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Molecular Cloning, Correlation of Genetic and Restriction Maps, and Determination of the Direction of Transcription of *gnd* of *Escherichia coli*

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Expression of the gene *gnd* of *Escherichia coli*, which encodes 6-phosphogluconate dehydrogenase, is regulated by growth rate. Using deoxyribonucleic acid from the specialized transducing phage λ h80 d*gnd his* as the source of *gnd*, we cloned restriction fragments carrying the complete gene and portions of it on the plasmid vector pBR322. A hybrid plasmid carrying a 3.7-megadalton *Hin*dIII restriction fragment from the phage was prepared and found to be *gnd*$^+$. Through restriction mapping of this fragment and subcloning segments of it, we prepared a *gnd*$^+$ hybrid plasmid which carried only 1.85 megadaltons of *E. coli* deoxyribonucleic acid. A cleavage site for the restriction endonuclease *Pst*I was located on the genetic map of *gnd* by cloning adjacent *Eco*RI-*Pst*I restriction fragments and crossing the resulting hybrid plasmids with previously mapped *gnd* deletion and bacteriophage Mu insertion mutants. A maxicell experiment was used to determine the direction of transcription of *gnd*, to identify which *Eco*RI-*Pst*I fragment contains the *gnd* promoter, and to localize the beginning of the structural gene to a region about 850 ± 150 base pairs from the *Pst*I cleavage site. A fine-structure restriction map surrounding the *Pst*I cleavage site was prepared for endonucleases *Kpn*I, *Hin*cII, *Hae*III, *Hpa*II, and *Taq*I.

In *Escherichia coli* the level of 6-phosphogluconate dehydrogenase increases about fourfold with increasing growth rate (46), a pattern which is also observed for glucose 6-phosphate dehydrogenase (46), the chromosomally encoded β-lactamase (14), protein components of the translation apparatus such as ribosomal proteins (34), elongation factors (10, 11), and certain amino acyl tRNA synthetases (25, 28), and many other proteins of unidentified function (29). Although several models have been proposed to account for this metabolic regulation (20, 40), no specific mechanism has been elucidated, even for the best-studied systems, rRNA and ribosomal proteins (26, 27, for review). Indeed, more than one mechanism is probably involved. For example, when cells are shifted to a medium permitting a higher growth rate, there is a virtually instantaneous increase in the differential rate of synthesis of components of the translational machinery (28, 34), while there is a significant lag before the postshift rate of synthesis of 6-phosphogluconate dehydrogenase is attained (E. E. Farrish and R. E. Wolf, Jr., unpublished data). Moreover, since there is a similar lag before the postshift rate of total protein synthesis is reached, it may be that the mechanism regulating 6-phosphogluconate dehydrogenase synthesis is common to the regulation of the synthesis of many other *E. coli* proteins. Thus, study of the structure of *gnd*, the gene which encodes 6-phosphogluconate dehydrogenase, and elucidation of its mechanism of expression, should provide important information about the way in which *E. coli* is able to adjust its pattern of protein synthesis to accord with changes in its environment and thereby maximize its growth rate. Such studies should also provide insight into the mechanisms that regulate expression of genes for enzymes of central intermediary metabolism, about which virtually nothing is known.

Several earlier studies have served in the development of *gnd* as a model system, and some of them have facilitated the work to be described here. A genetic map of *gnd* has been prepared by mapping bacteriophage Mu insertion mutations against deletions which enter the structural gene from each end (44). The enzyme has been purified to homogeneity by a rapid, affinity chromatography method (47), and its physical and kinetic properties have been investigated (8, 42, 47). The enzyme has also been synthesized in vitro in a coupled transcription-translation system (13), using as template the DNA from a specialized transducing phage, λ *c*1857 *St*68 h80 d*gnd his* (λ h80 d*gnd his*) (45).

In the present communication we describe the cloning of restriction fragments of λh80 d*gnd his* which carry intact *gnd* and subgene portions of

it, using the plasmid cloning vector pBR322 (3). The hybrid plasmids were used to correlate the genetic and restriction maps of *gnd* and to locate the beginning of the structural gene. This work should facilitate the isolation and sequencing of the *gnd* control region and provide DNA probes for quantitative measurement of the effect of growth rate on the amount and rate of synthesis of *gnd* mRNA.

MATERIALS AND METHODS

Media and growth conditions. Minimal medium 63 (36) supplemented with carbon source (4 mg/ml), thiamine hydrochloride (1 μg/ml), amino acids (25 μg/ml), and agar (2%) was used for genetic tests. For the maxicell experiments, cultures were grown in M9 medium (24) containing glucose, 1% Casamino Acids (Difco), and tryptophan and starved in glucose M9 medium containing tryptophan, histidine, and magnesium chloride in place of magnesium sulfate. The standard broth, BTYEX63, was medium 63 supplemented with 1% tryptone (Difco) and 0.4% yeast extract (Difco). The standard rich plate was tryptone-yeast extract (TYE) agar (45). Gluconate bromothymol blue (BTB) indicator plates, prepared as described previously (46), were used to distinguish *gnd*$^+$ *edd* strains from *gnd edd* strains. Antibiotic concentrations were (micrograms per milliliter): ampicillin (Ap), 25; tetracycline, (Tc), 25; and chloramphenicol, 150. LBC broth and plates (45) were used for preparation and titration of lysates of P1 *virS*. Strains lysogenic for λ h80 d*gnd his* or Mu *cts*61 are temperature sensitive and were grown at 32°C; other strains were grown at 37°C.

Bacterial strains and plasmids. Table 1 shows the strains of *E. coli* K-12 used in this study. Strain RW233, a *relA* mutant, was used for preparation of S-30 extracts for in vitro protein synthesis and replaces the previously used (13) Rel$^+$ strain RW232. The *relA* mutation was introduced as follows. A P1 *virS* lysate was prepared on strain Q1 and used to transduce strain RW232 to growth on 1, 2-propanediol (Prd) minimal medium containing histidine. Prd$^+$ transductants were cloned, and their Rel character was assessed by measuring the effect of starvation for isoleucine, imposed by addition of valine (100 μg/ml), to 1-ml cultures growing in glucose minimal medium, on the rates of incorporation of [^{3}H]uridine and [^{14}C]leucine into trichloroacetic acid-precipitable material, using a protocol kindly supplied by M. Berman (Harvard University). Four of 14 Prd$^+$ transductants showed "relaxed" synthesis of RNA and one strain, RW233, was used. (The 28% linkage between *prd* and *relA* accords with previous results [32].) Growth of strain RW233 on glucose minimal medium is inhibited by a mixture of serine, methionine, and glycine (100 μg/ml each) as reported for other *relA* mutants (41).

Table 2 shows the properties of hybrid plasmids prepared during the course of this work.

Preparation of phage and plasmid DNA. λh80 d*gnd his* was purified from a heat-induced culture of strain RW182 by cesium chloride density centrifugation, and phage DNA was extracted as described in detail elsewhere (43).

Plasmid DNA, for use in transformation or restriction endonuclease analysis, was prepared from chloramphenicol-amplified (5) cultures by detergent lysis (16) and centrifugation in a cesium chloride-ethidium bromide density gradient (6) as described previously (43). Screening for hybrid plasmids was carried out on cleared lysates prepared from 1-ml cultures by the method of Post et al. (30).

Enzymes. Restriction endonucleases were purchased from either Bethesda Research Laboratories or New England Biolabs. T4 DNA ligase was purchased from Bethesda Research Laboratories. 6-Phosphogluconate dehydrogenase was purified as described previously (47).

TABLE 1. *Bacterial strains*

Strain	Genotype	Origin
W3110	F$^-$ prototroph	L. Soll
Q1	F$^-$ Δ(*pro-lac*)*X111 trpA36 argA glyA34 relA1 rpsL*	Derivative of BF266 (9); P. Primakoff
HB101/pBR322	F$^-$ *leu thi pro lacY rpsL hsdR hsdM*(pBR322)	M. Mann
RW181	F$^-$ *trpR lacZ trpA kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*attP2H*(P2 *c*5 *nip*1-*sbcB-his-gnd-rfb*)*1*	This laboratory, (43)
RW182	RW181(λ *c*I857 *St*68 h80, λ *c*I857 *St*68 h80 d*gnd his*)	This laboratory, (45)
RW187-1,-2,-11,-12	RW181(λ *c*I857 *St*68 h80 d*gnd his*) Δ(*gnd-attR-tonB*)	This laboratory (44)
RW223-1,-3	RW181(λ *c*I857 *St*68 h80 d*gnd his*) Δ(*imm-his-gnd*)	This laboratory (44)
RW229	RW181 *str-125*	This laboratory, (43)
RW231	RW229 *recA1*	This laboratory (43)
RW231/pBR322	RW231/pBR322	Ap^r Tc^r transformant of RW231 with pBR322 from HB101(pBR322)
RW232	F$^-$ *trpR* Δ(*lac*)*X174 kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*attP2H*(*P2 c*5 *nip*1)-*sbcB-his-gnd-rfb*)*1 rspL*	(13)
RW233	RW232 *prd*$^+$ *relA*	See text

TABLE 2. *Properties of hybrid plasmids prepared in this study*

Plasmid	Phenotype	Fragment cloned	Mol wt of cloned fragment (10^6)	Origin of cloned fragment	Site in pBR322
pMN1	Ap^r Tc^s Gnd^+	*Hin*dIII	3.7	λ h80 d*gnd his*	*Hin*dIII
pMN2	Ap^r Tc^s Gnd^{+a}	*Eco*RI + *Eco*RI/*Hin*dIII	2.7	pMN1	*Eco*RI/*Hin*dIII
pMN3	Ap^r Tc^r Gnd^{+b}	*Eco*RI	2.45	λ h80 d*gnd his*	*Eco*RI
pMN4	Ap^r Tc^r Gnd^{+b}	*Eco*RI	2.45	λ h80 d*gnd his*	*Eco*RI
pMN5	Ap^r Tc^s Gnd^+	*Bgl*II	2.55	pMN1	*Bam*HI
pMN6	Ap^r Tc^s Gnd^+	*Eco*RI/*Bgl*II	1.85	pMN5	*Eco*RI/*Bam*HI
pMN7	Ap^s Tc^r Gnd^-	*Pst*I/*Eco*RI	1.1	pMN1	*Pst*I/*Eco*RI
pMN8	Ap^s Tc^r Gnd^-	*Pst*I/*Eco*RI	0.7	pMN1	*Pst*I/*Eco*RI
pMN9	Ap^r Tc^s Gnd^{-c}	*Eco*RI/*Bgl*II	1.6	pMN6	*Eco*RI/*Bam*HI

[a] pMN3 was formed by deletion of the 1.0-Mdal *Eco*RI fragment of pMN1; see text for details.
[b] These two plasmids have the 2.4-Mdal *Eco*RI fragment inserted into pBR322 in opposite orientations.
[c] This plasmid lacks the 405-bp *Kpn*I fragment of pMN6; see text for details.

Mapping of restriction endonuclease cleavage sites. Restriction enzyme digestions were carried out by using the buffer and assay conditions recommended by the manufacturer. When DNA was to be digested with two enzymes, the first enzyme used was the one whose assay buffer was of lower ionic strength; e.g., *Pst*I before *Eco*RI. After the first reaction was terminated by heating to 65°C for 10 min, ingredients were added to complete the assay buffer for the second digestion, which was then carried out and terminated as with a single digestion. Digestion products were then analyzed by agarose gel electrophoresis as described by Shinnick et al. (35). Molecular weight (MW) markers were *Eco*RI or *Hin*dIII digest products of λ (12), λ h80 (12), and λ h80 d*gnd his* (43) DNA or both and parallel digests of pBR322 (37).

Small restriction fragments were separated and analyzed by electrophoresis in 5% acrylamide gels as described by Maniatas et al. (22) using a vertical apparatus. MW markers were *Hae*III or *Hpa*II digestion products of pBR322 (37) or both. Electroelution was used to isolate restriction fragments (e.g., *Eco*RI-*Pst* fragments of pMN7; pMN8) for secondary digestions. After the gel was stained briefly with ethidium bromide and illuminated with UV light, the area of the gel containing the desired fragment was cut out and transferred to a 1-ml Eppendorf pipette tip plugged with a Whatman glass fiber filter. A piece of dialysis tubing filled with TBE buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) was placed over the plugged end, and the tip was filled with buffer. Electroelution was carried out for 16 h at 100 V/cm in a tube gel apparatus, and the current was reversed for 10 min before removal of the eluate.

Transformation and transduction. Transformation was carried out by the method of Cohen et al. (7). Lysates of P1 *virS* were prepared, and transduction was carried out as described by Miller (23).

Enzyme assays. To measure the specific activity of 6-phosphogluconate dehydrogenase in strains carrying hybrid plasmids, we prepared sonic extracts from cultures growing exponentially in BTYEX63 broth as described by Wolf et al. (47). Activity of 6-phosphogluconate dehydrogenase in sonic supernatant fluids was assayed spectrophotometrically (47). Strain W3110 served as control. Protein was measured by the method of Lowry et al. (19), using bovine serum albumin corrected for moisture content (18) as the standard.

To measure the amount of 6-phosphogluconate dehydrogenase synthesized in vitro, we centrifuged the synthesis mixture at 5,000 × *g* for 20 min to remove a flocculent material which interferes with spectrophotometric measurement of the rate of change of absorbance at 340 nm. We then assayed the supernatant fluid for 6-phosphogluconate dehydrogenase activity in a final assay volume of 200 μl, using microcuvettes. That the supernatant fluid contains all of the 6-phosphogluconate dehydrogenase was determined by comparing the activity present in it to that present in the total synthesis mixture, by using the previously described radiochemical assay (13).

In vitro protein synthesis. S-30 extracts of strain RW233, the components of the synthesis mixture, and conditions for protein synthesis were prepared and used as described by Isturiz and Wolf (13). The amount of 6-phosphogluconate dehydrogenase synthesized in 60 min was proportional to λ h80 d*gnd his* DNA concentration in the range of 10 to 50 μg/ml. The effect of restriction of phage DNA on the amount of 6-phosphogluconate dehydrogenase synthesis was determined as follows. Six micrograms of DNA was digested with 15 U of endonuclease in a 25-μl reaction. Digestion was terminated by heating to 65°C for 10 min. A 5-μl portion was removed and subjected to agarose gel electrophoresis to assure that complete digestion had taken place. The components of the in vitro synthesis mixture were added to the remaining 20 μl to a final volume of 100 μl, and synthesis was carried out as described above. Mock digestions, lacking only enzyme, but containing the appropriate enzyme storage buffer, were carried out in parallel reactions.

Construction of recombinant plasmids. Target DNA (e.g., λ h80 *dgndhis* DNA) and pBR322 plasmid DNA were codigested with the appropriate restriction enzyme(s), heated to 65°C for 10 min, and brought up to correct ligase conditions by addition of 10× ligase salts (660 mM Tris-chloride [pH 7.6], 66 mM $MgCl_2$, 100 mM dithiothreitol, 5 mM ATP). Ligation was

carried out for 2 h at 15°C with 0.1 U of T4 DNA ligase. Strain RW231 was transformed with the ligation mixture as described above. Transformants were selected on TYE plates or on gluconate BTB indicator plates supplemented with the appropriate antibiotic(s). Gnd$^+$ transformants form large yellow colonies on the indicator plates, rather than the green color of typical *gnd*$^+$ *edd* strains, probably because the rate-limiting step in metabolism of gluconate in these strains is 6-phosphogluconate dehydrogenase (R.E. Wolf, Jr., unpublished data) and Gnd$^+$ transformants have high levels of the enzyme; strains with *gnd* plasmids form white-colored colonies.

Protein labeling in maxicells and analysis by SDS-polyacrylamide gel electrophoresis and fluorography. Plasmid-encoded proteins were specifically labeled in maxicells by the method of Sancar et al. (33). Cultures (5 ml) were grown in glucose M9 minimal medium containing 1% Casamino Acids and tryptophan to an absorbance at 580 nm of about 0.2 (2×10^8 cells per ml). Cells were irradiated for 80 s with a germicidal lamp at a fluence of 1.2 J/m^2 per s and then incubated in the dark for 16 h. The cells were collected by centrifugation, washed, suspended in an equal volume of glucose M9 medium lacking sulfate, and starved for 1 h. [^{35}S]methionine (New England Nuclear Corp.) was added to 5 μCi/ml, and incubation was continued for 1 h. Cells were then collected by centrifugation, washed, suspended in 0.2 ml of 1% sodium dodecyl sulfate (SDS), and lysed by boiling for 3 min. Samples were subjected to electrophoresis at 120 V for 6 to 7 h in a slab containing 15% polyacrylamide at a 20:1.0 ratio of acrylamide to bisacrylamide and 0.1% SDS as described by Maizel (21). [^{35}S]methionine-labeled proteins were visualized by fluorography (17) by using EN^3 HANCE (New England Nuclear Corp.); exposure was for 1 day at −80°C. The MWs of labeled proteins were estimated from the migration in an adjacent slot of unlabeled marker proteins (Bio-Rad Laboratories): phosphorylase b (MW, 94,000), bovine serum albumin (MW, 68,000), ovalbumin (MW, 43,000), carbonic anhydrase (MW, 30,000), soybean trypsin inhibitor (MW, 21,000), and lysozyme (MW, 14,300); 6-phosphogluconate dehydrogenase (MW, 52,000) was also run in a separate slot (47).

RESULTS

Effect of restriction endonuclease digestion on the in vitro template activity of λ h80 d*gnd his* DNA. The source of DNA for cloning *gnd* of *E. coli* was λ h80 d*gnd his*. Since the cloning vector was to be plasmid pBR322 (3), we first determined whether certain of the enzymes that have single cleavage sites in the plasmid also cleave the 13 to 14 megadalton (Mdal)-segment of the phage genome which contains bacterial DNA and whether such cleavage sites are in *gnd* by measuring the effect of restriction with a given enzyme on the capacity of the DNA to direct the in vitro synthesis of 6-phosphogluconate dehydrogenase. Table 3 shows that *Hin*dIII cleaved λ h80 d*gnd his* DNA into six bacterial DNA-containing fragments and that *Eco*RI cleaved it into three. In vitro synthesis of 6-phosphogluconate dehydrogenase was reduced 60% after cleavage of the DNA with *Hin*dIII and 80 to 90% after cleavage with *Eco*RI. *Bam*HI cleavage reduced template activity 20% even though the enzyme did not cut in the bacterial portion of the phage genome. We interpreted these results as indicating that the closer *gnd* was to the end of a restriction fragment, the more labile was the gene in the in vitro synthesis of 6-phosphogluconate dehydrogenase. Indeed, we have found that the rate of hydrolysis of [^{3}H]thymidine-λ h80 d*gnd his* DNA in the S-30 extract is increased substantially by prior digestion with *Hin*dIII (M. Rosen and R. E. Wolf, Jr., unpublished data). Moreover, we show below that cleavage sites on λ h80 d*gnd his* DNA for *Eco*RI are closer to *gnd* than are cleavage sites for *Hin*dIII.

TABLE 3. *Effect of restriction of λ h80 dgnd his DNA on in vitro synthesis of 6-phosphogluconate dehydrogenase*

Restriction endonuclease	No. of cuts in bacterial DNA[a]	Size of bacterial DNA-containing fragments (Mdal)	Template activity of restricted DNA (% mock-digested)
*Bam*HI	0	>16	80
*Hin*dIII	5	3.7, 3.1, 2.8, 1.6, 1.4, 1.1	30–40
*Eco*RI	2	13.7, 2.4, 5.3	10–20

[a] λ h80 and λ h80 d*gnd his* DNA were digested with the restriction endonuclease, and the products were analyzed by electrophoresis in a 0.8% agarose slab gel as described in the text and as shown in reference 43. Bands present in the λ 80 d*gnd his* DNA digest and absent in the λ h80 DNA digest contain bacterial DNA.

Cloning of a *Hin*dIII restriction fragment carrying intact *gnd*. The data presented in Table 3 suggest that of the enzymes tested, *Hin*dIII digestion of λ h80 d*gnd his* DNA would most likely generate the smallest *gnd*$^+$ restriction fragment. Accordingly, λ h80 d*gnd his* DNA was digested with *Hin*dIII and the fragments ligated with *Hin*dIII-digested pBR322. Since insertion of DNA into the *Hin*dIII site of pBR322 inactivates the gene for Tc^r, Ap^r transformants of strain RW229 were selected and then tested for being Gnd$^+$ and Tc^s. Several Gnd$^+$ Ap^r Tc^s transformants were recovered. One such transformant, strain RW229/pMN1, was prepared and assayed for 6-phosphogluconate dehydrogenase activity. The specific activity was about 20-fold higher than in the control strain W3110. This shows that strain RW229/pMN1 is *gnd*$^+$; the high level of activity accords with the relaxed

replication of the plasmid vector (3). Strain RW229/pMN1 contained plasmid DNA which was able to transform strain RW231 to Ap^r at high frequency, and all transformants were Gnd^+. Restriction enzyme digestion and agarose gel electrophoresis showed that pMN1 DNA has a molecular weight of 6.3×10^6 and is composed of two *Hin*dIII fragments (Table 4): one corresponds to pBR322 (2.6 Mdal); the other (3.7 Mdal) comigrates with the 3.7-Mdal bacterial DNA-containing fragment of *Hin*dIII-digested λ h80 d*gnd his* DNA (Table 3).

Restriction mapping of pMN1. A restriction map of pMN1 (Fig. 1) was established to determine what portions of the 3.7-Mdal *Hin*dIII fragment might be subcloned into pBR322. Such subclones might serve, among other things, to define further the boundaries of *gnd* and to localize restriction endonuclease cleavage sites within the gene. Thus, restriction enzymes chosen initially for mapping were those that would generate a fragment which could be inserted into one of the single restriction sites on pBR322. Restriction mapping of pMN1 was facilitated by preparation (see below) of a deletion plasmid, pMN2; cleavage sites present in pMN1, but absent in pMN2, must lie within the deleted region (Fig. 1).

Digestion of pMN1 with *Eco*RI generated three fragments: 2.85, 2.45, and 1.0 Mdal (Table 4). An *Eco*RI digest of λ h80 d*gnd his* DNA contained a 2.45-Mdal fragment but not a 1.0-Mdal fragment (Table 3). Thus, the 2.45-Mdal *Eco*RI fragment of pMN1 is wholly contained within the 3.7-Mdal *Hin*dIII fragment, and the 2.85-Mdal fragment contains pBR322 and the 0.25-Mdal fragment of the cloned region. To determine whether *gnd* is on the 1.0- or 2.45-Mdal fragment or whether one of the *Eco*RI sites is within *gnd*, pMN1 was digested with

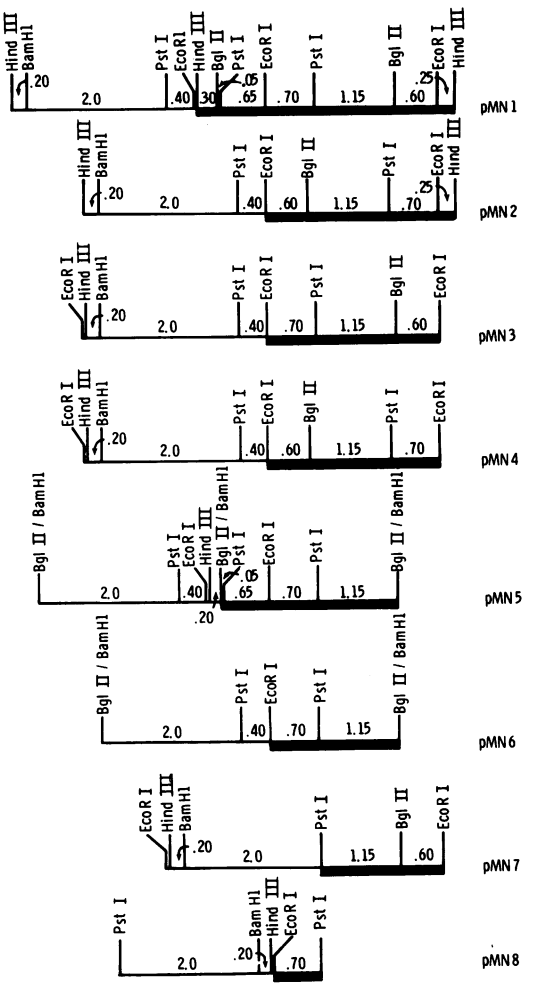

FIG. 1. *Restriction maps of hybrid plasmids constructed in this study. The darkened bar represents bacterial DNA; the light line represents a pBR322.*

TABLE 4. *MW of restriction fragments of hybrid plasmids*[a]

Enzyme	MW (10^6)							
	pMN1	pMN2	pMN3	pMN4	pMN5	pMN6	pMN7	pMN8
*Hin*dIII	3.7, 2.6	5.3			5.15			
*Eco*RI	2.85, 2.45, 1.0	2.85, 2.45	2.6, 2.45	2.6, 2.45		4.25		
*Bgl*II	3.75, 2.55	5.3					3.95	
*Pst*I	4.2, 1.35, 0.75	3.15, 2.15	3.95, 1.1	2.9, 2.15	3.15, 1.35, 0.65	3.15, 1.1		2.9
*Eco*RI/*Pst*I	2.45, 1.75, 0.70, 0.65, 0.40, 0.35	2.45, 1.75, 0.70, 0.40					2.2, 1.75	2.2, 0.7
*Eco*RI/*Bgl*II	2.85, 1.85, 0.70, 0.60, 0.30	2.85, 1.85, 0.60					3.35, 0.6	
*Bgl*II/*Pst*I		3.05, 1.15, 1.10						

[a] Hybrid plasmids were digested with restriction endonuclease(s), and the products were analyzed as described in the text. The data was used to prepare the restriction maps shown in Fig. 1.

*Eco*RI, and the mixture was religated and then used to transform strain RW231 to Ap^r on gluconate BTB indicator plates containing ampicillin. Gnd^+ Ap^r colonies appeared, and plasmid DNA was extracted and characterized by agarose gel electrophoresis. One plasmid, pMN2, contained the 2.85- and 2.45-Mdal *Eco*RI fragments and was missing the 1.0-Mdal fragment. Purified pMN2 transformed strain RW231 to Gnd^+ Ap^r at high frequency, and transformants had elevated levels of 6-phosphogluconate dehydrogenase. Thus, *gnd* lies wholly within the 2.45-Mdal *Eco*RI fragment. The 2.45-Mdal *Eco*RI fragment of pMN2 is present in the vector plasmid in orientation opposite to that in pMN1.

Further defining the boundaries of *gnd* by subcloning. Plasmids pMN3 and pMN4 (Fig. 1) were recovered from an experiment in which *Eco*RI fragments of λ h80 d*gnd his* DNA were ligated to *Eco*RI-digested pBR322, and the mixture was used to transform strain RW231 to Gnd^+ Ap^r Tc^r. Digestion of the two plasmids with *Eco*RI gave rise to fragments of 2.6 and 2.45 Mdal (Table 4), as predicted. Since *Pst*I cut pMN3 into fragments of 3.95 and 1.1 Mdal and pMN4 into fragments of 2.9 and 2.15 Mdal (Table 4), the two plasmids carried the *Eco*RI fragment of λ h80 d*gnd his* in opposite orientations. Strains carrying these plasmids also had high levels of 6-phosphogluconate dehydrogenase activity. These results demonstrate unequivocally that *gnd* lies within the 2.45-Mdal *Eco*RI fragment.

The restriction map of pMN1 suggested that cloning the 2.55-Mdal *Bgl*II fragment would be an important step in determining the boundaries of *gnd*. If a plasmid carrying this fragment could transform strain RW231 to Gnd^+, then *gnd* would be localized to the 1.85-Mdal *Eco*RI-*Bgl*II region, which is only about twice the minimum amount of DNA required to encode the 52,000-dalton 6-phosphogluconate dehydrogenase monomer (47). *Bgl*II fragments may be inserted into the *Bam*HI site of pBR322, which lies within the gene for Tc^r, because DNA cleaved with these enzymes has the same 5′-tetranucleotide extension. Accordingly, pMN1 was digested with *Bgl*II, and the fragments ligated to *Bam*HI-digested pBR322. Transformation of strain RW231 with this mixture gave rise to Gnd^+ Ap^r Tc^s transformants. Plasmid pMN5 isolated from one such transformant was not cleaved by *Bgl*II nor by *Bam*HI as anticipated since insertion of *Bgl*II fragments into a *Bam*HI site gives rise to a hybrid sequence not recognized by either enzyme (31). Digestion with *Pst*I gave rise to fragments of 3.15, 1.35, and 0.65 Mdal and digestion with *Eco*RI gave rise to fragments of 4.25 and 0.9 Mdal (Table 4), all as predicted from the map of pMN1. Strain RW231/pMN5 had high levels of 6-phosphogluconate dehydrogenase activity, and all Ap^r transformants of strain RW231 with purified pMN5 were Gnd^+. Therefore, since the 2.55-Mdal *Bgl*II fragment cloned in pMN5 and the 2.45-Mdal *Eco*RI fragments cloned in pMN3 and pMN4 carry intact *gnd*, the complete gene must lie within the region common to both fragments, i.e., the 1.85-Mdal *Eco*RI-*Bgl*II region.

To prove that this was the case, pMN5 was digested with *Eco*RI and self-ligated, and the mixture was used to transform strain RW231 to Ap^r. Cleared lysates prepared from 1-ml cultures of five Gnd^+ Ap^r Tc^s clones were analyzed by agarose gel electrophoresis and shown to contain plasmids whose MW was less than that of pMN5. Plasmid was purified from one of these strains, RW231/pMN6, and further characterized. Digestion with *Eco*RI gave rise to a single fragment of 4.25 Mdal, and digestion with *Pst*I generated fragments of 3.15 and 1.1 Mdal (Table 4), all as predicted from the map of pMN1. Thus, the 1.85-Mdal segment of the *E. coli* chromosome carried by pMN6 includes the complete *gnd* gene.

To determine whether the *Pst*I cleavage site in the 1.85-Mdal *Eco*RI-*Bgl*II region is in *gnd* or whether *gnd* lies entirely within the 1.15-Mdal *Pst*I-*Bgl*II region, we digested pMN1 with *Pst*I and then with *Eco*RI and then ligated the fragments to doubly digested pBR322. The mixture was used to transform strain RW231. Tc^r transformants were selected, and those carrying plasmid-containing inserts were identified by scoring for Ap^s, the *Pst*I cleavage site being in the gene for Ap^r. None of 50 Tc^r Ap^s transformants proved to be Gnd^+.

Cleared lysates were prepared from 12 Gnd^- Tc^r Ap^s transformants and analyzed by agarose gel electrophoresis. Strain RW231/pMN7 had a plasmid whose MW was ca. 3.95×10^6, the size predicted for a plasmid carrying the 1.75-Mdal *Eco*RI-*Pst*I fragment. Strain RW231/pMN8 contained a plasmid whose MW was ca. 2.9×10^6, the size predicted for a plasmid carrying the 0.7-Mdal *Eco*RI-*Pst*I fragment. The digestion patterns of both pMN7 and pMN8 (Table 4) accord with the predictions of the restriction map of pMN1 and demonstrate that the two adjacent portions of *gnd* have been cloned. Both plasmids transformed strain RW231 to Tc^r at high frequency, and all transformants were Gnd^- Ap^s. Cell extracts prepared from strains RW231/pMN7 and RW231/pMN8 had no detectable 6-phosphogluconate dehydrogenase activity. Thus, the *Pst*I cleavage site in the *Eco*RI-*Bgl*II region of pMN6 lies within *gnd*. Moreover, one

of the two plasmids, pMN7 or pMN8, must carry the *gnd* promoter region separated from part of the structural gene.

Correlation of the genetic and restriction maps of *gnd*. The *Pst*I cleavage site within *gnd* was located on the previously established genetic map of the gene (44) by in vivo recombination experiments (Fig. 2). Strains carrying the deletions that enter *gnd* from the *his*-proximal end (i.e., strains RW223-1 and RW223-3) and the *his*-distal end of the gene (i.e., strains RW187-1, -2, -11, -12) and the strains harboring *gnd*::Mu insertion mutations (i.e., JC2, JC3b, JC5a, JC6a, JC6b, and JC8b) were transformed with pMN7 and pMN8. Tc^r transformants were selected, cloned on the selective medium, and grown for about 20 generations in broth. Gnd^+ recombinants were selected by plating on gluconate minimal medium containing tryptophan, histidine, and citrate. pMN7 gave rise to Gnd^+ recombinants with each of the deletions that enter *gnd* from the *his* distal end, but not with the deletions that enter the gene from the *his* proximal end (Fig. 2). pMN8 failed to recombine with any deletion strain. To verify that recombinants were indeed gnd^+, we prepared cell extracts and assayed them for 6-phosphogluconate dehydrogenase activity. All Gnd^+ recombinants had levels of 6-phosphogluconate dehydrogenase equal to that of wild-type strain W3110, the amount of activity predicted to be present in a strain in which the *gnd* portion of the plasmid had recombined with chromosomal *gnd*. pMN7 gave rise to Gnd^+ recombinants with *gnd*::Mu mutants JC2, JC3b, JC6b, and JC8b, but not with JC5a or JC6a. These results suggest that the *Pst*I cleavage site lies between JC8b and the region of the gene defined by JC5a and JC6a. pMN8 failed to give rise to Gnd^+ recombinants with JC5a and JC6a ($<10^{-8}$), perhaps because the *Pst*I end of the cloned fragment is too close to the Mu prophages in the two mutants to permit crossing over.

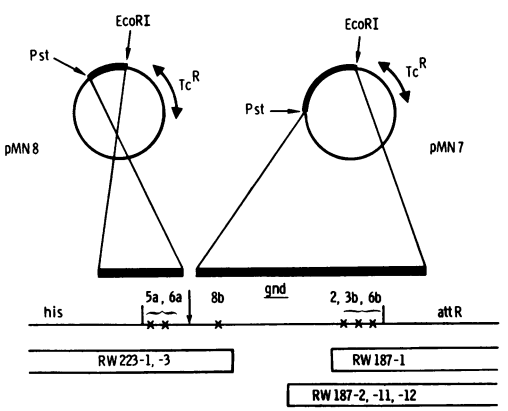

FIG. 2. *Location of the PstI cleavage site on the genetic map of gnd. Plasmids pMN7 and pMN8 were introduced into gnd deletion mutants (strains RW187-1, -2, -11, and -12 and RW223-1, and -3) and bacteriophage Mu insertion mutants (strains JC2, 3b, 5a, 6a, 6b, and 8b) and Gnd^+ recombinants were selected. Note that neither type of mutation reverted. The gnd::Mu mutations were previously mapped against the deletions shown (44).*

Direction of transcription of *gnd*. It was next of interest to determine which plasmid, pMN7 or pMN8, carries the *gnd* promoter region. This was accomplished through the use of maxicells (33). Maxicells result from UV irradiation of *recA* strains which carry high copy number plasmids such as pBR322. Because of the relatively small target size of the plasmid and its multicopy nature, chromosomal DNA is preferentially degraded in these cells. Thus, maxicells may be used to label plasmid-encoded proteins with little background labeling of host proteins. The rationale underlying our use of maxicells was that the plasmid carrying the *gnd* promoter and that part of the gene which codes for the aminoterminal portion of 6-phosphogluconate dehydrogenase should direct the synthesis of a polypeptide altered in size with respect to the normal monomer, whereas a plasmid carrying only that portion of the gene which codes for the carboxyl terminus would not make any polypeptide because it would lack a promoter. Accordingly, we analyzed by SDS-polyacrylamide gel electrophoresis and fluorography the size of the proteins synthesized in maxicells carrying pMN3, pMN7, and pMN8 (Fig. 3). Five bands were evident in the fluorograph of the pMN3 maxicells: a 52,000-dalton polypeptide which comigrated with authentic 6-phosphogluconate dehydrogenase and four other polypeptides which were also synthesized by pBR322-containing maxicells. The 37,000-dalton polypeptide is the product of the *tet* gene; the 31,000- and 27,000-dalton polypeptides are precursor and mature forms of β-lactamase, respectively (33); the coding region on pBR322 for the band at the bottom of the gel (<10,000 daltons) is unknown, but a band of similar MW has been observed previously (15). As predicted from the phenotypes of strains carrying pMN7 and pMN8, neither the two forms of β-lactamase nor 6-phosphogluconate dehydrogenase was synthesized by pMN7 or pMN8 maxicells. However, pMN7 maxicells, but not pMN8 maxicells, synthesized a unique polypeptide of 43,000 daltons. Thus, pMN7 carries the *gnd* control region and the beginning of the structural gene, with transcription proceeding in the counterclockwise direction as the map is drawn (Fig. 2).

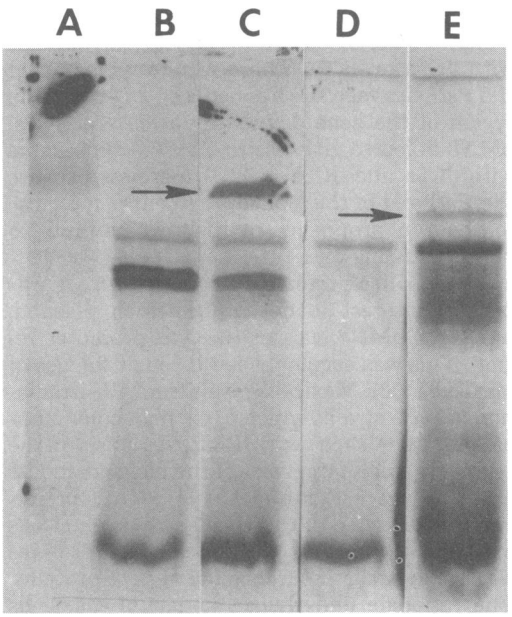

FIG. 3. *SDS-polyacrylamide gel electrophoresis of proteins labeled in maxicells. Strains were UV irradiated, grown in glucose M9 medium containing 1% Casamino Acids and tryptophan for 16 h, starved for sulfate, and labeled with [^{35}S]methionine (5 μCi/ml) for 1 h. Cells were lysed in boiling SDS, and the extracts were electrophoresed on a 15% SDS-polyacrylamide gel until the dye front reached the bottom of the gel. (A) strain RW231; (B) strain RW231/pBR322; (C) strain RW231/pMN3; (D) strain RW231/pMN8; (E) strain RW231/pMN7. Marker proteins and purified 6-phosphogluconate dehydrogenase were run in adjacent slots.*

Location of additional restriction sites in and around *gnd*. To facilitate a further definition of the boundaries of *gnd* and thus a more precise localization of the *gnd* control region, we mapped cleavage sites for *Kpn*I, *Hinc*II, *Taq*I, *Hae*III, and *Hpa*II surrounding the *Pst*I cleavage site in the cloned portion of pMN6 (Fig. 4). The sites were mapped by single and double digestions of pMN6, pMN7, and pMN8 with the various enzymes and subsequent analysis of the molecular weights of fragments after electrophoresis in 5% acrylamide gels. In some cases restriction fragments were eluted from gels and digested with one or two other enzymes, e.g., the 1,554-base pair (bp) *Eco*RI-*Kpn*I fragment of pMN6 and the *Eco*RI-*Pst*I fragments of pMN7 and pMN8.

The results of the maxicell experiment suggested that the *Pst*I-proximal *Kpn*I site, and probably the more distal *Kpn*I site are within *gnd*. Accordingly, a plasmid deleted for the 405-bp *Kpn*I-*Kpn*I fragment should be Gnd$^-$. Such a deletion plasmid was constructed by digesting pMN6 with *Kpn*I and self-ligating the mixture. (*Kpn*I does not cleave the pBR322 portion of the plasmid.) When strain RW231 was transformed with this mixture and plated on gluconate BTB indicator plates containing ampicillin, Gnd$^-$ Ap^r transformants appeared. Plasmid extracted from one such transformant, RW231/pMN9, was converted to linear form by *Kpn*I, and a single fragment was generated (data not shown). Cell extracts of strain RW231/pMN9 had no detectable 6-phosphogluconate dehydrogenase activity. Thus, at least the *Pst*I-proximal *Kpn*I cleavage site is in *gnd*, and the 320-bp

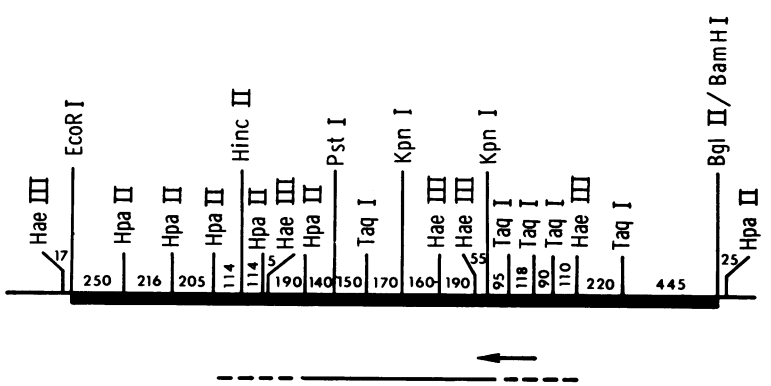

FIG. 4. *Restriction map of pMN6. Cleavage sites for HincII, KpnI, HaeIII, TaqI, and HpaII were mapped as described in the text. The heavy line represents the E. coli DNA portion of pMN6. The line below the map represents the location of ca. 1,000 bp of the gnd structural gene as calculated from the known size and reading frames of the 43,000-dalton fusion polypeptide observed in the pMN7 maxicells (see text and Fig. 3). The dashed line represents the uncertain location of the remaining 300 bp of gnd. The arrow shows the direction of transcription. The absolute order of the 216- and 205-bp HpaII fragments is unknown.*

*Pst*I-*Kpn*I region must lie wholly within the structural gene.

DISCUSSION

The work presented here should afford several benefits to the study of the mechanism of expression of *gnd*, a gene regulated by growth rate. For example, analysis of the DNA sequence surrounding the beginning of the structural gene might provide direct information about possible transcriptional or translational control mechanisms. Such sequencing studies are now feasible because we have prepared a hybrid plasmid, pMN7, which carries the promoter-proximal portion of *gnd*. The maxicell experiment (Fig. 3) and the fine structure restriction map of the *gnd* region (Fig. 4) allows us to locate the beginning of the structural gene to a 300-bp region. Assuming that polypeptides synthesized in maxicells are stable, we can estimate the distance between the *Pst*I cleavage site within *gnd* and the beginning of the gene, from the size of the unique, "fusion" polypeptide made by pMN7-containing maxicells. The fusion polypeptide should contain the aminoterminal amino acids of 6-phosphogluconate dehydrogenase to the junction of *gnd* and pBR322-DNA plus those additional amino acids that are encoded by pBR322 DNA beyond the *Pst*I cleavage site to the first in-place nonsense codon. Since the complete sequence of pBR322 is known (38), it is possible to determine precisely the number of additional amino acids which can be fused to the truncated 6-phosphogluconate dehydrogenase monomer. In one phase the fusion polypeptide would contain 103 amino acids from the carboxyl terminus of β-lactamase; translation in one of the other two reading frames would extend the truncated 6-phosphogluconate dehydrogenase polypeptide by either 29 or 9 amino acids. Based on the assumption that the MW of an average amino acid is 120, the distance between the *Pst*I cleavage site and the beginning of the *gnd* structural gene is about 1,048, 988, or 766 bp. The approximate boundaries of *gnd* are shown in Fig. 4.

An alternative interpretation of the results of the maxicell experiment is that the fusion polypeptide observed in pMN7 maxicells did not result from a *gnd-bla* fusion but rather from transcription initiating within pBR322 DNA and proceeding clockwise across the *Pst*I site or counterclockwise across the *Eco*RI site, with the true *gnd-bla* polypeptide being either too small or too unstable to be detected. However, this explanation is very unlikely since pBR322 does not contain either type of "readthrough" gene (38).

Another possible explanation for the presence of the 43,000-dalton polypeptide in pMN7 but not in pMN8-containing maxicells would be that the insert of pMN7 contains the promoter-proximal portion of another gene which is transcribed in the clockwise direction, with transcription proceeding across the *Eco*RI site into *tet*. However, this cannot be the case since the same 43,000-dalton polypeptide should also be observed in pMN3-containing maxicells. Finally, the 43,000-dalton polypeptide could not arise from a transcript originating in the cloned portion of pMN7 and proceeding counterclockwise across the *Pst*I site because that would require an open reading frame on the antisense strand of *gnd* of over 1,000 bp.

In vivo studies of *gnd* regulation should also profit from the present work. For example, it is important to determine the extent to which the amount and rate of synthesis of *gnd* mRNA varies as a function of growth rate. Such studies require an assay for *gnd* mRNA which is specific and quantitative, e.g., hybridization of labeled RNA to a DNA probe. Plasmid pMN6 should be a specific hybridization probe for *gnd* mRNA because (i) its *E. coli* DNA is only two times as large as the minimum amount necessary to encode the 6-phosphogluconate dehydrogenase monomer; and (ii) the rest of the cloned region is probably silent because the maxicell experiment (Fig. 3) showed that only a single protein, 6-phosphogluconate dehydrogenase, is encoded in the bacterial portion of pMN3 from which pMN6 was derived, with the assumptions that labeled proteins are stable in maxicells and that neither a methionine-deficient, nor a nontranslated RNA is encoded.

The location of the *Pst*I cleavage site on the genetic map of *gnd* (Fig. 2) and determination of the direction of transcription allows us to separate the *gnd*::Mu insertion mutations into promoter-proximal and promoter-distal groups. *gnd* mutants containing promoter-distal Mu insertion mutations are the mutants of choice for fusing *lac* to the *gnd* promoter (1); preparation of such *gnd-lac* fusion strains should permit the isolation of *gnd* regulatory mutants (2).

As a relaxed replicating plasmid, pBR322 is normally present in 25 to 30 copies per cell (5). Strains carrying *gnd*$^+$ derivatives of pBR322 have levels of 6-phosphogluconate dehydrogenase about 20-fold higher than normal strains. This proportionate increase in enzyme level with gene dosage suggests that expression of *gnd* is not subject to positive regulation by a factor present in limiting amount. Accordingly, it will be interesting to determine whether growth rate-dependent control of 6-phosphogluconate dehydrogenase levels still operates when high copy numbers of the gene are present and whether this high copy number affects the level of other *E. coli* proteins.

We previously described a method for the single-step purification of 6-phosphogluconate dehydrogenase (47). The method depended on the use of a cell extract prepared from a heat-induced culture of a λ h80 d*gnd his* double-defective lysogen. Cell extracts with similarly high specific activity of 6-phosphogluconate dehydrogenase can now be obtained more simply from strains carrying *gnd*$^+$ hybrid plasmids, and we have used one such strain for purification of 6-phosphogluconate dehydrogenase (R. E. Wolf, Jr., unpublished data). Thompson et al. (39) identified a strain in the Clarke-Carbon clone bank (4) which carries a *gnd*$^+$ hybrid plasmid, and deSilva and Fraenkel (8) used it for a similar purification.

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