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Characterization of Mutations Affecting the Osmoregulated *proU* Promoter of *Escherichia coli* and Identification of 5′ Sequences Required for High-Level Expression

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Expression of the *Escherichia coli proU* operon, which encodes an efficient uptake system for the osmoprotectant glycine betaine, is strongly increased in cells grown at high osmolarity. We isolated 182 independent spontaneous mutants with elevated expression of the chromosomal Φ(*proV-lacZ*) (Hyb2) fusion at low osmolarity. Genetic analysis demonstrated that eight of these mutant strains carried mutations closely linked to the fusion, whereas all others carried mutations that appeared to be in *osmZ*. All of the mutations resulted in increased but still osmoregulated expression of the Φ(*proV-lacZ*)(Hyb2) fusion. The *proU*-linked mutants carried an identical point mutation (*proU603*) which changes the −35 sequence of the *proU* promoter from TTGCCT to TTGACT and thereby increases the homology of the −35 region to the consensus sequence (TTGACA) of *E. coli* promoters. We also selected for mutants with decreased expression of the plasmid pOS7-encoded Φ(*proV-lacZ*)(Hyb2) fusion and isolated a plasmid with an IS*1* insertion (*proU607*) between the *proU* −10 and −35 regions. This insertion creates a hybrid promoter and drastically reduces expression of the fusion but does not abolish its osmotic regulation. Deletion analysis of chromosomal sequences 5′ to the *proU* promoter revealed that sequences located approximately 200 bp upstream of the −35 region were required for high-level expression. Removal of these sequences resulted in a 10-fold decline of Φ(*proV-lacZ*)(Hyb2) expression. Osmotic regulation was retained in deletion constructs carrying just 19 bp of chromosomal DNA 5′ of the promoter, showing that no sequences further upstream are required for the proper osmoregulation of *proU* transcription. Experiments with *himA* and *fis* mutant strains indicated that the IHF and FIS proteins are not required for the normal osmoregulation of *proU* expression.

Escherichia coli and *Salmonella typhimurium* can adapt to high-osmolarity growth conditions by a variety of mechanisms (for recent overviews, see references 8 and 55). One of these mechanisms is the intracellular accumulation of the osmoprotectant glycine betaine, which is either synthesized from exogenously provided choline (28) or taken up from the environment (43). Two glycine betaine porters have been identified: the low-affinity ProP system (5, 32, 34) and the high-affinity ProU system (2, 6, 24, 32).

ProU is a binding-protein-dependent transport system and is encoded by the *proU* operon, which consists of three structural genes, *proV*, *proW*, and *proX* (20, 31, 41, 52). The level of *proU* expression is sensitively determined by the osmolarity of the growth medium. The basal transcription of *proU* is very low and is strongly stimulated upon a sudden osmotic upshock. The increased steady-state level of *proU* expression during growth at elevated osmolarity is directly correlated with the osmolarity of the growth medium (3, 6, 8, 13, 19, 21, 32). Osmoregulation of *proU* expression is not dependent on the sensor/regulator protein pair EnvZ and OmpR, which mediate the reciprocal osmoregulation of the *ompC* and *ompF* porin genes (6, 21, 32). No classical regulatory protein has been identified for *proU*. Mutants with increased *proU* expression carry either *cis*-acting mutations closely linked to *proU* (12, 23, 31) or *trans*-acting mutations in *topA* or *osmZ* (23). The products of these genes, DNA topoisomerase I and the histonelike DNA-binding protein H-NS (H1a), respectively, are known to affect DNA topology, and mutations in these genes increase negative DNA supercoiling (18, 23, 24a, 30). Since such an increase was also observed in $topA^+$ $osmZ^+$ strains grown at high osmolarity, it has been suggested that changes in DNA topology are an important determinant for the osmoregulation of *proU* expression (23).

The osmotically stimulated uptake of K^+ and the concomitant synthesis of glutamate are among the first physiological responses of the cell to an osmotic upshift (14). In vivo, expression of the *proU* operon is strongly dependent on the presence of K^+ in the growth medium, and the intracellular accumulation of K^+-glutamate is implicated in the osmotic control of *proU* expression (14, 53). Stimulation of *proU* expression in vitro by elevated concentrations of K^+-glutamate in a coupled transcription-translation (S-30) extract has been reported (26, 46). A further 10-fold increase in *proU* expression was observed in an S-30 extract prepared from cells grown at high osmolarity, suggesting that a macromolecular factor might be involved in *proU* regulation (26). Recently, the reconstitution of K^+-glutamate-stimulated transcription from the *proU* promoter in a purified system containing only RNA polymerase holoenzyme, nucleotides, and a circular template DNA has been described by Prince and Villarejo (45). These authors have suggested that high concentrations of K^+-glutamate directly stimulate *proU* transcription by either increasing the affinity of RNA polymerase to the *proU* promoter or changing the microstructure of this promoter, thereby facilitating a productive interaction between RNA polymerase and the promoter. The *proU* promoter has been identified by mapping the transcriptional start site(s) and by analysis of a *cis*-acting mutation (20, 31, 52). The −35 (TTGCCT) and −10 (TAGGGT) sequences show homology to the consensus sequence of *E. coli* pro-

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TABLE 1. Bacterial strains, bacteriophage, and plasmids

Strain, phage, or plasmid	Description[a]	Source or reference
Strains		
MC4100	F⁻ Δ(*argF-lac*)*U169 araD139 rpsL150 deoC1 relA1 ptsF25 flbB5501 rbsR*	7
GM37	MC4100 Φ(*proV-lacZ*)(Hyb2)(λ*plac*Mu15)	32
BRE2074	GM37 *proU601*	23
EF027	MC4100 Δ(*proU*)*600*	32
HSK42	MC4100 *polA1*	47
MC1000	Δ*lacX74 araD139* Δ(*ara-leu*)*7697 galU galK strA*	25
RJ1617	MC1000 *fis-767*(Kan^r)	25
MC251	*ara* Δ(*lac-pro*) *nalA metB argE*(Am) Rif^r *himA*(Δ82::Tn*10*)	16
JML1	MC4100(λp1048)	This study
JML3	EF027(λpOS7)	This study
JML21	GM37 *proU603*	This study
JML81	GM37 *himA*(Δ82::Tn*10*)	This study
JML100	EF027(λpOS7-Δ540); see Fig. 4	This study
JML101	JML100 *himA*(Δ82::Tn*10*)	This study
JML102	JML3 *himA*(Δ82::Tn*10*)	This study
Bacteriophages		
λp1048	Φ(*tyrT'-lacY*$^+$)*1048*	4
λC17c_{I90}	Tester phage for multiple λ lysogens	1
λRZ5	*'bla 'lacZ lacY*$^+$ *attP*$^+$	40
λpOS7	λRZ5 with Φ(*proV-lacZ*)(Hyb2) *bla*$^+$	This study
Plasmids		
pMLB1034	*'lacZ lacY' bla*$^+$	51
pOS7	Φ(*proV-lacZ*)(Hyb2) *bla*$^+$	32
pJL1.55	pOS7 with Tn*5* insertion upstream of the *proU* promoter	J. M. Lucht
pJL9	pJL1.55 with a *Hpa*I-*Hpa*I deletion	This study
pJL21	pOS7::IS*1* (*proU607*)	This study

[a] Genes marked with a prime are incomplete. The λ*plac*Mu15 prophage carries a kanamycin resistance gene.

moters; however, the *proU* −10 region deviates from the consensus (TATAAT) sequence at three positions (22). Inhibition of *proU* transcription in vitro by antibodies directed against σ^{70} demonstrated that *proU* transcription does not depend on the presence of an alternative sigma factor of RNA polymerase (26). The molecular mechanism by which *proU* expression is regulated in vivo is still unclear. We set out to define the *cis*-acting DNA sequences that are required to achieve full and osmoregulated transcription of the *proU* operon and made a deletion and mutation analysis of the *proU* 5' region. Here, we report the identification of sequences in this region that are required for high-level expression and characterize mutations that affect the *proU* promoter.

MATERIALS AND METHODS

Media and growth conditions. Bacteria were grown aerobically at 37°C in LB medium or minimal medium A (MMA) with 0.2% glucose as the carbon source (33, 51). The osmolarity of liquid MMA was elevated by the addition of NaCl as indicated. Agar plates spread with 0.1 ml of a 10-mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in dimethylformamide were used to distinguish between LacZ⁻ and LacZ⁺ phenotypes. Lactose MacConkey medium (51), BS agar plates containing bromothymol blue and salicin (50), and MMA-based BTB lactose plates (33) were prepared as described. Kanamycin, tetracycline, and ampicillin were added to media at 30, 5, and 50 μg/ml, respectively unless indicated otherwise. Recombinant M13 phages or plasmids carrying *proU* promoter mutations were propagated in strains growing in LB or DYT (33) medium from which NaCl had been omitted.

Genetic procedures and construction of bacterial strains. The bacteria, phages, and plasmids used are described in Table 1. All strains were *E. coli* K-12 derivatives. Standard techniques were used for the propagation of bacteria and bacteriophages, for generalized transduction with phage P1*vir*, and for lysogenization of λ specialized transducing phages (51). To transfer plasmid-encoded Φ(*proV-lacZ*)(Hyb2) fusions into the bacterial chromosome, the hybrid genes were first recombined in vivo onto phage λRZ5 (40), and the resulting LacZ⁺, specialized transducing phages were integrated as lysogens into the bacterial chromosome at *attB* by selecting Amp^r (25 μg/ml) colonies. The presence of a single-copy lysogen was verified by testing the sensitivity of the lysogens against the indicator phage λC17c_{I90} (1).

Isolation of mutants with altered Φ(*proV-lacZ*)(Hyb2) expression. To isolate spontaneous mutants with increased expression of the chromosomal Φ(*proV-lacZ*)(Hyb2) fusion, strain GM37 (Table 1) was streaked on lactose MacConkey plates or BTB lactose plates and incubated at 37°C for several days. Lac⁺ papillae were picked and purified by restreaking on the same indicator media. In a previous study (23), such mutants were shown to carry either mutations closely linked to the fusion or mutations in the unlinked *osmZ* gene. OsmZ mutants express the normally cryptic *bgl* operon. Of the 182 mutants that we isolated, 154 were Bgl⁺ on BS indicator plates and thus are likely to carry mutations in *osmZ*. We confirmed this directly for a number of the Lac⁺ Bgl⁺ mutants by transductional mapping of the mutations to the *osmZ* region with the aid of a Tn*10* insertion closely linked to *osmZ* (23). To determine whether the remaining 28 Lac⁺ Bgl⁻ mutants carried mutations linked to the *proV-lacZ* fusion, a P1*vir* lysate was prepared on these strains and used to transduce strain MC4100 to Kan^r in the presence of X-Gal. The transductants were then tested for their Lac phenotype by streaking single colonies onto lac-

tose MacConkey plates. We found that the mutation was tightly linked to the *proU* region in eight of these mutants, since the majority of the transductants obtained from these strains showed the mutant phenotype (Lac^+ at low osmolarity and further inducible) and only a few showed the wild-type phenotype (Lac^- at low osmolarity but osmotically inducible). The *lac* fusion rescued from the other 20 mutants showed in each case wild-type regulation. Representative examples of these strains were subjected to a more detailed genetic analysis by three-factor crosses, which demonstrated that they carried mutations in the *osmZ* region. Various levels of *bgl* expression for different *osmZ* alleles have been noted (23), and it is possible that the Lac^+ Bgl^- mutants described here are also *osmZ* alleles.

Spontaneous mutants showing reduced expression of the pOS7-encoded Φ(*proV-lacZ*)(Hyb2) fusion (Table 1) were enriched by two cycles of cycloserine selection of strain JML1(pOS7) growing in MMA with 0.2% lactose as the carbon source (33). Mutants were identified by their Lac^- phenotype on lactose MacConkey indicator plates. Of 37 mutants analyzed, 31 showed β-galactosidase activities identical to that of the parent strain, indicating that they had acquired mutations affecting lactose uptake or utilization rather than expression of the fusion. The Lac^- phenotype of these strains is possibly due to the loss of the $lacY^+$ λp1048 prophage. Two strains had no detectable β-galactosidase activity, suggesting that mutations affecting synthesis or activity of the ProV′–β-galactosidase hybrid protein had occurred. Three strains carried chromosomal mutations that reduce the copy number of pOS7, since only reduced amounts of plasmid DNA were isolated from them, and when plasmids were retransformed, all transformants showed the parental Lac^+ phenotype. One strain carried a mutationally altered plasmid, pJL21, since the Lac^- character of the original mutant was recovered upon retransformation of pJL21.

Methods used with nucleic acids and construction of plasmids. Routine manipulations of nucleic acids were all as described previously (48). DNA sequencing (49) was performed with the Sequenase 2.0 kit, using the conditions recommended by the supplier (U.S. Biochemical, Bad Homburg, Federal Republic of Germany). *proU* promoter mutations were sequenced after subcloning a 1.7-kb *Eco*RI-*Bam*HI fragment (see Fig. 4) into M13mp18 (38), using a primer (5′-TATTTGAACGCTCGCTGTGG-3′) complementary to nucleotides 758 to 739 of the *E. coli proU* operon (31). The junction between the left side of IS*1* and *proU* sequences in plasmid pJL21 was determined by sequencing of alkali-denatured double-stranded DNA by using a primer (5′-GAAGCCACTGGAGCACC-3′; kindly provided by K. Heller) complementary to nucleotides 63 to 47 of the insertion element IS*1* (39). The junction between the right side of IS*1* and *proU* sequences was determined after subcloning a *Pst*I-*Ssp*I restriction fragment containing the IS*1-proU* joint into M13mp19 (38). A derivative of M13mp19 carrying the chromosomal material from plasmid pOS7 [Φ(*proV-lacZ*)(Hyb2)]; see Fig. 4) on a 1.7-kb *Eco*RI-*Bam*HI fragment was used to construct a nested set of unidirectional deletions progressively removing sequences 5′ to the *proU* promoter. Single-stranded DNA of the recombinant phage was linearized at the unique *Eco*RI site, digested with T4 DNA polymerase, and religated as described previously, (9). The deletion endpoints were determined by DNA sequence analysis. The shortened *Eco*RI-*Bam*HI inserts were excised from the M13mp19 recombinant phages and separately ligated into the 6.2-kb *Eco*RI-*Bam*HI vector and *′lacZ* backbone of plasmid pOS7. These constructions yielded a set of $LacZ^+$ Φ(*proV-lacZ*)(Hyb2) fusion plasmids that differ only in the amount of chromosomal DNA present 5′ to the *proU* promoter (Fig. 4). We refer to these deletion derivatives according to the nucleotide numbering (31) of the first base pair of chromosomal DNA still present, e.g., Δ311. Plasmid pJL9 was constructed as follows. A derivative of pOS7 with a Tn*5* insertion in chromosomal sequences upstream of *proU* (20, 31) (the Tn*5* is positioned approximately 300 bp 3′ to the unique *Eco*RI site of pOS7; unpublished data) was cleaved with *Hpa*I and religated. This manipulation removed all material between the *Hpa*I site in Tn*5* (10) that is proximal to the *Eco*RI site in pOS7 (Fig. 4) and the most 3′ *Hpa*I site in *lacZ* (51). The resulting $LacZ^-$ plasmid, pJL9, carries approximately 300 bp of chromosomal DNA from sequences upstream of the *proU* operon and a 2,271-bp fragment of the *′lacZ* and *lacY′* genes.

In vivo cloning of chromosomal *proU-lacZ* fusions. We used the strategy of Saarilahti and Palva (47) to cross the chromosomal Φ(*proV-lacZ*)(Hyb2) fusion by in vivo homologous recombination onto the acceptor plasmid pJL9. The mutationally altered Φ(*proV-lacZ*)(Hyb2) fusions were transduced with phage P1*vir* into the *polA* strain HSK42 (Table 1), and the transductants were transformed with plasmid pJL9 by selecting for colonies resistant against a low concentration of ampicillin (25 μg/ml). In the resulting transformants, plasmid pJL9 is integrated into the bacterial chromosome via homologous recombination at the Φ(*proV-lacZ*)(Hyb2) locus. A P1*vir* lysate was then prepared on each of these mutant strains and used to transduce the $PolA^+$ Lac^- strain MC4100 (Table 1) to resistance against a high concentration of ampicillin (200 μg/ml) in the presence of X-Gal. In such a $PolA^+$ strain, the plasmid can excise from the transduced chromosomal DNA segment by homologous recombination, thereby generating autonomously replicating plasmids. These plasmids either are identical to pJL9 ($LacZ^-$) or carry the intact Φ(*proV-lacZ*)(Hyb2) fusion ($LacZ^+$) of the mutant strains. $LacZ^+$ transductants were recovered with a frequency of 1%.

Preparation of protein samples, immunological detection of ProV–β-galactosidase hybrid proteins, and β-galactosidase assays. To visualize the ProV–β-galactosidase (Hyb2) fusion protein, whole cell extracts were prepared from 5-ml overnight cultures grown in MMA supplemented with various amounts of NaCl as described previously (51). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7% acrylamide) and transferred to a nylon membrane (Immobilon; pore size, 0.45 μm; Millipore). The bound proteins were then probed with a rabbit antiserum raised against β-galactosidase. The formed antigen-antibody complex was visualized with a second goat anti-rabbit immunoglobulin G alkaline phosphatase-coupled antibody (Sigma) (48). Specific β-galactosidase activity, expressed as micromoles of 2-nitrophenyl-β-D-galactopyranoside (ONPG) cleaved per minute per milligram of protein, was assayed as described previously (32).

RESULTS

Isolation of regulatory mutations that increase *proU* expression. Strains GM37 carries the chromosomal Φ(*proV-lacZ*)(Hyb2) protein fusion, which is expressed from the osmotically regulated *proU* promoter (31, 32). Consequently, the Lac phenotype of strain GM37 strongly depends on medium osmolarity: Lac^- at low osmolarity and Lac^+ at high osmolarity. We took advantage of the Lac^- character of

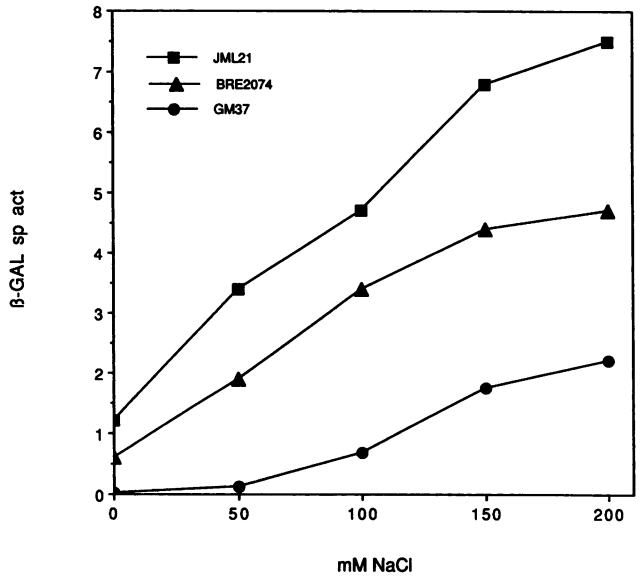

FIG. 1. Effect of *cis*-linked mutations on Φ(*proV-lacZ*)(Hyb2) expression. Cells of the parent strain GM37 and two mutant derivatives, BRE2074 (*proU601*) and JML21 (*proU603*), were grown overnight in glucose MMA with the indicated amounts of NaCl, and the specific β-galactosidase (β-gal) activities (expressed as micromoles of ONPG cleaved per minute per milligram of protein) were determined. All three strains carry the same *lac* fusion as a single copy in the chromosome at the *proU* locus.

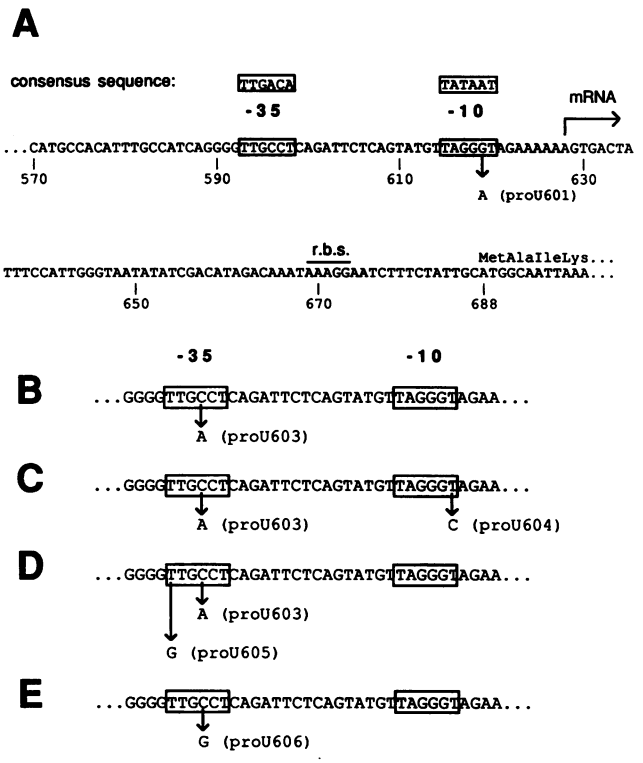

FIG. 2. DNA sequence of *proU* promoter mutations. (A) Nucleotide sequence of the *E. coli proU* promoter region. Positions of the −35 and −10 sequences, the transcriptional initiation site, the putative ribosome-binding site (r.b.s.), and the beginning of *proV* are indicated (20, 31, 52). The nucleotide alteration of the previously described *proU601* mutation (31) is marked by an arrow. (B) Nucleotide alteration in the −35 region of the *proU603* allele. (C to E) Secondary mutations recovered during the initial sequence analysis of the *proU603* mutation.

GM37 at low osmolarity and isolated 182 independent spontaneous Lac$^+$ mutants. In all mutants, expression of the Φ(*proV-lacZ*)(Hyb2) fusion was still osmoregulated, as judged by their Lac phenotypes on lactose MacConkey plates without or with 200 mM NaCl. Genetic analysis demonstrated that eight strains carried mutations tightly linked to the *proU* region. In all likelihood (see Materials and Methods), the remaining 174 mutant strains carry mutations in *osmZ*, the structural gene for the histonelike DNA-binding protein H-NS (H1a) (18, 23, 24a, 30) and were not characterized further.

To analyze the osmoregulated expression of the eight mutant strains carrying alterations closely linked to the Φ(*proV-lacZ*)(Hyb2) fusion, we grew these strains at different medium osmolarities and quantitated the specific β-galactosidase activities. Identical enzyme activities were found for all mutant strains; the data for one representative mutant, JML21, are shown in Fig. 1. At low osmolarity, the level of Φ(*proV-lacZ*)(Hyb2) expression was approximately 40-fold higher in strain JML21 than in strain GM37; at high osmolarity, it could be induced to higher levels in the mutant than in the parent strain GM37. The strength of Φ(*proV-lacZ*)(Hyb2) expression of the newly isolated mutants differed from that of the previously characterized (23, 31) mutant strain BRE2074 (Fig. 1), which carries a point mutation (*proU601*) in the −10 region of the *proU* promoter (Fig. 2A).

Increased expression in the mutants results from *proU* promoter mutations. To characterize these *proU*-linked mutations at the molecular level, we cloned the Φ(*proV-lacZ*)(Hyb2) fusion from the eight mutant strains by in vivo homologous recombination into a multicopy plasmid (Materials and Methods) and sequenced the *proU* promoter region. In each mutant, a single, identical point mutation was present in the *proU* promoter. This mutation (*proU603*) alters the −35 region from 5′-TTGCCT-3′ to 5′-TTGACT-3′ (Fig. 2B), thereby increasing the homology of the *proU* −35 region to the consensus sequence (5′-TTGACA-3′) of σ^{70}-dependent *E. coli* promoters (22).

We have previously reported that multicopy plasmids carrying the *proU601* mutation in a Φ(*proV-lacZ*)(Hyb2) fusion plasmid rapidly acquire secondary mutations that strongly decrease the expression of the fusion (31). Similar observations were made during sequence analysis of the *proU603* alleles. Recombinant M13mp18 phages carrying the *proU* promoter regions of the various mutants were originally propagated under standard conditions in DYT medium containing 86 mM NaCl (33). When these clones were sequenced, a variety of mutations in the *proU* promoter were recovered. Several clones carried the *proU603* allele and an additional mutation in either the −10 (*proU604*; Fig. 2C) or −35 (*proU605*; Fig. 2D) region. These second-site mutations affect base pairs that are highly conserved among most *E. coli* promoters and are known to be important determinants of promoter strength (22, 35). In addition, a point mutation (*proU606*; Fig. 2E) located in the −35 region was recovered which had occurred at the same position as the *proU603* base pair change. Likewise, this mutation decreases the homology score (35) of the *proU* promoter. We consider it likely that these types of mutations are secondary suppressors of the *proU603* mutation, because

only the *proU603* allele was recovered when the recombinant M13 clones were propagated in DYT medium without NaCl.

A IS*1* insertion between the −10 and −35 regions of *proU* does not abolish osmoregulation. Plasmid pOS7 carries the Φ(*proV-lacZ*)(Hyb2) fusion and all sequences required in *cis* for the proper osmoregulated expression of *proU* (31, 32). Because of the high copy number of plasmid pOS7, strain JML1(pOS7) exhibits a Lac$^+$ phenotype on MacConkey lactose plates. We isolated 37 Lac$^-$ derivatives of JML1 (pOS7) after cycloserine enrichment and found three mutants in which the mutation was located on the plasmid (see Materials and Methods). Two of these strains had no detectable β-galactosidase activity, and the third had reduced but osmotically inducible activity. Restriction analysis showed that the plasmid, pJL21, isolated from the latter strain had acquired an insertion of approximately 760 bp close to the *proU* promoter region. DNA sequence analysis of both junctions between the newly inserted material and the *proU* sequences demonstrated that a copy of IS*1* (39) was integrated between the −10 and −35 regions of the *proU* promoter (*proU607*; Fig. 3A).

Examination of the Lac phenotype of strain JML1(pJL21) on lactose MacConkey plates without or with 200 mM NaCl showed that expression of the Φ(*proV-lacZ*)(Hyb2) fusion was still osmoregulated: the strain is Lac$^-$ at low osmolarity but Lac$^+$ at high osmolarity. This characteristic was also evident when the specific β-galactosidase activities in strains carrying either the parental plasmid pOS7 or the mutant plasmid pJL21 were determined (Table 2). Plasmid-directed, high-level synthesis of the Φ(*proV-lacZ*)(Hyb2)-encoded hybrid protein can result in aberrantly low specific β-galactosidase activities as a result of intracellular aggregation of the large hybrid protein (31). We therefore performed Western immunoblot experiments with whole cell extracts to directly visualize the amount of hybrid protein produced in strain EF027 carrying either pOS7 or pJL21, and we found that the IS*1* insertion in pJL21 led to strongly reduced but still osmoregulated synthesis of the hybrid protein (Fig. 3B).

The IS*1* insertion into the *proU* promoter disrupts its integrity. Inspection of the DNA sequence at the insertion point revealed the presence of a hexameric sequence at the right end of IS*1* with homology to the consensus −35 region (15). This putative −35 region could possibly form a novel hybrid promoter in connection with the authentic *proU* −10 region located 18 bp further downstream (Fig. 3A). We mapped the 5′ end of the *proU* mRNA by primer extension and detected an osmoregulated transcript in cells carrying plasmid pJL21 that was identical to that detected in cells carrying the wild-type *proU* promoter on plasmid pOS7 (31). In both cases, several bands differing in length by one nucleotide were found, with the major band corresponding to the A nucleotide at position 628 (Fig. 2A). As expected, the amount of the pJL21-directed *proU* transcript was much lower than that directed by the parent plasmid, pOS7 (data not shown).

Deletion analysis of *proU* upstream sequences. Plasmid pOS7 carries a chromosomal DNA segment of 592 bp upstream of the *proU* −35 region (31; Fig. 4). A deletion analysis was carried out to determine whether sequences involved in *proU* expression or its osmoregulation are present in this DNA segment. Starting from the unique *Eco*RI site in pOS7, we constructed a nested set of unidirectional deletions that progressively remove sequences 5′ to the *proU* promoter. The exact deletion endpoints were determined by DNA sequencing (Fig. 4). To avoid any

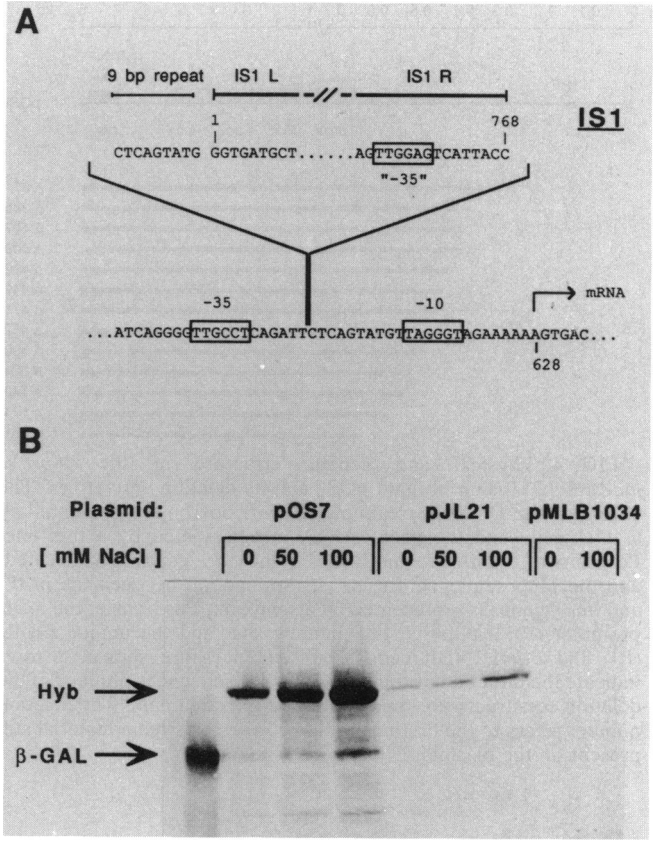

FIG. 3. Analysis of an IS*1* insertion into the *proU* promoter. (A) DNA sequence of the IS*1* insertion point in the *proU* promoter (*proU607*) on plasmid pJL21. The *proU* mRNA start site is indicated. The 9-bp duplication generated by the IS*1* insertion and a putative −35 region near the right end of IS*1* are marked (15, 39). (B) Western blot analysis of the ProV–β-galactosidase hybrid protein. Cells of strain EF027 carrying plasmid pOS7 (wild type), pJL21 (pOS7::IS*1*), or the *'lacZ* fusion vector pMLB1034 (51) were grown overnight in glucose MMA with the indicated amounts of NaCl. Total cellular proteins were separated by SDS-PAGE, transferred to a nylon membrane, and probed with anti-β-galactosidase antiserum. Positions of the hybrid protein (Hyb) and the marker protein β-galactosidase (β-gal) are indicated by arrows.

complications that might arise in studying the expression of the multicopy Φ(*proV-lacZ*)(Hyb2) fusion, we recombined in vivo the different constructs into phage λRZ5 and integrated the resulting Lac$^+$ specialized transducing phages as single-copy lysogens into the *E. coli* chromosome at *attB* (Materials and Methods). Specific β-galactosidase activities in the lysogens were determined in cultures grown at low (MMA)

TABLE 2. Effect of an IS*1* insertion on Φ(*proV-lacZ*)(Hyb2) expression carried by a multicopy plasmid[a]

Plasmid	β-Galactosidase activity in MMA with:		
	0 mM NaCl	50 mM NaCl	100 mM NaCl
pOS7	2.65	9.51	18.46
pJL21 (pOS7::IS*1*)	0.35	0.76	1.72

[a] Cells of strain JML1 carrying the indicated plasmids were grown overnight in glucose MMA with the indicated amount of NaCl, and the specific β-galactosidase activity (expressed as micromoles of ONPG cleaved per minute per milligram of protein) was determined.

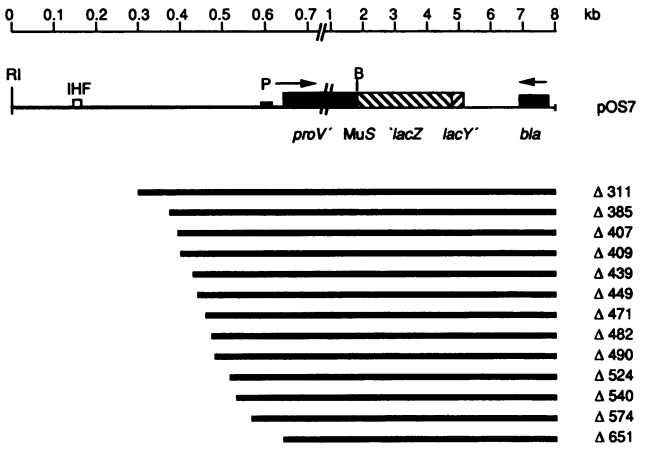

FIG. 4. Physical and genetic structure of the Φ(*proV-lacZ*)(Hyb2) fusion plasmid pOS7 and its deletion derivatives. The chromosomal DNA upstream of the *proU* operon is represented by a thick line, and the vector DNA is represented by a thin line. Positions of the *proV'*, *'lacZ*, *lacY'*, and *bla*$^+$ genes are indicated, and the MuS sequence present at the junction between the *proV'* and *'lacZ* genes is represented by a solid box. Positions of the *proU* promoter (P), a putative IHF-binding site, and the unique *Eco*RI (RI) and *Bam*HI (B) recognition sequences are shown. Arrows indicate the direction of transcription. The sequences retained in the deletion constructs are represented by the solid bars. The deletion number refers to the first nucleotide of *proU* upstream material still present on the plasmid.

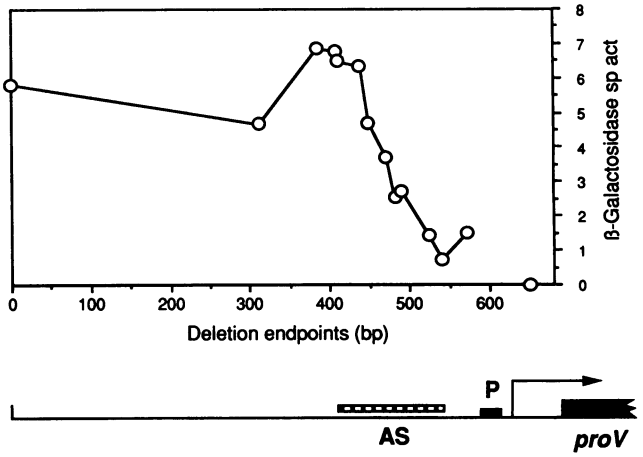

FIG. 5. Effects of upstream deletions on Φ(*proV-lacZ*)(Hyb2) expression. λ specialized transducing phages carrying the hybrid genes with progressive deletions of sequences 5' of the *proU* promoter (see Fig. 4) were integrated as single-copy lysogens into the bacterial chromosome at *attB*. Specific β-galactosidase activity (expressed as micromoles of ONPG cleaved per minute per milligram of protein; the values are taken from Table 3) of strains grown overnight in glucose MMA with 200 mM NaCl is plotted against the deletion endpoints. The relative positions of the *proU* promoter (P), the transcriptional start site, the beginning of the *proV* structural gene, and the activating sequences (AS) are indicated at the bottom.

or high (MMA with 200 mM NaCl) osmolarity. Osmoregulation of Φ(*proV-lacZ*)(Hyb2) expression was observed in all constructs with deletion endpoints 5' to the *proU* promoter (Table 3). The most extensive deletion, Δ574, carries just 19 bp of chromosomal DNA upstream of the *proU* −35 region, and thus no sequences further upstream are required for osmoregulation of *proU* expression. However, the absolute level of uninduced and osmotically induced Φ(*proV-lacZ*)(Hyb2) expression was strongly affected by sequences upstream of the *proU* promoter (Table 3). The progressive deletion of DNA sequences 3' to 407 bp resulted in a gradual decline of Φ(*proV-lacZ*)(Hyb2) expression (Fig. 5). Thus, the sequences between 407 bp and the *proU* −35 region at 593 bp are required for optimal functioning of the *proU* promoter. Removal of these 5' sequences resulted in an approximately 10-fold decline of Φ(*proV-lacZ*)(Hyb2) expression (Fig. 5).

TABLE 3. Effect of deletions 5' to the *proU* promoter on the osmoregulated expression of the Φ(*proV-lacZ*)(Hyb2) fusion[a]

Prophage[b]	β-Galactosidase activity in MMA with:		Induction factor
	0 mM NaCl	200 mM NaCl	
λpOS7	0.056	5.82	104
λpOS7-Δ311	0.055	4.65	84
λpOS7-Δ385	0.06	6.87	115
λpOS7-Δ407	0.2	6.77	34
λpOS7-Δ409	0.161	6.46	40
λpOS7-Δ439	0.092	6.31	69
λpOS7-Δ449	0.065	4.68	72
λpOS7-Δ471	0.028	3.69	132
λpOS7-Δ482	0.012	2.5	208
λpOS7-Δ490	0.022	2.71	123
λpOS7-Δ524	0.021	1.4	66
λpOS7-Δ540	0.021	0.70	33
λpOS7-Δ574	0.035	1.49	43
λpOS7-Δ651	0	0	

[a] Cells of strain EF027 carrying the indicated prophages were grown overnight in glucose MMA with the indicated amount of NaCl, and the specific β-galactosidase activity (expressed as micromoles of ONPG cleaved per minute per milligram of protein) was determined.

[b] Phage λpOS7 [Φ(*proU-lacZ*)(Hyb2)] and its derivatives carrying deletions 5' to the *proU* promoter (see Fig. 4) are present as a prophage integrated at *attB* in strain EF027 [Δ(*proU*)*600*].

Influence of FIS and IHF proteins on *proU* expression. It was recently shown that FIS protein binds to an upstream activating sequence present in stable RNA operons, thereby activating transcription from downstream promoters (37). We therefore tested whether a deletion mutation in *fis* affects *proU* expression. The specialized transducing phage λpOS7 [Φ(*proV-lacZ*)(Hyb2)], carrying the entire region upstream of the *proU* promoter, was lysogenized into the Δ*fis* strain RJ1617 and its *fis*$^+$ parent strain MC1000. No difference in expression of the *lac* fusion was found at low or at high osmolarity (data not shown).

It has been noted (20) that a sequence upstream of the *proU* promoter (Fig. 4) matches the consensus sequence for binding of IHF. Since this protein is known to affect expression of several genes (11), we tested the influence of IHF mutations on expression of the chromosomal Φ(*proV-lacZ*)(Hyb2) fusion. A deletion mutation in the structural gene (*himA*) for the α subunit of the IHF protein was introduced into the fusion strain GM37, resulting in the otherwise isogenic strain JML81. When the specific β-galactosidase activities of cultures of GM37 and JML81 were determined, we found osmotic induction of enzyme activity in both strains, but the induced level of β-galactosidase activity was reduced about twofold in the *himA* strain (Table 4). Identical results were obtained when a *himD* deletion that prevents the synthesis of the β subunit of IHF or a *himA*

TABLE 4. Effect of IHF on Φ(*proV-lacZ*)(Hyb2) expression[a]

Strain	Relevant genotype	Specific β-galactosidase activity in MMA	
		− NaCl	+ 200 mM NaCl
GM37	Φ(*proV-lacZ*)(Hyb2) *himA*$^+$	0.012	4.27
JML81	Φ(*proV-lacZ*)(Hyb2) Δ*himA*	0.013	1.75
JML3	λpOS7[Φ(*proV-lacZ*)(Hyb2)] *himA*$^+$	0.030	6.40
JML102	λpOS7[Φ(*proV-lacZ*)(Hyb2)] Δ*himA*	0.032	4.83
JML100	λpOS7-Δ540 [Φ(*proV-lacZ*)(Hyb2)] *himA*$^+$	0.013	1.01
JML101	λpOS7-Δ540 [Φ(*proV-lacZ*)(Hyb2)] Δ*himA*	0.005	0.38

[a] Cells were grown overnight in glucose MMA with the indicated amount of NaCl, and the specific β-galactosidase activity (expressed as micromoles of ONPG cleaved per minute per milligram of protein) was determined. The data shown are mean values of two independent experiments. The strains used carry the same *proV-lacZ* fusion, either at the *proU* locus (GM37 and JML81) or on a prophage (all other strains) integrated at *attB*.

himD double mutant strain was tested (data not shown). We also tested the influence of a *himA* deletion on the expression of the Φ(*proV-lacZ*)(Hyb2) fusion in a lysogen that carries deletion Δ540 and consequently lacks the putative IHF-binding site upstream of *proU* (Fig. 4). Again, only a small decrease in expression of the *lac* fusion was observed (Table 4). From these results, we conclude that the IHF protein has only a minor effect on *proU* expression, which is not mediated by the putative IHF-binding site positioned upstream of *proU*.

DISCUSSION

Transcription of the *proU* loci of *E. coli* and *S. typhimurium* is strongly stimulated by a sudden upshift of medium osmolarity, and its steady-state level is proportionally linked to the osmolarity of the environment (3, 6, 8, 13, 19, 21, 32). In addition, optimal *proU* expression in *S. typhimurium* requires anaerobic growth conditions (36), suggesting that regulation of *proU* expression is quite complex. We have selected a large number of independent spontaneous mutants that exhibit increased expression of a chromosomal *proU-lac* protein fusion at low osmolarity. Only a minor fraction of these mutants (8 of 182) had alterations linked to the *proU* locus and were shown to carry point mutations in the *proU* −35 region. Our analysis of the remaining strains indicates that they carried lesions in the unlinked *osmZ* gene. This gene has recently been shown to encode the histonelike DNA-binding protein H-NS (H1a), which is tightly associated with the *E. coli* nucleoid and has profound effects on DNA structure and the expression of a number of genes with diverse functions (18, 23, 24a, 30). Our results thus contrast with those of Druger-Liotta et al. (12), who carried out an identical selection in *S. typhimurium* and found only mutants (60 of 60) with alterations tightly linked to the *proU* locus. These differences might result from the different types of *lac* fusion used for the selection (protein versus operon fusion) or the species studied, although *S. typhimurium* is known to possess an H-NS (H1a) protein closely related to that of *E. coli* (24a, 29).

The eight *proU* promoter mutants characterized in this study still show osmoregulated *proU-lac* expression, but both the basal and induced levels of expression are significantly higher than those of their parent strain. The identical point mutation (*proU603*) present in the *proU* −35 region of these mutants readily explains the stronger expression of the fusion. The increasing level of Φ(*proV-lacZ*)(Hyb2) expression in strains carrying either the *proU* wild-type or the mutant *proU601* or *proU603* promoters (Fig. 1) is reflected by their increasing homology to the consensus sequence of σ^{70}-dependent *E. coli* promoters (22, 35). Although only two point mutations in the *proU* promoter have as yet been fully characterized, the properties of the *proU601* and *proU603* alleles demonstrate that alterations in the *proU* −10 and −35 regions do not abolish osmotic regulation. This finding indicates that the particular sequences present at the *proU* −10 and −35 regions are not the prime determinants for the osmotic regulation of *proU* transcription. This view is supported by the regulatory features of the *proU607* allele, in which the authentic −35 region and some spacer sequences have been replaced by IS*1* sequences. The hybrid promoter created by the IS*1* insertion functions only inefficiently, probably because of the increased spacing between the −10 and −35 region, the greater deviation of its −35 region from the consensus promoter sequence (22, 35), and the displacement of 5′ chromosomal sequences required for the high-level expression of *proU*.

Two different laboratories (20, 41) reported ambiguities in the assignment of the *proU* promoter, whereas we (31) and Stirling et al. (52) identified the promoter sequence shown in Fig. 2A. The positions of the secondary suppressor mutations that we recovered during the initial sequence analysis of the *proU603* promoter up-mutation emphasizes the assignment of these sequences as the main *proU* promoter. Although we have not analyzed the effects of these secondary mutations on *proU* transcription, they all affect base pairs known to be important for promoter function (22, 35). It is unclear why these secondary suppressor mutations are selected, but the osmolarity of the medium used to propagate recombinant plasmids or M13 phages apparently plays a decisive role. Most likely, the cell does not tolerate very high level transcription driven from the mutated and osmotically induced *proU* promoter. Our inability to isolate any *cis*- or *trans*-acting mutations that result in high-level, constitutive expression of a *proU-lac* fusion suggests that no classical repressor protein for the *proU* operon exists, unless such a protein is of central importance for other functions as well. This possibility has already been raised by other studies (12, 23); however, the rapid accumulation of secondary mutations that we found during our studies (31; this report) might indicate that constitutive mutants are strongly counterselected.

To determine whether any sequences upstream of the *proU* −35 region are involved in osmoregulation, we constructed a nested set of deletions which progressively remove chromosomal sequences 5′ of the *proU* promoter. Since the results of regulatory studies using multicopy plasmids carrying the *proU* promoter can sometimes be difficult to interpret (41, 52), we analyzed single copies of the deletion constructs integrated into the bacterial chromosome. Removal of sequences up to 19 bp 5′ of the −35 region, Δ574 (Fig. 4), still allowed osmoregulated expression of the Φ(*proV-lacZ*)(Hyb2) fusion, showing that no sequences further upstream are essential for this process. This result is similar to that found for the expression of *osmB*, the structural gene for a lipoprotein. Transcription directed by the *osmB* P2 promoter is osmotically stimulated, and this regulation is unaffected by a deletion in which just 10 bp 5′ of the *osmB* P2 −35 region are retained (21, 27). There are no close similarities between the *osmB* and the *proU* promoter sequences. What are the minimal sequences required for the osmoregulation of *proU* transcription? The still osmoregu-

lated deletion construct Δ574 carries chromosomal DNA sequences which extend from 19 bp 5′ of the −35 region (position 574; Fig. 2A) to 1,582 bp (31) in the first gene, *proV*, of the *proU* operon. The minimal sequences required for osmoregulation are certainly much shorter than these 1,008 bp. Park et al. (42) reported that a 253-bp *E. coli proU* DNA segment cloned into a multicopy promoter-probe plasmid conferred osmoregulated gene expression. This fragment carries 173 bp upstream of the −35 region and ends 52 bp downstream of the −10 region, just within the putative ribosome-binding site of *proV* (position 672; Fig. 2A). These data, together with our results, indicate that the sequences required for osmoregulation of *proU* are confined to a 99-bp chromosomal segment that contains the *proU* promoter and the transcriptional initiation site but no part of the *proU* structural genes.

Deletion analysis of chromosomal sequences 5′ of *proU* revealed that its maximal expression requires the presence of the DNA segment extending approximately 200 bp upstream of the −35 region. The successive shortening of these sequences results in a gradual decline of *proU-lacZ* expression (Fig. 5), indicating that no single site within this region is responsible for its positive effect on *proU* expression. The position of the sequences required for the full expression of *proU* matches that a DNA segment displaying features of intrinsically bent DNA (20). Whether this is just a coincidence or whether it points to a functional role of the DNA bend for efficient *proU* expression is unclear at present. Sequence distributions associated with DNA curvature upstream of a number of procaryotic promoters with upstream activation of transcription have been described (44), and the binding of accessory proteins such as FIS and IHF to sequences upstream of promoters is implied in the activation of transcription (37, 54). Our results with a *fis* mutant show that FIS does not play any role in *proU* expression. We observed a reduction in *proU* expression in *himA* or *himD* mutants, but these effects were independent of the presence of a putative IHF-binding site upstream of the *proU* operon (20). We consider it likely that this reduction is an indirect effect, since *himA* mutations decrease the level of DNA supercoiling (17), a determinant of *proU* expression (23). We do not know whether the sequences 5′ to the *proU* promoter serve only a general activating function for transcription or whether they mediate the input of specific regulatory stimuli other than osmolarity (36). The reconstitution of *proU* transcription in a purified in vitro system has recently been reported, and it has been suggested that intracellular K^+-glutamate concentration is the only signal that mediates osmotic regulation of *proU* (45). The role for the *osmZ*-encoded histonelike DNA-binding protein H-NS (H1a) for *proU* expression remains to be elucidated. In addition, it is not yet clear whether other factors, such as DNA-binding proteins that might recognize the *proU* upstream sequences, make important contributions to the finely tuned regulation of *proU* expression observed in vivo.

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