

In Vivo and *in Vitro* Structural Analysis of the *rplJ* mRNA Leader of *Escherichia coli*

PROTECTION BY BOUND L10-L7/L12*

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Shane C. Climie† and James D. Friesen‡

From the Department of Medical Genetics, University of Toronto, Ontario, M5S 1A8 Canada

The *rplJL-rpoBC* operon of *Escherichia coli* is regulated in part at the level of translation by an autogenous mechanism (feedback regulation) that involves ribosomal protein L10-L7/L12. Feedback regulation occurs as the result of L10-L7/L12 binding to a site on the untranslated leader region of the *rplJ* mRNA that is located more than 100 nucleotides upstream from the translation start site. Previous studies have indicated that the secondary structure of the *rplJ* leader region is important for efficient translation and feedback regulation. We have done chemical modification experiments to examine the secondary structure of approximately 200 nucleotides of the *rplJ* leader region, and we propose a secondary structure that is consistent with the experimental data. RNA structure was probed *in vitro* by treating samples of total cellular RNA with diethyl pyrocarbonate and *in vivo* by treating log-phase cultures with dimethyl sulfate. Modified bases were detected by primer extension using three different oligonucleotide primers. The proposed structure includes five double-stranded regions designated I to V, separated by single-stranded segments numbered 1 to 5. We have also identified specific nucleotides in the *rplJ* mRNA leader that are protected by purified L10-L7/L12 from methylation by dimethyl sulfate *in vitro*. The protected bases are located within a bulge-loop of region IV, a portion of the mRNA that has been shown genetically to be necessary for feedback regulation.

shown that regulatory r-proteins act at specific mRNA target sites to bring about translational repression *in vitro* and *in vivo* (Yates and Nomura, 1981; Baughman and Nomura, 1983; Johnsen *et al.*, 1982; Deckman and Draper, 1985, 1987; Climie and Friesen, 1987). The general features of this regulation have been reviewed by Lindahl and Zengel (1982, 1986), Nomura *et al.* (1984), and Draper (1987).

The *rplJL-rpoBC* operon of *E. coli* encodes ribosomal proteins L10 and L7/L12, and the β and β' subunits of RNA polymerase. The *rplJ* and *rplL* genes (encoding r-proteins L10 and L7/L12, respectively) are autogenously regulated at the level of translation by L10 or a complex of one L10 and four L7/L12 molecules (L10-L7/L12) (Dennis and Fiil, 1979; Holowachuk *et al.*, 1980; Brot *et al.*, 1980; Fukuda, 1980; Yates *et al.*, 1981; Johnsen *et al.*, 1982). Sequences located 80–200 nucleotides upstream from the translation start site are necessary for feedback regulation (Friesen *et al.*, 1983; Climie and Friesen, 1987) and efficient translation of the mRNA (Fiil *et al.*, 1980). RNase protection experiments have localized the L10-L7/L12 binding site to a region of the mRNA located more than 120 nucleotides upstream from the translation start site (Johnsen *et al.*, 1982), and several mutations that affect either translation (Fiil *et al.*, 1980) or feedback regulation (Friesen *et al.*, 1983; Climie and Friesen, 1987) are located within the L10-L7/L12 binding site. These findings have provided the framework for a model to describe the regulation of the *rplJL-rpoBC* operon (Christensen *et al.*, 1984). The model is based on the possible existence of alternate secondary structure configurations within the untranslated leader region of the *rplJ* mRNA. Under repressing conditions, L10-L7/L12 is thought to bind to the mRNA about 180 nucleotides upstream from the translation start site. It was proposed that L10-L7/L12 binding causes a change in the secondary structure of the RNA such that the region flanking the Shine-Dalgarno sequence becomes involved in the formation of a stem structure that prevents further translation of the message.

We have previously determined the secondary structure of a small portion of the *rplJ* mRNA that makes up part of the L10-L7/L12 binding site. We also showed that base pairing in this region is required for feedback regulation and that the primary sequence *per se* is less important for regulation (Climie and Friesen, 1987). In this paper we present the results of further structure-probing experiments and propose a secondary structure for a much larger region of the *rplJ* leader, including the translation start site. We also show that ribosomal protein L10-L7/L12 can protect specific nucleotides within the L10-L7/L12 binding site from methylation by dimethyl sulfate *in vitro*. The mRNA secondary structure was examined *in vitro* by chemical modification of total cellular RNA using diethyl pyrocarbonate (DEP) and *in vivo* by

The regulation of ribosomal protein (r-protein)¹ synthesis in *Escherichia coli* occurs in part at the level of translation by an autogenous mechanism termed feedback regulation. At least one of the gene products of each r-protein operon can act as a translational repressor to prevent the synthesis of most or all of the r-proteins encoded by its polycistronic mRNA (Dennis and Fiil, 1979; Lindahl and Zengel, 1979; Dean *et al.*, 1981a, 1981b; Yates and Nomura, 1980, 1981; Yates *et al.*, 1981). Both genetic and biochemical studies have

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† Current address: Dept. of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

‡ To whom correspondence should be addressed: The Hospital for Sick Children, Research Institute, 555 University Ave., Rm. 9116A, Toronto, Ontario, M5G 1X8 Canada.

¹ The abbreviations used are: r-protein, ribosomal protein; DEP, diethyl pyrocarbonate.

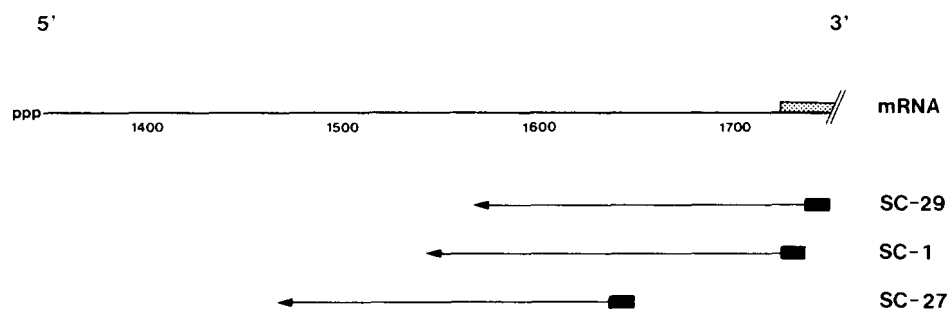


FIG. 1. **Primer-extension analysis of chemically modified RNA.** The mRNA is numbered according to the DNA sequence of Post *et al.* (1979). Solid rectangles represent oligonucleotides used for primer extension. Arrows show the extent of the RNA sequence examined with each oligonucleotide. The 3' end of oligonucleotides SC-29, SC-1, and SC-27 hybridize at positions 1741, 1723, and 1633, respectively. The stippled rectangle represents the 5' end of the *rplJ* coding region which starts at position 1721 (Post *et al.*, 1979).

treating log-phase cultures with dimethyl sulfate. Modified bases were detected by primer extension, and the results obtained using the two methods were in good agreement, suggesting that the proposed structure is similar to that which is found in rapidly growing cells.

EXPERIMENTAL PROCEDURES

Materials—[γ - 32 P]ATP (specific activity 3000 Ci/mmol) was obtained from Du Pont-New England Nuclear. Dimethyl sulfate was from Eastman Chemical Co., and DEP was from Sigma. Restriction endonucleases, T4 DNA ligase, and polynucleotide kinase were supplied by Bethesda Research Laboratories and New England Biolabs. Reverse transcriptase was from Life Sciences or Promega Biotec. Placental ribonuclease inhibitor (RNasin) was from Promega Biotec, and RNase-free DNase was purchased from Bethesda Research Laboratories. Chemicals for electrophoresis were obtained from Eastman, and chemicals for oligonucleotide synthesis were from Bachem and Applied Biosystems.

Oligonucleotide Synthesis—Oligonucleotide SC-1 (5'-CTTGAA-GATTTAAAGCC-3') was synthesized using phosphate triester chemistry (Miyoshi *et al.*, 1980) on a Bachem manual DNA synthesizer. Oligonucleotides SC-29 (5'-CAGCAACAATCGCTTGTGTTG-3') and SC-27 (5'-CGTCTTAATTACGGTGAGC-3') (Climie and Friesen, 1987) were synthesized on an Applied Biosystems 380B DNA synthesizer using phosphoramidite chemistry (Beaucage and Caruthers, 1981).

Bacterial Strains and Plasmid Construction—Strain MC1000 (*araD139 ara[ABOIC], leu-7679, Δ lac[POZY]X74, galU, galK, rpsL, relA, spoT*; Casadaban and Cohen, 1980) was used as a source of RNA for structure mapping experiments and as the recipient for plasmid-mediated transformations. Plasmid pJF4458 was used for *in vitro* transcription of *rplJ* mRNA using T7 RNA polymerase. The plasmid was constructed by ligating a 670-base pair *XhoI*-*XmaI* fragment from pNF2588 into plasmid pT7-2 (U.S. Biochemical) that had been digested with *SalI* and *XmaI*. The resulting plasmid (pJF4458) carries the entire 375-nucleotide *rplJ* regulatory region and 260 nucleotides of the *rplJ* coding sequence.

RNA Preparation—Total cellular RNA was purified from log-phase cultures of strain MC1000 and used for structure probing with DEP. 500-ml cultures were grown at 37 °C in L-broth (Lennox, 1955) to an optical density of 0.4 (O.D.₆₀₀ = 0.4). The cells were poured into chilled centrifuge bottles containing 10 mg of chloramphenicol and then pelleted by centrifugation in a Sorvall GS-3 rotor (8000 rpm, 5 min). Cell pellets were suspended in 2 ml of lysis buffer (10 mM Tris, pH 7.3, 10 mM KCl, 5 mM MgCl₂). Lysozyme was added to a concentration of 3 mg/ml, and the suspensions were frozen in a dry ice/ethanol bath. The suspensions were thawed by warming at 65 °C, and RNA was purified by repeated extraction with hot phenol. Phenol-extracted RNA was precipitated by the addition of 3 volumes of 95% ethanol. RNA pellets were suspended in 0.3 M sodium acetate and reprecipitated with 95% ethanol. The resulting pellets were suspended in water, and the RNA concentrations were determined by spectrophotometry.

Plasmid pJF4458 was used as a template for *in vitro* transcription to produce *rplJ* mRNA for use in methylation-protection experiments. The plasmid was linearized by digestion with *EcoRI*, extracted with phenol, and then ethanol-precipitated. RNA synthesis was car-

ried out in a 50- μ l reaction that included 1 μ g of template DNA, 40 mM Tris-HCl, pH 8.0, 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine, 5 mM dithiothreitol, 400 μ M nucleotide triphosphates, 10 units of RNasin, and 10 units of T7 polymerase. The reaction was incubated at 37 °C for 30 min and then treated with 5 units of RNase-free DNase (Bethesda Research Laboratories) for an additional 10 min. The reaction mixture was extracted once with phenol and three times with ether and then precipitated by adding 0.1 volume of sodium acetate followed by 3 volumes of cold 95% ethanol. The RNA concentration was estimated by electrophoresing a sample on a 2% agarose gel and comparing the intensity of the band to known amounts of ribosomal RNA following ethidium bromide staining.

RNA Structure Mapping—Structure mapping was done using the chemical modification reactions of Peattie and Gilbert (1980). DEP was used to modify RNA under a broad range of denaturing conditions *in vitro*, and dimethyl sulfate was used to modify RNA *in vivo*. For DEP modifications, 60- μ g samples of total cellular RNA were ethanol-precipitated, and the resulting pellets were suspended in 300 or 400 μ l of buffer A (50 mM sodium cacodylate, pH 7.0, 20 mM MgCl₂) or buffer B (50 mM sodium cacodylate, pH 7.0, 1 mM EDTA). The RNA samples were renatured by incubating at 37 °C for 20 min prior to modification. DEP modifications were done under native, semi-denaturing, and fully denaturing conditions by incubating the reactions at different temperatures in the presence and absence of magnesium (see figure legends). The reactions were stopped by adding 150 μ l of 3 M sodium acetate, and the carbethoxylated RNA was precipitated by adding 1 ml of cold 95% ethanol. The RNA was re-precipitated twice, and modified bases were detected by primer extension as described below. Dimethyl sulfate modifications were done *in vivo* at 37 °C by treating log-phase cultures of MC1000 as described below.

Modified bases were detected by primer extension using 5'- 32 P-end-labeled oligonucleotides whose 3' ends hybridized to the mRNA at positions 1633 (SC-27), 1723 (SC-1), and 1741 (SC-29) of the DNA sequence of Post *et al.* (1979). Oligonucleotides were end-labeled in 50- μ l reactions that included 60 pmol of DNA, 100 mM Tris, pH 8.0, 8 mM MgCl₂, 4 mM dithiothreitol, 50 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol), and 10 units of polynucleotide kinase. The reactions were incubated at 37 °C for 30 min then extracted once with phenol and two times with ether. The labeled oligonucleotides were diluted by adding 200 μ l of water, and 0.5–1.0 pmol was added to 100 μ l (60 μ g) of chemically modified RNA. The mixtures were precipitated by the addition of 350 μ l of 95% ethanol, and the resulting pellets were suspended in 7.5 μ l of RT buffer (50 mM Tris, pH 8.3, 40 mM NaCl, 0.5 mM EDTA) and then incubated at 50 °C for 20 min. The mixtures were cooled slowly to room temperature, and the primer-extension reactions were initiated by adding 4 μ l of R mix (2.5 mM deoxynucleoside triphosphates, 0.6 μ g/ml actinomycin D, 3 mM dithiothreitol, 30 mM MgCl₂) and 5 units of avian myeloblastosis virus reverse transcriptase. The reaction mixtures were incubated at 45 °C for 1 h and the reactions were stopped by ethanol precipitation using 1.5 μ l of 3 M sodium acetate and 40 μ l of 95% ethanol. Primer-extension products were run on 5% denaturing polyacrylamide gels (Maxam and Gilbert, 1977) for 2.5–4 h at 1300 V. DNA sequencing ladders were generated with the same oligonucleotide primers and used for size markers. Gels were exposed to Kodak XAR-5 film at –80 °C for 18–24 h with an intensifying screen.

The FOLD program (Zuker and Stiegler, 1981) was used to help determine a secondary structure that was consistent with the exper-

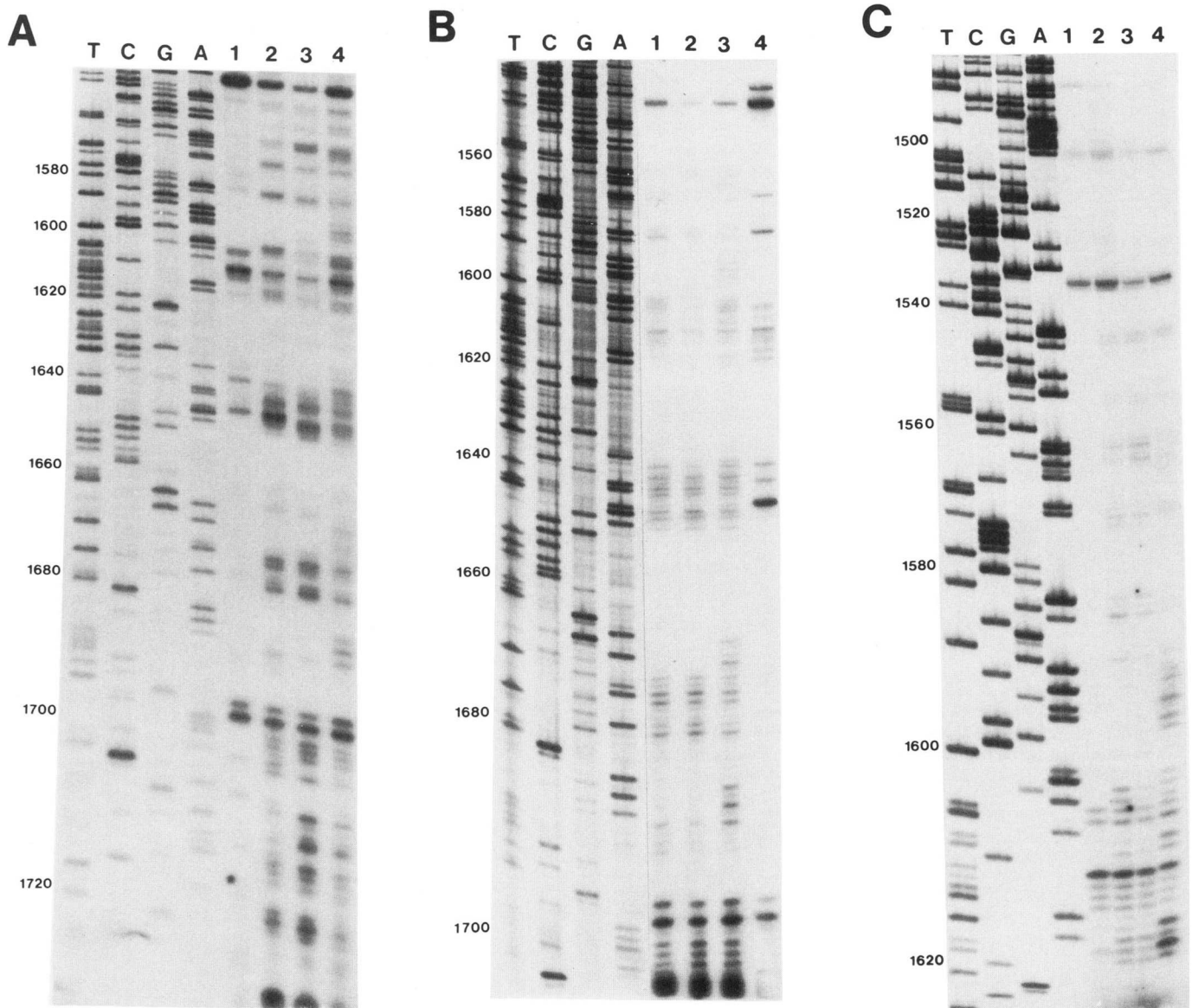


FIG. 2. DEP modification of *rplJ* mRNA. Autoradiography showing sites of DEP modification in the *rplJ* leader region. RNA samples were modified under native (37 °C, 10 mM MgCl₂), semi-denaturing (37 °C, 1 mM EDTA), and fully denaturing conditions (85 °C, 1 mM EDTA) as described under "Experimental Procedures." Modified adenosines were detected by primer extension using three different oligonucleotides as indicated below. Primer extension was also carried out on unmodified control samples. **A**, modified bases detected by primer extension using oligonucleotide SC-29: lane 1, unmodified control sample; lanes 2, 3, and 4, RNA modified under native, semi-denaturing, and fully denaturing conditions respectively. **A**, G, C, and T are dideoxy sequencing ladders generated from a DNA template using the same oligonucleotide primer. **B**, same as **A**, except that primer extension and DNA sequencing were carried out with oligonucleotide SC-1: lanes 1, 2, and 3, RNA modified under native, semi-denaturing, and fully denaturing conditions; lane 4, unmodified control sample. **C**, modified bases detected with oligonucleotide SC-27: lane 1, unmodified control sample; lanes 2, 3, and 4, RNA modified under native, semi-denaturing, and fully denaturing conditions. Note that modified bases are detected as bands that migrate slightly ahead of the corresponding adenosines in the DNA sequencing ladder.

imental data. We used implementations of the program that run on IBM PC-AT and DEC Micro Vax II computers.

RNA Structure Probing *in Vivo*—Total cellular RNA was modified *in vivo* by treating log-phase cultures of strain MC1000 with dimethyl sulfate. 10 ml cultures were grown at 37 °C in L-broth. When the optical density of a culture reached 0.3 (O.D.₆₀₀ = 0.3) 50 μ l of dimethyl sulfate was added, and the culture was shaken vigorously for 2 min. The dimethyl sulfate reactions were quenched by pouring cultures into ice-cold centrifuge tubes that contained 1 ml of 3 M sodium acetate and 1 mg of chloramphenicol. The cells were collected immediately by centrifugation in a Sorvall SS-34 rotor (6000 rpm, 5 min). The cell pellets were suspended in 2 ml of lysis buffer, and RNA was extracted as described above. RNA was also prepared from control cultures that were treated in the same manner except that

dimethyl sulfate was not added. Modified bases were detected by primer extension as described above.

Methylation-Protection of mRNA by L10-L7/L12—For methylation-protection experiments, 1- μ g samples of *in vitro* transcribed *rplJ* mRNA were precipitated, and the pellets were suspended in 50 μ l of dimethyl sulfate buffer (50 mM sodium cacodylate, pH 7.5, 20 mM MgCl₂). The RNA was allowed to renature by incubating at 37 °C for 20 min. Purified L10-L7/L12 (a gift from Dr. A. Liljas, Uppsala, Sweden) was diluted in dimethyl sulfate buffer and allowed to renature by incubating at 37 °C for 20 min. Renatured RNA and L10-L7/L12 were mixed together (1 μ g of mRNA plus increasing amounts of L10-L7/L12 as described in the legend to Fig. 8) in a total volume of 60 μ l and then incubated at 37 °C for 20 min prior to dimethyl sulfate modification. Protein concentrations were estimated by dye binding

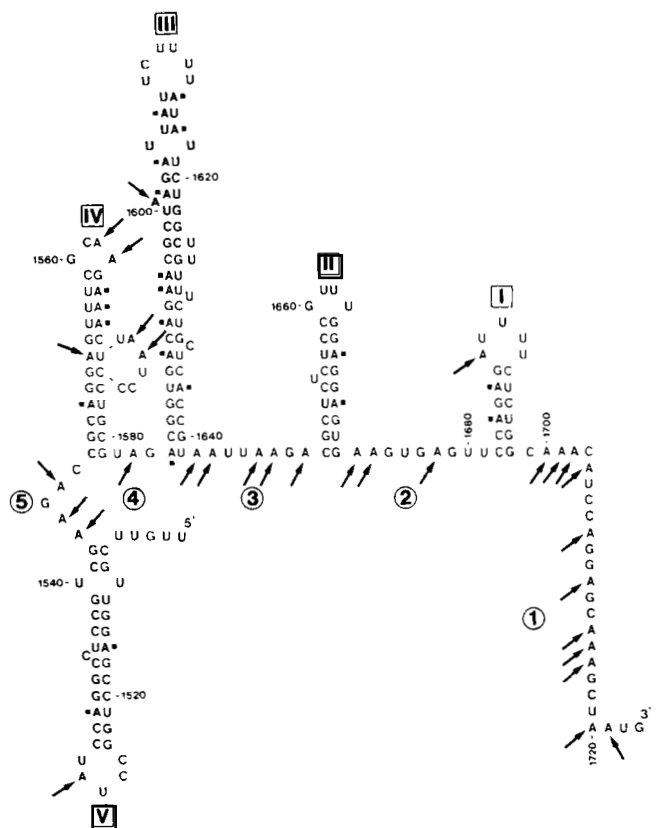


FIG. 3. **Summary of DEP-modification data.** Schematic diagram summarizing the results of DEP-modification experiments. The results of several DEP-modification experiments are superimposed on the secondary structure model of the L10 leader region. Adenosines that were modified under native conditions are indicated with arrows. Solid squares indicate the position of adenosines that were refractory to DEP modification under native conditions. Double-stranded regions are designated I–V, and single-stranded segments are numbered 1–5.

using a Bio-Rad protein assay kit with bovine serum albumin as a reference. Dimethyl sulfate modifications were done by adding 1 μ l of dimethyl sulfate and 20 μ g (1 μ l) of tRNA to the RNA-protein mixtures followed by incubation at 37 °C for 5 min. The reactions were stopped by adding 30 μ l of dimethyl sulfate stop buffer (1 M Tris acetate, pH 7.5, 1 M 2-mercaptoethanol, 1.5 M sodium acetate, 0.1 mM EDTA, 0.2 mg/ml tRNA), followed by ethanol precipitation. RNA pellets were suspended in 200 μ l of 0.3 M sodium acetate and then extracted once with phenol and two times with ether. The methylated RNA was ethanol-precipitated, the pellets were suspended in reverse transcriptase buffer (50 mM Tris, pH 8.3, 40 mM NaCl, 0.5 mM EDTA), and modified bases were detected by primer extension as described above.

RESULTS

Chemical Probing of mRNA Secondary Structure—Dimethyl sulfate and DEP were used as chemical probes to examine the secondary structure of approximately 220 nucleotides of the untranslated leader region of the *rplJ* mRNA. This region includes both the translation start site and the L10-L7/L12 binding site, which is located about 180 nucleotides upstream from the initiation AUG. The stacking of adenosines was probed *in vitro* by treating samples of total cellular RNA with DEP (Leonard *et al.*, 1971; Ehrenberg *et al.*, 1976; Peattie and Gilbert, 1980; Inoue and Cech, 1985) over a broad range of denaturing conditions. The base-pairing of adenosines and cytidines was examined *in vivo* by treating log-phase cultures with dimethyl sulfate (Lawley and Brookes, 1963; Singer, 1975; Peattie and Gilbert, 1980; Inoue and Cech,

1985). Chemically modified bases were detected by primer extension using three different end-labeled oligonucleotides (Youvan and Hearst, 1979; Inoue and Cech, 1985; Moazed *et al.*, 1986). The use of three different oligonucleotide primers allowed us to obtain overlapping data for many of the approximately 220 nucleotides of the *rplJ* leader which were examined (see Fig. 1).

The DEP reactions were carried out under native, semi-denaturing, and fully denaturing conditions. Under native conditions, only those adenosines located in non-base-paired regions of the RNA were susceptible to chemical attack. When RNA secondary structure was melted under fully denaturing conditions, most or all of the adenosines became reactive. Thus it was possible to assign adenosine residues to particular structural features of the mRNA using chemical modification data in conjunction with the FOLD program of Zuker and Stiegler (1981).

Fig. 2 shows the results of a DEP-modification experiment done under native, semi-denaturing, and fully denaturing conditions. Carbethoxylated adenosines were detected by primer extension using the three oligonucleotide primers described under "Experimental Procedures." Unmodified RNA samples were used as a control in the primer-extension reactions to identify artifactual bands that arose as the result of spontaneous breaks, reverse transcriptase pause sites, or strong secondary structure features. The portion of the *rplJ* mRNA that was probed includes 52 adenosine residues. 31 of the 52 adenosines were accessible to the reagent under native (37 °C, 20 mM MgCl₂) or semi-denaturing conditions (37 °C, 1 mM EDTA), and therefore these residues were assigned to single-stranded segments and loop regions. The remaining adenosines became susceptible to DEP attack only under denaturing conditions (80 °C, 1 mM EDTA) and were assigned to helical regions of the mRNA. The DEP-probing results are summarized in Fig. 3. It should be noted that products of the primer-extension reactions terminate at the nucleotide preceding the site of chemical modification. Consequently, the cDNA products migrate slightly ahead of the corresponding adenosines in the DNA sequencing ladder used for size reference. In some cases a small number of modified guanosines was also detected in DEP-modification experiments. This type of modification, which has been previously observed (Inoue and Cech, 1985), probably occurs as a side reaction. However, guanosine modifications are not well characterized, and therefore they were not considered when assigning the secondary structure.

Additional DEP-modification experiments were carried out over a broad range of denaturing conditions by conducting the carbethoxylation reactions at 30, 55, 70, and 85 °C in the presence and absence of magnesium. This approach allowed the relative stability of individual A-U pairs to be compared in the presence and absence of magnesium. The results of one such experiment are shown in Fig. 4 and are summarized in Fig. 5. Removal of magnesium had the general effect of reducing the temperature at which stem-forming adenosines first became susceptible to DEP modification. Adenosines that were modified at 30 °C were assigned to single-stranded segments and loop regions as described above. Adenosines located in stem-forming regions were modified only at higher temperatures when base pairing was disrupted. When modifications were done in the presence of 20 mM magnesium, adenosines in region I and region II were resistant to carbethoxylation up to a temperature of 85 °C. Adenosines located in the lower portion of region III were also resistant to modification up to 85 °C, while those located in the upper portion of region III became modified at lower temperatures, suggest-

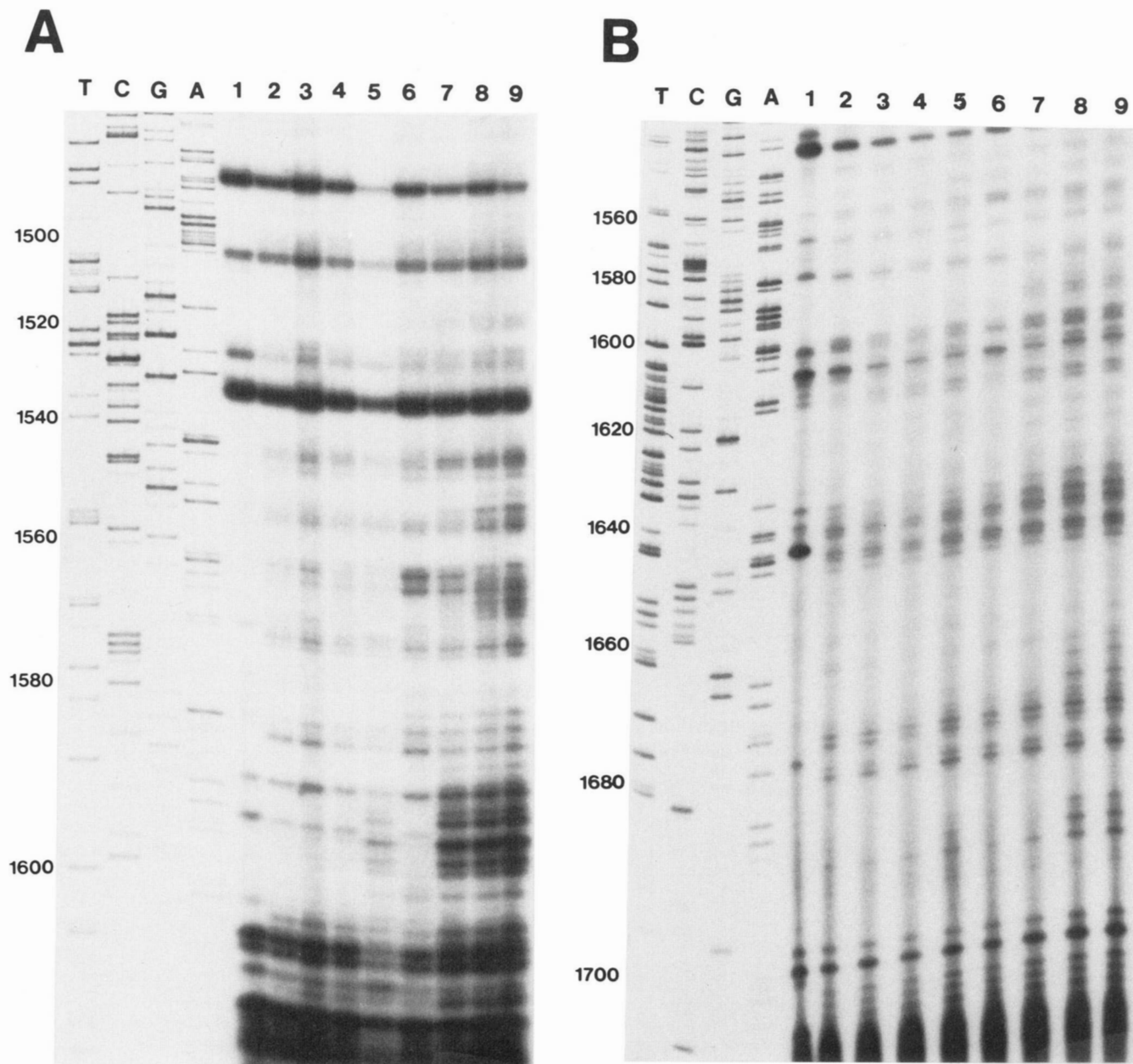


FIG. 4. DEP modification in the presence and absence of magnesium. Adenosines were modified over a broad range of denaturing conditions in the presence and absence of magnesium as described under "Experimental Procedures." A, modified bases detected by primer extension using oligonucleotide SC-27. B, modified bases detected using oligonucleotide SC-1. For A and B, RNA samples were treated as follows: lane 1, unmodified control; lanes 2, 3, 4, and 5, RNA modified at 30, 55, 70, and 85 °C, respectively, in the presence of 10 mM magnesium; lanes 6, 7, 8, and 9, RNA modified at 30, 55, 70, and 85 °C in the absence of magnesium. Modified bases were detected by primer extension.

ing that they were less stably paired. Base-paired adenosines located in region IV were highly resistant to carbethoxylation, with the exception of A1554 which was reactive at 30 °C, suggesting that it might be only transiently paired or single-stranded.

Only one of the two adenosines located in the helical portion of region V could be reliably probed with DEP due to the presence of artifact bands that migrated at the same position as A1531. A1517 became reactive at 70 °C in the presence of magnesium, suggesting that it was base-paired, while A1527 was reactive at 30 °C, consistent with it being located in the hairpin loop.

When DEP-modification reactions were carried out in the absence of magnesium, those adenosines that have been as-

signed to base-paired regions became accessible to the reagent at lower temperatures than when they were probed in the presence of magnesium. Adenosines located in unstructured segments or loops were reactive at 30 °C, while those located in helical regions became modified only at higher temperatures. Since magnesium has the general effect of stabilizing base pairs, a magnesium-dependent change in melting behavior (and susceptibility to DEP attack) is characteristic of paired adenosines (Kjems *et al.*, 1985).

In Vivo Modification of RNA—Nick and Gilbert (1985) have shown that dimethyl sulfate can enter growing bacterial cells rapidly and methylate DNA. Using this approach, they were able to detect nucleotides in the *lac* operator that were protected by *lac* repressor from methylation by dimethyl

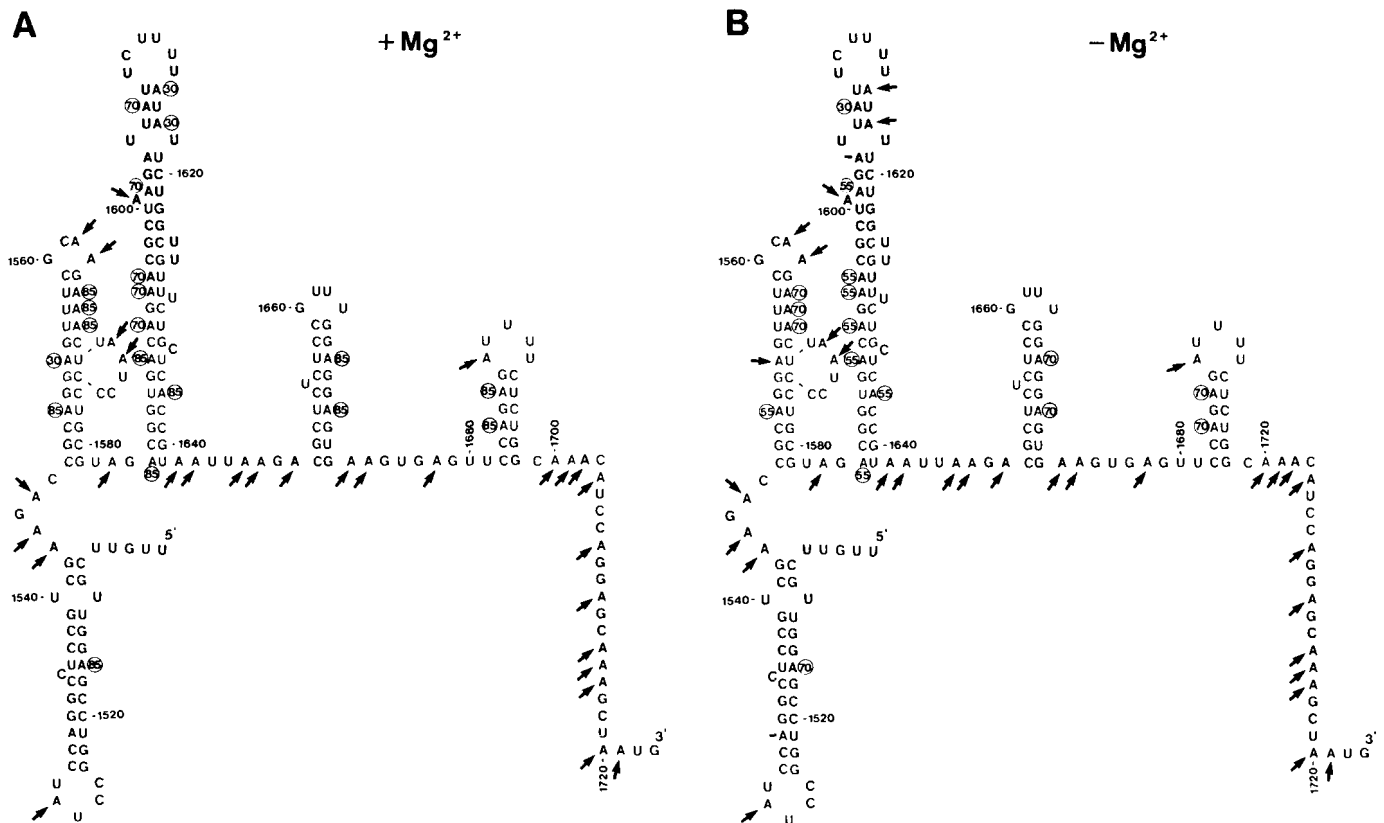


FIG. 5. Summary of DEP modifications done in the presence and absence of magnesium. DEP-modification results from several experiments are superimposed on the secondary structure model of the L10 leader region. RNA samples were modified at increasing temperatures in the absence of magnesium (A) and in the presence of 10 mM magnesium (B). The temperatures at which bases first became moderately reactive are indicated by circled numbers. Adenosines that were highly reactive at 30 °C are indicated by arrows.

sulfate *in vivo*. We used a similar approach to determine which bases within the untranslated leader region of the *rplJ* mRNA were susceptible to dimethyl sulfate modification *in vivo*. Log-phase cultures of strain MC1000 were treated with 0.5% dimethyl sulfate for 2 min at 37 °C. The dimethyl sulfate reactions were quenched with sodium acetate, and RNA was extracted immediately. Methylated adenosines and cytidines were detected by primer extension as described above. Adenosines and cytidines located in single-stranded portions of the mRNA were susceptible to dimethyl sulfate attack, while those located in helical regions were resistant to methylation at their respective N-1 and N-3 positions as the result of base pairing. Under the experimental conditions the doubling time of the culture was 25 min, allowing us to examine the mRNA structure when r-protein synthesis was occurring near the maximal rate. The results of an *in vivo* dimethyl sulfate modification experiment are shown in Fig. 6, and summarized in Fig. 7. All of the adenosine and cytidine residues located in segment 1 were susceptible to dimethyl sulfate attack, suggesting that the mRNA around the translation start site was in a single-stranded configuration under conditions of rapid growth. Similarly, adenosines and cytidines located in segments 2–5 were susceptible to methylation as were those residues assigned to the various loops. Adenosines and cytidines located in the proposed helical regions were resistant to dimethyl sulfate modification. Some of the residues located in the paired regions of stems I, II, and III showed moderate reactivity, but the extent to which those bases were modified was considerably less than that seen for bases in single-stranded regions. The results are consistent with the DEP modifications that were carried out *in vitro* using purified

total cellular RNA. The results are also consistent with previous dimethyl sulfate modification experiments carried out *in vitro* (Climie and Friesen, 1987). Thus, it appears that the secondary structure assumed by the *rplJ* mRNA *in vitro* is very similar to that which is found in rapidly growing cells.

Methylation-Protection—Previous studies have identified a portion of the *rplJ* mRNA that is protected from RNase digestion by ribosomal protein L10-L7/L12 (Johnsen *et al.*, 1982). Several mutations that abolish feedback regulation are located within the protected region (Friesen *et al.*, 1983; Climie and Friesen, 1987), and mRNAs encoded by some of these mutants bind purified L10-L7/L12 less tightly than wild-type message (Christensen *et al.*, 1984). We carried out *in vitro* methylation-protection experiments to identify nucleotides in the leader region that were protected from dimethyl sulfate modification by ribosomal protein L10-L7/L12. Protein-mRNA mixtures were treated with dimethyl sulfate, and modified bases were detected by primer extension. Preliminary results using RNase T₁ indicated that the mRNA transcribed *in vitro* had the same secondary structure as cellular mRNA (results not shown). The results from a methylation-protection experiment are shown in Fig. 8. Purified ribosomal protein L10-L7/L12 protected adenosines in the bulge-loop of region IV (A1571, A1572) from methylation by dimethyl sulfate. No other adenosines or cytidines were strongly protected, and there was no apparent change in the pattern of base methylation in the vicinity of the translation start site.²

DISCUSSION

The *rplJL-rpoBC* operon of *E. coli* is regulated autogenously at the level of translation by ribosomal protein L10-L7/L12.

² S. C. Climie and J. D. Friesen, unpublished observations.

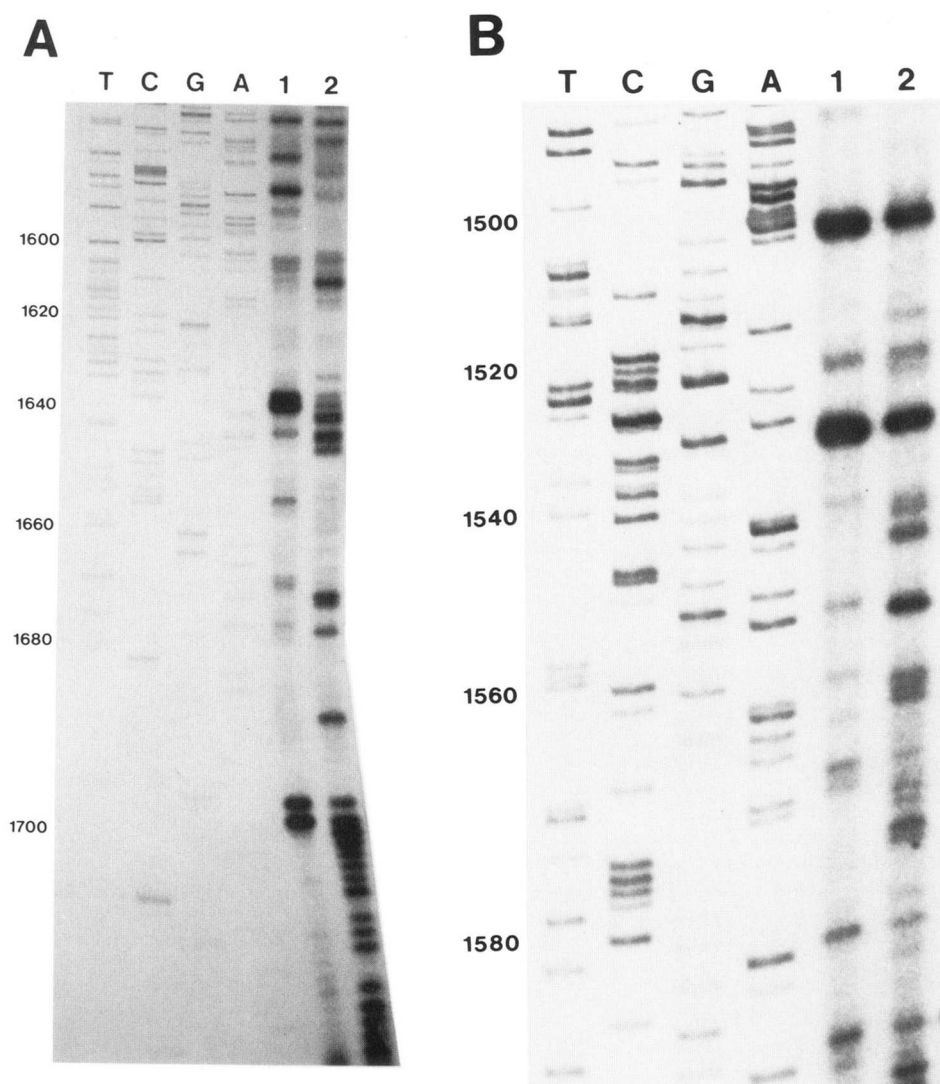


FIG. 6. *In vivo* dimethyl sulfate modification of *rplJ* mRNA. Adenosines and cytidines were modified *in vivo* by treating cultures with dimethyl sulfate as described under "Experimental Procedures." Modified RNA was extracted from cells, and methylated adenosines and cytidines were detected by primer extension. Representative results are shown: A, lane 1, unmodified control; lane 2, dimethyl sulfate-treated sample; A, G, C, and T are dideoxy sequencing reactions as described in the preceding figures. Primer extension analysis was done using oligonucleotide SC-29. B, lanes are the same as in A, but primer extension was done using oligonucleotide SC-27.

Previous studies have indicated that the secondary structure of the 375-nucleotide untranslated leader region is important for feedback regulation and for efficient translation of the mRNA (Friesen *et al.*, 1983; Climie and Friesen, 1987; Fiil *et al.*, 1980). According to the model of Christensen *et al.* (1984), feedback regulation occurs as the result of a change in the secondary structure of the mRNA leader that is induced by L10-L7/L12 binding. In this paper we describe the results of RNA structure-probing experiments that were carried out *in vitro* using DEP and *in vivo* using dimethyl sulfate. In both cases the target of chemical modification was total cellular RNA. The use of total cellular RNA as a target for chemical modification combined with primer-extension analysis of the reaction products allowed us to overcome some of the problems that are associated with the more common approach of using purified, end-labeled RNA molecules for structure analysis. Most importantly, the primer-extension analysis allowed us to examine the secondary structure of part of the *rplJ* leader that is located more than 350 nucleotides away from the 5' end of the message. The procedure also allowed us to probe the structure of intact, full length mRNA molecules transcribed *in vivo*. We propose a secondary structure for part of the *rplJ* mRNA leader extending from the L10-L7/L12 binding site through the translation start site. We also report the identification of nucleotides in the leader that are protected by L10-L7/L12 from methylation by dimethyl

sulfate *in vitro*. The protected nucleotides are located within the region of the mRNA leader that has previously been shown to include the L10-L7/L12 binding site (Johnsen *et al.*, 1982), and which also has been shown genetically to be important for feedback regulation (Climie and Friesen, 1987).

The results from structure-probing experiments were used in conjunction with the FOLD program (Zuker and Stiegler, 1981) to arrive at the secondary structure shown in Fig. 3. The proposed structure includes five double-stranded regions designated I to V, separated by single-stranded segments of various lengths. Segment 1 is at least 23 nucleotides in length, and it includes the translation start site. All of the adenosine residues in segment 1 were modified under native and denaturing conditions *in vitro* by DEP (See Figs. 3 and 5), and all of the adenosines and cytidines were modified by dimethyl sulfate *in vivo* (see Fig. 7), suggesting that they are located in an unstructured portion of the mRNA. Similarly, adenosines located in unpaired segments 2 to 5 were modified under native conditions by DEP *in vitro*, while adenosines and cytidines were modified by dimethyl sulfate *in vivo*.

Region I is comprised of a 6-base pair stem region and a 5-base hairpin loop. The structure includes nucleotides 1682–1698 of the sequence of Post *et al.* (1979). Adenosines assigned to the helical region were resistant to DEP modification under native conditions but they became reactive at higher temperatures (see Figs. 3 and 5). A1688 was reactive under all of the

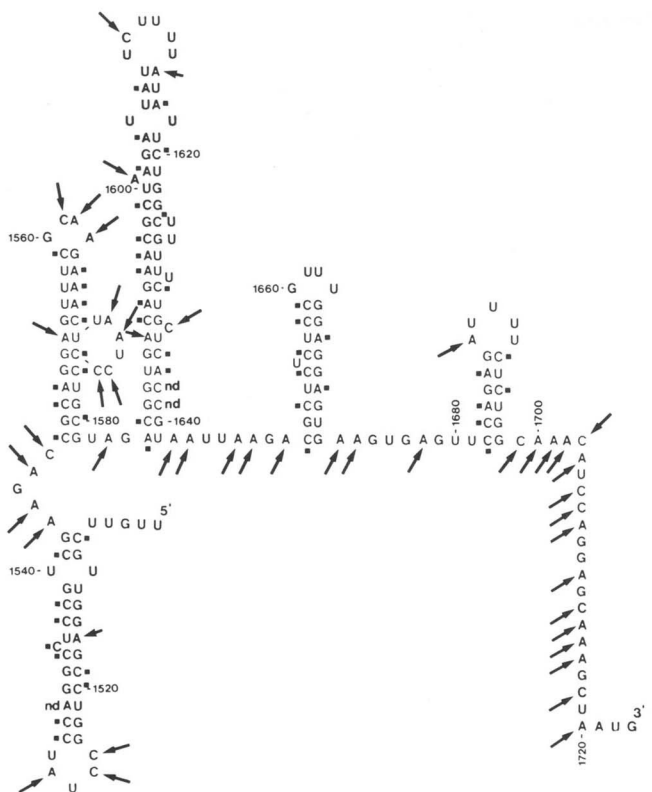


FIG. 7. Summary of *in vivo* dimethyl sulfate modification. The results of several *in vivo* dimethyl sulfate-modification experiments are superimposed on the proposed secondary structure of the L10 leader region. *Arrows* indicate the positions of adenosines and cytidines that were modified by dimethyl sulfate. *Solid squares* indicate the positions of adenosines and cytidines that were resistant to dimethyl sulfate modification. *nd*, the reactivity of these bases could not be determined because of artifactual bands that migrated at those positions.

conditions tested and it was thus assigned to the hairpin loop. All four of the cytidines and two adenosines assigned to the helical portion of region I were resistant to dimethyl sulfate modification *in vivo*, while A1688 was reactive (see Fig. 7), consistent with it being located in the hairpin loop.

Region II includes nucleotides 1650–1672. The proposed structure includes a 9-base pair helical region that is interrupted by a bulged uridine, and a 4-base hairpin loop. Again, adenosines located within the proposed helical region were resistant to DEP modification under native conditions, while adenosines and cytidines were refractory to dimethyl sulfate modification *in vivo* (see Figs. 3, 5, and 7).

The secondary structure proposed for region III includes nucleotides 1585–1641. Those adenosines and cytidines located in the lower portion of region III showed modification behavior that was consistent with the proposed structure assignments. Paired adenosines were modified only under denaturing conditions *in vitro*, while adenosines and cytidines were resistant to dimethyl sulfate modification *in vivo* (see Figs. 3, 5, and 7). The bulged cytidine located at position 1634 was modified by dimethyl sulfate *in vivo*. Some of the nucleotides that were assigned to base-paired regions showed a moderate susceptibility to dimethyl sulfate attack, but the extent of modification was much less than that seen for bases located in single-stranded segments. We are less certain about the structure of the upper portion of region III which includes nucleotides 1601–1621. The proposed structure is comprised almost entirely of adenosine and uridine residues. Five of the six base pairs that are shown in the proposed secondary

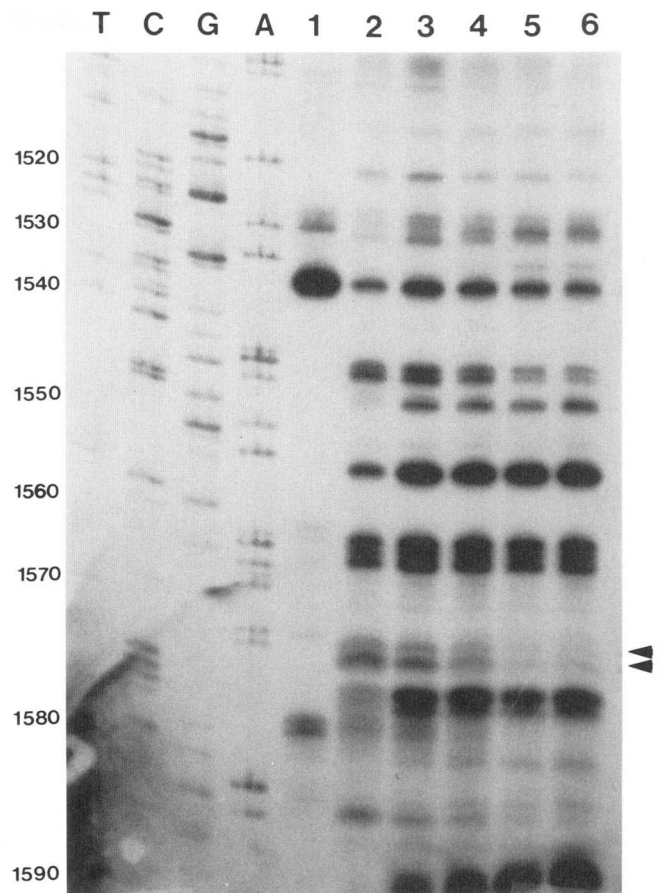


FIG. 8. Methylation-protection of *in vitro* transcribed *rplJ* mRNA. 1- μ g samples of L10 mRNA transcribed from pJF4458 were incubated with increasing concentrations of L10-L7/L12 *in vitro*. The 60- μ l samples were then treated with dimethyl sulfate, and primer extension was carried out using an end-labeled oligonucleotide. The primer-extension products were run on a 5% denaturing gel. *Lane 1*, unmodified control; *lanes 2-6*, dimethyl sulfate-treated samples incubated with increasing amounts of L10-L7/L12 (6 mg/ml) as follows: *lane 2*, no protein; *lane 3*, 1 μ l of L10-L7/L12; *lane 4*, 2 μ l of L10-L7/L12; *lane 5*, 5 μ l of L10-L12/L12; *lane 6*, 10 μ l of L10-L7/L12. *T*, *C*, *G*, and *A* refer to dideoxy DNA sequencing lanes that were generated using the same oligonucleotide primer. Numbers on the left are from the nucleotide sequence of Post *et al.* (1979). Arrowheads show the positions of A1571 and A1572 which are protected from dimethyl sulfate attack. A1571 and A1572 are located in the bulge-loop of region IV (see Fig. 3).

structure are A-U pairs. All of the adenosines located in this part of region III are at least partially reactive to DEP under native conditions, which suggests that they are not stably base-paired, or perhaps that they are in a single-stranded configuration. Also, a number of artifact bands that arose from this part of the RNA made it difficult to assign the structure with confidence. A1601 was assigned as a bulged base because it was usually modified under native conditions. In some cases, however, A1602 was observed to be reactive rather than A1601. This might reflect alternate pairing of these adenosines with U1621. As such, the assignment of A1601 as a bulged base should be regarded as provisional. Regardless of the proposed structure of the upper portion of region III, we have observed that an oligonucleotide-directed deletion of nucleotides 1605-1618 has little effect on the expression or feedback regulation of a *rplJ-lacZ* fusion plasmid that carries the entire *rplJ* regulatory region.

The structure of region IV was reported previously (Climie and Friesen, 1987). The results of DEP modifications carried

out at 37 and 90 °C are consistent with our previous DEP- and dimethyl sulfate-modification experiments. Adenosines located in the helical portion of region IV were not modified under native conditions or *in vivo* with the exception of A1554 (see Figs. 3, 5, and 7). A1554 has the potential to pair with U1569 but it was usually modified by DEP under native conditions and by dimethyl sulfate *in vivo*, indicating that it might be in a single-stranded configuration. Base pairing of A1554 with U1569 might be restricted as a result of the destabilizing effect of the adjacent bulge-loop (bases 1670–1675). Adenosines that were assigned to the hairpin and bulge-loops were modified under native and denaturing conditions. The results obtained from structure probing with dimethyl sulfate *in vivo* were also consistent with our previous *in vitro* findings.

The proposed structure of region V includes 32 nucleotides located from position 1511–1542 of the DNA sequence. DEP-modification experiments indicated that the adenosines located at positions 1517 and 1531 were resistant to carbethoxylation under native conditions while A1527 was modified. Accordingly, these nucleotides were assigned to helical and hairpin-loop regions, respectively (see Figs. 3 and 5). *In vivo* dimethyl sulfate-modification experiments gave results that were consistent with the proposed structure for region V (see Fig. 7). An exception was A1517, which was modified by dimethyl sulfate, a result that was not expected given the predicted base pairing with U1536.

Overall, the results from structure-probing experiments carried out *in vivo* and *in vitro* gave results that were in good agreement. This leads us to believe that the proposed secondary structure of the leader region of the *rplJ* mRNA is very similar to that which is found *in vivo* under conditions of rapid growth. The proposed structure is somewhat similar to the "Form I," or open structure proposed by Christensen *et al.* (1984) on the basis of theoretical considerations. Two features of the secondary structure of the *rplJ* leader are of particular interest. First, the lack of secondary structure around the translation start site might be an important requirement for efficient translation of the mRNA. There are a number of cases reported in which mRNA structure has been shown to influence translation initiation (Hall *et al.*, 1982; Coleman *et al.*, 1985; Cone and Steege, 1985; Schmidt *et al.*, 1987). Second, the L10-L7/L12 binding site includes a region of secondary structure that is necessary for feedback regulation. The possible role of RNA secondary structure at protein binding sites is considered below.

Methylation-protection experiments carried out using purified ribosomal protein L10-L7/L12 and *in vitro* transcribed *rplJ* mRNA allowed us to identify bases that are likely to interact with protein bound to the mRNA. Adenosines 1571 and 1572 which are located in the bulge-loop of region IV were the only bases that were strongly protected by L10-L7/L12 from methylation by dimethyl sulfate (see Fig. 8). The resistance of these nucleotides to dimethyl sulfate attack probably occurred as the result of close contact between L10-L7/L12 and the *rplJ* mRNA such that the N-1 position of the protected adenosines were not accessible to the reagent. It is possible that several other nucleotides within the L10-L7/L12 binding site were also in close contact with the protein and that they were not detected in the present study. The use of dimethyl sulfate as a probe limited the analysis to those adenosines and cytidines that are located in single-stranded portions of the RNA. Our previous work (Climie and Friesen, 1987) showed that the overall secondary structure of region IV is required for feedback regulation while the primary sequence of that region is less important. Several point mu-

tations located within the base-paired stem of region IV abolish feedback regulation. It was also shown that various deletions within region IV, including a deletion of the bulge-loop (bases 1570–1575) abolish feedback regulation. Those results, combined with the present observations, suggest that the specificity of L10-L7/L12 binding to the mRNA might be conferred in part by the single-stranded adenosine residues at positions 1571 and 1572. Bulged nucleotides have been identified as important structural features of other r-protein binding sites. Included among these are the L18 binding site on *E. coli* 5 S RNA (Peattie *et al.*, 1981; Douthwaite *et al.*, 1982), the S8 binding site on 16S RNA (Thurlow *et al.*, 1983; Gregory *et al.*, 1984), and the S4 binding site on 16 S RNA (Gregory *et al.*, 1984; Stern *et al.*, 1986). Binding of the bacteriophage R17 coat protein to the initiation site of the R17 replicase gene has also been shown to require a region of RNA secondary structure interrupted by unpaired residues (Carey *et al.*, 1983). In that case, little change in the binding affinity is seen when base-paired residues are altered in such a way that pairing is maintained. However, when base pairing is disrupted or single-stranded residues are altered, binding is reduced considerably (Romaniuk *et al.*, 1987).

It is interesting to note that we did not detect any difference in the pattern of dimethyl sulfate reactivity around the translation start site upon binding of L10-L7/L12. According to the alternate secondary structure model (Christensen *et al.*, 1984) one would predict that nucleotides in the vicinity of the Shine-Dalgarno sequence would become less reactive as the result of an L10-L7/L12 induced conformational change in the mRNA secondary structure. This result brings into question the mechanism by which L10-L7/L12 brings about translational repression. It is possible that the translation start site becomes weakly associated with upstream sequences, perhaps via a weak secondary or tertiary interaction, and that this association was not detected using the present techniques. In any event, further studies will be required to determine how L10-L7/L12 can act as a translational repressor when it binds to the mRNA 140 nucleotides upstream from the translation start site. The proposed secondary structure of part of the *rplJ* leader region and the identification of specific nucleotides that interact with r-protein L10-L7/L12 provide a good starting point for more detailed studies on the mechanism of feedback regulation in the *rplJL-rpoBC* operon.

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