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The use of the *luxA* gene of the bacterial luciferase operon as a reporter gene

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Summary. Bacterial luciferase can be assayed rapidly and with high sensitivity both in vivo and in vitro. Here we demonstrate that the N-terminal hydrophobic domain of the α catalytic subunit of the luciferase enzyme is indispensable for enzyme activity, although N-terminal translational fusions with full luciferase activity can be obtained. Bacterial luciferase is therefore ideally suited as a reporter enzyme for gene fusion experiments. A list of vectors for the convenient use of the luciferase marker genes to monitor gene expression in vivo are presented.

Key words: Bacterial luciferase – 5′*luxA* deletions – *luxA* gene fusions – Vectors

Introduction

Bacterial luciferase enzymes catalyse a light emitting reaction in luminous bacteria. The light emitting luciferase catalysed reaction is as follows:

$$RCHO + O_2 + FMNH_2$$
$$\rightarrow RCOOH + FMN + H_2O + h\nu (490\ nm),$$

in which R is an aliphatic moiety containing at least seven carbon atoms, FMN is a flavin mononucleotide, and $FMNH_2$ is reduced flavin mononucleotide. In bacteria, the oxidized flavin is efficiently reduced and continuously available to cytoplasmatic enzymes, such as luciferase. Upon external addition of the aldehyde substrate, which instantly penetrates living cells, the activity of luciferase can be followed in vivo by measuring light emission. Light can be monitored by a number of methods and with high sensitivity. Since a bacterial luciferase molecule gives rise to about one photon in the luciferase reaction, as little as 10^5 luciferase molecules can be detected by e.g. a luminometer. This level of detection is several orders of magnitudes lower than for any other non-light producing enzymatic reaction. Background bioluminescence in the assay is virtually zero, so the detection level of the reaction is only limited by the capacity of the instrument. With improvement in the instrumentation, a single *Escherichia coli* cell expressing the bacterial luciferase enzyme will soon be detectable.

The luciferase gene cluster from the marine microorganisms *Vibrio fisheri*, the *luxAB* structural genes from *V. harveyi*, and the firefly cDNA from *Photinus pyralis* have recently been introduced as reporter genes in procaryotic (Engebrecht et al. 1985; Legocki et al. 1986; Schmetterer et al. 1986; Carmi et al. 1987), as well as in eucaryotic organisms (Koncz et al. 1987; Ow et al. 1986).

The firefly luciferase enzyme catalyzes the ATP-dependent oxidation of a high molecular weight substrate, luciferin. This substance is only slowly transported through cell membranes, in contrast to the aldehyde substrate in the bacterial reaction. Therefore the bacterial luciferase system seems more suitable to the study of environmental or developmental changes in gene expression in living cells.

Presently, little is known about the function of the N-terminal part of the bacterial luciferase subunits and no vectors are available for the simple construction of in-frame translational luciferase fusions. In *V. harveyi* the structural *lux* genes are organized as an operon (for a review, see Ziegler and Baldwin 1981). Two of the five structural genes, namely the *luxA* and *luxB* genes, encode the luciferase enzyme, and these two genes have recently been cloned, sequenced and expressed in *E. coli* (Baldwin et al. 1984; Cohn et al. 1985; Johnston et al. 1986). In order to develop the bacterial luciferase enzyme as a reporter enzyme for transcriptional and translational studies in procaryotes and eucaryotes, we describe here the construction and function of a number of *luxA* transcriptional and translational gene fusions.

Materials and methods

Bacterial strains and plasmids. The *E. coli* strain HB101 (Boyer and Roulland-Dussoix 1969) was used as a recipient when amplifying plasmids. The *E. coli* strain BL21(DE3) (Studier and Moffatt 1986) was used for T7 promoter expression. T7 promoter plasmids used were T3/T7–19 (BRL catalogue number 5376), pET-3c, pET-3a and pET-7 (Rosenberg et al. 1987). Other cloning vectors used were pBR322 (Bolivar et al. 1977), pACYC184 (Chang and Cohen 1978), and M13 mp18 (Norrlander et al. 1983). Starting from the *V. harveyi luxAB* gene cluster in plasmid TB7 (Baldwin et al. 1984), a series of luciferase derivative plasmids were constructed, and are listed in Table 1.

Cloning methods. Bacteria culture media, procedures for DNA fragment isolation, for the use of restriction endonucleases and other DNA enzymes was as recommended by the manufacturers, or as described (Maniatis et al. 1982). Preparation of double stranded M13 replicative form was

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Table 1. Relevant *lux* vectors

Vector[a]	Acceptor plasmid	Gene[b]	Deleted added[c,d,e]	SD[f]	Reference
pLX101-a	M13, mp18	*luxA*	+150 bp	Yes	This paper
pLX102-a	M13, mp18	*luxA*	+45 bp	Yes	This paper
pLX103-a	M13, mp18	*luxA*	+15 bp	Yes	This paper
pLX104-a	M13, mp18	*luxA*	+7 bp	No	This paper
pLX105-a	M13, mp18	*luxA*	+1 bp	No	This paper
pLX106-a	M13, mp18	*luxA*	0 bp	No	This paper
pLX107-a	M13, mp18	*luxA*	+3 bp	No	This paper
pLX108-a	M13, mp18	*luxA*	+4 bp	No	This paper
pLX109-a	M13, mp18	*luxA*	+3 bp	No	Koncz et al. 1987
pLX110-a	M13, mp18	*luxA*	−1 bp	No	This paper
pLX111-a	M13, mp18	*luxA*	−6 bp	No	This paper
pLX112-a	M13, mp18	*luxA*	−11 bp	No	This paper
pLX113-a	M13, mp18	*luxA*	−11 bp	No	This paper
pLX114-a	M13, mp18	*luxA*	−12 bp	No	This paper
pLX115-a	M13, mp18	*luxA*	−16 bp	No	This paper
pLX116-a	M13, mp18	*luxA*	−27 bp	No	This paper
pLX117-a	M13, mp18	*luxA*	−28 bp	No	This paper
pLX118-a	M13, mp18	*luxA*	−31 bp	No	This paper
pLX119-a	M13, mp18	*luxA*	−59 bp	No	This paper
pLX120-a	M13, mp18	*luxA*	−69 bp	No	This paper
pLX203-a	pT3/T7–19	*luxA*	+15 bp	Yes	This paper
pLX207-a	pT3/T7–19	*luxA*	+4 **aa**	No(?)	This paper
pLX209-a	pT3/T7–19	*luxA*	0 **aa**	No	This paper
pLX218-a	pT3/T7–19	*luxA*	+4/−11 **aa**	No	This paper
pLX219-a	pT3/T7–19	*luxA*	+4/−20 **aa**	No	This paper
pLX203-ab	pLX203-a	*luxAB*	+15 bp	Yes	This paper
pLX207-ab	pLX207-a	*luxAB*	+4 **aa**	No(?)	This paper
pLX209-ab	pLX209-a	*luxAB*	0 **aa**	No	This paper
pLX218-ab	pLX218-a	*luxAB*	+4/−11 **aa**	No	This paper
pLX219-ab	pLX219-a	*luxAB*	+4/−20 **aa**	No	This paper
pLX303-a	pET-3c	*luxA*	0 **aa**	Yes	This paper
pLX304-a	pET-3c	*luxA*	+17 **aa**	Yes	This paper
pLX311-a	pET-3a	*luxA*	+14/−2 **aa**	Yes	This paper
pLX312-a	pET-3c	*luxA*	+15/−4 **aa**	Yes	This paper
pLX320-a	pET-3a	*luxA*	+14/−23 **aa**	Yes	This paper
pLX303-ab	pLX303-a	*luxAB*	0 **aa**	Yes	This paper
pLX304-ab	pLX304-a	*luxAB*	+17 **aa**	Yes	This paper
pLX311-ab	pLX311-a	*luxAB*	+14/−2 **aa**	Yes	This paper
pLX312-ab	pLX312-a	*luxAB*	+15/−4 **aa**	Yes	This paper
pLX320-ab	pLX320-a	*luxAB*	+14/−23 **aa**	Yes	This paper
pLX509-a	pACYC184	*luxA*	0 **aa**	No	This paper
pLX1-b	mp18	*luxB*	+24 bp	Yes	This paper
pLX302-b	pET-7	*luxB*	+24 bp	Yes	This paper
pLX502-b	pACYC184	*luxB*	+24 bp	Yes	This paper
pLX609-a1	pBR322	*luxA*	+3 bp, Bam	No	This paper
pLX609-a2	pBR322	*luxA*	+3 bp, HIII	No	This paper
pLX609-a3	pBR322	*luxA*	+3 bp, Sal	No	This paper
pLX603-b1	pBR322	*luxB*	+20 bp, Bam	Yes	This paper
pLX603-b2	pBR322	*luxB*	+20 bp, HIII	Yes	This paper
pLX603-b3	pBR322	*luxB*	+20 bp, Sal	Yes	This paper

SD, Shine and Dalgarno sequence; aa, amino acid; Bam, *Bam*HI; HIII, *Hin*dIII; Sal, *Sal*I

[a] The vectors were constructed by cloning the relevant *lux* sequence in the acceptor plasmid as indicated

[b] *luxA* or *luxB* gene present or both

[c] Deletions of the *luxA* gene are defined from the A in the ATG start codon, where natural *luxA* sequence upstream of the start codon is denoted by ± and deletions downstream of the start codon by − followed by the number of bp

[d] In the Lux α N-terminal fusions, the number of amino acids added and/or removed are denoted by +aa and −aa, respectively

as described (Bergström et al. 1982), and DNA sequencing was according to the dideoxy chain termination method (Sanger et al. 1977).

Construction of luxA *N-terminal deletions.* A *Sal*I–*Eco*RI fragment from plasmid TB7 (Baldwin et al. 1984; Cohn et al. 1985) was cloned into the M13 phage mp18. A series of N-terminal deletions was constructed by either exonuclease III treatment, followed by mung bean nuclease digestion, or by Bal31 exonuclease digestions. After the exonuclease digestions, the phage DNA was either religated, or deleted pieces were recloned into mp18. All new constructs were sequenced over the 5′ region of the gene to determine precisely where the deletion had occurred. In that way a *luxA* deletion library was obtained containing 55 different versions of the gene cloned into the M13 mp18 phage.

For further study we selected 19 such *luxA* N-terminal deletions, 10 of which are included in this paper and shown in Fig. 1. All upstream restriction sites originate from the mp18 polylinker sequence. The *luxA* sequence ends at an *Eco*RI site 58 bp downstream from the translational stop codon of the *luxA* gene.

The plasmids were given the prefix pLX followed by numbers, starting from 101, and the letter a (for *luxA*). The series continues to pLX120-a, in which 69 bp were deleted, counting the A of the first methionine (ATG) of the *luxA* polypeptide as +1 (Table 1).

The *luxA* gene derivatives from the pLX100-a plasmids (Fig. 2a) were cloned into pT3/T7–19 as either a *Sal*I–*Eco*RI or a *Hin*dIII–*Eco*RI fragment. In this way a new series of plasmids denoted pLX200-a etc. was constructed (Table 1, Fig. 2b). In order to obtain "natural" *luxA* N-terminal translational fusions, a vector series denoted pET-3a, pET-3b and pET-3c was exploited (Rosenberg et al. 1987). These vectors carried *Bam*HI sites in all 3 reading frames situated 12 amino acids downstream from the starting methionine codon of the gene *10* protein of phage T7. Chosen *luxA* fragments emanating from the pLX100-a plasmids were cloned into the above vectors and formed the pLX300-a series (Table 1, Fig. 2c).

The *luxA luxB* transcriptional unit was restored in both the pLX200-a and pLX300-a series of vectors by insertion of the *luxB* gene, excised as an *Eco*RI fragment from pLX1-b (Fig. 2f), into the *Eco*RI site of these plasmids. This produced plasmids pLX200-ab and pLX300-ab (Table 1, Fig. 2d, e).

In order to test whether truncated *luxAB* constructs, carrying 5′ deletions or additions, gave rise to luciferase enzymes with an altered specific activity, the pLX200-ab and pLX300-ab plasmids were transformed into the *E. coli* strain BL21 (DE3) (Studier and Moffat 1986), which carry the T7 RNA polymerase gene on the chromosome.

In parallel experiments the *luxA* and *luxB* genes were expressed from different replicons. In order to do this a plasmid that carried the *luxB* gene under the T7 promoter on plasmid pACYC184 was constructed as follows: the *luxB* structural gene was excised from pLX1-b as a *Bam*HI fragment and subcloned into the *Bam*HI site of the pBR322 derived plasmid pET-7 (Rosenberg et al. 1987) downstream

[e] Bam, HIII and Sal DNA linkers were added both 5′ and 3′ to the *lux* gene as indicated

[f] Natural SD present in the construct

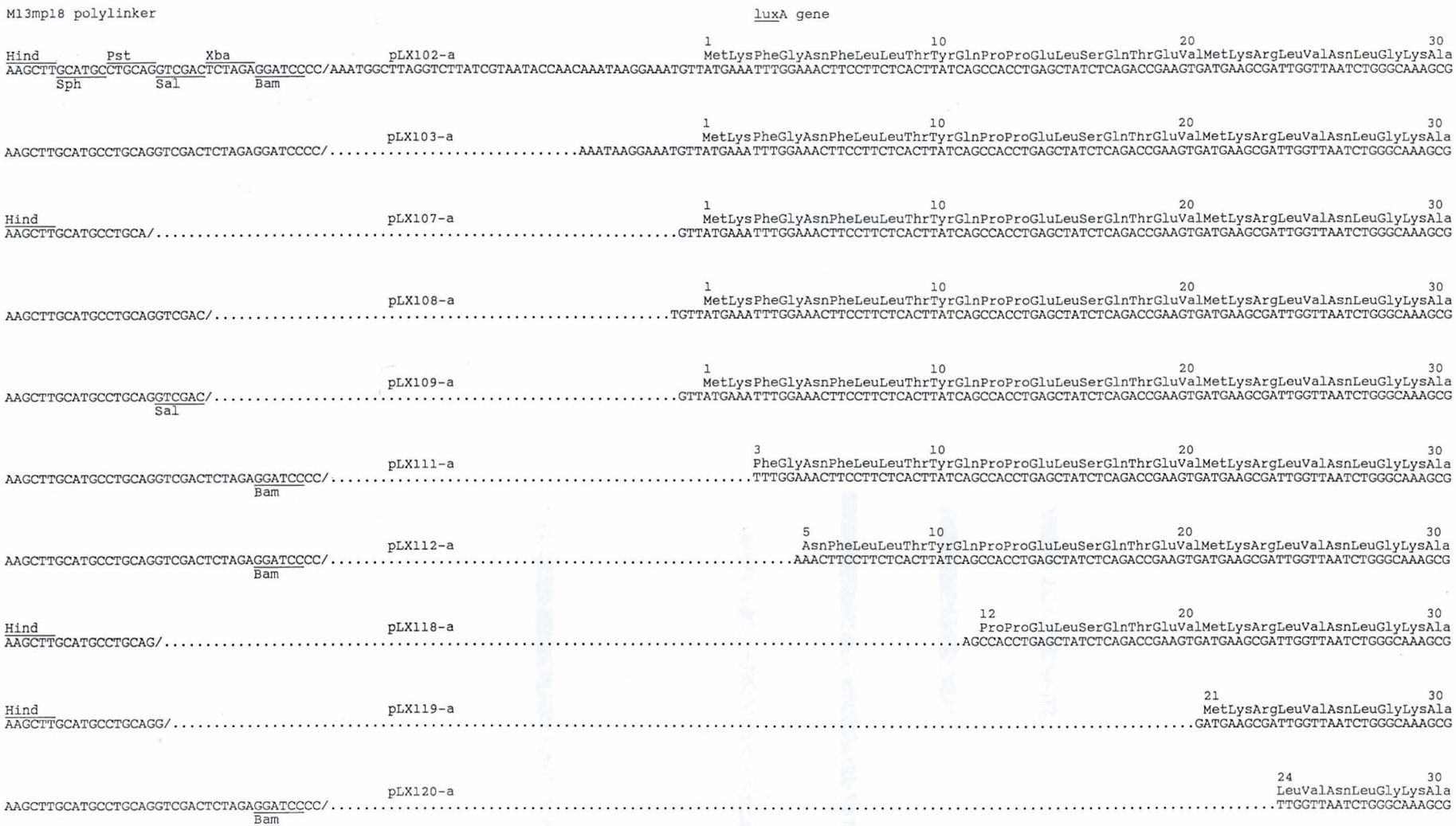

Fig. 1. Nucleotide sequence of 5′ end deletions of the *luxA* gene. The sequence is displayed in the conventional 5′-3′ orientation, with the deduced amino acids on top. Sequence to the left of the *slash* indicates the M13 mp18 polylinker sequence. Relevant restriction endonuclease sites are indicated: Hind; *Hin*dIII; Sph; *Sph*I; Pst, *Pst*I; Xba, *Xba*I; Bam, *Bam*HI; Sal, *Sal*I. The *dotted line* indicates the extension of each deletion, where each *dot* corresponds to one base pair

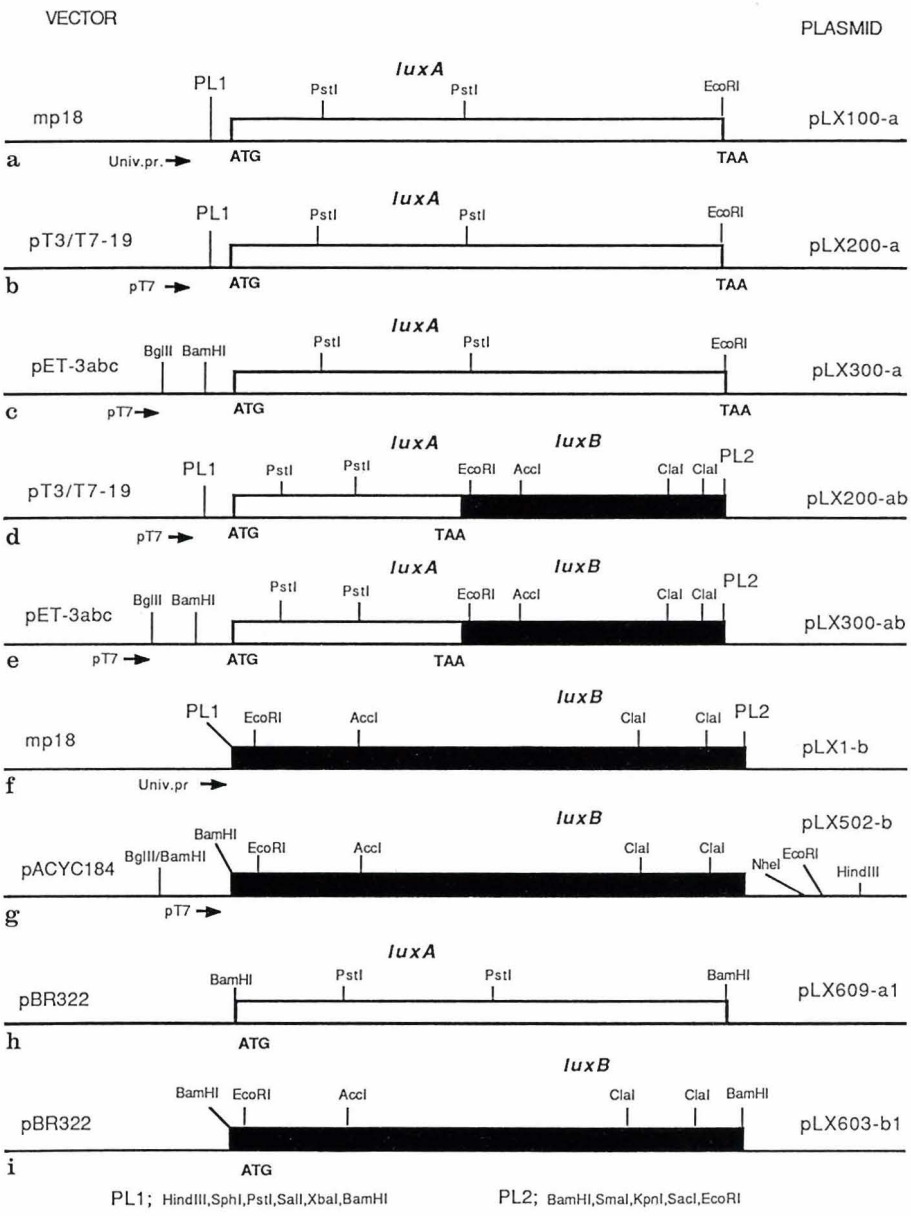

Fig. 2. Restriction endonuclease maps of plasmids carrying *luxA* gene derivatives. The specific vector used in each case is indicated to the left, and the name of the vector series is indicated to the right. PL1 and PL2, polylinker sequences with several restriction sites as indicated at the *bottom* of the figure; Univ. primer, M13 mp series universal sequencing primer site; pT7, recognition sequence for bacteriophage T7 RNA polymerase. (a) pLX100-a series, (b) pLX200-a series, (c) pLX300-a series, (d) pLX200-ab series, (e) pLX300-ab series, (f) pLX1-b, (g) pLX502-b, (h) pLX609-a series, (i) pLX603-b series

from the T7 promoter, resulting in plasmid pLX302-b. From this plasmid a *Bgl*II/*Hin*dIII fragment containing the entire *luxB* gene fused to the T7 promoter was cloned into the *Bam*HI/*Hin*dIII sites of pACYC184, creating pLX502-b (Fig. 2g).

This plasmid was then co-transformed with the compatible pLX203-a, pLX207-a and pLX209-a plasmids carrying the *ColE1* replicon. Alternatively, cells were co-transformed with plasmids pLX509-a and pLX302-b. Plasmid pLX509-a is a pACYC184 derived plasmid which carries the *luxA* gene, with the same 5′ end as in pLX109-a (Fig. 1), driven by the T7 promoter.

Finally, in order to facilitate further work on the *luxAB* operon or on the separated *luxA*, *luxB* gene system, we fused chosen *luxA* and *luxB* constructs with single starting ATG codons to *Bam*HI, *HindIII and Sal*I linkers and cloned them into plasmid pBR322, creating the pLX600-a or pLX600-b series (Fig. 2h, i, Table 1).

Measurement of luciferase activity. Competent BE21(DE3) cells (200 µl) were transformed with 50 ng of pLX200-ab or pLX300-ab plasmid DNA under standard procedures and plated on L-agar (Bertani 1951) containing 100 µg/ml ampicillin. Plates were incubated 12–15 hrs at 37° C. Since some of the plasmid deratives were not stable upon prolonged growth of the cells, even under selective conditions, the plates were photographed and the colonies visually inspected. Provided that all colonies from a particular transformation event displayed similar light emission, luciferase activity was assayed directly on resuspended transformed cells in the following way. With a sterile pipett, cells were transferred to ice cold lux buffer (50 mM Na-phosphate, pH 7; 50 mM 2-mercaptoethanol; 2% bovine serum albumin) to an OD_{595} of 1. Samples, 10 µl to 100 µl, were immediately taken from the different, resuspended transformed cells, mixed with 400 µl lux buffer and placed in a luminometer (Turner TD-20e). 1-Decanal substrate (Sigma

Table 2. Luciferase activities in transformed *Escherichia coli* BL21(DE3) cells and their extracts

Plasmid(s)	LU/OD_{595} in vivo	Relative luciferase activity[a]	LU/total protein (μg) in vitro	Relative luciferase activity[a]	Ratio A/B (Western)[b]	Relative amount of total protein	Specific luciferase activity[c,d]
pLX203-ab	419551	100	52235	100	117/100	100	100
pLX207-ab	28949	6.9	1836	3.5	26/100	110	12.2
pLX209-ab	31047	7.4	3669	7.0	7/100	95	105
pLX218-ab	0	0	0	0	0/100	101	0
pLX219-ab	0	0	0	0	0/100	95	0
pLX303-ab	524439	100	64167	100	149/100	100	100
pLX304-ab	540171	103	92750	145	165/100	99	146
pLX311-ab	18880	3.6	3594	5.6	169/100	85	6.6
pLX312-ab	10.5	0.002	5.5	0.009	185/100	78	0.011
pLX320-ab	0	0	0	0	175/100	71	0

LU, light units

[a] Luciferase activity in pLX203-ab was taken as 100%, and was compared to the other pLX200-ab plasmids on a relative basis. Analogically, the pLX303-ab plasmid was taken as 200% when compared to the pLX300-ab series

[b] In Western blot scans, the value of the luciferase α polypeptide was related to the corresponding β polypeptide in the same lane

[c] In the pLX200 series, calculated by dividing the relative luciferase activity, obtained from the in vitro measurements, with the ratio of the Lux α and Lux β polypeptides, and then by dividing by the relative amount of total protein loaded on the gel

[d] In the pLX300 series, calculated by dividing the relative in vitro luciferase activities with the amount of total protein loaded, since the α subunit was in excess in these experiments

0-7384), prepared as a sonicated 1:1000 dilution in lux buffer, was then injected into the luminometer. The total light produced during the first 10 s after injection of the substrate was recorded. The thus recorded in vivo activity was given as light units (LU)/ml cells at an OD_{595} of 1.0 (Table 2).

For the in vitro measurements of luciferase activity, 500 μl aliqouts of the same resuspended cells were sonicated, the extracts were centrifuged, and luciferase activity in 1–100 μl of the extracts were measured as described (Koncz et al. 1987). Essentially, the extract was mixed with 400 μl lux buffer, placed in a luminometer, and the luciferase reaction was started by injecting 500 μl of light reduced FMN in tricine buffer (200 mM, pH 7.0), and 10 μl of the diluted 1-decanal. The height of the light peak produced during the first 10 s of the reaction was taken as the luciferase activity. Luciferase activity in vitro was given as LU/total protein (μg) in the extracts (Table 2). Total protein was measured by the Bradford assay (Bradford 1976).

Immunoblotting. Resuspended transformed cells (20 μl, see above) were boiled in 2 × SDS-sample buffer, and the proteins were separated on a 10% SDS-polyacrylamide gel at 150 V for 4 h (Laemmli 1970). The separated proteins were transferred to a nitrocellulose filter by electrophoresis, as described (Koncz et al. 1987; Tobin et al. 1979), and the Lux α and Lux β protein bands were developed by the alkaline phosphatase system (Promega Scandinavian Diagnostic Services, Box 40, Falkenberg, Sweden) after being sandwiched by anti-Lux α and anti-Lux β IgG antibodies, as described (Koncz et al. 1987). The Western blot filters were photographed and quantitatively scanned in a spectrophotometer.

Results

Plasmids carrying N-terminal luxA *deletions*

In order to make use of *luxA* as a marker gene, 10 out of 55 M13 mp18 clones, carrying different resected and modified *luxA* gene derivatives, were chosen for the construction of transcriptional and translational fusions and were denoted pLX101-a–pLX120-a (Table 1, Figs. 1 and 2a).

Plasmids of the pLX100-a family were classified into three different groups, based on whether or not they retained the ribosomal binding site (SD sequence, Shine and Dalgarno 1974) and the translational initiation codon (ATG) of the *luxA* coding region. Thus, in plasmids of the first group, both the SD site and the ATG of the *luxA* gene are present. This plasmid group is exemplified by plasmids pLX102-a and pLX103-a (Fig. 1).

In the second group of plasmids, the SD sequence is partially or completely removed, but the ATG of the *luxA* gene is retained, as shown in plasmids pLX107-a, pLX108-a, and pLX109-a (Fig. 1), while in the third group both the SD sequence and the ATG codon of the *luxA* gene are deleted. The *luxA* deletions extend from the first amino acid, as in pLX111-a, to amino acid 24, as in plasmids pLX112-a–pLX120-a (Table 1, Figs. 1 and 2a). These plasmids, as well as plasmids of the second group, are suitable for the isolation of translational gene fusions. By exploiting the *Pst*I site in the plasmids pLX107-a, pLX108-a, and pLX109-a, translational gene fusions to the *luxA* gene can be made in all three reading frames. In contrast, the plasmids of the first group can be used primarily to construct transcriptional gene fusions.

Construction of plasmids carrying transcriptional or translational gene fusions

Starting with the pLX100-a series, two types of *luxA* gene fusions were constructed and inserted behind a phage T7 promoter element. From plasmids pLX103-a and pLX109-a the *luxA* gene was subcloned into plasmids pT3/T7–19 (BRL catalogue number 5376) or pET-3c and pET-3a (Rosenberg et al. 1987), resulting in plasmid derivatives pLX203-a, pLX303-a (cf. pLX103-a, Fig. 1) and pLX209-a (cf. pLX109-a, Fig. 1) (Table 1, Figs. 2b, c and 3).

Similarily to the transcriptional fusions, two different

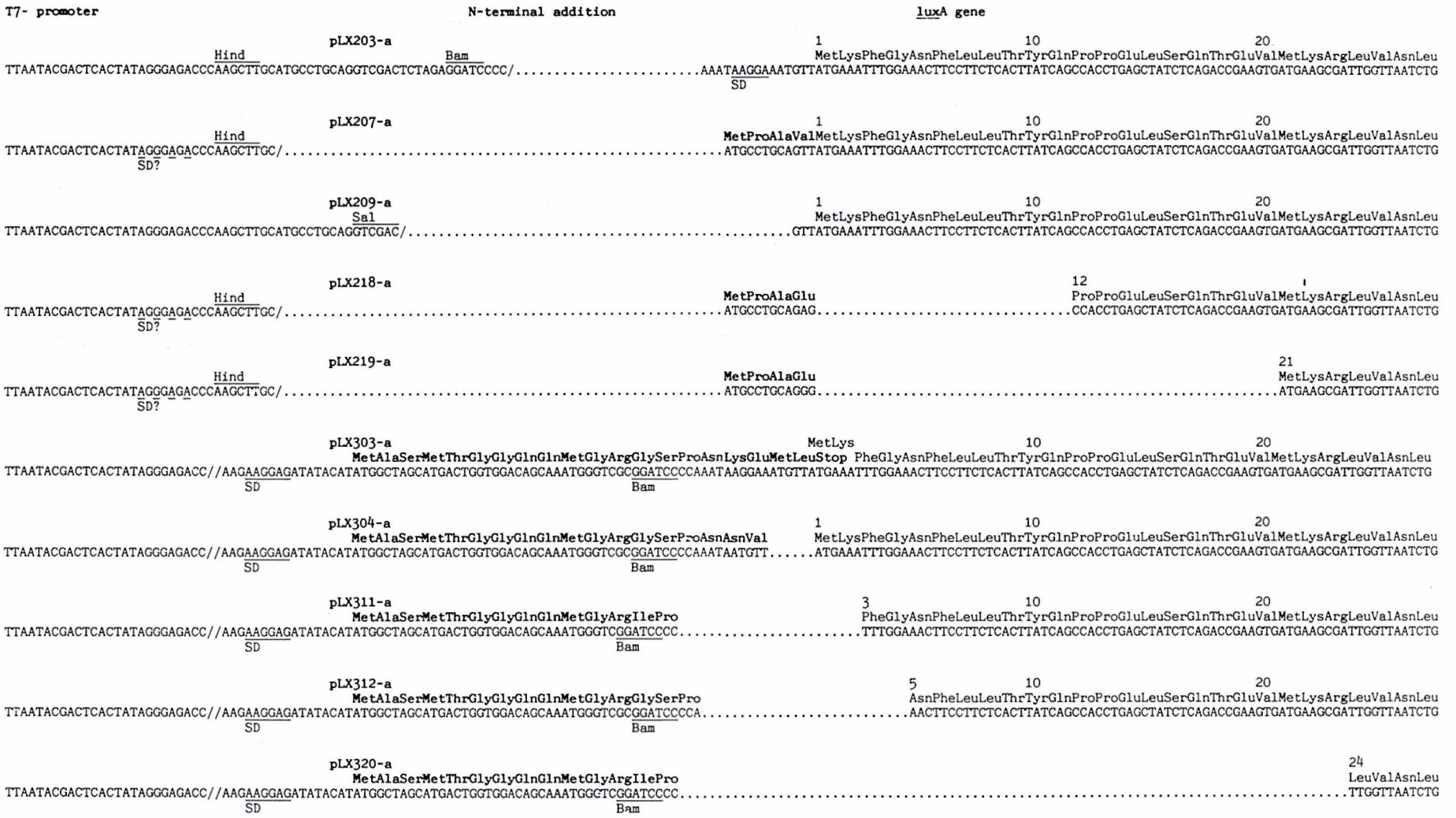

Fig. 3. Nucleotide sequence of transcriptional, and translational *luxA* gene fusions. Deduced amino acids are written on the top of the sequence. Amino acids added to the Lux α polypeptide are in *bold* writing. SD, natural Shine and Dalgarno sequence; SD?, presumed Shine and Dalgarno sequence. Hind, *Hin*dIII; Bam, *Bam*HI; Sal, *Sal*I

translational fusions were also created. In plasmids pLX207-a and pLX304-a either the mp18 polylinker sequence, or DNA sequences originating from the phage T7 major capsid protein and from a DNA linker, were fused in-frame with the natural ATG codon of the *luxA* gene (Fig. 3). This step resulted in Lux α subunits which carry 4 additional amino acids in the first case and 17 extra amino acids in the latter, 11 of which are derived from the phage protein and 6 amino acids from the linker.

In further translational fusions 5′-end deletions of the *luxA* gene, lacking the natural initiation ATG, were linked in-frame with the above polylinker sequence or to the T7 phage/DNA linker sequences. In plasmids pLX218-a, and pLX219-a the 4 amino acids originating from the mp18 polylinker were linked to codons 12 and 21 of the *luxA* gene, respectively, without restoring the SD site. In plasmids pLX311-a, pLX312-a and pLX320-a, *luxA* gene deletions extending to corresponding amino acid positions 3, 5 and 24 were fused to the T7 DNA sequences of the pET-3abc vector (Fig. 3). These plasmids also carry the gene *10* SD sequence from the phage T7. Consequently the expression of the transcriptional and translational *luxA* fusions are controlled by the same T7 promoter in all the above constructs.

In a final cloning step, the original *luxAB* transcriptional unit was restored in all the transcriptional and translational fusions by inserting the *luxB* gene from plasmid pLX1-b (Fig. 2f) in the *Eco*RI site located 59 bp downstream of the *luxA* translational stop codon. This gave rise to plasmid derivatives pLX203-ab–pLX219-ab and pLX303-ab–pLX320-ab, respectively (Table 1, Figs. 2d, e).

Expression of transcriptional fusions

Escherichia coli BL21(DE3) (Studier and Moffatt 1986), carrying in its chromosome the structural gene of the bacteriophage T7 RNA polymerase, were transformed with the plasmids pLX203-ab, pLX209-ab and pLX303-ab. Luciferase activities, normalized for the same number of cells, or in protein extracts, to the same amount of total protein, were highest in transformants harbouring plasmids pLX303-ab and pLX203-ab. Since these luciferase activities originate from the wild type (wt) enzyme they were considered as 100% when compared to other luciferase derivatives in the same plasmid series. Transformants carrying the pLX209-ab plasmid displayed 13 times lower luciferase activity than cells carrying pLX203-ab (Table 2). When luciferase activity was calculated for equal amounts of Lux αβ protein, obtained by quantitative scanning of protein Western blots, the specific luciferase activities were found to be the same in the transcriptional fusions expressing the wt luciferase enzyme (Fig. 4, Table 2).

The observed difference in relative luciferase activities between the pLX203-ab and pLX209-ab transformed cells (Table 2) is most likely due to less efficient translation of the Lux α polypeptide in the ribosome binding site deficient pLX209-ab *luxAB* transcript (Figs. 3, 4, Gold et al. 1981). The highest relative luciferase activity in the pLX303-ab containing cells can probably be explained by the presence of double SD sites in the 5′ *luxAB* leader transcript, and by the overlap of the stop codon of the short leader phage polypeptide with the start codon of the *luxA* gene (Fig. 3). This overlap will result in translational coupling, where ribosomes translating the short polypeptide will re-initiate translation at the *luxA* start codon without falling off the mRNA.

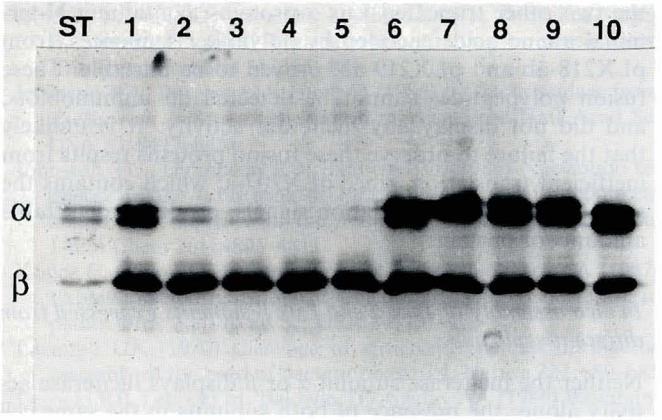

Fig. 4. Western blot from a 10% SDS-polyacrylamide gel separation of total protein extracted from cells expressing different luciferase plasmid derivatives. *ST*, purified *Vibrio harveyi* luciferase. Lane 1, pLX203-ab; 2, pLX207-ab; 3, pLX209-ab; 4, pLX218-ab; 5, pLX219-ab; 6, pLX303-ab; 7, pLX304-ab; 8, pLX311-ab; 9, pLX312-ab and 10, pLX320-ab. α and β indicate bands on the gel that react with luciferase anti-α and anti-β antibodies respectively. A double α band is seen in all lanes, including the control lane with purified luciferase (ST). The reason for this is not clear, but it could be that the flavin substrate is still bound to the α subunit in the upper band

Functional studies on translational fusions

Plasmids pLX207-ab and pLX304-ab exemplify two translational fusions retaining the natural *luxA* translational initiation codon. The N-terminal addition of four amino acids encoded by the polylinker sequence upstream from *luxA* in pLX207-ab not only reduced in vivo luciferase activity by more than 90%, but also decreased the specific activity of the luciferase enzyme 8 times. The observed ratio of the Lux α/Lux β protein subunits (Fig. 4, Table 2) shows that the modified SD site in pLX207-ab leads to reduced translation of the *luxA* gene, while the drop in the specific activity of the enzyme indicates alteration of Lux α subunit function or stability.

In contrast, the addition of 11 amino acids from the major capsid protein of phage T7 to the N-terminus of Lux α resulted in a slight increase in the specific activity of the luciferase enzyme. However, if the natural ATG initiation codon of *luxA*, along with the second codon, was deleted before the addition of the coding region of the capsid peptide, a 15-fold loss in both relative and specific luciferase activities was observed, as shown for pLX311-ab (Fig. 3, Table 2). The removal of two additional *luxA* codons, as in plasmid pLX312-ab, resulted in a reduction of luciferase activity to about 0.01% of the wt activity.

In position 21 of the *luxA* coding sequence there is an in-frame ATG and therefore it is possible that translation of the luciferase α subunit may be re-initiated at this position. However, this is unlikely since no corresponding shorter Lux α polypeptide was detected on the protein gel. The fusion protein encoded by pLX320-ab, was found to be stable (see Fig. 4, Table 2), however cell extracts containing this Lux α polypeptide showed no luciferase activity.

In contrast to stable T7 capsid protein-Lux α fusions,

the two other truncated Lux α proteins, containing N-terminal amino acids encoded by polylinker sequences, from pLX218-ab and pLX219-ab, proved to be unstable. These fusion polypeptides cannot be detected on immunoblots, and did not display any luciferase activity. It is unlikely that the failure to observe these fusion proteins results from inefficient translation, since pLX207-a, which contains the same transcription-translation signals, produced detectable amounts of protein.

In vivo assembly of Lux α and Lux β subunits expressed from different replicons

Neither the luciferase subunit α or β displays luciferase activity alone; the presence of both subunits in the same cell is required in order to catalyse the luciferase reaction. When working with the luciferase reporter gene system, one can take advantage of this finding. In complementation experiments, only cells in which the two subunits are present simultaneously, will display luciferase activity.

In the *V. harveyi* luciferase operon, the *luxA* and *luxB* genes are normally co-transcribed, i.e. when the translation of the *luxA* gene is completed, the *luxB* gene is still being transcribed. Therefore it is possible that assembly of the luciferase α and β subunits occurs during the synthesis of the Lux β subunit.

To examine whether a functional luciferase enzyme is synthesised in vivo when expressed from separated *luxA* and *luxB* transcriptional units, we determined luciferase activities in cells transformed pairwise with *luxA* and *luxB* plasmids. The experiments were designed in such a way that cells were expressing different levels of either the Lux α or Lux β subunit. When luciferase activities were normalised to the subunit present in limiting amounts, similar specific enzyme activities were obtained from transformed cells containing the different plasmids, regardless of whether the α or the β subunit was present in limiting amounts (data not shown). When comparing these luciferase activities on a molar basis with wild-type luciferase enzyme activity expressed from a single transcriptional unit, no significant difference in activity was seen between luciferase enzymes synthesised from the single transcriptional unit or from two transcriptional units. This therefore confirmed previous luciferase enzyme subunit assembly experiments made in vitro (Meighen et al. 1971a, b; Nicoli et al. 1974; Meighen and Bartlet 1980), which showed that separate, purified α and β subunits will assemble, and reach up to 80% of maximal activity in 24–48 h.

Discussion

Potential of the luciferase system

Recently, several laboratories have realized the potential of using the bacterial and firefly luciferase systems (Engebrecht et al. 1985; Legocki et al. 1986; Schmetterer et al. 1986; Carmi et al. 1987; Koncz et al. 1987; Ow et al. 1986) as a tool to answer intricate biological questions. The sensitivity, ease of detection, and most importantly, the unique possibility of assaying luciferase activity without destroying the host cells, in contrast to other widely used reporter enzymes such as β-galactosidase and more recently β-glucuronidase (Jefferson et al. 1986), opens novel ways to study complex genetic interactions in vivo. Thus, the movement of procaryotic cells can be followed, e.g. in chemotaxis, phagocytosis and symbiosis, and the survival and spreading of particular bacterial strains in natural ecosystems can be readily traced.

Up to now more advanced applications of the luciferase system in molecular genetics have been hampered by the fact that:

A. Convenient plasmid vectors, allowing for transcriptional and translational gene fusions to the luciferase genes were not available.

B. It was not known whether modification of the N-terminal segment of the catalytic Lux α subunit, as would be necessary to make fusion proteins, altered luciferase activity.

A series of *luxA* 5′-end deletion derivatives with or without the ribosome binding site were constructed. These *luxA* gene derivatives made it possible to create different transcriptional fusions, represented by plasmids pLX203-ab, pLX207-ab and pLX303-ab (Figs. 2, 3).

Formation of a short coding region upstream of the natural ribosomal binding site of *luxA*, as in pLX303-ab, is also possible and may even positively effect *luxA* expression due to translational coupling. The absence of a properly spaced SD site in pLX209-ab, on the other hand, caused a significant reduction of *luxA* translation (Fig. 3, Table 2).

Addition of short coding sequences upstream of the natural *luxA* initiation codon, provides new initiation codons for translational fusions. Two types of translational fusions were compared. In plasmids pLX207-ab, pLX218-ab and pLX219-ab an artificially created 12 bp DNA sequence was fused to either the intact *luxA* gene or to 5′-end truncated *luxA* genes. In plasmids pLX304-ab, pLX311-ab, pLX312-ab and pLX320-ab, a DNA sequence corresponding to 11 N-terminal amino acids, representing the natural N-terminal region of the T7 major capsid protein of phage T7, was fused to an intact or truncated *luxA* gene giving rise to a Lux α protein with an extra N-terminal of 15 or 17 amino acids (Fig. 3). One such fusion, pLX304-ab, which retained the translational start codon of *luxA*, was slightly more active than the native α protein in vivo, and at the same time displayed a higher specific activity of luciferase in vitro (Table 2). It is not yet clear whether this enhanced activity could be due to an altered conformation.

There appears to be a striking difference in the stability of Lux α fusion proteins. This stability seems to be dependent on the nature of the N-terminal extensions. Those constructs having the synthetic polylinker encoding a four amino acid extension are apparently unstable. This instability is even more pronounced when the 4 amino acid extension is followed by one of the truncated Lux α polypeptides missing 11 or 20 of the N-terminal amino acids of Lux α (Figs. 3, 4; pLX218-ab, pLX219-ab). In contrast Lux α polypeptides with the 11 amino acids from the N-terminal part of the T7 coat protein added to their N-terminal end, appear to be stable. Stable fusion protein allowed an exact comparison of the specific luciferase activities obtained from the various truncated Lux α polypeptides. This stability was essential in order to test whether the Lux α polypeptide was suitable as a reporter enzyme in translational fusions.

The experiments described here show that as much as 17 amino acids can be added to the N-terminal part of the intact α polypeptide without altering the activity of the luciferase αβ complex. However, deletion of only two N-

terminal amino acids of Lux α reduces luciferase activity by 95%. Moreover, the removal of four amino acids decreases the enzyme activity by more than 10000-fold (Table 2). These results indicate that the N-terminal domain of the Lux α polypeptide plays an important role in luciferase function. Despite this, the luciferase enzyme can easily tolerate the addition of 17 amino acids to its α subunit while retaining full activity.

A possible limitation of the bacterial luciferase as a reporter enzyme is the fact that its function is dependent on the correct assembly of the two subunits. However the luciferase subunits can assemble to an active enzyme, not only when co-synthesised on the same transcriptional unit, but also when synthesised from two separate transcriptional units from two different replicons. Furthermore, luciferase assembly in vivo is not affected when one of the subunits, whether it is α or β, is in excess over the other. Therefore the luciferase reporter genes can be separated on different replicons, or in different cells, which can be advantageous in many applications.

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