FEBS Letters 377 (1995) 140–144

The human β_2-adrenergic receptor expressed in *Schizosaccharomyces pombe* retains its pharmacological properties

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Received 26 October 1995

Abstract **We have developed a rapid and efficient expression system to study the human β_2 adrenergic receptor (huβ_2AR) in the fission yeast *Schizosaccharomyces pombe*. This was achieved by cloning the huβ_2AR gene, modified by replacement of the 5′ untranslated and a small part of the N-terminal coding sequence (first 14 amino acids) with the corresponding region of the yeast *Saccharomyces cerevisiae* STE2 (α-factor receptor) gene. The gene was then placed under the control of a *S. pombe* constitutive promoter for alcohol dehydrogenase (adh). Huβ_2AR expression was assessed by immunoblot analysis of the chimeric protein with an anti-STE2 serum raised against a dodecapeptide homologous to the N-terminal amino acids of STE2 and ligand binding was assayed using [^{125}I]cyanopindolol. We demonstrate here that the chimeric receptor expressed in *S. pombe* exhibits the same characteristic ligand specificity and affinity as that of the authentic huβ_2AR. This system constitutes a convenient alternative to existing methods for studying seven transmembrane domain receptors due to its simplicity and high reproducibility.**

Key words: Human β_2-adrenergic receptor; Yeast vector; *Schizosaccharomyces pombe*; Membrane protein; Heterologous expression

1. Introduction

Eukaryotic cells can sense a wide variety of external stimuli and respond to them through conformational changes of plasma membrane receptors following specific ligand binding, which in turn influences the activity of downstream effector molecules.

The mammalian β adrenergic receptors (βAR) are probably the most intensively studied cell surface receptors of the nervous system. They are members of a class of ligand binding receptors containing seven transmembrane domains that cause activation of adenylate cyclase. These receptors catalyze the binding of GTP to a regulatory protein, Gs, on the inner face of the plasma membrane, thereby activating it. Gs then stimulates adenylate cyclase to produce cAMP [1].

The yeast *S. cerevisiae* also contains similar 7-span membrane receptors [2,3], STE2 (α factor receptor) and the STE3 (a factor receptor) proteins. Their transducing pathways, induced by pheromone binding (α or a factor), involves a G protein [2,3]. In fact, the expression of a mammalian $G_s\alpha$ subunit in mutant yeast lacking the corresponding endogenous protein (encoded by GPA1) restores viability [4].

Most of the β adrenergic receptors, identified by gene cloning and sequence analysis, have been expressed in eukaryotic cells [5]. The human β_2AR gene was also expressed in *E. coli* [6,19] and in *S. cerevisiae* [7]. The huβ_2AR expressed in *E. coli* retains its pharmacological profile and is capable of coupling to a G protein in an in vitro reconstitution system [8].

King et al. have programmed *S. cerevisiae* to express the huβ_2AR gene. They have shown that the yeast's intrinsic signalling pathway could be activated in a β-adrenergic-agonist-dependent manner in cells expressing the human protein. This suggests evolutionary similarities may facilitate the expression of membrane bound protein in yeast. However, their expression technique is laborious and requires several manipulations including coexpression of the LAC9 gene, a transcriptional transactivator protein, and induction with galactose at late exponential phase of cell growth [9].

In this paper we report the expression of the huβ_2AR gene in the fission yeast *Schizosaccharomyces pombe*. We show that this organism is a more efficient host than *S. cerevisiae* and a convenient tool to explore expression and binding capacity of this class of human receptors.

2. Materials and methods

2.1. Strains and Media

E. coli K514 (hsrk, hsmk derivative of strains C600) was used [10]. *Schizosaccharomyces pombe* (*S. pombe*) leu1-32,h$^-$ strain was grown in either rich medium (YEA) or synthetic minimal medium [11].

2.2. Plasmid construction

Two different expression vectors containing the huβ_2AR gene were constructed. pEVP11, an *S. pombe* expression vector that contains a polylinker region under the control of the *S. pombe* constitutive promoter for alcohol dehydrogenase (adh) (Fig. 1A) was used in each case.

For one construct, pEVβAR (Fig. 1B) the whole cDNA huβ_2AR gene (gift from Dr. Lefkowitz) was removed from the eukaryotic vector pkSV10 [12] by *Sac*I–*Bam*HI digestion and placed directly into a similarly digested region of the pEVP11 polylinker region.

The other construct replaces the N-terminal region of the huβ_2AR gene with the N-terminal region from the *S. cerevisiae* STE2 gene [2]. Briefly, a synthetic oligonucleotide (Fig. 2A) was made corresponding to 11 bp of noncoding and 42 bp of coding sequence from the STE2 gene. This sequence was cloned into the *Bam*HI and *Aat*II sites of the plasmid YIP5 [13]. The resulting plasmid, YIP5-oligo, was then digested with *Ssp*I and *Aat*II to open it up and a *Aat*II–*Sal*I fragment isolated from the huβ_2AR-pkSV10 clone was ligated into this plasmid, filling in the mismatched *Sal*I–*Ssp*I sites. The recombinant plasmid yi-STE2β_2 is shown in Fig. 2B. A *Bam*HI–*Bam*HI fragment was then isolated from the yi-STE2β_2 plasmid and inserted into the *Bam*HI site of the polylinker region of the expression vector pEVP11 resulting in pEVSTE2βAR (Fig. 1B).

2.3. Fractionation of yeast membranes

For the isolation of the inner and outer membranes, yeast cells were grown in EMM medium with 40 μM alprenolol to an OD_{600} value of 2. Then the cells were harvested by centrifugation at 6,000 × *g* and the

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SSDI 0014-5793(95)01330-X

pellet was resuspended in 5 ml of SCE buffer (1 M Sorbitol, 0.1 M sodium citrate, 60 mM EDTA, pH 7.0) with the addition of 1% β-mercaptoethanol and 0.05 mg/ml Zymolase 100T (Kirin, Japan). After 1 h of incubation at 37°C, spheroblasts were harvested by centrifugation at 3,500 × g and resuspended in 2 ml lysis buffer (50 mM Tris-HCl pH 7.4 5 mM EDTA) with the addition of 1 mM PMSF and 20 μg leupeptin. Spheroblasts were lysed at 4°C with glass beads using a Braun homogenizer (Braun Melsungen, Germany). After removing the glass beads the suspension was centrifuged at 85,000 × g for 30 min. The pellet was resuspended in 150–200 μl of lysis buffer and the protein concentration was measured according to Bradford [14].

2.4 Binding assays

Five to ten μg of yeast membranes were spotted onto nitrocellulose filters (8 cm in diameter, Schleicher and Schuell, Germany) and were incubated in 10 ml binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) supplemented with 2% bovine serum albumin (BSA), for 1 h at room temperature by gentle shaking. Then the filters were incubated for 90 min. in the same buffer with ^{125}I-labeled cyanopindolol, ([^{125}I]CYP, 2000 Ci/mmol., Amersham, UK) at a final concentration of 20 pM. Filters were washed four times for 15 min each with 10 ml of binding buffer supplemented with 0.2% Tween 20, air dried and then exposed for 12–16 h at −70 °C to X-ray film with an intensifying screen. The binding assay was also performed in solution at the same protein concentration and incubated in 1 ml of binding buffer for 30 min at 37°C. Reactions were stopped by filtration on Whatman GF-C filters followed by one wash with 20 ml of cold buffer.

B_{max} and K_d values were determined by varying [^{125}I]CYP concentrations (2–300 pM) in the presence of 10 μM alprenolol. Displacement of ^{125}I]CYP (present at a concentration of 20 pM) with various concentrations of unlabeled competitors was also measured.

2.5 Immunoblots

An antibody raised against a dodecapeptide constructed from the N-terminal sequence of STE2 (a gift from Dr. F. Scavizzi) was used in immunoblot analysis to detect the chimeric receptor protein produced by pEVSTE2βAR transformed cells (Fig. 3). Briefly, membranes from lysed yeast were solubilized in Laemmli's sample buffer containing 10% SDS and run on a 12% polyacrylamide gel [15]. Proteins were transferred onto a nitrocellulose membrane using a mini trans-blot cell (BioRad) in 25 mM Tris-HCl, pH 7.4, 192 mM glycine at 150 mA for 1 hour [16]. This membrane was then blocked using phosphate-buffered saline (PBS) containing 2% BSA and 0.05% Tween 20 for 30 min. A 1:1000 dilution of the rabbit anti-STE2 serum in blocking buffer was incubated with the membrane for 1.5 h at room temperature. The membrane was then washed three times with the same buffer and incubated with a 1:1000 dilution of goat anti-rabbit alkaline phosphatase antibody for 1.5 h. Bands were detected by colorimetric reaction after three washes in blocking buffer, a rinse in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and then a 1 h incubation at room temperature in the dark with 10 ml AP buffer containing 66 μl of NBT (nitroblue tetrazolum) and 33 μl of BCIP (5-bromo-4-chloro-3-indonylphosphate).

3. Results

3.1. Plasmid construction

We chose two cloning strategies directed towards a stable expression of the huβ$_2$AR gene in *S. pombe*. In both recombinant plasmids, the cloned huβ$_2$AR gene was under the control of the *S. pombe* constitutive promoter for alcohol dehydrogenase (adh) in the expression vector pEVP11 (Fig. 1A). One approach used an unmodified huβ$_2$AR cDNA sequence, which contained the entire coding sequence followed by 250 bp that included the RNA transcription termination signal cloned into the *S. pombe* expression vector pEVP11 (plasmid pEVβAR, Fig. 1B). The other used a N-terminally modified huβ$_2$AR gene. In this instance, a specific STE2 oligonucleotide sequence (Fig. 2A) corresponding to the N-terminal region of STE2, replaced the 5′ untranslated region and the coding sequence for the first 22 N-terminal amino acids of the huβ$_2$AR gene. This gene was then cloned into the *S. pombe* expression vector (plasmid pEVSTE2βAR, Fig. 1B). This procedure is consistent with the strategy used to express the huβ$_2$AR gene in the yeast *S. cerevisiae* [7]. The transformed cells were grown in glucose media

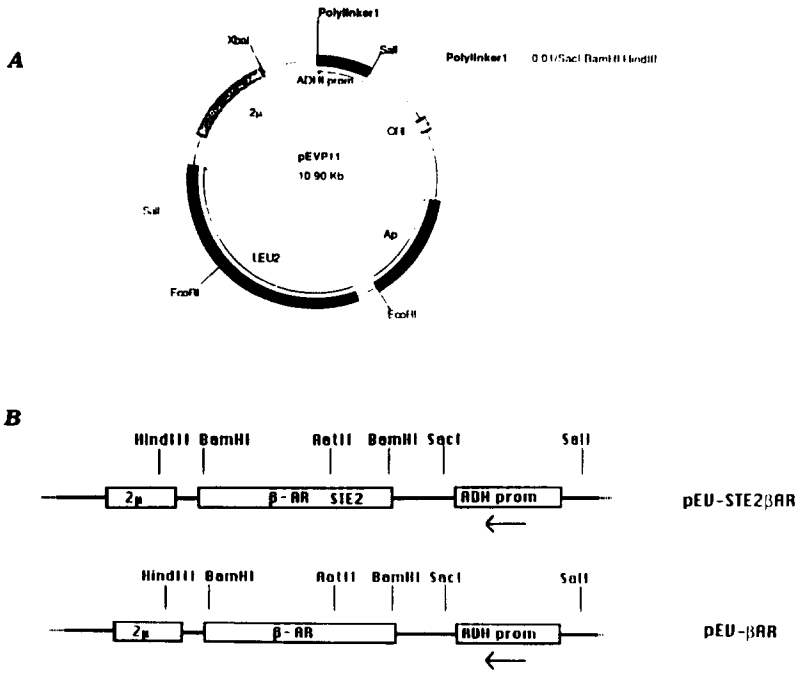

Fig. 1. Yeast expression vectors. (A) Yeast plasmid pEVP11. (B) The recombinant plasmid pEVSTE2βAR constructed by inserting, at the *Bam*HI site of the polylinker, the *Bam*HI–*Bam*HI fragment isolated from YISTE2βAR. The recombinant plasmid pEVβAR contains the whole huβ2AR gene cloned into the *Sac*I–*Bam*HI sites of the pEVP11 polylinker.

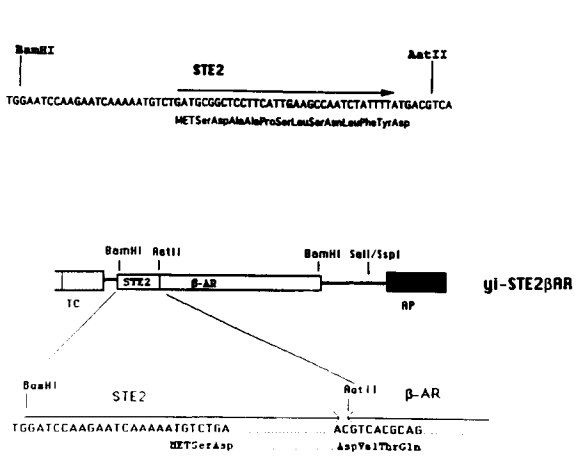

Fig. 2. Construction of yeast plasmid yI-STE2βAR. (A) A synthetic oligonucleotide corresponding to 11 bp of noncoding and 42 bp of coding sequence from the STE2 gene [6]. (B) The plasmid yI-STE2βAR was constructed by inserting of *Bam*HI–*Aat*II fragment containing the synthetic oligonucleotide and then by inserting a *Aat*II–*Sal*I fragment containing the huβ_2AR.

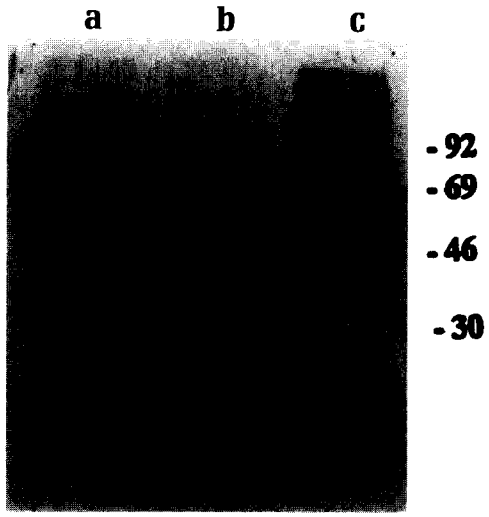

Fig. 3. Immunoblots with a rabbit polyclonal antibody against the first 12 amino acids of the STE2 receptor. (Lane a) Nontransformed cells. (Lane b) The control strain containing the plasmid pEVP11. (Lane c) *S. pombe* cells transformed with plasmid pEVSTE2βAR.

until late exponential phase and the cell membranes were screened by immunoblot analysis and ligand binding.

3.2. Immunoassay

The expression of the huβ_2AR receptor in the yeast *S. pombe* was first detected by immunoblot analysis of extracts from cells transformed with pEVP11 and pEVSTE2β_2AR plasmids (Fig. 3). Total cellular proteins were separated by SDS PAGE and then blotted onto nitrocellulose sheets and reacted with a rabbit polyclonal antibody against the first 12 aminoacids of the STE2 receptor present in the chimeric protein STE-β_2AR (Fig. 2A). Immune complexes were detected by successive incubation with alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (Ig G).

S. pombe cells that contained the plasmid pEVSTE2βAR synthesized two novel polypeptides that are recognized by anti-STE2 antibodies (Fig. 3, lane c). The upper band had an electrophoretic mobility similar to that of the authentic human β_2-adrenergic receptor glycoprotein, molecular weight 64 kDa [17]. Since the theoretical molecular weight for the nonglycosylated full length β_2AR protein is 46 kDa [18], the lower band shown in Fig. 3 lane c, might correspond to that protein.

No such polypeptides were produced in nontransformed cells (Fig. 3 lane a) or in the control strain containing the plasmid pEVP11 (Fig. 3 lane b).

3.3. Receptor pharmacology

To determine the pharmacological profile of the receptor encoded by the chimeric huβ_2AR gene, yeast transformants carrying the vector pEVP11 or the pEVSTE2βAR construct were examined. Transformants were tested for the presence of β_2-adrenergic binding sites by using the antagonist [^{125}I]CYP in saturation experiments [6].

Crude membranes prepared from cells transformed with pEVSTE2βAR showed specific and saturable binding of [^{125}I]CYP (Fig. 4) whereas no binding was detected using membranes from transformants obtained with the vector alone. Data analysis indicated a single class of binding sites with a K_d of 50 μM and densities of 7.5 pmol/mg (Fig. 5).

No appreciable ligand binding could be detected with membranes from the pEVβ_2AR transformants (data not shown). This observation could conceivably reflect folding-stability and/or processing (e.g. glycosylation) problems of the human receptor protein which lacks the *S. cerevisiae* STE2 N-terminal sequence. The affinities of a series of adrenergic ligands in competition binding studies with [^{125}I]CYP confirmed the structural integrity of the β_2-adrenergic binding sites detected in the pEVSTE2βAR transformants. The ligands included two agonists (isoproterenol and epinephrine) and the antagonist alprenolol. The competition curve are depicted in Fig. 6.

3.4. Filter assay

S. pombe membrane preparations were used to determine the binding of [^{125}I]CYP to receptor proteins immobilized on nitrocellulose filters. Different concentrations of membrane proteins

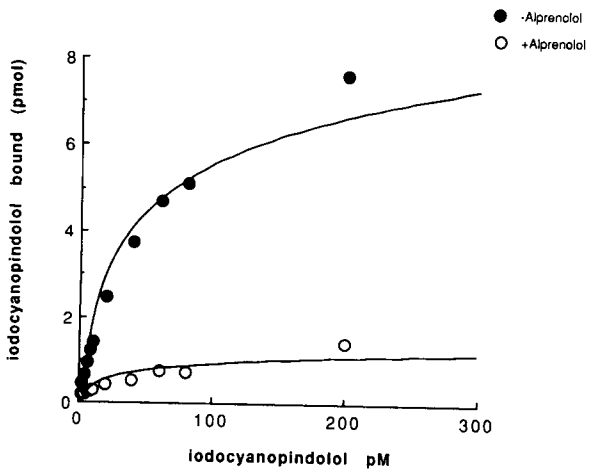

Fig. 4. [^{125}I]CYP binding to crude membranes prepared from cells containing the fusion gene. Results correspond to two independent experiments performed in triplicate. Specific binding was defined as the amount of total binding (●) minus nonspecific binding measured in the presence of 10 μM (−)alprenolol (○).

(5, 15, 25 μg) prepared from cells transformed with the pEVp11, pEVβ_2AR and pEVSTE2βAR plasmids were spotted onto nitrocellulose filters. As shown in Fig. 7 there is a strong binding signal with pEVSTE2βAR clone and the intensity of the autoradiograph signal appears to be proportional to the amount of protein spotted. An unlabeled agonist (alprenolol) competes with the radioactive ligand for the binding to the membranes (Fig. 7B). Membranes from the cells transformed with pEVp11 do not exhibit any binding activity. Five μg of pig lung membrane proteins, which contain a high concentration of β_2-adrenergic receptors, were spotted onto each of the filters to provide an internal control (marked with an arrow in Fig. 7) [19]. Contrary to the saturation experiments, in the filter assay, membranes from the cells carrying the pEVβAR plasmid show binding activity, although weaker than that of the membranes from cells transformed with pEVSTE2βAR. This confirms the importance of the *S. cerevisiae* STE2 N-terminal sequence to express the human protein, and shows that the filter assay is probably a more sensitive method than the ligand binding assay performed in solution to identify receptor ligand complexes.

4. Discussion

Traditionally, most molecular biologists interested in yeast have directed their attention to one particular species, *S. cerevisiae*. More recently the powerful genetic and molecular methods developed to study this organism have been successfully applied to another yeast species, *S. pombe*. A useful set of plasmids has been constructed for cloning in *S. pombe* [20]. We cloned the huβ_2AR gene into the plasmid pEVP11 [21] under the control of the *S. pombe* adh promoter [22]. The adh gene is expressed constitutively in glucose and glycerol/ethanol-grown cells; it is estimated that 0.5–2% of the total soluble protein is alcohol dehydrogenase.

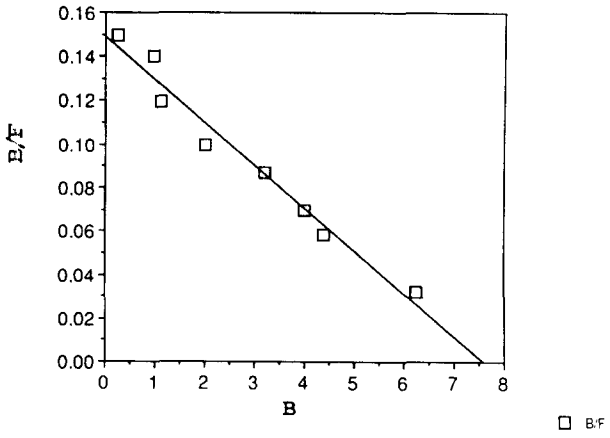

Fig. 5. Scatchard analysis of the binding isotherm.

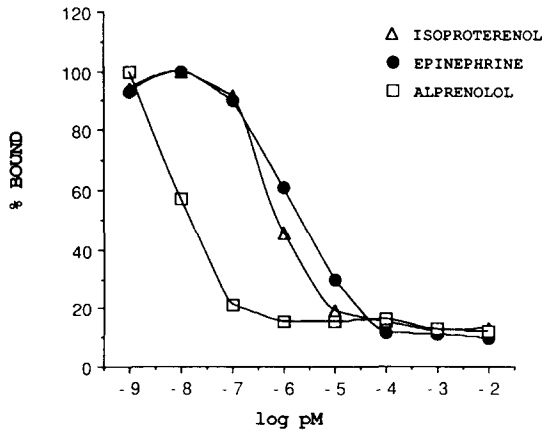

Fig. 6. Displacement of [^{125}I]CYP with various concentrations of agonists.

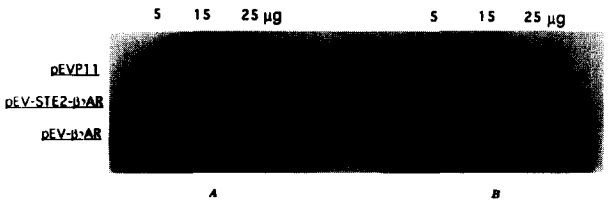

Fig. 7. [^{125}I]CYP binding to different concentrations of membrane proteins spotted onto nitrocellulose filters, without (A) and with (B) an unlabeled competitor (alprenolol). Arrow (△) indicates an internal positive control: five μg of pig lung membrane proteins.

S. pombe cells transformed with the recombinant plasmid pEVβAR, carrying the complete human receptor coding sequence show poor expression of the huβ_2AR protein as demonstrated by (^{125}I)CYP binding on filters. This might be due to: (i) an inefficient synthesis of the protein in yeast, or (ii) instability of the heterologous protein (possibly due to inefficient membrane insertion or folding). It has been shown previously that the modification of the N-terminal region of a protein alien to yeast may improve its expression [23]. King et al. have shown that the replacement of the 5′ untranslated and part of the N-terminal coding sequence of the huβ_2AR gene with the corresponding region of the STE2 (α factor receptor) gene has improved the expression of the huβ_2AR gene in *S. cerevisiae* [7] We show that the same sequence is functional and necessary to express and translocate the foreign protein in *S. pombe* cells.

Immunoblot analysis of membrane proteins synthesized in transformants carrying the pEVSTE2βAR plasmid revealed a protein of the same size as the native huβ_2AR protein, suggesting that proper post-translational processing occurred. In addition, a smaller protein is produced, consistent with the size of the nonglycosylated form. A combination of heterologous glycosylated and nonglycosylated products has been previously observed in yeast [24]. The results reported here confirm that the huβ_2AR protein is translocated to the membrane of *S. pombe* retaining its ligand binding properties.

The K_d value reported for the pEVSTE2βAR expression system is close to those obtained in mammalian expression systems, with values ranging from 21 pM for transfected L-cells [25] to 134 pM for the human A 431 cell line [26]. Furthermore, the 3 subtype-selective antagonists and agonist compete with [^{125}I]CYP binding with a rank order of potency (alprenolol > isoproterenol > epinephrine) which is consistent with published data [27].

Previous papers have reported the use of microbial hosts for the expression of neurotransmitter receptors [19,28,29]. Human β_2-adrenergic receptors expressed from fusion genes in *E. coli* and *S. cerevisiae* retain the 'native' ligand binding profiles [6,17,19]. In particular, in the *S. cerevisiae* cells it was observed that maximal expression required several manipulations includ-

ing: (i) coexpression of a modified huβ_2AR gene and a transcription transactivator protein (the LAC9 gene product) [9]; (ii) growth to late exponential phase; (iii) induction by galactose for an additional 36 hours.

In this report we show that the eukaryote *S. pombe* is a particularly attractive candidate for the expression of mammalian receptors with 'native' properties since (i) it is easily manipulated; (ii) the expression of the receptor gene is constitutive; (iii) the protein inserts into the cellular membrane; (iv) it possesses a signal transducing machinery with remarkable similarities to mammalian G protein coupled systems. Recent results demonstrate that signals initiated by the mating factors of *S. pombe* are also transmitted through a G protein to the effector(s) [29].

Most recently Sander et al. have expressed the human D_3 dopamine receptor in *S. cerevisiae* and *S. pombe* [30]. By measuring the binding of a dopaminergic antagonist (spiperone) to membranes prepared from the yeasts they find that recombinant receptor production is at least 3 times higher in *S. pombe* than in *S. cerevisiae*.

Morever the fission yeast utilizes a G protein α-subunit to positively transmit the signal from the mating factor receptor to effectors [31]. This resembles the role of G_α during signal trunsduction in mammalian cells and therefore makes *S. pombe* a closer model system for investigating mammalian receptor function.

Studies are now in progress to establish functional coupling of the recombinant huβ_2AR receptor to the G-protein mating pathway in *S. pombe*.

Acknowledgements: We thank Dr. R. Lefkowitz for the generous gift of the human β_2-adrenergic receptor cDNA clone and Dr. F. Scavizzi for the antiserum against STE2. Our cordial thanks go to Dr. A.B. Stoler and Dr. T. Wagner for critical reading the manuscript. This work was funded through contract number 118907-1016/261 of the 'Programma Nazionale di Ricerca' consigned by the 'Ministero dell'Università e della Ricerca Scientifica e Tecnologica' to EniChem SpA and through Progetto Finalizzato Ingegneria Genetica, C.N.R.

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