NOTES

Rapid Tagging and Integration of Genes in *Giardia intestinalis*† †

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We developed a series of plasmids that allow C-terminal tagging of any gene in its endogenous locus in *Giardia intestinalis*, with different epitope tags (triple hemagglutinin [3HA] and triple Myc [3Myc]) and selection markers (puromycin, neomycin, and a newly developed marker, blasticidin). Using these vectors, cyclin B and aurora kinase were tagged, expressed, and localized.

The protozoan *Giardia intestinalis* is a human parasite responsible for considerable morbidity both in humans and in cattle (1, 9, 15). Due to its high divergence from other eukaryotes, many antibodies useful for cell biology will not recognize *Giardia* proteins. The process of developing gene-specific antibodies is long and laborious, typically requiring several months. A faster approach is to express a version of the gene tagged with a well-characterized epitope by integrating a construct into the chromosome. Integration of constructs into the *Giardia intestinalis* genome has been achieved previously (6, 7, 11) but not for the purpose of tagging a gene. Our goal was to facilitate integration of a tagged *Giardia* gene into the genome and to keep expression as close to endogenous levels as possible.

A construct was designed for knocking in a tagged version of cyclin B (*GiardiaDB* identification [ID] number GLS0803_3977) into the *Giardia* genome. This strategy is derived from the gene tagging originally developed with *Schizosaccharomyces pombe* (2, 4) and since then adapted to other organisms, such as *Trypanosoma brucei* (10). The C-terminal part of the gene of interest is cloned in frame to a C-terminal triple-hemagglutinin (HA) epitope tag (3HA) (Fig. 1A) and appended to the NotI/KpnI fragment from the plasmid *pyG-GFP* (11) containing the puromycin resistance cassette to yield the plasmid *pc-cycB-3HA-PAC*. The plasmid was then linearized using an original NruI site, ethanol precipitated, and resuspended in distilled water, and 10 μg of linear DNA was introduced into *Giardia* strain WB-C6 by electroporation as previously described (11). Transfected cells were selected using puromycin (10 μg/ml), and after 7 to 9 days of selection, resistant cells were recovered. Cells were harvested, lysed in SDS sample buffer supplemented with protease inhibitors, and analyzed by Western blotting using an anti-HA antibody (HA-7, 1:3,000; Sigma). A single band matching the predicted full-length cyclin B size of 43 kDa was observed (Fig. 1B, anti-HA panel); mock-transfected cells showed no signal. The blot was also stripped and reprobed for tubulin (TAT1 antibody, 1:5,000) to verify equal loading (Fig. 1B, antitubulin panel).

Separately, a second plasmid, *pc-cycB-3HA-BSR*, was constructed by combining the 5’-*γ*-giardin region of *pyG-GFP* and the blasticidin resistance gene (5) from the plasmid *pBOSH2BGFP* (Clontech) using overlap PCR (see the supplemental material for more details). This plasmid is identical to *pc-cycB-3HA-PAC* except that the blasticidin coding sequence replaces that of puromycin. This new plasmid was linearized using NruI and introduced into *Giardia* cells. After 9 to 11 days of selection with 75 μg/ml blasticidin, resistant cells were observed and were subsequently harvested and analyzed by Western blotting. Again, a single band at 43 kDa was observed (Fig. 1B, *pc-cycB-3HA-BSR* lanes). Transfection efficiencies using blasticidin were ~50% less than those observed using puromycin. However, with respect to the levels of cyclin B or the growth rate of the cells, we could not find any difference between cells transfected with *pc-cycB-3HA-PAC* or *pc-cycB-3HA-BSR* and then selected using puromycin or blasticidin, respectively.

Given that only full-length cyclin B was observed and that none of the plasmids carried either a promoter or the cyclin B
N terminus, we concluded that our construct integrated into the genome of *Giardia intestinalis* precisely as expected. To confirm this conclusion, we isolated genomic DNA from both pc-cycB-3HA-PAC- and pc-cycB-3HA-BSR-transfected cells and from mock-transfected cells and performed PCR analysis. We used a common 5′ primer that binds in the cyclin B gene but used either a 3′ primer binding the cyclin B gene or a 3′ primer binding immediately upstream of the drug resistance marker. Importantly, use of this last primer should yield a product only if the cells carry a tagged version of cyclin B and this construct has integrated at the correct locus; circular plasmid does not yield a product. After PCR, all cells yielded an ~1-kb product when both cyclin B primers were used (Fig. 1C); however, the untransfected cells did not yield any product when the 3′ plasmid-specific primer was used. In contrast, the pc-cycB-3HA-PAC- and pc-cycB-3HA-BSR-transfected cells showed an ~1.5-kb product, as expected (Fig. 1C). Taken together, these data suggest that the constructs integrated into the *Giardia* genome at the correct locus.

Having successfully integrated a tagged copy of the cyclin B gene into the *Giardia* genome, we wanted to expand this technique to other genes. As an example, aurora kinase (*GiardiaDB* ID number GL50803_5358) was tagged at its C terminus using the same protocol used for cyclin B (see the supplementary material for the cloning description). The plasmid pcAUK-3HA-NEO was linearized using BsmBI and transfected into *Giardia* cells as before. After selection, a single band of approximately 38 kDa was observed (Fig. 2A, left panel); conversely, no signal was observed in the mock-transfected cells. Cells expressing tagged aurora kinase and tagged cyclin B were then stained using anti-HA antibodies, and the localization of both proteins was visualized (Fig. 2B). Cyclin B localized predominantly to the cytoplasm, while aurora kinase showed a nuclear localization, with a slight enrichment at the nuclear rim, which agrees exactly with the previously published aurora localization (3). Thus, for the case of aurora kinase, introducing the C-terminal epitope tag did not affect the localization of the protein.

To determine if different tagged genes could be introduced into *Giardia*, two cyclin-dependent kinases, Cdk1 (GL50803_8037) and Cdk2 (GL50803_16802), were PCR amplified and fused to a C-terminal triple Myc (3Myc) tag (see the supplemental materials for oligonucleotide and cloning details). The two plasmids, pcCdk1-3Myc-BSR and pcCdk2-3Myc-BSR,
were linearized using PshAI and BglII, respectively, transfected into *Giardia* cells carrying integrated pc-cycB-3HA-PAC, and selected using blasticidin. After 10 days of selection, resistant cells were harvested, lysed, and analyzed by anti-HA Western blotting; blots were also probed for tubulin to verify equal loading. MW, molecular weight markers. (B) Cells were fixed, permeabilized, and probed with anti-HA followed by a tetramethyl rhodamine isocyanate (TRITC)-labeled secondary antibody. 4',6-Diamidino-2-phenylindole (DAPI) was used to counterstain the cell nuclei. Wt, wild type.

were linearized using PshAI and BglII, respectively, transfected into *Giardia* cells carrying integrated pc-cycB-3HA-PAC, and selected using blasticidin. After 10 days of selection, resistant cells were harvested, lysed, and analyzed by Western blotting using an anti-Myc antibody (71D10, 1:1,000; Cell Signaling Technologies). Both 3Myc-tagged Cdk and 3HA-tagged cyclin B could be readily detected (Fig. 3A). In a separate experiment, cells were lysed under nondenaturing conditions using RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1% deoxycholate) supplemented with protease inhibitors and tagged Cdk2 purified using an anti-Myc antibody matrix (Sigma). Immunoprecipitated proteins were released by boiling the beads in SDS sample buffer and analyzed by Western blotting (Fig. 3B). We found that, as expected from the general model of cyclin/Cdk function (8, 12), 3Myc-tagged Cdk2 binds to 3HA-tagged cyclin B (Fig. 3B) but does not bind actin (Fig. 3B, bottom panel; actin antibody used at 1:3,000 dilution). These results suggest that the C-terminal epitope tags on cyclin B and Cdk2 do not disrupt their activity, since they retain the ability to specifically interact with each
other. Together with the aurora kinase localization, our data suggest that although protein tagging for every protein must be determined empirically, our tagging method per se does not alter the protein’s levels or localization.

Overall, our results indicate that the tagged genes are integrated into the genome and expressed at endogenous levels. We created several new vectors containing different epitopes, such as the 3Myc tag, and different drug markers (maps and plasmid are available upon request), including neomycin resistance (13, 14, 16), and have introduced the blasticidin marker in Giardia and shown it to be usable for the selection of stable cell lines. Integration into the Giardia genome has been previously demonstrated (6, 7, 11); however, this technology improves the endogenous tagging of genes by significantly simplifying cloning and by expressing genes under their native promoter and in their native chromosomal environment.

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