

Molecular cloning of a bovine MSH receptor which is highly expressed in the testis

Mirko Vanetti^a, Christiane Schönrock^b, Wolfgang Meyerhof^b, Volker Höllt^{c,*}

^aPhysiologisches Institut, Ludwig Maximilian Universität München, Munich, Germany

^bInstitut für Zellbiochemie und klinische Neurobiologie, Universität Hamburg, Hamburg, Germany

^cInstitut für Pharmakologie und Toxikologie, Otto von Guericke Universität, Leipzigerstraße 44, Magdeburg, Germany

Received 27 May 1994

Abstract

A novel G protein-coupled receptor (BDF3) was isolated from bovine genomic DNA by a combined approach of polymerase chain reaction (PCR) and hybridization techniques. The predicted amino acid sequence is 317 amino acids in length and displays 80% homology to the human α -MSH receptor MC1. Stably transfected into CHO-K1 cells, BDF3 mediates an increase of intracellular cAMP-levels following incubation with NLE- α -MSH, a potent α -MSH analog. The stimulation with ACTH_{1–10} is only moderate and γ -MSH is ineffective. Northern blot analysis of bovine tissues revealed that the BDF3 gene is highly expressed in the testis as a single 2.3 kb mRNA species, suggesting an involvement of the BDF3 receptor in spermatogenesis.

Key words: G-protein coupled receptor; α -MSH; POMC; Testis; Adenylate cyclase; PCR; spermatogenesis

1. Introduction

Proopiomelanocortin (POMC) is a precursor for β -endorphin, ACTH and MSH occurring in the pituitary, in the brain and in the periphery [1]. Tissue-specific proteolytic cleavage of POMC leads to a cell specific mixture of bioactive peptides called melanocortins displaying a wide array of biological effects. Beside the known actions on glucocorticoid production in the adrenals and melanocyte growth and pigmentation, melanocortins have been shown to affect learning, attention and memory [2], analgesia [3], thermoregulation [4] and release of PRL and LH from the pituitary [5]. Little is known about the role of melanocortins in the periphery. However, melanocortins which have been found in testis [6,7] are supposed to act in a paracrine/autocrine way as growth factors and to play a role in maintaining testis cell function and differentiation [8]. MSH and ACTH act through G protein-coupled receptors resulting in an increase of adenylate cyclase activity [9], increase of intracellular calcium and activation of protein kinase C [10]. Four subtypes (MC1–MC4) belonging to the melanocortin receptor subfamily have been cloned from human and mouse tissues [11–14]. However, one or two more members of this melanocortin subfamily are predicted from Southern blot analysis [11]. Here we report the cloning and stable expression in CHO-K1 cells of the bovine melanocortin receptor BDF3 which is expressed in large amounts in bovine testis.

2. Materials and methods

2.1. Polymerase chain reaction, cloning and sequencing

Fragments of putative G protein-coupled receptors between transmembrane domain (TM) III and VI were cloned as follows: 100 ng of bovine genomic DNA (Stratagene, Heidelberg, Germany) was amplified in a total volume of 50 μ l containing 50 mM KCl, 10 mM Tris pH 8.3, 2 mM MgCl₂, 200 mM dNTPs, 0.01% gelatine, 25 ng each degenerate primer against TM III and VI [15] and 1 unit Taq I Polymerase (Perkin-Elmer, Überlingen, Germany) by 60 cycles of PCR. Cycle conditions were 1 min 30 s 95°C, 2 min 55°C and 3 min 72°C with additional 6 s of extension time each cycle of PCR. PCR products were purified with Primerase columns (Stratagene), blunted with Klenow polymerase and cloned into pBluescriptSK[–] (Stratagene). Sequencing of the cloned DNA was performed by the chain termination method [16] with a Sequenase 2.0 kit (USB, Bad Homburg, Germany).

2.2. Library screening and subcloning

Clone 517–3 was ³²P-labelled by the random primer technique [17] and used as a probe to screen a bovine genomic library constructed in phage λ DASH II (Stratagene, Heidelberg, Germany). Hybridization conditions were 16 h at 37°C in 20% formamide/5 $\times$ SSC/5 $\times$ Denhart's solution/1% SDS and 100 mg/ml salmon sperm DNA. Filters were washed 30 min at 42°C in 2 $\times$ SSC/1% SDS and 15 min at 60°C in 0.5 $\times$ SSC/1% SDS. Out of 5 $\times$ 10⁵ phage clones screened, two identical clones containing ~20 kb inserts were isolated and verified by Southern blotting [18]. The DNA region of interest was subcloned into pBluescriptSK[–] by a one-step procedure: 500 ng DNA of the genomic clone were cleaved with HindIII together with 250 ng DNA of the recipient vector pBluescriptSK[–]. The enzyme was heat-inactivated at 70°C for 10 min, the DNA precipitated, resuspended and ligated *o/n* at 22°C. Bacterial clones containing 1.9 kb HindIII fragments (pBDF3) were detected by hybridization using clone 517–3 as a probe.

2.3. Stable expression of BDF3 receptors in CHO-K1 cells

A 1180 bp EcoRI/StuI fragment of pBDF3 was subcloned into expression vector pcDNA I (Invitrogen, San Diego, USA) at EcoRI/EcoRV sites. 2 μ g of the derived expression plasmid pBDF3–2E was cotransfected with 0.1 μ g of the plasmid pcDNA I neo (Invitrogen) into CHO-K1 cells (ATCC, MD, USA) using Lipofectin (BRL, Eggenstein, Germany). Stable transfectants were selected in NUT-F12 medium containing 400 μ g/ml G418 (BRL) and 10% foetal calf serum (FCS). Clones expressing the highest levels of specific mRNA were

*Corresponding author. Fax: (49) (391) 67 34 40.

selected and tested for cAMP-accumulation following incubation of the cells with melanotropic peptides.

2.4. cAMP assay

Accumulation of cAMP was measured as follows: 1.5×10^5 stably transfected CHO-K1 cells were seeded in 22 mm twelve-well dishes with NUT-F12 medium containing 10% foetal calf serum. The cells were then washed twice with serum-free NUT-F12 medium (SFM) and pre-treated with SFM containing 200 μ M isobutyl-methyl-xanthine for 30 min at 37°C. Thereafter, the melanotropic peptides [Nle⁴,D-Phe⁷]- α -MSH, γ -MSH and ACTH_{1–10} (Saxon, Hannover, Germany) were added in a concentration of 10^{-6} M and the incubation continued for 30 min at 37°C. The reaction was terminated by removing the medium, scraping and sonicating the cells in 1 ml ice-cold HCl/EtOH (1 vol 1 N HCl/100 vol EtOH). After evaporation of the solution, the residue was dissolved in TE buffer and the cAMP content measured by a radioassay kit (Amersham, Braunschweig, Germany).

2.5. RNA preparation and Northern blot analysis

Total RNA was isolated by the acid guanidinium isothiocyanate method [19]. Poly(A) RNA was isolated from total RNA using the QuickPrep mRNA purification kit from Pharmacia (Freiburg, Germany). Aliquots of 10 μ g poly(A) RNA were denatured by glyoxal, separated by electrophoresis, transferred to Nytran membranes

(Schleicher and Schuell, Dassel, Germany) and hybridized under high-stringency conditions according to standard methods [18] with a [³²P]UTP-labeled cRNA probe transcribed from clone 517–3. Washing conditions were 30 min at 50°C in 1 $\times$ SSPE/0.1% SDS. Nylon membranes were exposed to X-ray films for 3–72 h.

3. Results and discussion

Bovine genomic DNA was subjected to PCR amplification using degenerate primers to transmembrane domain III and VI of G protein-coupled receptors [15]. PCR products were cloned and sequenced. Out of 40 clones analyzed, clone 517–1 of 450 bp was found to be the bovine variant of the orphan receptor dRDC4 previously cloned from dog [15], and clone 517–40 of 500 bp was identical to the bovine β_2 adrenergic receptor [21]. In addition, 9 clones were found to encode fragments of novel putative G protein-coupled receptors.

1	GCTTGGGGCCATGCTTGGGCCGACATTGTCACGCCAGGGAGGGAGGTGTGAGGCCCTCCAGGGAGCCATGAGTTGAGCAGGACCCCT	91
	M p a l g s Q r r L l G S L	14
	GAGAGCAAGCACCCCTTCCTGCTCCCTGCGGGACG ATG CCT GCA CTT GGC TCC CAG AGG CGG CTG CTG GGT TCC CTT	168
N#	c t p p a t l p f t L A p N# r t g p q C L e	37
	AAC TGC ACG CCC CCA GCC ACC CTC CCC TTC ACC CTG GCC CCC AAC CGG ACG GGG CCC CAG TGC CTG GAG	237
	<u>v s s l D G L F L S*</u> L G L V S L V E N V L V V	60
	GTG TCA TCC CTG GAC GGG CTC TTT CTC AGC CTG GGG CTG GTG AGT CTC GTG GAG AAC GTG CTG GTA GTG	306
	<u>a a I</u> a K N R N L H S P M y Y <u>F I C C L A V S</u>	83
	GCT GCC ATT GCC AAG AAC CGC AAC CTG CAC TCC CCC ATG TAC TAC TTT ATC TGC TGC CTG GCT GTG TCT	375
	<u>D L l V S v s n V L E T a v m</u> p L L E a G V L	106
	GAC TTG CTG GTG AGC GTC AGC AAC GTG CTG GAG ACG GCA GTC ATG CCG CTG CTG GAG GCC GGT GTC CTG	444
	a t q a A V v Q Q L D N <u>v I D V l i C G S M v</u>	129
	GCC ACC CAG GCG GCC GTG GTG CAG CAG CTG GAC AAT GTC ATC GAC GTG CTC ATC TGC GGA TCC ATG GTG	513
	<u>S S L C F L G a I A v</u> D R Y I S* I F Y A L R Y	152
	TCC AGC CTC TGC TTC CTG GGT GCC ATT GCT GTG GAC CGC TAC ATC TCC ATC TTC TAC GCC CTG CGG TAC	582
	H S v V T* l P <u>R A W R I i a a I W v a S i l t</u>	175
	CAC AGT GTT GTG ACA CTG CCC CGA GCG TGG AGG ATC ATT GCG GCC ATC TGG GTG GCC AGC ATC CTC ACC	651
	<u>S l L F I t Y Y</u> n H k v i L L C L V g <u>l F i A</u>	198
	AGC CTG CTC TTC ATC ACC TAC TAC AAC CAC AAG GTC ATC CTG CTG TGC CTC GTT GGC CTC TTC ATA GCT	720
	<u>M L a L M A v L Y v H M l a</u> R A C Q H a r G I	221
	ATG CTG GCC CTG ATG GCC GTC CTC TAC GTC CAC ATG CTG GCC CGG GCC TGC CAG CAT GCC CGG GGC ATT	789
	A r L q K R q R p i h Q G F G L K G A a T <u>L T</u>	244
	GCC CGG CTC CAG AAG AGG CAG CGC CCC ATT CAT CAG GGC TTT GGC CTC AAG GGC GCT GCC ACC CTC ACC	858
	<u>I L L G v F F L C W G P F F L H L s L I V L C</u>	267
	ATC CTG CTG GGC GTC TTC TTC CTC TGC TGG GGC CCC TTC TTC CTG CAC CTC TCG CTC ATC GTC CTC TGC	927
	P Q H P T C G C I F <u>K N F N L F L a L i i c n</u>	290
	CCC CAG CAC CCC ACC TGT GGC TGC ATC TTC AAG AAC TTC AAC CTC TTC CTG GCC CTC ATC ATT TGC AAC	996
	<u>a i v D P L I Y A F</u> r S* Q E L R k T* L Q E V L	313
	GCC ATT GTG GAC CCC CTC ATC TAT GCC TTC CGC AGC CAG GAG CTC CGG AAG ACG CTC CAA GAG GTG CTG	1065
	q C S* W OPA	317
	CAG TGC TCC TGG TGA GGGTGGCAGTGCCGTCGTGTGCCCCAGGCCGTGAGGCCAGGGCAGTCCCTTGACAAAGAGGATCGGCTAG	1151
	ACCATCCCTGAAGGTGAGGGTGACAGGCCT	1182

Fig. 1. Nucleotide and predicted amino acid sequence of the *EcoRI/StuI* fragment encoding the bovine receptor BDF3. The presumptive transmembrane domains (I–VII) are underlined and are assigned on the basis of a hydrophobicity plot [27]. Amino acid residues of BDF3 that differ from human and murine MSH (MCI) receptors [11] are indicated with small letters. Potential N-linked glycosylation sites at the N-terminal region (N#), phosphorylation sites at the C-terminal region and cytoplasmatic loops (S* and T*) and a potential palmitoylation site at the C-terminus (C) are indicated.

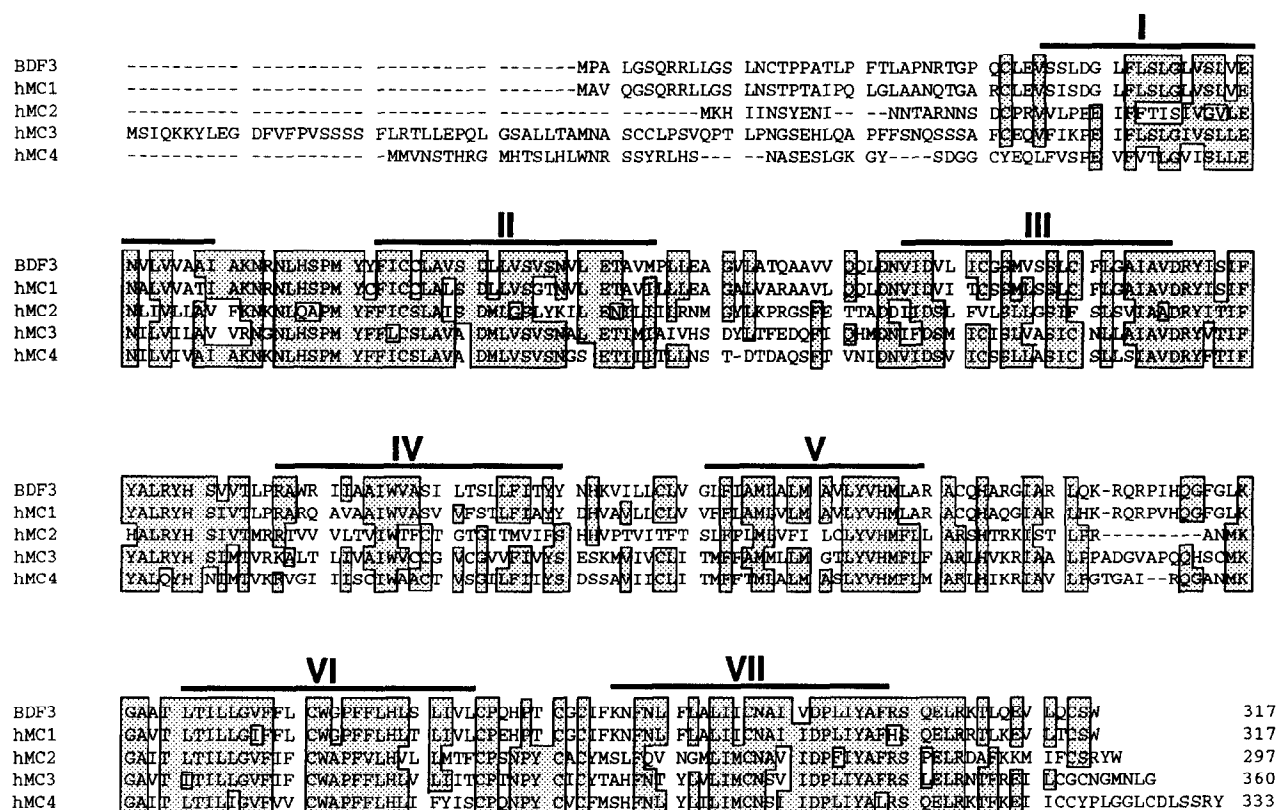


Fig. 2. Alignment of amino acid sequences of the bovine receptor BDF3 and other members of the melanocortin receptor subfamily cloned from man [14]. Identical amino acid residues are boxed. The positions of transmembrane regions (I–VII) are indicated by bold lines over the sequences.

One of these fragments, clone 517–3, was used to isolate from a bovine genomic library a 1.9 kb *Hind*III fragment named BDF3 with an open reading frame of 951 bp encoding a protein of 317 amino acids (Fig. 1). Hydrophobicity analysis revealed seven highly hydrophobic regions characteristic of G protein-coupled receptors. The predicted protein contains two potential N-linked glycosylation sites (Asn¹⁵ and Asn²⁹) at the N-terminus, seven potential phosphorylation sites [20] at the intracellular loops and C-terminus (Ser⁷¹, Ser¹⁴⁵, Ser¹⁵⁴, Thr¹⁵⁷, Ser³⁰², Thr³⁰⁸ and Ser³¹⁶) and a conserved Cys³¹⁵ at the C-terminus which serves as a potential palmitoylation site and probably anchors the C-terminus at the cell membrane [22].

The bovine receptor BDF3 has a very short C-terminus and lacks some amino acid residues which are conserved among most members of the G protein-coupled

receptors [23]; thus, the cysteine residue at the first extracellular loop is missing, thought to form a disulfide bond with a cysteine residue at the second extracellular loop; moreover, conserved proline residues at transmembrane domain (TM) IV and V are missing and the amino acid motif NP--Y at TM VII which is strongly conserved in all cloned G protein-coupled receptors is replaced by the motif DP--Y which occurs also in the human thrombin receptor and the RH3 and RH4 opsins from fruit flies [24].

A homology search on an EMBL protein database reveals the highest similarity of BDF3 to MC1, the α -MSH receptor recently isolated from human melanoma cells [11,12], and to the murine MSH-R [11] indicating that the bovine receptor BDF3 is closely related to this subtype of the melanocortin subfamily. The homology between the bovine BDF3 receptor and other

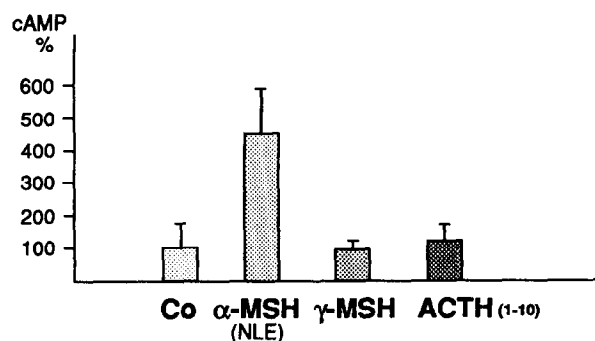


Fig. 3. cAMP accumulation in CHO-K1 cells expressing the bovine receptor BDF3 following stimulation with melanotropic agonists. Cells stably expressing the bovine receptor BDF3 were treated for 30 min with serum-free medium containing 200 μ M isobutyl-methyl-xanthine to inactivate cellular phosphodiesterase activities and cAMP accumulation was measured in the absence (CON) or presence of 10^{-6} M [Nle⁴,D-Phe⁷]- α -MSH (NLE- α -MSH), γ -MSH and ACTH₁₋₁₀ for a further 30 min. Values are means of triplicate determinations and expressed in percent of control. Wild-type CHO cells did not respond to any of the peptides (data not shown).

members of the human melanocortin family [14] is weaker, as depicted in Fig. 2.

Two CHO cell clones, stably expressing high levels of BDF3 mRNA were tested for cAMP accumulation following exposure to melanotropic peptides (Fig. 3). Consistent with previous reports of cloned human MSH receptor stably transfected into HEK 293 cells [11], treatment of transfected cells with 10^{-6} M [Nle⁴,D-Phe⁷]- α -MSH [25] a potent α -MSH analog produced a 4.6-fold increase of intracellular cAMP level, whereas γ -MSH was not effective. However, in contrast to the human MSH receptor, the elevation of cAMP levels following ACTH₁₋₁₀ was only moderate. Thus, these results, as well as the homology to the human α -MSH receptor confirm that BDF3 is a bovine α -MSH receptor.

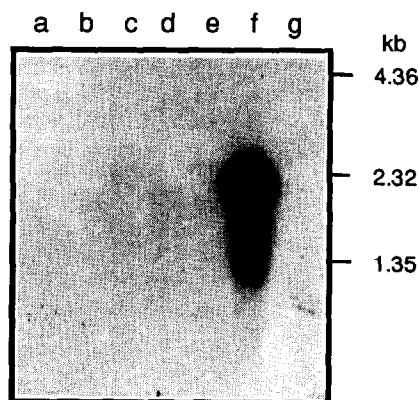


Fig. 4. Northern blot analysis of receptor BDF3 mRNA in bovine tissues. 10 μ g aliquots of poly(A) RNA were subjected to electrophoresis, blotted on nylon membranes and hybridized with a ³²P-labelled 517–3 cRNA probe. (a) kidney (medulla), (b) brain, (c) endometrium (day 0), (d) thymus, (e) thyroidea, (f) testis, (g) corpus luteum.

Northern blot analysis revealed that high levels of BDF3 mRNA are present in bovine testis, but none in kidney (medulla), endometrium, thyroidea and corpus luteum. Faint signals were seen in brain and thymus (Fig. 4). The occurrence of POMC mRNA and POMC-derived peptides in testis [6,7] and the ability of ACTH/MSH to stimulate cell growth in cultured Sertoli cells [26], suggests that melanocortins may be involved in the maintenance of spermatogenesis in the adult testis and may explain the high level of BDF3 receptor mRNA in this tissue.

In contrast, no transcripts for MSH receptors could be detected in human testis [11]. Future experiments should be done to find out if this reflects a real species-specific difference or may be due to age-dependent testicular activity.

Acknowledgements: The study was supported by the Deutsche Forschungsgemeinschaft (SFB 220).

References

- [1] Höllt, V. (1993) Handbook of Experimental Pharmacology 104/I, 307–346.
- [2] De-Wied, D. and Jolles, J. (1982) *Physiol. Rev.* 62, 976–1059.
- [3] Walker, J.M., Akil, H. and Watson, S.J. (1980) *Science* 210, 1247–1249.
- [4] Murphy, M.T., Richards, D.B. and Lipton, J.M. (1983) *Science* 221, 192–193.
- [5] Khorram, O., Bedran-de-Castro, J.C. and McCann, S.M. (1984) *Proc. Natl. Acad. Sci. USA* 81, 8004–8008.
- [6] Jingami, H., Nakanishi, S., Imura, H. and Numa, S. (1984) *Eur. J. Biochem.* 142, 441–447.
- [7] Margioris, A.N., Liotta, A.S., Vaudry, H., Bardin, C.W. and Krieger, D.T. (1983) *Endocrinology* 113, 663–671.
- [8] Ackland, J.F., Schwartz, N.B., Mayo, K.E. and Dodson, R.E. (1992) *Physiol. Rev.* 72, 731–787.
- [9] Tatrow, J.B., Entwistle, M.L., Lester, B.R. and Reichlin, S. (1990) *Cancer Res.* 50, 1237–1242.
- [10] Buffey, J., Thody, A.J., Bleeher, S.S. and Mac-Neil, S. (1992) *J. Endocrinol.* 133, 333–340.
- [11] Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D. (1992) *Science* 257, 1248–1251.
- [12] Chhajlani, V. and Wikberg, E.S. (1992) *FEBS Lett.* 309, 417–420.
- [13] Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J. and Yamada, T. (1993) *J. Biol. Chem.* 268, 8246–8250.
- [14] Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J. and Yamada, T. (1993) *J. Biol. Chem.* 268, 15174–15179.
- [15] Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.J., Dumont, J.E. and Vassart, G. (1989) *Science* 244, 569–572.
- [16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [17] Feinberg, A.P. and Vogelstein, B.C. (1982) *Anal. Biochem.* 132, 6–13.
- [18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning*, CSH Laboratory Press, 2nd Edn., Cold Spring Harbor, New York.
- [19] Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 1556–1559.

- [20] Kennelly, P.J. and Krebs, E.G. (1991) *J. Biol. Chem.* 266, 15555–15558.
- [21] Kobilka, B.K., Dixon, R.A., Frielle, T., Dohlmann, H.G., Bolanowski, M.A., Sigal, I.S., Yang-Feng, T.L., Francke, U., Caron, M.G. and Lefkowitz, R.J. (1987) *Proc. Natl. Acad. Sci. USA* 84, 46–50.
- [22] O'Dowd, B.F., Hnatowich, M., Caron, M.G., Lefkowitz, R.J. and Bouvier, M. (1989) *J. Biol. Chem.* 264, 7564–7569.
- [23] Savarese, T.M. and Fraser, C.M. (1992) *Biochem. J.* 283, 1–19.
- [24] Findlay, J. and Eliopoulos, E. (1990) *Trends Pharmacol. Sci.* 11, 492–499.
- [25] Cody, W.L., Mahoney, M., Knittel, J.J., Hruby, V.J., Castrucci, A.M. and Hadley, M.E. (1985) *J. Med. Chem.* 28, 583–580.
- [26] Gizang-Ginsberg, E. and Wolgemuth, D.J. (1985) *Dev. Biol.* 111, 293–305.
- [27] Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.* 157, 105–132.