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Expression and Characterization of the Human rpS3 in a Methylotrophic Yeast *Pichia pastoris*

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A human ribosomal protein S3 (rpS3), which also functions as a DNA repair enzyme (UV endonuclease III), was expressed in a methylotrophic yeast, *Pichia pastoris,* and biochemically characterized. UV endonuclease activity was previously characterized, and this activity of mammalian rpS3 was found to be non-specific upon purification and storage. Under the *Pichia* expression system, the subcloned cDNA of the human rpS3 gene revealed a peptide of 42 kDa by SDS-PAGE and Western blot. The secreted form of human rpS3 rendered no endonuclease activity while the intracellular form showed UV specific endonuclease activity by the nick circle assay.

Key words: rpS3, *Pichia pastoris*, endonuclease activity, nick circle assay

A ribosomal protein S3 (rpS3) was known to function as UV endonuclease III, a DNA repair enzyme, which corrects DNA lesions in pyrimidine dimers and AP (apurinic/apyrimidinic) sites induced by UV irradiation (5). Both human and murine rpS3 have an associated endonuclease activity which produces a nick at 3' side of AP site *via* a β-lyase mechanism (5). This endonuclease activity is altered in xeroderma pigmentosum (XP) group-D individuals, in which a rare, autosomal human disease is characterized clinically by hypersensitivity to UV irradiation, leading to a high incidence of sunlight-induced skin cancers (8). As the first stage of excision repair in the mammalian system, this enzyme also cleaves the phosphodiester bond between the pyrimidine dimer (5). Purified rpS3 (also as UV endonuclease III) is very difficult to obtain in appropriate amount for the biochemical study and its innate form isolated from murine MPC-11 cells tends to lose its substrate specificity upon purification and storage (10). Therefore, an effective expression system for the rpS3 gene is required for the study.

As an eukaryotic yeast, *Pichia pastoris* has developed into a highly successful protein expression system, and its increasing use is favorable in such characteristics as its ability to produce foreign proteins either intracellularly or extracellularly, easy manipulation in *E. coli* or *Saccharomyces cerevisiae,* simple techniques for the molecular manipulation of *P. pastoris* similar to those of *Saccharomyces* processing (gene disruption, gene replacement, and transformation by complementation), and the performance of many eukaryotic post-translational modifications as well as the commercially available kit (1). There are two genes in *P. pastoris* that code for alcohol oxidase (*AOX1* and *AOX2*), in which this enzyme oxidizes methanol as a sole carbon source to formaldehyde using oxygen. Since alcohol oxidase has poor affinity for oxygen, *P. pastoris* compensates for this fact by high expression of this enzyme. Under the promoter of alcohol oxidase, heterologous protein expression in *Pichia* can be induced by methanol.

The *AOX1* gene (responsible for over 30% of the total soluble protein in cells grown with methanol as the carbon source) has been isolated and a plasmid-borne version of the *AOX1* promoter is used to derive the expression of the gene encoding the heterologous protein. With 97% homology to *AOX1*, *AOX2* causes slower growth on methanol (Mut^s strain) than *AOX1*(2, 7).

In this study, the cDNA of the human rpS3 gene was subcloned in a methylotrophic yeast, *Pichia pastoris,* under the *Pichia* expression system, and the expressed protein was characterized for endonuclease activity by nick circle assay.

Materials and Methods

Materials

pT7-7 (2.5 kb) which contains cDNA of the human rpS3 gene (732 bp) was kindly provided by Stuart Linn at the

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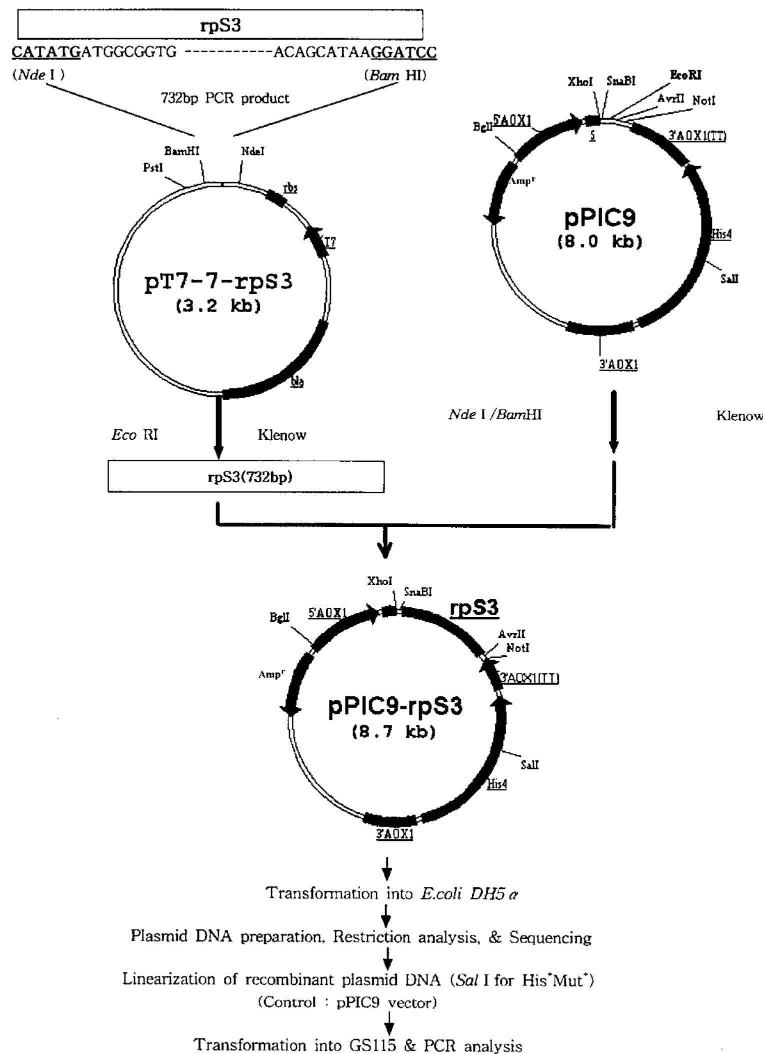

Fig. 1. Schematic diagram of pPIC9-rpS3 construction and transformation of *P. pastosis* GS115.

University of California, Berkeley (Fig. 1). *Pichia* expression kit was purchased from Invitrogen.

Preparation of supercoiled PM2 phage [^{3}H-thymidine] DNA

PM2 phage and its host strain, *Pseudoalteromonas espejiana* (*BAL 31* and *BAL 31-14*), were kindly donated by Dr. Stuart Linn, University of California at Berkely, U.S.A. For labeling PM2 DNA with ^{3}H-thymidine, the thymidine auxotroph strain (*BAL 31-14*) was used. Growth and maintenance of the host strain were carried out as described previously (3), and the supercoiled PM2 phage [^{3}H-thymidine] DNA was purified as described (9).

Cell culture of Pichia

The *Pichia* host strain, GS115 (*his4* genotype; Mut$^+$ phenotype, referring to the wild type ability in methanol utilization) was grown on complex YPD medium and on minimal medium supplemented with histidine until transformation. As a control expression strain, GS115/His$^+$ Mut^S Albumin (Mut^S phenotype refers to the mutant type ability of slow methanol utilization) has a cloned serum albumin gene and secretes albumin (67 kDa) into the medium.

Subcloning of the rpS3 gene into the Pichia expression vector and transformation

Cloned cDNA of the human rpS3 gene in pT7-7 (3.2 kb) was cleaved at *Nde*I and *Bam*HI restriction sites, and the cohesive ends of the digested DNA became blunt-ended with the Klenow enzyme. *A Pichia* expression vector for secretion, pPIC9 (8.0 kb), was digested with *Eco*RI and treated with the Klenow enzyme. These DNA fragments were electroeluted for blunt-end ligation. Ligated products were transformed into *E. coli* DH5α, and the transformants were screened for the desired recombinant DNA. This recombinant DNA was sequenced using the 5'-AOX1 primer for expression in the correct orientation. Control vector plasmid pPIC9 and the recombinant plasmid

pPIC9-rpS3 were propagated in *E. coli* DH5α, and the recovered DNA was linearized with *Sal*I. The resulting linear DNAs were used to transform GS115 according to the Lithium Chloride transformation method as previously described (3). By patching on a minimal dextrose (MD; YNB 1.34%, biotin $4 10^{-5}$%, dextrose 1.0%) plate and a minimal methanol (MM; YNB 1.34%, biotin $4 10^{-5}$%, methanol 0.5%) plate, Mut^+ phenotype transformants were readily distinguished from Mut^s phenotype transformants (7). GS115/albumin (His^+Mut^+) on MD and MM plates were used as a control for selection of the Mut^+ phenotype. Transformants (GS115-rpS3; His^+Mut^+ phenotype) growing normally on both MD and MM medium were retained for further characterization.

PCR analysis of Pichia integrants

Pichia recombinants were confirmed by PCR for the integration of the rpS3 gene into the *Pichia* genome. Genomic DNAs from Mut^+ *Pichia* recombinant clones and the DNAs from the GS115 transformed with the parent plasmid (pPIC9) were isolated (12). Amplification of the rpS3 gene is carried out with 5'- and 3'-AOX primers (5'-AOX1, 5'-GACTGGTTCCAATTGACAAGC-3'; 3'-AOX1, 5'-GCAAATGGCATTCTGACATCC-3'). The PCR reaction mix was as follows : 100 ng of genomic DNA, 5.0 μl of 10 × PCR buffer, 5.0 μl of 2.5 mM dNTPs, 25 pmol of 5'-AOX1 primer, 25 pmol of 3'-AOX1 primer, and 1.25 U of Taq polymerase (Takara), in a total volume of 50 μl. A thermal cycler (Takara PCR thermal cycler MP) was programmed and run for 1 cycle of hot start at 94°C for 2 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and 1 cycle of final extension at 72°C for 10 min. Ten μl out of 50 μl of the PCR reaction was analyzed on a 1xTAE, 0.8% agarose gel.

Expression of recombinant Pichia strains (GS115-rpS3)

A control strain (GS115/Albumin) was tested for expression conditions. Individual colonies were inoculated in 25 ml of BMGY medium (Yeast extract 1%, peptone 2%, potassium phosphate, pH 6.0, 100 mM, YNB 1.34%, biotin 4 × 10^{-5}%, glycerol 1%). Growth was performed at 30°C in a shaking incubator (280 rpm) until the cultures reached an OD_{600} of 0.6. The cells were centrifuged at 2,000 g for 5 min, and the cell pellets were suspended in 200 ml of BMMY medium (yeast extract 1%, peptone 2%, potassium phosphate, pH 6.0, 100 mM, YNB 1.34%, biotin 4 × 10^{-5}%, methanol 0.5%). Cells were grown in a shaking incubator, and methanol was added every 24 hr to a final concentration of 0.5%. After 24 hr, 48 hr, 72 hr, 96 hr, and 120 hr of incubation, cells were pelleted, and the culture media were harvested. Cell lyates were prepared using acid-washed glass bead (size 0.5 mm; Sigma). All details followed the manufacturer's instruction (Catalog no. K1710-01). Experimental outline illustrates the whole procedure in Fig. 1.

Analysis of protein expression

Coomassie-stained SDS-PAGE and Western blot were performed for the expressed rpS3. Proteins in the supernatant were separated by SDS-PAGE on 12% polyacrylamide gels and stained with Coomassie blue or transferred to a nitrocellulose membrane (BioRad). The membrane was blocked with non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) and incubated with anti-rabbit rpS3 monoclonal antibody followed by peroxidase-conjugated monoclonal rabbit IgG (Sigma). The blot was subjected to Western analysis using chemiluminescence system (Boehringer Mannheim).

Preparation of damaged DNAs

To analyze the endonuclease enzyme activity of the expressed rpS3 under the *Pichia* expression system, substrate PM2 [^{3}H]DNA was UV-irradiated in 10 mM of Tris-HCl (pH 7.5) and 0.02% glycerol at 250 J/m^2 under a UV crosslinker (UVP).

Endonuclease asssay (Nick circle assay)

The filter-binding nick circle assay with nitrocellulose filters was used to determine endonuclease activity as previously reported (6). Briefly, UV irradiated- or normal ^{3}H-labeled PM2 DNA was incubated with the cell lysate or supernatant. The enzyme reaction buffer consisted of 40 mM Tris-Cl (pH 8.0), 70 mM KCl, 0.01% Triton X-100, 3 mM EDTA and 5 mM 2-mercaptoethanol. The whole reaction mixture (total 100 μl) contained 10 μl of 10x reaction buffer, 4.5 μl of UV irradiated- or unirradiated-PM2 DNA (1 nmol), 5 μl of protein solution, and 80.5 μl of distilled water. The reaction mixture was incubated at 37°C for 30 min, denatured with 100 μl of denaturation buffer (0.3 M K_2HPO_4, pH 12.4) for 5 min, renatured with 50 of renaturation buffer (1 M KH_2PO_4, pH 4.0) for 5 min, and added with 100 μl of 5 M NaCl. The reaction mixture was diluted to 1 ml with filtration buffer [50 mM Tris-Cl (pH 8.2), 1 M NaCl] and filtered through nitrocellulose membrane (0.45 nm). The membrane was washed with 5 ml of filtration buffer, then with 5 ml of 2 × SSC. The washed membrane was dried and counted by scintillation counter.

Results and Discussion

Expression of rpS3 under the Pichia expression system

The pPIC9 vector contains the *Pichia HIS4* gene, but does not contain an yeast origin of replication (2); therefore, His^+ colonies arising after transformation of GS115 (*his4*; responsible for a defect in the synthesis of histidine) are attributable to integration of the vector into the *P. pastoris* genome. The pPIC9 vector was constructed commercially

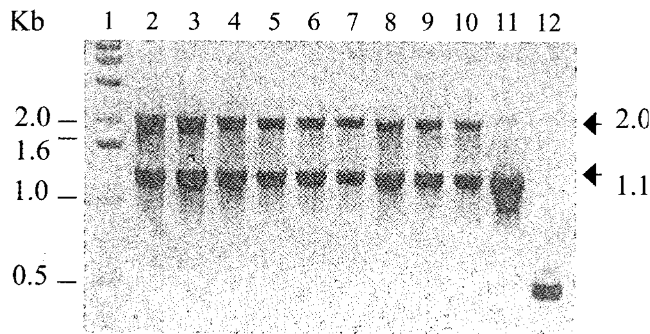

Fig. 2. Screening of the *Pichia pastoris* transformants by PCR. Lane 1, DNA Size Marker (Boehringer Mannheim, type/Kb), Lanes 2-10, Chromosomal DNA from GS115-pPIC9-rpS3, Lane 11, pPIC9-rpS3 plasmid DNA, Lane 12, Chromosomal DNA from GS115-pPIC9

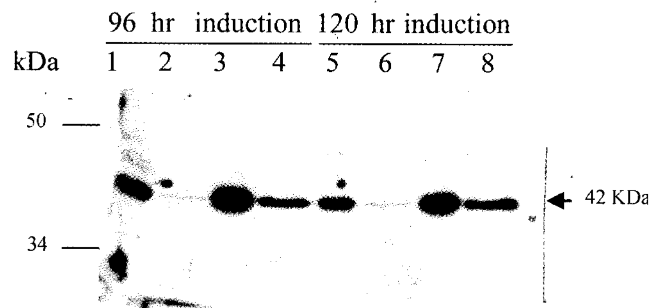

Fig. 4. Western blot analysis of rpS3 expression. Supernatant and cell lysate were taken at 96 hr and 120 hr after induction with methanol, electrophoresed through a 12% acrylamide gel, and followed by Western blotting as described in Materials and Methods. Lanes 1 and 5, Cell lysate from GS115-pPIC9, Lanes 2 and 6, Supernatant from GS115-pPIC9, Lanes 3 and 7, Cell lysate from GS115-pPIC9-rpS3, Lanes 4 and 8, Supernatant from GS115-pPIC9-rpS3

for the secretion of the protein expressed from heterologous gene, and recombinant *P. pastoris* strains designated as GS115-pPIC9-rpS3 were generated as described under Materials and Methods. The recombinant plasmid pPIC9-rpS3 was linearized by digestion with Sal I and transformed into the *P. pastoris* strain GS115. In this case, transformation of the GS115 strain produced only His$^+$ Mut$^+$ transformants. The phenotype of these transformants is normal growth (Mut$^+$) on medium containing methanol as the sole carbon source, due to non-disruption of the major alcohol oxidase gene, *AOX1*. To identify integrants, amplification of the gene of rpS3 was performed by PCR using 5'-AOX1 and 3'-AOX1 primers (Fig. 2). Nine clones (lanes 2-10) which had the integrated rpS3 gene were identified. pPIC9-rpS3 plasmid (lane 11) and chromosomal DNA from GS115-pPIC9 (lane 12) were used as positive and negative controls, respectively.

For rpS3 expression, 5 clones were examined in shake flask cultures. After 24 hr, 48 hr, 72 hr, 96 hr and 120 hr induction with 0.5% methanol, aliquots of 5 cultures were centrifuged, and the supernatants were evaluated by SDS-PAGE (Fig. 3) and Western blotting (Fig. 4). Through SDS-PAGE analysis, two clones (Fig. 3; lanes 3 and 4) were identified to express a secreted protein with the highly modified (glycosylated) form (2) of rpS3 (about 42 kDa in contrast to its calculate mass of 26.7 kDa). Control GS115-pPIC9 cells grown under the same conditions did not produce secreted rpS3 proteins in this molecular weight range. The protein concentrations of GS115-pPIC9-rpS3 and GS115-pPIC9 were measured by Bradford method. Different protein quantities of GS115-pPIC9-rpS3 (12.4 μg/ml) from GS115-pPIC9 (6.4 mg/ml) were expected due to the effect of heterologous human rpS3 gene expression besides the endogenous yeast rpS3 of GS115.

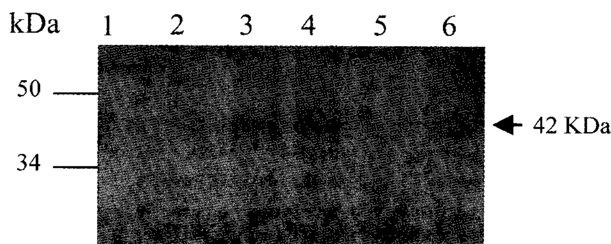

Fig. 3. SDS-PAGE analysis of supernatant from *Pichia pastoris* cultures. Samples were taken at 96 hr after induction with methanol, electrophoresed through a 12% acrylamide gel, and stained with Coomassie Brilliant blue. Lane 1. Supernatant from GS115-pPIC9; Lanes 2-6. Supernatant from GS115-pPIC9-rpS3 #1-5.

To confirm the presence of the expressed protein, secreted rpS3 was screened by Western blot analysis with anti-rabbit rpS3 monoclonal antibodies (Fig. 4). Consistent with the results obtained by SDS-PAGE analysis, secreted rpS3 was detected in the GS115-pPIC9-rpS3 supernatant (lanes 4 and 8), but not in the GS115-pPIC9 supernatant (lanes 2 and 6). To investigate the quantities of endogenous and non-secreted rpS3, the corresponding cell extracts were prepared and tested. In both GS115-pPIC9 and GS115-pPIC9-rpS3, endogenous rpS3 was detected (lanes 1, 5 and 3, 7). However, higher intensities were shown in the GS115-pPIC9-rpS3 cell lysate (lanes 3 and 7). It is thought that non-secreted human rpS3 was retained in the cell lysate and added to the endogenous rpS3 of GS115.

Endonuclease activity of secreted and endogenous rpS3

When PM2 DNA is irradiated with UV light, closed cir-

Table 1. Radioactivity from rpS3 enzyme assay

	CPM of unirradiated DNA	CPM of UV-irradiated DNA
**Control cpm	249	277
Supernatant from GS115-pPIC9-rpS3	363	227
Cell lysates from GS115-pPIC9-rpS3	368	484

*Total cpm count from substrate PM2 DNA was 730
**Radioactivity derived from innate nicks present in substrate PM2 DNA

Table 2. UV specific endonuclease activities in cell lysate and supernatant

PM2 DNA	Nicks on DNA in control assay	Nicks on DNA in enzyme assay		Enzyme activity (unit/ml)		UV specific enzyme activity (unit/ml)	
		supernatant	cell lysate	supernatant	cell lysate	supernatant	cell lysate
Un-irradiated	0.41	0.68	0.93	45.3*	62*	-	-
UV-irradiated	0.81	0.61	3.72	40.6	248	0	186

*non-specific activity

cular DNA containing lesions can be converted to nicked DNA by this endonuclease. Under this assay condition, any intact closed circular DNA is renatured after alkaline treatment (pH 12.4), while nicked DNA is not renatured. This denatured DNA remains on the nitrocellulose membrane after filtration and can be detected using scintillation counter. From the radioactivity, the nicks produced by endonuclease activity are calculated according to the method described by Kuhnlein *et al.* (9) The total radioactivity of 1 nmol of PM2 DNA was measured either by spotting DNA or by irreversible denaturation of DNA by treating with buffer (pH 13.2). The results of the radioactivity from the endonuclease assay were shown in Table 1. In Table 2, the calculated nicks and the enzyme activity were presented. One unit of enzyme activity is defined as the amount which produces 1 fmole strand breaks per min. By these results, endonuclease activity was revealed in the fraction of the cell lysate of GS115-pPIC9-rpS3, but not in the supernatant fraction (Table 2). Non-specific endonuclease activity found in the non-UV irradiated substrate was perhaps due to the other nuclease activities. UV specific activity was calculated by subtracting the endonuclease activity of non-UV irradiated DNA from that of UV irradiated DNA. From these results, it is predicted that pPIC9 vector secretes some of heterologously expressed human rpS3 proteins into the medium (Fig. 3, lanes 2-6; Fig. 4, lanes 4 and 8), and these secreted proteins lose the endonuclease activity in the medium as murine rpS3 loses specific activity upon purification and storage (Tables 1 and 2).

Non-secreted forms of heterologously expressed human rpS3 proteins remained in the yeast cell and added to the endogenous enzyme activity of yeast GS115 rpS3 in the cell lysates (Fig. 4, lanes 3 and 7; Tables 1 and 2).

In this report, the human rpS3 gene was expressed in *Pichia* and tested for UV-specific endonuclease activity. Because of the inactive secreted form of human rpS3 in the medium and the interference of endonuclease activity from endogenous yeast rpS3 in GS115, purification of the heterologously expressed human rpS3 is required, and thus the construction of intracellular expression system using histidine-tagging *Pichia* vectors is in progression.

Acknowledgments

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