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Poising of *E. coli* RNA polymerase and its release from the $\sigma 38$ C-terminal tail for *osmY* transcription

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Summary

Bacteria must adapt their transcription to overcome the osmotic stress associated with the gastrointestinal tract of their host. This requires the $\sigma 38$ (rpoS) form of RNA polymerase. Here, CHIP experiments show that activation is associated with a poise and release mechanism *in vivo*. A C-terminal tail unique among σ factors is shown to be required for *in vivo* recruitment of RNA polymerase to the promoter region prior to osmotic shock. CTD tail-dependent transcription *in vivo* can be mimicked by using the intracellular signaling molecule potassium glutamate *in vitro*. Following signaling, the barrier to elongation into the gene body is overcome and RNA polymerase is released to produce *osmY* m-RNA.

Introduction

As bacteria have limited mobility they need to respond rapidly and effectively to environmental stress. Although stress response pathways are diverse, most rely on transcription by RNA polymerase that uses various σ factors other than the major housekeeping factor $\sigma 70$. The major stress RNA polymerase typically contains the product of the *rpoS* gene, $\sigma 38$, a smaller, but closely related σ factor. The $\sigma 38$ holoenzyme has the potential to transcribe many stress response genes, but each stress induces a unique pattern of expression^{1; 2; 3}.

As a gastroenteric bacterium, *E. coli* is subject to diverse stresses and environments, both inside and outside a human host. Many such environments are associated with increased salinity and osmolarity. Examples include the lower gastrointestinal tract of animal hosts, expelled fecal matter and seawater. To avoid the loss of water and survive, *E. coli* couples increased $\sigma 38$ transcription of osmo-protective genes with reductions in $\sigma 70$ transcription of genes that are unnecessary or harmful in hyper-osmotic environments^{2; 3; 4; 5; 6}.

Hyper-osmolarity and most other stresses lead to increased amount of $\sigma 38$ protein beyond the low basal level⁷. This makes a major contribution to activation. Because stressors yield unique (but overlapping) patterns of transcripts other signaling events must make important additional contributions³. No sets of “master” repressors or activators have been identified that regulate these groups of promoters. Potassium glutamate, which accumulates rapidly upon osmotic shock, appears to be involved in both activation of $\sigma 38$ osmotic transcription^{8; 9; 10; 11; 12} and repression of bulk $\sigma 70$ transcription⁵.

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In the case of the strongly induced *osmY* promoter, and many other $\sigma 38$ responsive genes, potassium glutamate strongly activates transcription *in vitro*. However, potassium glutamate is not required for the binding of $\sigma 38$ RNA polymerase to the *osmY* promoter. Supercoiled *osmY* DNA can be engaged by $\sigma 38$ RNA polymerase in the absence of potassium glutamate, but the transcription complex is inactive. Potassium glutamate allows the poised RNA polymerase to escape into elongation mode⁹. Such a poise and release mechanism of activation has the potential to be quite common in *E. coli*. A CHIP-CHIP study found that nearly a quarter of all *E. coli* $\sigma 70$ promoter transcription complexes consisted of poised RNA polymerases; this raised the possibility that environmental cues might be required to activate RNA polymerase release¹³. However, evidence for the physiological significance of poise and release mechanisms is limited. It has not been established *in vitro* for $\sigma 70$ RNA polymerase at any of these genes. The *osmY* poise and release pathway has only been studied using supercoiled DNA *in vitro*. DNA supercoiling density varies significantly *in vivo*, including changes induced by stresses met via $\sigma 38$ ^{2; 14; 15; 16; 17}, so the relevance of the mechanism *in vivo* is not assured.

The events responsible for activation of osmotic promoters are not well characterized. Several lines of evidence point to the central involvement of the C-terminal region of $\sigma 38$ in osmotic induction. Mutations in the $\sigma 38$ CTD alter osmotic induction of transcription^{18; 19}. The CTD consists of 2 regions, a DNA-binding region that resembles $\sigma 70$ and a C-terminal non-conserved region (see below). The DNA-binding segment of the CTD can contact -35 DNA^{20; 21}, which at $\sigma 38$ promoters is very poorly conserved^{3; 22}; removal of this DNA sequence at several osmotic promoters strongly reduces transcription^{19; 23}. At the *osmY* promoter $\sigma 38$ RNA polymerase appears to contact this -35 DNA and then makes extensive contacts with DNA further upstream⁹. *In vitro*, potassium glutamate disrupts these far upstream contacts, which is thought to contribute to the release of $\sigma 38$ RNA polymerase for transcription. The role of the $\sigma 38$ CTD in establishing these upstream contacts and in contributing to the regulation of initiation is very unsettled.

Given the degree of uncertainty about the *osmY* induction mechanism and the possible general relevance of the proposed poise and release mechanism, we have undertaken studies of its nature and relevance *in vivo*. To learn whether poise and release can occur *in vivo* CHIP studies are used to look directly for poised RNA polymerases at the *osmY* promoter and at their potential release upon hyper-osmotic shock. The role of the CTD of $\sigma 38$ in activation is also addressed in CHIP studies. An integration of *in vivo* studies with those using potassium glutamate to activate *in vitro* is attempted. The outcome strongly supports the relevance of the *osmY* poise and release mechanism and extends our knowledge of the role of the σ factor in directing it.

Results

RNA polymerase collects near the *osmY* promoter prior to osmotic stress

Chromatin immunoprecipitation (ChIP) was used to explore whether RNA polymerase occupies the *osmY* promoter *in vivo* prior to any osmotic stress. CHIP has been used successfully for genome-wide studies and has been applied to individual genes²⁴. The procedure is commonly used to give a snapshot of protein:DNA complexes *in vivo*. The cellular complexes are cross-linked by adding formaldehyde to cells and shearing the chromosome to obtain smaller than gene size fragments with attached proteins. DNA fragments containing RNA polymerase are collected using antibody against the core beta' subunit. To determine how much *osmY* DNA is bound by RNA polymerase, the DNA is de-proteinized and the amount of *osmY* DNA is determined by quantitative PCR. Our procedure follows that described previously for individual $\sigma 70$ and $\sigma 38$ transcription units.²⁵ The only modifications were minor changes in the growth conditions and the protocol for DNA shearing.

Samples of *E. coli* are grown in rich media to an approximate OD₆₀₀ of 0.8, corresponding to late exponential phase, where σ^{38} levels are just beginning to rise. The samples are cross-linked, immuno-precipitated to collect RNA polymerase and attached DNA, the DNA is released and then the amount of the sample that consists of *osmY* DNA is determined by use of specific primers in quantitative PCR. The goal is to compare the amount of RNA polymerase associated with DNA near the *osmY* promoter to the amount within the transcribed *osmY* gene body.

To do this, two pairs of primers were used, each yielding a 190 base pair product. One pair (called the “promoter” pair) is centered at the *osmY* promoter and extends beyond it within the non-coding leader region (see figure 1, bottom panel). The other pair (termed “downstream”) is centered on the transcribed region within the coding gene body located approximately 700 nucleotides beyond the transcription start site. As the 2 PCR (P and D in figure 1) products are the same size and are amplified from the same immuno-precipitated sample any differences in signal should reflect differences in the numbers of associated RNA polymerases at the time of cross-linking *in vivo*. The results are typically taken to represent a snapshot of average density of RNA polymerases within each region during the steady-state situation that applies *in vivo*.²⁴

The quantitation of the promoter fragment showed an enrichment when using beta' antibody (gray bar at left in figure 1A; 0.65% of input, comparable to prior CHIP enrichments²⁶) compared to controls lacking antibody (dark bar; less than 0.05% of input). When normalized to the signal at this uninduced promoter (set at 100% relative signal in figure 1A) this background signal is less than 5% of the experimental signal. We infer that detectable amounts of RNA polymerase specifically cross-links to the *osmY* promoter region fragment in the absence of osmotic stress.

The same samples were probed in parallel using primers that quantified the amount of RNA polymerase within the downstream *osmY* gene body. In this case the amount of cross-linked DNA was significantly less, approximately 35% of the uninduced promoter signal strength (gray bar in Figure 1A, right). The background signal was again low (black bar in figure 1A, right). We conclude that there is approximately a 3 times greater RNA polymerase density associated with the *osmY* promoter fragment compared to the downstream gene body fragment. Prior CHIP experiments that show a very similar pattern interpreted the excess RNA polymerase associated with the promoter region to be poised there¹³, consistent with the poising observed previously *in vitro* at the *osmY* promoter⁹.

Release of RNA polymerases into the *osmY* gene body upon osmotic stress

To learn if osmotic stress triggers the release of RNA polymerases from the promoter region into the gene body the CHIP experiments were repeated 8 minutes after the addition of 400mM NaCl. The addition of this concentration of salt is known to induce the osmotic transcription program^{2; 27} which is confirmed by *osmY* m-RNA measurements before and after the addition of salt (figure 1A inset). An 8 minute time frame was chosen because it is long enough for transcriptional induction of *osmY*, yet not so long as to allow the unchallenged cells to reach much higher optical density than the challenged cells. CHIP experiments have not been reported previously in the presence of added salt. The time of sonication required to give equivalent DNA shearing was slightly shortened in salt stressed samples. However, the total amount of DNA recovered after immuno-purification was not significantly different from that obtained in the absence of salt, indicating that salt does not grossly interfere with cross-linking. The CHIP experiment assays the amount of RNA polymerase associated with *osmY* promoter and gene body regions to compare directly to the results obtained prior to osmotic shock.

The results (figure 1B) show that the distribution of RNA polymerases changes after osmotic shock. Instead of RNA polymerase collecting at the *osmY* promoter, as occurs prior to shock (figure 1A), the RNA polymerases now occur at roughly equal densities within the promoter and gene body (gray bars in figure 1B). That is, the addition of NaCl to the culture media has effected a re-distribution of RNA polymerases from the promoter region to the gene body. We infer that osmotic stress increases the probability that RNA polymerase will be released into elongation mode.

The data also shows that a greater amount of RNA polymerase is associated with the promoter region after osmotic shock (approximately 4 times greater than the uninduced comparison). Hyper-osmotic shock is known to increase the total amount of $\sigma 38$ within the cell^{7; 10; 28} and this was confirmed under the conditions of this experiment (data not shown). We suspect that the increase in promoter occupancy is a consequence of the greater amount of $\sigma 38$ holoenzyme present after addition of salt.

Taken together these 2 observations suggest the following effect of an increase in external osmolarity on *osmY* transcription. During unstressed growth some RNA polymerase is at or near the *osmY* promoter and little leaks into the gene to transcribe. Upon addition of salt the restriction that poises RNA polymerase there is relieved, allowing it to be released for elongation of transcript. In addition, because under osmotic conditions a higher level of RNA polymerase is present in both promoter and downstream regions it is apparent that more RNA polymerases have been recruited to initiate *osmY* transcription. In this view salt has 2 effects that jointly activate; salt leads to the production of more $\sigma 38$ holoenzyme that can be recruited to the *osmY* promoter region and also facilitates the ability of these RNA polymerases to initiate and be released into elongation mode.

Activation of RNA polymerase *in vivo* in the absence of increasing $\sigma 38$ levels

The above model suggests that there are independent contributions to activation made by the increase of $\sigma 38$ levels and by other effects of salt. When $\sigma 38$ is expressed from a heterologous plasmid-based promoter the increase in levels induced by increase in osmolarity have been reported to be nearly abolished, presumably by a lack of effective translational control²⁹. This would allow one to assess *osmY* activation by salt in the absence of higher $\sigma 38$ levels. Figure 2A shows that *osmY* transcription can be activated by salt when $\sigma 38$ is expressed from a plasmid in cells lacking $\sigma 38$ on the chromosome (lane 1 vs 2). The level of $\sigma 38$ in the cell is not increased after the addition of salt (Figure 2B). The data demonstrate that *osmY* transcription is induced by osmotic upshift in the absence of increasing $\sigma 38$ protein levels.

We repeated the CHIP experiments in this context to see if RNA polymerase was still poised when expression was via a plasmid. Figure 2C shows that under non-stressed conditions the signal at the promoter (gray bar at the left) is far above the background signal in the absence of RNA polymerase antibody (dark bar). This indicates that some RNA polymerase has also collected at the promoter in this plasmid expression-based system. The amount of polymerase present downstream of the promoter is approximately 35% of the amount at the promoter (gray bar at the right of Figure 2C) but still well above background levels (dark bar). Overall, the data are very similar to those obtained when $\sigma 38$ is expressed from the chromosome (figure 1A). The high ratio of RNA polymerase at the promoter compared to within the gene is consistent with the accumulation of poised RNA polymerases under non-stressed conditions.

The experiment was repeated after addition of salt. Figure 2D shows that osmotic shock leads to the preferential accumulation of RNA polymerase within the downstream gene body at the expense of the promoter (see gray bars). That is, in contrast to the situation pertaining prior to salt induction (figure 2C) RNA polymerase appears to flow more freely out of the promoter and into elongation, indicating that osmotic shock helps release poised RNA polymerases.

We note that the amount of RNA polymerase at the *osmY* promoter appears to decrease slightly after osmotic shock (to the 67% level comparing the promoter in figures 2C and D). This minimal change contrasts with the 4-fold increase seen when expression of $\sigma 38$ is in its normal chromosomal context (figure 1AB). The difference is likely due to plasmid-based expression not leading to an increase in $\sigma 38$ levels, as occurs in the chromosomal context (figure 2B). Note, however that the signal at the promoter in stressed cells tested via plasmid expression is significantly less than that from chromosomal expression (figures 1B and 2D). This is surprising because there is several-fold more $\sigma 38$ in the plasmid context (not shown); we don't know why plasmid-based expression seems to be less efficient at directing RNA polymerase to the promoter, but apparently not all the plasmid expressed protein is available to support promoter binding. Nonetheless, the data as a whole confirm that salt treatment of cells can relieve the restriction on release of RNA polymerases poised at the *osmY* promoter.

The non-conserved $\sigma 38$ CTD is generally required for osmotic transcription *in vivo*

Several lines of evidence have implicated the C-terminal region of $\sigma 38$ as a central player in *osmY* transcription. These include genetic¹⁹, *in vitro* transcription^{18; 30} and footprinting studies⁹. Both conserved and non-conserved regions of the CTD have been proposed to be involved. The C-terminal domain of $\sigma 38$ contains residues that are quite similar to those of $\sigma 70$ in conserved region 4 that contacts DNA²⁰ (shaded in figure 3A). Beyond amino acid R 314 the sequences diverge and are not obviously related. To investigate systematically the role of the CTD *in vivo* a series of C-terminal truncations were constructed; these progressively shortened the nonconserved region. The loss of CTD tail residues did not change the expression levels of $\sigma 38$ protein, either before or after salt induction (figure 3B).

These truncated versions of $\sigma 38$ were tested for their ability to support salt-activated transcription *in vivo* in a context lacking chromosomally expressed protein. In initial experiments 4 versions of $\sigma 38$ were tested for support of *osmY* m-RNA synthesis: 1-330 (full length protein), 1-322, 1-320 and 1-319. The results show that truncation protein 1-322 supports wild type levels of *osmY* m-RNA, but the more extensive truncations support reduced transcription (figure 3C). In the absence of salt transcription was very low for all constructs and essentially undetectable for 1-320 and 1-319 (not shown). A more extensive set of $\sigma 38$ truncations were made and assayed for *osmY* m-RNA *in vivo* (black bars in figure 3D). The shortest deletion to show a significant loss of activity is 1-320 and removal of most of the non-conserved tail leads to a nearly total loss of *osmY* activity (construct 1-316). Overall, the data demonstrate that amino acids at or near position Q320 are important for *osmY* transcription. We note that this residue and the region surrounding it is non-conserved compared to $\sigma 70$ (figure 3A).

To learn if the requirement for this region of the $\sigma 38$ CTD is general or specific for *osmY*, m-RNA was assayed for several other salt-inducible promoters. This basically involves dividing common preparations of cellular RNA and assaying with different gene-specific primers. The collective results for the *osmY*, *otsB* P1, *gadA*, *gadB* and *cfa* P2 promoters are shown in figure 3D. In aggregate, the data show that the majority of transcription is lost at all promoters when using truncations in the series from 1-321 to 1-318. (The levels of transcription in the absence of salt ranged from very low to not detectable for these constructs.) There are some differences in how sensitive different promoters are to truncation within this region, but for all promoters the small segment is required to maintain significant transcription levels. This highlights the general importance of the tail residues surrounding amino acid Q320 for general salt-induced transcription.

The role of the σ 38 non-conserved CTD as assessed by ChIP

The general requirement for a small segment of the σ 38 CTD for transcription could have several sources. Two prominent possibilities would be a role in recruiting RNA polymerase to the promoter or a role in allowing a poised RNA polymerase to escape into elongation mode. To evaluate these possibilities CHIP studies were done using selected CTD truncations. These compared the full length 1-330 protein with truncations 1-319 and 1-314. 1-319 was chosen as it is the first truncation in which a majority of transcription is lost (see figure 3D). 1-314 removes the entire non-conserved region and is essentially transcriptionally inactive¹⁸. The experiments basically followed the procedure described above for wild type protein expressed from a plasmid in cells lacking chromosomally expressed σ 38.

The role of the σ 38 CTD in bringing RNA polymerase to the *osmY* promoter was assessed using CHIP assays of unstressed cells. Figure 4A compares the amount of RNA polymerase brought to the *osmY* promoter by the 3 versions of σ 38. The data show a progressive reduction in RNA polymerase occupancy as the CTD is progressively shortened. Specifically, the 1-319 protein binds at the 40% level and the 1-314 protein binds at the 30% level compared to the full-length 1-330 σ 38. The comparisons show that the CTD tail has a role in directing uninduced promoter occupancy by σ 38 RNA polymerase. When experiments are done in the absence of σ 38 (no plasmid) the level of binding is comparable to the 30% binding using the 1-314 construct (not shown). These levels are significantly above the background obtained with no antibody; the result suggests that some RNA polymerase can associate with the *osmY* promoter in the absence of σ 38, perhaps via other σ factors. However, the main point is that truncation of the σ 38 CTD leads to a lowering of the amount of RNA polymerase associated with the *osmY* promoter. It appears that the CTD tail is used to recruit RNA polymerase at the *osmY* promoter *in vivo*.

The data also help evaluate the role of the CTD tail in directing the release of RNA polymerase from the *osmY* promoter. When the downstream region is probed, one sees reduced amounts of RNA polymerase within the downstream gene body compared to at the promoter for all 3 proteins (figure 4A). The residual association within the gene body for the truncation mutants does seem to represent escape from the promoter as the level in a σ 38 null context is below the 5% level (checkered bars in figure 4A), which could include significant contributions from other forms of holoenzyme. But overall, there is no convincing evidence that truncation of the CTD tail leads to either excess retention of RNA polymerase at the promoter or its promiscuous release into elongation mode. However, minor effects on release cannot be excluded.

Further information comes from repetition of the CHIP experiment after salt induction. Again, the procedure follows that described for wild type σ 38, described above. The results are fully consistent with truncation reducing the ability of σ 38 to bring RNA polymerase to the *osmY* promoter. Full length σ 38 binding is slightly reduced at the promoter compared to prior to induction (at 67% level; textured bar in figure 4B), while the 2 truncations lead to further reductions (gray and black bars). Induction leads to proportionally less RNA polymerase associated with the downstream gene body in the case of truncated proteins (figure 4B). There appears to be no defect in the ability of those RNA polymerases that bind to the promoter to be released into the gene body by salt treatment. Taken together with the results from unstressed cells the data show that the primary reason that CTD truncations lower transcription is that they fail to bring the full complement of RNA polymerase to the *osmY* promoter.

The role of the CTD can be reproduced *in vitro* using potassium glutamate activation

The CTD of σ 70 can be used to regulate transcription by serving as a target for activator proteins^{31; 32; 33}. No such evidence yet exists for σ 38 promoters. The results presented here demonstrate a role for the CTD in general σ 38 transcription and the CHIP experiments suggest

that this role is to recruit RNA polymerase to the *osmY* promoter. We have previously demonstrated that the *osmY* promoter can be activated to transcribe *in vitro* by the addition of potassium glutamate to a system which contains only $\sigma 38$, core RNA polymerase, NTPs and DNA, without the presence of any additional factors⁹. Activation by potassium glutamate has been observed at many promoters but the effect is not universal and some promoters are inhibited^{5; 6; 9; 34}. We now wish to learn if the *in vivo* behavior of $\sigma 38$ CTD truncations can be reproduced by potassium glutamate activated transcription *in vitro* in the absence of other factors.

The experiment was done using the *osmY*, *cfa* and *otsB* promoters. Each promoter can be activated by potassium glutamate, with maximal transcription occurring between added 300 and 500 mM. This is shown for the *osmY* promoter in figure 5A, lanes 1–4. Truncation of σ to 1-322 retains the full response (figure 5A, lanes 5–8). Using 400 mM potassium glutamate each of the 3 promoters was transcribed with each of 8 forms of $\sigma 38$ RNA polymerase. As an example, figure 5B shows that truncation 1-319 is defective at transcribing the *osmY* and *cfa* P2 promoters under these conditions. These *in vitro* results using potassium glutamate activation may be compared to the same data *in vivo* presented in figure 3C above; they are essentially the same demonstrating that the effect of this CTD truncation is the same in the 2 cases.

The full data set for potassium glutamate activated transcription is presented in figure 5C. As the CTD is truncated general reductions are observed beginning with protein 1-321 and extending to protein 1-318. The data may be compared to transcription studies *in vivo* using the same set of promoters and constructs (figure 3D). Although there are some minor differences the effects of truncation are very similar *in vivo* and using potassium glutamate to activate *in vitro*. We conclude that potassium glutamate activation *in vitro* mimics cellular activation at these promoters; there is no suggestion that activators or repressors are needed to interact with the CTD as part of activation.

The $\sigma 38$ CTD is required to recruit core RNA polymerase into holoenzyme

These experiments indicate that the effect of the $\sigma 38$ CTD tail is general rather than promoter specific. This derives from transcription experiments, both *in vivo* and *in vitro*. In addition, the CHIP experiments suggest that the CTD is needed to recruit the RNA polymerase to the *osmY* promoter. Taken together, this raises the possibility that the CTD is required for $\sigma 38$ to bind core RNA polymerase. Furthermore, the major core binding determinants for $\sigma 70$ are known and do not rely centrally on the $\sigma 70$ extreme CTD³⁵. In that case the contacts between σ and core involve very extensive complementary surfaces. But $\sigma 38$ and $\sigma 70$ have completely dissimilar sequences in the extreme CTD (figure 2A) and have different interactions with the CTD as a whole³⁶ and so a central role of this $\sigma 38$ region in core binding cannot be excluded. However, a previous report¹⁸ suggested that the CTD was not required for $\sigma 38$ to bind RNA polymerase. In that study heterologously expressed mutant 314 and wild type proteins were found to cause similar growth defects; this was interpreted to mean that they both were likely to bind core RNA polymerase and hinder growth.

To directly test the role of the extreme CTD in the interaction of $\sigma 38$ with core a pulldown assay was used. In this assay his-tagged $\sigma 38$ is pre-incubated with core polymerase. After 10 minutes magnetic Ni-NTA beads are added to the reaction and any $\sigma 38$ holoenzyme that forms is pulled down. To remove core protein that is not bound tightly to σ 2 washes are carried out. Finally, the proteins are eluted and run on an SDS PAGE gel to observe whether holoenzyme forms. The procedure was modified to include potassium glutamate and no significant effects were seen at 400 mM (not shown).

The procedure was repeated with selected CTD truncations to learn if the CTD is needed for $\sigma 38$ and core to bind each other and form holoenzyme.

Figure 6 shows the result using wild type protein 1-330 and truncations 1-319 and 1-314. The amount of core is represented by the upper band and the amount of σ by the lower band. The result shows that there is a progressive loss of core binding to σ as the CTD is truncated; 1-319 binds core less well than wild type and 1-314 shows nearly background levels. These results are similar to those obtained in transcription where 1-319 shows partial reductions (figures 3D and 5C) and 1-316 is inactive. The data are also fully consistent with the CHIP results, which showed that the CTD was needed to collect the RNA polymerase and bring it to the *osmY* promoter, where it is poised to activate transcription. Taken together with the *in vitro* transcription experiments with potassium glutamate the data suggests, but by no means proves, the view that the release of the poised RNA polymerase for transcription requires no factors other than RNA polymerase itself, DNA and potassium glutamate.

Discussion

These CHIP data have provided evidence that hyper-osmotic stress induces a poise and release mechanism that helps activates *osmY* transcription. The master stress regulator $\sigma 38$ is shown to contain a unique tail element that recruits RNA polymerase to the *osmY* promoter region *in vivo* in the absence of stress. The addition of salt to cells is shown to cause the release of RNA polymerase from this region into the main gene body for transcription. We show that tail-dependent transcription *in vivo* can be mimicked *in vitro* by the addition of potassium glutamate; this compound is known to accumulate intracellularly when cells are salt-shocked. The activation does not require a macromolecular activator or repressor to interact with the $\sigma 38$ CTD tail, as can occur with $\sigma 70$ ^{37; 38}. Because CHIP studies have suggested that poising of RNA polymerase may be very common in the bacterial genome¹³, the poise and release mechanism has the potential to be of widespread significance. We believe that the poising at *osmY* is likely by RNA polymerases that are engaged at the promoter and have difficulty escaping because our prior studies found such a state *in vitro* that could be triggered to support elongation upon addition of potassium glutamate.⁹

Hyper-osmotic stress is one of many stresses that are countered by transcription using forms of bacterial RNA polymerase containing σ factors other than the “housekeeping” $\sigma 70$. $\sigma 38$ is by far the most common of these as it mediates the response to a very wide variety of stresses. Outside the laboratory stress is likely the norm, as osmotic and other stresses exist normally in the gastrointestinal tract of animal hosts, in expelled fecal matter and in diverse marine environments. The various alternative forms of σ often have overlapping promoter specificities and thus the interplay amongst them can be quite complex^{13; 25; 39}. That is, the effect of occupying a promoter with one form of RNA polymerase has the consequence of inhibiting recognition by other forms of RNA polymerase. In the example of *osmY* this means that the poising of $\sigma 38$ RNA polymerase in the absence of osmotic shock can interfere with inappropriate promoter usage by the other forms of RNA polymerase. It is possible that this type of repression is a significant attribute of the use of poise and release mechanisms. In the case of *osmY*, potassium glutamate, which triggers release, is an osmotic-specific signal, but other poised RNA polymerases could respond to other environmental cues.

The current data show that the CTD tail of $\sigma 38$, which is directly adjacent to the -35 binding region and is not at all related to $\sigma 70$, is needed to bind RNA polymerase and recruit it to the *osmY* promoter. This was unexpected in view of prior growth rate experiments¹⁸ that were interpreted as suggesting that the tail was not needed for this function. Nonetheless, in a direct test, the short CTD segment was removed from $\sigma 38$ and then RNA polymerase failed to bind in a pull-down assay. The same simple mutant severely reduced polymerase binding to *osmY*

in vivo, as measured by the CHIP assay. By contrast, $\sigma 70$ does not rely centrally on a single determinant as it has an extensive surface that helps it attract core polymerase and bind it tightly³⁵. Even though $\sigma 38$ overall binds core much less well than $\sigma 70$ ⁴⁰, its ability to bind the beta flap near the promoter -35 region is much stronger than that of $\sigma 70$ ⁴¹. Thus, it appears that $\sigma 38$ differs from $\sigma 70$ in that it has evolved to rely primarily on a localized determinant for forming RNA polymerase holoenzyme.

This reliance on a localized $\sigma 38$ CTD determinant for making effective contact to core could possibly play a central role in the osmotic control mechanism. The adjacent region of the CTD has been implicated in induction and contacts -35 DNA^{9; 18; 19; 20; 21}. The network of interactions between the σ factor, the -35 DNA and the RNA polymerase core⁴² is typically disrupted to effect the release of core into elongation mode⁴³. The extreme localization of core binding determinants within this network in the case of $\sigma 38$ may allow potassium glutamate to easily switch the network to an alternate state and release the poised RNA polymerase upon accumulation after osmotic shock.

The *osmY* promoter is subject to repression by global transcription factors Lrp, CRP and IHF and global regulators such as H-NS and Fis co-regulate other osmotic promoters⁴⁴. At the *osmY* promoter, Lrp and IHF are reported to repress from locations centered more than 50 base pairs upstream from the promoter -35 region⁴⁴, well beyond the normal range of repressor action by steric hindrance⁴⁵. We showed previously that supercoiled DNA can poise $\sigma 38$ RNA polymerase in an inactive complex at the *osmY* promoter *in vitro* and in doing so the upstream DNA appears to wrap around the RNA polymerase⁹. It is possible that this mechanism of poising makes the promoter a potential target for somewhat remote regulation that interferes with the ability to wrap DNA.

Under non-stressed conditions the amount of $\sigma 38$ ^{7; 28} and other alternative members of the $\sigma 70$ family is quite low. One expects that the amounts of holoenzyme could be even lower as $\sigma 70$ has the highest affinity for core. The effect of poising RNA polymerase at the *osmY* promoter could be a way to collect limited $\sigma 38$ holoenzyme at this promoter to mark it for selective activation *in vivo* upon receipt of the osmotic signal. $\sigma 54$ promoters are also bound by poised RNA polymerases, although in these cases the signaling is via macromolecular activators⁴⁶. By contrast, *E. coli* $\sigma 70$ RNA genes have not yet been shown to be subject to poise and release mechanisms. However, the potential clearly exists; viruses regulate $\sigma 70$ holoenzyme in this manner (for example⁴⁷) and slow promoter escape⁴⁸ can limit the $\sigma 70$ transcription rate *in vivo*^{49; 50}. The use of genomic probing methods such as CHIP^{13; 24; 25; 39} has provided unexpected insights into mechanisms *in vivo* and the combination of such studies with *in vitro* reconstruction, as done here for *osmY*, is needed to discover the extent of the variety of transcription mechanisms used by *E. coli* and other bacteria.

Materials and Methods

Plasmids and proteins

Plasmids carrying *cfa P2*, *otsB P1*, and *osmY* promoters were previously described¹⁹. Plasmid pRpoS⁵¹, was mutated to remove the C-terminal His tag by addition of an ochre stop codon (TAA) after amino-acid 330 of $\sigma 38$ and its truncations were used for *in vivo* experiments (Invitrogen). Plasmid pETF⁵² was used for purification of N-terminally HIS tagged full-length and truncated $\sigma 38$ for *in vitro* experiments.

Truncations of the CTD of $\sigma 38$ (rpoS) were constructed by site specific insertion of stop codons into the desired sites on plasmids pETF and pRpoS as described in the Quikchange site directed mutagenesis protocol (Invitrogen). $\sigma 38$ proteins were purified as described⁵¹.

Media and growth conditions

Escherichia coli K12 (from the ATCC) was used for chromosomal ChIP experiments. In plasmid expression experiments a $\sigma 38$ null derivative of *E. coli* K12, RJ4099, was used⁵³. Bacteria were grown overnight in 5 ml of Luria broth (LB) at 37°C. The cells were diluted 1:100 and grown to an approximate optical density of 0.8. Osmotically challenged cultures were collected 8 min after the addition of 400 mM NaCl.

Chromatin Immunoprecipitations

ChIP experiments were done following the protocol of Raffaella et al.²⁵ with slight modifications. Overnight cultures of *E. coli* were subcultured 1:100 into 100 mL LB media. Upon reaching OD₆₀₀ of 0.8 the cultures were either crosslinked (1% final concentration of formaldehyde for 10 minutes at room temperature) or 400 mM final concentration of NaCl was added for 8 minutes. If salt was added bacterial samples were crosslinked immediately following the 8 minute salt shock. The crosslinking was quenched by addition of 100 mM glycine for 20 minutes at 4°C. Samples were washed 3 times in TBS buffer, and frozen at -70°C until use.

To process the samples further they were resuspended in 250 μ L ChIP IP buffer (10 mM Tris-HCl, 300 mM NaCl, 2% Triton X-100) and the DNA was sheared with a Bioruptor (Diagenode) water bath sonication system. All sonications were done in a 4°C water bath. Non-stressed samples were sonicated 8 times for 30 seconds, while salt stressed samples were sonicated for 7 times 30 seconds, yielding average DNA fragments of approximately 500 bp in length, as determined by agarose gel electrophoresis. After sonication an additional 500 μ L of ChIP IP buffer was added, and cellular debris were removed by centrifugation, and the lysates were pre cleared by incubation with protein G beads for 3 hours. 50 μ L of the original sample was saved as a total DNA input control, and the remaining sample was split and Immunoprecipitated by β' antibody (Neoclone® catalog number W0001) or used as a mock experiment without the presence of antibody. Samples were incubated for 1 hr at 4°C with 30 μ L of Protein G Sepharose beads. Washes of the IP reactions consisted of two washes each with IP buffer, High Salt IP buffer (600 mM NaCl, 100 mM Tris-HCl, 2% Triton X-100), LiCl buffer (250 mM LiCl, 100 mM Tris-HCl, 2% Triton X-100, and one final wash with buffer TE to remove the salts. The beads were eluted in ChIP elution buffer (150 μ L of TE buffer with 1% SDS) by overnight incubation at 65°C. Eluted DNA fragments were purified using a qiagen PCR purification kit.

Primers of 24 bp length for the *osmY* promoter (TCCCTTCCTTATTAGCCGCTTACG; ATCGGTAGAATGACAATCGACGGC) and the *osmY* gene body (ACACCGCCACCACAGTGAAATCA; CGCTTTTCACACCATCTACCGCTT) were designed to have an equal amplification product length to avoid differences in fluorescent intensity. The primers to the promoter were centered around the promoter, and the downstream primers were approximately 700 nucleotides after the transcription start site. Each pair of primers was tested for melting temperature, specificity, and amplification efficiency and were found to be specific for *osmY* and suitable for ChIP assays (each having an E factor greater than 1.75, determined according to⁵⁴). Quantification of ChIP signal was done using a Qiagen Quantitect SYBR Green PCR kit and an ABI 7300 qPCR instrument. Duplicate or triplicate qRT PCR reactions were used for each data point. All ChIP values reflect the percent of DNA pulled down as compared to input DNA collected before antibody immunoprecipitation. Each ChIP experiment from chromosomally expressed $\sigma 38$ and full length $\sigma 38$ expressed from the chromosome was repeated at least 4 separate times. Each experiment using truncated $\sigma 38$ was repeated at least twice.

***In vitro* transcription**

In vitro transcription experiments were as described⁹. 200 nM σ 38 and 50 nM core RNA polymerase were pre-incubated at 37 °C for 10 min in 1x buffer B (50 mM Tris-HCl at pH 7.9, 3 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 100 µg/ml BSA, variable amount of potassium glutamate as indicated). Unless otherwise indicated 400 mM neutralized potassium glutamate was present. 2.5 nM of supercoiled plasmid DNA was then added and the incubation continued for 15 min. 200 µM of NTP mix (50 µM ATP, 50 µM TTP, 50 µM GTP, 10 µM CTP, and 200 nCi P³² labeled CTP) was then added. After 12 min the reaction was stopped and samples were run on 6% PAGE with 1x TBE at 21 Watts for 70 min. Radioactive bands were visualized and quantified by phosphorimager analysis. Each experiment was conducted at least 3 times. Core RNA polymerase was from Epicentre.

***In vivo* m-RNA**

Primer extension experiments were as described¹⁹. *E. coli* K12 (from the ATCC) was grown overnight in 5 ml of Luria Broth with glucose (LB) at 37 °C. The cells were diluted 1:100 and grown to an optical density of 0.5. Osmotic challenge cultures were harvested either immediately before or 10 min after the addition of NaCl to a final concentration of 400 mM. This concentration of NaCl led to a delay in cell growth. Cells were harvested by centrifugation and stored at -70°C. RNA was extracted from the pellets using the Qiagen RNeasy kit. Total amount of RNA of each reaction was normalized, and the RNA from each promoter (*osmY*, *otsB*, *cfa*, *gadA*, and *gadB*) was detected by primer extension. Primers for *otsB*, *cfa*, and *gadB* promoter were previously described¹⁹. The *osmY* primer (AGGGAGAGACTTCACCCTCTACAGAAG) and the *gadA* primer (CTCCGCGATAGTAGAAATGGCCTTT) were used here for the first time. 10 µl reactions for primer extension analysis contained 10 µg of total RNA, 10 nM labeled primer, reverse transcriptase buffer (Promega), 5 units of reverse transcriptase, and 0.2 mM dNTPs. Urea stop dye was added and samples run at 21 watts on a 6% polyacrylamide sequencing gel. Radioactive bands were visualized and quantified by phosphorimager analysis. RNA samples were prepared at least three separate times, and the average was taken.

Western Blot

The procedure for western blotting for σ 38 was as described (14, 15). 50 mls of K12 *E. coli* and RJ4099 null strain carrying plasmids expressing either full-length or truncated versions of σ 38 were grown O.D.600 of 0.8. All plasmid expressing samples were grown in LB media without the addition of IPTG. Samples of each strain were then split into 2 separate tubes. One sample was spun down and kept frozen for subsequent protein measurements and NaCl was added to the remaining sample to a final concentration of 400 mM and the sample was left to incubate at 37° C for 8 minutes. Total cellular protein was extracted from sonicated cells and samples were diluted so that the overall total of protein would be the same for each sample. The σ 38 antibody was from NeoClone. The goat anti-mouse immunoglobulin G conjugated with Horseradish Peroxidase was from Promega.

Core pulldown assay

3 µg of N-terminally his tagged σ 38 or truncated variants were incubated with 1.5 µg of core RNA polymerase (Epicentre) for 10 minutes at room temperature to allow holoenzyme formation. 10 µl of NTA magnetic beads suspended NPI-20 buffer (Qiagen) were added to each sample and they were incubated on a rocker at room temperature. After 15 minutes the supernatant was removed and beads were washed twice with NPI-20 buffer. Σ 38 and associated core were eluted from the NTA beads with NPI-250 elution buffer, containing 250 mM imidazole. SDS loading dye was added, and the samples were run on 8% SDS-PAGE. Gels were silver-stained using the Biorad silverstain plus kit.

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Figure 1. RNA polymerase is poised at the *osmY* promoter region prior to osmotic stress, and can be released upon addition of salt

A. CHIP in late exponential phase at the *osmY* promoter. Gray bars denote samples enriched with β' antibodies. Black bars denote the no antibody negative control. The results in the promoter and downstream gene body are indicated.

Inset: Transcription of *osmY* is induced by osmotic stress. A radiograph comparing the *osmY* in vivo mRNA levels before (lane 1) and after (lane 2) salt stress as assayed by primer extension using an radio-labeled primer to *osmY*.

B. CHIP after the addition of 400 mM NaCl for 8 minutes. Gray bars denote samples enriched with β' antibodies. Black bars denote no antibody negative control.

Shown below is a schematic of the locations of the 190 base pair PCR fragments used for CHIP analysis. The promoter fragment (P) is centered near the transcriptional start site (+1) and overlaps with the long untranslated leader region. The downstream fragment (D) is centered near position +700 and is wholly included within the *osmY* gene body (boxed).

Figure 2. Plasmid expressed σ^{38} induces osmotic transcription at the *osmY* promoter by releasing RNA polymerases for elongation

A. in vivo *osmY* mRNA levels before (lane 1) and after (lane 2) salt stress.

B. σ^{38} protein levels from plasmid expression before (lane 1) and after (lane 2) salt stress.

C. CHIP in a σ^{38} plasmid expression context prior to salt shock. Gray bars denote samples enriched with β' antibodies. Black bars denote no antibody negative control. The results in the promoter and downstream gene body are indicated.

D. CHIP after the addition of 400 mM NaCl for 8 minutes. Gray bars denote samples enriched with β' antibodies. Black bars denote no antibody negative control.

A.

$\sigma 70$ REAKVLRMRFGIDMNTDYTLEEVGKQFDVTRERIRQIEAKALRKLHPSRSEVLRSLDD
 $\sigma 38$ KQREVLARRFGLLGYEAAATLEDVGREIGLTRERVRQIQVEGLRRLREILQTQGLNIEALFRE
 $\sigma 38$ 1-319 KQREVLARRFGLLGYEAAATLEDVGREIGLTRERVRQIQVEGLRRLREILQT
 $\sigma 38$ 1-314 KQREVLARRFGLLGYEAAATLEDVGREIGLTRERVRQIQVEGLRRLR

B.

C.

D.

Figure 3. Residues near Q320 of the $\sigma 38$ CTD are critical for in vivo transcription of $\sigma 38$ promoters

A. Sequence comparison of the CTDs of *E. coli* $\sigma 70$ and $\sigma 38$. Areas of highest conservation are shaded.

B. Western blot of $\sigma 38$ and truncated variants 1-319 and 1-314. Lane 1, 3, and 5 show the amount of $\sigma 38$ protein under non-osmotic conditions for full length, 1-319, and 1-314 variants, respectively. Lanes 2, 4, and 6 show the amount of $\sigma 38$ after addition of salt for full length, 1-319, and 1-314 variants.

C. *osmY* m-RNA under osmotic conditions. Cells were grown to late exponential phase and osmotically induced by the addition of 400mM NaCl. Full-length $\sigma 38$ (FL) and truncated variants 1-322, 1-320, and 1-319 are shown.

D. In vivo transcription using $\sigma 38$ CTD truncations on 5 $\sigma 38$ promoters. The endpoint of each truncation is shown below the graph. In each set the promoters used from left to right were: *cfa*, *otsB*, *gadA*, *gadB*, and *osmY*. Transcription is normalized to the full-length in each set.

Figure 4. Deletions of the σ^{38} CTD decrease RNA-polymerase binding in vivo

A. CHIP using full-length σ^{38} (textured bars), truncations 1-319 (black bars) and 1-314 (gray bars), and the σ^{38} null strain not supplemented by plasmid expressed protein (checkered bars). The results in the promoter and downstream gene body are indicated.

B. CHIP after salt addition using full-length σ^{38} (textured bars), and truncations 1-319 (black bars) and 1-314 (gray bars).

Figure 5. potassium glutamate activated transcription *in vitro* mimics m-RNA production *in vivo*

A. *in vitro* transcription of *osmY* by either full-length $\sigma 38$ (lanes 1–4) or 1-322 (lanes 5–8). Potassium glutamate was titrated into the transcription reactions: Lanes 1 and 5 have 200 mM; lanes 2 and 6 have 300 mM; lanes 3 and 7 have 400 mM; lanes 4 and 8 have 500 mM.

B. *In vitro* transcription of *osmY* and *cfa* P2 promoters under osmotic conditions comparing full-length $\sigma 38$ and 1-319.

C. *In vitro* transcription using $\sigma 38$ CTD truncations on three $\sigma 38$ promoters. In each set the promoters are from left to right: *cfa* P2, *osmY*, and *otsB*. Transcription is normalized to the full-length (wt) band in each set.

Figure 6. σ 38 residues near Q320 are important for holoenzyme formation *in vitro* His-tagged σ 38 was incubated with core RNA polymerase and bound by NTA beads and washed. A silver stain after 8% PAGE is shown. The upper bands represent core (β and β' subunits) and the lower band are σ 38. The length σ 38 and 1-319 and 1-314 are compared.