

Essential site for growth rate-dependent regulation within the *Escherichia coli gnd* structural gene

(translational control/gene fusions/gene regulation)

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ABSTRACT Expression of *gnd* of *Escherichia coli*, which encodes the hexose monophosphate shunt enzyme, 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44), is subject to growth rate-dependent regulation: the level of the enzyme is directly proportional to growth rate under a variety of growth conditions. Previous results obtained with strains carrying transcriptional fusions of *gnd* to the structural genes of the lactose operon suggested that the growth rate-dependent regulation of *gnd* expression is at the post-transcriptional level. To characterize the regulation further, we prepared with phage MudII a set of eight independent *gnd-lac* gene (protein) fusions. We showed through genetic analysis and DNA sequencing that each fusion joint was located within the 6PGD-coding sequence between the first and second base pair of a codon, the reading frame required for production of a hybrid 6PGD- β -galactosidase. Strains harboring the *gnd-lac* fusion plasmids produced proteins whose mobility in a NaDodSO₄/polyacrylamide gel agreed with the molecular weights predicted from the DNA sequence for the respective hybrid proteins. The level of β -galactosidase was high and relatively growth rate-independent in the fusion whose fusion joint was at codon 48. The level of β -galactosidase in the other seven fusion strains whose fusion joints were located further downstream in the 6PGD-coding sequence showed the same dependence on growth rate as 6PGD in a normal strain. β -Galactosidase levels were not affected by the presence of a *gnd*⁺ gene in *trans* to any of the fusions. The results suggest that all sites necessary for growth rate-dependent regulation of 6PGD level lie in *gnd* upstream from codon 118 and that an essential site of negative control lies within the coding sequence, between codons 48 and 118.

Steady-state growth of bacterial cells derives from a finely tuned balance between the rates of production of energy and carbon skeletons and the rates of synthesis of macromolecules and their small molecule substituents. This balance is achieved by regulating at the genetic level the rates of accumulation of individual proteins and by regulating at the protein level the activities of key enzymes in the various anabolic and catabolic pathways. The genetic regulatory processes, induction and repression, that control expression of genes for carbon source assimilation and for small molecule biosyntheses are understood in considerable molecular detail. Much less is known about the mechanism(s) for regulating the expression of genes that encode essential functions. In particular, virtually nothing is known of a fundamental genetic regulatory process, the coordination between the rates of synthesis of individual proteins and the rate of total protein synthesis.

We have been studying as a model system for growth rate-dependent regulation of gene expression the *Escherichia coli* gene, *gnd*, which codes for the hexose monophosphate shunt enzyme, 6-phosphogluconate dehydrogenase (6PGD; EC

1.1.1.44). Like many *E. coli* proteins, including the components of the translational machinery, the level of 6PGD is proportional to the cellular growth rate (1-3). However, the components of the translational machinery and the remainder of *E. coli* proteins, including 6PGD, are members of different regulatory units. For example, rRNA (4) and r-proteins (5) begin to accumulate at an increased rate almost immediately after a nutritional shift-up, whereas 6PGD and total protein show a distinct lag before their rates of accumulation increase (6). Similarly, synthesis of rRNA and r-proteins is strongly gene dosage-independent and often autoregulated (7-9), whereas synthesis of 6PGD, and presumably most other *E. coli* proteins, is gene dosage-dependent and not autoregulated (6).

Recently we determined the DNA sequence of *gnd* and identified the sites for transcription and translation initiation (10). Analysis of the sequence ruled out several previously proposed transcriptional and translational control models for growth rate-dependent regulation of 6PGD level. Several regions of dyad symmetry were observed within or near the ribosome-binding site, providing the potential for regulating the frequency of translation initiation on *gnd* mRNA. Indeed, the properties of *gnd-lac* operon (transcriptional) fusions prepared with MudI fusions accord with this suggestion in that the levels of β -galactosidase are independent of growth rate in *gnd-lac* operon fusion strains (11). Thus, the growth rate-dependent regulation of 6PGD level appears to be carried out by a post-transcriptional mechanism (11).

Here we describe the preparation with phage MudII of *gnd-lac* gene (protein) fusions and their genetic and physiological properties. We prove by DNA sequencing and by analyzing the proteins produced by strains carrying the fusions that the coding sequences of *gnd* and *lac* are fused in frame at different sites in the *gnd* structural gene. The patterns of growth rate dependence of β -galactosidase level in the fusion strains suggest that a site essential for growth rate-dependent regulation of *gnd* expression lies within the 6PGD-coding sequence.

MATERIALS AND METHODS

Genetics and Physiology. Media and growth conditions have been described (3). Lysates of phage MudII (12) were prepared by heat induction of strain MAL315 (12). Isolation and characterization of phage MudII-induced *gnd* mutants of strain HB351 $\Delta(\text{argF-lac})U169 \text{ zeb-1::Tn10 } \Delta(\text{edd-zwf})22$ (11) employed the same methods used earlier for isolation of *gnd::MudI* mutants (11), except that enrichment was carried out with 200 μg of D-cycloserine per ml. λ *pgnd-lac* phages carrying the respective gene fusions were prepared and used

Abbreviations: 6PGD, 6-phosphogluconate dehydrogenase; bp, base pair(s).

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for mapping of fusion joints by previously described methods (11).

Physiological experiments were carried out as described (3) by using appropriately supplemented 3-(*N*-morpholino)-propanesulfonic acid minimal medium. Growth rates are expressed as the specific growth rate constant (k), which was calculated from the expression $k = \ln 2 / \text{mass doubling time}$ (in hours). β -Galactosidase activity was assayed in cells permeabilized by treatment with chloroform and NaDodSO₄ and expressed in Miller units (13). The specific activity of 6PGD was determined in sonic extracts (3).

Cloning and DNA Sequencing of *gnd-lac* Gene Fusions. λ *pgnd-lac* phages carrying the gene fusions were precipitated from lysates with polyethylene glycol and purified on glycerol block gradients (14). Phage DNA was extracted by a NaDodSO₄/phenol treatment (14) and passed over Elutip-d columns (Schleicher & Schuell). The DNA was digested with *Eco*RI. The *Eco*RI fragments were then ligated (14) to plasmid pMLB524 (15) that had been digested with *Eco*RI and treated with bacterial alkaline phosphatase (14). Strain HB351 was transformed (14) with the ligation mixtures and the transformed cells were plated on tryptone/yeast extract (TYE) medium containing ampicillin (Ap) and 5-bromo-4-chloro-3-indolyl β -D-galactoside. Plasmids were purified from Lac⁺ transformants and shown by restriction mapping to harbor the respective *gnd-lac* gene fusions. The names of the plasmids signify the fusion alleles they carry.

To obtain templates for determining the DNA sequence of gene fusion joints, plasmids carrying the respective fusions were prepared from strain NF3079 [*dam-3* Δ (*lacX74*)], digested with *Bam*HI and *Bcl* I, and the resulting fragments were ligated into the *Bam*HI site of phage M13mp8 replicative form II DNA. The ligation mixtures were used to transform strain KK2186, and recombinant phages were identified and cloned as described by Messing *et al.* (16). Lysates of phage M13 and its derivatives were prepared by the method of Schreier and Cortese (17) as modified by New England Biolabs. The hybridization test of Barnes (18) was used to identify recombinant phages that have the Mu *s* end of the fusion adjacent to the universal priming site; such phages carry the sense strand of *gnd*. We had previously prepared a phage, mKD14 (10), which carries the sense strand of *gnd*, by cloning the *Bcl* I-*Pst* I fragment of *gnd* into the *Bam*HI and *Pst* I sites of M13mp8. This phage was used to identify a recombinant M13 phage, mHB560-9, that carries the anti-sense strand of *gnd* DNA. DNA from phage mHB560-9 was then used as the hybridization probe to identify recombinant phages that carry the respective *gnd-lac* fusion fragments in the desired orientation. DNA from these latter phages was used as the templates for dideoxy sequencing (19).

Electrophoretic Analysis of Hybrid Proteins. Strains containing the fusion plasmids were grown in L broth. Portions of exponential-phase cultures were lysed and subjected to electrophoresis on 10% NaDodSO₄/polyacrylamide gels (20). Coomassie blue (R250) was used to visualize the proteins.

RESULTS

Rationale. The level of β -galactosidase in *gnd-lac* operon fusion strains is independent of growth rate, whereas the level of 6PGD is directly proportional to growth rate (11). The

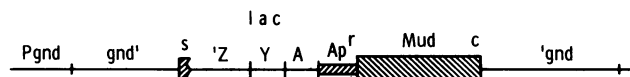


FIG. 1. Genetic structure of phage MudII fused to *gnd*.

interpretation of these data was that growth rate-dependent regulation of 6PGD level is carried out by a post-transcriptional mechanism or that the regulation requires a site near the promoter-distal end of the gene (11). We have used the properties of *gnd-lac* gene fusions prepared with phage MudII to distinguish between these two possibilities and to aid in the further characterization of the growth rate-dependent regulation.

The structure of phage MudII is shown in Fig. 1. A lactose operon without its promoter, operator, Shine-Dalgarno sequence, and the first 22 base pairs (bp) of the *lacZ* structural gene is immediately adjacent to 117 bp of Mu DNA at the *s* end of the phage (21). Expression of the *lac* genes occurs when the phage inserts between the first and second base pair of a codon of a transcriptionally active gene in the orientation that places the *s* end of the phage closer to the gene's promoter. Thus, in a *gnd-lac* gene fusion, production of a hybrid 6PGD- β -galactosidase would depend on transcription initiating at the *gnd* promoter and on translation initiating at the *gnd* ribosome-binding site. Expression of the hybrid gene would be subject to regulation by all regulatory sites in *gnd* that lie upstream from the fusion joint. Accordingly, the pattern of response of β -galactosidase level to changes in growth rate in a set of *gnd-lac* gene fusions can be used to map the site(s) in *gnd* responsible for growth rate-dependent regulation of 6PGD level. For example, if the level of β -galactosidase in all of the members of a set of gene fusion strains were to vary with growth rate like 6PGD, then the sites necessary for the regulation must lie upstream from the site defined by the most promoter-proximal fusion joint. On the other hand, if β -galactosidase levels were independent of growth rate in the fusion strains with promoter-proximal fusion joints but were proportional to growth rate in fusions with more distal fusion joints, then an essential regulatory site could be assigned to the region bracketed by the two classes of fusions. Finally, if β -galactosidase levels were independent of growth rate in all gene fusions, then the regulation would likely require a site downstream from the 6PGD-coding region.

Isolation and Genetic Characterization of *gnd-lac* Gene Fusions. Eight independent mixtures of MudII lysogens of strain HB351 were prepared and enriched for Gnd⁻ mutants with D-cycloserine. The cultures were plated on lactose MacConkey indicator plates containing ampicillin at 30°C. Lac⁺ Ap^r colonies were picked and tested for their Gnd phenotype and for the temperature sensitivity associated with phage MudII lysogens. At least one Ap^r, Lac⁺, Gnd⁻, temperature-sensitive clone isolated from each pool proved by enzyme assay to lack detectable 6PGD activity and hence to be a *gnd* mutant (strains HB516, HB520, HB533, HB543, HB550, HB560, HB570, and HB580).

The fusions were characterized genetically (22). First, a previously described genetic method (11) was used to show that the Lac⁺ phenotype of the fusion strains was due to the insertion of phage MudII into *gnd* and that no other MudII

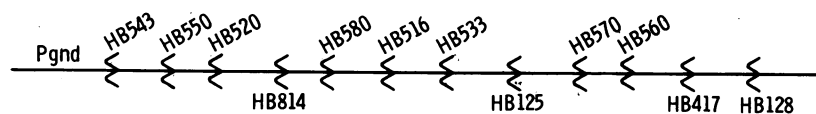


FIG. 2. Genetic map of *gnd-lac* gene fusion joints. Gene fusion joints are listed above the line and operon fusion joints are listed below the line. Gene fusions were prepared with phage MudII as described in the text. The preparation of the operon fusions and the relative order of their fusion joints has been described (11).

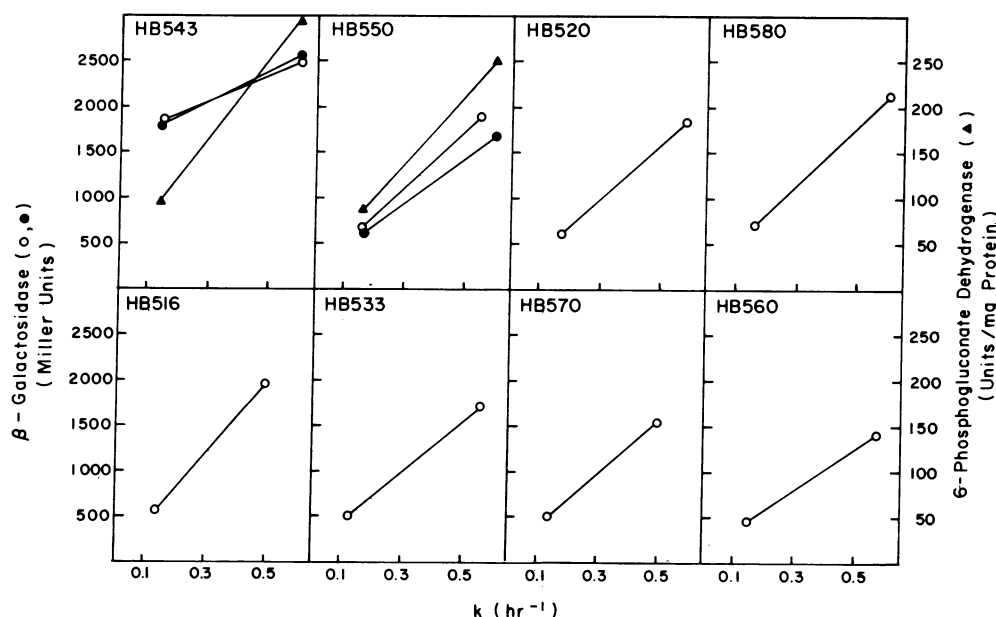


Fig. 3. Growth rate dependence of β -galactosidase activity in *gnd-lac* gene fusion strains. Fusion strains lacking and harboring $F'gnd^+$ episomes were grown in acetate and glucose 3-(*N*-morpholino)propanesulfonic acid minimal medium, permeabilized with chloroform and NaDodSO₄, and assayed for β -galactosidase activity. The F' -containing strains were also assayed for 6PGD activity. Each point represents the average value obtained from two assays per culture of two or more independent cultures. Each panel is marked with the fusion allele that was characterized. The order of panels is the same as the order of the fusion joints. \circ , Haploid fusion strains; \bullet and \blacktriangle , fusion strains carrying the $F'T80\ his^+gnd^+$ episome.

prophages were present in the strains (22). Next, the *c* end of MudII was removed from each fusion and replaced with phage λ by the genetic method of Komeda and Iino (23). Lac^+ λ specialized transducing phages were then prepared from each fusion strain and used in genetic crosses to position the fusion joints on the genetic map of *gnd* (Fig. 2). A previously described genetic method (11) was used to determine whether *lac* is fused to *gnd* in the gene fusion strains. The results showed that *lac* is indeed fused to *gnd* in seven of the gene fusions—namely, HB550, HB520, HB580, HB516, HB533, HB570, and HB560 (22). However, the genetic data obtained with fusion HB543, the fusion with the most promoter-proximal fusion joint, did not rule out the possibility that in this fusion *lac* is fused to an upstream gene (22).

Growth Rate Dependence of β -Galactosidase Levels in *gnd-lac* Gene Fusions. Fig. 3 shows the effect of growth rate on β -galactosidase levels in cultures of the gene fusion strains grown in acetate and glucose 3-(*N*-morpholino)propanesulfonic acid minimal media. Expression of β -galactosidase was growth rate-dependent in the seven strains that were shown by the genetic tests described above to carry *gnd-lac* fusions: the level of the enzyme was 3-fold higher when the strains were grown on glucose than when they were grown on acetate. This shows that all sites necessary for growth rate-dependent regulation lie upstream from the site defined by the fusion joint of strain HB550. In contrast, the level of β -galactosidase in acetate-grown cultures of the strain with the HB543 fusion was about 3-fold higher than in the acetate cultures of the other fusions and the level was increased only about 0.3-fold by growth on glucose—i.e., expression of the HB543 fusion was relatively growth rate-independent.

The presence of a *gnd*⁺ gene in *trans* had no effect on the level of β -galactosidase in any of the eight fusion strains (Fig. 3 and ref. 22). This demonstrates that expression of the fusions is not autoregulated and that the growth rate independence of β -galactosidase level observed with fusion HB543 was not due to the absence of 6PGD.

In addition to assaying β -galactosidase in permeabilized

cells (Fig. 3), we also measured the activity of the enzyme in cell extracts prepared by sonic treatment (data not shown). We found that a significant fraction of the total β -galactosidase activity could be removed from the extracts by centrifugation at $10,000 \times g$ for 10 min. For example, 95% of the activity in sonic extracts of strains with the HB543 fusion, and 65% of the activity in HB550 fusion strains, was present in the pellet fraction after centrifugation, while the supernatant fluids contained the remaining activity and normal amounts of soluble protein. Thus, the *gnd-lac* fusion proteins and perhaps other fusion proteins as well have a strong tendency to aggregate or to associate with sedimentable cell debris.

DNA Sequencing of Fusion Joints. The different patterns of expression of β -galactosidase in strains containing the HB543 and HB550 fusions raised the possibility that the region of *gnd* delimited by the two fusion joints is essential for growth rate-dependent regulation. The likelihood of this interpretation would be greatly increased by proving that *lac* is fused in frame to *gnd* in the fusion strains. This was accomplished by determining the precise location in *gnd* of the respective fusion joints by DNA sequencing. We first prepared a set of plasmids carrying the fusions by cloning *EcoRI* fragments from the *gnd-lac* transducing phages (see Fig. 4) into the *lac* fusion cloning vector pMLB524 (15). The unique *Bam*HI site that is located at the junction between *Mu s* and *lacZ* in each fusion facilitated the subsequent cloning of the fusions from the plasmids into phage M13mp8. We digested each fusion plasmid with *Bam*HI and *Bcl* I [*Bcl* I cleaves in the *gnd* leader (10); see Fig. 4] and ligated the mixtures into *Bam*HI-cleaved phage M13mp8. Recombinant M13 phages were picked. Those carrying the fusion joint adjacent to the universal priming site were identified by the hybridization method described above. We then sequenced from the priming site across the 117 bp of *Mu s* DNA (21) into *gnd* and compared the sequence with the known DNA sequence of *gnd* (10). Fig. 5 shows the position of the fusion joints in *gnd*. Each fusion, including the HB543 fusion, is located between the first and second base pair of a codon in the 6PGD-coding

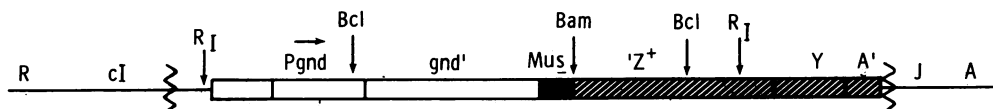


Fig. 4. Genetic and restriction map of a λ *pgnd-lac* gene fusion phage.

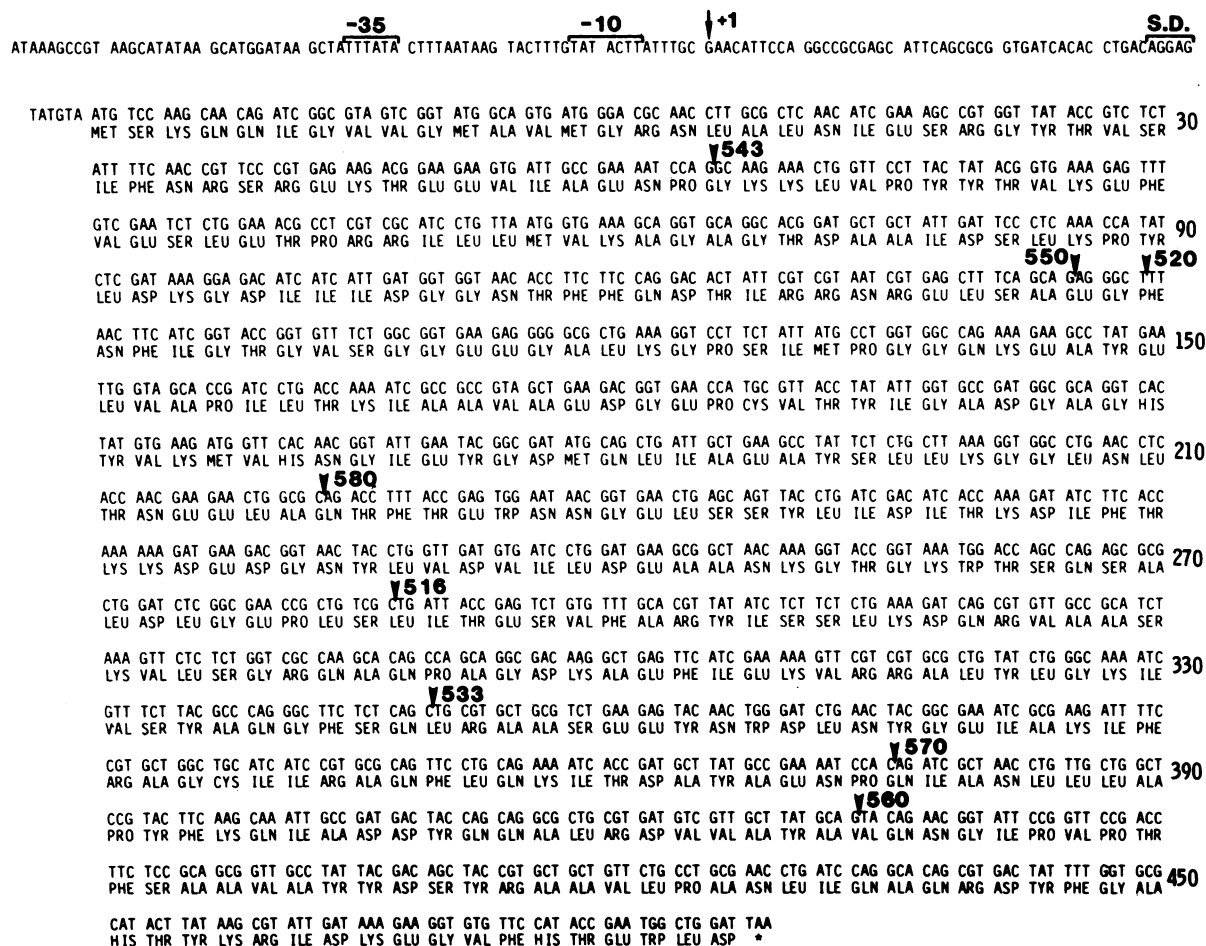


Fig. 5. Location of *gnd-lac* gene fusion joints in the 6PGD-coding sequence of *gnd*. The -10 and -35 regions of the *gnd* promoter, the start of transcription (+1), and the Shine-Dalgarno sequence (S.D.) preceding the *gnd* structural gene are noted (10). The numbers in the right margin refer to the codons in the 6PGD-coding region.

sequence and in the orientation and reading frame for production of a hybrid 6PGD- β -galactosidase. The order of the respective fusion joints on the DNA sequence is the same as on the genetic map.

Characterization of Proteins Produced by *gnd-lac* Gene Fusions. We carried out an experiment to show that each *gnd-lac* gene fusion does, in fact, produce a hybrid β -galactosidase polypeptide whose synthesis originates at the 6PGD start codon and hence is subject to regulation by any *gnd* control sequences that lie upstream from the fusion joint. Cell extracts were prepared from strains carrying the fusion plasmids and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis. Fig. 6 shows that each extract contained a unique protein, appearing in the region of the gel between native β -galactosidase and the β , β' subunits of RNA polymerase. Moreover, there was a high correlation (0.99 by linear regression analysis) between the relative mobility of the unique bands and the logarithm of the molecular weight predicted for each 6PGD- β -galactosidase hybrid polypeptide from the DNA sequence.

DISCUSSION

Genetic, physical, and biochemical criteria were used to show that each of the phage MudII-induced fusion strains carries a *gnd-lac* gene (protein) fusion (Figs. 2, 5, and 6). The level of β -galactosidase produced from gene fusion HB550 and the six other gene fusions with more promoter-distal fusion joints showed the same dependence on growth rate as 6PGD level in both the absence and the presence of a *gnd*⁺ gene in *trans* (Fig. 3). We conclude that all sequences

necessary and sufficient for growth rate-dependent regulation of 6PGD level lie 5' to the HB550 fusion joint and that *gnd* is not autoregulated. Moreover, the fact that the fusion joints of several growth rate-regulated gene fusions (e.g., HB550 and HB520) are upstream from the operon fusion joints (e.g., HB814; see Fig. 2) rules out the possibility mentioned above that the growth rate-dependent regulation requires a control site in the promoter-distal portion of *gnd*.

Compared to the growth rate-"inducible" phenotype of fusion HB550, strains containing the HB543 fusion are "derepressed." These phenotypes and the locations of the respec-

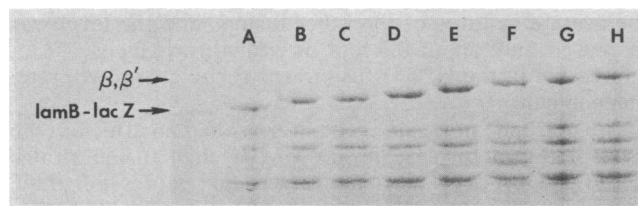


Fig. 6. 6PGD- β -galactosidase hybrid polypeptides produced by strains containing *gnd-lac* gene fusion plasmids. Cell extracts were prepared from strains containing *gnd-lac* gene fusion plasmids (pMLB524 derivatives) and run on a 10% polyacrylamide gel. The extracts were loaded according to the map order of the fusion joints. The high molecular weight region of the gel is shown. Arrows show the positions of a *lamB-lacZ* hybrid protein that was run on an outside lane of the gel and the β , β' subunits of RNA polymerase. Lanes: A, NF3079/pHB543; B, NF3079/pHB550; C, NF3079/pHB520; D, NF3079/pHB580; E, NF3079/pHB516; F, NF3079/pHB533; G, NF3079/pHB570; H, NF3079/pHB560.

tive fusion joints suggest that an essential site (or a portion thereof) for growth rate-dependent regulation of *gnd* expression lies in the 6PGD-coding sequence between codons 48 and 118 and that the site is one of negative control. Alternatively, the derepressed phenotype of the HB543 fusion might be a consequence of the particular *gnd-lac* fusion joint, not a reflection of the regulation of the gene. This seems unlikely in view of the following fact. *gnd* contains a sequence of 13 bp (T-G-C-C-G-A-A-A-T-C-C-A) that is directly repeated in the structural gene. The HB543 fusion joint is 1 bp downstream from one of the repeated sequences; the HB570 fusion joint is 1 bp downstream from the other (Fig. 5). However, the growth rate dependence of β -galactosidase is different in strains containing the two fusions (Fig. 3). Thus, the DNA sequence at the fusion joint of the HB543 fusion cannot be solely responsible for its unique, derepressed phenotype.

The properties of *gnd-lac* operon fusions described previously (11) suggested that the growth rate-dependent regulation of 6PGD level is due to a post-transcriptional control mechanism. According to this interpretation the region between codons 48 and 118 would be a site of translational control. However, we previously qualified the interpretation of the operon fusion data by pointing out that possible polar effects of the operon fusion joints (11), in combination with the fact that expression of *lacZ* is translationally coupled to *trpB* in phage MudI-induced operon fusions (24), might have obscured the "true" growth rate dependence of *gnd* transcription. To examine this possibility we measured the growth rate dependence of galactoside transacetylase in the *gnd::MudII* strains carrying the HB543 and HB550 fusions. The rationale was that in these strains expression of the transacetylase would be dependent on *gnd* transcriptional control signals—i.e., that the *gnd-lacZ* protein fusions were also *gnd-lacA* operon fusions (see Fig. 1). Expression of *lacA* was growth rate-independent in both fusion strains (unpublished results). This supports the conclusion that growth rate-dependent regulation of *gnd* expression is due to a translational control mechanism.

We know of no other example of translational control in bacteria that requires a regulatory site that lies deep within the protein-coding region of the regulated gene. However, several types of negative control of translation have been recognized that involve the ribosome-binding region of mRNA. For example, several proteins are known to act as specific translational repressors by binding to the leader region of a given mRNA (reviewed in ref. 25). In addition, evidence has been presented that RNA molecules can act in *trans* to block translation by hybridizing to the ribosome-binding site of mRNAs (26, 27). Finally, the formation in *cis* of secondary structures in mRNA that sequester the ribosome-binding site can also reduce the translation initiation frequency (28–31). Indeed, translational regulation of *rplJ* mRNA appears to derive from the effect of the binding of the L10-L7/L12 ribosomal protein complex on the formation of alternative secondary structures of the leader (32). It is also possible that mRNA codons that are recognized by tRNAs that are present in low relative molar amounts (33) or by modified tRNAs (34) could be sites for translational control.

Theoretically, any of these general mechanisms of translational control could use a coding sequence, rather than a ribosome-binding site, as a target for regulation and thus any could be responsible for the growth rate-dependent regulation of 6PGD level. By facilitating the isolation of mutants altered in the regulation, the *gnd-lac* gene fusions described here should be useful in elucidating the mechanism of the growth rate-dependent regulation of *gnd* expression and the role of the internal regulatory region.

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