Correspondence

Cell-free expression of a GFP fusion protein allows quantitation *in vitro* and *in vivo*

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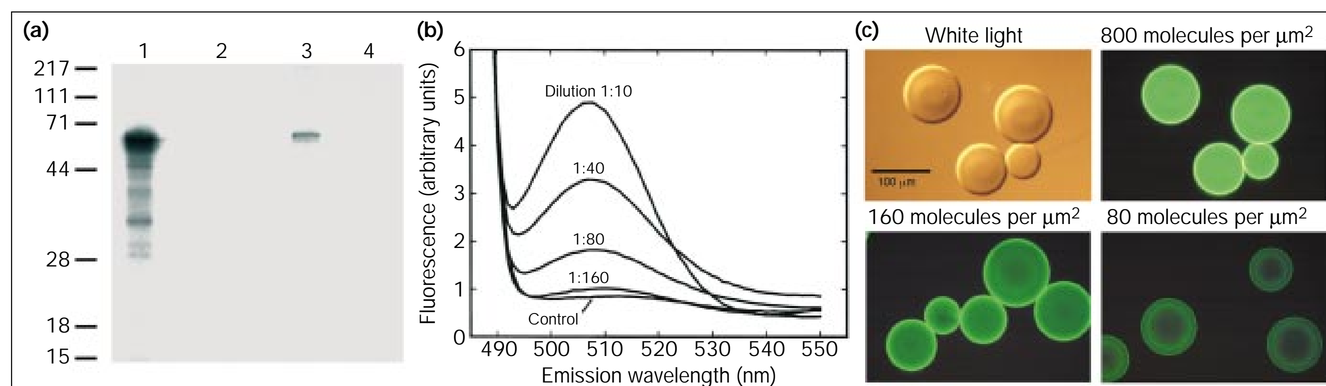
The green fluorescent protein (GFP) of *Aequorea victoria* is frequently fused to other proteins to serve as a reporter for gene expression or the localization of proteins *in vivo*. We report that when a GFP fusion protein is translated *in vitro* under standard conditions, the GFP portion folds efficiently and becomes fluorescent. This provides a convenient method for monitoring *in vitro* translation efficiency of a fusion protein, and to screen for improved mutants of GFP. In addition, quantitation of the translation product combined with fluorescence microscopy of the product immunoprecipitated onto beads allows the determination of the density of the fusion protein in microscopic images.

A fusion of the tobacco mosaic virus 30 kDa movement protein [1] to the amino terminus of the S65T mutant of GFP [2] was translated using a standard rabbit reticulocyte lysate system incorporating ^{35}S-labeled methionine of known specific radioactivity. The products were electrophoresed using SDS and polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography (Fig. 1a, lane 1). The major product exhibited the expected mobility; minor products of greater mobility are presumed to arise from internal initiation or premature termination. Scintillation counting, combined with knowledge of the number of methionine residues per molecule of the fusion protein, showed that each microliter of the translation reaction contained 3.8×10^{-15} moles of fusion protein.

The GFP part of the fusion protein folded properly, as shown by its fluorescence emission spectrum (Fig. 1b). A clear signal was readily detected in as little as 2 µl of the translation reaction diluted 100-fold, corresponding to a fusion protein concentration of approximately 4×10^{-11} M.

The fusion protein was immuno-precipitated onto protein G–sepharose beads using an anti-GFP antibody. Only the major translation product and a minor product, presumed to arise from internal initiation 20 amino-acids downstream from the amino

Figure 1



(a) Autoradiogram of translation products separated by SDS–PAGE. The cDNA encoding the fusion protein was excised from the pTMVM:Gfus plasmid [1] and inserted into pSP64T [6]. SP6 transcript (3 µg) was added to 60 µl rabbit reticulocyte lysate (Promega) containing [^{35}S[methionine. The control sample had no transcript added. Reactions were allowed to proceed for 2 h at 25°C, and were then kept at 16°C for 4 h. To remove unincorporated [^{35}S]methionine, the samples were diluted with phosphate-buffered saline (PBS) and filtered four times in Millipore ultrafree-MC low binding regenerated cellulose filter units with a nominal molecular weight limit of 30 kDa, which had been prewashed with 1% bovine serum albumin (BSA), 1% sucrose. Lane 1, translation product; lane 2, control sample (no RNA); lane 3, immunoprecipitated translation product; lane 4, immunoprecipitated control sample (no RNA). Each lane contains the equivalent of a 0.25 µl sample of the original reaction. Markers (in kDa) on the left. **(b)** Fluorescence emission spectra of fusion protein translated *in vitro*, diluted to various extents in PBS, and of a control (no RNA) sample. The excitation wavelength was 475 nm. A Hitachi F-2000 fluorescence spectrophotometer was used, with a 10 nm bandpass for both the excitation and emission monochrometers. Fluorescence was stable for at least 3 days at 4°C. **(c)** White light and fluorescence micrographs of protein G–sepharose beads used to immunoprecipitate varying amounts of fusion protein translated *in vitro*. Each immunoprecipitation reaction contained 1 µl protein G–sepharose 4 fast-flow bead slurry (Pharmacia), containing about 630 beads, and 0.5 µl anti-GFP antibody (Clontech), in 500 µl PBS containing 0.2% Triton X-100 (Sigma). The reactions were shaken at 4°C for 16 h, and the beads were washed once with PBS containing 0.2% Triton X-100. Scintillation counting showed that the efficiency of the immunoprecipitation was about 60% regardless of the amount of translation reaction added to the beads. The beads were examined with a Nikon Optiphot2-UD microscope equipped with a B-2A FITC filter set (450–490 excitation filter, 505 dichroic mirror, 520 barrier filter) using an objective with a numerical aperture of 0.50, and were photographed on Kodak Ektachrome P1600 film. Beads used to immunoprecipitate a control reaction (no RNA) showed no fluorescence (not shown).

terminus, were recognized by the antibody (Fig. 1a, lane 3).

Fluorescence from the beads was easily detectable by fluorescence photomicroscopy when the amount of translation reaction added to an immunoprecipitation corresponded to as little as 1 nl per bead (Fig. 1c). This is equivalent to about 2×10^6 protein molecules per bead, giving a surface density of about 80 molecules per μm^2. Even a density as low as about 8 molecules per μm^2 could be seen in the microscope (data not shown). The true density of fluorescent molecules may be even lower than indicated, as the fraction of synthesized molecules that adopted a fluorescent conformation is not known. Each bead is somewhat larger than a eukaryotic cell (90 μm in diameter), suggesting that a similarly small number of GFP-fusion molecules could be detected in a living cell, particularly if the protein were localized; autofluorescence from a cell, however, might place a lower limit on this number [3].

Cell-free expression of GFP seems ideal for the rapid development of new GFP mutants with brighter fluorescence or shifted spectra, based on the recently determined crystal structure [4,5]. Large numbers of mutants could be screened rapidly by using cell-free coupled transcription–translation of cDNA clones in microtiter plates. In addition, the fusion of GFP to other proteins provides a simple nonradioactive method of measuring translation efficiency, which could expedite the optimization of translation of any protein of interest. Furthermore, if radiolabeled amino acids are incorporated, fluorescence intensity can be correlated with protein quantity, allowing the determination of the density of GFP-fusion protein molecules in a microscopic image of a living cell, a task which cannot easily be accomplished by analysis *in vivo*.

References

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Gazetteer

Medline

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