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Growth Rate-Dependent Regulation of 6-Phosphogluconate Dehydrogenase Level in *Escherichia coli* K-12: β-Galactosidase Expression in *gnd-lac* Operon Fusion Strains

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The level of 6-phosphogluconate dehydrogenase is positively correlated with the cellular growth rate. To determine whether growth rate-dependent regulation of expression of *gnd*, which encodes this enzyme, is carried out by a transcriptional mechanism, the structural genes of the lactose operon were fused to and brought under the control of the *gnd* promoter through the use of phage Mu *d*1(Ap^r *lac*). Four independent *gnd*::Mu *d*1(Ap^r *lac*) operon fusion strains were isolated. After the Mu *d*1 prophage was replaced with λp1(209), Lac$^+$ specialized transducing phages carrying the *gnd-lac* fusions were prepared. These phages were used to demonstrate that the *lac* genes were fused to the *gnd* promoter by crossing them with *gnd* promoter deletion mutants and with polar phage Mu *c*ts-induced *gnd* mutants. A genetic map of the fusion joints was deduced. The level of β-galactosidase in each fusion strain was the same in cells growing on acetate as in cells growing on glucose (with specific growth rate constants of 0.1 and 0.55 h^{-1}, respectively) and was unaffected by the presence of a *gnd*$^+$ gene in *trans*. Our results suggest that a post-transcriptional mechanism mediates growth rate-dependent regulation of *gnd* and that this regulation is not autogenous. Models for regulation are discussed with respect to these results and the physiology and DNA sequence of *gnd*.

6-Phosphogluconate (6PG) dehydrogenase catalyzes a reaction of the hexose monophosphate shunt. The amount of this enzyme in *Escherichia coli* is regulated by the cellular growth rate and is about fourfold higher in cells growing on glucose than in cells growing on acetate (17). Most of the protein components of the translational machinery (the "core"), as well as a number of other *E. coli* proteins, show a similar positive correlation between relative amount and growth rate (13). However, *gnd*, which encodes 6PG dehydrogenase, is clearly not a member of the same regulatory network as the core genes since after a nutritional shift-up the rate of accumulation of the various components of translation increases considerably before the rate of accumulation of 6PG dehydrogenase increases (7). Furthermore, *gnd* is not subject to stringent control (7).

A genetic map of *gnd*, which is comprised of *gnd*::Mu *c*ts mutations and deletions that enter the gene from each end, has been prepared previously (16). The complete *gnd* gene and subgene portions of it have been cloned on plasmid pBR322, and a fine-structure restriction map of the *gnd* locus has been prepared (12). These two maps have been correlated by genetic crosses between the hybrid plasmids and the *gnd* deletion and *gnd*::Mu *c*ts mutants (12). The direction of transcription of *gnd* is counterclockwise with respect to the circular genetic map of *E. coli* as it is usually drawn (12). The approximate start of the structural gene has been located on the *gnd* restriction map (12).

More recently, we have determined the complete nucleotide sequence of *gnd* and its flanking control regions and identified the precise starting points of transcription and the structural gene (M. S. Nasoff and R. E. Wolf, Jr., manuscript in preparation). The *gnd* locus is monocistronic, and there are 56 bases in *gnd* mRNA before the start codon. An analysis of the sequence of *gnd* did not reveal an obvious mechanism for growth rate-dependent regulation of *gnd*, but several of the previously proposed transcriptional and translational control models (7) were ruled out.

Thus, an important, basic question is at what level (transcriptional or post-transcriptional) is the growth rate-dependent regulation of *gnd* exerted? Our initial attempts to answer this question by DNA-RNA hybridization experiments were unsuccessful. Accordingly, we turned to an alternative method, the preparation of strains carrying *gnd-lac* operon fusions. In a *gnd-lac* operon fusion, synthesis of β-galacto-

sidase depends upon transcription that initiates at the *gnd* promoter and is subject to any regulation that takes place at the *gnd* promoter or any other site of transcriptional regulation (e.g., an attenuator) that lies between the promoter and the fusion joint. Thus, the rationale for this approach is that if the level of β-galactosidase in a *gnd-lac* operon fusion strain responds to changes in the steady-state growth rate like 6PG dehydrogenase, then growth rate-dependent regulation takes place at the level of transcription. On the other hand, if the level of β-galactosidase does not respond like 6PG dehydrogenase, then regulation is mediated by a post-transcriptional mechanism or by a transcriptional mechanism whose site of action is distal to the fusion joint.

In this study we used phage Mu *d*1 (Ap^r *lac*) (referred to below as Mu *d*1) (5) to isolate four independent *gnd-lac* operon fusion strains. We describe genetic mapping of the fusion joints and genetic proof that *lac* is fused to the *gnd* promoter. We also show that β-galactosidase levels do not vary with growth rate in the fusion strains, regardless of whether a wild-type *gnd* gene is also present. We suggest that a post-transcriptional mechanism is probably responsible for the growth rate-dependent regulation of *gnd* and that transcription of *gnd* is not subject to autogenous regulation.

(A preliminary report of the results has appeared previously [H. V. Baker II and R. E. Wolf, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H15, p. 115].)

MATERIALS AND METHODS

Media and growth conditions. Minimal medium 63 (10) supplemented with a carbon source (4 mg/ml), thiamine hydrochloride (1 μg/ml), amino acids (25 μg/ml), and agar (2%) was used for genetic tests. Sodium citrate (1.25 mg/ml) was added to enhance growth of *tonB* mutants. LB broth (10) was the standard broth used. Tryptone yeast extract (TYE) agar (10 g of tryptone [Difco Laboratories] per liter, 5 g of yeast extract per liter, 8 g of sodium chloride per liter, 1.5% agar) was the standard nutrient agar medium used. The following antibiotic concentrations were used: ampicillin, 25 μg/ml; tetracycline, 25 μg/ml; chloramphenicol, 100 μg/ml; and kanamycin, 50 μg/ml. TBYCM broth (10 g of tryptone [Difco] per liter, 5 g of NaCl per liter, 10 g of yeast extract per liter, 0.01 M $MgSO_4$, and 0.005 M $CaCl_2$) and MC buffer (0.1 M $MgSO_4$, 0.005 M $CaCl_2$) were used to prepare lysates of bacteriophages P1 and Mu *cts*. TMB broth (10 g of tryptone [Difco] per liter, 5 g of NaCl per liter, 0.01 M $MgSO_4$) and 0.01 M $MgSO_4$ were used for the preparation and dilution, respectively, of phage λ lysates. Physiological experiments were carried out as described previously (17) by using appropriately supplemented morpholinepropanesulfonic acid minimal medium. Growth rates are expressed as the specific growth rate constant (k), which was calculated from the following expression: k = ln2/mass doubling time (in hours). Gluconate bromthymol blue (BTB) indicator plates were prepared as described previously (18) and were used to distinguish gnd^+ *edd* strains from *gnd edd* strains. The Lac phenotype of fusion strains and phages was assessed on lactose MacConkey indicator plates. Glucose minimal plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml, tryptophan, histidine, and sodium citrate (called glucose-XG plates) were used to score the Lac phenotypes of Gnd^+ recombinants in the crosses designed to determine whether *lac* was fused to *gnd* in a given fusion strain. Glucose minimal plates containing 40 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml were used in the selection of $phoA^+$ strains (14). Strains lysogenic for phage Mu *d*1 were grown at 30°C; other strains were grown at 37°C.

Bacterial strains and scoring for *gnd*. Table 1 shows the strains of *E. coli* K-12 used in this study. *gnd* mutants of an otherwise wild-type strain have no readily scorable phenotype. Hence, genetic studies of *gnd* are carried out in strains blocked in the Entner-Doudoroff pathway of gluconate metabolism (e.g., by an *edd* mutation) (8). Thus, gnd^+ *edd* strains can use gluconate as the sole carbon source and form green colonies on gluconate BTB indicator plates, whereas *gnd edd* strains cannot grow on gluconate minimal medium and form white colonies on the indicator plates; edd^+ strains form yellow colonies on the indicator plates, regardless of the *gnd* phenotype (16).

Phage Mu *d*1-induced fusions have generally been prepared in strain MC4100. Accordingly, we wanted to prepare *gnd-lac* fusions in a strain MC4100 background and thus needed first to prepare an *edd* mutant of this strain. However, strain MC4100 turned out to have a defect in gluconate metabolism; it grew considerably slower than other edd^+ strains on gluconate minimal medium and formed white colonies on gluconate BTB indicator plates. This defect is not due to a mutation in *edd* or to a gene linked to it (unpublished data). Revertants with apparently normal metabolism of gluconate were obtained as follows. Yellow papillae appeared on colonies of strain MC4100 after 3 to 4 days of incubation on gluconate BTB indicator plates. Several papillae were cloned twice on the indicator plates. These papillae bred true and grew faster than strain MC4100 on gluconate minimal medium. One such revertant, MC4100-Y, was chosen, and strain HB11 was derived from it in the following way. We first positioned a Tc^r marker near an *edd-zwf* deletion by transducing strain RW181 to growth on TYE plates containing tetracycline with a P1CM *clr100* lysate prepared on strain AE1031. About one-third of the Tc^r transductants retained the *edd-zwf* deletion since they formed white colonies when they were streaked onto gluconate BTB plates and failed to grow on gluconate minimal medium. A P1 lysate was then prepared on one of these strains, strain RW181-21, and was used to transduce strain MC4100-Y to Tc^r. Strain HB11 was one of the Tc^r transductants that formed green colonies on gluconate BTB indicator plates. To verify that the *edd-zwf* deletion of strain RW181-21 had been introduced, strain HB11 was scored for the unselected marker, *zwf*, by using an enzyme assay. Strain HB11 contained no detectable glucose 6-phosphate (G6P) dehydrogenase, and its level of 6PG dehydrogenase was approximately the same as the level in other *E. coli* K-12 strains (see below).

Most physiological studies of *gnd* have been carried

TABLE 1. Bacterial strains[a]

Strain(s)	Genotype and phenotype[b]	Source, derivation, and/or reference(s)
AE1031	Hfr *leu-6 his-1 arg-6 lacY1 gal-6 zeb-1*::Tn*10 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 supE44 metB1 thyA*	P. M. Silverman
RW181	F⁻ *trpR lacZ*(Am) *trpA9605*(Am) *kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*sbcB-his-gnd-rfb*)	16
RW181-21	RW181 *zeb-1*::Tn*10*	P1(AE1031) × RW181
RW187-1,-2,-11, and -12	F⁻ *trpR lacZ*(Am) *trpA9605*(Am) *kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*sbcB-his-gnd-rfb*) λ c1857 *St68 h80 d gnd his* Δ(*gnd-attR-tonB*)*1,2,11,12*	12, 16
JC2	F⁻ *trpR lacZ*(Am) *trpA9605*(Am) *kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*sbcB-his-gnd-rfb*) λ c1857 *St68 h80 d gnd his* Δ(*attR-tonB*) *gnd-2*::Mu cts61 Nal^r	This laboratory
JC6b	F⁻ *trpR lacZ*(Am) *trpA9605*(Am) *kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*sbcB-his-gnd-rfb*) λ c1857 *St68 h80 d gnd his* Δ(*attR-tonB*) *gnd-6b*::Mu cts61 Nal^r	This laboratory
JC7	F⁻ *trpR lacZ*(Am) *trpA9605*(Am) *kdgR*^{c} Δ(*edd-zwf*)*22* Δ(*sbcB-his-gnd-rfb*) λ c1857 *St68 h80 d gnd his* Δ(*attR-tonB*) *gnd*::Mu cts61 Δ(Mu cts61) Nal^r	This laboratory (12,16)
W3110	F⁻ prototroph	L. Soll
W3110-17	W3110*zeb-1*::Tn*10* Δ(*edd-zwf*)*22*	P1(RW181-21) × W3110
MC4100	F⁻ *araD139* Δ(*argF-lac*)*169 flbB ptsF relA rpsL thi deoC*	M. Casadaban (5)
MC4100-Y	Revertant of MC4100 to normal growth on gluconate	See text
HB11	MC4100-Y *zeb-1*::Tn*10* Δ(*edd-zwf*)*22*	P1(RW181-21) × MC4100-Y
MAL103	F⁻ Mu cts *d*1 (Ap^r *lac*) Mu cts Δ(*proA-lac*)*X111 rpsL*	M. Casadaban (5)
HB123	HB11 *gnd-123*::Mu cts *d*1 (Ap^r *lac*)(Lac$^+$)	Mu *d*1 × HB11
HB125	HB11 *gnd-125*::Mu cts *d*1 (Ap^r *lac*)(Lac$^+$)	Mu *d*1 × HB11
HB128	HB11 *gnd-128*::Mu cts *d*1 (Ap^r *lac*)(Lac$^+$)	Mu *d*1 × HB11
HB417	HB11 *gnd-417*::Mu cts *d*1 (Ap^r *lac*)(Lac$^+$)	Mu *d*1 × HB11
HB814	HB11 *gnd-814*::Mu cts *d*1 (Ap^r *lac*)(Lac$^+$)	Mu *d*1 × HB11
EF1	F⁻ Δ(*sbcB-his-gnd-rfb*)	This laboratory (phage P2-induced deletion of strain W3110)
HB126	HB125 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1(EF1) × HB125
HB129	HB128 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1(EF1) × HB128
HB418	HB417 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1 (EF1) × HB417
HB815	HB814 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1(EF1) × HB814
HB127	HB126 *gnd-125*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209)(Lac$^+$)	λp1(209) × HB125
HB1210	HB129 *gnd-128*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209)(Lac$^+$)	λp1(209) × HB129
HB419	HB418 *gnd-417*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209) (Lac$^+$)	λp1(209) × HB418
HB816	HB815 *gnd-814*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209)(Lac$^+$)	λp1(209) × HB815
MPh30	F⁻ *araD* Δ(*argF-lac*)*169 phoAK114*::Tn*5*	J. Beckwith
HB350	W3110-17 Δ(*argF-lac*)*169 phoAK114*::Tn*5*	P1(MPh30) × W3110-17
HB351	F⁻ Δ(*argF-lac*)*169 zeb-1*::Tn*10* Δ(*edd-zwf*)*22*	P1(W3110) × HB350
HB352	HB351 *gnd-128*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209)(Lac$^+$)	P1(HB1210) × HB351
HB360	HB351 *gnd-125*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209)(Lac$^+$)	P1(HB127) × HB351
HB380	HB351 *gnd-814*::ΔMu cts *d*1 (Ap^r *lac*)::λp1(209)(Lac$^+$)	P1(HB816) × HB351
HB354	HB352 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1(EF1) × HB352
HB361	HB360 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1(EF1) × HB360
HB381	HB380 *zeb-1*$^+$ *edd*$^+$ *zwf*$^+$	P1(EF1) × HB380
SG2204	HfrH *his*::Tn*10 thyA*	S. Gottesman
HB355	HB351 *his*::Tn*10*	P1(SG2204) × HB354
EF4	Δ(*his-gnd-rfb*)*1448 ara-9*/F$_{T80}$ *his*$^+$ *gnd*$^+$	This laboratory
HB356	HB355/F$_{T80}$ (*his*$^+$ *gnd*$^+$)	EF4 × HB355

[a] All strains except EF4 are *E. coli* K-12 strains; strain EF4 is *Salmonella typhimurium* strain SB6448 into which the *E. coli* episome was introduced.

[b] The notations used are those of Bachmann and Low (1).

TABLE 2. Bacteriophages

Phage	Bacterial genes carried	Source
P1CM *clr100*		L. Rosner via J. Hays
λ *cI*		Laboratory stock
λ *vir*		Laboratory stock
λp1(209)	::(+Mu') *trp'BA'* ΔW209 *lac'OZYA'*	M. Casadaban[a]
λHB125	*gnd'-125*::*trp'CBA'* ΔW209 *lac'OZYA'*	This study
λHB123	*gnd'-123*::*trp'CBA'* ΔW209 *lac'OZYA'*	This study
λHB128	*gnd'-128*::*trp'CBA'* ΔW209 *lac'OZYA'*	This study
λHB417	*gnd'-417*::*trp'CBA'* ΔW209 *lac'OZYA'*	This study
λHB814	*gnd'-814*::*trp'CBA'* ΔW209 *lac'OZYA'*	This study

[a] See reference 4.

out with strain W3110. Accordingly, *gnd-lac* fusions isolated in strain HB11 were introduced into strain HB351, which was prepared as follows. First, an *edd-zwf* deletion was introduced into strain W3110 by the method described above to form strain W3110-17. The *lac-169* deletion could then be introduced because it is linked to *phoA*::Tn*5* in strain MPh30. Thus, strain W3110-17 was transduced to Kan^r with a P1 lysate prepared on strain MPh30, and the transductants were scored for Lac$^-$ on lactose MacConkey indicator plates. The last step of the construction was to repair the *phoA*::Tn*5* mutation by transducing the strain to growth on glucose minimal plates containing 5-bromo-4-chloro-3-indolyl phosphate with a P1 lysate prepared on strain W3110 and scoring for the blue color which is indicative of *phoA*$^+$ strains on these plates (14) and for Kan^s and Lac$^-$.

Bacteriophages and phage methods. Table 2 lists the bacteriophages used. P1CM *clr100* (designated P1) lysates were prepared by heat induction of lysogens and used for generalized transduction as described by Miller (10). Lysates of phage Mu *d*1 were prepared by heat induction of strain MAL103, as described by Casadaban and Cohen (5). Lysates of λ *cI*, λ *vir*, and λp1(209) were prepared by the plate method (10). High-titer lysates of plaque-forming λ phages carrying the various *gnd-lac* fusions were prepared as described by Berman and Beckwith (2). The λpl(209) lysogenic derivatives of the fusion strains were induced by irradiation with UV light. The lysates were titrated with strain MC4100-Y on lactose MacConkey plates and contained 10^5 to 10^7 PFU/ml, 8 to 20% of which were Lac$^+$ (Red) (i.e., the putative λ p*gnd-lac* phages). Lac$^+$ plaques were cloned, and lysates were prepared by the plate method.

Specialized transductions with λ p*gnd-lac* fusion phages were carried out as follows. Recipient cells were grown to saturation, washed in 0.01 M $MgSO_4$, and infected at a multiplicity of infection of 1 with high-frequency transducing lysates. Cells and phage were allowed to adsorb at 30°C for 20 min. F-top agar (2.5 ml) (10) was then added, and the mixture was plated onto gluconate minimal medium.

Mutagenesis by bacteriophage Mu *d*1 and methicillin enrichment for *gnd* mutants. Cultures of strain HB11 were mutagenized with phage Mu *d*1 by the liquid infection method of Casadaban and Cohen (5). Each pool contained more than 15,000 independent Mu *d*1 lysogens, the number calculated from the Poisson distribution to give a 0.95 probability that a *gnd*::Mu *d*1 mutant would be present, assuming that the *E. coli* genome contains 3,000 genes. A modification of a previously reported method (16) was then used for enrichment of *gnd* mutants. After prior growth at 30°C in glucose minimal medium containing ampicillin, the cells were washed, diluted to a density of about 5×10^7 cells per ml in gluconate minimal medium, and incubated at 30°C until the culture reached a density of about 2×10^8 cells per ml. Methicillin was added to a concentration of 10 mg/ml, a concentration shown in pilot experiments to lyse growing cultures of Mu *d*1 lysogens. The cultures were incubated overnight, washed, diluted appropriately, and spread onto gluconate BTB indicator plates. White (Gnd$^-$) colonies which appeared after incubation at 30°C for 2 days were cloned, and their phenotypes were further characterized.

Conversion of Mu *d*1 prophages to λp1(209) prophages. The method of Komeda and Iino (9) was used. Strains carrying *gnd*::Mu *d*1 fusions were spread onto H plates (10), and drops of a lysate of λp1(209) were applied. Turbid centers of plaques were picked and streaked onto either lactose MacConkey plates at 42°C to select directly for the desired recombinants or TYE plates at 30°C. Lac$^+$ clones from the 42°C plates were scored for Ap^s and resistance to λ *cI*. To obtain the replacement derivatives without selection, λp1(209) lysogens were identified by picking single colonies from the TYE plates with a toothpick and testing the clones for the presence of λ immunity by streaking them across lysates of λ *cI* and λ *vir*. Most λp1(209) lysogens prepared in this manner were Ap^r and thus still retained the Mu *d*1 prophage. The desired segregants were obtained readily by restreaking onto lactose MacConkey plates at 30°C and scoring Lac$^+$ clones for Ap^s, temperature resistance, and λ immunity.

Introduction of *gnd-lac* fusions into the genetic background of strain W3110. Strain HB351 was transduced to growth on lactose minimal medium with P1 lysates prepared on three of the *gnd*::Mu *d*1 mutants, strains HB1210, HB127, and HB816. Transductants were cloned once on the selective medium and then tested for the stability of the Lac phenotype by streaking onto lactose MacConkey plates and for the Gnd phenotype by streaking onto gluconate BTB indicator plates. Most of the transductants of each cross turned out to be stably Lac$^+$ Gnd$^-$ and immune to λ. One such transductant from each cross was chosen, and the *edd-zwf* deletion was repaired by transduction to

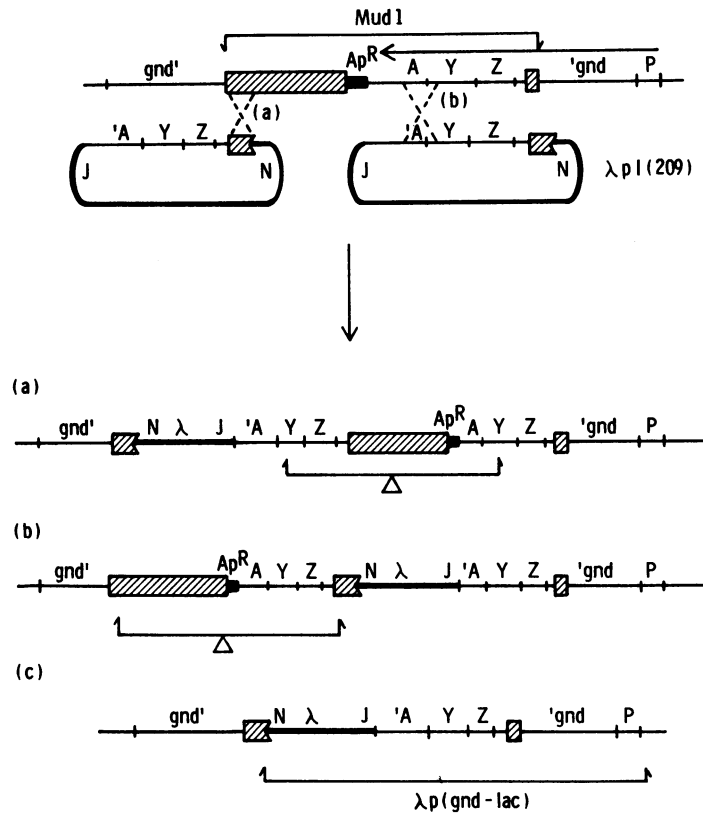

FIG. 1. Scheme for conversion of a *gnd*:::Mu *d*1 lysogen to a *gnd*::λp1(209) lysogen and formation of a Lac$^+$ transducing phage carrying a *gnd-lac* operon fusion. The top line shows a Mu *d*1 (Ap^r *lac*) prophage inserted into *gnd* such that transcription initiating at the *gnd* promoter proceeds across the S end of the phage (small cross-hatched area) into the *lacZYA* genes. λp1(209), which lacks the λ phage attachment site, can integrate into the Mu *d*1 prophage by general recombination, using Mu-Mu homology (crossover a) or *lac-lac* homology (crossover b). Excision of a Mu *d*1 prophage by homologous recombination at *lac* (a) or Mu (b) sequences yielded a *gnd*::λp1(209) (Lac$^+$) fusion (c). Induction of the prophage and illegitimate recombination can result in the formation of a λ p*gnd-lac* (Lac$^+$) transducing phage. Symbols: —, bacterial DNA; →, direction of transcription, with promoter shown as P; cross-hatched areas, bacteriophage Mu DNA; black box, Ap^r gene; dark line, bacteriophage DNA; Δ, possible deletion after homologous recombination.

growth on gluconate minimal medium with a P1 lysate prepared on strain EF1 to yield strains HB354, HB361, and HB381.

Bacterial matings. Episome transfer experiments were carried out by the plate mating technique of Miller (10).

Measurements of enzyme specific activity. The activities of 6PG dehydrogenase and G6P dehydrogenase in sonic extracts were assayed spectrophotometrically as described by Wolf et al. (17); 1 enzyme unit was equivalent to 1 nmol of NADPH formed per min at 25°C. β-Galactosidase activity was assayed spectrophotometrically in sonic extracts by measuring the rate of *o*-nitrophenol formation in the assay system described by Miller (10); 1 enzyme unit was equivalent to 1 nmol of *o*-nitrophenol formed per min at 28°C. The protein content was determined by the method of Bradford (3), using immunoglobulin G as the standard. Measurements were made with a Gilford model 250 spectrophotometer equipped with a constant-temperature circulating water bath.

RESULTS

Strains with *gnd-lac* operon fusions were prepared by the method of Casadaban and Cohen (5). This method makes use of specialized transducing bacteriophage Mu *d*1, whose structure is shown in Fig. 1. Bacteriophage Mu *d*1 has the following features that facilitate construction of operon fusions: (i) phage Mu *d*1, like phage Mu itself, is a translocatable element that can insert at random sites in the *E. coli* chromosome and hence is a nonspecific mutator; (ii) pools of random, phage Mu *d*1-induced mutants can be prepared easily because the phage carries a gene for ampicillin resistance; (iii) Mu *d*1 carries the structural genes of the lactose operon, but not the promoter, and therefore expression of the *lac* genes occurs only when the prophage is located in the proper orientation in a transcrip-

tionally active gene. Aside from the simplicity of Mu *d*1, there were two reasons for making fusions via this phage rather than with in vitro methods and bacteriophage λ (11) or a plasmid (6). Since Mu *d*1 inserts at random, it is possible to isolate fusion strains that have fusion joints at many different sites within a gene; this feature of the method could be of considerable importance if the target site for growth rate-dependent regulation of *gnd* were within the structural gene. Second, the fusions would be present in the cells in a single copy and at the same position on the chromosome as *gnd* itself, thereby eliminating possible complications due to gene dosage effects.

Isolation of *gnd*::Mu *d*1 mutants. Eight independent pools of Mu *d*1 lysogens of strain HB11 were prepared, enriched for Gnd$^-$ mutants with a concentration of methicillin high enough to overcome the prophage-encoded β-lactamase activity, and plated onto gluconate BTB indicator plates containing ampicillin at 30°C. Gluconate-negative, Ap^r colonies appeared in seven of the selections at a frequency of about 6×10^{-3} per cell plated and were tested further by streaking onto gluconate minimal medium and lactose MacConkey indicator plates at 30°C and on TYE plates at 30 and 42°C. Four independent gluconate-negative, Ap^r, Lac$^+$, temperature-sensitive clones were identified (strains HB125, HB128, HB417, and HB814). These clones were shown by enzyme assay to have no detectable 6PG dehydrogenase activity and hence are Gnd$^-$.

To determine whether the entire phenotype was due to the presence of a Mu *d*1 prophage at *gnd*, the four mutants were transduced to growth on gluconate minimal medium at 30°C with P1 lysate prepared on strains W3110-17 [Δ(*edd-zwf*) *gnd*$^+$] and EF1 [*edd*$^+$ Δ(*his-gnd*)]. All of the Gnd$^+$ transductants, which were selected by using the former lysate, were Lac$^-$, Ap^s and temperature resistant, whereas all of the Edd$^+$ transductants were Lac$^+$, Ap^r, and temperature sensitive. Therefore, the phenotypes of the mutants were due to the presence of a Mu *d*1 prophage located at *gnd*, and there were no other Mu *d*1 prophages in these strains. However, as discussed below, this fact does not prove that expression of the lactose operon is under the control of the *gnd* promoter in these strains.

Replacement of the Mu *d*1 prophages with λp1(209) and preparation of lambda phages carrying the *gnd-lac* fusions. Fusion strains lysogenic for phage Mu *d*1 are unsuitable for physiological studies and for genetic selections based on the Lac phenotype because they are temperature sensitive and because the prophage can transpose to additional sites on the chromosome. To overcome these drawbacks, we used the approach of Komeda and Iino (9) (Fig. 1), in which the Mu *d*1 prophage was replaced with λp1(209). An additional benefit of this method is that it provides a simple way to prepare specialized lambda transducing phages carrying the fusions for subsequent mapping of the fusion joints.

An Edd$^+$ Tc^s transductant of the four fusion strains was infected with phage λp1(209). With three of the strains (strains HB126, HB418, and HB815), the infected cells were streaked onto lactose MacConkey plates at 42°C, thereby selecting directly the desired Mu *d*1-deleted derivatives.

It was also possible to effect the replacement by an indirect screening method that reduced the possibility of selecting a Mu *d*1-induced deletion mutant. Strain HB129 was infected with phage λp1(209), and the infected cells were streaked onto TYE plates at 30°C. Mu *d*1, λp1(209) dilysogens were identified by screening clones for the composite phenotype (viz., Lac$^+$, Ap^r, temperature sensitive, and immune to phage λ). The dilysogens were unstable and segregated the desired λp1(209) monolysogens at a frequency of about 0.2 per cell.

Figure 1 shows the recombination events that can lead to the formation of transducing phages carrying the *gnd-lac* fusions. UV-induced lysates were prepared from each of the four λp1(209) lysogens (strains HB127, HB1210, HB419, and HB816), and plaque-forming transducing phages carrying the fusions were isolated as described above. The numbers of plaque-forming units in the primary lysates were low (10^5 to 10^7 PFU/ml), but about 8 to 20% of the plaques were Lac$^+$. The transducing phages were cloned, and higher-titer lysates of each were prepared. The phages were given the same designations as the original fusion strains (i.e., λHB125, λHB128, λHB417, and λHB814).

Genetic proof of *gnd-lac* fusion. Bacteriophage Mu often forms deletions adjacent to its site of insertion (15). Therefore, before conducting physiological studies with the fusion strains, we had to prove that transcription of the *lac* genes was dependent on the *gnd* promoter. A genetic method for doing this is shown in Fig. 2. The rationale for this method is that if a mutation that blocks expression of *gnd* (e.g., a promoter mutation) also blocks expression of the *lac* genes in a putative *gnd-lac* fusion strain, then *lac* must be fused to *gnd* in that strain.

Strains RW187-1, -2, -11, and -12 carry deletions that enter *gnd* from the promoter-proximal end and terminate at different sites within the gene (12). These mutants were infected with the fusion transducing phages. Gnd$^+$ recombinants were selected on gluconate minimal medium containing tryptophan, histidine, and citrate and scored for the Lac phenotype by streaking on

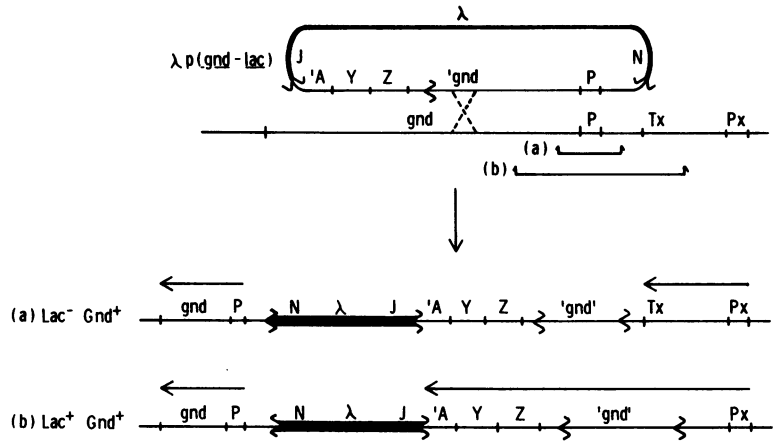

FIG. 2. Genetic proof of *gnd-lac* fusion. A Lac$^+$ transducing phage carrying a presumptive *gnd-lac* fusion was used to infect strains RW187-12, -1, -2, and -11, which contain deletions entering *gnd* from the promoter-proximal side. A single reciprocal recombination event between homologous *gnd* gene sequences generated a *gnd*$^+$ transductant. (a) Gnd$^+$ Lac$^-$ transductants resulted when the *lac* genes were displaced from a transcriptionally active promoter. (b) Gnd$^+$ Lac$^+$ transductants resulted when the *lac* genes were displaced from the *gnd* promoter and were fused to another transcriptionally active gene oriented in the same direction as *gnd*. Gnd$^+$ Lac$^-$ transductants could also arise in a cross with a type (a) deletion if the λ p*gnd-lac* transducing phage carried a portion of gene "X" and a second homologous recombination event occurred there. Symbols: —, bacterial chromosome; ←, direction of transcription, with promoter shown as P; dark line, lambda phage gene; ⦚, fusion joint.

glucose-XG plates (Table 3). Transducing phages λHB128, λHB417, and λHB125 were able to transduce strain RW187-12 to Gnd$^+$, and most of the transductants were Lac$^-$. Thus, the *lac* genes were under the control of the *gnd* promoter in the strains from which the transducing phages were derived. (The few Gnd$^+$ Lac$^+$ transductants obtained in the various crosses were unstable, as shown by the segregation of white [Lac$^-$] colonies on glucose-XG plates; these transductants probably resulted from the integration of a second fusion phage, either within the first phage or at *lac*.)

Gnd$^+$ recombinants were not recovered in the crosses between the λHB814 fusion phage and the promoter deletion strains, presumably because the fusion joint in strain HB814, from which λHB814 was derived, lies within the re-

TABLE 3. Mapping of *gnd-lac* fusion joints[a]

Recipient	Phage					
	λp1(209)	λHB128	λHB417	λHB125	λHB814	λHB123
RW187-12	0	+	+	+	0	0
RW187-1	0	+	0	0	0	0
RW187-2	0	+	0	0	0	0
RW187-11	0	0	0	0	0	0
JC2	0	+	NT	+	0	NT
JC6b	0	+	NT	+	0	NT
JC7	0	+	NT	+	+	NT
HB814	0	+	+	+	0	0
HB125	0	+	+	0	0	0
HB123	0	+	+	0	0	0
HB417	0	+	0	0	0	+
HB128	0	0	0	0	0	+

[a] Strains were infected with λp *gnd-lac* fusion phages. *gnd*$^+$ recombinants were selected and scored for the Lac phenotype as described in the text. +, Recovery of *gnd*$^+$ transductants; 0, no transductants were detected ($<10^7$); NT, not tested. In each cross with strains RW187-12,-1,-2, and -11 and strains JC2, JC6b, and JC7, some of the *gnd*$^+$ transductant were Lac$^+$, whereas most or all of the *gnd*$^+$ transductants of strains HB814, HB125, HB123, HB417, and HB128 were Lac$^-$, except for crosses with λHB123, which gave predominantly Lac$^+$ transductants. See the text for details.

gion deleted in the mutants. As an alternative approach in determining whether *lac* is fused to *gnd* in strain HB814, we crossed the λHB814 fusion phage with mutants JC2, JC6b, and JC7, which carry bacteriophage Mu *c*ts-induced *gnd* mutations that lie within the region that is missing in the promoter deletion strains (12, 16). The rationale for this cross was similar to the rationale described above, namely, that if a polar mutation in *gnd* (e.g., a *gnd*::Mu *c*ts mutation) can reduce *lac* expression when it is introduced into a fusion strain, then the *lac* genes must be fused to the *gnd* promoter in that strain. The results of the crosses are shown in Table 3. Crosses between λHB128 and λHB125 and the *gnd*::Mu mutants gave rise to Gnd^+ Lac^- transductants. Our results show that the mutations in strains JC2, JC6b, and JC7 are polar and confirm the map positions of these mutations. Gnd^+ Lac^- transductants were also recovered when the λHB814 fusion phage was crossed with strain JC7. From these results we conclude that *lac* is fused to *gnd* in strain HB814.

Mapping of fusion joints. The data in Table 3 place the fusion joint in strain HB814 as most promoter proximal and the fusion joint in strain HB128 as most promoter distal, but the relative order of the fusion joints in strains HB417 and HB125 could not be determined. To complete the map of fusion joints, we transduced the four original fusion strains (HB814, HB125, HB417, and HB128) to Gnd^+ with the fusion phages. This mapping strategy is analogous to selecting for recombination between a point mutation (*gnd*::Mu *d*1) and a deletion (the fusion phage). The results are shown in Table 3, and the complete map of the fusion joints is shown in Fig. 3.

The only caveat to using these results to determine the order of the fusion joints is that if a given *gnd-lac* fusion phage also carries a portion of *gnd* distal to the fusion joint, then it could recombine with point mutations on either side of the fusion joint. Indeed, λHB123, a transducing phage obtained from a sibling of strain HB125, appears to carry only the promoter-distal portion of *gnd* (Table 3).

It is also interesting that most of the Gnd^+ transductants obtained when the λHB814, λHB125, λHB417, and λHB128 fusion phages were crossed with the *gnd*::Mu *d*1 mutants were Lac^-. This result was unexpected, since both donor and recipient were Lac^+ and only a single crossover event in *gnd* was selected, but it can be explained by proposing that a second recombinational event occurred on the promoter-proximal side of the Mu *d*1 insertion. In fact, the Gnd^+ Lac^- transductants were λ *c*I sensitive. Thus, there was high negative interference in these crosses, perhaps because of the IS*5* element located about 500 base pairs upstream from *gnd* (B. A. Jordan, M. S. Nasoff, and R. E. Wolf, Jr., manuscript in preparation). This hypothesis is supported by the fact that λHB123 gave predominantly Gnd^+ Lac^+ transductants when it was crossed with strains HB128 and HB417, presumably because it lacked the promoter-proximal portion *gnd* and IS*5*.

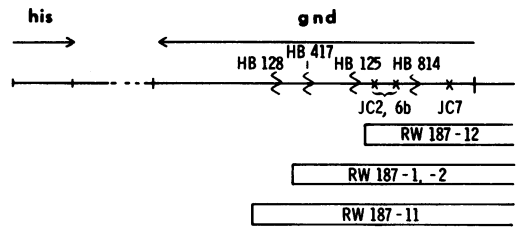

FIG. 3. Genetic map of *gnd-lac* fusion joints. This map was derived from the data in Table 3. Symbols: ⦚, location of *gnd*::Mu *d*1 (Ap^r *lac*) insertion mutations; X, location of phage Mu *cts61*-induced *gnd* mutations (the relative order in strains JC2 and JC6b has not been determined); ═, portion of *gnd* deleted in the promoter deletion strains; ←, direction of transcription.

Effect of growth rate on β-galactosidase levels in operon fusion strains. Most physiological studies of growth rate-dependent regulation of the level of 6PG dehydrogenase have been carried out with strain W3110, whereas the fusions were isolated in the genetic background of strain MC4100. Accordingly, we compared the effects of growth rate on the levels of the enzyme in these two strains. Although strain MC4100 grew at about twice the rate of strain W3110 under all nutritional conditions used, the absolute specific activities of the enzyme were nearly identical in the two strains for each carbon source tested. The regulation of G6P dehydrogenase was also similar in the two strains.

The specific activity of β-galactosidase was measured in operon fusion strains growing on glucose and acetate morpholinepropanesulfonic acid minimal media; G6P dehydrogenase was measured as a control. Some measurements were made with the fusions in the strain MC4100 genetic background, whereas others were made after the fusions had been introduced by P1 transduction into strain HB351, a derivative of strain W3110 (see above for construction of strains HB354, HB361, and HB381). Figure 4 shows that the level of β-galactosidase was invariant in each fusion strain, whereas the level of G6P dehydrogenase showed the normal positive correlation with growth rate. Furthermore, the level of β-galactosidase was not affected by the genetic background (Fig. 4).

The level of β-galactosidase in the fusion strains might have been invariant because

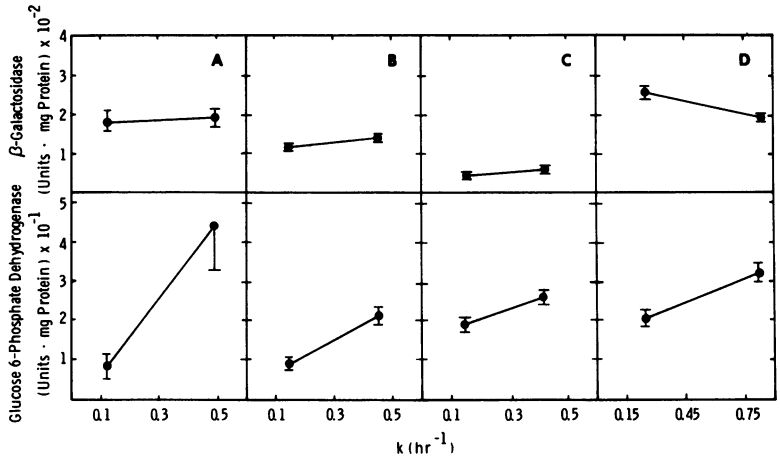

FIG. 4. Growth rate dependence of β-galactosidase and G6P dehydrogenase in *gnd-lac* operon fusion strains HB354 (A), HB381 (B), HB361 (C), and HB419 (D). Each point represents the average value obtained from two assays per culture from two or more independent cultures. Error bars represent the 95% confidence intervals of the means. Cultures were grown in acetate and glucose morpholinepropanesulfonic acid minimal media. Sonic extracts were prepared and assayed for β-galactosidase and G6P dehydrogenase as described in the text.

growth rate-dependent regulation is dependent upon the action of 6PG dehydrogenase itself at the *gnd* promoter or at some other site between the promoter and the fusion joint. To test this possibility, we introduced an F *gnd*$^+$ episome into fusion strain HB355 and measured the β-galactosidase levels in cultures growing on glucose and acetate. Figure 5 shows that the presence of 6PG dehydrogenase had no effect on the level of β-galactosidase in the merodiploid strain.

DISCUSSION

The most important result of the work described here is that the level of β-galactosidase did not vary with growth rate in four independent *gnd-lac* operon fusion strains, nor did it vary when wild-type *gnd* was also present. This result sheds light on the mechanism of growth rate-dependent regulation of *gnd* only if synthesis of β-galactosidase in these strains is dependent on transcription originating at the *gnd* promoter (i.e., only if in these strains *lac* is fused to *gnd* and not to another gene). The genetic methods used to verify that the structural genes of the lactose operon are fused to the *gnd* promoter were based on the methods devised by Berman and Beckwith (2) for *tyrT-lac* fusions. Lac$^+$ transducing phages λHB128, λHB417, and λHB125 were able to transduce to Gnd$^+$ (Table 3) strain RW187-12, which carries a deletion that enters *gnd* from the promoter-proximal end (12, 16), and most of the transductants were Lac$^-$. The simplest interpretation of these results is

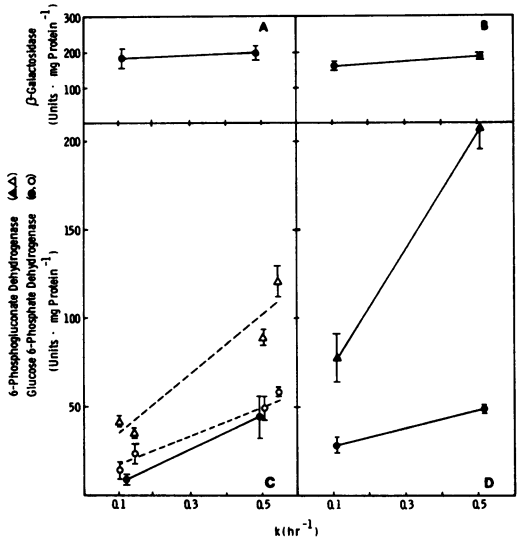

FIG. 5. Effect of 6PG dehydrogenase on the β-galactosidase level in a *gnd-lac* operon fusion strain. (A and C) Solid lines indicate β-galactosidase (A) and G6P dehydrogenase (C) levels in fusion strain HB354. Dotted lines indicate 6PG dehydrogenase and G6P dehydrogenase levels in the control strain, strain W3110. (B and D) β-Galactosidase level in strain HB356, which carries an F′ *his*$^+$ *gnd*$^+$ episome and a *gnd-lac* fusion. Each point represents the average of two assays per culture from two or more cultures. Error bars represent the 95% confidence intervals of the means. Cultures were grown in acetate and glucose morpholinepropanesulfonic acid minimal media. Sonic extracts were prepared and specific activities were measured as described in the text.

that the strains from which the transducing phages were derived carried *gnd-lac* fusions and that the fusion joints are promoter distal to the endpoint of the deletion. According to this interpretation, the Gnd^+ transductants arose by a single reciprocal recombination event in the region of homology in *gnd* on the phage and on the chromosome, as shown in Fig. 2; such transductants would be Lac^- because the recombination event would displace *lac* from the wild-type *gnd* promoter and fuse it to the promoter deletion. (Note that these transductants would have been Lac^+ if the *gnd* promoter deletion had actually fused *gnd* to a transcriptionally active gene oriented in the same direction.) An alternative explanation is that in the three strains *lac* is fused to a gene located on the promoter-distal side of *gnd* and that the transducing phages derived from these strains carry the fusions and the region of *gnd* missing in the deletion. If this were true, then the Gnd^+ Lac^- transductants would have arisen by two homologous recombination events, one within *gnd* and the other on the promoter-proximal side of the gene. However, this interpretation can be ruled out because in strain RW187-12 *gnd* is carried on a λ h80 d*gnd his* prophage integrated at *att*φ80 and the *tonB-gnd* deletion in this strain removes all of the potential homology between the fusion phage and the chromosome on the promoter-proximal side of *gnd* (16). Thus, we conclude that *lac* is fused to *gnd* in strains HB128, HB814, and HB125 and their derivatives.

No Gnd^+ transductants were obtained when Lac^+ transducing phage λHB417 was crossed with the *gnd* promoter deletion mutants (Table 3). However, the availability of strains JC2, JC6b, and JC7, which carry phage Mu *c*ts-induced *gnd* mutations, enabled us to demonstrate fusion of the *lac* genes to the *gnd* promoter in strain HB417 and to map the fusion joint. We first showed that the Mu *c*ts-induced *gnd* mutations in strains JC2, JC6b, and JC7 are polar. When these strains were crossed with the λHB128, λHB417, and λHB125 transducing phages, most of the Gnd^+ transductants were Lac^- (Table 3); i.e., when the mutations were placed by recombination between the *lac* genes and the *gnd* promoter, they were able to block expression of the lactose operon and hence must be polar. (The *gnd* mutations in strains JC2 and JC6b were expected to be polar since they were shown previously to be due to the presence of a Mu *c*ts prophage [16]. Strain JC7 does not carry a complete Mu *c*ts prophage in *gnd* (16), and hence the polar, nonreverting *gnd* mutation is probably a deletion.)

Strains JC2, JC6b, and JC7 were crossed with the λHB417 transducing phage. Two Gnd^+ transductants, both of which were Lac^-, were obtained in the cross with strain JC7, and no Gnd^+ transductants of the other two strains were obtained (Table 3). Since the polar *gnd* mutation in strain JC7 was able to block expression of the lactose operon on the λHB417 phage, then the *lac* genes must be fused to the *gnd* promoter in the strain (strain HB417) from which the transducing phage was derived. The inability of the λHB417 phage to transduce strains JC2 and JC6b to Gnd^+ and the low frequency of recovery of Gnd^+ transductants of strain JC7 show that the fusion joint in strain HB417 is close to, but promoter distal to, the site defined by the *gnd* mutation in strain JC7 and promoter proximal to the sites in strains JC2 and JC6b. The conclusion that *lac* is fused to *gnd* in strain HB417 could be incorrect only if the Gnd^+ Lac^- transductants of strain JC7 obtained with the λHB417 phage arose by two recombination events, one on either side of the *gnd* mutation. This is not likely since strain JC7 carries a deletion that removes most of the potential homology on the promoter proximal side of *gnd* (16).

The most reasonable interpretation of the fact that the level of β-galactosidase does not vary with increasing growth rate in strains with *gnd-lac* operon fusions is that the growth rate-dependent regulation of the level of 6PG dehydrogenase is carried out by a post-transcriptional mechanism. (The variation in the absolute level of β-galactosidase in the four fusion strains is probably due to a polar effect of the fusion joint in several of them.) However, it is also possible that a transcriptional mechanism is involved in the growth rate-dependent regulation and that the target site for this regulation is in the portion of *gnd* that lies beyond the fusion joint in strain HB128.

There is an important caveat in using measurements of β-galactosidase levels in *gnd-lac* operon fusion strains growing at different rates as an index of the effect of growth rate on *gnd* transcription: the yield of β-galactosidase from *gnd-lac* mRNA might vary as a function of growth rate, either because of an inherent property of *lacZ* mRNA itself or because of an effect of growth rate on the degree of polarity in fusions with putative polar fusion joints. The first possibility is not likely to affect the interpretation since β-galactosidase levels in *tyrT-lac* operon fusion strains vary like tRNA (2), but no information is available about the second possibility.

If growth rate-dependent regulation of *gnd* expression is indeed mediated by a post-transcriptional mechanism, what control models accord with the known physiology (7) and DNA sequence (Nasoff and Wolf, manuscript in preparation) of the gene? An analysis of the DNA

sequence with respect to the starting point of transcription showed that there are several potential secondary structures in *gnd* mRNA which could mask the ribosome binding site and reduce translational efficiency if they formed in vivo. Two of these structures involve sequences of the leader and the first two codons of the structural gene, whereas another, with a very long stem and a large loop, includes sequences of the central portion of the structural gene and most of the leader. Thus, growth rate-dependent regulation of *gnd* expression could be carried out by regulating the rate of formation on the stability of one of these structures. Alternatively, because of inefficient ribosome binding, the yield of 6PG dehydrogenase monomers from *gnd* mRNA might be regulated directly by the cellular ribosome concentration, which increases with increasing growth rate. One of the regions of dyad symmetry in *gnd* mRNA could also be a binding site for a putative translational repressor.

Another possible model is that the functional half-life of *gnd* mRNA is inversely proportional to the growth rate. For this model to be consistent with the results obtained with the fusion strains, the target for regulation would have to be toward the 3′ end of the message, since the 5′ ends of *gnd-lac*$^+$ fusion mRNA and normal *gnd* mRNA are the same. Indeed there is a rho-independent transcription terminator at the end of *gnd*, which is part of a longer region of dyad symmetry that might be the site for message inactivation.

Determining which region of *gnd* is the target for growth rate-dependent regulation and whether secondary structures within *gnd* mRNA are important may be accomplished by preparing *gnd-lac* gene fusion strains (4), mapping the fusion joints, and determining the effect of growth rate on β-galactosidase levels in the various strains.

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