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# Determination of the Precise Location and Orientation of the *Escherichia coli dnaE* Gene

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The minimal region required for expression of the *dnaE* gene of *Escherichia coli* has been determined relative to a detailed restriction endonuclease map. This has been accomplished by analysis of Bal 31 exonuclease-generated deletions from the termini of the *E. coli* DNA contained in plasmid pMWE303, a plasmid that we have previously demonstrated to contain the *dnaE* gene (M. M. Welch and C. S. McHenry, J. Bacteriol. **152**:351–356, 1982). The competence of these deletion-containing plasmids in expressing the α subunit of DNA polymerase III holoenzyme has been determined by their ability both to complement a *dnaE* mutant and to direct the synthesis of a complete α subunit. The carboxyl-terminal coding region of *dnaE* has been identified through the detection of partial α polypeptides encoded by plasmids containing deletions from one end of the gene. This approach has permitted the precise determination of both termini of the *dnaE* gene and the determination of the orientation of the gene within the *E. coli* chromosome.

The DNA polymerase III holoenzyme is the major replicative enzyme in *Escherichia coli* as determined by both biochemical and genetic criteria. It contains at least seven different subunits: α, ε, θ, τ, β, γ, and δ (for a review, see reference 14). Three of these proteins, α, ε, and θ, constitute the catalytic core of the holoenzyme (15). This core, termed DNA polymerase III, is only active on duplex DNA that has been artificially activated by nuclease treatment. The full complement of subunits is required for efficient replication of natural chromosomes in vitro.

Many of the DNA polymerase III holoenzyme subunits have been shown to be encoded by essential *E. coli* genes. The *dnaN* gene, the structural gene for the β subunit, is located at 83 min on the *E. coli* genetic map distal to *dnaA* in the same operon (3, 18). The *dnaZ* and *dnaX* genes, which encode the γ and δ subunits, map adjacent to one another at 11 min (5, 7, 10, 11, 14, 23, 24). The structural gene for the ε subunit (*mutD* [*dnaQ*]) is located at 5 min (4, 8, 9; R. DiFrancesco, S. K. Bhatnagar, A. Brown, and M. J. Bessman, J. Biol. Chem., in press). The *dnaE* gene (4 min) has been known for several years to be a structural gene for a DNA polymerase III subunit, since defects in this enzyme have been correlated with lesions in *dnaE* (6). We have recently demonstrated that it encodes the α subunit (22).

Since most of the structural genes for the DNA polymerase III holoenzyme map at distinct genetic loci, an important question pertains to the mechanism by which their synthesis is coordinated to ensure the proper assembly of the limited number of polymerase complexes per cell. Here, we present the structure of the *dnaE* gene, including a detailed restriction endonuclease map and an assignment of the orientation of the gene within the *E. coli* genetic map, a necessary step in approaching this important regulatory problem.

## MATERIALS AND METHODS

**Bacterial strains and media.** The *E. coli* strains used in this study were UTH2 ($F^-$ *thr leu* $B1^-$ *thy lac strR met tonA* $SuII^+$ *dnaE486 srl-1300*::Tn*10 recA56*) (22) and CSR603 (*recA1 uvrA6 phr-1*) (19). The permissive temperature for strains carrying *dnaE486* is 30°C; 43°C is the nonpermissive temperature.

**Preparation and manipulation of DNA.** Cells were treated with $CaCl_2$ and transformed essentially as described previously (13). Transformants were plated in soft agar onto LBT (22) plates or similar plates containing ampicillin (100 μg/ml). All enzymes were used in accordance with the instructions of the manufacturers except as noted. General procedures used in the manipulation of DNA have been described previously (13, 20).

**Bal 31 exonuclease deletion procedure.** In a typical experiment, linear plasmid DNA (ca. 40 μg, created by the specified restriction endonucleases) was treated with 2.5 U of Bal 31 exonuclease (Bethesda Research Laboratories) in 12 mM $CaCl_2$–12 mM $MgCl_2$–0.6 M NaCl–1 mM EDTA–20 mM Tris-hydrochloride (pH 8.1, 700 μl, 30°C). At various times, portions were removed and added to 50 mM EDTA–50 mM Tris-hydrochloride (pH 8, 100 μl); this mixture was extracted with phenol and precipitated with ethanol. A portion of this material was digested with restriction endonuclease *Hin*fI and subjected to polyacrylamide gel electrophoresis. This permitted selection of the time point that had the desired amount of digestion for further analysis. The 15-min time point of digestion from the *Bam*HI terminus was selected (ca. 750 bases removed). Digestions that proceeded for 20 min from the *Cla*I terminus under these conditions removed no detectable DNA; a second digestion (sixfold-higher DNA concentration, 1.5 min) was required to remove ca. 300 bases.

Bal 31 exonuclease-digested DNA (4 μg) was treated with T4 DNA polymerase (16 U) in the presence of deoxynucleoside triphosphates to increase the quantity of blunt-ended molecules. After heat inactivation of the polymerase, the designated oligonucleotide linker containing the recognition site for a restriction endonuclease was added (200-fold molar excess). Treatment with T4 DNA ligase (800 U, overnight at 14°C) resulted in a resealing of the circles, with the linker inserted at the site of the joint.

**Detection of proteins encoded by plasmids.** The "maxicell" system of Sancar et al. (19) was used with modifications as noted. A culture of strain CSR603 containing the designated plasmid was grown (37°C) to a density of $2 \times 10^8$ cells per

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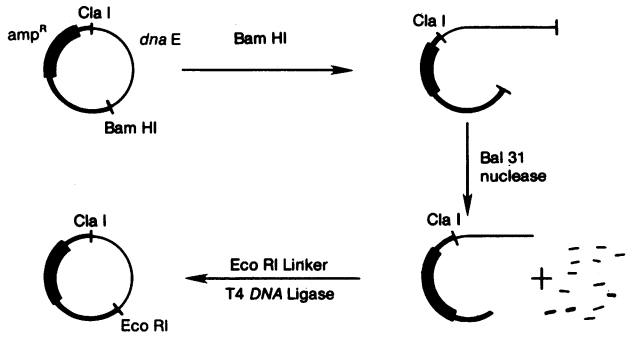


FIG. 1. Procedure used to generate deletions from the *Bam*HI restriction endonuclease site of *dnaE*-carrying plasmid pMWE303. Experimental details are described in the text. The oligonucleotide (10-mer) containing the restriction endonuclease *Eco*RI recognition site was prepared and treated with polynucleotide kinase according to the instructions of the manufacture (Collaborative Research).

ml. Cells (10 ml) were irradiated for 5 s with a UV germicidal lamp spaced at 14 cm. The irradiated cells were incubated for 1 h before the addition of cycloserine (final concentration, 0.1 mg/ml); a second portion of cycloserine was added after an additional 3 h (total concentration, 0.3 mg/ml). After overnight incubation, cells were suspended in sulfate-free minimal medium (5 ml) and incubated (1 h, 37°C) in the presence of [$^{35}$S]methionine (80 μCi; 1,470 Ci/mmol). The bacteria were then rapidly harvested, washed, suspended in sodium phosphate buffer (pH 7, 50 mM, 0.1 ml), and added to a lysis solution (110 μl of 0.5 M Tris-hydrochloride [pH 6.8], 0.2 M dithiothreitol, 2% sodium dodecyl sulfate). This material was placed in a boiling water bath for 15 min; cellular debris was removed by centrifugation. Aliquots of the supernatant (40 μl) were mixed with sample buffer (40 μl) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (12).

## RESULTS

**Generation of deletions from the *Bam*HI-proximal terminus of *dnaE*.** We have previously demonstrated that *dnaE* is encoded within a 4,600-base DNA fragment defined by two unique restriction endonuclease sites, *Bam*HI and *Cla*I (22). To precisely define the termini of the regions required for expression of this gene, we performed Bal 31 exonuclease digestions from each of these unique sites (Fig. 1). To define the terminus nearest the *Bam*HI site, plasmids incised with *Bam*HI were treated with sufficient Bal 31 exonuclease to permit removal of ca. 700 base pairs of *E. coli* DNA. The resulting partially digested plasmids were treated with T4 DNA polymerase to increase the proportion of blunt-ended termini and were ligated in the presence of a duplex oligonucleotide containing the recognition sequence for restriction endonuclease *Eco*RI. This provided a convenient site for further analysis of the deletions. To decrease the proportion of undigested plasmids, the product was again treated with *Bam*HI. The products were used to transform UTH2, a *dnaE486 recA* mutant.

**Determination of genetic activity of deletions from *Bam*HI terminus.** Initially, transformants were selected in the presence of ampicillin at the permissive temperature for the *dnaE* mutant. Individual colonies selected from the transformants were then classified by their ability to express the wild-type *dnaE* gene as judged by their ability to confer temperature resistance upon the mutant strain (Fig. 2).

To permit a correlation of the region required for genetic expression with the restriction endonuclease map, an analysis with restriction endonuclease *Hin*fI was conducted. The terminus of the *E. coli* segment was found by determining the length of the remaining terminal *E. coli Hin*fI-*Eco*RI fragment. The terminal fragment was identified by differential digestions with and without restriction endonuclease *Eco*RI. All terminal positions were confirmed with a second restriction endonuclease. For example, the termini of plasmids pDS7-5, pDS6-29, and pDS4-26 were also determined by the use of restriction endonuclease *Hin*cII (Fig. 2). The deletion of 1,020 base pairs (pDS6-29) does not affect the ability of the plasmid to complement *dnaE* mutants, whereas the deletion of an additional 10 bases (pDS7-5) destroys all activity. This permitted a very precise determination of one end of the *dnaE* gene (Fig. 2).

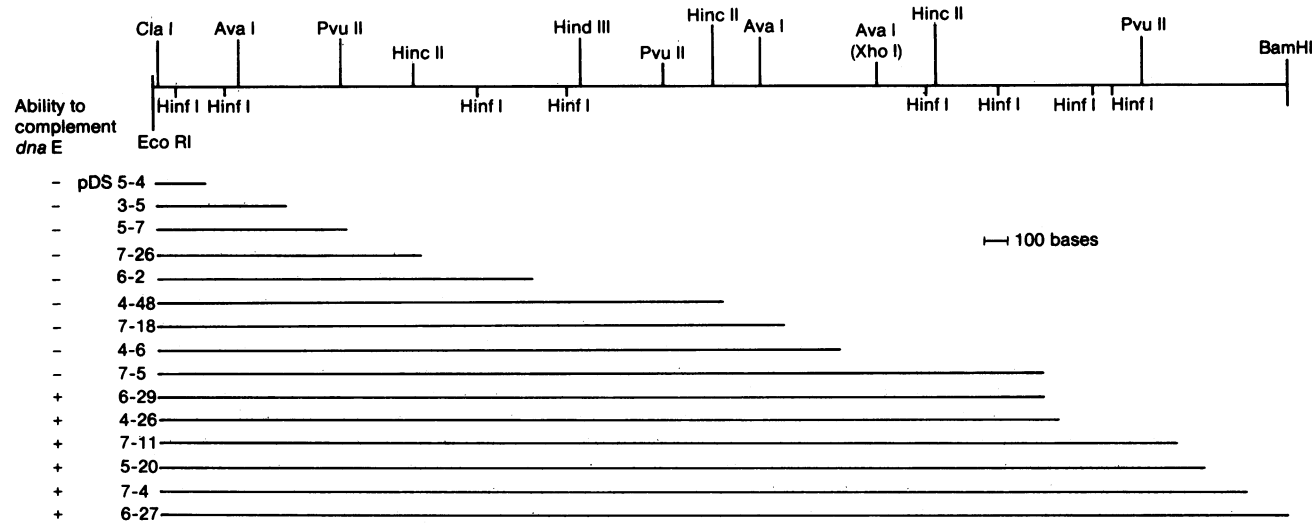


FIG. 2. Restriction endonuclease map and function of deletions from the *Bam*HI-proximal terminus of the *dnaE* coding region. The restriction endonuclease map was constructed by standard methods, including partial digestions of DNA terminally radioactively labeled at appropriate sites by the method of Smith and Birnsteil (21). The ordering of fragments was verified by their sequential disappearance as DNA was deleted from the plasmid. The ability of the *dnaE* gene to function was determined by the ability of plasmids to make cell growth possible at 43°C.

**Identification of proteins encoded by plasmids containing deletions from the *Bam*HI terminus.** The restriction endonuclease analysis of the constructed deletions demonstrated that the removal of 1,030 nucleotides from the *Bam*HI site of the cloned *E. coli* fragment destroyed its ability to complement *dnaE* mutants. To correlate these results with the protein product, we examined the proteins synthesized from the deletion-bearing plasmids by the maxicell technique (19). Consistent with our previous results (22), plasmids that complement *dnaE* mutants synthesize a 140,000-dalton protein, α. Plasmids that were unable to complement *dnaE* mutants did not direct the synthesis of α or any detectable partial polypeptides (Fig. 3). This suggests, but alone does not prove, that the *Bam*HI-proximal terminus of the *dnaE* gene is the amino-terminal coding region.

**Generation of deletions from the *Cla*I-proximal terminus of *dnaE*.** To permit analysis of the terminus of the *dnaE* gene nearest the *Cla*I site, it was necessary to first reorient the cloned restriction endonuclease fragment so that the creation of deletions could not destroy ampicillin resistance. This was required, since transformants were selected by their ability to confer ampicillin resistance. We wanted to be certain that all deletions containing a portion of the *dnaE* gene also contained an intact ampicillin resistance gene to avoid bias in our selection procedure. Therefore, the *Cla*I-*Eco*RI fragment from plasmid pDS4-26, a deletion plasmid that still expresses α, was excised and cloned into the corresponding sites of pBR322. The resulting plasmid, pDS100, contained *dnaE* aligned in the opposite orientation relative to the ampicillin resistance gene (Fig. 4).

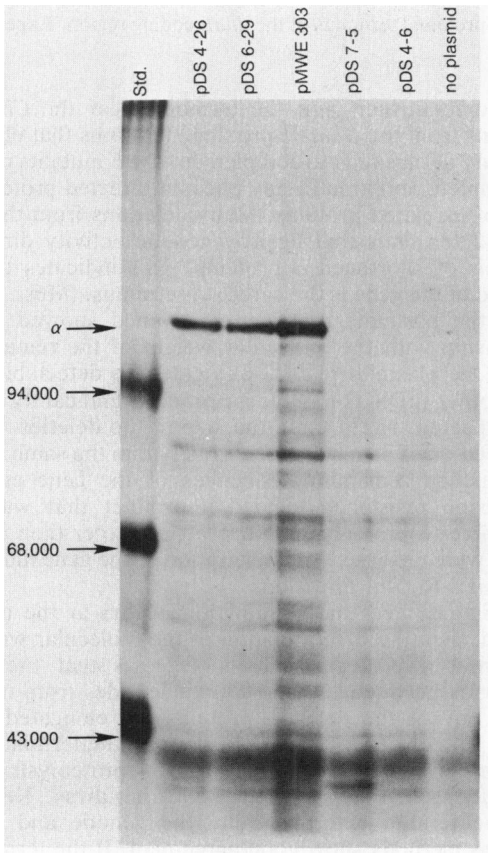


FIG. 3. Protein synthesis directed by plasmids containing deletions in or near the *Bam*HI terminus of the *dnaE* gene. Std., Standard.

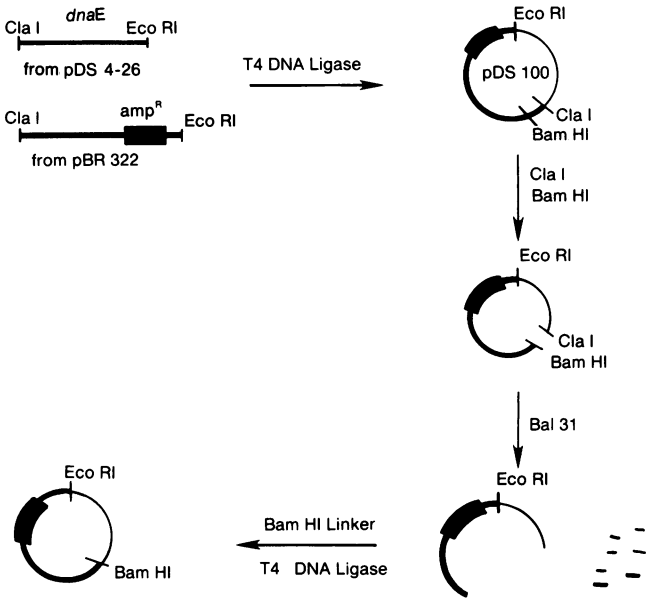


FIG. 4. Procedure used for reorientation of the *dnaE* gene and creation of deletions from the *Cla*I-proximal terminus. Plasmid pDS4-26 was digested sequentially with *Pst*I, *Eco*RI, and *Cla*I. The *E. coli Eco*RI-*Cla*I fragment was purified by agarose gel electrophoresis (0.7, 0.6 μg of ethidium bromide per ml) by a method described in a Schleicher & Schuell technical bulletin. The DNA within the desired band was electrophoretically transferred onto a DEAE membrane (NA45) and eluted with 1 M NaCl. The *Eco*RI-*Cla*I pBR322 molecule was also purified by a similar method except the *Pst*I digestion was deleted. The two DNA fragments were mixed (1 μg each) and joined by treatment with DNA ligase. The resulting plasmid pDS100 conferred temperature resistance upon UTH2 and yielded the predicted restriction endonuclease fragments upon reisolation from the transformed strain. Details pertaining to the generation of deletions were as described in the legend to Fig. 1.

Deletions were created by the digestion of pDS100 with *Cla*I and *Bam*HI, which resulted in a linear plasmid lacking a segment of pBR322 DNA. Digestion with Bal31 exonuclease and ligation in the presence of an oligonucleotide linker containing the recognition site for restriction endonuclease *Bam*HI created a family of plasmids containing variable deletions from the former *Cla*I site. The inclusion of *Bam*HI linkers provided a unique site to define the termini of the deletions (Fig. 4). In a manner similar to that described above, these were analyzed with restriction endonuclease *Hin*fI. Deletions ranging from fewer than 10 to 3,310 nucleotides were obtained (Fig. 5). These were analyzed for their ability to complement a *dnaE* mutant (Fig. 5). Correlation of the restriction endonuclease map of the deletions with their genetic function permitted us to assign the terminus of the *dnaE* gene to be within 10 to 300 nucleotides of the *Cla*I site of pMWE303.

An analysis of the proteins encoded by the deletion-containing plasmids revealed that all deletions that destroyed genetic activity also destroyed the ability of the plasmid to direct the synthesis of intact α (Fig. 6). However, unlike plasmids containing deletions from the *Bam*HI-proximal end, plasmids containing deletions from the *Cla*I end that inactivated *dnaE* were capable of directing the synthesis of incomplete α polypeptide chains. This confirms our

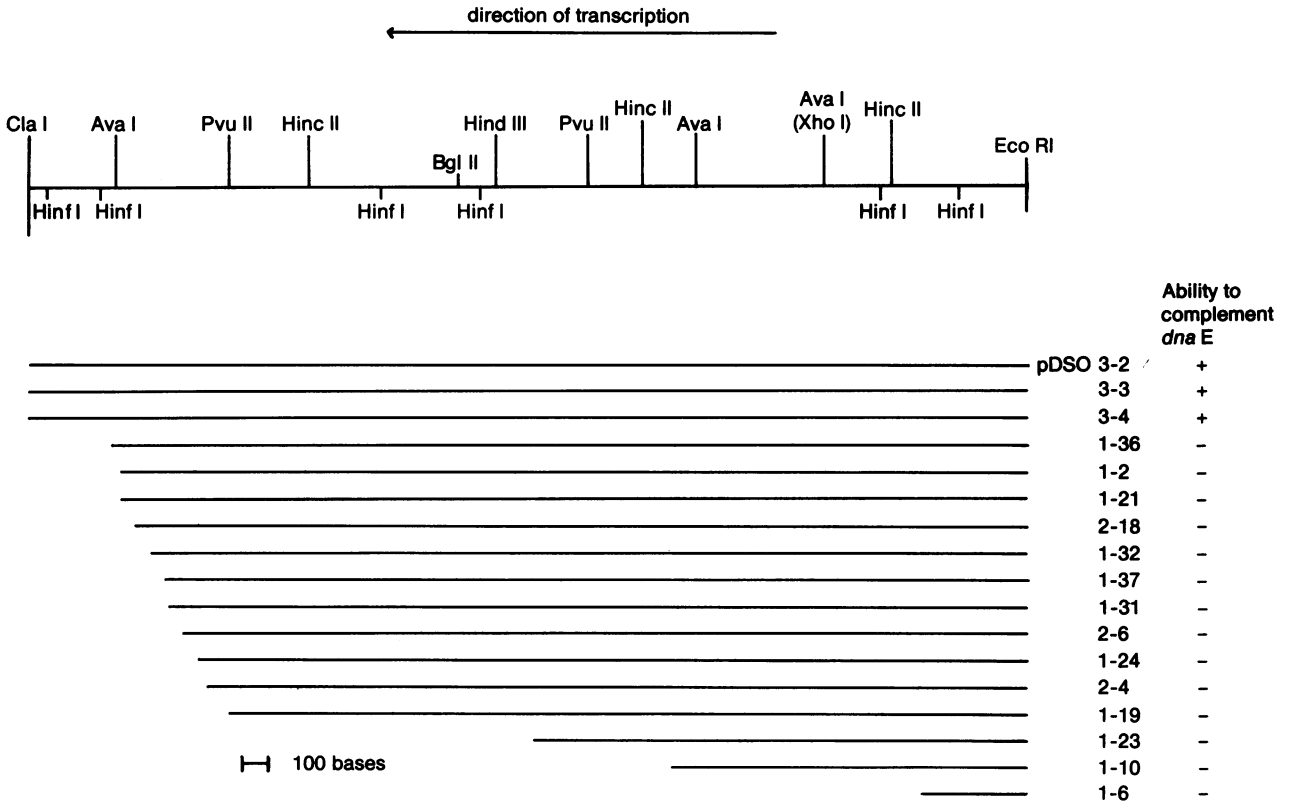


FIG. 5. Restriction endonuclease map and function of deletions from the *Cla*I-proximal terminus of the *dnaE* coding region. Experimental details were the same as described in the legend to Fig. 2.

earlier supposition that the *Cla*I-proximal region encodes the carboxyl terminus of α

Plasmids pDSO3-2, pDSO3-3, and pDSO3-4 contain deletions of fewer than 10 bases and direct the synthesis of complete α. Plasmids pDSO1-21 and pDSO2-4 encode α foreshortened by 9,000 and 19,000 daltons, respectively (Fig. 6). If the termination of protein synthesis occurred precisely at the deletion junction, the end of *dnaE* would be 236 and 500 nucleotides from the termini of these deletions, respectively. (A molecular weight of 114 for the average amino acid residue in a protein was used for the calculation. This was obtained from the amino acid composition of *E. coli* β-galactosidase, a protein of comparable molecular weight.) This would place the terminus of the *dnaE* gene at a site between 180 and 120 nucleotides from the *Cla*I site. If any readthrough occurred, generating fusion proteins, the terminus would have to be even closer to the *Cla*I site.

## DISCUSSION

In earlier studies, by successive subcloning, we limited the coding region for the *dnaE* gene to 4,600 bases of *E. coli* DNA (22). In this work we have performed Bal 31 exonucleolytic digestions from each of the unique restriction endonuclease sites that define the ends of this clone. Correlation of the restriction endonuclease maps of the resulting deletion-containing plasmids with their ability to complement a *dnaE* mutant has permitted us to define the termini of the region required for the expression of *dnaE* as being 1,020 to 1,030 bases from the *Bam*HI site and 10 to 300 bases from the *Cla*I site of pMWE303.

An analysis of the proteins synthesized from these plasmids has permitted us to identify the direction of transcription and to further limit the terminus near the *Cla*I site. Deletions from the *Bam*HI-proximal terminus that eliminate the ability of plasmids to complement *dnaE* mutants result in the complete abolition of any plasmid-directed protein synthesis of α-related proteins. Many deletions from the *Cla*I-proximal terminus that destroy genetic activity direct the synthesis of shortened α proteins. This indicates that the *Cla*I end of the gene is the carboxyl terminus. (Most, but not all, of the plasmids that were examined showed a clear relationship with the molecular weight of the remaining α chain. One plasmid (pDSO1-6) yielded no detectable product; another (pDSO2-6) gave a product significantly shorter than expected, based upon the size of the deletion. It may have been that fusions that occurred within the same reading frame as the remaining sequences of the gene encoding tetracycline resistance yielded a product that was very susceptible to proteolysis. No proteins larger than 140,000 daltons were observed.) Transcription of the gene must be in the *Bam*HI-to-*Cla*I direction.

If it is assumed that termination occurs at the deletion junction, then the determination of the molecular weight of the partial α protein products suggests that the actual terminus is between 120 and 180 nucleotides from the *Cla*I site. If any of the deletion proteins are elongated into a reading frame within pBR322 sequences, the terminus must be even closer to the *Cla*I site; if any proteolysis occurs subsequent to synthesis, it may be further away. Nevertheless, the termini determined by the genetic and protein synthesis methods show good agreement. If the terminus is 150 nucleotides from the *Cla*I site (the average value from both procedures used), the total length of the *dnaE* gene would be 3,520 nucleotides, sufficient coding capacity for a

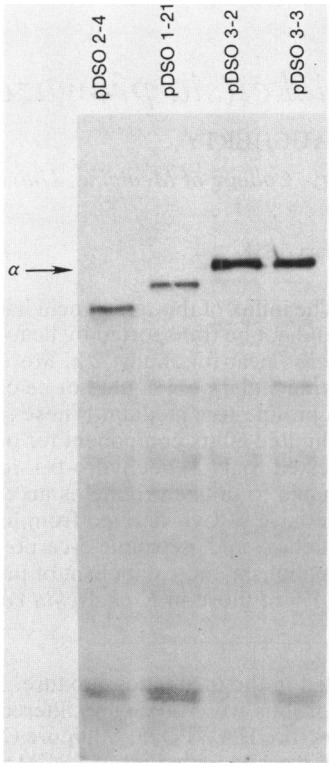


FIG. 6. Protein synthesis directed by plasmids containing deletions in or near the *Cla*I terminus of the *dnaE* gene.

protein of 134,000 daltons. This value is within experimental error of the 140,000 daltons determined from sodium dodecyl sulfate-polyacrylamide gels.

Additional information allows us to determine the orientation of the *dnaE* gene relative to the *E. coli* chromosome. Bendiak and Friesen (2) demonstrated that *dnaE* molecular clones contained a *Bgl*II site slightly further away from *firA* and *tsf* than the internal *Hin*dIII site. We have demonstrated that the *Bgl*II site is on the *Cla*I site of the *Hin*dIII site that maps within *dnaE*. Furthermore, Nishijima et al. (17) found that *pgsB*, a gene that maps on the same side of *dnaE* that *tsf* does, is carried on pLC26-43, the plasmid from which pMWE303 was derived. Within the *Bam*HI-proximal region of the *dnaE*-carrying *Cla*I-*Bam*HI fragment are sequences required for rescue of *pgsB1* (M. Welch and W. Dowhan, unpublished data; C. Raetz, personal communication). This information permits us to assign the direction of transcription of the *dnaE* gene toward increasing map units (toward *met*D [1]).

## ACKNOWLEDGMENTS

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