

Optimizing Functional versus Total Expression of the Human μ -Opioid Receptor in *Pichia pastoris*

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The expression of the EGFP–human μ -opioid receptor fusion protein in the methylotrophic yeast *Pichia pastoris* was optimized and monitored using both fluorescence and ligand-binding experiments. A set of parameters, including gene copy number, strain type, temperature, pH, and methanol inducer levels, was studied for its effect on the production of the recombinant protein. We show here that the expression level is optimal after 10 h of promoter induction and that the maximum is reached at a lower temperature and a higher pH than normally used. The optimized conditions have allowed a fourfold increase of the ligand-binding active form of the receptor, whereas the total expression level determined by EGFP fluorescence measurements was not modified. © 2002 Elsevier Science (USA)

Key Words: EGFP; fusion protein; μ -opioid receptor; expression; *Pichia pastoris*; GPCR.

The human μ -opioid receptor (HuMOR)² is the receptor subtype most identified with the analgesic properties of opiate drugs. μ opioid receptor agonists can modify pain in spinal and supraspinal analgesia and also have euphoriant actions and strong addictive properties (1). HuMOR is a G-protein-coupled receptor (GPCR) characterized by seven membrane-spanning domains with extracellular N-terminus and cytoplasmic C-terminus ends and has been demonstrated to be the unique

receptor of morphine drug (2). The pharmacological implications of HuMOR and of GPCRs in general has led to an increasing interest in their 3D structure. However, the generation of structural information on GPCRs, such as NMR or X-ray crystallography data, has been seriously hampered by the lack of sufficient amounts of purified material. HuMOR is naturally expressed at very low levels in cells (0.1 pmol/mg membrane proteins), and in order to express high quantities of HuMOR protein, several heterologous expression systems have been used (3). The amounts of functional receptor expressed vary from 0.36 pmol/mg of membrane protein in *Escherichia coli* (4) to about 10 pmol/mg in baculovirus-infected cells (5–7). We have previously reported (8) the expression of the HuMOR in *Pichia pastoris* with a typical μ -opioid receptor binding profile suggesting a proper folding of the protein in yeast membranes. Despite the initial low expression level found in *P. pastoris* (0.4 pmol/mg membrane proteins), this methylotrophic yeast has several advantages compared to other organisms. The expression is made under the strong, tightly controlled, and inducible promoter of methanol oxidase (AOX1), which is a key enzyme of the specific methanol utilization pathway and represents 30% of total protein when its expression is induced with methanol. The expression gene is integrated in the *P. pastoris* genome and recombinant strains are very stable. *P. pastoris* shows a rapid growth on low-cost media, and high cell densities may be obtained (up to 150 g/L dry cell weight). Moreover, the eukaryotic nature of this yeast is well suited to heterologous expression, i.e., protein processing, folding, and posttranslational modifications (9). It is also possible to produce isotopically labeled proteins (10, 11) which are essential for future NMR investigations. In addition to the μ -opioid receptor, expression of three other

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² Abbreviations used: HuMOR, human μ -opioid receptor; GPCR, G-protein-coupled receptor; EGFP, enhanced green fluorescent protein; BMGY, buffered minimal glycerol-complex medium; BMMY, buffered minimal methanol-complex medium; YPD, yeast peptone dextrose; S, sorbitol; PMSF, phenylmethylsulfonyl fluoride; MD, minimal dextrose; AOX1, alcohol oxidase 1.

GPCRs has been reported in *P. pastoris*: 5-hydroxytryptamine receptor, β 2-adrenergic receptor (12), and bovine opsin (13). The various advantages of using *P. pastoris* and the good expression levels obtained for these three receptors led us to try to optimize the expression level for the μ -opioid receptor. By using a combination of enhanced green fluorescent protein (EGFP) fluorescence measurement and binding experiments, we have studied the influence of gene copy number or strain type on the chimeric EGFP- μ -opioid receptor expression, as well as other culture parameters such as duration of methanol gene induction, temperature, pH, and methanol level.

MATERIALS AND METHODS

Bacterial and yeast strains. *E. coli* strain TOP 10 F' (F' $\{lacI^qTn10(Tet^R)\}$ *mcrA* Δ (*mrr-hsdRMS-mcrBC*)- Φ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-leu*)7697*galU* *galK* *rpsL* (Str^R) *endA1* *nupG*) was used for plasmid propagation. The chemical CaCl₂ method was used for *E. coli* transformation (14). For *P. pastoris* electrotransformation (15), four strains were used: wild type, GS115 (*his4*), SMD1163 (*his4 pep4 prb1*) (kind gift from Dr. H. Reiländer), and SMD1168 (*his4 pep4*).

Plasmids and DNA recombinant technology. Plasmids pPIC9K and pPICZ α A were from Invitrogen (San Diego, CA). Plasmid pPICZ α MF-EGFP-HuMOR-c-myc-his (Fig. 1) was constructed as follows: the double-strand oligonucleotide (5' AAGCTTCTCGAGAAAAGAGAGGCTGAAGCCATGG 3') was inserted into the *Hind*III and *Nco*I sites of the plasmid pGEM4Z-HuMOR (8), thus introducing a *Xho*I site into the pGEM4Z-HuMOR vector and leading to the pGEM4Z-HuMOR-*Xho*I construction. Then, the *Xho*I to *Xba*I fragment of the pGEM4Z-HuMOR-*Xho*I containing the HuMOR gene was cloned into the *Xho*I and *Xba*I sites of the pPICZ α A vector, leading to the pPICZ- α MF-HuMOR plasmid. This resulted in a vector containing in frame the *Saccharomyces cerevisiae* α -mating factor signal sequence (α MF, 89 amino acids), the HuMOR gene ending with a TAA codon, and both a c-myc epitope and a 6-histidine tag (his-tag) originally present in the pPICZ α A. The CCCTAACAG sequence between the HuMOR gene and the c-myc epitope (containing the stop codon and corresponding to the Pro-Stop-Asp sequence) was then transformed into a CCTCTAGAT sequence (corresponding to the Pro-Leu-Glu amino acid sequence) by PCR with a forward primer located before the *Eco*RI site of the HuMOR gene (5' GTGGTGGTGGCTGTGTTTCATCGTCT 3') and a reverse primer homologous to the 3' end of the HuMOR gene (5' GATCTAGAGGCAACGGAGTTTC 3'). This reaction suppressed the stop codon and introduced a new *Xba*I site at the end of the PCR product. This PCR product (0.3

kb) was double digested with *Eco*RI and *Xba*I and subsequently cloned into the *Eco*RI and *Xba*I sites of pPICZ- α MF-HuMOR, leading to the pPICZ- α MF-HuMOR-c-myc-his-tag vector. Unique restriction sites (*Xho*I, *Hind*III, *Nco*I, and *Kpn*I) were introduced into the 5' and 3' regions of the EGFP gene by PCR, using the commercially available vector pcDNA-EGFP (Clontech, Palo Alto, CA) as a matrix and the following primers: 5'-EGFP-forward, 5' CCCCCTCTCGAGAAAAGAGATGACGACGACAAGCTTAGCAAGGGCGAGGAG 3', and 3'-EGFP-reverse, 5' GGCCATGGTACCGCGTGGCACCAGCTCGTCCATGCC 3'. The obtained PCR product (761 bp), corresponding to the EGFP gene deleted of two codons at its 5' end (first ATG and Val residue) and of seven codons at its 3' end (corresponding to the Leu-Arg-Ser-Arg-Ala-Cys-Ile amino acid sequence), was then digested with *Hind*III and *Nco*I and subsequently cloned into the *Hind*III and *Nco*I sites of pPICZ- α MF-HuMOR-c-myc-his-tag, leading to the pPICZ- α MF-EGFP-HuMOR-c-myc-his-tag vector. To construct the pPIC9K- α MF-EGFP-HuMOR-c-myc-his plasmid, the 2-kb *Sac*I-*Age*I restriction fragment of vector pPICZ α MF-EGFP-HuMOR-c-myc-his was exchanged against the corresponding fragment of pPIC9K that contains a kanamycin-resistance gene. Plasmids were verified by DNA sequencing using the dideoxy-termination method (DNA sequencing kit; USB Corp., Cleveland, OH).

Media. *E. coli* transformants were selected on low-salt Luria Bertani medium, pH 7.5, containing 0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 0.5% (w/v) NaCl with 25 μ g/ml zeocin. *P. pastoris* liquid growth medium was BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M phosphate buffer, pH 7.5, 100 μ g/ml ampicillin). Receptor expression was induced after elimination of buffer by centrifugation (1000g, 10 min) and addition to the yeast pellet of a buffered methanol-containing medium (BMMY; 1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M salt buffer, methanol at different concentrations, 100 μ g/ml ampicillin). Ampicillin was added to avoid bacterial contamination.

Selection for multicopy transformants following electrotransformation. For zeocin selection of multicopy transformants, 10 μ g of plasmid pPICZ α MF-EGFP-HuMOR-c-myc-his was linearized with *Sac*I restriction enzyme, and the SMD1163 *P. pastoris* yeast strain was used for electrotransformation. Electrotransformed yeast cells (10^5 – 10^6) were then spread onto YPDS plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, 2% (w/v) agar) containing from 100 to 1000 μ g/ml zeocin. For Geneticin G418 selection, 10 μ g of plasmid pPIC9K-EGFP-HuMOR-c-myc-his was linearized with *Sac*I restriction enzyme, and recombinant SMD1163 yeast cells were selected onto MD plates (1.34% (w/v) yeast nitrogen base without amino acids,

0.4 mg/L biotin, 2% (w/v) dextrose, 1.5% (w/v) agar) for their ability to grow on histidine-deficient medium. His⁺ clones were pooled and diluted in sterile water, and 10⁵ cells were spread onto YPD plates containing from 0.1 to 4 mg/ml G418. For production of the receptor, yeast cells were grown at 30°C in BMGY until the late exponential phase and induction was achieved in BMMY (0.1 M phosphate buffer, pH 7.5, 0.5% MeOH) for 15 h at 30°C.

Isolation of crude membranes. Cells were harvested after induction by centrifugation and resuspended to an OD₆₀₀ of 200 in breaking buffer (50 mM Tris-HCl, 10 mM EDTA, pH 7.5) supplemented with protease inhibitors (17.4 µg/ml PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 20 µg/ml benzamide). An equal volume of glass beads (425–600 µm; Sigma, St. Louis, MO) was added to the suspension, and cells were broken by vigorous vortexing at 4°C for 30 min. Unbroken cells and particulate matter were eliminated by centrifugation (1000g, 10 min, 4°C). Membranes were pelleted from the supernatant at 100,000g for 1 h at 4°C and resuspended in the breaking buffer containing protease inhibitors. Membrane protein concentrations were determined using the Lowry method (16) and bovine serum albumin was used as a standard.

Fluorescence measurements. For calibration of EGFP–receptor fusion protein fluorescence in membranes, standard solutions of 0.1–1 µg · ml⁻¹ of rEGFP (Clontech) were used. Each EGFP standard solution was prepared in 50 mM Tris-HCl, 10 mM EDTA, pH 7.5 buffer and sonicated with fixed quantities of *P. pastoris* SMD1163 membranes. The fluorescence of the membrane suspension was measured using a Quantamaster spectrofluorimeter (Photon Technology International, South Brunswick, NJ). The sample was excited at 470 nm and an emission scan from 500 to 560 nm was performed. The fluorescence emission peak at 509 nm was plotted as a function of the amount of standard rEGFP and the corresponding regression line was calculated. In membranes expressing EGFP–receptor, the amount of receptor was calculated on the basis of the calibration curve. For time-course experiments, whole yeast cell samples were diluted 1/100 in 50 mM Tris-HCl, 10 mM EDTA, pH 7.5 buffer, and fluorescence at 509 nm was measured.

Determination of gene copy number. Genomic DNA from recombinant *P. pastoris* clones was prepared as follows: an overnight culture of yeast cells was harvested and resuspended to OD₆₀₀ of 200 in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2.5% SDS buffer. An equal volume of glass beads was added and cells were broken by vortexing 30 min at 4°C. The suspension was heated at 100°C for 1 min. After centrifugation (10,000g, 15

min) and elimination of the precipitate, 2 vol of isopropanol and 0.1 vol of 3 M potassium acetate were added to the supernatant. Genomic DNA was harvested by centrifugation (10,000g, 5 min) and washed with 70% ethanol. Finally, DNA was diluted in 1 ml of water containing 30 µg RNase A. The concentration and purity of the preparation were UV checked at 260 nm. For hybridization, 18 µg of genomic DNA from each clone was dropped onto a nylon membrane, fixed by UV radiation, and hybridized with respective gene-specific ³²P-labeled DNA probes, which were generated by a random-priming approach (17). Afterwards, radioactivity bound to the filter was quantified using a STORM phosphorimager (Molecular Dynamics, U.S.A.). Genomic DNA of the *P. pastoris* SMD1163 strain was used as a standard for determination of gene copy number.

Radioligand binding experiments. In order to determine *K_d* and *B_{max}* values, crude membrane preparations were diluted in 0.5 ml final volume of binding buffer (50 mM Tris-HCl, 10 mM EDTA, pH 7.5) with selected concentrations (0.1 to 3 nM) of [³H]diprenorphine (Amersham Pharmacia Biotech; 58 Ci/mmol sp act). Unlabeled diprenorphine, in the micromolar range, was used to determine nonspecific binding. Following a 1-h incubation period at 25°C, free ligand was removed by filtration onto Whatman GF/B filters, presoaked in 0.3% polyethyleneimine, and filter-bound radioactivity was determined using a Packard apparatus (Meriden, CT). For time-course experiments, whole cells were assayed in 0.5 ml final volume of binding buffer with 1 nM [³H]diprenorphine and nonspecific binding was determined with 1 µM unlabeled diprenorphine. Data were analyzed with Prism software (GraphPad Software, Inc., San Diego, CA).

Time-course experiments. For all the experiments, biomass was generated using recombinant yeasts bearing the αMF-EGFP-HuMOR-c-myc-his gene that were grown on BMGY, pH 7.5, at 30°C in a shake flask until late exponential phase (2 × 10⁹ cells/ml). After the biomass growth medium was changed to the inducing medium (BMMY), receptor expression was monitored every 2 h for 40 h both by binding and by fluorescence methods. For the temperature time-course experiments cells were changed from BMGY to BMMY medium (0.5% MeOH, pH 7.5) and expression was monitored at 15, 20, 25, and 30°C. To study methanol kinetics, BMGY was replaced with BMMY (pH 7.5) containing various amounts of methanol (0.5, 1, 2, 5%) and induction was carried out at 20°C. For the pH kinetics experiments, expression was monitored at different pH values in BMMY (0.5% MeOH) at 20°C. For the pH values 2, 3, 4, and 5 a 0.1 M citrate buffer was used; for pH 6, 7, and 8 a 0.1 M phosphate buffer was used, and for pH 9 and 10 a 0.1 M bicarbonate buffer was used. In order

to study the effects of μ -opioid ligands on receptor expression levels, morphine and diprenorphine were used at micromolar concentrations in the induction medium (BMMY, 0.5% MeOH, pH 7.5, 20°C).

RESULTS AND DISCUSSION

Expression of the human μ -opioid receptor. In a previous study (8), we showed that the human μ -opioid receptor fused to the prepropeptide of the α MF could be expressed in the methylotrophic yeast *P. pastoris* and displayed a typical μ -opioid receptor pharmacological profile with a $B_{\max}$ value of 0.4 pmol/mg of membrane protein. In order to follow the receptor total expression level reached during induction and not only the ligand-binding receptor active form (determined using [3 H]diprenorphine binding), we have fused the receptor at its N-terminus with EGFP and at its C-terminus with a c-myc epitope (for immunoblot detection) and a hexahistidine tag (for further purification using nickel resin) (pPICZ- α MF-EGFP-HuMOR-cmyc-his; Fig. 1). The chimeric receptor was functional as determined by its

ligand-binding properties and displayed a normal μ -opioid receptor pharmacological profile. It was expressed as an active ligand-binding form, at a level of 1 pmol/mg of membrane protein. Moreover, the fluorescence properties of EGFP allowed us to demonstrate that the majority of the fusion protein was not detected through saturation binding experiments and that the exact expression level was 16 pmol/mg of membrane protein. These data were obtained when the recombinant strain was grown at 30°C on BMGY, pH 7.5, until the late exponential phase after which the culture was switched to the methanol induction medium (BMMY) containing 0.5% MeOH, pH 7.5, at 30°C for 15 h. In order to improve the human μ -opioid receptor production, we have examined a set of parameters that are known to influence protein expression. The expression was monitored using both ligand-binding experiments and fluorescence measurements, thus allowing a direct comparison between the total receptor expression level and its ligand-binding active form.

Use of *P. pastoris* strains. Four strains were examined for their ability to express the human μ -opioid receptor. *P. pastoris* wild type was used for the transformation of the plasmid with zeocin selection (pPICZ- α MF-EGFP-HuMOR-cmyc-his, Fig. 1), *P. pastoris* strains GS115 (*his4*) and two protease-deficient strains SMD1168 (*his4 pep4*) and SMD1163 (*his4 pep4 prb1*) were transformed either by zeocin selection or by histidine auxotrophic selection (pPICZ- α MF-EGFP-HuMOR-cmyc-his or pPIC9K- α MF-EGFP-HuMOR-c-myc-his plasmids, respectively). In a first attempt, recombinant clones were selected on low-antibiotic-concentration plates (0.1 mg/ml zeocin or 0.25 mg/ml G418), and 100 clones for each strain were examined for their ability to express the protein of interest. For all the strains used and for the different ways of selection, expression levels were in the range of that determined for a well-characterized clone expressing α MF-EGFP-HuMOR-c-myc-his protein (i.e., about 1 pmol/mg membrane protein determined by measuring the ligand-binding expression level and 16 pmol/mg membrane protein determined by fluorescence measurements). In this particular case the receptor was expressed in the SMD1163 yeast strain, which bears mutations in two protease genes and shows a substantial reduction in or elimination of three protease activities. Weiss *et al.* (12) have reported an eightfold increase of 5-HT_{5A} serotonin receptor expression when GS115 was replaced with the SMD1163 yeast strain. We can assess that the effect of yeast strains on a particular GPCR expression is not predictable. Nevertheless the intrinsic characteristics of the protease-deficient SMD1163 strain may be important for future purification procedures.

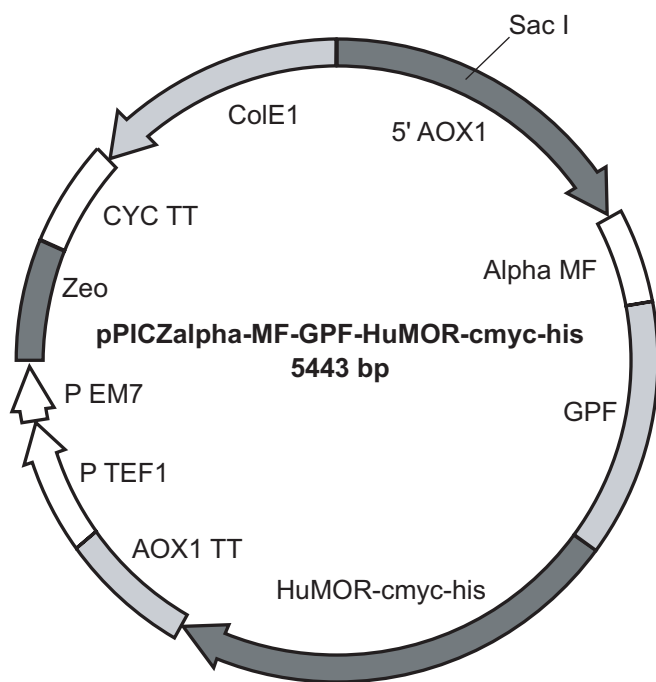


FIG. 1. Schematic overview of the plasmid coding for the EGFP-HuMOR gene. pPICZ α MF-EGFP-HuMOR-c-myc-his was used to study the optimization of the receptor expression and to produce zeocin-resistant strains. 5' AOX1 is the methanol-inducible promoter, Alpha MF is the α -mating factor secretion signal sequence from *S. cerevisiae*, AOX1 TT is the alcohol oxidase transcription termination region, P TEF1 is the promoter region of the elongation factor 1, P EM7 is the promoter conferring zeocin resistance, Zeo is the zeocin selectable marker, CYC TT is the transcription termination region, ColE1 origin allows replication and maintenance of the plasmid in *E. coli*, and SacI is the restriction site used to linearize the plasmid before electroporation into *P. pastoris*.

Effect of gene copy number. The generation of recombinant yeast strains with multiple heterologous gene insertion events may result in the expression of high amounts of heterologous protein (18). This multiple insertion phenomenon occurs spontaneously in a cell, at a low but detectable frequency. After transformation, because of the genetic linkage between the antibiotic gene and the expression cassette, presumed high gene copy number *P. pastoris* transformants can be selected for their ability to grow on medium containing high levels of antibiotics. In order to determine if increased genome-inserted gene copy number has a positive effect on expression levels, two kinds of transforming plasmids were used: pPIC9K- α MF-EGFP-HuMOR-c-myc-his, which contains the *E. coli* kanamycin (*kan*) gene that confers resistance to Geneticin G418 after selection of His⁺ transformants (able to grow on histidine-deficient media), and pPICZ- α MF-EGFP-HuMOR-c-myc-his, which contains the *Sh ble* gene which confers resistance to the zeocin drug and for which transformants may be directly selected after transformation on zeocin-containing plates. A single copy of *kan* or *Sh ble* gene integrated into the *P. pastoris* genome is known to confer resistance to a level of 0.25 mg/ml G418 and to a level of 0.1 mg/ml zeocin, respectively. The expression levels determined on resistant clones by fluorescence (15–20 pmol/mg membrane protein) or binding experiments (1 pmol/mg membrane protein) were in the range of data obtained with low-antibiotic-resistant strains. In order to make a correlation between antibiotic resistance and genetic events, the gene copy number of high-level-resistant strains was determined. In this experiment, genomic DNA from antibiotic-resistant clones was hybridized with three different random-prime-labeled probes. These probes proceeded from a 728-bp fragment from the EGFP gene, 902 bp from the HuMOR gene, and 708 bp from the AOX1 gene. Gene copy number was deduced from the radioactivity ratio between EGFP or HuMOR probes and AOX1 probe. We have found that yeast clones resistant to high zeocin levels (1 mg/ml) bear from 15 to 25 copies of the μ -opioid receptor gene integrated into their genome. This means that in our case a high gene copy number has no effect on expression level. The gene copy number effect on expression is unpredictable; one may observe in some cases a direct relationship but there are also instances in which an increase in copy number has few or no effects on expression (12, 18). In the following study, we have used a well-characterized SMD1163 recombinant clone (containing four copies of the gene of interest) in order to estimate the effect, during the induction step, of parameters such as temperature, pH, or methanol concentration on the expression levels of the human μ -opioid receptor.

Effect of temperature during induction period.

P. pastoris cells grow well at 30°C and induction of protein expression is usually carried out at 30°C. As temperature is known to affect protein expression (19), induction of HuMOR expression was examined at 15, 20, 25, and 30°C. Cells were collected every 2 h and analyzed by binding (Fig. 2A) and fluorescence (Fig. 2B). Interestingly, cell growth ceased after methanol induction. However, this was not accompanied by an increase in cell mortality. The recombinant strain was grown in glycerol in order to obtain a maximal cell

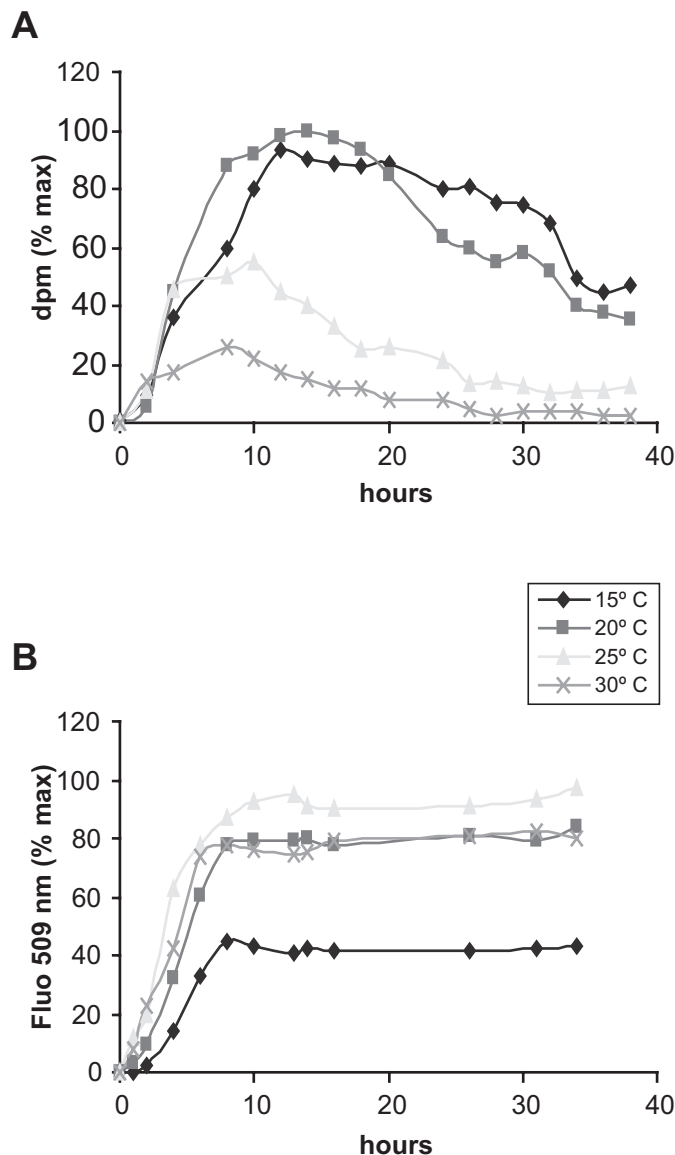


FIG. 2. Effect of temperature on HuMOR expression. (A) Effect monitored by binding experiments, (B) effect monitored by fluorescence measurements. Yeast cell biomass was made in a glycerol-containing medium, pH 7.5 at 30°C. Binding experiments were performed using [³H]diprenorphine and fluorescence measurements were made at 509 nm. Results are representative of several similar experiments and are expressed as percentages of maximum value.

density. After exchange of the medium, protein expression was induced with methanol. OD₆₀₀ was measured every 2 h and was constant during the induction period, indicating that there was no cell growth. For the four temperatures tested, fluorescence measurements and binding profiles were different. Nevertheless, we always observed an initial increase of expression followed by a maximum value. Beyond this maximum, the profiles develop differently: while fluorescence remained constant from 8 to 30 h after induction, the ability of the receptor to bind [³H]diprenorphine decreased significantly during the same period. As illustrated in Fig. 2A, maximum functional expression levels are nearly identical at 15 and 20°C and are two times higher than those at 25°C and four times higher than those at 30°C. For these four temperatures, a bell-shaped expression peak was observed, and its maximum shifted toward higher induction times as the temperature decreased. The significant increase in the ligand-binding active form of the receptor expression levels at lower temperatures may be due to improved protein folding at lower temperatures. Inducing the cells at a lower temperature could reduce the rate of protein synthesis and thus may allow more time for the nascent receptor peptide chain to fold properly into membranes (19). The decrease of binding observed in Fig. 2A, for all temperatures, may be indicative of protease action on the μ -opioid part of the fusion protein or of a denaturation of the expressed protein over time. At the same time (Fig. 2B), the fluorescence level appears to be constant after reaching its maximum value, indicating that EGFP is stable toward denaturation and proteases (20, 21). Further experiments were carried out at 20°C, the temperature for which the cells exhibited a maximum expression level, both by fluorescence and by ligand-binding experiments.

Effect of pH during the induction period. As *P. pastoris* is able to grow across a broad range of pH (from 3 to 7.5), we decided to study the influence of this parameter on the expression of the human μ -opioid receptor. Inductions were carried out at 20°C in BMMY (0.5% MeOH) at pH 2, 3, 4, and 5 with 0.1 M citrate buffer; at pH 6, 7, and 8 with 0.1 M phosphate buffer; and at pH 9 and 10 with 0.1 M bicarbonate buffer. All pH values were checked after induction, and the values were unchanged within 0.2–0.8 pH unit. No expression was observed at pH 2, 3, or 4 by binding experiments (Fig. 3A) and by fluorescence measurements (Fig. 3B). Fluorescence intensities determined at pH 6, 7, 8, 9, and 10 were in the same range and revealed that expression increased up to 10 h after induction and remained stable afterwards. At pH 5, fluorescence was half of that obtained at pH 6, 7, 8, 9, or 10 and followed a constant progression up to 30 h after induction and was stable

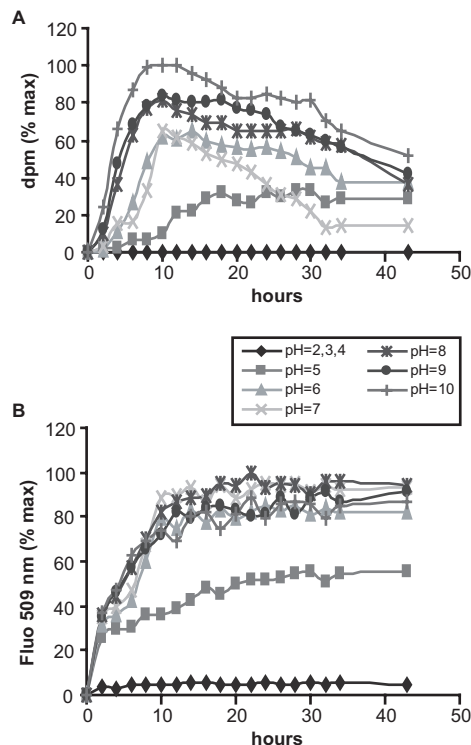


FIG. 3. Effect of pH on HuMOR expression. (A) Effect monitored by binding experiments, (B) effect monitored by fluorescence measurements.

afterwards. Ligand-binding profiles at different pH values showed also an increase up to 10 h, when the maximum expression level was observed. After 10 h, expression followed a slight but constant decrease, which was particularly fast during the pH 7 experiment. The ligand-binding experiment at pH 5 showed, as for fluorescence measurements, a constant progression of expression until 30 h after induction, when it reached a third of the value obtained at pH 10. Maxima of expression levels by binding were observed for high pH values and differences between maxima were much greater than those observed by fluorescence, thus showing once again a difference of behavior between the total amount of the receptor expressed within the cell and the fraction that is able to bind [³H]diprenorphine. EGFP fluorescence is known to be stable between pH 7 and pH 11.5, but to drop sharply above pH 11.5 and then to decrease between pH 7 and pH 4.5, retaining about 50% of fluorescence at pH 6 (21). These results were obtained from pH titration curves of purified EGFP but it is not clear whether these results are meaningful to a living yeast expressing a membrane protein fused with EGFP. The total expression level, as determined by fluorescence measurement, is maximal and stable over a wide range of pH and variations may be indicative, as with the temperature studies, of denaturation of the expressed protein at some pH values. Nevertheless, in *P. pastoris*

asci spores, only small variations in the internal pH values were found upon incubation of the asci in buffer ranging in pH from 3 to 10. For this range of external pH, the internal pH values were from 5.1 to 6.3 (22). In the same way, *S. cerevisiae* cells maintain their cytoplasmic pH around 6.3, with a pH variation of only 1 pH unit, when the external pH varies from 3.5 to 9.1 (23). We could assume that the sensitivity of the receptor to pH is extreme but we cannot exclude a pH-dependent protease sensitivity of the chimeric protein. Finally, by comparison of fluorescence measurements and binding experiments, we concluded that the use of an induction medium buffered at pH 10 is optimal for expression of the receptor.

Effect of methanol concentration. As the expression of the receptor is under the control of the AOX1 promoter, which is induced by methanol, it was essential to verify that the methanol concentration commonly used (0.5%) was optimal during the induction step. The strain carrying the EGFP- μ -opioid receptor fusion gene was grown on BMGY, pH 7.5, at 30°C and expression was induced with different levels of methanol in BMMY, pH 7.5, at 20°C. We observed by binding analysis (Fig. 4A) or fluorescence (Fig. 4B) the same expression profiles as before. The maxima were observed for the two methods after about 8 h of induction. From 8 h of protein expression, fluorescence remained stable, whereas ligand-binding receptor dosage showed a large decrease (up to 50% of the maximum). Total expression determined by fluorescence was maximal with 0.5% MeOH in the medium. Higher quantities of MeOH caused a drastic reduction of expression (up to 40% of maximum for 5% MeOH), in agreement with the known toxicity of MeOH at high concentration. When 0.5% of fresh MeOH was added after 10 h of induction we did not observe any increase of expression. In the same way, binding was maximum for 0.5% MeOH and was reduced at higher concentrations. After 30 h of induction, the binding value was half of the maximum. These experiments showed that, within the range of concentrations that we have studied, a 0.5% methanol concentration was optimal and never limiting during the induction period.

Effect of μ -opioid receptor ligands during induction. Many authors have reported the stabilization of GPCRs when ligands were added to the culture medium or during the purification steps (12, 24). In order to improve the stability of the EGFP-HuMOR protein during the induction period, we added opioid ligands (morphine and diprenorphine), at the micromolar range, to the medium. This experiment failed to exhibit any improvement of receptor expression determined either by fluorescence or by binding experiment. Nevertheless, the addition of opioid ligands during the purification

steps may be important for the stabilization of the protein.

Use of optimized parameters to express the fusion protein. The optimized conditions of induction were used to quantify the expression of the μ -opioid receptor, both by fluorescence and by binding experiments. After generation of biomass, three induction conditions were compared: 20°C, pH 10; 20°C, pH 7.5, and 30°C, pH 7.5. The cultures were performed in 0.5% MeOH in BMMY and yeast cells were collected after 10 h of induction. Expression levels determined on membrane fractions were 4, 2, and 1 pmol of receptor per milligram of membrane protein for the following conditions: 20°C, pH 10;

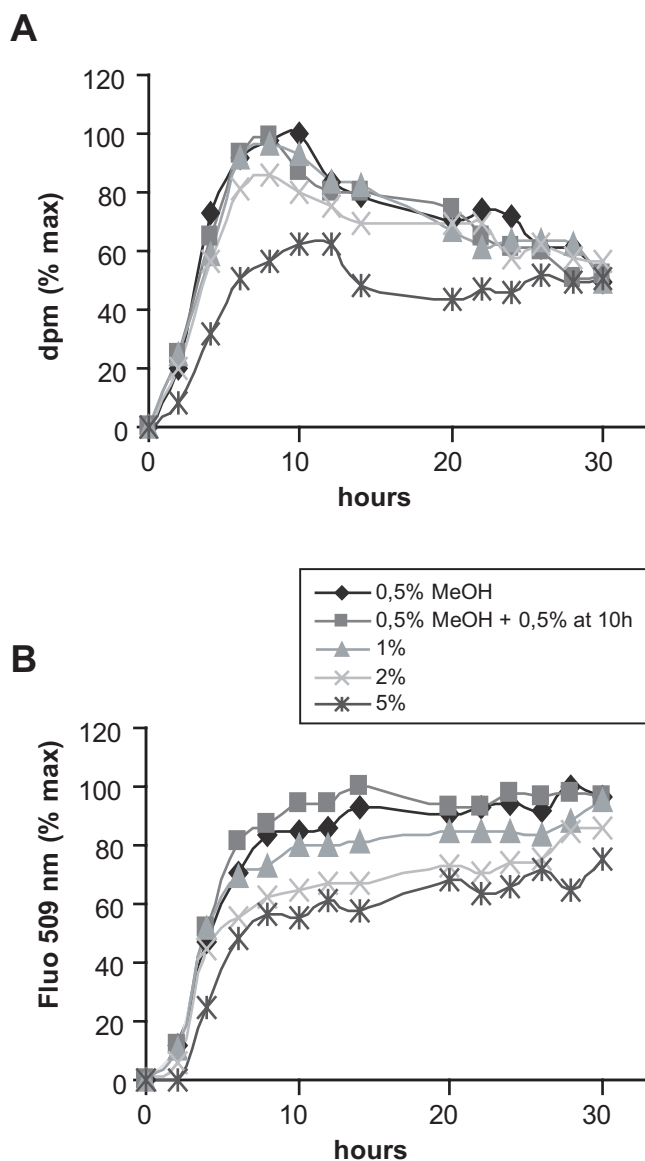


FIG. 4. Effect of MeOH concentration on HuMOR expression. (A) Effect monitored by binding experiments, (B) effect monitored by fluorescence measurements.

TABLE 1

Expression Levels Determined under Optimized Conditions by Binding or Fluorescence Experiments

Gene induction conditions	Production level by binding (pmol/mg) ^a	Production level by fluorescence (pmol/mg) ^a	K _d (nM) ^b
30°C, pH 7.5	1 ± 0.1	20 ± 1	0.45 ± 0.05
20°C, pH 7.5	2 ± 0.2	19 ± 1	0.50 ± 0.04
20°C, pH 10	4 ± 0.2	18 ± 1	0.48 ± 0.05

^a Assays were performed on membrane preparations and production levels are given in pmol/mg membrane protein.

^b K_d values were calculated from saturation binding data.

20°C, pH 7.5; and 30°C, pH 7.5, respectively (Table 1). In contrast, expression levels determined using fluorescence experiments (20 pmol/mg membrane protein) were identical for the three conditions. These results demonstrate that a fixed amount of receptor protein was synthesized after 10 h of induction and that the stability of the ligand-binding active form of the human μ -opioid receptor is dependent on the culture conditions during the induction step. SDS-PAGE and Western blotting experiments were also performed on samples (data not shown). After immunodetection with anti-myc and anti-EGFP antibodies, no reactive bands of small-size protein were revealed, indicating that the recombinant protein was not degraded in the samples taken after 10 h of induction. At the same time, K_d values for diprenorphine were unchanged for all the conditions used. We can assume that the total amount of expressed protein was nearly identical under each of these conditions but that stability was highly dependent on the induction conditions employed. The difference between the total versus the functionally active receptor found in the cell could be explained by a different intracellular localization of a portion of the receptor molecule population. Further work should involve the quantitation of receptor in plasma membrane versus intracellular membrane, such as ER and Golgi, using for instance differential centrifugation techniques. Furthermore, all studies were performed in shake flasks and from the perspective of fermenter production of the receptor, parameters such as methanol concentration or pH should be reinvestigated. In conclusion, the successful optimization of the μ -opioid receptor expression should allow large-scale production of the recombinant protein by fermentation technology and facilitate the purification and reconstitution steps that are currently under way.

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