Positive Autoregulation of the Vibrio fischeri luxR Gene

LuxR AND AUTOINDUCER ACTIVATE cAMP-CATABOLITE GENE ACTIVATOR PROTEIN COMPLEX-INDEPENDENT AND -DEPENDENT luxR TRANSCRIPTION*

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The LuxR protein is a transcriptional activator involved in regulation of the genes required for bioluminescence (lux) in the marine bacterium Vibrio fischeri. Transcription of the two divergently oriented lux operons (luxR and luxICDABEG) is activated by LuxR in the presence of a diffusible inducer (autoinducer). Transcription of the luxR gene is subject to both positive and negative autoregulation as well as activation by the cAMP-catabolite gene activator protein complex (cAMP-CAP). Transcription of luxR was studied using both luminescence in vivo as a reporter and primer extension analysis of mRNA synthesized in vivo. Mutation of the lux CAP-binding site resulted in a reduction in luminescence from the reporter and the complete loss of luxR positive autoregulation. Positive autoregulation was restored if luxR was provided in trans, demonstrating that LuxR and autoinducer activate luxR transcription in the absence of cAMP-CAP. By means of primer extension analysis, three sites of initiation of luxR transcription were demonstrated; initiation at two of these sites required cAMP-CAP. The quantity of all three transcripts was increased in the presence of LuxR and autoinducer when a plasmid with a wild-type CAP-binding site was used. Initiation at the cAMP-CAP-dependent site was not observed from a plasmid with a mutated CAP-binding site in the presence or absence of autoinducer even with luxR supplied in trans. Instead, with luxR supplied in trans, initiation at the cAMP-CAP-independent initiation site was specifically stimulated by LuxR and autoinducer. Thus, in the course of positive autoregulation, the LuxR protein activates transcription from two luxR promoters by a cAMP-CAP-dependent mechanism and a third promoter by a cAMP-CAP-independent mechanism.

The expression of the genes required for bioluminescence in the marine bacterium Vibrio fischeri, the lux genes, is controlled primarily by a complex set of transcriptional autoregulatory circuits. Since the cell density-dependent induction mechanism, termed autoinduction (Nealson et al., 1970), functions faithfully in Escherichia coli, the regulation of lux gene expression has been studied using the cloned system in E. coli (Engebrecht et al., 1983; Engebrecht and Silverman, 1986). By transposon insertion mutagenesis, the lux genes were shown to be organized in two divergently transcribed operons (Engebrecht et al., 1983). This arrangement was confirmed by DNA sequence analysis (Devine et al., 1988; Engebrecht and Silverman, 1987). The entire nucleotide sequence of the lux regulon from V. fischeri ATCC 7744 has been determined (Baldwin et al., 1989; Swartzman et al., 1990). Autoinduction is mediated by a positive-acting regulatory protein, LuxR, which activates transcription of both lux operons (luxR and luxICDABEG) after binding a diffusible species-specific inducer molecule, N-(3-oxohexanoyl)-homoserine lactone, called autoinducer (Eberhard, 1972; Eberhard et al., 1981; Kaplan and Greenberg, 1985; Shadel and Baldwin, 1991; Shadel et al., 1990b). Activation of both operons requires the luxR operator, located in the control region between the two operons, which is the binding site for the LuxR protein (Devine et al., 1989; Shadel and Baldwin, 1991). The luxR gene is required for the synthesis of autoinducer and is the first gene in the luxICDABEG operon. The luxR gene, which encodes the LuxR transcriptional activator, is located in the oppositely oriented operon. Thus, bidirectional stimulation of transcription of the lux operons by LuxR and autoinducer acts to simultaneously increase the synthesis of both molecules required for high levels of transcription of the luxICDABEG operon, LuxR and autoinducer (Shadel and Baldwin, 1991). This mechanism also leads to the establishment of a dual positive feedback loop since increased transcription of the luxICDABEG operon leads to the production of more autoinducer by increased levels of the LuxI protein, which in turn further activates LuxR and transcription of both lux operons. The end result of this positive feedback loop is the rapid and high level induction of transcription of the genes required for light production (luxCDABE) at a particular cell density, which corresponds to the point in the growth of the culture when autoinducer reaches a critical concentration. This positive feedback system is apparently limited to some degree by a negative autoregulatory loop through which luxR acts to inhibit its own expression. This luxR negative autoregulatory mechanism occurs at the transcriptional level by an autoinducer-dependent mechanism (Dunlap and Ray, 1989) and requires both the luxR operator and a negative regulatory element in the luxICDABEG operon (Shadel and Baldwin, 1991). In addition, a post-transcriptional negative autoregulatory mechanism has been proposed to limit the synthesis of the LuxR protein (Engebrecht and Silverman, 1986).

As pointed out above, the expression of luxR is positively autoregulated by an autoinducer-dependent mechanism...
LUXR Protein Activates Multiple luxR Transcripts

(Shadel and Baldwin, 1991). It is also well established that expression of luxR is activated by the adenosine 3’5’-cyclic monophosphate:catalase gene activator protein complex (cAMP-CAP), thereby placing autoinduction in V. fischeri under catalase repression control (Dunlap and Greenberg, 1985, 1988; Friedrich and Greenberg, 1983). Thus, luxR transcription is subject to the influence of two transcriptional activators. We present here a more detailed analysis of the mechanism of luxR positive autoregulation with emphasis on how the action of these two activators is coordinated in the control region of the lux regulon.

**EXPERIMENTAL PROCEDURES**

Enzymes and Chemicals—Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Life Sciences Inc. Restriction endonucleases and T4 DNA ligase were from New England BioLabs, Promega, and United States Biochemical Corp. Solutions of deoxyenzymes triphosphates (dCTP, dTTP, and dGTP) and adenosine triphosphate (ATP) were obtained from Pharmacia LKB Biotechnology Inc. The [α-32P]dATP (3000 Ci/mmole) was purchased from Du Pont-New England Nuclear.

Strains and Plasmids—The following strains of E. coli were used for measurement of luxR gene expression and RNA isolation: TS1 (hus flas luxZAM15) (Balaban et al., 1984), LE392 (husR516 luxM supE44 supF38 dacrI22 galk22 galT22 trpRS55), and CA8445.1A (HfrH thi dpr4). The strain CA8445.1A was a gift from Dr. Paul V. Dunlap (Woods Hole Oceanographic Institute, Woods Hole, MA). Uracil-containing single-stranded DNA was obtained using the E. coli strain C6256 (dut ung).

The V. fischeri luxR reporter plasmids used in this study are diagrammed in Fig. 1. All of the plasmids are derivatives of pACYC184 and contain the Vibrio harveyi luxA and luxB genes, which encode the α and β subunits of luciferase, located downstream of an intact V. fischeri luxR gene. This arrangement creates a luminescence reporter of luxR transcription. Most of the luxICDABEG operon has been deleted from each of these plasmids, leaving only a truncated luxI gene. This deletion allows the system to be controlled by the addition of synthetic autoinducer to the growth medium. The plasmids pGSI35 and pGSI18 both contain V. fischeri DNA extending from the 3’ end of luxR to a HinII restriction site in luxI, while the V. fischeri DNA in pGS164 extends to an engineered HpaI site near the beginning of luxI (Fig. 1). The plasmids pGSI35 and pGS164 have additional mutations in the lux control region that were introduced by site-directed mutagenesis. The plasmid pGSI35 harbors a 4-bp deletion in the CAP-binding site and pGS164 contains a mutation in the luxICDABEG operon promoter (Fig. 1).

The plasmid pPD749 was used to supply luxR in trans under control of the IPTG-regulated tac promoter and has been described (Dunlap and Greenberg, 1988). This plasmid also encodes a lacS allele and, therefore, luxR is tightly regulated by IPTG since adequate levels of lac repressor are present. The plasmid pPD749 was a gift from Dr. E. P. Greenberg (University of Iowa).

Site-directed Mutagenesis—Site-directed mutagenesis was done essentially by the method of Kunkel (Kunkel et al., 1987) except T4 gene 52 protein was added to the primer extension reaction at a concentration of 10 ng/μl. Uracil-containing single-stranded DNA was isolated from cultures of E. coli CJ236 containing the plasmid pVFS185. This plasmid contains a 3.7-kilobase SacI restriction fragment harboring most of luxR, all of luxI and luxC, and approximately half of luxD inserted into the phagemid vector pTZ18R.

Growth of Cultures and Measurement of luxR Transcription Using Bioluminescence in Vivo—The level of transcription of luxR was determined by monitoring adenosine-5’-triphosphate-luciferase activities and optical density at 590 nm. The growth conditions and selection of strains have been described (Shadel et al., 1990b). The luciferase activity in vivo and cell density (A590) were measured at various times throughout the growth of the culture. The units of luciferase activity are presented in Tables I and II as light units/ml and are related to absolute light intensity by a calibration factor of 1.67 × 107 quanta-s-1 light unit-1 that was determined by the method of Hastings and Weber (1963). So that luciferase activities of cultures could be compared at the same density and measurement of luxR transcription activity and optical density were plotted and the luciferase activity at a specific cell density determined graphically. The random error associated with measurement of luminescence is less than ±10% of the determined value. Plotting the data results in an error reduction; the values presented in Tables I and II are therefore accurate to better than ±10%.

**Isolation of Total Cellular RNA**—Total cellular RNA was isolated as described by Xiong et al. (1991). Cultures of E. coli containing the plasmid vector of interest were grown at 30 °C to an A600 of 0.7–1.2. The cultures were then cooled to 0 °C in an ice bath. Samples were removed from the cultures to 1.5-ml Eppendorf tubes and centrifuged in a microcentrifuge for 1 min to pellet the cells. The volume of the sample (0.9–1.1 ml) taken from cultures was determined so that A600 × ml values of samples to be compared were equal. The cell pellet was resuspended in 200 μl of STET buffer (Maniatis et al., 1982) followed by the addition of 200 μl of phenol:chloroform (1:1). The sample was then heated to 100 °C in a boiling water bath for 40 s and microcentrifuged for 3 min. The aqueous layer was removed to a fresh tube and extracted with 200 μl of chloroform. The aqueous layer was then again removed to a fresh tube and RNA was precipitated by adding 100 μl of 7.5 M ammonium acetate and 1 ml of ice-cold ethanol. After DNAase treatment on ice for 1 h, the samples were then precipitated with ethanol at −20 °C for 1 h and then washed with cold ethanol. The RNA pellet was then dissolved in 50 μl of Milli-Q water.

** Primer Extension of Transcripts Synthesized in Vivo**—The 5’ ends of the transcripts isolated as described above were mapped using AMV reverse transcriptase. An RNA sample (12 μl of the 50-μl stock; 10–15 μg) was placed in a 1.5-ml Eppendorf tube containing 1.5 μl of primer (20 ng/ml), and 2.5 μl of 5 × AMV buffer (0.5 M Tris–Cl, pH 8.3; 50 mM MgCl2; 200 mM KCl). The sample was heated to 100 °C in a water bath and allowed to cool slowly to 25–30 °C. The following were then added to the cooled sample: 2.5 μl of 5 × AMV buffer, 3.5 μl of Q water, 1 μl of deoxynucleoside mixture (25 μM each of dCTP, dGTP, and dTTP), 1.0 μl of [α-32P]dATP (3000 Ci/mmole), and 1.0 μl of AMV reverse transcriptase (4 units/μl). The samples were then incubated at 43 °C for 20 min. The reaction was terminated by the addition of 75 μl of water and extraction with 100 μl of phenol:chloroform (1:1). The aqueous phase was transferred to a fresh tube containing 12 μl of 3 M sodium acetate and 230 μl of ice-cold ethanol. The sample was then incubated at −20 °C for 15 min and then microcentrifuged at 4 °C for 15 min. The nucleic acid pellet was washed with ice-cold 100% ethanol, dried, and resuspended in 8 μl of water and tracking dye, and loaded on an 8% polyacrylamide DNA sequencing gel. A sequencing reaction generated using the same primer used in the corresponding primer extension reaction was also loaded on the gel to serve as a reference for mapping the ends of the primer extension products. The template DNA used had a BigIII site (AGATCT) that had been introduced for other purposes. The wild-type sequence, AAAAACT, is given in Fig. 3 with the sequence of the primer extended DNA indicated below in parenthesis for help in reading the gels.

Two primers were used in the primer extension reactions (R8 and PLMP1) and their locations are referenced relative to the deoxy- nucleoside sequence numbering scheme given in Devine et al. (1988). Both primers correspond to the message sequence immediately upstream of luxR. The R8 primer (5’-CGGACATTATGTTCCTTATA-3’) hybridized at the beginning of the luxR coding region corresponding to nucleotides 752–771 and the primer PLMP1 (5’-CTTTATCTTTAGCTTTC-3’) hybridized in the lux control region just upstream of the luxR coding region corresponding to nucleotides 779–796.

The yield of RNA and the yield of the primer extension products were not independently quantitated. We assume that the yield of each transcript should be the same for a particular extraction and that the relative yield of primer extended products from each transcript would likewise be the same. The primers and primer extension reactions were repeated at least twice for each aliquot of cells, and primer extension reactions were repeated with the different samples of RNA for each aliquot of cells. The critical comparisons were between cells that were grown in the presence of autoinducer and those grown in its absence. The differences in transcriptional activities were large enough to make the comparisons straightforward.

1 The abbreviations used are: CAP, catalase gene activator protein; AMV, avian myeloblastosis virus; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
RESULTS

The location of the CAP-binding site was originally proposed based on the knowledge that transcription of luxR is subject to CAMP-CAP activation (Dunlap and Greenberg, 1986; 1989) and the nucleotide sequence of the regulatory region of the lux operon (Engelbrecht and Silverman, 1987; Devine et al., 1988). The ability of the CAMP-CAP complex to bind to this site was later demonstrated by footprinting of the complex with DNA (Shadel et al., 1990a). The discovery that LuxR-autoinducer could activate transcription of luxR upon binding to a site upstream of the CAP-binding site (Shadel and Baldwin, 1991) posed obvious questions regarding the interplay of CAMP-CAP and LuxR-autoinducer in stimulating transcription of luxR.

Mutation of the lux CAP-binding Site Reduces luxR Transcription in Vivo—A mutation which was intended to remove the ability of CAMP-CAP to activate luxR transcription was introduced by site-directed mutagenesis into the luxR reporter vector pGS138. This mutation consisted of a 4-base pair alteration of the consensus recognition element for CAP (Fig. 1). The plasmid harboring the CAP-binding site mutation (pGS135) was otherwise identical to pGS138. The results presented in Table I demonstrate that the CAP-binding site mutation caused a 6-fold reduction in the level of luminescence observed compared to the wild-type control (pGS138). These results also demonstrated a functional role for the CAP-binding site in vivo which was previously identified by DNA sequence comparison to other known CAP-binding sites and DNase I footprinting in vitro (Devine et al., 1988; Shadel et al., 1990a). Results identical to those in the pGS135 experiment above were obtained using the wild-type luxR reporter (pGS137) in an E. coli crp strain that is devoid of CAP protein (Shadel, 1991), indicating that the CAP-binding site mutation in pGS135 completely abolished activation of luxR transcription by CAMP-CAP.

Positive Autoregulation of luxR Occurs Independently of CAMP-CAP Activation—The mechanism of LuxR positive autoregulation was investigated in the context of potential interaction with the CAMP-CAP activation system. It was of interest to distinguish whether activation by LuxR was independent of CAMP-CAP activation or if the presence of the intact CAMP-CAP system was required.

The ability of LuxR to stimulate transcription of luxR in the absence of CAMP-CAP binding was studied using the plasmid pGS135 which contained a mutated CAP-binding site (Fig. 1) and the plasmid pPD749 as a source of luxR in trans under control of the IPTG-regulated tac promoter. When autoinducer alone was added to cultures of E. coli transformed with pGS135 and pPD749, no increase in reporter activity was observed (Table II). However, when both autoinducer and IPTG were added, a significant increase in luminescence was observed (Table II). These results were markedly different from those obtained using the wild-type luxR reporter (pGS138), which exhibited a 3-fold stimulation of reporter activity when autoinducer alone was added to the culture and a 6-fold increase in reporter activity when both IPTG and autoinducer were added (Table II). The increase in the presence of autoinducer alone was due to LuxR produced from the wild-type luxR promoter on pGS138. Interestingly, the observed level of luminescence from the vector with the mutant CAP-binding site (pGS135) in the presence of IPTG and autoinducer was virtually the same as the level of luminescence observed from the wild-type vector in the absence of autoinducer and IPTG. Thus, in the absence of CAMP-CAP-dependent luxR transcription (pGS135), LuxR, provided in trans by IPTG induction, and autoinducer effectively substituted for CAMP-CAP and increased the level of transcription to the normal CAMP-CAP-activated level seen in the wild type.

Two Major luxR Transcripts Are CAMP-CAP-dependent—Primer extension analysis was used to map the 5' end of the luxR mRNA synthesized in vivo and to determine if the CAMP-CAP-regulated promoter was indeed the promoter activated by LuxR and autoinducer. Two major sets of transcripts were observed when RNA isolated from E. coli containing a plasmid with a wild-type luxR promoter (pGS138; Fig. 2, lanes 3 and 4) was used in a primer extension reaction that were not observed when RNA from E. coli harboring the CAP-binding site mutated plasmid (pGS135; Fig. 2, lanes 1 and 2) was used (Fig. 2, compare lanes 2 and 4). These sets of transcripts are labeled 1 and 2 in Fig. 2. Addition of
autoinducer to the cultures caused an increase in the level of both of these cAMP-CAP-dependent transcripts, 1 and 2 for the wild type (pGS138; compare lanes 3 and 4), but not for the mutant (pGS135; compare lanes 1 and 2). In addition, a third set of transcripts was induced by the presence of autoinducer and is labeled 3 in Fig. 2 (see Fig. 2B). The observed initiation sites for the various mRNA molecules were mapped using the corresponding DNA (with the AAAACT to AGATCT BglII alteration) sequencing reactions as a reference and are shown in Fig. 3. The 5′ end of transcript 2 corresponded to the identical initiation site for luxR mRNA mapped by other investigators (Engelbrecht and Silverman, 1987). However, transcript 1 was consistently the major cAMP-CAP-dependent transcript observed in our primer extension analyses, and it mapped to a position 6–8 nucleotides closer to the beginning of the luxR coding region (Fig. 3). Transcript 3 mapped to a position 7–10 nucleotides upstream of transcript 2 (Fig. 3).

In the Absence of cAMP-CAP Activation, LuxR and Autoinducer Stimulate luxR Transcription from a Promoter Distinct from the cAMP-CAP-Regulated Promoter—Since stimulation of luxR transcription by LuxR and autoinducer was also observed in the absence of cAMP-CAP-dependent activation (Table II), it was of interest to determine which of the identified transcripts were increased by the stimulatory action of LuxR and autoinducer under these conditions. A growth experiment was performed that was identical to that presented in Table II with the plasmid pGS164 used as the source of the wild-type luxR promoter and LuxR supplied in trans under control of the IPTG-regulated tac promoter. Total RNA was prepared from these cultures and used for primer extension analysis. The results of this analysis are presented in Fig. 4. One complicating factor in this experiment was the ability of the primer used in these reactions to hybridize to those transcripts synthesized from the luxR-overproducing plasmid pPD749, resulting in detection on the gel of additional extension products corresponding to the IPTG-induced transcripts from this plasmid. A separate growth experiment was performed in which IPTG alone was added to a culture of E. coli containing pGS135 and pPD749, so that only the IPTG-induced transcripts were expressed. The gel containing the primer extension products observed in this experiment is not shown, but based on the results of this experiment the locations of the IPTG-induced products from pPD749 are indicated by the arrows in Fig. 4. The IPTG-induced transcripts partially obscured the other cAMP-CAP-dependent transcript normally observed from the wild-type luxR promoter (transcript 2) and also made it difficult to determine if other specific luxR transcripts were induced by LuxR and autoinducer.

For the wild type (pGS164), the major cAMP-CAP-dependent transcript (transcript 1) was observed in the absence of IPTG and autoinducer and was increased by the addition of IPTG and autoinducer (Fig. 4, compare lanes 1 and 2). These results are comparable to those obtained using pGS138 as the source of wild-type luxR promoter in the presence and absence of autoinducer (Fig. 2). With pGS135 (mutant CAP-binding site; Fig. 4, lanes 3 and 4), the major cAMP-CAP-dependent transcripts were not observed with or without IPTG and autoinducer. Therefore, we may conclude that the stimulation of light production was dependent upon the presence of the wild-type luxR promoter and LuxR supplied in trans under control of the IPTG-regulated tac promoter.

**Table I**

<table>
<thead>
<tr>
<th>luxR reporter plasmid</th>
<th>luxR promoter mutation</th>
<th>Light units/ml</th>
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<tbody>
<tr>
<td>pGS138</td>
<td>None (wild type)</td>
<td>6.0</td>
</tr>
<tr>
<td>pGS135</td>
<td>4 bp mutation in CAP site</td>
<td>1.0</td>
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</tbody>
</table>

* Light units/ml values are from 1 ml samples of a culture of LE392 (A660 of 1.0).

*The mutation consists of a 4-bp change in the lux CAP-binding site (see Fig. 1).

**Table II**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Light units/ml</th>
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<tbody>
<tr>
<td></td>
<td>-AI</td>
</tr>
<tr>
<td>pGS138/pPD749</td>
<td>4.0</td>
</tr>
<tr>
<td>pGS135/pPD749</td>
<td>0.5</td>
</tr>
</tbody>
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* Values represent the peak light intensity emitted from a culture of LE392 at an A660 of 1.3. Autoinducer and IPTG were added to the cultures to a final concentration of 500 nM and 1 mM, respectively.

**Fig. 1.** Primer extension analysis of RNA isolated from E. coli LE392 containing lux plasmids with or without a mutated CAP-binding site. Primer extension reactions were done as described under "Experimental Procedures" using primer R8. RNA was isolated from samples of cultures with (+) or without (−) 1 μM autoinducer present from the beginning of the growth experiment. A, autoradiogram of the primer extension products obtained from this experiment. Lanes 1 and 2 contain primer extension reactions using RNA isolated from LE392 with pGS135 (mutant CAP-binding site). Lanes 3 and 4 contain primer extension reactions using RNA isolated from LE392 with pGS138 (wild-type control region). Also shown are sequencing reactions using the same primer used for the primer extension reactions and purified plasmid DNA containing the lux control region. Note that the plasmid DNA used for the sequencing markers contained a BglII site so the sequence AGATCT is apparent rather than the wild-type AGTTTT (see Fig. 3). The three most abundant transcripts observed are labeled 1, 2, and 3 in the figure. The sequence of the DNA is also given at the right edge of the figure for comparison with Fig. 3A. B, autoradiogram of the same primer extension gel as in A that was overexposed to more clearly show the three luxR transcripts.
of luxR transcription observed in the pGS135 growth experiment (Table II) was not occurring from the cAMP-CAP-dependent initiation sites.

Since stimulation of luxR transcription by LuxR and autoinducer was not affecting the cAMP-CAP-dependent transcripts in the absence of binding of cAMP-CAP (from pGS135 with the mutant CAP-binding site, lanes 3 and 4 of Fig. 4), it was assumed that the observed LuxR/autoinducer-stimulated transcription from pGS135 reported in Table II must be due to another promoter that was responsive to LuxR and autoinducer. It also seemed likely that the third set of transcripts observed in the wild type (pGS138, transcript 3; Figs. 2 and 3) might be produced from this promoter. In order to test this hypothesis, the primer extension reactions were performed using a different primer which would not hybridize to the IPTG-induced transcripts from pPD749 and therefore would be specific for mRNA originating only from the natural luxR promoters. The addition of IPTG and autoinducer to a culture of E. coli TB1 containing the vector with the mutant CAP-binding site (pGS135) and the IPTG-inducible luxR in trans (pPD749) caused the induction of a set of transcripts (Fig. 5, lanes 3 and 4) that corresponded exactly to the third set of transcripts observed for the wild type (pGS138; Fig. 2, lanes 3 and 4, and Fig. 3). The identical set of transcripts was observed when the same experiment was done using pGS164 (wild-type CAP-binding site) in an E. coli crp strain (Fig. 5, lanes 1 and 2). Thus, in the absence of cAMP-CAP activation, LuxR and autoinducer activate transcription from a previously unidentified promoter that directs initiation of transcription at a point located upstream of the initiation sites for the cAMP-CAP-dependent transcripts (transcript 3 in Fig. 3).

**DISCUSSION**

It has been long appreciated that autoinduction of bioluminescence in many marine bacteria is subject to catabolite repression (Nealon et al., 1972; Friedrich and Greenberg, 1983). In *V. fischeri*, the effects of catabolite repression are mediated through the activation of luxR transcription by the cAMP-CAP regulatory system (Dunlap and Greenberg, 1985, 1988). Transcription of luxR has also been shown to be both positively and negatively autoregulated (Engbericht and Silverman, 1986; Dunlap and Greenberg, 1988; Dunlap and Ray, 1989; Shadel and Baldwin, 1991). The elucidation of the positive autoregulatory mechanism for luxR transcription which occurs in addition to its role in regulation of the “rightward” lux operon (luxCDABECD) demonstrated that LuxR can stimulate transcription in a bidirectional fashion and that luxR transcription is influenced by two transcriptional activators, LuxR/autoinducer and CAP/cAMP (Shadel and Baldwin, 1991). In the current study, inactivation of the CAP-binding site in the lux control region allowed the effects of the cAMP-CAP regulatory system to be abolished and facilitated a more detailed analysis of the luxR positive autoregulatory mechanism.

Mutation of the lux CAP-binding site resulted in a substantial decrease in the observed level of luminescence from a luxR luminescence reporter vector and the complete loss of two major cAMP-CAP-dependent luxR transcripts as judged by primer extension analysis of mRNA synthesized in vivo (Table I and Fig. 2). These results demonstrate a functional role for the lux CAP-binding site in vivo which was previously suggested by DNA sequence comparison and characterized by DNase I footprinting in vitro (Devine et al., 1988; Shadel et al., 1990a). Positive autoregulation of luxR was lost as a result of a mutation in the CAP-binding site, but was restored when
a high level of LuxR protein was supplied \textit{in trans} to the CAP site-mutated plasmid (Table II). These results demonstrate that levels of LuxR necessary to activate \textit{luxR} transcription in the presence of autoinducer are not attained in the absence of cAMP-CAP and that \textit{luxR} positive autoregulation can occur by a cAMP-CAP-independent mechanism.

Primers extension analysis of \textit{luxR} transcripts produced \textit{in vivo} has allowed a more detailed examination of \textit{luxR} positive autoregulation. By using plasmids containing an intact CAP-binding site, \textit{luxR} transcripts were observed to initiate from three regions of the \textit{lux} control region (Fig. 3). One of these transcripts, designated transcript 2, had a 5’ terminus that corresponded to the initiation site of a \textit{luxR} transcript observed by Engebrecht and Silverman (1987) (Fig. 3). In addition, we consistently observed two other major \textit{luxR} transcripts, the most abundant one (transcript 1) beginning at a point 6–8 base pairs downstream of transcript 2 (Fig. 3). The third transcript, designated transcript 3, was present at much lower levels than transcripts 1 and 2 in the absence of LuxR and autoinducer (Fig. 2). All three of these transcripts were increased in response to LuxR and autoinducer (Fig. 2). A different pattern of transcripts was observed with RNA isolated from \textit{E. coli} containing a plasmid with a mutant CAP-binding site. Transcripts 1 and 2 were no longer observed in the presence or absence of autoinducer even with \textit{luxR} supplied \textit{in trans} and were therefore designated as cAMP-CAP-dependent transcripts. With \textit{luxR} supplied \textit{in trans}, transcript 3 was the only transcript observed to increase in the presence of autoinducer when the CAP-binding site was mutated or when RNA was isolated from an \textit{E. coli} \textit{crp} strain containing a wild-type reporter vector (Fig. 5). Several sequences were found in the \textit{lux} control region that exhibited similarity to the consensus sequences for \textit{E. coli} −35 and −10 promoter elements (Fig. 3). However, it is not possible, based on sequence comparison alone, to define which of these elements function as promoters responsible for initiation of the three \textit{luxR} transcripts. This is especially true since many sequences were found that could possibly function as the −10 element of a promoter (Fig. 3). In fact, multiple −10 elements would explain the observation of multiple transcripts from each of the three promoters.

A model describing positive autoregulation of the \textit{luxR} gene is presented in Fig. 6. In the presence of cAMP-CAP, all three transcripts are induced by LuxR and autoinducer (Fig. 6A), while in the absence of cAMP-CAP, only transcript 3 is induced (Fig. 6B). Thus, activation by LuxR and autoinducer occurs at one promoter by a cAMP-CAP-independent mechanism, while activation at the other \textit{luxR} promoters is cAMP-CAP-dependent. These results suggest at least two possible mechanisms for transcriptional activation by LuxR during positive autoregulation. In one case, the dependence of LuxR activation on cAMP-CAP at the cAMP-CAP-dependent promoters could be the result of a direct influence of LuxR on cAMP-CAP (e.g., by facilitating the binding of cAMP-CAP to the CAP-binding site). Given this situation, the activation by LuxR at the cAMP-CAP-independent promoter in the absence of cAMP-CAP would necessarily be occurring by a different mechanism than activation at the cAMP-CAP-dependent promoters. Another possibility is that cAMP-CAP is simply required for recognition of the cAMP-CAP-regulated promoters by RNA polymerase and LuxR is activating transcription of both the cAMP-CAP-dependent and -independent \textit{luxR} promoters by the same, more general mechanism (e.g., by effecting a localized increase in the concentration of RNA polymerase at the \textit{lux} control region).

The existence of a cAMP-CAP-independent mechanism for \textit{luxR} positive regulation may provide a means for autoinduction of \textit{lux} gene expression to occur under relatively good conditions.
nutrient conditions (high glucose). In the presence of glucose, activation of luxR transcription by cAMP-CAP would not be expected, consistent with the observed delay in the onset of luminescence in the presence of glucose (Dunlap and Greenberg, 1985). Under such conditions, the existence of a weak cAMP-CAP-independent promoter would allow luxR transcription to continue at a low rate and thus allow for the eventual accumulation of LuxR in the cell. If autoinducer has accumulated, signalling the presence of a critical concentration of V. fischeri, the dual positive feedback mechanism would be engaged and induction of the light producing enzymes would occur. The observation that luminescence in cultures of an E. coli crp strain containing the intact lux regulon increases in response to autoinducer demonstrates that LuxR does accumulate in the absence of CAMP-CAP (Dunlap and Greenberg, 1985).

CONCLUSIONS

Transcription of the V. fischeri luxR gene initiates at three sites in vivo. The synthesis of two of these transcripts is dependent on cAMP-CAP while synthesis of the third is not. In the presence of CAMP-CAP all three of the luxR transcripts increase in response to LuxR and autoinducer. In the absence of CAMP-CAP only the CAMP-CAP-independent transcript increases in response to LuxR and autoinducer. Therefore, positive autoregulation of the luxR gene involves induction of three luxR transcripts, induction of two of these transcripts requiring the presence of CAMP-CAP and induction of the third transcript occurring both in the presence and in the absence of CAMP-CAP.

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