Role of region 4 of the sigma 70 subunit of RNA polymerase in transcriptional activation of the *lux* operon during quorum sensing

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ABSTRACT

The mechanism of gene regulation used by Gram-negative bacteria during quorum sensing is well understood in the bioluminescent marine bacterium *Vibrio fischeri*. The cell-density dependent activation of the luminescence (*lux*) genes of *V. fischeri* relies on the formation of a complex between the autoinducer molecule, N-(3-oxohexanoyl) homoserine lactone, and the autoinducer-dependent transcriptional activator LuxR. LuxR, a 250 amino acid polypeptide, binds to a site known as the *lux* box centered at position –42.5 relative to the *luxI* transcriptional start site. During transcriptional activation of the *lux* operon, LuxR is thought to function as an ambidextrous activator capable of making multiple contacts with RNA polymerase (RNAP). The specific role of region 4 of the *Escherichia coli* sigma 70 subunit of RNAP in LuxR-dependent transcriptional activation of the *luxI* promoter has been investigated. Rich in basic amino acids, this conserved portion of sigma 70 is likely to be surface-exposed and available to interact with transcription factors bound near the –35 element. The effect of 16 single and 2 triple alanine substitution variants of sigma 70 between amino acid residues 590 and 613, was determined *in vivo* by measuring the rate of transcription from a *luxI-lacZ* translational fusion via β-galactosidase assays in recombinant *E. coli*. *In vitro* work was performed with LuxRΔN, the autoinducer-independent C-terminal domain (amino acids 157 to 250) of LuxR because purified, full length LuxR is unavailable. Single-round transcription assays were

performed in the presence of LuxRΔN and 19 variant RNAPs, one of which contained a C-terminally truncated sigma 70 subunit devoid of region 4. Results indicate that region 4 is essential for LuxRΔN-dependent *luxI* transcription with two specific amino acid residues, E591 and K597, having negative effects on the rate of LuxRΔN-dependent *luxI* transcription *in vivo* and *in vitro*. None of the residues tested were identified as having any effect on LuxR-dependent *luxI* transcription *in vivo.* These findings suggest that region 4.2 is most likely to be in close proximity to LuxR when bound to the *luxI* promoter. However, unlike the situation found for other ambidextrous activators, no single residue within region 4.2 of sigma 70 may be critical by itself for LuxR-dependent during transcriptional activation.

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CHAPTER ONE

Introduction

Quorum sensing in *Vibrio fischeri*

Many bacteria employ intercellular communication systems that rely on small signal molecules to control the expression of multiple target genes. In Gram-negative bacteria, the most intensively investigated signal molecules are N-acyl-L-homoserine lactones, often referred to as autoinducers. In a process known as 'quorum sensing', these autoinducer signal molecules are utilized by the bacterial cells to monitor their own population densities. This density-dependent regulatory system relies on two proteins, an autoinducer synthase and an autoinducer receptor protein. At low population densities, cells produce basal levels of autoinducer via the activity of the autoinducer synthase. As the cell density increases, autoinducer accumulates in the growth medium. On reaching a critical threshold concentration, sufficient numbers of autoinducer-receptor complexes form to result in the induction / repression of autoinducer-regulated genes (for reviews, see Fuqua *et al.*,1994; Salmond *et al*., 1995; Fuqua *et al*., 1996; Eberl, 1999, Withers *et al*., 2001).

The quorum sensing method of gene regulation in Gram-negative bacteria was first observed in the luminescent marine organism *Vibrio fischeri* (Nealson *et al*., 1970). This organism occurs free-living in sea water and is also the symbiont in the light organs of certain marine fishes and squids in particular, the Japanese pinecone fish (*Monocentris japonica*) and the Hawaiian squid (*Euprymna scolopes*) from which *V. ficheri* can be isolated as a pure culture. (Fuqua *et al*., 1996). Activation of the luminescence (*lux*) genes of *V. fischeri* relies on the formation of a complex between an autoinducer molecule, N-(3-oxohexanoyl) homoserine

lactone (N-3oxoC6-HSL), and an autoinducer-dependent transcriptional activator known as LuxR (Figure 1; Fuqua *et al.*,1994). The two essential genes involved in this activation process are therefore *luxI*, which encodes the autoinducer synthase and *luxR*, which encodes the LuxR activator. Light emission occurs only at high cell densities where the freely diffusible autoinducer is able to reach the critical threshold concentrations necessary to form LuxR-autoinducer complexes. Since light production is a very energy-expensive process, induction of the luminescence system is desired only when cells are in the high nutrient/cell density host-associated state. This autoinduction system therefore allows *V. fischeri* to discriminate between the host-associated state and the low nutrient/cell density, free-living state (for reviews, see McFall-Ngai & Ruby, 1991; Ruby & McFall-Ngai, 1992). It has recently been reported that in addition to the *lux* operon, the LuxR / autoinducer complex substantially increases the expression of other non-Lux proteins, indicating the presence of a quorum-sensing regulon in *V. fischeri* (Callahan and Dunlap, 2000). Two of these non-*lux* proteins, QsrP and RibB, have been characterized and found to be involved with the synthesis of a novel periplasmic protein and riboflavin, respectively. Although the role of these proteins in host association has not been determined, the direct involvement of quorum sensing with cellular physiology suggests that luminescence is part of a coordinated adaptational response to the host associated state.

Quorum sensing circuits homologous to the LuxR/LuxI system of *V. fischeri*, have been identified in a wide range of Gram-negative bacteria where they regulate various functions including plasmid conjugal transfer, biofilm formation, motility, antibiotic biosynthesis and the production of virulence factors in plant and animal pathogens (Eberl, 1999). The acyl homoserine lactone signaling molecules produced by all these LuxR/LuxI-like systems vary in the length and active groups of the acyl chain attached to the homoserine lactone ring (Fuqua *et*

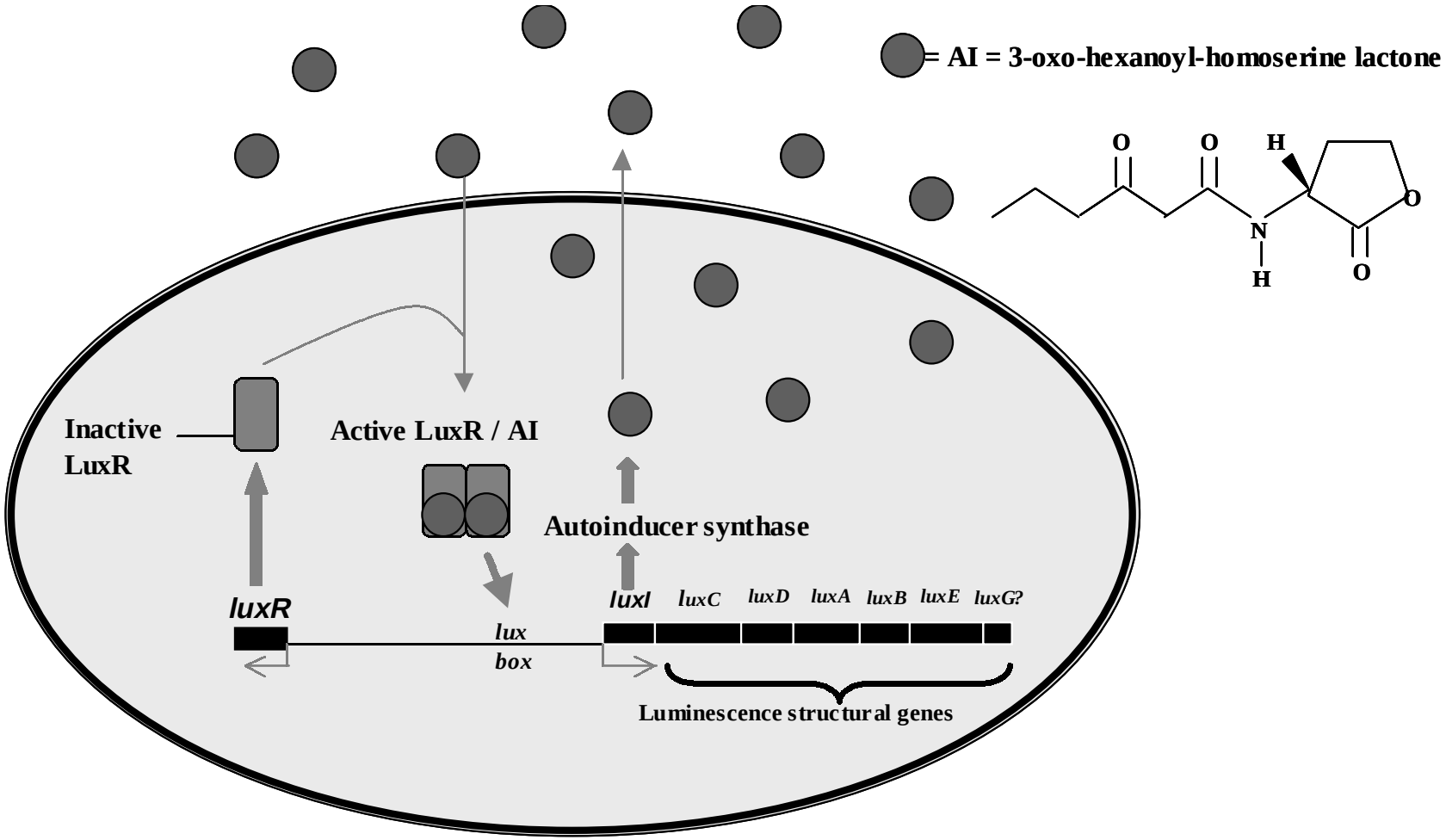

Figure 1: Model of quorum sensing in *Vibrio fischeri*. See text for details.

al.,1996). Understanding the precise molecular and biochemical function of LuxR and its homologues in their interactions with autoinducers, DNA targets and RNA polymerase (RNAP), may eventually allow these regulatory systems to be manipulated in such a way that certain density-dependent bacterial phenotypes can either be enhanced or eliminated.

The luminescence system of *Vibrio fischeri* serves as a model for studies of the molecular mechanisms involved in quorum sensing. The cloning of a 9-kb fragment of *V. fischeri* DNA that encodes all of the functions required for autoinducible luminescence in *Escherichia coli* was first reported by Engebrecht *et al*. in 1983. The luminescence genes are arranged in two divergent transcriptional units separated by an intergenic region roughly 155 bp long (Figure 2). One unit contains *luxR*, and the other unit, which is activated by the LuxR-autoinducer complex, contains the seven genes of the *lux* operon (*luxICDABEG*). The *luxI* gene encodes the193-amino acid autoinducer synthase that directs *E. coli* to synthesize N-3oxoC6-HSL. The other genes in the *lux* operon play mechanistic roles in light production; *luxA* and *luxB* code for the α and β subunits of luciferase and *luxC, luxD*, and *luxE* code for components of the fatty acid reductase complex required for synthesis of the aldehyde substrate for luciferase. The function of *luxG* is still unknown (Engebrecht & Silverman, 1984).

The transcriptional activator, LuxR

Studies of *luxR* mutations in recombinant *E. coli* have suggested that LuxR is a two domain polypeptide consisting of 250 amino acid residues (Figure 3; for a recent review, see Stevens and Greenberg, 1999). The membrane associated N-terminal domain (NTD) is involved in autoinducer binding and in the absence of autoinducer, it functions to block the activity of the C-terminal domain (CTD) which is involved in DNA binding and activation. LuxR is thought to

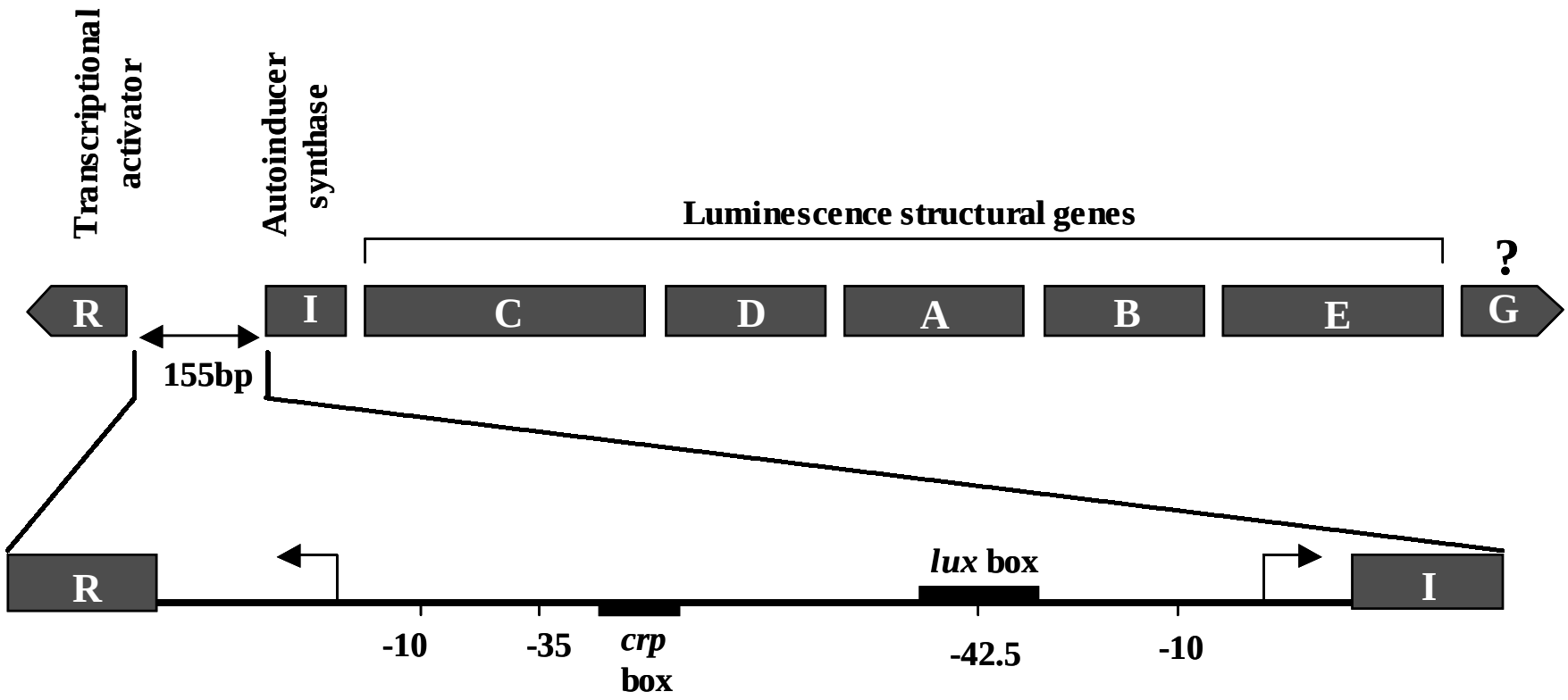

Figure 2: Model of the intergenic region between *lux*R and the *lux* operon (diagram not drawn to scale). See text for details. (Adapted from Stevens & Greenberg, 1999).

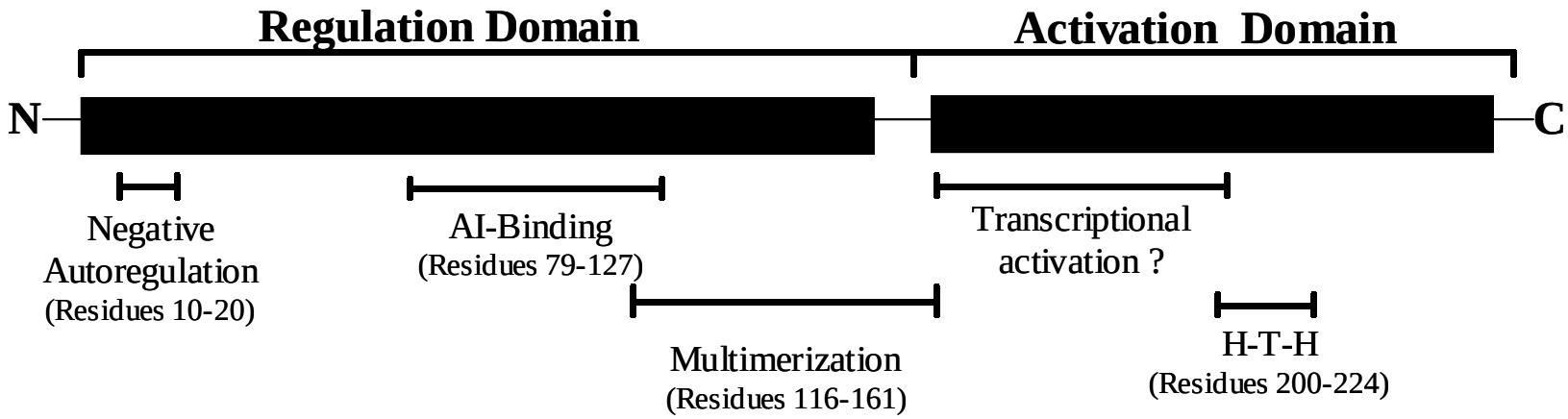

Figure 3: Model of the functional regions of LuxR. See text for details.

function as a multimer, presumably a dimer, with a region between residues 116 and 160 involved in dimerization (Choi & Greenberg, 1992a). The recent conversion of LuxR into a repressor, strongly suggests that LuxR binds specifically to a region of the *luxI* promoter DNA termed the *lux* box (Egland & Greenberg, 2000). This 20 bp intergenic region with a dyad symmetry is centered at the – 42.5 position relative to the *luxI* transcription start site (Egland and Greenberg, 1999). The CTD of LuxR shows sequence similarity to the helix-turn-helix (HTH)-containing domain of the LuxR-FixJ superfamily of transcription factors (Choi and Greenberg, 1992a; Fuqua *et al*., 1994; Egland and Greenberg, 2001). An amino acid sequence alignment between LuxR and other members of this superfamily suggests that the HTH motif of LuxR is between residues 200-224. Alanine-scanning mutagenesis of residues 190-224 identified numerous substitutions within the putative HTH (and the region immediately upstream of it), that resulted in LuxR proteins incapable of binding to the *lux* box within an artificial *lacZ* promoter (Egland and Greenberg, 2001). Substitution of numerous residues downstream of this HTH motif have also been found to be deficient in *lux* DNA binding (Trott and Stevens, 2001). It is not known which of these residues (if any) are involved in making specific protein-DNA interactions because active, full length LuxR has not been successfully purified so *in vitro* experiments demonstrating specific DNA binding have not been possible. Egland and Greenberg (2001) did identify two positive control variants of LuxR, W201A and I206A, which can bind to *lux* DNA but nevertheless fail to activate transcription of the *lux* operon. Since no positive control variants were identified downstream of residue 206, the 'activating region' of LuxR is thought to lie within and possibly upstream of the HTH motif.

Although active, full length LuxR has not been successfully purified, it has been possible to purify LuxRΔN, the cytoplasmic C-terminal domain (amino acids 157 to 250) of

LuxR (Stevens *et al*., 1994). Purified LuxRΔN functions as a signal-independent activator of the *luxI* promoter *in vitro* and in *E. coli* (Choi & Greenberg, 1991; Stevens *et al*., 1994). Unlike LuxR, LuxRΔN is only capable of binding to the *lux* box and the *luxI* promoter in synergy with *E. coli* σ^{70} holoenzyme RNA polymerase (RNAP) (Stevens *et al*., 1994). Since both proteins are necessary for promoter DNA binding, it is not possible to conclude that LuxRΔN is binding to the *lux* promoter directly. However, their requirement for one another to achieve protection of the *lux* promoter from DNaseI and to produce high levels of transcription at the *lux* promoter suggests that there may be some strong protein-protein interactions occurring between LuxRΔN and RNAP. LuxRΔN therefore serves as a useful tool in 'highlighting' potential contact points between LuxR and RNAP.

RNA polymerase and transcriptional activators

In *E. coli*, a multi-subunit RNAP holoenzyme consists of a core enzyme subunit structure (α_2, β, β') capable of elongation and termination of transcription and one of the several species of σ, which when bound to form holoenzyme, allows specific promoter recognition and efficient transcription initiation (Figure 4). The two α domains of RNAP each have two separately folded domains; the N-terminal domain (NTD; residues 8-241) and the C-terminal domain (CTD; residues 249-329) which are joined by an interdomain linker of at least 9 amino acid residues (Blatter *et al*., 1994). The αNTD interacts with β and β' and participates in α subunit dimerization (Kimura *et al*., 1994). The αCTD is known to make specific contacts with certain transcriptional activators and repressors and is capable of recognizing and binding to 'upstream elements' (UP elements) which are specific DNA sequences often referred to as the third recognition element in bacterial promoters (Ross *et al*., 1993; Gourse *et al*., 2000). It has

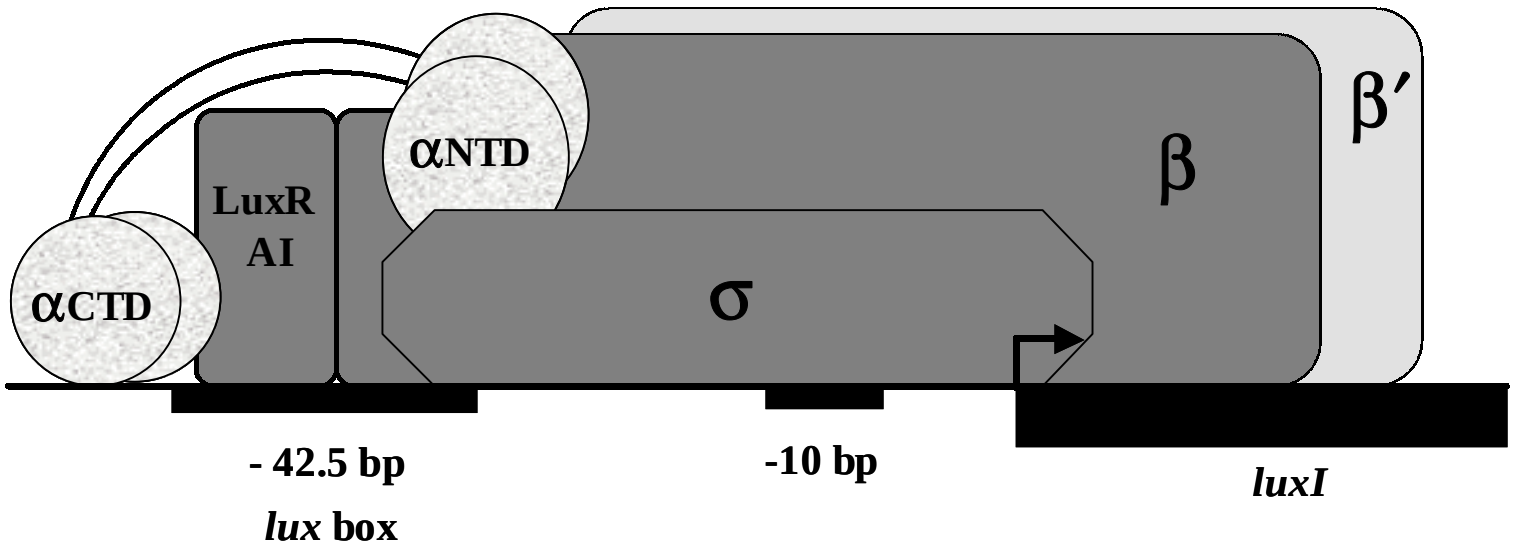

Figure 4: Model of the proposed interactions between RNAP and LuxR at the *lux* operon promoter. See text for details

recently been proposed that the αCTD recognizes and binds to a specific curved DNA conformation that results from the intrinsic bending of the AT-rich UP element rather then to the specific base sequence (Yasuno *et al*., 2001). The major recognition elements typically found in many *E. coli* bacterial promoters are two six-base-pair sequences located at positions –10 (consensus 5' TATAAT 3') and –35 (consensus 5' TTGACA 3') upstream of the transcriptional start site. Transcription inititation at these promoters is σ^{70}-dependent and requires specific contacts between σ^{70} and each of these hexamers (Gross *et al*., 1992).

Of the seven different sigma subunits in *E. coli*, the major housekeeping sigma is σ^{70} (Gross *et al*., 1998). The σ^{70} subunit is a 613 amino acid polypeptide which has been found to have many functions including binding to core RNAP, recognizing and clearing promoters, and interacting with transcriptional activators (Figure 5). Most primary "housekeeping" sigma subunits that have been analyzed from organisms other then *E. coli* show an astounding amount of amino acid similarity to σ^{70} (Lonetto *et al*., 1992). Regions 2 and 4 are the most highly conserved regions of σ^{70} and tend to be comprised of very basic amino acids. This is consistent with their role in DNA binding since a single α-helix within region 2.4 and a helix-turn-helix (H-T-H) motif in region 4.2 are thought to bind to the –10 and –35 regions of promoter DNA, respectively (Dombroski *et al*.,1992; reviewed by Gross *et al*.,1992). However, σ^{70} makes these critical contacts only when it is bound to core RNAP. Promoter binding by free σ^{70} is prevented by region 1.1 (Dombroski *et al*., 1992). Specific residues in regions 2.1, 2.2 and 3.1 are thought to interact with the β' subunit of core RNAP resulting in significant conformational movements in σ^{70} which somehow 'unmask' the DNA-binding regions to permit the formation of a closed promoter complex. (Sharp *et al*., 1999; Vuthoori *et al*., 2001; reviewed in Burgess and Anthony,

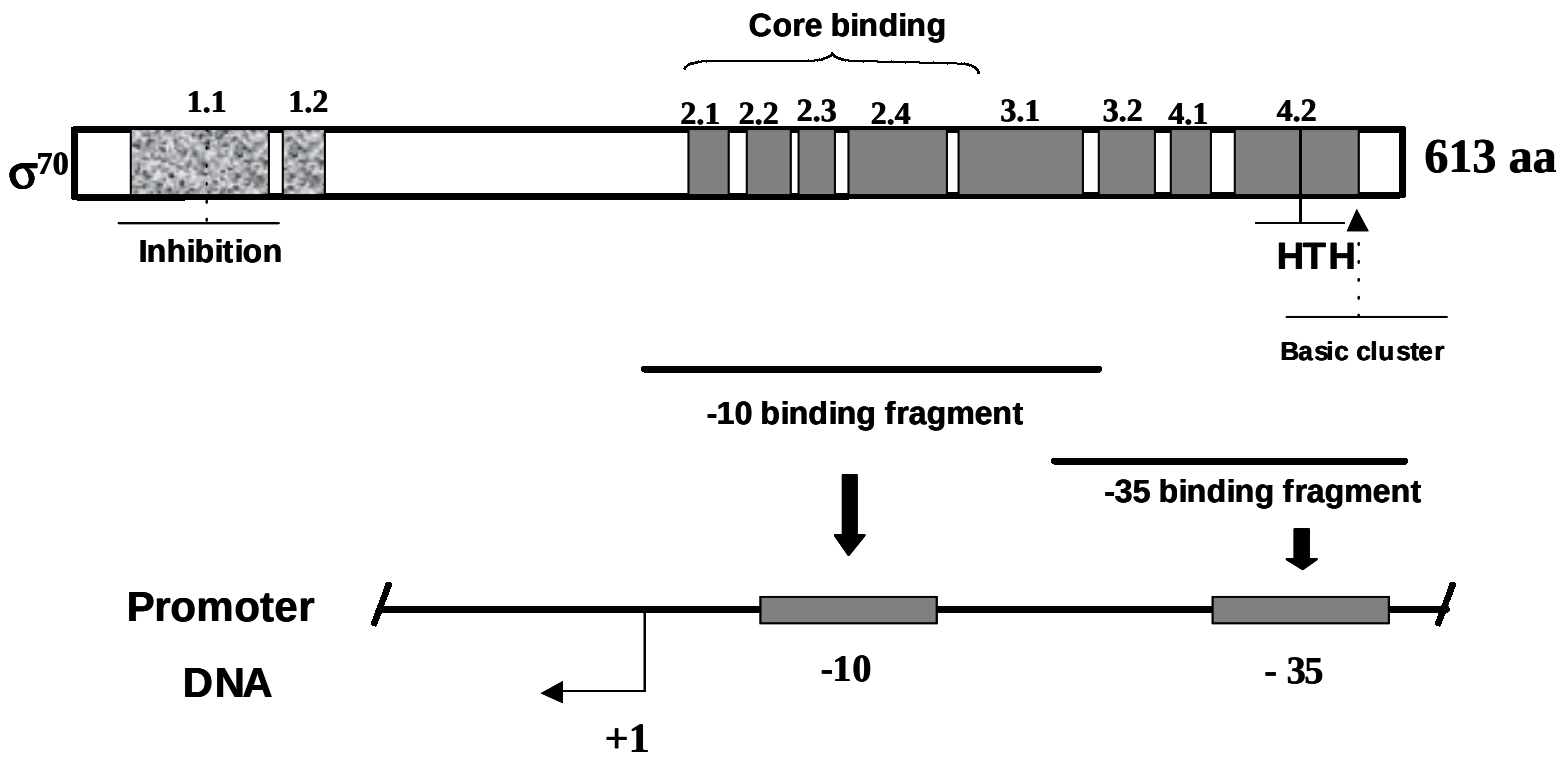

Figure 5: Model of the conserved regions of σ^{70} and functions related to DNA binding. The σ^{70} subunit of RNA polymerase holoenzyme (RNAP) is a 613 amino acid polypeptide. The top portion of the figure shows a linear representation of *E. coli* σ^{70} illustrating the location of the four highly conserved regions of the σ^{70} family. It has been proposed that a single α-helix within region 2 of σ^{70} and a helix-turn-helix (HTH) motif in region 4 of σ^{70} bind to the –10 and –35 regions of promoter DNA, respectively. Region 1.1 inhibits DNA binding of free σ^{70}. Specific residues in regions 2.1, 2.2 and 3.1 are thought to interact with the β' subunit of core RNAP. A region downstream of the HTH motif is rich in basic amino acid residues and is likely to be surface exposed and available to interact with Class 2 transcription factors. See text for further details and references. (Adapted from Lonetto *et al.*, 1998)

2001). In this holoenzyme -associated conformation, region 4 is positioned so that it can interact with upstream transcriptional activators (Burgess and Anthony, 2001).

Transcriptional activators can be involved in facilitating the initial recruitment of RNAP to the promoter allowing formation of a closed promoter complex , isomerization to an open complex (or DNA strand separation), early formation of a short transcript or the release of the sigma factor from the complex (for reviews, see Busby & Ebright, 1994; Ptashne & Gann, 1997; Rhodius and Busby, 1998, Vicente *et al*., 1999). Many transcriptional factors that bind to target promoters function by making direct contact with RNAP. *E. coli* transcription factors have been classified into 4 groups based on the RNAP subunit that serves as the contact site: Class 1 (α subunit), Class 2 (σ subunit), Class 3 (β subunit), Class 4 (β'subunit) (Table 1; Ishihama, 1993; Ishihama, 1997). Some transcription activators, like the cAMP receptor protein (CRP) and the fumarate/nitrate reduction regulator (FNR) are ambidextrous activators capable of interacting with either the α subunit and/or the σ subunit depending on the location of DNA binding relative to the promoter. At 'Class I' activator-dependent promoters, the activator binding site is located upstream of the –35 promoter region and the activator functions by making direct contact with the αCTD. At 'Class II' activator-dependent promoters, activator binding sites overlap and replace the –35 determinant for binding of RNAP. In these cases, the activator can contact the σ subunit, αCTD and αNTD (for reviews, see Busby & Ebright., 1994; Busby & Ebright., 1997; Ishihama, 1997; Rhodius & Busby, 1998). The lack of the usual –35 consensus sequence at Class II-activator-dependent promoters suggests that the activator provides protein-protein contacts which effectively compensate for the missing –35 DNA contact (Kumar *et al*., 1994). In fact, many σ-contacting transcription factors appear to specifically contact a target adjacent to the conserved helix-turn-helix motif in region 4.2 of σ^{70} during transcription initiation at certain

Table 1: Classification of *E. coli* transcription factors [a]. (Table adapted from Ishihama, 1997)

Class	Contact subunit	Transcription Factor
1	α	**^{me}Ada [b], AraC, CRP [b], FNR[b,c], GalR, LacI, MelR, NusA, OmpR, OxyR, SoxS, TrpR, TyrR, Mor (phage Mu)**
2	σ	**CRP [c], FNR [c], Ara C, PhoB, UhpA [c], ^{me}Ada [c], RhaS, AsiA (phage T4), cII (phage λ), Mor (phage Mu)**
3	β	**DnaA, NusA, Rho, ppGpp, nascent RNA**
4	β'	**NusA, Rho, Polyphosphate, nascent RNA**

[a] Although the site of DNA binding relative to the promoter does seem to influence which RNAP subunit the transcription factor might contact, some factors like CRP, FNR, and phage Mu Mor have been shown to behave as ambidextrous transcription factors, contacting multiple subunits of RNAP at the same time (reviewed in Rhodius & Busby, 1998). See text for further details and references.

[b] Class I promoters

[c] Class II promoters

promoters (Figure 6; Makino *et al*., 1993; Li *et al*., 1994; Lonetto *et al*., 1998; Landini *et al*., 1998; Rhodius & Busby, 2000; Oleknovich & Kadner, 1999; Landini & Busby, 1999; Bhende & Egan, 2000; Minakhin *et al*., 2001, Jishage *et al*., 2001; Dove & Hochschild, 2001). Rich in basic amino acids, this conserved region (mainly between amino acid residues 590 and 603) is likely to be surface-exposed and therefore available to interact with transcription factors bound near the –35 element (Figure 6).

It has been shown that σ^{70} is capable of initiating transcription from an activator-independent promoter that lacks the –35 consensus sequence as long as it has "an extended –10 consensus sequence" (Keilty and Rosenberg, 1987; Kumar *et al*., 1993). In fact, a truncated σ^{70} subunit (σ^{70}-529) lacking the carboxy-terminal amino acids 530 to 613 (and therefore the minus 35-recognition region) allowed RNA polymerase to efficiently initiate transcription at the P_{RE}# promoter (a constitutive derivative of the lambda P_{RE} promoter which contains a 'perfect' extended –10 consensus sequence) (Kumar *et al*., 1993). In a promoter containing an extended –10 consensus sequence, the required sequence in the –10 region is extended upstream to form the new consensus TGnTATAAT with the TG motif considered to be the important requirement for transcription inititation. To investigate the importance of region 4 of σ^{70} at other promoters lacking the –35 consensus sequence, Kumar *et al*. (1994) analyzed the ability of a series of truncated σ^{70} subunits (including σ^{70}-529), to initiate transcription at two Class II activator-dependent promoters: the PhoB-dependent P*pstS* and CRP-dependent P1*gal*. Despite the fact that neither promoter could act as an extended –10 sequence, transcription (in the presence of the respective activators and the truncated σ^{70} subunit lacking region 4.2 only) was successfully initiated from both promoters *in vitro*, indicating that the –35 recognition helix within region 4.2 was not essential. These results suggest that at promoters lacking both the –35 and the extended

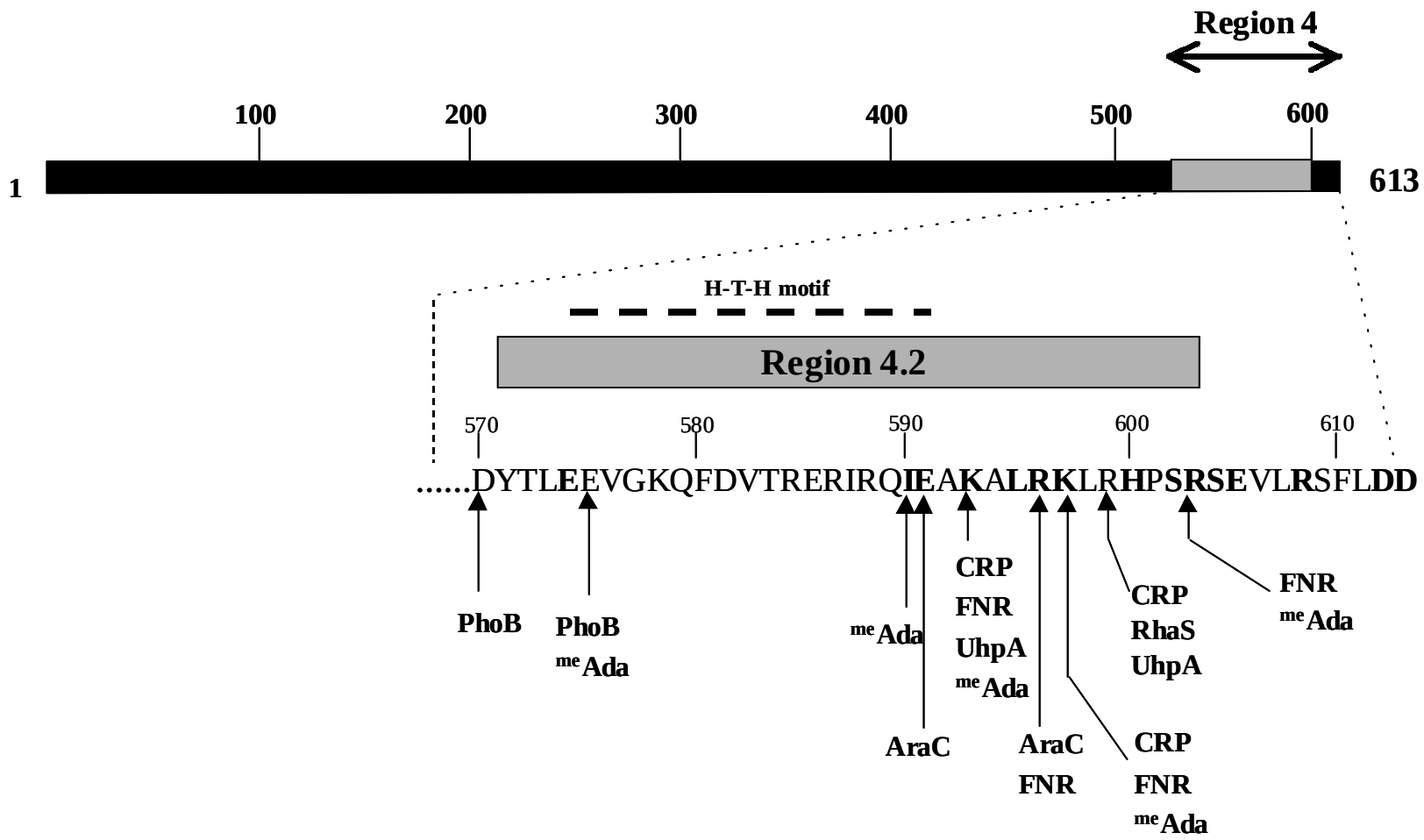

Figure 6: Model of the contact sites in region 4 of σ^{70} with Class 2 transcription activators. The bottom portion of the figure shows residues 570 to 613 of σ^{70} which includes region 4.2. The position of the predicted H-T-H motif is indicated and the sites of amino acid substitutions that modify activation by different Class 2 *E. coli* transcription activators are shown with arrows. Amino acid residues that are being tested in the work presented here are shown in boldface. See text for further details and references. (Adapted from Jishage *et al.*, 2001).

–10 consensus sequences, the reduced affinity of the mutant σ^{70} subunits for DNA may be compensated for by protein-protein contacts between RNAP and the respective transcription activators. In the study by Kumar *et al*., the location of the σ^{70}–activator contact site was proposed to be upstream of region 4.2.

CRP activator model

The classification system regarding Class I and Class II activator-dependent promoters is based on the extensive analysis of promoters stimulated by *E. coli* CRP (also known as catabolite gene activator protein or CAP) and the model for LuxR-dependent transcription activation at the *lux* promoter is based on the CRP model, it is important to review some of the key points regarding this activator. CRP is a global regulator of carbon utilization genes and functions by binding, in the presence of cAMP, to specific DNA target sites in or near target promoters and recruiting RNAP. CRP consists of a homodimer with each subunit consisting of 209 amino acids. It interacts with a 22 bp inverted repeat DNA sequence in which one CRP subunit interacts with one half of the DNA site and the other CRP subunit interacts with the other half. 'Class I' CRP-dependent promoters, such as the *lac* promoter, provide a DNA site for CRP that is located upstream of the DNA site for RNAP, approximately –61.5 relative to the transcriptional start site. Activation at these promoters is mediated by protein-protein interactions between activating region 1 (AR1; residues 156-164) of the downstream subunit of CRP and a target in the αCTD of RNAP which increases the affinity of RNAP for promoter DNA. At 'Class II' CRP-dependent promoters, like the P1*gal* promoter, the DNA site for CRP overlaps and replaces the –35 region and is centered near position – 41 relative to the transcriptional start site. Activation is due to two surfaced-exposed regions of CRP, AR1 and activating region 2 (AR2; amino acids 19, 21, 96 and

101). In this situation, AR1 of the upstream subunit of the CRP dimer makes protein-protein contacts with the αCTD while AR2 of the downstream subunit of the dimer makes protein-protein contacts with the αNTD of RNAP (Attey *et al*.,1994; Niu *et al.*, 1996).

AR1 and AR2 can be supplemented by a third, non-native activating region known as activating region 3 (AR3) which consists of amino acids 52-58 (Busby & Ebright., 1997). AR3 arises from the substitution of Lys-52 with a neutral or negatively charged residue. Located in the downstream subunit of the CRP dimer, AR3 is thought to make specific contacts with region 4.2 of the RNAP σ^{70} subunit since substitution of Lys-593, Lys-597 and Arg-599 of σ^{70} reduces or eliminates AR3-dependent transcription activation at the Class II promoter, p*melRcon*, *in vivo* and *in vitro* (Lonetto et al., 1998). Through the application of suppression genetics, Rhodius and Busby (2000) proposed that the inhibitory side chain, Lys-52 in AR3, clashes with Arg-596 in σ^{70}, however substitution of Lys-52 results in an 'activatory' AR3 with negatively charged residues Asp-53, Glu-54, and Glu-55, capable of interacting with the positively charged residues Lys-593, Lys-597 and Arg-599 of σ^{70}. The exact function of this contact during the transcription initiation process remains unclear. However analysis of a CRP (AR3)- σ^{70} contact required for transcription activation at the promoter, P1*gal*, indicates that CRP functions to recruit region 4 to the promoter –35 element (Bown *et al*., 2000).

LuxR-dependent transcription activation at the *lux* operon

The proposed model for the mechanism of LuxR-dependent transcription activation at the *lux* operon is similar to that of CRP at Class II type promoters (Figure 4). LuxR is thought to bind to the *lux* box as a homodimer and to function as an "ambidextrous activator" with each LuxR subunit interacting with a different region of RNAP. Support for this hypothesis comes

from the following key findings: both half-sites of the *lux* box are required for activation of the *luxI* promoter by LuxR and the monomeric LuxRΔN in *E. coli* (Egland and Greenberg, 1999); both LuxR and LuxRΔN require the αCTD of RNAP for efficient *luxI* transcription in *E. coli* and *in vitro* (Stevens *et al*., 1999); the location of the *lux* box at position –42.5 is critical for LuxR activity suggesting that it cannot function through contacts with the αCTD alone (Egland and Greenberg, 1999). Furthermore, the 20 bp *lux* box overlaps and replaces the –35 consensus sequence at the *luxI* promoter placing the proximal LuxR subunit in an ideal position to interact with region 4 of the RNAP σ subunit.

The focus of this research project is to determine the role of region 4 of σ^{70} in LuxR-dependent transcription activation at the *luxI* promoter. Using *in vitro* transcription assays and β-galactosidase assays on cell extracts, the effects of variant forms of σ^{70} containing alanine substitutions between amino acid residues 590 and 613 can be compared to wild-type σ^{70} by changes in the rate of *luxI* transcription. *In vitro* work can be performed with LuxRΔN and establishing a good correlation between the *in vitro* and *in vivo* data with LuxRΔN and the *in vivo* data with LuxR, will provide insight into the native activity of LuxR. An interesting feature of the *luxI* promoter is the presence of a –10 sequence that deviates from an extended –10 consensus sequence at only one position (Figure 7; Egland and Greenberg, 1999). This raises the possibility that contact between LuxR and region 4 of σ^{70} may actually occur at a region upstream of region 4.2 as is likely the case with wild-type CRP at the P1*gal* promoter (Kumar *et al*., 1994). Transcription assays using a carboxy-truncated σ^{70} (σ^{70}-529, which lacks region 4) were used to further examine the requirement of other residues within region 4 in LuxRΔN-dependent activation of the *lux* operon.

TGT ATG TGT CGT CGG CAT TTA TGT TTT TCA **T**AC CCA TCT CTT TAT CCT TAC CTA TTG TTT

lux R **+ 1**

GTC GCA AGT TTT GCG TGT TAT ATA TCA TTA AAA CGG TAA TGG ATT GAC ATT TGA TTC

TAA TAA ATT GGA TTT TTG TCA CAC TAT TGT ATC GCT GGG AAT ACA ATT ACT TAA CAT

AMS4

AAG C**AC CTG TA**G **GAT CGT ACA GGT** TTA GCG AAG AAA ATG GTT **TGT TAT AGT** CGA

lux box

Extended -10

ATA **A**AC GCA AGG GAG GTT GGT ATG ACT ATA ATG ATA AAA AAA TCG GAT TTT TTG GCA

+1 *luxI*

ATT CCA TCG GAG GAG TAT AAA GGT ATT CTA AGT CTT CGT TAT CAA GTG TTT AAG CAA

AGA CTT GAG TGG GAC TTA GTT GTA GAA AAT AAC CTT GAA TCA GAT GAG TAT GAT AAC

TCA AAT GCA GAA TAT ATT TAT GCT TGT GAT GAT ACT GAA AAT GTA AGT GGA TGC TGG

AMS5

Figure 7: Nucleotide sequence of the intergenic region between *luxR* and *luxI*. Sequences shown include the first 10 codons of the *luxR* gene and the first 69 codons of the *luxI* gene. The transcriptional start sites of the *luxI* and *luxR* promoter and the 20 bp *lux* box and 'extended' –10 consensus sequences of the *luxI* promoter are boldfaced, underlined and labeled underneath. The location of primers used for this study are underlined and also labeled underneath.

CHAPTER TWO

Materials and Methods

Plasmid and Strain Construction

The *E. coli* strains and plasmids used in this study are listed in Table 2. To construct pBdgjR2 and pBdgjΔN2, a 1-kb *Bam*HI fragment with *ptac-luxR* from pSC300 and a 0.6-kb *Bam*HI fragment with *ptac-luxRΔN* from pSC156, (Choi & Greenberg, 1991), were separately cloned into the *Bam*HI site of the cloning vector, pBBR1MCS-5 (Kovach *et al*., 1995). Restriction endonucleases were used according to the manufacturer's instructions (New England Biolabs, Beverly, MA). The DNA fragments containing *luxR* and *luxRΔN* were isolated and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), following electrophoresis through a 0.8% agarose gel. pBBR1MCS-5 was linearized with *Bam*HI at the multiple cloning site within the *lacZα* gene and analyzed by electrophoresis for successful digestion. Ligation of the *luxR* or *luxRΔN* genes into the cloning vector was completed using T4 DNA ligase (New England Biolabs, Beverly, MA) as per the manufacturer's instructions. The ligation reactions were subsequently transformed into *E. coli* DH5α and transformants carrying the recombinant plasmids were selected on Luria-Bertani (LB) agar containing 20 μg/ml gentamycin (Gn), 20 mg/ml 4-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) and 1 mM isopropylthiogalactoside (IPTG). Recombinant plasmid DNA was purified from the transformants by the alkaline lysis miniprep procedure (Sambrook *et al*., 1989) and the presence and orientation of the inserts in the vector was determined by size analysis of the restriction fragments following digestion with *Bam*HI and *Eco*RI respectively. Plasmids encoding wild-type σ^{70} (RpoD) and alanine

Table 2: Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant Characteristics	Source or Reference
***E.coli* strains**		
DH5α	*supE*44, Δ*lacU169* (ϕ80 *lacZ*ΔM15), *hsdR1, recA1, endA1, gyrA96 thi-1, relA1*	Hanahan, D., 1983
JM109	*recA, supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F' (*traD*36 *ProAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	Yanisch-Perron *et al.* 1985
CAG20154	derivative of strain C600: *galK, thi-1, thr-1, leu-6, lacY1, tonA21, supE44,* Ω (CAM) P_{trp}-*rpoD*	Lonetto *et al.* 1999
CAG20176	derivative of strain MC1061: *galK, galU, mcrB, hsdR, rpsL, thi-1,araD139 Δ(ara-leu)7679,* Ω (CAM) P_{trp}-*rpoD zgh::tn10*, ΔlacX74	Lonetto *et al.* 1999
GS162λ*luxI-lacZ*	derivative of strain MC400*: pheA905, thi-1,* Δ*lacU169, araD129, rpsL,* λ*luxI-lacZ* translational fusion inserted in chromosome at *att*λ	Urbanowski, M (unpublished result)
Plasmids		
pSC300	*luxR* in pKK223-3, Ap^r	Choi & Greenberg, 1992
pSC156	*luxR* with deletion of sequences encoding N-terminal amino acids 2-156 of *luxR* in pKK223-3, Ap^r	Choi & Greenberg, 1991
pAMS121	1-kb *Bam*HI fragment with *ptac-luxR* from pSC300 in *Bam*HI site of pSUP102, Cm^s Tc^r Mob$^+$	Stevens *et al.*, 1999
pAMS122	0.6-kb *Bam*HI fragment with *ptac-ΔluxR* from pSC156 in *Bam*HI site of pSUP102, Cm^s Tc^r Mob$^+$	Stevens *et al.*, 1999
pBBR1MCS-5	pBBR1MCS-based, Gn^r, Mob$^+$	Kovach *et al*. 1994, 1995.
pBdgjR2	1-kb *Bam*HI fragment with *ptac-luxR* from pSC300 in *Bam*HI site of pBBR1MCS-5, Gn^s Mob$^+$	**This study**
pBdgjΔN2	0.6-kb *Bam*HI fragment with *ptac-ΔluxR* from pSC156 in *Bam*HI site of pBBR1MCS-5, Gn^s Mob$^+$	**This study**
pAMS129	8.0-kb *Xba*I-*Sal*I fragment with *luxR' luxICDABEG* from pJE202 in *Xba*I-*Sal*I sites of pJRD215, Kn^r Sm^r Mob$^+$	Stevens *et al.*, 1999
pAMS1300	*luxI* promoter in pMP7, Ap^r	Stevens & Greenberg, 1997.
PGEX-2Tσ^{70} (and derivatives)	Ap^r , *rpoD* (derivatives carrying RNAP σ^{70} subunit single alanine substitutions at positions, 574, 590, 591, 593, 595, 596, 597, 599, 600, 602, 603, 604, 605, 608, 612, 613 and triple alanine substitutions at positions 593/596/597 and 599/600/603.	Lonetto *et al*., 1998
pSR/lacUV5	pBR322 derivative carrying lacUV5 promoter cloned upstream of transcription terminator	Savery *et al*., 1998
pML-P_{RE}#	derivative of pKK232-8 containing P_{RE} # promoter inserted between the *Bam*HI and *Hind* III site. The P_{RE} # promoter contains an extended –10 consensus sequence.	Kumar *et al*., 1993

substitution derivatives of RpoD were provided by C. Gross (UCSF) and are variants of pGEX2T in which the coding region of glutathione–S-transferase (GST) is fused to RpoD residues 8 to 613 and expressed from the IPTG inducible *tac* promoter (Lonetto *et al*., 1998). These plasmids carry ampicillin (Ap) resistance.

E. coli GS162 *luxI-lac*Z was provided by M. Urbanowski (University of Iowa) and was used as the host organism for *in vivo* studies. This strain contains a *luxI-lac*Z translational fusion (first 19 codons of the *luxI* gene fused via a synthetic *Bam*HI site to the eighth codon of *lacZ*) integrated as a single-copy lysogen in the *att*λ site of the chromosome (personal communication). A library of two-plasmid strains was constructed by the sequential transformation of either pBdgjR2 or pBdgjΔN2 and one of the plasmids encoding either wild-type GST-RpoD or an alanine substitution derivatives of GST-RpoD. Transformants harboring both plasmids were selected on LB agar medium containing 20 μg/ml Gn, 100 μg/ml Ap, and 20 mg/ml X-Gal.

β-Galactosidase assays

Assays were performed based on conditions described in Trott and Stevens (2001). To obtain cell extracts for the assay, overnight cultures of each transformant were grown at 30°C in LB containing the appropriate antibiotics. Each overnight culture was subcultured to an OD_{600} of 0.025 into LB with the appropriate antibiotics. In the case of the LuxR-containing strains, 200 nM of N-(3-oxohexanoyl) homoserine lactone was also added to the LB medium. Subcultures were grown at 30°C to a final OD_{600} of 0.5 and subsequently placed on ice. A small volume of each subculture (5 μl) was diluted 1:200 in Z buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$-$7H_2O$, 400 nM DTT, pH 6.5) and lysed using 50 μl

chloroform. β-galactosidase activity levels of each mutant strain was measured using the Tropix chemiluminescent reporter assay kit (Tropix, Bedford, MA). The β-galactosidase reaction was initiated by the addition of 100 μl of chemiluminescent substrate (1X) to 20 μl of the cell lysate in a luminometer tube. The reaction was left to proceed for 1 hour and terminated by the addition of 150 μl of light emission accelerator. A TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) was used to measure light output over a 4.0 sec integration period.

Purification of *E. coli* Glutathione-S-transferase (GST)-RpoD fusion proteins

GST-RpoD fusion proteins were purified using GST affinity chromatography as described by Lonetto *et al*., 1998. Protein concentration was estimated from the absorbance at 595nm using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the efficiency of the purification process (Sambrook *et al*., 1989). Purified protein was stored at -70°C.

Preparation of Promoter Fragments

A 289 bp polymerase chain reaction (PCR) product containing the *luxI* promoter and the first 210 bp of the *luxI* gene was amplified from supercoiled pJE202 (Engebrecht *et al*., 1983) and served as the test promoter fragment in the *in vitro* transcription assays. Purified pJE202 was obtained using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), and quantitated by comparison to a mass ladder (BioRad, Hercules, CA) following electrophoresis through a 0.8% agarose gel. Primers corresponding to nucleotides –79 to –62 (AMS4) and +210 to +193 (AMS5) with respect to the *luxI* transcriptional start site were used to obtain the PCR product (see Figure 7) (Engebrecht and Silverman, 1987). The 100 μl PCR reaction contained the

following reagents: 1 μM of each primer (Sigma-Genosys, The Woodlands, TX), 2 mM dNTPs (Promega, Madison, WI), 2.5 units *Taq*2000 Polymerase (Stratagene, La Jolla, CA), 1X *Taq*2000 Reaction Buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) (Stratagene, La Jolla, CA), 2 mM $MgSO_4$ (Fisher, Springfield, NJ), and 50 ng of supercoiled pJE202. A Sprint thermal cycler (Hybaid, Middlesex, UK) was programmed as follows: one cycle: 94°C for two minutes; 30 cycles: 94°C for one minute, 44°C for one minute, and 72°C for two minutes; and one cycle: 72°C for ten minutes. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and quantified by comparison to a mass ladder (BioRad, Hercules, CA) following electrophoresis through a 0.8% agarose gel.

Promoter fragments for use as internal controls in the *in vitro* transcription assays consisted of a 205 bp *lac*UV5 promoter fragment generated by PCR on supercoiled pSR/*lac*UV5 (Savery *et al.*, 1998) or a 244 bp PCR product containing P_{RE}# (a constitutive derivative of the lambda P_{RE} promoter) amplified from pML-P_{RE}# (Keilty and Rosenberg, 1987). Synthetic primers (Sigma-Genosys, The Woodlands, TX):
5'CCTATAAAAATAGGCGTATCACGAGGCCCT 3' and
5'GATGCCTGGCAGTTCCCTACTCTCGC 3' were used to obtain the *lac*UV5 PCR product, while primers: 5'AGCTGAACGGTCTGGTTATAGG 3' and
5'TAAACTGCCAGGGAATTCCC 3' were used to obtain the P_{RE}# PCR product. Preparation of the supercoiled plasmid templates and reaction conditions for each of these PCR reactions was exactly as described above except for the annealing temperature in the P_{RE}# PCR reaction which was set at 50°C instead of 44°C.

In vitro Transcription Assays

Single-round transcription assays were performed as described by Stevens and Greenberg (1997) with the following modifications: for transcription assays with the GST-RpoD proteins, the binding reactions (30 μl final volume) contained the *luxI* test promoter fragment (1 nM), the *lacUV5* control promoter fragment (1 nM), LuxRΔN (2 μM), and variant RNAP consisting of core RNA polymerase (30 nM) (Epicentre Technologies, Madison, WI) and GST-RpoD (each used at 400 nM except for GST-RpoD (597) and GST-RpoD (605) which were used at 56nM and 280nM respectively). At these concentrations, the control *lacUV5* promoter fragment was saturated for all of the RNA polymerases tested. Binding reactions for transcription assays with carboxy-truncated σ^{70}-529 contained the *luxI* test promoter fragment (1.5 nM), the P_{RE}# control promoter fragment (1.5 nM), LuxRΔN (10 μM), wild-type RNAP (20nM) (Epicentre Technologies, Madison, WI) or variant RNAP (provided to us by A. Ishihama, National Institute of Genetics, Japan) which was reconstituted by mixing core RNA polymerase (33.3 nM) with a 10-fold excess of σ^{70}-529, and incubated for 15 min at 30°C immediately before use in transcription assays (Kumar et al., 1994). Radiolabeled transcripts and control bands were visualized and quantified on a Molecular Dynamics phosphorimager using the software ImageQuant, v3.3.

Western Immunoblotting

A comparison of the expression levels of the plasmid-derived GST-RpoD and the chromosomally encoded wildtype RpoD in each of the variant *E. coli* GS162λ*luxI-lacZ* / pBdgjΔN2 strains was measured using western immunoblotting techniques as described in

Brahamsha and Greenberg (1988). Cell pellets were obtained from strains that were grown for the "β-Galactosidase assays" described above (final OD_{600} of 0.5). Each pellet was prepared by centrifugation of 0.5 ml aliquots of culture, careful removal of the supernatant and storage at -20°C. Prior to use, cell pellets were resuspended in 100 μl sample buffer (56 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.5 ml β-mercaptoethanol, 0.01% bromophenol blue) and heated for 5 min in a boiling water bath. A total volume of 25 μl of each sample was loaded on to an 8% SDS-polyacrylamide resolving gel. Monoclonal primary mouse antiserum to RpoD (Neoclone, Madison, WI) was used at a dilution of 1:1500 to probe for RpoD and GST-RpoD transferred to a nitrocellulose blot. Horseradish peroxidase-conjugated goat immunoglobulin G (IgG) to mouse IgG served as the secondary antiserum (1:2000 dilution) (Cappel, Aurora, OH) in the colorimetric detection system used. Spot densitometry was used to quantify and compare the amount of RpoD and GST-RpoD in each culture and was performed on an Alphaimager™ v3.3 using the software AlphaEase™ (Imgen Technologies, Alexandria, VA).

CHAPTER THREE

Results

Effects of changes in the σ^{70} subunit on LuxR- and LuxRΔN-dependent *luxI-lacZ* expression

Sixteen single and two triple alanine substitutions between amino acids 570 to 613 in the C-terminal domain of *E. coli* σ^{70} (RpoD) were examined for their effects on LuxR- and LuxRΔN-dependent *luxI-lacZ* expression. This region of RpoD is involved in the recognition of the –35 promoter element and in the action of certain transcriptional activators that bind near position –40. Plasmids encoding the variant GST-RpoD were initially introduced into *E. coli* CAG20176λ*luxI-lacZ* harboring either pBdgjR2 (expressing LuxR) or pBdgjΔN2 (expressing LuxRΔN). In the host strain *E. coli* CAG20176 (Lonetto *et al*., 1998), the chromosomal *rpoD*$^{+}$ allele is replaced by Ω(Cm)*trpP-rpoD*, which allows the GST-RpoD variants to be tested with reduced competition from the wild-type RpoD. The presence of tryptophan in the growth medium for this strain results in the repression of the chromosomal *rpoD* making cell growth dependent on the tryptophan competitor indole-3-acrylic acid (IAA) or a plasmid-derived GST-RpoD variant. A single-copy of a *luxI-lacZ* translational fusion (derived from strain *E. coli* GS162λ*luxI-lacZ*) was transferred into CAG20176. When grown in the presence of IAA and in the absence of a plasmid-derived GST-RpoD, strains harboring the LuxR- or LuxRΔN-containing plasmids exhibited increased levels of β-galactosidase activity consistent with the expected *luxI-lacZ* expression from an "activated" *luxI* promoter (data not shown). However, under *rpoD* repression conditions and complementation by the plasmid-borne, wildtype GST-RpoD, only basal levels of β-galactosidase expression were observed. These results indicated

that in *E. coli* CAG20176λ*luxI-lacZ*, the wildtype *gst-rpoD* was not able to completely complement the repressed, chromosomally- encoded *rpoD*. Growth of these sigma depletion strains had been reported as being problematic (Lonetto *et al*., 1998; Olekhnovich & Kadner, 1999) displaying abnormal growth upon reaching stationary phase and showing intolerance to high levels of GST- RpoD resulting from induction at the IPTG-inducible *tac* promoter. Similar complications arose with attempts to construct a three plasmid *in vivo* system in *E. coli* CAG20154 (Table 2) to test the effects of the variant forms of RpoD on *lux* operon expression instead of *luxI-lacZ* expression. Luminescence, rather then β-galactosidase activity, was to have served as the reporting system in this strain. However, for reasons that are not understood, the substitution of plasmid-encoded wild-type GST-RpoD for chromosomally encoded RpoD affected the cell's ability to luminesce. Furthermore, these three plasmid strains showed severe growth defects. Due to the many complications encountered with *E .coli* CAG20176 and CAG20154, the use of these strains for further studies was abandoned.

The establishment of a functional *in vivo* system was achieved with *E. coli* GS162λ*luxI-lacZ* (which carries the single-copy *luxI-lacZ* translational fusion). The plasmids encoding variant GST-RpoD proteins with the wild-type or eighteen alanine substitutions were introduced into two *E. coli* derivatives of this strain: (i) GS162λ*luxI-lacZ* harboring pBdgjR2 (expressing LuxR) and (ii) GS162λ*luxI-lacZ* harboring pBdgjΔN2 (expressing LuxRΔN). The plasmid-encoded RpoD proteins compete with the chromosome-encoded RpoD for assembly into RNAP holoenzyme. Previous studies involving the use of these plasmids had suggested that the level of plasmid-encoded GST-RpoD (uninduced) was approximately that of chromosomal RpoD in the haploid cell (Lonetto *et al*., 1998; Bhende & Egan, 2000). A RpoD variant that resulted in a two-fold decrease in *luxI-lacZ* expression would, therefore, constitute a reasonably large defect.

In the presence of LuxR, none of the variant forms of GST-RpoD that were tested exhibited a defect in *luxI-lacZ* expression (Figure 8). In comparison to the wildtype (WT) control, increases in *luxI-lacZ* expression were observed with GST-RpoD variants containing substitutions at residues 591 (145% WT), 600 (132% WT), 602 (139% WT), and 603 (130% WT). In the presence of LuxRΔN however, the most severe average decreases in β-galactosidase activity were observed with GST-RpoD variants containing substitutions at residues 591 (10% WT), 595 (3% WT), and 597 (5% WT) (Figure 9). More modest average decreases were observed with substitutions at residues 596, 602, and 603 with β-galactosidase levels at 63%, 61% and 32% of the WT control respectively. In general, a higher degree of variation from trial to trial was observed with these three strains. The triple alanine substitution variants, MA1 (593, 596, 597) and MA2 (599, 600, 603), also had slight defects in *luxI-lacZ* expression with levels of 78% WT and 76% WT, respectively. Interestingly, the effects of the alanine substitution at residue 597 and 603 produced more dramatic effects in the strains containing the single variant GST-RpoD compared to strains containing the triple variant GST-RpoD.

Since LuxRΔN is known to require RNAP in order to bind the *lux* box (Stevens *et al*., 1994), while LuxR can bind the *lux* box independently (Egland & Greenberg, 2000), it was not surprising to find residues in RpoD uniquely affecting LuxRΔN –dependent transcription. DNase I protection assays clearly show that LuxRΔN and RNAP have a very low affinity for *lux* promoter DNA when exposed to it independently of each other (Stevens *et al*., 1994). An interaction with RNAP (most probably in the form of direct protein-protein contacts) seems to be necessary for LuxRΔN to both bind and activate transcription at the *lux* promoter. It therefore follows that a potential interaction between RpoD and LuxRΔN, is likely to involve a broader

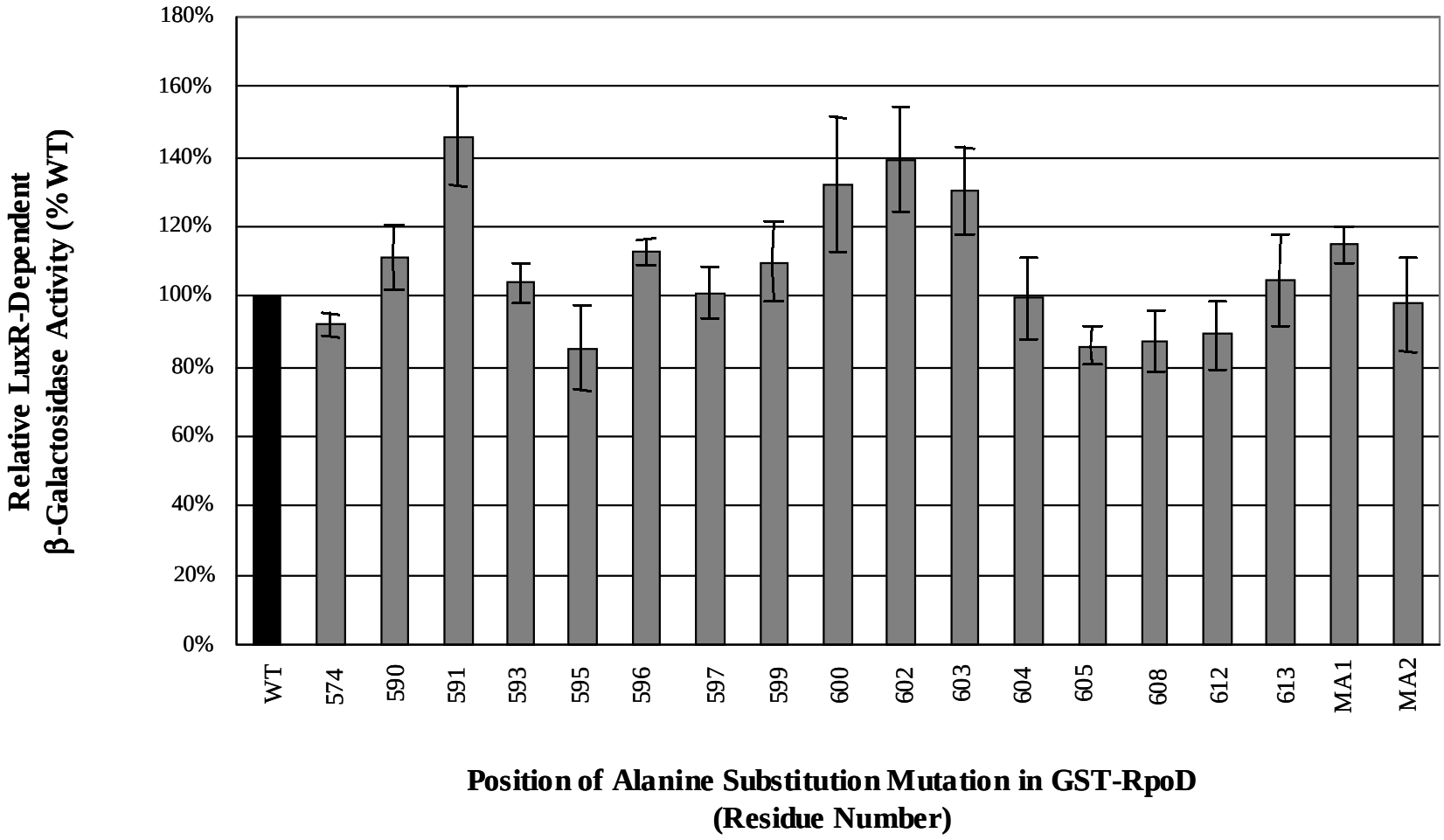

Figure 8: Effect of alanine substitutions in RpoD on LuxR-dependent *luxI-lacZ* expression in recombinant *E. coli* GS162λ *luxI-lacZ*. Values of β-galactosidase activity are averages of two independent assays and are presented relative to the average wild-type (WT) value (342 RLU ± 83RLU / OD_{600}). Error bars indicate the range on either side of the mean. The two triple variant GST-RpoD are represented by MA1 (residues 593, 596, 597) and MA2 (residues 599, 600, 603).

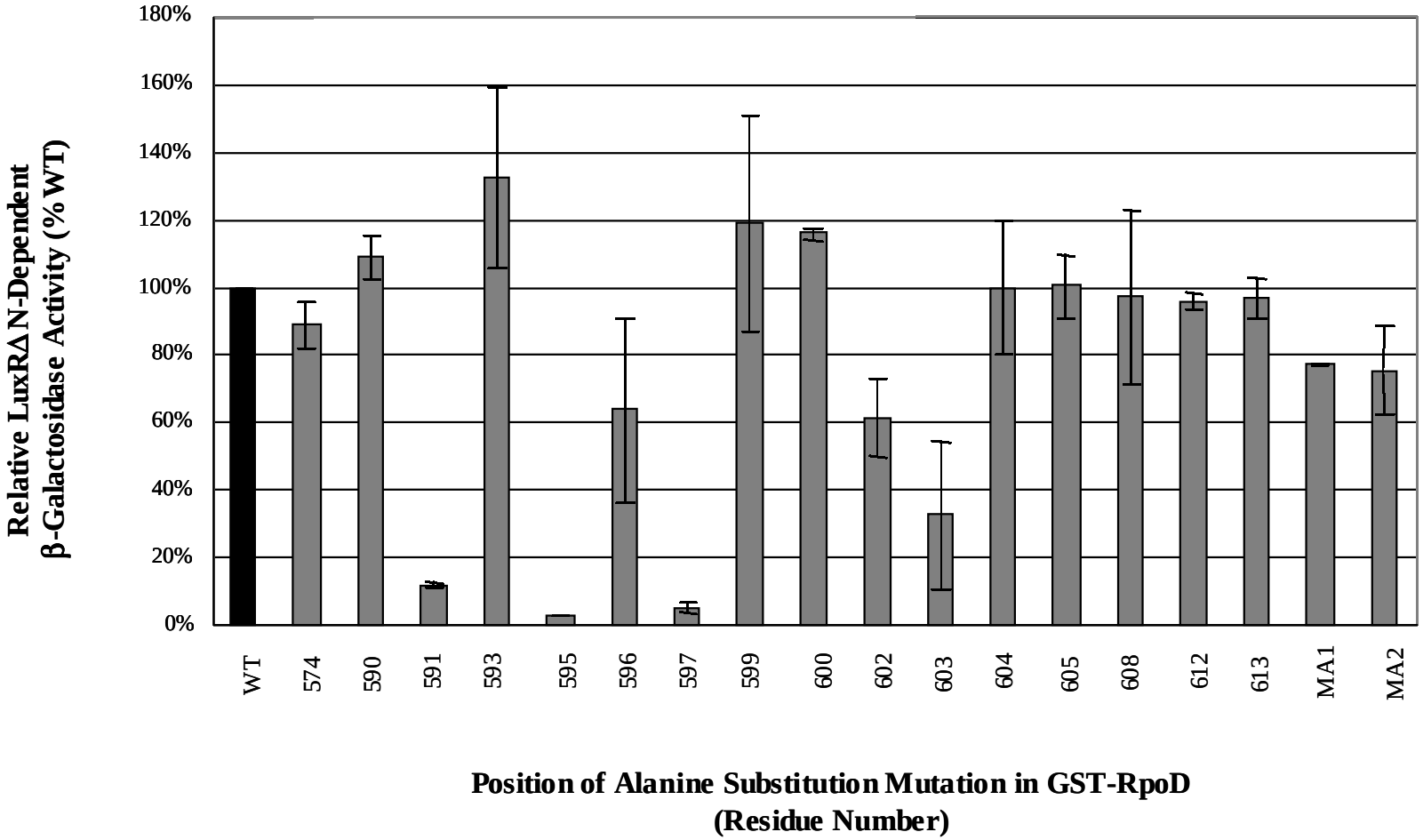

Figure 9: Effect of alanine substitutions in RpoD on LuxRΔN-dependent *luxI-lacZ* expression in recombinant *E. coli* GS162λ *luxI-lacZ* . Values of β-galactosidase activity are averages of two independent assays and are presented relative to the average wild-type (WT) value (129 RLU ± 4 RLU/ OD_{600}). Error bars indicate the range on either side of the mean. The two triple variant GST-RpoD are represented by MA1 (residues 593, 596, 597) and MA2 (residues 599, 600, 603).

interface, encompassing more amino acids compared to those that might be involved in interactions with LuxR.

The residues identified as playing a role in LuxRΔN-dependent activation of the *luxI-lacZ* gene fusion could be affecting the formation of a LuxRΔN–RNAP complex, the ability of this complex to bind the *luxI* promoter, the ability of the complex to successfully activate transcription or some other indirect process necessary for β-galactosidase expression. However, the magnitude of the decrease in β-galactosidase expression as a result of substitutions 591, 595 and 597 was especially surprising, since 50% of the holoRNAP was expected to contain wild-type, chromosomally-encoded RpoD. The low level of expression in these three strains was equivalent to the basal level of transcription from the *luxI* promoter in the absence of LuxRΔN (data not shown).

Comparison of the expression levels of plasmid-derived GST-RpoD and chromosomal wildtype RpoD in each *E. coli* GS162λ*luxI-lacZ* / pBdgjΔN2 variant strain

Western immunoblot analysis was used to gain insight into the relative abundance of wildtype and variant GST-RpoD compared to wild-type RpoD to further investigate the unexpectedly low levels of β-galactosidase expression in the LuxRΔN-containing strains discussed above. Equivalent amounts of proteins from whole-cell extracts from all LuxRΔN-containing strains tested for β-galactosidase activity, were separated by SDS-PAGE, transferred to a nitrocellulose blot and analyzed with monoclonal antisera raised against RpoD. A colorimetric detection system allowed visualization of the protein bands on the blot (Figure 10). In addition to RpoD and GST-RpoD, the presence of a protein larger then RpoD but smaller then GST-RpoD was also detected by the monoclonal antibody (Mab) to RpoD and is assumed to be a

proteolytic cleavage product of GST-RpoD. The relative mobilities of RpoD and GST-RpoD did not represent the expected molecular weight of each protein (approximately 70 kDa and 96 kDa, respectively). A 'control' western immunoblot (Figure 11) comparing the relative mobilities of RpoD from commercially purified RNAP holoenzyme (Epicentre Technologies, Madison, WI), a laboratory stock of purified RpoD (A. Finney, unpublished result), and RpoD from cell extracts of *E. coli* GS162l*luxI-lacZ* containing LuxRΔN but lacking GST-RpoD, confirmed that the bands analyzed corresponded to RpoD and GST-RpoD (Figure 11).

Spot densitometry performed on the western immunoblots (Figure 10) was used to quantify the minimum ratio of the amount of GST-RpoD to RpoD from each strain. A graphic representation of this analysis is shown in Figure 12. The minimum ratios between the GST-RpoD to wildtype RpoD in the 19 different strains showed an overall variation from 1 to 4.7. However, all strains except for those containing GST-RpoD variants with substitutions at residues 590, 593, 605 and MA2 (residues 599,600, 603) exhibited ratios of GST-RpoD to σ^{70} above 2. Since equal amounts of cell extracts were separated by SDS-PAGE (data not shown) and the levels of RpoD detected by the Mab to RpoD were fairly consistent from one strain to the other, the variation in the abundance of the GST-RpoD compared to RpoD seems to be due to a difference in wild-type GST-RpoD and variant GST-RpoD expression and/or stability. Taken together, the western immunoblot analysis does indicate that all but one of the strains (MA2) which had an effect on *luxI-lacZ* expression (591, 595, 596, 597, 602, 603 and MA1), expressed more GST-RpoD than wildtype RpoD. However, the ratio of GST-RpoD to wildtype RpoD in these strains was comparable to that of the WT and to other strains which showed no effects on *luxI-lacZ* expression (608, 612 and 613). This suggests that the defects observed in the *in vivo* analysis, are specific to the amino acid substitution in the variant GST-RpoD tested.

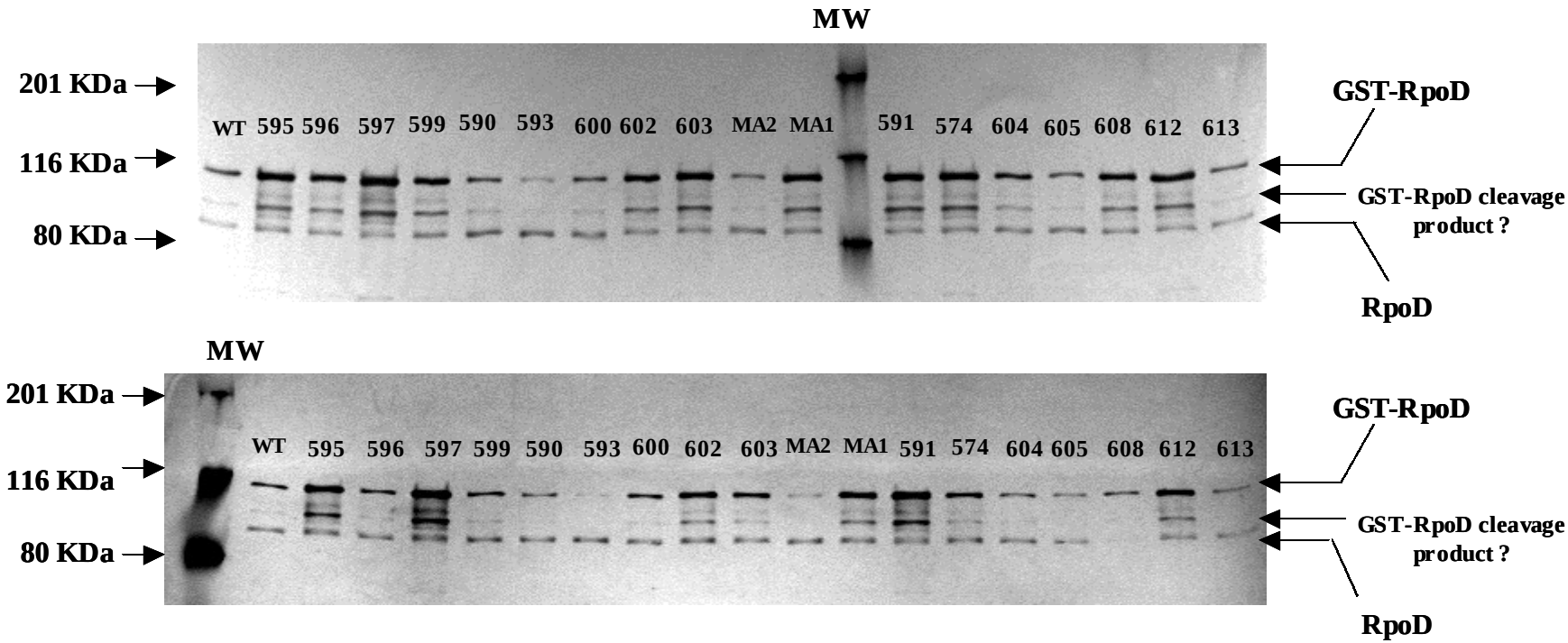

Figure 10: Western immunoblots to detect GST-RpoD and wildtype RpoD from cell extracts of LuxRΔN-containing strains used in β-galactosidase assays. The positions of the GST-RpoD and the wildtype RpoD bands are indicated by arrows on the right. The band representing the putative GST-RpoD proteolytic cleavage product is also indicated by an arrow. With the exception of the lanes showing wild-type GST-RpoD (WT) and the markings of the SDS-PAGE broad range molecular weight standard (MW), each lane is labeled at the top by the amino acid residue number in the GST-RpoD converted to alanine. The two triple variant GST-RpoD are represented by MA1 (residues 593, 596, 597) and MA2 (residues 599, 600, 603). The mobility of the molecular weight size standards (in kilodaltons) is indicated by arrows on the left. The use of equivalent amounts of total cell extracts (based on total protein content) was confirmed by SDS-PAGE. Results shown are representative of two independent analyses.

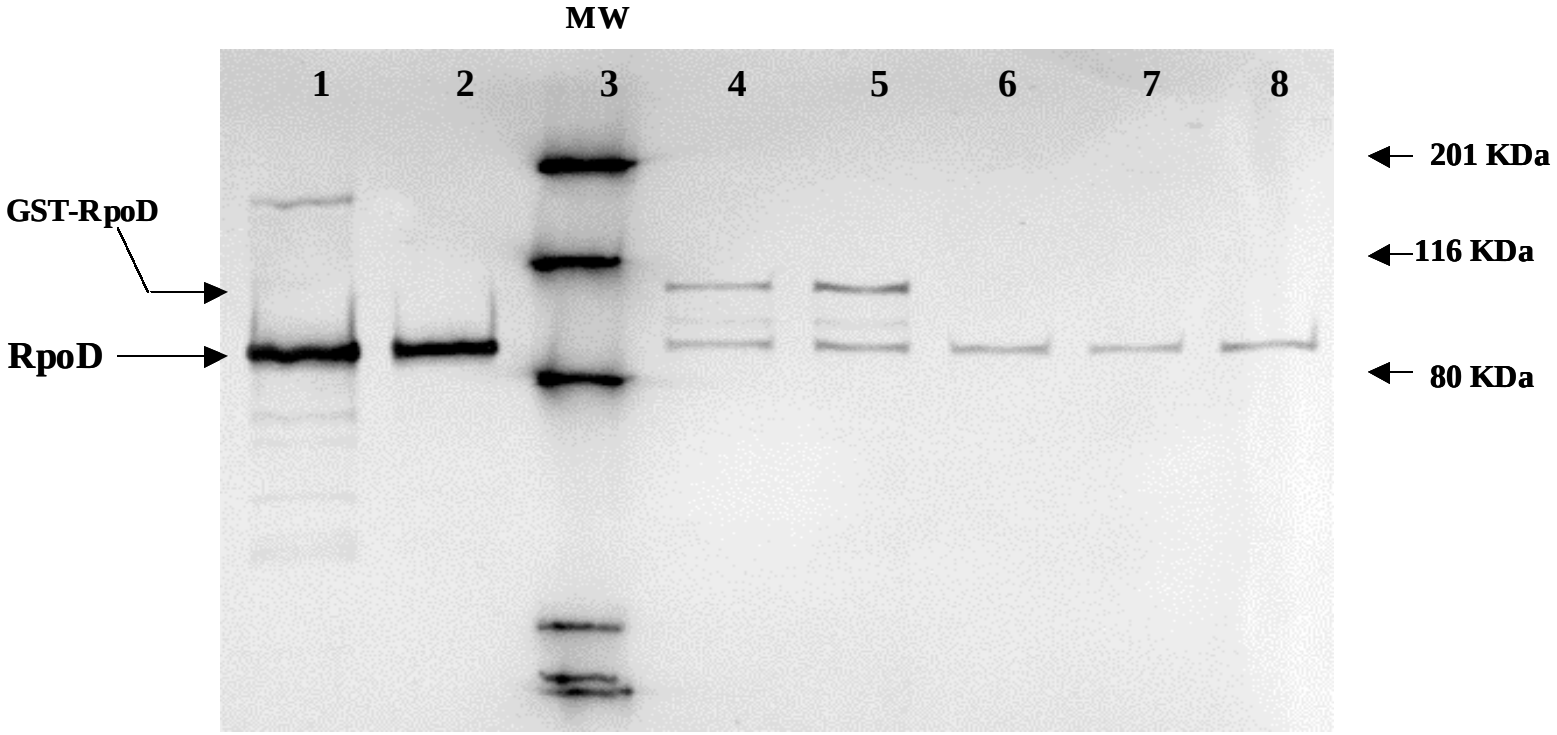

Figure 11: Western immunoblot comparing the mobility of RpoD and GST-RpoD relative to a molecular size standard. The positions of the GST-RpoD and the wildtype RpoD bands are indicated by arrows on the left. The mobility of the molecular weight size standards (in kilodaltons) is indicated by arrows on the right. **Lanes 1 and 2** show the strong reaction between the monoclonal anti-RpoD antibody and RpoD from commercially purified RNAP holoenzyme (Epicentre Technologies, Madison, WI) and a laboratory stock of purified RpoD (A. Finney, unpublished result), respectively. **Lane 3** shows the mobility of the Kaleidoscope Pre-stained Standards (Bio-Rad, Hercules, CA). **Lanes 4 and 5** show the anti-RpoD antibody reaction to cell extracts of *E. coli* GS162λ*luxI-lacZ* containing LuxRΔN and plasmid-borne wildtype GST-RpoD. **Lanes 6 and 7** represent the anti-RpoD antibody reaction to cell extracts of *E. coli* GS162λ*luxI-lacZ* containing *LuxR*ΔN only while **lane 8** represents cell extracts from *E. coli* GS162λ*luxI-lacZ* in the absence of both LuxRΔN and GST-RpoD.

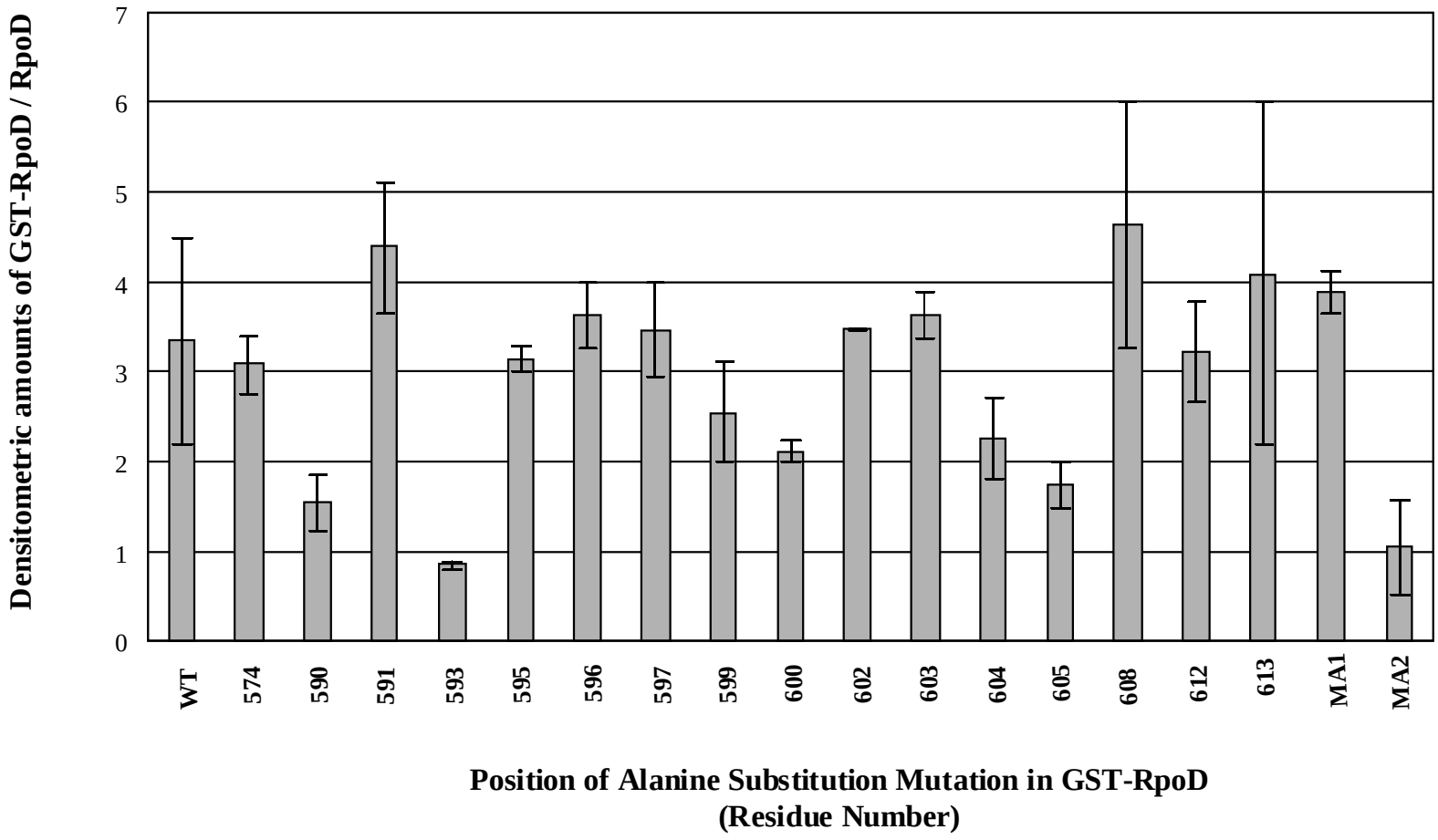

Figure 12: Comparison of the expression levels of plasmid-derived GST-RpoD and chromosomal wildtype RpoD in LuxRΔN-containing strains used in β-galactosidase assays. Spot densitometry of western immunoblots (Figure 11) was used to quantify the amount of GST- RpoD and wildtype RpoD expressed from each strain. Data are presented as the average ratio of GST- RpoD and wildtype RpoD. The two triple variant GST-RpoD are represented by MA1 (residues 593, 596, 597) and MA2 (residues 599, 600, 603).

Effects of changes in the σ^{70} subunit on LuxRΔN-dependent *in vitro luxI* expression

To more directly examine the effects of the σ^{70} variants on *luxI* transcriptional activation, *in vitro* single-round transcription assays were performed on linear template DNA containing the *luxI* promoter, with and without LuxRΔN, and in the presence of purified RNA polymerase reconstituted either with wild-type or variant GST-RpoD preparations (Figure 13). Only LuxRΔN was used for *in vitro* analysis since LuxR has not been purified to date. Transcription from the LuxRΔN-independent *lacUV5* promoter was used to measure the RNAP activities of the reconstituted enzymes. The amount of *luxI* transcript (normalized to the *lacUV5* transcript) for each RNAP with variant GST-σ^{70} subunit was analyzed relative to the equivalent value for the RNAP with GST-σ^{70} wildtype subunit and a graphic representation of this data is displayed in Figure 14B. RNA polymerase containing the GST-σ^{70} subunit with an alanine substitution at residue 597 showed the largest decrease in *luxI* transcription (70% of WT) while single substitutions at residues 591 and 593 and the triple substitution at residues 591, 596 and 597 (MA1) resulted in more modest decreases: 84% WT, 82% WT and 86% WT respectively. Modest increases of *luxI* transcription were observed with GST-RpoD variants 600 (114% WT), 603 (113%) and MA2 (599, 600 and 603) (115% WT). Residue 595 showed considerable variation in its effect on *luxI* transcription with a decrease (76% WT) in one trial and an increase (124% WT) in the second independent trial. The remaining GST-RpoD variants exhibited little or no defect in *luxI* transcription compared to wild-type.

A correlation seems to exist between the effects of substituted residues 591 and 597 on LuxRΔN-dependent *luxI-lacZ* expression *in vivo* and *luxI* transcription *in vitro* (Figures 14A and 14B). In both analyses, these substitutions result in a reduction of *luxI* expression compared to

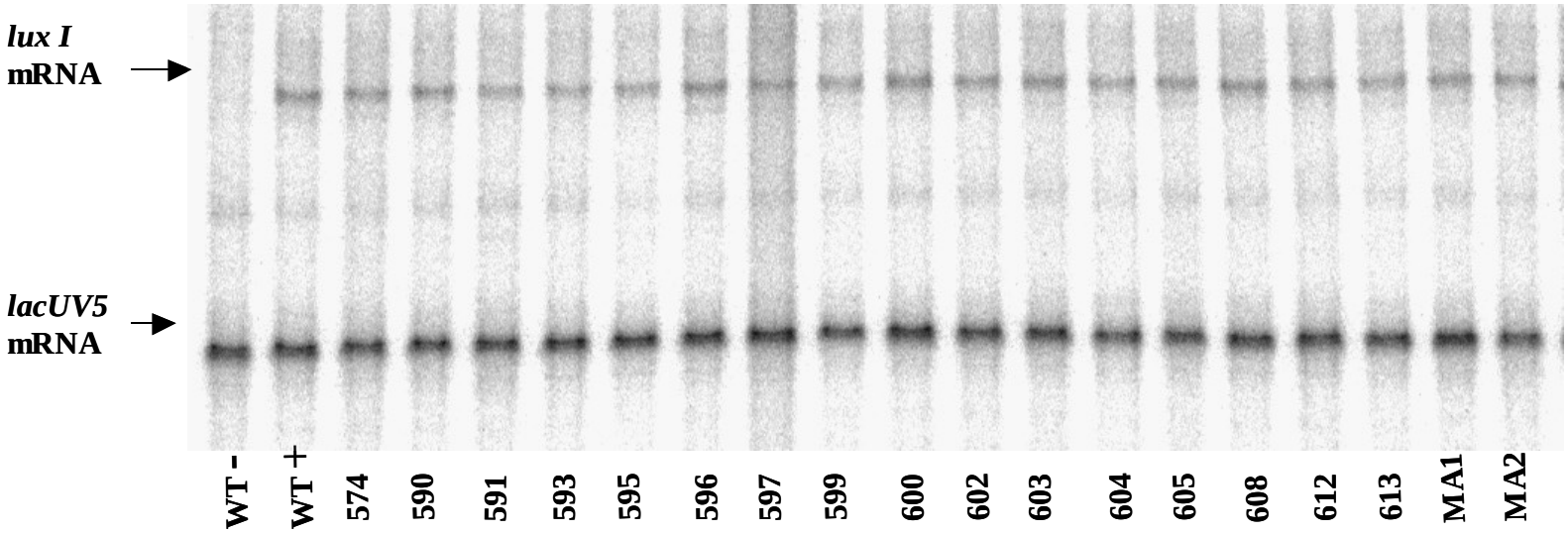

Figure 13: *In vitro* transcription of the *luxI* and *lacUV5* promoters by wild-type (WT) RNAP and variant RNAP's. Reactions were carried out in the presence of 2 µM LuxRΔN (except for the negative control in lane 1). Lanes 1 and 2 contain mRNA produced by core RNAP and wildtype GST-RpoD in the absence (-) and presence (+) of LuxRΔN, as labeled. The remaining lanes, labeled by the amino acid residue number in the RpoD converted to alanine, contain mRNA produced by variant RNAPs in the presence of LuxRΔN. The two triple variant GST-RpoD are represented by MA1 (residues 593, 596, 597) and MA2 (residues 599, 600, 603). The *lacUV5* promoter served as an internal control. Visualization and quantitation of radiolabeled transcripts was performed using a Storm phosphoimager and the software ImageQuant, v3.3. Results shown are representative of two independent assays.

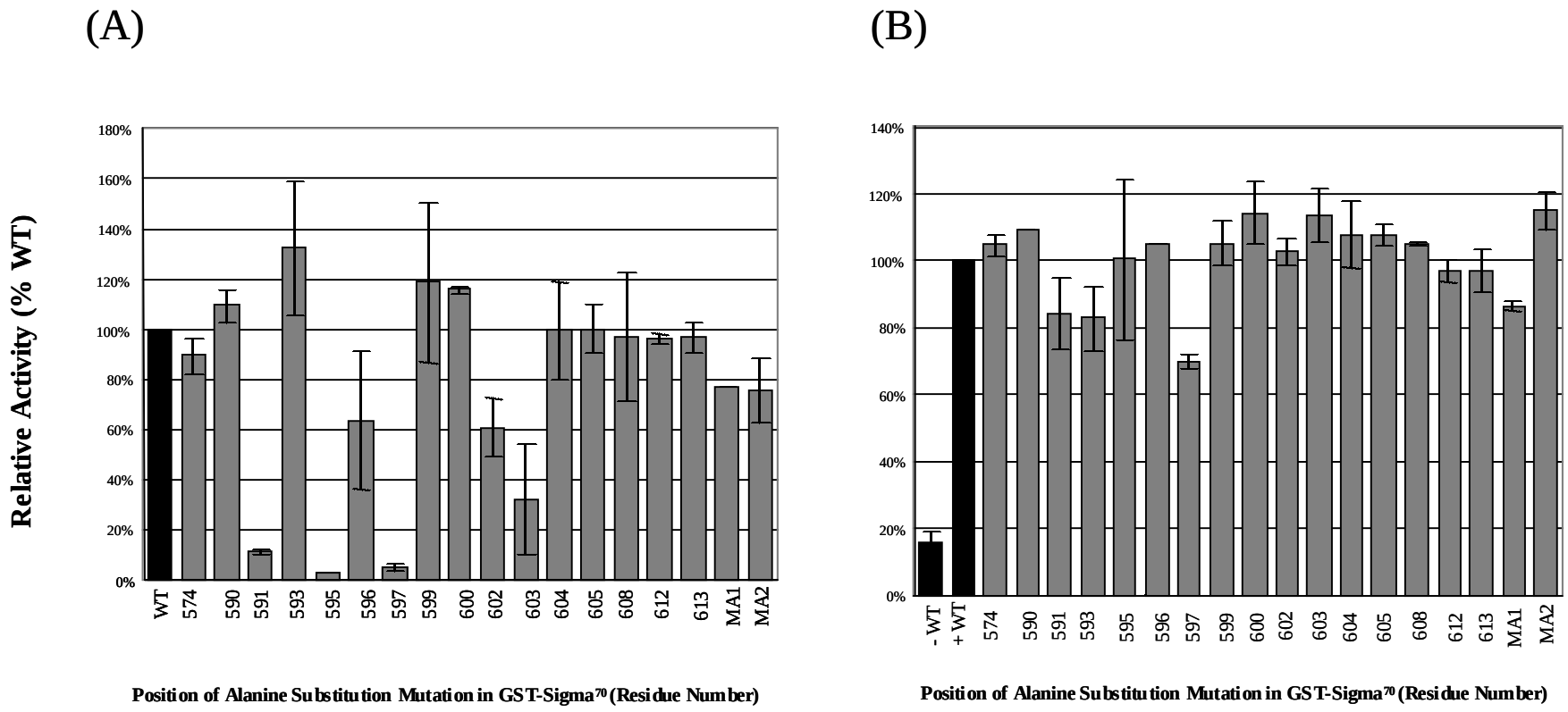

Figure 14: Comparison between (A) *in vivo* β-galactosidase and (B) *in vitro* transcription assays results. (A) Effect of alanine substitutions in RpoD on LuxRΔN-dependent *luxI-lacZ* expression in recombinant *E. coli* GS162λ *luxI-lacZ*. See Figure 9 for details. (B) Effect of alanine substitutions in GST-RpoD on LuxRΔN-dependent *in vitro* transcription from the *luxI* promoter. The amount of *luxI* mRNA normalized to the amount of *lacUV5* mRNA for each sigma variant is divided by the equivalent expression for wildtype GST-RpoD (0.325 ± 0.001) and is presented as a percentage of WT activity. The bars represent the average of two independent assays and the error bars show the range on either side of the mean.

wild-type although the magnitude of the *in vitro* defects are not as great as those observed *in vivo*. Some GST-RpoD variants (in particular those with substitutions at residues 593, 595, 596, 602 and 603) failed to show a good correlation between the *in vitro* and *in vivo* analyses. Differences in plasmid maintenance and stability in the two-plasmid *in vivo* system used may account for these discrepancies. Due to the inherent problems of the *in vivo* system, the *in vitro* transcription data is considered to be more reliable

Effects of a carboxy-truncated σ^{70} subunit (σ^{70}-529) on LuxRΔN-dependent *in vitro luxI* expression

Only substitutions at residues 591and 597 of σ^{70} showed consistent effects on LuxRΔN-dependent transcription *in vivo* and *in vitro* and the *in vitro* effects were not dramatic. Therefore, it is possible that region 4 of σ^{70} either does not play an essential role in LuxRΔN-dependent transcriptional activation at the *luxI* promoter with no one individual amino acid being critical for activation or that other amino acid residues or regions within σ^{70} play a more significant role. To examine this possibility, single round transcription assays were performed with RNAP containing a truncated σ^{70} subunit (σ^{70}-529), which lacks amino acids 529 – 613 and therefore has lost about four amino acids from region 3.2, as well as all of region 4 (Kumar *et al*., 1994). Lacking region 4.2 and, therefore, the –35 promoter recognition element, σ^{70}-529 does not allow significant initiation *in vitro* at normal, factor-independent promoters like *lacUV5*. However, σ^{70}-529 will initiate transcription from a synthetic promoter, like P_{RE}# (a constitutive derivative of the λ P_{RE} promoter) which has a perfect extended –10 consensus sequence and which served as a suitable internal control for this assay (Kumar *et al*., 1993). Figure 15 shows the results of transcription from the *luxI* promoter in response to wild-type σ^{70} RNAP and truncated σ^{70}-529

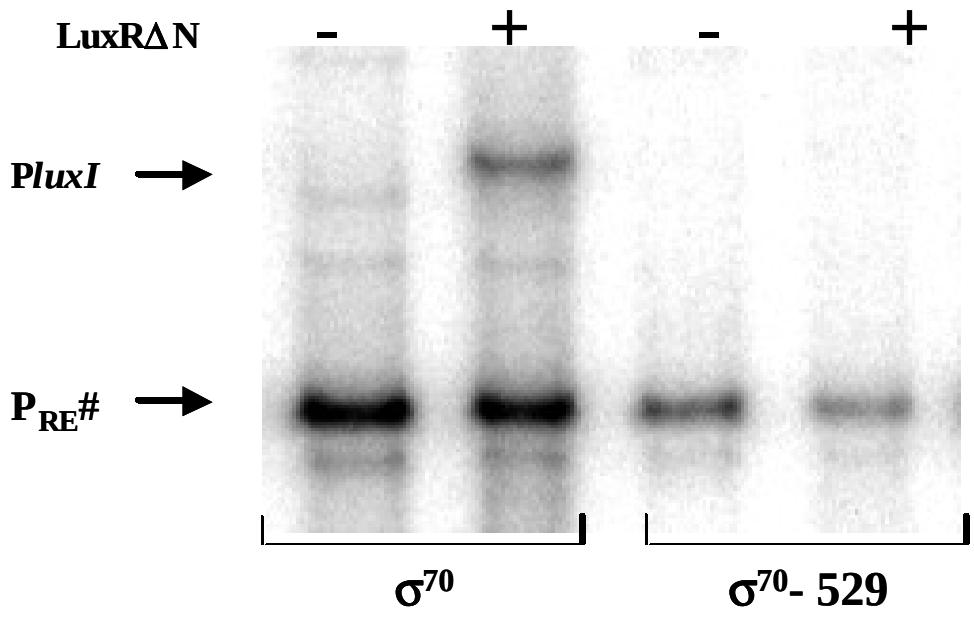

Figure 15: Transcription from the *luxI* promoter in response to wild-type σ^{70} RNAP and truncated σ^{70}-529 RNAP in the absence (-) and presence (+) LuxRΔN (10 μM). Single round transcription reactions were performed *in vitro* as described in the text. The minus 35-independent (extended –10) promoter P_{RE}# served as an internal control. The normal or truncated σ^{70} present in the holoenzyme used is shown below the tracks and the positions of the P_{RE}#- and P*luxI*-generated transcripts are marked with arrows on the left. Visualization and quantitation of radiolabeled transcripts was performed using a Storm phosphoimager and the software ImageQuant, v3.3. Results shown are representative of two independent assays.

RNAP in the absence (-) and presence (+) LuxRΔN. The truncated σ^{70}-529 RNAP did not allow transcription activation from the *luxI* promoter fragment indicating that the region 4 of σ^{70} is necessary for LuxRΔN-dependent *in vitro* transcription at the *luxI* promoter. Overexposure of the radiolabeled transcripts and control bands on the Molecular Dynamics phosphorimager confirmed the lack of a *luxI* transcript in the presence of LuxRΔN and σ^{70}-529 RNAP. In agreement with observations from previous studies (Kumar *et al.*, 1993; Kumar *et al*., 1994) the extended–10 promoter, P_{RE}#, was only transcribed about 20% as efficiently by the variant holoenzyme as by the normal holoenzyme. This decreased level of transcription is thought to be associated with the decreased binding of the variant σ^{70} to core enzyme. In fact, residues within region 4 of σ^{70} have been suggested to play a role in the interaction of σ^{70} with core (Sharp *et al*., 1999).

CHAPTER FOUR

Discussion

During transcriptional activation of the *luxI* (or *lux* operon) promoter from *Vibrio fischeri*, the LuxR-autoinducer complex is thought to bind to a DNA site known as the *lux* box (centred at position –42.5 bp relative to the *luxI* transcriptional start site) and initiate transcription through multiple contacts with RNAP (Figure 4; Egland & Greenberg, 1999; Stevens & Greenberg, 1999; Egland & Greenberg, 2000). In this study, alanine substitution mutations at 16 amino acid residues within region 4.2 of the σ^{70} subunit of *E .coli* RNA polymerase have been analyzed, *in vivo* and *in vitro*, for their effects on LuxR- and LuxRΔN-dependent transcription at the *luxI* promoter. Several amino acid residues within this region of σ^{70} have been shown to be necessary for the function of numerous transcriptional activators which bind near the –35 element of their respective promoters (Figure 6; Makino *et al*., 1993; Li *et al*., 1994; Lonetto *et al*., 1998; Landini *et al*., 1998; Rhodius & Busby, 2000; Oleknovich & Kadner, 1999; Landini & Busby, 1999; Bhende & Egan, 2000; Minakhin *et al*., 2001, Jishage *et al*., 2001; Dove & Hochschild, 2001). Due to its ease of manipulation compared to *V. fischeri*, the current analysis was performed using the heterologous system of *E. coli*. Region 4.2 of σ^{70} represents one of the most highly conserved regions within the σ^{70} family of proteins (Lonetto et al., 1992). Although the full genomic sequence of *Vibrio fischeri* is currently unavailable, an amino acid sequence alignment between RpoD from *Vibrio cholerae* and *E. coli* RpoD (data not shown) supports this fact. *E. coli* RpoD (a 613 amino acid protein) and *V . cholerae* RpoD (a 625 amino acid protein) show sequence identity >90% throughout region 4 and 100% identity between residues 572-612 of *E. coli* RpoD and residues 584-624 of *V. cholera* RpoD. Such

homology lends support to the value of analyzing this region of σ^{70} in attempting to understand the involvement of RpoD in LuxR-dependent transcriptional activation at the *luxI* promoter.

The *in vivo* analysis conducted in the present study resulted in the identification of at least 6 amino acid residues in σ^{70} which when substituted for alanine, seem to play a role in LuxRΔN-dependent *luxI* expression (residues E591, L595, R596, K597, S602 and R603). Interestingly, the substitution of residue K597A in the triple variant MA1 and residue R603A in the triple variant MA2, seemed to also result in defects in LuxRΔN-dependent *luxI* expression, although the magnitude of these defects were less dramatic. This suggests that the other substitutions in these triple variants somehow suppress the negative effects of K597A and R603A. Alternatively, the triple substitution as a whole may result in a conformational change that reduces the ability of this variant GST-RpoD to form a functional complex with core RNAP or *luxI* promoter DNA. In such a case, β-galactosidase activity for these triple substitution GST-RpoD variants would be expected to be higher then for a single substitution GST-RpoD variant specifically defective in transcriptional activation, because the triple variants would not compete successfully with wildtype RpoD for incorporation into RNAP holoenzyme. However, none of these residues showed similar defects on LuxR-dependent *luxI* expression. Rather, substituted σ^{70} residues S602A and R603A, seemed to slightly enhance LuxR-dependent *luxI* expression in the recombinant *E. coli* strain analyzed.

Alanine substitutions for L595, R596, K597 and R603 have been shown to affect transcriptional activation by a variety of proteins that bind near the –35 element. Specifically, activation of the *pmelRcon* promoter by CRP $AR3^{+}$ was reduced by alanine substitutions at σ^{70} residues K593, K597, and R599 both *in vivo* and *in vitro* (Lonetto *et al*., 1998; Rhodius & Busby, 2000). Through the application of suppressor genetics, residue R596A was found to

enhance activation of the *pmelRcon* promoter in the presence of a positive control variant of CRP AR3$^+$, suggesting the direct involvement of this residue in protein-protein interactions between σ^{70} and CRP AR3$^+$ (Rhodius & Busby, 2000). Activation of the *alkA* promoter by the methylated form of the Ada protein (^{me}Ada) was also reduced by σ^{70} variants K593A, K597A, and R603A, both *in vitro* and *in vivo* (Landini & Busby, 1999). Similarly, FNR-dependent transcription of the anaerobically-induced p*dms*A promoter was hindered, *in vivo* and *in vitro*, by σ^{70} variants K593A, R596A, K597A and R603A (Lonetto *et al*., 1998). While substitution of residue E591L of σ^{70} has been shown to have moderate effects on the activation of the *araBAD* promoter by AraC (Lonetto *et al*., 1998), residue L595 has not been so far been found to be defective for transcription at other Class II-type promoters.

The severity of the decrease observed in LuxRΔN-dependent *luxI-lacZ* expression in the presence of variant GST-RpoD 591, 595 and 599 was surprising. Only 50% of the RNAP holoenzyme (holoRNAP) in all the strains analyzed was expected to contain the GST-RpoD variant, with the remaining holoRNAP containing wild-type, chromosomally encoded σ^{70}. This assumed 1:1 ratio was based on previous reports which indicated that the level of plasmid-encoded GST-RpoD (uninduced) was approximately that of chromosomal RpoD in the haploid cell (Lonetto *et al*., 1998; Bhende & Egan, 2000). A reduction in β-galactosidase activity below 50% (relative to wild-type GST-RpoD) therefore suggested that the specific amino acid substitution in the variant σ^{70}subunit was either affecting the ability of the activator to reach the *luxI* promoter or indirectly disrupting some other aspect of β-galactosidase expression or LuxRΔN expression. However, if the amount of the plasmid-derived GST-RpoD (uninduced) compared to chromosomal RpoD was above the expected 1:1 ratio, these large decreases in β-galactosidase activity would more likely represent direct transcriptional activation defects due to

the amino acid substitution in the σ^{70}subunit. The use of western immunoblotting to analyze the ratio of the amount GST-σ^{70} to wild-type σ^{70} in each recombinant strain served to address the validity of this assumption. The results show that in the *E. coli* strain used for this study, contrary to previous findings, the amount of GST-σ^{70} available in all but two of the 19 strains analyzed *in vivo*, was at least double that of wild-type-σ^{70}. In numerous cases the GST-σ^{70} to wild-type-σ^{70} ratio was above 3 (Figure 12). The GST-fused RpoD proteins have been found to have a reduced affinity for core RNAP, and do not compete very well with the wild-type σ^{70} (W. Ross, personal communication). However, with ratios of GST-σ^{70} to wild-type σ^{70} above 2, the problem of reduced affinity may be overcome and the majority of the holoRNAP available in these strains may contain the variant GST-σ^{70} subunit rather then the wild-type σ^{70} subunit. Although comparing the amount of GST-σ^{70} to wild-type -σ^{70} does not provide direct evidence regarding the amount of GST-σ^{70} that is actually incorporated into functional holoRNAP in these strains, it does make it more likely that the observed defects in LuxRΔN-dependent *luxI-lacZ* expression with these six variant GST-σ^{70} subunits are due to the specific alanine substitutions involved.

In vitro transcription assays served as a means to confirm if the six variant GST-σ^{70} subunits defective in LuxRΔN-dependent *luxI-lacZ* expression were the result of specific defects in transcriptional activation at this promoter, or the result of more general transcription defects in the strains analyzed. Residues E591A and K597A had reduced LuxRΔN-dependent *luxI* expression *in vitro* and, therefore, correlated with the observed effects of these substitutions on LuxRΔN-dependent *luxI* expression *in vivo* (Figure 14A and 14B). Triple substitution MA1 (593, 596, 597) also exhibited a slight defect in *in vitro luxI* transcription, however as observed

with the *in vivo* analysis, the effects of residue K597A seemed to be somewhat suppressed in the presence of alanine substitutions at residues K593 and R596. Interestingly, the single substitution of residue K593A did exhibit a small defect in *in vitro luxI* transcription that was not observed *in vivo*, although this substitution did not seem to have a "compounding effect" on the defect observed with MA1. Substitution of residues L595, R596, S602 and R603 exhibited little or no defect or enhancement in *luxI* transcription *in vitro*, suggesting that the defects observed *in vivo* with LuxRΔN, represented a more general defect on transcription in the strains analyzed during the *in vivo* assays.

Establishing a suitable *in vivo* system to study the effects of the variant GST-fused RpoD proteins proved difficult, possibly owing to the overall importance of σ^{70} in transcription. Overexpression of σ^{70} is toxic and may account for the presence of a possible GST-σ^{70} cleavage product that reacted with the monoclonal anti-σ^{70} antibody and that seemed to be predominantly associated with strains containing a relatively high GST-σ^{70} to wild-type σ^{70} ratio (Figure 10 and 12). The variations observed in the amounts of GST-σ^{70} expressed by a single strain between one trial and another (Figure 12: WT, 591, 608, 612) does suggest that either the wild-type and variant GST-RpoD proteins or the plasmids encoding them, are not particularly stable in these strains. It is possible that these plasmids or GST-RpoD proteins are also differentially unstable in the presence of pBdgjΔN2. The effects of residue K593A on *in vivo* LuxRΔN-dependent *luxI* expression may have been 'masked' due to the lower amount of GST-σ^{70} available in this strain compared to wild-type σ^{70} (Figure 12). Unfortunately, it was not possible to conduct *in vivo* assays in strains in which expression of the chromosomal *rpoD* can be repressed allowing reduced competition with the plasmid-borne, GST-RpoD variants (see Results section).

Taken together the *in vivo* and *in vitro* analysis has highlighted two residues (E591 and K597) in the σ^{70} subunit which seem to play a role in LuxRΔN-dependent *luxI* expression. Both residues constitute charged amino acid residues that are likely to be surface exposed and therefore available to interact with transcriptional activators that bind close to the –35 promoter element. As described above, both residues have been found to be involved in transcriptional activation at other Class II promoters, specifically involving the transcriptional activators AraC (E591) and CRP, FNR and ^{me}Ada (K597). However, the defects displayed by these two substitutions were not dramatic *in vitro* and neither exhibited defects in LuxR-dependent *luxI* transcription *in vivo*. This raises two possibilities that are in contrast to the overall requirement of region 4 of σ^{70} by other transcriptional activators: (i) no one residue in this region is critical by itself or (ii) this region does not play an essential role in LuxR-dependent transcriptional activation at the *lux* operon. The importance of region 4 as a whole, on LuxRΔN-dependent *luxI* expression was confirmed with *in vitro* transcription assays conducted with σ^{70}-529 RNAP holoenzyme, which lacks amino acids 529-613 of the σ^{70}subunit and therefore is devoid of region 4. The failure of this variant holoRNAP to allow any transcription at the *luxI* promoter strongly indicates that at least LuxRΔN requires region 4 of the σ^{70}subunit for efficient transcriptional activation at this promoter. Based on the *in vitro* analysis performed with σ^{70}-529, it appears that the *luxI* promoter cannot serve as a –10 extended promoter despite the fact that the *luxI* promoter carries a –10 consensus sequence that deviates from a perfect –10 'extended' consensus sequence at only one position (Figure 7; Keilty & Rosenberg, 1997). Deletion of region 4 (and therefore the –35 promoter binding region) of the σ^{70} subunit has been shown be inconsequential to transcription at factor-independent promoters which do not contain a –35 consensus sequence but do have a perfect –10 'extended' consensus sequence (Kumar *et*

al., 1993; Kumar *et al*., 1994). Crude quantitative estimates (data not shown), comparing the amount of LuxRΔN-independent or basal *luxI* transcription in the presence of σ^{70}-529 holoRNAP and the wild-type σ^{70} holoRNAP, show a 2- to 5-fold decrease in basal *luxI* transcription with the variant holoRNAP that is not observed at the pRE# control promoter. It is possible therefore, that despite the presence of a 'near perfect' –10 extended consensus sequence, transcription at the *luxI* promoter requires some kind of direct or activator-mediated interaction between the promoter and region 4 of σ^{70}.

Conclusions

Region 4 of the σ^{70} subunit of RNAP is necessary for LuxRΔN-dependent *luxI* transcription *in vitro.* Amino acid residues E591 and K597 within region 4.2 seem to play a role in LuxRΔN-dependent *luxI* transcription *in vitro* and *in vivo* although neither showed any effects in LuxR-dependent *luxI* transcription *in vivo*. Since LuxRΔN is likely to require more contacts with RNAP then LuxR due to its inability to bind the *luxI* promoter independently, it is possible that residues E591 and K597 are involved in facilitating the binding of the LuxRΔN-RNAP complex to the *luxI* promoter. These residues highlight a part of region 4.2 of σ^{70} that is most likely to be in closest proximity to LuxR during transcriptional activation of the *luxI* promoter. Furthermore, due to the complications encountered with the *in vivo* system, the requirement of these residues in LuxR-dependent transcriptional activation cannot be completely ruled out. The 18 alanine substituted amino acid residues analyzed in this study represent a relatively small portion of region 4.2 (Figure 6) and it is possible that other residues within this region play a more significant role or that unlike other ambidextrous transcriptional activators like CRP and

FNR, LuxR does not rely on any one single amino acid in this region to efficiently activate transcription at the *lux* operon.

CHAPTER FIVE

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References

Available upon request