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Posttranscriptional Regulation of the Inducible Nonenzymatic Chloramphenicol Resistance Determinant of IncP Plasmid R26

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The inducible nonenzymatic chloramphenicol resistance (Cm^r) determinant of the IncP plasmid R26 was cloned on a 1,900-base-pair restriction endonuclease *Hin*dIII fragment. Transposon Tn*5* mutagenesis revealed that at least 1,400 base pairs is required for expression of Cm^r. There was no increase in the level of Cm^r when the copy number of the determinant was raised by cloning in pBR322 or pUB5572. Expression of Cm^r by cells carrying a lower-copy-number pUB5572*cml*$^+$ plasmid was inducible and thus indistinguishable from those with R26 itself. However, pBR322*cml*$^+$-carrying cells expressed Cm^r constitutively, possibly due to the activity of vector promoters or an elevated copy number. Transcriptional and translational *cml-lac* fusions were constructed. The operon (transcriptional) *cml-lac* fusion carried by the low-copy-number plasmid pUB5572 caused a low level of constitutive β-galactosidase activity, which could not be elevated by induction with chloramphenicol and was not affected by a coresident R26*cml*$^+$ element. In contrast, the gene (translational) *cml-lac* fusion expressed low-level β-galactosidase activity, which was elevated fivefold by prior exposure to chloramphenicol. We conclude that the regulation of Cm^r occurs posttranscriptionally.

The most commonly encountered mechanism of chloramphenicol resistance (Cm^r) in gram-negative bacteria is that mediated by the plasmid-encoded enzyme chloramphenicol acetyltransferase (CAT). This enzyme is expressed constitutively and results in inactivation of the drug by 3-*O*-acetylation, using acetyl coenzyme A as a cofactor (13, 16, 26, 29).

Plasmids have been identified in gram-negative bacteria that confer Cm^r by a mechanism which does not involve CAT (15, 20, 22, 23). The Cm^r determinant of the IncP plasmid R26 is of this type (15). It differs from CAT-mediated Cm^r determinants in that it specifies a low level of resistance. Also, it is inducible and does not involve drug inactivation or modification (15). Furthermore, the ribosomes in Cm^r cells are not resistant to inhibition by chloramphenicol in vitro (15). It is thought that resistance is conferred by a chloramphenicol-specific permeability barrier acting at the level of the cell envelope (15).

In this paper, we report the molecular cloning of the R26 Cm^r determinant and the mapping of the gene(s) responsible for resistance by restriction enzyme analysis and by transposon Tn*5* mutagenesis. Also, the mechanism of regulation was investigated by analyzing the properties of *cml-lac* gene and operon fusions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strains were C600 *thr leu lac supE*, XAc Su^- Δ(*lac-pro*)XIII *ara argE thi gyrA rpoB* (both obtained from N. Kleckner, Harvard University), and J5-3 *pro met* (8). The plasmids used are listed in Table 1.

Bacteriological media, chemicals, and antibiotics. The compositions of L broth, L agar, and M9 minimal medium were previously described (14). MacConkey agar (Oxoid Ltd.) was used for studying *cml-lac* fusions.

Antibodies and other inhibitors were incorporated into agar as follows (micrograms per milliliter): ampicillin (Beecham Laboratories), 50; chloramphenicol (Sigma Chemical Co.), 20; kanamycin (Sigma), 20; sulfathiazole (BDH), 20; tetracycline (Sigma), 10; trimethoprim (Wellcome Research Laboratories), 30.

Chemicals were purchased from Sigma or were the best grade available from BDH.

Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals or New England Biolabs and were used according to the instructions of the manufacturer.

DNA manipulations. Plasmid DNA was purified from cleared lysates (7) by centrifugation in ethidium bromide-cesium chloride density gradients. A small-scale method was employed for rapid screening of transformants, etc. (9). Agarose gel electrophoresis was performed as previously described (1). DNA fragments were excised from agarose gels by freeze-thawing, followed by extraction in 0.5 M NaCl–1 mM EDTA–10 mM Tris-hydrochloride (pH 7.8).

MIC determinations. The procedures for measuring the level of resistance to chloramphenicol by plate MIC determinations and growth and challenge tests in broth have been previously described (10).

Tn*5* mutagenesis. Chloramphenicol-sensitive derivatives of pDU1246 were isolated after insertion of transposon Tn*5* with λ *rex*::Tn*5* *c*I857 *b*221 *O*29 *P*80 (obtained from N. Kleckner, Harvard University) as a vector (9).

β-Galactosidase assays. The method for measuring β-galactosidase activity was described by Miller (21).

Cloning the R26 *cml* gene(s) in pBR322 and pUB5572. About 20 μg of plasmid R26 DNA was cleaved with *Eco*RI and ligated with *Eco*RI-cleaved pBR322. A chloramphenicol-resistant transformant was isolated which carried a plasmid with a 7,000-base-pair (bp) insertion in pBR322.

A 1,900-bp *Hin*dIII fragment from this element was then inserted into the *Hin*dIII site of pBR322 in both orientations, giving pDU1246 and pDU1247.

To lower the copy number of the cloned *cml* genes, the 1,900-bp *Hin*dIII fragment was inserted in the appropriate

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site of the R388-derived plasmid vector pUB5572 (Tp^r) to form pDU1249.

To facilitate the formation of *cml-lac* translational fusions at the *Bam*HI site in the *cml* region, the 1,900-bp *Hin*dIII *cml* fragment was introduced into a deletion mutant of pBR322 (pDU1252) which had lost the *Bam*HI site in the *tet* gene. This recombinant, pDU1253, has a single *Bam*HI site within the *cml* determinant.

Isolation of deletions in the cloned *cml* region. Deletions in *cml* were constructed in vitro by removing DNA located between restriction endonuclease cleavage sites. pDU1355 was constructed by partial digestion of pDU1246 with *Pst*I, excision of the appropriate-sized fragment from an agarose gel, ligation, and transformation into C600 selecting for Ap^r (Cm^s).

DNA was removed from either side of the *Bam*HI site in *cml*. Plasmids pDU1247 and pDU1246 were cleaved with *Bam*HI, and the DNA was ligated and transformed into C600 selecting for Ap^r (Cm^s) colonies to yield pDU1333 and pDU1334, respectively.

Construction of *cml-lac* fusions. Putative operon (transcriptional) *cml-lac* fusions in the pBR322*cml* plasmid pDU1246 were constructed by ligating the purified 7,700-bp *Bam*HI *lac* fragment of pMC903 (which carries the promoterless *lacZ* gene [5]) with partially *Bam*HI-cleaved pDU1246 DNA. Transformants of $XAcSu^-$ which were Ap^r Cm^s Lac^+ were obtained. Two clones were isolated that had the *lac* fragment inserted in the two possible orientations (pDU1290 and pDU1291) (Fig. 1).

The copy number of the *cml-lac* fusions was reduced by cloning the 8.9-kilobase (kb) *Hin*dIII fragment (having the *lac* insert in *cml*) into the *Hin*dIII site at pUB5572. These plasmids are called pDU1292 and pDU1293 (Fig. 1).

A gene (translational) fusion was constructed by inserting the purified 6.8-kb *Bam*HI fragment of pMC931 (5) (which has a *lacZ* gene sequence lacking a promoter, a ribosome-binding site, and a translational initiation codon) into the *Bam*HI site of pDU1253. Ap^r Cm^s transformants were obtained which had the *lac* fragment inserted in both possible orientations, giving pDU1294 and pDU1295.

RESULTS

Cloning and expression of the chloramphenicol resistance determinant of R26. The Cm^r determinant was cloned from

TABLE 1. Plasmids

Plasmid	Phenotypic markers[a]	Comments	Reference
R26	Cm^r Tc^r Ap^r Ka^r Su^r Hg^r Sm^r IncP		27
pBR322	Ap^r Tc^r		2, 3
pUB5572	Tp^r IncW	Derived from R388	6
pMC903	Ap^r Ka^r	Has BamHI fragment with promoter-less *lac* operon	5
pMC931	Ap^r Ka^r	Has *Bam*HI fragment with *lacZ* derivative suitable for constructing gene fusion	5
pDU1246	Ap^r Cm^r	pBR322 with 1.9-kb *Hin*dIII fragment from R26	This study
pDU1247	Ap^r Cm^r	pDU1246 with *Hin*dIII insert in reverse orientation	This study
pDU1249	Cm^r Tp^r	pUB5572 with 1.9-kb *Hin*dIII fragment from pDU1246	This study
pDU1252	Ap^r	pBR322 with a 1.1-kb deletion in the *tet* region	This study
pDU1253	Ap^r Cm^r	pDU1252 with 1.9-kb *Hin*dIII fragment from pDU1246	This study
pDU1256-pDU1280	Ap^r Ka^r	pDU1246*cml*::Tn*5* mutants	This study
pDU1290	Ap^r Lac^+	7-kb *Bam*HI fragment from pMC901 in *Bam*HI site of pDU1246	This study
pDU1291	Ap^r Lac^+	As for pDU1290 but with insert in reverse orientation	This study
pDU1292	Tp^r (Lac^+)	*cml-lac* construction from pDU1290 in *Hin*dIII site of pUB5572	This study
pDU1293	Tp^r (Lac^+)	*cml-lac* construction from pDU1291 in *Hin*dIII site of pUB5572	This study
pDU1294	Ap^r (Lac^+)	Φ(*cml-lacZ*$^+$) formed from 6.8-kb fragment of pMC931 in pDU1253	This study
pDU1295	Ap^r	6.8-kb *Bam*HI fragment of pMC931 in pDU1253 in reverse orientation	This study
pDU1333	Ap^r	Cm^s deletion of pDU1247	This study
pDU1334	Ap^r	Cm^s deletion of pDU1246	This study
pDU1335	Ap^r	Cm^s deletion of pDU1246	This study

[a] Resistance markers are abbreviated as follows: Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; Ka, kanamycin; Su, sulfonamides; Hg, mercuric ions; Sm, streptomycin; Tp, trimethoprim. Lac^+ indicates sufficient expression of β-galactosidase to form red or pink colonies on MacConkey agar. (Lac^+) indicates insufficient expression of β-galactosidase to form pink colonies on MacConkey agar.

plasmid R26 on a 1,900-bp *Hin*dIII fragment which was inserted into the *Hin*dIII site of pBR322, giving pDU1246 and pDU1247, and into the low-copy-number vector pUB5572, forming plasmids pDU1248 and pDU1249.

The phenotypes conferred by the three chimeric plasmids in *E. coli* J5-3 were compared with that of R26 by plate MIC determinations and by broth growth and challenge tests (Table 2, Fig. 2). The MIC conferred by the two pBR322*cml* plasmids was indistinguishable irrespective of whether the cultures had been induced by exposure to a subtoxic concentration of chloramphenicol or were uninduced (Table 2). In contrast, the pUB5572*cml* plasmids appeared to express high-level resistance to chloramphenicol only after induction, in a similar fashion to the parental plasmid R26. This behavior was confirmed by the broth growth and challenge tests (Fig. 2). Thus, the complete resistance determinant, including structural and regulatory regions, must be contained within the 1,900-bp *Hin*dIII fragment.

The observation that Cm^r expression by pBR322*cml* plasmids in MIC experiments was expressed constitutively (Table 2) was tested in the growth and challenge tests (Fig. 2). This constitutive expression could be due to a higher basal level of *cml* gene expression from the multiple copies or due to the influence of vector promoters (28) or both.

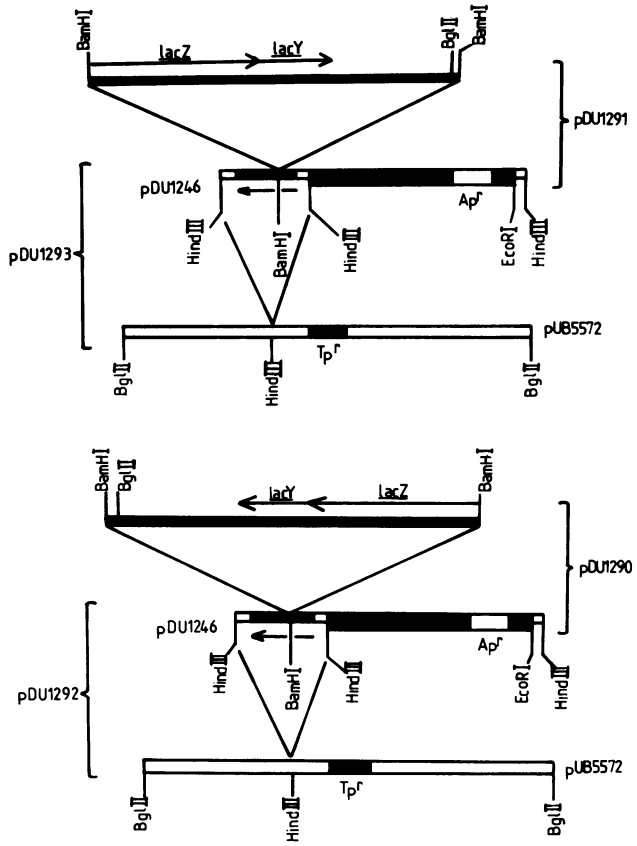

FIG. 1. Construction of transcriptional fusions and structure of recombinant plasmids. The upper narrow solid bar in each diagram represents the 7.7-kb *Bam*HI fragment from pMC903. The direction of transcription of the *lac* operon (when expressed) is indicated by the arrow. The broader part of pDU1246 represents pBR322 DNA, and the narrower part is R26 DNA. The Cm^r determinant is represented by the dark area within the narrow R26 part. The direction of transcription of *cml* is indicated by the lower arrow. The vector pUB5572 is depicted by the lower narrow open bar. The positions of the ampicillin resistance (Ap^r) and trimethoprim resistance (Tp^r) genes are shown.

TABLE 2. MIC determinations

Plasmid[a]	MIC (μg of chloramphenicol per ml)	
	Uninduced	Induced[b]
None	2	2
R26	15	30
pDU1246	30	30
pDU1247	30	30
pDU1249	15	30
pDU1256-pDU1279	2	2
pDU1280	20	20
pDU1333, pDU1334	2	2
pDU1335	5	5

[a] The host used throughout was *E. coli* K-12 J5-3.
[b] Induction was with 1 μg of chloramphenicol per ml added to exponentially growing cells 60 min before plating.

Mapping the chloramphenicol resistance region. A detailed restriction map of the 1,900-bp *Hin*dIII fragment in plasmids pDU1246 and pDU1247 was constructed (Fig. 3). To map the Cm^r determinant more precisely, transposon Tn*5* was inserted in pDU1246, and 25 independent Cm^s mutants were isolated. The site of each insertion was mapped by restriction enzyme analysis. The distribution of the insertions showed that at least 1,400 bp was required for expression of Cm^r (Fig. 3).

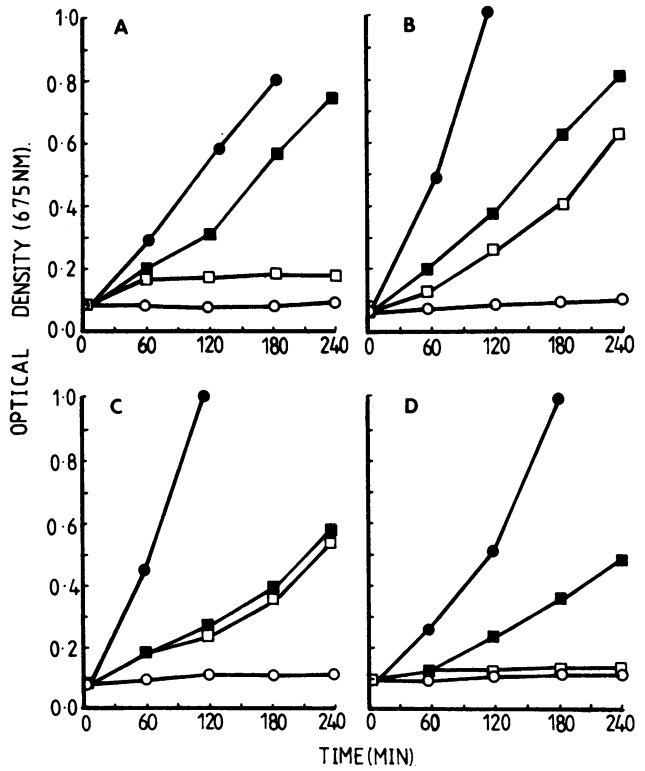

FIG. 2. Growth and challenge tests. Exponentially growing cultures of *E. coli* J5-3 carrying Cm^r plasmids either induced (■) or uninduced (□) with 1 μg of chloramphenicol per ml were challenged with 15 μg of chloramphenicol per ml at time zero. The plasmid-free J5-3 strain either unchallenged (●) or challenged (○) with 15 μg of chloramphenicol per ml is shown for reference. A, R26, B, pDU1246; C, pDU1247; D, pDU1249.

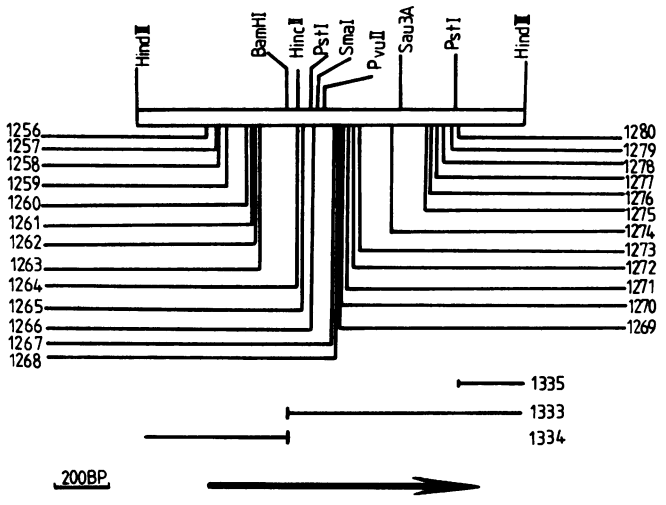

FIG. 3. Structure of the cloned chloramphenicol-resistant determinant. The broad horizonal bar represents the cloned 1.9-kb *Hin*dIII fragment. Restriction endonuclease cleavage sites are shown above the bar, and transposon Tn*5* insertions are shown below the bar. The structures of the deletion mutations are summarized at the bottom. The deleted DNA is represented by the horizontal lines. The arrow at the base of the diagram shows the inferred direction of transcription.

All but one of the pBR322*cml*::Tn*5* mutants was completely Cm^s (Table 2). The deletions constructed by removing DNA from either side of the *Bam*HI site (pDU1333 and pDU1334; Fig. 3) also conferred a Cm^s phenotype (Table 2). One Tn*5* insertion mutant (pDU1280) allowed expression of low-level Cm^r (Table 2). A possible explanation for this is that the Tn*5* insertion has damaged the carboxy terminus of a resistance protein. This idea was supported by the observation that a deletion which removed DNA to the right of the *Pst*I site (pDU1335) also expressed a low level of Cm^r (Fig. 3, Table 2). Assuming that the direction of transcription is from left to right (Fig. 3), the damage to the *cml* gene caused by the deletion in pDU1335 would be more extensive than that of the Tn*5* insertion in pDU1280. This is consistent with the observation that pDU1335 expressed a lower level of Cm^r than pDU1280 (Table 2).

Construction of *cml-lac* fusions and studies on the mechanism of regulation. To investigate the mechanism of regulation of the inducible Cm^r determinant, both gene (translational) and operon (transcriptional) *cml-lac* fusions were generated to allow expression of the *lacZ* gene from a *cml* promoter. In the case of operon fusions, the expression of β-galactosidase is dependent solely upon the provision of an exogenous promoter. The *lac* mRNA provides the ribosome-binding site and translational initiation codon to permit translation of β-galactosidase. A 7,700-bp *Bam*HI fragment from pMC903 which carries a promoter-deficient *lac* operon was inserted into the *Bam*HI site located within the *cml* region of the pBR322*cml* plasmid pDU1246. This construction caused a Cm^s mutant phenotype. Insertions of the *lac* fragment in both possible orientations were obtained (pDU1290 and pDU1291; Fig. 1). β-Galactosidase was expressed constitutively by both elements (Table 3).

However, since the expression of the Cm^r phenotype by multicopy pBR322*cml* plasmids was not inducible by chloramphenicol (Table 2, Fig. 2) and thus apparently not subject to normal regulation, it was considered prudent to examine the expresison of β-galactosidase under low-copy-number conditions. Thus, the *cml-lac* fusions were cloned from pDU1290 and pDU1291 into the low-copy-number vector pUB5572 (Fig. 1). The resulting pUB5572*cml-lac* fusion derivatives pDU1292 and pDU1293 also expressed β-galactosidase constitutively, albeit at lower levels than the pBR322-located fusions (Table 3). This level of β-galactosidase activity was not altered by the presence of the wild-type Cm^r determinant of R26 which expresses inducible chloramphenicol resistance (Table 3). This indicates that any transcriptional regulatory functions were not lacking in the operon fusions. Indeed, there is no evidence that expression of the *cml* gene(s) is regulated at the level of transcription.

A *lacZ* gene-carrying fragment of DNA derived from pMC931 which lacks a promoter, ribosome-binding site, and translational initiation codon was inserted into the *Bam*HI site located in the *cml* region of pDU1253. β-Galactosidase activity will be expressed only if a promoter, ribosome-binding site, and in-frame initiation codon are provided by *cml*. Thus, a hybrid chloramphenicol-β-galactosidase protein will be formed. Insertions in both orientations in the *Bam*HI site located in the *cml* gene of pDU1253 were isolated, forming pDU1294 and pDU1295, respectively. Only pDU1294 expressed detectable β-galactosidase activity (Table 4). Furthermore, the level of expression was increased fivefold after induction with chloramphenicol. This suggests that the regulation occurs at the level of translation. This induction occurred in the absence of a wild-type Cm^r determinant, suggesting that autogenous control is not involved. No increase in β-galactosidase activity was detected when the wild-type inducible Cm^r determinant was also present on plasmid pDU1249 (pUB5572*cml*$^+$). In fact, the absolute

TABLE 3. β-Galactosidase activity of putative transcriptional *cml-lac* fusions

Plasmid	β-Galactosidase activity (U)[a]	
	Uninduced	Induced[b]
pDU1291	500	470
pDU1290	1,200	1,200
pDU1293	13	13
pDU1291	20	20
pDU1293 + R26	18	18
pDU1292 + R26	22	25

[a] Numbers were obtained from at least three experiments
[b] Induction was with 1 μg of chloramphenicol per ml for 60 min before assay.

TABLE 4. β-Galactosidase activity of putative translational *cml-lac* fusions

Plasmid	β-Galactosidase activity (U)[a]	
	Uninduced	Induced[b]
pDU1295	0	0
pDU1295 + pDU1249*cml*$^+$	0	0
pDU1294 Φ(*cml'-lacZ*)	4	20 (x5)[c]
pDU1294 Φ(*cml'-lacZ*$^+$) + pDU1249*cml*$^+$	1.5	4.5 (x3)
pDU1294 Φ(*cml'-lacZ*$^+$) + pUB5572	2	10 (x5)

[a] Numbers were obtained from three independent experiments.
[b] Induction was with 1 μg of chloramphenicol per ml for 60 min before assay.
[c] Numbers in parenthesis are the induction ratios, the ratio of β-galactosidase in induced/uninduced samples.

level of β-galactosidase expressed by the pDU1294*cml-lacZ* and pDU1249*cml*$^+$ cells was lower than that expressed by pDU1294 alone. Also, the induction ratio was threefold in the heterozygous strains compared with the fivefold induction ratio observed in the pDU1294-carrying cells. In addition, the vector plasmid pUB5572 also reduced the level of β-galactosidase expressed by pDU1294 (Table 3). This may be due to an effect on the copy number of the pBR322-derived plasmid. This, combined with the presence of an induced wild-type Cm^r determinant which is thought to prohibit entry of drug into the cytoplasm (15), could cause the reduction in absolute levels of β-galactosidase and also the induction ratio seen in the heterozygous strain.

DISCUSSION

The IncP plasmid R26 specifies resistance to chloramphenicol by a mechanism that is inducible and which does not involve drug inactivation (15). Here we describe the molecular cloning and a genetic analysis of the R26 Cm^r determinant. The Cm^r phenotype was not inducible when the determinant was carried by the multicopy plasmid pBR322. However, when the genes were transferred to the low-copy-number plasmid pUB5572, resistance was expressed inducibly. Thus, the 1,900-bp-*Hin*dIII fragment present in pDU1246, pDU1247, and pDU1249 is sufficient to encode the *cml* structural gene(s) and regulatory functions. Furthermore, Tn*5* mutagenesis showed that at least 1,400 bp is required for Cm^r (Fig. 3).

The direction of transcription of the *cml* gene(s) can be inferred from the properties of a deletion mutant, pDU1335, and of a Tn*5* insertion mutant, pDU1280, which express low-level Cm^r (Table 2), and also from the behavior of a *cml-lacZ* gene fusion. The simplest explanation for the low-level Cm^r phenotype of the mutants is that the region encoding the carboxy terminus of a Cm^r protein has been damaged. If this is the case, then transcription of the *cml* gene(s) must be from left to right as the map is drawn in Fig. 3. The construction of a gene fusion between *cml* and *lacZ* (presumably resulting in the formation of a chloramphenicol-inducible hybrid chloramphenicol resistance-β-galactosidase protein) occurred only when the *Bam*HI fragment from pMC931 which carries the *lac* operon was cloned in one particular orientation in the *Bam*HI site located in *cml*. Since β-galactosidase activity expressed by the fusion was inducible by chloramphenicol, it can be concluded that the protein is expressed from the *cml* promoter and that the direction of transcription is as indicated in Fig. 3. The low level of β-galactosidase activity may be due to the fusion being out of frame. In this case, translational slippage would be responsible for the activity.

It seems likely that the *cml* promoter is quite weak. Operon fusions with *lacZ* located on a low-copy-number plasmid (eight copies per chromosome [5]) expressed 20 U of β-galactosidase. The higher activity of the pBR322*cml-lac* operon fusions (500 to 1,200 U) can be ascribed to the higher copy number of the plasmid (30 to 40 copies per chromosome (2, 3) combined with the activity of vector promoters, particularly in the case of pDU1290, where the strong outward-reading *tet* promoter of pBR322 could be involved (28). However, despite the apparent weak transcription of the *cml* gene and the failure of preincubation with chloramphenicol to increase the rate even when a wild-type inducible Cm^r determinant was present in the same cell, it is clear from resistance level determinations (Table 2, Fig. 2) that a single copy of the *cml* gene on plasmid R26 is sufficient to confer the maximum achievable level of resistance on *E. coli* cells. Increasing the copy number of the *cml* gene(s) did not result in an increase in resistance level. Furthermore, the multicopy pBR322*cml* plasmids apparently expressed Cm^r constitutively. Possibly, sufficient basal expression of the resistance determinant occurs to protect the cells from the inhibitory effect of the drug and to prevent induction.

Experiments with in vitro-constructed *lac* fusions have shed some light on the possible mechanism of regulation involved in the expression of Cm^r. The operon (transcriptional) fusion was not induced by chloramphenicol under any circumstances, even when a low-copy-number plasmid carrying a wild-type inducible determinant was present in the same cell. This precludes the possibility that transcription of *cml* is being regulated either positively or negatively by a regulatory protein. Similarly, autogenous regulation of transcription by the Cm^r protein is unlikely.

The data obtained with the *cml-lac* gene fusion indicate that induction occurs at the level of translation. Other systems in which translational regulation occurs include the ribosomal protein genes of *E. coli* (24, 25, 30), gene *32* of bacteriophage T4 (19), erythromycin resistance in gram-positive bacteria (17, 18), and inducible Cm^r mediated by CAT in *Bacillus pumilus* (11, 12) and *Staphylococcus aureus* (4). Expression of the ribosomal protein operons of *E. coli* and gene *32* of T4 is controlled by a subtle interplay between the gene products and the message. These proteins are capable of binding to single-stranded nucleic acid molecules, the ribosomal proteins to rRNA, and the gene *32* product to single-stranded DNA. They can bind to specific regions of their own mRNA to modulate translation autogenously.

The regulation of erythromycin resistance involves the formation of alternative secondary structures in the mRNA 5′ to the coding region for the resistance methylase. Ribosomes stalling in the presence of the drug cause conformational changes in the mRNA which exposes the hitherto sequestered ribosome-binding site for the methylase gene, thus permitting its translation. The expression of inducible CAT genes is thought to be controlled by a single secondary structure in the mRNA which sequesters the ribosome-binding site for the CAT gene. Induction by chloramphenicol may involve a chloramphenicol-mediated conformational change in 23S rRNA, permitting binding of the altered ribosome to that part of the message which is complementary to the CAT ribosome-binding site. This would leave the ribosome-binding site free to accept an unaltered ribosome to initiate translation (4). Experiments are in progress to elucidate the nature of the translation regulation involved in the control of expression of the R26 *cml* gene.

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