Overproduction and purification of the *luxR* gene product: Transcriptional activator of the *Vibrio fischeri* luminescence system

(sensory receptor/gene activation/bacterial pheromone/symbiosis functions)

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ABSTRACT Expression of *Vibrio fischeri* luminescence genes requires an inducer, termed autoinducer, and a positive regulatory element, the *luxR* gene product. A plasmid containing *luxR* under control of a *tac* promoter was engineered to overproduce this gene product. The overproduced *luxR* gene product was active *in vivo*, and its apparent monomeric molecular weight was indistinguishable from that of the protein encoded by *luxR* under control of its own promoter (M_r 27,000). The new *tac-luxR* construct directed the synthesis of large quantities of the *luxR* gene product in induced *Escherichia coli* cells lacking other *lux* genes. In the presence of the other *lux* genes, overexpression of the *tac-luxR* construct was not detected. The overproduced *luxR* gene product, which formed cytoplasmic inclusion bodies, was purified and used in subsequent studies. Nonequilibrium pH gradient electrophoresis indicated that the protein was basic, and the amino-terminal 15 amino acids were sequenced. DNA-binding activity was detected by membrane filter binding assays; under the conditions used, the binding was not *lux* DNA-specific. Binding of tritium-labeled autoinducer to the *luxR* gene product was not detected, and autoinducer enhancement of the binding of the *luxR* gene product to DNA could not be detected reproducibly.

Vibrio fischeri, a luminescent marine bacterium, can be isolated from seawater and has been identified as the bacterial symbiont in the light organs of certain marine fishes (1–3). The luminescence of *V. fischeri* is inducible; the inducer, *N*-(3-oxohexanoyl)homoserine lactone, termed "autoinducer," is a diffusible metabolite produced by *V. fischeri* that accumulates at equal concentrations in the culture medium and in cells during growth. When autoinducer reaches a critical concentration of a few molecules per cell, induction of the light-emitting enzyme luciferase and other enzymes involved in luminescence commences (4–7). At high cell densities, autoinducer can accumulate, and induction of the *V. fischeri* luminescence system will occur. Presumably this is the case in the light-organ symbiosis, where *V. fischeri* occurs at densities of $\approx 10^{10}$ cells per ml of light organ fluid. In low-cell-density habitats such as seawater, where *V. fischeri* exists at $<10^2$ cells per ml, autoinduction of luminescence would not be expected (1–3, 8). Thus, autoinducer serves as the chemical signal or pheromone in this cell density-dependent control system that provides a rapid and sensitive cellular response to changes in the external environment (4, 6).

The cloning of a fragment of *V. fischeri* DNA that encodes regulatory functions and enzymatic activities necessary for light production in *Escherichia coli* enabled a genetic characterization of the regulation of luminescence (9, 10). A positive regulatory element was identified, the product of *luxR*, that is required along with autoinducer to activate

transcription of a contiguous but divergently transcribed operon encoding five polypeptides required for luminescence (the *luxC,D,A,B,E* gene products) and one polypeptide for autoinducer synthesis (the *luxI* gene product). The *luxR* gene product can be considered the autoinducer receptor, since it is the only *V. fischeri* gene product required in *E. coli* for response to autoinducer (9, 10). Induction of luminescence also has been found to require cAMP receptor protein (CRP) and cAMP (11). There is an additional control mechanism not yet understood; the presence of either *luxI* (which is adjacent to *luxR*) or its product suppresses synthesis of the *luxR* gene product (10). This was an important consideration in our attempts to overproduce the *luxR* gene product.

We report here the construction of a plasmid that directs overproduction of the *luxR* gene product in *E. coli* and a procedure for purifying this overproduced protein. Using the material purified from *E. coli*, we established that the LuxR protein has DNA-binding activity, and, using the *luxR* expression vector, we demonstrated that there is a regulatory region within the *luxR* structural gene.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Culture Conditions. The *E. coli* strain used was JM109 (12). The plasmids used are listed in Table 1. Unless otherwise specified, *E. coli* cultures were grown in L broth (6) containing antibiotics (80 μg of ampicillin or 50 μg of chloramphenicol per ml) with shaking at 37°C. Cells from which the *luxR* gene product was purified were grown in 100-ml batches to an optical density at 550 nm of about 0.8; the inducer of the *tac* promoter, isopropyl β-D-thiogalactoside (IPTG), was added to a final concentration of 1 mM; and the cultures were incubated a further 3 hr.

Plasmid Purification and Constructions. Plasmids were purified as described (15). Manipulations of plasmid DNA were performed by following the procedures of Maniatis *et al.* (16) and Struhl (17). The transformation procedure used was that described by Hanahan (18).

For identifying ColE1 replicons containing *luxR* under control of new promoters, the other *lux* genes were supplied on a compatible replicon, and colonies on plates incubated at 30°C were screened for luminescence. These *lux* genes from a ColE1 replicon were subcloned into pACYC184 (a P15A replicon) by digestion of pJE455 with *Sal* I and ligation of a fragment of about 19 kilobases (kb) into the *Sal* I site of pACYC184. The resulting plasmid, pHK555, contained *luxI, C,D,A,B,E*, a *luxR* deletion mutation, and some mini-Mu DNA.

A plasmid containing *luxR* under its own control, pHK737, was constructed as a source of *lux* regulatory regions for use in DNA-binding assays. This plasmid was also used to identify the *luxR* gene product by *in vitro* transcription–

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Source (reference)
pACYC184	P15A replicon, Cm^r	B. Brahamsha (13)
pDR720	ColE1 replicon, Ap^r	Pharmacia
pUC8	ColE1 replicon, Ap^r	Amersham† (14)
pUC18	*lac* promoter vector, Ap^r, ColE1 replicon	M. Weiner (12)
pKK223-3	*tac* promoter vector, Ap^r, ColE1 replicon	Pharmacia
pJE455	*luxR*::mini-Mu, *luxIC,D,A,B,E$^+$*, Ap^r	J. Engebrecht*
pJE737	*luxR$^+$luxI'*, Cm^r	J. Engebrecht (10)
pHK555	*luxR$^-$luxIC,D,A,B,E$^+$*, Cm^r	This study
pPD737	*luxR$^+$luxI'*, Ap^r	P. Dunlap†
pHK737	*luxR$^+$,luxI'*, Ap^r	This study
pHK705	*lacZ'-luxR$^+$*, Ap^r	This study
pPD723	*luxR$^+$*, Ap^r	P. Dunlap†
pHK724	*luxR$^+$*, Ap^r	This study

Cm^r and Ap^r, chloramphenicol and ampicillin resistance.
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translation analysis. A *Hinc*II fragment from pJE737 of about 1.5 kb containing *luxR* and a small amount of *luxI* was cloned into the *Sma* I site of pDR720 by P. Dunlap in our laboratory, resulting in the recombinant plasmid pPD737. To produce pHK737, the *lux* DNA was removed from pPD737 by digestion with *Eco*RI and *Sal* I and then inserted into pUC8.

Polyacrylamide Gel Electrophoresis. The procedure of Laemmli (19) for NaDodSO$_4$/PAGE was used as described (20). The relative amount of the *luxR* gene product was determined with scanning densitometry. Standard fluorography procedures were used with molecular weight markers (Sigma) prestained with Remazol brilliant blue R (21). Protein concentrations were determined by the method of Bradford (22).

The charge of the *luxR* gene product was estimated by the nonequilibrium pH gradient electrophoresis technique of O'Farrell *et al.* (23). Samples were prepared as described (20) except that NaDodSO$_4$ was omitted. The standards used were broad- and low-pI calibration kit standards (Pharmacia).

Purification of the *luxR* Gene Product from *E. coli* (pHK724). The procedure for the purification of the *luxR* gene product was similar to that for other overproduced proteins that precipitate in cells (24, 25). Cells induced to overexpress *luxR* were harvested, the supernatant fluid was decanted, and the cell pellet was weighed (usually 0.6 g). The pellet was suspended in 2.4 ml of R buffer (50 mM Tris, pH 7.8 at 5°C/200 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/5% glycerol). A 0.24-ml volume of 50 mM EDTA in 10% Triton X-100 was added to the suspension, and then a fresh solution of lysozyme was added to a final concentration of 150 μg/ml. The suspension was incubated for 45 min, sonicated, and then centrifuged at 100,000 × *g* for 30 min. The resulting supernatant fluid was removed, and the pellet was suspended in 2.4 ml of 6 M guanidine hydrochloride in R buffer and incubated for 20 min. This was followed by centrifugation for 30 min at 100,000 × *g*. The resulting supernatant fluid was diluted to 1 M guanidine hydrochloride by a dropwise addition of RS buffer (R buffer containing 1 M NaCl and 50% glycerol), dialyzed in two steps against RS buffer containing decreasing concentrations of guanidine hydrochloride (0.5 M and 0.25 M), and then dialyzed twice against RS buffer without guanidine hydrochloride. All manipulations were at 4°C or on ice. The protein was stored at −70°C at a concentration of ≈0.8 mg/ml. For the amino-terminal sequence analysis (performed by the Cornell Biotechnology Institute Protein Sequencing Facility), the purified *luxR* gene product was dialyzed for 16 hr against 10 mM HCl.

DNA-Binding Assays. A membrane filter binding assay based on a previously described method (26, 27) was used to measure DNA-binding activity. The *luxR* gene product in RS buffer was diluted to 7.5 μg/ml in the buffer described by Riggs *et al.* (26) and was then added to a reaction mixture containing 200 nM autoinducer and 2–250 ng of [^{32}P]pHK737 (specific activity was adjusted so that ≈1000 cpm were added to each reaction mixture) in Riggs buffer (final volume, 1 ml). These reaction mixtures were incubated at 25°C for about 20 min. Samples (0.4 ml) were filtered and washed as described elsewhere (26), except that 25-mm mixed nitrocellulose-cellulose acetate filters, 0.45-μm pore size, (Millipore) were used. The amount of radiolabel retained on each filter was determined. The data have been corrected for background counts (the counts retained on filters in the absence of *luxR* gene product). The [^{32}P]pHK737 was prepared by digesting pHK737 with *Eco*RI and end-labeling with reverse transcriptase and [α-^{32}P]dATP (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq).

Autoinducer-Binding Assays. One of the methods used to measure autoinducer binding to the purified *luxR* gene product was equilibrium dialysis (28, 29). The procedure used two Plexiglas chambers separated by dialysis membrane. One side of the membrane contained ED buffer (10 mM Tris, pH 7.8 at 5°C/100 mM KCl/10 mM magnesium acetate/0.1 mM EDTA/and 0.1 mM dithiothreitol), 50 μg (*ca*, 2 μM) of *luxR* gene product or 50 μg of bovine serum albumin with or without 5 μg (0.01 μM) of closed circular pHK737 as indicated. The other side contained ED buffer with 0.1 μCi of tritiated autoinducer (30) at a concentration of 13 nM or 100 nM. Incubation was at 4°C with rotation at 25 rpm for 3 hr; equilibration was reached within this time. The amount of radioactivity in each chamber was determined.

The other procedure used to measure autoinducer binding was the membrane filter assay described above. However, for these assays tritiated autoinducer was present in the reaction mixtures at concentrations ranging from 0.02 nM (1 nCi) to 2 nM (100 nCi), and the amount of tritium retained on the filters was determined.

RESULTS

Construction of the *luxR* Expression Vector pHK724 and Overproduction of the *luxR* Gene Product. To overproduce the *luxR* gene product, a series of manipulations of the *lux* DNA was performed (Fig. 1). A plasmid (pJE737) obtained from J. Engebrecht containing *luxR* and part of *luxI* was used as a source of *luxR*. Expression of *luxR* is low in cells containing pJE737 in part because of the presence of *luxI'* or its truncated gene product (J. Engebrecht, personal communication). To increase expression of *luxR*, it was initially subcloned into pUC18 (Fig. 1). First, *luxI'* and the regulatory region of the *luxR* gene were removed by digestion of pJE737 with BAL-31 nuclease. The DNA was then inserted into pUC18 and used to transform *E. coli* JM109 (pHK555). Clones were selected for ampicillin and chloramphenicol resistance and screened for luminescence in the presence of IPTG. Luminescence was due to a functional *luxR* gene product and the intact *luxI,C,D,A,B,E* on pHK555. To ensure that *luxR* was under control of the *lac* promoter, luminous clones were then screened to obtain those that did not make light in the absence of IPTG. NaDodSO$_4$/PAGE of IPTG-induced *E. coli* containing one such plasmid, pHK705 (data not shown), and of *in vitro* transcription–translation products of pHK705 (Fig. 2) indicated, respectively, that expression of the *luxR* gene was poor (it could not be visualized on Coomassie blue-stained gels) and that a 29-kDa fusion product was synthesized.

To obtain a clone that did not encode a fusion protein, *luxR* was further subcloned into pKK223-3 (P. V. Dunlap and
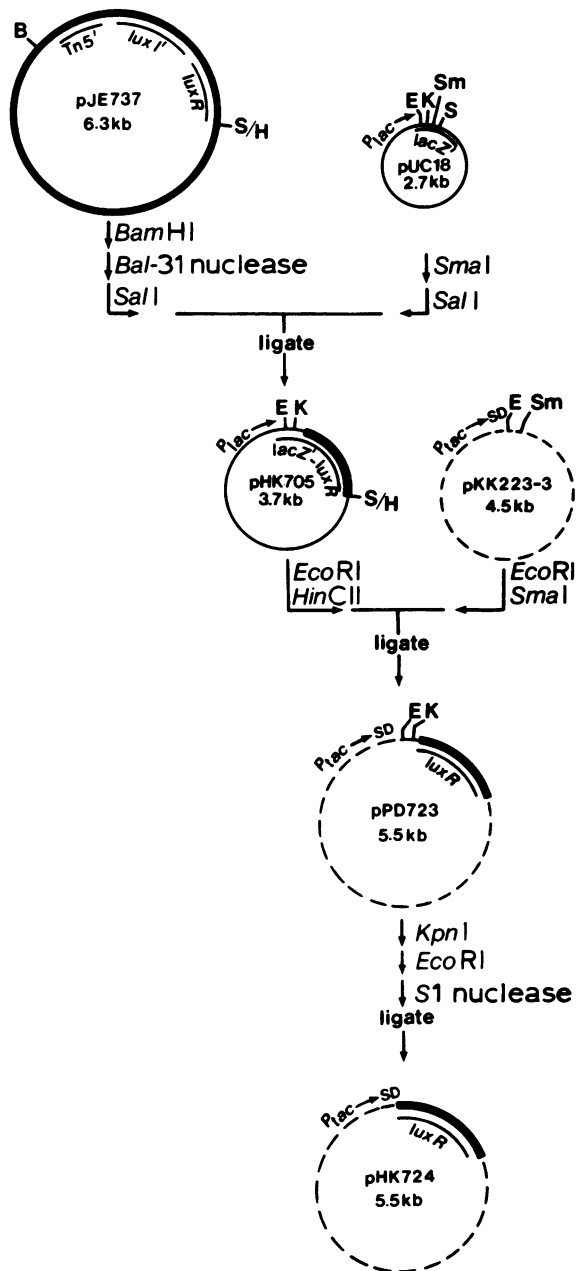
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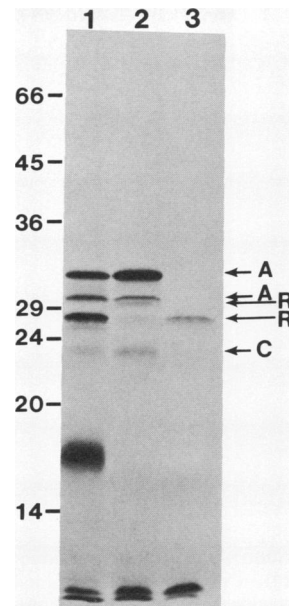*Proc. Natl. Acad. Sci. USA 84 (1987)*   6641

FIG. 2. Analysis of *in vitro* transcription–translation products. Radiolabeling of plasmid encoded-proteins synthesized *in vitro* was performed with L-[4,5-^{3}H]leucine (specific activity, 120 Ci/mmol) and a prokaryotic DNA-directed translation kit (Amersham). Fluorography of a NaDodSO$_4$/polyacrylamide gel displays proteins encoded by pHK737 (lane 1), pHK705 (lane 2), and pHK724 (lane 3). Arrows indicate gene products of interest. R, *luxR* gene product (27 kDa) in lanes 1 and 3: R', fusion protein of the *lacZ'–luxR* gene in lane 2: A and C, *bla* and *cat* gene products, respectively, which are encoded by vector DNA. Standards (left) are indicated in kilodaltons.

FIG. 1. The construction of pHK724. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; K, *Kpn* I; S, *Sal* I; Sm, *Sma* I. P_{lac} and P_{tac} are the *lac* promoter and the *tac* promoter, respectively. SD is the Shine–Dalgarno sequence (ribosome binding site).

E.P.G., manuscript in preparation). The *luxR* gene was excised from pHK705, removing the *lac* ribosome binding site and translation initiation sequence (ATG), and was cloned into a site downstream of the pKK223-3 *tac* promoter (a hybrid of the *lac* and *trp* promoters that is regulated by IPTG and the *lac* repressor; ref. 31), resulting in pPD723 (Fig. 1). Because pKK223-3 contains a ribosome binding site (a Shine–Dalgarno sequence) but lacks a translation initiation sequence, translation of *luxR* in pPD723 requires a *luxR* initiation sequence. Clones containing pPD723 together with pHK555 produced light under control of IPTG; however, expression of the *luxR* gene was low as measured by NaDodSO$_4$/PAGE of IPTG-induced *E. coli* (pPD723) (data not shown) in the presence or absence of pHK555. *In vitro* transcription–translation of pPD723 showed that the apparent monomeric molecular weight of the *luxR* gene product

was indistinguishable from that of the gene product encoded by pHK737 (data not shown).

Sequence information for the DNA between the ribosome binding site on the vector and the DNA cloned from pHK705 indicated that 26 base pairs (bp) of vector (pKK223-3 and pUC18) were present. To increase the efficiency of translation, 16 bp from this region were removed (Fig. 1). The resulting molecules were ligated and transformed into *E. coli* (pHK555). Clones recovered were more highly luminescent in the presence of IPTG than those containing pPD723 and produced relatively high levels of light in the absence of IPTG. When IPTG-induced cultures of *E. coli* containing one resulting plasmid, pHK724, and pHK555 were monitored for luminescence, an autoinducer response was observed (H.B.K., unpublished data). The monomeric molecular weight of the *in vitro* transcription–translation product of *luxR* on pHK724 was indistinguishable from that encoded by pHK737 (Fig. 2).

Overproduction of the *luxR* gene product was not observed by NaDodSO$_4$/PAGE of these luminescent cells containing both pHK724 and pHK555 when grown in the presence of IPTG. However, when IPTG-induced *E. coli* (pHK724) lacking pHK555 was analyzed by NaDodSO$_4$/PAGE, the *luxR* gene product was found to constitute ≈10% of the total cellular protein (Fig. 3) and synthesis of the *luxR* gene product was unaffected by the presence of pACYC184. It has been demonstrated previously that the synthesis of the *luxR* gene product is suppressed by the presence of *luxI* (10). Here we demonstrate suppression even when *luxI* is in trans and when *luxR* is not under control of its own promoter. Although the *luxI* gene product is involved in autoinducer synthesis (9, 10), addition of pure autoinducer (200 nM) together with IPTG to *E. coli* (pHK724) did not decrease synthesis of the *luxR* gene product (D. Kolibachuk and E.P.G., unpublished data).

Purification of the *luxR* Gene Product and Chemical Characteristics of the Purified Protein. Granules similar in appearance to cytoplasmic inclusion bodies were observed in cells of IPTG-induced *E. coli* (pHK724). Cytoplasmic inclusion bodies are insoluble precipitates of overproduced proteins sometimes observed in cells (25, 32). A characteristic of some of these insoluble overproduced proteins is their solubility in high concentrations of guanidine hydrochloride and poor solubility in detergents (24, 25). The overproduced *luxR* gene product exhibited these characteristics. The selective solubilization of the *luxR* gene product in 6 M guanidine hydro-
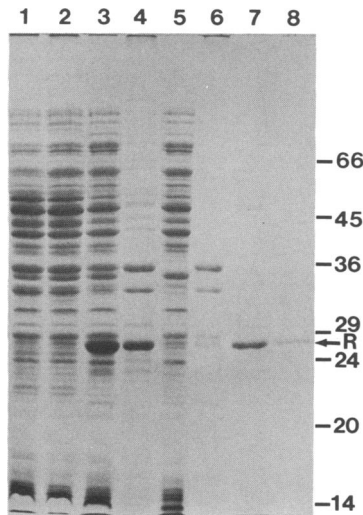
FIG. 3. Overproduction and purification of the *luxR* gene product from IPTG-induced *E. coli* (pHK724). Coomassie blue-stained NaDodSO₄/polyacrylamide gel shows stages of the purification. Lanes: 1 and 2, controls [*E. coli* containing the plasmid vector (pKK223-3) grown in the presence of IPTG (lane 1) and *E. coli* (pHK724) grown without IPTG (lane 2)]; 3, *E. coli* (pHK724) grown in the presence of IPTG; 4, pelleted material after centrifugation of sonicated *E. coli* (pHK724) grown with IPTG; 5, supernatant fluid after centrifugation of sonicated material; 6, insoluble proteins after guanidine hydrochloride treatment of the pelleted material; 7, guanidine hydrochloride-soluble material in RS buffer; 8, guanidine hydrochloride-soluble material in 10 mM HCl. The arrow points to the *luxR* gene product visible in lanes 3, 4, 7, and 8. Standards (right) are indicated in kilodaltons.

chloride from the material pelleted by centrifugation of sonicated detergent-treated cells formed the basis of our purification. Removal of the guanidine hydrochloride by slow dilution and dialysis in RS buffer resulted in a soluble, purified preparation of the *luxR* gene product that contained other polypeptides in minor amounts only (Fig. 3). Nonequilibrium pH gradient electrophoresis of the *luxR* gene product resulted in a band that migrated to the same relative position as the lentil lectin protein middle band (pI, 8.45). The amino-terminal sequence through residue 15 is shown in Fig. 4. This amino-terminal sequence is in agreement with that predicted by the DNA sequence of *luxR* (J. Devine, C. Countryman, and T. Baldwin, personal communication).

DNA and Autoinducer Binding Studies. Membrane filter binding assays were used to demonstrate that the purified *luxR* gene product bound to DNA; binding of this protein to DNA was saturable (Fig. 5), as was the binding of DNA to this protein (Fig. 6). Binding of DNA as a function of either DNA concentration or protein concentration was not reproducibly affected by autoinducer over a concentration range of 0–200 nM (data not shown). Competition experiments, in which increasing amounts of unlabeled vector, pUC8, or recombinant plasmid pHK737 (providing *lux* regulatory DNA) were added to the reaction mixture, indicated that, under the conditions used, binding was not *lux* DNA specific (data not shown).

Filter binding assays with various concentrations of tritiated autoinducer in the presence of the *luxR* gene product or the *luxR* gene product and *lux* regulatory DNA (provided on pHK737) did not reveal any binding of the *luxR* gene product

Met-Lys-Asn-Ile-Asn-Ala-Asp-Asp-Thr-Tyr-Arg-Ile-Ile-Asn-Lys
 1   2   3   4   5   6   7   8   9   10   11   12   13   14   15

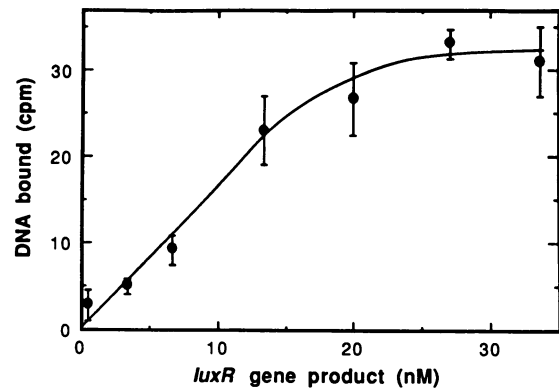FIG. 4. Amino-terminal sequence of the purified *luxR* gene product.

FIG. 5. DNA binding as a function of the amount of *luxR* gene product. Each reaction mixture contained 50 ng (0.02 nM) of [³²P]pHK737 and 200 nM autoinducer. Each point represents the average from duplicate filtrations, and the marker bars represent the range between the values.

to autoinducer. In addition, binding of the *luxR* gene product to autoinducer in the presence or absence of *lux* regulatory DNA (provided on pHK737) could not be detected by equilibrium dialysis. Either the purified protein had lost its ability to bind autoinducer, the experimental conditions were not appropriate for binding, the affinity constant was too low to detect binding in these assays, or the *luxR* gene product alone does not bind autoinducer.

DISCUSSION

The product of the *luxR* gene has been overproduced in *E. coli* (Fig. 3). This was accomplished by placing *luxR* under control of the *tac* promoter on pHK724 (Fig. 1). The overproduced protein was active *in vivo*, and the apparent monomeric molecular weight was indistinguishable from that produced by an unaltered *luxR* (Fig. 2). The cloning of *luxR* first involved transformation of *E. coli* containing the other *lux* genes on a compatible plasmid and screening for colonies that were luminous only when IPTG was present. Once a plasmid containing *luxR* under IPTG control was obtained, expression of *luxR* was optimized by construction of pHK724. *luxR* remained under IPTG control (Fig. 3); however, cells containing both pHK724 and pHK555 were luminous even in the absence of IPTG. The basal level of *luxR* synthesis directed by pHK724 was sufficient for *in vivo*
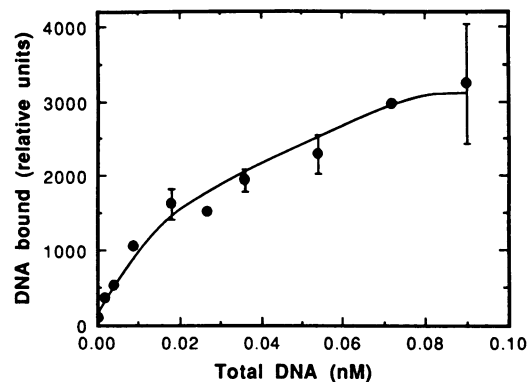

FIG. 6. Dependence of DNA binding on the concentration of [³²P]pHK737 DNA. Each reaction mixture contained 20 nM *luxR* gene product and 200 nM autoinducer. Bound DNA (relative units) was calculated from the specific activity of the DNA in each reaction mixture and the number of counts retained on the filters. Each point represents the average from duplicate filtrations, and the marker bars represent the range between the values.

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activity, presumably because this protein functions at very low concentrations. This sort of scheme, involving a screen for regulated activity *in vivo*, followed by optimization of expression, may be useful in attempts to produce large quantities of other transcriptional activators.

The *tac-luxR* construct in pHK724 directed the synthesis of large quantities of the *luxR* gene product in IPTG-induced *E. coli* lacking other *lux* genes (Fig. 3). In *E. coli* containing both this construct and pHK555 (*luxI,C,D,A,B,E*), the *luxR* gene product was not abundant enough to be visualized by gel electrophoresis. This is consistent with the conclusion of Engebrecht and Silverman (10), that the expression of *luxR* is low in the presence of *luxI*. We observed that the presence of *luxI,C,D,A,B,E* in low-copy number suppressed synthesis of the *luxR* gene product when *luxR* in high-copy number was controlled by the *tac* promoter and was not adjacent to *luxI*. These results suggest that the effector of *luxR* protein synthesis acts on a site within the *luxR* structural gene or it acts posttranscriptionally.

The overproduced *luxR* gene product was purified (Fig. 3) and used for an amino terminus amino acid analysis (Fig. 4). It was determined that the *luxR* gene product is basic, as is true of many other proteins that affect transcription (33). The purified *luxR* gene product exhibited DNA-binding activity (Figs. 5 and 6); however, specificity for *lux* DNA was not detected. Since no specific affinity for *lux* DNA was observed, it may be that the renaturation procedure used did not lead to correct refolding of the protein or that only a small percentage of the molecules folded correctly. However, no or low specificity of DNA binding, as measured by filter binding assays, is a problem not uncommon with purified transcriptional activators (26, 34, 35). It is also possible that the conditions used were not optimal for measurement of DNA-binding activity. Within this context, it is possible that other proteins are required for activity, perhaps RNA polymerase, as has been shown for cII protein of phage λ (36). Further characterization of the *luxR* gene product and its DNA-binding capacity will require an increase in its activity. In addition, genetic analyses can provide more precise information about binding sites for the LuxR protein, facilitating development of a specific DNA-binding assay.

The fact that no direct association of autoinducer with the *luxR* gene product was detected, either by filter binding assays or equilibrium dialysis in the presence or absence of DNA, provides further evidence to suggest that the native state of the protein was not recreated or perhaps conditions of the assays were not appropriate for the measurement of such interactions. It is also possible that the current model (9) in which the *luxI,C,D,A,B,E* operon is regulated by the direct interaction of autoinducer bound to the *luxR* gene product is incorrect.

The development of this rapid method for purification of large quantities of the *luxR* gene product has allowed a basic characterization of this protein and enabled us to demonstrate that this is a DNA-binding protein. It should now be possible to characterize the interaction of autoinducer and the *luxR* gene product biochemically and to determine the molecular mechanisms by which they serve to activate transcription.

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