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Replication Genes of Plasmid pE194—*cop* and *repF*: Transcripts and Encoded Proteins

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In vivo transcription of the replication region of plasmid pE194 yields two classes of mRNAs that encode Cop and RepF proteins, respectively. These transcripts are oriented 5′ to 3′ exclusively in the clockwise direction on the standard map. The *cop* region contains an open reading frame capable of encoding a 55-amino-acid protein that was demonstrated electrophoretically as a 6-kilodalton product synthesized in *Bacillus subtilis* minicells and chemically by N-terminal sequencing of a 116-kilodalton fusion protein with *Escherichia coli* β-galactosidase. Four transcripts derived from the *repF* region were found, of which the longest, approximately 720 nucleotides, had the length, orientation, and transcription start site necessary to code for the full-length RepF protein (216 amino acid residues), deduced from the DNA sequence. The 5′ ends of the shorter *repF* transcripts fall within the *repF* open reading frame. We propose that (i) *cop* specifies a protein rather than an RNA countertranscript, (ii) the Cop protein functions as a negative-acting element in pE194 replication by regulating synthesis of both RepF and of itself, and (iii) increased plasmid copy number can be explained in terms of *cop* region mutations that either reduce the intrinsic activity of Cop protein or the rate of its synthesis.

The replication of plasmid pE194 and several other small plasmids, mostly from gram-positive bacteria, is believed to proceed by a rolling-circle model (27), in which a site-specific nuclease, the RepF protein (or its functional homolog in other systems), produces a specific single-stranded nick that allows initiation of synthesis of the leading (i.e., plus) strand. The first phase of replication proceeds by polymerization onto the 3′ OH associated with the nick and displacement of the strand bearing the resultant 5′ phosphoryl group. After a round of synthesis and closure of the resultant product, single-stranded circles demonstrable by agarose gel electrophoresis are formed. For reviews of replication of this class of plasmids, see Gruss and Ehrlich (13) and Novick (22, 23).

The general region of plasmid pE194 responsible for replication control was tentatively identified by localization of a DNA sequence change in the high-copy-number *cop-6* mutant (14, 15). In other studies (28; R. J. Villafane, Ph.D. dissertation, New York University, New York, N.Y., 1985), it was noted that *Cfo*I fragment B of plasmid pE194, a 1,160-base-pair (bp) fragment that contains the sequence altered in the *cop-6* mutant, can support autonomous replication, and additional mutations conferring the high-copy-number phenotype were localized within this region by DNA sequence analysis. Additionally, transcripts of pE194 were examined, and it was reported that (i) two sets of transcripts were synthesized, *cop* and *repF*, both oriented in the same counterclockwise orientation on the standard circular map (15, 26); (ii) the *repF* transcripts that were detected were oriented in the antisense direction relative to the orientation expected for the *repF* message; and (iii) the *cop* gene was therefore likewise oriented in the antisense direction, and its action was mediated through a noncoding antisense countertranscript possibly acting on *repF*. A model for regulation of pE194 replication based on antisense *cop* and *repF* countertranscripts has been proposed (28; Villafane, Ph.D. thesis).

In the present studies, in vivo transcription from the replication region of plasmid pE194 is characterized by S1 nuclease protection using both 5′ end-labeled restriction fragments and M13-cloned fragments uniformly labeled with ^{32}P. The only detectable RNA present that was transcribed from the replication region had a 5′-to-3′ clockwise orientation; this orientation is opposite to that of the countertranscripts previously reported (28). Also, the clockwise transcripts possess the orientation, length, and 5′ transcription start site needed to function as messenger for RepF protein as well as for a 55-amino-acid residue, previously undescribed Cop protein. Based on these new findings, a model for pE194 replication control is proposed in which plasmid copy number is regulated by Cop protein acting as a negative regulatory element.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used, including their pertinent attributes and derivations, are summarized in Table 1.

Enzymes and reagents. Restriction endonucleases and other enzymes were purchased from New England BioLabs, Inc., except for *Cfr10*I, which was from Amersham Corp. Fine reagents were purchased from Sigma Chemical Co., and radioactive isotopes were from Dupont, NEN Research Products. Protein standards for calibration of polyacrylamide gels were purchased from GIBCO-BRL. Nutrient broth (NB) medium was from Difco Laboratories and was prepared according to the specifications of the manufacturer. 2YT medium (20) contained the following (per liter): tryptone (Difco), 8 g; yeast extract (Difco), 5 g; and NaCl, 5 g.

S1 mapping with ^{32}P-end-labeled restriction fragments. To prepare probes, pE194 DNA was digested with *Msp*I restriction endonuclease, end labeled, and secondarily cleaved with *Alu*I. The resultant four labeled fragments, *Msp*I-*Alu*I fragments A, B, C, and D (926, 391, 332, and 239 bp, respectively; Fig. 1), each labeled at its respective single

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TABLE 1. Bacterial strains, plasmids, and phage used

Strain, plasmid, or phage	Description
E. coli	
NM522	$r^- m^-$ transformable host for plasmid constructions before transfer to *B. subtilis* as well as for preparation of Cop–β-galactosidase fusion protein (12)
JFC102	$r^- m^-$ transformable host for constructions utilizing a phage M13-based cloning vector; derived from JM103 (19) by J. F. Cannon (personal communication)
B. subtilis	
BD170	Transformable host for studying expression of plasmid constructions (10)
BR151	Transformable host for studying expression of plasmid constructions (30)
CU403	Transformable host for studying polypeptides encoded by plasmid constructions (18)
Plasmids	
pMC1403	Source of promoterless *lacZ* cassette for plasmid constructions (5)
pBS42	*E. coli-B. subtilis* shuttle vector for construction pMME109 and pMME228; pBR322 and pUB110 origins, chloramphenicol resistance derived from pC194, and ampicillin resistance derived from pBR322 (2)
pE194	Starting plasmid for construction of *cop-101* (15)
pE194*cop-6*	High-copy-number plasmid mutant with initiator Met codon (ATG) of Cop protein replaced by ATA (15, 28, 29)
pE194*cop-101*	High-copy-number plasmid obtained by cleavage of pE194cop^+ with *Cfr10*I (T/CCGGA), filling in of the CCGG overhang with Klenow fragment of polymerase I, dGTP, and dCTP, ligation, transformation, and selection for erythromycin (10 μg/ml) resistance (this work)
pMM156	*E. coli-B. subtilis* shuttle vector obtained from pBS42 to which a promoterless β-galactosidase cassette was added; pBS42 was treated successively with *BamH*I, S1, and *EcoR*I to prepare an insertion site bounded by an *EcoR*I site and a flush end (prepn 1); a *lacZ* cassette was obtained by digestion of pMC1403 with *EcoR*I and *Dra*I (prepn 2); prepns 1 and 2 were ligated, and the resultant mixture was introduced into *E. coli*, followed by selection for chloramphenicol resistance and screening for Lac$^-$ phenotype (this work, constructed by M. Mayford)
pMME109	*E. coli-B. subtilis* shuttle vector obtained from pMM156 in which pE194 cop^+ including its promoter were (translationally) fused to *lacZ* of pMM156; pMM156 was treated with *BamH*I (prepn 1); pE194 cop^+ was treated with *Mbo*I and *Dra*I; the 1,176-nt fragment containing *cop* and its promoter were isolated (prepn 2); prepns 1 and 2 were ligated, followed by treatment with S1, phage T4 DNA ligase, transformation of *E. coli* NM522, selection for chloramphenicol resistance, and screening for the Lac$^+$ phenotype (this work)
pMME228	Same as pMME109, except that pE194 *cop-6* was used as the source of the *Mbo*I-*Dra*I fragment (this work)
Phage M13mp9	Phage vector used for cloning pE194 and some of its restriction fragments (19)

*Msp*I end, were fractionated by polyacrylamide gel electrophoresis and individually annealed with unfractionated RNA from *Bacillus subtilis* BD170(pE194*cop-6*). The 5′ termini of RNA transcripts were determined by the method of Berk and Sharp (4). Since the absolute orientation, length, and location of each of these four end-labeled fragments are known, the precise orientation and length of 5′ ends of protecting transcripts could be deduced unambiguously.

Construction of a *cop-lacZ* translational fusion for high-level synthesis of Cop protein. A 1,176-nucleotide (nt) DNA fragment containing the *cop* open reading frame and its promoter was obtained by digestion of pE194cop^+ and pE194*cop-6* DNA with both *Mbo*I and *Dra*I. The *Mbo*I-*Dra*I fragment from each source was ligated at its *Mbo*I site with plasmid pMM156 digested with *Bam*HI. The ligated complexes were treated with S1 nuclease to remove the 5′ pGATC overhang remaining on the unligated *Bam*HI end attached to the vector, and the resultant flush ends were ligated; transformation (10), selection for chloramphenicol resistance, and screening for Lac$^+$ transformants yielded plasmids pMME109 (cop^+) and pMME228 (*cop-6*). In the resultant constructions, the *cop* open reading frame was translationally fused at the T residue of its termination codon, TAA, to the second nucleotide of the Pro-9 codon of *Escherichia coli lacZ*. In a comparison of the two constructions that were made, pMME109 gave about fivefold higher β-galactosidase specific activity than did pMME228, so that cop^+ was used for preparation of the Cop–β-galactosidase fusion protein. The sequence of pMME109 at the splice junction was checked by determination of the DNA sequence with the dideoxy method (24).

Preparation of Cop–β-galactosidase fusion protein. *E. coli* cells carrying plasmid pMME109 were grown in NB medium containing chloramphenicol (30 μg/ml). The cell pellet (9 g) obtained from 6 liters of culture was suspended in 60 ml of 0.01 M Tris acetate buffer (pH 7.5) containing 0.01 M $MgCl_2$. The resultant suspension was disrupted with a French pressure cell and fractionated through the ammonium sulfate and DEAE steps described by Craven et al. (6) for purification of β-galactosidase. Peak fractions of β-galactosidase activity were pooled, precipitated with ammonium sulfate, and further fractionated by polyacrylamide (12%) gel electrophoresis. The gel was stained with Coomassie blue, and the prominent fusion protein-containing band was excised with a razor blade. The resultant protein sample in the polyacrylamide gel used for its fractionation was submitted for N-terminal analysis to the Macromolecular Structure Facility, Biochemistry Department, Michigan State University, East Lansing.

Construction of *cop-101*, a high-copy-number mutant of pE194. Wild-type plasmid pE194 DNA was digested with *Cfr10*I (Pu/CCGGPy), which cuts once, next to the recognition site for *Msp*I (nt 1129). The overhanging 5′ CCGG ends were filled in with the Klenow fragment of *E. coli* polymerase I plus dCTP and dGTP, resulting in replacement of the *Cfr10*I site, ACCGGT, with the sequence ACCGGCCGGT. The reaction mixture was ligated with phage T4 DNA ligase plus ATP and used to transform *B. subtilis* BR151. Transformants were selected on NB medium containing erythromycin (10 μg/ml) and checked for the high plasmid copy number phenotype by agarose gel electrophoresis. The *cop-101* mutation was verified by DNA sequencing.

Comparison of protein synthesis directed by pE194*cop-6* and pE194*cop-101* in *B. subtilis* minicells. Protein synthesis directed by *cop-6* and *cop-101* was analyzed with minicells of *B. subtilis* (18) as described by Shivakumar et al. (26). After

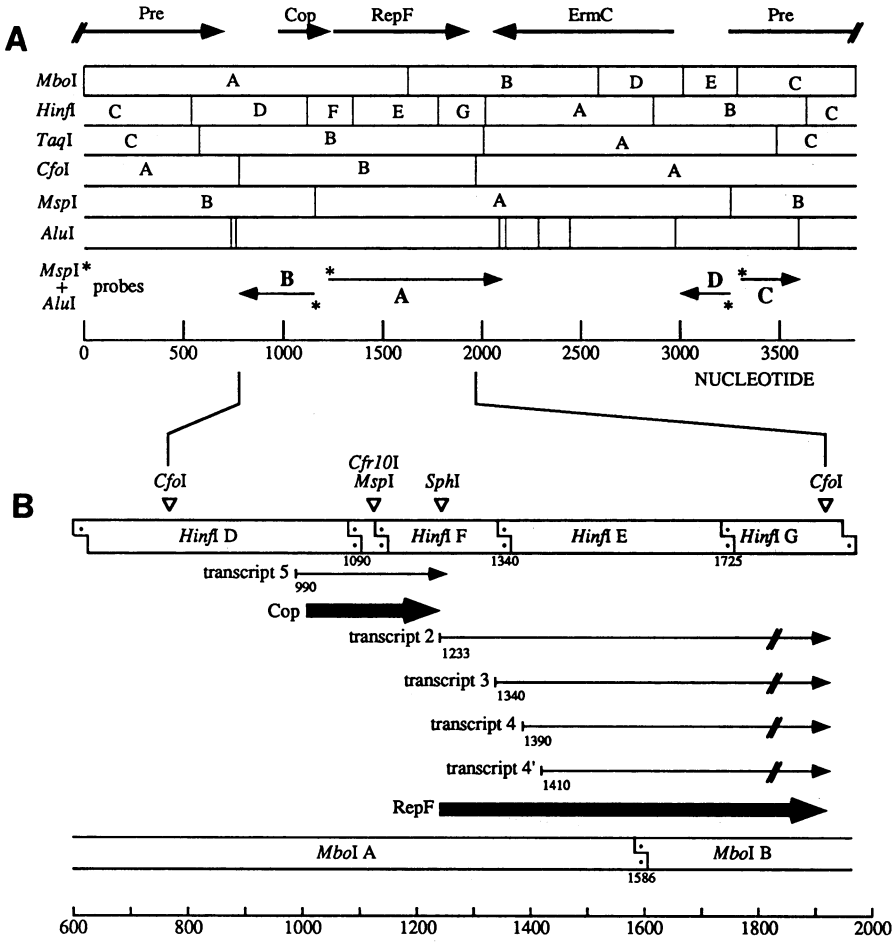

FIG. 1. Physical map of plasmid pE194 and replication region transcripts. (A) Map of plasmid pE194 showing pertinent restriction sites and open reading frames associated with experimentally demonstrable polypeptides. RepF is encoded by open reading frame CD, which is composed of two open reading frames that were consolidated on the basis of a revision of the DNA sequence (28; Villafane, Ph.D. dissertation). Open reading frame E, originally assigned to the same region (15), was oriented in the counterclockwise direction, reflecting the presence of a longer (318-nt) open reading frame; however, based on experimental data presented in this study, the major transcription and translation activity from this part of the plasmid is reinterpreted as shown, and codes for Cop. Of the two *Msp*I sites (C/CGG), one is recognized by *Bsp*MII (T/CCGGA) and the other is recognized by *Cfr10*I (A/CCGGT), allowing selective cleavage of pE194 DNA with either of these enzymes at a single site to form unit linear molecules. Primary digestion of pE194 DNA with *Msp*I followed by 5′ end labeling and secondary digestion with *Alu*I yielded four labeled DNA fragments A, B, C, and D) that were used as probes that demonstrated the *cop* transcript. (B) Summary of replication region transcripts. DNA fragments obtained by digestion with *Mbo*I, *Hin*fI, and *Msp*I restriction endonucleases were 5′ end labeled with ^{32}P and used as probes to locate 5′ ends of pE194 transcripts by S1 nuclease mapping. Transcripts are oriented 5′ to 3′, as shown by the arrows, and numbered 2, 3, 4, 4′, and 5, consistent with Fig. 6 and 7. Other numerals refer to nucleotide residues in the plasmid pE194 sequence.

transformation of *B. subtilis* CU403 with pE194*cop-6* and pE194*cop-101* DNA and selection (on medium containing 10 μg of erythromycin per ml) of cells carrying the desired plasmids, 2-liter cultures were prepared by growing cells at 30°C in Spizizen medium (1) supplemented with thymine and methionine (each at 50 μg/ml) to the late-log phase. Each culture was inoculated with 10 ml of an overnight culture cell suspension grown in LB medium, which contained, per liter, 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl. The pH was adjusted to 7.5 with NaOH, and after sterilization the culture was supplemented with thymine (50 μg/ml). Penicillin G (25,000 U/liter) was added to late-log-phase cell suspensions, and cells were incubated for an additional hour on a rotary shaker. Minicells were isolated and purified from the harvested cell pellet by two cycles of sucrose density gradient centrifugation (5 to 30% sucrose, 60-ml gradients Spinco SW-25.2 rotor, 20 min). To prepare labeled proteins, minicells were incubated as described by Shivakumar et al. (26) and fractionated by 15% polyacrylamide gel electrophoresis. The low-molecular-weight range mixture of proteins from GIBCO-BRL, used as standard, contained the following: ovalbumin, 43 kilodaltons (kDa); α-chymotrypsinogen, 25.7 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa; bovine trypsin inhibitor, 6.2 kDa; insulin α and β chains, 3 kDa.

Cloning of pE194 and its restriction fragments with phage M13mp9. Replicative-form M13mp9 DNA (19) (New England BioLabs) digested with *Acc*I and pE194 DNA digested with *Cla*I were mixed, ligated, and introduced into *E. coli* by transfection. Resultant Lac$^-$ plaque isolates were expanded to produce phage stocks that were screened for augmentation of single-stranded DNA size by reduced mobility on 1%

agarose gel electrophoresis. The orientation of plasmid pE194 DNA in the clone was determined by retardation analysis, in which DNA from a single plaque was tested for its ability to hybridize with single-stranded DNA from other Lac$^-$ plaques carrying inserts with the opposite orientation and thus able to form complexes with reduced electrophoretic mobility. Similarly, the two *Msp*I fragments, A and B, and the three *Taq*I fragments, A, B, and C, of plasmid pE194 were cloned in both orientations into the *Acc*I site in the M13mp9 multiple cloning site, yielding a total of 12 phage clones, 6 in each of the two possible orientations. The 5′-to-3′ clockwise orientation was assigned to the set of phage clones that annealed to the *ermC* message, whose 5′-to-3′ orientation has been represented as counterclockwise on the circular map (15, 26).

Orientation and sizing of pE194 in vivo transcripts by S1 mapping with uniformly ^{32}P-labeled M13-cloned DNA. The orientation, length, and localization of the transcripts of plasmid pE194 were determined by using S1 protection. Labeled probes were prepared by inoculating 0.5 ml of overnight growing cell suspensions of each of the 12 M13mp9 clones into 3 ml of 2YT medium supplemented with 0.1 mCi of $^{32}P_i$. After incubation for 4.5 h with vigorous shaking, virions were collected by precipitation with 20% polyethylene glycol–2.5 M NaCl. DNA was extracted from the precipitated virions with phenol and precipitated twice with ethanol. Total unfractionated RNA was purified from *B. subtilis* BD170 carrying pE194*cop-6*. ^{32}P-Labeled single-stranded DNA probes (2,000 cpm) and RNA (6.5 μg) were annealed in 20 μl of reaction mixture containing 80% formamide, 0.4 M NaCl, 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.8), and 0.001 M disodium EDTA. After incubation for 37°C for 4 h, 180 μl of S1 buffer (0.05 M sodium acetate [pH 4.6], 0.28 M NaCl, 4.5 mM $ZnSO_4$, 20 μg of tRNA per ml, 330 U of S1 nuclease per ml) was added to the annealed complexes, and the reaction mixture was incubated at 37°C for 30 min. Complexes were precipitated with 2 volumes of ethanol, dissolved in 10 μl of TE buffer (0.01 M Tris hydrochloride [pH 7.4], 0.001 M disodium EDTA), and fractionated by polyacrylamide gel electrophoresis, and annealed hybrids were visualized by autoradiography.

S1 mapping of transcript 5′ termini with ^{32}P-end-labeled restriction fragments. The 5′ termini of RNA transcripts were determined by the method of Berk and Sharp (4) with pE194 DNA fragments obtained by digestion with either *Mbo*I or *Hin*fI, respectively, and end-labeled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase.

RESULTS

Four distinct polypeptides encoded by plasmid pE194 have thus far been demonstrated experimentally. These include the following, in order of decreasing size: Pre (11, 26), ErmC methylase (26), RepF (28), and ErmC leader peptide (17). The origin of replication and the determinants for *cop* and *repF* were localized functionally by Villafane et al. (28) within *Cfo*I fragment B. In addition, the transcripts from this region, studied by Northern blot hybridization, were characterized as having the (same) counterclockwise and therefore antisense orientation with respect to *repF*. These RNAs were therefore postulated to function as countertranscripts for as yet undemonstrated transcripts of *repF*. Our experimental findings, presented below, are at variance with those of Villafane et al. (28) and lead to a different interpretation of the role of the *cop* and *repF* transcripts.

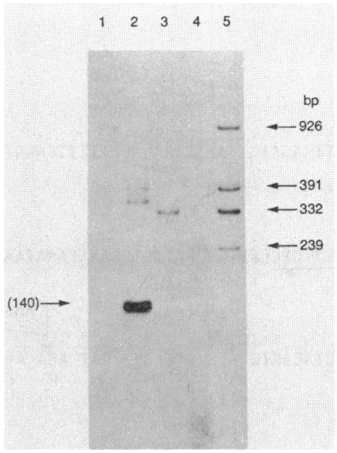

FIG. 2. Transcripts of plasmid pE194 demonstrable by S1 mapping with the DNA fragments obtained by digestion with *Msp*I and *Alu*I. The 5′ transcript ends were determined by S1 mapping in which the four probes were individually hybridized with total RNA from *B. subtilis*(pE194*cop-6*). Protection seen for these fragments was assigned as follows (lanes): 1, *Msp*I-*Alu*I-A, no protection by RNA; 2, *Msp*I-*Alu*I-B, protection of 140 nt by *cop* transcript; 3, *Msp*I-*Alu*I-C, no protection by RNA; 4, *Msp*I-*Alu*I-D, possible protection by *pre* transcript; 5, *Msp*I-*Alu*I fragments A, B, C, and D (unreacted probes).

S1 mapping of 5′ ends of *cop* region transcripts with *Msp*I. Inspection of the pE194 DNA sequence suggested the potential of the *cop* region for synthesis of an additional (i.e., fifth) polypeptide; we therefore examined the orientation of transcripts in the *cop* region. To prepare probes, the four overhanging 5′ ends generated by digestion of pE194 DNA with *Msp*I were labeled with ^{32}P, followed by secondary digestion with *Alu*I, to yield four separable fragments: *Msp*I-*Alu*I fragments A, B, C, and D. The relation of these DNA fragments to pE194 are shown schematically in Fig. 1 together with an updated summary of polypeptides that have been experimentally demonstrated and the replication region transcripts that are discussed in detail below.

Transcripts originating in the replication region of plasmid pE194 that traverse the *Cfr10*I (*Msp*I) restriction site should protect *Msp*I-*Alu*I fragment A if they are oriented in the counterclockwise direction. Alternatively, fragment B should be protected if the transcripts are oriented in the clockwise direction. No protection of fragment A was seen (Fig. 2, lane 1). Instead, protection of fragment B to the extent of 140 nt was seen (lane 2) by a clockwise transcript that produced an intense autoradiographic band. In addition a weaker 357-nt band was found in lane 2. If this band is due to protection of *Msp*I-*Alu*I fragment B by a replication region transcript, the initiation point for this transcript would have to be near nt 772 (Fig. 3). Such a transcript would have to traverse the leading strand origin between nt 860 and 930 proposed by Dempsey and Dubnau (8). This observation suggests additional possibilities for regulation of pE194 replication, and the significance of the 357-nt transcript remains to be determined. A diffuse weak band was seen for *Msp*I-*Alu*I fragment D (Fig. 2, lane 4, bottom). This band, less than 100 nt in length, may reflect protection by the transcript that encodes Pre (11).

Protein specified by the *cop* transcript. Examination of the DNA sequence in the *cop* region suggested the presence of an open reading frame that can encode a 55-amino-acid polypeptide (Fig. 3). The open reading frame is preceded

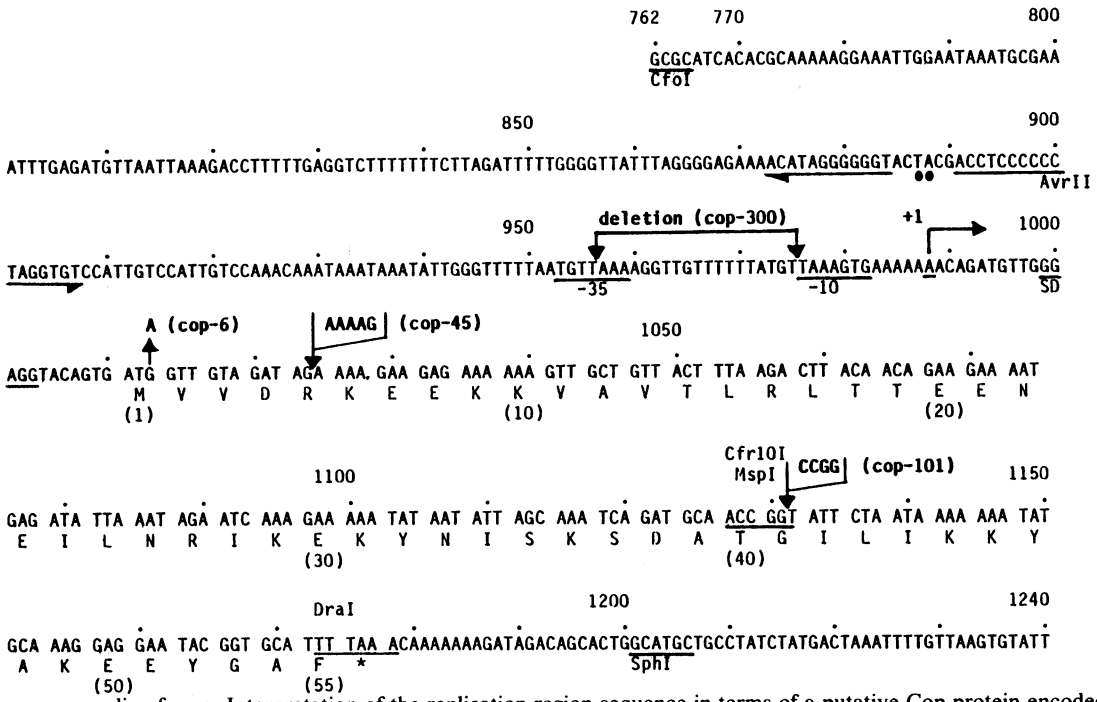

FIG. 3. Cop open reading frame. Interpretation of the replication region sequence in terms of a putative Cop protein encoded by nt 762 through 1240. *cop* mutations are shown, and their effects on synthesis of a putative Cop protein are discussed in the text. The G+C-rich dyad sequence discussed in the text begins at nt 873, and its center of symmetry is shown at nt 887 and 888.

upstream by a possible promoter with TGTTAA as a -35 sequence, an interval of 16 nt, TAAAGTG as a -10 sequence, a possible transcription start at A-988 or A-989, and GGAGG as a ribosome-binding site for the proposed open reading frame that starts 7 nt further downstream with ATG. Also shown are the locations of *cop* mutations in this region that have been sequenced: *cop-300*, *cop-45*, and *cop-101* by Villafane et al. (28) and *cop-6* reported by us (15); their significance in terms of *cop* function is discussed below.

To test whether a Cop protein is actually synthesized in vivo, the proposed *cop* open reading frame was inserted into plasmid pMM156 such that the resultant construction would be expected to contain the entire Cop protein fused in phase at the ninth codon, Pro-9, of β-galactosidase. The DNA sequence across the *cop-lacZ* junction of the construction was confirmed by DNA sequencing. The Cop–β-galactosidase fusion protein (Fig. 4) was purified and sequenced from its amino end to the extent of 12 amino acid residues. Results of the analysis (data not shown) indicated that a polypeptide with the expected Cop N-terminal sequence was synthesized by *E. coli* cells in vivo.

Demonstration of Cop-101 protein by differential labeling of proteins produced in minicells. From the deduced amino acid sequence (Fig. 3), the proposed Cop protein from either pE194*cop*$^+$ or pE194*cop-6* contains no Cys. A high-copy-number mutant, pE194*cop-101*, was obtained by cutting at *Cfr10*I, followed by filling in the overhanging 5′ CCGG sequences and ligating the resultant flush ends. This procedure would be expected to add four nucleotides to the *cop* open reading frame and to produce a frame-shifted Cop protein 61 amino acid residues long that contains two Cys residues: MVVDRKEEKKVAVTLRLTTEENEILNRIKE KYNISKSDATGRYSNKKICKGGIRCILNKKR. The underlined amino acid residues TG are encoded, respectively, by the two codons, ACC and GGC, derived from ACCGGT, the *Cfr10*I site in the wild type used for construction of the *cop-101* mutation. Minicells carrying either pE194*cop-6* or pE194*cop-101* were incubated with either [^{35}S]Met or [^{35}S]Cys, and the resultant four labeled protein preparations were fractionated by polyacrylamide gel electrophoresis. Autoradiograms were prepared (Fig. 5). Induced and uninduced [^{35}S]Met-labeled *cop-6* DNA (Fig. 5; lanes 1 and 2, respectively) were included for orientation to identify the 29-kDa methylase that is synthesized upon induction with erythromycin (band C). On longer exposure of similarly prepared labeled proteins (Fig. 5, lane 3), five prominent bands were seen (A, B, C, D, and F), corresponding to E1 through E5 described by Shivakumar and Dubnau. The pattern of labeling with [^{35}S]Met and [^{35}S]Cys was consistent with the extent to which these amino acids are present in the predicted sequence.

cop-101 gave a pattern of labeling (Fig. 5, lanes 5 and 6) that was clearly different from that found for *cop-6* (lanes 3

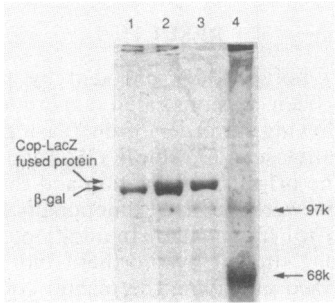

FIG. 4. Cop-LacZ fusion protein. Protein extracted from *E. coli* carrying plasmid pMME109 was partially fractionated and analyzed by analytical polyacrylamide gel electrophoresis and stained with Coomassie blue. Lanes: 1, β-galactosidase; 2, β-galactosidase plus Cop-LacZ fusion product; 3, Cop-LacZ fusion product, 4, standards (molecular masses in kilodaltons).

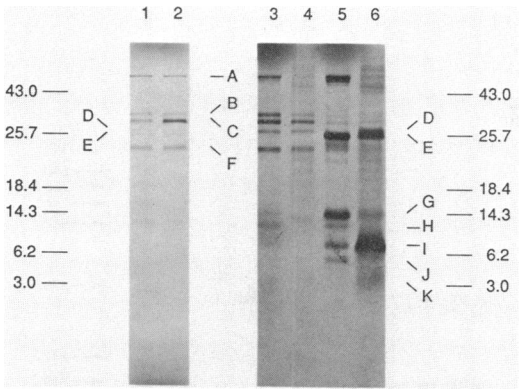

FIG. 5. Demonstration of the 6-kDa Cop-101 protein synthesized by minicells. Minicells obtained from *B. subtilis* CU-403 carrying either plasmid pE194*cop-6* or pE194*cop-101* were incubated with either [^{35}S]Met or [^{35}S]Cys. The resultant labeled proteins were fractionated by electrophoresis and visualized by autoradiography. Lanes: 1, [^{35}S]Met, *cop-6*, uninduced; 2, [^{35}S]Met, *cop-6*, induced (by erythromycin); 3, [^{35}S]Met, *cop-6*, induced; 4, [^{35}S]Cys, *cop-6*, induced; 5, [^{35}S]Met, *cop-101*, induced; 6, [^{35}S]Cys, *cop-101*, induced.

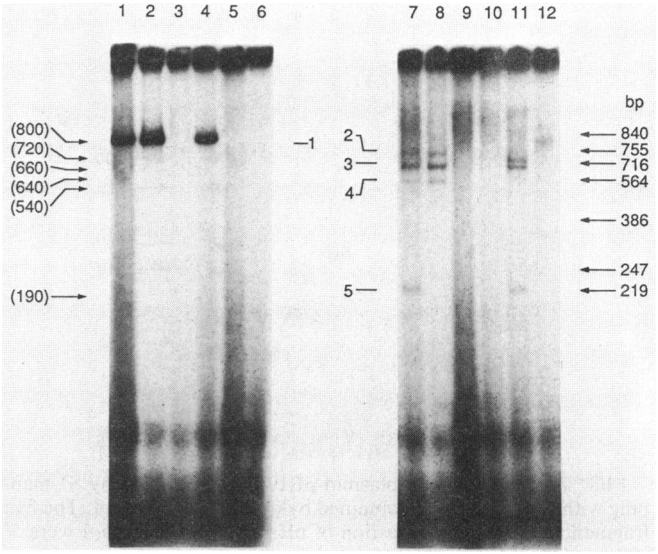

FIG. 6. DNA strand localization of plasmid pE194 transcripts. Single-stranded phage M13 DNAs containing the clockwise and counterclockwise strands of plasmid pE194 *cop-6* DNA, were uniformly labeled with $^{32}P_i$ and annealed to total RNA extracted from *B. subtilis* BD170(pE194*cop-6*). After digestion with S1 nuclease, the resultant fragments were fractionated by polyacrylamide gel electrophoresis and visualized by autoradiography. The numbers on the left of lane 1 refer to the calculated nucleotide lengths of transcripts 1 through 5. Numbers inserted between lanes 6 and 7 refer to transcripts as follows: pre (not shown); 1, *ermC*; 2, 3, and 4, *repF*; 5, *cop*. Mobilities of the seven fragments obtained by digestion of pE194 DNA with *Hin*fI are shown to the right of lane 12. DNA fragments in lanes 1 through 6 are in the clockwise orientation; those in lanes 7 through 12 are in the counterclockwise orientation. Lanes: 1 and 7, full-length pE194; 2 and 8, *Msp*I-A; 3 and 9, *Msp*I-B; 4 and 10, *Taq*I-A; 5 and 11, *Taq*I B; 6 and 12, *Taq*I-C.

and 4). Most pertinent was the presence of an intense band (lane 6) at a mobility corresponding to the predicted 6-kDa [^{35}S]Cys-containing *cop-101* product. The intense labeling of band J with [^{35}S]Cys in *cop-101* but not *cop-6* (lane 4) is consistent with appearance of two Cys residues in *cop-101*, resulting from the frame shift. The other bands of interest are D and E, possibly representing ErmC methylase initiated internally at Met-23 (222 amino acid residues [26]) and RepF (199 amino acid residues [28]).

Orientation of pE194 replication region transcripts. Having demonstrated the Cop protein and its transcript, we next searched for transcripts associated with *repF*. An overview of replication region transcription can be obtained by examining the protection of full-length single-stranded pE194 probes cloned in phage M13mp9. To determine more generally the relative orientations of all detectable in vivo transcripts of plasmid pE194, total RNA from *B. subtilis* (pE194*cop-6*) was annealed to single-stranded probes consisting of pE194 DNA cloned in phage M13 and uniformly labeled in vivo with $^{32}P_i$. The annealed preparations were digested with S1 nuclease, and the resultant fragments were fractionated by polyacrylamide gel electrophoresis and visualized by autoradiography. The single-stranded pE194 samples that were cloned and used as probes included the full-length plasmid, *Msp*I fragments A and B, and *Taq*I fragments A, B, and C, all in both orientations. The transcripts of plasmid pE194 that were detected by this method were numbered 1 through 5 (Fig. 6).

In the initial circular map of pE194 (15, 26), *ermC* was oriented 5′ to 3′ in the counterclockwise direction. Thus, the DNA strand from which the *ermC* message is transcribed, and therefore to which it would anneal, is oriented 5′ to 3′ in the clockwise direction. We noted (Fig. 6, lane 1) a strong band, transcript 1, corresponding to 800 nt based on electrophoretic mobility. This transcript appeared to be wholly contained in both *Msp*I fragment A (lane 2) and in *Taq*I fragment A (lane 4). Since *ermC* has an expected length in the range 905 to 910 nt and is the only apparent open reading frame wholly contained in both *Msp*I fragment A and *Taq*I fragment A, we ascribe the observed protection to the *ermC* message. We therefore infer that the absolute orientation of the DNA fragments used in lanes 1 through 6 is 5′ to 3′, clockwise on the circular map. No other transcripts having the same counterclockwise orientation were visualized.

The other readily demonstrable RNA transcripts of pE194, comprising a group of at least three ranging in size between 540 and 720 nt in length (transcripts 2, 3, and 4), were contained in *Msp*I fragment A and *Taq*I fragment B, and they annealed to the counterclockwise DNA strand of these fragments; the orientation of these transcripts therefore, is clockwise. In addition a small ca. 219-nt transcript 5 apparently straddled an *Msp*I site (absent in Fig. 6, lanes 8 and 9). Because it was wholly contained in *Taq*I fragment B (Fig. 6, lane 11) it must straddle the *Cfr10*I (*Msp*I) site and can therefore be identified as the full-length *cop* transcript, shown above to protect *Msp*I-*Alu*I fragment B (Fig. 2, lane 2).

To align these clockwise transcripts more precisely within the pE194 sequence, S1 mapping with the aid of end-labeled DNA fragments obtained by digestion with *Mbo*I and *Hin*fI was used. A consistent picture of the transcripts from this region was obtained (Fig. 1).

Detailed S1 mapping of 5′ *repF* transcript ends with *Mbo*I. Having established the general location of the *RepF* transcripts, we attempted to map them more precisely in relation to the open reading frame associated with *repF*. Digestion of pE194 DNA with *Mbo*I yielded five fragments. Results of S1 mapping studies with these fragments are shown in Fig. 7.

(i) *Mbo*I-A (Fig. 7, lane 1) showed protection of four size

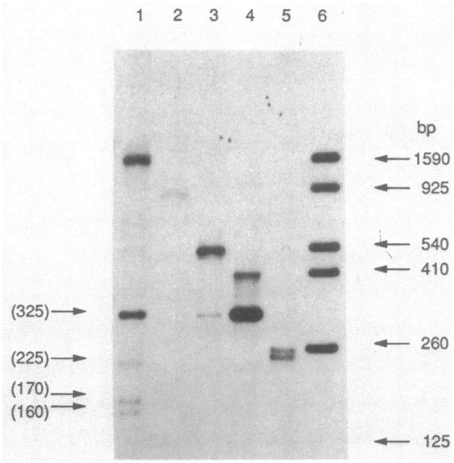

FIG. 7. Transcripts of plasmid pE194 demonstrable by S1 mapping with DNA fragments obtained by digestion with *Mbo*I. The five fragments obtained by digestion of pE194 DNA with *Mbo*I were 5′ end labeled and individually tested for the presence of transcription starts by S1 mapping. Protection for these fragments was assigned as follows (lanes): 1, *Mbo*I-A, protection of 325, 225, 170, and 160 nt by transcripts 2, 3, 4, and 4′, respectively, *repF*; 2, *Mbo*I-B, no protection by RNA initiated internally; 3, *Mbo*I-C, no protection by RNA initiated internally, possible transcription start within *pre* open reading frame (or cross-contamination from lane 4); 4, *Mbo*I-D, protection of 325 nt by transcript 1, *ermC*; 5, *Mbo*I-E, protection of 240 nt (nearly full length of *Mbo*I-E) by *pre* transcript; 6, *Mbo*I fragments A through E, size standards.

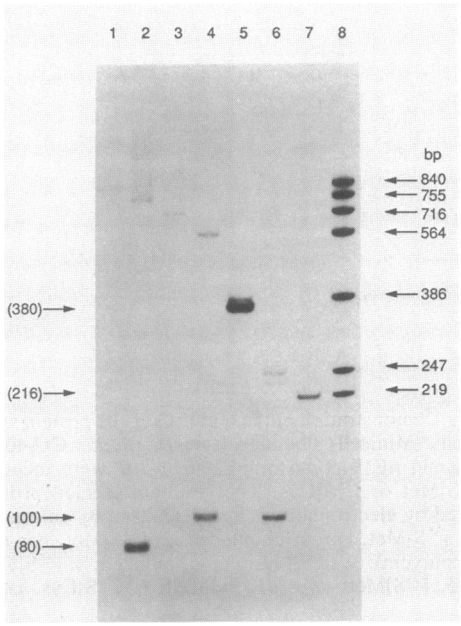

FIG. 8. Transcripts of plasmid pE194 demonstrable by S1 mapping with DNA fragments obtained by digestion with *Hin*fI. The seven fragments obtained by digestion of pE194 DNA with *Hin*fI were 5′ end labeled and individually tested for the presence of transcription starts by S1 mapping. Protection seen for these fragments was assigned as follows (lanes): 1, *Hin*fI-A, no protection by RNA; 2, *Hin*fI-B, protection of 80 nt by transcript 1, *ermC*; 3, *Hin*fI-C, no protection by RNA; 4, *Hin*fI-D, protection of 100 nt by transcript 5, *cop*; 5, *Hin*fI-E, protection of 350 nt by transcripts 4 and 4′, *repF*; 6, *Hin*fI-F, protection of 100 nt by transcript 2, *repF*; 7, *Hin*fI-G, no protection by RNA initiated internally; 8, *Hin*fI fragments A through G, size standards.

classes, which we designate as transcripts 2, 3, 4, and 4′. If these four transcripts are oriented in the clockwise direction, as we expect from the data shown in Fig. 6, they can only protect the *Mbo*I-A site at nt 1586 (*Mbo*I-A–*Mbo*I-B junction). The four transcripts therefore must originate within the replication region, as shown. Because of their localization and orientation we believe that they correspond to transcript bands 2, 3, and 4 shown in Fig. 6, but that the 20-nt difference between 4 and 4′ (Fig. 1) was not resolved by the lower-resolution gel that was used in Fig. 6. In relation to the physical maps of pE194 (Fig. 1), our findings suggest that transcript 2 corresponds to the full-length *repF* message and that transcripts 3, 4, and 4′ originate within the *repF* open reading frame. (ii) *Mbo*I-D (Fig. 7, lane 4) was protected to the extent of 325 nt, which we attribute to protection of the *Mbo*I-D site at nt 2515 (*Mbo*I-D–*Mbo*I-B junction) by the *ermC* transcript. (iii) *Mbo*I-E (Fig. 7, lane 5) appeared to be protected to the extent of nearly its full length, which we attribute to protection of the *Mbo*I site at nt 3185 (*Mbo*I-E–*Mbo*I-C junction) by the *pre* transcript, oriented in the counterclockwise direction. This observation predicts that the *pre* transcript must originate between nt 2924 (the *Mbo*I-D–*Mbo*I-E junction) and nt 3000. Independent evidence for the existence of such a transcript has been obtained in the context of protection of *Msp*I labeled ends (Fig. 2, lane 4). (iv) *Mbo*I-B, (Fig. 7, lane 2) did not appear to contain any mappable 5′ ends, and the weak protection seen for *Mbo*I-C (Fig. 7, lane 3) may be due to slight spillover from the lane 4 sample.

Detailed S1 mapping of 5′ *repF* and *cop* transcript ends with *Hin*fI. Our detailed S1 mapping studies of *repF* and *cop* make several predictions that can be tested by use of the seven DNA probes obtained by digestion of pE194 DNA with *Hin*fI. Results of studies with these fragments are shown in Fig. 8 and summarized in Fig. 1.

(i) *Hin*fI-A, -C, and -G (Fig. 8, lanes 1, 3, and 7, respectively) contained no mappable transcript ends. This observation is consistent with the expectation that *Hin*fI-A should be completely protected by the *ermC* transcript, that *Hin*fI-C should be completely protected by the *pre* transcript, and that there would be no transcription initiation sites expected in *Hin*fI-G. (ii) *Hin*fI-B (Fig. 8, lane 2) was protectable to the extent of 80 nt, which we attribute to protection of the *Hin*fI site at nt 2782 (the *Hin*fI-A–*Hin*fI-B junction) by the *ermC* transcript. Protection of the *Hin*fI site at the *Hin*fI-B–*Hin*fI-C junction by the *pre* transcript, which would result in protection of *Hin*fI-B to the extent of ca. 400 nt, was not seen. (iii) *Hin*fI-D (Fig. 8, lane 4) was protectable to the extent of 100 nt, which we attribute to the *cop* transcript, consistent with the findings shown in Fig. 2, lane 2. (iv) *Hin*fI-E (Fig. 8, lane 5) was protectable to the extent of 380 nt, nearly its full length, which we attribute to *repF* transcripts 4 and 4′. (v) *Hin*fI-F (Fig. 8, lane 6) was protectable to the extent of 100 nt, which we attribute to transcript 2 (full-length *repF*).

In summary, (i) all detectable transcription from the replication region of plasmid pE194 is oriented in the clockwise direction, i.e., the sense orientation with respect to the determinants that code for both Cop and RepF proteins; (ii) the expression of *cop* is probably mediated by a previously undescribed Cop protein, and (iii) no evidence for *cop* or *repF* antisense countertranscripts was found.

DISCUSSION

In the present studies we have attempted to distinguish between mechanisms of regulation of plasmid replication that depend on antisense countertranscripts, as in the case of pT181 from *Staphylococcus aureus* or ColE1 from *E. coli*, and mechanisms that depend on regulation by negative-acting protein(s) (for reviews, see Novick [23] and Scott [25]). Our findings are more easily explained by a mechanism that depends on a negatively acting Cop protein rather than on *cop* antisense countertranscripts for regulation of plasmid copy number.

In these studies we have demonstrated that the *cop* region probably encodes a protein composed of 55 amino acid residues, and we have obtained direct physical evidence by sequencing the amino terminus of the proposed Cop protein translationally fused to *E. coli* β-galactosidase. Several other mutations in the *cop* region that result in an elevated copy number have been sequenced at the DNA level (15, 28), and their behavior can be explained in terms of the way that they affect either the intrinsic activity of Cop or the rate at which it is synthesized.

Interpretation of copy number mutations in terms of their effects on Cop. With reference to Fig. 3, we can interpret the reported behavior of copy-number mutants as follows. (i) *cop-300* (28), a deletion of 19 nt (nt 957 through 975) would delete part of the -35 sequence and the nucleotides that intervene between -35 and -10 of the promoter for synthesis of the *cop* message. (ii) *cop-6* (15, 29) is an alteration in the initiation codon for Cop protein from ATG (Met) to ATA (Ile). Dreyfus et al. (9) observed a reduction to 1% of wild-type activity in a mutant of *GalE* in which the ATG initiator codon was similarly replaced by ATA. In our own studies, comparison of cells carrying β-galactosidase fused to *cop*$^+$ (ATG initiator codon) showed fivefold-higher specific activity than cells carrying the construction in which *cop-6* (ATA initiator codon) was used. (iii) *cop-45* (28), a duplication of the sequence AAAAG that begins at nt 1025, would produce a frame shift leading to premature termination and synthesis of a truncated 17-amino-acid mutant Cop protein with presumed absent or reduced activity. (iv) *cop-101*, duplication of the sequence CCGG that begins at nt 1131 (by filling in overhanging 5′ ends of the *Cfr10*I cleavage site), would produce a frame shift leading to synthesis of an extended, 61-amino-acid Cop protein containing two Cys residues that is presumed to have reduced activity.

Minicells carrying the *cop-6* and *cop-101* mutants of pE194 show different patterns of plasmid-directed protein synthesis. The patterns of minicell protein synthesis in *cop-6* and *cop-101* show marked differences: a relatively higher rate of the ErmC family of peptides in *cop-6* on one hand and a relatively higher rate of Cop and RepF in *cop-101* on the other. Thus, Cop and RepF are apparently overproduced in association with a (frameshift) mutation (*cop-101*) that modifies the primary structure of Cop but not in a mutant (*cop-6*) that replaces the initiator Met codon (ATG) with Ile (ATA), which we assume have not yet proven experimentally primarily acts by reducing the rate of synthesis of active Cop but not necessarily its intrinsic activity. Such a finding is consistent with a model in which Cop acts as a negative regulatory element of both RepF and its own synthesis.

Clockwise orientation of pE194 replication region transcripts. The use of DNA fragments labeled at a single end has enabled us to deduce unambiguously that the *cop* region transcripts of plasmid pE194 are predominantly, if not solely, oriented 5′ to 3′ in a clockwise direction. In our previous studies of the sequence of plasmid pE194 (15), we reported an open reading frame, E, oriented in the counterclockwise direction that contained the *cop-6* mutation at residue 1013. When read in the counterclockwise direction, as originally described, the *cop-6* mutation would convert the third position of a Thr codon (ACT) to ACC, leaving the Thr residue unchanged. It should be noted that the wild-type and *cop-6* mutant sequences were inadvertantly transposed. In terms of the open reading frame E that was described previously, this would produce no obvious functional alteration.

In our search for transcripts from the replication region we have consistently failed to find experimental evidence for such a countertranscript oriented in the counterclockwise direction. If the G+C-rich dyad sequence beginning at nt 873 (Fig. 3) does not serve as transcription terminator for counterclockwise transcription, what other functions might it serve? Dempsey and Dubnau (8) have suggested that the sequence between nt 860 and 930 might provide, instead, a site recognizable at the DNA level for the nicking action of *repF*, the first step in initiation of leading strand synthesis. (The plus origin of plasmid pE194 has been localized recently by Sozhamannan et al. [26a] within the 8-bp sequence containing nt 885 to 892 [TACTACGA].) It therefore appears likely that the transcripts characterized by Villafane et al. (28) were the same as those described above and that, owing to an error, their orientation was consistently inverted.

Similarities between replication regions of plasmids pE194, pLS1, and pADB201. The organization of the *cop-rep* region of pE194 described above resembles that of the replication regions from plasmids pLS1 (16) and pADB201 (3). In the case of pLS1, Lacks et al. (16) reported tandem open reading frames RepA (45 amino acid residues long) and RepB (210 amino acid residues long). Minton et al. (21) noted structural similarities at the amino acid level between RepB of pLS1 and RepF of pE194 (199 amino acid residues long); del Solar et al. (7) have reported mutations in *repA* that confer increased copy number. Similarly, Bergemann et al. (3) reported two tandem open reading frames for plasmid pADB201, polypeptides B (52 amino acid residues long) and A (196 amino acid residues long); they aligned polypeptide A from pADB201 with both RepB from pLS1 (210 amino acid residues) and RepF from pE194 (199 amino acid residues).

Does Cop protein function as a repressor? On the basis of plasmid construction studies, Gryczan et al. (14) reported that *cop* acted in *trans* as a dominant negative regulatory element in pE194 replication. In support of these observations we noted that cells carrying *cop*$^+$ fused to β-galactosidase showed fivefold-higher specific activity than cells carrying the construction in which *cop-6* was used. These observations would imply that the intracellular concentration of Cop protein and plasmid copy number are inversely related. RepA from plasmid pLS1 discussed above may have a function similar to that of Cop protein from plasmid pE194. del Solar et al. (7) analyzed the conformation of the RepA protein from pLS1 and, on the basis of an apparent helix-turn-helix motif, proposed that RepA has DNA-binding activity. On the basis of footprinting experiments they proposed that RepA binds to the promoter for the *repA-repB* operon, acting as a repressor.

RepB from plasmid pLS1 appears to be homologous to RepF in plasmid pE194. Indeed, Villafane et al. (28) have noted additional *cop* mutations (*cop-1*, *cop-85*, and *cop-93*) at the beginning of the *repF* open reading frame. The location of these mutations just one nucleotide upstream of the initiator Met of RepF could act directly on the *repF*

message to increase efficiency of translation initiation; however, *repF* appears to lack an obvious ribosome-binding site, so that the basis for such an effect cannot yet be ascribed to altered secondary structure around the ribosome-binding site or its complementarity with the 3′ end of 16S rRNA.

The patterns of protein synthesis in *cop-6* and *cop-101* differ markedly. The basis for this difference is not yet understood. Possibly, Cop functions at its maximum efficiency as a multisubunit protein whose associated form requires the integrity of a precise sequence of amino acids not directly related to its regulatory function. It will be interesting to test this hypothesis by comparing *cop* mutations that reduce the level of Cop peptide synthesis and those that disrupt its internal amino acid sequence, thereby preventing formation of the associated form. To the extent that Cop is functionally equivalent to RepA from plasmid pLS1, it is noteworthy that del Solar et al. (7) have proposed that RepA is physiologically active in a dimeric form. If that is so, the difference between the patterns of protein synthesis by *cop-6* and *cop-101* may reflect, in part, the biological consequences of the failure of this dimerization.

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