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Use of Transcriptional Fusions To Monitor Gene Expression: a Cautionary Tale

ÅKE J. FORSBERG,† GRAHAM D. PAVITT,‡ AND CHRISTOPHER F. HIGGINS*

Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

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Gene fusions are frequently used to facilitate studies of gene expression and promoter activity. We have found that certain reporter genes can, themselves, influence promoter activity. For example, the commonly used *luxAB* reporter genes can activate or repress transcription from a subset of promoters, generating data apparently at odds with those obtained with other reporter genes. These effects are probably related to an intrinsically curved DNA segment in the 5′ coding sequence of the *luxA* gene. Thus, caution must be observed when one is interpreting results obtained with a single reporter gene system such as *luxAB*.

For many genes, it is inconvenient or impossible to assay the gene product as a means of monitoring expression. This problem is frequently circumvented by generating transcriptional fusions between the promoter of interest and a reporter gene whose product is easy to assay. Reporter gene systems have now been refined for a multitude of uses, not only for studying gene expression but also for investigating many other aspects of cell biology. Among the most widely used reporter genes are *lacZ*, which encodes β-galactosidase (32), *cat*, which encodes chloramphenicol acetyltransferase (CAT) (3), *galK*, which encodes galactokinase (18), and *luxAB*, which encode bacterial luciferase (7). Luciferase is gaining considerable popularity as a reporter gene, as it offers unique advantages of high sensitivity and the facility to make real-time measurements without the need for cell disruption (14).

Vectors for integrating *cat* and *luxAB* gene fusions onto the chromosome. Two closely related vectors were constructed for generating gene fusions to *cat* or *luxAB*, which could be studied as multicopy plasmids or in single copy following integration onto the chromosome (Fig. 1). These vectors were based on the suicide plasmid pGP704, which has an R6K origin of replication and can replicate only when the π protein (*pir*) is provided in *trans* (21). pGP704 also contains the *mob* region from RP4, which enables it to be transferred by conjugation from *Escherichia coli* strains which express the genes required for mobilization. Plasmids pCH257 (for *luxAB* fusions) and pCH258 (for *cat* fusions) were derived from pGP704 as described in the legend to Fig. 1. By cloning a defined promoter fragment into the polylinker sites of pCH257 and pCH258, identical transcriptional fusions to the *luxAB* and *cat* genes, respectively, can be generated. These vectors allow promoter activity to be studied in the multicopy state (in the π-expressing *E. coli* strain S17-1λ*pir*) or when integrated onto the chromosome; integration can be achieved by conjugative transfer of the plasmid into a π-minus strain, selecting for the antibiotic resistance encoded by the plasmid. Integration occurs by homologous recombination between the cloned promoter and its chromosomal counterpart.

Multicopy plasmid fusions of the *leuP* and *leu-500* promoters to *cat* and *luxAB*. The *leu-500* promoter of *Salmonella typhimurium* has a single-base-pair mutation in the −10 region of the leucine operon promoter, *leuP* (9). This mutation renders the promoter inactive, although function can be restored in a strain carrying a *topA* (topoisomerase I) mutation (17). In *topA* strains, negative DNA supercoiling is increased, and *leu-500* is often regarded as a model for a DNA supercoiling-sensitive promoter (16).

The *leuP* and *leu-500* promoters were fused, separately, to the *cat* and *luxAB* reporter genes of pCH258 and pCH257 as follows. First, 237-bp DNA fragments containing the *leuP* or *leu-500* promoter were amplified by PCR, using Amplitaq *Taq* polymerase (Cetus). The templates were chromosomal DNA from *S. typhimurium* LT2 (*leuP*) or PM596 (*leu-500*). The primers were 5′-CATGCATCTAGACGCGCAGCCCAGCA CAATTAGC-3′ and 5′-CATGCAGAGCTCGAGCTGACT TAATGTTGAATGCCG-3′, corresponding to nucleotides −115 to −91 and +122 to +98 relative to the start point of transcription of the *leuABCD* operon (8), and tagged with *Xba*I and *Sac*I sites to facilitate subsequent cloning. These promoter fragments did not include the attenuator sequences downstream of the *leu* promoter which play a role in regulating *leu* operon expression (30). The amplified *leuP* and *leu-500* promoter fragments were cloned into the polylinker sites of pCH257 and pCH258 to generate *leuP-cat* (pCH248), *leuP-luxAB* (pCH241), *leu-500–cat* (pCH249), and *leu-500–luxAB* (pCH242) fusions. DNA sequence analysis showed that no errors were introduced during the PCR amplification and confirmed the identity of the cloned *leuP* and *leu-500* promoters in each plasmid. Each plasmid had the same translational start and stop signals within the cloned promoter fragment so that the possibility of different translational fusions was excluded.

Single-copy chromosomal fusions of the *leuP* and *leu-500* promoters to *cat* and *luxAB*. To integrate the *cat* gene fusions onto the chromosome, plasmids pCH248 and pCH249 were mobilized, separately, by conjugation from *E. coli* S17-1λ*pir* into the restriction-negative *S. typhimurium* strain CH1858 by mating on a filter as described previously (33). As the plasmids cannot replicate in *S. typhimurium* (no π protein), selection for the antibiotic resistance determinant carried by the plasmids forced chromosomal integration by homologous recombina-

* Corresponding author. Mailing address: Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom. Phone: (0)865-222423. Fax: (0)865-222431.

† Present address: Department of Microbiology, National Defense Research Establishment, S-901 82 Umeå, Sweden.

‡ Present address: National Institute of Child Health and Human Development, Bethesda, MD 20892.

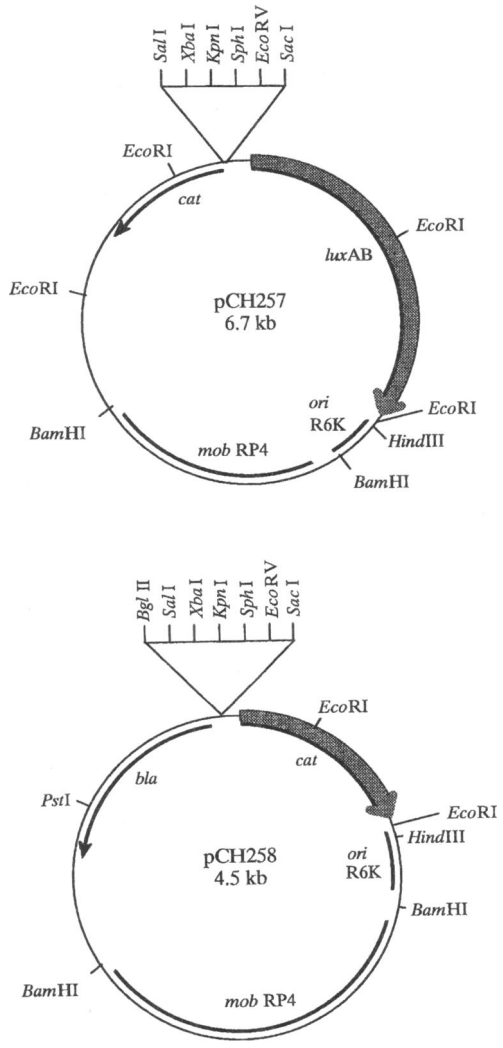

FIG. 1. Suicide plasmids which facilitate the generation of transcriptional fusions to *cat* (pCH258) or *luxAB* (pCH257). Only the unique cloning sites upstream of the reporter genes are shown. The plasmid for isolating *cat* gene fusions (pCH258) was constructed by inserting the promoterless *cat* gene from Tn*903* (excised from pCM7 [Pharmacia] by using *Hin*dIII and with the overhanging ends repaired with Klenow polymerase) into the *Sma*I site of pGP704. The plasmid for isolating *luxAB* fusions (pCH257) was constructed by inserting the promoterless *luxAB* genes from *Vibrio harveyi* (excised from pXL303ab [22] by using *Bam*HI and with the overhanging ends repaired with Klenow polymerase) and inserted into the *Sma*I site of pNQ705, a derivative of pGP704 in which the *bla* gene had been replaced with the *cat* gene of pACYC184 (22).

tion. The integrated plasmids were then transduced into the wild-type *S. typhimurium* strain LT2 by using phage P22-*int4* (29). For the *leu-500* plasmids, two possible outcomes of integration were possible, depending on whether the recombination event occurred 3′ or 5′ to the promoter mutation. If recombination was 5′ to the mutation, then the *cat* or *luxAB* reporter gene would remain under control of the mutant *leu-500* promoter (as required for the experiment). If, however, recombination was 3′ to the *leu-500* mutation, then the reporter gene would be under control of the wild-type promoter (*leuP*) and the chromosomal *leu* operon would be under control of the *leu-500* promoter. These latter, unwanted, recombinants would be leucine auxotrophs and could be readily identified and eliminated. The integration events were verified by Southern blotting (data not shown) and by direct sequencing of chromosomal DNA from amplified PCR fragments, using a 5′-biotinylated oligonucleotide primer homologous to the 5′ end of the *cat* gene and a second primer (nonbiotinylated) identical to that used for the initial amplification of the promoter fragment (see above). The PCR-amplified fragments were purified and sequenced by using streptavidin-coated magnetic Dynabeads (Dynal) and Sequenase 2.0 (U.S. Biochemical Corp.) as described previously (13). This direct sequencing of chromosomal DNA also allowed us to confirm the identity of the promoter (*leuP* or *leu-500*) upstream of the reporter gene in each integrated fusion. The chromosomal *cat* fusion strains were designated CH1951 and CH1952 (*leuP-cat* and *leu-500–cat*, respectively) (Table 1).

The nature of the reporter gene influences the *leuP* and *leu-500* promoters of *S. typhimurium*. Expression of the *leuP* and *leu-500* promoters fused to *cat* was assayed in the multicopy plasmid state (in *E. coli* S17-1λ*pir*, in which the plasmids can replicate), and in single copy following chromosomal integration (Table 2). Whether in multicopy or integrated onto the chromosome, the *leuP-cat* fusion (pCH248 and CH1951) was active, while no expression was observed from the *leu-500* promoter (pCH249 and CH1952). The absolute levels of expression were, as expected, lower in the single-copy state. The observation that the *leuP* promoter was active while the *leu-500* promoter was inactive was entirely consistent with previous studies on *leuP* and *leu-500* expression in their normal chromosomal contexts and on plasmids using the *galK* reporter gene (27).

In contrast, when cloned upstream of the *luxAB* reporter genes, the *leuP* and *leu-500* promoters were equally active (plasmids pCH241 and pCH242; Table 2). The activity of the *leu-500* promoter was unexpected, as this mutant promoter has never previously been found to be active in a *topA*$^+$ strain. The possibility that the difference between *luxAB* and *cat* as reporter genes was due to other transcriptional units on the plasmid (for example, through effects on local DNA topology [16]), seemed unlikely, as the *cat* and *luxAB* plasmids were so closely related. However, to exclude the possibility that expression from the mutant *leu-500* promoter was replicon or strain specific, the *leuP-luxAB* and *leu-500–luxAB* promoter-reporter gene arrays were excised from plasmids pCH241 and pCH242 as *Sal*I-*Eco*RI fragments and inserted into the low-copy-number, pSC101-based vector pHSG575 (36). This generated plasmids pCH255 and pCH256, in which the *leuP-luxAB* and *leu-500–luxAB* fusions are transcribed in the opposite orientation to the *lacZ′* gene of pHSG575. When pCH255 and pCH256 were transformed into *E. coli* DH5α or *S. typhimurium* LT2A, the *leuP* and *leu-500* promoters were, again, equally active, although the absolute levels of expression were somewhat higher in *S. typhimurium* than in *E. coli* (Table 2). Thus, the *leu-500* promoter is inactive in its chromosomal context and when on plasmids cloned upstream of the *cat* or *galK* reporter genes yet is active when *luxAB* is used as a reporter. Thus, the *luxAB* reporter genes appear to activate a normally inactive promoter.

The nature of the reporter gene also influences expression of the *proU* promoter. The *proU* locus of *S. typhimurium* encodes a high-affinity transport system for the osmoprotectant glycine betaine, and its transcription is induced by growth in media of high osmolarity (1, 6). A 317-bp DNA fragment encompassing the *proU* promoter was cloned upstream of the *lacZ* and *luxAB*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference(s)
S. typhimurium		
CH1858	*hsdLT hsdSA hsdSB dpp-101*::Tn*10*	This study
CH1951	Integration of pCH248 (*leuP-cat*) into *S. typhimurium* LT2A chromosome	This study
CH1952	Integration of pCH249 (*leu-500–cat*) into *S. typhimurium* LT2A chromosome	This study
LT2A	Wild type	B. N. Ames
PM596	*leu-500 ara-9*	15
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169* [φ80(Δ*lacZ*)M15]	10
MC4100	*araD139* Δ(*argF-lac*) *U196 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	2
S17-1λ*pir*	*E. coli* RP4-2 Tc::Mu-Km::Tn7 (λ *pir*)	5, 33
Plasmids		
pAV375	*proU-luxAB* fusion (943 bp of *proU*)	25
pAV382[a]	*proU-luxAB* fusion (317 bp of *proU*)	25
pAV399[a]	*proU-lacZ* fusion (317 bp of *proU*)	25
pAV400	pAV375 with the *proU* promoter fragment deleted	This study
pAV511	pAV400 with the *gyrB* promoter inserted (*gyrB-luxAB*)	This study
pCH241	*leuP-lux* (*leuP* promoter fragment cloned into pCH257)	This study
pCH242	*leu-500–lux* (*leu-500* promoter fragment cloned into pCH257)	This study
pCH248	*leuP-cat* (*leuP* promoter fragment cloned into pCH258)	This study
pCH249	*leu-500–cat* (*leu-500* promoter fragment cloned into pCH258)	This study
pCH255	*leuP-luxAB* fusion of pCH241 cloned into pHSG575	This study
pCH256	*leu-500–luxAB* fusion of pCH242 cloned into pHSG575	This study
pCH257	Suicide plasmid used to generate *lux* fusions	This study
pCH258	Suicide plasmid used to generate *cat* fusions	This study
pGP704	Amp^r suicide plasmid	21
pHSG575	Low-copy-number cloning vector, Cm resistance	36
pNQ705	Cat^r derivative of pGP704	21
pRS475	p*lac-lacZ* fusion plasmid	34
pRS528	*lacZ* expression plasmid	34
pSB188	p*lac-luxAB* fusion plasmid	26
pSJ31	*proU-lacZ* (317-bp *proU* fragment) in pRS528	25

[a] pAV382 and pAV399 are identical except for the reporter gene used.

reporter genes to generate plasmids pAV399 and pAV382, respectively (25). The *proU* promoter fragment corresponded to nucleotides −219 to +98 relative to the transcription start point of the *proU* operon (35). pAV399 and pAV382 were identical except for the *lacZ* and *luxAB* reporter genes. The plasmids were transformed into *E. coli* MC4100, and promoter activity was assayed (Table 3). The *proU* promoter exhibited different patterns of regulation depending on which reporter gene was present in the plasmid. Expression of the *proU-luxAB* fusion (pAV382) was repressed at low osmolarity and induced by growth at high osmolarity, with an induction ratio of nearly 10. In contrast, the *proU-lacZ* fusion (pAV399) was derepressed when grown at either low or high osmolarity such that expression was constitutive (an induction ratio of 1). As the only difference between the two plasmids was the reporter

TABLE 2. Effects of reporter genes on expression from the *leuP* and *leu-500* promoters[a]

Fusion	Plasmid	Strain	Reporter gene activity	
			Lux	CAT
leuP-cat	pCH248	*E. coli* S17-1λ*pir*		310
leu-500–cat	pCH249	*E. coli* S17-1λ*pir*		<5
leuP-cat		*S. typhimurium* CH1951		24
leu-500–cat		*S. typhimurium* CH1952		<5
leuP-lux	pCH241	*E. coli* S17-1λ*pir*	3,550	
leu-500–lux	pCH242	*E. coli* S17-1λ*pir*	2,820	
leuP-lux	pCH255	*E. coli* DH5α	6,550	
leu-500–lux	pCH256	*E. coli* DH5α	5,760	
leuP-lux	pCH255	*S. typhimurium* LT2A	12,130	
leu-500–lux	pCH256	*S. typhimurium* LT2A	9,800	

[a] Cells were grown to an optical density at 600 nm (OD_{600}) of 0.3 in nutrient broth (Difco) at 37°C. Luciferase (Lux) activity was assayed as described previously (25), expressed as millivolts of light output per 100-μl sample per OD_{600} unit. CAT activity was assayed by the spectrophotometric method of Shaw (31) and is expressed as the change in OD_{412} per minute at 25°C per OD_{600} unit of bacteria.

TABLE 3. Effects in strain MC4100 of reporter genes on *proU* promoter function[a]

Plasmid	Fusion	Osmolarity		Induction ratio
		Low	High	
pAV399	*proU-lacZ*	6,918	7,875	1.1
pAV382	*proU-lux*	360	3,342	9.3
pSJ31	*proU-lacZ*	23,500	23,200	1.0
pSJ31[b]	*proU-lacZ*	1,360	1,470	1.1

[a] Cells were grown at 30°C in nutrient broth (Difco) to an OD_{600} of 0.3. The culture was divided, and NaCl was added to half of the culture to a final concentration of 0.3 M (high osmolarity), while the other half of the culture was left untreated (low osmolarity). After 20 min of further incubation, Lux activity was assayed as described in the footnote to Table 2, and β-galactosidase activity was measured as described by Miller (20). This protocol for osmotic upshock has been shown to induce *proU* expression (1, 25, 26). As osmotic induction was by transient upshock on a divided culture, rather than continuous growth in media of high osmolarity, this excludes complications which could otherwise arise from variations in plasmid copy number.

[b] pSJ1 has been integrated onto the chromosome at λ*att* by using λRS45 as described elsewhere (34).

gene, the reporter gene must influence the function of the upstream promoter. The possibility that constitutive expression of the *proU-lacZ* fusion was a specific property of the replicon used was excluded by cloning the same *proU* promoter fragment into another *lacZ* reporter plasmid, pRS528 (34), generating pSJ31. *proU-lacZ* expression from pSJ31 was also constitutive, whether the fusion was maintained as a multicopy plasmid or was in single copy following integration onto the chromosome at λ*att* by homologous recombination (Table 3). Finally, the orientation of the promoter-reporter gene array with respect to the rest of the plasmid was not responsible for the effects seen: expression of the *proU-lux* and *proU-lacZ* reporter gene fusions was unaffected by recloning them in the vectors in the opposite orientations (data not shown).

The wild-type chromosomal *proU* gene is osmoregulated. It is important to note that although the *proU-luxAB* fusion is osmoregulated, and therefore appears to reflect normal regulation of the *proU* promoter more accurately than does the *proU-lacZ* fusion, it is in fact the *proU-luxAB* fusion which is abnormal. Osmotic regulation of the *proU* promoter requires a downstream regulatory element which maintains that promoter in a repressed state at low osmolarity (4, 24, 25). The promoter fragment used here did not include the downstream regulatory element, and expression would therefore be expected to be constitutive. This was the case for the *proU-lacZ* fusion. In contrast, when the *luxAB* reporter gene was used, the *proU* promoter was repressed at low osmolarity; *luxAB* represses the upstream promoter. Thus, as well as influencing *leu-500* expression, the *luxAB* reporter gene also influences *proU* expression. However, its effects on the two promoters are opposite: *luxAB* was found to activate the *leu-500* promoter but to repress the *proU* promoter.

The *luxAB* genes do not always behave anomalously. The *luxAB* genes are commonly used as reporters and generally give results consistent with those obtained with other reporter genes. This suggested that the anomalous effects of the *luxAB* genes on *leu-500* and *proU* may be specific to a subset of promoters. To assess this possibility, a control was performed with the *E. coli lac* promoter. We have previously described construction of the p*lac-luxAB* promoter fusion plasmid pSB188 (26). pRS475, carrying a p*lac-lacZ* transcriptional fusion, is described elsewhere (34). Neither plasmid showed any significant *lac* promoter function in the absence of inducer. When the *lac* promoter was induced by isopropylthiogalactopyranoside (IPTG), the degrees of induction were similar regardless of whether the promoter was fused to the *luxAB* or *lacZ* reporter gene (6 to 22,088 U with *lux* as the reporter; 9 to 22,000 U with *lacZ* as the reporter). Furthermore, when pRS475 was integrated onto the chromosome, induction was also retained, although the absolute level of expression was reduced (<3 to 600 U). Thus, the *luxAB* genes neither activated nor repressed the *lac* promoter, confirming that *luxAB* does not influence all promoters.

One feature common to both the *leu-500* and *proU* promoters is that they are sensitive to DNA supercoiling (11, 27). We therefore examined another supercoiling-sensitive promoter, that of the *gyrB* gene, which encodes the β subunit of DNA gyrase (19). A *gyrB* promoter fragment identical to that studied by Menzel and Gellert (19), extending from −110 to +68 relative to the translational initiation codon, was amplified by PCR from *E. coli* chromosomal DNA. This amplified fragment was cloned into the *Eco*RI site of plasmid pAV375 (25), replacing the *proU* promoter and generating plasmid pAV511. Coumermycin (20 μg ml^{-1}) induced expression of the *gyrB* promoter in pAV511 by 5.4-fold (219 to 1,183 U). This induction was due to increased transcription from the *gyrB* promoter, as plasmid pAV400, which was identical to pAV511 except that it lacked the *gyrB* promoter fragment, showed only 5.2 and 4.7 U of activity with and without coumermycin, respectively. The levels and patterns of expression and induction observed for the *luxAB* reporter system were similar to those obtained with the *galK* reporter gene (19). Thus, *luxAB* does not appear to have any undue influence on the *gyrB* promoter.

Conclusions. The data presented above show that different reporter genes, in otherwise identical plasmids, can provide very different indications of promoter activity. The *lac*, *cat*, and *galK* reporter genes, at least in the constructions that we have analyzed, appear to yield consistent results. However, for a subset of promoters, the *luxAB* reporter gene system appears to influence promoter function. We have found that the *luxAB* reporter genes can both activate and inactivate upstream promoters. For example, the *leu-500* mutant promoter is normally inactive, either on the chromosome or upstream of other reporter genes, yet was as active as the wild-type promoter (*leuP*) when cloned upstream of the *luxAB* reporter genes. In contrast, the *luxAB* reporter gene repressed a *proU* promoter fragment which was active when other reporter genes were used.

How might the *luxAB* genes influence the function of upstream promoters? We have shown previously that the first 200 nucleotides of the *lux* coding sequence contain tracts of A residues which can form a curve in the DNA (25). It is well known that curved DNA can influence promoter activity (see reference 37 for a review), and indeed, normal repression of the *proU* promoter requires a downstream curved DNA element (25). Additionally, the chromatin-associated protein H-NS interacts at curved sequence elements, including that in the *luxAB* genes, and can influence DNA topology (11, 12, 25, 38). It may be relevant that the two promoters at which *luxAB* has an anomalous effect (*proU* and *leu-500*) are both known to be sensitive to DNA topology (11, 27). An effect of *luxAB* on local DNA topology may account for the observation that some promoters are activated and others are repressed by *luxAB*. However, this is not a universal rule: the *gyrB* promoter is topologically sensitive yet unaffected by the *luxAB* genes. A resolution of this paradox will require a more detailed understanding of the mechanisms by which DNA topology influences promoter activity.

As the *luxAB* genes affect expression from a subset of promoters, care must be taken when this reporter gene system is used. The same may be true for other reporter genes which could, potentially, influence the activity of upstream promoters. If the advantages of the *lux* reporter system are to be retained, then it may be pertinent to remove the intrinsically curved DNA element by introducing multiple mutations without disrupting the coding sequence or by reconstructing a synthetic gene with altered codon usage. These results emphasize the caution which must be observed when one is interpreting results obtained with use of gene fusions, particularly when only a single reporter gene or fusion is used.

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