HCV and Hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV [8]. HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence prompted us to seek for determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered, of course, in a study on the hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: Can inflammation alone result in the development of HCC in such a high incidence (90% in 15 years) or multicentric nature in HCV infection?

The other role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in which severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, in vivo, by transgenic mouse technology.

Transgenic Mouse Lines Carrying the HCV Genome

As described above, the HCV proteins have been characterized chiefly using in vitro translation or cultured cells. Little is known, however, about the role of HCV or its proteins in the pathogenesis of hepatitis and subsequent liver diseases, cirrhosis and HCC. One of the major issues regarding the pathogenesis of HCV-associated liver lesion is whether the HCV proteins have direct effects on pathological phenotypes. Although several strategies have been used to characterize the hepatitis C viral proteins, the relationship between the protein expression and disease phenotype has not been clarified. For this purpose, several lines of mice have been established which were transgenic for the HCV cDNA. They include the ones carrying the entire coding region of HCV genome [9], the core region only [3, 10], the envelope region only [11], the core and envelope regions [12] and the core to non-structural (NS)2 regions [13]. Although detection of mRNA from the NS regions of the HCV cDNA has been reported [9], the detection of HCV NS proteins in the transgenic mouse liver has not been successful. The reason for this failure in detecting NS proteins is unclear, but the expression of the NS enzymes may be harmful to mouse development and may allow the establishment of only low-expression mice.

In terms of expression system, two different ways have been applied: transient and constitutive expression systems. One transgenic mouse line has been reported which expresses the HCV genes using a transient expression system. Wakita et al. [13] utilized the Cre/*lox*P system, by which a gene under silent can be switched on by the introduction of Cre recombinase. They established a transgenic mouse line that had the core, envelopes and NS2 genes of HCV in a silent state. After the injection of the recombinant adenovirus that had *Cre* recombinase in the mice, the HCV genes expressed transiently. These mice developed acute hepatitis, which was blocked by the administration of anti-CD4 and CD8 antibodies. This mouse system would provide a good animal model for acute hepatitis C and be useful for the study of immunological aspects of hepatitis. The possibility, however, that the greatly overexpressed HCV proteins had caused the death of hepatocytes and provoked the immune response thereafter still remains.

We have engineered transgenic mouse lines carrying the HCV genome were by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 14]. Established are four different kinds of transgenic mouse lines, which carry the core gene, envelope genes, the entire NS genes, or NS5A gene, respectively, under the same transcriptional regulatory element (fig. 1). Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [14]. The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins [11, 15], and the transgenic mice carrying the entire NS or NS5A gene have developed no HCC.

The core gene transgenic mice express the core protein of an expected size, and the level of the core protein in the liver is similar to that in chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is

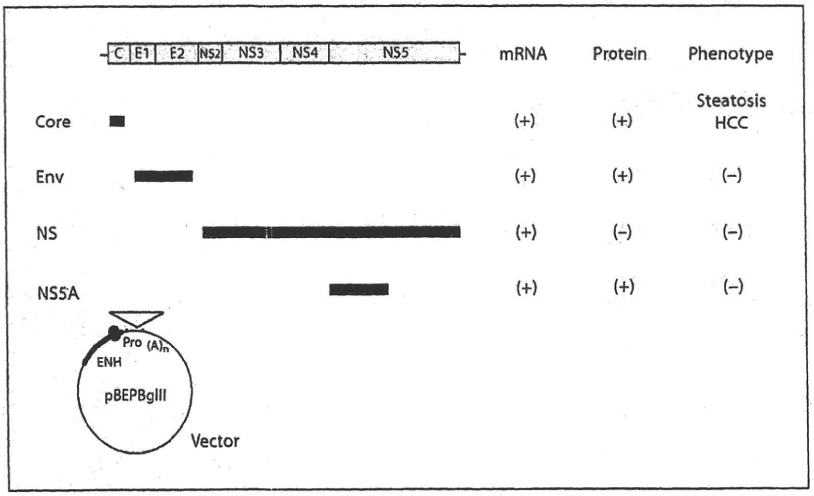

Fig. 1. Transgenic mouse lines carrying the HCV genome. Four different kinds of transgenic mouse lines, carrying the core gene, envelope genes, the entire NS genes, or NS5A of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene develop HCC after an early phase with hepatic steatosis in two independent lineages. HCV = Hepatitis C virus; HCC = hepatocellular carcinoma; Env = envelope genes; NS = non-structural genes.

one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [1]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene [9, 10, 12]. These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Enhancement of Oxidative Stress and Intracellular Signaling in HCV-Associated Hepatocarcinogenesis

It is difficult to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei [14]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation [16, 17]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice. These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of antioxidant system, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

In the absence of inflammation, thus, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged anymore by a physiological antagonistic system. This suggests that the inflammation in chronic HCV infection would have a characteristic different in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction [16, 18]. The dysfunction of the electron transfer system of the mitochondrion is suggested in as-

sociation with the presence of the HCV core protein [18, 19].

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated [20]. The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. In the downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [20, 21]. At far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Hepatitis C as a Metabolic Disease

Steatosis is frequently observed in chronic hepatitis C patients, and significantly associated with increased fibrosis and progression rate of fibrosis of the liver [22]. A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly up- or downregulated.

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver due to simple obesity. Carbon-18 monounsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased. This is also the case in the comparison of liver tissues from hepatitis C patients and simple fatty liver patients due to obesity [17]. The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are, at least, three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1 [23]. Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of microsomal triglyceride transfer protein (MTP) by HCV core protein [24]. This inhibits the secretion of very-low-density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last one involves by the sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, while neither SREBP-2 nor SREBP-1a is upregulated [25].

Steatosis, HCV and PA28γ

Interestingly, we found recently that a protein interacting with the core protein, proteasome activator (PA)28γ, is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC [3, 25]. Steatosis is defined as an accumulation of lipid droplets, a majority of which are triglycerides. Biosynthesis of triglycerides is mainly regulated by SREBP-1c. Transcription of SREBP-1c is controlled by a heterodimer of nuclear hormone receptors, liver X receptor (LXR)-α and retinoid X receptor (RXR)-α. Indeed, it has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees. Our study has demonstrated that the core protein enhances the binding activity of the LXR-α–RXR-α complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes [25]. The activation may be mediated by the direct interaction between the core protein and RXR-α [26] or by suppression of a corepressor such as Sp110b, a negative regulator of RAR-α, by sequestering it in the cytoplasm via interaction with the cytoplasmic core protein [27]. Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and the core gene transgenic mice [5]. As stated before, the MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein B, cholesterol and triglycerides. In the core gene transgenic mice, MTP-specific activity is significantly decreased. Therefore, the downregulation of MTP may be involved in the development of the steatosis cooperating with upregulation of SREBP-1c, although the precise role of HCV core protein is still unclear. Recently, it has been reported that the assembly and budding of HCV occur around the accumulated lipid droplets within the endoplasmic reticulum [28]. Furthermore,

increases in saturated and monounsaturated fatty acids enhance HCV RNA replication. These data suggest that regulation of lipid metabolism by the core protein plays crucial roles in the HCV life cycle. Obesity and hepatic steatosis often result in insulin resistance. However, 1- to 2-month-old core gene transgenic mice, which do not exhibit apparent steatosis and obesity, already exhibit insulin resistance due to a decrease in insulin sensitivity in the liver [23]. Moreover, the core gene transgenic mice have been shown to exhibit overt diabetes when fed a high-fat diet, while control mice do not. Binding of insulin to the insulin receptor triggers tyrosine phosphorylation of the IRS proteins, leading to the following signal transductions to increase glucose uptake and inhibit the net production of glucose in the liver. An inflammatory cytokine, TNF-α, is known to impair the insulin-signaling pathway via inhibition of tyrosine phosphorylation of IRSs. In fact, the overproduction of TNF-α has been reported to reduce the phosphorylation of IRS-1 and Akt in the core gene transgenic mice despite the absence of hepatic. In the latter study, moreover, hyperinsulinemia was cured by depletion of TNF-α, suggesting that upregulation of TNF-α contributes to the core protein-induced insulin resistance [23]. Our previous study has indicated that the core protein-induced overexpression of TNF-α is also dependent on the presence of PA28γ [25].

In relation to lipid metabolism, the core protein has also been found to interact with RXR-α [26]. RXR-α is one of the nuclear receptors which forms a homodimer or heterodimers with other nuclear receptors including peroxisome proliferator-activated receptor (PPAR)-α, and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation and proliferation. In fact, the core protein of HCV activates genes that have an RXR-α-responsive element as well as those with a PPAR-α-responsive element, in both mice and cultured cells [26]. Based on these results, we, then, examined the expression and function of PPAR-α in the liver of core gene transgenic mice.

PPAR-α Activation and 'Fatty Acid Spiral' in HCV-Associated Hepatocarcinogenesis

PPAR-α is one of PPAR genes, and plays a central role, as a heterodimer with RXR-α, in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs. On the other hand, a prolonged administration of PPAR-α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.

The level of PPAR-α protein was increased in the liver of core gene transgenic mice as early as 9 months. PPAR-α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR-α mRNA was not increased at any age. By the pulse-chase experiment, the stability of nuclear PPAR-α turned out to be increased in the presence of the core protein. In line with the increase of PPAR-α protein, target genes of PPAR-α were activated in the liver of core gene transgenic mice; these genes include cyclin D1, CDK4, acy-CoA oxidase, and peroxisome thiolase [29]. However, in general, the activation of PPAR-α leads to improvement but not aggravation of steatosis. Then, what is a function of PPAR-α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR-α activation in pathogenesis of steatosis and HCC, we made mating of core gene transgenic mouse with PPAR-α knockout (KO) mouse, and studied the phenotype. PPAR-α KO mice have reduced expressions of target genes of PPAR-α, and have mild steatosis in the liver as expected. It was unanticipated, however, that steatosis was absent in PPAR-α-null or -heterozygous core gene transgenic mice but present in PPAR-α-intact core gene transgenic mice at the age of 9 or 24 months [29]. 8-Hydroxydeoxyguanosine and peroxylipids, both of which are markers for oxidative stress, were decreased in PPAR-α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to an overproduction of oxidative stress, was also improved in PPAR-α KO core gene transgenic mice.

Finally, PPAR-α KO core gene transgenic mice did not develop HCC at the age of 24 months, while about one-third of PPAR-α-intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for PPAR-α gene did not develop HCC either [29]. When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR-α-heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core-gene (+) mice with a greater PPAR-α activation. It should be noted that steatosis was present only in core-gene (+) PPAR-α-heterozygous mice. In summary, steatosis and HCC developed in PPAR-α-intact but not in PPAR-α-heterozygous or PPAR-α-null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR-α would be important in hepatocarcinogenesis by HCV

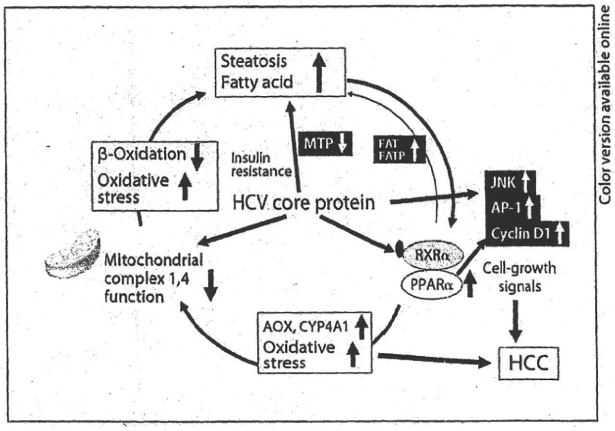

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Fig. 2. HCV core protein causes 'fatty acid spiral'. In HCV infection, the core protein induces steatosis via several pathways, leading to 'fatty acid spiral' in the presence of the mitochondrial complex-1 dysfunction and PPAR-α activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. HCV = Hepatitis C virus; HCC = hepatocellular carcinoma; JNK = c-Jun N-terminal kinase; AP-1 = activating protein-1; RXRα = retinoid X receptor-α; PPARα = peroxisome proliferator activated receptor-α; AOX = acyl-CoA oxidase; CYP = cytochrome P450; MTP = microsomal triglyceride transfer protein; FAT = fatty acid translocase; FATP = fatty acid transport protein.

core protein. In general, PPAR-α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR-α may exacerbate steatosis. A persistent activation of PPAR-α with 'strong' ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV infection. However, in HCV infection, the core protein induces steatosis through the above-mentioned pathways, leading to the 'fatty acid spiral' in the presence of the mitochondrial electron transfer system dysfunction [18, 19] and PPAR-α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

Conclusion

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein [30] has gained a wide popularity. They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the APC gene for inactivation, those in K-ras for activation and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer [30]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis.

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for the hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute. The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers. Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

Disclosure Statement

The authors declare that they have no financial conflict of interest.

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Hepatitis C Virus Core Protein Compromises Iron-Induced Activation of Antioxidants in Mice and HepG2 Cells

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One of the characteristics of hepatitis C virus (HCV) infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, the core protein of HCV was shown previously to induce the overproduction of reactive oxygen species (ROS) in the liver. In the present study, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, the augmentation of some antioxidants, including heme oxygenase-1 and NADH dehydrogenase, quinone 1, was compromised by the presence of the core protein. The attenuation of iron-induced augmentation of heme oxygenase-1 was also confirmed in HepG2 cells expressing the core protein. This attenuation was not dependent on the Nrf2 transcription factor. Thus, HCV infection not only induces oxidative stress but also hampers the iron-induced antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. ***J. Med. Virol. 82:776–782, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: oxidative stress; transgenic mouse; hepatocellular carcinoma; antioxidant; heme oxygenase-1

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem both in medical and socio-economical aspects [Saito et al., 1990]. Despite overwhelming evidence from epidemiological studies, the precise mechanism of hepatocarcinogenesis in HCV infection is still not fully understood. Recently, it has been shown that the core protein of HCV induces HCC in transgenic mice [Moriya et al., 1998; Naas et al., 2005; Machida et al., 2006]. Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [Farinati et al., 1995; Moriya et al., 2001; Choi and Ou, 2006]. Reactive oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [Fujita et al., 2008]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [Farinati et al., 1995; Chapoutot et al., 2000]. On the other hand, in chronic hepatitis C, HCC and fibrosis are closely associated with the amount of iron in the liver. Iron depletion both in the form of dietary iron restriction or phlebotomy improved hepatic inflammation and lowered serum aminotransferase levels in hepatitis C patients. Phlebotomy decreases the hepatic content of 8-OH deoxyguanosine, a marker of DNA damage, improved inflammation and fibrosis

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; HO-1, heme oxygenase-1; thiobarbituric acid reactive substances (TBARS); GST, glutathione-*S*-transferase; SOD, superoxide dismutase; GPx, glutathione peroxidase; NQO1, NAD(P)H dehydrogenase, quinone 1; AP-1, activator protein-1; NF-κB, nuclear factor-kappa B; Bach1, BTB and CNC homology 1.

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scores, and prevented HCC development [Kato et al., 2001].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, in association with iron accumulation, but no underlying mechanism is understood well yet. Hence, it is an important issue to understand the mechanism of oxidative stress augmentation, which may allow us to develop new tools of therapies for chronic hepatitis C. Iron accumulation and oxidant/antioxidant status were assessed with or without iron overloading in the liver of a transgenic mouse model of HCC in HCV infection. The expression levels of genes associated with the antioxidant system were also determined.

MATERIALS AND METHODS

Transgenic Mice and Cultured Cells

The production of HCV core gene transgenic mice has been described previously [Moriya et al., 1998]. Briefly, the core gene of genotype 1b HCV was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). Mice were cared for according to institutional guidelines approved by the institutional review board of the animal care committee, fed an ordinary chow diet (Oriental Yeast Co., Ltd, Tokyo, Japan), and maintained in a specific pathogen-free state. At least five mice were used for each experiment and the data were subjected to statistical analysis.

Determination of Iron in the Liver

Determination of Fe in the liver was performed by Shimadzu Techno-Research, K.K. (Kyoto, Japan) using Inductively Coupled Plasma apparatus, ICP8100 (Shimadzu Corp.). Briefly, samples were resolved by microwave (Microwave Resolution System ETHOS-TC, Milestone General, Inc., Tokyo, Japan) after the addition of nitric acid, and the volume was adjusted by the addition of H_2O. This solution was then subjected to the quantification of iron using ICP8100 (http://www.shimadzu.com/products/lab/ms/glossary/oh80jt00000008w4.html).

Iron Loading Experiments

For the short-term iron loading experiment, three doses of $FeSO_4$ solution (100 mg/kg BW, suspended in dH_2O) or vehicle only were administered to the core gene transgenic or control mice i.p., with intervals of 24 hr at the age of 6 months [Zhu and Miller, 2007]. For the long-term iron loading experiment, 50 mg/kg BW of $FeSO_4$ was administered to the core gene transgenic mice i.p., once a week for 3 months from the age of 3 months. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [Ruggieri et al., 2004]. For the iron loading experiments, hemin solution (10 mM in DMSO) was added to the culture medium at the final concentration of 5 μM, and the cells were incubated for 5 or 72 hr.

Evaluation of Oxidant and Antioxidant Systems

Lipid peroxidation in the liver was estimated spectrophotometrically using thiobarbituric acid reactive substances (TBARS) and is expressed in terms of malondialdehyde formed per milligram protein. In the cell culture experiment, the cells were examined for ROS production using chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Inc., Eugene, OR). For the evaluation of DNA damage in cells, apurinic/apyrimidinic (AP) sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

Real-Time PCR and Western Blotting

RNA was prepared from mouse liver tissues using TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The fluorescence signal was measured using ABI Prism 7000 (Applied Biosystems, Tokyo, Japan). Primers and probes for hepcidin (Unigene ID: Mm. 439939), catalase (Mm. 4215), glutathione-S-transferase (GST) (Mm. 1090), superoxide dismutase (SOD)1 (Mm. 01344233), glutathione peroxidase (GPx)1 (Mm. 1090), heme oxygenase (HO)-1 (Mm. 00516004), NAD (P)H dehydrogenase, quinone (NQO) 1 (Mm. 500821), activator protein (AP)-1 (Mm. 275071), nuclear factor-kappa B (NF-κB)1 (Mm. 256765), BTB and CNC homology (Bach)1 (Mm. 26147), and hypoxanthine phosphoribosyltransferase (Mm. 299381) were purchased as assays-on-demand (Applied Biosystems). Each cDNA prepared was used in triplicate for the real-time PCR procedures for each gene tested.

Western blotting was performed with an anti-HO-1 antibody (Stressgen Biotechnologies, Corp., Victoria, BC, Canada) or anti-Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Super Signal Femto (Pierce, Rockford, IL).

Statistical Analysis

Data are presented as the mean ± SE. The significance of the difference in means was determined by Mann–Whitney's U-test. $P < 0.05$ was considered significant.

RESULTS

Iron Accumulation in Ordinarily Fed Core Gene Transgenic Mice

The core gene transgenic mice develop HCC after an incubation period of approximately 16 months, in the absence of inflammation [Moriya et al., 1998]. During the incubation period, there is augmentation of oxidative stress with a concomitant activation of antioxidants and development of DNA damage in the liver [Farinati

et al., 1995]. For mice fed with normal chow, the concentration of total iron in the liver was higher in the core gene transgenic mice than in the control mice, and the difference became significant after the age of 12 months (Fig. 1A). The level of hepcidin mRNA, the product of which maintains iron homeostasis by a direct inhibition of ferroportin [Muckenthaler, 2008], was significantly higher in the core gene transgenic mice than in the control mice at the age of 3 and 15 months (Fig. 1B).

Iron Overloading to Core Gene Transgenic Mice

When the mice were overloaded with iron, the intrahepatic levels of iron markedly increased. In the short-term iron loading (for three consecutive days at the age of 6 months), the iron concentration in the iron-treated mice was more than twofold higher than that in the vehicle-treated ones in both the core gene transgenic and control mice, but there was no difference between the core gene transgenic and control mice. In the long-term iron loading (for 3 months from the age of 3 months), the iron concentration became significantly higher in the core gene transgenic mice than in the control mice (Fig. 1C). The hepcidin mRNA level was proportionally higher in the long-term iron-loaded mice than in the vehicle-treated mice and was significantly higher in the core gene transgenic than in the control mice ($P < 0.05$, Fig. 1D), suggesting that the positive feedback from iron to hepcidin is instrumental.

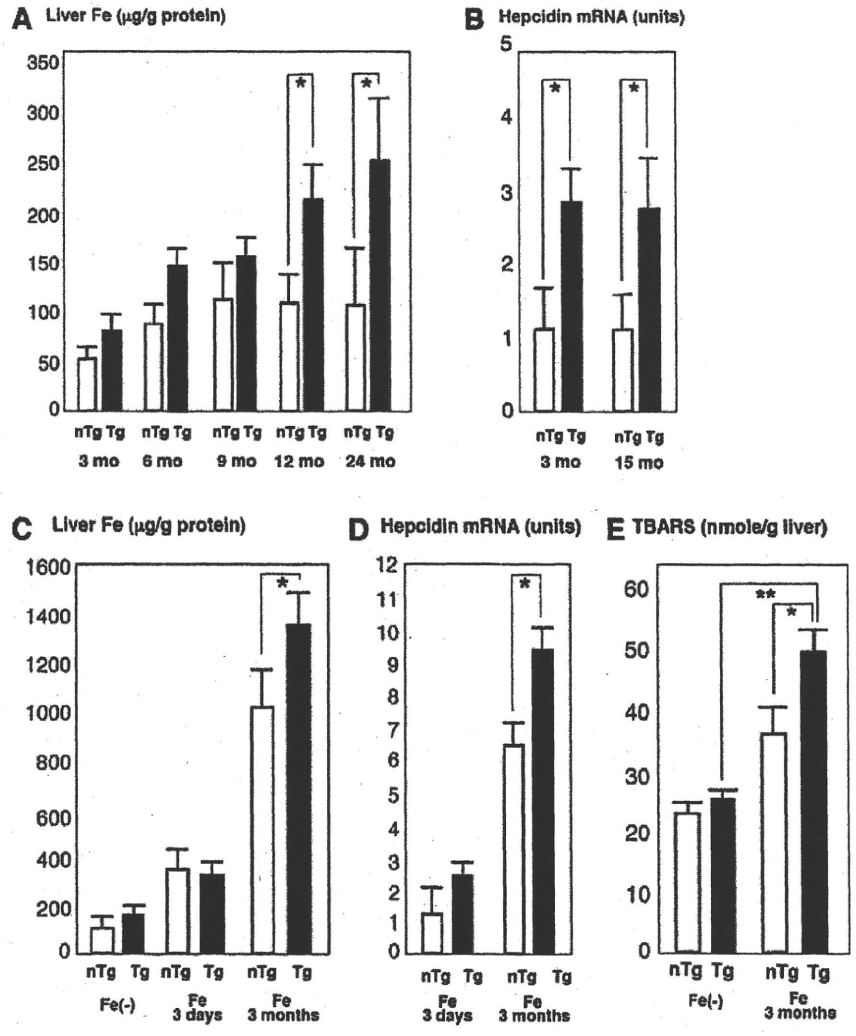

Fig. 1. **A**: Amounts of iron accumulated in the liver of mice fed with normal chow. **B**: Hepcidin mRNA level in the liver of mice fed with normal chow at 3 and 15 months. **C**: Amounts of iron in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. **D**: Hepcidin mRNA level in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. **E**: Oxidative stress in the liver of mice subjected to iron overloading for 0 or 3 months. The data represent means ± SE, n = 5 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$. nTg, nontransgenic control mice; Tg, transgenic mice; TBARS, thiobarbituric acid reactive substances.

Oxidative Stress and Iron Overloading in Mice

As described previously, ROS production in the liver of the core gene transgenic mice is already augmented at the young age, but the lipid peroxidation level is not higher than that in the control mice due to the concomitant activation of antioxidant system [Moriya et al., 2001]. This was also the case in the current experiment: there was no significant difference in the ROS level at the age of 6 months between the core gene transgenic and control mice that were treated with the vehicle, as determined by TBARS (Fig. 1E). However, after the long-term iron treatment, ROS levels in the liver of the core gene transgenic mice became significantly higher than that in the control mice (Fig. 1E, $P < 0.05$). After the long-term iron treatment, the AP site index, a marker for nuclear DNA damage, became significantly higher in the core gene transgenic mice than in the vehicle-treated core gene transgenic mice (5.2 ± 0.6 vs. 3.9 ± 0.3 nmol/g liver, $P < 0.05$), showing that iron overloading facilitates nuclear DNA damage through oxidative stress augmentation.

Impact of Iron Overloading on Antioxidants

The effect of long-term iron overloading on antioxidants was evaluated by real-time PCR analysis (Fig. 2). As already reported [Moriya et al., 2001], the levels of antioxidant enzymes, such as catalase, GST, or SOD1, were higher in the core transgenic mice than in the control mice before iron overloading. The levels of antioxidant mRNAs were higher in the long-term iron-treated mice than in the vehicle-treated mice. However, the magnitude of augmentation by iron overloading was different among the antioxidant genes. While catalase and GST genes were significantly more enhanced in the core gene transgenic mice than in the control mice by iron overloading ($P < 0.05$), there was less augmentation in the expressions of HO-1 and NQO-1 genes in the core gene transgenic mice than in the control mice. The level of HO-1 mRNA in the long-term-treated mice was significantly higher in the control mice than in the core gene transgenic mice ($P < 0.05$, respectively), in contrast to that of catalase, GST, or SOD1 gene. The level of NQO-1 mRNA in the liver was lower in the core gene transgenic than in the control mice, although the difference was not statistically significant.

To confirm this observation, the protein level of HO-1 was determined by Western blotting. As shown in Figure 3A, there was only a marginal expression of HO-1 protein in the untreated core gene transgenic and nontransgenic mice. After the short-term iron overloading, there was a marked induction of HO-1 protein but there was no significant difference between these two. However, after the long-term overloading, there was an attenuation in the levels of HO-1 protein in both the core gene transgenic and control mice; in particular, the HO-1 level was lower in the core gene transgenic mice than in the control mice. Thus, the long-term in vivo iron overloading compromised some antioxidants, such as HO-1 and NQO-1, which may lead to an augmentation of oxidative stress in HCV infection.

HO-1 and Iron in Cultured Cells

HepG2 cells expressing the core protein were treated with hemin as described in the Materials and Methods

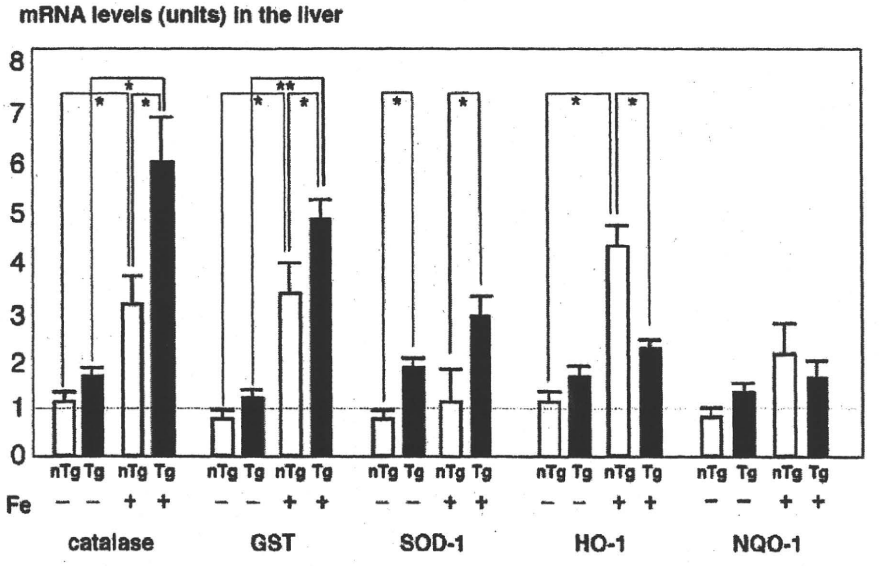

Fig. 2. Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. Levels of mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe−). Mice were sacrificed at 6 months and liver tissues were subjected to determination. The data represent means ± SE, n = 5 in each group. $^*P < 0.05$, $^{**}P < 0.01$. nTg, nontransgenic control mice; Tg, transgenic mice; GST, glutathione-*S*-transferase: SOD1, superoxide dismutase 1; HO-1, heme oxygenase 1; NQO-1, NAD(P)H dehydrogenase, quinone 1.

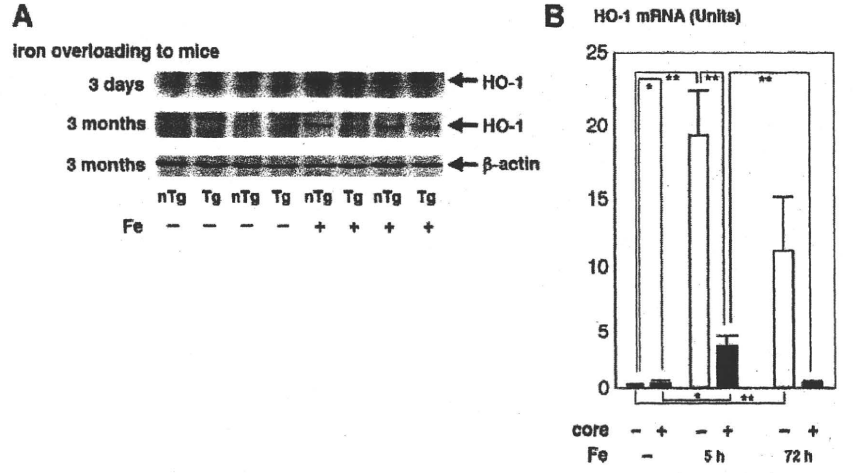

Fig. 3. A: Levels of HO-1 protein determined by Western blotting in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. B: Levels of HO-1 mRNA in core-protein-expressing or nonexpressing HepG2 cells subjected to iron loading for 0, 5, or 72 hr. nTg, non-transgenic control mice; Tg, transgenic mice; HO, heme oxygenase.

Section. Before the treatment, the HO-1 mRNA level was significantly higher in the core-expressing cells than in the control HepG2 cells (Fig. 3B, 1.00 ± 0.33 vs. 0.19 ± 0.10 arbitrary units, $P < 0.05$). After the short-term iron treatment (for 5 hr), the HO-1 mRNA levels increased in both cell lines but it was significantly higher in the control cells than in the core-expressing cells (Fig. 3B, $P < 0.01$). After the long-term iron treatment (72 hr), there was a decrease in the HO-1 mRNA levels in both cell lines compared with those after the short-term iron treatment, but the magnitude of decrease was marked in the core-expressing cells ($P < 0.05$, 5 hr core (+) vs. 72 hr core (+)). Thus, the core protein compromised the iron-induced enhancement of HO-1, and such an effect of the core is more prominent in the long-term iron overloading than in the short-term one. Thus, both in mice and cultured cells, the iron-induced induction of HO-1 was compromised by the presence of the core protein. Similar to the iron-overloading experiment in mice, iron treatment induced ROS production in cultured cells to a greater extent in the core-expressing cells than in control cells (data not shown).

Absence of Nrf2 Involvement in HO-1 Impairment by the Core Protein

To explore the mechanism underlying the differential responses to iron overloading in antioxidant gene expressions, the intracellular distribution of a transcription factor, Nrf2, which regulates the expression of HO-1 [Srisook et al., 2005; Farombi and Surh, 2006] was examined. For this analysis, the liver tissues from the core gene transgenic and control mice, either iron-overloaded or not, were subjected to subcellular fractionation, followed by detection by Western blotting. However, there was no decrease or rather an increase in the Nfr2 level localized in the nuclear fraction after the iron-overloading treatment in the core transgenic mouse liver (Fig. 4A), indicating that the attenuation of HO-1 expression is not dependent on Nrf2. Finally, no interaction was observed between the core protein and the Nrf2 protein as determined by coimmunoprecipitation using cultured cells (data not shown). Because transcription factors, other than Nrf2, such as AP-1 and NF-κB, may be responsible for the HO-1 gene expression [Ferrándiz and Devesa, 2008], and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the attenuation of HO-1 expression, changes in expression levels of these factors were determined by the real-time PCR. AP-1 was activated by the core protein while NF-κB was not in the liver of transgenic mice [Tsutsumi et al., 2002]. With the administration of iron, mRNA levels of AP-1 and NF-κB1 were increased slightly both in core gene transgenic and control mice (Fig. 4B), thereby not explaining the attenuation of iron-induced HO-1 induction by the core protein. Bach1 expression level was not changed significantly by iron administration in the core gene transgenic mice (Fig. 4B), negating the possibility that this repressor acts to inhibit the induction of HO-1 gene expression by iron.

DISCUSSION

Chronic hepatitis C is characterized by its prominent augmentation of oxidative stress [Choi and Ou, 2006; Fujita et al., 2008; Moriya et al., 2001]. Iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [Chapoutot et al., 2000; Moriya et al., 2001; Choi and Ou, 2006; Fujita et al., 2008]. In this study, iron was accumulated in the liver of the HCV core gene transgenic mice, which is destined to develop HCC after a certain period with increased

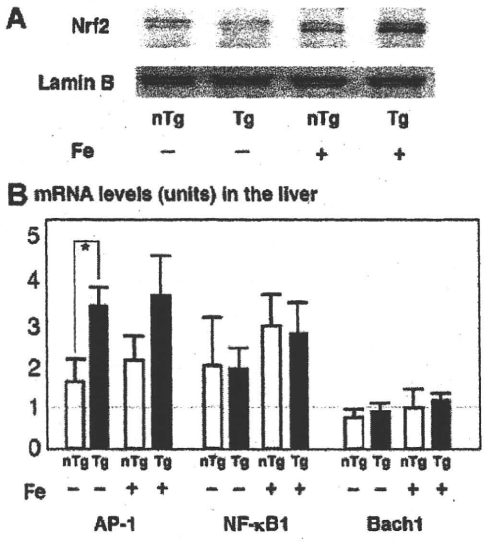

Fig. 4. **A**: Intracellular localization of Nfr2 in the liver of mice subjected to iron overloading for 0 or 3 months. The nuclear fraction was isolated from liver tissues and subjected to Western blotting with antibody to Nrf2 or lamin B (control). Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. **B**: Levels of transcription factor mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe−). Mice were sacrificed at 6 months and liver tissues were subjected to determination. The data represent means ± SE, n = 5 in each group. $^*P < 0.05$. nTg, nontransgenic control mice; Tg, transgenic mice; AP-1, activator protein-1; NF-κB1, nuclear factor-kappa B1; Bach1, BTB and CNC homology 1.

oxidative stress and steatosis [Moriya et al., 1998, 2001]. Some of the key antioxidant enzymes, HO-1 and NQO-1, were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or noniron-overloaded core gene transgenic mice.

The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates well the observation in chronic hepatitis C patients [Farinati et al., 1995; Chapoutot et al., 2000; Kato et al., 2001]. Recently, it has been reported that the expression level of hepcidin, which regulates iron metabolism by inhibiting iron absorption from the intestine and utilization from the reticuloendothelial system [Muckenthaler, 2008], is decreased in the liver of mice that are transgenic for the entire HCV genome [Nishina et al., 2008] or hepatitis C patients [Fujita et al., 2007], and is implicated in iron accumulation. ROS overproduction in the liver of the entire HCV genome transgenic mice is suggested to be responsible for the decrease in the hepcidin level [Nishina et al., 2008]. In the current study, however, the level of hepcidin mRNA was significantly higher in the liver of the core gene transgenic mice than that of the control mice despite the augmentation of oxidative stress. In the current model, hepcidin seems to be instructive in the regulation of iron homeostasis and cannot be a cause of hepatitis-C-associated iron accumulation. The reason for this dissociation between the two HCV mouse models is unclear, but the difference in the transgene construct may account for it: the core gene only or the full coding sequence [Moriya et al., 1998; Nishina et al., 2008].

It is interesting that the induction of HO-1 by iron overloading was compromised by the presence of the core protein, while the HO-1 mRNA level was higher in the core gene transgenic mice than in the control mice before iron treatment. This was also observed in the case of another antioxidant, NQO-1 [Nioi and Hayes, 2004], although its induction by iron in the control mice was not as marked as that of HO-1. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves pro-oxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted to bilirubin in mammals, both of which have been known to have very strong antioxidant activities [Stocker et al., 1987]. Thus, HO-1 is an antioxidant defense enzyme that converts potentially toxic heme into antioxidants. In addition, HO-1 has been also suggested to be a central antioxidant under the condition of glutathione depletion [Oguro et al., 1998]. Therefore, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly, in the case of iron overload, although it is hard to compare the abilities of antioxidants quantitatively [Srisook et al., 2005; Farombi and Surh, 2006]. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection. There have been some contradicting reports on the interaction between HCV and HO-1. Abdalla et al. [2004] reported that the HCV core protein down-regulated HO-1 expression, while Ghaziani et al. [2006] insist that HCV proteins including the core up-regulates HO-1 expression. HO-1 has recently been shown to suppress the propagation of HCV replicons [Zhu et al., 2008] and might be an essential molecule in the pathogenesis of HCV infection.

The mechanism underlying the compromise of iron-induced HO-1 gene augmentation by the core protein is unclear. HO-1 gene has a regulatory element that is controlled by a transcription factor Nrf2 stress [Srisook et al., 2005; Farombi and Surh, 2006]. Therefore, if the subcellular distribution of Nrf2 was altered, that is, nuclear transport were disturbed, it would explain this compromised phenomenon. However, this was not the case in the present experiment. The fact that the GST gene, the expression of which was augmented substantially by the iron overloading, also has a binding site for Nrf2 also negates the involvement of Nfr2 in the compromise by the core protein of iron-induced HO-1 gene augmentation. It has been suggested that complex intracellular signaling cascades mediate the expression of HO-1 in response to external stimuli. Transcription factors, other than Nrf2, such as AP-1 and NF-κB [Ferrándiz and Devesa, 2008] and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the HO-1 gene expression, but these factors did not explain

the current attenuation of HO-1 by the core protein under iron stimulation. Identification of the mechanism may lead to the development of new therapeutic devices with the relief of the core-induced compromise of the iron-induced augmentation of HO-1, which may strengthen the antioxidant system and suppress HCV replication.

ACKNOWLEDGMENTS

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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**.

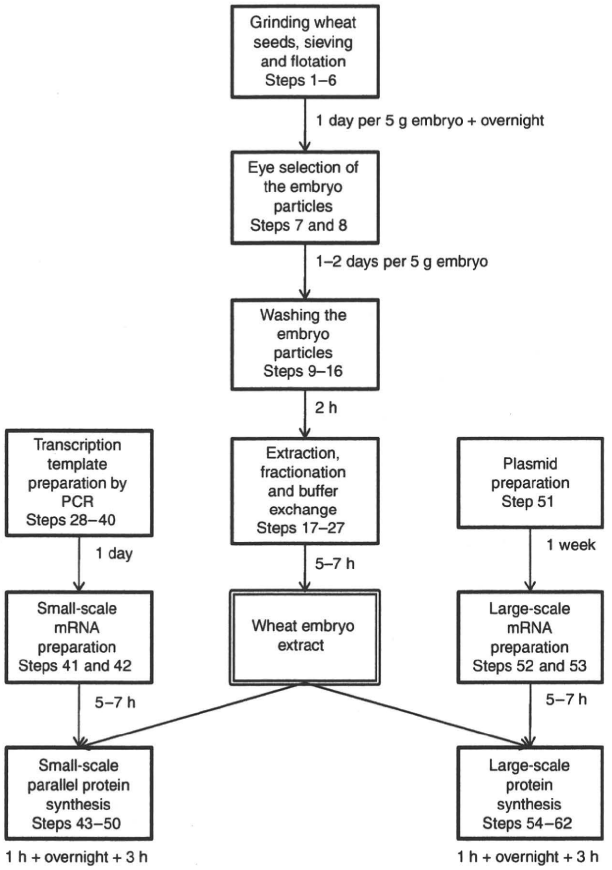

Figure 1 | Summary of steps involved in the procedure.

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

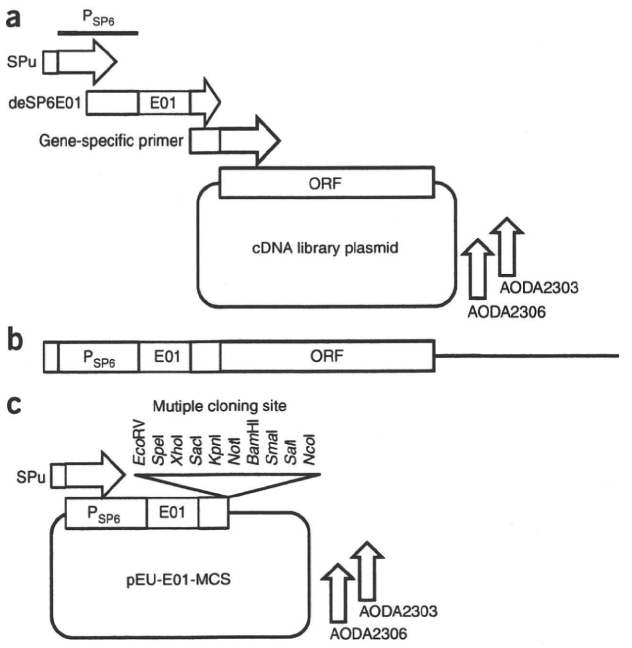

Figure 2 | Schematic representations of DNA molecules. (**a**) Positions of the primers used for the two PCR amplifications are indicated with a schematic representation of a typical cDNA library plasmid clone. The two downstream primers hybridize near the plasmid origin, and thus the library vector 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.

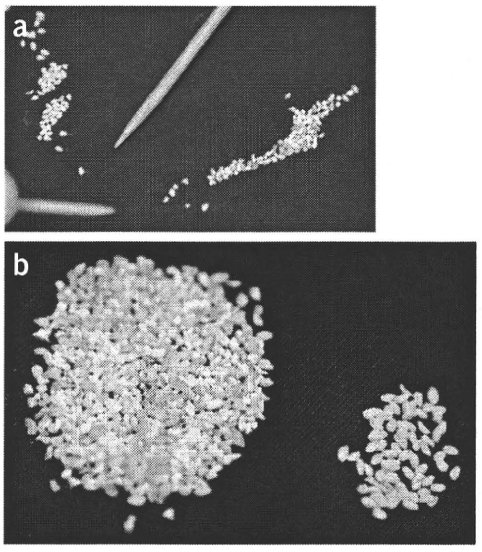

Figure 3 | Selection of good embryo particles by eye. (**a**) Selecting good particles using a toothpick. (**b**) Good particles (left) and bad particles (right).

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

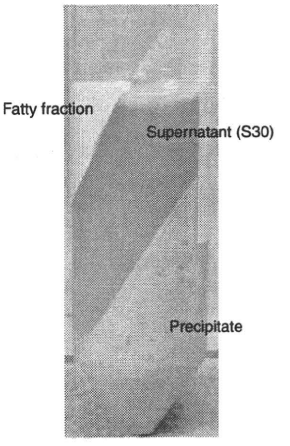

Figure 4 | An example of the sample after the first 30,000*g* centrifugation. The sample in Step 19 may be separated as in the figure (in which a conical tube is used).