bination-repaired plasmid topologies with the latest transformation technologies (1), it should be possible to routinely obtain transformation frequencies in the range of 10^6 – 10^7 per microgram of plasmid DNA. Such frequencies will greatly aid in library screening strategies that entail very large numbers of transformed yeast cells.

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Improved β -Glucuronidase Reporter for Mammalian Gene Expression Analysis

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Reporter gene assays have become essential for the study of genes and their corresponding regulatory elements. β -glucuronidase (GUS) is one of the most widely used reporter genes in plant genetic research and is used to a lesser extent in mammalian cells. The gene that encodes β -glucuronidase, *gusA*, was originally isolated from *Escherichia coli* by Jefferson et al. (2). GUS chemiluminescent assays are easy and non-isotopic. They allow the detection of amounts as low as 60 fg, with linear measurements over six orders of magnitude of enzyme concentration (1). The use of GUS as a reporter gene in plant genetics has been so successful largely because of the lack of endogenous GUS activity.

Like β -galactosidase (β -gal), GUS expression can be localized at the single cell level by histochemical staining, in the case of GUS, using 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc) as a substrate. Also like β -gal, GUS activity is endogenous to animal cells. However, for using GUS as a reporter gene in animal cells, the heat stability and neutral pH optimum of the microbial GUS allows discrimination between the two. The highly stable microbial GUS also allows aggressive treatment of cell extracts to further reduce the endogenous background (5).

These characteristics make GUS a good candidate for a primary reporter gene or an internal control reporter for normalization of gene expression analysis.

To improve the sensitivity of GUS as a reporter gene, we inserted GUS into pCEP4 (Invitrogen, San Diego, CA, USA) and made three modifications to the expression system (Figure 1A). First, we inserted a translational enhancer from the alfalfa mosaic virus coat protein mRNA leader. This translational enhancer element has demonstrated the ability to increase the translational efficiency of plant and animal mRNAs by as much as 35-fold when fused to the foreign coding sequences (3). Second, the translational initiation sequence was optimized with the placement of a purine at the -3 position (4). Lastly, the nuclear localization signal (NLS) from simian virus 40 (SV40) Large T antigen was added at the 5' end of the GUS coding region (6). The NLS was added to improve the detection of GUS.

The expression of pCEP4GUS and the modified version, pCEP4GUS2, was compared in COS-7 and A549 cells by chemiluminescent enzyme activity assays (GUS-Light™; Tropix, Bedford, MA, USA). The GUS activity was increased 4.5 ± 0.5 -fold in COS-7 cells with the modified construct and 2.6 ± 0.2 -fold in A549 cells (Figure 1B). Similar results were obtained with rat fetal lung primary cells (data not shown). Assays were also performed with the inclusion of a secreted alkaline phosphatase reporter gene as an internal control to exclude the possibility of increased transfection efficiency with the pCEP4GUS2 construct (data not shown).

For histochemical detection of GUS expression in cells, the substrate, X-gluc, has to go through a two-step reaction to produce the final colored precipitate, CIBr-Indigo. First, X-gluc is cleaved by GUS, and then the cleavage product, "X", undergoes oxidative dimerization to produce CIBr-Indigo. CIBr-Indigo is extremely insoluble and precipitates immediately, resulting in cellular localization (8). Thus, by concentrating GUS in the nucleus of the cells, the staining should be able to detect cells that express amounts of GUS previously undetectable. Ideally, this

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would increase the sensitivity of the GUS histochemical assay, which is essential for the determination of cell and tissue specificity. X-gluc histochemical staining of COS-7 cells was significantly enhanced with the pCEP4GUS2 construct (Figure 2A). Immunostaining was performed to determine the effectiveness of the nuclear localization sequence to localize the GUS enzyme in transfected cells. Two days posttransfection, cells were fixed and stained with a primary anti-GUS antibody (Molecular Probes, Eugene, Oregon, USA) and an fluorescein isothiocyanate (FITC) conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells were observed by fluorescent microscopy (Figure 2B).

GUS immunostaining was clearly localized to the nucleus in the cells transfected with pCEP4GUS2. Cells transfected with pCEP4GUS showed GUS immunostaining throughout the cytoplasm without any localized foci.

This does not prove that the nuclear localization of the reporter enzyme, GUS, directly results in the improved sensitivity of the X-gluc histochemical assay that was observed. Nevertheless, it does confirm that the incorporation of the viral nuclear localization sequence can encourage nuclear localization of the GUS gene product.

In summary, the effectiveness of the

GUS reporter gene has been enhanced with three modifications to the 5' untranslated region and the coding region. Reporter gene activity is increased greater than fourfold in transiently transfected COS-7 cells, as determined by chemiluminescence and X-gluc histochemical assays. The incorporation of the nuclear localization region from the SV40 large T antigen

Figure 1. An improved GUS construct for mammalian expression. (A) Schematic diagrams of the original and modified GUS constructs. Both constructs have the same pCEP4 backbone, pCMV promoter, SV40 polyadenylation signal and SV40 intron. The modified GUS construct includes an NLS, (from the SV40 large T antigen), a translational enhancer (from the alfalfa mosaic virus) and an optimized translational start sequence. (B) GUS activity of the original and modified GUS constructs (pCEP4 β -gal was used as a negative control) as determined by chemiluminescence assays on A549 and COS-7 cells. Cells were transiently transfected in 6-well plates with 1 μ g of DNA premixed with 12 μ g lipofectamine (Life Technologies, Gaithersburg, MD, USA). The GUS activity values are normalized to the original pCEP4GUS to indicate the fold increase in activity. The average relative light units (RLU) are expressed above the respective data bars. Results are from duplicate wells of 3 separate experiments. Error bars indicate the standard error.

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Figure 2. Histochemical staining of GUS and nuclear localization of GUS with pCEP4GUS2. (A) X-gluc staining of COS-7 cells transfected with (i) pCEP4GUS2, (ii) pCEP4GUS and (iii) pCEP4β-gal, as a negative control. Colors were modified slightly to enhance the color contrast. (B) Fluorescent micrographs of COS-7 cells immunostained with anti-GUS antibody following transient transfection with (i) pCEP4GUS2, (ii) pCEP4GUS and (iii) pCEP4β-gal.

successfully conferred nuclear localization of the gene product. However, it is not known whether the increase in GUS activity as observed by X-gluc staining results from enhanced translation or enzyme nuclear localization. The robust, easy and sensitive nature of the chemiluminescent GUS reporter gene assays, enhanced by the increased GUS activity of pCEP4GUS2, should encourage people to consider GUS as a strong candidate for a primary reporter gene or as an internal control reporter (7) for normalizing gene expression analyses.

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