

Role of the C-terminal domain of the α subunit of RNA polymerase in transcriptional activation of the *lux* operon during quorum sensing

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
in
Biology

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December 15, 2000
Blacksburg, Virginia

Keywords: *Vibrio fischeri*, quorum sensing, LuxR, RNA polymerase, alpha subunit,
transcriptional activation, luminescence, DNA binding
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ABSTRACT

Quorum sensing in Gram-negative bacteria is best understood in the bioluminescent marine microorganism, *Vibrio fischeri*. In *V. fischeri*, the luminescence or *lux* genes are regulated in a cell density-dependent manner by the activator LuxR in the presence of an acylated homoserine lactone autoinducer molecule (3-oxo-hexanoyl homoserine lactone). LuxR, which binds to the *lux* operon promoter at position -42.5 , is thought to function as an ambidextrous activator making multiple contacts with RNA polymerase (RNAP). The specific role of the CTD of RNAP in LuxR-dependent transcriptional activation of the *lux* operon promoter has been investigated. The effect of seventy alanine substitution variants of the α subunit was determined *in vivo* by measuring the rate of transcription of the *lux* operon via luciferase assays in recombinant *Escherichia coli*. The mutant RNAPs from strains exhibiting at least two fold increased or decreased activity in comparison to the wild-type were further examined by *in vitro* assays. Since full-length LuxR has not been purified to date, an autoinducer-independent N-terminal truncated form of LuxR, LuxR N, was used for *in vitro* studies. Single-round transcription assays were performed using reconstituted mutant RNAPs in the presence of LuxR N, and fourteen residues in the CTD were identified as having negative effects on the rate of transcription from the *lux* operon promoter. Five of these fourteen residues were also involved in the mechanism of both LuxR and LuxR N-dependent activation *in vivo* and were chosen for further analysis by DNA mobility shift assays. Results from these assays indicate that while the wild-type CTD is capable of interacting with the *lux* DNA fragment tested, all five of the variant

forms of the CTD tested appear to be deficient in their ability to recognize and bind the DNA. These findings suggest that CTD-DNA interactions may play a role in LuxR-dependent transcriptional activation of the *lux* operon during quorum sensing.

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ACKNOWLEDGEMENTS

I would like to thank A. Ishihama for providing purified mutant RNAPs and R. L. Gourse and R. H. Ebricht for providing strains and protocols. I would also like to recognize Tamas Gaal for his assistance throughout my entire project and Robert Blick for constructing the three-plasmid strains used in assays.

I also want to thank my advisor, Ann Stevens for all of her support, patience and encouragement during my three and a half years in the lab. Thanks for taking a chance on me and being a great mentor. I couldn't have picked a better advisor!

Also, thank you to the following people in the Stevens lab: Rob Blick, Melissa Fouratt, Debbie Johnson, Jen Meador-Parton, Guy Townsend, and Amy Trott for all their company, friendship and help during the years. I never would have made it without all of you!!

To my committee members: David Popham and Charles Rutherford, thank you for all of your help and suggestions. It was so nice having such a helpful and optimistic committee.

Last but not least, I want to thank my family for their constant love and support. I am so lucky to have all of you in my life!!

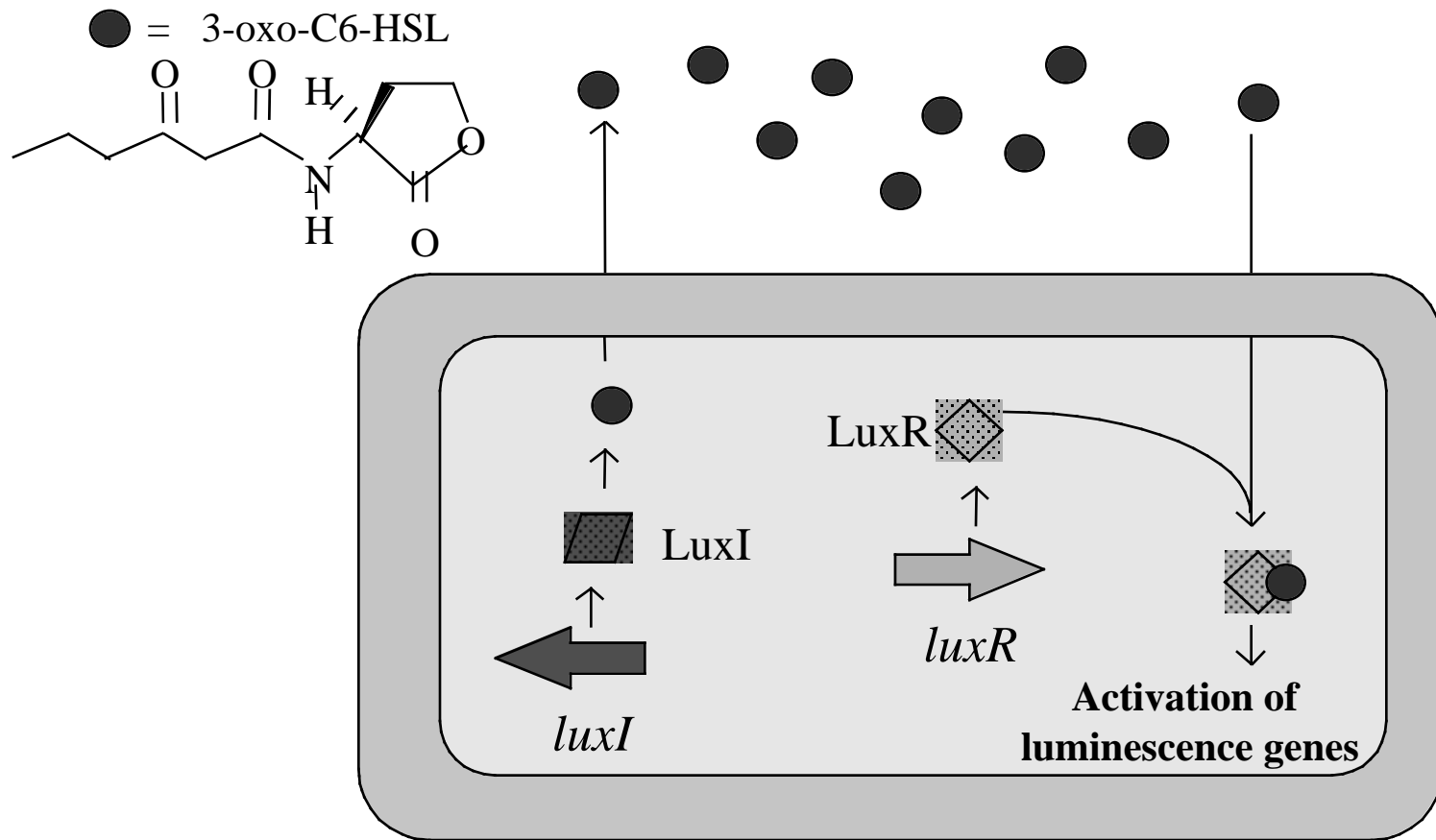
CHAPTER ONE

Literature Review

Quorum sensing in *Vibrio fischeri*

Many species of bacteria regulate gene expression in a cell density-dependent manner. This phenomenon is known as quorum sensing. Only when an intercellular signaling molecule, commonly termed autoinducer (AI), accumulates to a certain level will the expression of particular subsets of genes be activated. The bacteria produce, as well as respond to, the autoinducer and by this mechanism can sense their surroundings (for reviews, see Dunlap and Greenberg, 1991; Fuqua *et al.*, 1994; Fuqua *et al.*, 1996). In Gram-negative organisms, the autoinducer is an acyl-homoserine lactone with slight differences existing in the acyl side chain from species to species (Fuqua *et al.*, 1996). The best understood model for quorum sensing in Gram-negative bacteria is the regulation of the luminescence (*lux*) genes in *Vibrio fischeri*. This system is regulated by the autoinducer molecule, N-(3-oxohexanoyl) homoserine (3-oxo-C6-HSL) lactone (Eberhard *et al.*, 1981; Figure 1).

V. fischeri can be isolated as a pure culture from the light organs of the Japanese pinecone fish (*Monocentris japonica*) and the Hawaiian squid (*Euprymna scolopes*). These symbiotic relationships allow *V. fischeri* to grow to high densities in a nutrient-rich environment, and in turn, the bacteria provide light to these sea creatures for various behavioral activities such as feeding, mating, and counter illumination. Light production is very energy expensive to the bacterial cells, therefore expression of these genes is tightly controlled. Light emission occurs only at high cell densities within the light organs of animal hosts and not in the low nutrient/low cell density environment of open sea water (for reviews, see McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). Luminescence in *V. fischeri* is catalyzed by the enzyme luciferase, which is a mixed function oxidase. In the chemical reaction that generates light, luciferase oxidizes a reduced flavin and a long-



2

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

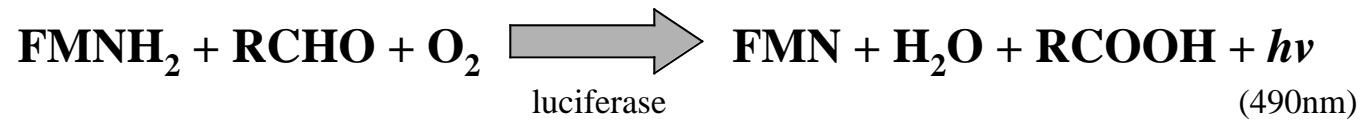
chain aldehyde (tetradecanal), producing oxidized flavin and a long chain fatty acid (tetradecanoic acid) (Rosson, R. A. and Nealson, K. H., 1981; Figure 2).

The *lux* operon, necessary for light generation, is composed of seven genes (*luxICDABEG*) (Engebrecht and Silverman, 1983). The first gene of the operon, *luxI*, encodes an autoinducer synthase. The remaining genes in the operon encode the proteins necessary for luminescence; *luxA* and *luxB* code for the α and β subunits of luciferase and *luxC*, *luxD* and *luxE* encode components of the fatty acid reductase complex needed for synthesis of the aldehyde substrate for luciferase. However, the function of *luxG* is still unknown at this time (Engebrecht and Silverman, 1984). Another essential gene involved in quorum sensing is *luxR* which is located in a separate transcriptional unit (Figure 3). The *lux* operon and *luxR* are separated by 155 base pairs (bp) and are divergently transcribed. The *luxR* gene codes for LuxR, the autoinducer-dependent transcriptional activator of the luminescence (*lux* operon) genes.

The regulatory protein, LuxR

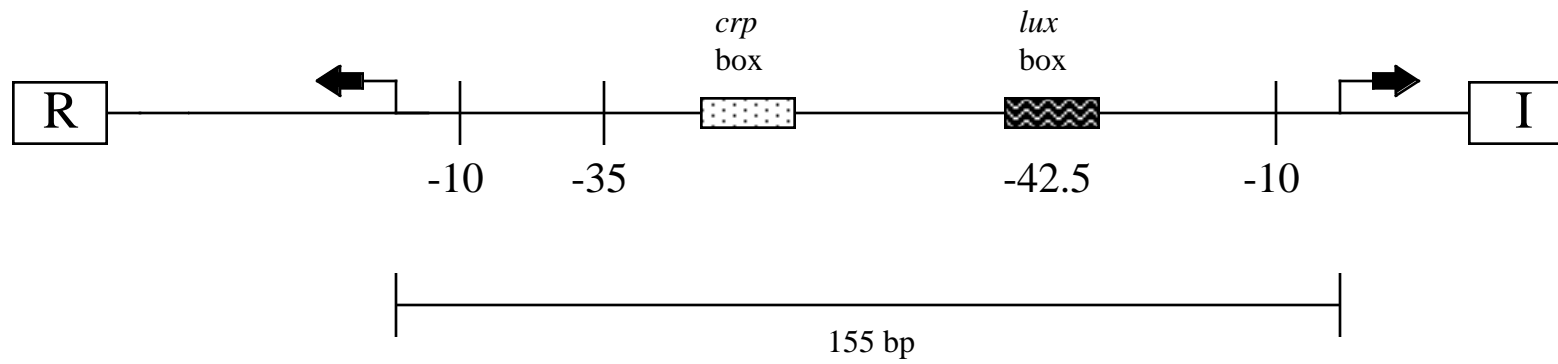
LuxR is a two domain polypeptide containing regulatory and activation regions (Figure 4). It consists of 250 amino acid residues. The N-terminal domain is involved in autoinducer binding and modulates the activity of the C-terminal domain (CTD) which is the activation region (Choi and Greenberg, 1991; Choi and Greenberg, 1992a). The CTD of LuxR has a helix-turn-helix motif that suggests involvement in DNA binding (Henikoff *et al.*, 1990). The location of the helix-turn-helix (HTH) motif of NarL, a member of the FixJ-LuxR family of transcriptional activators, has been resolved by crystal structure analysis (Baikalov *et al.*, 1996). Consequently, the HTH motif of LuxR is likely to be located between residues 200 and 224 (as opposed to 196-210, Choi and Greenberg, 1992a).

LuxR binds to a region of the DNA termed the *lux* box, which is 20 bp in length with a dyad symmetry (Devine *et al.*, 1989; Egland *et al.*, 2000). The *lux* box is centered



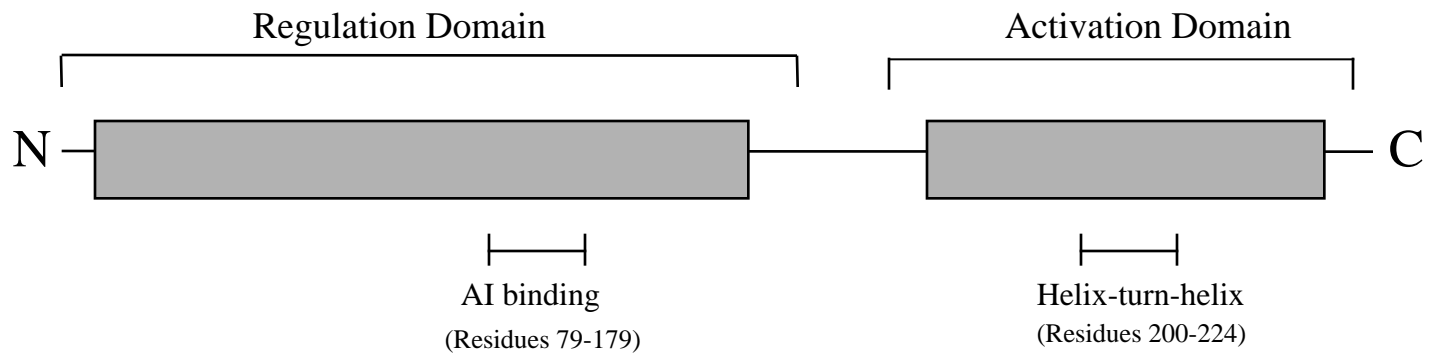
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Figure 2: Reaction catalyzed by luciferase



5

Figure 3: Model of the intergenic region between *luxR* and the *lux* operon (diagram not drawn to scale). See text for details.



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Figure 4: Model of the functional regions of LuxR. See text for details.

at the -42.5 position relative to the *luxI* transcription start site (Egland, K. A. and Greenberg, E. P., 1999). At the present time, efforts to purify full-length LuxR have been unsuccessful for a variety of reasons. Therefore, a N-terminally truncated form of LuxR, LuxR^N, which activates transcription independent of an autoinducer is used for *in vitro* studies (Choi and Greenberg, 1991). LuxR^N exists as a monomer in solution and binds the *lux* box and *luxI* promoter cooperatively with RNA polymerase (RNAP) as has been demonstrated through DNase I footprinting analysis (Stevens *et al.*, 1994). However, there is some evidence to suggest that LuxR functions as a homodimer and can bind to the *lux* box independently of RNAP (Choi and Greenberg, 1992b). Furthermore, it has been shown through genetic analysis that LuxR is able to bind specifically to the *lux* box in an artificial *lacZ* promoter (Egland *et al.*, 2000) and the LuxR homologs TraR and ExpR, which have been purified, have also been shown to bind to the DNA independently of RNA polymerase (Reverchon *et al.*, 1998; Zhu *et al.*, 1999).

RNA polymerase

Escherichia coli RNAP is composed of 5 subunits: α , α' , β , β' and ω . α interacts with promoter elements at positions -10 and -35 with respect to the transcriptional start site (Dombroski *et al.*, 1992). Each subunit has two separately folded domains; the N-terminal domain (NTD) and the C-terminal domain (CTD) which are joined by a flexible interdomain linker (Blatter *et al.*, 1994). The NTD is comprised of residues 8-241 while the CTD includes residues 249-329. The NTD interacts with α and α' and participates in subunit dimerization. The CTD makes contacts with transcriptional activators and repressors, and is capable of recognizing and binding to specific sequences of the DNA (Gourse, *et al.*, 2000).

The CTD binding site is the third recognition element in some bacterial promoters and is called the upstream element (UP element). The UP element is located upstream of the -35 element and is often A+T-rich (Ross *et al.*, 1993). It is responsible for the high

activity of rRNA promoters such as the *rrnB* P1 promoter. The UP element at this promoter is located at -59 to -38 with the consensus sequence 5'NNAAAWWTWTTTTNNNAAANN-3' (W = A or T, N = any base; Estrem *et al.*, 1998). Additional studies have shown that the UP element consists of two subsites, proximal and distal. As demonstrated by DNaseI footprinting, protects both of these sections of the UP element, one centered at -42 and the other at -52 (Newlands *et al.*, 1991; Ross *et al.*, 1993; Estrem *et al.*, 1998 and 1999). The upstream half of the UP element or the distal subsite positioned at -46 to -38 with the consensus sequence 5'-AAAAAARNR-3' (R = purine) was shown to increase transcription at the *rrnB* P1 promoter by 170-fold. The proximal subsite is positioned at -59 to -46 with the consensus sequence 5'-NNAWWWWWTTTTTN-3'. This region alone can increase transcription only about 16-fold. Together as a full UP element, the two consensus subsites increase transcription about 340-fold (Estrem *et al.*, 1999).

CAP/CRP activator model

One of the best understood model systems for studying interactions of an activator protein with RNAP and the DNA involves the CAP/CRP protein. The catabolite activator protein (CAP) or cyclic AMP receptor protein (CRP) is a global regulator of carbon utilization genes. CRP in *E. coli* has been shown to make contacts with RNAP mediated by the CTD, NTD and (Attey *et al.*, 1994; Belyaeva *et al.*, 1996). Promoters that require CRP for transcription activation have been grouped into two classes (I and II) based on the position of the location of the DNA site for CRP.

Class I CRP-dependent promoters such as the *lac* promoter contains a DNA site for CRP upstream of the promoter at approximately -61.5 relative to the transcriptional start site. A 7 amino acid surface-exposed turn which is contained in residues 156-162, referred to as the activation region 1 (AR1), is present in both subunits of the CRP dimer. At a Class I type promoter, transcription activation requires only the activating region of the downstream subunit of the CRP dimer. Protein-protein interactions between the activating

In addition to activator-RNAP contacts, interactions between the CTD and DNA next to the activator-binding site also contribute to the stability of the complex in both Class I and Class II type promoters (Gourse *et al.*, 2000). CTD-DNA interactions can involve either UP element like sequences or non-specific DNA sequences (Bokal *et al.*, 1995; Kolb *et al.*, 1993).

Mechanism of LuxR-dependent transcriptional activation

The proposed model for transcriptional activation of the *lux* operon, under investigation here, is similar to that in the CRP model at Class II type promoter. A LuxR multimer (presumably a dimer) is positioned to make contacts with the DNA (*lux* box) and with the CTD, NTD and subunits of RNAP (Figure 5). The CTD could also be involved in DNA binding. The interaction between an activator and RNAP cannot be predicted based solely on the position at which the activator binds the DNA. In order to establish whether a promoter can be classified as Class I or Class II, individual residues involved in making the contacts must be examined (Gussin *et al.*, 1992). By investigating several different promoters, universal interactions can be identified as well as unique contacts made by specific activators. This information can then be used to expand our knowledge and establish common trends in transcription activation among different promoters.

The focus of this research project is to establish the role of individual residues in the CTD in LuxR-dependent transcription of the *lux* operon. It has already been demonstrated that the CTD plays a role in this process (Stevens *et al.*, 1999). Through the *in vivo* and *in vitro* analysis of mutant RNA polymerases (for reviews, see Ishihama, A., 1993; Busby, S. and Ebright, R. H., 1994; Gourse *et al.*, 2000), residues in the CTD that are involved in the mechanism of activation can be identified by significant decreases or increases in the rates of transcription when compared to the wild-type. Further studies can then determine whether these residues are involved in protein-protein or

region of CRP and the CTD are necessary. The close physical proximity of the two has been exhibited by protein-protein photocross-linking of CRP and RNAP at the *lac* promoter (Chen *et al.*, 1994). DNase I footprinting has demonstrated that wild-type protects the DNA between the site at which CRP binds and the -35 element of the promoter. However, the footprint was not seen when this experiment was performed with RNAP derivatives containing C-terminally truncated . This experiment demonstrated that the CTD of RNAP does in fact interact with the DNA and thereby allows RNAP to bind more effectively at the promoter (Kolb *et al.*, 1993).

At Class II CRP-dependent promoters such as the *galP1* promoter, the DNA site for CRP is centered near position -41 and overlaps the -35 determinant for RNAP. In this case, RNAP contacts the promoter upstream as well as downstream of the CRP dimer. The upstream interaction is mediated by the CTD of RNAP and the downstream interaction is mediated by the remainder of RNAP (Attey *et al.*, 1994; Belyaeva *et al.*, 1996). Two mechanistic components are involved during activation of Class II promoters: anti-inhibition and direct activation. Anti-inhibition involves overcoming an inhibitory effect of the CTD. This is mediated by the upstream subunit of the CRP dimer and the CTD. It has been suggested that the inhibitory effect of CTD is due to the amount of energy it takes to prevent CTD from binding at its favored location, directly upstream of the -35 element. This anti-inhibition, similar to activation at Class I promoters, involves protein-protein interactions between the activating region 1 of CRP (AR1) and CTD of RNAP (Busby and Ebright, 1997). Additionally, direct activation is carried out by the protein-protein interaction between activation region 2 (AR2) of the downstream subunit of CRP and region 4 of the ⁷⁰ subunit. Transcription activation at Class II CRP-dependent promoters demonstrates how an activator can make multiple contacts with RNAP during initiation of transcription (Attey *et al.*, 1994). Activators having more than one interaction with RNAP have been described as being ambidextrous (Busby and Ebright, 1997).

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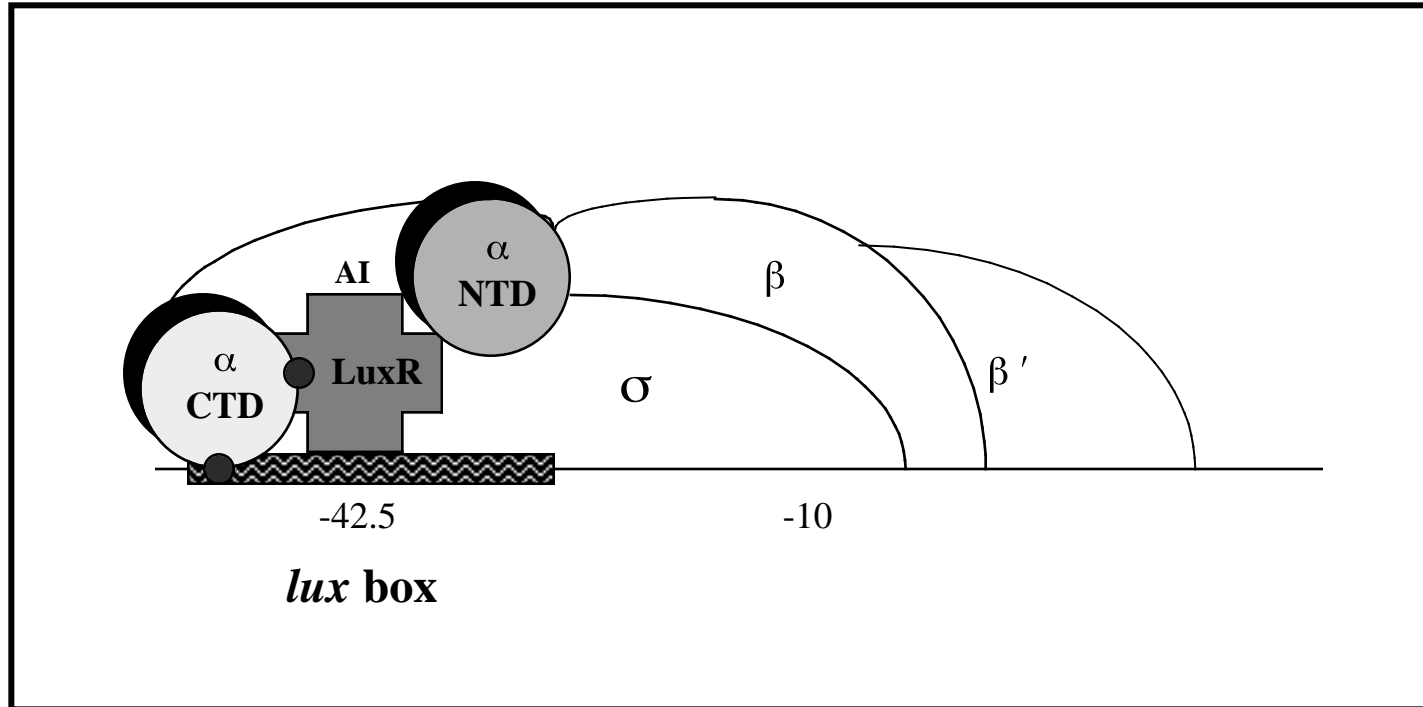


Figure 5: Model of the proposed interactions between RNAP and LuxR at the *lux* operon promoter.

protein-DNA interactions. Unfortunately, purification of full length LuxR has been unsuccessful to date; however *in vitro* work can be performed with a truncated form of LuxR (LuxR N) that is capable of the autoinducer-independent activation of the *luxR* operon. Experiments have been designed to establish a correlation between the *in vivo* and *in vitro* studies using LuxR N with the intention of then using the *in vivo* data acquired for full-length LuxR as a reflection of its native activity.

CHAPTER TWO

Materials and Methods

Strain construction

E. coli JM109 (Yanisch-Perron *et al.*, 1985.) was used as the host organism for *in vivo* studies due to its ease of manipulation compared to *V. fischeri*. A three-plasmid system was constructed by sequentially transforming three plasmids into JM109 (Stevens *et al.*, 1999). One plasmid (pAMS129) codes for the *lux* operon (*luxICDABE*) and kanamycin (Kn) resistance on a RSF1010-based vector (Stevens *et al.*, 1999). The second plasmid encodes LuxR N (pAMS122) or LuxR (pAMS121) under control of the isopropylthiogalactoside (IPTG) inducible *tac* promoter and chloramphenicol (Cm) resistance on a pACYC-based vector (Stevens *et al.*, 1999). The third plasmid codes for either wildtype (WT) or mutant subunits of RNAP under control of the IPTG inducible *lac* promoter and ampicillin (Ap) resistance on a pBR-based vector. The WT was encoded on plasmids pREII or pHTf1. Variant forms of these two parent plasmids code for alanine substitutions at positions 273-329 (Blatter *et al.*, 1994; Gaal *et al.*, 1996; Wood *et al.*, 1997; Kainz, M. and Gourse, R. L., 1998) and 255-271 in the CTD, respectively. (Tang *et al.*, 1995 and Savery *et al.*, 1998). Transformants carrying all three plasmids were selected on Luria-Bertani (LB) agar medium containing 100µg/ml Kn, 100µg/ml Ap, and 20µg/ml Cm.

Luminescence assays

Strains were grown overnight at 30°C in LB broth containing Kn, Ap and Cm at the appropriate concentrations and were then diluted 1:1000 in the same medium supplemented with 1 mM IPTG. Strains expressing LuxR from pAMS121 were also grown in the presence of 200 nM 3-oxo-C6 HSL (Sigma, St. Louis, MO). When this primary subculture had grown to an optical density at 600 nm (OD₆₀₀) of 0.1, it was diluted to an OD₆₀₀ of 0.025 into the same medium and grown to a final OD₆₀₀ of 0.5. Luminescence output from 20 µl of culture was measured over a 4-s integration period in a TD-20/20

luminometer (Turner Designs, Sunnyvale, CA) with a sensitivity range over several logs. Cells from 0.5 ml aliquots of each culture at an OD_{600} of 0.5 were also harvested via centrifugation and frozen at -70°C for use in luciferase assays.

Luciferase assays

Similar to measuring cellular β -galactosidase, the levels of luciferase found within cells can be quantitated (Rosson, R. A., and K. H. Neelson, 1981) and used as a more direct measure of transcriptional activation from the promoter of the *lux* operon. Cells harvested as described above were resuspended in 1 ml of lysis buffer (10 mM KPO_4 [pH 7.0], 10 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% bovine serum albumin [BSA], 50 μg of lysozyme per ml) and lysed via a single freeze-thaw step. Each luciferase reaction mixture contained the following final volumes of the reagents: 10 μl of crude cell extract, 10 μl of 1:1,000 diluted and sonicated *n*-decyl aldehyde (decanal; Sigma), 90 μl of assay buffer (10 mM KPO_4 [pH 7.0], 0.1% BSA, 1 mM DTT), and 100 μl of 50 μM reduced flavin mononucleotide (FMNH_2). The FMNH_2 was added directly to the tube containing the other reagents only after the tube was placed within the chamber of the luminometer. The luminescence emitted from the reaction was measured (3-s delay, 30-s integration time) with a TD-20/20 luminometer with a manual injection port.

Purification and reconstitution of wild-type and variant RNAP holoenzymes (Set 1)

Sixteen RNAPs containing the variants (residues 258-266, 268, 271, 273, 275, 297, 298 and 235) and the wild-type were purified, reconstituted according to the method of Igarashi and Ishihama, 1991 and provided to us. Other RNAPs of interest (Set 2) were purified and reconstituted as described below.

Purification of *E. coli* RNAP α subunit and derivatives (Set 2)

RNAP subunits containing a hexahistidine tag between the first and second codons were prepared using plasmid pHTT7f1NH. The plasmids carrying mutant *rpoA*

alleles were constructed by replacing the *HindIII-BamHI* fragment, which encodes the CTD, with fragments from plasmids encoding the different amino acid substitutions of interest (Tang *et al.*, 1994, Gaal *et al.*, 1996, Wood *et al.*, 1997). Our laboratory constructed pT7-his6 alpha 290, 292, 301, 302, 303, 307 and 314 and we received pT7-his6 alpha 278, 286, 291, 295, 296, 300 and the wild-type control pHTT7His6 from Richard L. Gourse's laboratory (Tang *et al.*, 1995; Savery *et al.*, 1998). The non-denaturing protocol used to purify the σ subunit of RNAP (containing alanine substitutions at the residues identified above) from recombinant *E. coli* was modeled from Tang *et al.*, 1994 and Gaal *et al.*, 1996. *E. coli* strain BL21 (DE3) containing pHTT7His6 or pT7 alpha series plasmids were grown at 37°C in LB plus 200µg/ml Ap overnight. The culture was diluted 1:100 into 100 ml of the same medium and grown at 37°C to an OD₆₀₀ of 0.5 (± 0.1). The culture was induced with 2 mM IPTG and an additional 200 µg/ml Ap was added. The cells were harvested by centrifugation (8 minutes at 5000 x g, 4°C) after growth at 37°C for three additional hours.

The cell pellet was resuspended in 4 ml of Buffer 3 (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 5 mM imidazole) and cells were lysed by sonication. The lysate was cleared by centrifugation (20 minutes at 16,000 x g, 4°C) and hexahistidine-tagged or derivative was purified by batch-mode metal ion-affinity chromatography. The lysate was adsorbed onto 1.5 ml of Ni²⁺-NTA agarose (Qiagen, Chatsworth, CA) in Buffer 3, the column was washed three times with 5 ml of Buffer 3 and eluted with 5 ml Buffer 4 (Buffer 3 plus 150 mM imidazole). Fractions (0.5 ml) were collected and those fractions of high yield and purity as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were pooled and stored in 50% glycerol at -70°C.

Purification of *E. coli* RNAP β , β' and σ^{70} subunits (Set 2)

The β , β' and σ^{70} subunits of RNAP were purified from inclusion bodies as described by Tang *et al.*, 1995. *E. coli* strains BL21 (DE3) transformed with plasmids pMKSe2, pT7 and pHTT7fl respectively were grown following the same protocol described above for strains expressing σ^{70} . However, a larger 500 ml volume was grown and inclusion bodies containing crude β , β' and σ^{70} were isolated by centrifugation (8 minutes at 5000 x g, 4°C) and resuspended in 20 ml lysis buffer (40 mM Tris-HCl, [pH 7.9], 300 mM KCl, 10 mM EDTA). Phenyl methyl sulfonyl fluoride (PMSF) (1 mM), lysozyme (0.2 mg/ml), and sodium deoxycholate (0.2%) were added one at a time to the cells at the final concentrations indicated. After incubation on ice for 10 minutes, the cells were disrupted by sonication until the OD₆₀₀ was about 20-30% the initial OD₆₀₀. After centrifugation (30 minutes at 16,000 x g, 4°C) the insoluble fraction was resuspended in 20 ml of lysis buffer with lysozyme (0.2 mg/ml) and octyl- β -glucopyranoside (0.2%). The solution was then sonicated and centrifuged as described above. The insoluble fraction was resuspended in 20 ml lysis buffer with octyl- β -glucopyranoside (0.2%) and DTT (1 mM). After a final treatment via sonication and centrifugation as described above the insoluble fraction was resuspended in 2.5 ml lysis buffer and stored in 25 μ l aliquots at -80°C. Protein concentrations of each subunit were measured with the Bradford assay (Bradford, M., *et al.*, 1976) using BioRad protein reagent (BioRad, Hercules, CA) and the purity was estimated by SDS-PAGE.

Reconstitution of RNAP holoenzyme (Set 2)

The protocol of Tang *et al.*, 1995 was followed to reconstitute RNAP holoenzyme from the purified subunits. Aliquots of β subunit (80 μ g), β' inclusion bodies (300 μ g) and σ^{70} inclusion bodies (600 μ g) were resuspended in 2 ml of guanidine HCl denaturing buffer (Buffer B; 6 M guanidine hydrochloride, 50 mM Tris-HCl, [pH 7.9], 10 mM MgCl₂, 10 μ M ZnCl₂, 1 mM EDTA, 10 mM DTT, 10% glycerol). The σ^{70} inclusion bodies (300 μ g) were separately resuspended in 200 μ l of guanidine HCl denaturing buffer. The two

mixtures were added to separate colloidion bags (25,000 MWCO; Schleicher & Schuell, Keene, NH) and dialysis was performed against 500 ml of Buffer C (50 mM Tris-HCl, [pH 7.9], 200 mM KCl, 10 mM MgCl₂, 10 μM ZnCl₂, 1 mM EDTA, 5 mM 2-mercaptoethanol, 20% glycerol) for 16 hours at 4°C. The used buffer was replaced with fresh buffer after 10-12 hours. After dialysis, the contents of each colloidion bag were separately centrifuged for 5 minutes at 10,000 x g to remove any precipitate that may have formed. The soluble fractions of the core enzyme and ⁷⁰ were subsequently combined in a conical tube (Sarstedt; Newton, NC) and incubated for 45 minutes at 30°C with no shaking.

Ni²⁺-NTA resin (100 μl) was washed three times with 1 ml of Buffer C (mixed by inversion followed by centrifugation at 4,000 x g). The reconstituted protein (1 ml) was added to the pre-washed resin, which was then mixed by inversion and slow shaking for 45 minutes at 4°C. The resin was pelleted via centrifugation (2 minutes at 16,000 x g, 4°C) and washed three times in Buffer C. The reconstituted RNA polymerase was eluted by adding an equal volume of Buffer C containing 150 mM imidazole and shaking for 45 minutes at 4°C. The resin was removed via centrifugation (2 minutes at 16,000 x g, 4°C) and the soluble protein was concentrated on Microcon 30K MWCO filters (Millipore; Bedford, MA) to approximately 50 μl. Glycerol was added to the reconstituted protein to a final concentration of 50% and the protein concentrations were estimated by the Bradford assay (Bradford, M., *et al.*, 1976).

***In vitro* transcription assays**

Purified pAMS1300 (Stevens and Greenberg, 1997) DNA was linearized with *Hind*III according to the manufacturer's instruction and electrophoresed on a 2% low melting point agarose gel. The appropriate DNA fragment was extracted from the agarose matrix using the Qiagen QIAquick gel extraction kit (Qiagen; Valencia, CA) and quantitated by comparison to a mass ladder (BioRad, Hercules, CA). Single-round transcription assays were performed from this template as previously described (Stevens and Greenberg, 1997). All reactions contained linearized pAMS1300, the *luxI* promoter-

containing DNA template (1.3 nM), LuxR N (10 μ M) and mutant RNA polymerase at an activity level equivalent to the wild-type (either 30 nM [Set 1] or 54 nM [Set 2]). The RNA-1 transcript produced from the template serves as an internal control for LuxR N-independent RNAP activity. Quantitation of radiolabelled transcripts was performed using a Storm phoshoimager (Molecular Dynamics, Sunnyvale, CA).

DNA Mobility shift assays

Three DNA templates were used during DNA mobility shift assays. To obtain a template which contained the *lux* operon promoter region, a portion of the plasmid, pJE202, containing the *luxI* regulatory region (Figure 6) (Engebrecht, J., and Silverman, M., 1984) was amplified via the polymerase chain reaction (PCR) with primers AMS4 (5'CGCTGGGAATACAATTAC3') and LuxR2A (5'AAAAAATCCGATTTTTTTATC T3') (Sigma-Genosys, The Woodlands, Texas). The 132-bp PCR fragment that was generated was ligated *into* pGEM T-easy vector (Promega, Madison, WI) and the resulting plasmids were transformed into *E. coli* DH5 (Hanahan, D., 1983). Strains containing the Ap resistant recombinant plasmids were selected for and screened for the presence of the desired construct via LacZ complementation and for the correct restriction endonuclease recognition sites. The nucleotide sequence of both strands of the cloned PCR product was verified using T7 and SP6 primers by the Virginia Tech Sequencing Facility. The final construct was designated pAHF100.

Two control DNA templates were also generated. A 228-bp region of plasmid pRLG4238 (Estrem *et al.*, 1998) containing the *rrnB* P1 promoter region with a strong UP

element was amplified via PCR with the vector-specific primers, 1620 (5' GCGCTACGGCGTTTCACTTC 3') and 3038 (5' CGTATCACGAGGCCCTTTCG 3') (Sigma Genosys, The Woodlands, Texas) to be used as a positive control for binding.

The 104-bp pUC multiple cloning site was amplified from pUC19 by using the universal forward and reverse primers (Yanisch-Perron *et al.*, 1985) for use as a negative control in the assay.

Radiolabeled DNAs for the mobility shift experiments were generated by incorporating ³²P-CTP (20 μM per 100 μl reaction) into PCR reaction mixtures. The reaction mixtures were subjected to two phenol-chloroform-isoamyl alcohol (25:24:1) extractions and an ethanol/sodium acetate precipitation. The pellets were then washed with 70% ethanol and resuspended in water. DNA concentrations were estimated by comparison to a mass ladder (BioRad, Hercules, CA).

Gel mobility shift assays were based on published procedures (Stevens *et al.*, 1994). Reaction mixtures (45 μl) contained 3 nM radiolabeled DNA, 50 mM NaCl, 1 mM MgCl₂, 0.002 mg/ml calf thymus DNA, acetylated bovine serum albumin (2 mg/ml), 1 mM DTT, 0.1 mM EDTA, 10 % glycerol in 40 mM Hepes (pH 7.4). Reactions were incubated for 15 minutes at 30°C and were run on 4% polyacrylamide gels containing 10% glycerol with recirculation of the running buffer (20 mM Hepes, 3 mM NaCl, 1 mM EDTA, pH 8.0). Radioactive bands were visualized using a Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA).

CHAPTER 3

Results and Discussion

Effect of alanine substitutions in α on LuxR Δ N and LuxR-dependent luminescence and luciferase levels

The effect of alanine substitutions in the CTD on the rate of transcription of the *lux* operon was first examined through *in vivo* luminescence assays. Strains containing three plasmids encoding (i) the *lux* operon, (ii) LuxR Δ N or LuxR, and (iii) wild-type, or one of sixty-nine variants of α with single alanine substitutions in the C-terminal domain between residues 255-329 were tested. In addition, a variant of α with alanine substitutions at 162-165 in the NTD was also included in our analysis. Simultaneous substitution of alanine for all four of these amino acid residues in NTD resulted in a defect in Class II CRP-dependent transcription (Niu *et al.*, 1996).

In the presence of LuxR Δ N, the variant forms of α CTD that resulted in at least a two-fold decrease (< 50% WT) in luminescence had mutations in residues: 257-258, 260, 262-266, 269, 271, 277-279, 281, 286-287, 289-291, 294-303, 307, 314, and 320. (Figure 7). In addition, α CTD variants with alanine substitutions at residues 268, 292, and 315 displayed more than two-fold higher (>200% WT) luminescence levels in comparison to the wild-type. In the presence of LuxR, variant forms of α that produced at least a two-fold decrease in luminescence had substitutions at residues: 255, 257, 260, 262-265, 269, 273, 276-279, 286, 288-291, 295-297, 299, 300, 303, 305, 314, 315, and 321 (Figure 8). Furthermore, there were also mutations at residues 209 and 211 that resulted in greater than a two-fold increase in luminescence levels in comparison with the wild-type. The NTD variant with a quadruple alanine substitution mutation had no effect on either LuxR Δ N or LuxR-dependent luminescence (Figure 7 and 8).

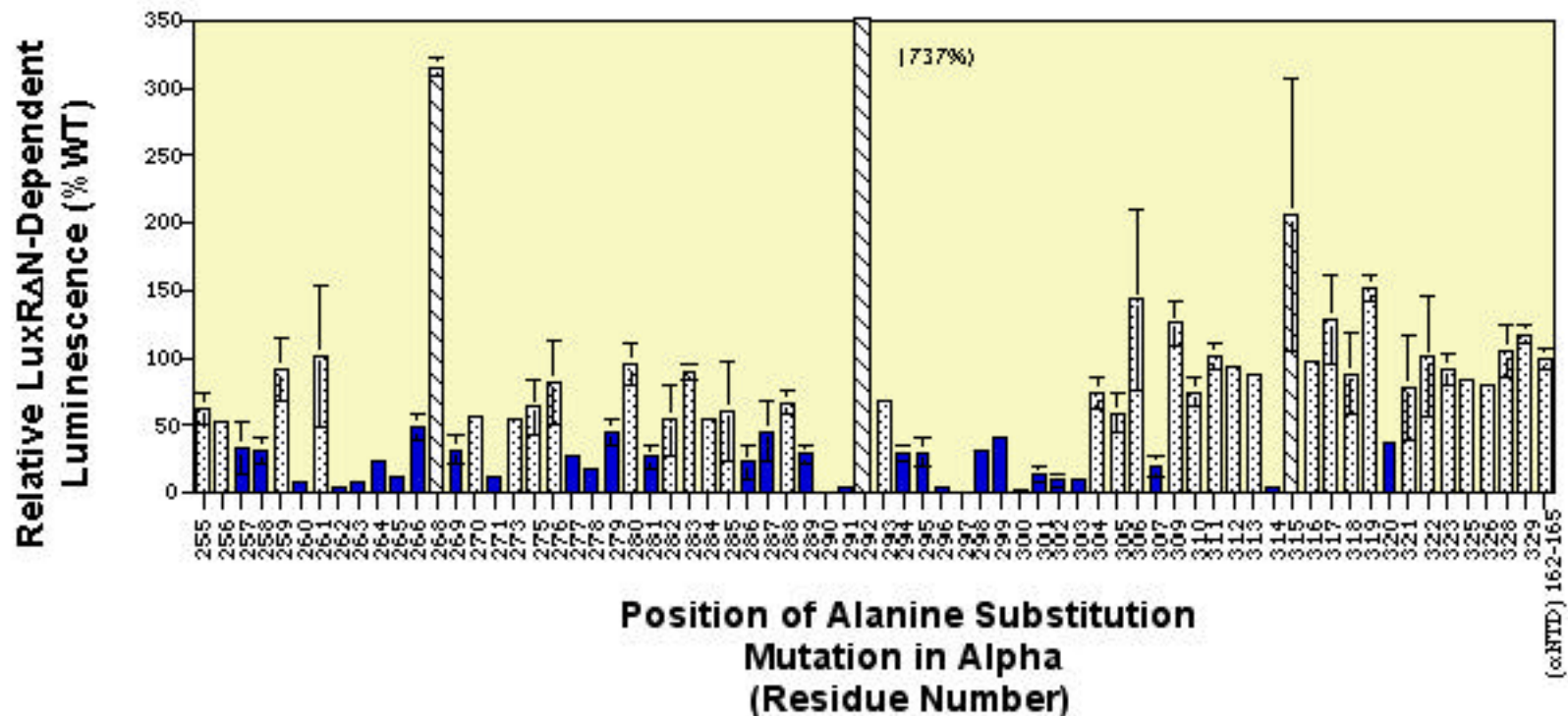


Figure 7: Effect of alanine substitutions in α on LuxR Δ N-dependent cellular luminescence levels in recombinant *E. coli*. The value for each variant form of α represents the average of two independent experiments. The error bars represent the range of each experiment from the mean. Luminescence output from strains containing either of the two wild-type controls, pHTf1 α or pRE11 α , was set to 100% for each experiment (data not shown). The solid columns highlight those strains with less than 50% wild-type levels of luminescence while the striped columns highlight those strains with greater than 200% wild-type levels of luminescence. The actual value for the α CTD variant at 292 is given in parentheses.

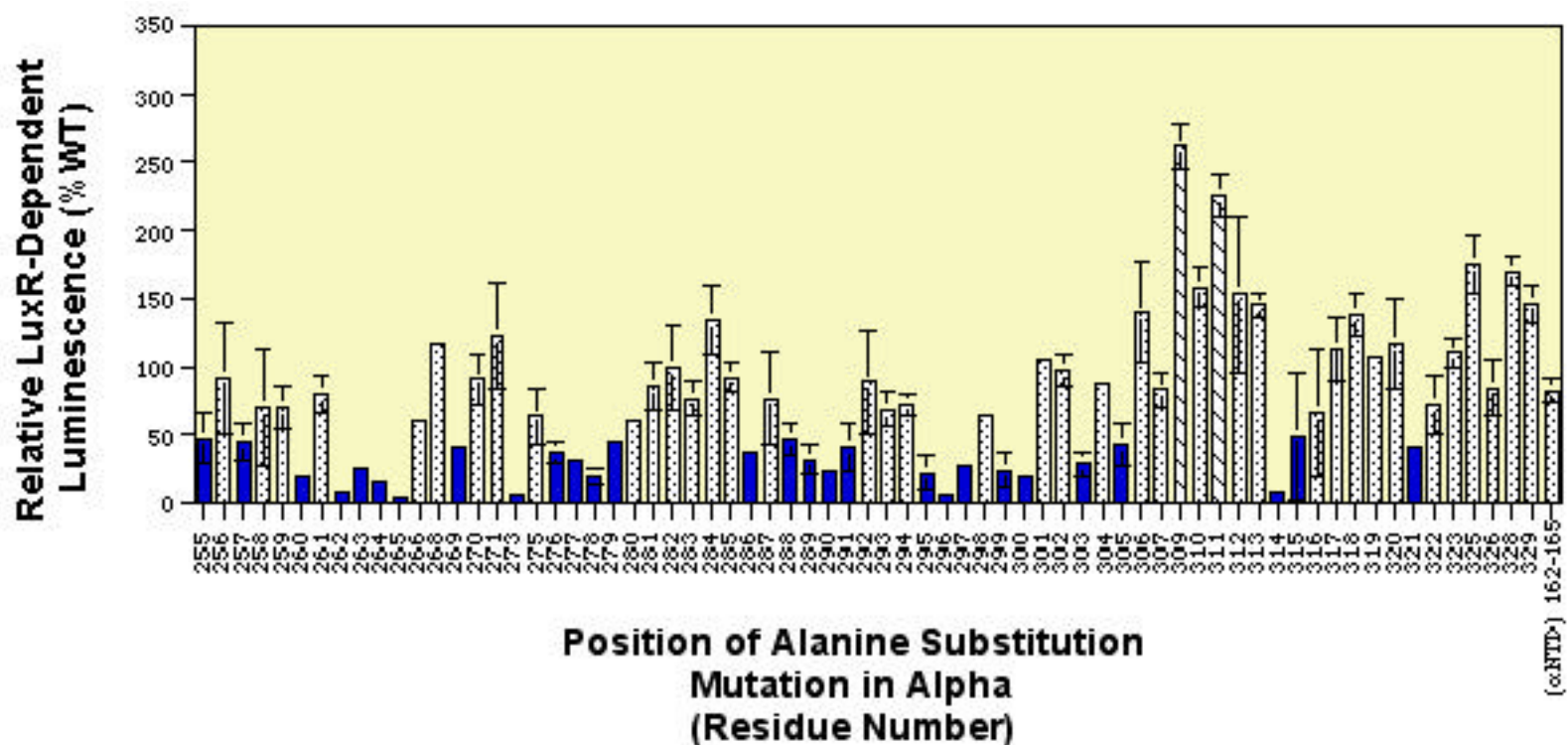


Figure 8: Effect of alanine substitutions in α on LuxR-dependent cellular luminescence levels in recombinant *E. coli*. The value for each variant form of α represents the average of two independent experiments. The error bars represent the range of each experiment from the mean. Luminescence output from strains containing either of the two wild-type controls, pHT1 α or pRE11 α , was set to 100% for each experiment (data not shown). The solid columns highlight those strains with less than 50% wild-type levels of luminescence while the striped columns highlight those strains with greater than 200% wild-type levels of luminescence.

Luminescence assays were used as a first screen to establish whether or not the variant forms of *luxI* had any significant effects on activation of the *lux* operon. However, other cellular metabolic functions that play an indirect role in the luminescence process might also be affected by the variant RNAPs. Therefore, while the luminescence assays do serve as a useful first screen to establish which strains should be further investigated, luciferase activity levels from cell extracts were also used as a more direct way to measure the rate of transcription from the *luxI* promoter in comparison to luminescence assays.

Strains expressing *luxI* variants with alanine substitutions at residue numbers 260, 262-263, 265, 271, 278, 286, 290-291, 296-298, 300-303, 307, and 314 had at least two-fold lower luciferase enzyme levels compared to the wild-type in the presence of LuxR N. While the strains expressing the *luxI* variant with a substitution at residue 292 had more than a two-fold higher luciferase level in comparison to the wild-type (Figure 9). In the presence of LuxR, the variant forms of *luxI* that resulted in at least a two-fold decrease in luciferase enzyme levels compared to the wild-type had mutations in residues 262, 265, 290, 295, 296, and 314 (Figure 10). There were no *luxI* variants that produced a two-fold increase in luciferase levels in the presence of LuxR. The *in vivo* luciferase assays performed also showed that the NTD variant acted essentially as wild-type. The position of amino acid residues shown to be involved in activation of the *lux* operon via the luciferase assays are shown on a space-filling model of *luxI* (Jeon *et al.*, 1995) in Figure 11.

The residues identified as playing a critical role in activation of the *lux* operon through luciferase assays represent a subset of those identified through luminescence assays. This is most likely due to additional indirect effects of the *luxI* variant on other cellular processes during the luminescence assays. Full-length LuxR and a truncated form of LuxR (LuxR N) are both used in the luminescence and luciferase assays so that comparisons could be made between the two. LuxR N is a monomer in solution and apparently has a lower affinity for the DNA compared to full-length LuxR. It can only bind to the *lux* operon promoter in synergy with RNAP (Stevens *et al.*, 1994).

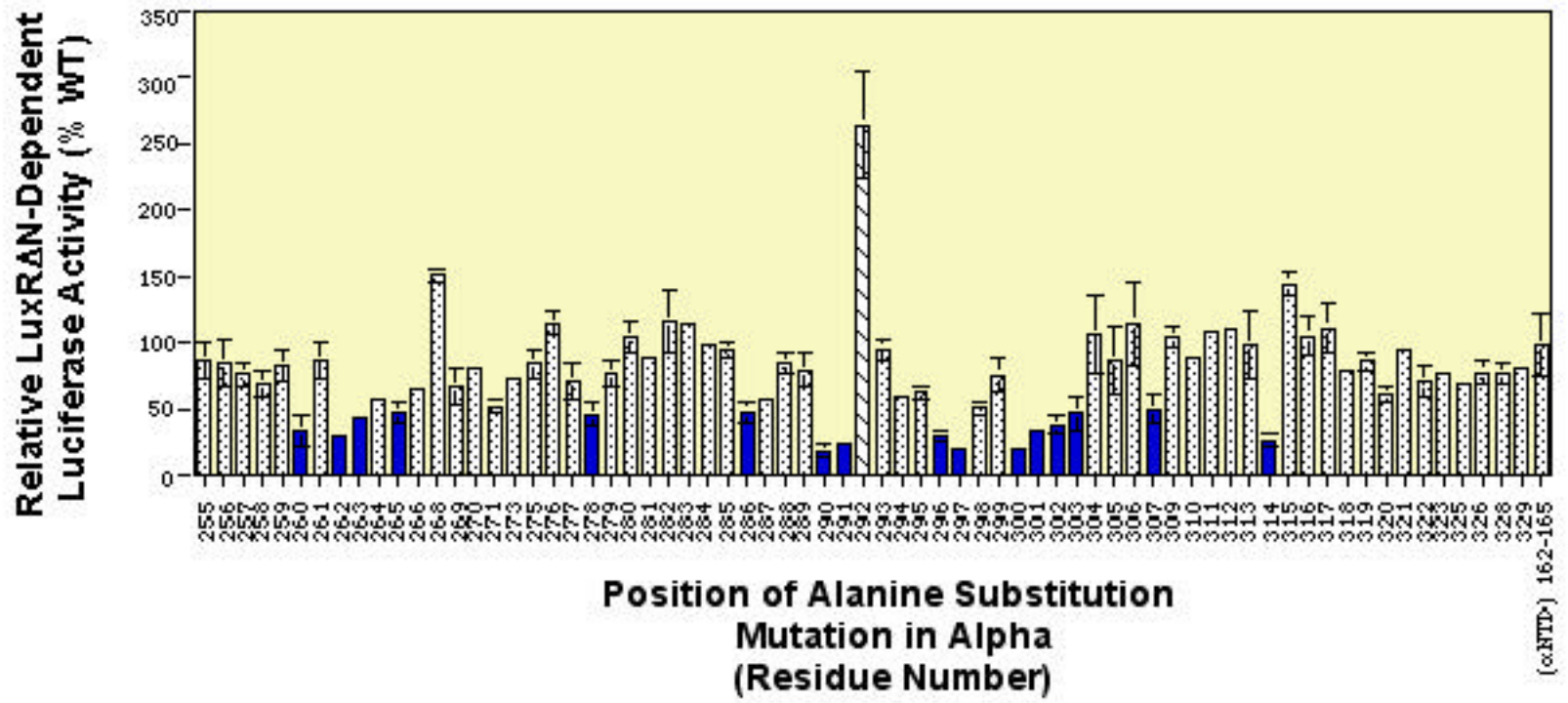


Figure 9: Effect of alanine substitutions in α on LuxR Δ N-dependent cellular luciferase levels in recombinant *E. coli*. The value for each variant form of α represents the average of two independent experiments with individual luciferase assays performed in quadruplicate. The error bars represent the range of each experiment from the mean. Luciferase activity from strains containing either of the two wild-type controls, pHTf1 α or pRE11 α , was set to 100% for each experiment (data not shown). The solid columns highlight those strains with less than 50% wild-type levels of luciferase activity while the striped columns highlight those strains with greater than 200% wild-type levels of luciferase activity.

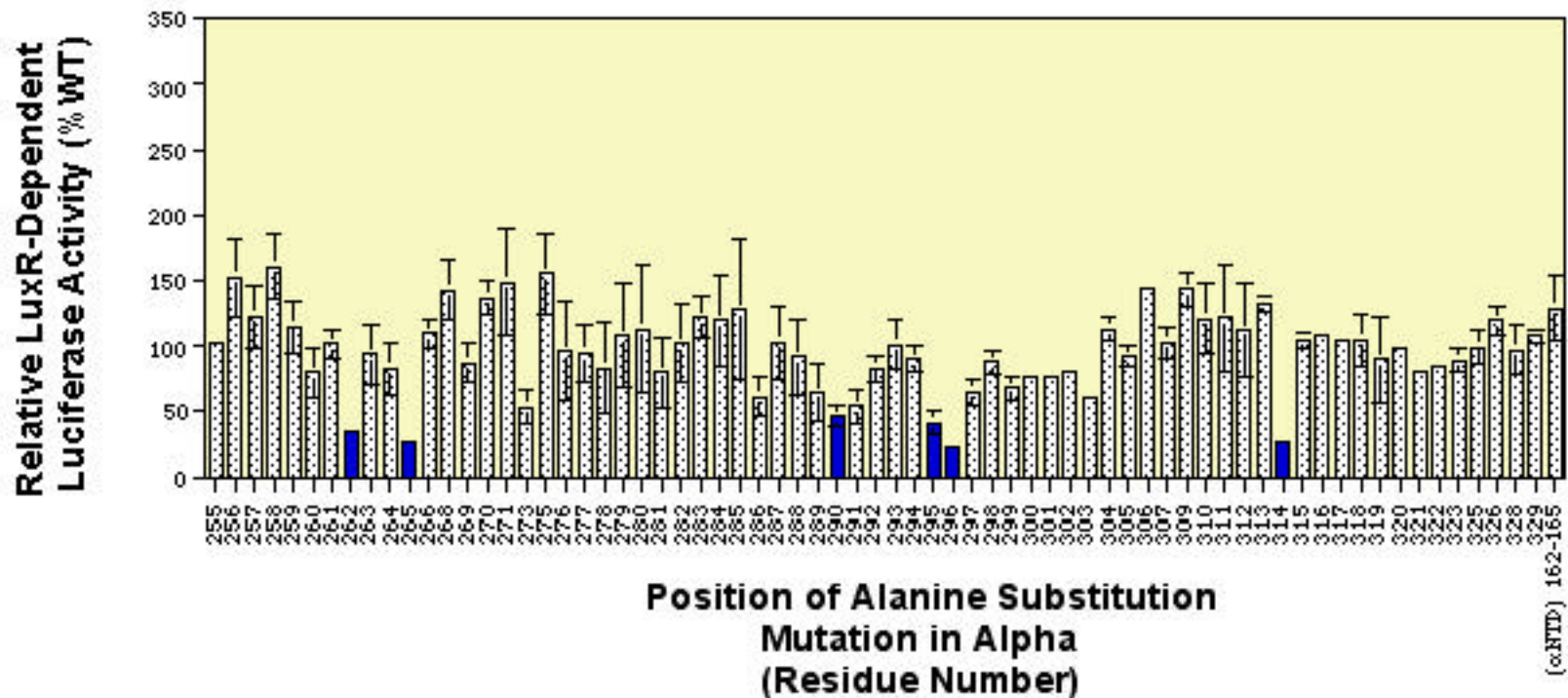


Figure 10: Effect of alanine substitutions in α on LuxR-dependent cellular luciferase levels in recombinant *E. coli*. The value for each variant form of α represents the average of two independent experiments with individual luciferase assays performed in quadruplicate. The error bars represent the range of each experiment from the mean. Luciferase activity from strains containing either of the two wild-type controls, pHTf1 α or pRE11 α , was set to 100% for each experiment (data not shown). The solid columns highlight those strains with less than 50% wild-type levels of luciferase activity.

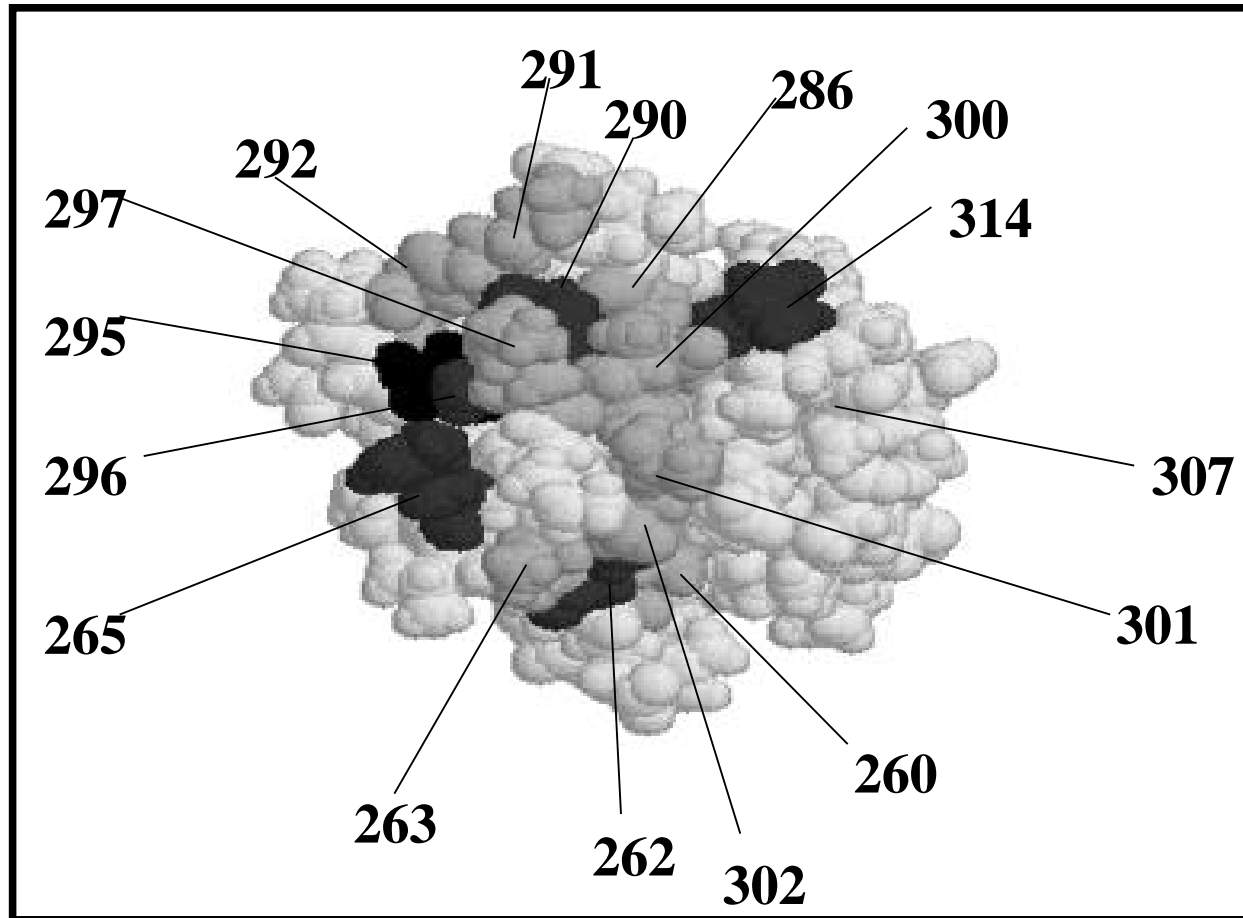


Figure 11: Space-filling model of the RNAP CTD highlighting amino acid residues of interest (Jeon *et al.*, 1995). The light gray residues are important during LuxR N-dependent activation. The dark gray residues were found to be important during activation of the *lux* operon in the presence of either LuxR or LuxR N. The sole black residue was involved only in LuxR-dependent activation.

Full-length LuxR is believed to bind to the DNA as a multimer, presumably a homodimer, (Choi and Greenberg, 1992b; Eglund, K. A. and Greenberg, E. P., 1999) and recent genetic evidence confirmed that LuxR can bind to the *lux* box in the absence of RNAP (Eglund *et al.*, 2000). The results reported here support these previous findings; more residues in the CTD are involved in LuxR N-dependent activation versus LuxR-dependent activation of the *lux* operon.

Effect of alanine substitutions in the α CTD on LuxR Δ N-dependent transcriptional activation of the *lux* operon *in vitro*

Those variants limiting or enhancing luciferase levels by at least two-fold were further analyzed via *in vitro* transcription assays. The analysis was carried out in two phases. In the first phase (Set 1), the activity of a set of existing mutant RNAPs (and a wild-type control) provided by Akira Ishihama was examined (Figure 12). In the second phase (Set 2), additional mutant RNAPs (and a wild-type control) of interest were reconstituted in house for use in the assays (Figure 13). So that data comparisons could be made between the two sets, the activities of the two wild-type RNAP controls were examined. Even though slightly different protein concentrations were used [30 nM (Set 1) vs. 54 nM (Set 2)], there was approximately equal levels of activity as defined by the amount of RNA-1 transcript. The *luxI*/RNA-1 transcript ratio was also calculated for both and similar values were observed (0.51 vs. 0.54). Thus, the protein concentrations used for both RNAP sets were within two-fold of one another and the level of LuxR N-dependent activation was roughly equal. Therefore, direct comparisons can be made between the two sets of results.

Since LuxR has not been purified to date, only LuxR N was used for the *in vitro* analysis. LuxR N is the truncated form of LuxR that cannot bind to the *lux* DNA alone, but rather binds cooperatively in the presence of RNAP (Stevens *et al.*, 1994). RNAP containing a truncated form of σ missing the C-terminal 94 amino acid residues (σ^{235}) was also used in the assays as an additional negative control. Using this variant of

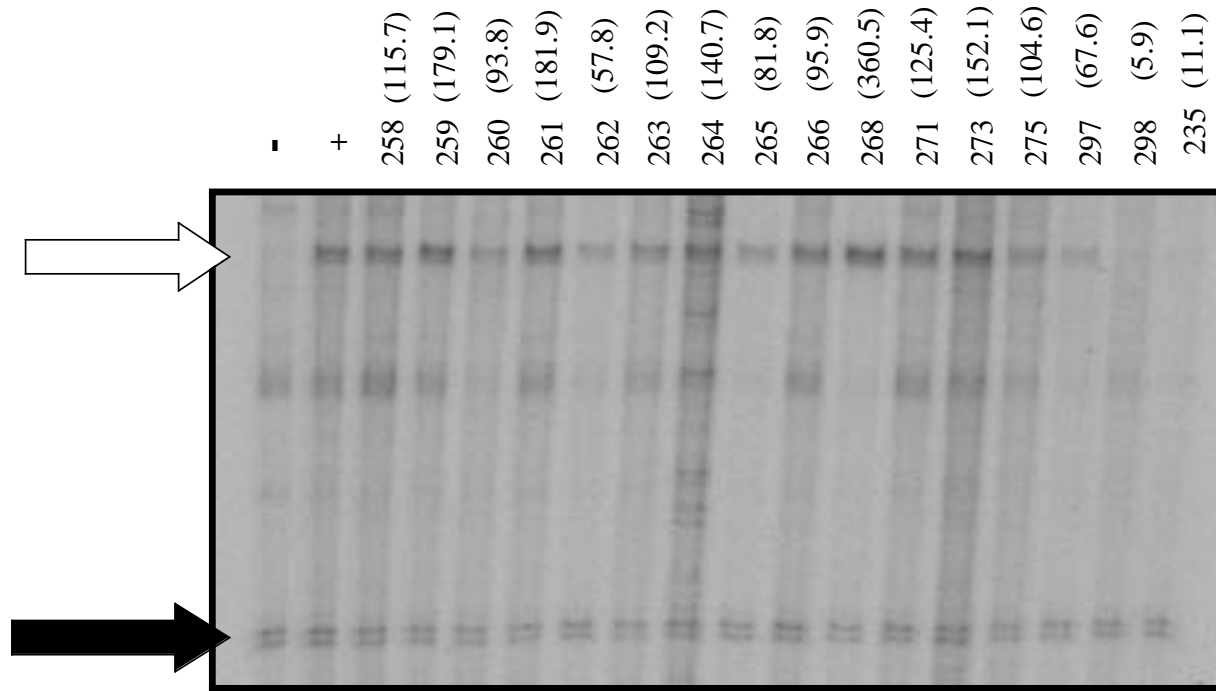


Figure 12: LuxR N-dependent *in vitro* transcription from the *luxI* promoter generated by wild-type or variant RNAPs (Set 1). Transcripts produced by wild-type RNAP (30 nM) in the absence (-) and presence (+) of LuxR N (10 μ M) are shown in the lanes labeled - and +, respectively. The lane labeled 235 illustrates results when a truncated form of RpoA missing the C-terminal 94 amino acid residues was used in the assays and serves as an additional control. The remaining lanes are labeled by residue number indicating the position of the alanine substitution in present in RNAP. The numbers in parentheses indicate the relative average value of the *lux* transcript in comparison to the wild-type from two independently run experiments. The white arrow points to the *lux* mRNA product, and the black arrow marks the RNA-1 transcript, which served as an internal control for LuxR N-independent activity and permits normalization of the activity of the variant RNAPs under investigation. Reactions were performed as described previously (Stevens and Greenberg, 1997). The template DNA was *Hind*III-linearized pAMS1300 (1.3 nM), and all reaction mixtures except the one in the first lane (-) contained 10 μ M LuxR N. RNAPs were purified as described in the text.

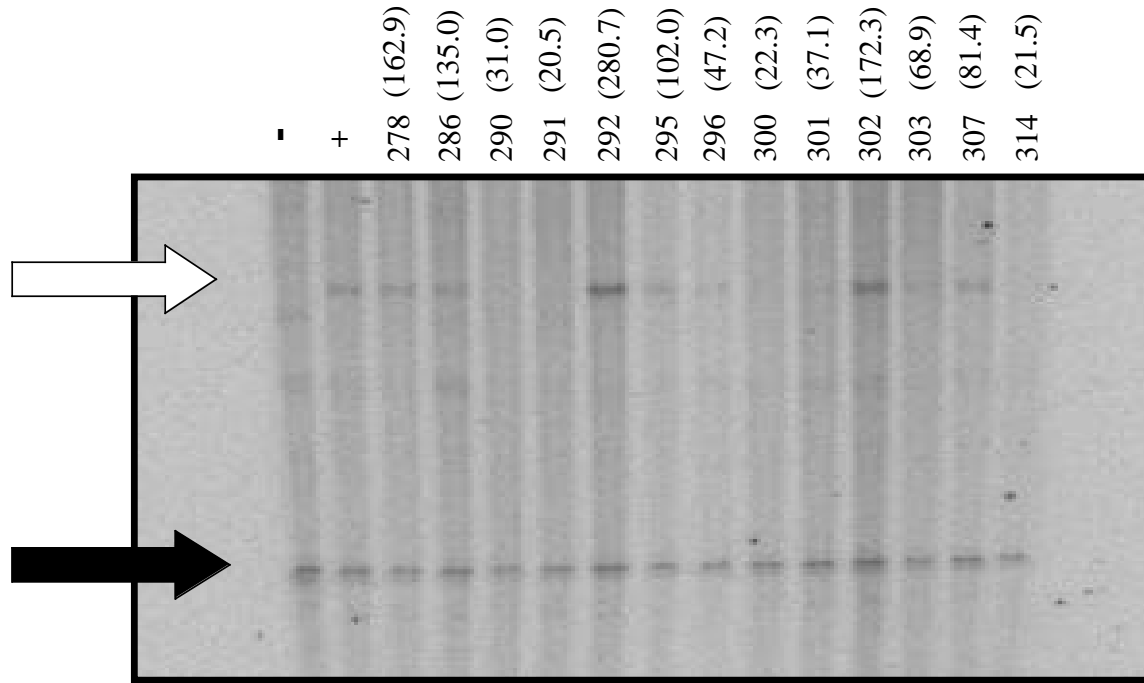


Figure 13: LuxR N-dependent *in vitro* transcription from the *luxI* promoter generated by wild-type or variant RNAPs (Set 2). Transcripts produced by wild-type RNAP (54 nM) in the absence (-) and presence (+) of LuxR N (10 μ M) are shown in the lanes labeled - and +, respectively. The remaining lanes are labeled by residue number indicating the position of the alanine substitution in present in RNAP. The numbers in parentheses indicate the relative average value of the *lux* transcript in comparison to the wild-type from two independently run experiments. The white arrow points to the *lux* mRNA product, and the black arrow marks the RNA-1 transcript, which served as an internal control for LuxR N-independent activity and permits normalization of the activity of the variant RNAPs under investigation. Reactions were performed as described previously (Stevens and Greenberg, 1997). The template DNA was *Hind*III-linearized pAMS1300 (1.3 nM), and all reaction mixtures except the one in the first lane (-) contained 10 μ M LuxR N. RNAPs were purified as described in the text.

, it has been previously established that the C-terminal region of the α CTD is essential for transcription of the *lux* operon (Stevens *et al.*, 1999). Several RNAPs containing α variants exhibited two-fold decreases in the rate of transcription from the *luxI* promoter in comparison to the wild-type (residues 290, 291, 296, 298, 300, 301, 314). Smaller decreases were also observed in residues 260, 262, 265, 266, 297, and 303 with values ranging from 57.8 – 95.9%. In addition, RNAP variants containing mutations in α at residues 268 and 292 produced greater than two fold higher rates of transcription from the *luxI* promoter. Somehow the substitutions at these sites have enabled RNAP to bind more effectively to the DNA in the presence of LuxR N. However, none of the mutant RNAPs tested were shown to display any LuxR N-independent activation of the *luxI* promoter (data not shown).

Overall, a good correlation exists between the *in vivo* luciferase and *in vitro* transcription assay results in 24 out of the 28 mutant RNAP variants tested (Figure 14 and 15). However, results using some RNAP variants (alanine substitutions at 278, 286, 298 and 302) do not show a strict correlation between the *in vivo* and *in vitro* analyses (Figures 14 and 15). This may in part be explained by differences in plasmid maintenance and stability in the three-plasmid system used in the *in vivo* assays. Despite these discrepancies, five α variants with mutations in residues 262, 265, 290, 296 and 314 were identified as having greater than two-fold effects *in vivo* (in the presence of either LuxR or LuxR N), and producing some degree of decreased rates of transcription *in vitro*. The function of these five variants in α was further examined via gel mobility shift assays.

Analysis of the interactions between the α CTD and the *luxI* promoter

Purified wild-type α has previously been shown to have the ability to bind to the UP element of the *rrnB* P1 promoter (Ross *et al.*, 1993). In this study, the ability of wild-type and five α variants to bind and recognize the UP element and the *luxI* operon promoter was examined. A complete shift of the DNA fragment containing the UP element was observed when 8-12 μ M of the wild-type α was used in the assays (Figure 16). A slightly

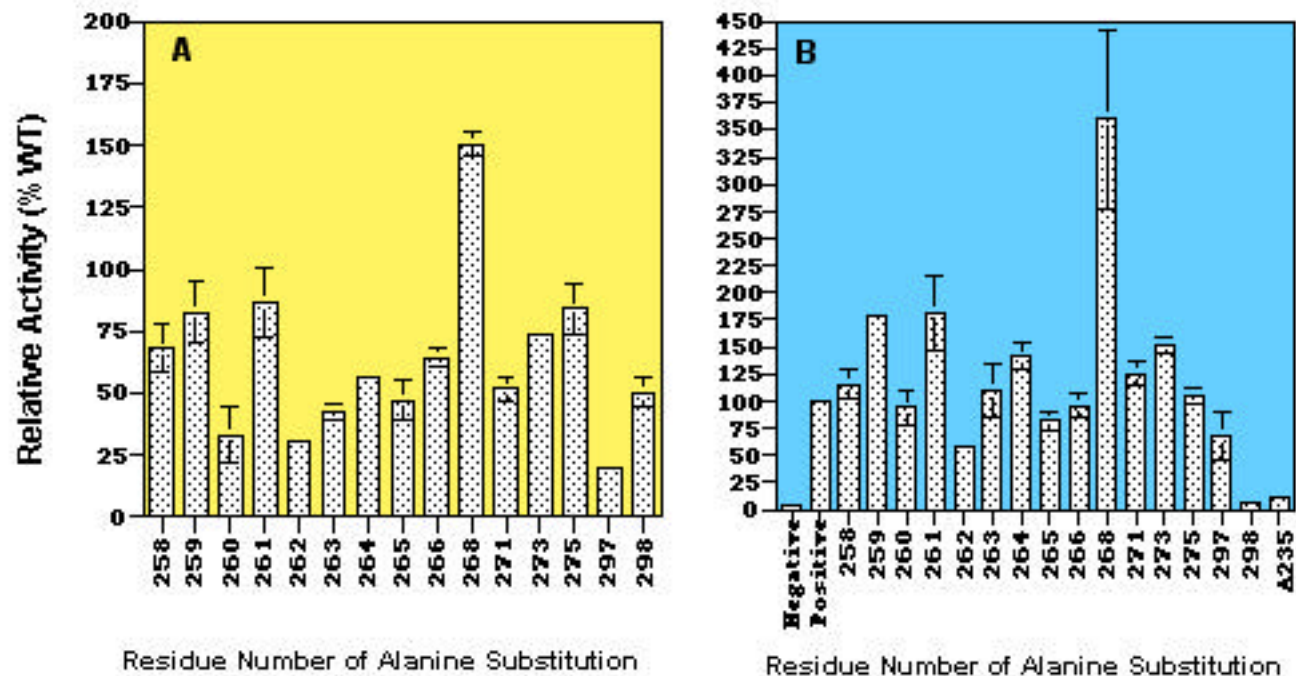


Figure 14: Comparison between (A) *in vivo* luciferase and (B) *in vitro* transcription assay results (Set 1). (A) The value for each alanine substitution mutant represents the average of two independent experiments with individual luciferase assays performed in quadruplicate. Luciferase activity from strains containing either of the two wild-type controls, pHTf1 α or pRE11 α , was set to 100% for each experiment (data not shown). (B) The *in vitro* transcription assay results are the average of two independent experiments. The error bars represent the range of each experiment from the mean.

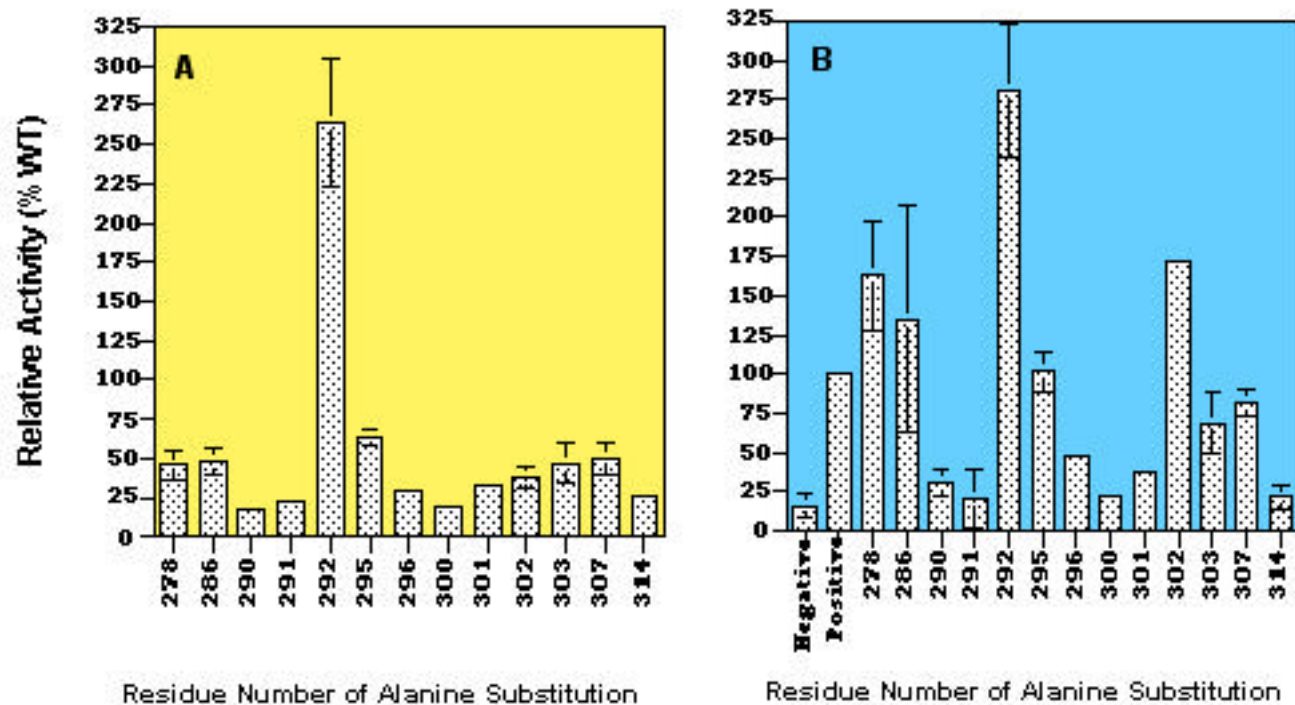


Figure 15: Comparison between (A) *in vivo* luciferase and (B) *in vitro* transcription assay results (Set 2). (A) The value for each alanine substitution mutant represents the average of two independent experiments with individual luciferase assays performed in quadruplicate. Luciferase activity from strains containing either of the two wild-type controls, pHTf1 α or pRE11 α , was set to 100% for each experiment (data not shown). (B) The *in vitro* transcription assay results are the average of two independent experiments. The error bars represent the range of each experiment from the mean.

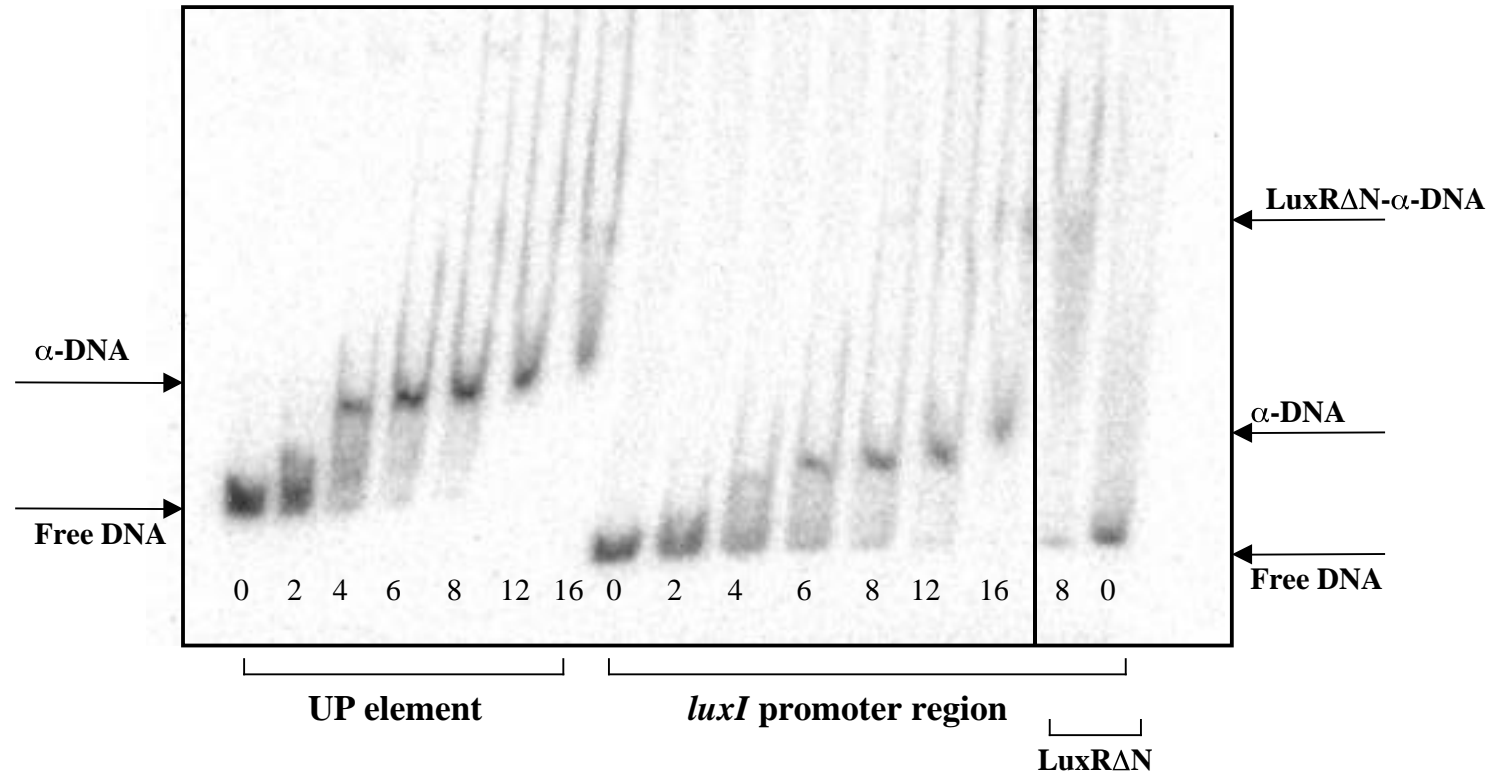


Figure 16: Autoradiogram of gel mobility shift assays with wild-type σ subunit. Interactions between purified wild-type RNAP and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of σ (μ M) used in the assays. The last two lanes show interactions when 10 μ M LuxR Δ N is added. The results shown are representative of two independent trials.

greater concentration was needed to obtain a similar shift with the *lux* DNA (Figure 16). Additionally, there was no shift observed with the negative control fragment containing the pUC multiple cloning site and WT (Figure 17). The control was included in the experiment to illustrate that was exclusively involved in making specific interactions in our assay conditions. Therefore, there is a possible binding site for in the *lux* DNA fragment used. However, apparently forms complexes with the *lux* DNA at a lower efficiency than it does with the strong UP element of the *rrnB* P1 promoter. By nucleotide sequence analysis, a region immediately upstream of the *lux* box had the potential binding site for differing from the consensus sequence for the proximal subsite of the UP element with only two mismatches (See Figure 18) (Estrem *et al.*, 1999). However, due to the high A+T nature of *Vibrio* DNA in the *lux* operon promoter (Figure 6), it is not surprising that could bind at this location or possibly another region in the DNA fragment used for the studies. Further *in vitro* analysis will be necessary to determine the precise binding site of on the *lux* DNA.

Our finding that could interact with *lux* DNA was not predicted based on DNase I protection assays performed by Stevens *et al.* using wild-type and -256 mutant RNAPs. The -256 RNAP contains a truncated form of missing the C-terminal 73 amino acid residues. It was shown that LuxR N-RNAP complexes can bind and protect the *luxI* promoter, but with less efficacy when the CTD is truncated in comparison to the wild-type. However, a prominent footprint of was not observed in either case (Stevens *et al.*, 1999). It has been stated that a footprint may not be detected by DNase I protection assays at promoters lacking a strong UP element because of weak binding of the CTD (Burns *et al.*, 1999). It has also been demonstrated that protection of UP elements in DNase I protection assays is not a true measure of their function (Ross *et al.*, 1998). Therefore, binding at the *luxI* promoter may be occurring, but it is not strong enough to obtain a visible footprint. Others have proposed that in the absence of CTD-DNA interactions the CTD still plays a critical role at many promoters by influencing the kinetics of open

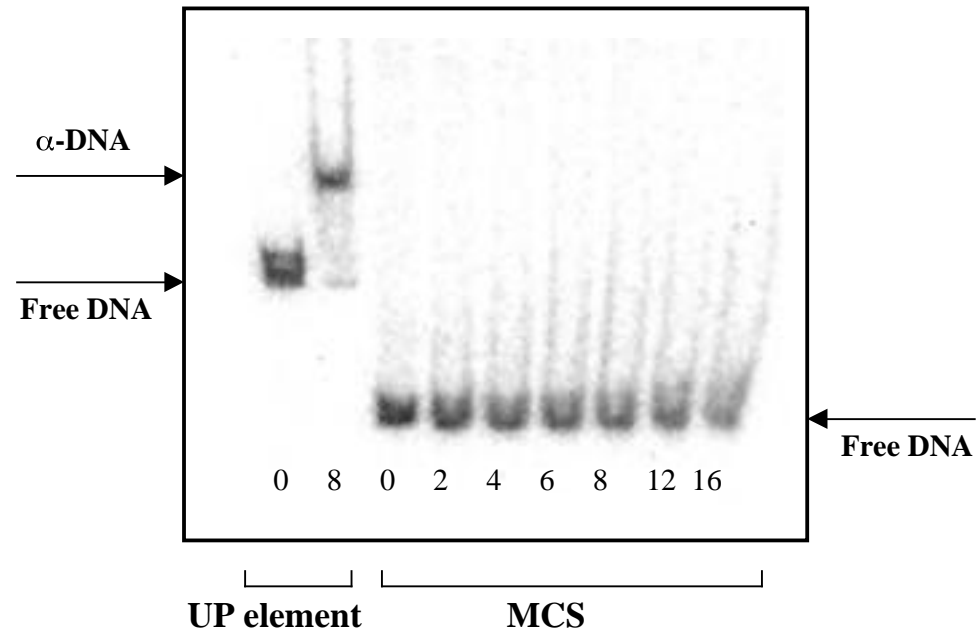


Figure 17: Autoradiogram of gel mobility shift assays with wild-type σ subunit and the pUC19 multiple cloning site. Interactions between purified wild-type RNAP σ and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 104-bp PCR fragment with the pUC multiple cloning site (MCS) amplified from pUC19. The numbers below each lane refer to the concentration of σ (μM) used in the assays. The results shown are representative of two independent trials.

Full UP
element

NNAAAWWTWTTTNNNAANN

Proximal
Subsite

AAAAARNR

**Region upstream
of *lux* box**

ACATAAGCA

Distal
Subsite

NNAWWWWT

Figure 18: Consensus sequences of the full UP element, proximal and distal subsites (Gourse *et al.*, 2000). The sequence of the region upstream of the *lux* box is shown as well. Underlining shows mismatches. W = A or T; R = A or G; N = no single base pair present in 70% of the population and no 2 bp make up 95% of the population.

complex formation, thereby driving transcription initiation (Burns *et al.*, 1999). This in part may allow us to reconcile our results with those of Devine *et al.* who found that eliminating the sequences upstream of the *lux* box and replacing them with vector sequences still allowed for LuxR-dependent transcription of the *lux* operon from this artificial promoter (Devine *et al.*, 1989).

The ability of the variants with alanine substitutions at residues 262, 265, 290, 296 and 314 to bind to the UP element and the native *luxI* promoter was examined to determine whether or not these residues play a role in the process of DNA binding (Figures 19-23). In all the experiments, the concentration of mutant alpha used ranged from 2-16 μ M. The variants at residues 262, 265, 296 and 314 all showed a complete inability of to bind to both the UP element and the *luxI* promoter (Figures 19, 20, 22, 23). The mobility shift assays were repeated using the variant at residue 314 at a slightly higher concentration range (12-32 μ M), since previous reports did not suggest that this variant would be unable to bind to the DNA (Gaal *et al.*, 1996; Fritsch *et al.*, 2000). However, no shift was observed for the variant at residue 314 under these conditions either. The variant at residue number 290 exhibited a severely weakened ability of to bind to both the UP element and the *luxI* promoter. Approximately, a 4-fold higher concentration of the protein was needed to observe a shift equivalent to wild-type (Figure 21). Therefore, all five variants (L262, R265, L290, G296, and L314) demonstrated a reduction in the ability for to interact with both the UP element and the *luxI* promoter DNA.

In an effort to establish whether protein-protein contacts might be affected in the variants, an attempt was made to study complex formation between purified wild-type and LuxR N. As previously observed, LuxR N cannot bind to the DNA alone (Stevens *et al.*, 1994; Figures 16 and 24). However, there is a super shift of the *luxI* promoter

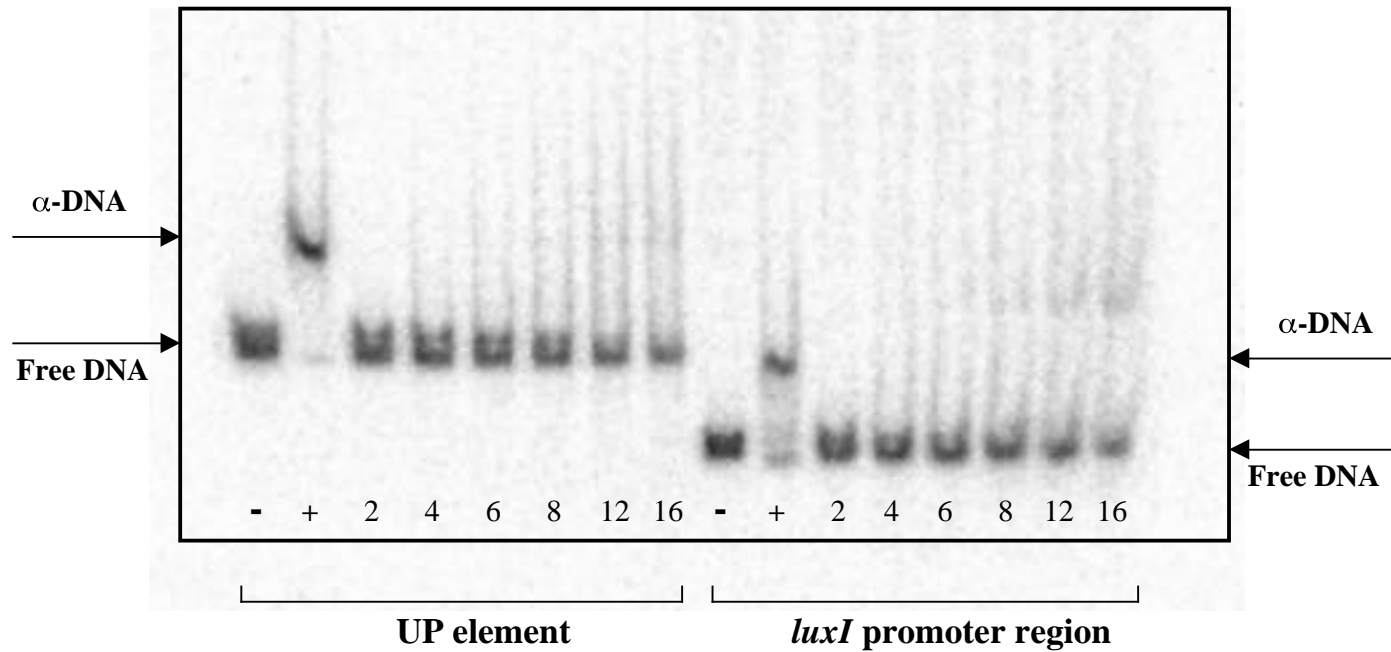


Figure 19: Autoradiogram of gel mobility shift assays with L262A variant. Interactions between purified L262A RNAP and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of variant (μM) used in the assays. The negative control (-) contains only DNA template. The positive control (+) contains 8 μM wild-type. The results shown are representative of two independent trials.

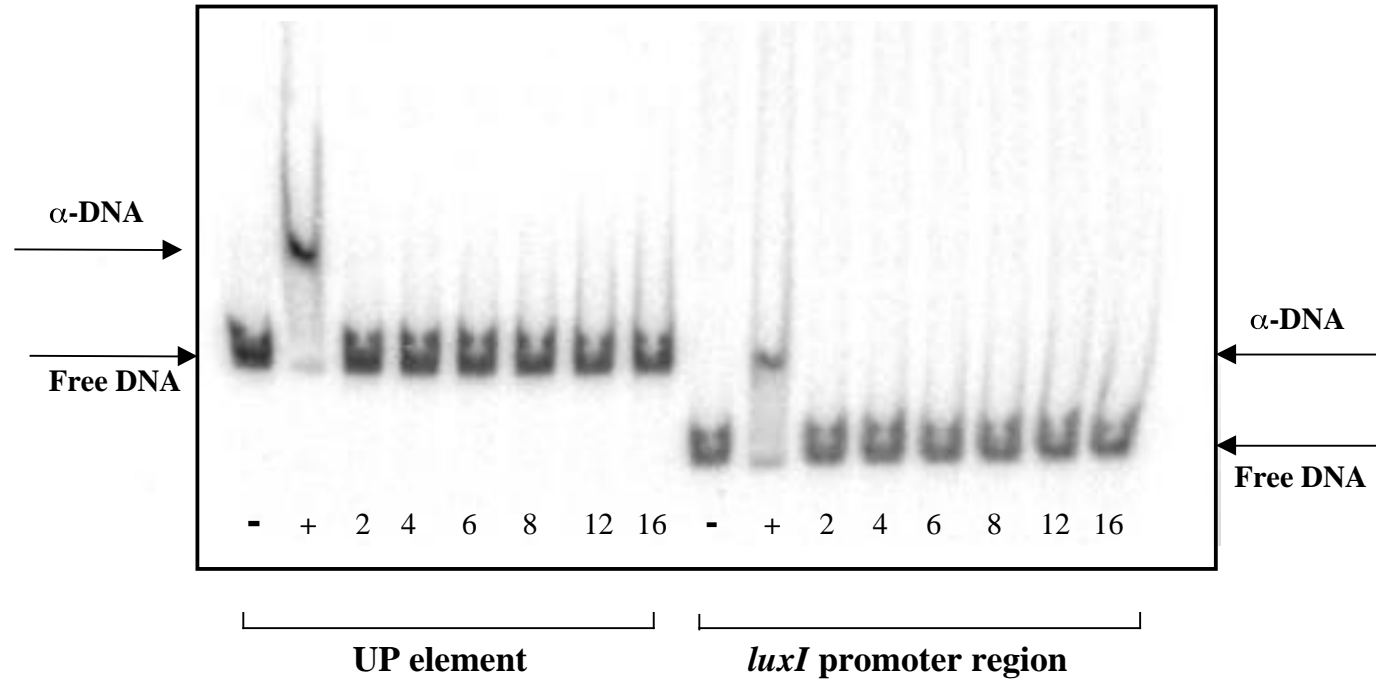


Figure 20: Autoradiogram of gel mobility shift assays with R265A variant. Interactions between purified R265A RNAP and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of variant (μM) used in the assays. The negative control (-) contains only DNA template. The positive control (+) contains 8 μM wild-type. The results shown are representative of two independent trials.

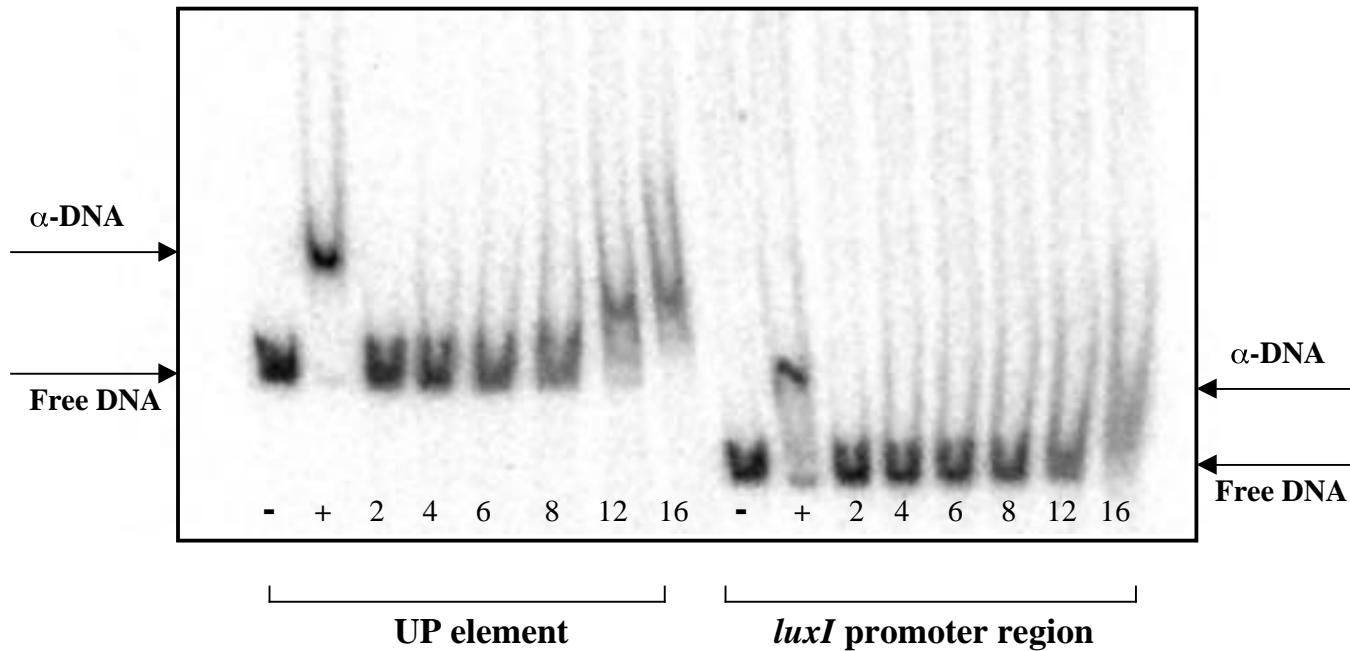


Figure 21: Autoradiogram of gel mobility shift assays with L290A variant. Interactions between purified L290A RNAP and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of variant (μM) used in the assays. The negative control (-) contains only DNA template. The positive control (+) contains 8 μM wild-type. The results shown are representative of two independent trials.

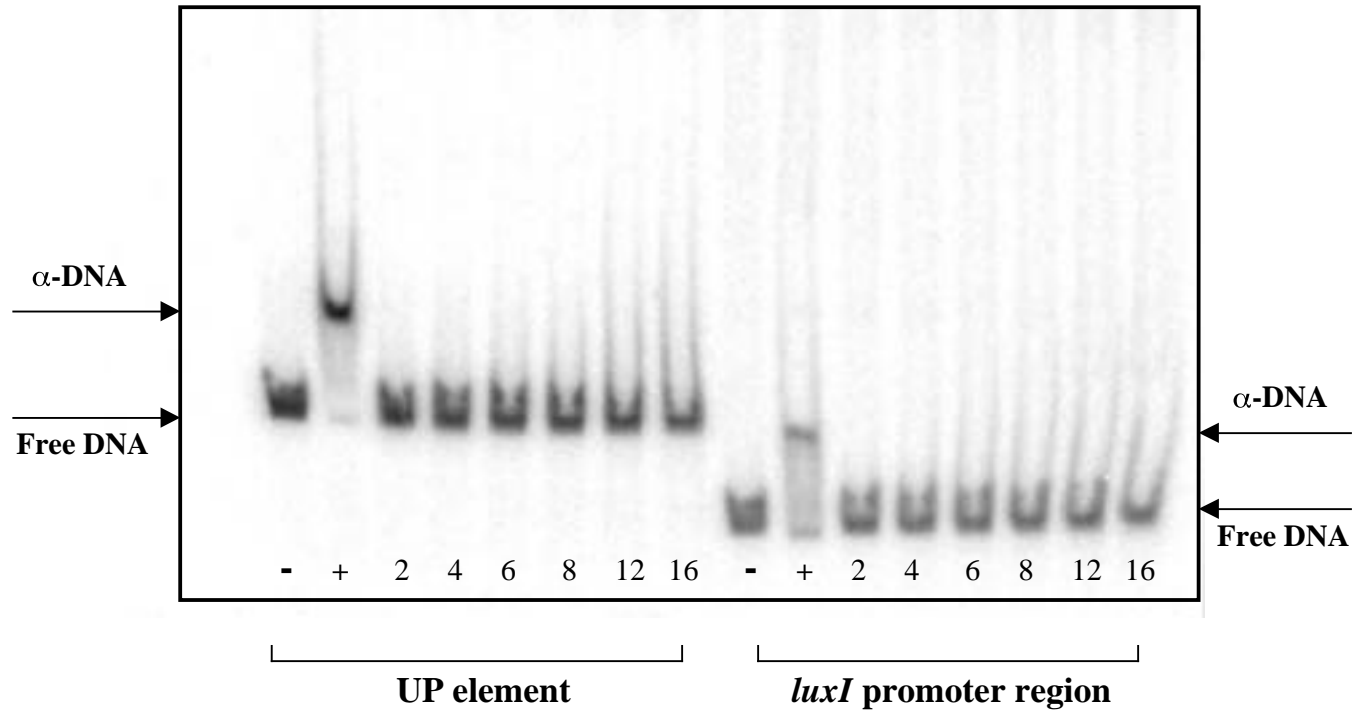


Figure 22: Autoradiogram of gel mobility shift assays with G296A variant. Interactions between purified G296A RNAP and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of variant (μM) used in the assays. The negative control (-) contains only DNA template. The positive control (+) contains 8 μM wild-type. The results shown are representative of two independent trials.

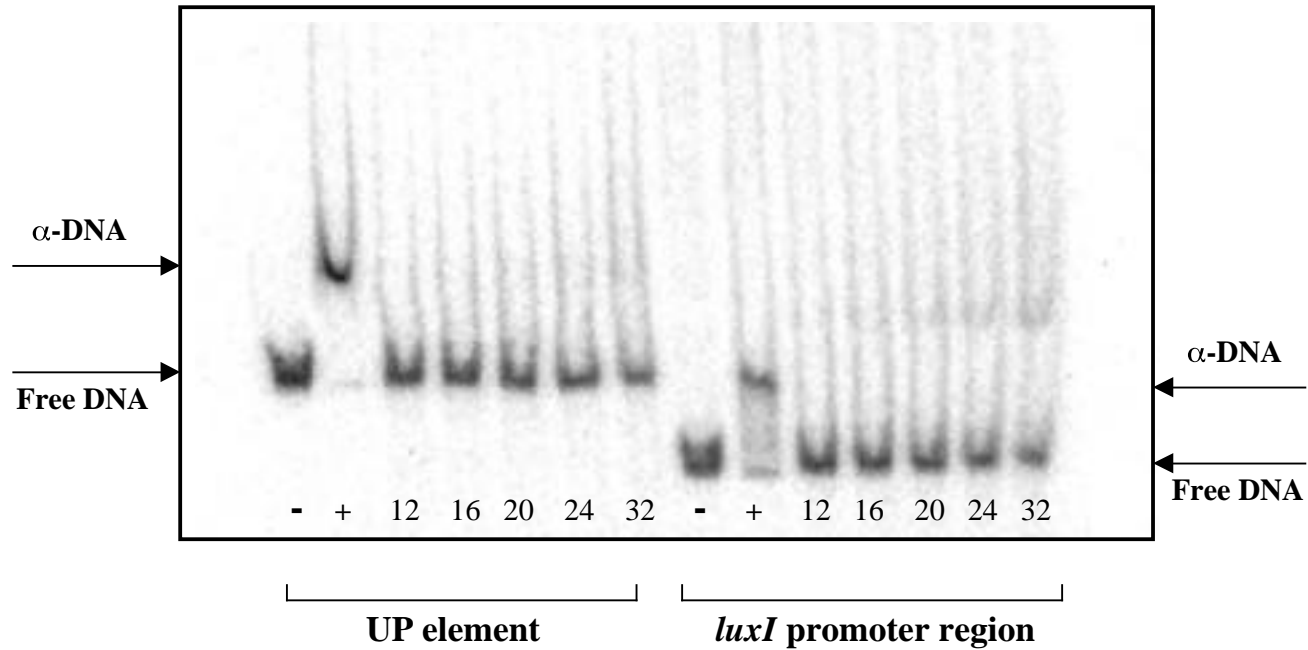


Figure 23: Autoradiogram of gel mobility shift assays with L314A variant. Interactions between purified L314A RNAP and (i) 228-bp PCR fragment containing a strong UP element from the *rrnB* P1 promoter amplified from pRLG4238 and (ii) 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of variant (μM) used in the assays. The negative control (-) contains only DNA template. The positive control (+) contains 8 μM wild-type. The results shown are representative of two independent trials.

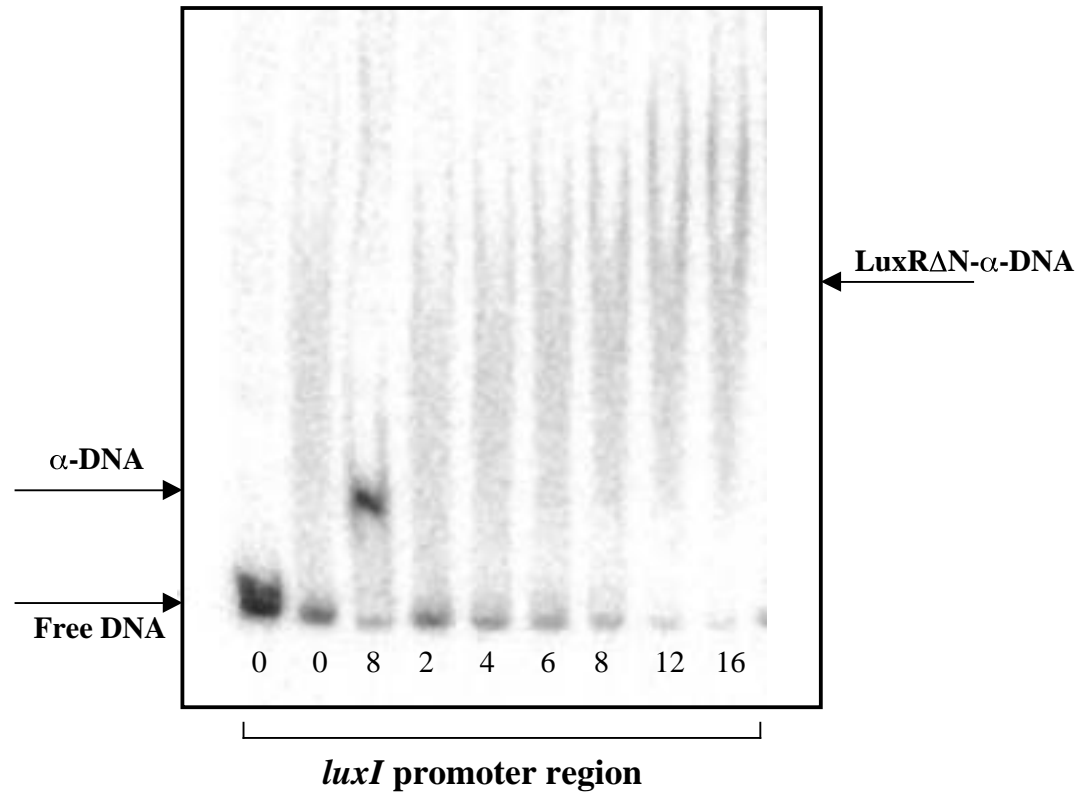


Figure 24: Autoradiogram of gel mobility shift assays with wild-type α subunit in the presence of LuxR N. Interactions between purified wild-type RNAP and the 132-bp PCR fragment with the *luxI* promoter region amplified from pAHF100. The numbers below each lane refer to the concentration of α (μ M) used in the assays. All lanes except 1 and 3 contain 10 μ M LuxR N. The results shown are representative of two independent trials.

DNA fragment observed in the presence of both LuxR^N and wild-type (Figures 16 and 24). The super shift occurred roughly at the same concentration of that was necessary to observe a shift of the DNA fragment with alone (Figure 16 and 24). Therefore, in our *in vitro* system, it appears that is assisting LuxR^N in binding, but LuxR^N does not seem to enhance binding. The LuxR^N-DNA complexes formed did not appear to be stable as evidenced by severe smearing (Figure 16 and 24). Given these difficulties, attempts to determine the effects of the alanine substitutions in on protein-protein interactions with LuxR^N were not extensively pursued.

Nevertheless, the fact that complexes formed between , LuxR^N and the *lux* DNA in the gel mobility shift analysis suggests that is binding to the *lux* DNA fragment in a position allowing it to interact correctly with LuxR^N. While LuxR^N does not bind to the *lux* DNA independently of , it is thought that full-length LuxR does (Egland, K. A., and Greenberg, E. P., 2000). Therefore, LuxR could be necessary in the recruitment of RNA polymerase to the *lux* promoter by positioning the DNA in a way that allows for a more efficient CTD interaction, or via direct protein-protein interactions with the CTD or other regions of RNAP such as region 4 of the ⁷⁰ subunit. Suppressor analysis using positive control mutants in LuxR could be used to define specific protein-protein contacts that are required in our system.

Conclusions

The amino acid residues in the CTD of RNAP that were found to be involved in both LuxR and LuxR^N-dependent transcriptional activation of the *lux* operon *in vivo* are 262, 265, 290, 296 and 314. These residues also appear to play a role in LuxR^N-dependent transcriptional activation *in vitro*. Of those residues we have identified as serving an important role in our system, residues L262, R265 and G296 are located within the DNA binding surface of the CTD (i.e. residues L262, R265, N268, C269, G296, K298, S299; Zou *et al.*, 1992; Tang *et al.*, 1994; Gaal *et al.*, 1996 and Murakami *et al.*, 1996).

Alternatively, other residues we have identified (L290 and L314) are close to a region in the CTD (residues 285-288 and 317) that compromises a determinant thought to make direct contacts with CRP at Class-II type promoters (Savery *et al*, 1998) and that has been shown *in vivo* to be involved in transcription activation by FNR (residues 289 and 317; Lombardo *et al*, 1991) and P2 Ogr (residues 286, 287, 289, 290 and 300; Wood *et al*, 1997) (Figure 25).

R265 and G296 are surface-exposed residues and have been found to be two of the most critical for direct DNA interaction (Gaal *et al*, 1996). L262 also plays a role in DNA binding, however this residue is not surface-exposed and may be involved indirectly due to resultant conformational changes of the CTD. This amino acid could be necessary in accurate protein folding to allow other residues to contact the DNA (Murakami *et al*, 1996). Alanine substitutions at residues 290 and 314 have also disrupted CTD-DNA at both the UP element and the *luxI* promoter. However, CTD-LuxR interactions should not be ruled out as having a possible role at the *luxI* promoter especially given the fact that 290 is still capable of binding to the DNA although at a lower efficiency. Residues 290 and 314 are both natively leucine, an aliphatic molecule with a hydrophobic side chain. Consequently, it does not seem likely that these residues are directly contacting the DNA. If these residues are not involved in making protein-protein contacts with LuxR, they may alternatively be necessary in allowing for either proper conformation or dimerization to occur.

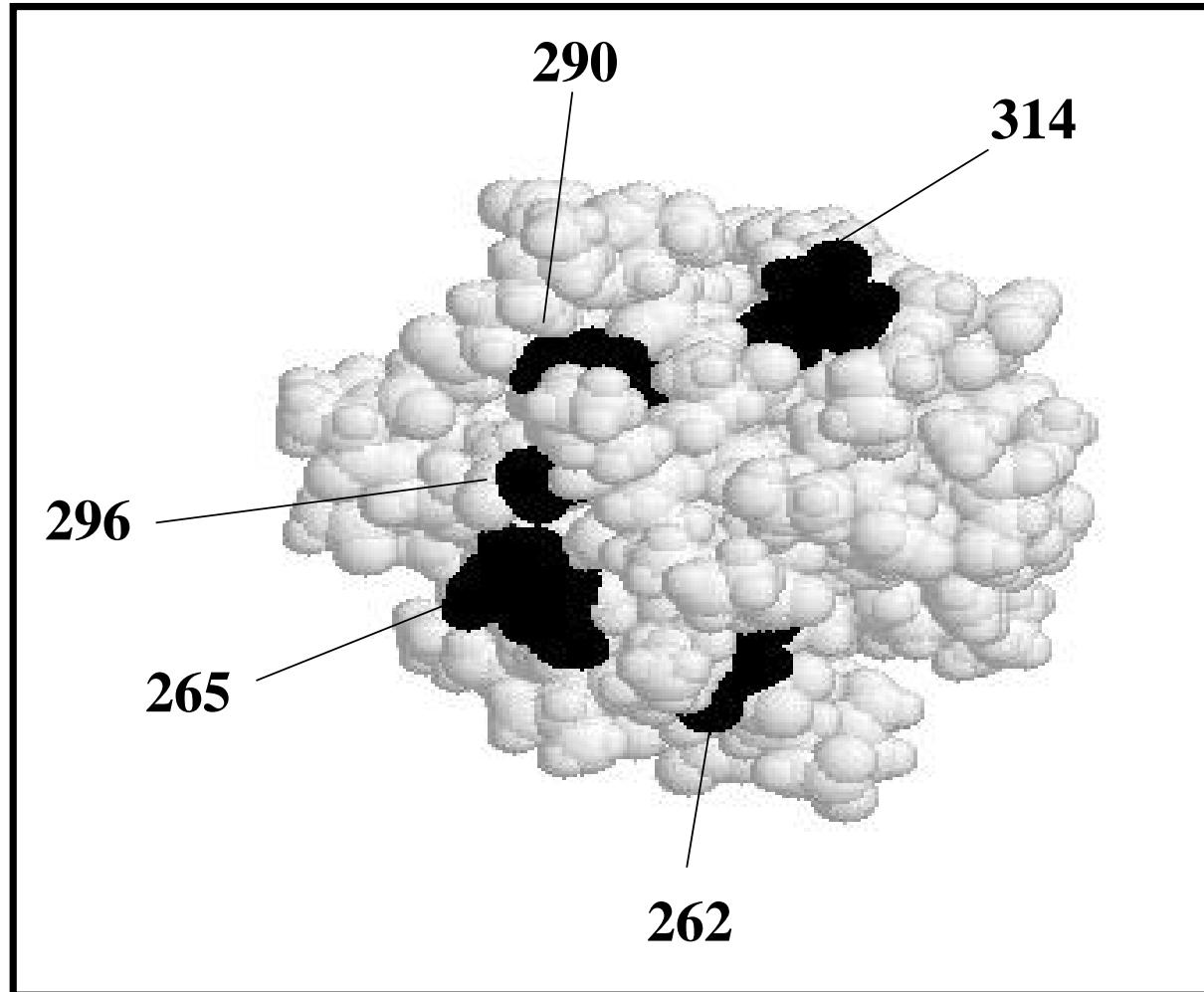


Figure 25: Space-filling model of the RNAP CTD highlighting the position of the five amino acid residues tested in the mobility shift assays (Jeon *et al.*, 1995).

CHAPTER 4

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REFERENCES

Available upon request