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Genetics

Control of ColE1 DNA replication: The *rop* gene product negatively affects transcription from the replication primer promoter

(operon fusion/primer transcription/repressor)

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ABSTRACT **A 600-base-pair region essential for ColE1 and pMB1 plasmid replication contains two promoters responsible for the synthesis of two RNA molecules central to copy number control. One promoter directs synthesis of the primer RNA precursor. The second promoter directs the synthesis of a small RNA molecule, RNA1, which acts in *trans* to inhibit processing of the RNA primer precursor. We have fused each promoter to the β-galactosidase structural gene contained in a λ phage. Expression of the RNA1 promoter in lysogens is not influenced by the presence of wild-type pMB1 or ColE1 plasmids residing in the cell. Transcription from the RNA primer promoter, however, is repressed by the product of a *trans*-acting plasmid gene product, which we have designated *rop* (for repressor of primer). The *rop* gene maps downstream from the replication origin in a region that encodes a polypeptide of 63 amino acids whose sequence is completely conserved in pMB1 and ColE1. We propose that this polypeptide is the *rop* gene product and that it regulates plasmid DNA replication by modulating the initiation of transcription of the primer RNA precursor.**

The small multicopy plasmid ColE1 and its close relative pMB1 have been extensively studied as model systems for the investigation of control of initiation of DNA replication (1).

Two regions of the ColE1 genome carry information involved in the control of initiation of DNA replication and plasmid copy number. The first is located in a symmetrically transcribed region approximately 400 nucleotides upstream from the replication origin (2, 3). This region codes on one strand for part of the primer precursor and on the other strand for a small RNA of 108 nucleotides, known as RNA1 (Fig. 1). The larger RNA is processed by RNase H to yield a molecule of 555 nucleotides, which serves as a primer for the initiation of DNA replication *in vitro* (4). The smaller RNA has been shown to inhibit DNA replication by interfering with the processing of the primer by interacting with the complementary structure in the primer precursor (5–7).

The second *trans*-acting regulatory element was mapped by Twigg and Sherratt (8) to a region bounded by two *Hae* II restriction sites 806 and 184 nucleotides downstream from the replication origin of pMB1 and designated the *Hae* II fragment C region. This region is located between the replication origin and the mobilization genes of ColE1 and is not essential for plasmid replication. It is not known whether this second regulatory element participates in the same inhibitory circuit in which RNA1 is involved or whether it controls plasmid copy number by a different mechanism.

Here we show that the *Hae* II fragment C region encodes a *trans*-acting element that inhibits transcription initiated at the primer promoter. We have localized the element to a region that can code for a 63 amino acid polypeptide. The sequence of this polypeptide is conserved in the related plasmids ColE1 and pMB1. We present evidence that this inhibitor controls the initiation of DNA replication by decreasing the amount of primer available for initiation of DNA synthesis.

MATERIALS AND METHODS

Strains, Enzymes, and General Techniques. *Escherichia coli* K-12 71/18, Δ[*lac pro*], F'lac I^qZ ΔM15 pro^+, was the bacterial strain used as a host for the β-galactosidase fusion phages (9). λ phage 132 (10) was obtained from Barbara Meyer.

General microbiological techniques were essentially as described by Miller (11) or by Brenner *et al.* (12). Restriction enzymes and ligase were obtained from V. Pirrotta and H. Cambier. Radiochemicals were purchased from Amersham. Minicell preparation and labeling were done as described by Reeve (13).

Construction of β-Galactosidase–Promoter Fusion Phages. Plasmid DNA (10 μg) digested by restriction endonucleases (*Hae* III, *Alu* I, and *Hpa* II) was ligated to a 7-fold excess of *Hin*dIII linkers. *Hpa* II-digested DNA fragments were made blunt-ended by incubation with DNA polymerase I Klenow subunit (Boehringer Mannheim) in the presence of 0.1 mM dCTP and dGTP.

Restriction fragments from the replication region were purified on a polyacrylamide gel and ligated to *Hin*dIII-digested λ 132 DNA (1:1 molar ratio).

After *in vitro* packaging, phage were recovered by plating on strain 71/18 in 5 ml of 0.7% agarose in L broth. Plates containing 100–1,000 plaques were screened by plaque hybridization (14) using nick-translated pBR322 plasmid as a probe. Purified plaques were further checked by hybridization to different *Hpa* II fragments from the replication region.

The orientation of the DNA fragments inserted into the *Hin*dIII site of phage 132 was determined by recombination with plasmids in which the plasmid is inserted in a known orientation (15). Occasionally, clones harboring DNA fragments that were known to contain an active promoter were identified by directly plating on MacConkey plates and screening for red plaques.

Construction of Lysogens and Assay for β-Galactosidase. Lysogens of 71/18 harboring fusion prophages were constructed by low multiplicity infection of a bacterial culture and selection on plates containing approximately 10^8 λ phage 1052 (*h80 att80 imm21* cI^{ts}). Immune colonies were purified twice in the absence of the selective phage and at least three independent colonies per lysogenization were analyzed for β-galactosidase activity in order to avoid picking lysogens carrying

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Abbreviation: bp, base pair(s).

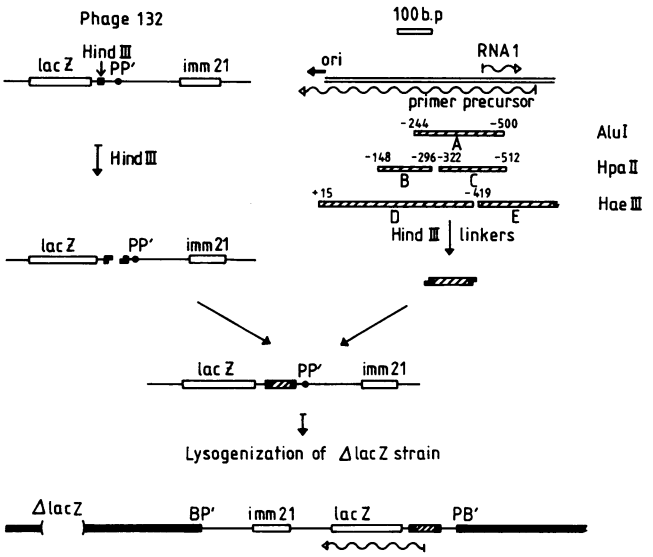


FIG. 1. Fusion of replication promoters to the β-galactosidase gene. The drawing shows a schematic representation of the strategy used to fuse restriction fragments that originate from the pMB1 replication origin to the β-galactosidase structural gene. The bars beneath the transcription map represent the restriction fragments that have been cloned in phage 132. Numbers above the bars refer to the distance in base pairs (bp) of the restriction sites from the replication origin. Phage (P, P') and bacterial (B, B') attachment sites are indicated. Wavy lines represent RNA transcripts.

multiple prophages. Derivatives harboring different plasmids were isolated after $CaCl_2$ treatment and transformation.

Quantitation of β-galactosidase synthesis was essentially as described by Miller (11).

Construction of pDL180. In order to delete the 180-nucleotide DNA fragment between the *Pvu* II and *Acc* I restriction sites immediately downstream from the replication origin, 1 μg of pBR322 DNA was partially digested with *Acc* I. After the ends generated by *Acc* I had been filled in, the plasmid DNA was digested to completion with *Pvu* II. The digested DNA was ligated at low concentration and used to transform 71/18 containing the fusion phage φBG34. The structure of those plasmids that were unable to repress β-galactosidase synthesis in this strain was determined by restriction endonuclease digestion.

DNA Sequence Analysis. The DNA sequence of the *Hae* II fragment C region in the ColE1 derivative pRSF2124 was obtained by the chemical degradation method of Maxam and Gilbert (16).

RESULTS

Phage 132 (Fig. 1) was constructed (10) to facilitate the identification of elements involved in transcriptional regulation. This vector includes a unique *Hin*dIII site preceding a functional β-galactosidase structural gene that lacks its natural promoter. When this vector is inserted into the bacterial chromosome, β-galactosidase expression is dependent on the activity of any upstream promoter sequence inserted at the *Hin*dIII site.

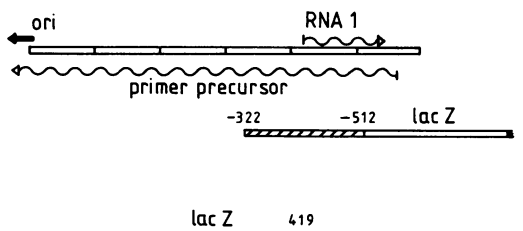
The advantages that a phage system offers for the study of promoter activities are particularly relevant when the promoters considered are involved in the control of plasmid replication functions. A phage system allows the integration of promoter–β-galactosidase fusions in single copy into the bacterial chromosome. As a consequence, promoter activity can be studied in the absence of possible interference by plasmid copy number effects or regulatory elements present in a plasmid vector.

Restriction fragments from the region essential for pMB1 plasmid replication (17) were inserted into the *Hin*dIII site of phage 132 to identify transcription signals that might be important for plasmid replication. Of all the DNA fragments tested, only fragments that contain the promoters for RNA1, P_{RNA1} (Fig. 1, fragments A and C), or for the RNA primer precursor, P_{primer} (Fig. 1, fragment E), were able to promote β-galactosidase synthesis, and they could do this only when they were inserted in the correct orientation.

Fig. 2 shows the relative strengths of the promoters in the replication region, measured as β-galactosidase activity in extracts of bacteria harboring a prophage with the relevant promoter fusion. The strength of the promoters measured *in vivo* is qualitatively consistent with the results obtained *in vitro* (4). The promoter for RNA1 is approximately 10 times stronger than the primer precursor promoter (compare lines A and B, column 1, Fig. 2).

To determine whether plasmid-encoded elements could control transcription starting from P_{RNA1} or P_{primer}, we measured β-galactosidase synthesis directed by these fusion phages in cells harboring the plasmids pMB3 or pBR322. pMB3 is a pMB1 derivative containing the Tn3 transposon (18).

β-Galactosidase synthesis directed by P_{RNA1} is not influenced by a resident pMB1 plasmid derivative (Fig. 2, line A, columns 1, 2, and 3). By contrast, β-galactosidase expression in fusion strains that contain the primer promoter is inhibited by a plasmid-encoded, *trans*-acting element specified both by pMB3 and by its smaller derivative pBR322 (Fig. 2, line B, columns 1, 2, and 3). When the transcriptional activity provided by the



FUSION		β galactosidase activity: no plasmid 1	+pMB3 2	+pBR322 3
φ BG 39	A	450±60	420±60	420±60
φ BG 34	B	65±10	27±5	25±5
132	C	23±5	20±5	20±5

FIG. 2. β-Galactosidase activity in bacterial strains lysogenized with different fusion prophages. β-Galactosidase units are defined as in Miller (11). Each activity is the mean of at least three measurements that did not differ by more than 15%.

Table 1. β-Galactosidase activities in lysogens

Plasmid present	Ref.	Replication origin	β-Galactosidase activity: φ132, no promoter	φBG34, RNA primer pMB1	φBG29, RNA primer ColE1
1. None			23	65	73
2. pBR322	19	pMB1	24	29	27
3. pAT153	8	pMB1		72	71
4. pDL180	This work	pMB1		46	
5. pac162	7	pMB1		34	
6. pac162 *svir11*	7	pMB1		30	
7. pCR1	20	ColE1		24	30
8. pNOP42II	3	ColE1		60	70
9. pDS4156	8	ColK		42	45
10. pDS4152	8	ColK		63	74
11. pJN59	21	CloDF13		45	
12. pNOP1	3	ColE1		26	
13. pUC8	J. Messing	pMB1		68	
14. pUC8-HpaII$_{309}$	This work	pMB1		28	

Lysogens containing no insert promoter (φ132) or the primer promoter from either pMB1 (φBG34) or ColE1 (φBG29) were transformed with a variety of plasmids containing replication origins as listed in the third column. Relevant genetic features of the residing plasmids are described in the text. The data are units of β-galactosidase activity as described by Miller (11) and are the means of at least three measurements that did not differ by more than 15%.

prophage above is considered (Fig. 2, line C), it is apparent that the β-galactosidase activity directed by the primer promoter in the presence of the inhibitor is not significantly above background levels.

Genetic Mapping of the *rop* Gene. The plasmid pac162 *svir11* contains the replication region of pMB1 with a single base pair alteration in the RNA1 coding sequence that reduces the ability of RNA1 to interact with the wild-type target site (7). However, pac162 *svir11* is capable of inhibiting β-galactosidase expression directed by the primer promoter (Table 1, compare lines 1, 5, and 6). Therefore, either RNA1 has two separate functions that can be independently modified or, alternatively, this molecule is not involved in controlling primer transcription. The plasmid pAT153 (8) is a pBR322 derivative in which the DNA fragment corresponding to the ColE1 *Hae* II fragment C has been deleted (Fig. 3). pAT153 does not code for the inhibitory activity (Table 1, line 3). This function can be restored by the insertion of a 576-bp *Sau*3A/*Acc* I fragment located downstream from the replication origin of pMB1 (Fig. 3). Thus, all the information essential for the inhibitory activity must be contained in this 576-bp DNA fragment. The gene that encodes the *trans*-acting negative regulator has been designated *rop* for repressor of primer.

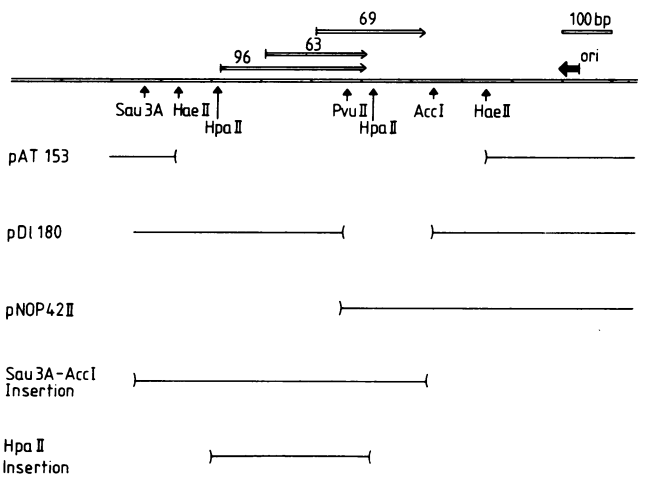


FIG. 3. Translation map of the region approximately 600 bp downstream from the replication origin in the plasmid pBR322 (19). Horizontal arrows represent open reading frames. Figures above the arrows are the lengths of the polypeptides (in amino acids) that could be coded for by the open reading frames. Beneath the translation map the limits of the DNA deletions and insertions that affect *rop* activity are marked by curved vertical lines.

Comparison of *rop* Activity in Different Plasmids. To investigate whether the ColE1 primer promoter was a target for the *rop* function, a phage was constructed that contained the β-galactosidase structural gene under the control of this promoter. This phage, φBG29, contains a *Hae* III fragment from nucleotides 420–834 upstream from the ColE1 origin inserted into the *Hin*dIII site of phage 132 (22). Like φBG34, φBG29 contains the primer promoter but not the RNA1 promoter. Various plasmids were tested for their ability to provide *rop* gene activity as measured by their effects on β-galactosidase synthesis in strains carrying either φBG34 or φBG29 as lysogens (Table 1). It is clear that the primer promoter from ColE1 does not differ appreciably from that of pMB1 in its sensitivity to the *rop* gene activity provided by either ColE1- or pMB1-derived plasmids (Table 1, lines 2 and 7). This result is not unexpected because the DNA sequence of the two promoters is highly conserved (19, 22).

Twigg and Sherratt (8) reported that the ColE1-related plasmid ColK could reduce the copy number of ColE1 derivatives from which the repressor encoded in the ColE1 *Hae* IIC region has been deleted. The ability of ColK to complement the ColE1 deletions was mapped to the vicinity of the ColK *Hae* II B fragment. We measured the *rop* gene activity derived from ColK derivatives that contain or lack the *Hae* IIB region. pDS4156 contains the *Hae* IIB region and shows weak but detectable *rop* activity on both ColE1 and pMB1 primer promoters (Table 1, line 9). pDS4152 has a deletion of the ColK *Hae* II fragment B and lacks *rop* activity (Table 1, line 10). The ColK *rop* gene is less active on ColE1 and pMB1 targets than the *rop* activity from the homologous plasmids (Table 1, compare lines 2 and 7 with line 9). Similarly, the multicopy plasmid CloDF 13 also encodes an inhibitor that is slightly active on the pMB1 target (Table 1, line 11).

Comparison of the DNA Sequence of the *rop* Genes in ColE1 and pMB1. A deletion of ColE1 sequences to the left of the *Pvu* II site in the *Hae* II C fragment (pNOP42II in Fig. 3) abolishes *rop* activity (Table 1, line 8). This result confirms the fact that the ColE1 *rop* gene maps in a position equivalent to that of the pMB1 *rop* gene, as expected (8). Inspection of the pMB1 DNA sequence (19) in the vicinity of the *Pvu* II site reveals the presence of two overlapping open reading frames. The first could encode two polypeptides of 96 and 63 amino acids, depending on which of two possible start codons is utilized (Fig. 4). The 96-amino acid polypeptide would initiate from an ATG codon at position 1,817 in pBR322. The shorter polypeptide would be translated starting from a GTG initiation codon that is preceded by a potentially active ribosome-binding site G-G-A-G-G [boxed in Fig. 4 (23)]. The second open reading frame could encode a very basic polypeptide of 69 amino acids starting at an ATG codon at position 2,010 of pBR322 (Fig. 4).

To investigate whether the amino acid sequences of these three potential polypeptides were conserved in ColE1, we partially determined the sequence of the *Hae* II C fragment of ColE1. The ColE1 plasmid whose sequence was determined was derived from pRSF2124 (24), which contains the Tn3 transposon inserted downstream from the replication origin. Sequence analysis revealed that in pRSF2124 the Tn3 transposon integrated into the ColE1 genome at a position corresponding to that between nucleotides 1,867 and 1,868 of pBR322. This position is 667 bp downstream from the ColE1 origin of replication and interrupts the potential 96-amino acid open reading frame of ColE1 (Fig. 4). However, pNOP1, a pRSF2124 derivative that contains Tn3 inserted in the same position of ColE1 DNA, displays full *rop* gene activity (Table 1, line 12). Consequently, the 96-amino acid open reading frame cannot encode the *rop* gene product.

In Fig. 4 the differences between the pMB1 and ColE1 sequences in the region encoding the 69- and 63-amino acid polypeptides are shown. Three single base deletions and 19 substitutions are present in the ColE1 sequence corresponding to the pMB1 fragment that encodes these polypeptides. Strikingly, each of the 11 substitutions in the sequence that encodes the 63 amino acid polypeptide are third position changes that do not alter the amino acid sequence. By contrast, the 12 substitutions in the potential gene encoding the 69-amino acid polypeptide cause 8 changes in the amino acid sequence. These considerations suggest that the 63-amino acid polypeptide is the more likely candidate for the *rop* function in pMB1 and ColE1.

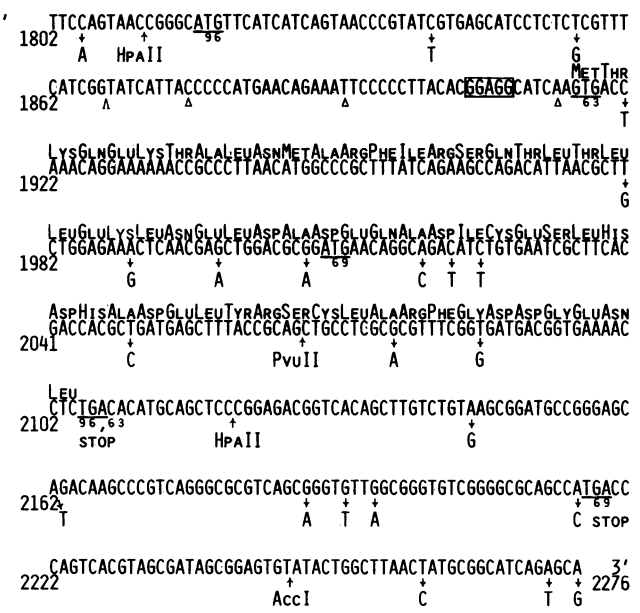


FIG. 4. Sequence of pBR322 and ColE1 DNAs in the *rop* gene region. Sequence changes in ColE1 in this region relative to pBR322 DNA are shown below the position in ColE1. Single-base deletions not found in ColE1 DNA are denoted by Δ. The location of the Tn3 insertion in pRSF2124 and its derivatives is denoted by Λ. The initiation and termination codons for the 63, 69, and 96 amino acid open reading frames are underlined. The locations of relevant restriction sites are noted. A potential ribosome-binding sequence immediately upstream from the GTG initiation codon of the 63 amino acid polypeptides is boxed.

Identification of the *rop* Gene Product. To assign the *rop* gene activity to a polypeptide encoded in the region immediately downstream from the replication origin, we examined polypeptides synthesized in minicells containing plasmids whose *rop* activity had been measured (Table 1). Minicells containing pBR322 synthesize a prominent polypeptide migrating with an approximate molecular weight of 6,500 (Fig. 5, lane 3, band C). In the absence of other information, however, the resolution of the gel system does not permit discrimination between polypeptides of 63 and 69 amino acids.

The plasmid pDL180 is a deletion derivative of pBR322 lacking the 180-bp *Pvu* II/*Acc* I fragment in the *rop* region (Fig. 3). This deletion removes most of the 69-amino acid encoding sequence but only 13 amino acids from the COOH terminus of the 96/63-amino acid encoding sequence (Fig. 4). In minicells pDL180 encodes a M_r 14,000 band (labeled A, Fig. 5, lane 2) not seen in minicells containing pBR322 (compare lanes 2 and 3, Fig. 5). Inspection of the DNA sequence of pDL180 indicated that an open reading frame of 128 amino acids is generated, resulting from a fusion of the 63-amino acid start codon with in-frame sequences terminating at a TGA codon at position +55 from the replication origin. The expected fusion polypeptide originating from the 63-amino acid start codon corresponds in size to the observed band A in Fig. 5, lane 2. The fusion polypeptide retained 50 amino acids from the NH_2 terminus of the 63-amino acid polypeptide. When tested for *rop* gene activity, pDL180 showed about half the activity of pBR322, suggesting that the fusion polypeptide retains substantial *rop* activity (Table 1, line 4). These results are consistent with the 63-amino acid polypeptide being the *rop* gene product and argue against a role for the 69-amino acid polypeptide.

Greater insight into the relationship between the 69- and 63-amino acid polypeptides and the *rop* gene product was provided by the following cloning experiment. The plasmid pUC8 is a 2,900-bp ampicillin-resistant pBR322 derivative that lacks *rop* activity (Table 1, line 13). Moreover, pUC8 does not direct syn-

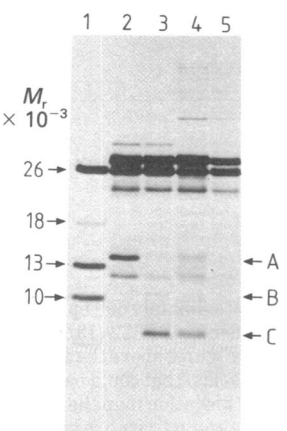


FIG. 5. Analysis of plasmid-encoded polypeptides in minicells. Minicells were isolated and labeled with [^{35}S]methionine as described by Reeve (13). Extracts were analyzed on a sodium dodecyl sulfate/20% polyacrylamide gel and autoradiographed. Lane 1, marker polypeptides consisting of labeled concanavalin A species (Amersham). Corresponding molecular weights are shown at left. Lanes 2–5, minicell extracts from pDL180 (lane 2), pBR322 (lane 3), pUC8-*Hpa*II$_{309}$ (lane 4), and pUC8 (lane 5). A, B, and C refer to the position of the fusion polypeptide from pDL180 and the expected positions of the 96-amino acid polypeptide and the 63-amino acid polypeptide, respectively.

thesis of a M_r 6,500 polypeptide in minicells (Fig. 5, lane 5). We constructed a derivative of pUC8 that contained the 309-bp *Hpa* II fragment between nucleotides 1,811 and 2,120 of pBR322 (shown schematically in Fig. 3; sequence shown in Fig. 4). This *Hpa* II fragment contains the 96- and 63-amino acid reading frames but not the 69-amino acid reading frame. pUC8-*Hpa*II$_{309}$ provides the *rop* gene activity when tested with the pMB1 primer promoter *in vivo* (Table 1, line 14). In minicells, this pUC8 derivative synthesizes a prominent polypeptide that comigrates with that synthesized from pBR322 (Fig. 5, lane 4, band C). Because this derivative cannot synthesize a 69-amino acid polypeptide we conclude that the M_r 6,500 product is 63 amino acids in length and is the product of the *rop* gene.

DISCUSSION

The activity of the promoters for RNA1 and the RNA primer precursor in the plasmids pMB1 and ColE1 were studied by fusion to a β-galactosidase gene with its own promoter deleted. β-Galactosidase, synthesized under the control of the RNA1 promoter, is not affected by any plasmid gene product.

By contrast, we have identified a gene (*rop*) that encodes an inhibitor of RNA primer transcription. When a plasmid carrying an intact *rop* gene was present in cells in which β-galactosidase was expressed under the control of the primer promoter, β-galactosidase synthesis was reduced to background levels.

The *rop* gene is located approximately 600 nucleotides downstream from the replication origin. Twigg and Sherratt (8) have shown that ColE1 and pMB1 plasmids deleted for this region show an increase in copy number. In addition, they showed that the copy number of these plasmids could be returned to wild-type levels by derivatives of the related plasmid ColK, which provides the deleted function in *trans*. We have tested these ColK derivatives for their *rop* activity on the primer promoters of pMB1 and ColE1 and concluded that ColK codes for an inhibitor that is active on the two promoters. The correlation between *rop* activity and control of copy number suggests the involvement of the *rop* gene product in the control of plasmid replication.

This mechanism of control of initiation of DNA replication is not dependent on RNA1, because we have shown that mutations in RNA1 do not affect the functioning of the *rop* gene. Therefore, it is likely that the *rop* control system utilizes a regulatory mechanism independent from that previously characterized (5–7).

It is not clear why ColE1 has two apparently different mechanisms to control the initiation of DNA synthesis. The localization of the *rop* gene in the region essential for plasmid mobilization may mean that the *rop* gene product has a role in transfer replication, possibly through repression of vegetative replication during the conjugation process. Alternatively, replication control in ColE1 may result from the cumulative effects of RNA1 and the *rop* gene product acting to inhibit functional primer formation at two distinct levels: RNA1 affecting primer maturation by interfering with RNase H processing, and the *rop* gene product affecting the frequency of primer transcriptional initiation.

DNA sequence analysis suggests that the *rop* gene product is a 63-amino acid polypeptide whose predicted sequence is entirely conserved in ColE1 and pMB1. The isolation of mutants in this region will be necessary to confirm this prediction.

That the *rop* gene is not involved in plasmid incompatibility is indicated by the fact that plasmids compatible with ColE1 such as ColK specify a *rop* function. The increased copy number of mutants with the *Hae* II C region deleted (8) suggests that the *rop* gene is involved in the control of initiation of DNA replication. The results presented here suggest that the *rop* gene product may modulate plasmid replication by limiting the amount of primer precursor available for RNase H processing.

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