identifying immunoreactive and protective antigens [56]. Whatever may be the approach used for the discovery of novel vaccine candidates, we do need to have an optimal recombinant protein synthesis system not only for the synthesis and characterization of these vaccine candidates in the initial stage, but also for the mass production of vaccine antigens for vaccination purpose in the later stage.

The first and the foremost barrier to the rapid discovery of vaccine candidates is the lack of an efficient system to synthesize quality malaria proteins. *Plasmodium falciparum* genes are A/T rich (average A/T content per coding sequence is 76%) and a number of them encode repeated amino-acid motifs [48], and these features have been proposed as the major factors limiting *P. falciparum* protein expression in conventional *Escherichia coli* cell-based systems [57-59]. Moreover, the presence of glycosylation machinery in eukaryotic cell-based protein expression systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses [60-62]. There are also constraints such as requirement of disulfide bond formation if the target protein requires it for its bioactivity, and requirement of preparation of large quantities of antigen for immunization. The above impediments are completely insurmountable in the case of eukaryotic cell-based expression systems such as yeast, baculovirus or Chinese hamster ovary cell. But, fortunately, we learnt that the wheat germ cell-free system could surmount most of the above stumbling blocks in the path of post-genome malaria vaccine candidate discovery [63].

4. Wheat germ cell-free protein synthesis system

Recently, the wheat germ cell-free protein synthesis system was established for practical use in protein production [64] and is now commercially available as simple protein synthesis kits including *in vitro* transcription system and translation system (CFS Co., Ltd, Matsuyama, Japan). The system is especially powerful when used for the production of eukaryotic proteins because of its eukaryotic nature [65,66]. Basically, two wheat germ cell-free protocols were established for practical use [65]. The first one is for small scale synthesis of proteins. It can be used to produce small quantities of proteins from a large number of cDNAs, in parallel, for testing synthesis and solubility of proteins and also for genome-wide biochemical annotation of gene products [67]. The second protocol is for the production of large quantities of proteins [68]. In this protocol, genes of interest are cloned into the pEU wheat germ expression vectors [69,70] and the mRNA is transcribed. Then, the mRNA is translated either using the bilayer or discontinuous batch translation method [65]. The bilayer method can produce hundreds of micrograms of protein. The discontinuous batch translation method can produce around 150 mg of a control protein in a reaction volume of 50 ml in 5 h and gives room for scale up [68]. These two methods have already been acknowledged as advantageous to yield good quality proteins, in the fields of structural and functional genomics of eukaryotes [66,71]. If we could establish a good manufacturing practice facility for production of recombinant proteins for clinical studies such as vaccine research using this system, then this system may be suitable for mass production of vaccine. The fact that both the wheat germ system and the malaria parasite do not have glycosylation machinery is an advantage for expressing malaria proteins without any inadvertent glycosylation in wheat germ system. Taken together, this system is versatile for both small and large scale production of quality malaria proteins.

We found from our initial studies that the wheat germ cell-free system can surmount most of the above impediments and hence accelerate the discovery of malaria vaccine candidates [63]. Initially, in order to test the suitability of wheat germ cell-free system for high-throughput expression of the parasite genes, we tried to express 124 genes encoding asexual blood-stage parasite proteins, selected from the malaria genome database, PlasmoDB (http://plasmodb.org/plasmo/). Seventy-five percent of genes (93 out of 124 genes) yielded soluble protein products. However, the extent of solubility among these 93 soluble proteins was on an average 65% and ranged from 26 to 100% [63]. The average yield of expressed protein estimated for each full size product was 1.9 µg per 150 µl of reaction mixture, an amount sufficient for preliminary antigen discovery studies using hyper-immune serum. We found that there was significant inverse correlation between yield and molecular size of the protein; the greater the size, the lower the protein yield. Because of this reason, it will be difficult to synthesize the full size of high molecular mass vaccine candidates, such as PfEMP1. There was also an inverse correlation between the protein yield and the abundance of low-complexity regions. In addition, solubility was inversely correlated with the isoelectric point (pI) value [63]. Similar observations have been also documented in *E. coli* cell-based expression system [58,59]. Surprisingly, we did not see any correlation among yield and A/T content, pI value or the existence of a transmembrane domain [63]. From these pilot experiments, we found that the wheat germ system can be used successfully for the expression of *P. falciparum* genes.

Later on, from many of our and our collaborator's proof-of-principle studies, we confirmed that wheat germ cell-free protein synthesis system is advantageous when it comes to synthesizing biologically active malaria proteins by directly using the native A/T rich malaria genes without any codon optimization (summarized in **Table 1**). First, the system was able to produce active malaria enzymes, such as *P. falciparum* dihydrofolate reductase-thymidylate synthase [72] and chitinase of *P. vivax* [73]. Localization of pyruvate kinase type-II isozyme was also identified in apicoplast using antibody raised against recombinant protein produced in this system [74]. In fact, all the above mentioned three genes were recalcitrant to expression in other protein expression systems. Second, the wheat germ cell-free system was able to produce a sufficient amount of good quality proteins (an important prerequisite

Table 1. Proof-of-principle studies for the production of biologically active malaria proteins using the wheat germ cell-free expression system*.

Gene ID‡	Gene name	Species§	Results¶	Ref.
Enzymes				
PFD0830w	Bifunctional dihydrofolate reductase-thymidylate synthase	Pf	Enzyme activities (DHFR, TS)	[72]
PVX_087680	Chitinase	Pv	Enzyme activities	[73]
PF10_0363	Pyruvate kinase 2	Pf	Ab (WB, IFA)	[74]
Sporozoite antigens				
PFC0210c	Circumsporozoite protein	Pf	Ab (WB, IFA)	[63]
PFD0215c	P52	Pf	Ab (IFA)	[75]
Merozoite antigens				
PF11_0344	Apical membrane antigen 1	Pf	Ab (WB, IFA)	[63]
PF14_0495	Rhoptry neck protein 2	Pf	Ab (IP, WB, IFA, IEM)	[76]
PY04764	Erythrocyte binding ligand	Py	Ab (WB, IFA, IEM)	[77]
Ookinete antigen				
PF10_0303	Pfs25	Pf	Ab (WB, IFA, TBA)	[63]

*The individual articles may be consulted for further details.
‡Detailed information is available at the PlasmoDB website (http://plasmodb.org/plasmo/).
§*Plasmodium* species, *Pf*; *P. falciparum*, *Pv*; *P. vivax*, *Py*; *P. yoelii*.
¶Results obtained by the contribution of recombinant proteins synthesized by the wheat germ cell-free system.
Ab: Antibody; DHFR: Dihydrofolate reductase; IEM: Immunoelectron microscopy; IFA: Immunofluorescence microscopy; IP: Immunoprecipitation; TBA: Transmission-blocking activity; TS: Thymidylate synthase; WB: Western blot.

for the malaria vaccine candidate discovery), such as a repeat-rich molecule, circumsporozoite protein [63], a cysteine-rich molecule, P52 (one of the target molecules for the generation of genetically attenuated live-sporozoite vaccine) [75], RON2 (merozoite rhoptry neck protein involved in the erythrocyte invasion process) [76], PyEBL (orthologue of EBA175 in a rodent malaria parasite, *P. yoelii*, that is responsible for the merozoite invasion into erythrocyte) [77] and cysteine-rich Pfs25 (a promising transmission-blocking vaccine candidate) [63]. AMA1 requires synthetic codon optimized gene in order to be expressed in *E. coli* cell-based system and the synthesized proteins also need a series of labor-intensive and technically complex refolding processes, because of its cysteine-richness, to obtain quality proteins [78]. Even this recalcitrant AMA1 could be expressed easily without any codon optimization in the wheat germ cell-free system [63].

Recently, we attempted to express 567 of *P. falciparum* cDNA clones selected from sporozoite, merozoite and gametocyte stages in a high-throughput format by the wheat germ cell-free system. Out of 567 genes, 478 (i.e., around 84%) yielded soluble protein products (unpublished). Our biochemical, immunocytochemical and biological analyses have revealed that the recombinant malaria proteins synthesized by this system are of high quality and, therefore, amenable for vaccine candidate assessment.

5. Expert opinion

In order to establish high-throughput genome-wide functional or immunoscreening methods for malaria vaccine candidate discovery, the choice of the recombinant protein synthesis method is the most crucial factor. There were many leading trials to achieve genome-wide expression of *P. falciparum* genes using the conventional *E. coli*-based protein synthesis system. Aguiar *et al.*, using *E. coli* cells, were able to express only 39 out of 292 malaria genes cloned in GST-fusion vector [57]. Mehlin *et al.* carried out an even more challenging trial in which 1000 genes encoding relatively small (< 450 amino acids) malaria cytosolic proteins were expressed in *E. coli* [58]. In this study, only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9 – 406 mg of protein per liter of culture medium. Vedadi *et al.* used another approach that used an engineered *E. coli* strain with genetically supplemented tRNAs that allows reading of high number of A/U codons in malaria mRNA [59]. They found marginal improvement in protein solubility, that is, 38 (20.9%) out of 182 proteins tested were soluble. Mu *et al.* [55] and Doolan *et al.* [56] attempted to express malaria recombinant proteins using an *E. coli* cell-free expression system in order to find potential vaccine candidates. Mu *et al.* [55] could express around 60% of the malaria genes (65 out of 108 genes) that are considered to be under immune selection pressure. They verified the expression of proteins via western blot using antibodies to the histidine tags incorporated into the C-terminus of the expressed proteins [55]. Recently, Doolan *et al.* reported the construction of the *P. falciparum* protein microarrays using *E. coli* cell-free protein synthesis system for high-throughput immunoscreening for vaccine candidate discovery [56]. In their study, they obtained > 90% efficiency in their effort to express 250

P. falciparum genes using *E. coli* cell-free *in vitro* transcription and translation reactions. And they have attributed their high success rate in this system, at least in part, to the fact that the system is supplemented with rare transfer RNAs to help translate A/T-rich genes and that the proteins are printed directly without purification on to microarray slides. The above experiments showed better productivity and throughput only in the *E. coli* cell-free system and not in *E. coli* cell-based system. Nonetheless, because the results based on the *E. coli* cell-free system study [55,56] were obtained using a relatively small number of *P. falciparum* genes, they do not truly represent the capacity of *E. coli* cell-free system for genome-wide *P. falciparum* gene expression.

It has been suggested that the eukaryotic translation and folding machinery have been optimized through evolution to facilitate co-translational domain folding of a multi-domain protein. In fact, the rate of peptide growth on ribosomes differs considerably between eukaryotes and bacteria, being five to ten times slower in eukaryotes. This slow rate of peptide growth in eukaryotic protein expression system contributes greatly towards correct conformational folding of a multi-domain protein or its epitope and their solubility [79,80]. Although the *E. coli* system is known to support folding of prokaryotic and small eukaryotic proteins, because of its prokaryotic nature, the proteins with multiple domains that are commonly found in eukaryotes such as malaria parasite proteins, when expressed either cell free or cell-based *E. coli* system, tend to fold incorrectly, resulting in the formation of inclusion bodies [79]. After all, the solubility and the functionality of the correctly folded malaria proteins and/or their epitopes are indispensable and critical factors that directly affect the success of our genome-wide search for potential vaccine antigens. Therefore, from this point of view, the eukaryotic-based system is greatly advantageous over the *E. coli*-based system.

Other cell-free systems derived from eukaryotic sources, including rabbit reticulocytes, yeast cells, tumor cells and insect cell lines, have also been widely used to produce eukaryotic multidomain proteins in active forms, but all suffer from low productivity [65]. One of the most convenient and promising eukaryotic cell-free translation systems for achieving high productivity and high solubility of proteins is conceivably the one based on wheat germ embryos [65].

Recently, Goshima *et al.* have again proved that wheat germ cell-free system is more advantageous than *E. coli* cell-free system, *E. coli* cell-based system, and MD2 and CHO cultured cell expression systems. In their study, they have successfully expressed human proteins on a whole-proteome-scale [66]. In their study, initially, on a trial basis, they expressed 26 cDNA clones for soluble protein (molecular mass; MM 10.4 – 101.3 kDa) and 24 clones for membrane protein (MM 7.0 – 80.1 kDa) in various expression systems, such as wheat germ cell-free system, *E. coli* cell-free system, *E. coli* cell-based system, and MD2 and CHO cultured cell expression systems. In this expression study, they found that the wheat germ cell-free system is the best of all the methods for achieving high-throughput, high-quality and high-productivity synthesis of recombinant proteins, and ease of handling. Moreover, of the 75 phosphatases expressed by them in wheat germ cell-free system, 58 (77%) showed biological activity and several cytokines containing disulfide bonds were produced in active forms. They also expressed 13,277 human proteins by this system. The success rate of recombinant protein synthesis was almost 100%. Although the protein-synthesis system exhibits nearly perfect efficacy, the yields of the proteins were variable [66].

In order to discover novel vaccine candidates, the malaria genome has to cross a lot of barriers, such as protein synthesis, selection of protein purification tags, characterization of protein molecules, selection of *in vitro* functional assay systems (i.e., growth inhibition assay or antibody-dependent cellular inhibition assay) and selection of adjuvant. From our experience of expressing around 500 malaria genes using wheat germ cell-free system (unpublished), we learnt that the recombinant malaria proteins synthesized are of high quality and, therefore, amenable for vaccine candidate assessment. Therefore, we indeed believe that this system is a key tool to overcome the first and the foremost barrier, that is, decoding of the malaria genome. From a malaria vaccine perspective, wheat germ system will facilitate production of malaria proteins on a whole-proteome-scale and construction of microarrays of malaria proteins. These microarrays will be an indispensable tool for profiling immune responses developed in non-immune, semi-immune and immune individuals, and identifying immunoreactive antigens that correlate with protection. In fact, we are now focusing on the establishment of microarray of malaria proteins. No doubt, production of malaria proteins on a whole-proteome-scale using wheat germ cell-free protein synthesis system is achievable and microarray of malaria proteins will be soon available for facilitating post-genomic high-throughput identification of novel vaccine candidates.

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Declaration of interest

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Malaria Ookinete Surface Protein-Based Vaccination via the Intranasal Route Completely Blocks Parasite Transmission in both Passive and Active Vaccination Regimens in a Rodent Model of Malaria Infection▽

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Malaria vaccines based on ookinete surface proteins (OSPs) of the malaria parasites block oocyst development in feeding mosquitoes and hence disrupt the parasite life cycle and prevent the disease from being transmitted to other individuals. To investigate whether a noninvasive mucosal vaccination regimen effectively blocks parasite transmission in vivo, *Plasmodium yoelii* Pys25, a homolog of the Pfs25 and Pvs25 OSPs of *Plasmodium falciparum* and *Plasmodium vivax*, respectively, was intranasally (i.n.) administered using a complement-deficient DBA/2 mouse malaria infection model, in which a highly elevated level of oocysts develops in feeding mosquitoes. Vaccinated mice developed a robust antibody response when the vaccine antigen was given together with cholera toxin adjuvant. The induced immune serum was passively transferred to DBA/2 mice 3 days after infection with *P. yoelii* 17XL, and *Anopheles stephensi* mosquitoes were allowed to feed on the infected mice before or after serum transfusion. This passive immunization completely blocked oocyst development; however, immune serum induced by the antigen or adjuvant alone did not have such a profound antiparasite effect. Further, when i.n. vaccinated mice were infected with the parasite and then mosquitoes were allowed to directly feed on the infected mice, complete blockage of transmission was again observed. To our knowledge, this is the first time that mucosal vaccination has been demonstrated to be efficacious for directly preventing parasite transmission from vaccinated animals to mosquitoes, and the results may provide important insight into rational design of nonparenteral vaccines for use against human malaria.

Malaria is one of the most important infectious diseases, and the levels of mortality and morbidity are high, especially among children in developing countries in Africa, Asia, and South America. Implementation of malaria control measures, such as antimalaria drug chemotherapy and insecticide-treated bed nets, has made a significant contribution to reducing the incidence of malaria in many parts of the world. However, these control measures may not be sufficient, and therefore new tools, including vaccines, should be included in a new malaria control campaign for local elimination and final eradiation of malaria from the globe (7). A promising strategy to counteract global malaria endemicity is to develop highly efficacious vaccines, and several promising candidates have been intensively investigated (7, 20); vaccines targeting asexual stages (i.e., sporozoite, hepatic, and erythrocytic stages) are designed to prevent infection and reduce disease severity, while vaccines that target the sexual stage, in which the parasite undergoes sporogonic development in anopheline mosquitoes, prevent vector-mediated transmission of the parasite from person to person (4, 8, 14, 17, 25). Although transmission-blocking vaccines do not directly prevent infection, they reduce parasite infectivity for the vector and consequently lower the mosquito infection rate and the frequency of transmission to humans. In addition, this strategy is believed to be particularly useful for controlling escape of mutants from vaccines designed based on antigens expressed at an asexual stage; therefore, transmission-blocking vaccines are increasingly being considered indispensable components of malaria vaccine strategies and are key components of malaria elimination (10, 11).

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Studies on rodent and human malaria concluded that an effector mechanism that is pivotal for blocking transmission is induction of antigen-specific serum antibodies in a vaccinated host, from which female mosquitoes, when they bite to obtain blood meal, coingest gametocyte pairs together with the induced antibodies (2, 3, 5, 9, 18, 24). The ingested antibodies seem to be stable in the mosquito midgut, at least in the time frame within which the transmitted gametocytes develop into ookinetes.

Parasite antigens expressed later at the postfertilization stage in the mosquito midgut, such as ookinete surface proteins (OSPs), including Pfs25 and Pvs25 from *Plasmodium falciparum* and *Plasmodium vivax*, respectively, are particularly important vaccine targets because they are likely to be concealed immunologically, if not concealed completely, from the mammalian host's immunosurveillance system, which suggests that there is a reduced driving force to produce the antigenic variations often observed for antigens expressed at prefertilization stages (6, 12, 23, 28). In addition, several recent studies indicated that in the malaria life cycle the ookinete-to-oocyst transition stage is one of the most vulnerable stages of parasite development, making the postfertilization stage of sporogonic development an ideal target for antitransmission vaccines.

The vast majority of pathogens invade through mucosal tissues and therefore can be controlled effectively by mucosal vaccines rather than parenteral vaccines. Notwithstanding the great merit of mucosal vaccines, most vaccines in use today are delivered parenterally (subcutaneously [s.c.] or intramuscularly). In spite of many arguments against the concept that vaccines against arthropod vector-borne human pathogens, such as malaria parasites, could be designed based on mucosal delivery, recent studies performed by us and other workers demonstrated that mucosal vaccines could be efficacious for prevention of arthropod-transmitted infections, because mucosal administration of foreign antigens mixed with a potent mucosal adjuvant, such as cholera toxin (CT), can induce strong systemic immunity (2, 3, 13). Here we extended our previous studies to test our hypothesis that the malaria OSPs are sufficiently immunogenic when they are administered by the intranasal (i.n.) route in the presence of a mucosal adjuvant, which should in theory effectively block parasite transmission to feeding mosquitoes when both passive and active vaccination regimens are used.

MATERIALS AND METHODS

Mice, vaccination, and antibody enzyme-linked immunosorbent assay (ELISA). Seven-week-old female DBA/2NCrj (DBA/2) mice were purchased from Japan SLC (Tokyo, Japan). Complement C5-deficient DBA/2 mice were used for live mosquito-feeding experiments, because the highly elevated levels of oocysts that developed in the mosquito midgut were useful for evaluation of transmission-blocking vaccine efficacy (26).

Mice were i.n. vaccinated once a week for 4 weeks with 25 μg of yeast-derived recombinant *Plasmodium yoelii* Pys25 synthesized and purified like Pvs25 as described previously (14) in the absence or presence of 1 μg of CT (Sigma-Aldrich). As a control, a group of mice were vaccinated with 1 μg of CT alone. For passive vaccination experiments, DBA/2 mice were intravenously vaccinated with 0.5 ml of pooled immune sera derived from mice vaccinated i.n. with Pys25 plus CT, with Pys25 alone, or with CT alone.

For ELISA of vaccine-induced immune sera, a flat-bottom 96-well microtiter plate (Immulon 4; Dynex Technology Inc., Chantilly, VA) was coated with recombinant Pys25 (0.5 μg/well in bicarbonate buffer, pH 9.6) and blocked with 1% skim milk in Tris-buffered saline containing 0.05% Tween 20. Immune sera serially diluted with the blocking buffer were applied to wells in duplicate (100 μl/well) and incubated for 2 h at 37°C, which was followed by addition of alkaline phosphatase-conjugated anti-mouse antibody for immunoglobulin (Ig) isotype and IgG subclass analysis. The alkaline phosphatase substrate (*p*-nitrophenyl phosphate [Sigma-Aldrich]) was added, and the absorbance at 490 nm was determined with a microplate reader (Bio-Rad Laboratories). The antibody concentration was determined based on known amounts of mouse Igs used as a standard. The statistical significance of differences in antibody concentration or absorbance was determined by Student's *t* test.

Parasite infection, blood feeding experiment, and assay of transmission blocking. For analysis of the parasite-killing effect of i.n. vaccination-induced immune sera, mice were intraperitoneally inoculated with 10^6 peripheral red blood cells that had been infected with *P. yoelii* strain 17XL, and the infected mice were maintained for 3 days until the level of parasitemia reached 9 to 10%, which was determined by microscopic examination of Giemsa-stained thin blood smear preparations. Then approximately 100 *Anopheles stephensi* mosquitoes that had been starved overnight were allowed to obtain a blood meal from the infected mice either before or 1 h after intravenous injection of immune sera that had been prepared from mice 1 week after the last i.n. vaccination with Pys25 plus CT, with Pys25 alone, or with CT alone. Fully engorged mosquitoes were maintained at 24°C for 1 week by giving them water containing 1.5% fructose and 1.5% sucrose. For each experimental group, mosquitoes were dissected, and their midguts were examined with a light microscope to count the number of oocysts.

For analysis of the direct parasite transmission-blocking efficacy of i.n. vaccination, mice vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone were infected as described above with the parasite 1 week after the last vaccination, and then mosquitoes were allowed to feed directly on the infected animals; this was followed by enumeration of the oocysts that developed.

The statistical significance of differences in the numbers of oocysts was determined by the Kruskal-Wallis test or the Wilcoxon-Mann-Whitney U test by using the software JMP (SAS Institute Inc.).

RESULTS

A significant level of specific serum IgG and IgM antibodies (mainly IgG) was induced in DBA/2 mice by i.n. vaccination with Pys25 plus CT (14,147 ± 4,241 μg/ml) but not by i.n. vaccination with Pys25 alone or CT alone (Fig. 1a, upper panel). Oral inoculation of 50 μg of Pys25, however, did not induce an antibody response even in the presence of 10 μg CT (data not shown). IgG1 was found to be the predominant serum IgG subclass, and almost no IgG2a was detected in mice vaccinated with Pys25 plus CT, an indication of the Th2 type of immune response induction (Fig. 1a, lower panel). Low but detectable levels of Pys25-specific serum IgA and IgE were seen in the group vaccinated with Pys25 plus CT but not in the group vaccinated with Pys25 alone or CT alone (Fig. 1b). Similar humoral immune responses were observed when outbred ddy mice were used for the immunization experiments (data not shown).

To evaluate the parasite transmission-blocking effect of the induced immune sera in vivo, *A. stephensi* mosquitoes were allowed to obtain a blood meal from DBA/2 mice that had been infected with *P. yoelii* 17XL before or after passive transfer of the immune sera of mice vaccinated i.n. with Pys25 plus CT, with Pys25 alone, or with CT alone as described in Materials and Methods. For all three immunization groups, large numbers of oocysts were observed in the mosquito midgut when the mosquitoes were allowed to feed before the immune sera were transferred (median for CT alone, 346 oocysts; median for Pys25 alone, 302 oocysts; median for Pys25 plus CT, 311 oocysts) (Fig. 2a). In contrast, when the mosquitoes received the blood meal after the immune sera were transferred, oocyst formation was completely blocked in the group vaccinated with Pys25 plus CT, but not in the group vaccinated with

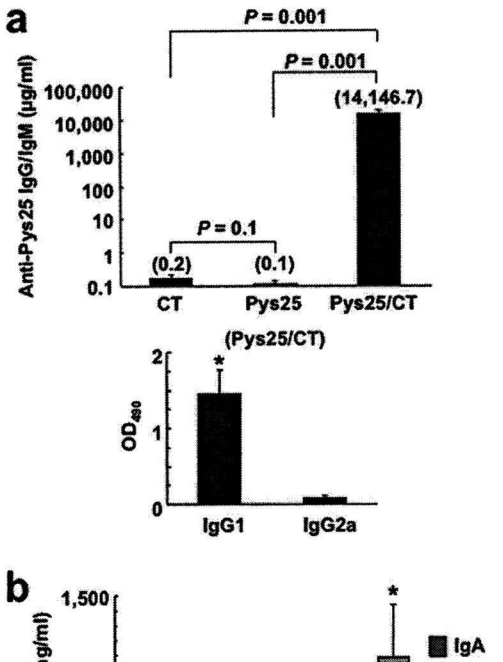

FIG. 1. Antibody responses in mice i.n. vaccinated with recombinant Pys25. DBA/2 mice (three or four mice/group) were vaccinated i.n. with a mixture of Pys25 (25 μg) and CT (1 μg), with Pys25 alone (25 μg), or with CT alone (1 μg) once a week for 4 weeks, and 1 week after the final vaccination serum antibody titers were analyzed by ELISA. (a) (Upper panel) Pys25-specific serum IgG and IgM (predominantly IgG) levels expressed as mean antibody concentrations in the serum. The error bars indicate standard deviations. (Lower panel) Immune sera (1/128,000) from mice vaccinated with Pys25 plus CT were also analyzed for the presence of IgG1 and IgG2a, and the results were expressed as mean optical densities at 490 nm (OD_{490}). The error bars indicate standard deviations. *, $P = 0.002$ for comparison of IgG1 and IgG2a. (b) Pys25-specific serum IgA and IgE levels expressed as mean antibody concentrations in serum (ng/ml). The error bars indicate standard deviations. *, $P < 0.01$ for a comparison of Pys25 plus CT and CT or Pys25. No significant differences between groups were observed for serum IgA. Statistical significance was determined by Student's *t* test.

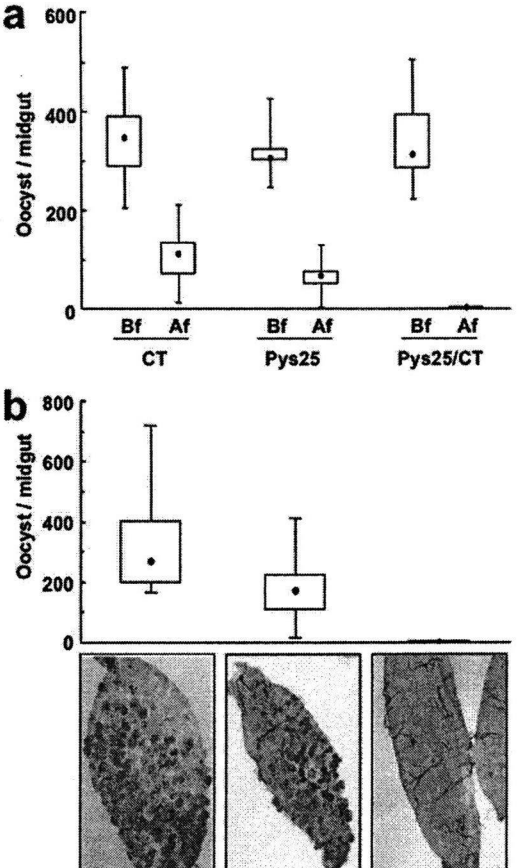

FIG. 2. Transmission-blocking vaccine efficacy. (a) Transmission-blocking effects of passively transferred immune sera on *P. yoelii* oocyst development in the *A. stephensi* mosquito midgut. Approximately 100 mosquitoes were allowed to feed on parasite-infected mice (9 to 10% parasitemia at the time of feeding) before (Bf) or after (Af) intravenous injection of immune sera derived from mice i.n. vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone. Data are expressed as the median numbers of oocysts per mosquito (dots in boxes), quartiles (boxes), and ranges (lines above and below boxes). Statistically significant differences were found in all three immunization groups compared for the treatment after intravenous injection of immune sera ($P < 0.0001$) but not for the treatment before intravenous injection of immune sera ($P = 0.726$) in an analysis performed using the Kruskal-Wallis test. There are also statistically significant differences between the treatment before intravenous injection of immune sera and the treatment after intravenous injection of immune sera for all three immunization groups ($P < 0.0001$, Wilcoxon-Mann-Whitney U test). (b) Transmission-blocking effects of active immunization on oocyst development. Mice were i.n. vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone and then were infected with the parasite. Mosquitoes were allowed to directly feed on infected mice, the numbers of oocysts were determined, and the data were expressed as described above for panel a. Statistically significant differences were found in all three groups compared by using the Kruskal-Wallis test ($P < 0.0001$). Light microscopic images of representative mosquito midguts from the active vaccination experiments are shown at the bottom.

CT alone (median, 109 oocysts) or with Pys25 alone (median, 65 oocysts). Although we do not know why the CT or Pys25 immune serum had a significant parasite-killing effect (for CT, 346 oocysts versus 109 oocysts; for Pys25, 302 oocysts versus 65 oocysts), no mosquitoes completely lacked oocysts when they were given CT or Pys25 immune serum (Table 1). The results demonstrated that i.n. vaccination with Pys25 plus CT induced antibodies which confer complete transmission-blocking immunity when a passive vaccination regimen is used.

Next, to evaluate the direct mucosal vaccine efficacy of Pys25, mosquitoes were allowed to obtain a blood meal directly from parasite-infected mice that had been vaccinated as described in Materials and Methods. The results demonstrated that vaccination with Pys25 plus CT completely blocked oocyst development, as we observed in the passive vaccination experiment, while significant numbers of oocysts were observed in mosquitoes that fed on CT-vaccinated mice (median, 269 oo-

TABLE 1. Prevalence of oocyst infection in *A. stephensi* mosquitoes

Type of immunization	Vaccine	No. of oocyst-positive mosquitoes/ total no. examined (% infection)[a]	No. of oocyst-positive mosquitoes/total no. examined (% infection)[a]	
			Before immune serum transfer[b]	After immune serum transfer[b]
Passive	CT		31/31 (100)	40/40 (100)
	Pys25		71/71 (100)	39/39 (100)
	Pys25 + CT		51/51 (100)	0/56 (0)
Active	CT	150/150 (100)		
	Pys25	90/90 (100)		
	Pys25 + CT	0/84 (0)		

[a] The percentages are the percentages of oocyst-positive mosquitoes based on the total numbers of mosquitoes examined.

[b] Approximately 100 mosquitoes were allowed to feed on parasite-infected mice (9 to 10% parasitemia at the time of feeding) before or after intravenous transfer of immune sera obtained from mice i.n. vaccinated with Psy25 plus CT, with Psy25 alone, or with CT alone.

cysts) or Pys25-vaccinated mice (median, 170.5 oocysts) (Fig. 2b). Vaccination with Pys25 alone had a weak but significant transmission-blocking effect compared with the effect observed for CT-vaccinated mice, suggesting that i.n. vaccination with the recombinant antigen alone might have some efficacy, even though the antibody levels for these two groups were not significantly different (Fig. 1). The oocyst prevalence was 100% for all vaccination regimens that we tested except both the passive and active Pys25-plus-CT regimens, for which the oocyst prevalence was 0% (Table 1). On the basis of our results, we concluded that i.n. vaccination with the malaria OSP was very efficacious when this OSP was combined with a mucosal adjuvant to block parasite transmission to mosquitoes.

DISCUSSION

Although most vaccines in use today are administered s.c. or intramuscularly, the advantages of mucosal vaccines are indisputable; they result in local immunity as well as systemic immunity, which, in general, is hard for parenteral vaccines to induce, and they provide a first line of defense against many infections that occur at or emanate from mucosal surfaces. They could prevent transmission of blood-borne pathogens by reuse of syringes; they may be safer and more cost-effective and thus have advantages for developing countries; and they are painless and therefore likely to be readily tolerated by small children and individuals with needle phobia (16). Although not all of the advantages attributed to mucosal vaccines mentioned above are directly relevant to the design of vaccines against malaria, and although there are some intrinsic technical difficulties that cannot be circumvented by development of effective mucosal vaccines (7), evaluation of the concept of designing mucosal vaccines for nonmucosal pathogens seems to be worthwhile.

To investigate malaria parasite OSP-based mucosal vaccines, we previously demonstrated that for two types of human malaria (*P. falciparum* malaria and *P. vivax* malaria) experimentally induced mouse immune sera specific for Pfs25 and Pvs25 (homologues of rodent Pys25) were very effective in blocking parasite transmission from patients' parasitized blood to mosquitoes in a membrane feeding assay (2, 3). In the present study we demonstrated that in a rodent malaria infection model, OSPs were immunogenic when they were administered i.n. (with levels of antigen-specific Igs reaching 15 mg/ml), and the induced immune serum was very effective in blocking parasite transmission. Most importantly, however, we demonstrated that vaccination directly prevented the transmission of a parasite from vaccinated animals to feeding mosquitoes. To our knowledge, this is the first demonstration that mucosal vaccination with malaria OSPs can directly prevent malaria transmission to mosquitoes in vivo. In the DBA/2 strain of mice lacking a component of the complement system, the number of oocysts formed is significantly increased in feeding mosquitoes (26); however, anopheline mosquitoes collected in field are usually not as heavily infected, and a single oocyst is commonly detected. Therefore, a more moderate antibody level may confer effective transmission-blocking immunity in humans (21).

Malaria vaccines targeting hepatic and erythrocytic stages suffer from antigenic variations mainly due to selection pressure from the host immune system. However, antigens expressed at a parasite sexual stage, such as OSPs, are immunologically concealed from the host immune system, and hence the chance that antigenic variations occur may be low. Indeed, OSPs of *P. falciparum* and *P. vivax* were shown to have minimal antigenic variations even in field isolates collected from remote regions of the world (19, 27). This is an important characteristic of ideal vaccines. On the one hand, sexual-stage antigens have disadvantages such as (i) the absence of an infection-induced booster effect and the resulting long-term immunity and (ii) the absence of direct protection of vaccinees from infection. Therefore, it is believed that a vaccine candidate should have multiple components and that at least one component should be a sexual-stage antigen (7). In such a vaccine formulation, preerythrocytic and/or erythrocytic antigens may function cooperatively with sexual-stage antigens for prevention of or reduction of infection and parasite transmission.

Mucosal administration, such as i.n. or oral administration, unlike parenteral immunization, of nonreplicating inert antigens with CT tends to induce Th2-type immunity, which is characterized by predominant induction of serum IgG1, induction of local secretory IgA, and in some cases induction of serum IgE in mouse models. Unlike what happens in other infectious diseases, which require induction of cell-mediated immunity (22), serum antibody, regardless of the Ig isotype, seems to be the predominant, if not only, protective arm of immunity that blocks malaria transmission. We do not know the mechanism of action of Pys25-specific antibodies in blocking parasite development in the midgut of a feeding mosquito, but binding of antibodies to the zygote surface and subsequent prevention of parasite development into the ookinete may be the most important blocking mechanism (14, 25). This antibody binding may occur within the midgut, and this may be independent of Ig isotypes. Thus, although IgE antibody is not the major antibody isotype present in a vaccinated host, it may contribute to blocking transmission. However, the induction of serum IgE may potentially lead to an allergic response in vaccinated individuals, and the data shown in Fig. 1b are relevant to this issue. Another important issue that needs to be

considered is the duration of protective antibodies. Recent findings relevant to the present work indicated that when *P. vivax* transmission-blocking vaccine candidate Pvs25 was injected s.c. with incomplete Freund's adjuvant into BALB/c mice, it induced a strong antigen-specific serum IgG response that was maintained for more than 6 months (our unpublished data). i.n. vaccination with Pvs25 plus CT induced a level of serum IgG comparable to that induced by s.c. vaccination formulated with incomplete Freund's adjuvant, but the level gradually decreased over 6 months. However, we found that i.n. vaccination with Pvs25 plus CT was generally more potent based on the magnitude and duration of the specific serum IgG response than s.c. vaccination with Pvs25 formulated with aluminum hydroxide (unpublished data).

In this study we used CT as a mucosal adjuvant; however, the use of CT for humans is hampered by the toxicity of this compound. Also, as mentioned above, issues related to the potential allergic response and the duration of antibodies need special consideration. Fortunately, however, nontoxic and thus safer adjuvants, but adjuvants that are as effective as CT, are being developed, making a mucosal malaria vaccine a feasible goal (1, 15). For example, we recently found that when a nontoxic subunit of CT, CTB, was fused to malaria OSP, it was efficacious by both the mucosal and s.c. routes for blocking parasite transmission (unpublished data). Thus, if the mucosal transmission-blocking vaccine efficacy data obtained with this rodent infection model can be reproduced in human clinical trials with guaranteed safety, OSP antigens formulated as noninvasive vaccines may become a powerful tool for use against human malaria.

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Practical cell-free protein synthesis system using purified wheat embryos

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Biochemical characterization of each gene product encoded in the genome is essential to understand how cells are regulated. The bottleneck has been and still is in how the gene products can be obtained. The wheat cell-free protein synthesis system we have developed is a powerful method for preparation of many different proteins at a time and also for preparation of large amounts of specific proteins for biochemical and structural analyses. Here, we show a method for preparation of the wheat embryo extract useful for the cell-free reactions, by which 5 ml of a high-activity extract is obtained in 4–5 d. We also describe the methods for small- and large-scale protein synthesis by hands-down operations with the use of mRNAs prepared by transcription of PCR products and pEU plasmids harboring the target cDNAs, which need 2–4 d excepting the time required for plasmid preparation.

INTRODUCTION

With much information on the genome and cDNA sequences of many different organisms, we can now obtain and deduce a lot of information about the gene products and their interactions with the aid of bioinformatics, genetics and cell biology. However, such pieces of information are in many cases a result of presumption, or an extrapolation from known facts. To understand specific biochemical phenomena at the molecular level, *in vitro* analyses of biochemically characterized samples are strictly required. Conventionally, biochemists had to purify their samples, i.e., proteins and other biomolecules, from living organisms. The recombinant expression technologies that emerged in the late twentieth century helped them greatly. However, although there is a lot of information and a lot of potential targets to be analyzed, the preexisting technologies do not meet the need to prepare sufficient sized samples of many different proteins.

Cell-free protein synthesis was developed in the middle of the last century. It entered the limelight as a method for the preparation of proteins when the continuous-flow cell-free method was developed by Spirin *et al.*[1] in 1988, in which fresh substrates were supplied into and byproducts were removed from the reaction chamber continuously during the reaction with the extract from *E. coli* or wheat embryos. Our group found that the instability that had been observed for the translation with wheat extracts prepared by conventional methods arose from intrinsic factors that catalytically inhibited translation[2], and developed a method to eliminate the catalysts. Here, we describe the method for preparation of the extract as well as the basic techniques for parallel preparation of many different proteins for functional analyses and those for a large-scale preparation that may be applicable to structural analyses and antigen preparation. These technologies are based essentially on the following three elemental technologies: a method for eliminating the contaminants from the endosperm in the wheat embryo extract, which keeps up the inherent robustness of the natural translation apparatus[2], the PCR-based high-throughput method for preparation of DNA templates (the 'split-primer PCR' method)[3] and a reaction format that fit to highly parallel operation (the 'bilayer' method)[4]. A more detailed history of the development of the wheat cell-free system has been reviewed elsewhere[5,6]. The most prominent advantage of the wheat cell-free method, as recognized by our group through the collaborations with many other groups, is the high quality of the produced proteins, particularly when cytosolic proteins from eukaryotic origins are produced[7] (see below in the 'Applications of the method' section).

The cell-free protein synthesis system from *E. coli* is also capable of both highly parallel protein production and mass production. The wheat system has an advantage over the *E. coli* system in the probability of producing human proteins in soluble forms as clearly demonstrated[7]. This is probably due to the eukaryotic nature of the wheat system. In addition, machines for production of proteins are commercially available for the wheat system, as below. On the other hand, the productivity per reaction time may be higher in the *E. coli* system than in the wheat system. In addition, the method for preparation of the cell extract at the laboratory level is less laborious in the *E. coli* system. As a result, the cost of the extract is lower in the *E. coli* system. Thus, if one wishes to produce polypeptides in insoluble forms, the *E. coli* system has a clear advantage both in the cost and the productivity. The *E. coli* system also has an advantage in the production of bacterial soluble proteins that can fold properly in bacterial cells, as the cost of the extract is lower. Although we do not have clear statistical data, it seems that the codon usage bias in the open reading frame (ORF) sequence to be translated affects the productivity much less severely in the wheat system[8].

The wheat cell-free protein synthesis system has been commercialized by CellFree Sciences (CFS). The products of CFS, including the wheat embryo extract (cat. nos. CFS-TRI-1240/1240H/1240G), have highly controlled qualities, and they come with detailed protocols when purchased. Thus, we start with a method for preparation of the extract, for those who are hesitant to purchase the extract. As the extract is stable at −80 °C for years, it is less convenient to prepare small amounts of the extract at a time. In addition, we have already described the small-scale method several times elsewhere[9,10]. Thus, we show here a large-scale method[11]. The extract prepared by this protocol fits to the CFS protocols. We then describe the 'bilayer' methods for small-scale high-throughput parallel protein synthesis in a microtiter plate and those for larger scale preparation, which are essentially the same as that in the CFS protocols and are most convenient at present. A diagram showing the procedure is in **Figure 1**.

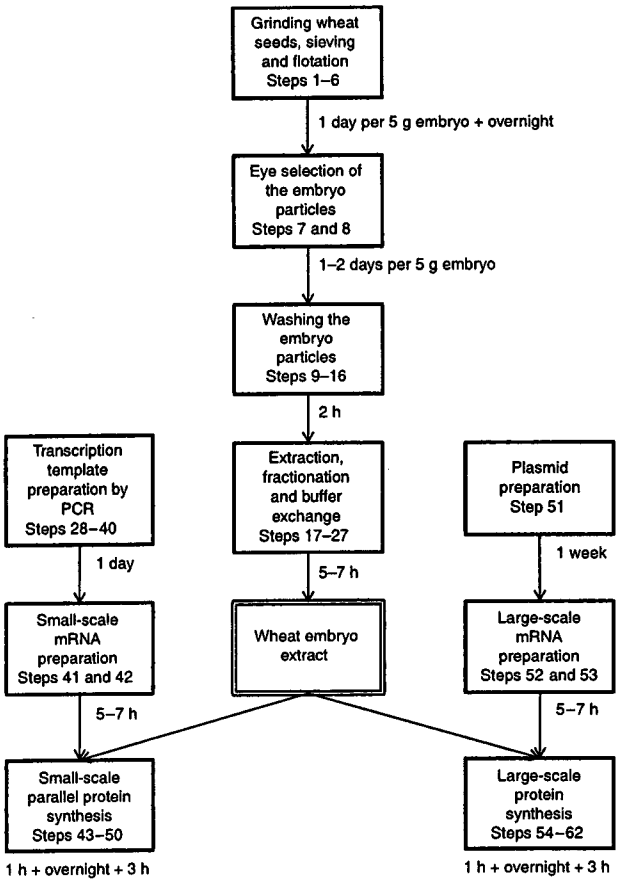

Figure 1 | Summary of steps involved in the procedure.

Experimental design

Preparation of unwashed embryo particles. The primary (unwashed) embryo particles are prepared by crushing wheat seeds with a mill followed by sieving and selection. Typically, 1 kg of wheat seeds gives around 1 g of crude embryo particles.

The most important step in the preparation of a good extract is to obtain an ensemble of good embryo particles that attach minimal amounts of endosperm to be washed out in the next step. This is possible at present only by selecting the particles apparent to human eye. This eye selection is the toughest step for laboratory workers. Thus, we use conventional methods to select the particles crudely by flotation before selection by eye. However, this raises the problem of organic-solvent waste containing carbon tetrachloride. Therefore, we now use the extract supplied by CFS for ordinary experiments. Nevertheless we describe here the method including the flotation selection step, expecting that the researchers who can use carbon tetrachloride in the laboratory may be able to perform the experiments themselves. The flotation steps may be omitted if more time and effort can be invested in the eye selection. We have not tested if other liquids could be used for the purpose, such as pure methylene chloride that has a density close to the one used in the present procedure and high-density aqueous solutions of polymers and/or salts.

Preparation of the extract. The embryo particles should be washed extensively before being crushed. This will eliminate translation inhibitors that come from endosperm. Conventionally, we crushed up to several grams of the washed embryo particles with mortar and pestle under liquid nitrogen in a cold room[2,9,10]. Here, we describe a patented method using a food processing mill/mixer, which has facilitated a larger-scale preparation of the extract with higher activities and has eliminated the risk of choking in the cold room associated with the use of liquid nitrogen[11]. While we used a popular food processor, a conventional Waring Blender can also be used. Although we usually start with 60 g of unwashed embryo particles, the amount of the starting material can be reduced down to several grams. The smallest amount that could be crushed properly is dependent on the size of the blender cup. A machine that can grind green tea may be useful for smaller-scale experiments, although we have not tested any. Once a large amount of the extract is prepared, it can be stored at −80 °C for at least a year.

Conventionally, we prepared the extract in the extraction buffer (EB, see below). However, we found that the buffered substrate mixture as below (BSS) is useful and more convenient than the EB, because preparation of the translation reaction mixture can be simpler, which is important for high-throughput applications.

The translational activator sequences. The mRNA molecules to be translated in the wheat cell-free system should have a translational enhancer sequence in the 5′-untranslated region (UTR)[3,12]. We have observed that the introduction of a cap structure at the 5′ end of mRNA molecules by a standard transcription method does not work well unless the mRNA concentration to be used for translation is optimized for each preparation. The 3′-UTR of the mRNA molecules should be at least as long as 500 nt, while no sequence preferences have been observed. Thus, 3′-UTR can contain the sequence from the vector in which the ORF to be translated is subcloned.

Template preparation by PCR. We describe a method for preparation of transcription-ready PCR fragments with a 5′-enhancer sequence and a long 3′ sequence[3], by which many different cDNA clones could be amplified in parallel. The PCR method comprises two steps. In the first PCR, a 5′ gene-specific primer and the AODA2306 primer (see **Table 1** and **Fig. 2a,b**) are used. The gene-specific primer should be designed for each gene to be expressed so that it hybridizes with the first 20 bases of the ORF to be amplified as in **Table 1**. It should introduce a short leader sequence. The

TABLE 1 | Oligodeoxyribonucleotides.

Name	Sequence	Convenient stock concentration (μM)
AODA2303	5′-GTCAGACCCCGTAGAAAAGA-3′	1
AODA2306	5′-AGCGTCAGACCCCGTAGAAA-3′	0.1
deSP6E01	5′-GGTGACACTATAGAACTCACCTAT CTCCCCAACACCTAATAACATTCAAT CACTCTTTCCACTAACCACCTATCTAC ATCACCACCCACCACCACCAATG-3′	0.1
SPu	5′-GCGTAGCATTTAGGTGACACT-3′	1
Gene-specific primer	5′-CCACCCACCACCACCAATGNNN NNNNNNNNNNNNN-3′	0.1

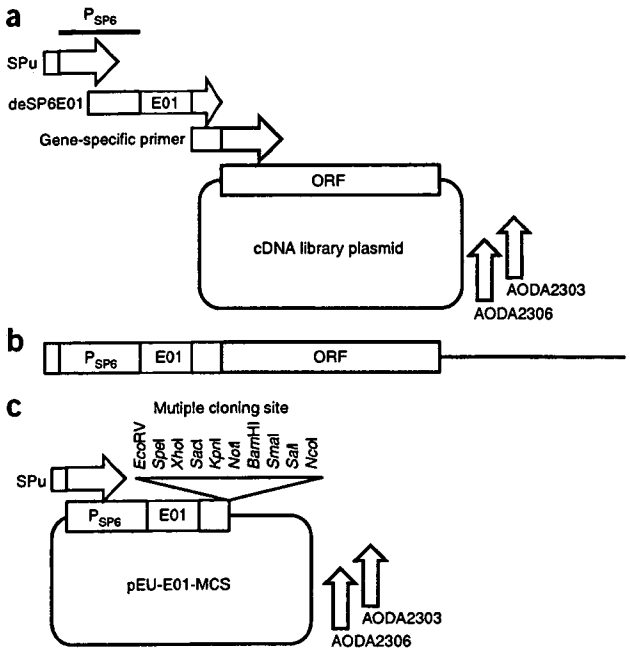

Figure 2 | Schematic representations of DNA molecules. (a) Positions of the primers used for the two PCR amplifications are indicated with a schematic representation of a typical cDNA library plasmid clone. The two downstream primers hybridize near the plasmid origin, and thus the library vector should have the same sequence, and the ORF of the library clone should be inserted in this direction. The gene-specific primer and AODA2306 are used for the first PCR, and the other three primers are used for the second PCR. (b) An illustration of the transcription-ready template DNA that should be generated after the second PCR. (c) An illustration of pEU-E01-MCS, which is provided in the CFS kits and is useful for cloning of the cDNA sequence to be expressed in the wheat cell-free translation system.

AODA2306 primer is designed to hybridize within the replication origin region of the pUC plasmids, which is present in many cDNA library vectors. In the second PCR, AODA2303, deSP6E01 and SPu are used as primers. AODA2303 hybridizes three bases closer to the ORF than the position for AODA2306. deSP6E01 is a long oligonucleotide containing a partial SP6 promoter sequence lacking the 5′ five bases, the E01 sequence, and the leader sequence that is also in the 5′ region of the gene-specific primer. This primer is used in a lower concentration. The E01 5′ enhancer sequence used here can be substituted with the E02 sequence[12] or by the Ω sequence from tobacco mosaic virus. SPu contains the 5′ 14 bases of the SP6 promoter sequence in the 3′ part (the 3′ 9 bases overlaps with the 5′ region of deSP6E01). It is important to split the promoter sequence so that no primer has a complete promoter sequence because this dramatically reduces the possibility of generating nonspecific amplification of transcribable sequences. This 'split-primer' method is not required if the cDNA clones are inserted into pEU as described below.

Small-scale parallel protein synthesis. The PCR products can be transcribed into mRNA by a simple enzymatic reaction. The transcription products can be transferred directly into the translation mixtures. The bilayer method described here is suitable for parallel translation of many different mRNA samples, as it can be performed in microtiter plate wells and is much more efficient than a simple batch reaction. The reaction mixture containing the extract is slightly heavier than the substrate solution, and these two solutions can form a bilayer. Translation starts within a small space with concentrated initiation factors and ribosomes, forming polysomes. As the reaction proceeds, the byproducts are gradually diluted into the upper substrate solution, and the fresh substrates gradually diffuse into the reaction site. Thus, it is very important not to mix the two layers. Robots performing this procedure by parallel operations are available from CFS.

Conventionally, we adjusted the concentrations of the ingredients in the starting reaction mixture that should be layered under the substrate mixture to those of the components in the BSS substrate mixture. However, we have found that this is not necessary, probably because low-molecular-weight compounds will diffuse rapidly into the reaction mixture from the substrate mixture. In addition, it has been found to be unnecessary to remove the white insoluble material generated during the transcription reaction. The protocol shown here is thus quite simple: just mixing three solutions, including the extract, the transcription product and the creatine kinase solution. This simplicity has made this protocol more useful for high-throughput parallel production of many different proteins.

The concentrations of magnesium and potassium ions can affect the translation efficiency, whereas the transcription buffer contains a higher concentration of magnesium ions and no potassium ions, which may cause inefficient translation. However, we have observed no problem in the efficiency of translation. This may be because small ions can exchange rapidly between the reaction and substrate mixtures. It is also possible that the magnesium pyrophosphate precipitate may be serving as a buffer of magnesium concentration.

The pEU expression vector. The pEU vector contains an SP6 promoter, a translational enhancer and a multiple cloning site (**Fig. 2c**). This vector is suitable for large-scale expression of the subcloned ORF in the wheat cell-free system. Control pEU plasmids, such as that harboring the GFP cDNA, are available on request. Various pEU plasmids with inserted tags are also available. We are also ready to distribute a Gateway destination vector of pEU on request: various entry vectors for the human cDNA clones are also available from the National Institute of Technology and Evaluation, Japan[7].

Large-scale protein synthesis. We describe here the method for the bilayer mode large-scale protein synthesis with an mRNA solution prepared by direct transcription of the pEU plasmid template harboring the ORF sequence to be translated. For large-scale synthesis, more productive methods are available (see below). However, the present method is the simplest to be performed and thus fits with automation. The transcription template can also be prepared by PCR amplification of the plasmid sequence with the SPu and AODA2303 primers. We recommend the direct transcription method here just because the method is simpler than that including a PCR step.

Control reactions that should be added. pEU-E01-DHFR, which is available from CFS and from our laboratory, can be used for a positive control reaction for large-scale protein synthesis. For the small-scale experiment, an aliquot from the large-scale transcription product from pEU-E01-DHFR can be used. When only one sample is tested, the sample with no mRNA (water should be

added instead of the mRNA solution) may be used as a negative control, which will help to identify the band of the synthesized protein on the electrophoresis gel among the other bands arising from the components of the extract. If plural different mRNA samples are translated, we usually perform no negative control reaction because each band can be identified by comparison with the other lanes.

Applications of the method

Examples of expressed proteins. We have already tested many different proteins for the productivity in the wheat cell-free system. An encompassing list of successful proteins would be so large that we could not show here. Lists of the successful examples at the time point of 2006 are elsewhere[5]. In such examples, the details of the methods for translation were different from the exact one presented here: as far as we know, success in production of a protein does not depend on the details and the reaction modes described below. Recently, 13,361 human cDNA clones were tested if they were translated in the wheat cell-free system by a bilayer protocol. It was found that 12,996 produced a detectable amount of the polypeptide and that 12,682 were detectable in the soluble fraction, of which 3,040 contain at least one predicted transmembrane domain[7]. Therefore, we believe that most cDNA sequences can be translated into the polypeptide sequences, unless the polypeptide interferes severely with the translation machinery. We have observed that many of the eukaryotic protein kinases can be produced in the active forms in the wheat translation system, while this group of proteins is recognized to be generally difficult to be produced in bacterial systems[5]. In particular, human calcium/calmodulin-dependent protein kinase II delta produced by this method readily phosphorylated novel natural substrates within a HeLa cell extract[13]. We have also produced plant RNA ligase, production of which has been reported to be difficult using bacterial expression systems[14]. It has been suggested that the wheat system has an advantage over bacterial systems in proper folding of multidomain proteins from eukaryotic origins, in particular those with a domain with a high β-strand contents[15]. This may be a result of coevolution of protein sequences and the protein synthesis apparatus, which might have been the origin of various protein functions in eukaryotic cells that confer the dazzling complexity of the eukaryotic organisms. On the other hand, some prokaryote-specific proteins might be produced in an inactive form in the wheat system: we have observed that a bacterial protein with a deep trefoil knot structure is not produced in its fully active form[16]. cDNAs with highly biased codon usages are also difficult to be expressed in bacterial systems. We have already expressed many *Plasmodium* cDNAs that have very high A/T contents successfully[8]. Proteases are generally difficult to be produced *in vitro* because of their activity. However, the wheat cell-free system sustains the translation activity even at 4 °C, and we have already confirmed that some proteases can be produced successfully (our unpublished data). Formation of proper quaternary structures has been observed for several proteins. The crystalline particles of polyhedrin were observed when its mRNA was translated[17]. A heterodimer enzyme was found to fold properly only when synthesized simultaneously in a reaction mixture containing the mRNA molecules for both subunits[18], whereas another heterodimer enzyme had its activity even when each subunit was synthesized separately and mixed with each other after purification[19]. There are only a limited number of examples of disulfide containing proteins and membrane proteins that were expressed efficiently as below. It is very difficult to introduce sugar modifications onto proteins, in part because the endoplasmic reticulum is absent from the extract and, in part, because the extract contains enzymes that degrade sugars.

The PCR method, fusion proteins and high-throughput production. The PCR method presented here can be modified in many ways. In fact, the templates for the human proteins above were amplified from *in vitro* recombinants produced in the Invitrogen Gateway system without transformation[7]. The templates for fusion proteins can be produced easily by PCR, and more than 500 different fusion proteins have been tested for their solubility and activities virtually by one person[15]. This was possible because proteins produced in the wheat system were generally quite stable. The stability is due to the lack of the 26*S* proteasome-dependent protein degradation activity[20]. It was also possible to manually produce many different proteins with different N-terminal sequences for a systematic analysis of the N-end rule in the wheat cell-free system[21]. N-terminal small tags, such as 6× His tag, can be fused to any protein by simply changing one primer used in the present PCR protocol. Larger tags such as glutathione-*S*-transferase can also be fused by including a small amount of the DNA fragment encoding the tag. These fusion technologies were successfully used for a high-throughput parallel assay of many different transcription factors encoded in cDNA library clones[22]. This type of rapid parallel assay and screening of many different proteins, we believe, will become very important for the post-genomic researches. A basic technology for construction of a protein chip with the proteins fused to a DNA-binding protein has also been developed[23]. The use of PCR for rapid template preparation was also shown to have a potential to accelerate protein engineering[24]. Most of these high-throughput applications have been performed according to older and more complicated protocols than the one that we show here. The present small-scale protein synthesis protocol can be applied to the parallel production of hundreds of different proteins just by performing it in parallel, using a multichannel pipette or the machine mentioned below.

Reaction modes for the cell-free translation. There are several reaction formats for translation. Although we present here the method for translation in the bilayer mode, the other formats are also possible with the extract prepared by the present procedure. Different reaction formats have been summarized elsewhere[9,10]. The batch mode translation, in which the reaction is performed in a homogeneous solution, is useful for testing the activity of the extract and mRNA preparations. For the batch mode synthesis, a fourfold concentrated solution of the substrates (4× BSS, see below) is useful for preparation of the starting mixture in 1× BSS with creatine kinase. In this case, the test can be more sensitive and quantitative if a radiolabeled amino acid is included in the reactions. The dialysis mode translation, in which the reaction is performed within a dialysis bag or a dialysis cartridge with continuous dialysis against the substrate solution, is generally more efficient than the bilayer method. The discontinuous batch (or 'repeat-batch') method[10], in which the buffer/substrate is forcibly exchanged during the translation reaction repeatedly, is very productive, although it needs a machine to be performed. A machine that performs the discontinuous batch mode translation reaction

for gram-scale synthesis is available from CFS, and the protocols for the machine are available in the CFS website. Machines for the bilayer mode parallel translation reactions for high-throughput applications are also available from CFS.

Amino acid labeling for protein structural analyses. The wheat translation system has also been applied to structural biology. This utilizes the high productivity and ease of amino-acid-specific labeling. NMR heteronuclear single quantum coherence (HSQC) spectra could be obtained by measuring the translation product with uniformly labeled amino acids almost without purification: only after a buffer exchange and removal of the precipitant[25]. This is very useful for high-throughput assessing the 'foldedness' of the structural biology samples[26]. The wheat cell-free system has already been modified to fit to a large-scale screening of proteins that are suitable for NMR-based structure determination[26,27]. Amino-acid-specific isotope labeling is also possible with only two transaminase inhibitors added to the translation reaction that prevent scrambling of the isotope caused by metabolic reactions[28–30]. This method may be useful also for the labeling with the stereo-array isotope labeling (SAIL) amino acids, which is expected to accelerate NMR structural analyses[31]. For these applications, the dialysis method may be more useful than the present bilayer method. Selenomethionine substitution of methionine residues in proteins is also easy with the wheat cell-free system, and an X-ray structure of a restriction enzyme has been determined[32].

Modification of the extract. The extract is resistant to many chromatography resins and ultrafiltration membranes. Therefore, it is possible to pretreat the extract with glutathione sepharose or with a metal-chelating resin in order to remove the binders arising from the wheat embryos. Such extracts are available from CFS. It is also possible to freeze-dry the extract without a severe loss of activity, and the freeze-dried extract is stable at least 3 years at −20 °C. We believe that the protein synthesis machinery within embryo cells in natural situations is preserved in a dehydrated state in the winter before imbibition in the spring. We are developing educational experiment kits including a freeze-dried extract, which can be stored stably even in a household freezer that most high schools may be equipped with. It may help the students to feel and understand the relationships between genetic information and protein function and between life and matter.

Other options. Many other applications are possible by modifying the materials added to the reaction. A cofactor-binding protein has been synthesized in the presence and absence of the cofactor, and it was found that both holo-forms and apo-forms could be produced, which was useful for the study of the architecture of the enzyme[10,33]. Disulfide bond formation may be inefficient in the wheat cell-free system because of the presence of DL-dithiothreitol (DTT) in the reaction mixture. By omitting DTT from the substrate mixture, disulfide bonds in some proteins may be formed, although the efficiency of protein synthesis are limited[7,34]. Some membrane proteins have been synthesized in their active forms in the presence of liposomes or some detergents[35–37]. It was also possible to select some functional sequences from a random pool of mRNA molecules, through which the E01 sequence used in the present protocols has been obtained[12]. The lack of the proteasome activity[20] may help us to reconstitute intracellular multicomponent molecular systems, such as protein degradation systems, without purification of each component. We believe that the wheat translation system may be useful also for synthetic biology purposes.

MATERIALS

REAGENTS

- Unsterilized wheat seeds (strain 'Chihoku': any strain may be used): dried after harvesting, unbaked and containing no pesticides or insecticides (as one may be exposed to the drugs when crushing the seeds)
- Nonidet P-40 (NP-40; Nacalai Tesque, cat. no. 23640-94) ! **CAUTION** Harmful (wear gloves).
- Cyclohexane (Wako Pure Chemicals, cat. no. 034-05001) ! **CAUTION** Highly flammable, harmful and dangerous for the environment (wear gloves and handle the reagent in a fume hood).
- Carbon tetrachloride (Wako Pure Chemicals, cat. no. 039-01271) ! **CAUTION** Toxic and dangerous for the environment. Use of this reagent is tightly regulated in Japan. Wear gloves and handle the reagent in a fume hood. Confirm and obey local regulations associated with the use and disposal of the reagent.
- 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; Nacalai Tesque, cat. no. 17514-15) ! **CAUTION** Irritant.
- Potassium hydroxide (Nacalai Tesque, cat. no. 28616-45) ! **CAUTION** Corrosive (wear gloves and protecting glasses).
- Potassium acetate (Nacalai Tesque, cat. no. 28405-05)
- Magnesium acetate tetrahydrate (Nacalai Tesque, cat. no. 20821-85)
- Calcium chloride (Nacalai Tesque, cat. no. 06729-55)
- DL-Dithiothreitol (DTT; Wako Pure Chemicals, cat. no. 049-08972)
- Standard 20 L-amino acids (Wako Pure Chemicals or Nacalai Tesque)
- Milli-Q water (freshly prepared with a Millipore system, Millipore)
- Elix water (produced with a Millipore system, Millipore)
- Sodium acetate (Nacalai Tesque, cat. no. 31119-65)
- Acetic acid (Nacalai Tesque, cat. no. 00212-56) ! **CAUTION** Flammable (handle in a fume hood).
- Ethanol (Nacalai Tesque, cat. no. 14713-95) ! **CAUTION** Highly flammable (handle in a fume hood).
- Sephadex G-25 Fine (GE Healthcare, cat. no. 17-0032-01)
- TaKaRa *Ex* Taq (Takara Bio, cat. no. RR001A)
- Plasmid preparation kit (QIAGEN Plasmid Midi Kit, QIAGEN, cat. no. 12143)
- ATP, disodium salt (Sigma, cat. no. A3377)
- GTP, sodium salt (Sigma, cat. no. G8877)
- CTP, disodium salt (Sigma, cat. no. C1506)
- UTP, trisodium salt (Sigma, cat. no. U6625)
- Spermidine (Rnase-free, Sigma, cat. no. S0266) ! **CAUTION** Corrosive (wear gloves).
- Creatine phosphate (Wako Pure Chemicals, cat. no. 030-04584, or Roche, cat. no. 621722)
- Creatine kinase (Roche, cat. no. 127566)
- SP6 RNA polymerase (HC) (80 U μl^{-1}, Promega, cat. no. P4084)
- RNasin Ribonuclease Inhibitor (20–40 U μl^{-1}, Promega, cat. no. N2511)
- Oligodeoxyribonucleotides listed in **Table 1** (Invitrogen)
- Liquid nitrogen ! **CAUTION** Wear nonpermissible gloves; obey local regulations.

EQUIPMENT

- Rotor Speed Mill PULVERISETTE 14 (Fritsch)
- Sieve shaker (A-3 PRO, Fritsch) with 710-, 850- and 1,000-μm mesh sieves (The Iida Testing Sieves, Iida Manufacturing)
- Sonicator (W-113 Ultrasonic Cleaner, Honda Electronics)
- Fume hood
- Blender (KC-4811W Mill & Mixer, Twinbird)
- Amicon Ultra-15 (10 kDa, Millipore, cat. no. UFC9 010 08)
- Toothpicks
- Mesh skimmer
- Kimwipe sheets
- Kim Towels
- Corner trash bags ('Gomipon', Kokubo): alternatively, nylon stockings may be useful

- Hitachi CR21G centrifuge with an R10A3 rotor (Hitachi, cat. no. 904308D0) with 500-ml centrifuge bottles (cat. no. 330437A)
- TOMY GRX-220 centrifuge with a TA-24BH rotor (TOMY), with 50-ml round-bottom centrifuge tubes (cat. no. 3177-9500; capped with DS3119-0029 centrifuge tube closures)
- 50-ml injection syringe (Terumo, cat. no. SS-50ESZ)
- Cotton wool
- Aluminum foil
- Air incubator (MIR-153, SANYO)
- UV spectrophotometer (Beckman DU600, Beckman)
- Submarine-type and slab gel electrophoresis systems for agarose and SDS-polyacrylamide gel electrophoresis, respectively
- UV light and camera (Toyobo)
- PCR machine (PCR Thermal Cycler MP, Takara Bio)
- 96-well microtiter plate (Techno Plastic Products AG, cat. no. 92096)
- 6-well plate (Techno Plastic Products AG, cat. no. 92006)
- pH meter
- Autoclave

REAGENT SETUP

Cyclohexane/carbon tetrachloride mixture Mix 2.4 volume of carbon tetrachloride with one volume of cyclohexane. Handle and store it in a fume hood at room temperature (20–25 °C). The mixture can be reused several times.

0.5% (vol/vol) NP-40 solution Dissolve NP-40 in Milli-Q water. Store it at room temperature (stable for years).

3 mM amino acid mixture Dissolve all of the 20 standard amino acids into Milli-Q water. For storage, seal the container tightly to avoid air oxidation and store it frozen at −20 °C (stable for at least a year).

2× EB Mix 80 mM HEPES-KOH (pH 7.6), 200 mM potassium acetate, 10 mM magnesium acetate, 4 mM calcium chloride, 0.6 mM amino acids and 8 mM DTT freshly at 4 °C. ▲ **CRITICAL** Use it in a few days.

5× Transcription buffer (TB) Mix 400 mM HEPES-KOH (pH 7.8), 80 mM magnesium acetate, 10 mM Spermidine and 50 mM DTT. Store it in aliquots at −20 °C (stable for at least a year).

3 M Sodium acetate Add 3 M acetic acid to a 3 M sodium acetate solution and adjust to pH 5.2. Sterilize the solution by autoclaving or by filtration. Store it at room temperature (stable at least a year).

70% (vol/vol) ethanol Mix 35 ml of ethanol and 15 ml of Milli-Q water. Store at −20 °C (stable at least a year).

100 mM ATP, CTP, GTP and UTP Dissolve the powder of the salt of the nucleotide in water and adjust pH of the solution between 7 and 8.5. Measure the absorbance at 260 nm and adjust the concentration to 100 mM by adding water according to the molecular extinction coefficient of 15.4, 9.0, 11.4 and 9.9×10^3 cm^{-1} M^{-1} for ATP, CTP, GTP and UTP, respectively. Store each solution frozen at −80 °C (stable for a year).

NTP solution Mix equal volume of 100 mM ATP, CTP, GTP and UTP. Store it frozen at −80 °C (stable for a year).

20 mg ml^{-1} Creatine kinase Dissolve the powder in water and store it in aliquots at −80 °C (stable at least a year).

4× Buffered substrate solution (BSS) 120 mM HEPES-KOH (pH 7.6), 400 mM potassium acetate, 10.8 mM magnesium acetate, 1.6 mM spermidine, 10 mM DTT, 1.2 mM amino acids, 4.8 mM ATP, 1 mM GTP and 64 mM creatine phosphate. Store it in aliquots at −20 °C (stable for 2 months).

1× BSS Dilute 4× BSS with Milli-Q water. Prepare just before use; can be stored for a few days at −20 °C.

EQUIPMENT SETUP

Rotor speed mill Set the rotor speed to 7,000 r.p.m. The mill and the sieve shaker will produce a lot of fine powder of flour, which floats around in the air and contains inhibitors of protein synthesis. Thus, these equipments may be better kept away or spatially isolated from the biochemical laboratory.

A-3 PRO shaker Set the amplitude to 2.0 mm, sieving time to 2 min and interval to 1 s. This may also be isolated from the biochemistry laboratory.

The blender Use the larger cup (200 ml mixer cup) for 60 g embryo. The smaller (70 ml) cup may be useful for the preparation from smaller amounts (less than around 20 g) of the purified embryo particles.

Sonicator Add ice to the water in the top bucket. Set the frequency to 45 kHz.

PCR thermal cycler Program 1: 94 °C for 4 min; 30 cycles of 98 °C for 10 s, 55 °C for 1 min and 72 °C for 1 min per 1-kb amplified sequence; and 72 °C for 5 min. Program 2: 94 °C for 4 min; 5 cycles of 98 °C for 10 s, 55 °C for 1 min and 72 °C for 1 min per 1-kb amplified sequence; 35 cycles of 98 °C for 10 s, 60 °C for 40 s and 72 °C for 1 min per 1-kb amplified sequence; and 72 °C for 7 min.

PROCEDURE

Preparation of unwashed embryo particles ● TIMING 2–3 d per 5 g embryo particles from 5 to 6 kg seeds

1| Grind the wheat seeds in the mill at the rate of 100 g per min. Repeat this four times.

! **CAUTION** Wear protectors if needed, because fine powder will drift around in the air.

2| Shake the sample in the sieve shaker.

3| Collect the particles on the 850- and 710-μm sieves in a dish.

4| Let the sample fall onto another dish from around a 50-cm height repeatedly to remove seed-coat fragments.

5| Pour the particles into a beaker containing around 1 l of cyclohexane/carbon tetrachloride mixture in the fume hood and stir the mixture thoroughly. Do not leave the embryo particles in the solvent too long.

6| Collect the floating particles with a mesh skimmer as fast as possible after the particles are separated, and put them on Kimwipe sheets in the fume hood to remove the solvent overnight.

■ **PAUSE POINT** The embryo particles can be stored at 4 °C for several years.

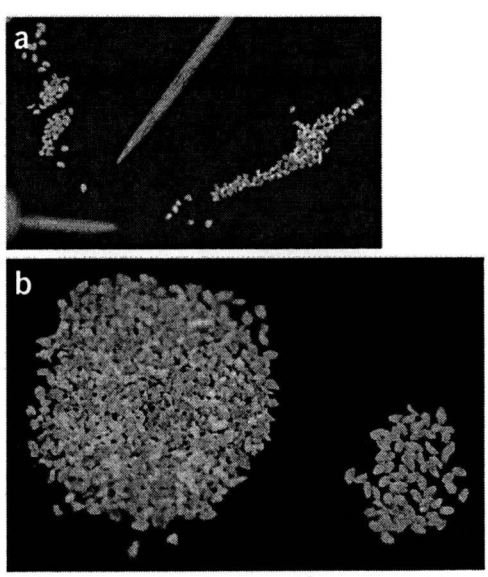

Figure 3 | Selection of good embryo particles by eye. (a) Selecting good particles using a toothpick. (b) Good particles (left) and bad particles (right).

7| Spread the particles on a clean paper or plastic sheet on a desk. Select only those particles with yellow color with a minimum amount of white matter attached to them using a toothpick, carefully investigating each particle by eyes: remove brownish particles and the particles with much white matter coming from endosperm (**Fig. 3**).
▲ **CRITICAL STEP** The white matter contains the catalytic inhibitors of protein synthesis.

8| Store the selected particles at 4 °C until use.
■ **PAUSE POINT** The embryo particles can be stored at 4 °C for several years.

Preparation of the extract ● TIMING **1 d**

9| Put 60 g of embryos in a corner trash bag. A smaller amount may also be washed successfully, although we have only a little experience. Wash the particles in a stream of 5-l cold Elix water.

10| Dip the bag in cold Elix water (4 °C, typically 700 ml) and knead it gently. Do not knead it too much.

11| Change the water and repeat Step 10 several times more until no white matter disperses out through the bag.

12| Change the water to 500 ml of 0.5% NP-40 solution (4 °C) and sonicate the sample for 5 min with gentle stirring.

13| Wash in an Elix water stream (typically 3 liters, not chilled) until no bubbles can be seen, and sonicate twice in cold Elix water.

14| Wash the particles five times more in a beaker with 800 ml each of cold Milli-Q water.

15| Take out the embryo particles and wrap them in Kimwipe sheets, which are further wrapped with a sheet of Kim Towel, in order to remove water.

16| Repeat this wiping a few times until no more water can be removed.

17| Put the washed embryo particles from 60 g of the starting unwashed embryos (around 120 g) in the larger (mixer) cup of the blender and add 90–120 ml of 2× EB (4 °C). We have confirmed that 10 g of the washed embryo particles could also be processed successfully with 10 ml of 2× EB in the smaller cup.

18| Run the blender for 30 s three times.

19| Centrifuge the sample at 30,000*g* at 4 °C for 30 min in a TA-24BH rotor with the GRX-220 centrifuge (**Fig. 4**). Collect the supernatant, i.e., the middle layer between the fatty material and the precipitate.

20| Centrifuge the sample again at 30,000*g* at 4 °C for 15 min in a TA-24BH rotor with the GRX-220 centrifuge and collect the supernatant in a tube.

21| Pass the sample through a G-25 column pre-equilibrated with 1× EB. A 40-ml column may be prepared in a 50-ml injection syringe with cotton wool at the bottom, which can be hung at the rim of a 500-ml centrifuge bottle in it, loaded with maximum of 20 ml of the sample, capped with aluminum foil and centrifuged at 750*g* for 5 min at 4 °C in a R10A3 rotor.

22| Pass the sample through a G-25 column pre-equilibrated with 1× BSS.

23| Measure the absorbance of the extract at 260 nm in a 1-cm path length cuvette (a several hundred-fold dilution will be needed), which may be more than 150. If the absorbance is, e.g., 160, then the concentration of the extract is 160 AU ml^{-1}, where '1 AU' is the amount of the extract that gives the absorbance of 1 at 260 nm in a 1-cm

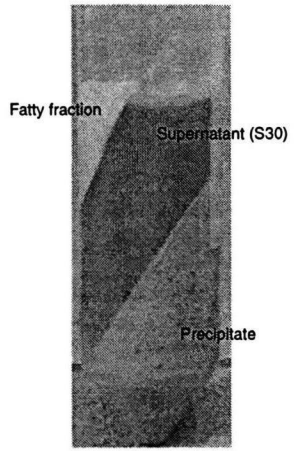

Figure 4 | An example of the sample after the first 30,000*g* centrifugation. The sample in Step 19 may be separated as in the figure (in which a conical tube is used).

path if dissolved in a 1-ml solution. The manuals from CFS use 'OD' instead of 'AU.'
? TROUBLESHOOTING

24| Concentrate the extract to around 300 AU ml^{-1} by ultrafiltration with 10 kDa Amicon Ultra-15 cartridges. Eight cartridges can be centrifuged at once in the TA-24BH rotor.

25| Pass the sample through a G-25 column pre-equilibrated with 1× BSS again.

26| Measure the absorbance at 260 nm of the extract and adjust the concentration to 240 AU ml^{-1}.
? TROUBLESHOOTING

27| Aliquot the sample and freeze each tube quickly in liquid nitrogen. Store them at −80 °C. Do not store the extract at −20 °C.
■ **PAUSE POINT** The extract is stable for at least a few years at −80 °C.

Template DNA preparation for small-scale parallel protein synthesis ● TIMING 1 d
28| Mix 0.5 ng of the plasmid containing the target cDNA sequence in a PCR tube on ice with 10 nM of the target-specific primer, 10 nM of the AODA2306 primer, 200 μM each of dNTP, the buffer supplied by the supplier and 0.0125 U μl^{-1} of *Ex* Taq DNA polymerase in a 20-μl reaction mixture (**Fig. 2**). When many different reactions are performed in parallel, mix the materials other than the plasmid and the target-specific primer in one tube and dispense this mixture to the plasmid/target-specific primer solution. Set the sample(s) in PCR Thermocycler and run it with Program 1.

29| Check the amplified DNA by agarose gel electrophoresis.
■ **PAUSE POINT** PCR products may be stable at −20 °C for years.
? TROUBLESHOOTING

30| Mix 5 μl of the first PCR product (without any purification) in a 200-μl PCR tube with 100 nM each of the SPu and AODA2303 primers, 1 nM of the deSP6E01 primer, 200 μM each of dNTP, the buffer supplied by the supplier and 0.0125 U μl^{-1} of *Ex* Taq DNA polymerase in a 50-μl reaction mixture (**Fig. 2**). For parallel preparation, mix everything other than the first PCR product in one tube and dispense it into each PCR tube before putting in the first PCR product. Put the sample(s) in PCR Thermocycler and run it with Program 2.

31| Add 5 μl of 3 M sodium acetate (pH 5.2) and 140 μl of ethanol to each of the second PCR solutions. Mix the samples well. Incubate them at −20 °C for 10 min.

32| Centrifuge the samples at 15,000*g* for 15 min at 4 °C in a standard microcentrifuge.

33| Remove the supernatant and add 300 μl of chilled 70% ethanol.

34| Centrifuge the samples at 15,000*g* for 5 min at 4 °C.

35| Remove the supernatant carefully and thoroughly and dry the samples with the tube lids open covered with Saran Wrap.

36| Add 10 μl of Milli-Q water to each sample and dissolve the pellets well.

37| Analyze 1 μl of the samples by agarose gel electrophoresis. Estimate the concentration of the DNA by comparing with the bands of the molecular weight marker run in the same gel. Adjust the concentration of each sample around 0.25 μg μl^{-1}.
■ **PAUSE POINT** PCR products are stable at −20 °C for years.
? TROUBLESHOOTING

mRNA preparation for small-scale parallel protein synthesis ● TIMING 5–7 h
38| Prepare a solution containing 6 μl of 5× TB, 3 μl of 25 mM NTP, 48 U of RNase inhibitor and 48 U of SP6 RNA polymerase per 16 μl using Milli-Q water. This gives the 1.5× final buffer concentration. Dispense 16 μl of this solution in each well of a microtiter plate. Add 4 μl of the second PCR solution and incubate the plate at 37 °C for 4–6 h. White insoluble material will be generated during transcription.

39| Analyze the product (1 μl) in a 1% (wt/vol) agarose gel stained with ethidium bromide.
■ **PAUSE POINT** The sample can be stored at −80 °C for weeks. Transportation with dry ice is not recommended.
? TROUBLESHOOTING

Small-scale parallel protein synthesis ● TIMING 1 h + an overnight reaction + 3 h

40| Keep the mRNA tubes in room temperature. Thaw the extract in water and put it on ice immediately after it has thawed. Thaw the creatine kinase solution on ice. Thaw 1× BSS on ice and mix it well. Spin down the thawed solutions.

41| Dispense 10 μl of 240 OD per ml extract and 0.8 μl of 1 mg ml^{-1} creatine kinase into each well of a microtiter plate on ice. This microtiter plate can be replaced with standard microtubes or PCR tubes if the number of the samples is small.

42| Resuspend the mRNA solutions, which contain white insoluble material, and transfer 10 μl of each suspension into each well of the microtiter plate. Mix the samples gently by pipetting, avoiding bubbles.

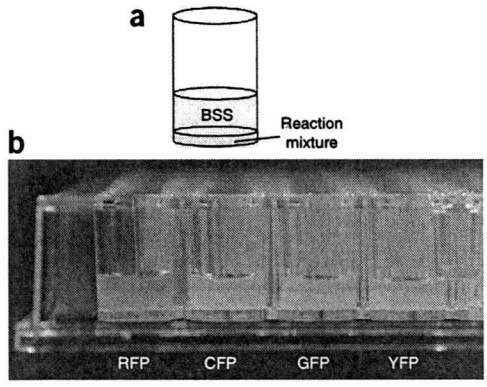

Figure 5 | A schematic representation of the bilayer to be formed at the start of the translation reaction (**a**) and a typical result of translation (**b**). (**a**) In the small-scale protein synthesis, the reaction mixture is layered under the buffered substrate solution (BSS). The two solutions will mix together gradually during incubation. (**b**) Fluorescent proteins synthesized in microtiter plate wells.

43| Dispense 206 μl of 1× BSS in each well of a flat-bottomed microtiter plate.

44| Take each of the mRNA/extract mixtures into a micropipette tip so that no air is at the end of the tip. Insert the tip at the bottom of a microtiter well containing 1× BSS carefully with holding the mixture within the tip and then carefully pump out the mixture under the buffer without mixing, avoiding bubbles, so that the mRNA/extract mixture and the buffer form a bilayer (**Fig. 5a**). Do not mix the samples.
▲ **CRITICAL STEP** Do not mix the samples. It is very important at the start of the reaction that the starting reaction mixture forms a distinct layer that forms a clear boundary with the upper BSS liquid.

45| Seal the wells to avoid evaporation. Be careful not to shake the plate too much.

46| Leave the plate in the air incubator at 15 °C for 20 h.

47| Analyze the sample (3–5 μl) on a standard SDS gel.
? TROUBLESHOOTING

Transcription of a pEU plasmid harboring a target ORF sequence ● TIMING 5–7 h excepting Step 48

48| Subclone the target ORF into pEU (**Fig. 2c**) and prepare the plasmid using a standard plasmid preparation kit, such as QIAGEN Plasmid Midi Kit. Dissolve the plasmid in the standard TE buffer. Determine the concentration and purity of the DNA sample by measuring the absorbance values at 260 and 280 nm. If the A_{260}/A_{280} ratio is not between 1.70 and 1.85, then further purify the sample by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation with rinsing the pellet with 70% ethanol. Adjust the concentration to 1 μg μl^{-1} with TE.
▲ **CRITICAL STEP** It is recommended to always perform the appended purification steps, because most plasmid preparation kits use an RNase, and because even a small amount of RNase would inhibit the transcription and translation.
■ **PAUSE POINT** Plasmids can be stored for years at −20 °C.

49| Mix 25 μg of plasmid DNA in 250 μl of the transcription buffer containing 50 μl of 5× TB, 25 μl of 25 mM NTP, 250 U of RNase inhibitor and 250 U of SP6 RNA polymerase and incubate this mixture at 37 °C for 6 h. White insoluble material will be generated during transcription. Perform the control reaction with pEU-E01-DHFR or pEU-E01-GFP.

50| Check the sample in an agarose gel. Transcription stops partially at the plasmid replication origin.
■ **PAUSE POINT** The transcription product can be stored at −80 °C for several weeks. Transportation with dry ice is not recommended.
? TROUBLESHOOTING

Large-scale protein synthesis ● TIMING 1 h + an overnight reaction + 3 h

51| Put the mRNA tubes in room temperature. Thaw the extract in water and put it on ice immediately after it has thawed. Thaw the creatine kinase 20 mg ml^{-1} solution on ice. Thaw 1× BSS on ice and mix it well. Spin down the thawed solutions.