Journal of Bioscience and Bioengineering
VOL. 133 No. 6, 587–595, 2022

www.elsevier.com/locate/jbiosc

Triple gene expressions in yeast, *Escherichia coli*, and mammalian cells by transferring DNA fragments amplified from a mother yeast expression plasmid

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Received 22 December 2021; accepted 4 March 2022
Available online 2 April 2022

Escherichia coli*, *Saccharomyces cerevisiae*, and mammalian culture cells are standard host organisms for genetic engineering and research, thus various plasmid vectors have been developed. However, the vectors are designed only for a single host owing to their host-specific genetic elements such as promoters and selection markers. In this study, we developed a yeast expression plasmid that enables the expression of the same gene in *E. coli* and mammalian cells via the transfer of PCR products amplified from the plasmid as a template. The yeast plasmid YHp26352 was constructed to contain the following regions sequentially: yeast *TDH3* promoter (*TDH3*p), red fluorescent protein (eEmRFP), SV40 terminator (SVpA), *E. coli* origin (ori), ampicillin resistant gene (AmpR), mammalian cytomegalovirus promoter (CMVp), *E. coli srlA* promoter (*srlA*p), and yeast selection marker *URA3*, which expressed eEmRFP in yeast. To express eEmRFP in mammalian cells, an end-promoter DNA fragment encompassing the eEmRFP-SVpA-ori-AmpR-CMVp region was amplified by PCR and directly used for transfection to mammalian culture cells, resulting in gene expression in mammalian cells through non-homologous end joining. Homologous recombination-mediated circularization was carried out for *E. coli* cloning and expression by attaching a short overlapping sequence to the 5′-end of a PCR primer, which was used to amplify the eEmRFP-SVpA-ori-AmpR-CMVp-*srlA*p fragment, after which *E. coli* transformation was performed. Proof-of-concept experiments were performed by expressing GFP-fused human synaptobrevin VAMP1, and wild-type and codon-changed CLuc luciferase genes in yeast, *E. coli*, and HEK293 cells. This is the first all-in-one plasmid applicable for expression in three host organisms.

[**Key words:** *Saccharomyces cerevisiae*; *Escherichia coli*; HEK293; Homologous recombination; Non-homologous end joining; End-promoter; YHp; Gene synthesis; Codon optimization]

Many organisms such as bacteria, yeasts, and insect and mammalian cells, are used as recombinant DNA expression hosts for research and application. To express a desired gene in different hosts, the plasmid should be equipped with organism-specific expression machinery such as promoters and terminators, and oftentimes selection markers and replication sequences. Generally, *Escherichia coli* plasmid vectors are used for initial cloning, after which an inserted gene is cloned to construct an expression plasmid designed for the desired host organism a process known as sub-cloning. However, these expression plasmids can only be applied to the corresponding host organisms. For example, commercially available plasmids such as pET, pYES, and pcDNA are used specifically in *E. coli*, *Saccharomyces cerevisiae*, and mammalian cells, respectively (1–6). Nevertheless, the vector exchange systems have been developed to change the expression host after cloning in *E. coli*. For example, gateway cloning utilizes a site-specific recombination enzyme to exchange a plasmid insert with the destination vectors (7). Moreover, Gibson assembly, Golden gate assembly, and In-Fusion cloning kits are also available to reduce cloning efforts in *E. coli* (8–10). Regardless, the availability of various subcloning tools suggests that *E. coli* plasmid construction is still a time-consuming task for gene engineering laboratories.

Shuttle plasmids are also known to manipulate gene in different hosts. For example, pNCMO2, pAUR123, and pEBMulti are shuttle vectors between *E. coli-Brevibacillus*, *E. coli-S. cerevisiae*, and *E. coli*-mammalian cells, respectively (11–13). These shuttle plasmids are usually used only for cloning without gene expression in *E. coli*, and subsequently transferring the plasmid to a different host for gene expression. If the same gene can be expressed in different hosts with little effort, genetic and protein analyses can be more easily facilitated.

For example, the mammalian α-synuclein gene that causes neurodegenerative diseases has been studied by expression in *E. coli* (14,15), *S. cerevisiae* (16,17), human culture cells (18,19), and

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2022.03.002

TABLE 1. Strains and plasmids.

Strain	Genotype and plasmid structure	Reference and remarks
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachamann et al. (36)
RAK3599	*MAT***a**/α *ura3Δ0/URA3 LEU2/leu2Δ0*	Nonklang et al. (34)
RAK17606	*MAT***a**/*MAT***a** *ura3Δ::LYS4/ura3Δ::LYS4 his3/his3 lys4/lys4 cir*^{0} YHp[*TDH3*p-yEmRFP-*URA3*] (YHp17606)	Misumi et al. (33)
RAK20326	*MATα ho trp1 his3 leu2-3,112 pho3-1 pho5-1 can1 cir*^{0}	BY1847 (NBRP)
RAK20616	*MATα ho trp1 his3 leu2-3,112 pho3-1 pho5-1 can1 cir*^{0} YHp[*LEU2-TDH3*p-yEmRFP-*URA3*] (YHp20616)	This study
RAK21140	*MAT***a**/*MAT***a** *ura3Δ::LYS4/ura3Δ::LYS4 HIS3/his3 lys4/lys4 cir*^{0}	Misumi et al. (33)
RAK22598	*MAT***a**/*MAT***a** *ura3Δ::LYS4/ura3Δ::LYS4 his3/his3 lys4/lys4 cir*^{0} YHp[*TDH3*p-eEGFP-*URA3*] (YHp22598)	This study
RAK22601	*MATα ho trp1 his3 leu2-3,112 pho3-1 pho5-1 can1 cir*^{0} YHp[*LEU2-GAL1*p-DsmCherry-bG lopA/*GAL10*p-eEGFP-SVpA-CMVp-*URA3*] (YHp22601)	This study
RAK22714	DH5α strain containing pAmp-srlAp-eEmRFP	Nakamura et al. (31)
RAK26350	*MAT***a**/*MAT***a** *ura3Δ::LYS4/ura3Δ::LYS4 HIS3/his3 lys4/lys4 cir*^{0} YHp[*TDH3*p-eEmRFP-ori-AmpR-*srlA*p-*URA3*] (YHp26350)	This study
RAK26352	*MAT***a**/*MAT***a** *ura3Δ::LYS4/ura3Δ::LYS4 HIS3/his3 lys4/lys4 cir*^{0} YHp[*TDH3*p-eEmRFP-SVpA-ori-AmpR-CMVp-*srlA*p-*URA3*] (YHp26352)	This study
RAK26396	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cir*^{0}	BY4741 *cir*^{0}
RAK28849	*MAT***a**/*MATα his3Δ1/his3Δ1 lue2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ 0 cir*^{0}	BY4743 *cir*^{0}
RAK28872	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cir*^{0} YHp[*GAL10*p-eEmRFP-SVpA-ori-AmpR-*srlA*p-*URA3*] (YHp28872)	This study
RAK29360	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YHp[*TDH3*p-eEGFP-SVpA-ori-AmpR-CMVp-*srlA*p-*URA3*] (YHp29360)	This study
RAK32834	*MAT***a**/*MATα his3Δ1/his3Δ1 lue2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ 0 cir*^{0} YHp[*TDH3*p-eEGFP-ehVAMP1-SVpA-ori-AmpR-CMVp-*srlA*p-*URA3*] (YHp32834)	This study
RAK33792	*MAT***a**/*MATα his3Δ1/his3Δ1 lue2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ 0 cir*^{0} YHp[*TDH3*p-hCLuc-SVpA-ori-AmpR-CMVp-*srlA*p-*URA3*] (YHp33792)	This study
RAK33793	*MAT***a**/*MATα his3Δ1/his3Δ1 lue2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ 0 cir*^{0} YHp[*TDH3*p-eCLuc-SVpA-ori-AmpR-CMVp-*srlA*p-*URA3*] (YHp33793)	This study

mice (20). DNA repair genes have also been studied in *E. coli*, yeasts, and mammals because of their highly conserved sequences from prokaryotes to humans (21–23). Selecting of host organisms is often required in genetic engineering to produce recombinant proteins. Accordingly, various organisms are used as protein production hosts, including *E. coli*, *Bacillus*, *Corynebacterium*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, and insect and mammalian culture cells. In all cases, plasmid vector exchange must be performed for expression in each host. If gene expression in each organism can be performed easily from a single plasmid, genetic analyses and selection of protein production hosts become simple. In this study, we developed a novel plasmid and gene expression system applicable to different host organisms by placing host-specific promoters downstream of a gene of interest, referred to as the end-promoter layout.

As an initial host for cloning and expression, we selected the yeast *S. cerevisiae* because it allows easy plasmid construction and gene synthesis through homologous recombination (HR). A simple and accurate oligonucleotide assembly method can be used for gene synthesis in yeast (24–27). Both sets of forward- and complementary-strand oligonucleotides of a gene are mixed and directly used to transform yeast with a linearized vector plasmid. This yeast oligonucleotide assembly method does not require any preparation of DNA processing enzymes or PCR assembly, which often produces mismatches and deletions (28).

For gene expression in mammalian cells, the mammalian promoter and terminator should be attached to a gene of interest. Previously, we successfully expressed a gene in mammalian cells by transfecting a linear DNA fragment amplified by PCR (29). Subsequently, linear DNA fragments that were introduced into mammalian cells were circularized by non-homologous end joining (NHEJ) (30). If a mammalian promoter is placed at one end and the other end is a start codon of a gene of interest, transfection of the DNA fragment containing a mammalian promoter and a coding sequence can be joined, allowing expression in mammalian cells.

For gene expression in *E. coli* from a yeast plasmid, PCR-mediated cloning, known as the one-round PCR method, can be applied (31). This method utilizes a 12-bp overlapping sequence for HR in *E. coli*. HR occurs efficiently between 12-bp sequences, even in a *recA* mutant, such as DH5α and HB101 strains (31). Similar to the concept of mammalian cell expression, circularization of a linear DNA fragment with a *E. coli* promoter at the end is used to join a coding sequence of interest.

In the proof-of-concept experiments, synthesized *E. coli* codon-optimized human vesicle-associated membrane protein 1 gene (ehVAMP1), the so called synaptobrevin, and wild-type or codon-changed *Cypridina* luciferase genes were expressed in yeast, *E. coli*, and HEK293 cells. Here we show that ehVAMP1 localization analysis can be performed on the three host cells. In addition, the expression of wild-type and *E. coli* codon-optimized *Cypridina* luciferase gene was compared among *E. coli*, yeast, and human cells. Thus, the plasmids constructed in this study could be useful for gene expression analyses in different host organisms and genetic analyses of different host organisms.

MATERIALS AND METHODS

Yeast and *E. coli* strains The *S. cerevisiae* and *E. coli* strains used in this study are listed in Table 1. The RAK numbers denote our laboratory stock names. Yeast strains were cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or drop-out SD media (0.17% yeast nitrogen base without amino acids and ammonium sulfate [United States Biological, Salem, MA, USA], 0.5% ammonium sulfate, 2% glucose, and necessary nutrients) at 28–30 °C (32). SD-U and SD-L media lacked uracil or leucine, respectively, with the necessary nutrients. The *E. coli* media were used LB (0.5% yeast extract, 1% peptone, and 0.5% NaCl) and LB supplemented with 1% sorbitol (LBsor). *E. coli* cells were cultured at 37 °C with shaking. LBamp plates (LB containing 1.5% agar and 100 μg/mL ampicillin) were also used for *E. coli* transformant selection.

For yeast transformation, *S. cerevisiae* competent cells were prepared as previously reported (33). Briefly, yeast cells were incubated in YPD overnight, and 1 mL of the culture was mixed with 9 mL fresh YPD and incubated for 5 h at 30 °C with shaking. Then, the cultured cells were centrifuged at 1660 ×*g* for 1 min, washed once with 10 mL sterile water, suspended in 200 μL of water, and mixed with 500 μL of the

transformation solution (prepared by mixing of 2400 μL of 60% polyethylene glycol 3350 [Sigma P4338; Sigma-Aldrich, St. Louis, MO, USA], 100 μL of 4 M lithium acetate, and 200 μL of 5 mg/mL carrier DNA [Sigma D1626; Sigma-Aldrich]). Small portions of competent yeast cells were kept in a deep freezer, and approximately 100 μL of these were mixed with 5−7 μL of oligonucleotide mixture or 1−3 μL of insert fragments and 1−3 μL of PCR-amplified vector fragments, incubated at 42 °C for 30 min, and spread on SD drop-out plates.

For *E. coli* transformation, 20 μL of *E. coli* DH5α and HB101 competent cells prepared general method were mixed with 1 μL of PCR fragment and processed.

Mammalian culture cells HEK293 cells were obtained from the Center of Gene Research (Yamaguchi University, Japan) and maintained in RPMI-1640 supplemented with 10 % (*v*/*v*) fetal bovine serum and antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C (29).

HEK293 cells were transfected as described previously (29). Briefly, the cells were seeded at 2000 or 4000 cells/well in 200 μL medium in 96-well flat microplates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated for 20−24 h before transfection. Next, DNA transfection mixtures were prepared in a 96-well flat microplate by mixing 2.5 μL of 20 ng/μL PCR products with 2.5 μL of 50% (*w*/*v*) polyethylene glycol 3350 and 2.5 μL of 625 μg/mL baker's yeast transfer RNA (Sigma-Aldrich). The total volume was then adjusted to 25 μL with distilled water. Then, 0.25 μL of FuGENE HD (Promega, Madison, WI, USA) was mixed and incubated for 15−30 min. Finally, 20 μL of the mixture was transferred to the cell culture.

Gene synthesis and PCR The oligonucleotides assembly method has been used to synthesize genes in yeast (26). Oligonucleotides (Eurofins Genomics, Tokyo, Japan) used in the present study are listed in Supplementary Table S2. Briefly, 1 μL each of 100 μM forward and reverse oligonucleotides encompassing the genes to be synthesized were mixed with vector fragments prepared by PCR, containing the overlap sequences for HR at the ends; there were then, used for the transformation of a yeast strain.

PCR was performed using PrimeSTAR GXL (Takara Bio Inc., Shiga, Japan) or KOD FX neo-DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. For colony PCR, cells of a colony were picked and suspended in 5 μL of 20 mM NaOH and incubated at 100 °C for 10 min. Sterile water (15 μL) was then added to the suspension, after which 1 μL of the suspension was used as a template in a 10-μL PCR reaction mixture (33).

The concentrations of PCR-amplified DNA were measured using a Qbit fluorometer (Molecular Probes, Eugene, OR, USA) and the DNA sequence was confirmed by PCR amplification of the region. DNA sequencing was performed by Eurofins Genomics.

Plasmid Plasmids constructed in this study are listed in Table 1 and the Supplementary Table S1. Many of these plasmids are maintained in yeast cells. For the plasmid construction, total DNA of yeast strains isolated by colony PCR method as described above or the purification method with Zymolyase 100T (33,34) was used as a template to produce PCR-amplified DNA fragments. The DNA fragments amplified by PCR were used directly for the transformation without purification.

YHp20616 *LEU2* selection marker was inserted to the upstream of *TDH3*p-yEmRFP of the YHp17606 (33). The *LEU2* gene fragment was amplified using D+546(-40)-ScLEU2-402 and ScTDH3-698(+40)c-ScLEU2+1095c primers and the total DNA of RAK3599 strain as a template (34). Two vector DNA fragments were amplified by PCR using two pairs of the primer, 2micro2816c(D+546c)/2micro2899(REP2+30c), and TDH3-698(50)/2micro3329c(FLP1+30c), and the total DNA of RAK17606 strain as a template (33). The three DNA fragments were mixed and used to transform the RAK20326 yeast strain. Transformants were selected on an SD-L plate, resulting in the construction of RAK20616 strain bearing YHp20616 plasmid.

YHp22598 *E. coli* codon-optimized EGFP (a green fluorescent protein, eEGFP) was synthesized using the oligonucleotide assembly method (33,35). Oligonucleotides designed for eEGFP (Supplementary Table S2) were mixed with vector DNA fragments prepared by PCR using primer pairs of eEGFP+39c-TDH3-1c/2micro6021(FLP1+452) and FLP1+600c/eEGFP+687-711-YEpURA3+880c, and the RAK17606 DNA as a template, and used to transform the RAK21140 yeast strain (33). The transformants were selected on an SD-U plate. The eEGFP sequence was confirmed by sequencing, resulting in the RAK22598 strain bearing YHp22598 plasmid.

YHp22601 To simultaneously express EGFP and mCherry in yeast, YHp22601 was constructed. Two vector DNA fragments were amplified by PCR using two pairs of primers, LEU2+1095c(35)/FLP1+452(50) and FLP1+600c/URA3+771c(35), and the RAK21132 strain as a template. An insert fragment containing GAL1p-Dsm Cherry-bGlopA/GAL10p-EGFP-SVpA-CMVp was amplified using ScLEU2+1095(-30)-bGlopA+190c and URA3+650 primers and the RAK13978 strain (Supplementary Table S1) as a template. These three PCR fragments were mixed and used to transform the RAK20326 yeast strain. The transformants were selected on an SD-L plate.

YHp26350 To insert the *E. coli* origin (ori), ampicillin-resistant gene (AmpR), and *E. coli srlA* promoter (*srlA*p) to the downstream of eEmRFP of YHp17606, *E. coli* plasmid fragment was amplified using TDH3-30-eEmRFP+1/URA3+880(-30)-srlA-1c primers and the RAK22714 strain as a template (31). Two DNA fragments were amplified by PCR using two pairs of primers, TDH3-1c40/FLP1+430(40) and FLP1+600c/URA3+880c(60), and the RAK17606 yeast strain as a template. The three PCR fragments were mixed and used to transform the RAK21140 yeast strain. The transformants were selected on an SD-U plate.

YHp26352 For mammalian cell expression, SV40 poly-A terminator (SVpA) and CMV promoter (CMVp) were inserted into YHp26350. The eEmRFP-SVpA-ori-AmpR-CMVp fragment was amplified using TDH3-30-eEmRFP+1/eCMV-1c(35) primers and the RAK26254 strain (Supplementary Table S1) as a template. The *srlA*p fragment was amplified using eCMV-30-srlA-255/URA3+880(-30)-srlA-1c primers and the RAK22714 strain as a template. Two vector DNA fragments were amplified by PCR using two pairs of primers, TDH3-1c(40)/FLP1+430(40) and FLP1+600c/URA3+880c(60), and the RAK17606 yeast strain as a template. These four PCR fragments were mixed and used to transform the RAK21140 yeast strain. The transformants were selected on an SD-U plate.

YHp28872 To insert the *GAL10* promoter (*GAL10*p) into YHp26350, the *GAL10*p fragment was amplified using RAF1+546(-30)-ScGAL1-1c/eEmRFP+30c-ScGAL10-1c primers and the RAK22601 strain as a template. Two vector DNA fragments were amplified by PCR using two pairs of primers, 2micro2816c(D+546c)/2micro2899(REP2+30c) and 2micro3329c(FLP1+30c)/eEmRFP+1, and the RAK26350 yeast strain as a template. These three PCR fragments were mixed and used to transform the RAK26396 yeast strain. The transformants were selected on an SD-U plate.

YHp29360 For the expression of eEGFP, eEGFP was inserted into YHp26352. The eEGFP fragment was amplified using TDH3-30-eEGFP+1/SVpA+30c-eEGFP+711c primers and the RAK22598 strain as a template. Two vector DNA fragments were amplified by PCR using two pairs of primers, SVpA+1(35)/ScREP2+1 and TDH3-1c(45)/FLP1+1(35), and the RAK26352 yeast strain as a template. These three PCR fragments were mixed and used to transform the BY4741 yeast strain (36). The transformants were selected on an SD-U plate.

YHp32834 The eh*VAMP1* gene was synthesized using the oligonucleotide assembly method (see RAK31954 in Supplementary Information). First, the eh*VAMP1* was inserted downstream of the eEGFP of YHp29360. Next, a vector fragment and eh*VAMP1* fragment were amplified by PCR using two pairs of primers, ScTDH3-1c/ScREP2+40c and ehVAMP1mRv1+1/ScFLP1+40c(40), respectively, and the RAK31954 strain as a template. Thereafter, the eEGFP fragment was amplified using TDH3-133/ehVAMP1mRv1+25c-eEGFP+708c primers and the RAK29360 strain as a template. Finally, these three PCR fragments were mixed and used to transform the RAK28849 strain. The transformants were selected on an SD-U plate and the sequence of eEGFP-eh*VAMP1* was confirmed, resulting in the construction of RAK32834 bearing YHp32834.

YHp33792 The eEmRFP of YHp26352 was replaced with the wild-type *Cypridina noctiluca* luciferase sequence (hCLuc). hCLuc was amplified by PCR using a primer pair of TDH3p-25-hCLuc+1/SVpA+25c-hCLuc+1662c and pCL-sv plasmid [ATTO 3512034, Genbank accession number AB159608] as a template (37,38). Two vector fragments were amplified by PCR using primer pairs of SVpA+1(40)/ScFLP1+40c(40) and TDH3-1c(40)/REP2+40c(40) and DNA from RAK26352 as a template. The three fragments were finally mixed and used to transform the RAK28849 strain. The transformants were selected on an SD-U plate, and the hCLuc sequence was confirmed, resulting in the construction of RAK33792 strain bearing YHp33792.

YHp33793 *E. coli* codon-optimized *Cypridina* luciferase (eCLuc) was synthesized using the oligonucleotide assembly method with YHp26352 vector fragments. Oligonucleotides designed for eCLuc (Supplementary Table S2) were mixed together with two vector fragments prepared as described above and used for the transformation of RAK28849 strain. Transformants were selected on an SD-U plate and the sequence of eCLuc was confirmed, resulting in the construction of RAK33793 strain bearing YHp33793.

Gene expression in *E. coli* from a yeast plasmid For eEmRFP and eEGFP expression in *E. coli*, eEmRFP-SVpA-ori-AmpR-CMVp-*srlA*p and eEGFP-SVpA-ori-AmpR-CMVp-*srlA*p fragments were amplified using *srlA* promoter-sequence-attached srlA-12-eEmRFP+1 primer (5′-AAGGAGAGAACAatggtgagcaaaggtgaagaggataatatg-3′) or srlA-15-eEGFP+1 primer (5′-CTGAAGGAGAGAACAatggtgagcaaaggtgaagaactgt-3′) and the counter primer srlA-1c(35) (5′-gttctctccttcaggatttattgttttattacca-3′), and RAK26352 strain for RFP or RAK29360 for GFP as templates, respectively. The fragments were used to transform the *E. coli* HB101 strain. For luciferase gene expression in *E. coli*, the primers attached to the 12-base *srlA* promoter sequence, srlA-12-hCluc+1 (5′-AAGGAGAGAACAatgaagaccttaattcttgccgttg-3′) or srlA-12-eCluc+1 (5′-AAGGAGAGAACAatgaaaaccctgatcctggctgttg-3′), and the counter primer srlAp-1c were used to amplify CLuc-ori-AmpR-srlAp fragments from RAK33792 or RAK33793 templates, respectively. The fragments were used to transform the *E. coli* DH5α strain. For eEGFP-ehVAMP1 expression, the eEGFP-eh*VAMP1*-SVpA-ori-AmpR-CMVp-*srlA*p fragment was amplified using the *srlA* promoter-sequence-attached srlA-15-eEGFP+1 primer and the counter primer srlA-1c(35) using RAK32834 yeast strains as a template. The fragments were used to transform the *E. coli* DH5α strain.

Gene expression in mammalian cells from a yeast plasmid For eEmRFP and eEGFP expression in mammalian cells, eEmRFP-SVpA-ori-AmpR-CMVp and eEGFP-SVpA-ori-AmpR-CMVp fragments were amplified using eEmRFP+1 (5′-atggtgag caaaggtgaagaggataatatg-3′) or eEGFP+1(30) (5′-atggtgagcaaaggtgaagaactgtttacc-3′) primers and the counter primer eCMV-1c(35) (5′-ggtggcgaccggtagcgctag cggatctgacggtt-3′), and RAK26352 or RAK29360 yeast strains as templates, respectively. For luciferase expression in HEK293 cells, CLuc-SVpA-ori-AmpR-CMVp

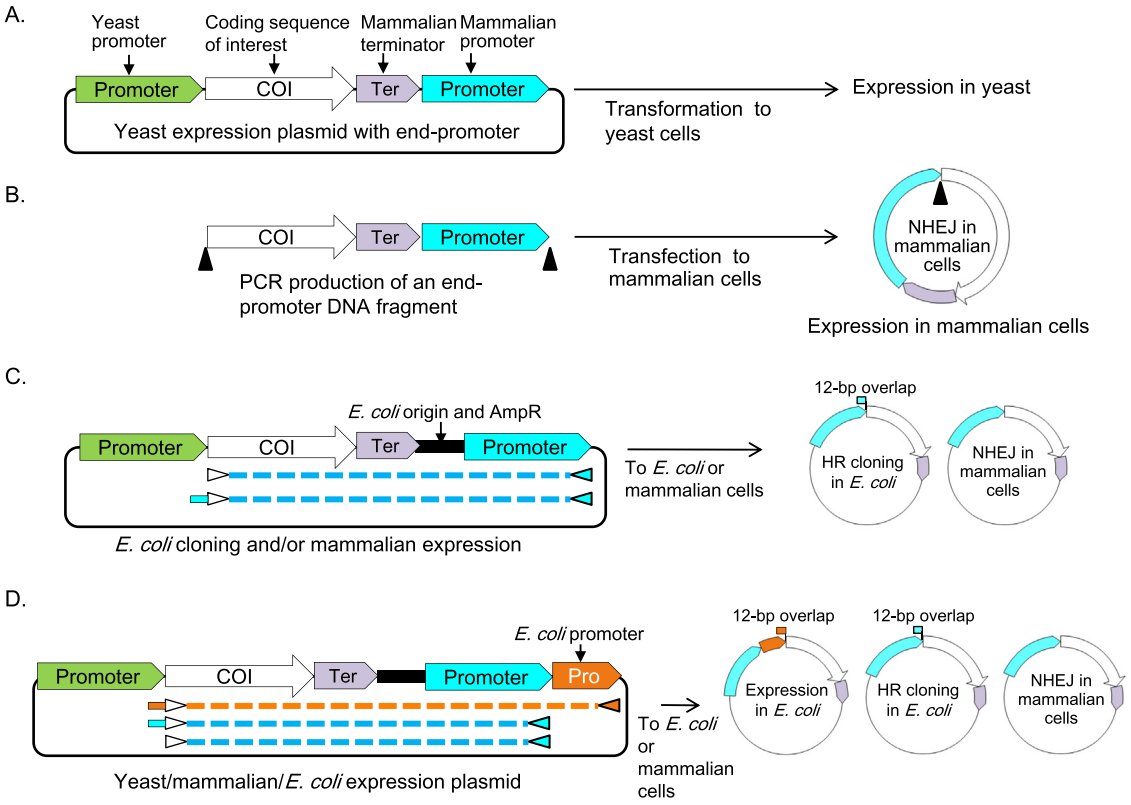

FIG. 1. Plasmid design for yeast/mammalian/*E. coli* expression. (A) Yeast expression plasmid with end-promoter construct. (B) End-promoter fragment. Both DNA ends of the fragment are joined by the NHEJ mechanism and expressed in mammalian cells. (C) Yeast expression plasmid with the end-promoter layout and *E. coli* replication origin and ampicillin-resistant gene (AmpR) for *E. coli* cloning and mammalian cell expression. The PCR product with a 12-bp overlap sequence (dotted blueline with blue bar) is amplified by PCR and used for *E. coli* transformation. The 12-bp overlap sequences at the ends are joined by HR and produced an expression plasmid for mammalian cells. The PCR product without the overlap sequence can also be used for mammalian cell expression via NHEJ. (D) The triple expression plasmid for yeast, mammalian cells, and *E. coli*. The *E. coli* promoter is placed downstream of the mammalian promoter. The PCR product with a 12-bp overlap sequence (dotted orange line with a short orange bar) is introduced to *E. coli* to form the *E. coli* expression plasmid. The mammalian expression plasmid is also constructed in *E. coli* using a PCR fragment (dotted blue line with an overlap). The mammalian cell expression fragment is also produced by PCR (dotted blue line without an overlap). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fragments were amplified by PCR and used for the transfection. A DNA fragment containing hCLuc was amplified using hCluc+1 (5′-atgaagaccttaattcttgccgttgcatta-3′)/CMV-1c (5′-ggtggcgaccggtagcgctagcgga-3′) primers and the RAK33792 (hCLuc) template, and an eCLuc DNA fragment was amplified using eCluc+1 (5′-atgaaaaccctgatcctggctgttgctctg-3′)/CMV-1c primers and the RAK33793 (eCLuc) template. For eEGFP-ehVAMP1 expression, eEGFP-eh*VAMP1*-SVpA-ori-AmpR-CMVp fragment was amplified using eEGFP+1(30)/CMV-1c primers and the RAK32834 DNA as a template. The DNA concentrations of the PCR-amplified fragments were measured, and the diluted DNA was directly used for the transfection without purification process. To calculate transfection efficiency, more than 200 cells were counted.

Microscopic observation For fluorescence microscopy observation, yeast and *E. coli* transformants were grown on SD-U and LBamp plates, respectively, and observed under an Axio Imager A1 fluorescence microscope (Carl Zeiss Meditec AG, Oberkochen, Germany) equipped with 43HE and 38HE filter sets for RFP and GFP, respectively. In addition, HEK293 cells were cultured for one day after transfection, after which fluorescence images of the transfected cells were visualized using a fluorescence microscope (AXIO Observer.Z1; Carl Zeiss Meditec) with a narrow-based excitation filter and red-shifted emission filter (Ex: 545/30, LP:570, Em: 610/75; Chroma Technology Corp, Bellows Falls, VT, USA) for RFP, and an Edow GFP BP filter, and emission filter (Ex: 470/40, LP:495, Em: 525/50; Chroma Technology) for GFP.

Luciferase assay Yeast cells were incubated in 1 mL of YPD for 24 h at 30 °C with shaking while *E. coli* cells were incubated in 1 mL of LBsor for 24 h at 37 °C with shaking. Thereafter, the cell cultures were centrifuged, and 5 μL of the supernatant was used for the luciferase assay. In *E. coli*, intracellular luciferase activity was measured. Briefly, 1 mL of *E. coli* culture was centrifuged, and the cell pellet was suspended in 100 μL sterile water, after which the cell suspension was placed on ice and sonicated using a Handy Sonic sonicator (model UR-21P; TOMY Seiko, Tokyo, Japan) for 1 min. The disrupted cell suspension was then centrifuged, and 5 μL of the supernatant was used for the luciferase assay. The luciferase activity of HEK293 cells transfected with PCR fragments was measured one-day after transfection, with the HEK293 culture supernatant being used directly for the luciferase assay. The luciferase activity was measured using *Cypridina* Luciferin (NanoLight Technologies 305; Prolume Ltd, Pinetop-Lakeside, AZ, USA). Briefly, luciferin (500 μg) was dissolved in 1 mL ethanol with 2 μL of 3 M HCl. Next, the ethanol solution was diluted to 1:750 with 100 mM Tris–HCl (pH 7.0). This Tris buffer solution was used as the luciferin working solution. Thereafter, 5 μL of supernatant was mixed with 15 μL of the luciferin working solution, and luminescence was measured using a GloMax-20/20 luminometer (Promega). Luciferase activity was determined as the relative luminescence unit (RLU) per OD_{600} and per mL of fluid for yeast and *E. coli*. For HEK293 cells, RLU was determined per mL of culture fluid.

RESULTS

Concept of PCR-mediated gene expression in *E. coli* and mammalian cells from yeast plasmid In general, a promoter must be placed in front of a coding sequence for expression in cells. However, we previously found that both ends of the PCR-amplified DNA construct were *in vivo* ligated by NHEJ in mammalian cells (30). From this, we surmised that when a promoter is placed downstream of a coding sequence, both ends of the end-promoter construct will be ligated and become circular, resulting in the expression of the coding sequence in mammalian cells (Fig. 1B). Therefore, we designed an end-promoter layout in a yeast expression plasmid for mammalian cell expression (Fig. 1A).

Next, we determined the location of the sequences of *E. coli* plasmid replication origin and a selection marker to construct a yeast/mammalian cells plasmid in *E. coli* using PCR amplification from a yeast plasmid. Our previous study demonstrated that PCR

products containing a 12-bp overlapping sequence produced circular plasmids in *E. coli* because of HR occurring between 12-bp homologous sequences (31). Therefore, we inserted an *E. coli* plasmid replication origin and selection marker AmpR between the mammalian terminator and mammalian promoter for *E. coli* cloning (Fig. 1C). After PCR amplification from a coding sequence of interest (COI) to the mammalian promoter with a 12-bp overlapping sequence (dotted blue line (in the web version) shown in Fig. 1C), the PCR product was used for the transformation to *E. coli*, which was connected by HR in *E. coli* and formed a circular plasmid.

Lastly, to express a coding sequence in *E. coli*, we installed an *E. coli* promoter downstream of the mammalian end-promoter layout (Fig. 1D). The PCR-amplified fragment from the COI to the *E. coli* promoter with 12-bp overlapping (dotted orange line (in the web version) in Fig. 1D) could be expressed in *E. coli*. These designed plasmids are available for expression in yeast as it is and in mammalian cells and *E. coli* after PCR amplification and their introduction to the host.

Based on the above concept, gene expression scenarios in different hosts is shown in Fig. 2A. COI can be obtained by PCR from a template or synthesized by the yeast oligonucleotide assembly method (24,26). Select an expression plasmid; a dual expression plasmid is used for the expression in yeast and *E. coli*, and a triple expression plasmid is used for yeast, *E. coli*, and mammalian cells. After the transformation to yeast, COI is expressed in yeast by the yeast promoter. For the expression in *E. coli*, *srlA*p (a constitutive *E. coli* promoter)-containing DNA fragment is amplified and is used for the transformation to *E. coli*, resulting in the expression in *E. coli*. For the expression in mammalian cells, CMVp (a constitutive mammalian promoter)-containing PCR fragment is amplified and used for the transfection to mammalian cells directly via the NEHJ-mediated gene expression (30) or used for the construction of a mammalian expression plasmid in *E. coli*.

Mother yeast expression plasmids The YHp yeast plasmid was selected as the mother plasmid because *S. cerevisiae* allows easy plasmid construction and gene synthesis through the efficient HR. For gene manipulation, we constructed various YHp plasmids containing several modules (Fig. 2B, Table 2), including a *TDH3* promoter, *GAL1* promoter and/or *GAL10* promoter for yeast expression, *LEU2* or *URA3* for yeast cloning, β-globin poly-A terminator or SV40 poly-A terminator and CMV promoter for mammalian expression; ori, AmpR, and *srlA* promoter for cloning and expression in *E. coli*, eEGFP, eEmRFP, hCLuc, and eCLuc for protein localization or expression analysis in yeast/*E. coli*/mammalian cell lines. Yeast terminators were not inserted to these plasmids. All plasmids were maintained as stocks of yeast strains at −80 °C. Depending on the purposes, various fragment modules were amplified by PCR from the total DNA preparations of the yeast strains and used to transform for the expression in each host.

The basic plasmids, YHp26352 and YHp29360 YHp26352 and YHp29360 are basic plasmids because they have end-promoter modules for both mammalian and *E. coli* expression. The Ura$^+$ yeast colonies harboring YHp26352 and YHp29360 expressed RFP and GFP, respectively (Fig. 3). To investigate the

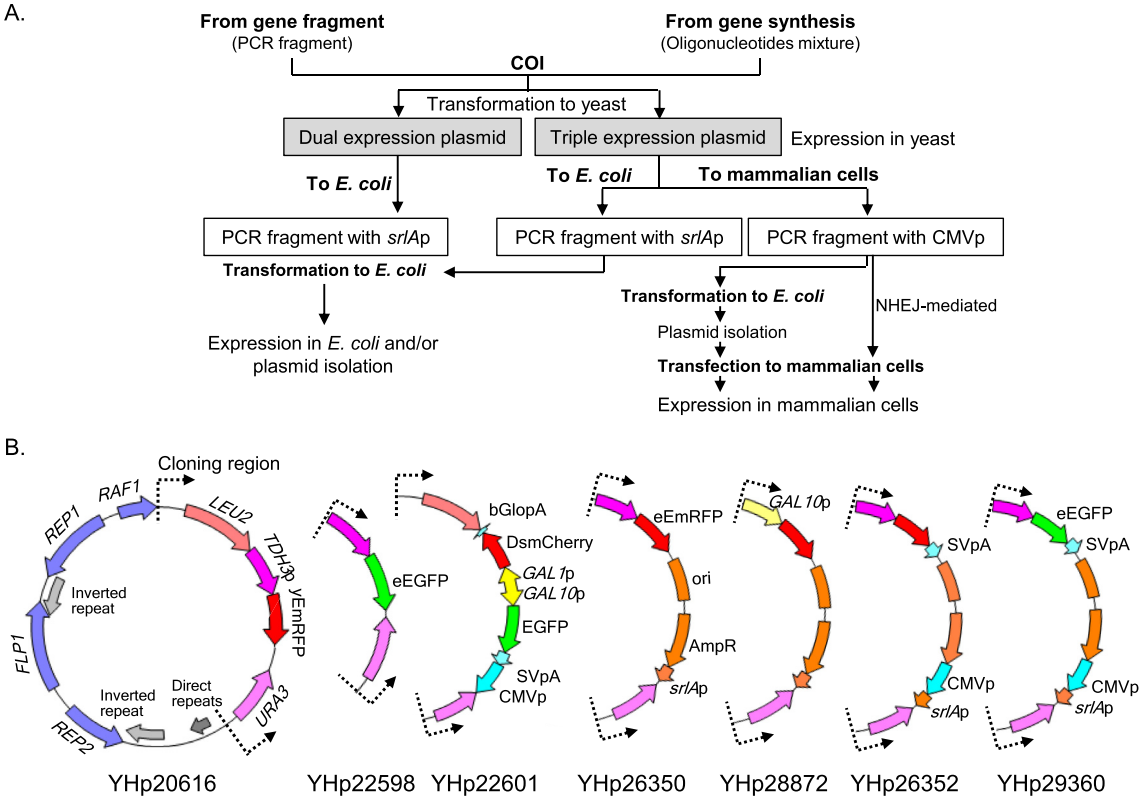

FIG. 2. Generation of expression constructs for yeast/*E. coli*/mammalian cells. (A) Flowchart of gene cloning for yeast/*E. coli*/mammalian cells expression constructs. COI can be prepared by PCR or gene synthesis in yeast. COI cloned in a mother plasmid is expressed in yeast. For *E. coli* expression, *srlA* promoter-containing fragment is amplified and used for the transformation. For mammalian cell expression, CMV promoter-containing fragment is amplified and used for the transformation to *E. coli* or the transfection to mammalian cells. (B) YHp plasmid series for expression in yeast, *E. coli*, and mammalian cells. The 2-μm plasmid backbone is shown on the left part of the dotted lines with blue arrows of coding sequences and inserted sequences are on the right side in each plasmid map. Components for expression and cloning in yeast/*E. coli*/mammalian cells are inserted (see text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

TABLE 2. Modules in YHp plasmids.

YHp plasmid no.	Yeast		Mammalian		*E. coli*			Coding sequence
	Promoter	Selection marker	Promoter	Terminator	Origin	Selection marker	Promoter	
20616	*TDH3*	*LEU2/URA3*	–	–	–	–	–	yEmRFP
22598	*TDH3*	*URA3*	–	–	–	–	–	eEGFP
22601	*GAL1/10*	*LEU2/URA3*	CMV	βGlobin/SV40	–	–	–	DsmCherry/EGFP
26350	*TDH3*	*URA3*	–	–	pUC	AmpR	*srlA*	eEmRFP
26352	*TDH3*	*URA3*	CMV	SV40	pUC	AmpR	*srlA*	eEmRFP
28872	*GAL10*	*URA3*	–	–	pUC	AmpR	*srlA*	eEmRFP
29360	*TDH3*	*URA3*	CMV	SV40	pUC	AmpR	*srlA*	eEGFP
32834	*TDH3*	*URA3*	CMV	SV40	pUC	AmpR	*srlA*	eEGFP-ehVAMP1
33792	*TDH3*	*URA3*	CMV	SV40	pUC	AmpR	*srlA*	hCLuc
33793	*TDH3*	*URA3*	CMV	SV40	pUC	AmpR	*srlA*	eCLuc

effectiveness of YHp26352 and YHp29360 for *E. coli* expression, DNA fragments were amplified using srlA-12-eEmRFP+1 primer; AAGGAGAGAACAatggtgagcaaaggtgaagaggataatatg, or srlA-15-eEGFP+1 primer; CTGAAGGAGAGAACAatggtgagcaa aggtgaagaactgt, and the counter srlA-1c(35) primer, and total DNA of RAK26352 strain (YHp26352) or RAK26360 strain (YHp29360) as templates, respectively. The PCR products were transformed into *E. coli* DH5α, and transformant colonies were picked and observed by the fluorescence microscopy (Fig. 3). The *E. coli* cells were red or green. For expression in mammalian cells, DNA fragments were amplified using eEmRFP+1 and CMV-1c primers and the DNA of RAK26352 strain or using eEGFP+1 and CMV-1c primers and DNA of RAK29360 strain as a template. The DNA fragments were directly transformed into HEK293 cells, resulting in visible fluorescence (Fig. 3). These results indicated that YHp26352 and YHp29360 plasmids expressed genes in yeast, and the transfer of the DNA fragments amplified from the plasmid templates allowed their expression in *E. coli* and mammalian cells.

Expression of a codon-optimized luciferase gene in yeast, *E. coli*, and mammalian cells Codon-optimized gene sequences are usually used for recombinant protein expression, as codon usage bias is known to affect the expression levels of genes in prokaryotes and eukaryotes (39). Therefore, to examine the expression levels of a gene with different codon sequences, *E. coli* codon-optimized and wild-type *Cypridina* luciferase genes with exactly identical amino acid sequences were expressed in yeast, *E. coli*, and mammalian cells, and their expression levels were compared.

We constructed the eCLuc sequence using the oligonucleotide assembly method in yeast. Wild-type-codon luciferase (hCLuc) was also prepared using the same vector (Tables 1 and 2; YHp33792). The luciferase activities of hCLuc and eCLuc in yeast culture supernatants were measured and compared. Results showed that hCLuc expression was higher than that of eCLuc in yeast cells (Fig. 4A). Next, the PCR fragment of hCLuc- or eCLuc-ori-AmpR-CMVp were amplified using NaOH-boiled yeast lysate as a template and used for transfection into HEK293 cells. Results revealed that the expression of hCLuc and eCLuc was similar to that in HEK293 cells (Fig. 4B), indicating that *E. coli* codon-optimized eCLuc can be used in mammalian cells similar to hCLuc. For expression in *E. coli*, PCR fragments of hCLuc- or eCLuc-ori-AmpR-CMVp-*srlA*p with a 12-bp overlap were introduced into the DH5α strain. No luciferase activity was detected in the culture supernatants of *E. coli* transformants, suggesting that *E. coli* could not secrete CLuc. However, by disrupting the transformed *E. coli* cells, hCLuc and eCLuc activities were detected at the same levels (Fig. 4C). These results suggested that yeast was sensitive to codon exchange, but HEK293 and *E. coli* were not significantly influenced by the codon-changed sequences of CLuc. We also compared the expression of yEmRFP and eEmRFP, yeast- and *E. coli*-codon optimized EmRFP, respectively, in yeast (Supplementary Fig. S1). Yeast cells expressing yEmRFP showed same red intensity as same as eEmRFP. In contrast with CLuc, EmRFP was found not to be a susceptible gene to codon exchange in yeast. Since eEmRFP expression in YHp plasmid is sufficiently high under microscopic observation, eEmRFP is available to localization analysis in yeast.

Protein localization analysis of a human gene in yeast, *E. coli*, and mammalian cells We next expressed human vesicle-associated membrane protein 1 (hVAMP1) in the three hosts. VAMP1 is a synaptic vesicle protein that is thought to play a role in vesicle targeting and fusion in all eukaryotic cells. hVAMP1 has been previously analyzed in yeast (40); therefore, we constructed *E. coli* codon-optimized h*VAMP1* (eh*VAMP1*) fused with eEGFP at the N-terminus based on YHp29360. eEGFP-ehVAMP1 expression was observed in yeast, in which the fusion protein was localized at the cell periphery and in a vesicular-like form (Fig. 5B), suggesting that hVAMP1 protein is localized to the cell membrane in yeast. Next, the same gene was expressed in *E. coli* by the transformation of the eEGFP-eh*VAMP1*-ori-AmpR-CMVp-*srlA*p PCR fragment with an overlap. The eEGFP-ehVAMP1 in DH5α was localized at the cell periphery (Fig. 5D), suggesting that the hVAMP1 protein may have an affinity for *E. coli* cell membrane. The same gene was also expressed in human HEK293 cells by transfection with the eEGFP-eh*VAMP1*-ori-AmpR-CMVp PCR fragment. The eEGFP-ehVAMP1 in HEK293 cells showed an ER/Golgi-like punctate structure similar to the published results (41,42) (Fig. 5F). The protein localization analysis of the same gene could be performed in three hosts, yeast, *E. coli* and human cells.

DISCUSSION

End-promoter construct with a host-specific promoter at the downstream of a coding sequence There are various shuttle plasmids for heterologous protein expression (3–6,11,13). For example, yeast plasmids such as YEp and YCp, are yeast-*E. coli* shuttle vectors that can be handled in both *E. coli* and yeast, allowing considerable progress in yeast research (43,44). There are similar shuttle vectors, such as yeast/bacterial/mammalian and yeast/*E. coli*/*Drosophila* triple shuttle vectors, which can be used in three hosts (45,46). However, the gene expression of these vectors is limited to a single host. Thus, although cloning can be performed in *E. coli* and yeast, gene expression is limited to mammalian cells or *Drosophila*, respectively. In the present study, we developed novel plasmids that can express a gene in yeast, *E. coli*, and mammalian cells by simply introducing PCR products into *E. coli* and mammalian cells after plasmid cloning in yeast. We believe this is the first vector based on the promoter switching concept through PCR and transformation.

We utilized two concepts that made it possible to develop a triple-expression vector. The first is the design of the end-promoter construct. We previously found that terminator-promoter-coding

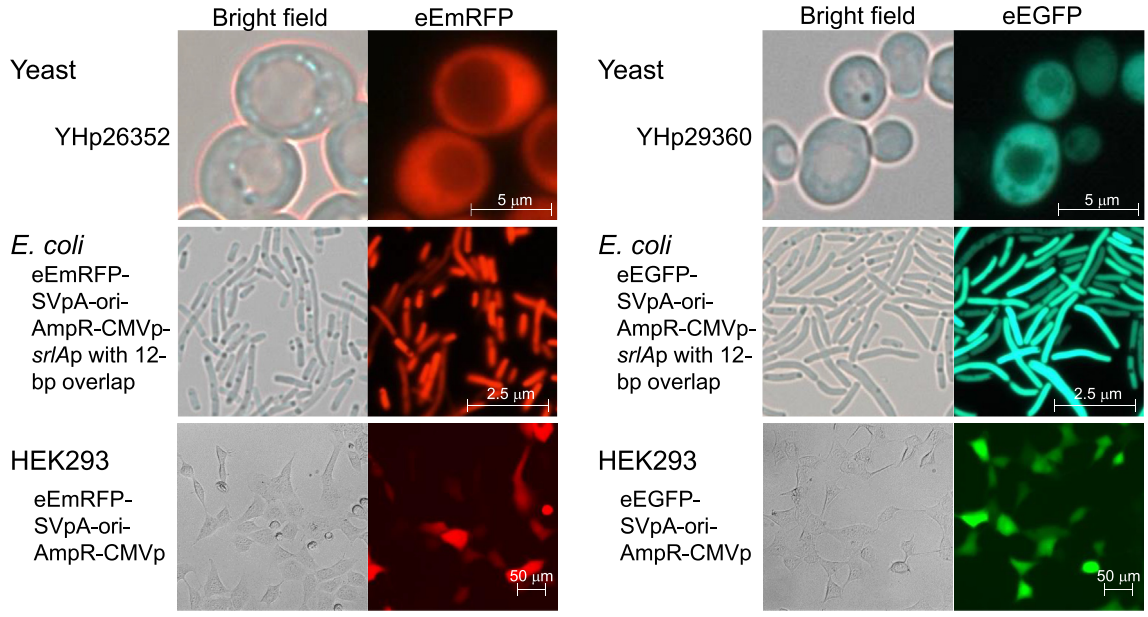

FIG. 3. Expression of the same gene sequence in yeast, *E. coli*, and mammalian cells. Plasmids or PCR fragments were introduced to yeast, *E. coli*, and human HEK293 cells. YHp26352 and YHp29360 were constructed in yeast, which showed red and green fluorescence, respectively. For *E. coli* expression, PCR fragments containing the indicated regions with 12-bp overlap sequences were introduced to *E coli*. Transformed *E. coli* cells showed red or green fluorescence. For HEK293 expression, PCR fragments encompassing the indicated region without overlap were introduced to HEK293 cells. RFP and GFP expressions were visualized by each PCR product transfection to HEK293 cells. Transfection efficiencies of RFP and GFP were 70 and 71%, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sequence fragments, named front-terminator, introduced in mammalian cells in that order formed circular like a plasmid by NHEJ (30). Similarly, a circular plasmid was constructed using a short overlap sequence in *E. coli* by HR (31). These findings inspired us to develop an end-promoter construct, where mammalian or *E. coli* promoters are placed downstream of a coding sequence, and each promoter can connect to the initiation codon of a coding sequence in mammalian or *E. coli* cells, resulting in gene expression. This concept can be applied to other hosts if there is a host-specific promoter, as well as to a plasmid with multiple end-promoters of a single host, one promoter used under specific conditions and another promoter regulated under different conditions for the expression. Thus, these vectors are useful tools for placing various promoters.

The second concept is to place the *E. coli* replication origin and selection marker between a terminator and an end-promoter for transfer from yeast to *E. coli*. By designing a short homologous

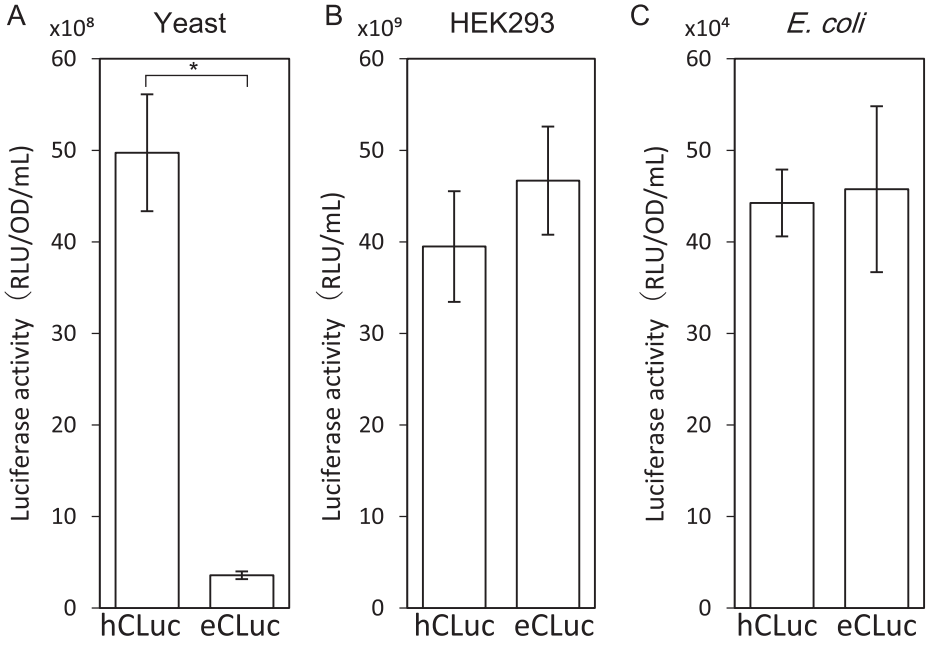

FIG. 4. Expression of wild-type and *E. coli* codon-luciferase in yeast, HEK293 cells, and *E. coli*. The wild-type hCLuc and *E. coli* codon-optimized eCLuc were expressed in the hosts, and extracellular luciferase activities were measured in yeast (A) and HEK293 cells (B), while intracellular activities were measured in *E. coli* (C). Asterisk indicates a significant difference ($P < 0.001$). Student's t-test was used to compare hCluc and eCluc. Values are means ± SD of three independent experiments in yeast and *E. coli*, and of nine independent experiments in HEK293.

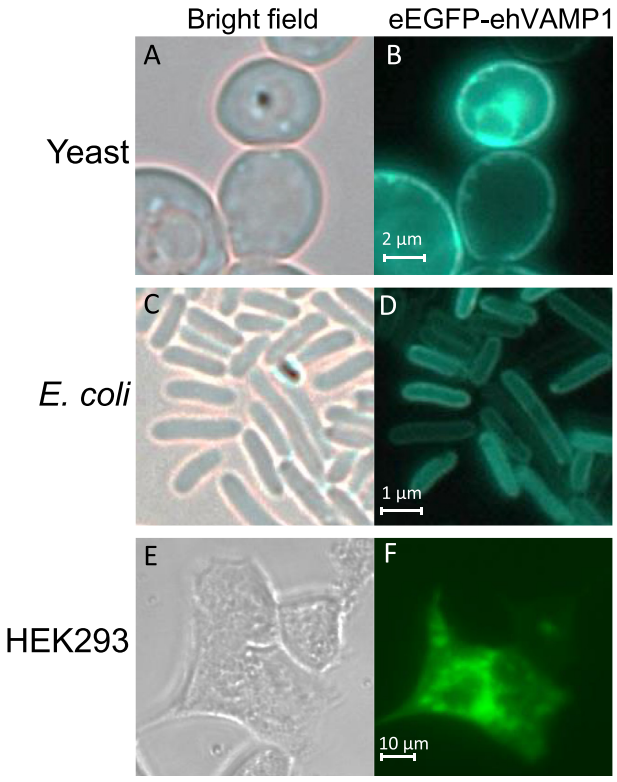

FIG. 5. Localization of ehVAMP1 in yeast, *E. coli*, and HEK293 cells. (A, B) Yeast cells bearing YHp32834 showed peripheral cell localization of the eEGFP-ehVAMP1 fusion protein. (C, D) The same gene was expressed in *E. coli* by transforming of a PCR product amplified from YHp32834. In *E. coli*, eEGFP-ehVAMP1 was also localized at the cell periphery. (E, F) HEK293 cells transfected with a PCR product amplified from YHp32834 showed punctate localization.

sequence (12–15 bp) between an end-promoter and a coding sequence, the linear DNA becomes a replicative and selectable circular plasmid in *E. coli*. As a result, by the two concepts, we constructed novel plasmids that enabled gene expression in yeast, *E. coli*, and mammalian cells.

Expression analysis of an identical gene sequence in multiple hosts To the best of our knowledge, the expression of the same gene sequence in yeast, *E. coli*, and mammalian cells has not been attempted often. When a heterologous protein is to be expressed in a host organism, codon optimization to the host is usually applied, and there are various programs available for codon optimization (47,48). Since it is difficult to prepare several types of codon-optimized genes, researchers usually prepare a single codon-optimized gene without examining its expression levels through actual experimentation. Obviously, the same expression marker gene such as a fluorescent protein available for various hosts is highly versatile because there is no need to prepare optimized gene sequences among hosts. In present study, eEmRFP and eEGFP designed with optimized *E. coli* codons, were expressed in the three hosts (Fig. 3). Therefore, our plasmids revealed that codon-exchanged eEmRFP and eEGFP could be used in three different hosts. Since a marker gene is required for several different organisms, the applicability of a single gene sequence to various host organisms provides valuable information.

eCLuc designed using *E. coli* codons was compared with the wild-type hCLuc. In yeast, eCLuc expression was lower than that of hCLuc. However, eCLuc showed the same luciferase activity as hCLuc in mammalian cells. Since both amino acid sequences are exactly the same, yeast may have more codon sensitivity to eCLuc, with mammalian cells likely having less sensitivity. Moreover, neither luciferase was secreted by *E. coli*. Both intracellular luciferase activities in *E. coli* were at the same level, indicating that eCLuc and hCLuc codons can be similarly used in *E. coli*.

Gene analysis in different hosts Human genes are often expressed and studied in yeast and *E. coli* or in other organisms such as, *Drosophila* and *Caenorhabditis elegans* (49,50). Model organisms are incredibly useful tools in genetic and molecular biology. Therefore, easy genetic manipulation using the same gene sequence in different hosts is helpful in gene analyses. In the present study, we expressed hVAMP1, a human synaptobrevin with a vesicular fusion function, in yeast and mammalian cells and *E. coli*. On the one hand, we observed that VAMP1 in HEK293 cells showed ER/Golgi-like punctate localization. On the other hand, in yeast and *E. coli*, the VAMP1 localized to the cell periphery, probably on the cell membrane, suggesting that eEGFP-ehVAMP1 has an affinity for yeast and *E. coli* cell membranes. The *E. coli* cell membrane mainly comprises phosphatidylethanolamine with phosphatidylglycerol and cardiolipin, which differs from yeast and human membrane structures containing phosphatidylcholine >50% of phospholipid in membrane (51–53). VAMP1 localization in *E. coli* suggests that it has the ability to localize to the hydrophobic lipid region regardless of the membrane composition.

In conclusion, we believe that the triple expression system in different hosts will be of great help in facilitating molecular biology and genetic engineering, especially in yeast, *E. coli*, and mammalian cells.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2022.03.002.

ACKNOWLEDGMENTS

We would like to thank Takahiro Ono, Nobutada Murakami, Motoyasu Tsuchiya and Satoko Nishioka for their technical assistance. We also thank the National BioResouce Project Japan (NBRP) for the BY1847 strain. This work was supported by the Research Center for Thermotolerant Microbial Resources Yamaguchi University (RCTMR, 2020-04, 2021-03) and Renaissance Center for Applied Microbiology Shinshu University (RCAM). The authors declare no conflict of interest.

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