The Escherichia coli proU Promoter Element and Its Contribution to Osmotically Signaled Transcription Activation

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The proU operon of Escherichia coli encodes a high-affinity glycine betaine transport system which is osmotically inducible and enables the organism to recover from the deleterious effects of hyperosmotic shock. Regulation occurs at the transcriptional level. KMnO₄ footprinting showed that the preponderance of transcription initiated at a single primary promoter region and that proU transcription activation did not occur differentially at alternate promoters in response to various levels of salt shock. Mutational analysis confirmed the location of the primary promoter and identified an extended −10 region required for promoter activity. Specific nucleotides within the spacer, between position −10 and position −35, were important for maximal expression, but every mutant which retained transcriptional activity remained responsive to osmotic signals. A chromosomal 90-bp minimal promoter fragment fused to lacZ was not significantly osmotically inducible. However, transcription from this fragment was resistant to inhibition by salt shock. A mutation in osmZ, which encodes the DNA-binding protein H-NS, derepressed wild-type proU expression by sevenfold but did not alter expression from the minimal promoter. The current data support a model in which the role of the proU promoter is to function efficiently at high ionic strength while other cis-acting elements receive and respond to the osmotic signal.

To counteract the injurious effects of hyperosmotic stress, Escherichia coli accumulates compatible solutes in a hierarchy dictated by their contribution to osmoprotection. Betaine compounds afford E. coli the greatest amount of recovery from the inhibitory effects of increased extracellular osmolarity (28), and glycine betaine accumulation is preferred over the accumulation of the alternative osmolytes proline, trehalose, and $K^+$ (12, 55). Transport of glycine betaine in E. coli is facilitated by proP (34) and by proU (32). The proU locus encodes a high-affinity binding protein-dependent transporter (32) containing three cistrons, proV, proW, and proX (18). Studies using transcriptional and translational proU-lacZ chromosomal fusions demonstrated a 60- to 100-fold induction by osmotic upshock (2, 3) with regulation at the level of transcription (32).

The initial response of E. coli to hyperosmotic stress is the active uptake of $K^+$ (12). Under normal physiological conditions, $K^+$ is transported by the constitutive, low-affinity Trk system (41), but under conditions of plasmolysis or $K^+$ deprivation, $K^+$ is also accumulated through the high-affinity kdp operon (27, 41). Intracellular $K^+$ concentration is an approximately linear function of extracellular osmolarity, although the absolute values obtained by different investigators varied considerably (15, 42). The level of proU expression correlates well with the accumulation of intracellular $K^+$ (55), leading to the hypothesis that the internal $K^+$ concentration is the signal for proU transcriptional activation.

Extensive searches for trans-acting mutations causing constitutive proU expression have identified only partially constitutive mutations which were still osmotically regulated. These mutations mapped to either the proU promoter region or the osmZ locus (1, 13, 22, 30). osmZ encodes H-NS (also known as H1, B1, or H1a), an abundant nucleoid-associated 15-kDa protein (52, 58) which binds and compacts DNA. Mutations found to be allelic to osmZ altered the expression of several other unlinked genes. A mutation in dnaX derepressed temperature-regulated pap pilus synthesis (16). One at pilG altered the frequency of fimA phase variation (22, 53), one at bgLY derepressed the bgICSB operon (10, 22) and one in the cur locus affected the metastable flu gene, altering surface properties and colony morphology (11).

The position of the proU transcriptional start site has remained somewhat ambiguous because of the differing results among the several independent groups who have mapped the 5' end of proU mRNA (9, 18, 30, 31, 54). Position 628 (18) is thought to be the main initiation site, but additional promoters have been suggested, centered at positions 474 and 438. In addition, regulatory elements 3' and 5' to the proposed start site have been identified (9, 30, 35). A downstream negative regulatory element extends into the first structural gene, proV (9). Approximately 10-fold derepression of a proU-lacZ fusion occurred under low-osmolarity conditions when this downstream element was deleted. proU of Salmonella typhimurium also has a similar downstream negative-acting sequence (35). A 200-bp segment of DNA upstream of the start site at position 628 seemed to affect gene expression positively and was necessary for maximal osmotic induction of proU in E. coli (30). For this study, we concentrated on the properties of the proU minimal promoter and assessed its contribution to osmotic signaling.

MATERIALS AND METHODS

Bacterial strains. E. coli strains are listed in Table 1. All strains containing lacZ fusions were derivatives of MC4100, except JM1701 and AP757, which were derived from W4680.

In vitro KMnO₄ footprinting. In vitro footprinting procedures were based on those previously described (37, 46). One microgram of either pBP33U(proU XmnI) fragment containing DNA from positions 377 to 786 or pHLaS (bla) was incubated with 2.7 mM ATP, 1 mM GTP, 0.7 mM CTP, and 1.4 mM UTP, 350 µg of rifampin per ml, and 2 U of RNA polymerase.

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(Boehringer Mannheim Corp.). After KMnO₄ modification, DNA plasmid templates were purified by GENE CLEAN (Bio 101, Inc.) and eluted in sterile water.

Primer extension, both 5’ and 3’ from the proU promoter and 5’ from the bla promoter, was performed as previously described (46), with the following modifications. Primers (U.S. Biochemical Corp.) were end labeled with [γ-32P]ATP (Amersham) and T4 polynucleotide kinase (Promega) and separated from the unincorporated nucleotides by a G-25 spin column. Five hundred nanograms of a purified template was combined with 150 ng of either end-labeled reverse (5’) or universal (3’) primer containing approximately 40,000 cpm. The reverse primer was annealed at 46°C, and the universal primer was annealed at 52°C. Annealed primers were extended with Klenow fragment (Boehringer Mannheim Corp.) and quenchd after incubation at 42°C for 10 min. Precipitated DNA was resuspended in 6 µl of water and 4 µl of stop solution (U.S. Biochemical Corp.) and then electrophoresed in a 6% polyacrylamide–urea gel (Amresco). The gel was dried and autoradiographed with Amersham Hyperfilm.

**proU mutagenesis.** The protocol used for obtaining mutations in the proU promoter region was a modification of that described by Kunkel (26). Klenow enzyme was substituted for T4 DNA polymerase to circumvent difficulties in polymerization due to DNA secondary structure, and single-stranded DNA was isolated with the pTZ vector system (U.S. Biochemical Corp.).

Two oligonucleotide primers for creating base substitutions were synthesized by the University of California Davis DNA Synthesis Laboratory. The oligonucleotide 5’TTCCTCAGATTGTTaggTgaaaagaaATCAATTTCC-3’ contained 94% of the correct nucleotide and 2% of each of the other three nucleotides at all adenine and guanine residues shown in lowercase. This 38-mer with 20 internal positions substituted was designed to create base substitutions in the −10 and poly(A) regions of the proU promoter. The oligonucleotide 5’-GCCTCAGAttgcAGATAtGtTGGGTAG-3’ contained 97% of the correct nucleotides and 1% of each of the other three nucleotides at the thymine and cytosine residues in lowercase. This 28-mer, with substitutions at nine positions, was designed to create proU spacer mutations. Both oligonucleotide primers were used with the parent plasmid pBP35CM; single-stranded DNA was isolated and sequenced to screen for mutations in the promoter region. The sequences of a representative set of the mutant plasmids are shown in Table 2.

**Media and growth conditions.** M9 minimal medium was supplemented with 0.2% glucose and 1 µg of thiamine per ml. For growth of strains containing lacZ fusions inserted into the trp locus, 30 µg of tryptophan per ml was also added. Unless otherwise stated, antibiotics were added to the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; tetracycline, 20 µg/ml; and chloramphenicol, 30 µg/ml. All strains were grown aerobically at 37°C. HY50 strains transformed with plasmids with mutant proU promoter fragments were prepared for chloramphenicol acetyltransferase (CAT) assays by diluting an overnight culture 1:20 into M9 glucose minimal medium containing ampicillin. Cultures were shocked with 0.5 M NaCl at 35 Klett units (early exponential phase), and samples were harvested at 80 Klett units.

Strains with lacZ fusions were prepared for kinetic analysis with a 1:100 dilution of an overnight culture into M9 glucose minimal medium without antibiotics so as not to interfere with protein expression and cell growth. Samples were taken from each flask at the indicated times, placed immediately on ice, and pelleted. Pellets were frozen at −70°C and subsequently resuspended in Z buffer without β-mercaptoethanol (33) in order not to interfere with protein determinations by Lowry assay. To determine the effect of the osmZ mutation on proU-, proP-, and bla-lacZ fusions, cultures were diluted 1:100 into M9 glucose minimal medium without antibiotics, shocked to 0.3 M NaCl after reaching exponential growth, and incubated overnight prior to assay.

**Plasmid constructions.** The 409-bp proU promoter fragment including DNA from positions 377 to 786 (XmnI-XmnI) was

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**TABLE 1. E. coli strains and plasmids**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>F' Δ(argF-lac)U169 thiA</td>
<td>5</td>
</tr>
<tr>
<td>GM50</td>
<td>MC4100 φ(proU-lacZ')3 (ApChemMu55)</td>
<td>32</td>
</tr>
<tr>
<td>HY50</td>
<td>(Kan'Cam-lacZ)</td>
<td>H. Yim</td>
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<tr>
<td>W4680</td>
<td>F' ΔlacZ Str' gal mel-4</td>
<td>47</td>
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<tr>
<td>TE2680</td>
<td>F' λ INRomD-rrmE1 Δ(lac)X74 rpsL galK2 recD1903; Tn10 Δ-lacZ</td>
<td>14</td>
</tr>
<tr>
<td>JM1700</td>
<td>F' MC4100 trpDC700::putPA1303; (Kan'Cam-lacZ)</td>
<td>This study</td>
</tr>
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<td>JM1701</td>
<td>F' W4680 trpDC700::putPA1303; (Kan'-1,700-bp proU-lacZ)</td>
<td>This study</td>
</tr>
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<td>F' MC4100 trpDC700::putPA1303; (Kan'-90-bp proU-lacZ)</td>
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</tr>
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<td>JM28</td>
<td>F' MC4100 trpDC700::putPA1303; (Kan'-720-bp bla-lacZ)</td>
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<td>AP575</td>
<td>F' W4680 trpDC700::putPA1303; (Kan'-90-bp lacUV5-lacZ)</td>
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<td>R. Kolter</td>
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<td>Plasmids</td>
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<td>Pharmacia</td>
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<td></td>
<td>pBP35CM 409-bp proU-CAT</td>
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<tr>
<td></td>
<td>pRB115 through pRB453 409-bp proU-CAT containing base substitutions</td>
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TABLE 2. proU mutational analysis and promoter sequence comparisons

<table>
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<th>Plasmid</th>
<th>Mutation</th>
<th>CAT Specific Activitya (nmoles min⁻¹ mg⁻¹)</th>
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<tr>
<td>pCM1 (no promoter)</td>
<td>-</td>
<td>3 5</td>
</tr>
<tr>
<td>pBP35CM (wt proU)</td>
<td>CAGAGTAAGGGGT</td>
<td>23 102</td>
</tr>
<tr>
<td>pBR115</td>
<td>CATGCTTCTAGGTT</td>
<td>3 4</td>
</tr>
<tr>
<td>pBR405</td>
<td>CAGAGTAAGGGGT</td>
<td>6 15</td>
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<td>pBR406</td>
<td>CAGAGTAAGGGGT</td>
<td>11 40</td>
</tr>
<tr>
<td>pBR415</td>
<td>CAGAGTAAGGGGT</td>
<td>15 30</td>
</tr>
<tr>
<td>pBR424</td>
<td>CAGAGTAAGGGGT</td>
<td>5 5</td>
</tr>
<tr>
<td>pBR429</td>
<td>CAGAGTAAGGGGT</td>
<td>5 5</td>
</tr>
<tr>
<td>pBR431</td>
<td>CAGAGTAAGGGGT</td>
<td>12 48</td>
</tr>
<tr>
<td>pBR436</td>
<td>CAGAGTAAGGGGT</td>
<td>8 10</td>
</tr>
<tr>
<td>pBR446</td>
<td>CAGAGTAAGGGGT</td>
<td>17 90</td>
</tr>
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</table>

−35 T T T

a CAT activity values are the average of at least three independent assays.

b The −10 region is underlined. Base substitutions are double underlined, and the deletion in pRB429 is noted by Ö, wt, wild type.

Footprinting of the proU and gap1 transcriptional start sites, +1, +10, and −35 regions have been identified. +1 coincides with position 626 of the E. coli proU operon published sequence (18). The proU promoter is putative (8). For the proU in vitro footprinting analysis, residues modified by KMnO₄ within regions of single strandedness (i.e., open complex) are in boldface. The boldface T residue is an actual site of modification, whereas boldface A residues represent modified complementary bases.

Also isolated for mutagenesis. The EcoRI-PstI fragment from pBP32 containing this region (38) was ligated into pTZ19U to form pBP35U. An 800-bp Sall-Sall promoterless CAT reporter gene (Pharmacia) was then ligated downstream of proU to form pBP35CM. Recombinant plasmids were screened for the correct orientation of the CAT cartridge on Luria-Bertani medium containing chloramphenicol after transformation into JM101.

For constructing single-copy transcriptional lacZ fusions, proU, bla, lacUV5, and proP promoter fragments were initially cloned into either the pRS550 or pRS551 expression vector (50) and transformed into E. coli DH5α. Strain JM100 contains the entire 1,700-bp proU fragment from pBP1 (39) ligated into pRS551 at EcoRI and BamHI sites. JM409 was constructed by directionally cloning the 409-bp XmnI proU fragment from pBP33U (37) into pRS551 with EcoRI and BamHI. To construct JM90 and JM2000, a 90-bp SspI-TagI proU fragment (positions 655 to 665) and a 2,000-bp BamHI-SspI proP fragment from pDC15 (8) were cloned into an intermediate vector, pTZ18U, prior to cloning into pRS550.

For constructing strain JM28, the bla promoter fragment, originally from pBR322, was obtained from pBlas5 (37) by isolating a 750-bp EcoRI-SphI fragment. It was then ligated into pDYES5 (pRS551 containing a portion of the multiple cloning region from pTZ18, including the SphI site), which was also digested with EcoRI and SphI. In order to obtain a single-copy chromosomal fusion of the lacUV5 promoter (43), we cloned the fragment from pRS475 (50), which does not have a 5′ flanking resistance gene, into pRS551, which does possess this marker. The lacUV5 fragment was isolated and then cloned into the recipient vector by digestion with EcoRI and EcoRV. Preliminary assays to confirm lacUV5 constructs prior to genetic manipulations were performed in the RS6177 background (50), which possesses an episomal lacI gene to achieve full repression of multicityc fusions. All plasmid constructs were verified by restriction enzyme digest, and in addition, the plasmid constructs containing the 90-bp proU and lacUV5 fragments were verified by DNA sequencing. The universal primer supplied by the manufacturer (U.S. Biochemical Corp.) was utilized for double-stranded sequencing of all recombinant plasmids because it annealed to the S′ region of lacZ, which is contained within the pTZ18U, pRS550, and pRS551 parent plasmids.

Genetic procedures. proU, bla, lacUV5, and proP promoter fragments fused to lacZ reporter genes in the multicity constructs described above were transferred to the E. coli chromosome in single copy by transformation of linearized plasmid DNA into the recD host TE6260 (14). All plasmids were linearized with XhoI, except pJM2000, which was linearized with ScaI.

To ensure that we indeed had single-copy chromosomal fusions of each of the promoter fragments, we transduced the proU, proP, and bla fusions contained within the trp operon into MC4100 by using the phage P1vir (49). The lacUV5-lacZ construct in JM575 was created by transducing the TE6280 chromosomal fusion into W4680, which contains a wild-type lacI gene for normal repression and an internal lacZ deletion (47). The 1,700-bp proU fragment from the proU fusion on the TE6268 chromosome was also transduced into W4680 to produce JM1701. Where possible, each genetic manipulation was checked for the expected phenotype by β-galactosidase assay.

Recombinant DNA techniques. Electrophoresis of DNA in agarose, ethanol precipitation of DNA, transformations, and phenol extractions were done by standard procedures (45). Double-stranded DNA sequencing and single-stranded DNA sequencing were performed with the Sequenase version 2.0 sequencing kit (U.S. Biochemical Corp.) according to the instructions provided. DNA for double-stranded sequencing was isolated with the Plasmid Midi kit (Qiagen).

Enzyme assays. Extracts were prepared for CAT assays by microcentrifugation of cells at 4°C and washing the pellets in cold TD2T buffer (50 mM Tris [pH 7.8], 30 μM dithiothreitol). Cell pellets were resuspended in cold TD2T, sonicated, and microcentrifuged, and the supernatant was transferred to a clean tube. CAT assays were performed with the crude extracts as previously described (48). CAT specific activity values were expressed in nanomoles per minute per milligram of protein. For β-galactosidase assays, resuspended cell pellets were diluted in L buffer and assayed for β-galactosidase activity as previously described (33). β-Galactosidase specific activity values were expressed in nanomoles per minute per milligram of protein. Protein quantities were determined by the Lowry assay (29). Assays for steady-state overnight values were performed as described above, except that pellets were not frozen prior to assay.

RESULTS

Identifying the proU open complex. KMnO₄ footprinting was used to identify the proU open complex and to simultaneously determine whether proU transcription initiation occurred at a single or multiple sites and whether osmotic conditions influenced promoter usage. Footprinting was performed with the 409-bp XmnI fragment containing all of the previously identified putative transcriptional start sites. RNA polymerase was bound to templates in the presence of all four nucleoside triphosphates and rifampin to form open complexes but pre-
vent translocation and elongation (4, 51). Templates were then modified with KMnO₄, which preferentially forms thymine glycols on single-stranded DNA (21). After the DNA was modified, it was purified and a vector-directed primer was annealed and extended with the Klenow fragment of DNA polymerase I. The Klenow fragment is able to insert a base across from the modified residue but is unable to continue DNA synthesis beyond the point of modification (7, 23). Extension products are visualized on a sequencing gel, and the 3′ sites of primer extension termination appear as hyperreactive bands. These bands identify a transcription bubble, or open complex, and thus correspond to a region where transcription is initiated.

On the noncoding strand, there were strongly hyperreactive bands at adenine residues 617, 622, and 624, corresponding to positions −11, −6, and −4, respectively, of the transcriptional start site at position 628 (Fig. 1A and Table 2). Analysis of the coding strand resulted in a weak band at the adenine residue at position 631, which corresponded to the +4 position (data not shown). A faint signal at position 650 (Fig. 1A) was approximately the same intensity as the nonspecific bands appearing across all lanes and did not correspond to any previously identified transcriptional start site. Additional bands at positions 467 and 468, most likely corresponding to the putative start site centered at position 474 (18), were the same intensity as the band at position 650 shown by the noncoding strand analysis (data not shown). Thus, this putative start site was minor compared with the set of strongly hyperreactive bands between position 617 and position 624. Although the procedure with in vitro KMnO₄ is not strictly quantitative, the preponderance of proU open complex was formed near position 628, confirming that this is the major region where transcription initiation occurs. The precise initiating nucleotide has been difficult to determine. Position 628 has the optimal 7-bp spacing between the −10 region and the start site of transcription for E. coli promoters (19). We will henceforth refer to position 628 as +1 for ease of discussion.

In vitro KMnO₄ footprinting studies were performed over a physiologically relevant range of K⁺ glutamate concentrations to determine how this ion pair affected open complex formation at the main proU promoter. Significant proU open complex formation, visualized as hyperreactive bands at positions −4, −6, and −11, occurred in the range of 120 to 520 mM, showing maximal open complex at 320 mM K⁺ glutamate (Fig. 1A). In contrast, the bla promoter formed significant open complex only between 120 and 320 mM, with a maximum at 220 mM K⁺ glutamate (Fig. 1B). Therefore, proU open complex occurred over a higher and broader range of K⁺ glutamate concentrations than bla. Furthermore, open complex formation was limited to the promoter region corresponding to position 628 (Table 2) at all K⁺ glutamate concentrations.

**Locating important residues in the proU promoter.** To test whether specific bases within the primary promoter region were responsible for osmotic inducibility, we mutagenized the 409-bp XmnI proU promoter fragment containing DNA from position −251 to position +158, fused to a promoterless CAT reporter gene (pBP35CM). The 409-bp construct was derepressed about 10-fold at low osmolarity, probably because of deletion of the downstream negative regulatory element, but can be osmotically induced to the same maximum level as a wild-type 1,700-bp fusion. A large number of proU promoter mutations were created simultaneously by dirty mutagenesis. Primers for constructing mutagenized promoter fragments were synthesized by incorporating a low percentage of each incorrect nucleotide at designated positions. All potential mutants were sequenced so that we could identify the mutations which did not affect expression as well as those with an altered phenotype.

Mutations that abolished expression identified residues important for transcription and confirmed the identification of this region as the primary promoter. A T-to-G transversion at position −12 in pRB424, moving the −10 sequence further from consensus, reduced reporter gene activity to the level of a control plasmid, pCM1, carrying a promoterless CAT gene (Table 2). Single base substitutions replacing the G at position −14 within the spacer region (pRB115, pRB429) also abolished expression, indicating that no upstream promoter contributed significantly to the activity. The flanking nucleotides at positions −13 (pRB415) and −15 (pRB446) were important for achieving maximal proU expression. Substitutions of the TT sequence at positions −23 and −24 also reduced activity, while a mutation at position −20 (pRB453) had little or no effect. These data indicate that specific nucleotide sequences between the −10 and −35 regions are important for promoter function. Mutations in the poly(A) region between the −10 region and the transcription initiation site either had no effect on gene expression or increased overall expression up to twofold (data not shown). The mutant analysis shows that a specific nucleotide sequence in the spacer region is required to obtain
optimum transcriptional activity. However, many of the mutants with reduced activity are still osmotically inducible.

Contribution of the minimal proU promoter to osmotic induction. Deletion analyses performed in other laboratories (9, 30, 35) identified regulatory elements 5' and 3' to the promoter region. To isolate the proU promoter in the absence of both upstream and downstream regulatory sequences, we constructed progressively smaller promoter fragments by utilizing convenient restriction enzyme cleavage sites. The 1,700-bp proU fragment in JMH90 contains all regulatory regions, since it is fully inducible in vivo. Approximately 30-fold induction was achieved 60 min after the addition of 0.3 M NaCl (Fig. 2A); by overnight assay, the induction was 60-fold (Table 3), consistent with our previous results for single-copy proU-lacZ transcriptional fusions (2). Strain JMH90 contains only the 90 bp from residue -63 to residue +27. This minimal promoter showed low-level constitutive expression with a specific activity of 500 nmol min⁻¹ mg⁻¹, approximately threefold greater than the fully repressed baseline activity of the 1,700-bp fragment. The 90-bp promoter was not significantly osmotically inducible (Fig. 2B). The slight apparent increase in specific activity in Fig. 2B was simply due to reduced protein synthesis in the salt-stressed cells and did not correspond to an actual increase in transcription (Fig. 3A).

The constitutive expression from the minimal promoter fusion was, however, resistant to inhibition by salt shock, since the β-galactosidase activity per minute per milliliter of culture increased at the same rate in the standard or salt-stressed medium (Fig. 3A). In comparison, synthesis of β-galactosidase activity directed from the bla and lacUV5 promoters was significantly reduced by high medium osmolarity in vivo (Fig. 3B and C).

**TABLE 3. Effect of an osmZ mutation on β-galactosidase activity from proU-, proP-, and bla-lacZ fusions**

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>β-Galactosidase activity (nmol min⁻¹ mg⁻¹)</th>
<th>osmZ⁺</th>
<th>osmZ205::Tn10</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>-NaCl</td>
<td>+NaCl</td>
</tr>
<tr>
<td>1,700-bp ϕ(proU-lacZ)</td>
<td></td>
<td>66</td>
<td>3,955</td>
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<tr>
<td>409-bp ϕ(proU-lacZ)</td>
<td></td>
<td>623</td>
<td>4,041</td>
</tr>
<tr>
<td>90-bp ϕ(proU-lacZ)</td>
<td></td>
<td>276</td>
<td>658</td>
</tr>
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<td>ϕ(proP-lacZ)</td>
<td></td>
<td>1,049</td>
<td>1,654</td>
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<tr>
<td>ϕ(bla-lacZ)</td>
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<td>284</td>
<td>260</td>
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</table>

* Specific activity values are the average of at least three independent experiments, each assayed in duplicate.

FIG. 2. proU promoter fragment containing only 90 bp was not osmotically inducible. (A) JMH90 (1,700-bp proU promoter fragment). Upon reaching exponential phase in M9 minimal medium, the cultures were split in two: control with no addition (○) and culture with NaCl added to 0.3 M (●). Samples were taken after osmotic upshift at the indicated times. a, β-galactosidase specific activity values from proU-lacZ fusions (nanomoles per minute per milligram). Data are representative sets from three independent experiments with each assay performed in duplicate.
proP, and coli

These described lacZ. Signal, proU the standing homology, 3-galactosidase activity was in interactions in requirement promoter to the proU to octide 628 consequences KMnO4 footprinting transcription in mutations in K+ expression. The KMnO4 footprinting analysis, which derepresses the osmotic response. The extended -10 APRE to transcription median -24 -24 was essential for -10 sequence TAGGCT matches the consensus sequence TTGACA at four of six positions. However, the proU –10 sequence TAGGTT shows only 50% homology to the canonical –10 sequence TATAA. In the case of proU, the extended –10 region may compensate for a poor –10 region or may afford proU resistance to the less-than-optimal binding conditions created by the influx of K+ ions during hyperosmotic shock. The designation of the bases between the –10 and –35 hexamers as spacers, implying that their only role is to properly separate the –10 and –35 hexamers, now seems inappropriate because of the mounting evidence for sequence specificity in this region.

Our goal in constructing a minimal proU promoter was to test its contribution to osmotically signaled transcription activation. Surprisingly, the 90-bp proU promoter in JM90 was not significantly osmotically inducible. How, then, are osmotic signals received? Previous deletion analyses have shown that no sequence upstream of position –251 is necessary for osmotic regulation or gene expression (9, 30). Therefore, it is the removal of sequences 3’ to position +158 within the proU gene that derepresses gene expression about 10-fold in our 409-bp construct in JM409. This region must also be required for H-NS repression, since a mutation in hns, which derepresses the wild-type 1,700-bp construct by sevenfold, had little effect on the basal level of expression from JM409. This observation refines an earlier in vivo study that localized this downstream H-NS-sensitive element to the region between position +123 and position +322 of the E. coli gene (9). Both contradict findings with S. typhimurium proU in which a promoter construct lacking all DNA downstream from position +98 was nonetheless derepressed sevenfold by a mutation in H-NS (36).

Direct binding of H-NS to a proU construct containing sequences from position –624 to position +208 has been demonstrated in vitro, with concomitant inhibition of transcription (57). The finding that the fragment extending to position +208 is sensitive to H-NS inhibition and that our JM409 construction terminating at +158 is essentially insensitive suggests that a target site for H-NS, leading to inhibition of

FIG. 3. proU fusion in JM90 was resistant to osmotic shock. Culture media, sampling, and assays monitoring gene expression were identical to those described in the legend to Fig. 2. (A) JM90 contains the 90-bp proU fragment fused to lacZ. (B) Strain JME28 contains the bla promoter fused to lacZ. (C) Strain AP575 contains the lacUV5 promoter fused to lacZ in strain W4680, which is lac+.

In vitro KMnO4 footprinting of the proU promoter identified a single region of strong open complex formation, corresponding to a start site at position 628, active at all concentrations of K+ glutamate. Increasing K+ glutamate mimics the in vivo consequences of high external osmolarity. Our mutational analysis strengthened this promoter identification since some mutations in region only .23 and –24 positions substantially reduced expression. The nucleotides at positions –14, –15, –23, and –24 are conserved within the putative promoter of proP, another gene that is stimulated by hyperosmotic conditions. The entire promoter region of proP shows a high degree of homology with proU (Table 2), reinforcing the idea that these residues are important for maintaining transcriptional activity during osmotic stress.

The proU promoter also has significant homology to galP1 (20). The TG motif at positions –15 and –14 has been shown to compensate for an inadequate or absent –35 region at altered X_pre and galP1 promoters (6, 24, 25). The dramatic reductions in activity associated with mutations at these positions in proU are similar in magnitude to those in X_pre and galP1 and emphasize their importance for promoter activity. These positions are part of a proposed consensus sequence for an extended –10 region (5’-ynthTGyTATAAT-3’) (25). The requirement for a pyrimidine at position –13 of the extended –10 consensus was confirmed by a T-to-G substitution which reduced proU-CAT expression in vivo by 70% (Table 2).

The TT sequence at positions –24 and –23 of proU may be analogous to the TT sequence of galP1 at positions –22 and –21, which, while not essential, is necessary for maximal expression (6). A proposed function of extended –10 regions is to strengthen RNA polymerase interactions with E. coli promoters (25). The proU –35 sequence TTGCT matches the consensus sequence TTGACA at four of six positions. However, the proU –10 sequence TAGGTT shows only 50% homology to the canonical –10 sequence TATAA. In the case of proU, the extended –10 region may compensate for a poor –10 region or may afford proU resistance to the less-than-optimal binding conditions created by the influx of K+ ions during hyperosmotic shock. The designation of the bases between the –10 and –35 hexamers as spacers, implying that their only role is to properly separate the –10 and –35 hexamers, now seems inappropriate because of the mounting evidence for sequence specificity in this region.

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gene expression at low osmolarity, is in the region between position +158 and position +208. Consistent with this prediction, we find that extending the JM409 construct to position +209 results in about 30% more repression in low-osmolarity medium. The effect of a mutation in hns is also 30% greater for the larger fragment (data not shown). This 51-nucleotide segment is not an obvious candidate for bent DNA, which has been shown to selectively bind H-NS (56). H-NS interaction with this region may therefore be more sequence specific, analogous to the sites upstream of the rcsA and darB promoters (171), or require cooperative interactions with other DNA regions.

A model in which H-NS is released as a consequence of intracellular accumulation of K+ glutamate is consistent with the body of knowledge on protein-DNA interactions as a function of salt concentration. Both specific protein-DNA binding and nonspecific protein-DNA binding are inhibited by increased salt concentrations (40, 44). During the purification of H-NS, it was observed that the protein could be dissociated from DNA by increasing the ionic strength to 500 mM NaCl, although it would coprecipitate with the DNA at a salt concentration of 140 mM (52). The model has been directly tested in vitro by demonstrating that increased K+ salt concentration reversed H-NS inhibition of proU transcription (57). On the other hand, the RNA polymerase-proU promoter interaction appears to be unusually resistant to high K+ concentrations, possibly because of additional protein-DNA interactions in the spacer region. The salt resistance allows for maximal expression of proU at K+ glutamate concentrations that inhibit expression from other promoters. Since the pool of RNA polymerase molecules is limited, inefficient binding to the majority of E. coli promoters under these physiological conditions would increase the fraction of polymerase molecules available to transcribe proU, contributing to activation of proU expression. The current data support a model in which the osmotic induction of proU is achieved by the K+-governed relief of H-NS repression in combination with a promoter-RNA polymerase interaction which functions efficiently at high intracellular levels of K+

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