Delineation of the Transcriptional Boundaries of the \( \text{lux} \) Operon of \textit{Vibrio harveyi} Demonstrates the Presence of Two New \( \text{lux} \) Genes*

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The 5' and 3' ends of the \( \text{lux} \) mRNA of \textit{Vibrio harveyi}, which extends over 8 kilobases, have been mapped, and two new genes, \( \text{luxG} \) and \( \text{luxH} \), were identified at the 3' end of the \( \text{lux} \) operon. Both \( S1 \) nuclease and primer extension mapping demonstrated that the start site for the \( \text{lux} \) mRNA was 26 bases before the initiation codon of the first gene, \( \text{luxC} \). The promoter region contained a typical -10 but not a recognizable -35 consensus sequence. By using \( S1 \) nuclease mapping, the mRNA was found to be induced in a cell density- and arginine-dependent manner. The DNA downstream of the five known \textit{V. harveyi} \( \text{lux} \) genes, \( \text{luxCDABE} \), was sequenced and found to contain coding regions for two new genes, designated \( \text{luxG} \) and \( \text{luxH} \), followed by a classical rho-independent termination signal for RNA polymerase. \( \text{luxG} \) codes for a protein of 233 amino acids with a molecular weight of 26,108, and \( \text{luxH} \) codes for a protein of 230 amino acids with a molecular weight of 25,326. The termination signal is active \textit{in vivo} as demonstrated by 3' \( S1 \) nuclease mapping, confirming that the two genes are part of the \textit{V. harveyi} \( \text{lux} \) operon. Comparison of the \( \text{luxG} \) amino acid sequence with coding regions immediately downstream from \( \text{luxE} \) in other luminescent bacteria has demonstrated that this gene may be a common component of the luminescent systems in different marine bacteria.

The regulation of luminescence in marine bacteria has been the target of intense investigation over the last few years. Structural genes responsible for light production have been isolated from several strains of luminescent bacteria, including \textit{Vibrio harveyi}, \textit{Vibrio fischeri}, and \textit{Photobacterium phosphoreum} (1). There are five common \( \text{lux} \) structural genes: \( \text{luxC}, \text{D}, \text{E} \) code for the reductase, transferase, and synthetase components, respectively, of a fatty acid reductase complex; and \( \text{luxA} \) and \( \text{B} \) code for the \( \alpha \) and \( \beta \) subunits of luciferase (2-6). The fatty acid reductase complex is responsible for producing an aldehyde substrate which, along with \( \text{O} \) and \( \text{FMNH}_2 \), is necessary for the light-emitting reaction catalyzed by luciferase. An additional gene, \( \text{luxF} \), has been found in the \( \text{P. phosphoreum} \) \( \text{lux} \) operon (7). Although its specific function is unknown, the protein exhibits 30% homology with the \( \beta \) subunit of luciferase.

The mode of regulation of only one bacterial \( \text{lux} \) system, that of \textit{V. fischeri}, has been well documented (4, 8). There are two (left and right) operons involved in the \textit{V. fischeri} \( \text{lux} \) system which are transcribed in opposite directions. The right operon contains the \( \text{luxA} \) gene, which is responsible for producing a small molecule (autoinducer) that causes induction of the luminescence system. This regulatory gene is followed by the five structural \( \text{lux} \) genes, \( \text{luxA-E} \), in the order \( \text{luxCDABE} \). The left operon contains the \( \text{luxR} \) gene which encodes a protein that has been proposed to function as a receptor for the autoinducer. This complex then stimulates transcription of the right operon. A positive feedback loop is therefore established, and autoinduction of the luminescent system is achieved in a cell density-dependent manner. The autoinducers of \textit{V. fischeri} and \textit{V. harveyi} have been purified and identified and have similar chemical structures (9, 10).

Analysis and expression of \textit{V. harveyi} \( \text{lux} \) DNA have shown that the \( \text{luxA-E} \) genes are arranged in the same order, \( \text{luxCDABE} \), as in the \textit{V. fischeri} \( \text{lux} \) system. There is no gene corresponding to the \( \text{luxI} \) gene of \textit{V. fischeri} immediately upstream from the \textit{V. harveyi} structural genes. Instead, the first open reading frame of greater than 40 codons is located more than 630 bases upstream from \( \text{luxC} \). It has the same relative position and orientation as the \( \text{luxR} \) gene of \textit{V. fischeri} (11), but this upstream \textit{V. harveyi} gene does not correspond in sequence to either regulatory \( \text{lux} \) gene of \textit{V. fischeri} (12, 13). The role, if any, of this gene in the regulation of the \( \text{lux} \) operon of \textit{V. harveyi} has yet to be determined. Although the structural genes of the luminescent systems as well as the structures of the autoinducers of both bacteria are comparable, the mechanism of regulation and/or the organization of the \( \text{lux} \) regulatory genes are different. Transposon mutagenesis of \textit{V. harveyi} resulting in \( \text{lux} \)-negative phenotypes has demonstrated that two linked regions of the genome are essential for luminescence (14). Region I contains the \( \text{luxCDABE} \) and \( \text{E} \) genes, whereas region II appears to have a regulatory function, suggesting that the regulatory genes of the luminescent operon of \textit{V. harveyi} are not linked to the structural genes.

The present work defines the boundaries of the transcriptional unit of the \( \text{lux} \) operon of \textit{V. harveyi} which extends over 8 kilobases and demonstrates that the \( \text{lux} \) mRNA is induced during development of luminescence. The 5' and 3' ends of the mRNA have been mapped, and two new \( \text{lux} \) genes, \( \text{luxG} \) and \( \text{luxH} \), have been located at the 3' end of the operon. Moreover, evidence has been obtained that \( \text{luxG} \) is found not only in \textit{V. harveyi}, but also in \textit{V. fischeri} and \textit{P. phosphoreum}.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) M21356.

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EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were from Boehringer Mannheim or Pharmacia LKB Biotechnology Inc. S1 nuclease, avian myeloblastosis virus reverse transcriptase, Klenow, T4 polynucleotide kinase, and T4 DNA polymerase were purchased from Pharmacia. [32P]-labeled nucleotide and deoxynucleotide triphosphates (>3000 Ci/mmol) were from ICN Biomedicals Canada Ltd., and [35S]AMP-S3 (1400 Ci/mmol) was obtained from Du Pont-New England Nucleic.

Growth Conditions and RNA Extraction—V. harveyi was grown in 1% NaCl complex or minimal medium (15) with or without added arginine (1.0 mg/ml). RNA was extracted as described previously (16) from uninhibited cultures of V. harveyi at OD595 = 0.3 and from induced cultures at OD660 = 1.5. RNA was extracted from V. harveyi grown in minimal medium as determined by the point of maximal light emission at OD595 = 0.3. In minimal medium the cells achieve maximum light emission at a lower OD595 value than that reached for cells grown in complex medium.

S1 Nuclease Mapping A modified procedure based on the one developed by Berk and Sharp (18) was used to map the 5' end of the lux mRNA. A SacI-BamHI 0.9-kbp double-stranded (ds) DNA fragment extending into luxC and 5' labeled at the BamHI site with T4 polynucleotide kinase and [γ-32P]ATP (0.02 pmol, 4.4 × 106 cpm/pmol) was coprecipitated with 50 μg of RNA. The pellet was resuspended in hybridization buffer (80% formamide, 40 mM PIPES, pH 6.6, 0.4 M NaCl, 1 mM EDTA), heated to 85 °C for 10 min, then slow cooled to 50 °C for 12-16 h. The hybrid mixture was then diluted to a final volume of 0.3 ml with 30 μl of 10 × S1 nuclease buffer (0.3 M NaOAc, pH 4.6, 1 M NaCl, 10 mM Zn(OAc)2, 50% glycerol) and H2O. S1 nuclease reactions were carried out for 30 min at 37 °C with the indicated amounts of enzyme. Reaction products were phenol extracted, precipitated with ethanol, and resuspended in an 80% formamide, 10 mM NaOH dye solution. Samples were then heat denatured and loaded onto a 6% acrylamide, 7 M urea sequencing gel. Samples were run at a sufficiently high temperature (50-55 °C) so that all secondary structure was eliminated in the urea gel. Under these conditions, the electrophoretic mobility of ssDNA is independent of base sequence, permitting a denaturing gel (17). Consequently, a sequence ladder of M13mp18 served as size standards.

To map the 3' end of the lux mRNA, the protocol just described was followed except that the probe used was a BamHI-Sac1 0.7-kbp dsDNA fragment located about 1.5 kbp downstream from luxE and 3' labeled at the BamHI site with [α-32P]dCTP (0.04 pmol, 8.8 × 106 cpm/pmol). The reaction products were electrophoresed on an 8% acrylamide, 7 M urea sequencing gel. The 3' end of the lux mRNA transcription was determined by a denaturing gel (17). Consequently, a sequence ladder of M13mp18 served as size standards.

Primer Extension Mapping—The 5' end of the mRNA was confirmed using primer extension analysis under conditions described by Lagace et al. (19). The probe, a 100-base Clai-SspI single-stranded (ss) DNA fragment 5' labeled at the SspI site with T4 polynucleotide kinase and [γ-32P]ATP (0.1 pmol, 7.8 × 106 cpm/pmol), was sealed in a glass microcapillary tube with RNA in a total volume of 10 μl containing 10 mM PIPES, pH 6.6, and 0.4 M NaCl. Hybridization was carried out at 55 °C for 12-16 h. The primer was extended with 15 units of avian myeloblastosis reverse transcriptase at 42 °C for 60 min in a total volume of 100 μl containing 50 mM Tris, pH 8.2, 10 mM dithiothreitol, 6 mM MgCl2, and 0.5 mM of the four dNTPs. The reaction products were phenol extracted, ethanol precipitated, resuspended in an 80% formamide, 10 mM NaOH dye solution, heat denatured, and resolved on an 8% acrylamide, 7 M urea sequencing gel.

DNA Sequencing—The sequence of the DNA found downstream from luxE was obtained using the dyeodeoxy chain termination method of Sanger et al. (20) according to procedures described previously (21). The cloning strategy for the downstream DNA is given in the text. Analysis of data was performed using the DNASIS and PROSIS programs of Hitachi Software Engineering Co., Ltd.

RESULTS

A set of polycistronic mRNAs which codes for the lux proteins has been identified in V. harveyi (16, 21), and it has been shown that the mRNAs detected by Northern blot analysis extending across luxD, B, A, and E are induced during development of luminescence. However, the mRNAs starting at luxC are not readily detected, and it could not be determined whether they are induced. Moreover, the exact upstream and downstream termini of the mRNA have not yet been determined.

Localization of the 5' End of the lux mRNA—To elucidate the 5' terminus of the lux mRNA, total mRNA was isolated from V. harveyi, hybridized to a DNA probe encompassing the 5' region of the luxC gene, and treated with S1 nuclease. The probe used was a SacI-BamHI restriction fragment (see Fig. 1), 5' 32P labeled at the BamHI site. As shown in Fig. 2, only mRNA isolated from V. harveyi cells after induction of luminescence partially protected this fragment, demonstrating that the 5' terminus of the induced lux mRNA occurs between the SacI and BamHI sites. Since arginine is known to augment luminescence in minimal medium (22), the S1 nuclease patterns of mRNA obtained from V. harveyi grown in the presence and absence of arginine in minimal medium were also investigated. Only mRNA from cells grown in minimal medium containing arginine protected the DNA probe, and the same sized fragment was obtained as during induction in complex medium. The partially protected DNA fragment was sized on a sequencing gel to determine the 5' end of the mRNA accurately. The S1 nuclease reactions were carried out with 20, 50, 100, and 300 units of enzyme (Fig. 3a). The sharpest band can be seen when 50 units of S1 nuclease are used (lane 2, Fig. 3a), placing the start of the message 16 nucleotides before the initiation codon of the luxC gene. However, start sites ranging from 11 to 26 nucleotides in front of luxC can be measured depending on the amounts of S1 nuclease used. With 20 units of S1 nuclease, a minor band (arrow, Fig. 3a) can be seen corresponding to a start site 26 nucleotides in front of luxC (position +1, Fig. 3c). Because of the somewhat ambiguous nature of the S1 nuclease mapping results, the 5' end of the mRNA was also determined by primer extension mapping (Fig. 3b). A 100-base ssDNA fragment (Clai-SspI) 32P labeled at the 5' end was hybridized to RNA and was extended with reverse transcriptase. The 100-base primer migrated as predicted with respect to the M13mp18 sequence ladder (data not shown), whereas the...
New Genes Encoded by lux mRNA

FIG. 2. Induction of V. harveyi lux mRNA. 5' S1 nuclease mapping was performed with RNA extracted from V. harveyi grown under various conditions using a Sacl-BamHI dsDNA fragment 5' 32P labeled at the BamHI site as the probe and 50 units of S1 nuclease. Lane 1, V. harveyi grown in complex medium and harvested before induction of luminescence; lane 2, V. harveyi grown in complex medium and harvested after induction of luminescence; lane 3, V. harveyi grown in minimal medium; lane 4, V. harveyi grown in minimal medium + 1.0 mg/ml arginine. No S1 nuclease products could be detected when RNA isolated from E. coli was hybridized to the same probe, as shown in lane 5. The DNA sequence of M13mp18 in lanes marked A, C, G, and T is given as a size standard.

primer extension products migrated as a doublet with sizes of 184 and 185 bases. From the size of the larger fragment (Fig. 3b), the 5' end of the message could be assigned to nucleotide +1 (Fig. 3c) in agreement with the results obtained by S1 nuclease mapping using the lowest amount of enzyme. It is interesting to note that an increase in S1 nuclease concentration results in the removal of the AT-rich 5' end by as much as 13 nucleotides. Upon examining the DNA sequence, a putative -10 promoter sequence can be recognized just upstream from the message start site (Fig. 3c). There does not, however, appear to be any recognizable -35 promoter consensus sequence.

Nucleotide Sequence of Two New Genes Found within the lux Operon—Since the mRNAs extend 2-3 kilobases downstream from the luxE gene, the last known gene of the lux operon, the downstream region was sequenced (using the strategy outlined in Fig. 4) in order to determine the specific 3' terminator site and whether or not other lux genes are encoded in this area. The DNA sequence downstream from luxE was found to contain two previously unrecognized genes, luxG and luxH, transcribed in the same direction as luxE (Fig. 5). The first gene, luxG, starts just one nucleotide after the stop codon of the luxE gene, and consequently the Shine-Dalgarno (23) sequence of luxG resides in the 3' end of the coding region of the luxE gene. This gene codes for a protein of 233 amino acids with a molecular weight of 26,108. The second gene, luxH, starts 22 nucleotides after luxG and codes for a protein of 230 amino acids with a molecular weight of 25,326. The first 150 codons of luxG were compared with an open reading frame found just downstream from the luxE gene of a different genera of luminescent bacteria, P. phosphoreum (Fig. 6). A 39% homology found between the amino acid sequences demonstrates that luxG is common to both V. harveyi and P. phosphoreum.

3' End of the lux Operon as Determined by Nucleotide Sequencing and S1 Nuclease Mapping—Analysis of this DNA resulted in the recognition of a putative rho-independent termination signal for RNA polymerase (24) located about 40

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2 R. Soly, unpublished results.
3516 New Genes Encoded by lux mRNA

FIG. 6. Comparison of V. harveyi and P. phosphoreum luxG amino acid sequences. The first 150 and 151 amino acids of luxG from V. harveyi and P. phosphoreum, respectively, were compared and found to contain 39% homology. The numbered positions refer to the V. harveyi luxG sequence. Maximum homology was obtained by inserting a space (-) within the luxG sequence of P. phosphoreum.

nucleotides downstream from the stop codon of luxH (Fig. 7c). It contains a classical GC-rich hairpin loop followed by a string of Ts. In order to determine if this signal is active in vivo, the 3’ end of the lux mRNA was mapped using S1 nuclease. A DNA probe 32P labeled at the 3’ end with Klenow was hybridized to total RNA isolated from V. harveyi cells and found to contain 39% homology. The numbered positions refer to the V. harveyi luxG sequence. Maximum homology was obtained by inserting a space (-) within the luxG sequence of P. phosphoreum.

FIG. 7. Mapping the 3’ end of the lux mRNA. a, 3’ S1 nuclease mapping of V. harveyi RNA using a BamHI-SacI dsDNA fragment 3’ 32P labeled at the BamHI site as the probe. The amounts (in units) of S1 nuclease used are as follows: lane 1, 20; lane 2, 50; lane 3, 100; lane 4, 300. Lane 5 is the control with E. coli RNA using 50 units of S1 nuclease. The DNA sequence reactions (A, C, G, and T) of M13mp18 served as size standards. b, 3’ S1 nuclease mapping of V. harveyi RNA isolated from cells before (lane 1) and after (lane 2) induction of luminescence was performed using the same BamHI-SacI dsDNA probe and 50 units of S1 nuclease. Lane 3 is the control using E. coli RNA. The arrow indicates the protected fragment obtained after S1 nuclease digestion. c, nucleotide sequence found downstream of luxH. Two rho-independent termination signals for RNA polymerase are found on opposite strands and converge end on end. The first one, starting 38 nucleotides after the coding region of luxH, has an energy of -17.6 kcal. The 3’ end of the lux mRNA terminates at one of two nucleotides within the run of Ts found after the hairpin loop, as indicated by the arrows. The second termination signal found in the opposite strand has an energy of -11 kcal and is located 19 nucleotides after the end of an open reading frame that extends at least 400 bp to the right. Nucleotides are numbered starting from the initiation codon of luxH as given in Fig. 5.

Just downstream from the termination signal for the lux operon is found an analogous, rho-independent termination signal in the complementary strand (Fig. 7c). The 3’ termination of an open reading frame that extends at least 400 bp occurs 19 nucleotides upstream from this signal. When a probe (BamHI-SacI, 3’ labeled at the SacI site) was hybridized to RNA isolated from uninduced and induced cultures of V. harveyi, no message could be detected (data not shown). It appears then that the mRNA corresponding to this gene is in low abundance and is not coordinately induced with light production, indicating that this open reading frame is not likely to code for a lux gene involved in the luminescent system. The presence of the two termination signals in opposite strands with converging coding regions at the end of luxH along with in vivo verification of the 3’ end confirms that the end of luxH is the 3’ terminus of the lux operon.
DISCUSSION

In this paper, the transcriptional end points of the mRNA from the lux operon of V. harveyi have been defined, and two new genes encoded by the lux mRNA have been identified. S1 nuclease and primer extension mapping were the two techniques used to map the 5' end of the mRNA and gave identical results providing that the amount of S1 nuclease was carefully controlled. Just upstream from the startpoint a -10 but no corresponding -35 recognition sequence for RNA polymerase could be found. This may suggest that a regulatory protein is required for proper transcription initiation by the RNA polymerase. The promoter region for the right operon of the V. fischeri luminescent system also lacks a -35 consensus sequence and is believed to require a positive regulator for transcription (12).

Previous studies using Northern blots have shown that polycistronic messages of varying lengths exist for the V. harveyi lux operon (16, 21). Although it is clear that the mRNAs extending across the luxI, A, B, and E genes and downstream DNA were induced, those starting at the luxC gene were not readily detected. Nor could it be determined whether or not they were induced during the development of luminescence. By application of S1 nuclease in these experiments, it has been possible to show that the mRNA originating at the luxC gene is indeed induced, consistent with the synthesis of all the proteins within this operon being coregulated during induction of light emission. Similarly, S1 nuclease mapping has shown that arginine causes an increase in the lux mRNA level in V. harveyi grown in minimal medium, indicating that arginine acts to stimulate luminescence at the transcriptional level.

The DNA located downstream of the luxE gene was sequenced in an effort to understand why the mRNA extends beyond the last known gene of the lux operon. Two new genes were found, designated as luxG and luxH. Downstream from luxH, two classical rho-independent termination signals for RNA polymerase on opposite strands and separated by less than 30 bp could easily be identified. The termination signal for the lux mRNA, which has an energy of -17.6 kcal, was confirmed in vivo using 3' S1 nuclease mapping. The other termination signal, with an energy of -11 kcal, is located just after the end of a convergent open reading frame coding for a protein of unknown function. The next best candidate that could exhibit a hairpin loop structure in the downstream region after luxE has an energy of only -6.9 kcal and lies within the luxH gene. The presence of the termination signal immediately after luxH along with the induction of the corresponding mRNA with light production provide strong evidence that the luxG and luxH genes are part of the lux operon.

Elucidation of the functions of the luxG and luxH genes may provide a key to understanding the role of luminescence in bacteria. Homologies between the proteins coded by luxG and luxH genes and sequences of proteins of known functions have not yet been detected. The presence of these genes in free living bacteria is not essential for light production since clones containing only the luxC, D, A, B, and E genes are able to emit light (16, 25), and transposon mutagenesis has failed to produce any Lux-negative phenotypes with insertions in these genes (14). It is possible that luxG and luxH are regulatory proteins, but this is difficult to test because Escherichia coli is unable to support regulated light generated by V. harveyi DNA (16). Preliminary sequence data have shown that V. fischeri contains a homologous gene to luxG at the same relative position in the operon. This result suggests that the luxG gene product is not essential for regulation by autoinduction since clones containing only the luxCDABE and regulatory genes of V. fischeri are able to produce regulated light in E. coli (5). Transposon mutagenesis of V. harveyi (14) has provided further evidence that luxG and luxH are not required for regulation since disruption of the transcription of downstream genes does not affect induction of the reporter gene, β-galactosidase. Comparison of the first 150 amino acids of luxG with an amino acid sequence found just downstream from luxE in P. phosphoreum demonstrated 39% identity. luxG is therefore common to and located in the same relative position in the lux operons of V. harveyi, V. fischeri, and P. phosphoreum. It is possible that luxG and luxH may produce proteins that fine tune the expression or properties of the light-emitting reaction without affecting induction of light production. Alternatively, it is possible that the downstream genes are required for an essential function relating the lux system to the survival and/or symbiosis of luminescent bacteria in the marine environment.

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E Swartzman, C Miyamoto, A Graham and E Meighen

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