

A Mutational Analysis Defines *Vibrio fischeri* LuxR Binding Sites[∇]

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***Vibrio fischeri* quorum sensing involves the LuxI and LuxR proteins. The LuxI protein generates the quorum-sensing signal *N*-3-oxohexanoyl-L-homoserine lactone (3OC6-HSL), and LuxR is a signal-responsive transcriptional regulator which activates the luminescence (*lux*) genes and 17 other *V. fischeri* genes. For activation of the *lux* genes, LuxR binds to a 20-base-pair inverted repeat, the *lux* box, which is centered 42.5 base pairs upstream of the transcriptional start of the *lux* operon. Similar *lux* box-like elements have been identified in only a few of the LuxR-activated *V. fischeri* promoters. To better understand the DNA sequence elements required for LuxR binding and to identify binding sites in LuxR-regulated promoters other than the *lux* operon promoter, we have systematically mutagenized the *lux* box and evaluated the activity of many mutants. By doing so, we have identified nucleotides that are critical for promoter activity. Interestingly, certain *lux* box mutations allow a 3OC6-HSL-independent LuxR activation of the *lux* operon promoter. We have used the results of the mutational analysis to create a consensus *lux* box, and we have used this consensus sequence to identify LuxR binding sites in 3OC6-HSL-activated genes for which *lux* boxes could not be identified previously.**

Quorum sensing and response in bacteria involves the production and detection of diffusible signal molecules. Many species of *Proteobacteria* use acyl-homoserine lactones (acyl-HSL) as quorum-sensing signals (2, 10–13, 22, 28). Acyl-HSL quorum sensing was first described for the marine luminescent bacterium *Vibrio fischeri*, where the seven-gene luminescence (*lux*) operon is activated by a transcription factor called LuxR when it is bound by the quorum-sensing signal *N*-3-oxohexanoyl-L-HSL (3OC6-HSL) (6, 8, 9, 25). The 3OC6-HSL synthase is LuxI (20). The activation of the *lux* operon involves LuxR binding to a 20-base-pair inverted repeat centered at –42.5 from the transcriptional start site of the *lux* operon (5, 7, 25). LuxR is an ambidextrous activator that requires contact with the carboxy-terminal domain of the RNA polymerase α subunit and region 4 of the σ^{70} subunit (7, 16, 23).

For other bacteria, LuxR homologs often bind to promoter elements with sequence similarity to the *lux* box (12). The *Agrobacterium tumefaciens* TraR protein has been studied in considerable detail. A TraR crystal structure has been solved (29), and the *lux* box-like element to which TraR binds has been subjected to extensive mutational analysis (27). The binding of TraR to target DNA and subsequent activation of transcription involves many bases in the binding site. Some bases are involved in direct contact with TraR, some with the ability of TraR to bend DNA, and some with the ability of RNA polymerase to bind to the promoter (27). For LuxR, the available evidence indicates that the two distal bases of the *lux* box are not critical, but little else is known about *lux* box sequence requirements for LuxR binding and transcriptional activation (5).

We recently performed a transcriptome analysis and identified 10 promoters in addition to the promoter of the *lux* operon that are activated by LuxR directly (1). Of these 10, only 1 had an identifiable *lux* box. To begin to understand elements in LuxR binding sites that are required for LuxR-DNA interactions and to develop an understanding of how LuxR might bind to quorum-controlled promoters for which a *lux* box cannot be identified, we performed a mutational analysis of the *lux* box in the promoter of the *lux* operon. This analysis has enabled us to redefine a minimal *lux* box. By using the minimal *lux* box-like sequence, we have identified LuxR binding sites for 3OC6-HSL-activated genes other than those in the *lux* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. We used *Escherichia coli* DH12S (Invitrogen, Carlsbad, CA) cells for cloning and for analysis of mutant *lux* box activities. *Vibrio fischeri* ES114 (4) was used as the source of DNA for amplification of the *lux* operon promoter. *Escherichia coli* cells were grown in Luria-Bertani broth (19) without added sodium chloride at 30°C with shaking, and *V. fischeri* cells were grown at 28°C in L-marine medium (3) with shaking. Plating was on media with 2% agar. Chloramphenicol and kanamycin were used for plasmid maintenance at 25 and 50 $\mu\text{g/ml}$, respectively. Where indicated, we used 3OC6-HSL (Sigma Chemical Co., St. Louis, MO) at a final concentration of 2.3 μM . The 3OC6-HSL was prepared as a concentrated stock in acidified ethyl acetate (100 μl of glacial acetic acid per liter). The stock was added to culture vessels, the ethyl acetate was removed by evaporation under a light stream of nitrogen, and then the culture medium was added.

The *luxR* expression vector used in our experiments was pHV402, which contains *luxR* under the control of its own promoter and contains a chloramphenicol resistance marker (14). We constructed our *lux* box mutant vectors in pPROBE-*gfp*[LVA]. This vector contains a promoterless short-half-life *gfp* and a kanamycin resistance marker (17).

DNA manipulations. For purification of chromosomal DNA, PCR products, and plasmids, we used Qiagen kits (Germantown, MD) according to the manufacturer's procedures. For PCR amplifications, we used an Expand Long Template system (Roche, Indianapolis, IN). We obtained T4 polynucleotide kinase, T4 DNA ligase, and EcoRI from New England Biolabs (Ipswich, MA), and BamHI was obtained from Roche. All primers were synthesized by Integrated

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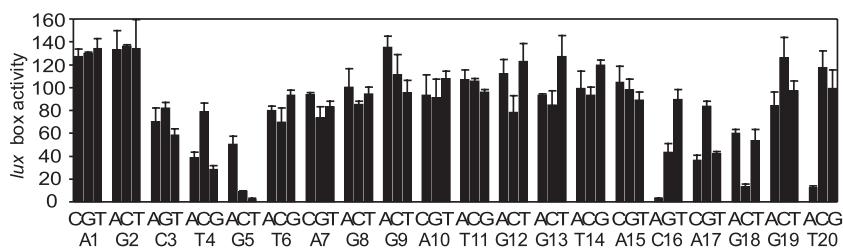


FIG. 1. Effects of single-base-substitution *lux* box mutations on promoter activity. Every nucleotide in the *lux* box was replaced with the three other ones. The sequence of a wild-type *lux* box and the nucleotide numbering system used are shown on the bottom, and activity is expressed as percent of wild-type activity. Experiments were performed with recombinant *E. coli* cells containing the LuxR expression vector pHV402 and a plasmid containing a *lux* promoter-*gfp* transcriptional fusion with a *lux* box nucleotide substitution as indicated. Cultures were grown with 3OC6-HSL. The results represent the means of the results of three independent experiments, and the error bars represent standard deviations from the means.

DNA Technologies (Coralville, IA), and primer sequences are available upon request.

Site-directed mutagenesis and construction of transcriptional fusions. We used site-directed mutagenesis to introduce nucleotide substitutions in the *lux* box of a native *V. fischeri* *lux* operon promoter. Briefly, we used primers containing restriction enzyme sites on their 5' extremities in conjunction with primers that were completely or partially complementary to the *lux* box sequence to amplify the *luxI* promoter. Appropriate primers were used to amplify the upstream and downstream flanks of the promoter. The two flanks were purified and used as templates in a crossover reaction using the external primers. The final PCR products were digested with EcoRI and BamHI and ligated to similarly digested pPROBE-*gfp*[LVA] by using standard techniques (19). The restriction sites for EcoRI and BamHI are located in a multicloning site upstream of a promoterless *gfp*. Promoters cloned into EcoRI and BamHI-digested pPROBE-*gfp*[LVA] will function to initiate *gfp* transcription. The resulting plasmids were then used to transform *E. coli* cells in the presence or absence of the *luxR* expression vector pHV402 by electroporation, as previously described (19). To construct transcriptional fusions containing promoters of non-*lux* genes, a similar method was used. However, amplification of the promoter regions was performed as a single step since no internal primers were used and no nucleotide substitutions were introduced.

DNA sequencing and sequence analysis. The *lux* promoters were PCR amplified and sequenced at either the University of Iowa DNA Facility or the University of Washington DNA Sequencing and Gene Analysis Center. Sequenced promoters were compared to the wild-type *lux* promoter sequence by using ClustalW (<http://clustalw.genome.jp>) (24). All plasmids used in this study possessed *lux* operon promoters that were identical to the wild-type promoter with the exception of the base substitutions introduced.

Analysis of promoter activity in recombinant *E. coli* cells. Plasmids containing transcriptional fusions were introduced into *E. coli* cells in the presence or absence of the *luxR* expression vector pHV402, as indicated in the figure legends. Overnight cultures of the recombinant *E. coli* cells were used to inoculate fresh cultures with or without 3OC6-HSL, as indicated, at a starting optical density (600 nm) of 0.1. When the optical density reached 1.2 ± 0.2 (mean \pm standard deviation), fluorescence was measured with a GENios Pro 96-well plate reader (TECAN, Research Triangle Park, NC). We chose an optical density which corresponds to late-logarithmic or early stationary growth because the differential in fluorescence between active and inactive promoters was at a maximum at this point. We used a fusion of the wild-type promoter to *gfp* as a positive control and the promoterless *gfp* plasmid pPROBE-*gfp*[LVA] as a negative control. The results are the averages of the results of three independent experiments, and the bars in the figures show the range of values obtained.

DNA mobility shift assays. Gel-shift experiments were performed as previously described (25). Specific probes were generated by PCR amplification of promoter regions using the transcriptional fusion plasmids as templates. The nonspecific probe was obtained by PCR amplification of non-*V. fischeri* DNA from the multicloning site of the mini-CTX plasmid (15). Probes were purified and labeled at both ends using [γ ³²-P]ATP (Amersham Biosciences, Piscataway, NJ) and T4 polynucleotide kinase (New England Biolabs). The binding reaction mixtures contained 1 fmol of each probe, LuxR at 7 nM, and 3OC6-HSL at 10 μ M in 20 μ l of DNA binding buffer (20 mM Tris-HCl [pH 7.4 at 22°C], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, and 5% glycerol).

RESULTS

Effects of single- and multiple-nucleotide substitutions on *lux* box activity in recombinant *E. coli* cells. We constructed promoters containing *lux* boxes with single- and double-nucleotide substitutions. These promoters were used to drive *gfp* transcription in recombinant *E. coli* cells. Promoter activity was followed by measuring *gfp* expression in the presence of 3OC6-HSL. In the first analysis, we tested all possible single-substitution *lux* box mutations (Fig. 1). In the presence of LuxR and 3OC6-HSL, most substitutions at most positions were well tolerated. Substitutions at positions 3, 4, 5, 16, 17, and 18 resulted in significant loss of activity, as did a specific mutation of T20 to A. Position T20 overlaps the -35 RNA polymerase binding region (7). Thus, the T-to-A mutation at this position might have affected RNA polymerase binding rather than LuxR activity.

The analysis of single-base substitutions in the *lux* box indicates that positions 3 to 5 and 16 to 18 are important for appropriate LuxR binding but, individually, the other bases are not. To learn more about the nature of the LuxR interactions with positions 3 to 5 and 16 to 18, we analyzed mutant *lux* boxes with double mutations in these critical positions that retained their dyad symmetry (Table 1). The activity of promoters with these symmetrical mutations was greatly reduced compared to the activity of the wild-type promoter or promoters with single substitutions at one of these positions. We believe that this evidence suggests that C3, T4, G5, C16, A17, and G18 represent areas where LuxR interacts physically with the DNA. If a loss of activity in the single-base-change analysis resulted from disruption of dyad symmetry, one would expect that the double mutants in which dyad symmetry was maintained would show higher activity than the single-substitution *lux* box mutants. This was not the case. As a control for these experiments, we examined *lux* boxes with double mutations in bases in which single substitutions did not appear to affect LuxR activity. These mutations also maintained dyad symmetry, and *lux* boxes with these mutations showed substantial activity (Table 1). This is consistent with the idea that specific interactions of LuxR with these bases are not critical for LuxR function.

Does a palindrome with conservation at positions 3 through 5 and 16 through 18 retain activity? To address the question of whether a palindrome with conservation at the critical posi-

TABLE 1. Double mutations in *lux* box critical bases that conserve the palindrome cause severe loss of promoter function

<i>lux</i> box nucleotide substitution(s)	Percent (\pm SD) of wild-type activity
Critical-base substitutions^a	
C3A.....	70 (12)
G18T.....	54 (9)
C3A, G18T.....	2.4 (0.5)
C3G.....	82 (5)
G18C.....	14 (2)
C3G, G18C.....	0.1 (0.4)
C3T.....	58 (6)
G18A.....	60 (3)
C3T, G18A.....	21 (1)
T4A.....	39 (5)
A17T.....	42 (2)
T4A, A17T.....	<0.1
T4C.....	79 (7)
A17G.....	84 (5)
T4C, A17G.....	21 (4)
T4G.....	28 (3)
A17C.....	36 (5)
T4G, A17C.....	<0.1
G5C.....	9 (1)
C16G.....	43 (8)
G5C, C16G.....	0.3 (0.3)
Noncritical-base substitutions	
A7T.....	84 (5)
T14A.....	99 (15)
A7T, T14A.....	67 (12)
A10C.....	94 (18)
T11G.....	96 (2)
A10C, T11G.....	77 (4)
A10T.....	108 (6)
T11A.....	107 (8)
A10T, T11A.....	91 (0.4)

^a The critical bases are positions 3 to 5 and 16 to 18 of the *lux* box.

tions retains activity, we created a single *lux* box with changes in the 14 positions shown by the experimental results depicted in Fig. 1 to tolerate substitutions. The mutant *lux* box was designed such that dyad symmetry was maintained, purines were substituted for purines, and pyrimidines were substituted for pyrimidines. A promoter with this *lux* box showed no detectable activation by LuxR and 3OC6-HSL (Fig. 2). Thus, at least some of the residues in regions flanking positions 3 to 5 and 16 to 18 are important for *lux* box function. The results are in some ways reminiscent of those obtained for the *lux* box-like element to which the *A. tumefaciens* TraR protein binds (27), where bases equivalent to the *lux* box positions 3 to 5 and 16 to 18 make direct contact with the transcription factor and the intervening bases serve as a noncontacted spacer. The spacer region undergoes bending upon TraR binding, and this bending is thought to be important for high-affinity TraR-DNA binding.

Identification of a 3OC6-HSL-independent *lux* box. The data shown in Fig. 1 establish that in the presence of LuxR and 3OC6-HSL, many mutant promoters retain considerable activity. However, these results do not demonstrate that activity is dependent upon 3OC6-HSL. Therefore, we tested the dependence of these promoters on 3OC6-HSL by performing control experiments in which the recombinant *E. coli* strains were grown without added 3OC6-HSL. In almost every case, the

promoter activity was absolutely dependent upon the presence of the signal (data not shown), but there were three notable exceptions. Two of these promoters, one with a C16T *lux* box mutation and the other with a G18A mutation, showed limited activity, and the activity was not only 3OC6-HSL independent, but it was also LuxR independent (Fig. 3A). We suggest that these promoters were better recognized by RNA polymerase than was the wild-type promoter. The more-interesting case was the G19T mutation. The promoter with this mutation was exquisitely dependent upon LuxR, but the LuxR response was 3OC6-HSL independent (Fig. 3A). This is an unexpected phenotype, and we do not have an explanation for how a *lux* box mutation can result in 3OC6-HSL-independent LuxR activity. One idea is that this mutation creates a high-affinity binding target for LuxR. However, the results of in vitro DNA binding experiments are not consistent with this model. The results presented in figure 3B show that binding of purified LuxR to both the wild-type and the G19T promoter depends on 3OC6-HSL. The absence of LuxR binding to the nonspecific probe in the reactions indicates that binding is DNA sequence specific.

Construction of a minimal *lux* box consensus sequence and identification of *lux* boxes for 3OC6-HSL-regulated promoters.

In a previous publication, we used microarray technology to identify 10 non-*lux* transcriptional units that are directly activated by LuxR and 3OC6-HSL (1). Curiously, only one of these transcripts had an identifiable *lux* box promoter element (1). Based on the results of our single-substitution mutational analysis (Fig. 1) we propose that the minimal sequence defining a *lux* box is [N₃]YR[N₁₀]YRDNB, where N is any nucleotide; Y is C or T; R is A or G; D is A, G, or T; and B is C, G, or T. Not all sequences that conform to this set of rules will function as a *lux* box, but we believe that most *lux* boxes will conform to this sequence. We searched the nine LuxR-activated promoters for which we could not previously identify a

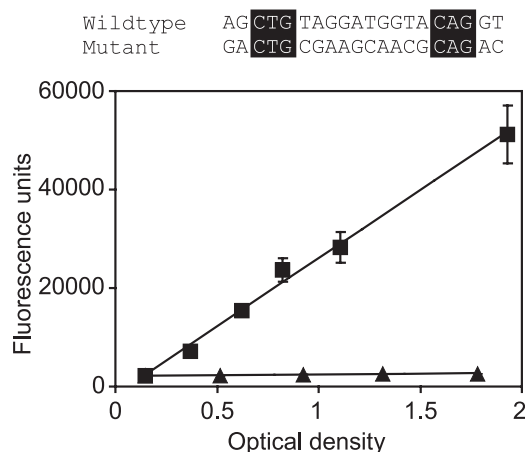


FIG. 2. Activity of a palindromic *lux* box containing substitutions in all nucleotides except those at positions 3 to 5 and 16 to 18. An alignment of the wild-type and mutant *lux* box sequences is shown. The unchanged nucleotides are boxed. Transcription was monitored in recombinant *E. coli* cells containing pHV402 and the appropriate *lux* promoter-*gfp* fusion plasmid. The activities of the wild-type *lux* box (squares) and the mutant *lux* box (triangles) are shown as a function of the optical density of the culture (at 600 nm). The results represent the means of the results of three independent experiments, and the error bars represent standard deviations.

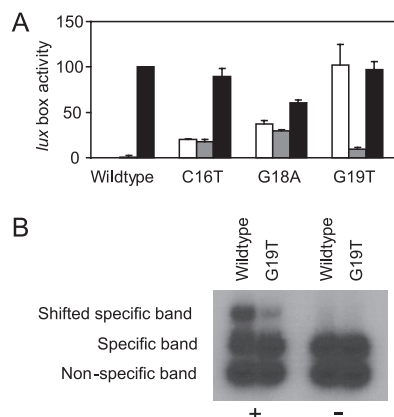


FIG. 3. 3OC6-HSL-independent activity of *lux* box mutants. (A) In vivo transcriptional-fusion analysis of selected *lux* box mutants. Transcription was monitored as green fluorescent protein fluorescence in *E. coli* cells containing a *lux* promoter-*gfp* fusion plasmid with the indicated *lux* box. White bars are results for cells containing the LuxR expression vector pHV402 grown without 3OC6-HSL, gray bars are results for cells without pHV402 grown with 3OC6-HSL, and black bars are results for cells containing pHV402 grown with 3OC6-HSL. Data are presented as percent of the activity in cells with the wild-type *lux* box plasmid and pHV402 grown with 3OC6-HSL. The results are the means of the results of three independent experiments, and the error bars show standard deviations. (B) In vitro binding of purified LuxR to the wild-type *lux* box and the G19T mutant *lux* box with 3OC6-HSL (+) or without 3OC6-HSL (–). Specific probes were generated by PCR amplification of promoter regions using the transcriptional-fusion plasmids as templates. The nonspecific probe was obtained by PCR amplification of the multicloning site of the mini-CTX plasmid (15).

lux box by using the minimal *lux* box sequence described above and Regulatory Sequence Analysis Tools (<http://rsat.ulb.ac.be/rsat/>) (26). Our minimal *lux* box is highly degenerate. So, as expected, multiple sites were found in each promoter region. Thus, we applied the following additional criteria: First, because we learned that mutant *lux* boxes with substitutions in more than one of the critical bases at positions 3 to 5 and 16 to 18 showed little or no activity, we filtered the search results so that a minimum of 5 of these 6 bases were conserved. We then rated the remaining candidate *lux* boxes according to two criteria: (i) the number of bases that formed a palindrome and (ii) the number of bases that were the same as in the wild-type (*luxI*) *lux* box. All promoters searched contained *lux* boxes with at least 8 bases forming a palindrome and 11 bases conserved with the wild-type *lux* box (Fig. 4), and these sequences were studied further.

To test whether putative *lux* boxes were required for LuxR activation of the quorum-sensing-controlled promoters, we generated two promoter fusions to *gfp* for five of the transcriptional units, VF1161, VF1725, VF1978, VFA0090, and VFA1058. One fusion extended from downstream of the ATG codon of the first gene in each unit to immediately upstream of the predicted *lux* box. The other fusion lacked either part of or the entire putative *lux* box (Fig. 5A). We measured promoter activity in recombinant *E. coli* cells with or without a LuxR expression vector. In all cases, there was LuxR-dependent activation of promoters with full *lux* box-like elements and there was not LuxR-dependent activation

VF1161	–124	AACTGTAAAATCGGACAGGT
VF1246	–178	ACCTGTAACCTTCTTACAATT
VF1247	–244	AATTGTAAGAAGTTACAGGT
VF1349	–125	TACTGTAAAATATTACATCT
VF1725	–68	ACCTGTAAAATCTATCAGTT
VF1978	–156	TCCTGTAGTTTATTGCGGCT
VFA0090	–69	AAGTGATATATTTTACAGTT
VFA0622	–264	ACCTGTTTTATTACAGTT
VFA0894	–65	ACCTGTAAAATCAGGAAGGT
<i>luxI</i>	–68	AGCTGTAGGATGGTACAGGT
<i>qsrP</i>	–70	ACCTGTAATAAACGACAGGA

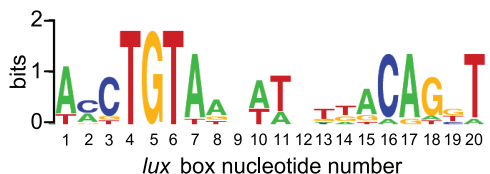


FIG. 4. Predicted *lux* boxes found in the promoters of 3OC6-HSL-activated genes. The distances of the *lux* box elements from the translational start sites of the downstream open reading frames are indicated. The sequence logo shown was constructed based on the *lux* boxes found using WebLogo (<http://weblogo.berkeley.edu/>).

of promoters with deletions in the *lux* box-like sequences (Fig. 5B). It is possible that some of the *lux* box deletions constructed have affected RNA polymerase binding to the promoter because the *lux* box overlaps the –35 region (7). Nevertheless, our data indicate that the regions deleted are required for promoter activity and that the activity of the construct containing the *lux* box-like element is strictly dependent upon LuxR. These data support the view that the elements we identified are involved in LuxR-driven promoter activation.

DISCUSSION

The induction of *V. fischeri* luminescence by LuxR and 3OC6-HSL serves as a quorum-sensing paradigm. We understand many details about how 3OC6-HSL is synthesized by *V. fischeri*, how it exits and enters cells, how it interacts with the transcription factor LuxR, and how LuxR interacts with its binding site in the promoter region of the *lux* operon (10, 22, 28). However, we know relatively little about elements of the binding site, the *lux* box, that is required for LuxR activation of the *lux* operon. We recently learned that a number of genes other than the *lux* genes are activated by LuxR and 3OC6-HSL (1). Although evidence indicates that they are activated by LuxR directly, there is no apparent *lux* box in the promoter regions of most of these genes (1). The systematic mutational analysis described here has revealed information about the roles of different elements in the *lux* box and has allowed us to identify LuxR binding targets in quorum-sensing-activated *V. fischeri* genes other than the *lux* genes.

Our analysis of mutant *lux* boxes with single-base substitutions (Fig. 1) indicates that LuxR makes direct contact with the *lux* box in the regions of nucleotides 3 to 5 and 16 to 18. Single-base substitutions in these regions reduce activity substantially. Residual activity could be a consequence of the affinity of the unaltered half site to interact with one monomer of a LuxR dimer. In support of this, mutants with nucleotide substitutions in both half sites in these critical regions (nucleotides 3 to 5 and 16 to 18), but

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