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Engineering of monomeric bacterial luciferases by fusion of *luxA* and *luxB* genes in *Vibrio harveyi*

(In vitro mutagenesis; recombinant DNA; enzyme subunit fusion; reporter gene)

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SUMMARY

Luciferase (Lux)-encoding sequences are very useful as reporter genes. However, a drawback when applying *Vibrio harveyi* Lux as a reporter enzyme in eukaryotic cells, is that it is a heterodimeric enzyme, thus requiring simultaneous synthesis of both Lux subunits to be active. To overcome this disadvantage, *luxA* and *luxB* genes encoding the A and B subunits of this light-emitting heterodimeric Lux, were fused and expressed in *Escherichia coli*. Comparative analysis of four fused monomeric Lux enzymes by in vivo enzyme assay, immunoblotting and partial enzyme purification, showed that the fused Lux were active both as AB or as BA monomers, albeit at different levels (up to 80% activity for AB and up to 2% for BA, as compared with the wild type binary A + B construct). One of the LuxAB fusion proteins was stably expressed in calli of *Nicotiana tabacum*, and displayed very high Lux activity, thus demonstrating its potential as a reporter enzyme in eukaryotic systems.

INTRODUCTION

Genes with an easily assayable product, so-called reporter genes, are important tools for the study of mechanisms underlying the regulation of gene expression. Enzymes catalyzing light-emitting reactions, such as firefly luciferase or bacterial luciferases, have recently been shown to be excellent reporter enzymes (Ow et al., 1986; Engebrecht et al., 1985; Schmetterer et al., 1986; Carmi et al., 1987;

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Abbreviations: aa, amino acid(s); bp, base pair(s); BSA, bovine serum albumin; ds, double stranded; f (in fab or fba), fused subunits; HPLC, high-performance liquid chromatography; IgG, immunoglobin G; IPTG, isopropyl-β-D-thiogalactopyranoside; Lux, luciferase(s); lux buffer, see Table II, footnote c; *luxA*, *luxB*, genes encoding LuxA or LuxB luciferase subunit polypeptides, respectively; *luxAB*, *luxBA*, genes encoding LuxAB or LuxBA fusion proteins, respectively; oligo, oligodeoxyribonucleotide; pT7, bacteriophage T7 gene *10* promoter; RBS, ribosome-binding site; RF, replicative form; SD, Shine and Dalgarno (1974) site; SDS, sodium dodecyl sulfate; ss, single strand(ed); wt, wild type.

Legocki et al., 1986; Shauer et al., 1988). The *V. harveyi* Lux is encoded by *luxA* and *luxB* cistrons, which are part of the *lux* operon, and encode the A and B subunits of a heterodimeric mixed-function oxidase (Baldwin et al., 1984). This enzyme, along with an enzyme-bound flavin mononucleotide cofactor, catalyzes the oxidation of a long-chain aldehyde. During the reaction blue-green light (490 nm) is emitted, which offers a very sensitive assay for enzyme reactivity (Ziegler and Baldwin, 1981). Because the long-chain aldehyde substrate can readily enter living cells, this reporter enzyme allows in vivo analysis of gene expression. This is of particular interest when studying gene regulation in multicellular eukaryotes. By expressing the *luxA* and *luxB* cistrons from a divergently transcribed dual promoter, the *V. harveyi* Lux was shown to be active in plants (Koncz et al., 1987). However, a major drawback with this approach was the fact that, to obtain quantitative results, both Lux subunits had to be produced at equivalent levels, or one of them had to be produced in excess over the one used as a reporter. In such a case though, the number of photons emitted in the light reaction was directly related to the level of the limiting subunit.

To simplify the use of the Lux system as a reporter enzyme system in eukaryotic cells, to eliminate the need for two open promoters for simultaneous expression of both *lux* genes, and also to test whether dicistronic prokaryotic genes can be converted to monocistronic genes, four different chimeric genes with fused *luxA* and *luxB* cistrons were constructed and their activity was tested in *E. coli* hosts. One of the monomeric *luxAB* genes was also tested in tobacco callus tissue. The results show that monomeric fused AB or BA proteins have Lux activity, albeit at different levels.

MATERIALS AND METHODS

(a) Bacterial strains

The *E. coli* strains HB101 (Boyer and Roulland-Dussoix, 1969) and JM101 (Messing et al., 1981) were used as recipients in plasmid transformation and for propagating plasmid and phage M13 vectors. The *E. coli dam*$^-$ strain TG17 (Grundström and Normark, 1985) was used in the in vitro mutagenesis experiments, and finally the *E. coli* strain BL21(DE3), carrying the T7 RNA polymerase-encoding gene on the chromosome (Studier and Moffat, 1986), was used as a host for expression of genes fused to the T7 promoter. The *E. coli* strain SM10 (Simon et al., 1983) was used for conjugation between *E. coli* and *Agrobacterium*, and the *Agrobacterium* strain 3101[pMP90RK] was used to mobilize the *lux* genes into plant cells as described (Koncz and Schell, 1986).

(b) Plasmids

Plasmids used for construction and expression of *lux* gene fusions are listed in Table I. More specifically, as expression vectors plasmids pET-7 (Rosenberg et al., 1987), pT3/T7-18 and pT3/T7-19 (BRL cat. Nos. 5379 and 5376), which carry the bacteriophage T7 promoter ϕ10 were used. Plasmid pACYC184 (Chang and Cohen, 1978) was used to introduce *luxA* or *luxB* expression cassettes into cells already containing a ColE1 replication origin-based vector. Vector M13mp18 (Norrander et al., 1983) was used in the in vitro mutagenesis experiments of *lux* gene sequences, and when subcloning these *lux* sequences, plasmid pIC20H (Marsh et al., 1984) carrying a different polylinker region, was used.

Appropriate *lux* sequences, which made possible the construction of the different *lux* gene fusions, were obtained from plasmids pLX104-a, pLX109-a, and pLX1-b (Olsson et al., 1988). In complementation experiments either plasmids pLX509-a or pLX502-b (Olsson et al., 1988), containing the *luxA* and *luxB* genes cloned in plasmid pACYC184 under the transcriptional control of the T7 promoter, were used to supply free LuxA and LuxB subunits. Plasmid pLX509-a was constructed in two steps. First, the *luxA* gene from plasmid pLX109-a, after treatment of the cohesive ends with DNA polymerase, was subcloned into the *Bam*HI site of the T7 promoter plasmid pET-7 (Rosenberg et al., 1987), forming plasmid pLX309-a. In the second step the *luxA* gene, now linked to pT7, was excised from this plasmid as a *Bgl*II-*Hin*dIII fragment and inserted between the *Hin*dIII and *Bam*HI sites of plasmid pACYC184 to create plasmid pLX509-a. The construction of plasmid pLX502-b, producing free LuxA subunits has been described (Olsson et al., 1988).

To synthesize the Lux fusion protein in plant cells, the binary plasmid pPCV702.hygro, carrying the CaMV 35S promoter and the bacterial *hyg* gene as a plant-selectable marker was exploited (C.K., unpublished; F. Sitbon, unpublished). The *luxAB* gene from plasmid pLX702-fab was linkered with *Bam*HI at its 5′-end and fused to the 35S promoter giving rise to plasmid p35SluxF2 (Table I). To synthesize wt Lux enzyme in tobacco cells, the *luxA* gene was also cloned in pPCV702.hygro, and the *luxB* gene was inserted in a derivative of this plasmid denoted pPCV702.kana, that carried the bacterial neomycin-phosphotransferase-encoding gene (Herrera-Estrella et al., 1983a) as a plant-selectable marker (Table I). These constructs were mobilized to *Agrobacterium* as described (Koncz and Schell, 1986) and introduced into the plant by the leaf disc method (Horsch et al., 1985).

(c) Cloning methods

Bacterial culture media, procedures for plasmid purification, DNA fragment isolation, for the use of restriction endonucleases and other DNA enzymes, were as recommended by the manufacturers, or as described (Olsson et al., 1988; Maniatis et al., 1982). Nucleotide sequencing was carried out according to the dideoxy chain-termination method (Sanger et al., 1977).

TABLE I

Plasmids and M13 phage used in the present study

Plasmid (or phage)	Characteristics[a]	Reference
pT3/T7-18	Phage T3 and T7 promoter	BRL cat. no. 5379
pT3/T7-19	Phage T3 and T7 promoter	BRL cat. no. 5376
pET-7	Phage T7 promoter	Rosenberg et al. (1987)
pACYC184	P15a replicon	Chang and Cohen (1978)
M13mp18	Phage M13mp derivative	Norrander et al. (1984)
pIC20H	Multiple cloning sites	Marsh et al. (1984)
pLX104-a	*luxA* gene on M13mp18	Olsson et al. (1988)
pLX109-a	*luxA* gene on M13mp18	Olsson et al. (1988)
pLX1-b	*luxB* gene on M13mp18	Olsson et al. (1988)
pLX309-a	*luxA* gene on pET-7	This paper
pLX509-a	*luxA* gene expressed from T7 promoter on pACYC184	This paper
pLX502-b	*luxB* gene expressed from T7 promoter on pACYC184	Olsson et al. (1988)
pLX203-ab	wt *luxA* and *luxB* genes on pT3/T7-19.	Olsson et al. (1988)
pLX209-ab	wt *luxA* and *luxB* genes on pT3/T7-19. *luxA* gene without SD site	Olsson et al. (1988)
pLX701-a	Mutagenized *luxA* gene in pT3/T7-19	This paper
pLX702-a	Mutagenized *luxA* gene in pT3/T7-19	This paper
pLX701-fab	Hybrid *luxAB* gene in pT3/T7-19	This paper
pLX702-fab	Hybrid *luxAB* gene in pT3/T7-19	This paper
pLX21-b	C-term. deleted *luxB* gene in pT3/T7-18	This paper
pICLX104-a	*luxA* gene from pLX104-a in pIC20H	This paper
pICLX109-a	*luxA* gene from pLX109-a in pIC20H	This paper
pLX703-fba	Hybrid *luxBA* gene in pT3/T7-19	This paper
pLX704-fba	Hybrid *luxBA* gene in pT3/T7-19	This paper
pPCV702.kana	Binary plant expression vector carrying kanamycin resistance and CaMV 35S open promoter	C. Koncz
pPCV702.hygro	Binary plant expression vector carrying hygromycin resistance and CaMV 35S open promoter	F. Sitbon
p35S*luxF*2.hygro	*luxAB*2 fusion from pLX702-fab linked with *Bam*HI on the 5′-end cloned in pPCV702.hygro	F. Sitbon
p35S*luxA*. kana	*luxA* gene from pLX109-a in pPCV702.kana	This paper
p35S*luxB*.hygro	*luxB* gene from pLX1-b in pPCV702.hygro	This paper
pMp90RK	Ti-plasmid derivative	Koncz and Schell (1986)

[a] Only characteristics most relevant for this work are shown.

(d) Construction of the *luxAB* gene fusions

The ss DNA, amplified in an *E. coli dam*$^-$ strain, was prepared from the M13-derived plasmid pLX109-a. This plasmid carries the complete *luxA* gene, the intergenic region between the *luxA* and *luxB* genes, and sequences from the 5′ coding region of the *luxB* gene. The ss DNA template was annealed to two oligo primers to generate *luxAB* gene fusions. The ss DNAs were made ds by in vitro synthesis using the Klenow large fragment of *E. coli* DNA polymerase I. The newly synthesized ds DNA was transformed into *E. coli* strain JM101 and the mutagenized clones were isolated by differential plaque hybridization, using the respective mutagenesis primer as a probe (Burke and Olson, 1986). Thus, by means of a 33-mer primer (5′–CTCAAAGAAAAA-CAGCTGATGAAATTTGGATTA–3′), the intergenic spacer sequence, including the *luxA* stop codon, between the *luxA* and the *luxB* genes was deleted. By a second 31-mer primer (5′–AAAAA-CAGTACTTAATATTTTCTCAAAAGGA–3′), an addition of a single bp to the intergenic spacer sequence shifted the *luxB* gene to the same reading frame as the *luxA* gene, and a single bp mutation converted the *luxA* stop codon TAA to a tyrosine codon TAC.

Several pLX109-a-derived clones were isolated and the nt sequences through the fused regions were determined. The new *luxAB* sequences were isolated as *Sal*I-*Eco*RI fragments from the ds RF-form of the mutagenized phages, and these fragments were subcloned to plasmid pT3/T7-19. In this way plasmids pLX701-a and pLX702-a were formed, which contained sequences derived from the 33-mer and 31-mer mutagenesis primers, respectively. The missing 3′ part of the *luxB* coding sequences in these mutagenized *lux* constructs was then added by the cloning of a *luxB* *Eco*RI fragment from plasmid pLX1-b into the single *Eco*RI sites of pLX701-a and pLX702-a, creating plasmids pLX701-fab and pLX702-fab.

The detailed construction of these fused *luxAB* genes is outlined in Fig. 1. Fig. 3A shows the nt sequence of the mutated intergenic regions, as well as the expected aa sequences of the interconnecting polypeptides joining the LuxA and LuxB subunit domains of fused LuxAB enzymes, encoded by plasmids pLX701-fab and pLX702-fab.

(e) Construction of *luxBA* gene fusions

Two different *luxBA* gene fusions were constructed by fusing two *luxA* sequences, slightly different in their 5′ ends, to the 3′ end of the *luxB* gene. This was accomplished by first removing DNA corresponding to the 10 C-terminal aa of the *luxB* gene, by subcloning a *Bam*HI-*Cla*I fragment from plasmid pLX1-b into plasmid pT3/T7-18 to obtain plasmid pLX21-b. In parallel, *luxA* gene derivatives carrying 5′-noncoding sequences of different lengths were subcloned from plasmids pLX104-a and pLX109-a as *Sph*I-*Eco*RI fragments into the polylinker of plasmid pIC20H, forming the plasmids pICLX104-a and pICLX109-a, respectively. Finally, in-frame *luxA* gene fusions to the 3′ deleted end of the *luxB* gene were obtained by the cloning of the *luxA* *Sph*I-*Hin*dIII fragments from plasmids pICLX104-a and pICLX109-a into the *Sph*I-*Hin*dIII sites of plasmid pLX21-b (see Fig. 2). Sequence joints between the *luxB* and *luxA* coding regions of plasmids pLX703-fba and pLX704-fba, as well as the deduced aa sequence of the interconnecting peptides in LuxBA fused proteins, are depicted in Fig. 3B.

(f) High-performance liquid chromatography

Solvents were delivered at a flow rate of 0.2 ml/min. Crude protein extracts, or standard protein mixes were introduced off-column via an Altex mod. 210 sample injection valve at 4 °C. Steric exclusion chromatography utilized two linked LKB TSK 3000 PW columns eluted isocratically with 0.15 M Na_2HPO_4/NaH_2PO_4 + 0.1 M NaCl pH 6.6. Fractions (0.9 ml) were collected and stored cold for further analysis.

RESULTS AND DISCUSSION

(a) Generation of *luxAB* gene fusions

In-frame translational *luxAB* gene fusions were constructed by primer mismatch mutagenesis, as outlined in Fig. 1. The first primer was designed in such a way that the *luxAB* gene fusion, represented by plasmid pLX701-fab, will encode a monomeric Lux in which the N-terminal A domain and the

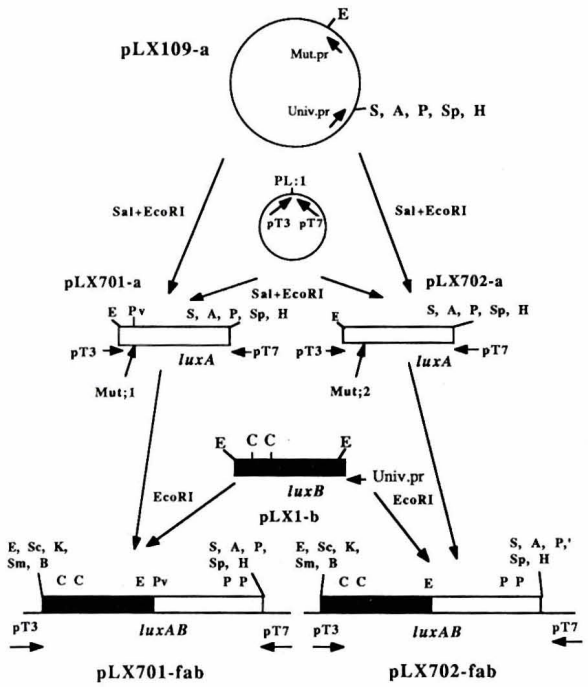

Fig. 1. Construction of the fused *luxAB* genes. Plasmid names and restriction sites are indicated in bold letters. Long arrows show different cloning steps, where DNA was excised with restriction enzymes as indicated. All restriction sites in different polylinkers are shown only in the final constructs. The *luxA* coding sequences are represented by open boxes, and the *luxB* coding sequences by filled boxes. Abbreviations: PL:1, polylinker sequence; A, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I. Univ. pr., M13 universal sequencing primer; Mut. pr., primers used for in vitro mutagenesis; pT3 and pT7, promoters for phage T3 and T7 RNA polymerase, respectively; Mut;1 and Mut;2, in vitro generated mutants.

C-terminal B domain is separated by a single aa. To alter the original *lux* sequences as little as possible, the primer sequence was chosen so that the intergenic sequence separating the *luxA* and *luxB* coding regions was deleted and that the TAA stop codon of the *luxA* gene was converted to a leucine codon CTG. In this process the natural RBS (SD site) of the *luxB* gene was removed. However, unexpectedly some free LuxB subunits were still synthesized in *E. coli* cells carrying the pLX701-fab plasmid (Fig. 4, lanes 1 and 7). These Lux subunits are most likely initiated from an RBS-like sequence in the 3′ part of the *luxA* coding sequence.

To generate a second *luxAB* gene fusion, a synthetic primer was designed to convert the stop codon of the *luxA* gene to a tyrosine codon, TAC, and to add a single nt to the intergenic spacer sequence, linking the *luxA* and *luxB* reading frames. When expressed in plasmid pLX702-fab, a monomeric Lux was synthesized, in which the A and B domains are separated by 10 aa. Since the original RBS preceding the *luxB* coding sequence was left intact in this construct, initiation of synthesis of free LuxB subunits could be expected in the *E. coli* cells. This was verified in the immunoblot analysis (Fig. 4, lanes 2 and 8).

(b) Generation of *luxBA* gene fusions

To test whether monomeric Lux enzymes, translated in reverse order from the original *lux* operon, i.e., with N-terminal B and C-terminal A domains still able to fold into an active enzyme, *luxA* sequences were fused to a 3′-end-deleted derivative of the *luxB* gene. As a first step in this construction, the 3′ coding region of the *luxB* gene was removed by subcloning the *Bam*HI-*Cla*I fragment of plasmid pLX1-b (Olsson et al., 1988) into the *Bam*HI-*Acc*I sites of plasmid pT3/T7-18 (Fig. 2). To verify that this deletion did not negatively affect the function of the Lux enzyme, the C-terminally modified *luxB* gene was expressed from the T7 promoter of plasmid pLX21-b in *E. coli* BL21(DE3) cells, and the truncated LuxB subunit was synthesized. When an intact A subunit was supplied to the same cells from the compatible plasmid pLX509-a, the truncated B subunit assembled with the intact A subunit into a functional enzyme (not shown). A similar analysis performed with other 3′-end deletion derivatives of the *luxB* gene (Sugihara and Baldwin, 1988; O.O., unpublished data) also confirmed that the C-terminal end of the LuxB subunit does not influence the stability or activity of the heterodimeric Lux.

In a second step, two *luxA* gene constructs with slightly different 5′-ends (Figs. 2 and 4) were fused in-frame to the deleted 3′-end of the *luxB* gene, forming plasmids pLX703-fba and pLX704-fba. The in-frame translational *luxBA* fusions carried by these plasmids differ from each other only in the length of the sequences connecting the *luxB* and *luxA* coding regions. These sequences, 45 and 27 bp in size, were derived from the polylinker of vector M13mp18. The RBS of the endogenous *luxA* sequence was removed to minimize the translation of free LuxA polypeptides. However, the connector sequences display

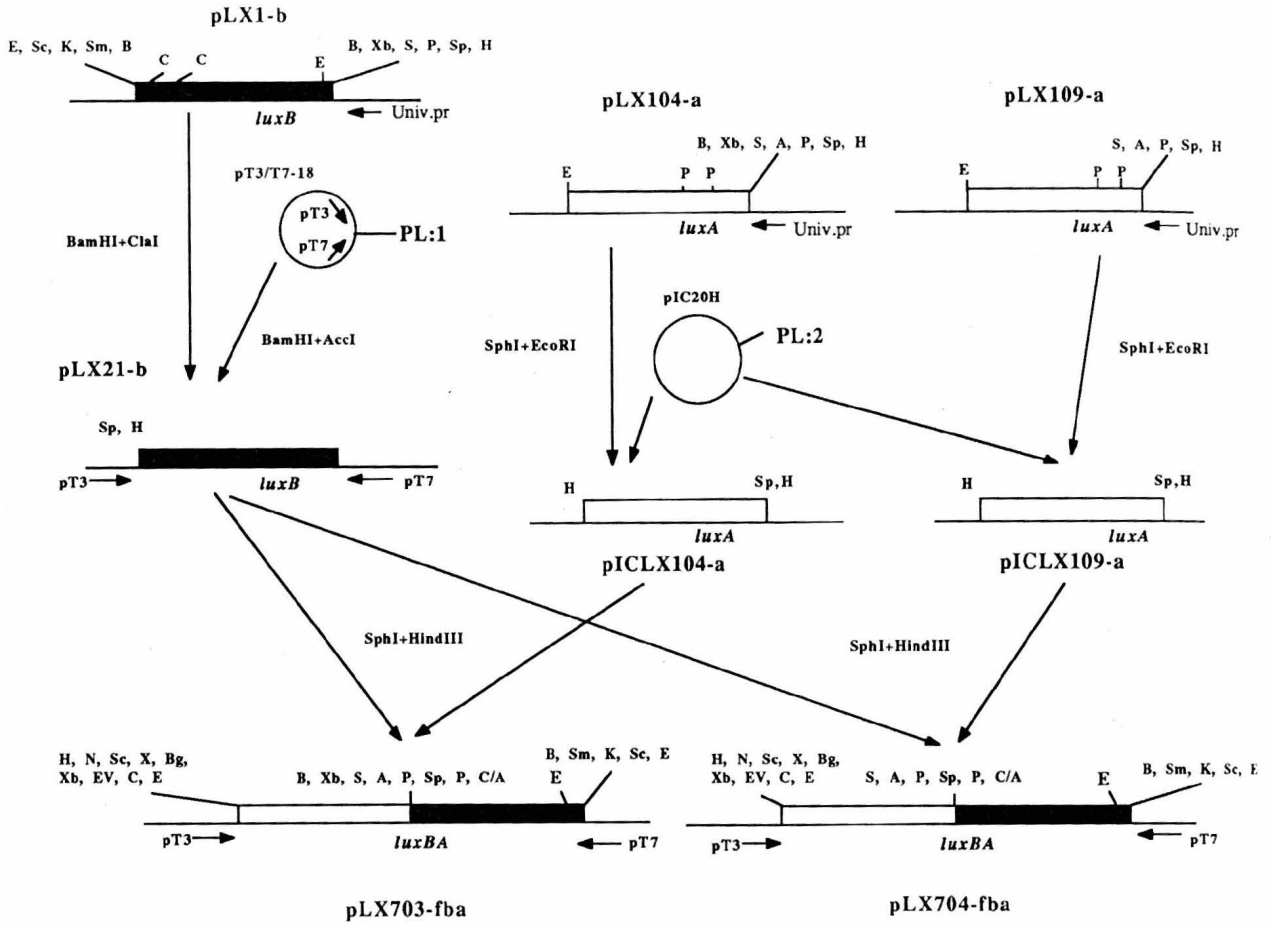

Fig. 2. Construction of the fused *luxBA* genes. Symbols are the same as in Fig. 1. Other abbreviations: PL : 2, polylinker sequence; Bg, *Bgl*II; EV, *Eco*RV; N, *Nru*I; Sc, *Sac*I; X, *Xho*I. C/A denotes a fused *Cla*I-*Acc*I site where both are destroyed.

a certain homology to the consensus SD sequence (Fig. 3B) and therefore some initiation of translation of LuxA subunit from the *luxBA* transcripts can be expected. In Western blots (Fig. 4, lanes 3 and 9) a band corresponding to the A subunit can indeed be observed.

(c) Synthesis of LuxAB and LuxBA monomeric Lux enzymes

To analyse the monomeric LuxAB and LuxBA proteins, plasmids carrying *lux* gene fusions under the transcriptional control of the T7 promoter were introduced to competent *E. coli* BL21(DE3) cells by transformation. This *E. coli* strain contains a *lac* promoter driving the transcription of a T7 RNA polymerase-encoding gene, which can be induced by IPTG (Studier and Moffat, 1986). To monitor the synthesis of fused Lux proteins, crude total protein extracts were prepared from *E. coli* BL21(DE3) transformants and similar amounts of cells were fractionated on SDS–polyacrylamide gels. Following electrophoretic transfer of proteins to nitrocellulose filters, the blots were treated with anti-LuxA and anti-LuxB antibodies and the Lux protein bands were visualized by the alkaline phosphatase system.

Cells harbouring plasmids pLX203-ab and pLX209-ab (Olsson et al., 1988) and expressing the wt heterodimeric Lux enzyme, were used as controls (Fig. 4, lanes 5, 6, 11 and 12). The *luxA* gene in the pLX209-ab construct lacks an RBS, hence the lower level of LuxA polypeptide in lanes 6 and 12. In Fig. 4, lanes 1 to 4, the appearance of novel proteins of approx. 75 kDa, which reacted with the Lux antibodies, could be seen. These proteins were present

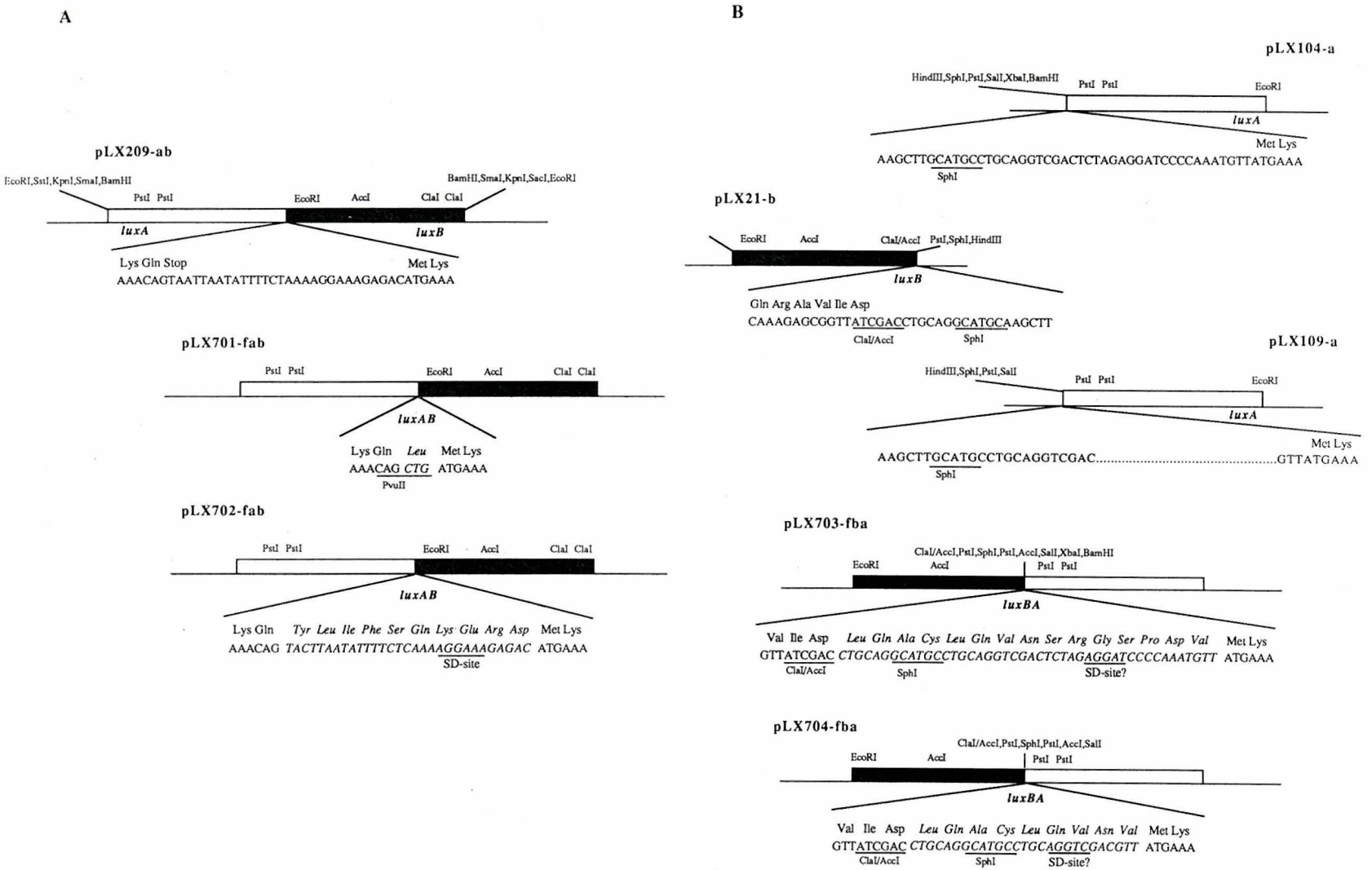

Fig. 3. Nucleotide sequence of linkers connecting the fused *lux* domains. Deduced aa sequence of linker peptides separating the original Lux polypeptides is in italics. (**A**) The *luxAB* gene fusions. Corresponding wt nt sequence in pLX209-ab is shown for comparison. (**B**) The *luxBA* gene fusions. Corresponding wt nt sequences in pLX104-ab, pLX109-ab and pLX21-b are shown for comparison. Restriction sites relevant for the constructions are indicated. SD site?, possible Shine and Dalgarno site.

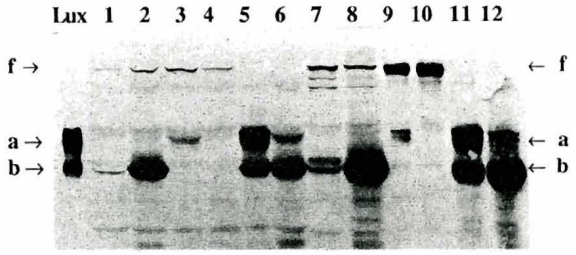

Fig. 4. Immunoblot analysis of LuxAB, LuxBA, LuxA, and LuxB proteins. A 20 μl sample of cells used in the Lux assay was boiled in SDS sample buffer (100 mM Tris · HCl pH 6.8/10 mM DTT/10% glycerol/1% SDS/0.1% bromphenol blue) and the proteins were separated on a 0.1% SDS–12% polyacrylamide gel (Laemmli, 1970) and transferred to a nitrocellulose filter by electrophoresis in transfer buffer (10 mM $NaHCO_3$/3 mM Na_2CO_3/20% methanol) as described (Dunn, 1986; Tobin et al., 1979). After treating the filter with rabbit anti-LuxA and anti-LuxB IgG, the Lux proteins were visualized by alkaline phosphatase conjugated goat anti-rabbit IgG (Promega Protoblot™). The relative amount of Lux subunits and fusion proteins loaded on the gel was determined by quantitative scanning of the stained Lux protein bands. Lux, purified wt *V. harveyi* luciferase enzyme; f, fused Lux enzyme; a, wt LuxA subunit; b, wt LuxB subunit. Lanes: 1–12, extracts from cells carrying *lux* plasmids; 1–6, non-induced cells; 7–12, induced cells; 1 and 7, pLX701-fab; 2 and 8, pLX702-fab; 3 and 9, pLX703-fba; 4 and 10, pLX704-fba; 5 and 11, pLX203-ab; 6 and 12, pLX209-ab (see Table I). Arrowheads show positions of fused and wt luciferase polypeptides. Molecular sizes (in kDa) of LuxA: 40.1; LuxB: 36.6; LuxAB and BA: 76.5–77.8 kDa.

in extracts of cells harboring lux gene fusions, but were missing in control samples. The amount of 75-kDa proteins increased markedly after IPTG induction (Fig. 4, lanes 7 to 10) indicating that these proteins were indeed translated from the transcripts derived from the T7 promoter-driven *luxAB* and *luxBA* gene fusions. In addition to fused Lux proteins, free A or B Lux subunits were also detected in different extracts. The amount of free subunits increased with IPTG induction which confirmed that these subunits were also translated from the fused *lux* gene transcripts.

Plasmid pLX702-fab, containing the RBS of the *luxB* gene, produced a much higher amount of B subunit than plasmid pLX701-fab lacking this SD sequence. The fact that pLX701-fab also slightly promoted the synthesis of B subunit, suggested that sequences in the *luxA* coding region served as a weak RBS (Fig. 3A). Translation of the A subunit from *luxBA* transcripts probably involved putative SD sites upstream from the *luxA* coding sequence (Fig. 3B).

(d) Activity of LuxAB and LuxBA monomeric Lux enzymes

Cell aliquots producing the different Lux fusions were divided into two parts. One part was analysed by immunoblotting as described (see legend to Fig. 4), while Lux activity was assayed on the other part. The data summarized in Table II show that all four fusions displayed Lux activity, albeit at different levels. LuxAB fusions were found to be more active than LuxBA fusions. An attempt to compare the activity of the different fusions on a relative basis was done on the assumption that the fused Lux proteins contributed to all Lux activity in the *E. coli* cells (Table II).

However, as we have recently shown, N-terminally extended LuxA proteins also join the B subunit to form functional enzymes (Olsson et al., 1988). Therefore it was conceivable that assembly of fused LuxAB proteins with free B subunits as well as that of fused LuxBA proteins with free A subunits to produce AB_2 or BA_2 heterodimers could occur, and that the observed Lux activity in our experiments is partly mediated by these complexes. In such a case, the relative concentration of fused AB or BA proteins and of free subunits, and/or the efficiency of their assembly could influence the activity observed with different constructs.

To be able to differentiate between these possibilities we did three different sets of experiments.

First, we tested whether the over-production of free LuxA subunits from plasmid pLX509-a, and free LuxB subunits from plasmid pLX502-b could alter the light-emitting activity of cells containing different fused *lux* gene constructs. Hence, *luxAB* plasmids and pLX502-b, as well as *luxBA* plasmids and pLX509-a, were cotransformed into *E. coli* BL21(DE3) cells. Lux activity, and the synthesis of fused Lux proteins, as well as free subunits, was monitored as described above. When compared to control cells which synthesized amounts of Lux fusion proteins similar to the double transformants, no significant stimulation of enzyme activity was observed with the addition of the free subunits (not shown).

Second, protein extracts were prepared from cells

TABLE II

Relative and normalized activities of luciferase enzymes

Plasmid[a]	Lux[b]	mV/A_{595}[c]	Relative activity[d]	Normalized activity[e]
pLX701-fab	AB1	175	3.6	18
pLX702-fab	AB2	1580	33	80
pLX703-fba	BA1	67	1.4	1.6
pLX704-fba	BA2	1.6	0.03	0.07
pLX209-ab	A + B	4845	100	100

[a] Plasmids carrying different *lux* genes, expressed in *E. coli* BL21(DE3) hosts. 'f' stands for fusion and 'a' and 'b' for LuxA and LuxB subunits. See also Table I and Figs. 1 and 2 for more details.

[b] Luciferase fusion expressed from respective plasmids. See Fig. 3 for details on connector region linking the *lux* genes. pLX209-ab was used as a control, since it lacks a RBS and the *luxA* gene in this construct has the same 5'-end as the two *luxAB* fusions. Therefore similar amounts of Lux proteins are synthesized in cells carrying these plasmids.

[c] Lux activity was assayed directly in living cells as described (Olsson et al., 1988). Essentially, 200-μl aliquots of competent *E. coli* BL21(DE3) cells were transformed with 50 ng of plasmid DNA, plated and grown on selective LB-agar plates. Cells (10 to 100 μl), resuspended in lux buffer (50 mM Na · phosphate pH 7.0/50 mM β-mercaptoethanol/2% BSA) to a density of A_{595} = 1, were mixed with 400 μl lux buffer, and placed in a luminometer (LKB1250). The peak value of Lux-mediated light emission produced during the first 10 s of the light reaction was recorded, after initiating the reaction by injecting 50 μl of a 0.1% sonicated solution of *n*-decanal substrate (Sigma D-7384). Lux activities were determined as mV/ml cells at A_{595} = 1.0.

[d] Relative activities were obtained by dividing by the activity obtained from wt pLX209-ab-containing cells, which was taken as 100%.

[e] Normalized Lux activities were obtained by relating the relative activities to the total amount of luciferase protein loaded on the gel. The LuxA band from pLX209-ab cells was used as the internal control (Fig. 4, lane 6), and was related to the amount of fused protein in the 75 kDa bands (Fig. 4, lanes 1–4). It was assumed that 90% of the relative luciferase activity obtained was contributed by the monomeric luciferase enzyme (see Table III).

containing *lux* gene fusions and size-fractionated on an HPLC LKB-TSK-3000 PW column. Each extract was separated into 30 fractions, with decreasing molecular sizes in later fractions. Lux activity was assayed from each fraction (Table III). As can be deduced from Table III, 82–94% of total Lux activity eluted from fractions 17 and 18, which contained proteins of 70–80 kDa, i.e., the fused monomeric luciferases. The rest of the Lux activity was located in fraction 15. This fraction contained proteins of about 110–120 kDa, thus corresponding to heterodimeric complexes formed between fused Lux proteins and free subunits. No other protein fraction displayed enzymatic activity, indicating that, if fused Lux monomers did assemble into homodimers, such dimers were either inactive, or present in such low amounts that they were not detected under these conditions. Unfortunately, the amount of specific Lux proteins loaded on the HPLC column was not high enough to allow later visualization of the Lux proteins in the different fractions on an immunoblot. Attempts to apply more protein on the column resulted in overloading.

Third, the *luxAB* gene from the most active fusion (pLX702-fab) was cloned under a strong plant promoter and introduced into the plant cell by leaf-disc transformation (Horsch et al., 1985). A number of transformed calli were assayed. They all displayed Lux activity, although a clonal variation was seen, as is normal in this type of experiments. However, as can be deduced from Table IV, when compared to corresponding constructs separately expressing the wt Lux subunits, either as a mean value from many different calli, or as a maximum value obtained from especially active calli, it is evident that the LuxAB2 fusion is nearly as active as the wt enzyme. Since the eukaryotic cell does not recognize RBS, and thus cannot initiate endogenous protein synthesis, this strongly indicates that the fused luciferase is active as a monomeric enzyme in tobacco cells.

Taken together these data show that the monomeric luciferases are active either as monomers, or to a much lesser extent as monomers associated with one free A or B subunit. However, since about 90% of total Lux activity resides in the monomeric enzyme (Table III), for all practical purposes the presence of free Lux subunits in the *E. coli* cells expressing a high Lux activity (pLX702-fab; Table II) can be

TABLE III

Relative luciferase activity in HPLC-fractionated crude *Escherichia coli* extracts

Plasmid[a]		Luciferase activity (in %)[b]						
	Fraction No.	13	14	15	16	17	18	19
	Lux[a]							
pLX701-fab	AB1	0	0	18	0	62	20	0
pLX702-fab	AB2	0	0	13	0	70	17	0
pLX703-fba	BA1	0	0	6	0	85	9	0
pLX704-fba	BA2	0	0	18	0	71	11	0
pLX209-ab	A + B	0	0	0	0	96	4	0
Molecular ratios			approx. 120				approx. 70	

[a] See Table II.

[b] The distribution of Lux activity after HPLC fractionation of crude extracts containing indicated fusion. Each fraction was assayed as described (Koncz et al., 1987; Olsson et al., 1988). Essentially, a sample was mixed with 400 μl lux buffer, placed in a luminometer, and the Lux reaction was started by injecting 500 μl of light-reduced flavin mononucleotide in tricine buffer, and 10 μl of the 0.1% *n*-decanal solution. The height of the light peak produced during the first 10 s of the reaction was taken as the Lux activity, which was given a value (in %) of the total activity, obtained from the sum of all fractions in each separation. The approximative molecular sizes of the proteins were determined by comparing the elution profile with a set of standard proteins separated under identical conditions.

neglected. Furthermore, preliminary data, obtained by dynamic fluorescent measurements of the fusion proteins using laser techniques, confirm that the fused Lux proteins are active as monomeric enzymes, and need not to associate with other Lux subunits to be active (A.E., in preparation).

(e) Importance of linker polypeptide length in enzyme subunit fusions

Assuming that the fused proteins are enzymatically active as monomers, a comparison of the measured activities of the different AB and BA constructs (Table II) indicates that the length of the peptide region linking the *luxA*- and *luxB*-coding sequences plays a role in determining the activity of the fused protein. The only difference between the two LuxAB fusions is the number of aa separating the original A and B polypeptide subunits. In pLX701-fab only 1 aa separates the two subunits, whereas in pLX702-fab, which is about five times more active, 10 aa separate the two subunits. A similar general conclusion is reached when comparing the two BA fused proteins. Constructs pLX704-fba and pLX703-fba are identical except that in the pLX703-fba construct the region connecting B with

TABLE IV

Luciferase activity in tobacco calli

Plasmid[a]	Activity (av.)[b]	% of wt[c]	Activity (max.)[d]	% of wt[c]
None	1		2	
p35S*luxF*2	450	56	1500	63
p35S*luxA*.kana + p35S*luxB*.hygro	800	100	2400	100

[a] Plant expression vectors. See Table I for more details.

[b] Average luciferase activity from 25 individual calli. To determine Lux activity in tobacco tissue, 1-month-old callus was homogenized in lux buffer, cell debris was removed by centrifugation and the supernantant was assayed for Lux activity as described in footnote b, Table III. Activity is given as mV/mg total protein in the crude plant extracts. Total protein was measured by the Bradford (1976) assay.

[c] Relation between wt and fusion protein luciferase activities in %.

[d] The highest Lux activity value obtained from 25 individual calli. Activity measurements were as in footnote b.

A is approx. 6 aa longer. The specific activity of pLX703-fba is 23-fold higher than that of pLX704-fba.

A few examples of other fused monomeric enzymes can be found in the literature, and experiments with these fused proteins also indicated that in these monomeric enzymes the length of the connecting polypeptide influenced the enzymatic function. The subunits of *E. coli* glycyl-tRNA synthetase were fused by in vitro mutagenesis and linked by a connector region of 6 aa. Different aa changes in the connector region did not influence the activity of fused glycyl-tRNA synthetase subunits (Toth and Schimmel, 1986). Another gene fusion occurs naturally between the anthranilate synthase component II and the indole-3-glycerol phosphate synthase. These enzymes are encoded by *trpG* and *trpC* genes in *E. coli*, but synthesized as a single bifunctional protein by the *TRP3* gene in yeast and by the *trp1* gene in *Neurospora* (Crawford et al., 1987). The bifunctional enzymes contain a connector region of 11 aa between the subunit domains. Since the aa sequence of the yeast connector peptide is not identical to that of *Neurospora*, it is very likely that only the length of the connector peptide is important for the activity of these bifunctional enzymes. A final example is tryptophan synthase, which is a heterodimer encoded by the *trpB* and *trpA* genes in prokaryotes (Zalkin et al., 1984; Crawford, 1975; Hütter et al., 1986) but by a single gene in yeast (Zalkin and Yanofsky, 1982). Sequence alignment of prokaryotic and yeast genes revealed that the yeast enzyme is translated in reverse order in yeast and that the subunits are connected by a 66-aa polypeptide. A deletion removing 18 aa of the connector region caused complete inactivation of the enzyme, while a replacement of this deletion with 14 different aa partially restored the activity. A spontaneous duplication of 16 aa in the connector region resulted in a further increase in enzymatic activity, highlighting the importance of the length of connecting peptides in determining the activity of fused enzymes.

Up to now no naturally occurring fused Lux enzymes have been found. However, in analogy to the examples of naturally existing fusions, the activity of the Lux monomeric enzymes is also affected by the distance spacing the two subunits. This could indicate that heteromeric enzyme subunits in general can be fused to bifunctional monomeric enzymes provided a linker of the correct length can be found. If so, this would be a novel approach in protein engineering, and allow for the creation of multifunctional 'super enzymes' by means of enzyme subunit fusions.

(f) Use of the monomeric Lux enzyme as a reporter enzyme

Monocistronic bacterial genes coding for enzymes such as β-galactosidase (Helmer et al., 1984), neomycin phosphotransferase (Herrera-Estrella et al., 1983a,b; Fraley et al., 1983), or chloramphenicol acetyl transferase (Herrera-Estrella et al., 1983a), have been very useful as 'reporter genes' for the structure-function analysis of both prokaryotic and eukaryotic gene expression regulation mechanisms. The usefulness of heteromeric enzymes as reporter enzymes, on the other hand, is limited by the fact that they are coded for either by polycistronic mRNAs or by separate transcripts, as is also the case for *V. harveyi* luciferase.

However, since the herein described monomeric AB Lux enzymes expressed by plasmids pLX701-fab and pLX702-fab display high light-emitting activity (Table II), these fused *luxAB* genes can now be applied as reporter cassettes in studies of gene expression. The fused *luxAB* genes were constructed from a trimmed-down version of the *luxA* gene (Fig. 3, Olsson et al., 1988), and therefore have no extra ATG codons upstream from the natural *luxA* methionine start codon. The *lux* fusion cassettes can thus, without further modifications, be subcloned into an appropriate vector for expression in eukaryotic cells.

Accordingly, not only have we already expressed the fused *luxAB2* gene (by pLX702-fab in Table II) in plant cells (Table IV), but we have also successfully synthesized the same active monomeric Lux enzyme in mammalian and yeast cells (O.O., unpublished data).

(g) Conclusions

We have constructed a monocistronic *lux* gene that can be used as a reporter gene in both pro- and eukaryotic systems. Since this artificial Lux is about 80% as active as wt A + B Lux, it combines the advantages of great sensitivity of light assay, and the

possibility of monitoring gene expression in a non-destructive manner in living cells or organisms, with the benefits of using a single gene as a reporter gene.

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