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Versatile Luciferase Reporter Plasmids for Transcription Studies in Diverse Eukaryotic Cells

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We have developed a couple of new luciferase reporter plasmids with very low background reporter activities. One can be used to measure the promoter strength, after insertion of some promoter fragment into the reporter plasmid, and the other, with very low basal promoter activities, aids in studying eukaryotic transcriptional regulators. The latter reporter plasmid contains such *cis* elements as a 17 nucleotide long initiator, Sp1-binding sites, GAL4 binding sites, and binding sites for a certain *Drosophila* homeodomain proteins. In an attempt to construct an improved reporter plasmid by facilitating transcriptional termination and minimizing any interference by cryptic promoters which may be present in the reporter plasmid DNA, we have inserted transcriptional termination-related signals, a three tandem repeat of SV40 polyadenylation signal (AAA) and the putative transcriptional termination signal (UMS) of the mouse *c-mos* gene, into just upstream of the initiator, and the promoter activities were measured by a transient expression assay employing the *Drosophila* Schneider line 2 cells. As expected, the basal promoter activities decreased maximally when both transcription termination related elements were inserted. Moreover, the reporter plasmid with the two elements allowed more sensitive measurement of transcriptional activation than the reporter plasmid without them. These reporter plasmids can be used for studying transcriptional regulators of higher organisms including mammals as well as *Drosophila melanogaster*.

KEY WORDS: Reporter, Luciferase, Transcription, Initiator, *Drosophila*

A simple and popular eukaryotic transcriptional assay is the transient transfection method employing the cultured cells. Since the transient transfection assay requires a reporter plasmid, which represents the promoter strength as the reporter enzyme activity, the transfection assay can be improved by using a reporter plasmid which allows a sensitive and accurate measurement of test promoter activities. Although the chloramphenicol acetyltransferase (CAT) has been widely used as the reporter, the firefly luciferase has been increasingly preferred. There are several advantages for using the luciferase instead of the CAT as the reporter. First, no radioactive substrate is required for the luciferase assay. Second, the sensitivity of the luciferase assay is about 100 times higher than that of the CAT assay (Schwartz *et al.*, 1990). Third, while

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the CAT reaction may take hours (Han *et al.*, 1989), the luciferase reaction takes less than a minute for the same transfection experiment. Fourth, the luciferase assay can be performed easily with a luminometer or even with a scintillation counter (Fulton and Van Ness, 1993). Therefore, the luciferase assay is more sensitive and efficient than the CAT assay.

A reporter plasmid necessarily contains a vector fragment as well as such elements as a promoter, a polyadenylation signal, and a reporter cDNA. If the vector fragment contains cryptic promoters by any chance, the reporter activities can not represent the genuine strength of the promoter in concern. One approach to overcome this problem is to use a minimal vector such as the 2 kb long pGAP4 (Han and Manley, 1993) to reduce the probability of having any cryptic promoter in the vector. However, even though the probability may have been reduced, there still remains the possibility that the cryptic promoters remain in the minimal vector. This problem may be solved by inserting the transcriptional termination-related signals between the vector fragment and the test promoter, since any transcription which is initiated or elongated past the polyadenylation signal downstream of the reporter cDNA can be terminated at the transcriptional termination signal just upstream of the test promoter, thereby the luciferase mRNA with only the proper 5′ end is produced.

In this study, we have developed a couple of novel versatile luciferase reporter plasmids which can be used for measuring the promoter strength and studying eukaryotic transcription factors respectively. These plasmids contain the transcriptional termination related signals, the AAA (a three head-to-tail tandem repeat of the SV40 polyadenylation signal; Maxwell *et al.*, 1989, Fridovich-Keil *et al.*, 1991) and the UMS (a DNA fragment of the mouse *c-mos* proto-oncogene which contains the putative transcriptional termination signal; McGeady *et al.*, 1986; Martin *et al.*, 1993), upstream of either the luciferase cDNA or the transcription factor binding sites. These signals, which are absent in the conventional reporter plasmids, were inserted to increase polyadenylation and transcriptional termination. Although the promoter activities were measured only in the *Drosophila* Schneider line 2 cells (S2 cells; Schneider, 1972) by a transient transfection assay, the new luciferase reporter can be used in mammalian cells as well, since most *cis* elements in the luciferase reporter either originated from or has been shown to be active in the mammalian cells.

Materials and Methods

Construction of the luciferase reporters

Construction of the luciferase reporters were performed by the standard methods (Sambrook *et al.*, 1989) with restriction enzymes, DNA polymerase I large fragment, mung-bean nuclease, and T4 DNA ligase. The luciferase reporters were constructed by ligating fragments of the pGAP4-SGNI-CAT (Han and Manley, 1993), pUBT-luc (kindly provided by Dr. R. de Martin), and pJFCAT1 (kindly provided by Dr. J. L. Fridovich-Keil). The details of the subcloning may be obtained upon request. All DNA samples for transfection were prepared with the Qiagen plasmid kit (Qiagen Co.) and dissolved in sterile TE buffer.

Transient transfection

Drosophila Schneider Line 2 cells (S2 cells) were maintained in the M3 medium (Lindquist *et al.*, 1982) + 10% insect medium supplement (Sigma Co.,) at about 2-4X10^6 cells/ml and split 1 to 10 every week. Before preparing cells in a 24 well plate for transfection, DNA-DDAB (dimethyldioctadecylammonium bromide) mixture was prepared. Appropriate amounts (total amount is about 100 to 200 ng) of DNA dissolved in a few μl of TE were transferred to microfuge tubes. Typical transfection contained 100 ng of a reporter plasmid and 100 ng of the pcopia-lacZ as an internal control representing the transfection efficiency. All experiments were performed in duplicate. DDAB suspension (250 μg/ml) was mixed with M3 in a 1 : 2 ratio and 30 μl was transferred to each of the DNA-containing microfuge tube and mixed well. While the mixture is sitting in a clean bench, the cell suspension

ready for splitting, and M3 media were mixed in a 1:4 ratio and 0.5 ml each of the diluted cell suspension was transferred to each well in a 24 well plate. Immediately after the plating, the DNA-DDAB mixture was transferred to the well and the cells were incubated for 3 days.

Preparation of cell extract

Three days after the transfection, the culture medium was removed by aspiration and 20 μl of CLR (cell lysis reagent, Promega) was added to each well. The plate was agitated on a shaker for about 5 minutes, cell extracts were transferred to microfuge tubes and spun at the maximal speed for 1 minute at room temperature and appropriate amounts of the supernatant were taken for assays.

β-galactosidase assay

Two μl of the supernatant was used for the β-galactosidase assay by a colorimetric method as described previously (Han and Manley, 1993).

Luciferase assay

The luciferase assay was performed according to the method described by Fulton and Van Ness (1993) with some modifications. Tewnty μl of luciferase assay buffer (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP; Promega Co.) was transferred to a microfuge tube and warmed up at room temperatrure. One μl of cell extract was mixed with the luciferase assay buffer and the chemiluminescence was measured within 40 seconds with a liquid scintillation counter. Normalized luciferase activities were calculated by determining the luciferase/β-galactosidase activity ratios and averaging the values from duplicate experiments.

Results

Construction of diverse luciferase reporter plasmids

We have attempted to construct a couple of reporter plasmids, one for the measurement of promoter strengths and the other for studying activities of transcription factors, which may allow sensitive and accurate measurements of test promoter activities in a wide range of host cells. To achieve this goal, we have constructed luciferase reporter plasmids which contain some or all of the various elements such as a mammalian initiator, Sp1-binding sites, GAL4-binding sites, the binding sites for some *Drosophila* homeodomain proteins (McGinnis *et al.*, 1984; Scott and Weiner, 1984), transcriptional termination-related signals, and the luciferase cDNA with the SV40 polyadenylation signal. The transcriptional termination-related signals used in this study are the UMS (a DNA fragment of mouse *c-mos* protooncogene containing a putative transcriptional termination signal; McGeady *et al.*, 1986; Martin *et al.*, 1993) and the A-trimer cassette (AAA, a 3 head-to-tail tandem repeat of the SV40 polyadenylation signal fragment; Maxwell *et al.*, 1989). Both signals have been shown to decrease background reporter activities very efficiently when the reporter plasmids, with either of the signal just upstream of the reporter cDNA, were transfected in mammalian cells (Salier and Kurachi, 1989; Maxwell *et al.*, 1989; Fridovich-Keil *et al.*, 1991). Although these two elements were tested in mammalian cells, they are expected to be functional in *Drosophila* cells as well, since the SV40 polyadenylation signal was shown to direct stable RNA synthesis efficiently in the *Drosophila* S2 cells (Angelichio *et al.*, 1991). When both the AAA and the UMS were inserted to decrease background reporter activities maximally, the AAA was located further upstream of the test promoter than the UMS, to make any transcript past the SV40 polyadenylation signal just downstream of the luciferase cDNA, be cleaved, polyadenylated, and terminated efficiently.

To construct a reporter plasmid for the measurement of promoter strengths, we constructed several luciferase reporter plasmids. They were constructed by inserting some *cis* elements into the minimal reporter construct pLUC, which contains the pGAP4 vector (Han and Manley, 1993) and the luciferase cDNA with the SV40 polyadenylation signal (Fig. 1). We have inserted combinations of the two fragments, the

AAA-UMS (T) and the TdT initiator (I), upstream of the luciferase cDNA, resulting in the pT-LUC, pI-LUC, and the pT-I-LUC, to find the optimal reporter construct (Fig. 1).

We inserted other *cis* elements as well since the reporter plasmids can be used to study transcriptional regulation as well as promoter strengths. They are SGN (S: Sp1 binding sites; G: GAL4 binding sites; N: *Drosophila* homeobox protein binding sites; Jaynes and O'Farrell, 1988; Han and Manley, 1993). The initiator of the mammalian terminal deoxynucleotidyltransferase (TdT) gene was used as a basal promoter (Smale and Baltimore, 1989; Han and Manley, 1993), and it has been shown that the transcription starts accurately at the genuine transcription start site in the *Drosophila* S2 cells as well as in the mammalian cells (Colgan and Manley, 1995). The basic structure of the resulting reporters is the pGAP4 plasmid-(AAA and/or UMS)-SGN-basal promoter(I)-luciferase cDNA(LUC)-SV40 poly(A) signal fragment(A)- (Figure 1).

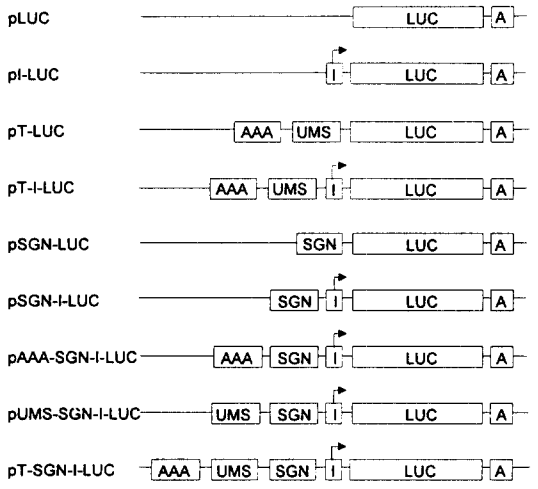

Fig. 1. The structure of the diverse luciferase reporter plasmids. The schematic displays features of the diverse luciferase reporter plasmids used in this experiment. A: the 240 bp SV40 BclI-BamHI fragment containing the polyadenylation signal. AAA: the A-trimer cassette which is a three tandem repeat of the 240 bp SV40 BclI-BamHI fragment containing the polyadenylation signal. T is composed of the AAA and the UMS. UMS: a ca 200 bp DNA fragment of the mouse *c-mos* proto-oncogene containing putative transcriptional termination signal. SGN: binding sites for Sp1(S), GAL4(G), and some *Drosophila* homeobox proteins(N). I: the TdT initiator, a mammalian basal promoter. LUC: the luciferase cDNA. The pGAP4 plasmid is used for construction of all the reporter plasmids shown above. They are not drawn in scale.

The UMS-AAA is required for a low basal promoter activity

We used the 2 kb pGAP4 plasmid (Han and Manley, 1993), which may fortuitously contain some undesirable *cis* elements, but in smaller number due to its smaller size compared to most of the other plasmids, for constructing diverse reporters. To test whether there is any cryptic promoter in the pGAP4 vector, we inserted the luciferase cDNA and the SV40 polyadenylation signal, but no eukaryotic promoter. The resulting plasmid (pLUC) was transiently transfected into the *Drosophila* S2 cells and the reporter activities were measured three days after the transfection. A significant level of luciferase activity was observed when the pLUC was transfected (Fig. 2), while no luciferase activity was observed when the luciferase reporter plasmid was omitted in the transfection (data not shown). However, when the AAA-UMS fragment (T) was inserted upstream of the luciferase cDNA in the pLUC resulting in the pT-LUC, there was an impressive 15 fold decrease in the luciferase activity. Although a small increase in luciferase activity was observed when the pI-LUC, which contains the TdT initiator upstream of the luciferase cDNA, was transfected, a similar

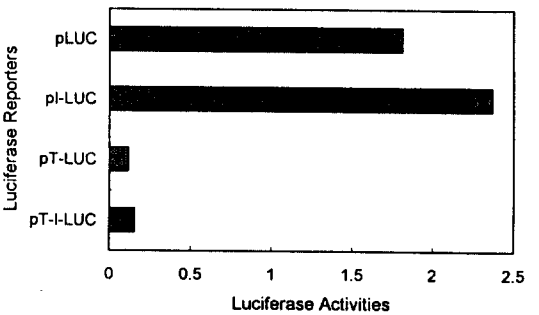

Fig. 2. The T(AAA-UMS) element inhibits background reporter activity of a reporter plasmid effectively. 100 ng each of the luciferase reporters shown was cotransfected into the *Drosophila* S2 cells with 100 ng of an internal control pcopiaLTR-*lacZ*. Normalized luciferase activities (arbitrary unit; see Materials & Methods) are shown.

reduction of luciferase activity was observed again when the pT-I-LUC, which contains the T fragment upstream of the TdT initiator in the pI-LUC, was transfected. This strong reduction of luciferase activities by the T element may have resulted from the termination of any transcript that was still elongated or initiated after the polyadenylation signal at the end of the luciferase cDNA. However, the decreased reporter activity is not due to any disturbance of translation, which may have started somewhere in the pGAP4 vector region, by the T insert, because there are two in-frame stop codons just upsteam of the genuine start codon of the luciferase gene. Therefore, the pT-LUC can be used for the measurement of promoter strengths by transfection after inserting a promoter fragment between the T element and the luciferase cDNA.

Reporter plasmids which contain the SGN (see above) can be used to study transcriptional regulation by transcription factors. When the SGN was inserted upstream of the luciferase cDNA in the pLUC, the luciferase activity was decreased to some extent compared to the pLUC (Fig. 3). It is not clear why the reporter activity was decreased. There was little difference in luciferase activities between the pSGN-LUC and the pSGN-I-LUC (Fig. 3). Although each of the UMS and the AAA has been used to decrease background transcription of a reporter plasmid (Martin *et al.*, 1993; Maxwell *et al.*, 1989; Fridovich-Keil *et al.*, 1991), we inserted either one or both of the two elements in the luciferase reporter to find the optimal reporter plasmid with the lowest background reporter activity. That is, we constructed four reporters, pSGN-I-LUC, pUMS-SGN-I-LUC, pAAA-SGN-I-LUC, pAAA-UMS-SGN-I-LUC (pTSGN-I-LUC), and compared the reporter activities (Fig. 3). Unexpectedly, each of the UMS and the AAA was not able to decrease basal reporter activities significantly. However, when both of the AAA and the UMS were inserted, the reporter activity was decreased by about 2 fold compared to the pSGNI-I-LUC, the reporter plasmid without the termination related signal. Therefore, it was essential to use both the AAA and the UMS to reduce basal promoter activities, at least in *Drosophila* cells.

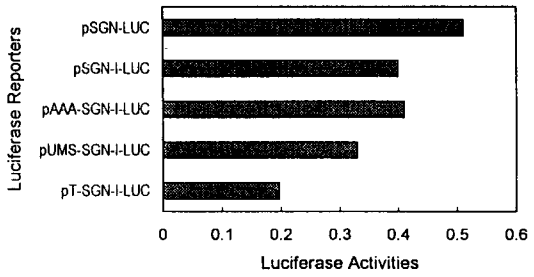

Fig. 3. The pT-SGN-I-LUC is a reporter plasmid with the lowest background reporter activity. Transfection was performed as described in Figure 2.

The AAA-UMS containing reporter plasmids can be used for accurate and sensitive measurement of promoter activities

We tested whether there is any increase in

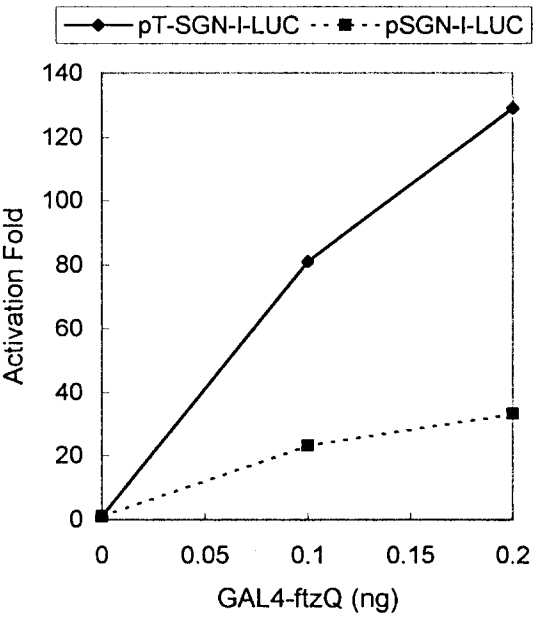

Fig. 4. The pT-SGN-I-LUC allows more sensitive measurement of transcriptional activation than the pSGN-I-LUC without the T element. Fifty ng each of the pT-SGN-I-LUC and the pSGN-I-LUC was cotransfected into the *Drosophila* S2 cells with 100 ng of an internal control pcopiaLTR-*lacZ* and various amounts (0, 0.1, 0.2 ng) of a transcriptional activator expression plasmid pAct-GAL4-ftzQ. Activation fold for each reporter plasmid = (normalized luciferase activity when the pAct-GAL4-ftzQ is cotransfected)/(normalized luciferase activity without the pAct-GAL4-ftzQ cotranscfection).

sensitivity in measuring transcriptional activation when we use the T element in the reporter plasmid. Either of the two reporter plasmids, the pT-SGN-I-LUC or the pSGN-I-LUC, and a transcriptional activator expression plasmid pAct-GAL4-ftzQ (Colgan *et al.*, 1993) were cotransfected. Then, the increased luciferase activities were compared to the observed luciferase activities when no transcriptional activator was cotransfected. There was a significant difference of approximately 4 fold in activation between the two reporter plasmids (Fig. 4). The increased sensitivity appears to be partly due to the decreased basal promoter activity, with less transcriptional noise, in the pT-SGN-I-LUC. This increased activation fold was reproduced when other transcriptional activators, such as the GAL4-VP16 and some *Drosophila* homeodomain proteins, were coexpressed (data not shown). Therefore, the pT-SGN-I-LUC is suitable for an accurate and sensitive measurement of promoter activities modulated by transcriptional regulators.

Discussion

There appear to be cryptic promoters, active in *Drosophila* cells, in a pGEM-based pGAP4 DNA

It is generally assumed that the prokaryotic plasmid DNA used for the construction of a reporter plasmid is neutral in terms of transcription. In other words, it is assumed that a short prokaryotic plasmid DNA does not contain any signals which may affect some stage of eukaryotic transcription (e.g., initiation, elongation, splicing, polyadenylation, and termination). However, it is possible that a plasmid DNA contains such signals just by chance. Among these *cis* elements, a cryptic promoter in a plasmid is likely to affect the reporter activity most severely, since the reporter plasmids contain the polyadenylation signal downstream of the reporter cDNA so that no capped mRNA may be elongated in the region of the plasmid DNA. The cryptic promoter in a plasmid, which may reduce the signal/noise ratio of the reporter activity, makes the proper assessment of the weak test promoter activity practically impossible. In this study, to decrease the possibility of containing a cryptic promoter in the prokaryotic plasmid DNA, which is required for the construction of a reporter plasmid, we used the short 2 kb long pGAP4 derived from the pGEM plasmid (Han and Manley, 1993). We have found out that even the short pGAP4 plasmid DNA appears to contain *cis* elements which interfere with some aspects of eukaryotic transcription in *Drosophila*, although we have not pinpointed where the *cis* element is and what stage of transcription it affects. Therefore, it is important to insert a transcriptional termination signal between a plasmid and a promoter, to block transcription from any cryptic promoter in a plasmid and to improve the signal/noise ratio, especially when the strength of a genuine promoter in the reporter plasmid is very low.

The pT-LUC with a very low background reporter activity is suitable for measuring very weak eukaryotic promoter activities

To maximally decrease the cryptic promoter activity in the pGAP4 plasmid, we inserted both the A-trimer cassette and the UMS upstream of the luciferase cDNA. The resulting plasmid pT-LUC, with very low background promoter activity, can be used for measuring very weak promoter activity after the insertion of a promoter between the T element and the luciferase cDNA.

The pT-SGN-I-LUC is suitable for studying eukaryotic transcription factors

The reporter plasmids can be applied for studying the activity of transcription factors as well as measuring promoter strengths. We have inserted some *cis* elements, SGN, upstream of a basal initiator, to test whether the T-containing reporter can be used for a sensitive measurement of the transcription factor activities. Unexpectedly, the basal promoter activity was decreased when the SGN element was inserted into the pLUC or the pI-LUC. It is not clear how the SGN decreases the basal promoter activity, although three explanations are possible. The SGN may have crippled a cryptic promoter by being inserted into a promoter by chance, or may have contained

binding sites for some endogenous transcriptional repressors, or may have facilitated the transcriptional termination. The first possibility is unlikely to be the case since deletion of a polylinker, where the SGN was inserted, did not result in a decrease of the reporter activity (data not shown). The second possibility is unlikely as well. If some endogenous transcriptional repressor binds to the SGN, we should have observed the luciferase activity of the pT-SGN-I-LUC to be lower than that of the pT-I-LUC. Moreover, since the luciferase activities of the pT-SGN-I-LUC and the pT-I-LUC are about the same, it is highly unlikely that there is any cryptic promoter in the SGN which lies downstream of the T element. The third explanation remains to be tested. However, it is not very unlikely that a transcription factor binding site also functions as a termination signal, since the CCAAT box in the adenovirus major late promoter was shown to be a termination signal (Connelly and Manley, 1989).

To construct a reporter plasmid with the lowest basal promoter activity, we inserted the AAA and/or UMS upstream of the SGN and measured the reporter activities. Unexpectedly, the AAA or the UMS alone reduced reporter activity very weakly, although both of them, the T element, reduced more strongly. This may have resulted because the two elements have mammalian origin but tested in *Drosophila* cells, or because the SGN has decreased cryptic promoter activity already. In any case, the T is required to reduce the fortuitous background transcription. The T element itself appears to contain no binding site for endogenous transcription repressors, since the reporter activity of the pT-SGN-I-LUC is increased more strongly by the GAL4-ftzQ transcriptional activator than that of the pSGN-I-LUC (see above). This increased sensitivity was not due to GAL4s fortuitous binding to the T element, since no transcriptional activation was observed when the pT-I-LUC was cotransfected with the GAL4-ftzQ activator expression vector (data not shown).

The pT-SGN-I-LUC offers a couple of advantages as a reporter plasmid for eukaryotic transcription study. First, it allows a sensitive measurement of transcriptional activation since the signal/noise ratio for a transcriptional activation is increased due to the presence of the T in the reporter plasmid. Second, the pT-SGN-I-LUC and its derivatives can be used for transcription studies in mammalian cells as well as in *Drosophila* cells. Both the TdT initiator, a mammalian basal promoter, and the (mammalian) SV40 polyadenylation signal work faithfully in *Drosophila* cells (Colgan and Manley, 1995; Angelichio *et al.*, 1991). In addition, the termination-related signals, the UMS and the AAA are derived from a mammal and (mammalian) SV40. Moreover, the transcription factor binding sites SGN can be used for binding a mammalian transcription factor Sp1 and the GAL4-fusion transcription factors which have been shown to be active in mammalian cells. Therefore, there is no reason why the pT-SGN-I-LUC should not work in mammalian cells and the reporter plasmid is expected to be applied in diverse eukaryotic systems.

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다양한 진핵생물 세포에서 전사 연구에 사용될 수 있는 Luciferase Reporter Plasmid의 개발

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우리는 두 가지의 새로운 luciferase reporter plasmid를 개발하였는데 그 하나는 이 plasmid에 promoter 조각을 삽입한 다음 그 promoter의 활성을 측정하는 용도로 사용될 수 있고, 다른 하나는 매우 낮은 basal promoter 활성을 갖고 있기 때문에 진핵생물의 전사 조절인자의 연구에 도움이 될 수 있다. 후자의 reporter plasmid에는 17염기쌍의 initiator와 Spl, GAL4, 그리고 일부 *Drosophila* homeodomain protein의 결합부위에 해당하는 *cis* elements 등이 들어 있다. 그리고 transcription termination을 촉진할 수 있는 signal을 initiator 앞에 삽입하여 이런 reporter plasmid에 존재할 수도 있는 cryptic promoter에서 시작된 transcript가 luciferase reporter cDNA로 진행되는 것을 방지하는 개선된 reporter plasmid를 제조하여 promoter 활성을 *Drosophila* Schneider line 2 cell을 이용한 transient transfection assay 방법으로 측정하였다. 여기에 사용한 termination 촉진 signal은 SV40 polyadenylation signal의 3연속 조각(AAA)과 transcription termination signal이 포함된 것으로 믿어지는 mouse *c-mos* 유전자의 일부조각(UMS)이다. 기대한대로 이 두 가지의 signal을 삽입하였을 때 basal promoter 활성이 최대한으로 감소하였으며 이 두 가지 signal이 삽입된 reporter plasmid를 사용하여 promoter의 활성을 보다 sensitive하게 측정할 수 있었다. 이 reporter plasmid는 *Drosophila melanogaster*뿐만 아니라 포유동물을 포함한 고등생물의 전사 조절인자 연구의 한 도구로 사용될 수 있을 것이다.