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High-throughput Fluorescence Labelling of Full-length cDNA Products Based on a Reconstituted Translation System

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Although recent advances in fluorescence-based technologies, such as protein microarrays, have made it possible to analyse more than 10,000 proteins at once, there is a bottleneck in the step of preparation of large numbers of fluorescently labelled proteins for the comprehensive analysis of protein–protein interactions. Here we describe two independent methods for high-throughput fluorescence-labelling of full-length cDNA products at their C-termini using a reconstituted translation system containing fluorescent puromycin. For the first method, release factor-free systems were used. For the second method, stop codons were excluded from cDNAs by using a common mismatch primer in mutagenic PCR. These methods yielded large numbers of labelled proteins from cDNA sets of various organisms, such as mouse, yeast and *Escherichia coli*.

Key words: cell-free protein synthesis, fluorescence labelling, proteomics, puromycin, release factor.

INTRODUCTION

There is currently great interest in profiling and evaluating the interactions of proteins encoded by large cDNA collections. Fluorescence-based technologies such as microarrays and microscopic techniques have been widely used for analysis of the expression, interaction and conformation of proteins (*1–4*). In particular, protein microarrays make it possible to search large numbers of protein–protein interactions in parallel (*5, 6*). Despite these successes, it is not always feasible to prepare large sets of fluorescently labelled proteins for comprehensive analysis. Conventionally, the proteins have been expressed in *Escherichia coli*, purified to homogeneity, and then fluorescently labelled by chemical modification methods, but the expression and purification of large numbers of proteins is a daunting task. As an approach to overcome this problem, a simple method for *in vitro* fluorescence-labelling of proteins using a derivative of puromycin was developed in our laboratory (*7–12*), and the method was also applied to the fluorescence labelling of newly synthesized proteins in living cells (*13*). Since our method relies on incorporation of a fluorescent puromycin analogue into the C-terminus of the full-length protein during protein synthesis on ribosomes, the expression and labelling steps are synchronized and a purification process before labelling is unnecessary. With the resulting fluorescently labelled proteins, we succeeded in detecting protein–protein and protein–DNA interactions by means of fluorescence polarization spectroscopy (*7*), protein microarrays (*11*), DNA microarrays (*9*) and fluorescence correlation spectroscopy (*9, 12*) in model experiments.

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Although puromycin-based labelling is very simple and rapid, the labelling efficiency is relatively low when fluorescent puromycin competes with release factors (RFs) for binding to the ribosomal A-site in the presence of a stop codon (Fig. 1A) (*7, 8*). The yield of fluorescently labelled proteins is enhanced by the use of mRNA without a stop codon (Fig. 1C) (*7, 10*), but many cDNA collections contain stop codons and their elimination usually requires a large set of oligo DNA primers. Agafonov *et al*. (*14*) reported that the inactivation of release factor 1 (RF1) leads to better incorporation of puromycin using template mRNA with a UAG stop codon (Fig. 1B). Here we describe two methods for high-throughput fluorescence labelling of full-length cDNA products using the PURE *E. coli* reconstituted *in vitro* transcription/translation (IVT) system (*15*), though we have previously used a highly efficient wheat germ IVT system (*9, 11, 16*). The PURE system includes highly purified *E. coli* ribosomes, tRNA and His-tagged recombinant proteins (translation factors, aminoacyl-tRNA synthetases, methionyl-tRNA transformylase and T7 RNA polymerase) (*15*). Recently, reconstituted IVT systems (*15, 17*) have been used for reassignment of a sense codon by omitting amino acids, aminoacyl tRNA synthetases and endogenous tRNA (*18–20*). We envisioned that fluorescently-labelled proteins could be easily obtained with the PURE DNA-direct IVT system by omitting RFs or the stop codons, and easily purified by affinity chromatography.

EXPERIMENTAL PROCEDURES

Synthesis of Fluorophore-dC-Puromycin—Protected deoxycytidine (dC) and Cy3- and fluorescein-phosphoramidites were purchased from Glen Research. The Cy3-dC-puromycin and fluorescein-dC-puromycin (Fig. 2)

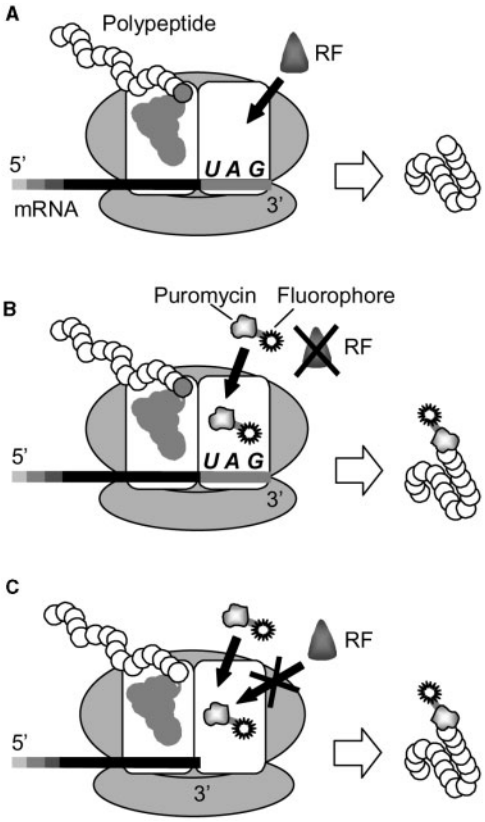

Fig. 1. **Schematic representation of the termination of translation and fluorescence labelling of proteins.** (A) In usual translation systems, RFs bind to ribosome with a stop codon positioned in the A site, and then the completed polypeptide chain is released from the ribosome. (B) In the absence of RF in a translation system, or (C) in the absence of any stop codon in mRNA (truncated messengers), the polypeptide cannot be released from the ribosome and the polypeptide-aminoacyl-tRNA-mRNA complex stalls at the P site of the ribosome. During this interval, fluorophore-dC-puromycin enters the A site and binds to the full-length polypeptide at the C-terminus. Then, the fluorophore-labelled polypeptide is released from the ribosome.

were synthesized by the standard solid-phase phosphoramidite method and purified by reverse-phase HPLC as previously described (*9–11*). The structures were confirmed by MALDI-TOF MS.

DNA Preparation—The template DNAs for IVT were prepared by multi-step PCR with KOD-plus DNA polymerase (Toyobo) or *Ex Taq* DNA polymerase (Takara) as follows. In each step, all PCR products were purified with a QIAquick PCR purification kit (Qiagen).

The mouse c-Jun (167-319 aa) gene with an N-terminal T7·tag (11 aa, MASMTGGQQMG) coding sequence (*9*) was amplified by PCR using a forward primer PT7tagF (5′-AAGGAGATATACCAATGGCTAGCATGAC TGGTGG-3′) and a reverse primer JunFLAGXAR (5′-CTTGTCGTCGTCGTCCTTGTAGTCGCCAAGCTTCA AAACGTTTGCAACTGCTGCGTTAGC-3′) containing a C-terminal FLAG-tag (8 aa, DYKDDDDK) coding sequence with no stop codon. Further, three c-Jun DNA fragments each containing a TAA, TAG, or TGA stop codon were prepared by 2nd PCR using the forward Universal primer (Post Genome Institute) and three kinds of reverse primers, FlagTAAAR (5′-TTTTTTTTTTACTTGTCGTCATCGTCCTTGTAG-3′), FlagTAGAR (5′-TTTTTTTTCTACTTGTCGTCATCGTCC TTGTAG-3′), or FlagTGAAR (5′-TTTTTTTTTCACTTGT CGTCATCGTCCTTGTAG-3′), respectively. Control DNA without a stop codon was similarly prepared by a second PCR using reverse primers, FlagAR (5′-TTTTTTTTCT TGTCGTCATCGTCCTTGTAG-3′).

Full-length yeast cDNAs were amplified from a *Saccharomyces cerevisiae* cDNA library (*21*) using a forward primer SC-T7F (5′-ATGGCTAGCATGACT GGTGGACAGCAAATGGGTCCGGAATTCCAGCTGACC ACC-3′) and a reverse primer SC-R (5′-CGATCCCC GGGAATTGCCATG-3′). The PCR products were re-amplified using the forward primer PT7tagF and reverse primers, tatR (5′-CGATCCCCGGGAATTGCCA TGTAT-3′) and/or tggR (5′-CGATCCCCGGGAATTGCCA TGCCA-3′). The PCR products were further amplified using the Universal primer and the reverse primer SC-R. The DNA sequence was confirmed with an ABI PRISM 3100 genetic analyser (Applied Biosystems).

The *E. coli* cDNAs were amplified from ASKA library (*22*) by PCR using a forward primer ORF-F3 (5′-AAGGAGATATACCAATGGATCCGGCCCTGAGGG CC-3′) and a reverse primer ORFFlag-R7 (5′-TCCACCCT TGTCGTCGTCGTCCTTGTAGTCTCCTTTACTGCGGCC GCATAG-3′). The PCR products were re-amplified using the Universal primer and ORFFlag-R7.

Fluorescence Labelling of Proteins—IVT was performed using the PURE system sometimes containing RF1 and RF2 in the presence of template DNA and 8 μM Cy3-dC-puromycin (or 16 μM fluorescein-dC-puromycin). A total volume of 10 μl was incubated at 37°C for 1 h in a microtube or in each well of a 96-well microplate. The reaction products were separated by 10–20% gradient SDS-PAGE or 15% SDS-PAGE and analysed with a Molecular Imager FX (Bio-Rad Laboratories) or by Western blot analysis using anti-FLAG M2 antibody (Sigma) or anti-T7·tag antibody (Novagen), and an ECL kit (Amersham Biosciences).

Pull-down Assay—The bait Pup2 was fused with the IgG-binding domain of protein A (ZZ domain). The cDNA of Pup2 was amplified by PCR using a forward primer (5′-CCGCGGGATCCCCGGAATTCCAGCTGACCACC-3′) containing a BamHI site and a reverse primer (5′-CACCCCTCGAGCGATCCCCGGGAATTGCCATG-3′) containing an XhoI site, and digested with BamHI and XhoI. The fragment was subcloned into the BamHI/XhoI site of pCMV-CBPzz vector (*10*). The ZZ-Pup2 template DNA was PCR-amplified with the forward primer PT7tagF and the reverse primer CMV-R (5′-GGATCT CCATTCGCCATTCA-3′), and the PCR products were further amplified using the Universal primer and CMV-R primer. The bait ZZ-Pup2 was synthesized by use of the PURE system, and mixed with the fluorescein-labelled Pre5 (prey protein) prepared as earlier. The complex was captured on IgG agarose beads. The beads were washed, and the bound protein was eluted with a buffer (0.1 M Tris–HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol) at 100°C for 5 min, separated by 15% SDS-PAGE, and analysed with a Molecular Imager FX.

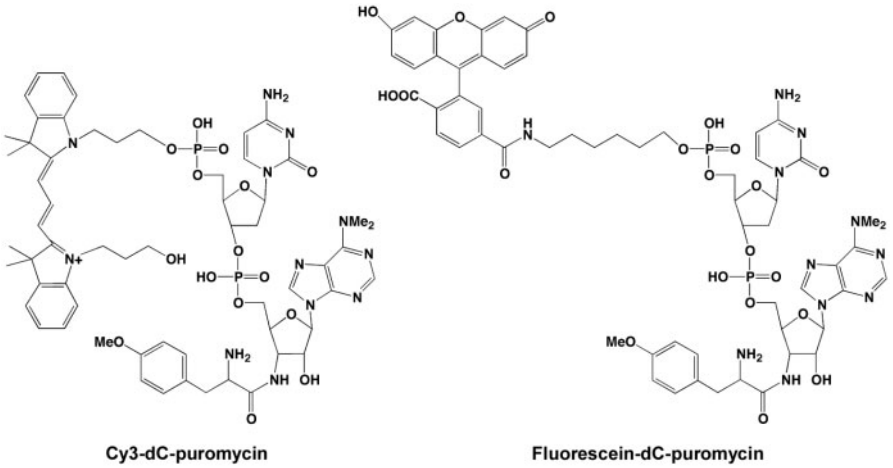

Fig. 2. **The structures of Cy3- and fluorescein-dC-puromycin**.

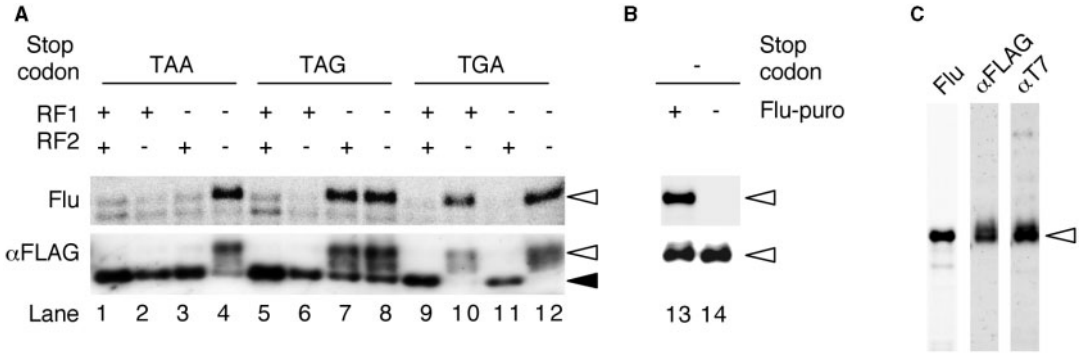

Fig. 3. **Fluorescence labelling of proteins in RF-free translation systems.** (A) Three DNA fragments encoding mouse c-Jun (216-318 aa) with FLAG-tag followed by a TAA, TAG or TGA stop codon (See Experimental procedures for details) were transcribed and translated in RF1 and/or RF2-free IVT systems containing fluorescein-dC-puromycin. The products were separated by 15% SDS-PAGE and detected with a fluorescence imager (Flu) or by Western blotting with anti-FLAG antibody (αFLAG). White and black arrowheads indicate labelled and non-labelled proteins, respectively. (B) Control c-Jun-KK protein was synthesized in the presence and absence of fluorescein-dC-puromycin (Flu-puro) and detected as described above. (C) A labelled c-Jun-KK protein was separated by 10–20% gradient SDS-PAGE and detected with a fluorescence imager (Flu) or by Western blotting with anti-FLAG antibody (αFLAG) and anti-T7·tag antibody (αT7).

RESULTS

C-Terminal Labelling of Proteins with Fluorescent Puromycin in RF-free Translation Systems—First we tested the fluorescence-labelling of cDNA containing a stop codon in the absence of RF (Fig. 1B) using the PURE *E. coli* reconstituted system (*15*). In *E. coli*, two codon-specific RFs, RF-1 (UAG, UAA) and RF-2 (UGA, UAA), have been shown to recognize stop codons (*23, 24*). Thus, we prepared cDNA fragments, each containing one of the three stop codons (TAG, TGA or TAA), as templates for fluorescence labelling with fluorescein-dC-puromycin (Fig. 2) in the absence of RF1 and/or RF2. As anticipated, fluorescein-labelled protein was specifically obtained from the TAA template in the absence of both RFs (Fig. 3A; lane 4), from the TAG template in the absence of RF1 (Fig. 3A; lanes 7 and 8), and from the TGA template in the absence of RF2 (Fig. 3A; lanes 10 and 12).

The mobility of the labelled proteins (Fig. 3A; white arrowheads) was slower than that of the non-labelled proteins (Fig. 3A; black arrowhead), probably because of the positive charge of the two additional Lys residues at the C-terminus derived from poly(A)$_8$ at the 3′-terminus of template mRNA, owing to ribosomal read-through at the stop codon in the absence of RF. The high concentration of magnesium ion, which is optimum for the transcription process driven by T7 RNA polymerase, is known to induce ribosomal read-through at the stop codon (*25*). We confirmed this interpretation by using a DNA template with no stop codon (Fig. 3B): the position of c-Jun-KK protein with two Lys residues (Fig. 3B; lanes 13 and 14) was consistent with that of the read-through products (Fig. 3A; white arrowheads). The attachment of fluorescein-puromycin did not affect the mobility of proteins (Fig. 3B; αFLAG; lanes 13 and 14).

When we used both N-terminal T7·tag and C-terminal FLAG tag sequences to detect a full-length protein in Western blotting analysis, no immature or truncated protein was detected (Fig. 3C).

These results indicated that the RF-free reconstituted translation system would be useful for the

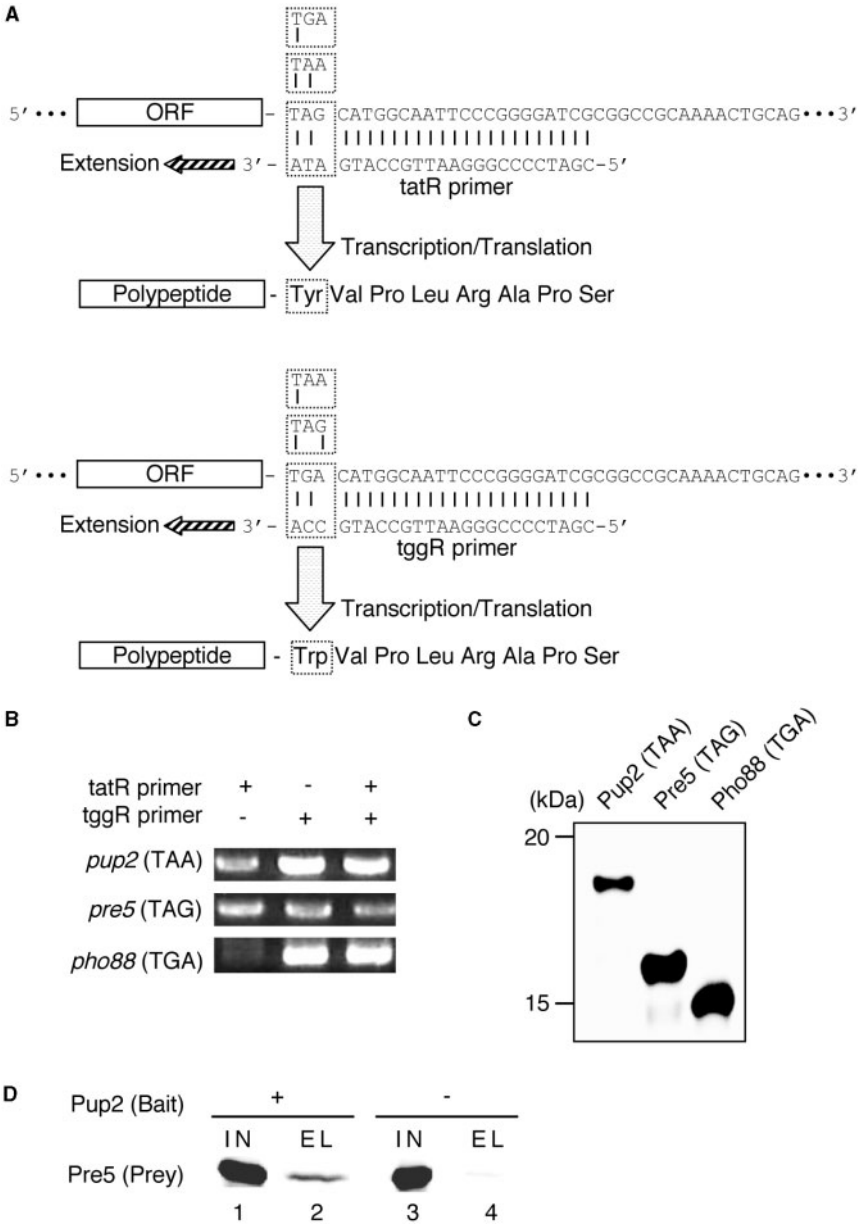

Fig. 4. **Fluorescence labelling of products of cDNAs whose stop codons have been eliminated by mutagenic PCR with common mismatch primers**. (A) The mismatch primers tatR (upper) and tggR (lower) replace the stop codon with a Tyr codon and a Trp codon, respectively. (B) The yeast cDNAs of *pup2*, *pre5*, and *pho88* were amplified with the tatR and/or tggR primers and analysed by agarose gel electrophoresis. The original stop codon of each cDNA is shown in parenthesis. (C) Fluorescein-labelled yeast proteins were synthesized using the PURE system, as described in the legend of Fig. 3. (D) Pull-down assay. Fluorescein-labelled Pre5 was mixed with the bait Pup2 fused with the ZZ domain (lane 1; IN, input), captured on IgG beads, washed, and eluted (lane 2; EL, elution). Lanes 3-4; negative control.

fluorescence-labelling of a large collection of full-length cDNA products containing stop codons.

Mutagenic PCR using Common Mismatch Primers for Replacing a Stop Codon with a Sense Codon in cDNA—As an alternative approach, efficient labelling with fluorescent puromycin can be achieved by using cDNA without a stop codon (Fig. 1C) (*7, 10*). For this purpose, a simple method for removing the stop codon from a large set of full-length cDNAs is required. Since many cDNA collections were cloned into a common vector (*21, 22*), we examined whether a stop codon could be replaced with a sense codon by mutagenic PCR using mismatch primers that hybridize with the common vector sequence.

We designed two primers, tatR and tggR, for mutating the stop codon in a yeast cDNA collection (*21*) to sense codons TAT and TGG, respectively (Fig. 4A). Three yeast cDNAs, *pup2*, *pre5* and *pho88*, with stop codons, TAA, TAG and TGA, respectively, were successfully amplified by mutagenic PCR with tatR and/or tggR primers (Fig. 4B). The mutations in all amplified sequences were confirmed by DNA sequencing. Only *pho88* with

TGA showed inefficient extension with the tatR primer, perhaps due to the two-nucleotide mismatch (Fig. 4A), while somewhat surprisingly, the *pup2* with TAA was efficiently amplified using tggR primer in spite of the two-nucleotide mismatch (Fig. 4B). All yeast cDNAs amplified with the mixed tatR and tggR primers were confirmed to be available for fluorescence labelling of the cDNA products with the PURE system containing both RF1 and RF2 (Fig. 4C).

Furthermore, we confirmed that the labelled proteins obtained in this way are suitable for protein-protein interaction analysis by means of pull-down assay of the known Pre5-Pup2 interaction (*26*). The bait Pup2 was synthesized as a fusion protein with the IgG-binding domain of protein A (ZZ domain) by use of the PURE system. The fluorescein-labelled Pre5 (prey protein) was bound to the ZZ-Pup2 protein captured on IgG agarose beads (Fig. 4D).

High-throughput Fluorescence Labelling of Proteins—Finally we attempted to perform fluorescence labelling of various proteins in a multi-well format. A complete set of *E. coli* ORFs was previously cloned by the authors (T. B. and H. M.) and their co-workers from a K-12 strain W3110 (*22*). We prepared template DNA of 162 *E. coli* genes by PCR and synthesized fluorescently labelled proteins with the PURE *E. coli* reconstituted system containing Cy3-dC-puromycin (Fig. 2) in 96-well microplates. One hundred and forty-five out of 162 full-length proteins (~90%) were successfully labelled with Cy3 (the results are shown in part in Fig. 5). We confirmed that the resulting Cy3-labelled proteins are useful for probing protein-protein interactions on *E. coli* proteome chips (unpublished results). Thus, high-throughput fluorescence labelling based on the PURE system is expected to be most useful for the large-scale analysis of protein–protein interactions.

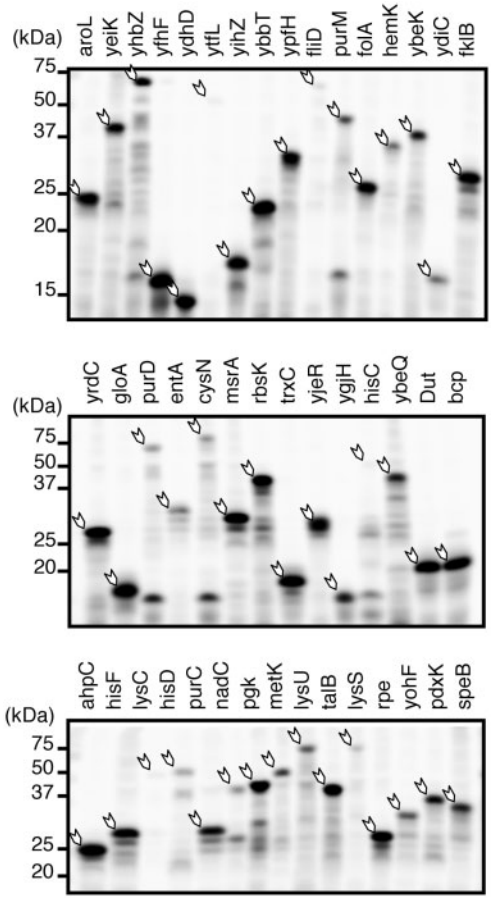

Fig. 5. **High-throughput fluorescence labelling of *E. coli* proteins.** The Cy3-labelled proteins were prepared using the PURE reconstituted *E. coli* translation system in a 96-well format and analysed by SDS-PAGE and with a fluorescence imager. White arrowheads indicate full-length proteins.

DISCUSSION

In this study, we have demonstrated that our method for C-terminal labelling of proteins based on a reconstituted translation system allows the efficient synthesis of large numbers of labelled proteins from full-length cDNA sets of various organisms, such as mouse, yeast and *E. coli*.

A demerit of our method is the addition of an artificial peptide [*e.g.* KK in Fig. 3 and (Y/W)VPLRAPS in Fig. 4] at the C-terminus of the full-length protein. Such additional amino acids may affect the structure and function of the protein, though it is also possible that the additional sequence may simply act as a peptide spacer between the fluorophore and the protein. Indeed, we previously confirmed that more than 30 proteins with C-terminal tag peptides and fluorescent puromycin retained their binding activities in the analysis of protein-protein and protein-DNA interactions (*9–12, 27–29*).

The use of the PURE reconstituted IVT system has advantages. First, the lack of tmRNA in the PURE system (*30*) might favour efficient C-terminal labelling, because nascent polypeptides are known to be released by SmpB-tmRNA complex (*31*) when ribosomes are stalled on mRNA in the absence of a stop codon. Second, supplements such as chaperonin GroEL can be easily added to the PURE system (*32*) for the synthesis of correctly folded proteins.

It should be noted that the RF-free reconstituted system may be useful not only for fluorescent puromycin labelling of proteins as described here, but also for ribosome-display (*33–35*) and mRNA-display (*10, 36–38*) systems.

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