Development of Expression Systems for the Production of Recombinant Human Serum Albumin Using the *MOX* Promoter in *Hansenula polymorpha* DL-1

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Abstract: To optimize the secretory expression of recombinant human serum albumin (HSA) under the control of methanol oxidase (*MOX*) promoter in the methylotrophic yeast *Hansenula polymorpha* DL-1, we analyzed several parameters affecting the expression of HSA from the *MOX* promoter. Removal of the 5′-untranslated region derived from HSA cDNA in the expression cassette led to at least a fivefold improvement of HSA expression efficiency at the translational level. With the optimized expression cassette, the gene dosage effect on HSA expression was abolished and thus, a single copy of the expression vector integrated into the *MOX* locus became sufficient for the maximal expression of HSA. Northern blot analysis revealed that the levels of HSA transcript did not increase any further upon increasing copy number. The *mox*-disrupted (*mox*Δ) transformant was constructed, in which the genomic *MOX* gene was transplaced with the HSA expression cassette, to examine the effect of the methanol oxidase-deficient phenotype of the host on HSA expression. The *mox*Δ transformant showed higher levels of HSA production in shake-flask cultures than the *MOX* wild-type transformant, especially at low concentrations of methanol and a twofold higher specific HSA production rate in fed-batch fermentation with an abrupt induction mode. The native prepro signal sequence of HSA secreted in *H. polymorpha* was correctly processed and the mature recombinant protein had a pI value identical to that of the authentic HSA. Our results suggest that the *H. polymorpha* expression systems developed in this study are suitable for large-scale production of recombinant albumin. © John Wiley & Sons, Inc. *Biotechnol Bioeng* **76**: 175–185, 2001.

Keywords: human serum albumin; *Hansenula polymorpha; MOX* promoter; copy number; 5′-untranslated region; methanol oxidase-deficient phenotype

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INTRODUCTION

Human serum albumin (HSA) is a single-chain nonglycosylated polypeptide with a molecular weight of 66.6 kDa. It is the most abundant protein in the blood, constituting 60% of the total protein content of plasma (Peters, 1975). Because the protein is clinically in great demand as a replacement fluid of plasma expander or as a compensator for blood loss, several attempts to produce large quantities of HSA have been made in a number of heterologous systems using recombinant DNA techniques (Goodey, 1993). Recombinant HSA was secreted at a high level in the correctly folded form in several yeast systems including *Saccharomyces cerevisiae* (Kang et al., 2000; Sleep et al., 1990), *Kluyveromyces lactis* (Fleer et al., 1991; Saliola et al., 1999) and *Pichia pastoris* (Sreekrishna, 1993). The recombinant proteins produced in *S. cerevisiae* and *P. pastoris* were shown to be equivalent structurally to the authentic HSA derived by blood fractionation (Dodsworth et al., 1996; Ohtani et al., 1998). Further, yeast-derived recombinant proteins had lower levels of structural heterogeneity than the blood-derived protein, suggesting that yeast is an excellent choice as a host system to produce recombinant HSA for pharmaceutical purposes.

Hansenula polymorpha has proven to be a promising host organism for the production of heterologous proteins along with the closely related yeast *P. pastoris* (Hollenberg and Gellissen, 1997; van Dijk et al., 2000). Most expression vectors in these methylotrophic yeasts have been developed

to exploit the powerful expression capacity of the promoters derived from the methanol-inducible genes, for example, the methanol oxidase gene (*MOX*) in *H. polymorpha* and the alcohol oxidase (*AOX*) genes in *P. pastoris.* Despite the substantial similarities of methylotrophic yeasts, however, the *H. polymorpha* system shows some unique features that distinguish it from the *P. pastoris* system. For example, unlike most of the other yeasts including *P. pastoris,* integrative transformation of *H. polymorpha* is usually mediated by nonhomologous recombination. Even plasmids bearing an autonomous replicating sequence integrate randomly into the host genome with high frequency, leading to tandem multiple integration of up to hundreds of copies (Gatzke et al., 1995; Gellissen et al., 1995). This ability to achieve stable multimeric integration of the expression cassette makes *H. polymorpha* an ideal host for the high-level expression of foreign genes. However, the high frequency of nonhomologous recombination poses potential complications in obtaining a precise insertion of the expression cassettes into a targeted site of *H. polymorpha* (Faber et al., 1992).

In the case of the integrative transformation of *P. pastoris,* the double crossover of the linear expression cassette containing the *AOX1* promoter and terminator at the *AOX1* locus frequently results in *aox1*Δ transformants, where the genomic *AOX1* gene is disrupted by the expression cassette. Comparative studies on foreign gene expression in the *aox1*Δ and the wild-type *AOX* transformants have suggested that *aox1*Δ strains were advantageous for the production of some proteins (Cregg et al., 1987; Romanos et al., 1991). During induction on methanol, these *aox1*Δ transformants do not simultaneously produce high levels of the AOX enzyme and the heterologous product. They are also characterized by having a much lower demand for O_2 and methanol than the wild-type strains. However, in the integrative transformation of *H. polymorpha,* such double homologous crossovers at the genomic *MOX* locus, leading to *mox*Δ transformants, are extremely rare. Although a special strategy has been recently developed to obtain the replacement of the *MOX* gene with expression cassettes (Agaphonov et al., 1995), the influence of *MOX* gene disruption on the production of heterologous proteins has not yet been analyzed in *H. polymorpha.*

In the present study, we report on the development of HSA expression systems in *H. polymorpha* using the methanol inducible *MOX* promoter. To optimize the secretory expression of HSA, we investigated several parameters affecting HSA expression, including the 5′-untranslated region (5′-UTR), gene dosage, and the integration site of the HSA expression cassette. We further analyzed the effect of *MOX* gene disruption by constructing the *mox*Δ transformant, in which the genomic *MOX* gene was replaced with the HSA expression cassette. We report here that the *H. polymorpha* expression systems developed in this study were able to produce correctly processed recombinant HSA at good yields both in shake-flask cultures and in fed-batch fermentations.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strains used in this study are *H. polymorpha* DL1-L (*leu2*) and DLT2 (*leu2 mox-trp3::ScLEU2*), which are derived from *H. polymorpha* DL-1 (ATCC 26012). The DLT2 strain is a *mox*-disruptant in which the whole coding region of the *MOX* gene and a partial N-terminal region of the adjacent *TRP3* gene (Agaphonov et al., 1995) were transplaced with the *S. cerevisiae LEU2* gene (Agaphonov MO, unpublished data). Plasmid pMOX3615 contains a 9 kb *Bam*HI fragment of the *H. polymorpha* DL-1 *MOX* gene, which was isolated based on the DNA sequence homology to the *H. polymorpha* CBS4732 *MOX* gene (Ledeboer et al., 1985). The 1.5 kb *Eco*RI/SalI *MOX* promoter and the 0.5 kb *Bgl*II/*Eco*RV *MOX* terminator, obtained from pMOX3615, were inserted into the multicloning sites of pBluescript KS^+ (Stratagene), generating pBMOX8 and pBKS-Tmox(I), respectively. The *Eco*RI and *Hin*dIII sites in the multiple cloning site of pBKS-Tmox(I) were removed by Klenow fragment treatment to result in pTmox(II). The 2.2 kb HSA cDNA fragment containing the whole coding region of HSA with its 5′ UTR was obtained from pBlue-HSAI (Kang et al., 1998). Plasmid pGLG61-135 is identical with pGLG61 (Sohn et al., 1999b), except that it contains the telomeric fragment TEL135 instead of TEL188 (Sohn et al., 1999a).

Construction of MOX-HSA Expression Cassettes I and II

To fuse the *MOX* terminator with HSA cDNA, the 0.5 kb blunt ended *Not*I/*Hin*dIII DNA fragment of the *MOX* terminator was obtained from pTmox(II) and inserted into the blunt-ended *Xho*I/*Hin*dIII sites of pBlue-HSAI (Kang et al., 1998), resulting in pHSA-Tmox. The HSA cDNA fused with the *MOX* terminator was generated as a 2.5 kb *Eco*RI/*Cla*I fragment from pHSA-Tmox and ligated with the 1.5 kb *Sal*I/*Eco*RI *MOX* promoter fragment, which was amplified by polymerase chain reaction (PCR) from pMOX3615. The ligated product was then inserted into the *Cla*I/*Sal*I sites of YIP5 (Struhl et al., 1979). The resultant plasmid YIP-HSA contained the MOX-HSA expression cassette I, which was composed of the HSA cDNA with its 5′-UTR under the control of the *MOX* promoter and terminator (Fig. 1A, MOX-HSAI).

To fuse the HSA cDNA devoid of its 5′-UTR directly to the *MOX* promoter, two DNA fragments were joined via PCR in three steps. At the first PCR, the 250 bp DNA fragment corresponding to the N-terminal part of HSA cDNA was amplified from pBlue-HSAI using two oligonucleotides, MOX/HSA-33mer (5′-CTAAAGTACAAAAACAAAATGAAGTGGGTAACC-3′; the translation initiation codon of HSA cDNA was underlined) and HSA(*Afl*III)-19mer (5′-GCTGACTCATCAGCAACAC-3′). In the second step, the 1.5 kb *MOX* promoter fragment containing ATG initiation codon was amplified from pBMOX8 using

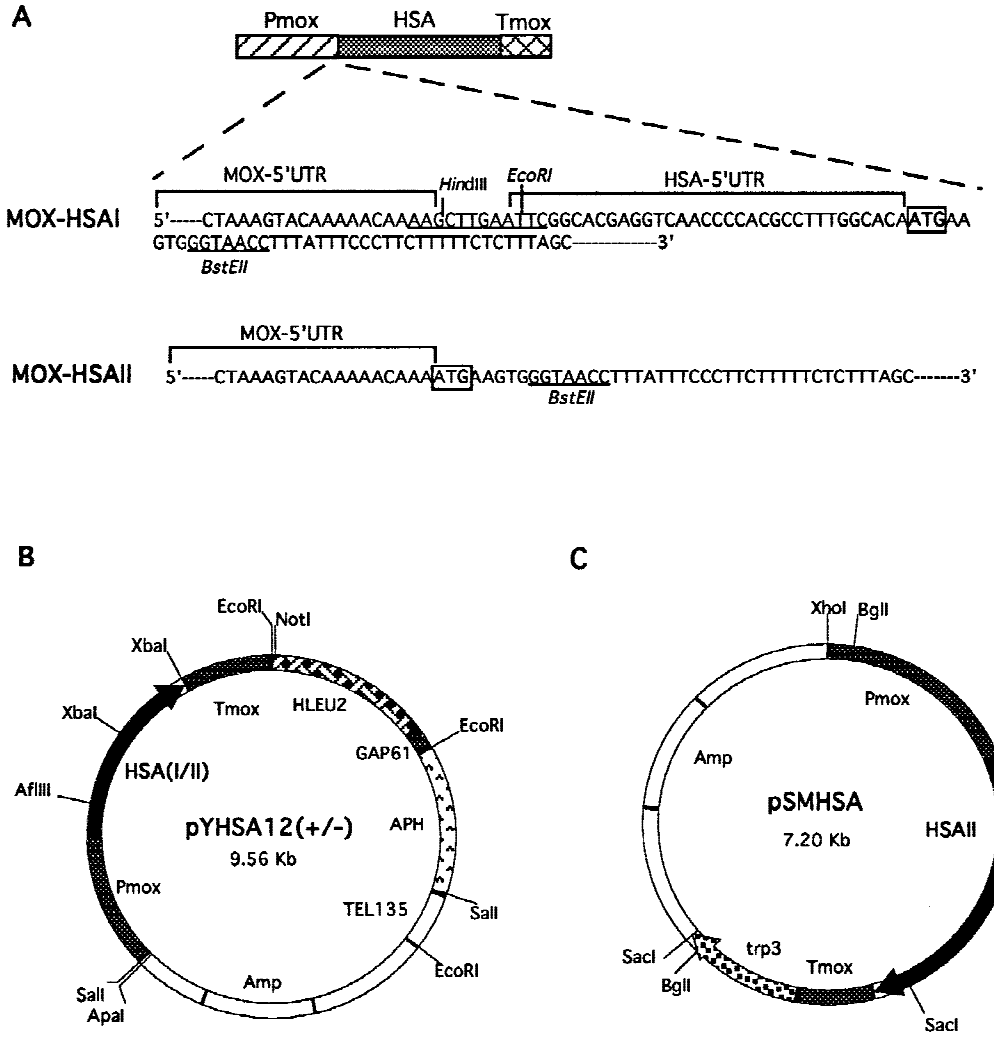

Figure 1. Generic plasmid base for the expression of HSA in *H. polymorpha.* (A) The 5′-untranslated region (5′-UTR) of HSA cDNAs in the MOX-HSA expression cassettes. The MOX-HSA expression cassette I was constructed with the HSA cDNA containing its 5′-UTR and an *Eco*RI site, which was generated during the cDNA construction (Kang et al., 1998). A *Hin*dIII site was introduced during the PCR amplification of the *MOX* promoter in this study. In the MOX-HSA expression cassette II, the HSA cDNA lacking its 5′-UTR was directly fused with the *MOX* promoter by the fusion PCR strategy. The initiation codon ATG of the HSA open reading frame is boxed. (B) Integration vectors for HSA expression. pYHSA12(+) and 12(−) contain the MOX-HSA expression cassettes I and II, respectively. Both vectors carry the telomeric fragment TEL135 as an autonomous replication sequence (Sohn et al., 1999b) and the *HLEU2* gene and the *APH* gene fused to the *GAPDH* promoter as selection markers (Sohn et al., 1999a). (C) Replacement vector pSMHSA. The vector contains the MOX-HSA expression cassette II and the partial *trp3* gene fragment of *H. polymorpha* derived from pSM1 (Agaphonov et al., 1995).

the reverse primer (5′-AACAGCTATGACCATG-3′) and MOX-19mer (5′-CATTTTGTTTTTGTACTTT-3′: the anticodon of start codon was underlined). In the last step, these two PCR products containing 16 bp complementary sequences at each end were annealed and used as a template for the reverse primer and MOX/HSA-33mer to amplify the 1.8 kb DNA fragment containing the *MOX* promoter and the N-terminal part of HSA cDNA. The final product of 1.8 kb DNA fragment was inserted into pT7Blue T-vector (Novagen) and sequenced to check any possible error generated during PCR. The resultant plasmid pT7Blue/PmoxHSA247 was digested by *Afl*III and *Sal*I to generate the 1.8 kb DNA fragment containing the *MOX* promoter fused with the N-terminal part of HSA cDNA. The 1.8 kb *Afl*III/*Sal*I fragment was ligated with the 2.2 kb *Afl*III/*Eco*RI DNA fragment containing the remaining part of HSA fused with the *MOX* terminator, which was obtained from YIP-HSA, and then inserted into the *Eco*RI/*Sal*I sites of pBluescript KS$^+$. The resultant plasmid pBHSA11 contained the HSA expression cassette II, which was composed of the HSA cDNA devoid of its 5′-UTR under the control of the *MOX* promoter and terminator (Fig. 1A, MOX-HSAII).

Construction of HSA Expression Vectors for *H. polymorpha*

To construct the integrative vector pYHSA12(−), carrying the MOX-HSA cassette II, the 4.0 kb *Apa*I/*Not*I cassette II generated from pBHSA11 was ligated with the 2.9 kb *Not*I/*Sal*I fragment of pGLG61-135, which contained the *H.*

polymorpha LEU2 gene (*HLEU2*), the modified *APH* gene, and TEL135. The ligated 6.9 kb *Apa*I/*Sal*I DNA fragment was inserted into the *Apa*I and *Sal*I sites of pBluescript KS^+, resulting in pYHSA12(−). The integration vector pYHSA12(+), which was identical to pYHSA12(−) except that it contained the MOX-HSA cassette I, was constructed by replacing the 4.0 kb *Apa*I/*Not*I cassette II in pYHSA12(−) with the 4.0 kb *Cla*I/*Sal*I cassette I derived from YIP-HSA (Fig. 1B). To construct the replacement vector pSMHSA (Fig. 1C), the 3.2 kb *Xho*I/blunt ended *Not*I DNA fragment containing the HSA cDNA fused with the *MOX* promoter was generated from pBHSA11 and ligated with the 4 kb *Xho*I/blut ended *Bgl*II fragment derived from pSM1 (Agaphonov et al., 1995).

Transformation of *H. polymorpha* and Screening of HSA-Expressing Transformants

Transformation of *H. polymorpha* and stabilization of the obtained transformants was carried out according to the DMSO-lithium acetate method as previously described (Sohn et al., 1999a). For the transformation of *H. polymorpha* DL1-L, Leu^+ transformants were selected in the minimal selective medium (0.67% yeast nitrogen base without amino acid and 2% glucose). The subsequent selection of the Leu^+ transformants for the G418 resistance was performed with minimum selective medium supplemented with different concentrations of G418 ranging from 0 to 4 mg/mL (Invitrogen). For the transformation of *H. polymorpha* DLT2, Trp^+ transformants were selected in the minimal medium complemented with leucine. The selected transformants were screened for the expression of HSA by colony immunoblotting. Transformants grown on YPD agar plate overnight were transferred onto nitrocellulose membranes (Schleicher & Schuell), which were then placed on a fresh YPM agar plate for 1 day. The membranes were washed with 20 m*M* Tris-HCl (pH 7.5) solution containing 100 m*M* NaCl and subsequently subject to Western blotting using the anti-HSA polyclonal antibody (Sigma) as previously described (Kang et al., 1998).

Shake-Flask and Fed-Batch Cultures

In shake-flask cultures, BYPM (1% yeast extract, 2% peptone, 2% methanol, and 0.1*M* potassium phosphate, pH 6.0) medium was used for the induction of HSA expression from the *MOX* promoter. Flasks (250 mL) containing 25 mL of BYPM medium were inoculated with 0.25 mL of the seed culture grown to the stationary phase in YPG (1% yeast extract, 2% peptone, and 2% glycerol) medium. Cultures were grown for 2 or 3 d at 37°C on a rotary shaker (200 rpm). Cell growth was measured as optical cell density (OD) at 660 nm using a spectrophotometer (Spectronic 21D, Milton Roy), in which OD 1 was equivalent to 0.3 g/L of dry cell weight of *H. polymorpha.*

Fermentations were carried out in a 5-L fermentor (Best Korea Fermentor) containing 2 L of the medium containing 10 g/L glycerol and 20 g/L yeast extract. The fermentor was inoculated with 100 mL of a preculture grown to the stationary phase on the medium containing 10 g/L glycerol, 13.4 g/L yeast nitrogen base without amino acid, and 4 g/L biotin. During fermentation, the pH was adjusted between 5.5 and 6.0 with 3N HCl and 3N NaOH. The temperature was maintained at 37°C and the level of dissolved oxygen was maintained above 10%. After consumption of initial glycerol in the medium at the end of the batch phase, a feeding of glycerol (30% w/v), supplemented with yeast extract (3:2 ratio), was initiated before the feeding of methanol. In the fed-batch fermentation with a gradual feeding change, glycerol feeding was continued up to cell density $OD_{660}150$ while maintaining the specific growth rate at 0.08–0.09 h^{-1}. While carrying out several sets of experiments to optimize the HSA production with *Hansenula polymorpha* DL-1 in high-cell density fermentations, we observed that the presence of even a small amount of glycerol was inhibitory in achieving the full induction from the *MOX* promoter in *H. polymorpha* DL-1 (Kang W. et al., unpublished results). Therefore, a rather low specific growth rate at around 0.09 h^{-1} was maintained during glycerol feeding. Then, methanol was added up to 4.0 g/L and the glycerol feeding rate was decreased to smoothly change the specific growth rate from 0.08 down to 0.02 h^{-1}. At the end of growth phase, only methanol was added at a rate of 0.015 g/L/OD/h. In the fed-batch fermentation with an abrupt feeding change, glycerol feeding was continued up to $OD_{660}200$ while maintaining the specific growth rate at 0.08–0.09 h^{-1}. After then, methanol feeding was started without the transition phase to the methanol fed-batch stage.

DNA, RNA, and Protein Analysis

Southern hybridization analysis for yeast genomic DNA was carried out as previously described (Sohn et al., 1999b). The integration copy number of transformants was determined by the quantitative reading of the Southern blot densitogram using an image-analysis system (GS-700, Bio-Rad). Total yeast RNA was isolated, fractionated on 1.2% formaldehyde/agarose gel, and subsequently blotted onto a nylon membrane (Schleicher & Schuell) as previously described (Kang et al., 1998).

For N-terminal amino acid analysis of recombinant HSA, the culture supernatant of recombinant *H. polymorpha* was fractionated on 8% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell). The band corresponding to HSA was excised and subjected to a Milligen/Biosearch M 6000 protein sequencer. For pI determination, the culture supernatant was fractionated on NOVEX pre-cast vertical IEF gel (pH 3–7) and analyzed by Western blotting with the HSA antibody. The concentration of HSA in culture supernatants was estimated by densitometric scans of Western blots (Kang et al., 2000) or silver-stained polyacrylamide gels with the authentic HSA (Sigma) as a standard. For Western blotting, 25–150 ng of HSA was loaded in each well, compared to

50–300 ng of HSA for silver staining. The standard curves with the authentic HSA within these ranges were linear and showed a relative standard deviation of less than 3.5%.

RESULTS

Effects of 5′-UTR and Gene Dosage on the Expression of HSA

In yeast, translation initiation is known to be particularly sensitive to the secondary structure of the 5′-UTR of mRNA (Donahue and Cigan, 1990). G+C-rich leaders of cDNA or restriction sites containing dyad symmetry introduced during cDNA constructions could have an inhibitory effect on the expression of foreign genes in yeast. To avoid any detrimental influence exerted by the 5′-UTR derived from the HSA cDNA, a set of HSA expression cassettes, under the control of the *MOX* promoter, were constructed using HSA cDNA with or without its 5′-UTR. The HSA 5′-UTR was retained in the MOX-HSA expression cassette I, while the HSA 5′-UTR was removed in the cassette II (Fig. 1A). Consequently, the HSA transcript generated from expression cassette II had a 5′-UTR identical to that of the *MOX* mRNA of *H. polymorpha* DL-1. Integrative expression vectors pYHSA12(+) or pYHSA12(−), which were identical except that they contained HSA expression cassettes I and II, respectively, were constructed to carry the G418 resistance cassette as a dominant selection marker and a telomeric fragment TEL135 as an autonomous replication sequence (ARS) (Fig. 1B). In this dominant selection system exploiting G418 resistance, transformants harboring various copy numbers of vectors integrated into the host's chromosome could be easily obtained by selecting transformants at different G418 concentrations (Sohn et al. 1999a).

Primary screening for the expression of HSA among the transformants harboring pYHSA12(+) or pYHSA12(−) was carried out by colony immunoblotting of individual transformants (data not shown). Southern blot analysis of the selected transformants harboring pYHSA12(−) revealed that the integration copy numbers of the vector generally increased in the range from one to seven with increasing G418 concentration in the selection medium (Fig. 2A). Identical results were also obtained in the Southern blot analysis of the transformants harboring pYHSA12(+) (data not shown). Comparison of the HSA levels in the culture supernatants of these transformants, grown in shake-flasks using BYPM medium (Table I), showed that a gene dosage effect on HSA production, apparently observed among the transformants of pYHSA12(+), was abolished among the transformants of pYHSA12(−). In the case of pYHSA12(+), multiple integrants bearing seven copies displayed at least fourfold higher yield compared to single-copy integrants. In the case of pYHSA12(−), however, multiple integrants produced equal or less HSA than single-copy integrants. It was noteworthy that the highest yield of HSA obtained from the integrants of pYHSA12(−) was twofold higher than that from integrants of pYHSA12(+), which suggested that the optimization of 5′-UTR in the HSA expression cassette significantly improved the expression of HSA from the *MOX* promoter. Interestingly, the result also indicated that after optimization, a single copy of expression cassette II became sufficient to achieve the maximal level expression of HSA from the *MOX* promoter in *H. polymorpha.*

Analysis of the Integration Site and the HSA Transcript in *MOX* Transformants

The site of integration is one of the most important factors that determines the expression efficiency of foreign genes in a heterologous host. The integrative vectors pYHSA12(+) and pYHSA12(−) were expected to be integrated mostly into the telomere locus, because they contain a telomeric ARS TEL135, which directs the integration of vectors exclusively into the ends of chromosomes (Sohn et al., 1999b). However, Southern blot analysis as shown in Figure 2 unexpectedly revealed that integration into the *MOX* locus occurred as frequently as integration into the telomere. When the *MOX* promoter fragment was used as a hybridization probe in Southern blotting, it was expected that single-copy integration of pYHSA12(−) into the telomere should show two bands corresponding to the *MOX* promoter: a 3 kb band originating from the genomic *MOX* promoter and an additional band of the 6.5 kb *Eco*RI fragment, containing the *MOX* promoter fragment derived from the expression cassette (Fig. 2B, bottom). However, in many transformants, the 3 kb signal of the genomic *MOX* promoter disappeared and new signals of 4.3 kb, 5.5 kb, and 6.5 kb appeared. This indicated that single or multiple copies of the vector were integrated into the *MOX* locus with a relatively high frequency in *H. polymorpha* DL-1 (Fig. 2B, top). Because this type of additive integration at the *MOX* locus did not lead to the disruption of the *MOX* gene, all the transformants retained *MOX* wild-type and had the methanol utilization positive (Mut^+) phenotype. The integrants at the *MOX* locus showed about twofold higher levels of HSA expression than the corresponding integrants at the telomere (Table I). To some extent this probably reflects the telomere positional effect on gene expression (Grunstein, 1998). Alternatively, the *MOX* locus itself might be the optimum site for the maximal performance of the *MOX* promoter employed in the expression cassette. The higher level of HSA expression at the *MOX* site could partly account for the unexpectedly high frequency of integration at the *MOX* locus, because the primary screening step of transformants for the HSA expression could favor integrants at the *MOX* locus.

Because increasing gene dosage is generally expected to lead to an increase in the level of the corresponding transcript, we analyzed the correlation between the copy number of the integrated vector and the level of HSA transcription (Table I and Fig. 3). In Northern blot analysis of integrants harboring pYHSA12(+), the steady-state level of HSA mRNA in the seven-copy integrant was about seven-

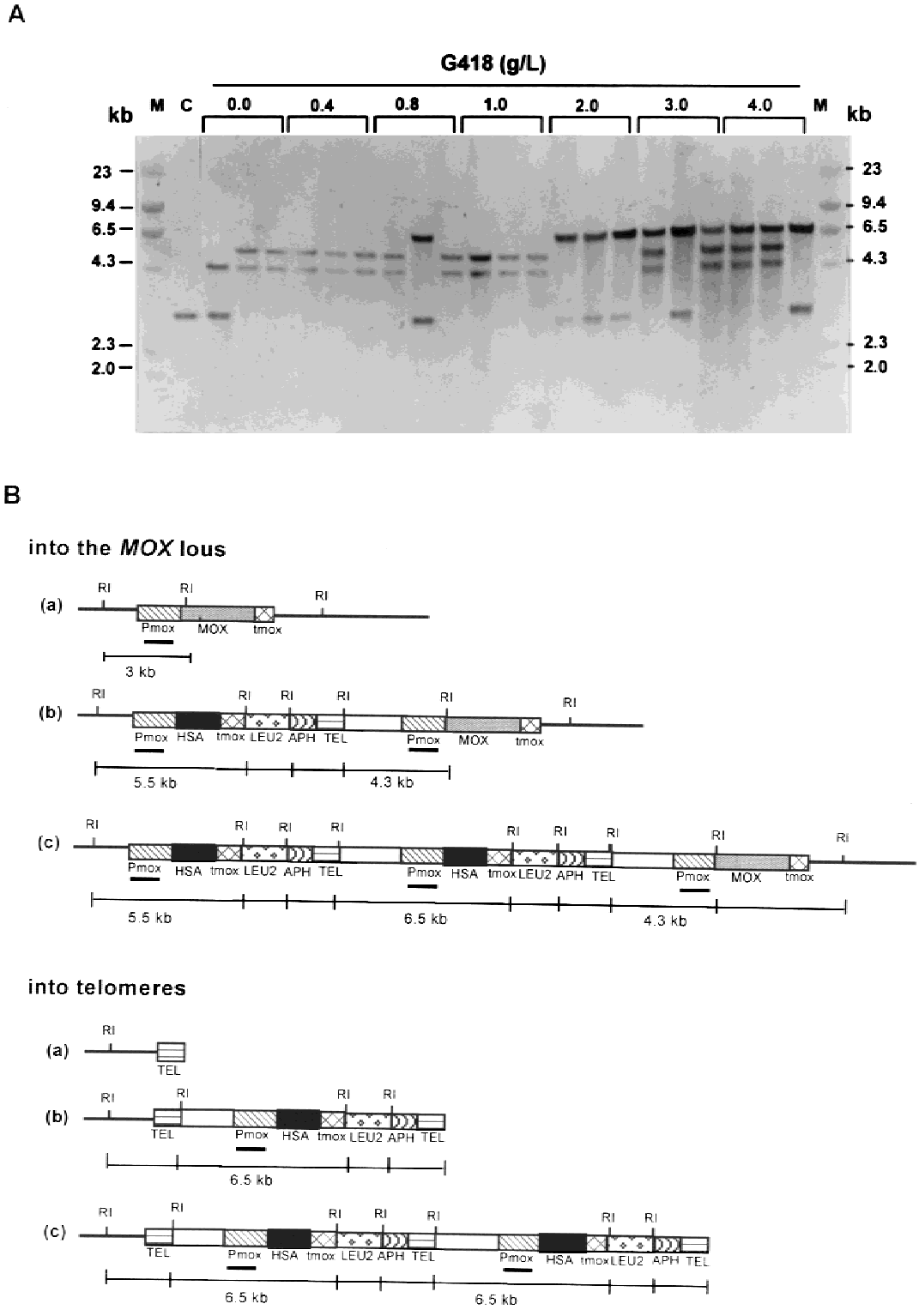

Figure 2. Integration pattern of transformants harboring pYHSA12(−). (A) Southern blot analysis. Genomic DNA of individual transformants picked from each plate containing different concentrations of G418 were digested with *Eco*RI and probed with the 1.5 kb digoxigenin-labeled *Sal*I/*Hind*III fragment of the *MOX* promoter obtained from YIP-HSA. Lanes M and C contain the digoxigenin-labeled ;λ *Hind*III size marker and the DNA from untransformed host cell, respectively. (B) Diagrams explaining the integration of pYHSA12(−) into the *MOX* locus or telomeres. Before integration (a), single-copy (b) and two-copy tandem (c) integrations were depicted. The probe used in the Southern blot analysis was represented a black box.

Table I. Effect of the 5′-UTR and the copy number of integrative vectors on the HSA expression in *H. polymorpha* transformants.

Vector	HSA 5′UTR	Transformants	Copy #	Integration locus	HSA mRNA[a]	HSA protein (mg/L)[b]
pYHSA12(+)	+	G0T2	1	tel	±	5
		G0T8	1	*MOX*	+	10
		G2T27	6	tel	++++++	15–20
		G4T5	7	tel	++++++	15–20
pYHSA12(−)	−	G0T7	1	*MOX*	+	40–50
		G3T8	2	*MOX*	+	40–50
		G4T9	3	*MOX*	+	40–50
		G0.8T3	1	tel	±	20–30
		G2T7	3	tel	±	20–30
		G3T5	6	tel	±	20–30

[a]The levels of HSA transcript after 24 h culture were analyzed by Northern blot analysis and presented as the relative value compared to the level observed in the transformant harboring single copy of HSA expression vector integrated into the *MOX* locus. Transformants were cultivated in a 250-mL baffled flask containing 25 mL BYPM medium.

[b]The levels of intact HSA protein in the culture supernatant after 48 h cultivation were analyzed by densitometric tracing of the 66-kDA HSA band in Western blot with the authentic HSA as a standard (Kang et al., 2000).

fold higher than the level of HSA detected in the single-copy integrant, which suggested a strong correlation between the transcript level and the gene dosage (Fig. 3, lanes 4 and 5). In contrast, the levels of HSA transcription in the integrants of pYHSA12(−) did not increase in proportion to the increased copy number, regardless of the integration site (Fig. 3, lanes 1–3). This would explain the lack of a gene dosage effect on HSA expression observed among transformants of pYHSA12(−). The fact that single-copy integrants of pYHSA12(−) produced about a fivefold higher level of HSA protein than the single-copy integrants of pYHSA12(+), in spite of similar levels of HSA transcript, strongly indicated that the optimization of 5′-UTR in the HSA expression cassette resulted in an improvement in expression efficiency at the translational level.

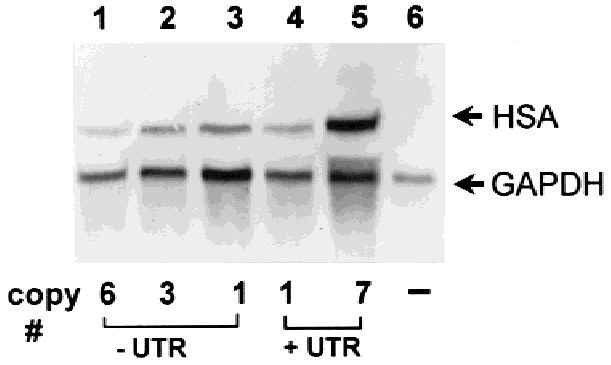

Figure 3. Northern blot analysis of HSA transcripts expressed in *H. polymorpha* transformants. Total RNAs from transformants, grown in YPM medium for 20 h, were fractionated on 1.2% formaldehyde gel, blotted to a nylon membrane, and hybridized with the 1.8 kb digoxigenin-labeled *Bst*EII/*Hind*III HSA cDNA fragment (Kang et al., 1998). Lanes 1 to 3, transformants harboring six copies (lane 1), three copies (lane 2), and one copy (lane 3) of pYHSA12(−), respectively. Lanes 4 and 5, transformants harboring one copy (lane 4) and seven copies (lane 5) of pYHSA12(+), respectively. Lane 6 contains the RNA sample from untransformed host cell. The *GAPDH* transcript (Sohn et al., 1999a) of *H. polymorpha* was used as an internal standard.

Construction and Analysis of *mox*-Disrupted Transformants

In a previous study, we observed that *MOX* wild-type transformants, with strong methanol oxidase activity, acted as a whole cell enzyme, to use up a large portion of methanol as a substrate for the enzyme reaction and not as an inducer (Kim et al., 1996). Further, the *MOX* protein was found to constitute more than 30% of the soluble cell protein in an *MOX* wild-type strain growing on methanol (Giuseppin et al., 1988). In an effort to overcome these limitations encountered in the *MOX* wild-type background, we constructed the *mox*-disrupted (*mox*Δ) transformant by replacing the genomic *MOX* gene with the HSA expression cassette II (Fig. 4). The replacement vector pSMHSA (Fig. 1C), carrying the truncated *trp3* gene of *H. polymorpha* (Agaphonov et al., 1995) and the MOX-HSA expression cassette II, was cleaved by *Bgl*I and then introduced into *H. polymorpha* DLT2 (*leu2 mox-trp3*::*ScLEU2*). Trp^+ transformants, which can only result from double homologous integration at the *MOX* site (Fig. 4A), were selected and examined for the leucine auxotrophic (Leu^-) and methanol-utilization negative (Mut^-) phenotype as well as for the ability to express HSA. Southern blot analysis of the Trp^+ transformants, with a probe of the *MOX* promoter, showed the generation of a 9.5 kb *Eco*RI fragment from the homologous recombination, confirming that the HSA expression cassette was correctly transplaced into the *MOX* locus (Fig. 4B, lanes 3–5).

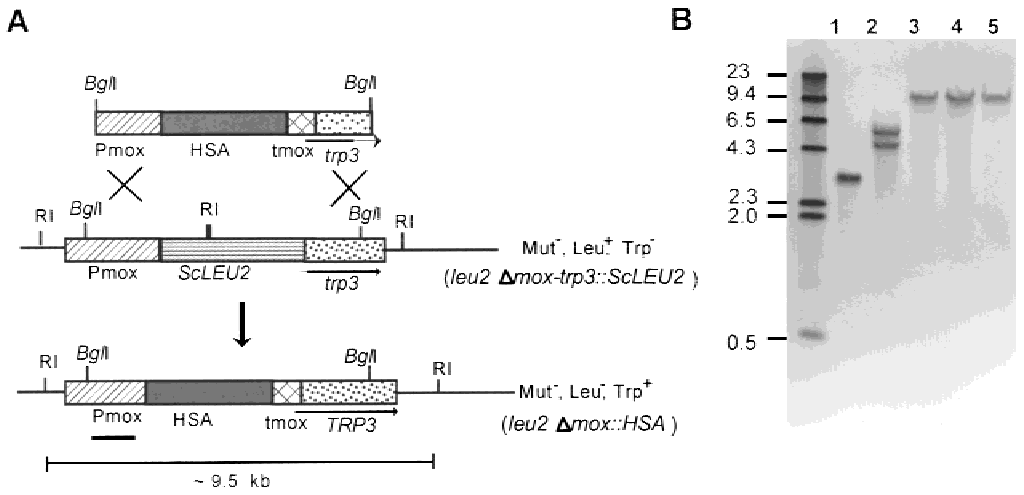

Figure 4. Transplacement of the *MOX* gene with the HSA expression cassette. (A) Double-crossover transplacement. Repair of *trp3* disruption was mediated by replacement with the HSA expression cassette II containing the partial *trp3* gene fragment. (B) Southern blot analysis. Chromosomal DNAs obtained from the Trp^+ transformants (lanes 3–5) were digested with *Eco*RI and hybridized with the DIG-labeled digoxigenin-labeled *Sal*I/*Hind*III fragment of the *MOX* promoter. Lane M contains the digoxigenin-labeled λ *Hind*III size marker. Lane 1 and lane 2 contain the DNA from untransformed host cell and the *MOX* transformant in which a single-copy of HSA expression cassette was integrated into the *MOX* locus, respectively.

The growth and the de-repressed levels of HSA expression from the *MOX* promoter for the *mox*Δ and the wild-type *MOX* transformants were comparable in shake-flask cultures using glycerol as the sole carbon source (Table II). When the induction of HSA expression by methanol was initiated at low cell density (0.25 OD_{660}) in BYPM medium, the *mox*Δ transformant showed lower production levels of HSA than the *MOX* transformants, primarily due to the much lower cell mass on the medium containing methanol as a sole carbon source. However, when the induction was initiated at high cell density (about 40 OD_{660}), the *mox*Δ transformant showed about a twofold higher yield of HSA production than the *MOX* transformant. Interestingly, the *MOX* transformant showed a lower level of HSA expression at high cell density induction than at low cell density induction, indicating that different induction modes should be considered when trying to achieve maximal levels of HSA expression for the *MOX* and *mox*Δ transformants. The superior productivity of the *mox*Δ transformant became more remarkable in the medium containing low concentrations of methanol, for example at 0.5%, which suggests that the Mut^- phenotype of the *mox*Δ transformants could offer advantages over the *MOX* transformants for the production of HSA under methanol-limited culture conditions.

We carried out preliminary fed-batch fermentations to evaluate the HSA expression system developed in this study. As indicated by the shake-flask cultures, comparisons of fed-batch fermentation profiles showed that the *MOX* and

Table II. Effects of carbon source and culture mode on the expression of HSA from the *MOX* and *mox*Δ transformants in shake-flask cultures.

		MOX		*mox*Δ	
Carbon source	Initial cell density (OD_{660})	Final cell density (OD_{660})	HSA[c] (mg/L)	Final cell density (OD_{660})	HSA[c] (mg/L)
Glyerol 2%	0.25[a]	50	25	50	20
Methanol 2%	0.25[a]	30	45	4	15
	40[b]	45	35	40	60
Methanol 1%	40[b]	45	40	40	60
Methanol 0.5%	40[b]	45	5	40	70

[a]The recombinant *H. polymorpha* cells were precultured in YPG medium for 1 day and then inoculated into BYPG or BYPM medium at a low culture density (0.25 OD_{660}). The cultivation was prolonged for 48 h.

[b]The cell mass accumulated in 25 mL YPG medium for 1 day was collected and resuspended in 25 mL BYPM medium containing different concentrations of methanol to induce the synthesis of rHSA.

[c]The levels of HSA protein in the culture supernatant were analyzed by densitometric tracing of the 66-kDA HSA band in silver-stained SDS-polyacrylamide gel with the authentic HSA as a standard.

*mox*Δ transformants required different induction modes to improve performance (Table III). The *MOX* transformant produced recombinant HSA at a higher level in the fermentation with a more gradual feeding change from glycerol to methanol than in the fermentation with an abrupt feeding change. This indicated that an adaptation phase for the cells to shift smoothly from glycerol to methanol growth was necessary for the *MOX* strain to achieve full induction from the *MOX* promoter. In contrast, the *mox*Δ transformant showed better productivity in the fermentation mode with an abrupt feeding change than with a gradual feeding change from glycerol to methanol. The specific production rate of the *mox*Δ transformant was observed to be twice as high as that of the *MOX* transformant. Nevertheless, the final level of HSA produced with the *mox*Δ transformant in the fed-batch fermentation with methanol induction was shown to be lower than that of the *MOX* transformant. While the synthesis of HSA was continuous throughout the induction phase in the *MOX* transformant, the expression time for HSA was relatively short in the *mox*Δ transformant. This is likely to be due to the inability of the *mox*Δ transformant to maintain metabolic activity long enough for recombinant protein production during the methanol induction phase.

Analysis of *H. polymorpha*-Derived rHSA

To achieve HSA secretion in *H. polymorpha*, we used the native prepro sequence of HSA and confirmed that the HSA sequence efficiently directed the secretion of HSA in *H. polymorpha* as it does in the other yeasts. Less than 15% of the total HSA expressed was detected in the intracellular fraction of the *H. polymorpha* transformants when analyzed by Western blotting (data not shown). Although the intact HSA of 67 kDa was secreted as the major dominant protein, the degradation products of 52 kDa and 45 kDa were also detected as minor forms of HSA in the culture supernatant of recombinant *H. polymorpha* (Fig. 5A). N-terminal amino acid sequencing of the mature HSA of 67 kDa confirmed the correct processing of the HSA-signal sequence during the secretion process in *H. polymorpha.* The N-terminal amino acid sequences of the 45 and 52 kDa degradation products were shown to have the same N-terminal sequences, D-A-H-K-S-(in single-letter amino acid code), as that of the 67 kDa intact protein, indicating that they were generated by C-terminal degradation. The generation of 45 kDa degradation products has been observed in all the yeast systems employed so far for HSA production. Another HSA fragment of 52 kDa was recently reported to be generated as a dominant degradation product, instead of the 45 kDa fragment, in recombinant *S. cerevisiae* under certain culture conditions (Kang et al., 2000). As reported in the case of the recombinant HSA produced by *S. cerevisiae* (Dodsworth et al., 1996), the *H. polymorpha*-derived recombinant protein also showed an average pI of around 4.8 on the isoelectric focusing gel, which is identical with that of authentic HSA (Fig. 5B). These results indicate that the recombinant HSA secreted from *H. polymorpha* was correctly processed.

Table III. Effect of induction mode on the expression of HSA from the *MOX* and *mox*Δ transformants in fed-batch fermentations with methanol feeding.

Induction mode	Strain	Specific HSA production rate[a] (mg/L · h · OD_{660})	Final cell density[b] (OD_{660})	Final HSA expression level[c] (g/L)
Gradual feeding change	*MOX*	0.16	270	1.3
	*mox*Δ	0.15	200	0.4
Abrupt feeding change	*MOX*	0.12	250	0.85
	*mox*Δ	0.34	200	0.6

[a]Specific HSA production rate was the average rate calculated for the expression time, which was defined as the period required to reach the maximum expression level of HSA after methanol induction.

[b]Cell growth was measured as optical cell density (OD) at 660 nm, in which OD 1 was equivalent to 0.3 g/L of dry cell weight of *H. polymorpha.*

[c]The HSA level was determined by densitometric tracing of the 66-kKa HSA band in SDS-polyacrylamide gel electrophoresis with the authentic HSA as a standard. Final cell density and HSA level were the values obtained at the end of the fermentation (96 h).

DISCUSSION

Hansenula polymorpha has drawn increasing attention as a promising alternative host system to the conventional yeast *S. cerevisiae.* Until now, production of heterologous proteins in this yeast has been mainly made based on the *H. polymorpha* CBS strain as a host system. We have developed the HSA expression system using the *MOX* promoter

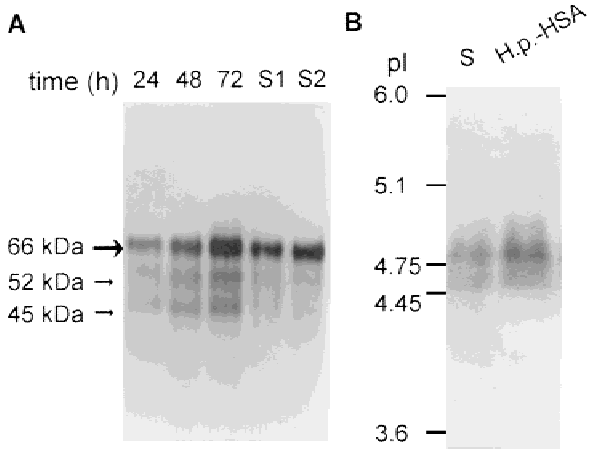

Figure 5. Analysis of recombinant HSA secreted in *H. polymorpha* DL-1. (A) SDS/polyacrylamide gel analysis. The culture supernatant (8 μL) of the *MOX* transformant grown in shake-flasks containing YPM medium buffered with 0.1*M* potassium phosphate (pH 6.0) was taken at the indicated times and then analyzed by silver staining. The two lanes, S1 and S2 contain 150 ng and 200 ng of the authentic HSA as standards, respectively. (B) Isoelectric focusing gel analysis. Authentic HSA (lane S) and the culture supernatant of *H. polymorpha* transformant (H.p.-HSA) were run on a horizontal polyacrylamide IEF gel (Novex 3-7 IEF gel) and analyzed by Western blotting with the polyclonal antibody against HSA. BioRad IEF standard was used as a marker for pI determination.

in the DL-1 strain, another representative strain of *H. polymorpha* (Levine and Cooney, 1973). Although the DL-1 strain is not suitable for classical genetic analysis due to its inability to mate and sporulate (Lahtchev K, personal communication), we have observed that the DL-1 strain showed some characteristics distinguishable from the CBS strain, which might be more useful for heterologous gene expression. For example, the DL-1 strain displays a higher growth rate, a faster adaptation to culture medium changes (data not shown), and a higher frequency of homologous recombination compared to the CBS strain (Agaphonov MO, personal communication). As a first step in optimizing HSA production using the *H. polymorpha* DL-1 strain, we optimized the 5′-UTR of HSA mRNA expressed from the *MOX* promoter of the DL-1 strain, which showed 91% nucleotide sequence identity to that of the CBS stain (data not shown). Our initial HSA expression cassette I generated HSA mRNA containing 5′-UTR derived from the HSA cDNA, which has a relatively high G + C composition (59%). Moreover, two restriction enzyme sites were artificially introduced into the 5′-UTR during the construction of the HSA cDNA (Kang et al., 1998) and the PCR amplification of the *MOX* promoter in this study. Removal of the 5′-UTR derived from the HSA cDNA and the restriction sites led to an approximate fivefold improvement in HSA expression at the translation level. This implies that the G + C-rich 5′-UTR tends to form an unfavorable secondary structure, which might be responsible for the lower expression of the MOX-HSA expression cassette I compared to the cassette II.

In general, one of most important factors for obtaining high-level expression is assumed to be the presence of multiple integrated copies of expression cassette. A direct correlation between gene dosage and expression level has been reported for many foreign proteins expressed in yeast. However, several cases were also reported in which a single- or double-copy integration of the expression unit resulted in the expression of recombinant products at maximum levels (Faber et al., 1996; Romanos et al., 1992; Sreekrishna, 1993). Although the nature of the gene and its protein product might be the major determinants of the gene dosage at which expression becomes limited, our present results show that an apparent gene dosage effect was abolished after the optimization of the MOX-HSA expression cassette. We have also made an identical observation during the course of developing the HSA expression system under the control of a strong constitutive promoter, namely, the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}) of *H. polymorpha.* The effect of gene dosage on HSA expression also became much weaker after optimizing the P_{GAP}-HSA expression cassette (Kang HA et al., unpublished result). These observations strongly support the notion that the gene dosage effect is largely dependent on the efficiency of the expression cassette. Analogously, the effect of gene dosage became more obscure in more optimized fermentor cultures than in shake-flask cultures in the production of mouse epidermal growth factor and tetanus fragment C using *P. pastoris* as a host system (Clare et al., 1991a; 1991b).

The limited HSA production in multicopy integrants could be explained by a blockage of the secretory pathway at high gene dosage, which might inhibit further secretion of HSA, as indicated in several secretory recombinant proteins (Wittrup et al., 1994). However, we did not detect the intracellular accumulation of recombinant HSA at high gene dosage (data not shown). Rather surprisingly, the levels of HSA transcription from the optimized expression cassette II did not increase any further with increased gene dosage. This is strikingly different to the case of expression cassette I, in which the levels of HSA transcripts showed a strong correlation with the copy numbers of the cassette up to at least the seven copy level. Comparable levels of HSA transcript were detected between the single-copy integrants of cassettes I and II, which excluded the possibility that no further increase of the HSA transcript level in multicopy integrants of cassette II would result from saturation of the transcription capacity of host cells. We have also made a similar observation that the HSA expression reached a maximum level at the two-copy integration of the P_{GAP}-HSA expression cassette and did not increase further at the transcription level upon increasing the copy number (Kang HA et al., unpublished result). At present, we do not understand the reason why the level of HSA transcript did not increase further once the maximum level of HSA protein was achieved in the *H. polymorpha* transformants.

Compared to the *MOX* wild-type strain, the *mox*Δ strain can be expected to have several advantages as the host system for the production of recombinant proteins, as previously reported for the *aox1*Δ strains of *P. pastoris.* While *MOX* strains with strong alcohol oxidase activity are extremely sensitive to transient high residual methanol concentrations (Swartz and Cooney, 1981), the *mox*Δ strain was found to be fairly insensitive to changes in the methanol concentration, which would facilitate the fermentation process on a large scale. Further, the synthesis of dihydroxyacetone synthase, another methanol inducible major protein, was reported to be much reduced in the absence of methanol oxidase (Faber et al., 1994). Consequently, the recombinant protein of interest may constitute a major portion of the total cellular protein in the *mox*Δ strain. However, as suggested in reports upon our preliminary fermentations, the inability of the *mox*Δ transformant to grow on methanol necessitates the development of some feeding strategies using mixed substrates to support growth and production from the *mox*Δ transformant during the prolonged methanol induction phase. In the case of *P. pastoris* Mut^- strain, in which both *AOX1* and *AOX2* genes were disrupted, the feeding of glycerol under growth-limiting conditions as an alternative carbon source was attempted during the methanol-induction phase (Chiruvolu et al., 1997).

In conclusion, by optimizing the expression cassette using the *MOX* promoter and the methanol-utilizing phenotype of the host strain, we have developed recombinant *H. polymorpha* strains that can direct high-level expression of correctly processed recombinant HSA from just a single integrated copy of the expression unit. The present systems

have a certain biotechnological advantage in that they can avoid the possible penalty of instability, which results from high-level expression as a result of tandem multiple integration (Gilbert et al., 1994). In particular, the development of the *mox*Δ transformant would alleviate the operational problems associated with the large amounts of methanol feeding required to grow the *MOX* transformants of *H. polymorpha*, such as the need for explosion-proof equipment and special technical infrastructure. To improve the yields of recombinant HSA from these *H. polymorpha* systems, efforts are currently being made to systematically optimize fermentation parameters.

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