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Quorum Sensing in *Vibrio fischeri*: Essential Elements for Activation of the Luminescence Genes

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LuxR is required for cell density-dependent activation of the *Vibrio fischeri* luminescence (*lux*) genes. It has not been possible to study full-length LuxR in vitro, but a polypeptide containing the C-terminal transcriptional-activator domain of LuxR (LuxRΔN) has been purified, and its binding to *lux* regulatory DNA has been investigated. By itself, LuxRΔN interacts with a region of *lux* regulatory DNA that is upstream of the *lux* box, which is a 20-bp element that is required for LuxR activation of the luminescence operon. Individually, neither the purified LuxRΔN nor RNA polymerase binds to the *lux* box region, but together the two proteins bind in synergy to the *lux* box-*luxI* promoter region. We show that binding of LuxRΔN to the upstream region is not a prerequisite for its synergistic binding with RNA polymerase to the *lux* box and the *luxI* promoter region. We also show that LuxRΔN and RNA polymerase are both required and sufficient for transcriptional activation of the *lux* operon. This argues against the hypothesis that LuxR functions to alleviate repression of the *lux* operon by another cellular factor. Rather, our data support the view that LuxR functions as an accessory factor that enables RNA polymerase to bind to and initiate transcription from the promoter of the *lux* operon.

Recent recognition that the phenomenon of quorum sensing and response is common to a number of gram-negative bacterial species has led to an increased interest in the model for this type of cell density-dependent control of gene expression: activation of the *Vibrio fischeri* luminescence genes (for recent reviews, see references 13, 14, 22, 28, and 32). In quorum sensing, a bacterial species takes a population census, and at sufficient cell densities, specific functions are induced. An intercellular signalling molecule termed the autoinducer is produced by the bacterial cells, and when it reaches a critical concentration, gene activation occurs. There are two genes essential for cell density regulation of *V. fischeri* luminescence: *luxI*, which codes for an autoinducer synthase; and *luxR*, which codes for an autoinducer-dependent activator of the luminescence genes. The *luxR* and *luxI* genes are adjacent and divergently transcribed, and *luxI* is the first of seven genes in the luminescence or *lux* operon (references 10–12, 24, 28, and 31 and Fig. 1). Thus, the *luxI* gene is positively autoregulated by LuxR in the presence of the autoinducer.

Although LuxR is the best understood member of a newly emerging family of transcription factors, relatively little is known about the mechanism by which it activates transcription from the promoter of the *lux* operon. The simple model is that LuxR is required for transcription initiation. However, there is some evidence that the situation might be more complicated, and it has been proposed that LuxR might serve to displace a repressor that otherwise binds to the *lux* box and blocks transcription initiation by RNA polymerase (RNAP) (33, 34, 36).

LuxR is a two-domain polypeptide consisting of 250 amino acid residues. The N-terminal domain binds to the autoinducer (17, 25, 29). In the absence of the autoinducer, the N-terminal domain inhibits the function of the C-terminal domain, which activates transcription of the *lux* operon (3, 4). Although full-length LuxR has not yet proven amenable for studies in vitro, we have described the interactions of a purified polypeptide containing the LuxR C-terminal domain (LuxRΔN) with *lux* regulatory DNA (30). In *Escherichia coli*, this C-terminal domain of LuxR activates transcription of the *lux* operon in an autoinducer-independent manner (3, 30). There is a 20-bp region of dyad symmetry, called the *lux* box, centered at −40 from the *luxI* transcriptional start site that is essential for LuxR activation of the luminescence operon in vivo (5, 6, 13, 16). Rather than binding to the *lux* box region by itself, purified LuxRΔN interacts with the *lux* DNA in a region upstream of the *lux* box (Fig. 1), which encompasses the cyclic AMP receptor protein (CRP)-binding site (roughly from 20 bp upstream to 40 bp downstream of the CRP-binding site). CRP is a transcriptional activator of *luxR* (8, 9). As is the case with LuxRΔN, RNAP alone does not bind to the *lux* box or −35 region of the *luxI* promoter. However, both LuxRΔN and RNAP together bind to the *lux* box and the *luxI* promoter (−54 through +6) cooperatively (30).

Here, we show that binding of LuxRΔN to the upstream region does not facilitate the LuxRΔN-RNAP interaction with the *lux* box region and that LuxRΔN is required for transcription from the *luxI* promoter. This finding is inconsistent with the hypothesis that LuxR functions by displacing a repressor of *luxI* transcription. We have also used the in vitro transcription assay to examine the thermal stability of LuxRΔN. This polypeptide is limited in its temperature range for activity, but it is remarkably resistant to thermal denaturation.

Is binding of LuxRΔN to the upstream DNA necessary for the LuxRΔN-RNAP interaction at the *lux* box? LuxRΔN by itself interacts with the *lux* DNA in a region encompassing the CRP-binding site; however, the significance of this interaction in activation of the *lux* promoter is unknown (30). We examined this issue in two ways. In the first analysis, we asked whether LuxRΔN and RNAP can bind to the *lux* box-*luxI* promoter region when CRP occupies the upstream region. In the second analysis, we asked whether LuxRΔN-RNAP can bind to the *lux* box-*luxI* promoter region on a fragment of DNA that does not contain the upstream LuxRΔN-binding site.

As shown with DNase I protection studies, high concentrations of CRP (780 nM) bind to the upstream region (26, 30).

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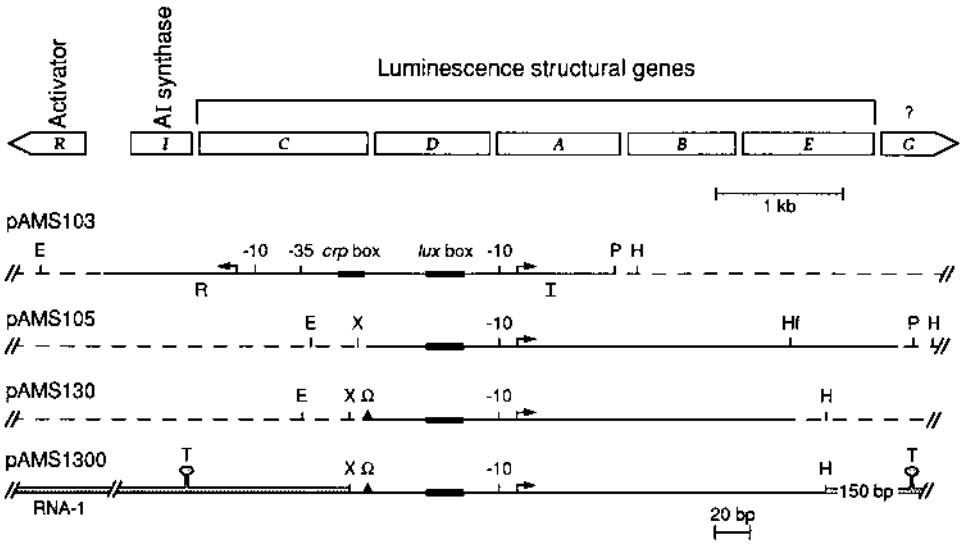

FIG. 1. Organization of the *V. fischeri lux* gene cluster and plasmids used as sources of DNA templates. (Top) The *lux* gene cluster. Arrowheads indicate the direction of transcription of *luxR* and of the *luxICDABEG* operon. The *luxR* gene codes for the transcriptional activator; *luxI* codes for autoinducer synthase; *luxC*, *-D*, and *-E* code for the fatty acid reductase required for synthesis of the aldehyde substrate for luciferase; *luxA* and *-B* code for the subunits of luciferase; and the function of *luxG* is unknown. (Bottom) Plasmids used. Construction of pAMS103 has been described elsewhere (30). A PCR product containing the *luxI* promoter and the first 210 bp of *luxI* was cloned into the *Hin*cII site of pUC19 to create pAMS105 (23, 37). The PCR product was amplified from pJE202 (10) by using primers corresponding to nucleotides −79 to −62 and +210 to +193 with respect to the *luxI* transcriptional start site (12). To construct pAMS130, a 272-bp *Eco*RI-*Hin*fI fragment from pAMS105 was cloned into the *Hin*cII site of pUC19 (37). This resulted in a 56-bp truncation of the *luxI* open reading frame and an insertion of 4 bp into the pUC multiple cloning site. The 265-bp *Hin*dIII-*Xba*I fragment of pAMS130 subcloned into *Hin*dIII-*Xba*I-digested pMP7 created pAMS1300 (19). Regions corresponding to pUC19 and pMP7 are depicted as dashed lines and double lines, respectively. The *lux* DNA is depicted by the solid lines. The solid boxes denote the locations of the *crp* box and the *lux* box. The transcriptional start sites and locations of the *luxR* and *luxI* promoter elements are shown. Restriction sites are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; P, *Pst*I; X, *Xba*I. //, sequences continue beyond the limits shown; Ω, the 4-bp insertion; stem-loop with T, T7 early transcriptional terminator. There are 150 bp between the *Hin*dIII site and terminator on the right end of pAMS1300.

Furthermore, these high concentrations of CRP block the binding of LuxRΔN-RNAP to the *lux* box-*luxI* promoter region (30). To examine the possibility that lower concentrations of CRP might bind to the upstream region without blocking LuxRΔN-RNAP binding to the *lux* box-*luxI* promoter region, we carried out DNase I protection experiments with lower concentrations of CRP. A 325-bp fragment containing all of the regulatory DNA between *luxR* and *luxI* served as the template for these experiments. Without LuxRΔN, a characteristic footprint was apparent at CRP concentrations as low as 22 nM (Fig. 2A, lanes 2 to 6). At 22 nM CRP, addition of LuxRΔN and RNAP resulted in the characteristic footprint on the *lux* box-*luxI* promoter region and the CRP footprint was still observed (Fig. 2A, lanes 16 to 19). Thus CRP binding to the upstream region does not in itself occlude binding of LuxRΔN-RNAP to the *luxI* promoter region. This is an indication that binding of LuxRΔN to the upstream DNA is not a prerequisite for the interactions of LuxRΔN-RNAP with the *lux* box-*luxI* promoter. Furthermore, the presence of CRP at 22 nM did not appear to have an appreciable influence on the affinity of LuxRΔN-RNAP for the *luxI* promoter region (Fig. 2A, lanes 15 to 19 versus lanes 21 to 26).

To examine the role of the upstream binding region in the interaction of LuxRΔN-RNAP with the *lux* box region more directly, we examined the DNase I cleavage pattern of a *lux* fragment without the upstream binding region. There was no footprint in the presence of purified LuxRΔN, nor was there a footprint in the presence of RNAP alone (Fig. 2B). Nevertheless, LuxRΔN and RNAP together footprinted a region identical to that which was footprinted by these two proteins together in full-length *lux* regulatory DNA (reference 30 and Fig. 2). Furthermore, we could not discern a difference in the amount of LuxRΔN required to achieve protection of this region from DNase I cleavage in assays with the truncated DNA fragment versus that required with the full-length fragment (compare Fig. 2A, lanes 21 to 26 with 2B, lanes 4 to 10). Thus, we conclude that the upstream sequences around the CRP-binding site are not essential for binding of LuxRΔN and RNAP to the *lux* box-*luxI* promoter region and that binding of LuxRΔN to the upstream element does not even appear to improve the efficiency of LuxRΔN-RNAP binding to the *luxI* promoter region.

Is LuxRΔN required for initiation of transcription from the *luxI* promoter? With the knowledge that the upstream binding of LuxRΔN is not a prerequisite for binding of LuxRΔN-RNAP to the *lux* box and *luxI* promoter region, we reasoned that a DNA molecule containing the *luxI* promoter but lacking the upstream region including the *crp* box and the *luxR* promoter would be sufficient as a template for in vitro assays to determine the influence of LuxRΔN on transcription initiation at the *luxI* promoter. To establish that LuxRΔN-dependent transcripts initiated from the *luxI* promoter, we used four different *lux* DNA templates that extend from a site just upstream of the *lux* box to sites internal to *luxI* (Fig. 1). Transcripts of the sizes predicted for *luxI* mRNA were observed, but only in the presence of both RNAP and LuxRΔN (Fig. 3). The LuxRΔN-RNAP-generated transcripts include the predicted 230-base mRNA produced from the *Eco*RI-*Hin*dIII fragment of pAMS105 (Fig. 3A, lane 2) and the 174-base mRNA from the *Xba*I-*Hin*dIII fragment of pAMS130 (Fig. 3A, lane 4). When the *Xba*I-*Hin*dIII fragment of pAMS130 was cloned into pMP7 (pAMS1300) and the vector was cut at its *Hin*dIII site, a 174-base LuxRΔN-dependent transcript and a 108-base LuxRΔN-independent transcript were observed (Fig. 3A, lane 6). The 108-base RNA, RNA-1, was generated from the plasmid origin of replication, and it serves as an internal control of RNAP

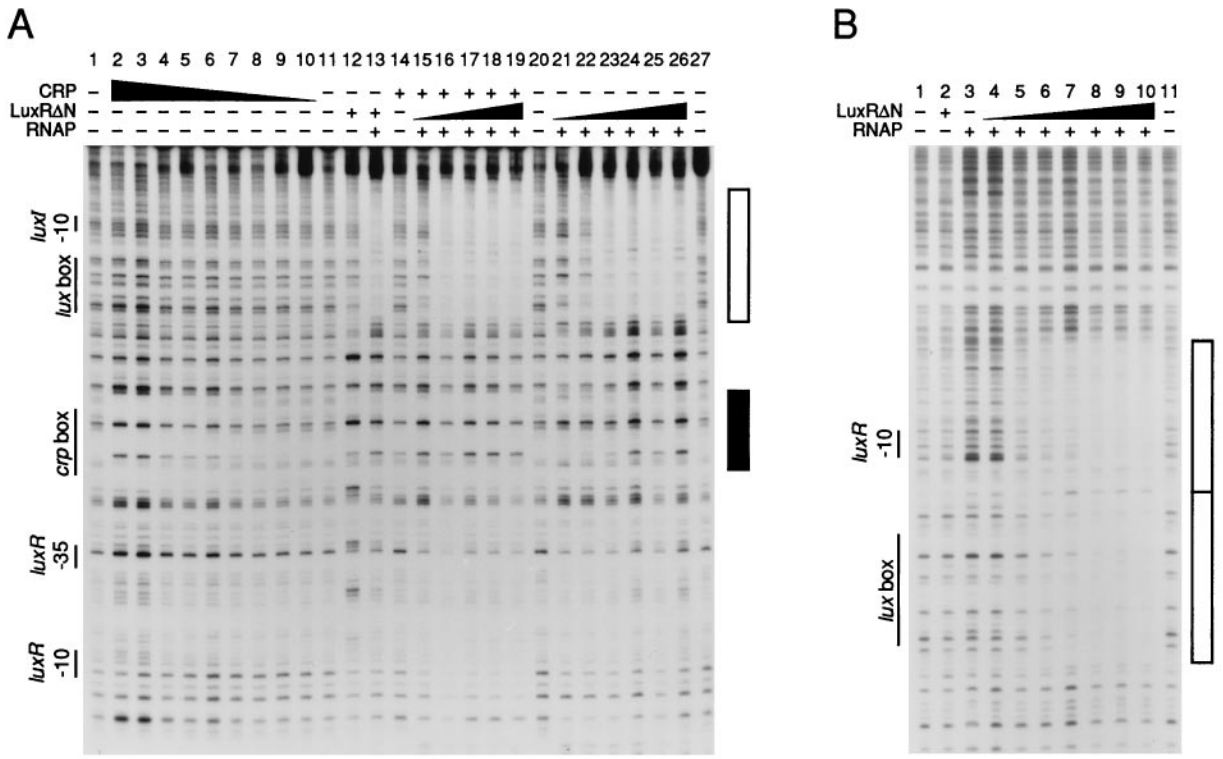

FIG. 2. DNase I protection analysis. (A) Influence of CRP on binding of LuxRΔN and RNAP to the *luxI* promoter region on a 325-bp *Eco*RI-*Pst*I fragment of *lux* regulatory DNA from pAMS103 (*luxI* coding strand), which includes the entire *lux* intergenic region. All of the reaction mixtures contained cyclic AMP (2 nM). Lanes: 1, 11, 20, and 27, no protein added; 2 to 10, CRP at 86, 43, 22, 11, 11, 5.5, 3.0, 1.5, and 0.75 nM, respectively; 12, LuxRΔN (6.1 μM); 13, LuxRΔN at 6.1 μM and RNAP at 40 nM; 14, CRP at 22 nM; 15 to 19, CRP at 22 nM, RNAP at 40 nM, and LuxRΔN at 0.51, 1.0, 2.0, 4.1, and 6.1 μM, respectively; 21 to 26, RNAP at 40 nM and LuxRΔN at 0.25, 0.51, 1.0, 2.0, 4.1, and 6.1 μM, respectively. (B) LuxRΔN and RNAP binding to a 332-bp *Eco*RI-*Pst*I fragment of *lux* regulatory DNA from pAMS105 (*luxI* coding strand), which is missing the CRP binding site. Lanes: 1 and 11, no protein added; 2, LuxRΔN at 8.1 μM; 3, RNAP at 40 nM; 4 to 10, RNAP at 40 nM and LuxRΔN at 0.25, 0.51, 1.0, 2.0, 4.1, 6.1, and 8.1 μM, respectively. The locations of the *luxI* −10 region, *lux* box, *crp* box, and *luxR* −10 and −35 regions are indicated by the solid lines. The regions protected by CRP or LuxRΔN and RNAP are indicated by the solid or open boxes. DNase I protection experiments were performed as described previously (30), except purified RNAP holoenzyme was purchased from Epicentre Technologies (Madison, Wis.), and reaction mixtures were modified slightly to contain (60 μl, final volume) ^{32}P-end labelled *lux* regulatory DNA (10,000 to 15,000 cpm), 50 mM NaCl, 1 mM $MgCl_2$, 2 mg of acetylated bovine serum albumin (New England Biolabs, Beverly, Mass.) per ml, 2.7 mM dithiothreitol, 0.4 mM EDTA, 6.7 mM sodium phosphate, and 10% glycerol in 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5).

activity (19, 21). A longer transcript with a length of about 324 bp is the predicted product of transcription from the *luxI* promoter if pAMS1300 is cut with *Xba*I rather than *Hin*dIII. Our results are consistent with this prediction (Fig. 3A, lane 8), and in fact, a 324-base *luxI* transcript was also produced from pAMS1300 by RNAP and LuxRΔN when the vector was maintained in a supercoiled state (data not shown). The amount of the *luxI* mRNA product was dependent upon the concentration of LuxRΔN (Fig. 3B). The level of LuxRΔN required to observe a stimulation in transcription from the *luxI* promoter was 2.5 μM. This is somewhat higher than the minimum concentration of LuxRΔN required for footprinting of the *lux* box-*luxI* promoter region in DNase I protection assays (reference 30 and Fig. 2). The in vitro transcription analyses shown in Fig. 3 are consistent with the conclusion that LuxRΔN is required for *luxI* promoter activity in the absence of any hypothetical repressors. The fact that *luxI* mRNA synthesis is limited by LuxRΔN concentration indicates that in vitro transcription assays can be used as a measure of LuxRΔN activity.

Activity and stability of LuxRΔN as a function of temperature. We used the in vitro transcription assay described above to monitor the activity of LuxRΔN over a temperature range of 10 to 50°C (Fig. 4A). The DNA template used for this experiment included the plasmid origin of replication so that we could monitor LuxRΔN-independent RNA-1 production as a measure of RNAP activity. LuxRΔN showed greatest activity between 25 and 30°C, and it was inactive at 45°C. The activity of RNAP was at a maximum over a higher temperature range (35 to 45°C). There is evidence that LuxR and other *lux* gene products are active in vivo at 30°C but not at 37°C (35, 36). This experiment shows that LuxR temperature sensitivity maps at least in part to the C-terminal domain of the polypeptide.

We were interested in whether inactivation of LuxRΔN at 37°C was due to denaturation of the polypeptide. Thus we incubated purified LuxRΔN at temperatures ranging from 30 to 100°C and then measured the activity with the in vitro transcription assay at 30°C (Fig. 4B). LuxRΔN was remarkably heat stable. Purified LuxRΔN incubated at 70°C for 30 min retained full activity in transcription assays. Some activity was retained even after treatment at temperatures up to 100°C (data not shown). LuxRΔN either must be capable of maintaining its structure at high temperatures, or it must readily fold back into an active molecule upon cooling.

To begin to investigate the basis of the heat stability of LuxRΔN and to confirm the predictions that this polypeptide should have a relatively high content of α-helical structure, because it contains a putative helix-turn-helix motif (1, 18, 28), we performed UV-circular dichroism (CD) spectroscopy (Fig. 4C). Spectra were obtained at 3°C and 30°C. The minima at 208 and 222 nm are characteristic of polypeptides having an

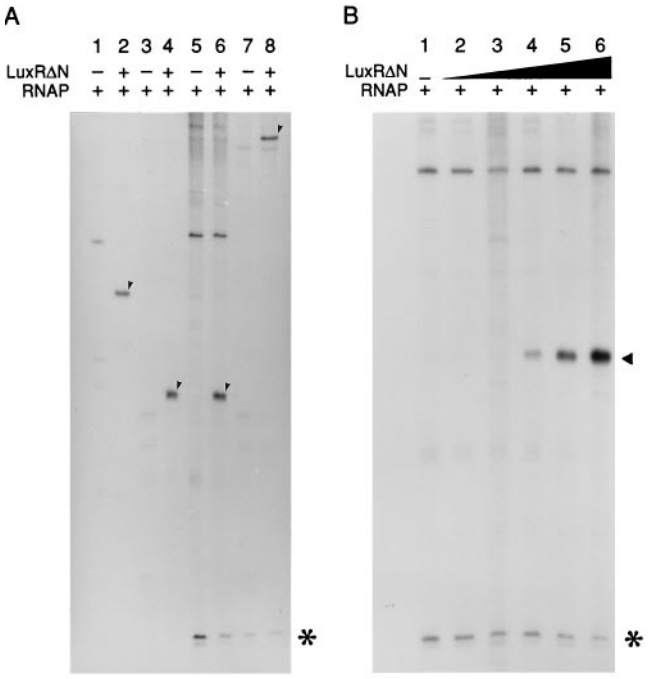

FIG. 3. LuxRΔN-dependent transcription initiation of the *lux* operon. (A) Transcript size is consistent with that predicted with four different templates. The following *lux* DNAs (by lane) were used as templates: 1 and 2, the 344-bp *Eco*RI-*Hin*dIII fragment from pAMS105; 3 and 4, the 256-bp *Xba*I-*Hin*dIII fragment from pAMS130; 5 and 6, *Hin*dIII-cut pAMS1300; and 7 and 8, *Xba*I-cut pAMS1300. RNAP and LuxRΔN (5.1 μM) were added as indicated. (B) Transcript abundance is dependent on LuxRΔN concentration. The DNA template was *Hin*dIII-cut pAMS1300. Lanes 2 to 6 contained LuxRΔN at 0.51, 1.0, 2.5, 5.1, 10.1 μM, respectively. The arrowheads point to the LuxRΔN-dependent mRNAs, and the asterisks mark the RNA-1 mRNA products. Controls with a *metE*- and *metR*-containing DNA template that produced transcripts of known length were used to estimate the sizes of the *luxI* transcripts (20). The reaction mixtures (30 μl, final volume) contained about 1.3 nM DNA template, 15 U of RNase inhibitor (United States Biochemical-Amersham Life Sciences Corp.), 0.75 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mg of acetylated bovine serum albumin (New England Biolabs) per ml, 2.7 mM dithiothreitol, 0.4 mM EDTA, 6.7 mM sodium phosphate, and 10% glycerol in 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5). After 20 min in the presence of LuxRΔN at 30°C, 40 nM (0.24 U) RNAP (Epicentre Technologies) was added. After an additional 20 min, transcription was initiated by addition of ATP, GTP, and CTP (each at 200 μM); 40 μM [α-^{32}P]UTP (2 μCi) (Amersham); and 50 μg of heparin per ml. Reactions were terminated after 20 min by addition of 7.5 μl of stop solution (3 M ammonium acetate, 0.25 M EDTA, and 15 μg of sonicated calf thymus DNA per ml). The resulting mRNA transcripts were precipitated in ethanol and resuspended in 6 μl of a loading buffer (5 M urea, 12.5 mM EDTA, and 0.025% each of xylene cyanol and bromophenol blue). Samples were incubated at 70°C for 5 min, placed on ice, and analyzed by electrophoresis in a 6% polyacrylamide–urea sequencing gel.

α-helical secondary structure. The mean residue ellipticities at 222 nm of −19,000 at 30°C and −20,500 at 3°C are consistent with a protein composed of 63 to 67% α-helical structure if the fraction of helix, f_H, is defined as $[\Theta]/-30{,}271$ at 222 nm with 11 amino acid residues per helix turn (2). A small but insignificant amount of destabilization of the α-helical structure occurred at 30°C, but was completely reversible by restoring the temperature to 3°C (data not shown). Therefore, it appears that a significant portion of the purified polypeptide is folded into a single conformation with a large amount of α-helix, which is consistent with the activity of a DNA-binding protein (1, 18, 28). This high level of α-helical structure could contribute to the ability of the LuxRΔN polypeptide to resist heat inactivation.

Conclusions. We have defined a minimal system required for transcription from the *V. fischeri luxI* promoter in vitro. The upstream LuxRΔN-binding region in the *V. fischeri lux* intergenic region is not required for the binding of LuxRΔN-RNAP to the *lux* box-*luxI* promoter region and is not required for the σ^{70}-RNAP-LuxRΔN-dependent transcription of *luxI*. This is in agreement with a single previous study of *lux* gene expression in *E. coli*, in which it was shown that to some extent, LuxR-dependent induction of the *lux* operon occurred with DNA constructs containing deletions of the upstream region through the first 2 bp of the *lux* box (6). Because there was little transcription initiation with RNAP in the absence of LuxRΔN or any other protein, our experiments provide evidence against the hypothesis that LuxR functions to induce luminescence simply by displacing a repressor at the *lux* box (33, 34, 36).

Although we present evidence that the upstream LuxRΔN-binding region is not required for transcriptional activation of the *lux* operon, we cannot rule out some other more subtle involvement of this region in regulation of the luminescence genes. For example, we know that CRP is required for activation of *luxR* transcription (8) and that *luxR* is autoregulated (9, 27). The *lux* box is required for *luxR* autoregulation (27), but we cannot rule out the possibility that the upstream sequence might also be involved in *luxR* autoregulation. Particularly because the upstream LuxRΔN-binding site overlaps the CRP-binding site, this possibility holds some allure. This is an appropriate point for a reminder that the DNase I protection experiments by necessity have all been done with a truncated LuxR, and it is possible that binding of the native protein to the upstream region might not occur.

The development of an in vitro transcription assay for measuring the activity of LuxRΔN has allowed us to begin to characterize this polypeptide biochemically. We have shown that the temperature range for its activity reflects the temperature range for luminescence gene activity in *E. coli* (Fig. 4). Whether the N-terminal autoinducer-binding domain of LuxR shows a similar temperature sensitivity remains to be determined. Of interest, LuxRΔN is quite resistant to heat inactivation (Fig. 4). An analysis of LuxRΔN by UV-CD spectropolarimetry indicates that it possesses a high degree of α-helical structure (Fig. 4). This could account for the resistance of the polypeptide to heat inactivation.

The α-helical content of LuxRΔN is consistent with a role as a DNA-binding protein, and we know that it can bind to the upstream sequence in the *lux* intergenic region (30). However, because both LuxRΔN and RNAP are required for protection of the *lux* box, at this time, we can provide no direct evidence that LuxRΔN makes any contact with the *lux* box. It seems likely that LuxRΔN does come in direct contact with the *lux* box, because single amino acid changes in the LuxR C-terminal domain can suppress mutations within the *lux* box (7, 28). Regardless of whether it does or does not bind to the *lux* box directly, there is a mutually dependent interaction of LuxRΔN and RNAP with the *lux* box and *luxI* promoter that does not appear to require any other LuxRΔN-DNA interactions. Therefore, one might view LuxRΔN as an accessory factor that possibly binds to RNAP in solution, enabling it to interact with the *luxI* promoter.

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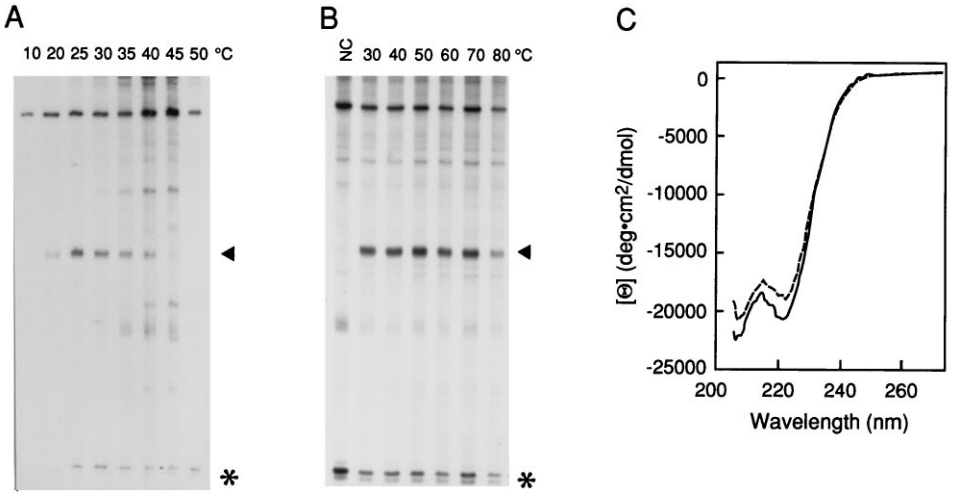

FIG. 4. Activity and stability of LuxRΔN as a function of temperature. (A) Activity as a function of temperature. The DNA template was *Hin*dIII-cut pAMS1300. Reaction mixtures contained RNAP (40 nM) and LuxRΔN (5.1 μM) and were incubated at the temperatures indicated. (B) Thermal stability of LuxRΔN. The DNA template was *Hin*dIII-cut pAMS1300. Lanes: 1, negative control (NC [no LuxRΔN added]); 2 to 7, LuxRΔN (5.1 μM) incubated for 30 min at 30, 40, 50, 60, 70, or 80°C, respectively, prior to addition to reaction mixtures. The arrowheads point to the *luxI* mRNA products, and the asterisks mark the RNA-1 mRNA products. The reaction mixtures were as described in the legend to Fig. 3. (C) Far UV-CD spectra of 1.7 μM (0.19 mg/ml) LuxRΔN at 3°C (solid line) and 30°C (dashed line). Data were acquired with an AVIV 62 DS spectropolarimeter with a 2-mm-pathlength cuvette at 0.5-nm intervals with a 2-s averaging time. The buffer consisted of 20 mM sodium phosphate, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% (vol/vol) glycerol, and 100 mM NaCl at pH 6.0. Exchange from the storage buffer was accomplished by using a Pharmacia Hi-Trap Desalting Column (Pharmacia Biotech Inc., Piscataway, N.J.). Spectra obtained with buffer alone were subtracted from the LuxRΔN spectra prior to smoothing over an interval of three data points. Protein concentrations were calculated by A_{280} measurements and by using an extinction coefficient of 13,940 for the polypeptide (15).

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