

UGUS, a reporter for use with destabilizing N-termini

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ABSTRACT

Due to constraints in vector construction, reporter polypeptides often carry N-terminal sequences of extraneous origin. Since protein half-life can be influenced by small determinants in the N-terminus, such foreign sequences can destabilize proteins and compromise results of reporter-based studies. We provide a real-life example of this problem (destabilizing sequences derived from a ribosomal protein) and show that it can be solved with the ubiquitin fusion technique, in which ubiquitin sequences are placed upstream of the reporter, in our case β -glucuronidase. Post-translational processing by characterized pathways removes the ubiquitin together with destabilizing sequences, generating a stable reporter whose N-terminus is constant.

Reporter genes are widely used to study promoter function. A little discussed technical problem may complicate such studies: the construction strategy often dictates that the reporter polypeptide bears at its N-terminus sequences 'left over' from other genes or derived by translating linker sequences or naturally untranslated regions. These sequences may affect reporter half-life. Thus, if extraneous N-terminal sequences contain processing signals, cleavage may expose a new amino acid on the N-terminus and open the way to degradation by the N-terminal rule pathway. Here half-lives can be as low as a few minutes (1). Other degradation pathways (2) are similarly controlled by small determinants and might be unintentionally activated. Comparisons of promoter strength made with fusion proteins of different half-lives are obviously meaningless; stable and unstable reporters may also show differences in apparent expression domains (3,4). Although it is in principle possible to produce promoter fragments with constant 5'-ends by PCR, the PCR products must be cloned, which is often difficult; furthermore, each case will have its combination of available unique restriction sites, and thus present its own challenges; finally many promoters contain extensive self-complementarities which make them unstable under amplification. Here we describe an alternative based on the ubiquitin-fusion technique which has been widely used to generate fusion proteins of biotechnological interest (7) such as N-terminally modified polypeptides, and also to increase their yield in the host system.

Our reporter is based on β -glucuronidase (β -GUS) encoding the *Escherichia coli* uidA gene (5). Since the natural uidA gene product localizes to endosomes in our system, *Dictyostelium discoideum*, we started with a gus gene modified by replacing five N-terminal codons with the core nuclear localization signal from SV-40 (6). Upstream of this signal are several codons derived from *Dictyostelium* ecmA gene. The N-terminus of the modified GUS gene and the first ubiquitin moiety in the ubi-4 gene of *Saccharomyces cerevisiae* (1) were amplified with primers designed to generate an overlap. The PCR products were then cleaned, annealed together and reamplified with primers complementary to the non-overlapping ends to generate the fusion U-GUS, where the fusion sequence is: GGTGGT/ATGAAAATT-TCTCGATCT/CCAAAAAAAAAAGAAAAGTTGAAGATC-CAGGAGGA/TGTAGAA, with successive portions from ubiquitin, ecmA, the NLS and uidA. The resulting polypeptide is correctly processed in *D. discoideum* (results not shown) and the predicted product is identical to the product of ecmAntGUS (D. Traynor, unpublished), which we have observed to produce an extremely stable β -GUS activity in *Dictyostelium* (MacWilliams, unpublished).

To facilitate the use of this cassette in combination with existing *Dictyostelium* vectors, many of which are designed for G418 selection, this cassette was introduced into a new vector backbone derived from pQE31 (Qiagen), with the addition of the hygromycin resistance gene driven by the actin 15 promoter (9). Derivatives of this vector were constructed containing each of the three polylinkers of the pDdGal vector (10) giving pHUGUS-1, -2 and -3, respectively (Fig. 1).

To compare the stability of the UGUS polypeptide with that produced by 'traditional' GUS constructs, we placed these under the control of the D19 (prespore) or the V18 (vegetative) promoters. D19 contains sequences from *Dictyostelium* PsA (10) and actin15 (12) promoters, with the latter donating TATA box, CAP site, 5' UTR and 13 N-terminal codons. V18 contains the V18 promoter (13) and 5' V18 sequences encoding a N-terminal fragment of 49 amino acids. In both cases, reporter half-life was estimated by inactivating the promoter and following the decrease in β -GUS activity over time. The PsA promoter was inactivated with caffeine (3); assays showed no, or very slow, decay for both forms of the β -GUS polypeptide (Fig. 2). As expected from reporter proteins with identical half-lives, the absolute activities observed with the two constructs were similar

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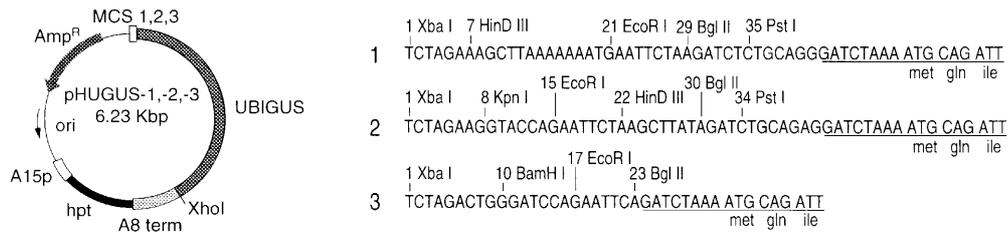


Figure 1. (A) pHUGUS-1, -2 and -3: actin 15 promoter (A15p) and hygromycin phosphotransferase (hpt) are derived from p49 (8); AmpR and replication origin (ori) are from pQE31 (Qiagen); actin 8 terminator and polylinkers are from pDDGal vectors (9). (B) Polylinker sequences. The UGUS cassette 5'-end is underlined and the first three ubiquitin amino acids are indicated.

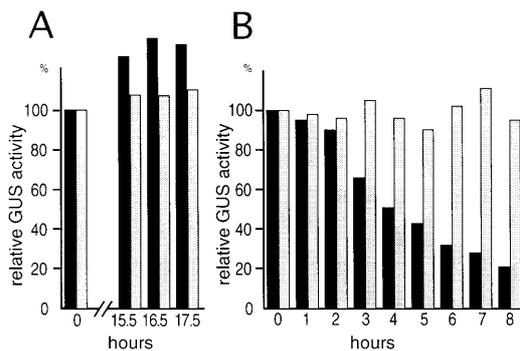


Figure 2. (A) The products of both D19GUS (black) and D19UGUS (grey) are stable: after promoter shut-off, neither activity decreases over 17.5 h. (B) After promoter shut off, the product of V18GUS (black) decays, but that of V18UGUS (grey) is stable.

(data not shown). The V18 promoter was inactivated by starvation (14); no loss of activity was observed with V18UGUS, while V18GUS showed, after a 2 h delay, a half-time of ~3 h. Consistent with this, their initial levels of activity were strikingly different, with the stable reporter giving 7-fold higher levels (not shown).

Thus, although the 'extraneous' actin N-terminus seems to have no effect, the 'extraneous' V18 N-terminus destabilizes

β -GUS. In the pHUGUS vector, this destabilization is eliminated, allowing the use of normal reporter protocols.

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