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SHORT COMMUNICATION

Construction of Stable T7 Expression System in *Saccharomyces cerevisiae* by Improving Nuclear Membrane Permeability with Viroporin HIV-1 Vpu

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Abstract
T7 expression system (T7 RNA polymerase / T7 promoter), derived from T7 bacteriophage, is one of the most extensively used protein expression systems, which is also an enabling tool in synthetic biology. However, in eukaryote, most of T7 expression system is transient expression system. This is mainly due to the absence of post-transcriptional processing of mRNAs transcribed by T7RNAP in eukaryotic cells, so they cannot effectively pass through nuclear membrane and enter cytoplasm. In this study, *Saccharomyces cerevisiae* was selected as host to construct stable T7 expression system, in which HIV-1 viroporin (Vpu) was used to improve the permeability of nuclear membrane. Results of NanoLuc® (Nluc) luciferase expression indicated that Vpu could effectively promote the transport of T7 transcripts and increase the amount of protein synthesized. The method of using viroporin to improve permeability of the nuclear membrane provides an effective tool for constructing a stable T7 expression system in eukaryote.

Keywords T7 expression system · *Saccharomyces cerevisiae* · Viroporin · Protein synthesis · Eukaryote

Introduction

T7 expression system, derived from T7 bacteriophage, consisting of T7 promoter and T7 RNA polymerase (T7 RNAP), is simple, efficient, orthogonal [1], which make it be an enabling tool in synthetic biology [1, 2], metabolic engineering [3, 4], biomedicine [5, 6], and enzyme engineering [7, 8]. T7 expression system has been extensively applied to synthesize protein in prokaryote and eukaryote. In prokaryote, the typical one is pET series of vectors (Novagen®), which has been used in *E. coli* for various proteins' synthesis [9]. In eukaryote, such as

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mammalian cells, many types of hybrid vaccinia virus-T7 RNA polymerase expression systems have been developed [10–19]; however, they are all transient T7 expression system and cannot express proteins stably with eukaryotic cell's division as it did in prokaryote, for the reason that eukaryotic viral vectors or plasmids, carrying T7 RNAP and T7 promoter-cognate transcription unite, will be lost with the cell division [1] or large amounts of offspring virus will kill host cells [20]. In addition, researchers also have used the trans-splicing mechanism of mRNA in kinetoplastida multicellular parasites to construct stable T7 expression system, which have shown promising results in trypanosomes; however, the trans-splicing mechanism is seldom happened in other eukaryotic species [1, 21, 22]. The absence of stable T7 expression system in eukaryote is due to no post-transcriptional processing for T7 RNAP-derived mRNA (i.e., the mRNAs transcribed by T7 RNAP lack 5′ cap and 3′ poly(A)-tail), which are very important for mRNA nuclear export and protein synthesis in eukaryote [23]. Therefore, how to transport T7 RNAP transcripts across the nuclear membrane to the cytoplasm is a key point for the construction of T7 expression systems, capable of stable (non-transient) expression in eukaryote.

In the viral life cycle, entry of virus into host cell and release of viral progeny particles as well as replication, assembly of the viral genome involve an important class of proteins, the viroporin [24, 25], such as SARS coronavirus small envelope protein (SCVE) [26], HIV-1 viral protein U (Vpu) [27], influenza A virus matrix protein 2 (M2) [28]. Investigations on viral life cycle revealed that viral viroporin could improve membrane permeability [25]. For example, expression of HIV-1 Vpu in *E. coli* can increase the ability of hygromycin, lysozyme, uridine, ONPG (2-Nitrophenyl β-D-*galactopyranoside*) to penetrate cell membrane [27].

In present paper, *Saccharomyces cerevisiae*, an important scientific and industrial microorganism, was selected as the model to construct the stable T7 expression system in eukaryote. Up to now, it has not been reported about the successful construction of T7 expression systems in *Saccharomyces cerevisiae*. Earlier studies showed that T7 RNAP could be expressed in *Saccharomyces cerevisiae* [29], the construction of T7 RNAP containing nuclear localization sequence (NLS) can import T7 RNAP into nucleus [30], and T7 RNAP can initiate the transcription of T7 promoter-driven gene on the plasmid in the nucleus and accumulate large amount of target gene mRNA [23]. Nevertheless, T7 promoter-driven target protein cannot be synthesized. During construction of the stable T7 expression system in *Saccharomyces cerevisiae*, we discovered that although the mRNA of hygromycin resistance gene (hygromycin b phosphotransferase gene, Hph gene, *hph*) transcribed by T7 RNAP lacks the 5′ cap structure, the hygromycin resistance protein can still be synthesized. It is speculated that the mRNA of hygromycin resistance gene can enter cytoplasm through nuclear membrane to synthesize proteins by osmotic diffusion [23]. Based on this discovery, in this paper, we introduce viroporin to construct stable T7 expression system, and the viroporin here was used to increase the amount of T7 RNAP-transcribed mRNA in the cytoplasm by improving the permeability of nuclear membrane. This study provides an effective tool for constructing a stable T7 expression system in eukaryote.

Materials and Methods

Strains, Media, and Reagents

Escherichia coli DH5α (purchased from Taihe Biotechnology (Beijing) Co., Ltd.) was used as host for gene cloning. *Saccharomyces cerevisiae* BY4741 (*MATa*; *his3Δ1*; *leu2*; *met15Δ*; *ura3-52*) [31] was used as host for gene expression.

Luria–Bertani (LB) medium with antibiotics (100 μg/mL kanamycin (Kana) or 100 μg/mL ampicillin (AmpR)) was used to culture *E. coli* DH5α and screen

recombinant *E. coli*. YPD medium (1% yeast extract, 2% peptone, and 2% D-glucose) was used to culture *Saccharomyces cerevisiae*. YPD medium containing 200 μg/mL geneticin (G418) was used to select *Saccharomyces cerevisiae* strains with KanMX marker, such as strains whose genome has been modified by linear DNA fragment derived from pMRI-31 (GenBank: KJ502281.1). *Saccharomyces cerevisiae* cells carrying plasmids derived from pESC-URA (GenBank: AF063585.2) were screened and cultured on a synthetic medium deficient in uracil (URA) with 2% D-glucose or 2% galactose (SD-URA and SG-URA, respectively). All antibiotics were purchased from Beijing Solarbio Science & Technology Co., Ltd. and all chemicals were purchased from Beijing Chemical and Reagent Co., Ltd.

All primers were synthesized by Ruibio Biotech (Beijing) Co., Ltd. Restriction enzyme *Sfi* I was purchased from Thermo-fisher Scientific. The enzyme for PCR amplification is KOD-Plus-Neo (TOYOBO, Japan). The PCR amplified gene was ligated to the linearized vector with the help of Seamless Assembly Cloning Kit (Clone Smarter, USA) by the method of homologous recombination in vitro.

Vpu gene (GenBank: D86068.1) and Nluc gene (Nano-Glo® Luciferase Assay System, Progma) were synthesized by General Biosystems (Anhui) Co., Ltd.

Plasmid Construction

Original vectors were pMRI-31 [32] and pESC-URA (Stratagene). Main plasmids used in this study are listed in Table 1. Map and sequence of main plasmids, complete lists of plasmids and primers, detailed information on all plasmid construction processes are provided in supplementary information (Fig. S1 ~ S6, Table S1 and S2).

Strain Construction

Saccharomyces cerevisiae strains in this study are listed in Table 1. Plasmids were transformed into *E. coli* DH5α by heat shock method [33]. Plasmid or gel-purified DNA fragments were transformed into *Saccharomyces cerevisiae* by using a MicroPulser electroporator (1,652,100, Bio-rad, USA) at a voltage of 1.5 kV in one 0.2-cm gap electroporation cuvette. pMRI-31-P_{gal}-NLS-T7RNAP-T_{CYC1} was digested by restriction enzyme *Sfi* I to generate a linear DNA fragment, treated with gel purification, and transformed into BY4741. NLS-T7RNAP element was integrated into the genome of BY4741, generating BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}). The recombinant strains were screened by YPD plate containing G418. The other strains were obtained by transforming corresponding plasmids into BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}). These 6 recombinant strains were screened by plate of SD-URA.

Strain growth Curves and Spotting Assays

Strain Growth Curve Determination

A single colony was picked from *Saccharomyces cerevisiae* sample streak plate and inoculated into 5 mL of the appropriate liquid medium. After being incubated at 30 °C for 24 h,

Table 1 List of main strains and plasmids in this study

Strains	Genotype	Plasmids	Source
-	-	pMRI-31-P_{gal}-NLS-T7RNAP-T_{CYC1}	This study
BY4741	*MATa*, *his3Δ1*, l*eu2Δ0*, *met15Δ0*, *ura3Δ0*	None	[31]
control	BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1})	pESC-URA	This study
y*hph*	BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1})	pESC-URA-P_{T7}-IRES-*hph*-T_{CYC1}-T_{T7}	This study
yVpu-*hph*	BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1})	pESC-URA-NLS-Vpu-P_{T7}-IRES-*hph*-T_{CYC1}-T_{T7}	This study
yVpu-EGFP	BY4741	pESC-URA-NLS-Vpu-*egfp*	This study
y*nluc*	BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1})	pESC-URA-P_{T7}-IRES-*nluc*-T_{CYC1}-T_{T7}	This study
yVpu-*nluc*	BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1})	pESC-URA-NLS-Vpu-P_{T7}-IRES-*nluc*-T_{CYC1}-T_{T7}	This study

OD_{600} value of *Saccharomyces cerevisiae* broth was adjusted to be the same one by dilution. One percentage of diluted broth was inoculated into SG-URA liquid medium for inducing expression, and hygromycin was added with the final concentration of 400 μg/mL. Two hundred microliters of broth was pipetted into a transparent 96-well plate every 12 h to measure OD_{600} value, and three parallels were carried out to reduce experimental error. The data were recorded and the growth curve of OD_{600} value versus time was plotted.

Spotting Assays of the Diluted Broth

An appropriate amount of the *Saccharomyces cerevisiae* broth cultured in the above medium was taken to adjust concentration of the strains to be consistent at initial OD_{600} value. Five microliters of the broth was taken after 1000-fold dilution and spotted on the SG-URA solid induction plates containing hygromycin with the concentration of 0 μg/mL, 50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL. After plates completely absorbed the broth solution, the plates were turned upside down and incubated at 30 °C. Growth of spotted colonies was observed after 2 ~ 3 days.

Vpu Localization Analysis

DAPI (4′,6-diamidino-2-phenylindole), a fluorescent dye for DNA, was used to label the location of the nucleus. One mg/mL of DAPI aqueous solution was diluted into 10 μg/mL of DAPI working solution with phosphate buffer saline (PBS) for the following experiment. Ten micrograms per milliliter of DAPI mixed with *Saccharomyces cerevisiae* solution at the ratio of 1:3 (v/v), and then, the mixture was dropped on glass slide, covered, and stained for 20 min at room temperature. We used a Leica TCS SP8 confocal laser-scanning microscope system (Germany) to observe and analyze localization of Vpu in the nucleus. Image of *Saccharomyces cerevisiae* cell's nucleus stained with DAPI was observed with 100 × oil immersion lens. Two preset settings of scan channel were chosen: one is for

DAPI; when DAPI is combined with double-stranded DNA, the maximum absorption wavelength of DAPI is 358 nm and the maximum emission wavelength is 461 nm; the other is EGFP; the absorption wavelength of EGFP protein is 488 nm and the emission wavelength is 516 nm.

Nluc Luminescence Intensity Assays

The cultured *Saccharomyces cerevisiae* was collected by centrifugation, washed by filter sterilized PBS for 2 ~ 3 times, and then resuspended in sterile PBS. Nano-Glo® luciferase assay system (Promega GmbH, Germany) was used for detection of NanoLuc® luciferase in this study. Desired amount of reconstituted Nano-Glo® Luciferase Assay Reagent was prepared by combining one volume of Nano-Glo® Luciferase Assay Substrate with 50 volumes of Nano-Glo® Luciferase Assay Buffer. The Nano-Glo® Luciferase Assay Reagent should be prepared freshly for each use. One volume of Nano-Glo® Luciferase Assay Reagent was added to 10 volumes of the sample and mixed for optimal consistency. Two hundred microliters of mixture was pipetted into white 96-well plate instantly; three parallels were carried out for each sample. Luminescence was measured immediately using the luminescence module of the EnSpire Multimode Plate Reader (PerkinElmer). Two hundred microliters of non-lysed samples were transferred to a transparent 96-well plate for determination of OD_{600}. To compare the difference between different *Saccharomyces cerevisiae* samples, the luminescence intensity was divided by value of OD_{600} to obtain the average luminescence intensity. All samples were diluted to appropriate concentrations prior to assay ($OD_{600} = 0.3 \sim 0.8$).

Results and Discussion

Principle of Utilizing the Viroporin to Construct a Stable T7 Expression System in Saccharomyces cerevisiae

In the process of constructing a T7 expression system for *Saccharomyces cerevisiae*, we first constructed yeast strain BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) as described in the "Material and Method" section, in which NLS-T7RNAP mRNA was exported into cytoplasm and synthesizes T7 RNAP protein containing NLS, and the T7 RNAP could be imported into nucleus with the aid of NLS (Fig. 1a). Hygromycin-resistant protein (Hph) gene was used as a reporter gene to construct T7 promoter-cognate transcription unit (Fig. 1a). T7 transcription unit included T7 promoter/terminator, internal ribosome entry site (IRES) structure [12, 13], Hph gene, eukaryotic terminator sequence (CYC1 terminator) that produces 3′ poly(A) tail, and the resultant plasmid pESC-URA-P_{T7}-IRES-*hph*-T_{CYC1}-T_{T7} (Plasmid 1 in Fig. 1a) was transferred into the BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) to obtain recombinant strain y*hph*. T7 RNAP with NLS transcribe its cognate transcription unit and synthesize *hph* mRNA (Fig. 1a), and the *hph* mRNA here lacks the 5′ cap. As a result, T7 RNAP-derived *hph* mRNA cannot cross nuclear membrane into cytoplasm to synthesize Hph protein (Fig. 1a). However, the recombinant strain y*hph* could grow in the medium with hygromycin concentrations of 150 μg/mL and 200 μg/mL, whereas the control without the *hph* gene could not (Fig. S7), suggesting that the recombinant strain *yhph* had expressed

Fig. 1 Principle of constructing stable T7 expression system in *Saccharomyces cerevisiae* by improving nuclear membrane permeability with viroporin. **a** Construction of stable T7 expression system in *Saccharomyces cerevisiae*. **b** Construction of stable T7 expression system in *Saccharomyces cerevisiae* using viroporin protein

hygromycin resistance (Hph) protein. Benton's study noted that in *Saccharomyces cerevisiae*, T7 RNAP protein was able to be transported across the nuclear membrane into nucleus by osmotic diffusion [25]. We were therefore inspired to speculate that the mRNA of *hph* transcribed by T7 RNAP might have been transported from the nucleus into the cytoplasm by osmotic diffusion; thus, Hph protein was synthesized. Based on this, if the nuclear membrane permeability could be improved, it would promote more T7 RNAP transcripts to be exported from the nucleus into the cytoplasm to synthesize proteins.

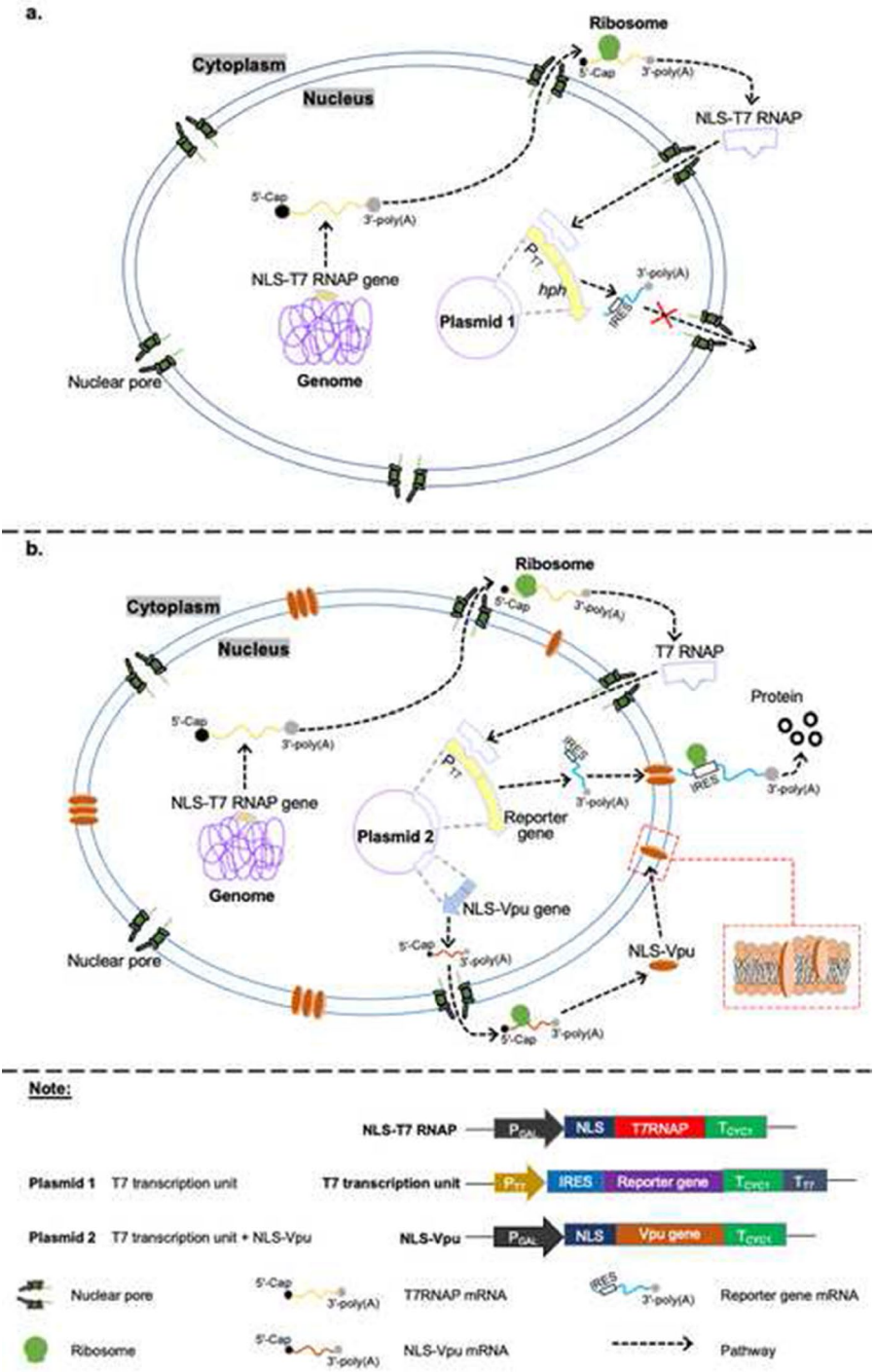
In view of above hypothesis, we introduced the viroporin in this study to improve the nuclear membrane permeability, and *Saccharomyces cerevisiae* was selected to construct a eukaryotic T7 expression system that could stably synthesize the protein. The research idea is shown in Fig. 1b. Protein of HIV-1 Vpu was selected to improve the permeability of *Saccharomyces cerevisiae* nucleus. Noticeably, NLS here was used to locate the viroporin into the nuclear membrane; thus, viroporin could play its role in improving the nuclear membrane permeability and increasing the amount of mRNA transcribed by T7 RNAP into cytoplasm, thereby prompting the synthesis of the target protein. Combination of NLS-T7RNAP integrated in genome and plasmids with NLS-Vpu and report genes (Plasmid 2 in Fig. 1b) resulted in the construction of viroporin-dependent T7 expression system (Vd T7 expression system).

Expression of Hygromycin-Resistant Protein (Hph) by Vd T7 Expression System

According to the assumption above, T7 RNAP expressed by strain BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) can be imported into the yeast nucleus, and this strain was used as host strain in Vd T7 expression system. Hygromycin resistance protein (Hph) gene was firstly selected to test the Vd T7 expression system, and the results are shown in Fig. 2. Figure 2a indicates that the growth of control strain (control) was hard to be observed in liquid medium containing hygromycin (400 μg/ml), while y*hph* and yVpu-*hph* show a noticeable growth rate, and the growth rate of yVpu-*hph* was higher than that of y*hph*. This indicates that both y*hph* and yVpu-*hph* synthesized Hph protein and that yVpu-*hph* expressed more Hph protein than y*hph* did. The growth of *Saccharomyces cerevisiae* spotting-colonies on hygromycin-resistant plates also showed the similar results (Fig. 2b). Above experimental results indicated that the introduction of HIV-1 Vpu can indeed increase the amount of reporter protein (Hph) that was transcribed by T7 RNAP, and that the previous hypothesis is feasible.

Vpu Nuclear Localization Analysis

To further confirm whether the NLS help viroporin locate into nuclear membrane or not, the recombinant strain yVpu-EGFP was constructed to label the subcellular location of


a.
Ribosome
Cytoplasm
Nucleus
5'-Cap
3'-poly(A)
NLS-T7 RNAP
NLS-T7 RNAP gene
Genome
Plasmid 1
PT7
hph
IRES
Nuclear pore
b.
Ribosome
Cytoplasm
Nucleus
T7 RNAP
Protein
NLS-T7 RNAP gene
Genome
Plasmid 2
Reporter gene
NLS-Vpu gene
NLS-Vpu
Nuclear pore
Note:
NLS-T7 RNAP
PGAL
NLS
T7RNAP
TCYC1
Plasmid 1 T7 transcription unit
T7 transcription unit
PT7
IRES
Reporter gene
TCYC1
TT7
Plasmid 2 T7 transcription unit + NLS-Vpu
NLS-Vpu
Vpu gene
Nuclear pore
T7RNAP mRNA
Reporter gene mRNA
Ribosome
NLS-Vpu mRNA
Pathway

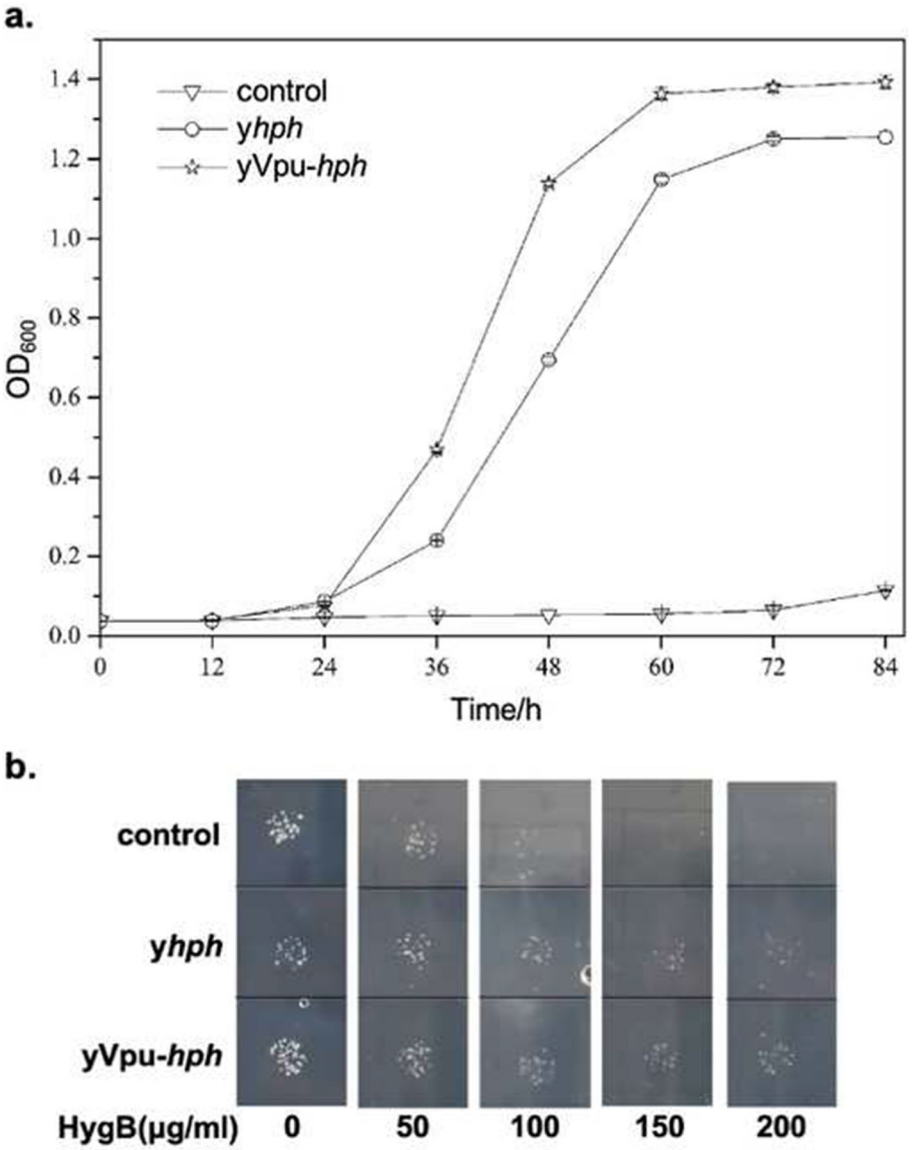


Fig. 2 Growth of *Saccharomyces cerevisiae* with Vd T7 expression system of Hph. **a** Growth curve of *Saccharomyces cerevisiae* with Vd T7 expression system of Hph (400 μg/ml). **b** Colony growth of *Saccharomyces cerevisiae* with Vd T7 expression system of Hph. The "control" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA; "y*hph*" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA-P_{T7}-IRES-*hph*-T_{CYC1}-T_{T7}; "yVpu-*hph*" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA-NLS-Vpu-P_{T7}-IRES-*hph*-T_{CYC1}-T_{T7}

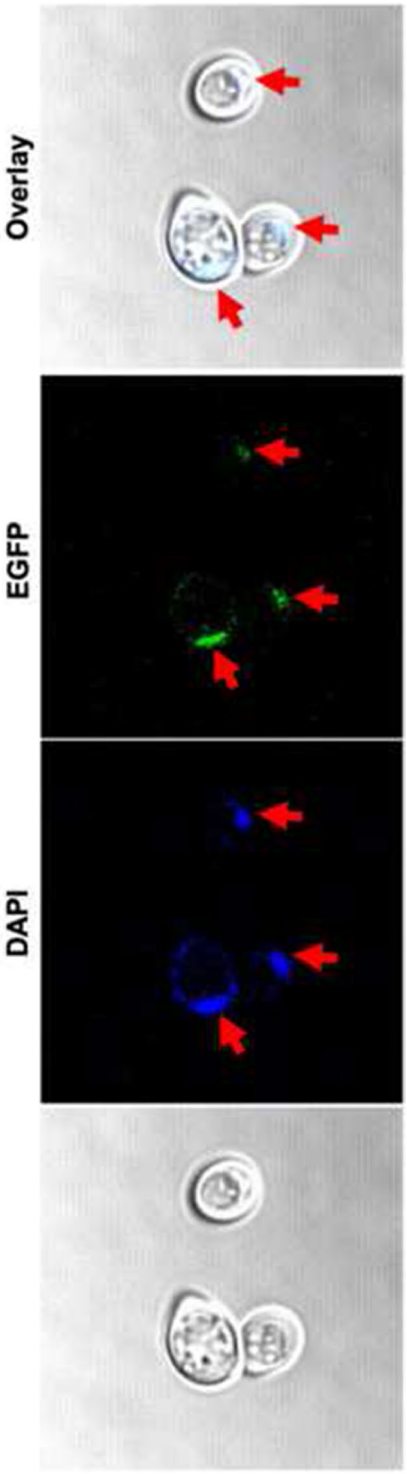


Fig. 3 Subcellular localization of Vpu observed by confocal microscopy. yVpu-EGFP was tested here. "yVpu-EGFP" indicates the *Saccharomyces cerevisiae* BY4741 with the plasmid of pESC-URA-NLS-Vpu-*egfp*

Vpu protein, in which fluorescent protein (EGFP) and NLS-Vpu were coexpressed as a fused protein. As shown in Fig. 3, the blue fluorescence of DAPI indicates the nuclear region and green fluorescence of EGFP exbibits the NLS-Vpu-EGFP expression region, which were coincided in the *Saccharomyces cerevisiae* cell. These results indicated that Vpu was associated with the nuclear membrane with the aid of NLS.

Expression of Luciferase Gene in Vd T7 Expression System

After verifying the feasibility of our hypothesis with Hph, the NanoLuc® (Nluc) luciferase was chosen to further investigate the protein expression capacity of Vd T7 expression system, and strains y*nluc* and yVpu-*nluc* were constructed and tested. As can be seen in Fig. 4, the luciferase luminescence of control strain (control) could barely be detected, while strain yVpu-*nluc* performed a significantly stronger luminescence intensity, the luciferase luminescence of yVpu-*nluc* was stronger than that of y*nluc*, and there was a significant difference between two samples ($p < 0.05$).

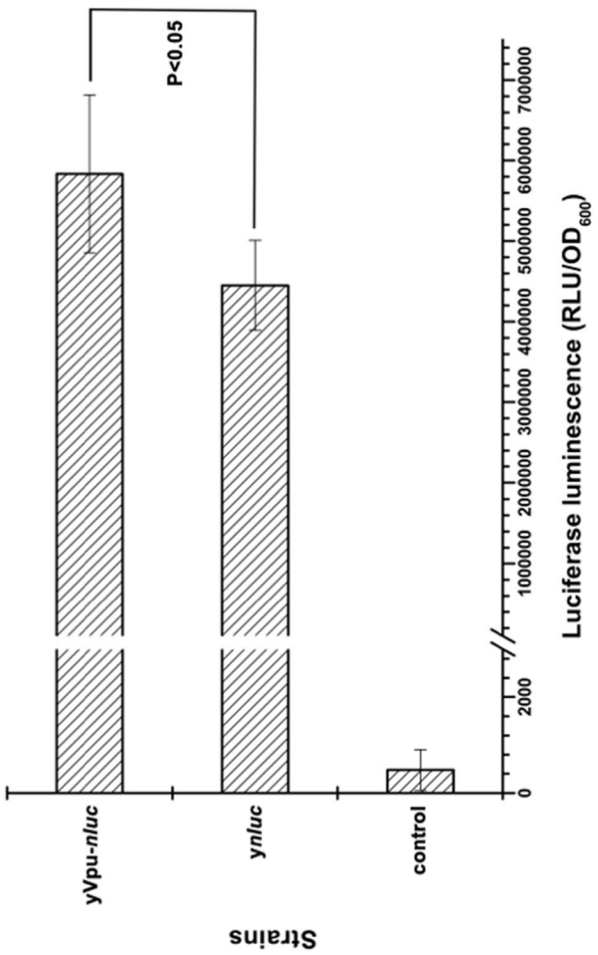


Fig. 4 The luciferase luminescence of *Saccharomyces cerevisiae* with Vd T7 expression system. The "control" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA; "y*nluc*" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA-P_{T7}-IRES-*nluc*-T_{CYC1}-T_{T7}; "yVpu-*nluc*" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA-NLS-Vpu-P_{T7}-IRES- *nluc*-T_{CYC1}-T_{T7}

The luminescence of luciferase showed that more Nluc luciferase was expressed with the help of Vpu, which is consistent with the result of Hph.

In order to explore the effect of the insertion of Vpu into nuclear membrane on *Saccharomyces cerevisiae* cell growth and normal metabolism, the OD_{600} values of the strain yVpu-*nluc* (with Vd T7 expression system) and y*nluc* (without Vd T7 expression system) were compared. The results showed that there was no significant difference in OD_{600} value between strains with and without Vd T7 expression system (Fig. 5), demonstrating that the insertion of Vpu into nuclear membrane did not severely affect *Saccharomyces cerevisiae* cell growth and normal physiological metabolism.

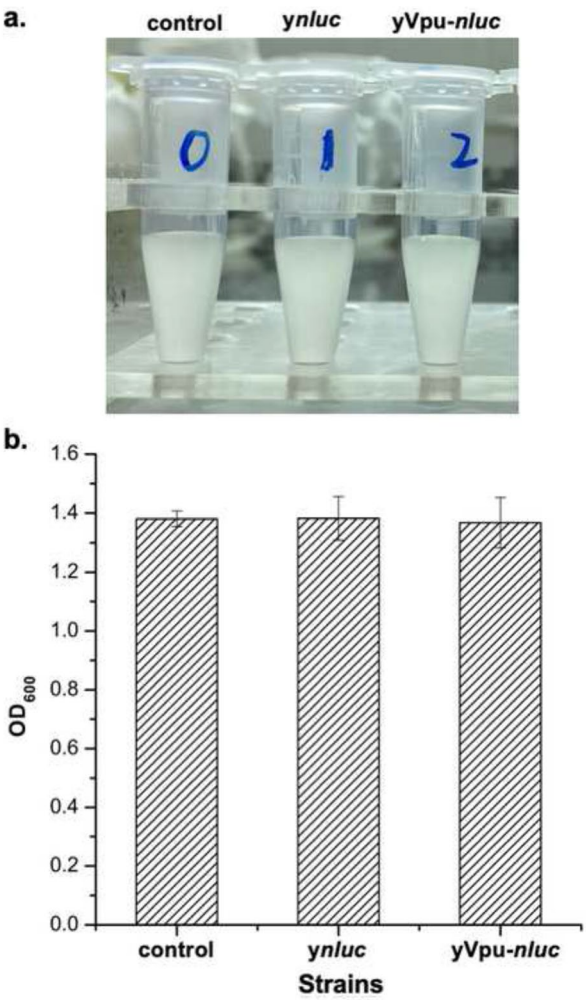


Fig. 5 Effect of Vpu protein on the growth of strains. **a** The culture of *Saccharomyces cerevisiae*; **b** the growth of *Saccharomyces cerevisiae*. The "control" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA; "y*nluc*" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA-P_{T7}-IRES-*nluc*-T_{CYC1}-T_{T7}; "yVpu-*nluc*" indicates the *Saccharomyces cerevisiae* BY4741(HO::P_{gal}-NLS-T7RNAP-T_{CYC1}) with the plasmid of pESC-URA-NLS-Vpu-P_{T7}-IRES- *nluc*-T_{CYC1}-T_{T7}

Conclusion

How to export T7 RNAP transcripts of nucleus into cytoplasm is a key point for the construction of stable T7 expression systems in eukaryotes. The present study constructed a stable T7 expression system in *Saccharomyces cerevisiae* in virtu of HIV-1 Vpu viroporin, in which the Vpu protein could increase nucleus permeability. Vpu localization analysis indicated that the Vpu could be located into nuclear membrane with the aid of NLS. The results of Nluc luciferase expression showed that the Vd T7 expression system can increase the production of luciferase protein without affecting cell growth and normal physiological metabolism. The method of using viroporin to improve the permeability of the nuclear membrane provides an effective tool for constructing a stable T7 expression system in eukaryotes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12010-021-03665-y.

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Data Availability The data is included in the article and supporting materials.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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