Identification of the operator of the lux regulon from the Vibrio fischeri strain ATCC7744

(bioluminescence / regulation / autoinduction / repression / activator)

JERRY H. DEVINE, GERALD S. SHADEL, AND THOMAS O. BALDWIN†

Department of Biochemistry and Biophysics, Texas A&M University, and the Texas Agricultural Experiment Station, College Station, TX 77843

Communicated by Charles J. Arntzen, April 10, 1989 (received for review November 2, 1988)

ABSTRACT Escherichia coli that carry a recombinant plasmid bearing the Vibrio fischeri lux regulon express luminescence that mimics the luminescence of V. fischeri. The lux regulon consists of two divergently transcribed operons, the rightward operon (luxCDABE genes) and the leftward operon (luxR gene). The luxR and luxI genes and the control region separating the two operons supply the primary regulatory control over the lux regulon; the regulatory mechanisms result in a dramatic increase in the rate of luciferase synthesis after induction, apparently due to a unique autoregulatory positive feedback mechanism, and in an enormous difference (>10^6) in levels of luminescence in cells before and after induction. The generally accepted model of primary regulation of bioluminescence in V. fischeri involves the interaction of the product of the luxR gene and N-(3-oxohexanoyl)homoserine lactone, the autoinducer produced by the enzyme encoded by luxI, the first gene of the rightward operon, with an operator sequence within the control region to stimulate transcription of the rightward operon in a positive feedback loop. We have used deletion mapping of a transcription reporter vector to determine the approximate location of the operator. By site-directed mutagenesis of the presumed operator, we have demonstrated that the 20-base-pair inverted repeat ACCCTTAGGATCGTA CAGGT (where the vertical line is the center of symmetry), which bears striking similarity to the recognition sequence for the pleiotropic repressor protein LexA, is the operator of the lux regulon. We also found that deletion of sequences upstream of the palindromic leads to increased transcription from the rightward promoter (Pp), indicative of a cis-acting element that represses transcription in the absence of the LuxR-autoinducer complex. Modifications of the palindromic that eliminate stimulation by LuxR-autoinducer of transcription from Pp have no effect on repression by the cis-acting mechanism(s), suggesting that the palindromic is not necessary for repression of the rightward operon. Thus, it appears that the large increase in transcription upon induction of the lux regulon is the result of at least two independent mechanisms, one positive and the other negative.

Luminescence from cultures of marine bacteria, which is strongly dependent on cell density, has been a subject of inquiry for many years (1). Kempner and Hanson (2) ascribed the lag in appearance of luminescence after inoculation of a broth culture to metabolism of inhibitors in the medium. However, later, the lag in appearance of luminescence was shown to be caused by a small dialyzable molecule produced by the bacteria. Nealon and his colleagues (3, 4) called this phenomenon "autoinduction." More detailed descriptions of autoinduction led to the isolation and structural elucidation of the autoinducer of Vibrio fischeri (5). This substance, N-(3-oxo-hexanoyl)homoserine lactone, has been synthesized and shown to function in a biological assay system (5, 6). Investigation of the effect of the synthetic autoinducer on expression of luminescence from a natural isolate of V. fischeri deficient in autoinducer synthesis confirmed that the autoinducer is freely diffusible and effective at low concentrations (7).

The genes required for regulated bioluminescence are located in a regulon that has been cloned from V. fischeri strains MJ-1 and ATCC7744. Light emission from bioluminescent marine bacteria or Escherichia coli carrying the lux regulon results from the activity of the enzyme bacterial luciferase, a flavin monooxygenase (for review, see ref. 8). Luciferase is a heterodimeric enzyme; the α and β subunits are encoded by the luxA and luxB genes, respectively. The aldehyde substrate is supplied by the products of the luxC, luxD, and luxE genes that form a fatty acid reductase complex responsible for the reduction of myristic (tetradecanoic) acid (9). The reduced flavin for the luciferase reaction can be supplied by E. coli enzymes. Through an extensive series of experiments employing transposon insertion mutagenesis, mini-Mud lac fusions, and protein programming in minicells, Silverman and coworkers (10, 11) have delineated the structural organization of the regulon. We have determined the nucleotide sequence of the entire lux regulon from V. fischeri (T.O.B., J.H.D., R. Heckel, T. C. Johnston, and J.-W. Lin, unpublished data). Based on the genetic studies and our sequence data, the physical map of the regulon presented in Fig. 1 is now firmly established.

E. coli that carry the lux regulon express the luminescence genes in a fashion that mimics the regulatory pattern observed in V. fischeri (10). Expression of the lux regulon is modulated by the cAMP-binding protein (CAP)–cAMP system (12, 13), by the autoinducer–LuxR system, and possibly by other regulatory processes superimposed upon these primary systems, including the SOS system and the heat shock response (14).

It has been suggested (10) that the primary level of control of the lux regulon is a positive feedback loop in which basal transcription of luxI leads to accumulation of a low level of autoinducer. The diffusible autoinducer provides a mechanism by which a strong coordinated response can be achieved from a population of cells. If LuxR is present, the LuxR–autoinducer complex stimulates transcription of Pp, the promoter of the rightward operon. Such stimulation would lead to increased levels of LuxI and autoinducer, which would further activate LuxR. Since the autoinducer is freely diffusible, the induction of one cell can lead directly to the induction of others. A positive feedback circuit can generate a large and rapid response to a small initial stimulus, but it is seldom employed in cellular systems because, once it has been initiated, it is difficult to control. Viral systems, however, often employ positive feedback after host-cell stability is no longer required. To prevent the excessive depletion of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.
a cell's resources by a positive feedback response, it is important to have a strong stopping mechanism and to apply this mechanism because the response has drained cellular reserves. It has been suggested that the LuxR–autoinducer complex functions at either the translational (15) or transcriptional (13) level in an autoregulatory fashion to slow production of LuxR, thereby limiting the positive feedback loop.

To understand the network of events that regulate bacterial bioluminescence, we have investigated the location and nature of the operator region of the lux regulon by first constructing deletions of the leftward operon extending into the control region. By supplying synthetic autoinducer and functional LuxR in trans, we tested the ability of the remaining sequences to interact with LuxR–autoinducer to stimulate transcription of the rightward operon. Site-directed mutagenesis confirmed that the operator is the 20-base-pair (bp) inverted repeat in the control region (16). A preliminary report of this work has been presented.6

MATERIALS AND METHODS

Materials. Primers for DNA sequencing were custom synthesized using an Applied Biosystems model 380B DNA synthesizer. Autoinducer, which was synthesized by the procedure of Eberhard et al. (5), was diluted 1:10,000 (wt/vol) in water and stored at 4°C. Cultures were supplemented with autoinducer by a 1:1000 dilution of the stock solution into culture medium. E. coli strain TB1 (17; hsdR, Δlac pro) was used for all expression experiments. The F0 transcription reporter vector, pJHD500 (6.7 kbp, Fig. 2), was constructed in a multistep process by combining 3.2 kbp of pBR322 DNA bearing the colEI origin and the bla ampicillin-resistance gene, 2.3 kbp of Vibrio harveyi luxAB DNA, and 1.2 kbp of V. fischeri DNA containing the intact luxR gene, a portion of the luxI gene, and the regulatory region.

Plasmids pJHD504 and -505 were derived from pJHD501, a variant of pJHD500 in which a single-base change created a unique Kpn I–Asp718 site immediately upstream of the 20-bp inverted repeat in the V. fischeri control region. Plasmids pJHD504 and -505 were constructed from pJHD501 by cutting with Ava I and Asp718 or Kpn I, respectively. The DNA was treated with Klenow fragment of DNA polymerase I and dNTPs, which filled-in the Ava I and Asp718 ends and blunted the 3’ ends generated by Kpn I with its 3’ → 5’ exonuclease activity. The fragments were then religated and used to transform E. coli strain TB1 to ampicillin resistance. Functional LuxR was supplied on the trans-complementing plasmid pAC102, a pACYC177-derived plasmid conferring kanamycin resistance and containing an intact luxR gene.

Site-Directed Mutagenesis. Site-directed mutagenesis was accomplished by a slight variation of the method of Kunkel et al. (18). Single-stranded uracil-containing DNA was used as template for the mutagenesis reactions; template was prepared from phagemid-infected cells rather than from M13 as described by Kunkel et al. (18). Cell Growth and Measurement of Bioluminescence. Growth measurements were initiated by 1:200 dilution of a fresh overnight culture into 40 ml of Luria broth (LB) containing ampicillin (100 mg/liter) in 250-ml flasks. The cultures were shaken at 100 rpm in a waterbath shaker controlled at 27–29°C. At various times, 2-ml aliquots were withdrawn and used immediately for determination of the absorbance at 600 nm. Luciferase activity in 1 ml of each sample was measured using a photomultiplier photometer (19) for which 1 light unit (Lu) = 9.8 × 109 quanta per second referenced to the light standard of Hastings and Weber (20). The substrate used was a freshly prepared suspension of 10 μl of decanal sonicated in 10 ml of LB. Assays in vivo were carried out by rapid injection of 1 ml of the substrate suspension into 1 ml of cell culture and measuring the peak intensity of the emitted light.

Determination of Nucleotide Sequence. Plasmid pHK705 (21), containing the regulatory functions of the lux regulon from V. fischeri strain MJ-1, was the generous gift of Peter Greenberg (University of Iowa). Double-stranded template DNA was prepared from overnight bacterial cultures using the alkaline lysis method (22) followed by treatment of the DNA pellet with RNase A and precipitation with PEG 8000. The

---

Footnotes:

---

**Fig. 1.** Physical map of the lux regulon of V. fischeri strain ATCC7744. kd, kDa.

**Fig. 2.** Plasmid map of the transcriptional reporter, pJHD500. Transcription from the rightward operon can be monitored with ease and sensitivity by measuring the light produced by cells bearing this or related plasmids upon addition of exogenous decanal.
Table 1. Primers used to sequence luxR from V. fischeri MJ-1

<table>
<thead>
<tr>
<th>Message equivalent primers</th>
<th>Primer sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>5’-AATGCCGAGCAGCATTACAG-3’</td>
<td>13–32</td>
</tr>
<tr>
<td>R4</td>
<td>5’-ATCTGTATATTCTAACTTACG-3’</td>
<td>159–158</td>
</tr>
<tr>
<td>R5</td>
<td>5’-GCTGTAATATAAAAAATCTCC-3’</td>
<td>301–320</td>
</tr>
<tr>
<td>R6</td>
<td>5’-GATATTATATATTTTACATGC-3’</td>
<td>442–461</td>
</tr>
<tr>
<td>R7</td>
<td>5’-CGAGAAGGAGACCTGCTGG-3’</td>
<td>585–604</td>
</tr>
<tr>
<td>M13</td>
<td>Reverse primer</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Message complementary primers</th>
<th>Primer sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>R8</td>
<td>5’-CCGCGATTATGTCTTCTATACCC-3’</td>
<td>19–34</td>
</tr>
<tr>
<td>R11</td>
<td>5’-GATTTCTATGGAAGATGTATG-3’</td>
<td>843–825*</td>
</tr>
<tr>
<td>R12</td>
<td>5’-GGGCAATTTGCTCTCGT-3’</td>
<td>737–718</td>
</tr>
<tr>
<td>R13</td>
<td>5’-CTCCGTGTTATAAATCGTG-3’</td>
<td>559–542</td>
</tr>
<tr>
<td>R14</td>
<td>5’-GGGAAATAAACCCAGTG-3’</td>
<td>374–357</td>
</tr>
<tr>
<td>R15</td>
<td>5’-GCTTCACTATTTTATGCTG-3’</td>
<td>202–185</td>
</tr>
</tbody>
</table>

Indicated primers were used to determine the sequence of both strands of the luxR gene of V. fischeri strain MJ-1 and to confirm the published sequence of the gene from strain ATCC7744 (16). The indicated nucleotide positions refer to the numbering system of Fig. 3 in which position 1 is the adenine of the second ATG. Negative numbers (primer R8) refer to positions on the 3’ side of position 1.

*The indicated location of primer R11 is beyond the published sequence of the lux regulatory region (16). The indicated positions are relative to the sequence presented in Fig. 3, as are the other primer locations.

purified plasmid DNA was then denatured with 0.2 M NaOH (23) and used as a template for sequencing by the dideoxynucleotide chain-termination method using modified T7 DNA polymerase (Sequenase) (24) and the primers listed in Table 1.

RESULTS

Nucleotide sequences of the regulatory regions of the lux regulons from V. fischeri strains ATCC7744 and MJ-1 have been reported (16, 25); in our report of the ATCC7744 sequence, we pointed out the existence of a 20-bp palindromic in a position that suggested a protein-binding role, potentially functioning in the regulation of the lux regulon. The reported MJ-1 sequence (25) within the control region was nearly identical to the ATCC7744 sequence, but there were major differences within the luxR coding region. Since major differences between the strains were not expected, we have determined the sequence of both strands of the luxR coding region from plasmid pHK705 (21). The correct sequence6 of the luxR gene from V. fischeri MJ-1 is presented in Fig. 3 and aligned with the published sequence (16) of the same region.

6These sequences have been deposited in the GenBank data base [accession nos. M25751 (for the MJ1 sequence) and M25752 (for the ATCC7744 sequence)].

Fig. 3. Comparison of the nucleotide and encoded amino acid sequences of the luxR genes of V. fischeri strains MJ-1 (upper lines) and ATCC7744 (lower lines). The position marked § in the MJ-1 sequence indicates the limit of the deletion used in the construction of plasmid pHK705 (ref. 21; see text). The amino-terminal sequence of Met-Gly is marked “?” due to the uncertainty regarding the correct translational start for this protein (see text).
from the ATCC7744 strain. Several points are worthy of mention. (i) Only 12 replacements were observed in the corrected MJ-1 luxR gene sequence and only 4 of those resulted in amino acid changes. (ii) Both strains have an in-frame ATG prior to the designated start of the protein. The start of the coding region of LuxR was proposed on the basis of the amino acid sequence of the overproduced protein reported by Kaplan and Greenberg (21). pHK724, the plasmid used to overproduce LuxR, was constructed by deleting sequences upstream of luxR, a process that may have altered or removed the original ribosomal binding signals. The possibility remains that the correct LuxR is initiated at the first ATG.

**Location of the Operator.** We have suggested that the 20-bp inverted repeat within the lux regulatory region, ATCGTAGGATCGTACAGGT (where the vertical line is the center of symmetry), might constitute a protein binding site and thereby serve a regulatory role (16). To test this suggestion, we have constructed deletions of the luxR gene up to and including portions of the palindrome. The effects of these deletions and the ability of functional LuxR added in trans to stimulate transcription of the rightward operon were evaluated by measuring the activity of the *V. harveyi* luciferase produced from the transcriptional fusions. The effects of the deletions on the ability of LuxR-autoinducer to stimulate transcription from the rightward promoter are presented in Fig. 4. Both pJHD504 and -505 displayed increased luciferase synthesis upon addition of autoinducer. The palindrome was intact in the former and lacked the AC sequence from the 5' end in the latter. The construct with the 2-base abbreviated palindrome, pJHD505, suffered a slight but significant (~30%) decrease in stimulation of transcription from $P_R$ by LuxR-autoinducer. The data presented in Fig. 4 demonstrate that sequences upstream of the 20-bp palindrome are not required for autoinducer-dependent transcriptional activation. Interestingly, the uninduced levels of luciferase synthesis from both plasmids were elevated at least 10-fold above the uninduced levels for pJHD500, suggesting either that upstream sequences have a strong repressive effect on transcription from $P_R$ or that there is a promoter within the pBR322 sequences that becomes apparent as luxR sequences are deleted. To discriminate between the two possibilities, the entire *V. fischeri* DNA insert was removed from the reporter vector. Cells carrying the resulting plasmid expressed luciferase activity at a level below the basal level of pJHD500, demonstrating that there is no strong promoter downstream of luxR in the reporter plasmid. It thus appears that upstream sequences in luxR have a repressive effect, in cis, on transcription from $P_R$.

To further evaluate the hypothesis that the 20-bp palindrome is the operator of the lux regulon, the central 12 bases of the palindrome were removed from the reporter plasmid by site-directed mutagenesis to generate plasmid pJHD506. Basal transcription from $P_R$ was repressed in this construction to approximately the level seen in pJHD500, but the system no longer responded to LuxR-autoinducer (Fig. 5). The fact that the basal transcription from $P_R$ was not altered by deletion of the central 12 bp of the palindrome demonstrated that the palindrome is not directly involved in repression of transcription from $P_R$ by upstream sequences (see Fig. 4).

**Construction and Properties of Operator Point Mutants.** As a more subtle probe of the role of the base sequence in the 20-bp palindrome, we generated two point mutants. The guanine at position 5 was changed to a cytosine (op5GC) and the cytosine at position 3 was changed to a thymine (op3CT) so that the mutant palindromes were ACCTCTAGGATCGTACAGGT (op5GC) and ACTCTTAGGAATCGTACAGGT (op3CT). Other than these single-base changes, the constructs were identical to pJHD500. Transcriptional activity from $P_R$ was monitored as described above (Fig. 6). Mutant op3CT appeared to be effectively unresponsive to autoinducer, whereas mutant op5GC showed an ~3-fold stimulation of transcription in response to the autoinducer; transcription from $P_R$ in pJHD500, the wild-type construct, was stimulated ~100-fold by LuxR-autoinducer. These results support the hypothesis that the 20-bp palindrome comprises a major portion of the LuxR-autoinducer binding site.

![Fig. 4](image1.png)

**Fig. 4.** In trans complementation of plasmids pJHD504 and pJHD505. Bioluminescence in vivo of *E. coli* TB1 cells bearing plasmid pAC102 and the deletion plasmids pJHD504 (circles) or pJHD505 (squares) at 28°C in LB containing carbenicillin (100 mg/liter) and kanamycin (25 mg/liter) is shown. Functional LuxR was added in trans by cotransformation with plasmid pAC102 that was constructed by insertion of the luxR/luxI* sequences (see Fig. 2) into the polylinker region of plasmid pAC9. Plasmid pAC9 was constructed by insertion of the *Hae* II fragment containing the polylinker and luxZ' regions of pUC9 into pACYC177 from which the ampicillin-resistance marker had been deleted through removal of a *Pst* I-*Hae* II fragment. Open symbols, cultures grown with autoinducer (AI) added at an OD$_{600}$ of 0.1; solid symbols, cultures grown without autoinducer. LU, light units.

![Fig. 5](image2.png)

**Fig. 5.** Effect of deleting 12 bp within the proposed operator. Cultures of *E. coli* TB1 bearing pJHD500 (squares) or pJHD506 (circles) were grown at 30°C in LB containing ampicillin (100 mg/liter). Open symbols, cultures that contain autoinducer; solid symbols, control cultures. LU, light units.
DISCUSSION

The results of the experiments reported here demonstrate a functional role for the 20-bp palindrome, ACCTGTTAGGA|CTGTACAGGT, located within the regulatory region between luxR and luxI (16). High-level transcription from PR required this palindrome, functional LuxR, and autoinducer.

Deletion of sequences on the 5' side of the palindrome up to and including the 5' AC of the palindrome resulted in an ∼10-fold increased level of transcription from PR in the absence of LuxR-autoinducer, but transcription from PR of the modified plasmid remained responsive to LuxR-autoinducer. LuxR-autoinducer stimulation of PR was effectively blocked by a single-base change, C → T, at position 3 of the palindrome and was greatly reduced by another mutation, G → C, at position 5 of the palindrome. Basal transcription was normal with both mutants as well as with the mutant in which the central 12 bp of the operator were deleted, demonstrating that the operator has no (or minimal) function in repressing transcription from PR in the absence of LuxR-autoinducer. We suggest that the palindrome is the primary binding site from which the LuxR-autoinducer complex functions to stimulate transcription from PR. Sequences upstream of the palindrome appear to function in cis, by an unknown mechanism, to repress transcription of PR in the absence of LuxR-autoinducer, thereby maintaining low basal levels of bioluminescence expression.

The lux regulon of V. fischeri possesses many features that are common to those of well-studied E. coli regulatory units, but its primary mechanism is a unique variation of positive feedback control. The lux regulon consists of two closely linked operons transcribed divergently from a common regulatory control region and governed by a transcriptional activator protein. The araC/BAD, malT-PQ, and asnc-A regulons of E. coli and the cl-cro regulon of phage λ have similar configurations. Of these four systems, only ara and cl-cro have been studied extensively.

The ara and mal systems have the greatest apparent similarity to lux. All three have the genes for their activator proteins isolated on one operon that is activated by the cAMP-binding protein-cAMP system. The similarity between lux and ara also includes autogenous regulation of the activator proteins, LuxR and AraC. Schleif and coworkers (26) have shown protein-mediated DNA looping to be a factor in the repression of uninduced transcription in the araBAD operon. DNA looping has also been invoked in the well-known E. coli lac operon (27). The results that we present here demonstrate the presence of sequences upstream of the primary operator that repress transcription from PR, but it is uncertain at this time if looping is involved in the lux system.

We are grateful to Rob Heckel, Vicki Green, and Mary Treat for their assistance with portions of this work and for their many suggestions. We are also grateful to Drs. Ryland Young and Miriam Ziegel for many helpful discussions and for critical reading of the manuscript. Dr. Robert McGregor of the Texas Agricultural Experiment Station Oligonucleotide Synthesis Facility prepared the oligonucleotides used in this study. This work was supported in part by grants from the National Science Foundation (DMB 85-10784) and the Robert A. Welch Foundation (A865).