ELSEVIER

FEBS Letters 344 (1994) 41–46

FEBS 13950

FEBS LETTERS

# Expression of the human $D_{2S}$ dopamine receptor in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*: a comparative study

Peter Sander, Sylvia Grünewald, Helmut Reiländer*, Hartmut Michel

*Max-Planck-Institut für Biophysik, Abt. Molekulare Membranbiologie, Heinrich-Hoffmann-Straße 7, 60528 Frankfurt/M, Germany*

Received 2 February 1994; revised version received 24 March 1994

**Abstract**

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were tested for heterologous expression of the human $D_{2S}$ dopamine receptor. The cDNA coding for the dopamine receptor was cloned into high copy number plasmids with inducible promoters. After transformation into the yeasts recombinant clones were examined for the presence of functional receptor by radioligand binding using the antagonist [$^3$H]spiperone. Subsequent Western blot analysis of positive recombinants with an antiserum raised against a peptide from the third intracellular domain of the receptor protein revealed the production of a protein with an apparent molecular mass of 40 kDa in both yeasts. Membranes harvested from recombinant yeast clones exhibited saturable binding of the dopaminergic antagonist [$^3$H]spiperone with $K_d$ values of 1.3 nM in *S. cerevisiae* and 0.25 nM in *S. pombe*. The rank order of potencies for several dopaminergic ligands to displace specific [$^3$H]spiperone binding to membranes were the same in both yeasts, whereas the affinities for ligands differed significantly.

*Key words:* *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*; $D_{2S}$ dopamine receptor; G-protein coupled receptor; Membrane protein; Heterologous expression

## 1. Introduction

The essential prerequisite for extensive biochemical, biophysical and structural analysis of G-protein coupled receptors is the availability of large quantities of homogeneous protein, which cannot be provided by natural sources. Hence cloned receptor cDNAs must be overexpressed in suitable heterologous expression systems [1].

The genetically well characterized unicellular eucaryote *Saccharomyces cerevisiae* has been used for the heterologous production of several G-protein coupled receptors [2,3] (Sander et al., in preparation). Recently, the expression of a bacterial membrane protein with seven transmembrane spanning regions in a second well characterized yeast was reported: bacteriorhodopsin had been functionally expressed in the fission yeast *Schizosaccharomyces pombe* [4]. The results presented were so convincing, that this unicellular organism was also considered as an alternative host for heterologous expression of G-protein coupled receptors from higher eucaryotes. We report here a comparative study using both yeast genera as expression systems for the heterologous production of a member of this receptor superfamily, the human $D_{2S}$ dopamine receptor.

The $D_2$ receptor was the first dopamine receptor to be cloned and is the best characterized one [5,6]. Two isoforms of the receptor are known ($D_{2L}$ and $D_{2S}$), which are produced by differential splicing [7–9]. Activation of the receptor results in various responses, including inhibition of adenylate cyclase, inhibition of phosphatidylinositol turnover, increase in $K^+$ channel activity and inhibition of $Ca^{2+}$ mobilization [6,10–13]. The distorted balance between dopamine and the dopamine receptors is thought to be responsible for the development of psychiatric and neurological disorders such as schizophrenia and Parkinson's disease [14,15]. Antagonism of postsynaptic $D_2$ receptors has long been regarded as the primary mechanism of the action of certain antipsychotic drugs. The major problems that arose with the use of dopamine antagonists were side effects resulting from drug coupling to other neuroreceptors [16]. The experimental confirmation of the three-dimensional structure of the $D_2$ dopamine receptor previously proposed by Dahl et al. [17], will not only significantly add to the understanding of the molecular mechanism of ligand binding, but will also give the possibility to design suitable and specific drugs based on the spatial requirements of the ligand binding pocket.

*Corresponding author. Fax: (49) (69) 96769 423.

*Abbreviations:* AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidinium salt; BSA, bovine serum albumin; DMF, dimethylformamide; NBT, Nitroblue-tetrazoliumchloride; mcs; multiple cloning site; PNGaseF, peptide-*N*-glycosidase F.


*SSDI* 0014-5793(94)00335-S

## 2. Materials and methods

### 2.1. Strains and transformations

*E. coli* strain XL-1 Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*$^-$ [F′*pro*AB, *lac*I$^q$Z$\Delta$M15, Tn10(tet$^r$)] (Stratagene) was used for propagation of the recombinant plasmids. For *E. coli* transformation, the $CaCl_2$ procedure was used as described [18]. *S. cerevisiae* strain YMTAB: (*MAT*$\alpha$, *GAL*$^+$, *leu2*-3,112; *ura3*$\Delta$5; *pra*::*HIS3*; *prb1*$\Delta$AV) was kindly provided by Prof. D.H. Wolf (University Stuttgart). The universal transforming strain *S. pombe* (*h*-, *ura4*- D18, *leu1*-32, *ade6*-704) was kindly provided by K. Maundrell (Glaxo, Geneva). Yeast cells were made competent for plasmid uptake by treatment with lithium acetate and polyethylenglycol [19,20].

### 2.2. Recombinant DNA technology and construction of recombinant plasmids

DNA isolation, restriction enzyme analysis, agarose electrophoresis and cloning procedures were performed using established techniques [18,21]. The plasmid pBSD2YSal used for the following cloning steps is described in detail elsewhere (Sander et al., in preparation). Plasmid YEp51, which bears the inducible GAL10 promoter for heterologous expression in *S. cerevisiae*, was used [22]. A *Sal*I-DNA fragment harbouring the $D_{2S}$ gene was isolated from pBSD2YSal and cloned into the *Sal*I site of vector YEp51. In the resulting plasmid, YEp51D2 (Fig. 1), the $D_{2S}$ gene is now located downstream of the inducible GAL10 promoter (YEp51). The vector YEp51 and the recombinant plasmid were used for the transformation of *S. cerevisiae* cells.

For the expression of the $D_{2S}$ receptor in *S. pombe* the plasmid pREP1 (kindly provided by K. Maundrell, Geneva) was used. This high copy number plasmid contains the inducible promoter and the transcriptional termination sequences of the *nmt1* gene from *S. pombe* separated by a multiple cloning site for the insertion of foreign genes [23]. The $D_{2S}$ gene was isolated from plasmid pBSD2YSal using appropriate *Bam*HI restriction sites flanking the gene and cloned into pREP1 digested with *Bam*HI and treated with calf intestinate phosphatase resulting in pREP1D2.

### 2.3. Yeast culture

Non-transformed *S. cerevisiae* and *S. pombe* strains were kept in YPD- and YE-medium, respectively. Transformed yeast always was cultured under selective pressure. For *S. cerevisiae* SC medium, for *S. pombe* MM medium containing the required supplements respectively was used to counteract the loss of transformed plasmids [24].

In *S. cerevisiae* the expression of the $D_{2S}$ dopamine receptor was induced by changing the carbon source in the media of the growing culture from 2% glucose to 2% galactose. Prior to induction the *S. pombe* transformants were grown in the presence of 1 $\mu$M thiamine. Induction of the *nmt1* promoter was achieved by washing the transformed cells with sterile water and resuspension in thiamine-free MM. Sixteen hours after induction yeast cells were harvested and membranes were isolated as described below.

### 2.4. Isolation of membranes from S. cerevisiae and S. pombe cells

250-ml cultures of yeast cells in the respective growth medium were harvested after overnight incubation by centrifugation. Cells were washed in water, centrifuged and the pellet was resuspended in 4 ml STED10-buffer (10 mM Tris-HCl pH 7.6; 1 mM EDTA; 1 mM DDT; 10% sucrose) supplemented with protease inhibitors (5 $\mu$g/ml leupeptin, 5 $\mu$g/ml chymostatin, 1 $\mu$g/ml pepstatin, and 100 mM Pefabloc). Glas beads (17 gr; mesh 400–600; Sigma) were added to the suspension and the cells were broken by vigorous vortexing at 4°C for 5 min. The homogenate was diluted with 15 ml of STED10-buffer plus protease inhibitors and centrifuged at 3,000 × *g* for 10 min to remove cell debris. Subsequently the membranes were pelleted in a Sorvall centrifuge at 35,000 × *g* for 45 min. The resulting membrane pellet was resuspended in 3–4 ml STED10-buffer and stored at −70°C for further experiments.

### 2.5. Western blot analysis

Membranes were diluted to a protein concentration of 2 $\mu$g/$\mu$l in STED10-buffer and incubated for 15 min at room temperature with an equal volume of SDS-loading buffer (2% w/v SDS, 5% v/v $\beta$-mercaptoethanol, 5% v/v glycerol, 62.5 mM Tris-HCl pH 6.8, 0.01% w/v Bromophenol blue). After a short centrifugation the membrane proteins were separated on a 10% SDS polyacrylamide gel [25]. For immuno blot analysis, proteins were transferred onto a polyvinylidendifluoride membrane (Immobilon P; Millipore) and processed as described [26]. The primary polyclonal antibody RD2 (directed against the receptor) was used at a dilution of 1 : 5000, the secondary anti-rabbit alkaline phosphatase coupled antibody at a dilution of 1 : 1000. Protein bands were visualized by enzymatic reaction in AP-buffer (100 mM NaCl, 100 mM Tris pH 9.5, 5 mM $MgCl_2$) with BCIP (stock solution: 50 mg/ml DMF) and NBT (stock solution: 50 mg/ml 70% DMF) as recommended by the suppliers.

### 2.6. Radioligand binding to recombinant human $D_{2S}$ receptor

Saturation binding measurements were performed on isolated membranes using antagonist [$^3$H]spiperone as a radioactive ligand (spec. activity 0.55–1.11 TBq/mmol; DuPont, NEN). Membranes equivalent to 10–30 $\mu$g protein were suspended in 500 $\mu$l binding buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl) and radioactive ligand was pipetted to the probe at a final concentration of 0.02–20000 pM. Binding assays were incubated for 45 min at 30°C. Nonspecific binding was determined in the presence of non-labeled (+)butaclamol (2 $\mu$M final concentration). Bound and free ligand were separated by rapid filtration (Whatman GF/F filters, soaked in 0.3% polyethyleneimine) and after 3 washes with ice-cold binding buffer, radioactivity bound to the filter was determined by liquid szintillation counting (rotiszint; Roth). $K_d$ and $B_{max}$-values were calculated by computer-aided non-linear regression analysis of the binding isotherms under equilibrium conditions.

## 3. Results and discussion

*S. cerevisiae* and *S. pombe* have successfully been used for the functional expression of various proteins. To compare the heterologous expression of the human $D_{2S}$ receptor in the two yeast genera, we constructed two recombinant plasmids named YEp51D2 and pREPD2 for the transformation of *S. cerevisiae* and *S. pombe*, respectively. In both constructs the coding region of the $D_{2S}$ receptor was placed under the transcriptional control of a strong, inducible promoter. In plasmid YEp51D2 which was used for transformation of *S. cerevisiae* the yeast *GAL10* promoter [22], and in plasmid pREP1D2 which was used transformation of *S. pombe*, the *nmt1* promoter [23], directed transcription of the foreign gene (Fig. 1). In both constructs the region for initiation of translation has been optimized for the expression in the yeasts, whereas the $D_2$ coding region has not been altered during construction.

Yeast transformants of both genera *S. cerevisiae* and *S. pombe* carrying the expression plasmids were assayed for their ability to bind the dopamine antagonist [$^3$H]spiperone. Recombinant clones which scored positive during this initial screening were then examined for the presence of the $D_{2S}$ dopamine receptor protein by Western blot analysis. As a control, transformants harbouring the plasmids without the inserted $D_{2S}$ cDNA were examined in parallel. The membrane proteins harvested after induction were separated on 10% SDS-polyacrylamid gels. After transfer of the membrane proteins onto Immobilon sheets, the blots were probed with polyclonal antiserum RD2 directed against a peptide occurring in the third intracellular loop of the human $D_{2S}$ receptor (Sander et al., in preparation).

Membrane preparations from recombinant *S. cere-*

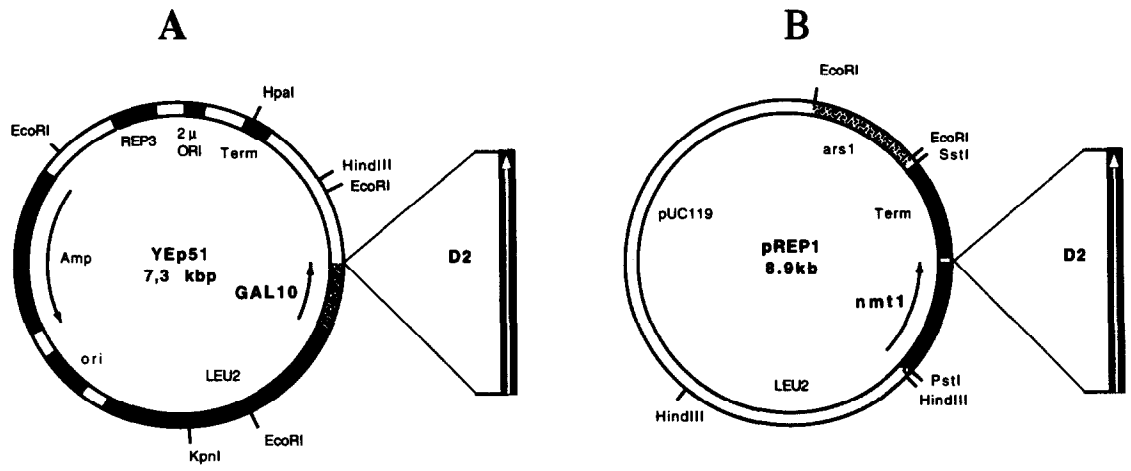


Fig. 1. Expression plasmids constructed for the heterologous expression of the human $D_{2S}$ dopamine receptor in *S. cerevisiae* and *S. pombe*. (A) Partial map of the expression plasmid YEp51D2 for *S. cerevisiae*. The human $D_{2S}$ receptor gene is under transcriptional control of the yeast *GAL10* promoter. (B) Partial map of the expression plasmid pREP1D2 for *S. pombe*. The *nmt1* promoter controls the heterologous expression of the human $D_{2S}$ receptor gene. ori, origin of replication for *E. coli*; Amp, ampicillin resistance gene; pUC119, *E. coli* plasmid pUC119; 2μ ORI, yeast 2μ origin of replication; Term, transcriptional termination region; LEU2, *LEU2* gene from *S. cerevisiae*.

*visiae* and *S. pombe* bearing only vectors YEp51 and pREP1 respectively, were totally devoid of stainable protein. As presented in Fig. 2A, the antibody specifically stained a protein with an apparent molecular mass of ≈40 kDa in both yeasts when transformed with the expression plasmid harbouring the $D_{2S}$ dopamine receptor gene. The apparent molecular mass determined by SDS-PAGE differs significantly from the molecular mass which can be calculated from the cDNA sequence (45.5 kDa) but is the same for both yeasts. It has been reported earlier, that the apparent molecular mass of the isolated and deglycosylated rat $D_2$ receptor is ≈7 kDa smaller than the molecular mass calculated from the corresponding cDNA sequence (47 kDa). An additional protein band migrating at about 43 kDa appears in the membrane preparation of receptor producing cells from *S. cerevisiae* might resemble core-glycosylated receptor protein. A band with a similar molecular mass does not appear in preparations from *S. pombe*, suggesting a more homogeneous population of heterologously expressed receptor protein. The Coomassie stain of a gel with the same membrane preparations did not show any obvious additional protein band in the range of 40 kDa but clearly demonstrates, that less protein was loaded with the preparations of *S. pombe* (Fig. 2B). However, the intensity of the immunologically stained protein in the

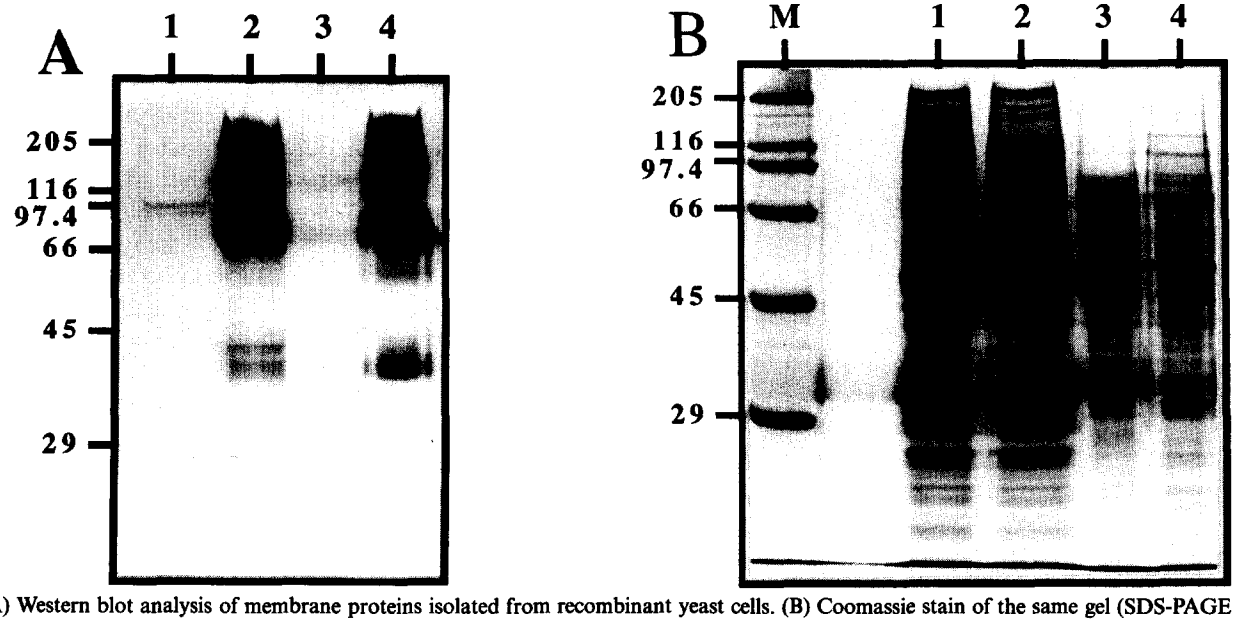


Fig. 2. (A) Western blot analysis of membrane proteins isolated from recombinant yeast cells. (B) Coomassie stain of the same gel (SDS-PAGE of membranes prepared from recombinant yeast clones). 1, *S. cerevisiae* YEp51; 2, *S. cerevisiae* YEp51D2; 3, *S. pombe* pREP1; 4, *S. pombe* pREP1D2; Lane M, marker proteins.

Western blot analysis indicated that the concentration of the heterologously produced receptor protein was remarkably higher in the fission yeast. Strong specific signals on the Western blot analysis that arise in the molecular mass range of ≈85 kDa in membrane preparations of both expressing yeasts most probably represent aggregated protein, which is produced in the cells due to the overexpression of the receptor. The treatment of membrane preparations with the glycosylase PNGaseF resulted in no significant changes in the western blot pattern (data not shown). From this we suggest, that the higher molecular mass proteins are not heavily altered by addition of carbohydrates. The regular signal pattern appearing specially in the membrane preparation of *S. pombe* transformants (Fig. 2A, lane 4) imply that the receptor aggregates in the form of multimers.

A primary function of a certain cell surface receptor is to recognize its appropriate ligands. Total binding experiments were conducted to determine the expression levels of the receptor proteins in the different transformed yeasts. The results of the experiments are depicted in Fig. 3, revealing that the receptor expression levels in the different yeast genera varied significantly. Membranes prepared from transformed *S. cerevisiae* bound ≈2.8 pmol ligand per mg of membrane protein whereas membranes prepared from transformed *S. pombe* bound up to ≈14.6 pmol per mg protein. The 3- to 5-fold higher receptor concentration monitored in the fission yeast could resemble an increased over all expression level or a better ratio of functional to non-functional receptor proteins.

To further establish the functional integrity of the $D_{2S}$ receptor expressed in both yeasts, recombinant clones were tested for the presence of dopamine binding sites using the antagonist [$^3$H]spiperone in saturation experiments. Membranes prepared from *S. cerevisiae* and

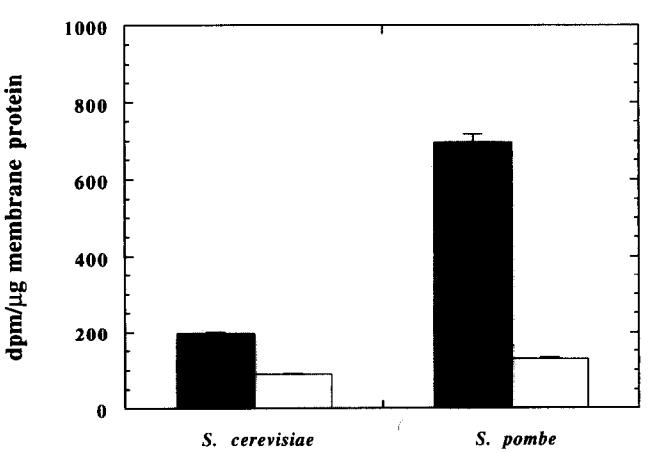


Fig. 3. Total binding of [$^3$H]spiperone to membranes prepared from *S. cerevisiae* or *S. pombe* cells expressing the human $D_{2S}$ dopamine receptor. Membranes were incubated with a final concentration of 5.1 nM [$^3$H]spiperone. Non-specific binding was monitored in the presence of 10 µM (+)butaclamol. ■, YEp51D2 for *S. cerevisiae*, pREP1D2 for *S. pombe*; □, YEp51 for *S. cerevisiae*, pREP1 for *S. pombe*.

Table 1
Pharmacological profiles for the $D_{2S}$ receptor expressed in *S. cerevisiae* and *S. pombe*

| Ligand | $K_i$ values | |
|---|---|---|
| | $D_{2S}$R in *S. cerevisae* | $D_{2S}$R in *S. pombe* |
| Apomorphine | $1.9 \times 10^3$ | $1.3 \times 10^3$ |
| (+)Butaclamol | 12.6 | 5.1 |
| Domperidone | 22.7 | 10.0 |
| Dopamine | $2.9 \times 10^5$ | $1.5 \times 10^5$ |
| Haloperidol | 63.4 | 28.2 |

$K_i$ values [nM] calculated from $IC_{50}$ obtained in the competition experiments depicted in Fig. 5. The $K_i$ values were calculated from the $IC_{50}$ values according to the equation $K_i = IC_{50}(1+C/Kd)$, where $C$ is the concentration of [$^3$H]spiperone (1.3 nM) and $K_d$ is the equilibrium dissociation constant of [$^3$H]spiperone for the heterologously expressed $D_{2S}$ receptor (1.3 nM for *S. cerevisiae* and 0.25 nM for *S. pombe*).

*S. pombe* cells transformed with YEp51D2 and pREP1D2, respectively, exhibited specific saturable binding of [$^3$H]spiperone while control cells transformed with the vectors did not show any specific binding. The $K_d$ and $B_{max}$ values were calculated from saturation isotherms (Fig. 4). The receptors expressed in yeast exhibited uniform affinity as indicated by the linear slope of the Scatchard plot, indicating a single class of binding sites. The affinities for [$^3$H]spiperone of the receptors expressed in *S. cerevisiae* ($K_d$ = 1.3 nM) and *S. pombe* ($K_d$ = 0.25 nM) were calculated from a non-linear fit.

The pharmacological profiles for the heterologously expressed $D_{2S}$ receptors in *S. cerevisiae* and *S. pompe* were determined by the replacement of [$^3$H]spiperone bound to the membranes with a series of ligands including two agonists (apomorphine and dopamine) and three antagonists ((+)butaclamol, domperidone and haloperidol). The corresponding inhibition constants (Table 1) were calculated from the resulting $IC_{50}$ values from the competition curves depicted in Fig. 5. The $K_i$ values for all chosen ligands differ by a factor 2 between the two yeasts, in general showing a higher affinitiy for the receptor expressed in *S. pombe*. Nevertheless, in both expression systems the compounds displayed the same rank order of potencies: (+)butaclamol > domperidone > haloperidol > apomorphine > dopamine, which is in agreement with results published earlier [27].

It should be noted, that the calculated $K_i$ as well as the $K_d$ values differ significantly from the values reported previously for the receptor in its natural environment or heterologously expressed in higher eucaryotic expression systems [7,8,28,29]. One possible explanation for this different binding behaviour may be minor conformational changes of the receptor protein forced by the lipid composition of the surrounding membranes. It already has been reported that the affinity of a certain receptor is very dependent on its environment. So for example, the

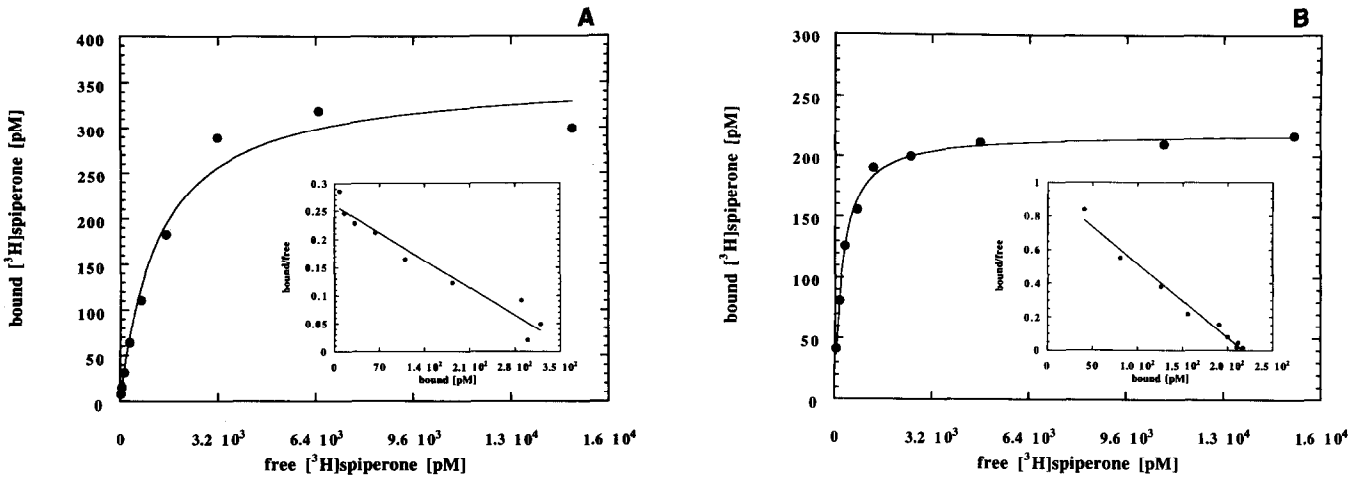


Fig. 4. Saturation isotherms of [³H]spiperone binding to membranes prepared from the yeasts *S. cerevisiae* (A) and *S. pombe* (B) transformed with plasmids YEp51D2 or pREP1D2, respectively. Membranes were incubated with [³H]spiperone as described in section 2, nonspecific binding was determined with 1 μM (+)butaclamol. *Inset*: scatchard transformation of the data. Data are representative of two independent experiments, with each point being measured in triplicate.

affinity of the serotonin $5HT_{1A}$ receptor which was heterologously expressed in *E. coli* remained 10- to 40-fold below that obtained for the receptor when expressed in mammalian systems [30]. However, affinities for the m1 muscarinic acetylcholine receptor expressed in *S. cerevisiae* were comparable to those for the same receptor in mammalian systems [3].

To our knowledge this is the first report of the heterologous expression of a G-protein coupled receptor in the fission yeast *S. pombe*. Our results indicate that the fission yeast *S. pombe* is even the more suitable organism for the heterologous expression of the human $D_{2S}$ dopamine receptor. Compared to the expression of the same receptor protein in the budding yeast *S. cerevisiae* the amount of receptor protein per mg membrane protein produced in *S. pombe* is notably higher. Additionally, the $K_d$ value for [³H]spiperone binding and the $K_i$ values for other specific ligands are lower, when the $D_{2S}$ dopamine receptor is expressed in the fission yeast. If in general the yeast *S. pombe* is the better expression system for G protein coupled receptors still has to be demonstrated. *S. pombe* has a signal transduction pathway which is similar to the pathway in *S. cerevisiae* [31]. It has been previously suggested, that the *gpa1* gene product encoding a $G_\alpha$ subunit in *S. pombe* functions as a positive mediator in transmitting the signal from the mating factor receptor to downstream effectors [32]. However, *GPA1* in *S. cerevisiae* encodes a $G_\alpha$ subunit playing a negative role in signal transduction (for review see [33]). The mode of function of the $G_\alpha$ subunit in *S. pombe* is,

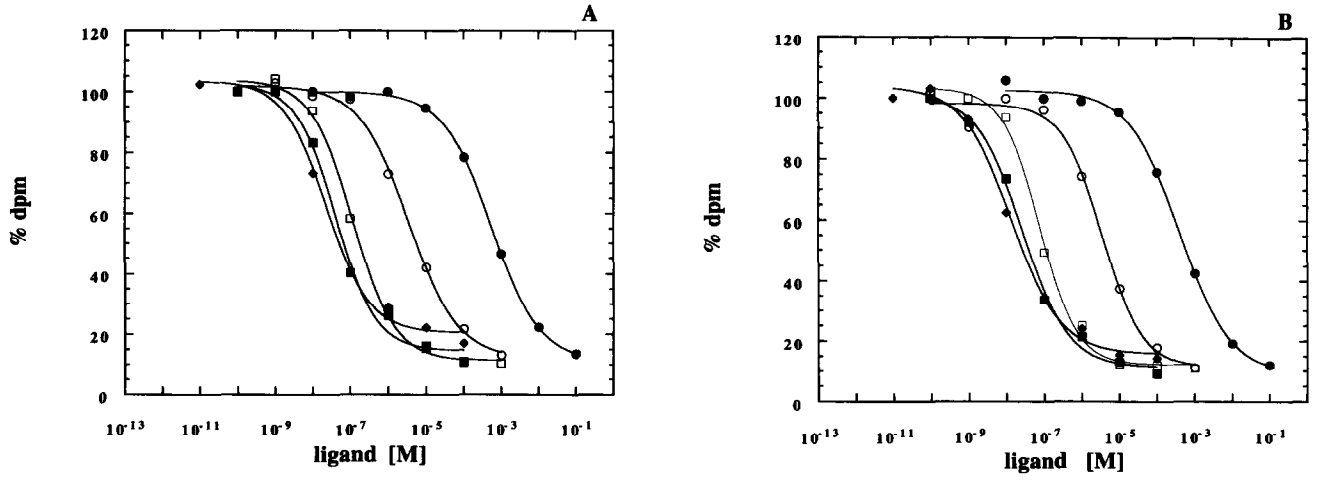


Fig. 5. Pharmacological profiles for the $D_{2S}$ dopamine receptor heterologously expressed in *S. cerevisiae* (A) and *S. pombe* (B). Competitive binding to membranes from recombinant clones of *S. cerevisiae* and *S. pombe* were performed at a final [³H]spiperone concentration of 1.3 nM. The 100% value corresponds to specific binding in the absence of any competitor and the 0% value corresponds to the nonspecific binding in the presence of 1 μM (+)butaclamol. Ligands: (○) apomorphine, (◆) (+)butaclamol, (■) domperidone, (●) dopamine, (□) haloperidol.

therefore, more like that of the mammalian homologue. Thus, it may be more suitable to establish a heterologous signal transduction pathway as an assay for drug screening in the fission yeast using a reporter protein under the control of a promoter which can be turned on by the transcription factors activated by agonist coupling.

*Acknowledgements:* The authors are grateful to Drs. M. G. Caron (Duke University, Durham) for providing the $D_{2S}$ receptor cDNA, K. Maundrell (Glaxo, Geneva) for providing the *S. pombe* strain and vector pREP1 and D. H. Wolf (University Stuttgart) for providing the *S. cerevisiae* strain. We also thank G. Maul for excellent technical assistance and Dr. P. Wood for critical reading of the manuscript. This work was supported by the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft (SFB 169) and the Fonds der chemischen Industrie.

## References

[1] Lester, H.A. (1988) Science 241, 1057–1063.
[2] King, K., Dohlman, H.G., Thorner, J., Caron, M.G. and Lefkowitz, R.J. (1990) Science 250, 121–123.
[3] Payette, P., Gossard, F., Whiteway, M. and Dennis, M. (1990) FEBS Lett. 266, 21–25
[4] Hildebrandt, V., Polakowski, F. and Büldt, G. (1991) Photochem. Photobiol. 54, 1009–1016.
[5] Civelli, O., Bunzow, J.R. and Grandy, D.K. (1993) Annu. Rev. Pharmacol. Toxicol. 32, 281–307.
[6] Dearry, A., Falardeau, P., Shores C. and Caron, M.G. (1991) Cell. Mol. Neurobiol. 11, 437–453.
[7] Dal Toso, R., Sommer, B., Ewert, M., Herb, A. Pritchett, D.B., Bach, A., Shivers, B.D. and Seeburg, P.H. (1989) EMBO J. 8, 4025–4034.
[8] Giros, B., Sokoloff, P., Martres, M.P., Riou, J.F., Emorine, L.J. and Schwartz, J.C. (1989) Nature 342, 923–926.
[9] Monsma, F.J., McVittie, L.D., Gerfen, C.R., Mahan, L.C. and Sibley, D.R. (1989) Nature 342, 926–929.
[10] Bigornia, L., Suozzo, M., Ryan, K.A., Napp, D. and Schneider, A.S. (1988) J. Neurochem. 51, 999–1006.
[11] Creese, I., Siblea, D.R., Hamblin, M.W. and Leff, S.E.A. (1983) Rev. Neurosci. 6, 43–71.
[12] Lacey, M.G., Mercuri, N.B., and North, R.A. (1987) J. Physiol. 392, 397–416.
[13] Vallar, L. and Meldolesi, J. (1989) Trends Pharmacol. Sci. 10, 74–77.
[14] Lee, T., Seeman, P., Rajput, A., Farley, I.J. and Hornykiewicz, O. (1978) Nature 273, 59–61.
[15] Seeman, P., Ulpian C., Bergeron, C., Riederer, P., Jellinger, K., Gabriel, E., Reynolds, G.P. and Tourtellotte, W.W. (1984) Science 225, 728–730.
[16] Reynolds, G.P. (1992) Trends Pharmacol. Sci 13, 116–121.
[17] Dahl, S.G., Edvardsen, O. and Sylte, I. (1991) Proc. Natl. Acad. Sci. USA 88, 8111–8115.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T.L. (1989) Molecular cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[19] Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (1993) in: Experiments with Fission Yeast, A Laboratory Course Manual, CSHL Press.
[20] Ito, H., Fukuda, J., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[21] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. (1989) Current Protocols in Molecular Biology, Wiley, New York.
[22] Broach, J.R., Li, Y., Wu, L.C. and Jayaram, M. (1983) in: Experimental Manipulation of Gene Expression, Academic Press, pp. 83–117.
[23] Maundrell, K. (1993) Gene 123, 127–130.
[24] Sherman, F. (1991) Methods Enzymol. 194, 3–21.
[25] Laemmli, U.K. (1970) Nature 227, 680–685.
[26] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci USA 76, 4350–4354.
[27] Hess, E.J. and Creese, I. (1987) in: Dopamine receptors (Creese, I. and Fraser, C.M., Eds.) Receptor Biochemistry and Methodology, Vol. 8, Liss, pp. 1–27.
[28] Grandy D.K., Marchionni, M.A., Makam, H., Stofko, R.E., Alfano, M., Frothingham, L., Fischer, J.B., Burke-Howie, K.J., Bunzow, J.R., Server, A.C. and Civelli, O. (1989) Proc. Natl. Acad. Sci. USA 86, 9762–9766.
[29] Todd, R.D., Khurana, T.S., Sajovic, P., Stone, K.R. and O'Malley, K.L. (1989) Proc. Natl. Acad. Sci USA 86, 10134–10138.
[30] Luyten, W.H.M.L. and Leysen, J. (1993) Trends Biotech. 11, 247–254.
[31] Nielsen, O. (1993) Trends Cell Biol. 3, 68–65.
[32] Obara, T., Nakafuku, M., Yamamoto, M. and Kaziro, Y. (1991) Proc. Natl. Acad. Sci. USA 88, 5877–5881.
[33] Kurjan, J. (1992) Annu. Rev. Biochem. 61, 1097–1129.