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Nucleotide Sequence of the *luxR* and *luxI* Genes and Structure of the Primary Regulatory Region of the *lux* Regulon of *Vibrio fischeri* ATCC 7744†

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ABSTRACT: The regulation of bioluminescence in *Vibrio fischeri* involves both an autoregulatory mechanism and the adenosine cyclic 3′,5′-phosphate/*crp* system. The *lux* regulon from *V. fischeri* strain MJ-1, consisting of two operons, L (left) and R (right), and at least seven genes, *luxR* (L operon) and *luxICDABE* (R operon) and the intervening region, functions in laboratory strains of *Escherichia coli* [Engebrecht, J., Nealson, K., & Silverman, M. (1983) *Cell (Cambridge, Mass.) 32*, 773–781]. The regulatory region, consisting of *luxR*, encoding the regulatory protein, and *luxI*, encoding a function required for synthesis of the autoinducer, and the intervening region of *V. fischeri* strain ATCC 7744 has been cloned and the nucleotide sequence determined. The regulatory protein is an M_r 28 518 polypeptide consisting of 250 amino acid residues; the I protein is an M_r 21 937 polypeptide consisting of 193 amino acid residues. The *luxR* gene, the only known gene of the L operon, is transcribed in the opposite direction to the direction of transcription of the other genes of the *lux* regulon. There are 218 base pairs that separate the 5′ end of the open reading frame of the *luxR* gene from the 5′ end of the open reading frame of the *luxI* gene, the first gene in the rightward operon. In this region, there are both an apparent catabolite repressor protein binding site and an inverted repeat structure that may serve as protein binding sites for the regulation of bioluminescence.

Regulation of bioluminescence in marine bacteria has been a subject of inquiry for many years (Harvey, 1952). The first semiquantitative description of this phenomenon was presented in 1968 by Kempner and Hanson, who ascribed the lag in appearance of luminescence following inoculation of a broth culture to metabolism of inhibitors in the medium (Kempner & Hanson, 1968). Nealson et al. showed that the lag in appearance of luminescence was not due exclusively to inhibitors of the luminescence system but that the bacteria produce and secrete into the medium a compound responsible for induction of bioluminescence, and described this phenomenon as "autoinduction" (Nealson et al., 1970; Rosson & Nealson, 1981). A more detailed physiological and biochemical description of the process of autoinduction led to the isolation and structural elucidation of the autoinducer of *Vibrio fischeri* (Eberhard et al., 1981). This substance, *N*-(3-oxohexanoyl)homoserine lactone, has been synthesized and shown to function in a biological assay system (Eberhard et al., 1981; Kaplan et al., 1985). Investigation of the effect of synthetic autoinducer on expression of luminescence from a natural isolate of *V. fischeri* deficient in autoinducer synthesis confirmed that the autoinducer is both freely diffusable and effective at very low concentrations (Kaplan & Greenberg, 1985).

Following the initial cloning of *luxA* and *luxB* encoding the α and β subunits of luciferase from *Vibrio harveyi* B392 (Baldwin et al., 1984; Cohn et al., 1983), a *Sal*I fragment from *Vibrio fischeri* strain MJ-1 was cloned and shown to express the entire regulon, including genes necessary for the autoinduction phenomenon, in *Escherichia coli* strain ED8654 (Engebrecht et al., 1983). By transposon insertion mutagenesis and polypeptide synthesis in minicells, seven genes in two operons of the *lux* regulon were defined (Engebrecht & Silverman, 1984, 1986). Their organization is shown in Figure 1. The rightward operon contains *luxA* and *luxB*, which encode the α and β subunits of luciferase, as well as *luxC*, *luxD*, and *luxE*, which encode proteins required for synthesis of the aldehyde substrate, and also *luxI*, which encodes a function required for synthesis of the autoinducer molecule. The only known gene in the leftward operon, *luxR*, encodes a regulatory protein.

Earlier investigations showed that catabolite repression played a role in the regulation of bioluminescence (Nealson et al., 1970, 1972; Friedrich & Greenberg, 1983). Dunlap and Greenberg (1985), using a series of *crp* and *cya* mutants in *E. coli*, have supplied compelling evidence that the adenosine cyclic 3′,5′-phosphate (cAMP)[1]/CRP complex is indeed im-

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[1] Abbreviations: CRP, catabolite repressor protein or cyclic AMP binding protein (CAP); cAMP, adenosine cyclic 3′,5′-phosphate; bp, base pair(s); SDS, sodium dodecyl sulfate.

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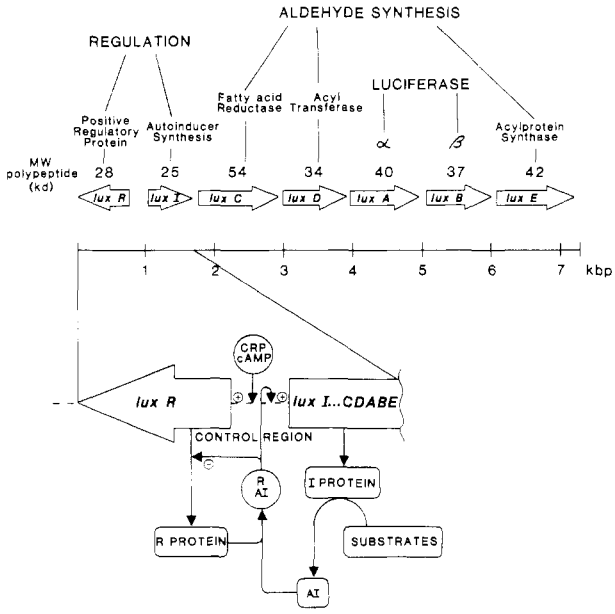

FIGURE 1: Physical map and proposed regulation of the *lux* regulon of *Vibrio fischeri* strain ATCC 7744. The proposed regulatory loops are based on the work of Greenberg and his colleagues (Kaplan et al., 1985; Kaplan & Greenberg, 1985, 1987; Friedrich & Greenberg, 1983; Dunlap & Greenberg, 1985) and of Silverman and Nealson and their colleagues (Engebrecht et al., 1983; Engebrecht & Silverman, 1984, 1986).

portant in regulation of bacterial bioluminescence.

A model has been proposed to describe regulation of bacterial bioluminescence at the level of the regulatory protein/autoinducer (Engebrecht et al., 1983), but this model does not consider the effects of cAMP/CRP. We propose in Figure 1 a model incorporating current evidence concerning the effects of cAMP/CRP. In this model, it is suggested that it is the buildup of cAMP which activates transcription of *luxR*. The regulatory protein interacts with basal levels of autoinducer and, in the presence of cAMP/CRP, potentiates transcription of the rightward operon, allowing further increase in autoinducer concentration and the rapid increase in transcription of luciferase (*luxA* and *luxB*).

Any model for the regulation of expression of bioluminescence in marine bacteria must incorporate both the autoinducer/*luxR* protein system and the cAMP/CRP system. An understanding of the mechanism of regulation of bioluminescence at the molecular level clearly will require knowledge of the structures of the molecules involved. We present here the nucleotide sequence and the encoded amino acid sequence of the *luxR* and *luxI* proteins and the nucleotide sequence of the presumed regulatory region of the *lux* regulon and discuss its relevance to investigations of the regulation of bacterial bioluminescence.

Materials and Methods

Materials. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Boehringer Mannheim. Acrylamide, *N,N*-methylenebis(acrylamide), and urea were from Schwarz/Mann Biotech. DNA polymerase I (Klenow fragment) was purchased from United States Biochemicals. Deoxynucleoside and dideoxynucleoside triphosphates were from Pharmacia. M13 universal and reverse sequencing primers were purchased from New England Biolabs. Additional primers were custom synthesized by using an Applied Biosystems Model 380B DNA synthesizer. All other reagents were of the highest grade commercially available.

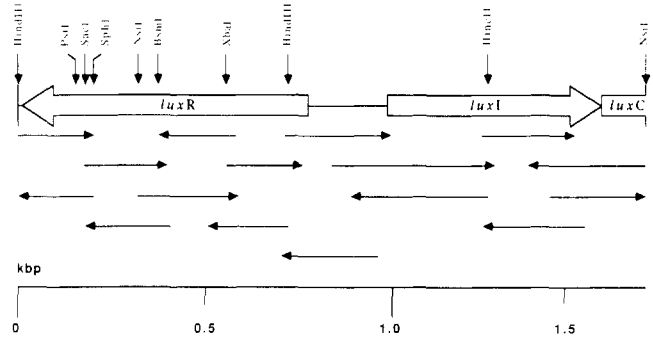

FIGURE 2: Strategy for determination of the sequence of the regulatory region of the *lux* regulon. The arrows indicate the direction and length of the sequence determined. Sequences originating from designated restriction sites were determined from subclones using the M13 universal primer. Sequences originating from other regions were determined by using oligonucleotide primers synthesized as described under Materials and Methods.

Cloning and Sequencing. The plasmid pVfB1 (Boylan et al., 1985), containing the intact bioluminescence regulon from *Vibrio fischeri* strain ATCC 7744, was the generous gift of Dr. E. A. Meighen (McGill University, Montreal). DNA fragments to be sequenced were cloned into either M13 mp18 and mp19 (Yanisch-Perron et al., 1985) or the phagemid vectors pTZ18R and pTZ19R (from Pharmacia) using standard methods (Maniatis et al., 1982). DNA sequencing was performed on single-stranded phage (or phagemid) DNA and double-stranded phagemid DNA using modifications of the dideoxynucleotide chain termination method (Sanger et al., 1977; Hattori & Sakaki, 1986).

Sequence Analysis. Nucleotide sequence data were analyzed by using software from Intelligenetics, Inc. on a Digital VAX Model 750 computer. The secondary structure of the deduced peptide sequence was predicted according to the method of Chou and Fasman (1974, 1978).

Results and Discussion

We report here the nucleotide sequence of a 1684 bp *Hin*dIII–*Nsi*I fragment carrying the regulatory region of the *lux* regulon of *Vibrio fischeri* strain ATCC 7744, the source of the *V. fischeri* luciferase with which most biochemical studies have been performed. Physical mapping of the genes involved in bioluminescence has previously been performed in *E. coli* with a cloned DNA fragment from *V. fischeri* strain MJ-1 (Engebrecht et al., 1983; Engebrecht & Silverman, 1984). We have also determined the nucleotide sequences of the *luxA* and *luxB* genes from both MJ-1 (M. L. Treat and T. O. Baldwin, unpublished results) and ATCC 7744 (J.-W. Lin and T. O. Baldwin, unpublished results) and find 87 differences in 2174 bp (4%) of the *luxA* and *luxB* structural genes between the two strains, indicating that the two strains are indeed closely related. While it is likely that there are differences between the regulatory regions of ATCC 7744 (the strain that we have analyzed) and strain MJ-1, it would appear that the mechanisms of regulation should be directly comparable. The strategy by which the sequence was determined is presented in Figure 2. The entire 1684 bp of sequence presented in Figure 3 were determined from both strands of DNA.

The sequence of amino acids encoded by the *luxR* gene is presented in Figure 4, and the encoded amino acid composition is in Table I. The open reading frame extends 750 bases (from position 770 to position 21 in Figure 3), encoding 250 amino acid residues. The molecular weight of the encoded polypeptide is 28 518, in good agreement with the molecular weight determined by SDS gel electrophoresis of the polypeptide

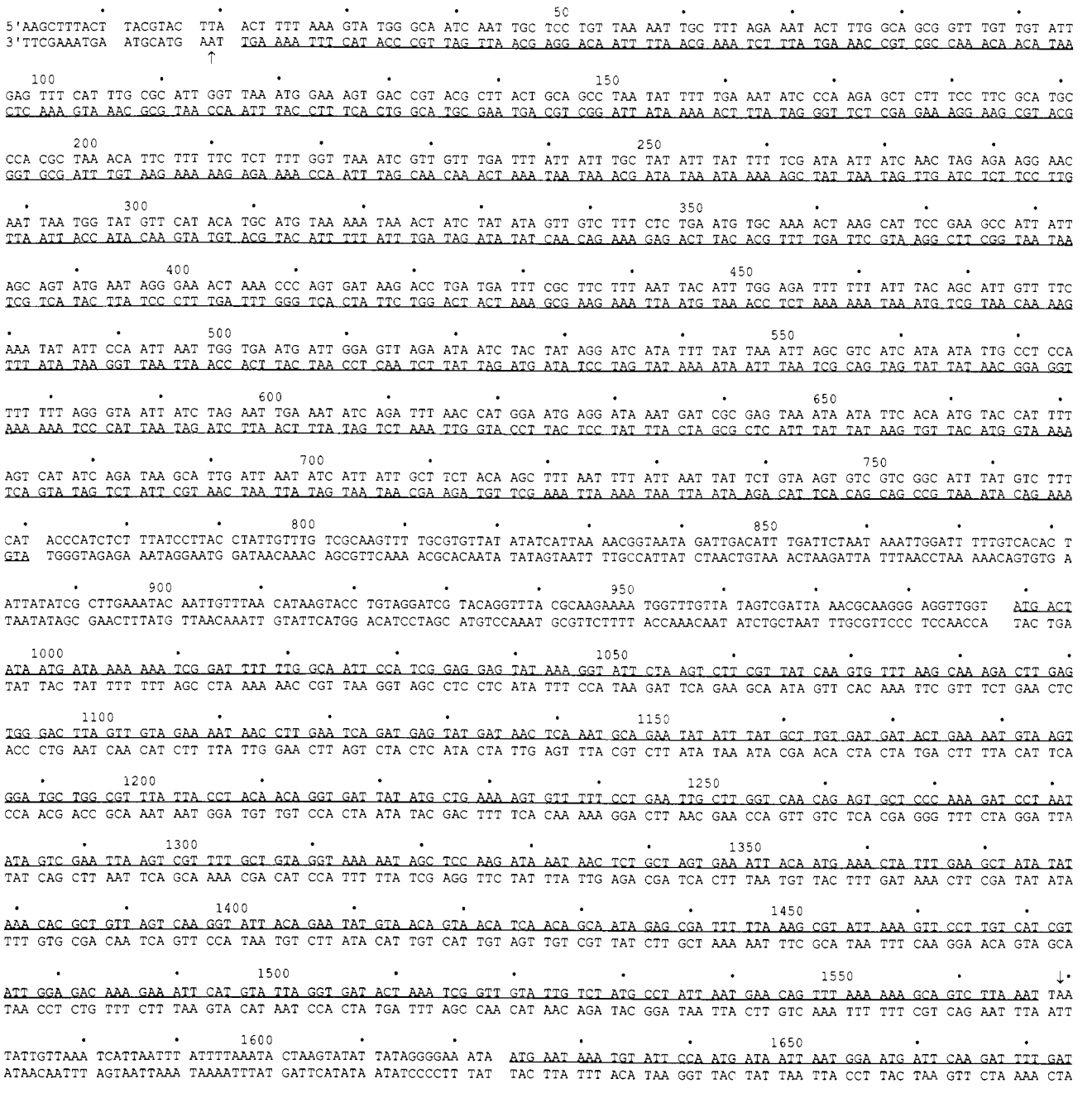

FIGURE 3: Complete nucleotide sequence of the 1684 bp *Hin*dIII–*Nsi*I fragment carrying the regulatory region of the *lux* regulon from *V. fischeri* strain ATCC 7744. The open reading frames encoding the *luxR* protein (bases 770–21) and the *luxI* protein (bases 989–1567) are underlined; the stop codon for each is indicated by an arrow.

product produced in minicells carrying the *luxR* gene (Engebrecht & Silverman, 1986).

The identity of the sequence presented in Figure 4 as that of the *luxR* gene is confirmed by the partial amino acid sequence of the regulatory protein from *V. fischeri* strain MJ-1 reported by Kaplan and Greenberg (1987). The amino-terminal 15 residues were reported and are identical with the sequence reported here, with the exception of position 3, which is aspartic acid in the ATCC 7744 strain and asparagine in the MJ-1 strain. To clarify this apparent discrepancy, we have determined the sequence of the region of plasmid pHK705 (Kaplan & Greenberg, 1987; supplied to us by Kaplan and Greenberg) encoding the amino-terminal region of the regulatory protein. Our results confirm the results of the amino acid sequence determination (Kaplan & Greenberg, 1987) and reveal a single difference between the strains in this region; the GAC codon, encoding Asp at position 3 in the ATCC 7744 strain, was AAC, encoding Asn, in the MJ-1 strain.

The amino acid composition of the *luxR* protein is similar in many respects to other proteins of ca. 28 000 molecular weight. The proportions of hydrophilic, hydrophobic, and neutral residues are similar to those of such proteins as trypsin and subtilisin. While the isoelectric points of the α and β subunits of luciferase, calculated from the amino acid compositions (Cohn et al., 1985; Johnston et al., 1986), are very low, 4.8 and 5.2, respectively, the isoelectric point calculated from the amino acid composition of the *luxR* protein presented in Table I is relatively basic, about 8.6, consistent with its proposed role as a DNA binding protein. Kaplan and Greenberg (1987) found that the *luxR* protein was insoluble

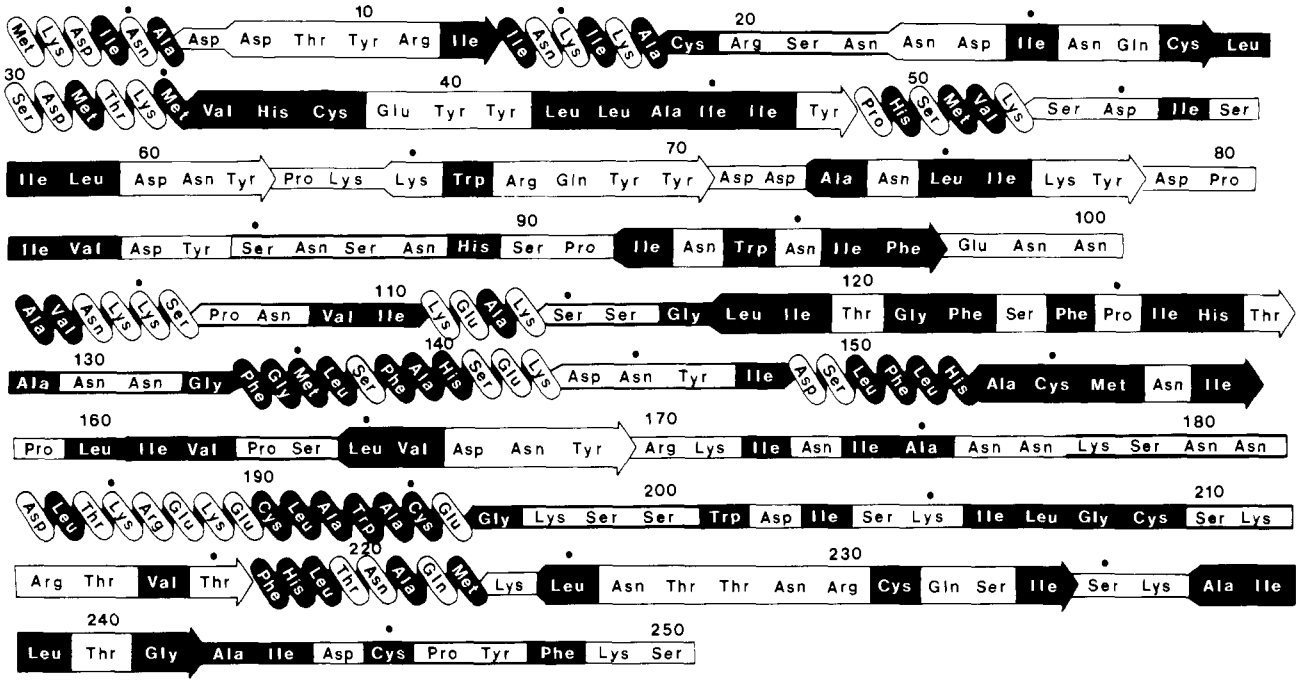

FIGURE 4: Amino acid sequence of the *luxR* protein and the secondary structure determined by the method of Chou and Fasman (1974, 1978). Predicted α-helical regions are given in the helical motif, predicted β-sheet regions are in the broad arrows, turns are designated by heavy lines, and random regions are designated with narrow lines. The residues on white backgrounds are those most often in contact with solvent water; those on black backgrounds are those most often buried in globular proteins (Swanson, 1984).

Table I: Amino Acid Compositions of the *luxR* and *luxI* Proteins

	luxR	*luxI*
lysine	23	16
histidine	7	3
arginine	7	7
aspartic acid	17	10
asparagine	28	11
threonine	11	10
serine	24	17
glutamic acid	7	16
glutamine	4	6
proline	9	7
glycine	7	8
alanine	15	10
cysteine	9	3
valine	8	15
methionine	7	5
isoleucine	26	15
leucine	17	17
tyrosine	12	8
phenylalanine	8	7
tryptophan	4	2
molecular weight	28 518	21 937

if overproduced in *E. coli* and purified the protein by differential solubilization from the cell pellet. The amino acid composition of the protein does not appear to be particularly hydrophobic nor do there appear to be long stretches of hydrophobic residues in the amino acid sequence (Figure 4). It is not uncommon for proteins that are overproduced in *E. coli* to precipitate (Stanley, 1983). For example, overproduction of the σ subunit of *E. coli* RNA polymerase (Gribskov & Burgess, 1983), the *gal* repressor (Majumdaret et al., 1987), and the Tn501 mercuric reductase regulatory protein (O'Halloran & Walsh, 1987) leads to precipitation of the proteins in *E. coli*.

We have analyzed the sequence of the *luxR* protein for secondary structure using the method of Chou and Fasman (Chou & Fasman, 1974, 1978; Figure 4). Regions of high α-helix potential are indicated by the helical motif, regions of probable β structure are indicated by the broad arrows, turns are indicated by heavy lines, and random-coil regions are indicated by lighter lines. Amino acid residues that are more often found in contact with water in globular proteins are indicated on white backgrounds, and residues that are more often buried within globular proteins are indicated on black backgrounds (Swanson, 1984). This treatment does not predict any long α-helical regions or suggest any particularly hydrophobic domains in the protein. There are, however, several interesting clusterings of basic residues, especially residues 10–20, 64–67, 101–114, and 185–188. According to the proposed model of the mechanism of regulation of the *lux* regulon, the *luxR* protein must bind both the autoinducer and DNA. The autoinducer is a hydrophobic molecule and thus is expected to have a hydrophobic binding site on the *luxR* protein. Several DNA binding proteins have been found to display a helix–turn–helix motif within the DNA binding region of the protein (Pabo & Sauer, 1984; Von Hippel, 1979). While the secondary structure proposed in Figure 4 is highly speculative, it is interesting that the region from residues 101 to 114 is proposed to have the helix–turn–helix motif and it also has four lysyl residues within the proposed helical regions. Short of determination of the three-dimensional structure of the *luxR* protein, the best available test of the proposed function of this region of the *luxR* protein in DNA binding is by analysis of the ability of mutants in this region to regulate the expression of bioluminescence.

The sequence of amino acids encoded by the *luxI* gene is presented in Figure 5. The amino acid composition of the encoded polypeptide is given in Table I. The isoelectric point of the *luxI* protein, calculated from the amino acid composition, is ca. 6.1, higher than the p*I* values calculated for the α and β subunits. The molecular weight of the *luxI* protein, 21 937, determined from the encoded amino acid composition (Table I) is somewhat less than the apparent molecular weight determined by SDS gel electrophoresis (25 000; Engebrecht & Silverman, 1986).

Downstream from *luxI* is another open reading frame, separated from the *luxI* stop codon by 53 bp, that is in the

```
                                                10                                  20
Met  Thr  Ile  Met  Ile  Lys  Lys  Ser  Asp  Phe  Leu  Ala  Ile  Pro  Ser  Glu  Glu  Tyr  Lys  Gly

                                                30                                  40
Ile  Leu  Ser  Leu  Arg  Tyr  Gln  Val  Phe  Lys  Gln  Arg  Leu  Glu  Trp  Asp  Leu  Val  Val  Glu

                                                50                                  60
Asn  Asn  Leu  Glu  Ser  Asp  Glu  Tyr  Asp  Asn  Ser  Asn  Ala  Glu  Tyr  Ile  Tyr  Ala  Cys  Asp

                                                70                                  80
Asp  Thr  Glu  Asn  Val  Ser  Gly  Cys  Trp  Arg  Leu  Leu  Pro  Thr  Thr  Gly  Asp  Tyr  Met  Leu

                                                90                                 100
Lys  Ser  Val  Phe  Pro  Glu  Leu  Leu  Gly  Gln  Gln  Ser  Ala  Pro  Lys  Asp  Pro  Asn  Ile  Val

                                               110                                 120
Glu  Leu  Ser  Arg  Phe  Ala  Val  Gly  Lys  Asn  Ser  Ser  Lys  Ile  Asn  Asn  Ser  Ala  Ser  Glu

                                               130                                 140
Ile  Thr  Met  Lys  Leu  Phe  Glu  Ala  Ile  Tyr  Lys  His  Ala  Val  Ser  Gln  Gly  Ile  Thr  Glu

                                               150                                 160
Tyr  Val  Thr  Val  Thr  Ser  Thr  Ala  Ile  Glu  Arg  Phe  Leu  Lys  Arg  Ile  Lys  Val  Pro  Cys

                                               170                                 180
His  Arg  Ile  Gly  Asp  Lys  Glu  Ile  His  Val  Leu  Gly  Asp  Thr  Lys  Ser  Val  Val  Leu  Ser

                                               190
Met  Pro  Ile  Asn  Glu  Gln  Phe  Lys  Lys  Ala  Val  Leu  Asn
```

FIGURE 5: Amino acid sequence of the protein encoded by the *luxI* gene from *V. fischeri* strain ATCC 7744 (see Figure 3).

```
                                                ←luxR
    Thr Asp Asp Ala Asn Ile  Asp Lys Met          SD
3'  TCA CAG CAG CCG TAA ATA CAG AAA GTA TGGGTAG  AGAAATAGGA  ATGGATAACA  AACAGCGTTC  AAAACGCA
5'  AGT GTC GTC GGC ATT TAT GTC TTT CAT ACCCATC  TCTTTATCCT  TACCTATTGT  TTGTCGCAAG  TTTTGCGT

                                                                    •cap binding site
    CA  ATATATAGTA  ATTTTGCCAT  TATCTAACTG  TAAACTAAGA  TTATTTAACC TAAAAACAGT GTGATAATAT  AGCG
    GT  TATATATCAT  TAAAACGGTA  ATAGATTGAC  ATTTGATTCT  AATAAATTGG ATTTTTGTCA CACTATTATA  TCGC

    AACTTT ATGTTAACAA ATTGTATTCA TGGACATCCTx AGCATGTCCA  AATGCGTTCT  TTTACCAAAC  AATATCAGCT
    TTGAAA TACAATTGTT TAACATAAGT ACCTGTAGGAx TCGTACAGGT  TTACGCAAGA  AAATGGTTTG  TTATAGTCGA

    AATTTAGCGT  TCCCTCCAAC  CA    TAC TGA TAG TAC TAT TTT TTT AGC CTA AAA AAC CGT TAA GGT 5'
    TTAAATCGCA  AGGGAGGTTG  GT    ATG ACT ATC ATG ATA AAA AAA TCG GAT TTT TTG GCA ATT CCA 3'
                  SD              Met Thr Ile Met Ile Lys Lys Ser Asp Phe Leu Ala Ile Pro
                                  luxICDABE→
```

FIGURE 6: Sequence of the regulatory region between the left and right operons of the *lux* regulon. The translational starts for *luxR* and *luxI* are indicated by the amino acid sequence above and below (respectively) the double-stranded DNA sequence. Potential promoter sequences (−10 and −35) are indicated for both operons. The potential cap binding site is indicated above the DNA sequence; the underlined sequence extends beyond the limits of the proposed cap binding domain. Two regions of sequence symmetry are indicated by underlining. One region, which contains the suggested −35 sequence of the rightward operon, has a 2-fold symmetry that is indicated by the "x". The second region of sequence symmetry is a true palindrome, in that the sequence symmetry is within each strand, not between strands, as the term palindrome has been used. The function of this 9-base repeat, if any, is not known.

position predicted for the *luxC* gene. The *luxC* gene encodes the reductase component of the fatty acid reductase complex that supplies the aldehyde substrate to luciferase (Boylan et al., 1985). The open reading frame extends 61 bp (20 amino acid residues) to the end of the sequence presented here. The molecular weight of the polypeptide (54 000; Engebrecht & Silverman, 1986) suggests a total of ca. 500 amino acid residues.

Dunlap and Greenberg utilized plasmids with *lacZ* fusions (mini Mud insertions) in *luxR* or *luxC* and a series of *E. coli* mutants with lesions in the genes for adenylate cyclase (Δ*cya*) or cAMP regulatory protein (Δ*crp*, *crp**) to examine the involvement of cAMP/CRP in *lux* regulation (Dunlap & Greenberg, 1985). They demonstrated that in the absence of the regulatory protein, the rightward operon is transcribed at very low (preinduction) levels, suggesting that the *luxR* product is a positive regulator of bioluminescence expression. It was also shown that cAMP/CRP stimulated transcription of the leftward operon (*luxR*) and repressed transcription of the rightward operon. The regulatory protein/inducer function activated transcription of the rightward operon, but fully induced levels of transcription were attained only with intact cAMP/CRP function. The requirement for cAMP/CRP could be overcome, however, if regulatory protein was supplied in trans at very high levels (P. Dunlap and P. Greenberg, unpublished results). It thus appears that cAMP/CRP activates transcription of the leftward operon and facilitates induction of the rightward operon by the regulatory protein. Kaplan and Greenberg determined that only one to two molecules of autoinducer per cell are necessary for the induction response in the presence of the regulatory protein; this amount of autoinducer could easily be supplied by preinduction levels of transcription of the rightward operon (Kaplan & Greenberg, 1985).

The existence of the consensus CRP binding site (deCrombrugghe et al., 1984; see Figure 6) was expected on the basis of earlier work (Dunlap & Greenberg, 1985), and gel retardation assays suggest that cAMP/CRP indeed binds to the

luxR–luxI intergenic region (data not shown). This regulatory scheme resembles that of the *E. coli* arabinose regulon in which a regulatory protein encoded by *araC*, in the presence of the inducer arabinose, acts as a positive regulator of genes encoding proteins for the catabolism of arabinose. Binding of cAMP/CRP to regulatory sites stimulates transcription of *araC* and is further necessary for correct occupation of the *araC* binding sites such that the promoter is accessible to RNA polymerase and thus transcription of *araBAD* (Martin et al., 1986). Why light expression should be under the control of a general regulator of sugar metabolism is a point of curiosity.

Engebrecht and Silverman (1986) suggest that the *lux* regulatory protein represses its own synthesis at the translational level, and sequences necessary for this means of regulation lie within the C-terminal one-third of the structural gene. The 20 bp palindrome centered at −62 with respect to the translational start of *luxI* is a potential site for binding of a dimeric regulatory protein, and bases 459–478 of *luxR* (bases 292–311 of Figure 3) bear striking sequence similarity to the palindrome; 12 of the 20 base pairs are identical. The significance of this apparent duplication with respect to *lux* regulation remains to be investigated.

Added in Proof

After this work was accepted for publication, we were supplied with a copy of a manuscript by Engebrecht and Silverman (1987) that reported the sequence of the same region from *V. fischeri* strain MJ-1 that we report here for *V. fischeri* ATCC 7744. The encoded amino acid sequences of the *luxI* genes are identical, but there are several differences in the *luxR* sequences, although both are 250 residues in length. Four-point mutations resulted in changes in MJ-1 relative to ATCC 7744 of Asp → Asn at position 3 (discussed in this paper), Ser → Thr at position 115, Lys → Glu at position 211, and Ser → Asn at position 250. The ATCC 7744 sequence beginning with residue 20 was Arg-Ser-Asn-Asn-Asp, while the same region from MJ-1 was Arg-Ala-Tyr-Asp. The ATCC 7744 sequence beginning with residue 40 was Tyr-Tyr-Leu-Leu, while the same region from MJ-1 was Tyr-Tyr-Leu-Thr-Leu. We have carefully rechecked the sequence of the ATCC 7744 *luxR* gene and are confident of our assignments.

Acknowledgments

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