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Optimized expression of serotonin receptors in mammalian cells using inducible expression systems

Research Article

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Abbreviations: 5-HT, 5-hydroxytryptamine; IFN, interferon; IL, interleukin; PK, protein kinase.

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Summary

Efficient expression of neurotransmitter receptor proteins in a pure and active form has become an indispensable tool for modern pharmaceutical research. Different expression systems for producing receptor proteins have been used with varying success, but the expression levels are often low or not stable over a long period of time. We evaluated different inducible expression systems for the stable, high-level expression of several serotonin receptors. Using the human interleukin-6 promoter, which is inducible by a variety of biological and chemical agents, only modest expression levels were obtained. Most likely, this is due to a down-regulation of the receptors by the inducing agents used. More successful was the type I interferon-inducible Mx promoter, with which high-level and stable expression of four different serotonin receptors was obtained for several months. Finally, the tetracycline-inducible expression system was also tested and resulted in a still higher expression, with induction levels varying from 10- tot 700-fold.

I. Introduction

In the last few years, molecular biology has had an enormous impact on the pharmaceutical industries. In their search of new pharmaceutics, the molecular target at which the drug is aimed, plays a pivotal role. Often, such targets are species-specific receptors, which are not readily available in sufficient quantity, especially those of human origin. Human receptors are preferred because homologous receptors from animals do not necessarily have the same characteristics. Therefore, cloned human receptors have become an essential and indispensable instrument in modern pharmaceutical companies (Luyten and Leysen, 1993).

Serotonin (5-HT, 5-hydroxytryptamine) is a phylogenetically ancient neurotransmitter which is widely distributed in the brain and the peripheral tissues, and which has been implicated in a wide variety of behavioral and physiological processes (Boess and Martin, 1994; Hoyer *et al.*, 1994). Molecular cloning studies have shown the existence of 14 different genes, each encoding a distinct 5-HT receptor subtype (Lucas and Hen, 1995). In view of the development of highly selective and potent therapeutic agents, thousands of compounds need to be screened, not only for the effect on the receptor of interest, but also for their possible interactions with other related receptor subtypes. For this reason, efficient and long-term stable expression of these neurotransmitter receptor proteins in an active form has become an indispensable tool. As this goal is not always achieved with various receptor subtypes, most probably due to toxic effects and counter-selection on

the producing host cells, we have addressed this problem using inducible expression systems.

Over the years, many regulatable expression systems have been developed and evaluated, ranging from heat-shock- and heavy-metal-ion-inducible systems to the more recently developed tetracycline (tet)- and ecdysone-inducible systems.

In this paper, we evaluate the efficiency, the advantages and the drawbacks of three different inducible systems: (i) the human interleukin-6 (IL-6) promoter; (ii) the murine Mx-promoter; and (iii) a tet-controlled expression system. Furthermore, and in contrast to recent review papers (Clackson, 1997; Saez *et al.*, 1997; Burcin *et al.*, 1998; Gingrich and Roder, 1998; Rossi and Blau, 1998), we have studied these different expression systems in one single cell line for the production in an active form of the same or comparable receptor proteins. This provides a more realistic and reliable comparison of the different systems.

A. The human interleukin-6 promoter

IL-6 is a multifunctional cytokine that plays an active role in immunological responses, inflammation, bone metabolism, reproduction, neoplasia, and aging. It can be expressed in a variety of cell types, including epithelial cells and fibroblasts, T cells, monocytes, macrophages and some tumors (for a review, see Hirano *et al.*, 1990). We have previously isolated the corresponding cDNA as well as a genomic clone, including a 1.2-kb fragment of the 5'-flanking region, which contains all elements necessary for its induction (Haegeman *et al.*, 1986; Ray *et al.*, 1988; Dendorfer *et al.*, 1994). The IL-6 gene can be activated by various agents, including cytokines such as tumor necrosis factor (TNF) and IL-1, lipopolysaccharide, bacteria and viruses, 12-O-tetradecanoyl-phorbol 1-3-acetate (TPA), dsI:C and/or cycloheximide (CHX) (Dendorfer *et al.*, 1994; Vanhoenacker *et al.*, 1994; Haegeman and Fiers, 1995).

B. The murine Mx promoter

The Mx protein is capable of mediating resistance in mice to influenza, measles and vesicular stomatitis viruses (Staeheli *et al.*, 1986b; Meier *et al.*, 1988; Pavlovic *et al.*, 1990; Zürcher *et al.*, 1992). Mx gene expression is strictly controlled at the transcriptional level by type I interferon (IFN) (Staeheli *et al.*, 1986a; Horisberger *et al.*, 1990). The promoter of the murine Mx1 gene, which was first characterized by Hug and coworkers (1988), contains an Sp1 binding site next to the TATA box, and a copy of the highly conserved "IFN stimulation response element" (ISRE) at position -131 to -120, relative to the start site of transcription (Staeheli *et al.*, 1984; Reid *et al.*, 1989). After binding of type I IFN to its specific cell-surface receptor, the IFN-stimulated gene factor ISGF-3α is activated by tyrosine phosphorylation and associates with ISGF-3γ; this complex then translocates to the nucleus, where it binds to the ISRE sequence, resulting in activation of transcription (reviewed in David, 1995).

C. The tetracycline-inducible system

In the original tet system of Gossen and Bujard (1992), the *E. coli* tet repressor (TetR) has been fused to the activation domain of Herpes virus VP16, thus creating the transactivator protein tTA. Transcriptional activation results from binding of tTA to tet operator sequence elements (tetO), flanking a minimal RNA polymerase II promoter which drives the gene of interest. When the antibiotic tetracycline, or its analogs, binds to the TetR subunit, it abolishes DNA binding and hence activation by tTA. Correspondingly, only low background transcription was observed in the presence of tet, and a dramatic induction of reporter gene expression (up to 100,000-fold) was obtained upon withdrawal of tet (Gossen and Bujard, 1992). After chemical mutagenesis of the TetR, a mutant tTA has been created that displays the reverse properties of the original transactivator, i.e. rtTA, switching on the expression of reporter proteins upon addition of the antibiotic (Gossen *et al.*, 1995). Hereby, three orders of magnitude of induction were obtained with a variety of tet analogs, of which doxycyline (dox) was found to be most efficient.

II. Results

A. Use of the human IL-6 promoter

In previous studies, we have already shown that heterologous expression of SV40 T antigen under control of the IL-6 promoter was equally well induced as the endogenous IL-6 protein in the human cell lines MG63 and HeLa H21 (Vanhoenacker *et al.*, 1994).

Here, we have used the same human (h)IL-6 promoter fragment for the stable, tightly-regulated and high-level expression of serotonin receptors in the mouse fibrosarcoma cell line L929. To that end, L929 cells were transfected with the expression plasmids pIL6-5HT1A or pIL6-5HT2A, in which the cDNAs for the human 5-HT_{1A} and 5-HT_{2A} receptors, respectively, were placed under the control of the hIL-6 promoter. From each transfection experiment, 24 individual G418-resistant colonies were selected, and tested for receptor expression, using radioligand binding assays, before and after induction with the combination of 110 IU/ml IL-1, 2 mM N^6,2'-O-dibutyryladenosine 3':5' cyclic monophosphate (dbcAMP) and 10 µM Ca^{2+}-ionophore (A23187). First, no measurable receptor expression could be demonstrated. This was not due to a failure of promoter stimulation, as the endogenous IL-6 gene was shown to be highly induced in the same

experiment. Furthermore, Southern-blot experiments confirmed the stable incorporation of the respective cDNAs in the genome, and for several clones the presence of receptor-specific mRNA could be demonstrated by RT-PCR (data not shown). However, as it became evident from the literature, certain neurotransmitter receptors, like the human thyroid-stimulating-hormone receptor (hTSHR) and the human 5-HT_{1A} receptor, could be desensitized by cAMP-dependent kinases (Harrington *et al.,* 1994; Tezelman *et al.,* 1994); for this reason we have attempted to circumvent this possible desensitization. To this end, various clones were re-induced with the combination of IL-1/staurosporine/poly(rI).poly(rC) (110 IU/ml; 6 µM; 50 µg/ml). Measurable receptor expression could be demonstrated only for the h5-HT_{2A} receptor (**Table 1)**. The expression levels thus obtained were rather low; this could, most likely, be a consequence of the desensitizing kinase activities, mediated by the inducing agents used. Therefore, this possible negative regulatory effect was further investigated using Mx promoter-controlled expression of these receptors (see section C).

Table 1. IL-6 promoter-controlled expression of the human 5-HT_{2A} receptor

clone number	[^{125}I] 5-I-R91150 binding (fmol/mg protein)	
	-	+
1	2	63
2	17	92
3	15	85
4	15	166
5	19	115
6	35	115
mock	23	29
rat frontal cortex	512	

L929 cells were cultured and induced as described in Materials and Methods; membrane preparation and radioligand binding studies were also carried out as indicated before. '-' stands for noninduced; '+' for induction with 110 IU/ml IL-1; 6 µM staurosporine; 50 µg/ml poly(rI).poly(rC) for a period of 24 hours at 37°C. Rat frontal cortex was used as a positive control.

B. Mx promoter-controlled expression of serotonin receptors

Using a 1,600-bp fragment of the Mx1 promoter, heterologous expression of human growth hormone has been obtained in VERO cells upon induction with human type I IFN (Lleonart *et al.,* 1990). We have extended the usefulness and inducibility of this promoter for heterologous expression in the murine cell line L929 using the bacterial chloramphenicol acetyltransferase (CAT) as a reporter system; later on we have also achieved strictly IFN-controlled expression of five different human serotonin receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_{1B}, 5-ht_{1E} and 5-ht_{1F}) in this cell line (Vanhoenacker *et al.,* 1997). **Figure 1** shows the results of different selected clones, expressing the 5-HT_{1B} or the 5-HT_{2A} receptor. The expression levels for the 5-HT_{1B} receptor varied from 300 fmol/mg protein up to 3,000 fmol/mg protein, with induction ratios of induced versus noninduced expression ranging from 6- to 40-fold. For the 5-HT_{2A} receptor, the expression levels were similar, although here the induction ratios were slightly higher (7- to 49-fold). An overview of the maximum expression levels obtained for the different serotonin receptors, tested so far, is given in **Table 2**. B_{max} values varied from 700 fmol binding/mg protein for the 5-ht_{1F} receptor, 3,100 fmol/mg protein for the 5-HT_{2A} receptor, 3,300 fmol/mg protein for the 5HT_{1B} receptor, 9,800 fmol/mg protein for the 5-ht_{1E} receptor, and up to 10,400 fmol/mg protein for the 5-HT_{1A} receptor.

Table 2. Overview of Mx promoter-controlled expression of serotonin receptors in L929 cells

Receptor	B_{max} (fmol/mg protein) (IFN-induced levels)
5-HT_{1A}	10,400
5-HT_{1B}	3,300
5-ht_{1E}	9,800
5-ht_{1F}	700
5-HT_{2A}	3,100

Receptor-expressing L929 cells were cultured and induced for 24 hours with 1,000 U/ml mIFNb. Membrane preparation and radioligand binding studies were carried out as indicated before. B_{max} values were derived as described in Leysen *et al.,* 1996.

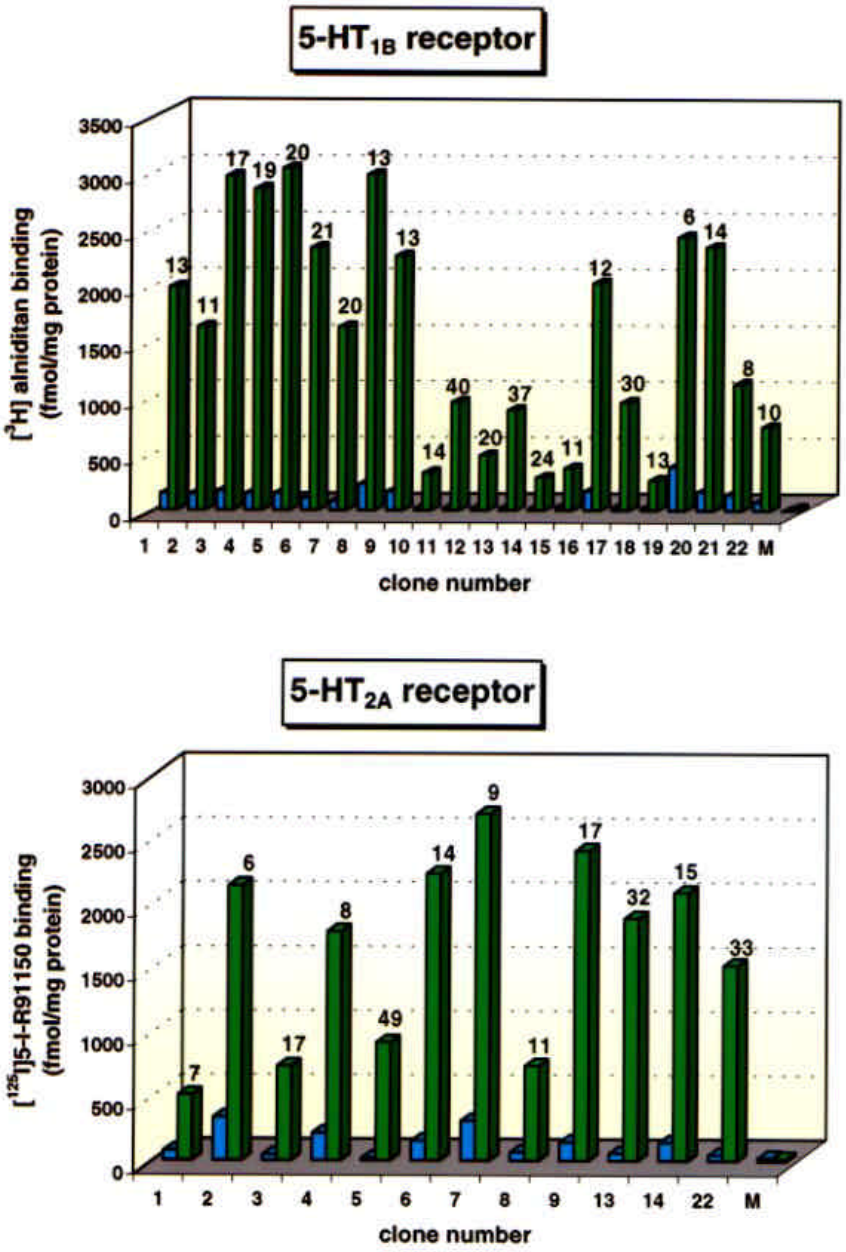

Figure 1. Mx promoter-controlled expression of the 5-HT_{1B} and 5-HT_{2A} receptor subtypes. Parallel subconfluent monolayers of different G418-resistant transfectants, grown in 60 cm2 plates, were either left noninduced (blue bars) or were induced with 1,000 U/ml mIFNβ (green bars) for 24 hr at 37°C. Receptor binding was measured as described before. The induction rate is shown by a number above the bars. M stands for mock-transfected cells.

In our hands the expression levels remained stable for at least one year in continuous culture, and therefore we feel that these serotonin receptor-expressing cells are a reliable source of subtype-specific receptor material for characterizing the pharmacological profile of therapeutic agents as well as for functional studies. Taking into account that the expression levels obtained with most of these serotonin receptors are equal to or far better than values published in the literature (Hamblin *et al*., 1992; Zgombick *et al.*, 1992; Van Huizen *et al.*, 1993; Grotewiel and Sanders-Bush, 1994; Harrington *et al*., 1994; Langlois *et al*., 1996), we are persuaded that this system meets the requirements of the present-day pharmaceutical industry.

C. Do dbcAMP and/or Ca-ionophore negatively affect serotonin receptor expression?

As the Mx promoter has turned out to be satisfactory for stable expression of serotonin receptors in L929 cells, we further investigated whether the conditions, used previously for the induction of the hIL-6 promoter, were deleterious for the expression of those receptors. Therefore, four different cell clones, expressing the 5-HT_{2A} receptor under control of the Mx promoter, were induced with either IFN alone, or with IFN in combination with dbcAMP, or with dbcAMP and the Ca-ionophore together. The results of the radioligand binding studies are shown in **Table 3**. For all four clones, dbcAMP alone has only a small negative effect, while the addition of dbcAMP + Ca-ionophore leads to a huge reduction in radioligand binding as compared to the induction with IFN alone. The most plausible explanation is that this proceeds via a kinase-dependent down-regulation. PKA-dependent down-regulation has already been demonstrated for the β_2-adrenergic receptor (Liggett *et al.,* 1993) and a PKC-mediated down-regulation of the β_1-adrenergic receptor and the α_{2A}-adrenergic receptor has been described recently (Li *et al.,* 1998; Liang *et al.,* 1998). These results may thus explain why only a modest detection of receptors was obtained using the inducible hIL-6 promoter, and studies to further investigate the presumed down-modulation are currently being performed.

Table 3. Influence of dbcAMP and Ca-ionophore on IFN-induced expression of the human 5-HT_{2A} receptor in L929 cells

	[^{125}I] 5-I-R91150 binding (fmol/mg protein)		
mIFNb (1,000 U/ml)	+	+	+
dbcAMP (2mM)	-	+	+
Ca-ionophore (10 µM)	-	-	+
clone 5	982	489	110
clone 13	2,270	1,875	353
clone 14	1,166	885	301
clone 22	1,345	976	262

Different clones of Mx promoter-controlled 5-HT_{2A} receptor-expressing cells were induced for 24 hours with the indicated reagents. Cells were then stored at -70°C and membrane preparation and radioligand binding studies were performed as described before.

D. Tetracycline-inducible expression of neurotransmitter receptors

As IFN is species-specific and not always readily available in large quantities, the Mx promoter-controlled expression system cannot easily be extrapolated to other cell types. Therefore, we have also evaluated the tet-regulated system for the inducible expression of the serotonin 5-HT_{1B} receptor and the 5-ht_{1F} receptor. To this end, a number of reasons led us to choose the recently developed "reverse" system in which the VP16 activation domain is fused to a mutant tetracycline repressor protein (rtTA), for direct induction by the antibiotic.

First, we transfected the DNA coding region of rtTA into the cell line L929; selected cell clones were tested for rtTA expression by transient transfection with the vector pUHC13-3, in which the firefly luciferase gene is under control of a minimal CMV promoter, flanked by 7 tet operator sequences. Using this approach several well-regulated $rtTA^+$ cell clones were retained; some of them were further stably transfected with the vectors pTet-5HT_{1B} or pTet-5ht_{1F}, in which the cDNA coding for the human 5-HT_{1B} or the 5-ht_{1F} receptor, respectively, is positioned under the control of the minimal CMV promoter and the tet operator sequences. After selecting the appropriate colonies, cells were induced for 24 hours with 1 µg/ml dox and assayed for receptor expression by radioligand binding. The results are shown in **Figure 2**. For the 5-HT_{1B} receptor, the expression levels varied between 4,000 and 20,000 fmol/mg protein with induction ratios of 50- to 200-fold; for the 5-ht_{1F} receptor, expression levels of 9,500 fmol/mg protein were obtained with an induction ratio of 700-fold. These expression levels are higher than the values reported in the literature so far. Taking into account that the inducing agent is inexpensive and readily available, and considering the fact that this system may be less cell type-dependent, it can become a valuable alternative to the high-level production of neurotransmitter receptor proteins using Mx promoter-driven expression.

III. Discussion

Many different subtypes of (serotonin) receptors have been identified and cloned in the last few years; for the characterization of the pharmacological profile of candidate drug compounds, sufficient amounts of these receptors need to be available in a biologically active form. As tissue material is not always readily available and as it usually contains a variety of different receptor subtypes, heterologous expression of cloned receptor subtypes for screening programs has become a real necessity for a modern pharmaceutical company.

Different constitutive expression systems have been used to produce receptor proteins, but with varying success, as in many cases expression levels are low and/or

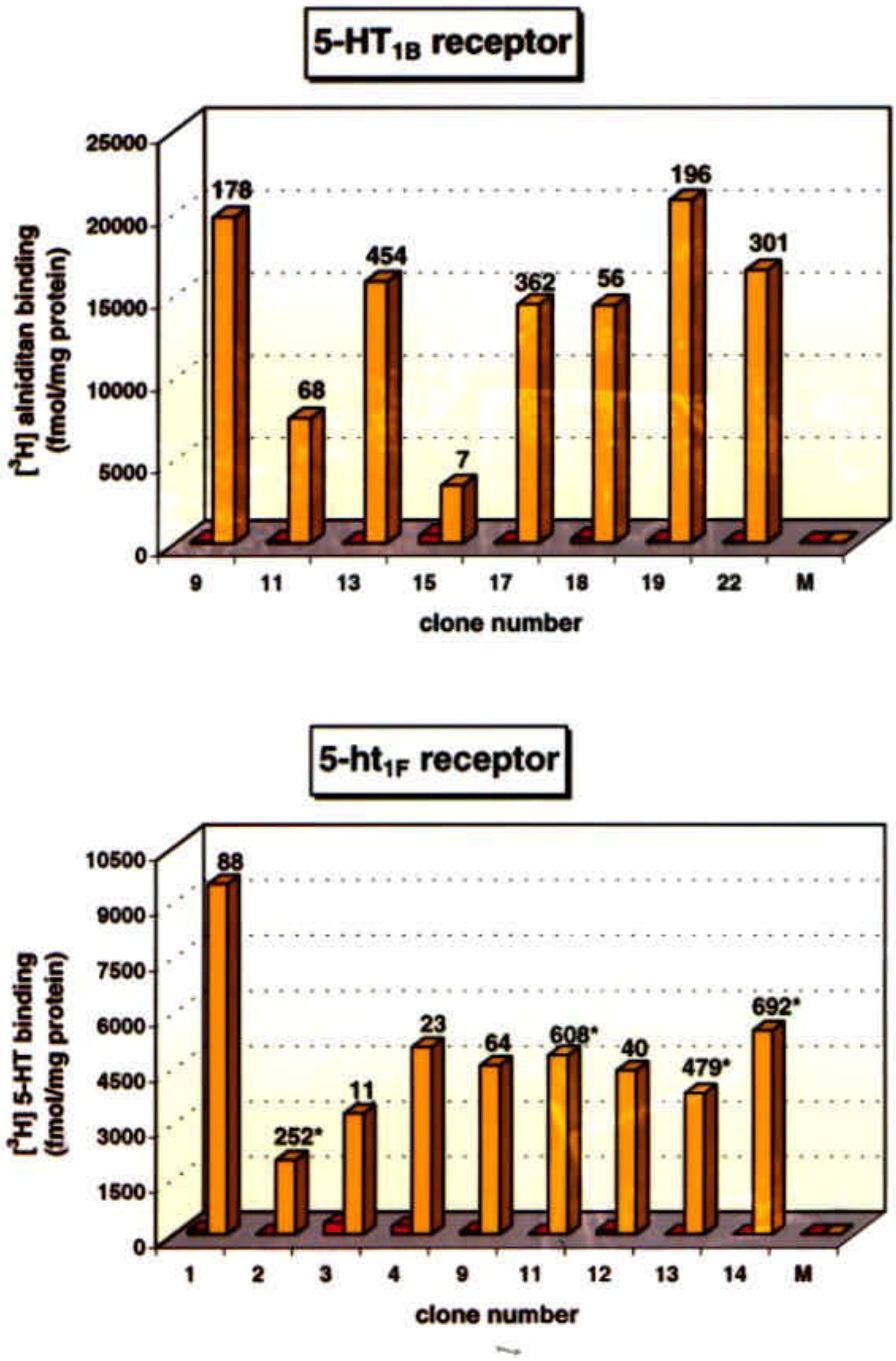

Figure 2. Dox-controlled expression of the 5-HT_{1B} and 5-ht_{1F} receptor subtypes in a L929 $rtTA^{+}$ cell line. Parallel subconfluent monolayers of different hygromycin-resistant transfectants, grown in 60 cm^2 plates, were either left noninduced (white bars) or were induced with 1µg/ml dox (black bars) for 24 hr at 37°C. Receptor binding was measured as described. The induction rate is shown by a number above the bars; in case the expression level of the noninduced cells was below the detection limit, the induction ratios (marked *) were obtained by taking the average expression level of the mock-transfected cells (M) as a background value.

not stable over a long period of time (Zaworski *et al.*, 1995). Therefore, we have addressed this problem by using 'inducible' expression systems as a possible valuable alternative and have evaluated the efficacy, advantages and disadvantages of three different inducible systems (i.e. the hIL-6 promoter, the murine Mx-promoter and the tet-inducible system) for the expression of serotonin receptors in the murine cell line L929.

First, we obtained no or only low level expression, as determined by radioligand binding assays, with the hIL-6 promoter, which was already successfully used for heterologous protein production in earlier studies (Vanhoenacker *et al.,* 1994). During subsequent experiments using the Mx promoter, however, we demonstrated that some of the inducing agents used may be deleterious for serotonin receptor expression, probably by activating kinase pathways which may result in receptor phosphorylation and down-regulation. Currently, immunocytochemical studies are under investigation to further explore this phenomenon.

On the other hand, the use of the murine Mx promoter, which is inducible by type I IFN, proved to be very successful. With this promoter system, we were able to generate five different, biologically active serotonin receptors; their expression levels, after induction, ranged from 700 fmol/mg protein for the 5-ht_{1F} receptor to up to 10,400 fmol/mg protein for the 5-HT_{1A} receptor. In addition, the same promoter system was also found to be successful for the expression of two dopamine receptor variants, i.e. the human dopamine D_3 receptor and dopamine D_4 receptor (data not shown). As the expression levels measured remained stable for a long period of time (i.e. more than 1 year), Mx promoter-driven expression may be considered as a valuable and reliable system for the generation of proteins of pharmaceutical interest. Furthermore, it should be noted that this system has also been successfully used to express toxic compounds and carry out analytical studies (Vandevoorde *et al.,* 1997; Boone *et al.,* 1999). This system has, however, two major drawbacks. First, the inducing agent IFN is species-specific and thus not always readily available; extrapolation to other cell types is not obvious, although CHO and NIH3T3 cells were also found to be responsive to mouse (m)IFNβ, regarding Mx promoter-driven expression of reporter genes (our unpublished results). Second, the inducing agent IFN is a cytokine and thus available in only limited amounts, which makes the system rather expensive and less attractive, if large scale industrial production is envisaged.

Another valuable alternative is the reverse tet-inducible expression system, by which very high expression levels of the serotonin 5-HT_{1B} and 5-ht_{1F} receptors were obtained, reaching to 20,000 fmol/mg protein and 9,500 fmol/mg protein, respectively. Background expression was rather low for most of the clones tested, which resulted in induction ratios of up to 700-fold. Due to the simplicity and the low cost of the inducing agent used, no major problems are expected for industrial exploitation or for its application to other cell types. However, it should be taken into consideration that this expression system involves two-components and, thus, requires the establishment of stable rtTA-expressing clones to allow a profound induction upon addition of dox. For L929 cells, for example, we have tested 48 G-418 resistant clones several times by transient transfection with pUHC13-3 in order to select at least several strictly regulatable and inducible cell clones. Although this extensive testing is labor-intensive and time-consuming, we feel that it is essential in order to obtain well-regulated expression of the gene of interest at a later stage. It is, however, not always possible: as with HEK293 cells, a more substantial leak in expression was obtained (data not shown).

In summary, inducible expression has proven to be a good option to obtain high-level, stable expression of neurotransmitter receptors, which could be readily used for comparative binding studies and adequate drug screening.

IV. Materials and Methods

A. Cell lines

MG63 (human osteogenic sarcoma), HeLa H21 (human cervix carcinoma) and L929 (mouse fibrosarcoma) cells were cultivated in a controlled environment (37°C, 5% CO_2, 98% humidity) in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 10% FCS or 5% FCS/5% NCS, respectively.

B. Recombinant DNA constructs

For the construction of pIL6-5HT_{1A}, the complete cDNA of the human 5-HT_{1A} receptor was isolated as a *Tfi*I (filled in with Klenow DNA polymerase)/*Bam*HI fragment from pSP64 5HT_{1A} (Vanhoenacker *et al.,* 1997) and cloned into a *Xho*I (filled in with Klenow DNA polymerase)/*Xho*II opened pBLHIL6CAT vector (Vanden Berghe *et al.,* 1993). Hereby the coding region for the 5-HT_{1A} receptor was placed directly under control of the hIL-6 promoter. The construction of pIL6-5HT_{2A} was similar. A *Stu*I/*Bam*HI fragment from pUC18 5HT_{2A} (Vanhoenacker *et al.,* 1997), containing the cDNA coding for the human 5-HT_{2A} receptor, was ligated to the same pBLHIL6CAT fragment as used for the construction of pIL6-5HT_{1A}.

For the construction of pTet-5HT_{1B} and pTet-5ht_{1F}, a *Nhe*I/*Bam*HI fragment containing the complete cDNA coding for the human 5-HT_{1B} and 5-ht_{1F} receptor, respectively, was cloned into a *Xho*I/*Bam*HI opened pUHD10-3 vector (Gossen and Bujard, 1992).

pPHT was constructed by inserting the hygromycin gene as an *Xho*I/*Xba*I fragment between the *Xho*I and *Xba*I sites of pPNT (Tybulewicz *et al.,* 1991).

C. Transfection procedure

Stable transfections were essentially performed as described previously (Vanhoenacker *et al.,* 1994). The pSV2neo plasmid (Southern and Berg, 1982) and the pPHT plasmid (see above) provided a resistance gene, and

transfectants were selected by G418 (400 µg/ml) or hygromycin-B (250 U/ml), respectively, for a period of three weeks. The selective medium was renewed every 7 days.

D. Induction of promoters

For induction, L929 cells were plated at a density of 4 x 10^4 cells/ cm^2. The inducing agents and conditions used for induction of the hIL-6 promoter and the murine Mx promoter were described previously (Vanhoenacker *et al.,* 1994; Vanhoenacker *et al.,* 1997). In case of the tet-system, cells were induced for 24 hours with 1µg/ml dox. After the induction period, cells were stored at -70°C for membrane preparation and assaying by radioligand receptor binding (Leysen *et al.*, 1996).

E. Radioligand binding studies

Binding experiments with membrane preparations of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{1B} and 5-ht_{1E} receptor-expressing cells were performed essentially as described previously (Vanhoenacker *et al.,* 1997). The 5-ht_{1F} expression levels were determined by radioligand binding with [^{3}H]5-HT; non-specific binding was measured in the presence of a 200-fold excess of 5-HT.

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