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rpoZ, Encoding the Omega Subunit of *Escherichia coli* RNA Polymerase, Is in the Same Operon as *spoT*

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Highly purified *Escherichia coli* RNA polymerase contains a small subunit termed omega. This subunit consists of 91 amino acids with a molecular weight of 10,105. We previously reported the cloning and sequencing of the gene encoding omega, which we call *rpoZ* (D. R. Gentry and R. R. Burgess, Gene 48:33–40, 1986). We constructed an *rpoZ* insertion mutation by placing a kanamycin resistance cassette into the coding region of the *rpoZ* gene. Purified RNA polymerase from strains carrying this mutation lacked detectable omega. We found that the insertion mutation conferred a slow-growth phenotype when introduced into most strains. We mapped the position of *rpoZ* on the *E. coli* chromosome by genetic techniques and by examining the restriction map of the whole chromosome and found that *rpoZ* maps around 82 min, very close to *spoT*. We determined that the slow-growth phenotype of the insertion mutant is suppressed in *relA* mutants and that the *rpoZ* insertion results in a classical SpoT$^-$ phenotype. This finding strongly suggests that *rpoZ* is upstream of *spoT* in the same operon and that the slow-growth phenotype elicited by the insertion mutation is due to polarity on *spoT*.

It is widely accepted that *Escherichia coli* RNA polymerase contains the beta, beta-prime, and two alpha subunits (which together constitute core polymerase) as well as one of at least three sigma factors (sigma-70, sigma-32, and sigma-54) which determine promoter specificity (5). It is likely that different sigma factors with additional promoter specificities will be discovered, and still other proteins have been found associated with core and holoenzyme (core polymerase plus a sigma factor). With the exception of the NusA protein, the function of these proteins in transcription, if any, has yet to be determined. One of these proteins is the omega subunit. Whereas many of the proteins reported to bind polymerase were identified by using less stringent forms of purification and apparently do not bind polymerase tightly (11, 13, 14), omega is present in highly purified core polymerase and holoenzyme isolated by standard procedures (4). The likelihood of omega being a mere contaminant is, in our opinion, highly unlikely. However, a physiological function for omega remains to be found.

We previously reported the cloning and sequencing of the gene encoding the omega subunit, which we named *rpoZ* (12). *rpoZ* encodes a protein of 10,105 daltons. We determined that two promoters are located just upstream of the coding region of *rpoZ*. We did not determine the position of the 3′ end of the *rpoZ* transcript. In this paper, we report the construction of an insertion mutation in *rpoZ* as well as evidence that *rpoZ* is in the same operon as *spoT* (a gene whose product is involved in regulation of the stringent response).

When bacterial cells are subjected to amino acid starvation, transcription of genes involved in translational machinery is greatly decreased (23). This effect, termed the stringent response, is mediated by the peculiar nucleotides guanosine 5′-triphosphate 3′-diphosphate and guanosine 5′-diphosphate 3′-diphosphate [abbreviated (p)ppGpp] (6). (p)ppGpp is synthesized by the ribosome-associated product of the *relA* gene after amino acid starvation in response to uncharged tRNA. An inverse correlation exists between the cellular level of (p)ppGpp and growth rate (18). An increase in (p)ppGpp levels also occurs during energy deprivation by a *relA*-independent pathway (8). *spoT* was initially identified as the gene locus responsible for the inability to form pppGpp after amino acid deprivation as well as an impaired ability to degrade ppGpp (17). It has since been determined that the *spoT* gene encodes an 80,000-dalton pyrophosphatase which degrades both ppGpp and pppGpp (1). The behavior of *spoT* mutants indicates that *spoT* is intimately involved in regulation of the response to both amino acid and energy deprivation (8).

MATERIALS AND METHODS

Bacterial strains, growth conditions, and bacteriological techniques. Table 1 lists the sources, genotypes, and derivations of strains used.

For ppGpp assays, cells were grown in minimal MOPS (morpholinepropanesulfonic acid) medium as described elsewhere (22) except that the P_i concentration was lowered to 0.2 mM as suggested by Bochner and Ames (3). $^{32}P_i$ (Dupont, NEN Research Products, Boston, Mass.) was added at a concentration of 50 μCi/ml at least three doublings before the cultures reached an optical density at 600 nm of 0.2 to 0.3.

P1 transductions were performed as described by Miller (21). We had difficulty obtaining P1 lysates from strains derived from CAG732, presumably because the *recBC* background of this strain. We found that introducing a plasmid carrying *recBC* (9; kindly provided by Graham Walker) allowed us to obtain a suitable P1 lysate for transduction experiments.

For determination of doubling times, cells were grown both in MOPS medium (22) supplemented with 20 amino acids (40 μg/ml), thiamine (1 μg/ml), and 0.4% glucose and in LB medium (21). Where appropriate, antibiotics were added to the following concentrations: kanamycin, 50 μg/ml; streptomycin, 100 μg/ml; chloramphenicol, 20 μg/ml; and tetracycline, 15 μg/ml.

Biochemical techniques. ppGpp levels were determined by analysis of cell extracts, using polyethyleneimine-cellulose

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TABLE 1. Bacterial strains used

Strain	Genotype	Source[a]
CAG732	*recB21 recC22 sbc-15 thr leu thiA lacY ara xyl mtl pro his arg rpsL tsx supE37*	C. Gross
MG1655	F^-	C. Gross
W3350A	*galK galT rpsL* Lac^-	W. Dove
CAG1690	*thi-1 argE his-4 proA strA31 thr leu lacY mtl xyl ara galK*	C. Gross
CAG1691	CAG1690, *relA*	C. Gross
CAG5051	HfrH, *nad*::Tn*10 thi-1 spoT supQ80*	C. Gross
CAG5052	KL227, *btu*::Tn*10 metB1 relA*	C. Gross
CAG5053	KL208, *zbc*::Tn*10 relA*	C. Gross
CAG5054	KL96, *trp*::Tn*10 thi-1 relA*	C. Gross
CAG5055	KL16, *zed*::Tn*10 thi-1 relA*	C. Gross
CAG18385	*gltC10 metB thi lac rpsL zia-204*::Tn*10*	C. Gross
CAG18346	*trpA zdc-203*::Tn*10 zdd-230*::Tn*9 his-85 thyA714 ilv-632 deo-70 pro-48 arg-59 tsx-84 rac trpR55*	C. Gross
CAG18177	*metA btu*::Tn*10*	C. Gross
CSH7	*lacY strA thi*	CSH[b]
CSH64	KL14, *thi*	CSH
DG100	CSH64, *zia-204*::*Tn*10	CSH64 × CAG18385; selection for Tet^r
DG101	CSH64, *btu*::*Tn*10	CSH64 × CAG18177; selection for Tet^r
DG1	CAG732, *rpoZ*::*kan*	This work
DG2	W3350A, *rpoZ*::*kan*	W3350A × DG1; selection for Kan^r
DG5	CSH7, *rpoZ*::*kan*	CSH7 × DG2; selection for Kan^r
DG5C	DG5, *zdd*::Tn*9*	DG5 × CAG18346; selection for Cm^r; scoring of Tet^s
DG6	MG1655, *rpoZ*::*kan*	MG1655 × DG5; selection for Kan^r
DG8	CAG1690, *rpoZ*::*kan*	CAG1690 × DG5; selection for Kan^r
DG9	CAG1691, *rpoZ*::*kan*	CAG1691 × DG5; selection for Kan^r

[a] All crosses were performed by P1 transduction.
[b] CSH, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

thin-layer chromatography as described by Cashel et al. (7). Cells were extracted as described by Bochner and Ames (3). Spots corresponding to ppGpp were localized by exposing the developed polyethyleneimine sheets to X-ray film, cut out, and counted in a scintillation counter.

Hfr mapping. The Hfr mating procedure and most of the strains we used were provided by Mitch Singer, in the laboratory of Carol Gross. We used a set of Hfrs containing Tn*10* insertions at known positions 20 to 30 min from the origin of each Hfr (Fig. 1). The strategy was to mate the Hfr to our insertion mutant (see below) and to score tetracycline-resistant transconjugants for loss of the insertion. A marker lying between the origin of transfer and the selected marker should recombine at a rate of between 50 and 60% except near the selected marker (where the frequency would be higher) or near the origin (where it would be lower) (19). A 0.5-ml sample of Hfr cells grown to early log phase in LB medium was mixed with 0.5 ml of a fresh overnight culture of the recipient. This mixture was spotted on a membrane filter (Millipore Corp., Bedford, Mass.), washed two times with 5 ml of M9 (21) salts, placed on a prewarmed LB plate, and incubated for 30 min at 37°C. The filters were then placed in 2 ml of M9 salts, vortexed vigorously for 2 min, and spread on selective plates. The matings involved DG100 and DG101 used DG5C as a recipient and were spread on tetracycline-chloramphenicol LB plates. All other matings used DG5 as a recipient and were spread on tetracycline-streptomycin LB plates. Transconjugants were purified once on selective plates and then scored for kanamycin resistance.

RESULTS

Construction of an *rpoZ* insertion mutation. We constructed an insertion in *rpoZ* by using the scheme outlined in Fig. 2. *rpoZ* is located on the 3-kilobase (kb) *Eco*RI-*Sal*I insert in pDRG4 and is further localized on a 1.1-kb *Eco*RV fragment within that insert. An approximately 1.5-kb *Bam*HI fragment from plasmid pUC4k (Pharmacia Fine Chemicals, Piscataway, N.J.), which carries a gene encoding kanamycin resistance, was inserted into the *Bam*HI site located in *rpoZ*. The resulting plasmid (pDRG4::kan) was linearized with *Eco*RI and used to transform CAG732 (*recBC sbc*) to kana-

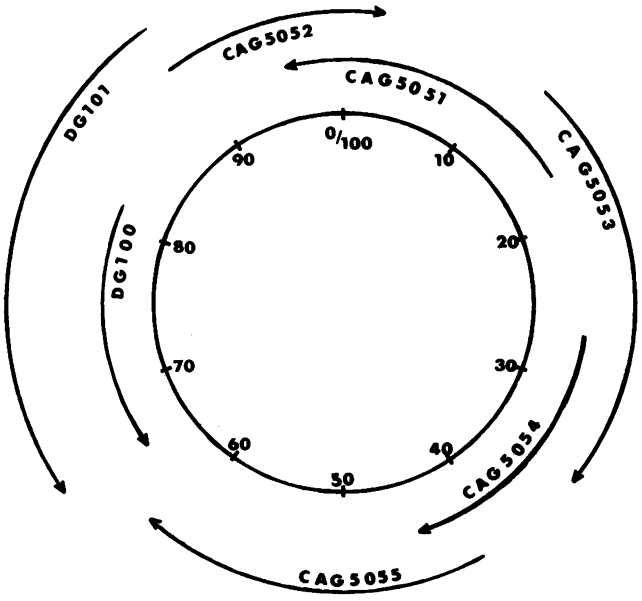

FIG. 1. Hfr strains used in mapping *rpoZ*. The 100-min genetic map of *E. coli* is represented by the complete inner circle. Each arrow represents an Hfr strain, arrowheads indicate the origins of transfer, and the ends indicate the positions of the Tn*10* insertions.

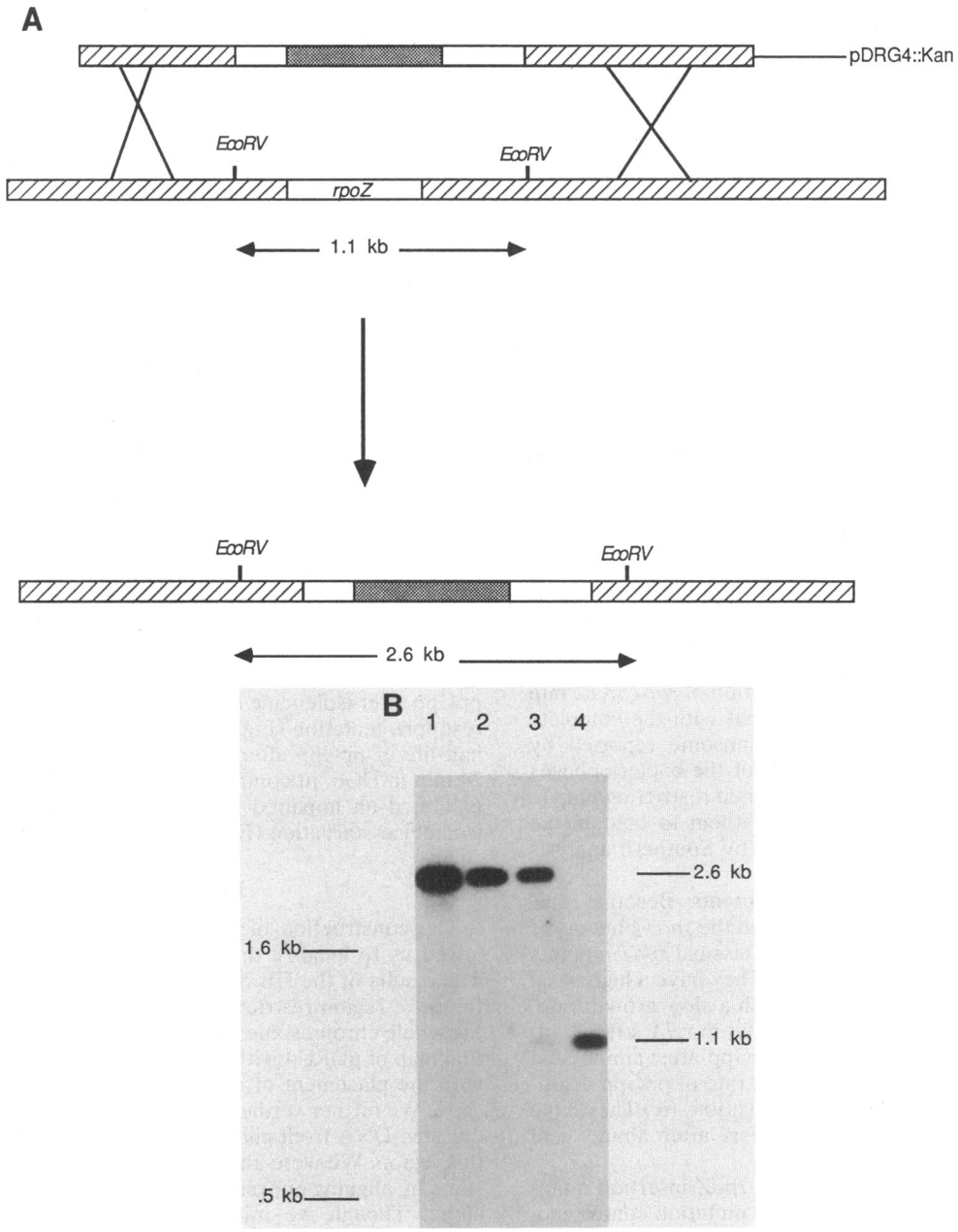

FIG. 2. Construction of an *rpoZ* insertion mutation. (A) Crossing linear pDRG4::kan onto the chromosome. pDRG4::kan was made as described in the text. Insertion of the kanamycin resistance cassette, which has no *Eco*RV site, converts a 1.1-kb *rpoZ*-containing *Eco*RV fragment into a 2.6-kb fragment. Symbols: ▭, kanamycin cassette; ▩, *rpoZ*; ▨, *E. coli* DNA. (B) Southern analysis of insertion mutants. Genomic DNA from *rpoZ*$^+$ and candidate *rpoZ* insertion mutants were digested with *Eco*RV, fractionated on a 0.8% agarose gel, blotted onto nitrocellulose, and probed with the nick-translated *Eco*RV fragment from pDRG4 as described by Maniatis et al. (20). Lanes: 1 to 3, *Eco*RV-digested genomic DNA from three candidate insertion mutants; 4, *Eco*RV-digested CAG732 genomic DNA. Positions of markers are indicated on the left; estimated sizes of *rpoZ*-specific fragments are indicated on the right.

mycin resistance. The *recBC sbc* strain was used to increase the efficiency of recombination of the introduced linear DNA (24). Insertion of the kanamycin cassette into *rpoZ* was verified by Southern analysis, with the *Eco*RV fragment from pDRG4 used as a probe. The transformation yielded eight colonies, all of which contained the correct insertion. Figure 2 shows the altered *Eco*RV fragment in three of these eight isolates. The insertion was moved from one isolate, DG1, into strain W3350A to make DG2. RNA polymerase isolated from DG2 contains no full-length omega as determined by Coomassie-stained 15% polyacrylamide-sodium dodecyl sulfate gels or by Western blot (immunoblot) analysis using polyclonal antibodies raised against omega (data not shown).

Hfr mapping. The results of the Hfr matings are shown in Table 2. The first set of matings performed were those using strains CAG5051 to CAG5055. The low but reproducible percentage of Kan^s recombinants obtained by using CAG5052 as a donor suggested that *rpoZ* was probably distal to the Tn*10* insertion in that strain. We constructed DG100

TABLE 2. Results of Hfr matings

Hfr[a]	Recipient	Kan^s/total	Selection[b]
CAG5051	DG5	0/50	Tet-Strep
CAG5052	DG5	7/50	Tet-Strep
CAG5053	DG5	0/50	Tet-Strep
CAG5054	DG5	0/50	Tet-Strep
CAG5055	DG5	0/50	Tet-Strep
DG100	DG5C	49/55	Tet-Cm
DG101	DG5C	30/47	Tet-Cm

[a] See Fig. 1 and Table 1 for position of origin of transfer and site of Tn*10* insertion.
[b] Tet, Tetracycline; Strep, streptomycin; Cm, chloramphenicol. See Materials and Methods for concentrations.

TABLE 3. Effect of *relA* allele on growth of *rpoZ::kan* mutants

Strain	Genotype[a]	Doubling time (min) after growth on[b]:	
		LB	Glucose + amino acids
CAG1690	*relA*$^+$ *rpoZ*$^+$	30	41
DG8	*relA*$^+$ *rpoZ::kan*	48	52
CAG1691	*relA rpoZ*$^+$	29	47
DG9	*relA rpoZ::kan*	29	45

[a] See Table 1 for complete genotype.
[b] See Materials and Methods for compositions of media.

and DG101 to narrow the region further. The very high percentage of Kan^s recombinants resulting from the mating with DG100 indicated that *rpoZ* was probably within a few minutes of *zia-204* (81 min) (2).

We searched the literature for genes in the region of 81 to 82 min and found that the restriction map of the region around *pyrE* (15) matched the restriction map of our original phage clone and that a plasmid found to complement *spoT* should also contain *rpoZ* (1). Figure 3 shows an alignment of pDRG4 with part of the plasmid that was shown to complement *spoT*, pGA1. The region that complemented *spoT* lay directly downstream of *rpoZ*. The position of *rpoZ* at 82 min on the *E. coli* genetic map is consistent with the complete restriction map of the *E. coli* chromosome reported by Kohara et al. (16). We obtained two of the bacteriophages that were used to determine the published restriction map in this region, 7F3 and 2A6, and found them to contain the 1.1-kb *Eco*RV *rpoZ*-specific fragment by Southern analysis (data not shown).

***spoT* phenotype of *rpoZ* insertion mutants.** Because *rpoZ* maps very close to *spoT*, we determined the *spoT* phenotype conferred by the insertion mutation. Classical *spoT* mutants have the following characteristics. (i) They have a high basal level of ppGpp which is correlated with a slow growth rate. The slow growth rate can be suppressed in *relA* strains. (ii) They show a high induced level of ppGpp after amino acid starvation. (iii) They have an impaired rate of ppGpp degradation after relief from amino acid starvation. (iv) They have a diminished rate of pppGpp synthesis after amino acid starvation (10).

Early in our characterization of the *rpoZ* insertion mutation, we found that in most strains the mutation conferred a lower growth rate than was found in isogenic *rpoZ*$^+$ strains. This growth defect could not be suppressed by supplying a plasmid containing only the *rpoZ*-bearing *Eco*RV fragment. Most of the strains that grew normally with the insertion mutation had known *relA* mutations. We directly tested the possibility that the growth defect could be suppressed in *relA* backgrounds by putting the insertion mutation into isogenic *relA*$^+$ and *relA* strains. Strain DG9 (*relA rpoZ::kan*) grew with about the same doubling time as did the *rpoZ*$^+$ strains, CAG1690 and CAG1691, whereas DG8 (*rel*$^+$ *rpoZ::kan*) grew with a doubling time 11 to 18 min longer than that of its *rpoZ* parent, depending on the culture medium (Table 3).

We monitored the levels of ppGpp accumulation after starvation for isoleucine and recovery in both DG6 and its wild-type parent, MG1655. DG6 exhibited both a higher burst of ppGpp synthesis and a slower rate of decay of ppGpp after isoleucine starvation and subsequent isoleucine resupplementation (Fig. 4). From Fig. 4B, we calculate the half-life of ppGpp after isoleucine resupplementation to be 24 min in DG6, in contrast to 0.7 min in MG1655. DG6 also exhibited an impaired ability to accumulate pppGpp after isoleucine starvation (Fig. 5).

DISCUSSION

The construction of an *rpoZ* insertion mutation has allowed us to localize the gene on the *E. coli* chromosome. The results of the Hfr mating experiments, the alignment of the *rpoZ* region restriction map with the restriction map of the whole chromosome (16), and the alignment of the restriction map of pDRG4 with that of pGA1 (Fig. 3) are consistent with the placement of *rpoZ* at about 82 min, very close to *spoT*. We further verified this conclusion by probing *Eco*RV-digested DNA from phage carrying inserts shown to map in this region. We were unable to localize *rpoZ* on the chromosome by aligning our restriction map with the published map alone. Though we initially singled out this region as a possible position for *rpoZ*, there were some inconsistencies between our map and that of Kohara et al. (16). First, the *Pvu*II and *Bam*HI digestion patterns reported for this region

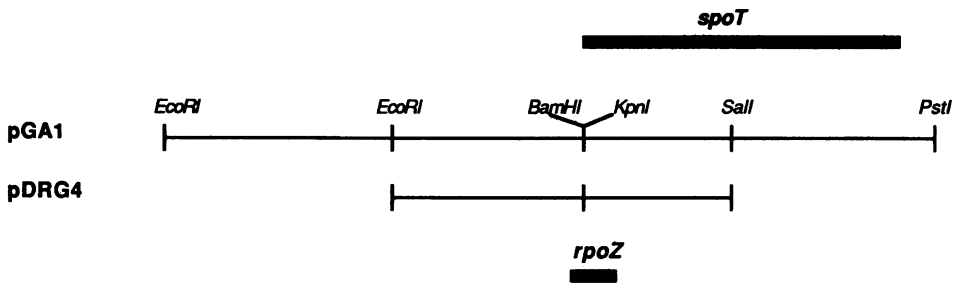

FIG. 3. Alignment of the *rpoZ* gene region with part of a *spoT* complementing plasmid. Plasmid pGA1 was reported by An et al. (1) to complement *spoT* mutations. The bar marked *spoT* represents the region identified in pGA1 responsible for complementation. pDRG4 contains *rpoZ*, whose sequence is found in the region indicated by the bar labeled *rpoZ*.

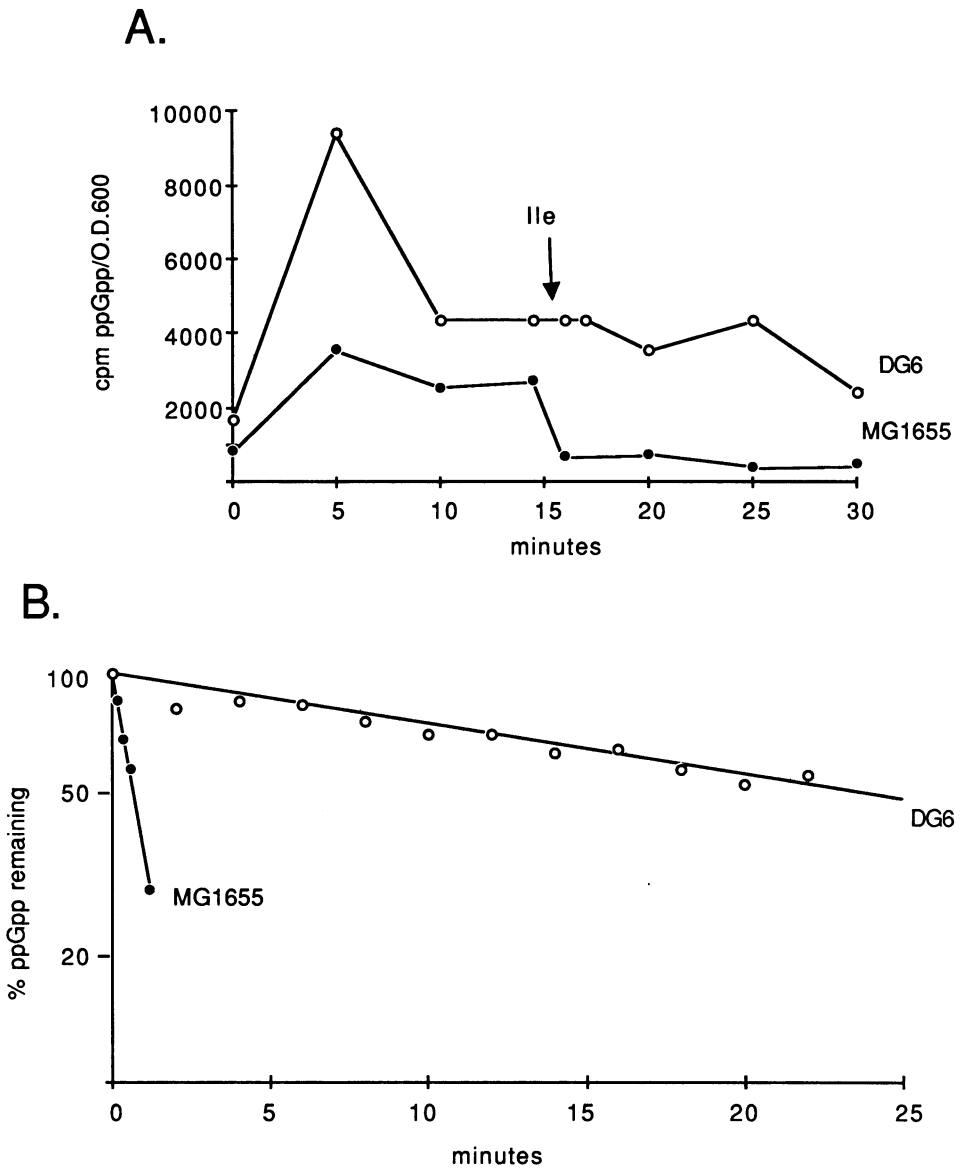

FIG. 4. ppGpp levels after induction of the stringent response. (A) Accumulation and decay of ppGpp during isoleucine starvation and resupplementation. Cultures of MG1655 (wild type; ●) and DG6 (*rpoZ*::kan; ○) were labeled with $^{32}P_i$ as described in the text. Isoleucine starvation was induced at $t = 0$ by the addition of valine to 0.5 mg/ml. Portions (0.1 ml) were removed from the culture at the indicated times and extracted with formic acid as described by Bochner and Ames (3). At $t = 15$ min, (arrow), isoleucine (0.5 mg/ml) was added to the cultures. Portions (10 μl) of the formic acid extracts were subjected to polyethyleneimine-cellulose thin-layer chromatography. Counts per minute incorporated into ppGpp were measured as described in the text. (B) Exponential decay of ppGpp after isoleucine resupplementation. Cultures of MG1655 (●) and DG6 (○) were grown as described above. Isoleucine starvation was induced for 15 min, and ppGpp levels were measured from extracts taken at various intervals after the addition of isoleucine. ppGpp levels from each extract were measured from 10-μl samples chromatographed in triplicate. Levels are plotted as the percentage of counts corresponding to ppGpp at $t = 0$.

are inconsistent with ours. Second, Kohara et al. were unable to get an unambiguous restriction pattern for *Eco*RV in this region. It is interesting to note that, upon close inspection, the *Bam*HI site reported to be around position 3880 appears to be a printing artifact rather than an intentional mark. Omission of this site would result in our *Bam*HI restriction map aligning perfectly with the published map.

The placement of *rpoZ* just upstream of *spoT* suggested to us an immediate explanation for the slow-growth phenotype exerted by our insertion mutation. Mutations in *spoT* cause an increased basal level of ppGpp in the mutant cell. This increased level of ppGpp is strongly correlated with a decrease in growth rate. Because mutations in *relA* decrease basal levels of ppGpp, a *relA spoT* double mutant grows better than a single isogenic *spoT* mutant (17). We have shown this relationship to be true for *rpoZ*::*kan* and *relA rpoZ*::*kan* double mutants as well. This relationship has also been determined independently by Michael Cashel, using an identical construction (M. Cashel, personal communication; see below). Further, our insertion mutation confers defects in ppGpp metabolism which are typical of *spoT* mutants. Our interpretation of these data is that *rpoZ* is in the same operon as *spoT* and that our insertion mutation exerts a polar effect on *spoT* expression. *rpoZ* and *spoT* cannot be the same gene

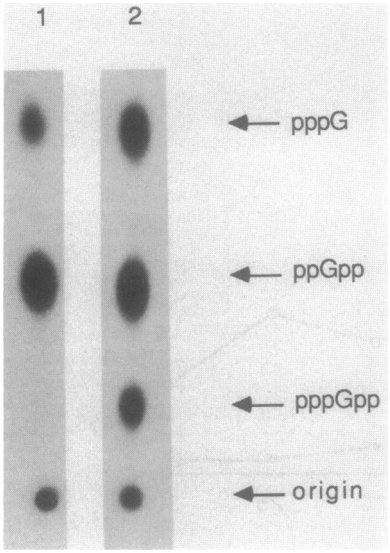

FIG. 5. Spotless phenotype of an *rpoZ* insertion mutant. Autoradiogram of time points taken 14.5 min after isoleucine starvation. Lanes: 1, DG6; 2, MG1655.

for several reasons. First, the plasmid shown previously to complement *spoT* mutations has the N-terminal coding region of *rpoZ* deleted. Second, our sequence of *rpoZ* clearly shows that it codes for a 10,000-M_r protein, whereas *spoT* has been reported to code for an 80,000-M_r protein (1). Finally, as discussed below, sequencing data reveal that *spoT* and *rpoZ* are distinct, though closely spaced, genes (Cashel, personal communication).

During this investigation, we learned that Michael Cashel's laboratory had sequenced the region around the *spoT* gene and found that an open reading frame identical to *rpoZ* is located immediately upstream of *spoT* (Cashel, personal communication). These sequence data confirm our conclusion that *rpoZ* is in the same operon as *spoT*. We failed to identify *spoT* from our own sequence data because there are no ATG codons in the region in which translation of the *spoT* gene is likely to be initiated (Cashel, personal communication). It is therefore probable that the translation of *spoT* is initiated with a codon other than ATG.

The placement of *rpoZ* in the same operon as *spoT* is intriguing. Because omega is a protein looking for a function, this placement is even more interesting. A most obvious hypothesis for the function of omega is that is plays a role in modulating the response of RNA polymerase to ppGpp. In vivo experiments are complicated by the fact that the insertion mutation is so polar on *spoT*. Since the slow-growth phenotype of our insertion mutant is completely suppressed in a *relA1* background, it is highly likely that the phenotype is due to the polar affect of the insertion and not to the absence of omega. Further, because the insertion mutant exhibits a classical SpoT phenotype, it is unlikely that omega is required for the inhibitory action of ppGpp. We are currently constructing strains which have *spoT* under the control of a regulatable promoter and strains in which all or most of *rpoZ* is deleted but the *rpoZ* promoter region and *spoT* are intact. Preliminary results of in vitro experiments indicate that, qualitatively, transcription of a ppGpp-sensitive promoter is inhibited equally by ppGpp, using RNA polymerase containing or lacking omega. We are continuing experiments in vitro, using both purified polymerase and S30 extracts. As an alternative to omega playing a part in the modulation of transcription by ppGpp, the presence of *rpoZ* in the same operon as *spoT* could simply be another example of a gene encoding an RNA polymerase subunit being found in a complex operon containing ribosome-associated proteins (5).

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