Leader peptides of inducible chloramphenicol resistance genes from Gram-positive and Gram-negative bacteria bind to yeast and Archaea large subunit rRNA

Robert Harrod and Paul S. Lovett*

Department of Biological Sciences, University of Maryland–Baltimore County, Baltimore, MD 21250, USA

Received January 30, 1997; Revised and Accepted March 5, 1997

ABSTRACT
catA86 is the second gene in a constitutively transcribed, two-gene operon cloned from Bacillus pumilus. The region that intervenes between the upstream gene, termed the leader, and the catA86 coding sequence contains a pair of inverted repeat sequences which cause sequestration of the catA86 ribosome binding site in mRNA secondary structure. As a consequence, the catA86 coding sequence is untranslatable in the absence of inducer. Translation of the catA86 coding sequence is induced by chloramphenicol in Gram-positives and induction requires a function of the leader coding sequence. The leader-encoded peptide has been proposed to instruct its translating ribosome to pause at leader codon 6, enabling chloramphenicol to stall the ribosome at that site. Ribosome stalling causes destabilization of the RNA secondary structure, exposing the catA86 ribosome binding site, allowing activation of its translation. A comparable mechanism of induction by chloramphenicol has been proposed for the regulated cmlA gene from Gram-negative bacteria. The catA86 and cmlA leader-encoded peptides are in vitro inhibitors of peptidyl transferase, which is thought to be the basis for selection of the site of ribosome stalling. Both leader-encoded peptides have been shown to alter the secondary structure of Escherichia coli 23S rRNA in vitro. All peptide-induced changes in rRNA conformation are within domains IV and V, which contains the peptidyl transferase center. Here we demonstrate that the leader peptides alter the conformation of domains IV and V of large subunit rRNA from yeast and a representative of the Archaea. The rRNA target for binding the leader peptides is therefore conserved across kingdoms.

INTRODUCTION
The basis for the modulated expression of many bacterial and eukaryotic genes involves the control of mRNA translation, frequently as a complement to transcriptional control. A novel cis-acting form of translational control has recently been suggested as the basis for the regulation of several bacterial and eukaryotic genes, which relies on principles unlike those seen in other examples of translational regulation (1–4). Genes such as cat and cmlA, which specify inducible chloramphenicol resistance in Bacillus subtilis and Escherichia coli respectively, and eukaryotic genes, which include the arg-2 gene of Neurospora crassa, the CPA1 gene of Saccharomyces cerevisiae, pp48 of cytomegalovirus, the human β2-adrenergic receptor gene and several other examples (1), appear to be controlled by a fundamentally similar mechanism. In each, the regulated gene is the downstream gene in a two-gene operon and it is the act of translation of the upstream gene that can activate or depress translation of the downstream gene. The role of the upstream gene appears to reside in its peptide product. Current evidence indicates that the amino acid sequence of the peptide product of the upstream gene enables it to interact in cis with its translating ribosome (1–5). This interaction, either alone or in conjunction with a small effector molecule, interferes with a normal catalytic function of the ribosome, preventing its release from the mRNA.

We have studied the regulation of two classes of chloramphenicol-inducible chloramphenicol resistance genes: the cat genes in Gram-positive bacteria and the cmlA gene in Gram-negatives. Both classes are induced by chloramphenicol through the translation attenuation mechanism (6). In this form of translational control, a single transcript spans a short upstream leader coding sequence (the upstream gene) and a downstream chloramphenicol resistance coding sequence (the downstream gene). The region in the mRNA that intervenes between the two coding sequences contains a secondary structure domain that sequesters the ribosome binding site (RBS) for the (downstream) chloramphenicol resistance coding sequence. As a consequence, the chloramphenicol resistance coding sequence cannot be translated because its RBS is unavailable for translation initiation. It has been shown that during induction of catA86 by chloramphenicol, the RNA secondary structure domain becomes destabilized (7), and this is also presumed to be the basis for induction of the cmlA gene (8).

Activation of translation of the catA86 gene in B. subtilis can be achieved by stalling a ribosome, through selective amino acid deprivation, at a specific codon within the leader coding sequence...
A ribosome is stalled with its aminoacyl site at leader codon 6 (9). Significant translational activation of cat results only when a ribosome is stalled with its aminoacyl site at leader codon 6 (9). This places the stalled ribosome adjacent to the secondary structure, but at a location that does not interfere with the entrance of a second ribosome at the cat RBS, designated RBS-C. It is therefore presumed that chloramphenicol induces cat translation only when it stalls a ribosome at leader codon 6.

Chloramphenicol is an inhibitor of peptidyl transferase, the ribosome activity that forms peptide bonds. However, the antibiotic does not act only on ribosomes transcribing a particular coding sequence. Genetic evidence has shown that leader codons 1–5 determine the site specificity of ribosome stalling during cat induction by chloramphenicol (10–12). Recently it was found that addition of a synthetic peptide (MVKTD) to ribosomes, a peptide which corresponds to the product of leader codons 1–5, inhibits ribosomal peptidyl transferase (13,14). The reverse sequence (DTKVM) is not inhibitory, nor are C- or N-terminal truncations of MVKTD. Amino acid substitutions in the 5mer peptide that correspond to missense mutations known to block cat induction in vivo prevent the 5mer peptide from inhibiting peptidyl transferase. Comparable results have been obtained using the N-terminal 8mer peptide (MSTSKNAD) encoded by the leader for the cmlA gene (15). It is inferred that during leader translation in vivo, a ribosome will pause at the induction site due to activity of the leader peptide.

Peptidyl transferase activity appears to require participation of 23S (large subunit) rRNA sequences (16). In vitro studies demonstrated that synthetic cat leader 5mer peptide (MVKTD) and synthetic cmlA leader 8mer peptide (MSTSKNAD) bind to extracted or in vitro transcribed E. coli 23S rRNA and alter conformation of the rRNA uniquely in the vicinity of the peptidyl transferase center (17). Here we demonstrate that both peptides bind to large subunit rRNA extracted from yeast and a representative of the Archaea. Thus, the rRNA target for the inhibitor peptides is conserved across kingdoms.

**MATERIALS AND METHODS**

**Organisms and extraction of rRNA**

*Saccharomyces cerevisiae* FY250 (trp1 his3 ura3 leu2) was grown at 30°C in YPD medium, consisting of 1% yeast extract, 2% peptone and 2% glucose. *Halobacterium halobium* strain R1 was grown at 37°C in a medium consisting of 0.3% yeast extract, 0.5% tryptone, 0.2% sodium chloride, 0.2% potassium chloride and 25% sodium chloride (18). Cells (2 l) were grown to late log phase (100 Klett U, filter 66), harvested by centrifugation and resuspended in 100 ml suspension buffer (20 mM Tris–HCl, pH 7.6, 10 mM magnesium chloride, 100 mM ammonium chloride and 6 mM β-mercaptoethanol). Yeast cells were washed twice with cold suspension buffer (17) and disrupted in a French pressure cell (15 000 p.s.i.). *Halobacterium halobium* cells began lysis upon resuspension and were therefore immediately disrupted as described above. Debris was removed from the lysates by centrifugation at 30 000 g for 15 min. The supernatant fractions were centrifuged through a 1.1 M sucrose cushion in suspension buffer. The ribosome pellet was rinsed with suspension buffer, resuspended and centrifuged at 105 000 g. Pellets were resuspended
Peptide synthesis

Peptides were synthesized on an ABI model 432 peptide synthesizer and were purified by reverse phase HPLC as previously described (13).

Nuclease probing of peptide effects on rRNA

rRNA was incubated with peptides as previously described (17), using peptide:large subunit RNA ratios of 6000:1. Preincubation was for 10 min at 0°C, after which time T1 or V1 ribonucleases was added to a final reaction volume of 20 µl (17). Digestion was at 0°C for 10 min and was terminated by addition of 100 µl phenol and 100 µl water. Samples were centrifuged and re-extracted with chloroform. RNA was precipitated in 3 vol cold ethanol + 0.3 M sodium acetate. The precipitate was pelleted, washed with 100 µl 70% ethanol, pelleted and resuspended in 5 µl water. This RNA served as template for primer extension analysis by AMV reverse transcriptase as previously described (17,19).

RESULTS

Footprinting the peptide on yeast large subunit rRNA using T1 and V1 endoribonucleases

T1 endonuclease cleaves RNA immediately 3' of G residues at single-stranded sites (20). V1 nuclease cleaves RNA at various nucleotides that are hydrogen-bonded (21). Hence, these enzymes have been used in determinations of the secondary structure of rRNA (19). To examine peptide effects on the conformation of yeast rRNA, total RNA was extracted from 80S ribosomes and mixed with peptides at peptide:large subunit RNA ratios of ∼6000:1. The peptide–RNA complex was digested with T1 or V1 endonuclease using enzyme concentrations empirically found to cleave each RNA molecule only a few times. Sites of cleavage were determined using specific DNA oligomers as primers for reverse transcriptase. Each cleavage site is detected as a site of incorporation of the RNA sequence into the cDNA. In the region of rRNA affected by the peptide is shown in Figure 2. No changes in enzymatic cleavage were observed using control peptides, such as reversemers or the cmlA leader 7mer and 9mer, which are not inhibitory for peptidyl transferase.

Footprinting the peptide on large subunit rRNA from Halobacterium using T1 and V1 endoribonucleases

Large subunit rRNA from Halobacterium (18) was preincubated with the various peptides and probed with T1 and V1 endonucleases. DNA oligomers that prime synthesis in each of the six rRNA domains were separately used to assess peptide effects on susceptibility to the enzymes (Fig. 3). Peptide-induced changes in RNA susceptibility to the enzymes were observed only in rRNA domains IV and V (Fig. 4). Control peptides that do not inhibit peptidyl transferase, such as the reversemers, did not detectably alter the conformation of the rRNA (Fig. 3).

Comparison of the results obtained with E.coli rRNA with those of Saccharomyces and Halobacterium

Table 1 summarizes the individual rRNA nucleotides in E.coli, Saccharomyces and Halobacterium whose susceptibility to T1 or V1 nuclease becomes altered in the presence of the 5mer and 8mer peptides. An effect observed at a specific nucleotide in the rRNA of one organism typically did not show a peptide-altered response to the nuclease probe at the corresponding nucleotide of the other organisms, although generally a nearby nucleotide was affected. By presenting the data in this fashion, patterns of susceptibility become evident. The pattern is more apparent when the changes in the RNAs of the three organisms are placed on a

Figure 2. Sites of peptide-induced alterations of nuclease susceptibility in yeast large subunit rRNA. Large subunit rRNA consists of six domains. Peptide-induced alterations were observed only in domains IV and V. Each site of a peptide-induced altered response is designated with a letter and number. The letter corresponds to the nucleotide at the site of alteration and the number refers to the specific nucleotide in yeast rRNA. Symbols designate the type of response: open circle, loss of V1 susceptibility; open triangle, loss of a T1 cleavage site; solid diamond, new site of T1 cleavage. Four primers were used to analyze this region of the rRNA by reverse transcriptase. Primer L-369 is complementary to nt 2461–2478. Primer L-371 is complementary to nt 2902–2917. Primer L-376 is complementary to nt 2192–2210. Primer L-377 is complementary to nt 2461–2478. Primer L-371 is complementary to nt 2902–2917. Primer L-376 is complementary to nt 2192–2210. Primer L-377 is complementary to nt 2461–2478. Primer L-371 is complementary to nt 2902–2917. Primer L-376 is complementary to nt 2192–2210. Primer L-377 is complementary to nt 2461–2478. Primer L-371 is complementary to nt 2902–2917. Primer L-376 is complementary to nt 2192–2210. Primer L-377 is complementary to nt 2461–2478. Primer L-371 is complementary to nt 2902–2917. Primer L-376 is complementary to nt 2192–2210. Primer L-377 is complementary to nt 2461–2478. Pr...
**Table 1.** Comparison of sites in large subunit rRNA from *E. coli*, *S. cerevisiae* and *H. halobium* whose susceptibility to T1 or V1 nuclease is altered by MVKTD and MSTSKNAD

<table>
<thead>
<tr>
<th>E. coli</th>
<th>S. cerevisiae</th>
<th>H. halobium</th>
<th>E. coli</th>
<th>S. cerevisiae</th>
<th>H. halobium</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1955 ◇</td>
<td>U2297</td>
<td>U1981</td>
<td>G2061 ◇</td>
<td>G2402</td>
<td>G2087 ◇</td>
</tr>
<tr>
<td>G1973</td>
<td>G2315</td>
<td>G1999 ◤</td>
<td>C2084</td>
<td>U2425 ◤</td>
<td>G2110</td>
</tr>
<tr>
<td>G1980</td>
<td>G2322</td>
<td>G2006 ◤</td>
<td>U2085</td>
<td>U2426 ◤</td>
<td>C2111</td>
</tr>
<tr>
<td>A1981 ◦</td>
<td>A2323 ◥</td>
<td>A2007 ◥</td>
<td>C2416</td>
<td>A2781</td>
<td>G2435</td>
</tr>
<tr>
<td>G1999 ◥</td>
<td>U2019</td>
<td>A2015</td>
<td>A2418</td>
<td>G2783</td>
<td>C2437 ◥</td>
</tr>
<tr>
<td>U1991</td>
<td>U2333 ◥</td>
<td>U2017</td>
<td>U2423</td>
<td>A2788</td>
<td>U2442 ◥</td>
</tr>
<tr>
<td>U1993 ◥</td>
<td>U2335</td>
<td>U2019</td>
<td>G2428</td>
<td>C2793 ◥</td>
<td>A2447</td>
</tr>
<tr>
<td>C1996</td>
<td>C2338 ◥</td>
<td>C2022</td>
<td>A2430 ◥</td>
<td>A2795</td>
<td>A2449</td>
</tr>
<tr>
<td>C2001</td>
<td>U2343 ◥</td>
<td>U2027 ◥</td>
<td>A2432</td>
<td>A2797 ◥</td>
<td>A2451</td>
</tr>
<tr>
<td>G2002</td>
<td>A2344 ◥</td>
<td>U2028</td>
<td>U2438</td>
<td>U2803</td>
<td>U2457</td>
</tr>
<tr>
<td>A2005 ◥</td>
<td>A2347</td>
<td>G2031</td>
<td>A2448</td>
<td>A2813 ◥</td>
<td>A2467</td>
</tr>
<tr>
<td>A2013 ◥</td>
<td>A2355</td>
<td>A2039</td>
<td>C2456</td>
<td>C2821</td>
<td>G2473</td>
</tr>
<tr>
<td>G2018</td>
<td>A2360</td>
<td>U2044 ◥</td>
<td>A2459</td>
<td>G2824</td>
<td>C2477 ◥</td>
</tr>
<tr>
<td>A1991</td>
<td>C2361</td>
<td>A2045</td>
<td>U2460</td>
<td>U2825 ◥</td>
<td>U2479</td>
</tr>
<tr>
<td>U2022</td>
<td>G2363</td>
<td>U2048 ◥</td>
<td>C2466</td>
<td>U2831 ◥</td>
<td>G2485</td>
</tr>
<tr>
<td>A2030 ◥</td>
<td>A2371 ◥</td>
<td>C2056</td>
<td>U2471</td>
<td>C2836 ◥</td>
<td>A2490</td>
</tr>
<tr>
<td>G2032</td>
<td>C2373 ◥</td>
<td>G2058</td>
<td>U2473</td>
<td>U2837</td>
<td>C2492</td>
</tr>
<tr>
<td>A2033 ◥</td>
<td>A2374</td>
<td>A2059</td>
<td>C2475</td>
<td>C2840</td>
<td>C2494 ◥</td>
</tr>
<tr>
<td>U2039</td>
<td>G2380 ◥</td>
<td>G2065</td>
<td>G2488</td>
<td>C2853</td>
<td>U2507 ◥</td>
</tr>
<tr>
<td>G2040</td>
<td>G2381</td>
<td>A2066 ◥</td>
<td>G2495</td>
<td>A2860 ◥</td>
<td>C2513</td>
</tr>
<tr>
<td>U2041 ◥</td>
<td>C2382</td>
<td>G2067 ◥</td>
<td>C2496</td>
<td>U2861</td>
<td>U2514</td>
</tr>
<tr>
<td>A2042</td>
<td>A2383</td>
<td>G2068 ◥</td>
<td>C2498</td>
<td>C2863</td>
<td>C2516 ◥</td>
</tr>
<tr>
<td>C2043</td>
<td>G2384 ◥</td>
<td>C2069</td>
<td>A2503</td>
<td>A2868</td>
<td>A2521</td>
</tr>
<tr>
<td>G2048</td>
<td>A2389 ◥</td>
<td>A2074</td>
<td>U2506</td>
<td>U2871</td>
<td>U2524</td>
</tr>
<tr>
<td>C2050 ◥</td>
<td>C2391</td>
<td>G2076</td>
<td>G2509</td>
<td>G2874</td>
<td>G2527</td>
</tr>
<tr>
<td>A2054</td>
<td>G2395</td>
<td>A2080 ◥</td>
<td>C2510</td>
<td>C2875</td>
<td>U2528</td>
</tr>
<tr>
<td>G2057 ◥</td>
<td>A2398</td>
<td>C2083</td>
<td>A2059</td>
<td>A2400</td>
<td>A2085 ◥</td>
</tr>
</tbody>
</table>

*The correspondence between rRNA nucleotides in yeast, Archaea and bacteria is from Egebjerg et al. (23). Symbols represent the following changes in ribonuclease cleavage: (i) open triangle, protection from ribonuclease T1; (ii) filled triangle, enhanced susceptibility to T1 digestion; (iii) filled diamond, a new site of T1 cleavage; (iv) open circle, protection from V1 digestion. The nucleotide positions of peptide-induced alterations of nuclease cleavage are enlarged and in bold. Absolutely conserved nucleotides are enlarged and in bold. Thus, when the letter and number of a nucleotide are in bold, this indicates an absolutely conserved nucleotide whose susceptibility to a nuclease is altered by the peptides. Nucleotide sequence information for *E. coli* 23S rRNA, *H. halobium* 23S rRNA and *S. cerevisiae* 26S rRNA was taken from Egebjerg et al. (23), Mankin and Kagramanova (18) and Hogan et al. (22).*

A likely explanation for the observed differences in nuclease susceptibility of the three rRNAs, with or without added peptide, resides in the differences in the primary nucleotide sequence and the resulting differences in secondary structure (24). The primary sequence of large subunit rRNA differs, somewhat to substantially, among members of the three kingdoms depending on the particular region of large subunit rRNA that is examined; the peptidyl transferase center, comprising domain V, shows the highest conservation of primary sequence. We suggest that the differences in the primary sequence of the rRNAs, and the resulting differences in secondary structure, cause differences in susceptibility to the nuclease probes.

**DISCUSSION**

The model suggested to explain translation attenuation regulation of *catA* and *cmlA* proposes that a ribosome translating the leader becomes stalled at a specific codon when the inducing antibiotic chloramphenicol is also present (6). Chloramphenicol is an inhibitor of translation in bacteria, presumably by interfering with peptidyl transferase. However, the antibiotic is not known to block translation at specific nucleotide sequences in mRNA nor at specific amino acid sequences in the nascent peptide. Stall site selection must therefore be a function of the leader. Genetic evidence suggested that induction might depend on the amino acid sequence of the leader peptide specified by codons upstream of the site of ribosome stalling (9–11,25). Our recent results strongly support the idea that the nascent leader peptide is the selector of the site of stalling through *cis*-effect on its translating ribosome (13–15).

**Peptidyl transferase inhibition**

Peptidyl transferase activity is intimately associated with large subunit rRNA. It remains unknown if the catalytic activity is due to the RNA alone or to RNA maintained in proper conformation...
Figure 3. Footprinting the 5mer and 8mer peptides on Halobacterium large subunit rRNA using endonuclease probes. Peptides used and the designation of symbols are as in Figure 1. (A) Probing the left half of domain V and the right half of domain IV using T1 (left panel) or V1 (right panel) endonuclease. The DNA primer was HH23 V/IV (see Fig. 4). (B) Probing the right half of domain V using T1 (left panel) or V1 (right panel) endonuclease. The DNA primer was HH23V (see Fig. 4).

Figure 4. Sites of peptide-induced alterations of nuclease susceptibility in H. halobium large subunit rRNA. Peptide-induced alterations in secondary structure were observed only in domains IV and V. Nomenclature and symbols are as in Figure 2. Primers used to analyze the sites of reverse transcriptase termination were: HH23IV (complements nt 1876–1893), HH23V/IV (complements nt 2554–2571) and HH23VI (complements nt 2801–2819).

by protein or, conversely, if the RNA confers correct structure to catalytic ribosomal proteins (26). The cat and cmlA leader peptides, which are inhibitory for peptidyl transferase, bind to large subunit rRNA and modify the secondary structure of domains at the peptidyl transferase center. These findings are consistent with the previous connection between rRNA and peptidyl transferase activity (16). It is also interesting that the peptidyl transferase of S. cerevisiae cytoplasmic ribosomes is insensitive to chloramphenicol but is inhibited by the leader peptides, whereas H. halobium and E. coli peptidyl transferase activity is inhibited by the antibiotic and by the leader peptides (14). Susceptibility of peptidyl transferase to the leader peptides therefore appears independent of susceptibility to chloramphenicol.

Large subunit rRNAs from yeast, Archaea and bacteria do not show identical leader peptide-induced alterations of RNA conformation, although in each case the RNA domains affected by the peptides are the same. Indeed, the regions within domains IV and V that show an altered conformation in response to the peptides are very similar (Fig. 5). Consequently, we presume that within the rRNA there exists a conserved structure or sequence that serves as the peptide binding target. Binding produces a change in conformation that is determined by the initial secondary structure within each of the rRNAs tested. This reasoning argues that conformational alterations caused by peptide binding might, and perhaps should, differ somewhat among the three rRNAs examined.

Our results with rRNAs from the three organisms do not necessarily identify the target for peptide binding, but rather the effects produced on the rRNA as a consequence of binding a peptidyl transferase inhibitor peptide. It remains unknown whether the basis for peptidyl transferase inhibition is due only to
Figure 5. Summary of all peptide-induced changes in rRNA susceptibility to T1 and V1 ribonucleases placed on the map of E.coli 23S rRNA domains IV–VI. Using E.coli 23S rRNA domains IV–VI, we can place all peptide-induced changes in conformation of E.coli, yeast and Archaea rRNA on this common representation of the peptidyl transferase center. In one representation, the actual sequence of rRNA nucleotides is shown, with E, S and H noting the nucleotides altered in E.coli, Saccharomyces and Halobacterium. In the other representation, the sites of the changes are depicted as solid circles. All data shown are taken from Table 1.

binding of an inhibitor peptide to its rRNA target or to the resulting conformational changes that occur in the rRNA.

Observations that place the nascent peptide at the peptidyl transferase center

Nucleotides in domain IV of 23S rRNA can be crosslinked to nucleotides in domain V (27). This observation suggests that in the three-dimensional model of the rRNA, domain IV may fold onto domain V, forming a channel through which the nascent peptide passes (28). The peptide-induced conformational alterations are in the rRNA domains that likely form the walls of such a peptide transit channel. Furthermore, the peptide-induced conformational alterations are observed at those regions to which a nascent peptide can be crosslinked concurrent with its synthesis (29). Our footprinting data are therefore consistent both with a model for rRNA tertiary structure and with those nucleotides that can be contacted by a peptide as it passes from the P site through the ribosome during translation.

Peptide inhibitors of peptidyl transferase also inhibit translation termination

In addition to its role as an inhibitor of peptidyl transferase, the catA86 5mer peptide is an inhibitor of translation termination (12,30). Tate et al. (31) have suggested that peptidyl transferase and translation termination might be two different functions of the same enzyme. If this idea were correct, it would provide an
explanation for the bifunctional inhibitory activity of the leader peptides. 

The translation of several eukaryotic genes is inhibited by short, sequence-specific, upstream open reading frames (uORFs) (2–4). It has been proposed that the uORFs may encode peptides that interact in cis with their translating ribosomes and prevent ribosome release from the mRNA at the uORF stop codon; specifically, the peptides are suggested to inhibit translation termination. A ribosome ‘stalled’ in this manner could serve as a block to ribosome scanning to downstream ORFs on the same transcript. Given the similarity between these observations and those we have made with the catA86 and cmrA leader peptides, we suspect that the mechanism of action of the cis-acting eukaryotic translational repressors may parallel that of bacterial leader peptides.

ACKNOWLEDGEMENTS

We thank Alexander Mankin for H. halobium, Steve Swift for S. cerevisiae and Beth Rogers for comments on the manuscript. Portions of this investigation were supported by grant GM-42925 from the National Institutes of Health.

REFERENCES