli*, *Dictyostelium*, and *Xenopus* rRNAs. This model (Fig. 5) differs in many respects from one advanced by Branlant *et al.* (3), which was based solely on the sequence protected by protein L1 in *E. coli* 23S RNA, whereas it is similar to another proposed by the same laboratory (4) from a comparison of L1-specific sequences in 23S RNAs from both *E. coli* and *B. stearothermophilus*. Our scheme for the L1 binding region consists of three distinct structural elements. First, this region is bounded by a long, base-paired stem that is only partially conserved in primary sequence but is highly conserved in secondary structure owing to compensatory base changes (residues 2093–2110 and 2179–2196 in *E. coli* 23S RNA). Second, we find two very highly-conserved base sequences that comprise the arms of a loop extending from the main stem to another, shorter, base-paired section. The corresponding segments from *E. coli*, *Dictyostelium*, and *Xenopus* share almost perfect homology in this region (see boxed sequences in Fig. 5). The third element consists of a nonconserved segment above the upper loop closure that varies markedly in length, primary structure, and secondary structure in the three organisms. The longest fragment from *Dictyostelium* recovered in association with protein L1 after digestion of the L1–23S RNA complex spans virtually all of the region drawn as a loop in Fig. 5*B*, including the two

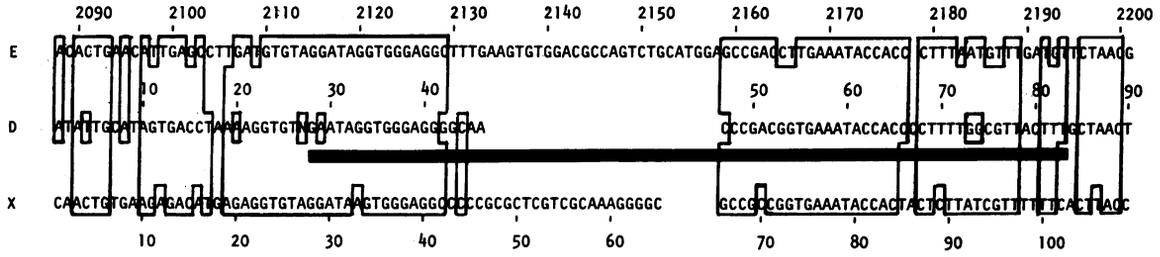

FIG. 4. Sequences from *Dictyostelium* 26S rDNA and *Xenopus* 28S rDNA corresponding to the segment of *E. coli* 23S that encodes the protein L1 binding site. (*E*), *E. coli* (21); (*D*), *Dictyostelium*; (*X*), *Xenopus*. Gaps have been introduced to maximize sequence identities (boxed regions). ■, Portion of the *Dictyostelium* rDNA that codes for the *Dictyostelium* rRNA region protected by protein L1.

highly-conserved sequences, but it contains only the 3′ portion of the main stem (see also Fig. 4). This may indicate that there are accessible bases on the 5′ side of the stem in the heterologous complex that are either less tightly coupled to the protein or more susceptible to nuclease attack than in the homologous complex.

Specific protein–RNA interactions recently have been implicated in the control of ribosomal protein synthesis (6, 7, 25–28). Nomura *et al.* (28) have proposed a model in which certain ribosomal proteins that bind to rRNA early in ribosome assembly are also capable of associating specifically, although less strongly, with the polycistronic mRNAs from which they are translated. With the assumption that the latter interaction blocks translation of the mRNA, an autoregulatory circuit can be established. In rRNA deficiency, protein–mRNA association takes place, and synthesis of the relevant proteins declines or stops; in rRNA abundance, however, the protein preferentially enters the assembly pathway, leaving the mRNA free for translation. For example, it has been found that synthesis of L1 and L11, which are encoded within the same operon, is suppressed both *in vitro* and *in vivo* when the former protein is present in excess (6, 7). In the absence of the L11 promoter region, how-

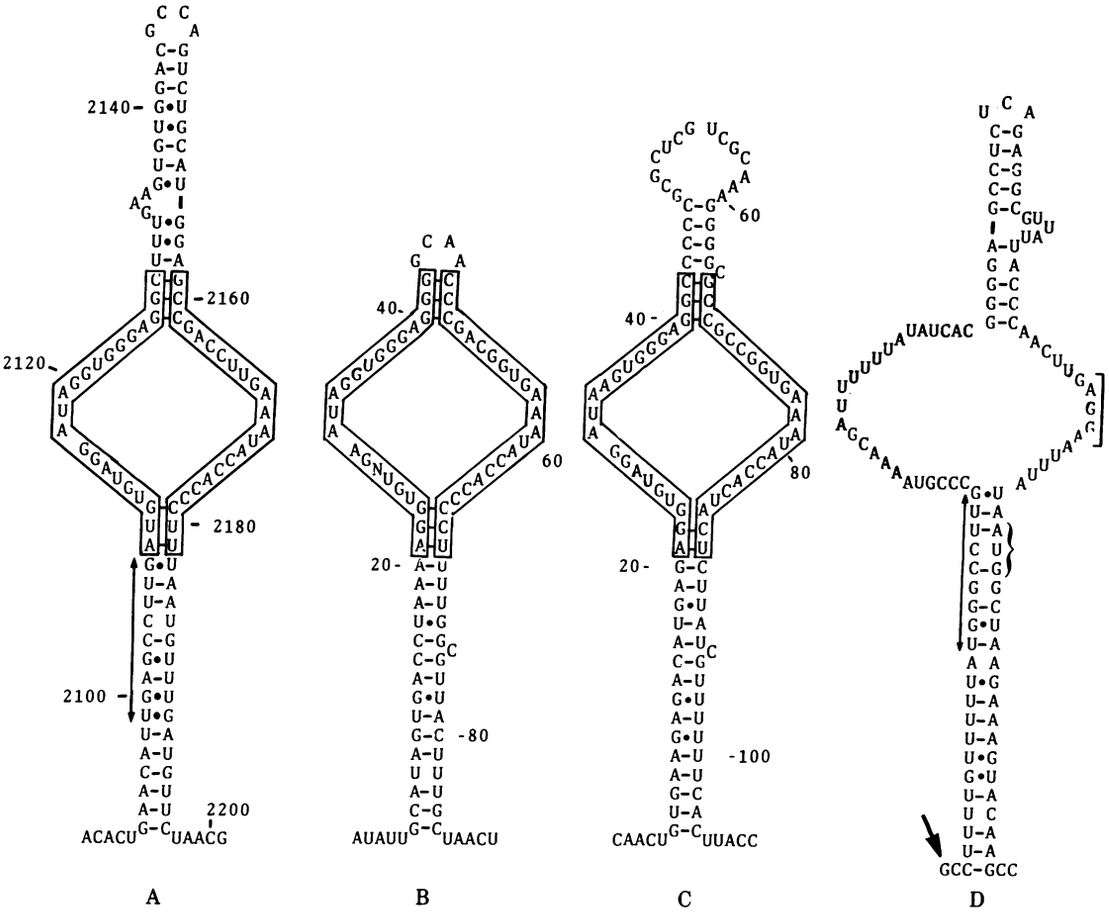

FIG. 5. Possible secondary structure of the L1 binding site. (*A*) L1 binding site in *E. coli* 23S RNA. (*B*) L1-specific region of *Dictyostelium* 26S RNA. (*C*) Analogous segment from *Xenopus* 28S RNA. (*D*) Initial portion of the L11–L1 mRNA. Highly conserved sequences in the rRNAs are boxed (*A–C*). The vertical bars in *A* and *D* point out a sequence homology at similar positions in *E. coli* 23S RNA and the L11–L1 mRNA. In *D*, the first base of the L11 gene transcript is indicated by a bold arrow, the ribosome binding site for the mRNA by brackets, and the initial AUG codon of the L11 message by a brace. The primary structure of the L1 binding site in *E. coli* was reconstructed from ref. 3 by using the sequence of 23S rDNA (21). The sequence of L11 mRNA was inferred from the sequence of the L11 gene (24). Although there are several plausible secondary structures for the L11 mRNA, we have shown one that emphasizes similarities to the L1 binding site on rRNA.

ever, L1 fails to regulate its own production (28). If in fact L1 does exert its regulatory influence by the model described above, the same domain of the protein is likely to be involved in its association with mRNA and rRNA. Consequently, the protein binding sites in both RNAs should then exhibit similar structural features. Therefore, we have examined the sequence determined by Post *et al.* (24) for the L11 gene, the first in the L11–L1 operon, for resemblance in either primary or secondary structure to the L1 binding region of rRNA.

Fig. 5*D* depicts a hypothetical secondary structure for the initial portion of the L11 mRNA that contains a long stem accommodating 17 possible base pairs. The first base of the transcript is two residues upstream from the beginning of the stem. A sequence of nine residues, indicated by a vertical bar on the left side of the base-paired region, occurs with only a single base change in a similar position in the L1-specific stem from *E. coli* 23S RNA. The AUG codon for fmet-tRNA that defines the start of the L11 cistron is located within the sequence comprising the right side of the stem, whereas the Shine–Dalgarno ribosome binding sequence lies just above it in the loop. Thus, if L1 binds to the proposed stem and loop structure in L11 mRNA, it would be in a perfect position to block translation of the message. It should be noted that the mRNA lacks the highly-conserved sequences that occur in the loop of the L1 binding region of rRNA. This difference could reduce the affinity of L1 for the mRNA relative to rRNA as envisioned in the model. Nomura *et al.* (28) have identified possible binding regions for S4 and S7 in mRNA sequences from the operons that code for and are regulated by these two proteins. In particular, they have pointed out similarities between the proposed structures and the known binding sites for S4 and S7 in 16S rRNA. Thus, there are structural features in at least three of the ribosomal protein mRNAs subject to "feedback repression" that might compete for the binding of regulatory ribosomal proteins. Together these observations lend support to the notion of autogenous regulation of ribosomal protein synthesis. The discovery of structurally similar regions in rRNA and ribosomal protein mRNA, some of which may play a role in the regulation of gene expression, raises the possibility that the messengers may have evolved from or coevolved with segments of the rRNA.

Although we cannot yet say with certainty why regions of common primary and secondary structure have persisted in prokaryotic and eukaryotic rRNAs, it is likely that they underlie essential aspects of ribosome assembly, function, or regulation. Whereas it is clear that *E. coli* protein L1 can interact with a site in *Dictyostelium* 26S RNA that strongly resembles its normal site of attachment in *E. coli* 23S RNA, it would be of interest to determine if a ribosomal protein of similar binding specificity exists in the *Dictyostelium* ribosome and if so, whether it participates in ribosome assembly. The question of L1 function is even more perplexing as this protein is not prominently associated with any of the major physiological activities of the 50S ribosomal subunit, although it has been implicated in subunit association (ref. 29; J. M. Lambert and R. Traut, personal communication) and can be crosslinked to elongation factors Tu and G along with many other 50S subunit proteins (30, 31). In fact, there are reports of viable *E. coli* mutants in which L1 is either not stably associated with or completely lacking from the *E. coli* ribosome (32). Therefore, the main significance of interactions represented by the L1–RNA complexes discussed here may be related to the regulation of ribosomal protein synthesis and assembly. Indeed, it will be of great interest to see if autogenous regulation of ribosomal protein synthesis has a part to play in eukaryotic cells.

From a study of prokaryotic L1 binding sites, Branlant *et al.* (33) have suggested a secondary structure model in general agreement with that proposed here, which in addition includes probable base pairing between residues 2120–2124 and 2174–2178 of the *E. coli* sequence.

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