Regulation of Plasmid pE194 Replication: Control of cop-repF Operon Transcription by Cop and of repF Translation by Countertranscript RNA

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The cop-rep region of plasmid pE194 contains two tandem structural genes, cop and repF, as well as the plus and minus origins of replication. The two structural genes comprise an operon whose expression is repressed by the binding of Cop protein to a 28-bp inverted complementary repeat sequence that overlaps the cop-repF promoter. From its position relative to the promoter and the experimentally determined footprint made by the Cop protein, the 28-bp inverted complementary repeat sequence is presumed to function as the cop operator. The intercistronic region between cop and repF is 80 nucleotides (nt) long and is transcribed bidirectionally: in the forward direction as part of the synthesis of the cop-repF message (ca. 900 nt), and in the reverse direction to yield a countertranscript ca. 65 nt long. The proposed countertranscript RNA (ctRNA) can form a single stem-and-loop structure that includes the single SphI sequence from the loop-forming segment. Enlargement of the proposed loop from 6 to 14 nt by insertion of a SphI-BamHI adapter at the SphI site or contraction of the proposed loop down to 4 nt, by cutting with SphI followed by blunting with S1 nuclease, yields mutants with an increased copy number. By gel retardation and DNaseI footprinting analysis, Cop protein was shown to bind to the promoter region of cop; no binding by Cop protein at the 5' end of repF was detected. Two major transcripts were synthesized in vitro using cop-repF region DNA as a template, the tandem cop-repF transcript, and the ctRNA. Addition of purified Cop protein to an in vitro transcription reaction mixture reduced only the rate of cop-repF transcription but not that of ctRNA. These observations suggest that regulation of repF occurs at two levels: (i) with Cop protein acting as a repressor of cop-repF mRNA transcription and (ii) with ctRNA acting as a repressor of RepF translation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains, plasmids, and phages used in this study are listed in Tables 1 and 2; PCR primers are tabulated below. Plasmid copy number was determined as described previously (21) with [3H]thymidine-labeled DNA fractionated by agarose gel electrophoresis. The label incorporated into the pE194 and chromosomal DNA fractions was counted, and the copy number was calculated for the molecular masses of pE194 and B. subtilis chromosomal DNA, with 2.4 × 10^6 and 2.5 × 10^6 Da, respectively, used as normalizing factors.

Cloning, overexpression, and purification of Cop protein. The cop gene was cloned in plasmid pET3a (15, 18), purchased from Novagen (Madison, Wis.), and overexpressed in Escherichia coli. The cop gene and its promoter were obtained by amplification using PCR with oligonucleotides 2920 and 2921 as upstream and downstream primers, respectively. The resultant PCR product was blunted with S1 exonuclease, cut with Rsal restriction endonuclease, and ligated with plasmid pET3a that was prepared by cutting with NdeI and blunting with S1

Plasmid pE194 belongs to a group of small plasmids that replicate using a rolling circle intermediate whose initiation site nick defines the plus replication origin; for reviews and reports of previous work on replication of plasmid pE194 and of a related tetracycline resistance plasmid, pLS1, see references 3, 4, 14, 16, and 19. In our previous study (1), we showed that the replication region of plasmid pE194 contains two tandem open reading frames that specify the proteins Cop and RepF. Mutations that reduce Cop function and result in an elevated plasmid copy number have been selected in vivo or constructed in vitro (1, 9, 20, 21). Analysis of the products synthesized in Bacillus subtilis minicells under the direction of such mutant plasmids shows increased rates of synthesis of both Cop and RepF proteins (1), suggesting that the synthesis of these proteins is coordinately regulated. The proximity of cop and repF suggests possible mechanisms for their coordinate regulation.

Villafane et al. (20) obtained four copy number mutations, namely, ts1, cop-1, cop-83, and cop-93, located downstream of the cop open reading frame, apparently associated with the repF open reading frame; it was not clear how their action could be mediated by the transcriptional repressor activity of Cop. In the present studies, we prepared purified Cop protein, demonstrated its role, in vitro, as a transcriptional repressor, and characterized its interaction, physically, with its proposed operator. In the same transcription reaction, we also observed the synthesis of a ca. 65-nucleotide (nt) transcript that corresponds to the negatively acting countertranscript RNA (ctRNA) which has been observed in other replication systems (4). Two mutants, cop-112 and cop-116, constructed in these studies, alter the loop segment of the proposed ctRNA. Since the nucleotide alterations in these two mutants fall between the structural genes for Cop and RepF, and within the proposed ctRNA DNA template, it appears likely that some of the cop mutants, e.g., cop-1 described by Villafane et al. (20), act by reducing either the rate of synthesis or the intrinsic regulatory function of the short ctRNA.
TABLE 1. Bacterial strains, plasmids, and phages used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, or phage</th>
<th>Description and/or derivation</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>Contains IPTG-inducible T7 RNA polymerase; used as host for plasmid pET3a</td>
<td>18</td>
</tr>
<tr>
<td><em>E. coli</em> JHK102</td>
<td>From <em>E. coli</em> BL21(DE3) by transformation with plasmid pJHK102 DNA</td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>Host for phage M13mp18, DNA sequencing</td>
<td>12</td>
</tr>
<tr>
<td><em>B. subtilis</em> BR151</td>
<td>Transformable host for testing phenotypic expression of <em>cop</em> mutants</td>
<td>22</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pE194+</td>
<td>Wild type</td>
<td>21</td>
</tr>
<tr>
<td>pET3a</td>
<td>Used for overexpression cloning under T7 promoter control in <em>E. coli</em></td>
<td>18</td>
</tr>
<tr>
<td>pJHK102</td>
<td>Used to overexpress <em>Cop</em>. Derived from pET3a by insertion of blunted PCR product (primers 2920 and 2921, pE194 as DNA template), cut with <em>Rsa</em>I and inserted at blunt <em>Nde</em>I site in plasmid pET3a</td>
<td>This work</td>
</tr>
<tr>
<td>Phage M13mp18</td>
<td>Used for DNA sequencing</td>
<td>12</td>
</tr>
</tbody>
</table>

deexonuclease. A transformant strain, *E. coli* JHK102 carrying plasmid pJHK102, grown in Luria-Bertani broth containing ampicillin (200 μg/ml), was induced with IPTG (isopropyl-β-D-thiogalactospyranoside) (0.4 mM) for 10 min before rifampin (200 μg/ml) was added. The culture was incubated for an additional 3 h and harvested by centrifugation. Cop protein was purified with Sephadex G-200-120, DEAE Sephadex, and heparin agarose as described for plasmid pLS1 RepA protein (5) to yield 0.9 mg per liter of culture. The purified Cop protein was divided into aliquots and stored at −70°C until used.

**Gel retardation.** Probes for studying the binding of Cop protein to pE194 DNA were obtained by PCR mixture primed with DNA oligonucleotide pairs that defined the sequences to be tested for their ability to bind Cop protein. The resultant PCR products were 5' end labeled with [γ-32P]ATP (specific activity, 6,000 Ci/mM). Gel retardation assays were performed as described by Garner and Revzin (8), adapted to the present study by inclusion of Cop protein (1 μg) and DNA probe (10,000 cpm). The reaction mixture was incubated for 20 min at 20°C, and poly(dI-dC) (2 μg) was added to dissociate nonspecific complexes. The resultant mixture was fractionated by polyacrylamide gel electrophoresis (PAGE; 5% gel in 1× Tris-borate-EDTA buffer.

**DNase I footprinting.** DNA probe containing the *cop* promoter was obtained by PCR in a form labeled at a single end by using 5'-end-labeled oligo 2920 and unlabeled oligo 2921 as upstream and downstream primers, respectively. The DNase I footprinting reaction was performed in a volume of 50 μl and contained 32P-labeled DNA probe (30,000 cpm), 0.01 U (2 μg) of RNase-free DNase from Promega (Madison, Wis.), Cop protein (10, 100, or 1,000 ng), and other components as described previously (7). The resultant product was fractionated by PAGE (8% gel in 1× Tris-borate-EDTA buffer containing 8 M urea) before being subjected to autoradiography.

**Reverse transcriptase mapping.** The transcription start site was determined by using reverse transcriptase as described by Stern et al. (17). The reaction was performed in a volume of 12.5 μl and contained total-cell RNA (5 μg), avian myeloblastosis virus reverse transcriptase (2.5 μg), 5' 32P-end-labeled primer (10,000 cpm), and other components, as described above. The resultant product was fractionated by PAGE (8% gel in 1× Tris-borate-EDTA buffer containing 8 M urea) before autoradiography.

**Runoff transcription.** Defined RNA transcripts from *cop-repF* region sequences were synthesized by using *B. subtilis* RNA polymerase holoenzyme as described by Levin and Chamberlin (10). The reaction was performed in a volume of 20 μl containing DNA template obtained by PCR of defined *cop-repF* region sequences (20 ng), *B. subtilis* RNA polymerase holoenzyme (3 U),

TABLE 2. *cop* mutants relevant to the present studies

<table>
<thead>
<tr>
<th><em>cop</em> mutation</th>
<th>Coordinate (nt)</th>
<th>Sequence alteration and expected effect</th>
<th>Plasmid copy no.</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cop</em></td>
<td></td>
<td>Wild type</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td><em>cop-6</em></td>
<td>1014</td>
<td>G→A, Met-1→Ile-1, missense</td>
<td>83</td>
<td>1, 9, 20, and 21</td>
</tr>
<tr>
<td><em>cop-101</em></td>
<td>1133</td>
<td>Insertion of CGG→frameshifted Cop</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td><em>cop-102</em></td>
<td>1151</td>
<td>Insertion of A, Tyr-47→Och-47, nonsense</td>
<td>92</td>
<td>This work</td>
</tr>
<tr>
<td><em>cop-103</em></td>
<td>1162</td>
<td>A→G, Glu-51→Gly-51, missense</td>
<td>109</td>
<td>This work</td>
</tr>
<tr>
<td><em>cop-104</em></td>
<td>1142</td>
<td>Deletion of A, frameshifted Cop</td>
<td>132</td>
<td>This work</td>
</tr>
<tr>
<td><em>cop-105</em></td>
<td>1141</td>
<td>T→C, Ile-44→Thr-44, missense</td>
<td>110</td>
<td>This work</td>
</tr>
<tr>
<td><em>cop-112</em></td>
<td>1202</td>
<td>Deletion of 4-base SphI overhang with S1 nuclease, loop contraction, 6→4 nt</td>
<td>143</td>
<td>This work</td>
</tr>
<tr>
<td><em>cop-116</em></td>
<td>1202</td>
<td>Insertion of SphI-BamHI linker loop enlargement, 6→14 nt</td>
<td>157</td>
<td>This work</td>
</tr>
<tr>
<td><em>cop-1</em></td>
<td>1258</td>
<td>C→G, ATC→ATG, formation of new Met codon in repF upstream of Met-1</td>
<td>202</td>
<td>20</td>
</tr>
<tr>
<td><em>cop-45</em></td>
<td>1024</td>
<td>Insertion of AAAAG, frameshifted Cop</td>
<td>85</td>
<td>20</td>
</tr>
<tr>
<td><em>cop-300</em></td>
<td>957</td>
<td>Deletion of 19 nt (coordinates 957 to 975), inactivation of <em>cop</em> promoter</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>
FIG. 1. DNA sequence of the replication region of plasmid pE194, showing cop, the cop-repF intercistronic region, and repF. Also shown are the locations (nucleotide positions) of mutations discussed, as well as the locations of PCR primers used to prepare DNA probes. Protein bands corresponding to the gene products encoded by the open reading frames for Cop and RepF were described previously. SD, Shine-Dalgarno sequence; ori, origin of replication.
and other components, as described above. [α-32P]GTP (specific activity, 3,000 Ci/mM, 10 μCi) was used as labeled substrate. The resultant product was fractionated by PAGE (8% gel in 1X Tris-borate-EDTA buffer containing 8 M urea) before autoradiography.

**DNA primers for PCR.** Oligonucleotides having the following sequences were synthesized by the University of Wisconsin Biotechnology Facility and were used in pairwise combinations to obtain defined DNA fragments for use, as indicated above:

- 2920, 5'-CCC CTA GGT GTC CAT TGT CCA TTT (24-mer, nt 897 to 920, sense); 5371, 5'-TGG GAG GTA CAG TGA TGG TTT (21-mer, nt 997 to 1017, sense); 5515, 5'-ATG GTT GTA GAT AGA AAA GAA (21-mer, nt 1011 to 1031, sense); 6394, 5'-GCA ACC GTT ATT CTA ATA AAA (21-mer, nt 1125 to 1145, sense); 20394, 5'-CAT CAC TGT ACC TCC CAA CAT CTG (24-mer, nt 1013 to 990, antisense); 2921, 5'-CAG CAT GCC AGT GCT GTC TAT C (22-mer, nt 1209 to 1188, antisense); 5720, 5'-AGT TTC TTT TAT TAC ATT TTC (21-mer, nt 1285 to 1265, antisense).

**RESULTS**

**Mutations that affect copy number and their map locations.**

To analyze the biochemical function of Cop protein in the regulation of pE194 copy number, we obtained cop mutants and determined the associated sequence alterations. The mutants were either selected with tyllosin, as described previously (21), or constructed (cop-101, cop-112, and cop-116) as described in Table 2. The resultant mutant plasmid DNA was sequenced by conventional methods as described previously (1), with an appropriate primer. The results are summarized in Fig. 1. Most of the mutations that lead to a high copy number can be explained in terms of their effect in reducing either the rate of Cop protein synthesis or the intrinsic activity of Cop protein; however, mutations that are located downstream of cop have been described (20) and additionally, two such mutants, cop-112 and cop-116, have been constructed in the present studies. As we discuss below, their action can be interpreted either in terms of reduction of the rate of ctRNA synthesis, by altering the structure of the ctRNA promoter, or by altering the nucleotide sequence of ctRNA and therefore its intrinsic activity in regulating repF.

**Overexpression of Cop protein in E. coli.** To study the interaction of Cop protein with its DNA target sequence, Cop protein was overexpressed in E. coli and purified to apparent homogeneity (Fig. 2). To prepare Cop⁺ protein (active) and

![FIG. 2. Overexpression of Cop protein. E. coli BL21(DE3) carrying plasmid pHK102 was used to prepare Cop protein. Lane 1, molecular standards; lane 2, crude extract (S-100) from uninduced cells; lane 3, crude extract (S-100) from induced cells; lane 4, peak fraction from Sephadex G-200; lane 5, peak fraction from DEAE Sephadex; lane 6, peak fraction from heparin agarose.](http://jb.asm.org/)
mobility characteristic of the 12-kDa standard (CytC) rather than of the more slowly moving 6-kDa standard (aprotinin). In some preparations, Cop 1 migrated with the mobility expected of a trimer (data not shown).

**Gel retardation analysis of complexes between cop-repF region DNA and Cop protein.** To localize the site(s) of Cop binding in the cop-repF region, prospective DNA target sequences were prepared by PCR with selected primers, as shown in Fig. 1. Results of the gel retardation studies, shown in Fig. 3A, indicated the presence of a binding site(s) in DNA samples used in lanes 2, 4, and 10 and the absence of binding sites in the DNA samples used in lanes 6 and 8. The retardation seen with probe pairs 2920-2921 and 2920-5720 and the absence of retardation seen with probe pairs 5515-5720 and 5515-2921 allow us to identify a binding site for Cop protein upstream of the initiator Met residue of the Cop protein open reading frame, probably in the cop promoter region. This was supported by observing retardation with probe pair 2920-2039, as well. These observations suggest the absence of a binding site for Cop protein at the 5' end of the repF open reading frame. No retardation of any of the probes was seen when Cop-101 protein, similarly purified, was used (Fig. 3B). Lane pairs 1 and 2, 5 and 6, and 7 and 8, but not 3 and 4 or 9 and 10, contain a slowly moving DNA band which we presume to be artifactual, whose mobility was unaffected by added Cop protein.

**DNaseI footprint analysis of Cop protein interaction with its target DNA sequence.** The interaction of Cop protein with the DNA sequence around the cop promoter was examined by using DNA synthesized by PCR with primer pair 2920-2921 in which primer 2920 was 5' 32P-end labeled prior to PCR. The resultant analysis showed a footprint of Cop protein localized precisely over a 28-bp inverted complementary repeat se-

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**FIG. 4.** (A) Interaction of Cop protein with its target DNA sequence by DNaseI footprinting. DNA probe covering the cop promoter was prepared by PCR using as primers upstream, 5' 32P-end-labeled oligo 2920 and downstream, oligo 2921 (unlabeled). The end-labeled DNA probe was incubated with Cop protein and DNaseI, and the resultant partial digest was fractionated by PAGE (8% polyacrylamide DNA sequencing gel containing 8 M urea, in 1× Tris-borate-EDTA). Duplicate DNA sequence determinations were performed (11) with independently amplified DNA samples. The 5' A at the bottom of the figure corresponds to nt 952, and the 3' G corresponds to nt 999 in Fig. 1. Lane 1, no Cop protein, control; lane 2, plus Cop protein, 10 ng; lane 3, plus Cop protein, 100 ng; lane 4, plus Cop protein, 1 μg; lane 5, G+A sequencing lane; lane 6, T+C sequencing lane. (B) Summary of footprinting data (schematic). Line a, cop promoter elements; line b, dyad symmetric element, Cop operator; line c, DNaseI footprint, sequence protection by Cop protein.

**FIG. 5.** Localization of transcription starts in the cop-repF region. Total cell RNA was extracted and annealed to 5' 32P-end-labeled primers 5720 and 2921, respectively (see Fig. 1). The primers were extended with reverse transcriptase, and the resultant products were fractionated by PAGE (8% polyacrylamide containing 8 M urea, in 1× Tris-borate-EDTA buffer). (A) Extension products using primer 5720. (B) Extension products using primer 2921. (The lane 2 samples, cop+, have low intensity presumably due to the low copy number. The lane 4 products migrated more slowly than the products in lanes 3 and 5, which was expected since cop-101 [lane 4] was obtained by fill-in of the Cfr-10I site in cop, adding 4 nt, whereas the mutant products in lanes 3 and 5 resulted from a transition [G→A, cop-6; lane 3] and a single base insertion [A, cop-102; lane 5]. The weak lane 6 intensities are not consistent with the band intensity expected of a high-copy-number plasmid; however, the objective of these determinations was to locate the transcription start rather than to measure copy number, which was done more appropriately as described above.) Lane 1, DNA standards bearing 5' terminal labeled with 32P were prepared from MspI-digested plasmid pBR322 DNA; lane 2, RNA template from pE194 cop'; lane 3, RNA template from pE194 cop-6; lane 4, RNA template from pE194 cop-101; lane 5, RNA template from pE194 cop-102; lane 6, RNA template from pE194 cop-105. DNA standards (nucleotides) are indicated on the left.
sequence that covers the cop promoter region, as shown in Fig. 4A and summarized in Fig. 4B. The presence of transcripts with 5’ termini corresponding to the 5’ end of repF was reexamined by reverse transcriptase analysis of in vivo transcripts. The results, shown in Fig. 5, indicate that most of the transcripts that are demonstrable by this method are initiated from the cop promoter. Several weak bands are seen; however, they represent only minor components. In view of the observations of cop RNA and ctRNA it is not clear how the discrete mRNA fragments associated with repF that we previously described (1) might be formed as part of a regulatory mechanism, except that they reflect the mode of degradation of the cop-repF message.

Analysis of in vitro transcription products. The absence of Cop protein binding sites other than in the cop promoter region does not exclude the existence of other promoters elsewhere in the cop-repF region that might participate in the regulation of cop and/or repF. Three DNA sequences selected to include both cop and repF were used as templates for transcription. The results are shown in Fig. 6. Transcription using DNA template 2920-5720 yielded two prominent transcripts, one ca. 310 nt long corresponding to cop and the second ca. 65 nt long. Transcription with template 2920-2921 showed only the cop transcript (expected length, 221 nt) but not the 65-base transcript, and transcription with template 5515-5720 showed only the short 65-nt transcript. We interpret the 65-nt transcript as ctRNA using a promoter located between coordinates 1279 and 1252 and initiating transcription at coordinate 1244. Failure to synthesize a ctRNA transcription product with the 2920-2921 template can be explained in terms of the absence of the information needed either to initiate or to propagate ctRNA synthesis by the test template. In contrast, the 2920-5720 and 5515-5720 templates both include the putative ctRNA promoter in its entirety and support the synthesis of ctRNA.

In vitro transcription of cop mRNA and ctRNA and the effect of added Cop protein. The effect of Cop protein on cop mRNA and ctRNA was tested by addition of purified Cop protein to the transcription reaction mixture. Results, shown in Fig. 7, show that in vitro transcription of the cop transcript was indeed inhibited, as expected from the footprinting studies, whereas that of ctRNA was not.

Mutagenic alteration of the ctRNA loop. Interaction between complementary loops in ctRNAs and their respective targets characterizes the action of several replication systems (13, 14). Nordström et al. (13), summarizing previous studies, have noted that a six-membered loop provides the most efficient interaction between ctRNA and its target. Loops smaller or larger than 6 nt paired less efficiently, resulting in reduced repression of replication and, therefore, higher plasmid copy number. Noting that plasmid pE194 contains its single-occurrence SphI site within the proposed ctRNA loop segment, we modified the DNA sequence in two ways. After cutting with SphI, we either blunted the SphI overhangs with SI nuclease and religated the plasmid, yielding cop-112 (plasmid pJK122), or inserted a self-annealing SphI-BamHI adapter, 5’GATCCATG, yielding cop-116 (plasmid pJK121). The modifications were verified by DNA sequencing, loss of the SphI site in both plasmids, and in the case of the insertion, pJK121, appearance of a BamHI site absent in the parent plasmid pE194'. In pJK122, we would expect loop contraction from 6 nt, 5’UGCCAG (Fig. 8A), down to 4 nt, 5’CAGU
**DISCUSSION**

Our previous studies of transcription of the cop-repF region of plasmid pE194 (1) demonstrated experimentally that cop and repF comprised two tandem genes oriented in the same (clockwise) direction relative to the circular map of the plasmid and that they specified two protein products. Parallel findings consistent with this interpretation were reported by del Solar et al. (6), who inferred the existence of the pE194 Cop protein on the basis of sequence analysis and noted its homology to RepA from their model system, plasmid pLS1. These findings suggested the need for a revision of the model proposed by Villafane et al. (20) in which it was postulated that the cop transcript was oppositely oriented relative to repF and that it functioned as a tRNA. In view of the finding of tRNA in several replication systems, these observations left open the question whether tRNA played a role in the replication of plasmid pE194. In a recent report on the replication of plasmid pLS1, del Solar and Espinosa (2) have inferred the evidence of tRNA in several other plasmids, including pE194, for which we have obtained experimental verification.

Two important questions remained unanswered. (i) What functional significance could be ascribed, if any, to the finding of two sets of transcripts, one corresponding to cop and the other to repF? And (ii) could some other transcription products function as tRNA? Villafane et al. (20) propose a ca. 350-nt tRNA whose synthesis was initiated as proposed for the tRNA described above. This tRNA was proposed to terminate at the inverted complementary repeat sequence spanning nt 970 to 1000, which contains the plus origin. The RNA that they observed experimentally and reported in support of their model was not the tRNA transcript but the cop transcript whose synthesis was shown to be oriented in a direction opposite to that proposed (1). Failure to find transcription initiation by several methods, both in vivo and in vitro, other than at the cop promoter suggests that cop and repF are transcribed as a single unit that is then processed or degraded. This endonucleolytic cleavage that occurs in the repF region appears to have some degree of specificity in view of the discrete set of fragments that is produced (1); however, it is unclear yet whether the fragmentation pattern that was seen plays a role in the regulation of either cop or repF. Since the proposed tRNA is complementary to part of the intracistronic region of the cop-repF message, the association of tRNA with this region might affect the specificity and rate at which nucleolytic cleavage of the message will occur.

Redefinition of the cop gene allowed us to explain the behavior of some of the elevated-copy-number mutants obtained both by Villafane et al. (20) and in our studies as falling within either the cop promoter region or cop open reading frame (1); however, elevated-copy-number mutations that map downstream of the cop open reading frame and do not appear to involve cop directly in any obvious way were obtained. The behavior of these mutants can be interpreted in terms of their negative effect on the pE194 tRNA. Thus, cop-1 (nt 1258, CG—AGC) and cop-85, and cop-93 (nt 1258, GC—AT), map between the −35 and −10 sequences of the proposed tRNA promoter. In contrast, ts1 (nt 1235, GC—AT) maps within the unpaired 5' end of the proposed tRNA. Finally, an increased copy number was obtained by an alteration in the proposed loop region of the tRNA, by either increasing the loop size from 6 to 14 nt (cop-116) or contracting the loop size from 6 to 4 nt (cop-112).

**ACKNOWLEDGMENTS**

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**REFERENCES**

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