The Prepro-Peptide of *Mucor* Rennin Directs the Secretion of Human Growth Hormone by *Saccharomyces cerevisiae*

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An aspartic proteinase, *Mucor pusillus* rennin (MPR), of filamentous fungus *Mucor pusillus*, is efficiently secreted from a transformant of *Saccharomyces cerevisiae* containing the intact MPR gene. To test the usefulness of the MPR leader peptide in secretion of heterologous proteins from yeast cells, several plasmids encoding the fusion proteins composed of different parts of the NH₂-terminal region of prepro-MPR and human growth hormone (hGH) were constructed. The parts of the leader peptide upstream of hGH were the whole prepro-peptide followed by the NH₂-terminal region of mature MPR in *JGH1*, the intact pre-sequence and a part of the pro-sequence in *JGH2*, and the putative signal sequences of the NH₂-terminal 18 and 22 amino acids in *JGH3* and *JGH7*, respectively. When the hGH genes fused to these leader sequences were expressed in yeast cells under the control of the yeast *GAL7* promoter, proteins of various sizes immunoreactive with the anti-hGH antibody were secreted into the medium. Among the plasmids mentioned above, *JGH2* directed the greatest secretion of the protein of 23 kilodaltons in size, which contained the expected NH₂-terminal amino acid sequence of an additional eight amino acids derived from the pro-peptide of MPR. The addition of the *GAL10* terminator downstream of the hGH gene in *JGH2* resulted in a greater than three- to fivefold increase in the secretion, whereas the insertion of the *GAL4* gene, which is a positive regulator for the *GAL* system, had no significant effect. The improved yield of the total protein of hGH secreted into the medium reached approximately 10 mg/liter.

The yeast *Saccharomyces cerevisiae* has been widely used as a host for expression of heterologous proteins because of the ease of handling it, the availability of strong controllable promoters, and its lack of pathogenicity. Although some mammalian proteins were efficiently produced in the intracellular fraction (11, 15, 23), the recovery of biologically active proteins from yeast cells was rather difficult because of the accumulation of insoluble proteins and the contamination of yeast proteins. Therefore, the development of a secretion system for heterologous proteins in yeast cells has been expected to be useful for industrial production and protein engineering studies. Secretion of a protein usually requires a leader peptide at the amino terminus of the primary translation product. Successful secretion of heterologous proteins in yeast cells by using the leader peptides of yeast secretory proteins, such as α-factor and invertase, has been reported (5, 27, 30). When several genes from filamentous fungi encoding their secretory proteins were expressed under the control of yeast strong promoters, most of the proteins were efficiently secreted by yeast cells (3, 26, 29). Therefore, some leader peptides of fungal secretory proteins may also be useful for the secretion of heterologous proteins by yeast cells.

An aspartic proteinase extracellularly produced by a filamentous fungus, *Mucor pusillus* (2), is widely used as the milk coagulant in industrial cheese production. We previously reported the cloning and sequencing of the structural gene of the enzyme *M. pusillus* rennin (MPR) (28). The gene encodes a prepro-enzyme composed of 361 amino acids of the mature MPR and an additional NH₂-terminal sequence of 66 amino acid residues. When the gene was expressed in *S. cerevisiae* cells under the control of the yeast *GAL7* promoter, the mature enzyme was efficiently excreted into the medium at a concentration of approximately 200 mg/liter (29). Furthermore, we recently found that MPR was excreted in a form of pro-enzyme containing the pro-peptide of 44 amino acids, which was then processed after the secretion mainly by autocatalytic proteolysis but also by proteinases of the yeast host (16).

In this paper, we describe the secretion of human growth hormone (hGH) from yeast cells by using the prepro-peptide of MPR as the leader peptide. The results indicate that the prepro-sequence of MPR is effective for the secretion of heterologous proteins by yeast cells.

**MATERIALS AND METHODS**

**Strains and plasmids.** *S. cerevisiae* MC16 (α leu2 his4 ade2) was used as the host. *Escherichia coli* JM105 (Δlac pro) thi rpsL endA sgcB15 hisD4 F' traD36 proAB lacZΔM15 and *E. coli* CJ236 (mut-1 ung-1 thi-1 relA-1 Cm') were used as the hosts for plasmid constructions or site-specific mutagenesis.

Plasmid JP1 in which the cloned MPR gene was placed under the control of the *GAL7* promoter was described previously (29). Plasmids pYE1016 (25), pHG41 (20), and pHG107 (13) contain the region covering the *GAL10* transcriptional terminator, the *GAL4* gene, and hGH cDNA, respectively. Plasmid pJDB207 (4) was used as a shuttle vector for *E. coli* and *S. cerevisiae*.

**Construction of hGH expression plasmids.** DNA was manipulated by standard methods (21). Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase were purchased from Takara Shuzo Co., Ltd. Oligonucleotides were synthesized automatically on a Beckman System 1 Plus DNA synthesizer. Site-specific mutagenesis was carried out with an Amersham oligonucleotide.
otide-directed in vitro mutagenesis system or a Bio-Rad Mutagen kit in vitro mutagenesis kit.

Four different plasmids, JGH1, JGH2, JGH3, and JGH7, containing the fused genes composed of different lengths of the 5'-terminal region of the MPR gene and hGH cDNA, were constructed by using plasmids JP1, pJDB207, and pHGH107 (see Fig. 1).

Site-specific mutagenesis was used to construct the correct sequence between the MPR sequence and the hGH sequence. For generation of an EcoRI restriction site at the position coding for amino acids 39 and 38 in the pro-fragment of MPR, site-specific mutagenesis was performed with a 1.2-kilobase-pair (kb) BamHI-SalI fragment of JP1 carried on M13mp18 as a single-stranded DNA. A synthetic oligonucleotide, 5'-GCATCAGAATCTTGGATAC-3', was used, and the resulting base change was confirmed by nucleotide sequencing. For construction of plasmids JGH3 and JGH7, site-specific deletion mutagenesis was carried out with a single-stranded DNA containing a BamHI fragment (ca. 1.5 kb) of JGH2 on M13mp18. Synthetic oligonucleotide, 5'-TATAGTTGGGAATGCAAAAGAAGCAG-3' for a 36-base-pair (bp) deletion and 5'-GTGTATAGTGGGAATTAGTGGCAGTGCTGAAGCAG-3' for a 24-bp deletion, were used as the mutagenesis primers. In the resulting mutated genes, the sequences for the MPR pre-peptides of amino acids 66 to 49 in the 36-bp deletion and 66 to 45 in the 24-bp deletion were precisely fused to the mature hGH sequence (see Fig. 2).

Plasmids JGH4 and JGH5 in which the GAL4 gene and the GAL10 terminator sequence, respectively, were inserted into JGH2, were constructed as follows. A DNA fragment containing the GAL4 gene was prepared by excision from pHG41 by HindIII plus BamHI digestion, and the resulting 2.9-kb fragment was integrated between HindIII and BamHI upstream of the GAL7 promoter of JGH2. A 0.82-kb BamHI-EcoRV fragment containing the GAL10 terminator sequence from pYF1016 was integrated between PvuII and BamHI downstream of the hGH gene of JGH2.

Culture conditions. S. cerevisiae MC16 was transformed by the method of Ito et al. (18). SD medium containing 0.67% yeast nitrogen base (Difco) and 2% glucose supplemented with 20 mg of adenine sulfate per liter was used as the selective medium. YPD medium containing 2% Bacto-Peptone (Difco), 1% yeast extract (Difco), and 2% glucose and YPGal medium containing 3% galactose instead of glucose in YPD medium were used for the cultivation of yeast transformants. The transformants were first cultured aerobically to the early stationary phase in 50 ml of YPD medium in a 500-ml Erlenmeyer flask on a reciprocating shaker (140 strokes per min) at 30°C for 24 h. The cells were harvested by centrifugation and suspended in the original culture volume of YPGal medium for induction of the GAL7 promoter. Cultivation was continued aerobically at 30°C.

Plasmid stability. Plasmid stability was determined by checking leucine auxotrophy. After cultivation for 10 generations in YPD medium or YPGal medium, about 2,000 yeast cells were plated onto both the selective SD plate and the nonselective YPD plate. The percentage of colonies on the selective medium versus that on the nonselective medium was taken as the index of plasmid stability.

Immunoblot analysis of hGH produced by yeast cells. Proteins secreted into the culture medium were precipitated by the addition of cold trichloroacetic acid to 10%, and after centrifugation, the pellet was washed with ethanol-ether (1:1). The pellets were then dissolved in 50 mM Tris hydrochloride, pH 8.0. For analysis of the protein in the cellular fraction, the cells were suspended in 1.2 M sorbitol-20 mM EDTA-20 mM 2-mercaptoethanol-50 mM KH2PO4 (pH 6.8)-0.3% Zymolyase T-100 (Seikagaku Kogyo Co.) and incubated at 30°C for 60 min. The supernatant obtained by centrifugation at 1,000 × g for 5 min was defined as the periplasmic fraction. The pellet dissolved in 1.2 M sorbitol-50 mM KH2PO4, pH 6.8, was defined as the intracellular fraction.

These fractions were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel (10 to 20% gradient or 17.5% gel) electrophoresis and analyzed by Western blotting (immunoblotting) by using a rabbit anti-hGH polyclonal antibody (UCB Bioproducts) or a rabbit anti-MPR polyclonal antibody prepared by the method of Etoh et al. (10) with the Bio-Rad immunoblot assay kit (goat anti-rabbit IgG (H + L)-horseradish peroxidase conjugate (GAR-HRP)). The amounts of hGH were quantified by measuring the staining intensity of yeast hGHS on nitrocellulose filters with a TOYO densitometer, using pituitary-derived hGH (UCB Bioproducts) as a standard.

Determination of NH2-terminal amino acid sequence of 23-kDa hGH. A yeast strain harboring JGH5 was cultured aerobically at 30°C for 2 days in 2 liters of YPGal medium. After the cells were removed by centrifugation, the supernatant was adjusted to pH 7.5 and then it was directly applied to a DEAE-TOYO PEARL 650 column (TOSOH Co.; 6 by 15 cm) equilibrated with 20 mM Tris hydrochloride, pH 7.5. After the column had been washed with the same buffer, the adsorbed proteins were eluted with the same buffer containing 400 mM NaCl. The relative content of hGH was monitored by quantitative dot immunoblotting on a nitrocellulose filter with the anti-hGH antibody and the Bio-Rad immunoblot assay kit, as described above. The fractions containing hGH were pooled, and the proteins were concentrated by ultrafiltration using a Diaflow YM5 membrane (Amicon). The sample was then subjected to gel filtration chromatography by using a fast protein liquid chromatography system equipped with a Superose 12 column (Pharmacia-LKB Biotechnology Inc.; 1.6 by 50 cm). Proteins were eluted with the same buffer containing 100 mM NaCl. The fractions containing hGH were pooled, and the proteins were applied to a fast protein liquid chromatography system equipped with a Mono Q anion-exchange column (Pharmacia; 5 by 50 mm). The adsorbed proteins were then eluted with a linear gradient of 0.1 to 0.5 M NaCl in the same buffer. The hGH fractions were concentrated by using a Centricon 10 tube (Amicon) and subjected to SDS-12% polyacrylamide gel electrophoresis. The 23-kilodalton (kDa) hGH was recovered from the gel by the method of Hunkapiller et al. (17). The purified 23-kDa hGH obtained in this way was subjected to automated Edman degradation on an Applied Biosystems gas-phase sequencer equipped with an on-line amino acid phenylthiohydantoin analyzer.

RESULTS

Construction of MPR-hGH expression vectors. The NH2-terminal 22 amino acids of prepro-MPR were assumed to be a functional signal peptide for the secretion of pro-MPR from yeast cells (16). However, comparison of the sequence with those of other signal peptides in yeast cells suggested that the NH2-terminal 18 amino acids of prepro-MPR were also able to play a role as a secretion signal. Furthermore, the pro-region of MPR was expected to play some role in the secretion. Therefore, the sequences encoding the NH2-terminal region of different sizes from prepro-MPR were
fused upstream of the hGH gene in order to examine their effects on hGH secretion. The construction of plasmids JGH1, JGH2, JGH3, and JGH7, which were designed to express these fusion genes in yeast cells, is shown in Fig. 1. The constructed plasmids directed synthesis of the fused hGH proteins containing the whole prepro-sequence and an NH$_2$-terminal part of mature MPR (−66 Met to 156 Val) with an additional 3 amino acids derived from the linker (JGH1), the whole pre-sequence and a part of the pro-sequence (−66 Met to −40 Lys) with 3 amino acids derived from the linker (JGH2), and the NH$_2$-terminal regions of 18 amino acids (−66 Met to −49 Ala) (JGH3) and 22 amino acids (−66 Met to −45 Ala) (JGH7) of the prepeptide (Fig. 2).

Expression and secretion of hGH. *S. cerevisiae* MC16 was transformed with each of the plasmids mentioned above, and the transformants were cultured first in YPD medium and then transferred to YPGal medium for inducing the expression of the fused genes. After 2 days of cultivation in YPGal
medium, the culture supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. When the gels were subjected to Western immunoblotting, the proteins reacting with the anti-hGH antibody were detected in the culture media of all yeast strains harboring plasmids JGH1, JGH2, JGH3, and JGH7 (Fig. 3a, lanes 1 to 4). The proteins immunoreactive with the anti-MPR antibody were detected only in the culture supernatant of the strain harboring JGH1 (Fig. 3b).

A major protein secreted by the strain harboring JGH2 seemed to be slightly larger than those secreted by the strains harboring JGH3 and JGH7 (Fig. 3a, lanes 3 through 5). To make the difference clear, long-distance electrophoresis using a 17.5% gel was carried out. The molecular size of the protein secreted by JGH2 was estimated to be 23 kDa, while that secreted by JGH7 was estimated to be 22 kDa, which is identical to pituitary-derived hGH (Fig. 3c). Both the 22- and 23-kDa proteins did not react with the anti-MPR antibody. These results suggested that the pre- and prepropeptides from MPR were functional as leader peptides for hGH secretion by yeast cells.

Many protein bands were detected with both the anti-hGH and the anti-MPR antibodies in the supernatant from the strain harboring JGH1 (Fig. 3a and b, lane 1). The fused protein directed by JGH1 was expected to contain two sites for N-linked glycosylation in the NH2-terminal region of mature MPR (Fig. 2). It has been reported that the carbohydrates added to glycosylation sites in some heterologous proteins were very heterogeneous in size when they were expressed in yeast cells (9, 22). Therefore, the immunoreactive smearing bands larger than 43 kDa (the expected size of the fused protein) may be hyperglycosylated forms of this fused protein. Other immunoreactive bands smaller than 43 kDa in size were also observed (Fig. 3a and b, lanes 1). It seems possible that the MPR-hGH fusion in JGH1 is subjected to complexed proteolytic cleavage and glycosylation in the yeast secretory pathway. Slight amounts of immunoreactive proteolytic fragments were also detected in the supernatants from yeast strains harboring JGH2, JGH3, and JGH7 (Fig. 3a and c; Table 1).

The total amounts of the immunoreactive proteins secreted into the culture medium were estimated by scanning the bands in the gels by densitometry (Table 1). The most efficient secretion of the hGH proteins was observed in the yeast strain harboring JGH2. In contrast, the lower levels of secretion were observed in yeast strains harboring JGH3 and JGH7. These results indicated that the NH2-terminal portion of the pro-peptide of MPR played an important role in facilitating the secretion of hGH from yeast cells.

**NH2-terminal amino acid sequence analysis of 23-kDa hGH.**

The size of the 23-kDa hGH secreted from yeast cells was about 1 kDa larger than that of pituitary-derived hGH. Therefore, we determined the NH2-terminal amino acid sequence of the 23-kDa hGH. The 23-kDa hGH was purified from the YPGal culture medium by successive rounds of chromatography and gel electrophoresis. The NH2-terminal sequence was determined to be Arg-Pro-Val-Ser-Lys-Asn by automated sequence analysis (Fig. 2b). The result showed that the processing of the signal peptide occurred between −45 Ala and −44 Arg of the prepro-peptide of MPR (Fig. 2c).
and that the 23-kDa hGH was the fused hGH containing the additional eight amino acids derived from the NH₂-terminal part of the propeptide of MPR with the linker peptide.

**Effects of the GAL4 gene and the GAL10 terminator sequence on the production of secreted hGH.** The GAL7 promoter of *S. cerevisiae* is useful as a controllable promoter giving high-level expression of the downstream gene in yeast cells. Galactose inducibility in this system is modulated by interaction between the GAL4 and the GAL80 proteins. The GAL4 protein is a positive regulatory factor required for the activation of transcription (14, 19). On the other hand, the transcriptional terminator sequence is required for optimal mRNA accumulation when the gene is placed under the control of a strong promoter (7). In order to examine a possible stimulatory effect of the increased copy number of the *GAL4* gene, the gene was introduced into JGH2 to generate an expression plasmid, JGH4. Another plasmid, JGH5, in which the transcriptional terminator sequence of *GAL10* was introduced downstream of the hGH gene on JGH2, was also constructed (Fig. 4).

*S. cerevisiae* MC16 was transformed with JGH2, JGH4, and JGH5, and then these transformants were cultured in YPD medium or YPGal medium. The culture supernatants were analyzed by Western blotting with the anti-hGH antibody, and the amounts of secreted hGH were compared (Fig. 5). No detectable immunoreactive protein was found in any of the transformants when they were cultured in YPD medium (Fig. 5, lanes 1 to 3). When cultured in YPGal medium, all the transformants produced the 23-kDa protein as a major product in the supernatants. Although JGH4 caused a slightly higher level of secretion of hGH in the

**FIG. 3.** Western blot analysis of hGHs secreted by yeast strains harboring four different plasmids. Yeast cells were cultured at 30°C for 2 days in YPGal medium, and the supernatants were collected by centrifugation. The supernatants were subjected to SDS-polyacrylamide gel electrophoresis using a 10 to 20% gradient gel, and protein bands were detected by immunoblot using the anti-hGH antibody (a) and by immunoblot using the anti-MPR antibody (b). Long-distance SDS-polyacrylamide gel electrophoresis using a 17.5% gel was also done, and hGH was detected by immunoblot using the anti-hGH antibody (c). (a) and (b) Lanes 1, Culture supernatant of yeast strain harboring JGH1 (0.4 ml); lanes 2, JGH2 (0.4 ml); lanes 3, JGH3 (0.6 ml); lanes 4, JGH7 (0.6 ml); lanes 5, JPH (0.1 ml); lane 6, pituitary-derived hGH (0.6 μg) as a control. Molecular weight standards used were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,000). (c) Lane 1, Pituitary-derived hGH (1 μg) as a control; lane 2, JGH2 (0.2 ml); lane 3, JGH7 (0.6 ml).

**TABLE 1.** Secretion of hGHs by yeast cells containing MPR-hGH fusion genes.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Product size (kDa)</th>
<th>hGH secretion (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGH1</td>
<td>&gt;45, 43, 34, 30, 29, 23</td>
<td>0.93-2.6</td>
</tr>
<tr>
<td>JGH2</td>
<td>23, 20, 16</td>
<td>1.7-2.8</td>
</tr>
<tr>
<td>JGH3</td>
<td>22, 15</td>
<td>0.12-0.20</td>
</tr>
<tr>
<td>JGH7</td>
<td>22, 15</td>
<td>0.38-0.69</td>
</tr>
</tbody>
</table>

*S. cerevisiae* MC16 cells were transformed with the indicated plasmids and cultured at 30°C for 2 days in YPGal medium. The size and the amount of the excreted hGH were measured as described in Materials and Methods.

The structures of the plasmids are shown in Fig. 2.

**FIG. 4.** Structures of plasmids for expression of hGH by using the GAL4 gene or the GAL10 terminator sequence. See the legend to Fig. 2 for abbreviations. GAL4, GAL7-p, and GAL10-t represent the GAL4 gene, the GAL7 promoter, and the GAL10 terminator sequence of yeast cells, respectively. A DNA fragment containing the GAL4 gene from pHG41 was integrated upstream of the GAL7 promoter of JGH2, yielding JGH4. A DNA fragment containing the GAL10 terminator sequence from pYFI016 was integrated downstream of the hGH gene of JGH2, yielding JGH5.
1-day culture after the induction, degradation of the product occurred later and the total amount of the secreted hGH proteins directed by JGH4 was smaller than that directed by JGH2. On the other hand, the level of hGH secretion directed by JGH5 was three- to fivefold higher than that directed by JGH2 (Fig. 5, lanes 6 and 9). A large amount of the degraded 16-kDa hGH was also produced in this case. The other plasmid possessing both the GAL4 gene and the GAL10 terminator sequence gave almost the same level of hGH secretion as JGH5 (data not shown). Thus, the addition of a transcriptional terminator sequence led to the increased production of secreted hGH in yeast cells.

Accumulation and degradation of hGH secreted by yeast cells. To examine in more detail the features of hGH secretion by the yeast strain harboring JGH5, the time course of the secretion was monitored before and after the induction by galactose. The strain harboring JGH5 was cultured first in YPD medium and then transferred to YPGal medium. The culture supernatants were taken at appropriate intervals, and the amounts and the sizes of the proteins reacting with the anti-hGH antibody were measured.

hGH was secreted efficiently after galactose induction, and the total amounts of the hGH proteins in the culture medium reached approximately 10 mg/liter at 48 h after the medium shift (Fig. 6). Although only the 23-kDa hGH was observed in the early stage, prolonged cultivation caused increases in the proportion of the degraded hGHS of 20 and 16 kDa (Fig. 5 and 6). The total amounts of the hGH proteins decreased concomitantly with the appearance of the degraded hGHS. When localization of hGH in the medium and the cells was analyzed after 2 days of cultivation in the presence of galactose, 60% of hGH was found in the culture medium (Table 2). Most of the intracellular hGH was 23 kDa in size, and the amount of the degraded hGHS was less than 5% (Table 2). In all cases of yeast strains harboring other plasmids, JGH1, JGH2, JGH3, JGH4, and JGH7, over 50% of the synthesized hGH was also excreted into the culture medium and no accumulation of hGH in the periplasmic space was detected (data not shown). These results suggested that hGH was excreted efficiently into the culture medium and that its degradation occurred mainly after secretion by both endo- and exoproteases of yeast cells during the prolonged cultivation.

Figure 6 also shows the influence of hGH expression on cell growth. The growth rate of the strain harboring JGH5 was comparable to that of the untransformed strain in YPD medium. However, the growth rate of the transformed strain was slower than that of the untransformed strain after galactose induction. When the yeast cells were cultured in only YPGal medium from the beginning, the deleterious effect on cell growth was more apparent (data not shown). We also measured the stability of plasmids JG1, JGH2, and JGH5 in each yeast transformant during cultivation. More than 90% of the cells retained their plasmids even after 10 generations of growth in YPD medium. However, in the case of YPGal medium culture, the proportion of the cells retaining their plasmids was 71% for JG1, 53% for JGH2, and 32% for JGH5.

TABLE 2. Production of hGHS by yeast strains harboring JGH5

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Size (kDa)</th>
<th>hGH (mg/liter)</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>23</td>
<td>6.8</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Periplasm</td>
<td>23</td>
<td>1.5</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Intracellular</td>
<td>25</td>
<td>0.2</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

* S. cerevisiae MC16 cells harboring JGH5 were cultured at 30°C for 2 days in YPGal medium.
*a Fractionation was carried out as described in Materials and Methods. 
*b The values are the means of those obtained by three independent experiments.
for JGH5. These data indicated that the production of a large amount of hGH had a deleterious influence on cell growth and plasmid stability in yeast cells.

**DISCUSSION**

Although all four plasmids, JGH1, JGH2, JGH3, and JGH7, allowed secretion of the proteins immunoreactive with the anti-hGH antibody, the amounts of the hGH proteins excreted into the medium were considerably different depending on the plasmid (Fig. 3, Table 1). The NH2-terminal region of hGH is rich in hydrophobic amino acids, which may interact with membranes in the yeast cells. In the case of the direct fusion between the MPR pre-peptide and mature hGH (JGH3 and JGH7), such an interaction may prevent the cleavage of the pre-peptide and the following export of hGH across the endoplasmic reticulum membrane. It seems possible that the reduced secretion by these plasmids is due to the efficiency of processing of the signal peptide on the endoplasmic reticulum membrane.

Both JGH3 and JGH7 caused secretion of the 22-kDa hGH, which was the same size as pituitary hGH. The amount of the secreted hGH directed by JGH7 was larger than that directed by JGH3. Although the NH2-terminal sequence of the 22-kDa hGH directed by JGH3 or JGH7 could not be determined because of the low levels of secretion, it might be intact hGH without any additional amino acid. These data suggested that the signal peptides of 18 and 22 amino acids were both processed at their junctions in yeast cells, but the efficiency was higher for the signal of 22 amino acids. This is consistent with the fact that the signal processing of prepro-MPR during its secretion from yeast cells occurred between amino acids 22 and 23 from the NH2-terminus (16).

JGH1 yielded many immunoreactive proteins in the culture medium. Most of them were larger in size than the expected fused protein of 43 kDa, but a few were smaller. There are three possible sites for cleavage by KEX2 proteinase (8, 12): two sites in the pro-sequence of MPR and one site in the hGH-coding sequence. There also are two sites for N-linked glycosylation in the pro-sequence of MPR in the fusion protein directed by JGH1. Furthermore, the excreted hGHS may be degraded in the culture medium by extracellular proteinases. Further work, such as the endoglycosidase H treatment of the secreted hGHS and the use of a kex2 mutant strain, is required to characterize these immunoreactive proteins.

The highest efficiency of the leader sequence for hGH secretion was observed with JGH2. The junction between the pre- and pro-sequences of MPR was conserved in this construction, which might assure effective processing of the signal peptide for secretion. In addition, a part of the pro-sequence of MPR at the NH2-terminus of the processed product may play some role to facilitate its transport in the cells. Although removal of the extra amino acids of the pro-peptide is required to obtain hGH with the same NH2-terminus as native hGH, it may be achieved by the introduction of an artificial process site, such as Ile-Glu-Gly-Arg recognized and cleaved by blood coagulation factor Xa (24), just before the hGH sequence.

Two possibilities were examined for the purpose of increasing the production of the secreted hGH directed by JGH2. First, the GAL4 gene was introduced into the same multicopy plasmid. Abe et al. (1) reported that amplification of the GAL4 gene by using a multicopy plasmid caused about a fivefold increase in the intracellular production of the Ela protein of human adenovirus under the control of the GAL7 promoter. Although we failed to observe a significant stimulatory effect of GAL4 on the extracellular production of hGH, it caused a slight enhancement of secretion in an early stage of growth in a 1-day culture. It seems possible that GAL4 actually increased the efficiency of transcription but failed to maintain its effect possibly because of instability of the plasmid or some other reasons. Second, the GAL10 terminator sequence was introduced downstream of the hGH gene (JGH5). The presence of such a transcriptional termination signal has been reported to be important for efficient expression of genes in yeast cells (1, 7). Our results also showed a three- to fivefold increase in the secretion level. The increased production of secreted hGH presum-ably resulted from more efficient transcription and translation. The amount of the secreted hGH directed by JGH5 reached 10 mg/liter. This indicates that the use of the leader peptides of MPR along with the GAL7 promoter may be useful for the construction of the effective secretion vectors for yeast cells.

The ratio of the 16-kDa hGH to the 23-kDa hGH after 2 days of cultivation in the strain harboring JGH5 was higher than that in the strain harboring JGH2 (Fig. 5, lanes 7 and 9). We can find no clear explanation for these phenomena. It is possible that the greater expression of hGH directed by JGH5 caused a more deleterious effect on the host cells, which might result in leakage of larger amounts of extracellular proteases and increased degradation of the secreted hGH.

In order to achieve higher levels of excretion of hGH in this system, the following two problems remain to be solved. First, high-level expression of hGH interferes with cell growth and plasmid stability. Similar phenomena have also been reported when human erythropoietin and gamma interferon were produced by yeast using multicopy plasmids containing the TRP1 gene as a selective marker (6, 9). Second, the excreted hGH is degraded primarily by yeast extracellular proteases during the prolonged cultivation. For the solution of these problems, the increase of plasmid stability by the integration of the expression units into the yeast genome and the increase of secretion ability of cells by isolation of a supersecreting mutant and a protease-defective mutant might be important.

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**LITERATURE CITED**


