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Evidence for Involvement of Proteins HU and RpoS in Transcription of the Osmoresponsive *proU* Operon in *Escherichia coli*

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Transcription of the *proU* operon of *Escherichia coli* is induced several hundred-fold upon growth at elevated osmolarity, but the underlying mechanisms are incompletely understood. Three *cis* elements appear to act additively to mediate *proU* osmoresponsivity: (i) sequences around a promoter, P1, which is situated 250 bp upstream of the first structural gene *proV*; (ii) sequences around another (σ^{70}-dependent) promoter, P2, which is situated 60 bp upstream of *proV*; and (iii) a negative regulatory element present within the *proV* coding region. These three *cis* elements are designated, respectively, P1R, P2R, and NRE. *trans*-acting mutants with partially derepressed *proU* expression have been obtained earlier, and a vast majority of the mutations affect the gene encoding the nucleoid protein HNS. In this study we employed a selection for *trans*-acting mutants with reduced *proU*$^+$ expression, and we obtained a derivative that had suffered mutations in two separate loci designated *dpeA* and *dpeB*. The *dpeB* mutation caused a marked reduction in promoter P1 expression and was allelic to *rpoS*, the structural gene for the stationary-phase-specific sigma factor of RNA polymerase. Expression from P1 was markedly induced, in an RpoS-dependent manner, in stationary-phase cultures. In contrast to the behavior of the isolated P1 promoter, transcription from a construct carrying the entire *proU* *cis*-regulatory region (P1R plus P2R plus NRE) was not significantly affected by either growth phase or RpoS. The *dpeA* locus was allelic to *hupB*, which along with *hupA* encodes the nucleoid protein HU. *hupA hupB* double mutants exhibited a pronounced reduction in *proU* osmotic inducibility. HU appears to affect *proU* regulation through the P2R mechanism, whereas the effect of HNS is mediated through the NRE.

The *proU* locus in *Escherichia coli* and *Salmonella typhimurium* encodes an active transport system for glycine betaine and L-proline and has been shown to play an important role in the adaptation of these organisms to growth in media of elevated osmolarity (4). Genetic and molecular characterization of *proU* has shown that it is composed of three genes (designated, in order, *proV*, *proW*, and *proX*) (5, 10) whose products constitute a binding-protein-dependent porter system that is a member of the large family of traffic ATPases or ABC transporters described for both prokaryotes and eukaryotes (1, 16).

Transcription of *proU* is induced several hundred-fold upon growth in high-osmolarity media (4, 9), making this quantitatively the most prominent example in which a mechanical stimulus controls gene expression. However, the mechanism of *proU* regulation is as yet poorly understood, and no *trans* mutant in which osmotic regulation of *proU* is completely abolished has been obtained.

Our studies with *E. coli* on the *cis* regulation of *proU* have shown the presence of two promoters, P1 and P2, with the sequences immediately around each conferring five- and eight-fold osmotic inducibility on transcription initiated from the respective promoters (6). The *cis* elements or mechanisms mediating such osmoresponsivity have been referred to in the present study as P1R and P2R, respectively (and are to be distinguished from the promoters P1 and P2 themselves). Transcription from the P2 promoter is known to be initiated by the σ^{70} form of the RNA polymerase holoenzyme (35). A negative regulatory element (NRE) residing in the sequence downstream of P2 (within the first structural gene, *proV*) is also necessary for repression of *proU* expression at low osmolarity and constitutes the third *cis*-regulatory element for *proU* (6). The three mechanisms identified (P1R, P2R, and NRE) appear to contribute additively to *proU* osmoresponsivity. The results of recent studies with *S. typhimurium* by Overdier and Csonka (29) and Owen-Hughes et al. (31) have been consistent with the conclusions for P2R and NRE above, although this organism appears to lack the promoter P1 (and the corresponding mechanism, P1R).

trans-acting regulatory mutants with partially derepressed *proU* expression have been isolated by several investigators, and the overwhelming majority of such mutations are alleles of the *hns* (previously called *osmZ*) gene, which encodes the abundant nucleoid protein HNS. The following lines of evidence indicate that binding of HNS to the NRE is associated with repression of *proU* expression at low osmolarity. (i) *proU* expression is derepressed in *hns* mutants (6, 17, 23, 24, 31) and in *cis* mutants in which the NRE is deleted (6, 24, 29, 31), whereas constructs lacking both HNS and the NRE are derepressed to the same extent as either of the single mutants (6, 24, 31). These experiments also demonstrated that P2R regulation is not significantly affected in *hns* mutants (6, 24, 31). (ii) HNS exhibits preferential binding to bent DNA (31, 34), and a bent-DNA motif has been demonstrated in the 200-bp segment immediately downstream of the *proU* P2 promoter, that is, in the region corresponding to the NRE (31). Lucht et al. (24) have recently also demonstrated the existence of high-affinity HNS-binding sites in this same region. (iii) That HNS does indeed directly repress *proU* transcription in a purified in vitro system (from a template that includes the NRE) has been shown recently by Ueguchi and Mizuno (35).

In this paper, we report the use of a selection strategy to isolate *trans*-acting regulatory mutants with reduced *proU*$^+$

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TABLE 1. Strains of *E. coli* K-12

Strain	Genotype[a]	Source or reference
CAG12017	*zba-3054*::Tn*10*	33
CAG12148	*tsx-247*::Tn*10*	33
CAG18455	*trpB83*::Tn*10*	33
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*	Lab stock
OHP252	*thr leuB6 lacY1 thi tonA21 supE44 hupA*::Cm^r *hupB*::Kan^r	18
RH90	MC4100 *rpoS359*::Tn*10*	22
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)	9
GJ144	λpp*roU-lac*9 lysogen of GJ134	11
GJ1001	MC4100 *proU610*::λp*lac*Mu55(Kan) [φ80d *supF*(Ts)]	From MC4100, in two steps
GJ1003	GJ1001 *hns-201*	This study
GJ1054	GJ134 *dpeA dpeB*/pHYD272	This study
GJ1055	Tp^s derivative of GJ1054	This study
GJ1064	GJ134 *dpeA zba-2101*::Tn*10*dKan	This study
GJ1075	GJ134 *dpeB zfi-2102*::Tn*10*dKan	This study
GJ1083	GJ134 *hns-201 trpB83*::Tn*10*	This study
GJ1084	GJ134 *hupB*::Kan^r	This study
GJ1085	GJ134 *hupA*::Cm^r	This study
GJ1086	GJ134 *hupA*::Cm^r *hupB*::Kan^r	This study
GJ1087	GJ134 *dpeA hupA*::Cm^r *zba-2101*::Tn*10*dKan	This study
GJ1089	GJ134 *dpeA hns-201 trpB83*::Tn*10 zba-2101*::Tn*10*dKan	From GJ134, in two steps
GJ1098	GJ134 *dpeA dpeB tsx-247*::Tn*10 zfi-2102*::Tn*10*dKan	This study
GJ1099	GJ134 *rpoS359*::Tn*10*	This study
GJ1100	GJ144 *hupB*::Kan^r	This study
GJ1101	GJ144 *hupA*::Cm^r	This study
GJ1103	GJ144 *hupA*::Cm^r *hupB*::Kan^r	This study
GJ1105	GJ144 *dpeA zba-2101*::Tn*10*dKan	This study
GJ1107	GJ144 *hupA*::Cm^r *dpeA zba-2101*::Tn*10*dKan	This study

[a] The nomenclature for genetic symbols follows that described by Bachmann (3), with the exception that the locus referred to as *osmZ* in the published linkage map has been designated *hns* here. Allele numbers are indicated if they are known. All strains are F$^-$. The *dpeA* and *dpeB* mutations have been redesignated *hupB200* and *rpoS337*, respectively, on the basis of data described in the text.

expression. One such mutant was demonstrated to have two unlinked mutations, which were allelic to *hupB* and *rpoS*, that affected *proU-lac* transcription. Our data are discussed in terms of a model in which multiple mechanisms, each composed of distinct *cis* elements and *trans*-acting factors, come together to contribute to the 400-fold osmoresponsivity of this operon.

MATERIALS AND METHODS

Chemicals and media. All antibiotics, media constituents, and chemicals were obtained from commercial sources. The media used in this study include MacConkey (Difco), Luria-Bertani (LB) (27), glucose-minimal A (27), and the low-osmolarity K medium with Casamino Acids as a C source (9).

Antibiotics were used at the following final concentrations (micrograms per milliliter) in LB: ampicillin, 50; chloramphenicol, 50; kanamycin, 50; tetracycline, 15; and trimethoprim, 50. The concentrations of antibiotics used in glucose-minimal A- and MacConkey-based media were one-half and one-third, respectively, of those used in LB. 3,4-Dehydro-DL-proline (DHP) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were used in glucose-minimal A plates at final concentrations of 0.3 mM and 25 μg/ml, respectively.

Bacterial strains and phages. All bacterial strains were derivatives of *E. coli* K-12 and are listed in Table 1. Phage P1*kc* was from our laboratory collection. The phage λ1105, used as a vector for transposition of Tn*10*dKan, has been described earlier (36).

Plasmids. The plasmids pHYD272, pHYD275, pHYD283, and pHYD284 have been described earlier (6) and are a panel of *proU*-bearing derivatives of the low-copy-number promoter-cloning vector pMU575 that contains *lacZ*$^+$ as the reporter gene and encodes Tp^r as the antibiotic resistance marker (2). The size and extent of DNA from the *proU* chromosomal locus cloned into each of these plasmids and the salient functional elements carried in the cloned region are described in the legend to Fig. 1. In particular, it may be noted that the *cis* element that mediates fivefold osmoresponsivity of promoter P1 expression, P1R, is operationally defined as that which is present in the region of *proU* DNA and carried by the plasmid pHYD275; similarly, the *cis* element that mediates eightfold osmoresponsivity of promoter P2 expression, P2R, is that which is carried by the *proU* DNA in plasmid pHYD284 (6).

Plasmids pJ004 and pHYD248 are pMU575 derivatives carrying, respectively, the phage T7A$_0$ promoter and a promoter from plasmid pBR322 upstream of the *lacZ* reporter gene and were constructed in this laboratory by R. Mishra and K. Rajkumari, respectively. The multicopy plasmids pK01 (Amp^r) and pMW1 (Amp^r), carrying the wild-type *hupA* and *hupB* genes, respectively, have been described by Huisman et al. (18).

Tagging of transposon insertions close to a mutation of interest. Two mutations that decreased *proU*::*lac* expression were identified in this study, and similar strategies were adopted in order to obtain new transposon insertions cotransducible with each of them. Random transpositions of Tn*10* dKan (a Tn*10* derivative encoding Kan^r) onto the chromosome of the wild-type strain MC4100 were generated following infection with λ1105 as described by Way et al. (36). A P1*kc* lysate prepared on a population of such clones was then used in transduction into the *proU*::*lac*-bearing mutant strain. Kan^r transductants were selected on lactose indicator plates, on which the Lac phenotype of the clones that had also acquired the wild-type allele (occurring at an approximately 1% frequency) was distinguishable from that of the remainder. The former were purified, and P1*kc* lysates prepared on each of

them were used again in individual transductions into the mutant strain in order both to determine linkage between the transposon insertion and the locus affecting *proU* expression and to obtain strains in which the transposon was now linked to the mutation of interest.

Isolation of an *hns* mutant with increased *proU* expression. Following nitrosoguanidine mutagenesis, a population of cells of the *proU*::*lac* strain GJ1001 was plated on MacConkey agar plates, and the colonies were scored for their Lac phenotype. The parental colonies are Lac$^-$ on this medium. One of the Lac$^+$ clones (designated GJ1003) was shown to have a mutation unlinked to *proU* that caused a two- to threefold derepression of *proU*::*lac* expression. The mutation was subsequently shown to be cotransducible with the *trp* locus at 27 min and also to confer a Bgl$^+$ phenotype, properties characteristic of mutations in the *hns* (*osmZ*) locus (17, 23). With the aid of a linked Tn*10* insertion (from CAG18455) (33), the *hns* mutation (designated *hns-201*) was transduced into GJ134, and the resultant strain was designated GJ1083.

Other genetic techniques. The methods for nitrosoguanidine mutagenesis (27), P1*kc* transduction (9), conjugation (27), and transformation (25) were as described earlier. Spontaneous plasmid-cured derivatives of strains carrying any of the Tp^r plasmids were obtained after growth of the strains for several generations in antibiotic-free medium followed by screening of single colonies from these cultures for their Tp^s phenotype.

Enzyme assays. Unless otherwise specified, cultures for β-galactosidase assays were grown at 30°C in media supplemented as appropriate with trimethoprim or other antibiotics. The specific activity of β-galactosidase was measured by the sodium dodecyl sulfate-chloroform method described by Miller (27), and the values are expressed in the units defined therein. Each value reported is the mean of at least three independent experiments, and the variation between individual measurements was typically less than 10%.

Catalase activity in strains was qualitatively monitored on agar plates, as described by Mulvey et al. (28), by placing a drop of H_2O_2 (30%, vol/vol, in water) directly on a colony and observing the rate of O_2 bubble formation. Catalase isoenzymes HPI and HPII were also detected by activity staining following electrophoresis of cell extracts in 8.5% nondenaturing polyacrylamide slab gels, as described previously (12, 28).

RESULTS AND DISCUSSION

Isolation of a mutant with decreased *proU* expression. The strain GJ134/pHYD272, which is merodiploid for *proUp* (being *proU*$^+$ on the chromosome and carrying a *lac* operon fusion to the full-length *proU* *cis*-regulatory region on a low-copy-number plasmid), was used to obtain *trans*-acting mutations that reduced *proU* expression. It was mutagenized with nitrosoguanidine and plated on glucose–minimal A–X-Gal medium supplemented with DHP and 0.2 M NaCl, thus imposing a selection for ProU$^-$ colonies (9). The colonies were also screened for their Lac phenotype on these plates, with the expectation that derivatives that both failed to express *proU-lac* and were DHP^r would harbor mutations that reduced *proU*-promoter activity in *trans*.

The mutation(s) conferring the DHP^r Lac$^-$ phenotype in one such mutant (GJ1054) was unlinked to *proUp* both on the chromosome and on pHYD272 (data not shown). To monitor the mutational effects on the three *proU* regulatory mechanisms (P1R, P2R, and NRE), a derivative of GJ1054 cured of pHYD272 (GJ1055) was obtained and transformed afresh with plasmid pHYD272, pHYD275, pHYD283, or pHYD284. The results of β-galactosidase specific activity measurements in these derivatives are presented in Fig. 1, in which they are compared with those obtained for similar plasmid transformants of the parental strain, GJ134, and may be summarized as follows. (i) Expression from promoter P1 is considerably reduced in the mutant at all osmolarities of the growth medium, although the same fivefold osmotic inducibility of the residual expression is still observed (see below). (ii) *lac* expression from plasmid pHYD284 (expressing the P2R regulatory mechanism) is lower in the mutant strain compared with that in the GJ134 derivative. (iii) Overall *proU* expression from a plasmid (pHYD272) possessing all three mechanisms is reduced in the mutant compared with that in GJ134, with the maximal difference observed in K medium supplemented with 0.15 to 0.2 M NaCl. (iv) Finally, *proU* regulation appears to be affected to the same extent in GJ1055/pHYD283 (possessing P1R plus P2R) or GJ1055/pHYD284 (possessing P2R alone) as that in GJ1055/pHYD272 (possessing P1R plus P2R plus NRE), suggesting that the reduction in *proU* expression observed in the mutant is not mediated through the NRE.

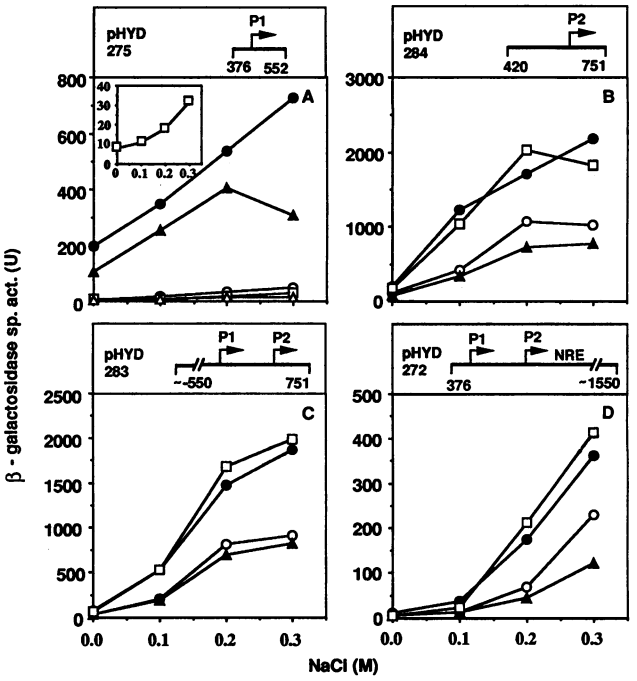

FIG. 1. Specific activities of β-galactosidase in *proU-lac* strains as a function of medium osmolarity. Derivatives of GJ134 (wild type) (●), GJ1055 (*dpeA dpeB*) (○), GJ1064 (*dpeA*) (▲), and GJ1075 (*dpeB*) (□) carrying the various Tp^r plasmids (as indicated for each panel) were grown to mid-log phase in K medium containing different concentrations of NaCl. Also included in panel A are results for GJ1099/pHYD275 (*rpoS*::Tn*10*) (△). Inset, enzyme activity values for GJ1075/pHYD275 replotted on an expanded ordinate scale. At the top of each panel is a schematic depiction of the extent of *proU* DNA carried by the corresponding plasmid, delimited by the *proU* nucleotide numbers that are marked for its left and right ends. (The frame of reference for all nucleotide position numbers in *proU* is the first base of the *Eco*RV site immediately upstream of the operon [10]. In this numbering scheme, the transcription initiation sites of P1 and P2 and the translation initiation site of *proV* are at positions 438, 628, and 688, respectively.) Also marked are the locations of promoters P1 and P2 and the NRE on the various plasmids, as appropriate. As mentioned in the text, P1R and P2R refer to the *cis* elements in the *proU* DNA fragments carried by plasmids pHYD275 and pHYD284, respectively, that confer osmoresponsivity to the corresponding promoters.

GJ1054 has two mutations affecting *proU-lac* expression. Transposon-tagging experiments were used to demonstrate that GJ1054 harbors mutations in two discrete chromosomal loci, *dpeA* and *dpeB* (named for "decrease in *proU* expression"), that affected *proU-lac* expression. A P1*kc* lysate prepared on a population of Tn*10*dKan-mutagenized MC4100 cells was used first to transduce GJ1055/pHYD272 to Kan^r on MacConkey agar plates supplemented with 0.1 M NaCl, and in this manner a Kan^r insertion 70% cotransducible with a locus designated *dpeA*$^+$ (that rendered the corresponding transductants Lac$^+$ on these plates) was identified. This transposon insertion (designated *zba-2101*::Tn*10*dKan, on the basis of the mapping data below) was then used to construct a *dpeA* derivative of GJ134 that was designated GJ1064. GJ1064 was DHP^r on NaCl-supplemented plates, indicating that the *dpeA* mutation does also reduce chromosomal *proU*$^+$ expression in the strain.

Analysis of *proU-lac* expression as a function of osmolarity from each in the panel of Tp^r plasmids transformed into GJ1064 (Fig. 1) indicated that the *dpeA* mutation affected the P2R regulatory mechanism to much the same extent as that observed in the original mutant, GJ1055. Unexpectedly, however, *lac* expression from the P1 promoter on plasmid pHYD275 was only marginally affected in the GJ1064 derivative, in contrast to the pronounced decrease observed in the corresponding GJ1055 derivative. In a complementary experiment, it was shown that normal *lac* expression from the P1 promoter continued to be severely depressed in the *dpeA*$^+$ transductant of GJ1055/pHYD275 (data not shown).

In order to tag the second locus (*dpeB*) in GJ1055 that affected promoter P1 expression, the phage P1*kc* lysate on the MC4100-random Kan^r population was used as a donor in transducing GJ1055/pHYD275 (bearing P1R alone) to Kan^r on MacConkey agar plates with 0.05 M NaCl. One Lac$^+$ colony obtained in this cross had a Kan^r insertion (designated *zfi-2102*::Tn*10*dKan) 70% linked to *dpeB*$^+$. A *dpeB* single-mutant derivative of GJ134 (GJ1075) was constructed and then used to show that this mutation alone is sufficient to curtail *lac* expression from plasmid pHYD275 and that *lac* expression from none of the other three Tp^r plasmids in the panel is significantly affected by it (Fig. 1). Significantly, although the absolute level of expression from pHYD275 was reduced in the *dpeB* strain, the fivefold osmotic inducibility from the P1 promoter was still preserved as in the original mutant, GJ1055 (Fig. 1A, inset).

The double mutant GJ134 *dpeA dpeB* (designated GJ1098) was constructed by sequential P1*kc* transductions with the aid of linked transposon markers, and the profiles of *proU-lac* expression from the various Tp^r plasmids introduced into it were indistinguishable from the corresponding derivatives of the originally isolated mutant strain, GJ1055 (data not shown). These results indicated that *dpeA* and *dpeB* are the only two mutations present in GJ1055 that affect *proU* regulation.

***dpeA* is allelic to *hupB*.** The *dpeA* mutation was mapped, with the aid of both the linked *zba-2101*::Tn*10*dKan insertion and the panel of mapping strains of Singer et al. (33), to the 10-min region on the chromosome, to the vicinity of the *hupB* locus (data not shown; a gene order of *tsx-247*::Tn*10*–*zba-2101*::Tn*10*dKan–*dpeA*–*zba-3054*::Tn*10* was established). The *hupA* and *hupB* genes encode the α and β subunits, respectively, of the nucleoid protein HU. In strains with a mutation in either *hupA* or *hupB*, homodimers of the residual functioning subunit can be formed without much phenotypic consequence (7, 18). We considered the possibility that *dpeA* represents a *hupB* mutation, and the following lines of evidence indicate that this is indeed the case.

TABLE 2. Osmolarity-dependent β-galactosidase specific activity in derivatives of GJ134/pHYD272 and GJ144

Genotype[a]	Sp act (U) at an NaCl concn (M) of[b]:			
	0	0.1	0.2	0.3
wt (P)	4	31	194	316
wt (C)	3	10	84	232
dpeA (P)	5	14	66	211
dpeA (C)	3	5	12	32
hupB (P)	3	13	52	191
hupB (C)	3	7	42	121
hupA (P)	4	11	42	237
hupA (C)	3	8	60	168
p*hupB*$^+$ (P)	13	50	344	639
dpeA/p*hupB*$^+$ (P)	7	30	267	572
p*hupA*$^+$ (P)	8	43	237	584
dpeA/p*hupA*$^+$ (P)	10	12	85	171
hupB hupA (P)	6	8	24	83
dpeA hupA (P)	10	19	50	80
hupB hupA (C)	4	6	12	20
dpeA hupA (C)	4	6	11	19
hns (P)	13	70	453	802
dpeA hns (P)	15	56	241	432

[a] The relevant mutations or plasmids in isogenic derivatives of GJ134/pHYD272 and GJ144 are listed. P and C, location of the *proU-lac* fusion on the plasmid (in GJ134/pHYD272) or the chromosome (in GJ144), respectively. Strain designations and full genotypes are given in Table 1. The *hupA*, *hupB*, and *hns* mutations refer to the *hupA*::Cm^r, *hupB*::Kan^r, and *hns-201* alleles, respectively. p*hupA*$^+$ and p*hupB*$^+$ refer to the presence in the strains of plasmids pK01 and pMW1, respectively. wt, wild type.

[b] Specific activities of β-galactosidase were measured in cultures grown to mid-log phase in K medium supplemented with NaCl at the indicated concentrations.

(i) The linkages of *hupB*::Kan^r in OHP252 to *tsx-247*::Tn*10* in CAG12148 (22%) and to *zba-3054*::Tn*10* in CAG12017 (99%) were comparable to the corresponding values obtained for each of the latter two markers and *dpeA* (24% and 96%, respectively; data not shown).

(ii) Introduction of a defined *hupB*::Kan^r null mutation by transduction from OHP252 into strains carrying *lacZ* operon fusions to the full-length *proU cis*-regulatory region (that is, either into GJ144, which carries a chromosomal *proU-lac* fusion, or into GJ134/pHYD272) had the same effect on osmotic regulation of *proU-lac* expression as did the *dpeA* mutation itself (Table 2). The effect of the *hupA*::Cm^r allele on plasmid-borne or chromosomal *proU-lac* regulation in *hupB*$^+$ strains was similar to that of *hupB*::Kan^r in *hupA*$^+$ strains (Table 2).

(iii) The multicopy *hupB*$^+$ plasmid pMW1, when transformed into GJ1064/pHYD272, restored the profile of *proU-lac* regulation to that seen in the pMW1 derivative of the wild-type strain, GJ134/pHYD272 (Table 2). Comparison of data from the *dpeA*$^+$ and the *dpeA* strains after introduction of plasmid pK01 indicated that the *hupA*$^+$ plasmid did not correct the mutant phenotype (Table 2).

(iv) It is known that *hupA hupB* double mutants (such as OHP252) grow slowly even in rich media and that their cultures exhibit filamentation and a significant frequency of anucleate cells (7, 18). A *hupA dpeA* double mutant was constructed and was found to exhibit all these phenotypes (data not shown). Expression of *proU-lac* from pHYD272 in *hupB hupA* double mutants was similar to that in *dpeA hupA* double mutants and was significantly less than that in any of the three single mutants (Table 2). The corresponding double-mutant derivatives of the chromosomal *proU-lac* fusion strain also exhibited a profound reduction in magnitude of osmoresponsive *proU-lac* expression (Table 2).

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                    -35          Spacer          -10          +1
glgS P2 (E.c)  TatTt  ACGCA cgTtATgTtTaaacGca CTACACTGAT  tggg G
proUP1 (E.c)   TtaTc  ACGCA aaTaATtTgTggt Gat CTACACTGAT actct G
proUP1 (S.t)   aaaTc  gCGCA aaTa tTaca acat Gtc CTACACT c Aa tacga G
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FIG. 2. Alignment of the *E. coli* (*E.c*) *proU* P1 and *glgS* P2 promoter sequences, upstream of the transcription initiation site (+1). Identical nucleotides are shown in uppercase, and the −10 and −35 regions are marked. Also shown is the corresponding region (designated P1) of the *S. typhimurium* (*S.t*) *proU* sequence (30), for which the bases that are identical with the pair above are in uppercase; the +1 start site of transcription marked in this instance is putative.

In control experiments, two plasmid pMU575 derivatives (pJ004 and pHYD248) bearing *lac* fusions to non-*proU* promoters resulted in β-galactosidase specific activity levels in *dpeA* and the various *hup* mutants (including the *hupA hupB* double mutant) that were similar to the corresponding levels obtained with derivatives of the wild-type parent GJ134 at all osmolarities tested (data not shown). The possibility that the observed changes in *proU-lac* expression in the mutants were an artifactual consequence of alterations in factors such as growth rate, cell size, or plasmid copy number was therefore excluded.

Taken together, these results suggest an activating role for HU in (the P2R mechanism of) *proU* regulation. In an observation that was consistent with this notion, GJ134/pHYD272 derivatives carrying either $hupB^+$ or $hupA^+$ on multicopy plasmids exhibited higher *proU-lac* expression than the haploid $hupB^+$ $hupA^+$ parent did (Table 2).

***proU-lac* expression in a *dpeA hns* double mutant.** We also examined the effects of *hns* mutation, singly and in combination with *dpeA*, on *proU* regulation. As expected, a mutation in *hns* caused partial derepression of *lac* expression from plasmid pHYD272 (Table 2). A strain with mutations in both *dpeA* and *hns* exhibited an induction profile for *proU* that was roughly the algebraic sum of the effects of each single mutation (Table 2), with neither gene being epistatic over the other. This result suggests that HU and HNS influence *proU* regulation through distinct mechanisms; in light of the earlier evidence that HNS acts through the NRE, this result also supports the conclusion drawn above that HU activates *proU* expression through the P2R mechanism.

***dpeB* is allelic to *rpoS*.** Conjugational and transductional mapping experiments were used to place *dpeB* at 59 min, where it was cotransducible 59% with *mutS* and 42% with *cysC*; three-factor analyses of data from two crosses established a gene order of *mutS–dpeB–zfi-2102*::Tn*10*dKan–*cysC* (data not shown). The map position of *dpeB* led us to examine whether it is an allele of *rpoS*, the gene encoding the stationary-phase sigma factor (σ^s or RpoS) of RNA polymerase in *E. coli* (13). The following lines of evidence indicate that *dpeB* represents an *rpoS* mutation and that the *proU* P1 promoter is under RpoS control.

(i) *E. coli* expresses two catalase isoenzymes, HPI and HPII, and it is known that HPII synthesis is under RpoS control (13, 28). The absence of HPII in *rpoS* mutants can be demonstrated either by a qualitative plate assay on colonies or by activity staining after electrophoresis of cell extracts (28). When a P1*kc* lysate prepared on a *dpeB zfi-2102*::Tn*10*dKan donor strain was used to transduce GJ134/pHYD275 to Kan^r, a perfect correlation was demonstrated between segregation of the catalase-negative phenotype (as assayed by the qualitative plate method) and that of the Lac^- phenotype amongst the transductants. Furthermore, in a comparison between the *dpeB* mutant GJ1075 and the isogenic wild-type strain GJ134 in the electrophoretic activity-staining assay, GJ1075 was found specifically to lack catalase HPII activity, whereas it had normal levels of HPI activity (data not shown).

(ii) A known *rpoS* null allele from RH90 (22), when transduced into GJ134/pHYD275, had the same effect on *proU-lac* expression as did the *dpeB* mutation (Fig. 1).

No consensus sequence motif for RpoS-controlled promoters has so far been identified (13), but we note that a 5-base stretch and 9-base stretch of nucleotides, situated in the −35 and −10 regions respectively, are identical in the *proU* P1 promoter and the *glgS* P2 promoter (also under RpoS control) (14) (Fig. 2). The *S. typhimurium proU* sequence also exhibits significant conservation with this pair of shared stretches (Fig. 2).

Growth-phase-dependent expression from *proU* P1 promoter. As is characteristic of many RpoS-controlled promoters (13), the *proU* P1 promoter in plasmid pHYD275 exhibited pronounced induction of expression as cultures of the wild-type ($dpeB^+$) strain grown in LB medium reached the stationary phase (Fig. 3). In cultures grown in K medium, promoter P1 expression was also higher in stationary phase than in the log phase, but the magnitude of induction was not as marked nor was the slope as steep as in LB (data not shown). Growth-phase-dependent induction of P1 was not observed in the *dpeB* mutant strain (Fig. 3).

A comparison of our observations with those of Kaasen et al. (21) and Lange et al. (22) indicates that regulation of the *proU*

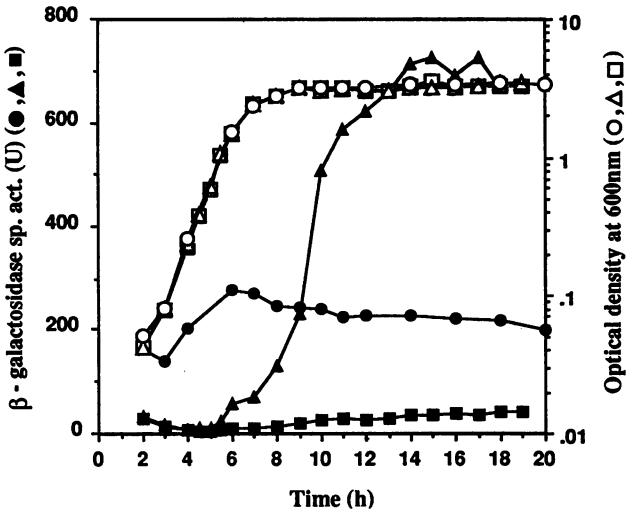

FIG. 3. Growth-phase-dependent variation in *proU-lac* expression. pHYD275 derivatives of GJ134 (wild type) (△ and ▲) and GJ1075 (*dpeB*) (□ and ■) and the pHYD272 derivative of GJ134 (○ and ●) were grown in LB. A_{600} and β-galactosidase specific activities were monitored for each culture at various stages of growth, and the values have been plotted as a function of time since inoculation.

P1 promoter is similar to that of the promoters for the *otsBA* operon (involved in osmoregulatory trehalose biosynthesis) and the *osmY* gene (of unknown function) with respect to three major features: (i) osmotic inducibility, (ii) inducibility in stationary phase, and (iii) RpoS dependence of stationary-phase inducibility but not of osmotic inducibility. The *rpoS*-mediated 100-fold induction of *proU* P1 is the highest known so far for any stationary-phase-induced promoter.

Growth phase and RpoS have little effect on full-length *proU* *cis*-regulatory region. Although the effect of *dpeB* (*rpoS*) on the isolated P1 promoter in plasmid pHYD275 was pronounced, that on the other three Tp^r plasmids in the panel (in particular, pHYD272, which carries the full-length *cis* region, P1R plus P2R plus NRE) was very much less marked (Fig. 1). Kaasen et al. (21) have also shown earlier that an *rpoS* mutation does not significantly affect expression of a normal chromosomal *proU-lac* fusion.

Furthermore, growth phase also had little effect on *lac* expression from plasmid pHYD272 in the $rpoS^+$ strain GJ134; the changes in β-galactosidase specific activity across the various time points of incubation of the culture were less than twofold (Fig. 3). The plasmids pHYD284 (expressing P2R alone) and pHYD283 (expressing P1R plus P2R) also exhibited similar, relatively insignificant variations in *lac* expression as a function of growth phase (data not shown).

It is intriguing that the isolated P1 promoter is both *rpoS* dependent and stationary phase inducible, whereas overall transcription from the full-length *proU* regulatory region (that is, P1R plus P2R plus NRE) is largely unaffected by either RpoS or growth phase. Two considerations lead us to suggest that, notwithstanding the findings mentioned above, the P1 promoter and its regulation are relevant to native *proU* expression. (i) The *dpeB* (*rpoS*) mutation was identified in a selection for derivatives with reduced expression of the chromosomal $proU^+$ operon. (ii) The present data on P1 regulation are consistent with the overlap that has been demonstrated between osmotic and stationary-phase responses for the promoters of several other loci in *E. coli* (15, 20–22). It appears, therefore, that there may be a level of complexity in *proU* regulation, as far as growth phase is concerned, which needs to be delineated in future studies.

Concluding remarks. Our model for *proU* regulation (6) differs from those proposed by other workers mainly in that it invokes distinct modular *cis* elements (P1R, P2R, and NRE) that together account for *proU* osmoresponsivity. Data from this study and from earlier work cited above indicate that regulation at each of these *cis* elements is mediated by distinct *trans*-acting proteins: RpoS for promoter P1, HNS for NRE, and HU for P2R.

Four additional points bear consideration. (i) The available data do not exclude the possibility that additional upstream sequences (in the vicinity of P2) are also necessary for the HNS- and NRE-mediated repression of *proU* expression. (ii) Lucht and Bremer (23) have reported that mutants defective in the nucleoid protein IHF exhibit a twofold decrease in *proU* expression, and our findings obtained by using the panel of Tp^r plasmids indicate that this effect is also mediated through P2R (data not shown). These observations are consistent with those from other study systems which indicate that HU and IHF perform similar, and often interchangeable, functional roles as DNA-binding and nucleoid proteins in *E. coli* (8, 19, 26). (iii) The mechanisms by which HU or IHF might affect P2R regulation are not known; given their pleiotropic roles in cellular metabolism, their effects on *proU* may be indirect. (iv) Finally, we suggest that the effects of potassium glutamate and DNA supercoiling on *proU* regulation, as postulated by other workers (17, 31, 32), need to be understood in the context of the multiple mechanisms outlined in our model.

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REFERENCES

1. **Ames, G. F.-L., C. S. Mimura, and V. Shyamala.** 1990. Bacterial periplasmic permeases belong to a family of transport proteins operating from *Escherichia coli* to human: traffic ATPases. FEMS Microbiol. Rev. **75**:429–446.
2. **Andrews, A. E., B. Lawley, and A. J. Pittard.** 1991. Mutational analysis of repression and activation of the *tyrP* gene in *Escherichia coli*. J. Bacteriol. **173**:5068–5078.
3. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54**:130–197.
4. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**:121–147.
5. **Dattananda, C. S., and J. Gowrishankar.** 1989. Osmoregulation in *Escherichia coli*: complementation analysis and gene-protein relationships in the *proU* locus. J. Bacteriol. **171**:1915–1922.
6. **Dattananda, C. S., K. Rajkumari, and J. Gowrishankar.** 1991. Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. J. Bacteriol. **173**:7481–7490.
7. **Dri, A.-M., J. Rouviere-Yaniv, and P. L. Moreau.** 1991. Inhibition of cell division in *hupA hupB* mutant bacteria lacking HU protein. J. Bacteriol. **173**:2852–2863.
8. **Goodman, S. D., S. C. Nicholson, and H. A. Nash.** 1992. Deformation of DNA during site-specific recombination of bacteriophage λ: replacement of IHF protein by HU protein or sequence-directed bends. Proc. Natl. Acad. Sci. USA **89**:11910–11914.
9. **Gowrishankar, J.** 1985. Identification of osmoresponsive genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. J. Bacteriol. **164**:434–445.
10. **Gowrishankar, J.** 1989. Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. J. Bacteriol. **171**:1923–1931. (Erratum, **172**:1165, 1990.)
11. **Gowrishankar, J., P. Jayashree, and K. Rajkumari.** 1986. Molecular cloning of an osmoregulatory locus in *Escherichia coli*: increased *proU* gene dosage results in enhanced osmotolerance. J. Bacteriol. **168**:1197–1204.
12. **Gregory, E. M., and I. Fridovich.** 1974. Visualization of catalase on acrylamide gels. Anal. Biochem. **58**:57–62.
13. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in E. coli. Cell **72**:165–168.
14. **Hengge-Aronis, R., and D. Fischer.** 1992. Identification and molecular analysis of *glgS*, a novel growth-phase-regulated and *rpoS*-dependent gene involved in glycogen synthesis in *Escherichia coli*. Mol. Microbiol. **6**:1877–1886.
15. **Hengge-Aronis, R., R. Lange, N. Henneberg, and D. Fischer.** 1993. Osmotic regulation of *rpoS*-dependent genes in *Escherichia coli*. J. Bacteriol. **175**:259–265.
16. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8**:67–113.
17. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in S. typhimurium and E. coli. Cell **52**:569–584.
18. **Huisman, O., M. Faelen, D. Girard, A. Jaffé, A. Toussaint, and J. Rouvière-Yaniv.** 1989. Multiple defects in *Escherichia coli* mutants lacking HU protein. J. Bacteriol. **171**:3704–3712.

19. **Hwang, D. S., and A. Kornberg.** 1992. Opening of the replication origin of *Escherichia coli* by DnaA protein with protein HU or IHF. J. Biol. Chem. **267:**23083–23086.
20. **Jenkins, D. E., S. A. Chaisson, and A. Matin.** 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. **172:**2779–2781.
21. **Kaasen, I., P. Falkenberg, O. B. Styrvold, and A. R. Strøm.** 1992. Molecular cloning and physical mapping of the *otsBA* genes, which encode the osmoregulatory trehalose pathway of *Escherichia coli*: evidence that transcription is activated by KatF (AppR). J. Bacteriol. **174:**889–898.
22. **Lange, R., M. Barth, and R. Hengge-Aronis.** 1993. Complex transcriptional control of the σ^s-dependent stationary-phase-induced and osmotically regulated *osmY* (*csi-5*) gene suggests novel roles for Lrp, cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in the stationary-phase response of *Escherichia coli*. J. Bacteriol. **175:**7910–7917.
23. **Lucht, J. M., and E. Bremer.** 1991. Characterization of mutations affecting the osmoregulated *proU* promoter of *Escherichia coli* and identification of 5′ sequences required for high-level expression. J. Bacteriol. **173:**801–809.
24. **Lucht, J. M., P. Dersch, B. Kempf, and E. Bremer.** 1994. Interaction of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. **269:**6578–6586.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mendelson, I., M. Gottesman, and A. B. Oppenheim.** 1991. HU and integration host factor function as auxiliary proteins in cleavage of phage lambda cohesive ends by terminase. J. Bacteriol. **173:**1670–1676.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Mulvey, M. R., P. A. Sorby, L. Barbara, B. L. Triggs-Raine, and P. C. Loewen.** 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. Gene **73:**337–345.
29. **Overdier, D. G., and L. N. Csonka.** 1992. A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **89:**3140–3144.
30. **Overdier, D. G., E. R. Olson, B. D. Erickson, M. M. Ederer, and L. N. Csonka.** 1989. Nucleotide sequence of the transcriptional control region of the osmotically regulated *proU* operon of *Salmonella typhimurium* and identification of the 5′ endpoint of the *proU* mRNA. J. Bacteriol. **171:**4694–4706.
31. **Owen-Hughes, T. A., G. D. Pavitt, D. S. Santos, J. M. Sidebotham, C. S. J. Hulton, J. C. D. Hinton, and C. F. Higgins.** 1992. The chromatin-associated protein H-NS interacts with curved DNA to influence topology and gene expression. Cell **71:**255–265.
32. **Prince, W. S., and M. R. Villarejo.** 1990. Osmotic control of *proU* transcription is mediated through direct action of potassium on the transcription complex. J. Biol. Chem. **265:**17673–17679.
33. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:**1–24.
34. **Tanaka, K., S. Muramatsu, H. Yamada, and T. Mizuno.** 1991. Systematic characterization of curved DNA segments randomly cloned from *Escherichia coli* and their functional significance. Mol. Gen. Genet. **226:**367–376.
35. **Ueguchi, C., and T. Mizuno.** 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12:**1039–1046.
36. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:**369–379.