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Molecular Characterization of the Promoter of *osmY*, an *rpoS*-Dependent Gene

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The *osmY* gene, which encodes a periplasmic protein with an apparent M_r of 22,000, is induced by both osmotic and growth phase signals. We demonstrate here that *osmY* expression is regulated at the level of transcription and that transcription initiates 242 nucleotides upstream of the *osmY* open reading frame. Relative to the transcriptional start site, 5′ deletions up to −36 did not inhibit *osmY* expression. 3′ deletions that extended into the untranslated leader region affected the overall level of *osmY*::*lacZ* expression but did not affect inducibility. 5′ and 3′ deletions that extended past the transcriptional start region essentially abolished *osmY* expression, suggesting that there is a single promoter region. A putative promoter was identified, and its −10 region, TATATT, closely resembles the σ^{70} consensus −10 sequence, TATAAT. However, we show that *osmY* is not absolutely dependent on a functional σ^{70} for its expression. Since *osmY* expression does require *rpoS* (R. Hengge-Aronis, R. Lange, N. Henneberg, and D. Fischer, J. Bacteriol. 175:259–265, 1993), which encodes a stationary-phase sigma factor, σ^S (K. Tanaka, Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi, Proc. Natl. Acad. Sci. USA 90:3511–3515, 1993), $E\sigma^S$ may be the form of RNA polymerase responsible for transcription of *osmY*.

Bacteria in nature are exposed to a variety of environmental stresses such as heat, UV radiation, toxic chemicals, nutrient deprivation, and fluctuations in osmolarity. Cells respond to these conditions by inducing the synthesis of specific sets of proteins that are characteristic of each stress (13). These proteins are thought to be important for adaptation to the new environment as well as for protection against future potentially lethal exposures to that stress. The enteric bacterium *Escherichia coli* is normally found in the gut of vertebrate animals but can also survive and grow in seawater. Both are environments where nutrients are scarce and the osmolarity is high.

Exposure to high osmolarity induces the expression of many genes or operons (7) and more than 20 proteins (as determined by two-dimensional gel electrophoresis) (4, 18, 22). Blocking expression of the osmotically induced proteins demonstrates their importance to hyperosmotic stress protection (22). Normally, *E. coli* becomes significantly more resistant to extreme osmotic challenge if it is preexposed to a moderate osmotic stress (22). If protein synthesis is prevented by adding chloramphenicol during the preexposure, the cells do not develop resistance to extreme challenge (22).

Many osmotically inducible genes have been identified by transposon mutagenesis to create reporter gene fusions. Early studies generated fusions to β-galactosidase (*lacZ*) (5, 14), while recent work has used protein fusions to *phoA* which are specific for proteins exported to the cell envelope (15, 46). Genetic loci responsive to hyperosmotic stress are distributed throughout the *E. coli* genome (7, 15, 46), raising the question of how these genes are regulated. As yet, no central regulatory protein for the hyperosmotic stress stimulon has been found. Some of the loci within the stimulon are known to be controlled by specific regulatory factors. For example, the *kdpABC* operon and the *ompC*/*ompF* genes are positively regulated by two-component regulatory systems (12, 20, 32–34, 36, 41, 45). The expression of the *proU* operon is increased several hundred-fold by hyperosmotic stress, but no specific *trans*-acting regulator has been found (10, 19, 30). Models for *proU* regulation invoke changes in supercoiling (9, 19) or elevated intracellular K^+ concentration as the signal for *proU* transcriptional activation (23, 37, 38, 42). Finally, several osmotically inducible genes have been found to respond to a second signal: they are expressed when cells enter or approach stationary phase. These genes are dependent upon *rpoS*, a sigma factor (43) induced early in stationary phase and required for the expression of a group of carbon-starvation-inducible proteins (reviewed in reference 16). The mechanism of *rpoS* regulation is unclear. The cellular concentration of RpoS protein does not appear to increase when *E. coli* is exposed to increased osmolarity, and many *rpoS*-dependent genes are not osmotically inducible (18). Thus, within the *rpoS* regulon there exists a subset of genes which are also induced by increased osmolarity.

osmY was originally characterized as an osmotically inducible gene encoding a periplasmic protein of unknown function (46). R. Hengge-Aronis and her colleagues independently identified this gene as the carbon-starvation-inducible gene *csi-5* (18). *osmY* has been shown to be dependent upon *rpoS* for expression by both osmotic and growth phase stimuli (18), adding to the list of dual-signal genes that includes *otsBA* (trehalose synthesis enzymes) (17, 25), *treA* (trehalase) (17), and *osmB* (an outer membrane lipoprotein) (17, 24). The *rpoS* gene product, σ^S, is required for the expression of these genes and others induced during stationary phase (26, 29). The requirement may be for direct recognition of promoters by an RNA polymerase in which σ^S replaces σ^{70} or indirect recognition through σ^S-dependent synthesis of a positive activator protein.

Understanding how these genes are regulated is fundamental to a comprehensive knowledge of cellular stress adaptation. In this article, we present evidence that *osmY* is regulated at the transcriptional level by both hyperosmolarity and growth phase signals and that one promoter is responsive to both

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
HYD200	MC4100 *osmY*::Tn*phoA*	46
HYD202	RH90 *osmY*::*lacZ*	This study
HYD205	MC4100 *osmY*::*lacZ*	This study
HYD285	UQ285 *osmY*::Tn*phoA*	This study
HYD503	UQ285 *lacUV5*::*lacZ*	This study
MC4100	F$^-$ Δ(*arg-lac*)*U169* *araD139 deoC1* *rpsL150 relA1* *flbB5301 ptsF25*	6
RB285	UQ285 *proU*::Tn*phoA*	This study
RB4100	MC4100 *proU*::Tn*phoA*	This study
RH90	MC4100 *rpoS359*::Tn*10*	26
TE2680	F$^-$ λ$^-$ IN(*rrnD-rrnE*)/ Δ(*lac*)*X74 rplS galK2* *recD*::Tn*10d*-Tet *trpDC700*::*putPA1303*:: (Kan^s Cam^r *lac*)	11
UQ285	Thermosensitive *rpoD* derivative of P90A5 (F$^-$ *relA*$^+$ *stA*$^+$ *argG* *lac thi* [λ *c*I857 *h80* *st68* d*lac*$^+$])	B. Bachmann[a] (21)
Plasmids		
pTZ-based vectors	pUC derivative with T7 promoter and f1 origin	USB[b]
pDY1.4	*osmY* gene cloned into pTZ18R	46
pRS551 and pRS550	pBR322 derivative, promoterless *lacZ* operon fusion vector	40

[a] *E. coli* Genetic Stock Center.
[b] USB, United States Biochemicals.

signals. The role of σ^S in *osmY* regulation is apparently direct, since *osmY* expression is not dependent on a functional σ^{70}.

MATERIALS AND METHODS

Strains and recombinant DNA techniques. Bacterial strains and plasmids used in this study are listed in Table 1. All DNA manipulations were performed by standard genetic and molecular techniques (28, 31, 39). DNA sequencing was performed on single-stranded and double-stranded templates derived from pTZ-based vectors (United States Biochemicals, Cleveland, Ohio) by using the Sequenase 2.0 kit (United States Biochemicals). DNA-modifying enzymes and kits were purchased from Boehringer Research Laboratories (Indianapolis, Ind.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Amersham Corp. (Arlington Heights, Ill.), Promega Corp. (Madison, Wis.), and Pharmacia (Piscataway, N.J.). Radiolabeled nucleotides [α-^{35}S]dATP (1,000 Ci/mmol), [α-^{32}P]UTP (3,000Ci/mmol), and [γ-^{32}P]ATP (3,000Ci/mmol) were purchased from Amersham Corp. The oligonucleotide used for primer extension analysis, 5′-CAGTCTTGTCATA GTCATCGATTTATTCC-3′, was purchased from Operon Technologies Inc. (Alameda, Calif.). Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Media and growth conditions. Cultures were grown in Luria-Bertani medium or M9 minimal medium (31) containing thiamine (1 μg/ml) and 0.2% glucose. Fusion strains grown in M9 were supplemented with 41 μg of tryptophan per ml. Hyperosmotic stress was accomplished by the addition of 5 M NaCl to the final desired concentration; for β-galactosidase and alkaline phosphatase assays, stress was 0.4 M NaCl for 1 h and for RNA isolations, stress was 0.5 M NaCl for 30 min. IPTG (isopropyl-β-D-thiogalactopyranoside) (United States Biochemicals) was added to 1 mM. Ampicillin, kanamycin, and chloramphenicol were used at concentrations of 100, 40, and 20 μg/ml, respectively.

Primer extension and S1 analysis of transcripts. Whole-cell RNA was prepared from *E. coli* MC4100 as described by Aiba et al. (1). To map the 5′ end of *osmY* transcripts by S1 nuclease analysis, a DNA fragment containing the first 20 codons of the *osmY* open reading frame (ORF) and 0.6 kb of upstream sequences was cloned into a vector which contains a T7 promoter (pTZ18R [United States Biochemicals]). A set of RNA probes were constructed by first linearizing the plasmid by digestion with *Cla*I, *Eco*RV, or *Hin*dIII and then transcribing with T7 RNA polymerase to generate ^{32}P-labeled RNA probes 67, 263, and 729 nucleotides long, respectively. Approximately 10^6 cpm of the labeled *Hin*dIII transcript was mixed with 50 μg of total-cell *osmY* mRNA in 20 μl of RNA hybridization buffer (80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.5], 0.4 M NaCl, 1 mM EDTA), heated to 85°C for 5 min, and hybridized at 45°C overnight. Two hundred microliters of S1 digestion buffer (300 mM NaCl, 50 mM Na acetate [pH 4.6], 10 mM $ZnCl_2$) was added to the hybridization reaction mixture and digested with 2,000 U of S1 nuclease (Sigma Chemical Co.) for 15 min at 37°C. Following phenol-chloroform extraction and ethanol precipitation, the digested products were resuspended in loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA, 95% formamide) and resolved on a 6% polyacrylamide–7 M urea sequencing gel. ^{32}P-labeled *Cla*I, *Eco*RV, and *Hin*dIII runoff transcripts were used as RNA size markers, and a sequencing reaction using the M13 control DNA and the universal primer from the Sequenase kit (United States Biochemicals) was used as a DNA size marker.

Primer extension analysis was performed with a synthetic oligonucleotide which anneals to *osmY* transcripts at positions 234 to 263 (Fig. 3). Ten picomoles (about 100 ng) of primer was end labeled by using T4 polynucleotide kinase from Pharmacia and 25 pmol of [γ-^{32}P]ATP (3,000 Ci/mmol, 10 mCi/ml) according to Pharmacia protocols. After the unincorporated nucleotides were removed by passing the labeled primer through a G-25 spin column (28), the primer was ethanol precipitated and resuspended in 50 μl of RNase-free hybridization buffer (50 mM Tris [pH 8.3], 100 mM KCl). Two microliters (approximately 0.2 pmol) of radiolabeled primer was mixed with 25 μg of ethanol-precipitated total-cell RNA which had been resuspended in 8 μl of hybridization buffer. The mixture was heated at 95°C for exactly 1 min and allowed to hybridize at 50 to 55°C for 30 min. Six hundred units of Superscript reverse transcriptase from Bethesda Research Laboratories was added and incubated at 44°C for 45 min. The RNAs in the samples were then digested with DNase-free RNase for 20 min at 37°C. The extended DNA products were phenol-chloroform extracted, precipitated with ethanol, and resuspended in 5 μl of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA, 95% formamide). The products were resolved on a 6% polyacrylamide–7 M urea sequencing gel. A standard sequencing reaction was performed with the same primer on a DNA template containing the relevant portion of *osmY* and compared with the primer extension products on the same gel.

Construction of *osmY* promoter deletions and *osmY-lacZ* fusion strains. Deletions were generated either by using the Promega exonuclease III/S1 nuclease system on pDY1.4 (46)

or by restriction enzyme digestion. Restriction products that had 5′ or 3′ overhanging ends were converted to blunt ends with T4 DNA polymerase. *Eco*RI or *Bam*HI linkers were ligated to blunt ends, digested with appropriate restriction enzymes, and ligated to pRS551 or pRS550 (40).

To generate single-copy fusions on the *E. coli* chromosome, we modified the method described by T. Elliott (11) for transferring *lac* fusions onto the chromosome of *Salmonella typhimurium*. Plasmids containing the *osmY-lacZ* fusions were linearized by *Xho*I, electrophoresed on an 0.8% agarose gel, and purified by GeneClean (Bio 101, La Jolla, Calif.). Between 0.5 and 1.0 μg of the DNA was used to transform TE2680 (11), and the transformants were selected for kanamycin resistance. This strain efficiently recombines linear pRS550- and pRS551-based plasmids into its chromosome, because it carries a *recD*::Tn*10* mutation and because it has sequences inserted into the *trp* operon that are homologous to sequences in the pRS plasmids. To ensure that the fusion strains were kanamycin resistant as a result of a double-recombination event into the chromosome and not as a result of the recircularization of the plasmids, they were screened for sensitivity to chloramphenicol and ampicillin. P1*vir* lysates were made from the fusion strains and used to transduce the fusions into MC4100 (6).

Expression of *osmY* in a temperature-sensitive *rpoD* strain. Overnight cultures grown in M9 minimal medium plus 0.2% glucose were diluted to 20 Klett units with fresh medium and incubated at 33°C until they had doubled twice. Cultures were divided into three flasks and incubated under three conditions: no stress at 33°C, a 0.4 M NaCl salt stress at 33°C, and a 0.4 M NaCl stress at 44°C. Timed aliquots were taken from each flask, and alkaline phosphatase-specific activity was assayed.

Enzyme assays. Alkaline phosphatase assays were performed as previously described (46). β-Galactosidase assays were performed essentially as described by Miller (31), except that cell debris was removed by centrifugation prior to determination of A_{420}. Enzymatic activity is presented either as Miller units (31) or as specific activity defined as $\{A_{420}/[(\text{time of assay in minutes}) \times (\text{milligrams of protein})]\} \times 1{,}000$. Protein determination was by the Lowry method (27) using lysozyme as a standard.

RESULTS

The *osmY* gene is transcriptionally activated. Previous studies in our laboratory have shown that *osmY* expression increases when *E. coli* is exposed to high osmolarity or grown in rich medium (46). In these studies, *osmY* expression was determined by assaying the alkaline phosphatase activity from the chimeric protein produced from an *osmY*::Tn*phoA* fusion and by examining the levels of radiolabeled OsmY protein (46). However, these methods measure gene expression as the sum of transcription and translation and do not reveal whether a gene is regulated at the transcriptional or translational level.

To determine whether *osmY* is transcriptionally regulated, we constructed an operon fusion in single copy on the chromosome. To achieve this construction, we used vectors pRS550 and pRS551 and strain TE2680. The pRS plasmids contain a promoterless *lacZ* gene with its own ribosome binding site, ampicillin and kanamycin resistance genes, and strong transcriptional terminator sequences just upstream of *lacZ* to prevent spurious transcription (40). Strain TE2680 contains a *recD*::Tn*10* mutation and sequences within the *trp* operon that are homologous to pRS551 and pRS550 (11). Mutations in the *E. coli recD* gene inactivate the major DNA exonuclease and slow degradation of linearized DNA. Because linearized plasmids cannot replicate, only those cells that integrate the linearized plasmid into the chromosome can stably maintain the kanamycin resistance gene and grow on kanamycin plates. The *osmY* promoter sequences were cloned into pRS-based vectors just upstream of the *lacZ* gene. The plasmids were linearized at a point just upstream of the kanamycin resistance gene with the restriction enzyme *Xho*I and transformed into TE2680. Colonies were selected for kanamycin resistance, and a P1*vir* lysate was produced from kanamycin-resistant strains. The *osmY*::*lacZ* operon fusions were then transduced into MC4100, a strain known to express *osmY* properly (18, 46). Transcription from the *osmY* promoter was determined by assaying the β-galactosidase activity of the MC4100-based fusion strain under inducing and noninducing conditions. As shown in Fig. 1A, expression from the wild-type *osmY*::*lacZ* fusion increased when induced by osmotic stress or by entry into stationary phase.

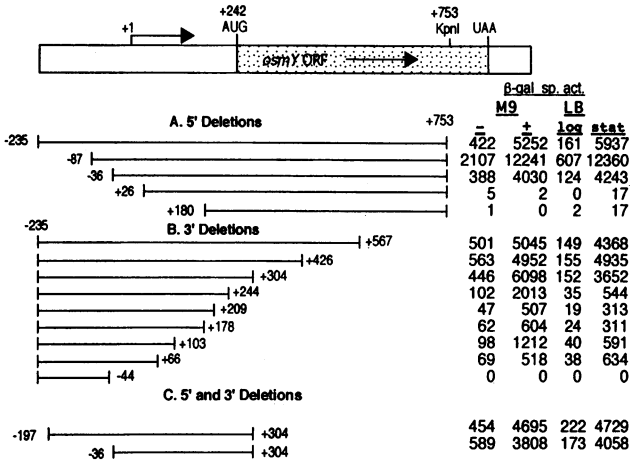

FIG. 1. Expression of *osmY*::*lacZ* fusions and deletion analysis of the *osmY* promoter region. A series of 5′ and 3′ deletions of the *osmY* locus were used to generate transcriptional *lacZ* fusions on the *E. coli* chromosome. A graphic representation of the *osmY* locus is shown at the top. The transcriptional start site (+1) identified by S1 nuclease and primer extension mapping is indicated. The AUG start codon and UAA stop codon for the *osmY* ORF are indicated and numbered relative to the transcriptional start site. Deletions that were cloned into the transcriptional fusion vectors, crossed onto the *E. coli* chromosome, and assayed for transcriptional activity are shown below. Numbers next to the lines indicate the extent of the deletions relative to the transcriptional start site. The specific β-galactosidase activity (see Materials and Methods) associated with each fusion construct is indicated on the right. β-Galactosidase activities from the strains were assayed from cells grown in four conditions: in M9 medium in the presence (+) and absence (−) of 0.4 M NaCl for 60 min and in Luria-Bertani medium during logarithmic (log)- or stationary (stat)-phase growth.

Mapping the 5′ end of *osmY* transcripts. Because the transcriptionally regulated *osmY* promoter is induced by two signals, osmotic stress and growth phase in rich medium, it is possible that *osmY* is expressed by two promoters. To locate the *osmY* promoter(s), we mapped the 5′ end of *osmY* mRNA by S1 nuclease protection assays as well as by primer extension analysis. S1 nuclease protection assays were performed by using synthetic antisense transcripts generated by T7 RNA polymerase. A 708-bp *Pst*I-to-*Eco*RI DNA fragment, containing the first 20 codons of the *osmY* ORF and 0.6 kb of upstream sequences, was cloned into a plasmid containing a T7 RNA polymerase promoter. The fragment was oriented such that runoff transcripts generated by T7 RNA polymerase

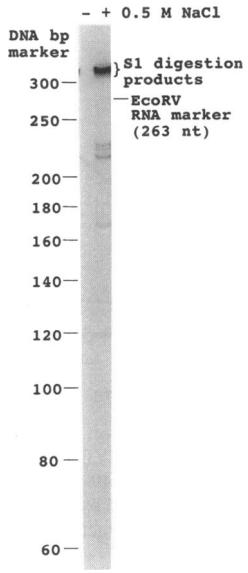

FIG. 2. S1 mapping of the *osmY* transcriptional initiation site. Total RNA was extracted from strain MC4100 grown in standard M9 medium (−) or in M9 medium supplemented with 0.5 M NaCl (+). RNA (50 μg) was hybridized to an antisense radiolabeled RNA probe generated by T7 runoff transcription and digested with 2,000 U of S1 nuclease. The antisense RNA probe was complementary to the first 20 codons of the *osmY* ORF and 0.6 kb of upstream sequences. The digestion products were analyzed on a 6% polyacrylamide–7 M urea sequencing gel; the gel was then dried and autoradiographed. The DNA marker is a DNA sequencing ladder of the M13mp18 DNA sequence using the universal sequencing primer. The radiolabeled *Eco*RV RNA marker was generated by runoff transcription using T7 RNA polymerase on a linear DNA template cut with *Eco*RV. The major S1 digestion products are indicated. nt, nucleotides.

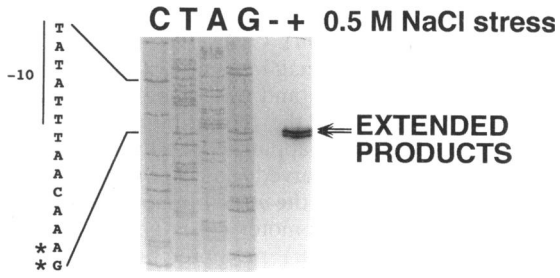

FIG. 4. Primer extension analysis of the *osmY* transcriptional initiation site. A ^{32}P-labeled oligonucleotide primer complementary to the *osmY* coding strand was annealed to 50 μg of total RNA from strain MC4100 grown in standard M9 medium (−) or in M9 medium supplemented with 0.5 M NaCl (+). The primer was extended with reverse transcriptase, and the products were electrophoresed on a 6% polyacrylamide–7 M urea sequencing gel. The DNA sequencing ladder was prepared by using the same primer to sequence a recombinant plasmid template containing the relevant portion of *osmY*. The nucleotides that are the start site of transcription are indicated (asterisks). A putative −10 region is also indicated.

would be complementary to *osmY* mRNA. ^{32}P-labeled antisense RNAs were annealed to RNA isolated from cells grown in minimal media, at standard or high osmolarity. The annealed duplex RNAs were then digested with S1 nuclease, and the protected RNA fragments were analyzed on a DNA-sequencing gel with DNA molecular weight markers and a limited number of RNA molecular weight markers. Protected RNA products were observed only with the RNA samples isolated from hyperosmotically stressed cells (Fig. 2). The most prominently protected products migrated slightly above the 300-bp DNA marker. This approximate size of these products suggests that the site of transcriptional initiation is located approximately 250 bases upstream of the *osmY* ORF (Fig. 2). Several minor bands are apparent in Fig. 2, but their intensities are significantly less than that for the major protected product. Assuming that the S1 protection products reflect the amount of *osmY* RNA present, this result confirms our fusion gene analysis showing that *osmY* transcription is inducible.

To precisely locate the transcriptional start site, we conducted primer extension analysis on RNA isolated from cells grown in four conditions: minimal and rich media at standard and high osmolarity. A primer complementary to the sequence +234 to +262 (Fig. 3) was hybridized to *osmY* mRNA, extended with reverse transcriptase, and analyzed on a polyacrylamide-urea denaturing gel (Fig. 4). Very low levels of extension products were synthesized from RNA isolated from cells grown in standard medium. These products could be detected only on overexposed autoradiograms. When cells were osmotically stressed, a very intense extension product was detected (Fig. 4). The size of the extension product indicated

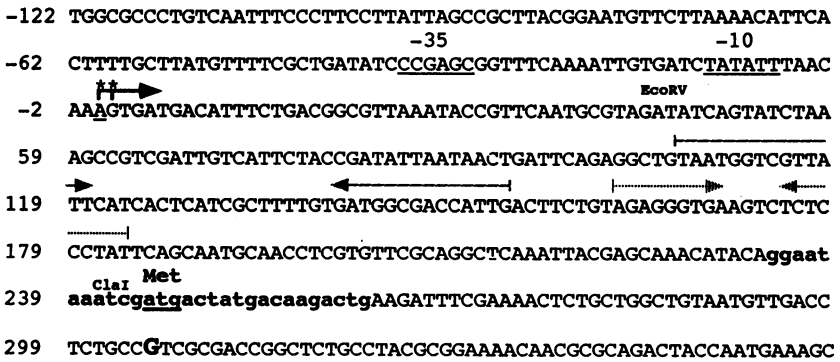

FIG. 3. DNA sequence of the *osmY* promoter region. The location of the transcriptional initiation site for the *osmY* promoter is indicated (double-starred arrow), and the A nucleotide is designated +1. The −10 and −35 sequences of a putative *osmY* promoter are underlined. The translational initiation codon (Met) and the inverted repeat sequences (arrows) are indicated. The sequence for which a complementary primer was generated for primer extension analysis is lowercased. The G nucleotide at position 305 is the first nucleotide synthesized by T7 RNA polymerase for antisense transcripts in S1 nuclease protection assays.

that the initiation site is 242 nucleotides upstream of the first AUG start codon in the *osmY* ORF (Fig. 4). Therefore, the *osmY* transcript has an unusual, long untranslated region at its 5′ end. This result confirms and further defines the approximate mapping position for the transcriptional start site obtained by the S1 nuclease mapping. Several very minor extension products were also observed (data not shown).

Analysis of deletions into the *osmY* promoter. To determine whether more than one promoter exists and to identify the sequences important for *osmY* promoter function, 5′ and 3′ deletions around the transcriptional start site identified above were generated by exonuclease III digestion or by cutting DNA at convenient restriction enzyme sites. The deletions were cloned into the pRS550- and pRS551-based expression vectors to create *lacZ* operon fusions. They were then recombined onto the chromosome so that the fusions would be present in single copy. As shown in Fig. 1, 5′ deletions that extended past −36 to +26 or +180 essentially abolished transcription. Therefore, promoter activity was associated with DNA proximal to the major transcriptional start site defined by 5′ mapping techniques, and sequences downstream of +26 do not have promoter activity. No promoter activity was associated with the 5′ ends of the other minor bands observed by S1 nuclease mapping and primer extension methods. 5′ deletions up to −36 do not significantly decrease the transcription from, or affect the induction of, the *osmY* promoter. One 5′ deletion to −87 did increase expression of the *osmY*::*lacZ* gene fusion, but this construct still responded to inducing signals.

Unlike the 5′ deletions, some of the 3′ deletions had intermediate effects on *osmY*::*lacZ* expression. Deleting 3′ sequences that were within the *osmY* ORF, to base +304, did not significantly alter *osmY*::*lacZ* expression. However, deletions that were within the untranslated leader region, from +244 to +66, did reduce expression of the fusion genes from 3- to 10-fold, depending upon the location of the 3′ deletion. While these deletions decreased overall expression, they were still responsive to high salt concentration or stationary-phase signals. Deleting to −44 completely abolished promoter activity, indicating that no promoter activity exists in the DNA upstream of −44. The combined deletion results indicate that sequences 5′ of the designated promoter are not essential for promoter activity or activation while 3′ sequences may be required for maximal promoter activity, although they do not alter inducibility. These data further support a model of a single promoter, responsible for both osmotic and growth phase induction, located near the major transcriptional start site defined by 5′ transcript mapping.

***osmY* expression does not require a functional σ^{70}.** Mapping the 5′ end of the transcript and deletion analysis allowed us to search for and define a putative *osmY* promoter. Just upstream of the major transcriptional start site is a potential −10 region, TATATT, that resembles the σ^{70} consensus −10 sequence, TATAAT, at five of six positions. Assuming an 18-bp spacer sequence, the −35 sequence is CCGAGC, which has poor homology to the σ^{70} consensus −35 sequence, TTGACA. σ^{70}, the product of the *rpoD* gene, is the housekeeping σ factor for RNA polymerase during vegetative growth. However, expression of *osmY* is dependent upon an intact *rpoS* gene product (Fig. 5) (18). When the *osmY*::*lacZ* fusion was present in MC4100 (HYD205), β-galactosidase activity was quickly and strongly induced by the addition of 0.4 M NaCl (Fig. 5). However, when the fusion was transduced into RH90 (MC4100 *rpoS359*::Tn*10* [26]), only a slight increase in β-galactosidase activity was observed (Fig. 5). Stationary induction of the fusion was also dependent upon an intact *rpoS* (data not shown). These results strongly agree with the results obtained

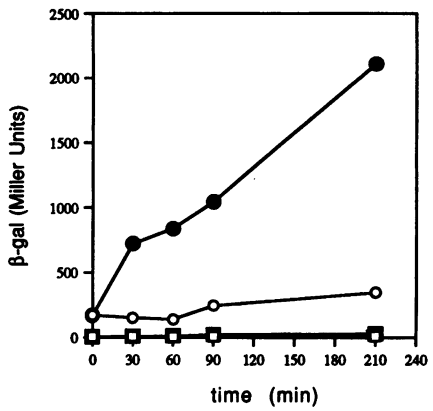

FIG. 5. *rpoS*-dependent expression of *osmY*. Strains HYD205 (*osmY*::*lacZ rpoS*$^+$) (circles) and HYD202 (*osmY*::*lacZ rpoS*::Tn*10*) (squares) were grown in standard M9-glucose minimal media (open symbols) or were osmotically stressed by the addition of 0.4 M NaCl at time zero (filled symbols). The β-galactosidase (β-gal) activities were then determined.

by R. Hengge-Aronis and her group, who observed that the expression of *csi-5* (*osmY*) in response to either osmotic or growth phase signals requires *rpoS* (18). RpoS is an active RNA polymerase σ factor (43) that could be acting directly in *osmY* promoter recognition or indirectly through synthesis of a positive activator under the control of $E\sigma^S$. In the latter case, *osmY* expression would require both σ^S and σ^{70}. For this reason, and because of the homology between the *osmY* −10 region and the σ^{70} −10 consensus sequence, we wished to determine whether *osmY* transcription is dependent upon σ^{70}.

An *osmY*::Tn*phoA* fusion was transduced into UQ285, a strain that contains a temperature-sensitive *rpoD*. The resultant strain, HYD285, was salt stressed and grown at permissive (33°C) and nonpermissive (44°C) temperatures. As controls, another osmotically inducible fusion, *proU*::Tn*phoA*, was transduced into UQ285 to produce RB285 and a *lacUV5*::*lacZ* operon fusion was transduced into UQ285 to produce HYD503. The in vitro expression of *proU* is dependent upon a functional σ^{70} subunit (23). When HYD285 (UQ285 *osmY*::Tn*phoA*) was osmotically stressed by the addition of 0.4 M NaCl, alkaline phosphatase activity from OsmY-PhoA increased significantly at both the permissive and the nonpermissive temperatures (Fig. 6), indicating that *osmY* induction was unimpaired by inactivation of σ^{70} at the higher temperature. In HYD285, expression of *osmY*::Tn*phoA* in standard osmolarity medium was twofold greater than in strain HYD200 (*rpoD*$^+$ *osmY*::Tn*phoA*). Although this affected the osmotic induction ratio, the kinetics and overall levels of *osmY*::Tn*phoA* expression were not dramatically altered. In the control experiments, expression of *proU*::Tn*phoA* was induced by salt stress at 33°C but not at 44°C, and expression from the *lacUV5* promoter could be induced by IPTG at 33°C but not at 44°C (Fig. 6). This confirms that σ^{70} was inactivated at the higher temperature in this strain. Therefore, a functional σ^{70} RNA polymerase subunit is not required for *osmY* expression, and it is, therefore, likely that *rpoS* mediates *osmY* transcription directly.

DISCUSSION

In response to hyperosmotic and nutrient starvation conditions, *E. coli* undergoes morphological and physiological changes to attain a resistant state. To gain a comprehensive

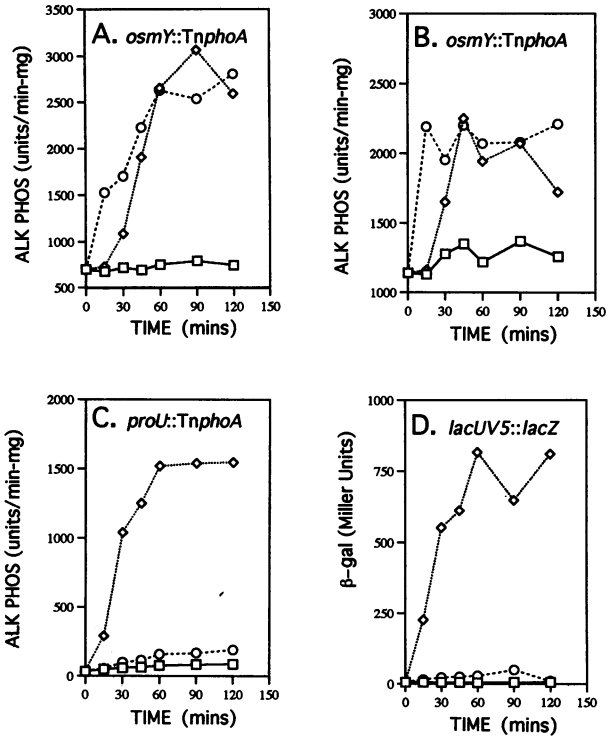

FIG. 6. σ^{70}-independent expression of *osmY*. Strains HYD200 (*rpoD*$^+$ *osmY*::Tn*phoA*) (A), HYD285 [*rpoD*(Ts) *osmY*::Tn*phoA*] (B), and RB285 [*rpoD*(Ts) *proU*::Tn*phoA*] (C) were grown in M9-glucose minimal media, stressed with 0.4 M NaCl at time zero, and incubated at either the permissive (33°C) or the nonpermissive (44°C) temperature. Symbols for panels A to C: □, 33°C with no additions; ◇, 33°C with 0.4 M NaCl; ○, 44°C with 0.4 M NaCl. Strain HYD503 [*rpoD*(Ts) *lacUV5*::*lacZ*] (D) was grown in Luria-Bertani medium and was treated with 1 mM IPTG at either the permissive (33°C) or the nonpermissive (44°C) temperature. Symbols for panel D: □, 33°C with no additions; ◇, 33°C with 1 mM IPTG; ○, 44°C with 1 mM IPTG. ALK PHOS, alkaline phosphatase; β-gal, β-galactosidase.

understanding of the mechanisms involved in this response, we have investigated *osmY*, a gene that is induced by increased osmolarity and nutrient starvation (18, 46). In this article, we present evidence that *osmY* is transcriptionally regulated and that its expression may involve direct interaction between the *osmY* promoter and RpoS, the putative stationary-phase sigma factor.

Transcriptional regulation was clearly demonstrated by examining *osmY* transcript levels and by assaying β-galactosidase activity from *osmY*::*lacZ* transcriptional fusions. Our gene fusion results agree with those found for *csi-5*, a carbon-starvation-induced gene that is allelic with *osmY* (18). Hengge-Aronis and her colleagues found that the *csi-5* transcriptional fusion was osmotically and growth phase induced (18).

Results from this study suggest that *osmY* regulation is achieved primarily through a single *osmY* promoter region that is located between positions −36 and +1, as shown in Fig. 1. Within this region, we did not detect the so-called gearbox sequence, CGGCNAGTA (2), that is found near the −10 region of several gene promoters that are induced as cell growth rate decreases (reviewed in reference 44). Because the gearbox region does not resemble a typical −10 consensus sequence for Eσ^{70}-dependent promoters, it was hypothesized that gearbox promoters may be recognized by an alternate sigma factor such as σ^S (43). However, not all gearbox promoters require *rpoS* for their expression (3), and not all *rpoS*-dependent genes have a gearbox promoter (for a review, see reference 16). The *osmY* promoter appears to belong to the latter category; its expression is dependent upon *rpoS* (Fig. 5) (18), and it does not have a gearbox promoter.

Thus far, a definitive consensus sequence for *rpoS*-dependent promoters has not been detected (for a review, see reference 18). In vitro, the *rpoS* gene product exhibits σ activity on a variety of σ^{70}-type promoters (43). One promoter that is positively regulated by *rpoS*, *fic* (43), is effectively transcribed by Eσ^S but not by Eσ^{70} (43). The *fic* promoter region contains a putative −10 sequence, TATACT, that is homologous to the consensus σ^{70} −10 region, TATAAT, but it does not contain a recognizable σ^{70}-type −35 element (43). Likewise, the *osmY* promoter contains a −10 sequence, TATATT, that is highly homologous to the consensus σ^{70} −10 region, but its −35 region does not resemble the consensus −35 sequence. The putative −35 regions for both genes are GC rich. Therefore, RpoS may recognize −10 sequences that are similar to the σ^{70} consensus −10 sequence, but it may recognize a different −35 motif, one that is GC rich.

Sequences around the promoter do not appear to affect the regulation of *osmY*. When sequences 5′ to the putative promoter were deleted, *osmY* was still capable of being expressed and induced. Increased expression was observed for the 5′ deletion at −87, but it was still regulated normally. Thus, either a negative regulatory site was removed or a positive acting site was inadvertently constructed. 5′ deletions to −36 would have also eliminated a negative acting site, but these deletions had normal levels of expression. Thus, it is unlikely that a negative acting site exists upstream of the promoter. For 3′ deletions, the expression of *osmY* varied significantly, depending upon the location of the deletion. Deletions within the *osmY* ORF all had approximately equal levels of β-galactosidase activity, whereas deletions that extended into the untranslated leader sequence had decreased levels of expression. This pattern of expression could indicate that a positive activating site was eliminated by these deletions. Several potential inverted repeat sequences are found downstream of the *osmY* promoter, which could be a binding site for an activator. However, such an activator would be nonspecific and not involved in *osmY* signal recognition, since all the 3′ deletions from +567 to +66 were still inducible. Another possibility is that constructs within the *osmY* ORF had an indirect positive effect on the expression of *lacZ*. Constructs that retain portions of the *osmY* ORF may have enhanced *lacZ* translation by increasing the local concentration of ribosomes near the *lacZ* ribosome binding site. Those deletions that extended into the untranslated leader sequence would not have actively translating ribosomes and would not have this activating effect. Finally, removing sequences within the untranslated leader could remove sequences important for *osmY* mRNA stability. As mentioned above, several inverted repeat sequences are located within the untranslated region; these could form stem-loop RNA structures that help stabilize *osmY* transcripts. Deleting these sequences would decrease translation of *osmY* without affecting induction. Therefore, sequences in the untranslated leader may be involved with maximal expression but not with induction.

If RpoS directly interacts with a single *osmY* promoter, how do the two different signals induce *osmY* expression? During nutrient starvation conditions, *osmY* expression could be induced by increased levels of σ^S. This would be similar to the mechanism used by several heat shock response genes whose expression is dependent upon the concentration of the heat

shock sigma factor σ^H (reviewed in reference 35). Under most conditions in strain MC4100, expression of *osmY* gene fusions parallels the expression of an *rpoS*::*lacZ* gene fusion; both are induced in rich medium as cells enter stationary phase, both are induced when yeast extract is the sole nitrogen source in M9 mineral medium, neither is induced at stationary phase when cells are grown in M9 mineral medium, and neither is induced by ammonia starvation (26, 46; our unpublished results). Thus, conditions that induce *rpoS* also induce *osmY*. However, *osmY* expression increases with hyperosmotic stress, while the level of RpoS protein apparently does not (18). To induce *osmY* under hyperosmotic conditions, a mechanism similar to that used by *proU* (reviewed in reference 8) could be used. Specifically, *osmY* could be activated by changes in K^+ levels or supercoiling without the need to increase RpoS concentration. Finally, RpoS could be modified during hyperosmotic stress so that its affinity for certain promoters, such as *osmY*, *osmB*, and *otsBA*, is increased.

This study suggests that σ^S is the principal regulator for *osmY* expression during osmotic stress and entry into stationary phase. Additionally, we have found that *osmY* expression is regulated primarily by a single promoter and that this promoter does not require a functional σ^{70}. Therefore, the molecular mechanism that σ^S utilizes to induce *osmY* may be to directly recognize and transcribe the *osmY* promoter. Clearly, more studies are warranted to understand the exact molecular mechanisms of this regulation.

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