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Expression of β_2-adrenoceptors in halobacteria

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Abstract Halobacteria are halophilic representatives of the recently defined domain, the Archaea. *Halobacterium salinarium* belongs to this group of microorganisms and contains large amounts of bacteriorhodopsin in its membrane. Bacteriorhodopsin is a seven-transmembrane protein that consists of bacterio-opsin (BO), and the chromophore retinal, which is covalently attached to BO. We have investigated whether the expression machinery for BO can be utilized for synthesis of the human β_2-adrenoceptor (β_2-AR), a protein with a similar seven-transmembrane-helix topology. An expression vector for BO synthesis was modified to express β_2-ARs under the control of BO regulatory elements in *H. salinarium*. Homologous recombination into the genome was verified by polymerase chain reactions. Northern blots revealed transcripts of the calculated size and significant amounts of epitope-tagged β_2-ARs were detected in Western blots. However, binding of the β-AR antagonist ^{125}I-cyanopindolol revealed low levels of functional receptors, and the ligand binding properties of these receptors were altered when compared to native receptors. Expression of chimeras containing larger amino terminal portions of BO did not result in higher receptor levels. Expression of β_2-AR in *Haloferax volcanii*, another member of halobacteria, was achieved with a vector carrying the ferredoxin promoter. The levels of functional receptor as determined by ^{125}I-cyanopindolol binding were 180 fmol/mg protein. The β-AR ligands isoprenaline and propranolol showed affinities expected for functional β_2-ARs. Thus, functional human β_2-ARs were expressed in halobacteria, constituting a first approach for expression of a eukaryotic protein in the domain of Archaea.

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Key words β_2-Adrenoceptor · Bacteriorhodopsin · *Halobacterium salinarium* · *Haloferax volcanii* · Heterologous expression

Abbreviations *BO* bacterio-opsin · *BR* bacteriorhodopsin · *β_2-AR* β_2-adrenoceptor · *β_2-ARmyc* myc-tagged β_2-AR · *NT-β_2-ARmyc* myc-tagged β_2-AR with a BO portion proximal to the first transmembrane helix · *H1-β_2-ARmyc* myc-tagged β_2-AR with an aminoterminal BO portion including the first transmembrane helix · *^{125}I-CYP* [^{125}I]-(-)Iodocyanopindolol · *MOPS* 3-(N-morpholino)propanesulfonic acid · *SDS-PAGE* sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Adrenoceptors, which mediate the physiological effects of catecholamines, are members of the large family of G protein-coupled receptors. The β_2-adrenoceptor (β_2-AR) is often studied as the prototypical receptor of this family. Binding of appropriate agonists such as isoprenaline to the β_2-AR results in activation of the stimulatory G protein G_s, which in turn enhances the activity of the effector enzyme adenylyl cyclase and generates the second messenger cAMP. The cloning of the cDNA of the β_2-AR as well as many other receptors of this family has resulted in a model of the transmembrane topography that predicts seven transmembrane α-helices, with the N-terminus located extracellularly and the C-terminus in the cytosol. This overall topography is supported by mapping of the epitopes with biochemical (Dohlman et al. 1987) and immunological (Wang et al. 1989) methods. More recently, low resolution electron diffraction data have been obtained on rhodopsin, a G protein-coupled receptor for light perception (Schertler et al. 1993; Unger and Schertler 1995). These data are compatible with the proposed transmembrane arrangement of these receptors.

A similar transmembrane topology is found in bacterio-opsin (BO), the protein moiety of bacteriorhodopsin (BR).

BR is a light driven proton pump that constitutes the major membrane protein of *Halobacterium salinarium* (formerly called *H. halobium*), an archaebacterium which grows in salt ponds (Woese et al. 1990). The membranes of this halobacterium contain two-dimensional crystals of BR, the so-called purple membrane, that covers up to 80% of the cell surface, which corresponds to about 300 000 BR molecules per cell. BR catalyzes the light-dependent translocation of protons resulting in the generation of a transmembrane proton gradient which is utilized by a proton-translocating ATPase for ATP synthesis (Oesterhelt and Tittor 1989; Tittor 1991). The three-dimensional structure of BR has been elucidated by the use of electron diffraction (Henderson et al. 1990). Similarly to the proposed structure of G protein-coupled receptors, it contains seven transmembrane α-helices which are arranged in a circular manner and form a hydrophilic pocket.

Since G protein-coupled receptors and BR share this overall topology, the three-dimensional structure of BR has been used as a template for the modelling of G protein-coupled receptors (Trumpp-Kallmeyer et al. 1992; IJzerman et al. 1992; Pardo et al. 1992; Hoflack et al. 1994). However, there is no sequence similarity between the halobacterial opsins and the G protein-coupled receptors (Baldwin 1993; Zhang and Weinstein 1994; Soppa 1994). Furthermore, the available structural data suggest that while BR and rhodopsin share the overall topology there are clear differences in the packing of the helices (Schertler et al. 1993). Thus, at the present state it is not clear what types of relationship exist between BR and G protein-coupled receptors.

Membrane receptors are the primary targets of many drugs, and therefore there is great interest in the development of new expression systems. β_2-ARs have been successfully expressed in a variety of cell types, including transient and stable expression in various eukaryotic cell lines (Dixon et al. 1987; Kobilka et al. 1987; Bouvier et al. 1988; Lohse, 1992), baculovirus-infected insect cells (George et al. 1989), and yeast (King et al. 1990). Using *Escherichia coli* as a host, several techniques resulted in expression of functional β_2-ARs (Marullo et al. 1989; Galli et al. 1990; Chapot et al. 1990; Freissmuth et al. 1991). These data suggested that expression of these receptors might be possible in a wide range of host cells.

Consequently, we investigated whether it might be possible to express β_2-ARs in halobacteria, which contain large amounts of bacteriorhodopsin. We chose these organisms, because, first, *H. salinarium* tolerates the highest expression of a seven-transmembrane protein so far. Second, archaebacteria have unique characteristics. They have recently been grouped into a new domain, now called the Archaea (Woese et al. 1990; Woese 1994). Some of the features of Archaea are similar to those of Eukarya, particularly several components of the transcription system (Baumann et al. 1995). Therefore, heterologous expression of eukaryotic genes appears more advantagous in Archaea than in Bacteria.

Recently, techniques have been developed for the homologous expression of halobacterial proteins. Overexpression of three integral membrane proteins has been achieved by use of the *E. coli-Halobacterium* shuttle vector pWL102ΔBA (Ferrando et al. 1993) or a similar vector (Heymann et al. 1993). Expression of recombinant BO in a *H. salinarium* BR^- strain resulted in synthesis of up to 300 000 molecules BR per cell. Recombinant halorhodopsin (Heymann et al. 1993) and sensory rhodopsin I (Ferrando-May et al. 1993) were expressed at approximately 200 000 and 100 000 molecules per cell, respectively. Thus, this shuttle vector appears to be useful for homologous expression in halobacteria. This led us to investigate the potential of this vector system for the expression of heterologous proteins such as the β_2-AR.

In this study we describe the expression of β_2-ARs in *H. salinarium* with the shuttle vector for BO expression as well as in *Haloferax volcanii*. In the latter halobacterial host seven-transmembrane-helix proteins have not been detected.

Materials and methods

Materials. GF/C filters were obtained from Whatman. Mevinolin was a kind gift from Merck, Sharp and Dohme. ^{125}I-CYP was from DuPont NEN, alprenolol, isoprenaline, novobiocin, polyethylene glycol 600 and propranolol were from Sigma. Restriction enzymes and Taq-polymerase were obtained from Boehringer Mannheim. The anti c-myc antibody Ab-1 was from Oncogene Science.

Vector constructions. The oligonucleotides used for the various constructions are listed in Table 1. In order to generate a vector for the expression of the human β_2-AR in halobacteria, the cDNA of the β_2-AR (Fig. 1A) was cloned into pEF1290 (Ferrando et al. 1993), a vector containing the full gene cassette for expression of BO (Fig. 1E) in the shuttle vector pWL102ΔBA (Lam and Doolittle 1989; Ferrando et al. 1993). This was done as follows: polymerase chain reaction (PCR) with the two primers H1 and H2 and pEF1290 as template gave a PCR product containing about 450 bp of BO 5′-sequences including the first 15 codons and a short multiple cloning site at the 3′-end.

Table 1 Oligonucleotides used for vector constructions

Oligonucleotide	Sequence
Bacteriorhodopsin	
H1	5′-GTG TAG CGG TCA CGC TG-3′
H2	5′-ATG CAT GCG GCC GCG ATA TCT AGA CCA TGG CCT GCG ATA CCC CC-3′
BS3	5′-ATG GGT GCA ACC GTG AAG-3′
β_2-Adrenoceptor	
β1	5′-ATG GGG CAA CCC GGG AAC-3′
β22	5′-AGA TCT GCG GAG TCC ATG CC-3′
BO/β_2-Adrenoceptor fusion primers	
FUS1A	5′-CAT GAC GAT GCC CAT GCC ACG TCC GGT GAT CTG GGC-3′
FUS1B	5′-GGC ATG GGC ATC GTC ATG-3′
FUS2A	5′-CAG ACG CTC GAA CTT GGC CCC TTT CAC GAG GAA ATA-3′
FUS2B	5′-GCC AAG TTC GAG CGT CTG-3′
β_2-Adrenoceptor/myc-tag	
myc	5′-GTC GCT GGT CGC GGC CGC TTA GAG ATC TTC CTC AGA TAT CAG CTT CTG CTC GGG CCC CAG CAG TGA GTC ATT TGT-3′

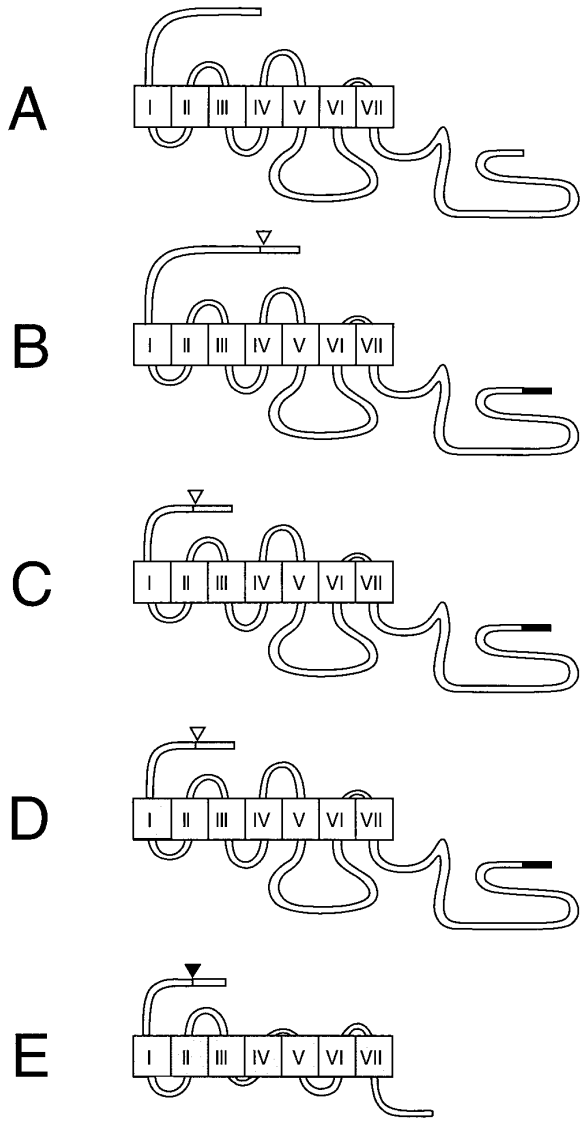

Fig. 1A–E Hybrid receptors and their β_2-AR and BO origins. **A** Wild-type β_2-AR; **B** β_2-ARmyc, containing 15 amino acids from the BO (*grey*) amino terminus and the myc-tag (*black*) at the carboxy terminus; **C** NT-β_2-ARmyc, chimeric receptor with the amino terminus (amino acids 1–34) proximal to the first transmembrane segment of β_2-AR exchanged for the corresponding region of BO (amino acids 1–20, grey), and the myc-tag (*black*) at the carboxy terminus; **D** H1-β_2-ARmyc, chimeric receptor with exchange of the N-terminus including the first transmembrane helix of β_2-AR (amino acids 1–58) replaced by the corresponding region of BO (amino acids 1–44, grey), and the myc-tag (*black*) at the carboxy terminus; **E** BO. The *arrow* indicates the putative processing site. The cytoplasmic part of the receptors is located *at the bottom* of each figure

*Bam*HI (5′) and *N*coI (3′) flanked the BO sequences whereas *Eco*RV and *Not*I were at the 3′-end. This PCR product was cut with *Bam*HI and *Not*I and cloned into pEF1290 which had been digested with the same enzymes to remove the major portion of BO. Insertion of a 1.2 kbp *N*coI-*Dra*I fragment from pBC-β_2-AR (Kobilka et al. 1987) into the *N*coI-*Eco*RV-sites places the receptor initiation ATG at codon 16 of the fusion protein. Thus, in the new plasmid pPS1100 (Fig. 2A) the amino acids 1–15 of BO are fused to the complete coding region of β_2-AR, and the regulatory elements of BO located 5′ and 3′ to the coding sequences are retained as in pEF1290.

Second, a myc-tag (EQKLISEEDL is an epitope of the human c-myc proto-oncogene, Evan et al. 1985; Munro and Pelham 1986) was fused to the carboxy terminus of β_2-AR in this halobacterial expression vector (β_2-ARmyc, Fig. 1B). For this purpose, a PCR product was generated with the two primers β1 and myc and the template pPS1100, and was inserted into pPS1100 after cutting both the insert and the vector with *Hpa*I and *Not*I. This plasmid, termed pPS1110, codes for 15 amino acids of BO at the amino terminus and the myc-tag at the carboxy terminus of the β_2-AR.

Third, two vectors were constructed that encoded chimeric β_2-ARs containing larger amino terminal portions of BO. In one construct, the entire extracellular N-terminus of the β_2-AR was replaced by the corresponding BO segment (NT-β_2-ARmyc, Fig. 1C). Two overlapping PCR products (a and b) were generated using (a) pEF1290 as the template and the primers BS3 and FUS1A, and (b) pPS1100 and the primers FUS1B and β22. The two resulting PCR products (a and b) were then used as overlapping templates together with the primers BS3 and β22. This resulted in a PCR product coding for the chimeric receptor which was cut with *Mlu*I and *Pst*I, and inserted into the corresponding sites of pPS1110. The resulting plasmid (coding for NT-β_2-ARmyc, Fig. 1C) was termed pPS2110. The second construct was made in which the BO region was more extended and included the first transmembrane helix (H1-β_2-ARmyc, Fig. 1D). The construction strategy was similar to the one described above, but the primers FUS2A and FUS2B were used instead of FUS1A and FUS1B, respectively. The resulting plasmid was termed pPS3110.

Finally, the *Eco*RI-*Hind*III fragments containing the entire coding regions of pPS1100, pPS1110, pPS2110 and pPS3110 were cloned into the *E. coli – Halobacterium* shuttle vector pWL102ΔBA (Lam and Doolittle 1989; Ferrando et al. 1993) encoding the selection marker 3-hydroxy-3-methylglutaryl coenzyme A reductase, which confers resistance to mevinolin. The resulting vectors are analogous to the vector pEF191 (Ferrando et al. 1993) and were used for transformation of *H. salinarium* strain NRL (Oesterhelt and Stoeckenius 1974) or strain Flx37 (Spudich and Spudich 1982).

In addition, analogous vectors were constructed for the expression of the various β_2-AR variants in eukaryotic cells. To generate these vectors, a *Mlu*I-*Sal*I fragment containing the halobacterial sequences coding for the fusion proteins was excised from pPS1100, pPS1110, pPS2110 and pPS3110, respectively. These fragments were cloned into pBC-SK-dhfr (Lohse 1992) cut with *Eco*RV and *Sal*I. The resulting vectors were used for transfection of HEK-293 cells.

In order to express a myc-tagged β_2-AR in insect cells, an *Eco*RI-*Not*I fragment from pPS1110 was cloned into the corresponding sites of the baculovirus expression vector pVL1393 (Summers and Smith 1987). Recombinant baculoviruses were generated and used for the expression of the myc-tagged β_2-AR in Sf9 cells by the methods described earlier (Müller et al. 1993).

A vector for the expression of the β_2-AR in *Hf. volcanii* was constructed on the basis of the shuttle vector pJAS35 (unpublished). This vector contains origins for *E. coli* and *Hf. sp*. Aa 2.2, novobiocin and ampicillin resistance markers and the ferredoxin promoter for expression of the halobacterial dihydrofolate reductase. The *N*coI-*Asp*718 fragment of the vector coding for dihydrofolate reductase was replaced by an *N*coI-*Pvu*II fragment containing the β_2-AR coding region from pPS1100. In the resultant vector the ferredoxin promoter controls the expression of an β_2-AR which contains two additional amino acids (Met-Pro) at the amino terminus (Fig. 2B).

In all constructs, sequences obtained by PCR were verified by automated sequencing.

Halobacterial strains. H. salinarium NRL is a wild-type strain (Oesterhelt and Stoeckenius 1974). *H. salinarium* Flx37 (Spudich and Spudich 1982) is a mutant from *H. salinarium* S9 (Wagner et al. 1981), deficient in BR, halorhodopsin (10% of wild-type) and the carotenoid pigments. *Hf. volcanii*, strain WR340, was kindly provided by M. Mevarech, Tel Aviv University.

Transformation of halobacteria. H. salinarium was grown at 40°C in support medium, which consists of basal salt (4.28 M NaCl, 80 mM $MgSO_4$, 10 mM trisodium citrate, 27 mM KCl, 1.4 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4), 0.3% Bacto Yeast extract (Difco), and

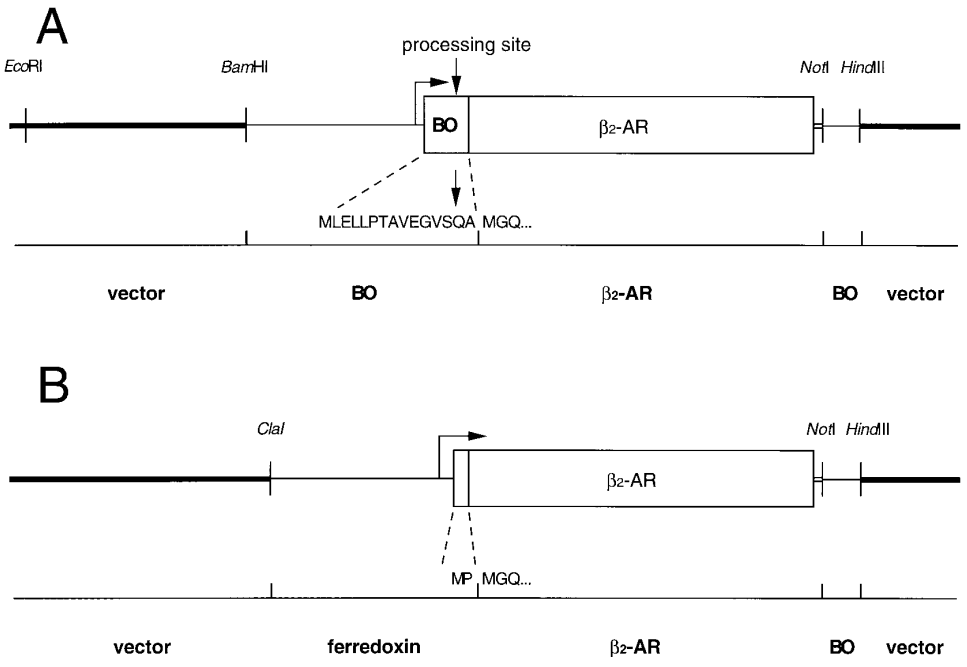

Fig. 2A,B Insertion of the β_2-AR cDNA into the halobacterial shuttle vectors. The open boxes denote the sequences coding for amino acids of BO (*grey*) or β_2-AR (*white*), respectively. Vector sequences are indicated by *bold lines*, halobacterial sequences by *thin lines* and the 3′-non-coding sequence of the β_2-AR by a *double line*. *Horizontal arrows* indicate the transcriptional start sites. **A** Vector for the expression of the β_2-AR in *H. salinarium*. The amino terminal 15 amino acids of BO and the first amino acids of the β_2-AR (proper) sequence are given in the one-letter-code, the vertical arrow indicates the processing site in BO. **B** Vector for the expression of the β_2-AR in *Hf. volcanii*. In this construct, two aditional amino acids were added at the amino terminus of the β_2-AR

0.5% Bacto tryptone (Difco). Support plates were made with medium containing 1.2% Bacto agar (Difco). Transformation of *H. salinarium* strains NRL or Flx37 and *Hf. volcanii* were essentially performed as described (Cline et al. 1989). *H. salinarium* cells were propagated in 35 ml of support medium for two weeks at exponential growth to a density of about 10^9 cells/ml (90–95 Klett units) and harvested by centrifugation for 5 min, 3000 *g* at room temperature. The cell pellet of 2 ml original cell culture was suspended in 200 µl sphaeroblasting solution (2 M NaCl, 27 mM KCl, 50 mM Tris-HCl, pH 8.75, 15% sucrose). Sphaeroblasting of the cells was achieved by adding 1/10 volume of 0.5 M EDTA, pH 8.75, to the cells in sphaeroblasting solution. After 10 min 4 µg of plasmid DNA were added in 20 µl sphaeroblasting solution for transformation of the sphaeroblasts. Aggregation of the cells was initiated after 5 min by addition of 1 volume of 60% polyethylene glycol 600 in sphaeroblasting solution. After another 10 min the cells were transferred into 1 ml of regeneration medium (support medium containing 15% sucrose). After incubating the cells overnight at 40°C, aliquots were plated onto support plates containing 1.2% Difco agar and incubated at 40°C under microaerobic conditions. Selection on support plates containing 25 µM mevinolin resulted in the appearance of transformants after 3–10 weeks which expressed the selection marker 3-hydroxy-3-methylglutaryl coenzyme A reductase encoded on the expression vector. For *Hf. volcanii*, a different basal salt (3.22 M NaCl, 174 mM $MgSO_4$, 33 mM KCl, 4.8 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.2) and sphaeroblasting solution (0.8 M NaCl, 27 mM KCl, 50 mM Tris-HCl, pH 8.2, 15% sucrose) were used. Transformants were visible on support plates containing 160 nM novobiocin, an inhibitor of wild-type gyrase, after one week growth at 40°C.

Conditions for protein expression. H. salinarium and *Hf. volcanii* cells were grown after 1:100 inoculation in support medium as described for transformation. *H. salinarium* cells were harvested after five days of incubation under microaerobic conditions as described for BR expression (Oesterhelt and Stoeckenius 1974) in the presence of 25 µM mevinolin and 10 µM alprenolol. In control cells, expression of BR was indicated by the purple color of the cells. *Hf. volcanii* cells were harvested after 2–3 days growth in the presence of 160 nM novobiocin and 10 µM alprenolol.

Amplification of DNA fragments by PCR. PCR was performed in Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl with 10 ng template DNA using the foreward or reverse primers (0.5 µM each) listed in Table 1, and 0.2 mM dNTP in 28–30 cycles at 50–55°C, with respect to the primer sequences.

In situ colony screening. Total DNA from bacterial colonies was transferred onto nitrocellulose filters as described by Grunstein and Hogness (1975). DNA of colonies carrying the cDNA of interest were identified with ^{32}P-labelled probes specific for the β_2-AR or for BO. These probes were generated by PCR using [α-^{32}P]dCTP.

SDS-PAGE and Western blotting. SDS-PAGE and Western blots were essentially performed as described (Laemmli 1970; Towbin et al. 1979). Receptors tagged with the myc-epitope (EQKLISEEDL, Evan et al. 1985; Munro and Pelham 1986) were detected with the monoclonal c-myc antibody Ab-1 (Oncogene Science, dilution 1:100 in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20, 0.5% nonfat powdered milk) as described by the supplier and signals were visualized by enhanced chemiluminescence.

Northern blot analysis. Transformed *H. salinarium* cultures were inoculated 1:100 into 35 ml support medium containing 25 µM mevinolin and 10 µM alprenolol, and were incubated for five days at 40°C. 5 ml of these cultures were used for RNA preparation using the method described by Chomczynski and Sacchi (1987). Glyoxylation of the RNA, separation on a 1.2% agarose gel in MOPS buffer and transfer onto nitrocellulose filters were done as described by Sambrook et al. (1989). Specific mRNAs were detected using β_2-AR-specific or BO-specific probes (1×10^6 cpm/ml) prepared and labelled by PCR as described above. The size of the transcripts was compared with an RNA ladder (Gibco BRL, sizes: 9.5 kbp, 7.5 kbp, 4.4 kbp, 2.4 kbp, 1.35 kbp, 0.78 kbp). The intensity of the signals was determined by densitometric scanning of the autoradiographies.

Receptor expression in HEK-293 cells. For control purposes, various β_2-AR constructs were also transiently expressed in human embryonic kidney HEK-293 cells. These cells were grown in DMEM/F-12 medium (Gibco) with 10% fetal calf serum and transfected by a calcium phosphate precipitation technique (Chen and Okayama 1987). Two days later the cells were harvested and crude membranes were prepared as described earlier (Lohse et al. 1990).

Radioligand binding assay. Binding assays were done using bacterial (10–100 µg) or eukaryotic cells or membranes (10 µg of protein). ^{125}I-CYP was present at 35 pM, and other β_2-AR ligands were added

as indicated. Various buffer systems were used as indicated in the figure legends: basal salt or sphaeroblasting solution (*H. salinarium*), sphaeroblasting solution (*Hf. volcanii*) and 50 mM Tris-HCl, pH 7.4. At the end of the incubations, separation of bound and free ligand was achieved by filtration over GF/C-filters followed by seven 4 ml-washes with the respective buffer. Radioligand binding data were analyzed with an updated version of the non-linear curve-fitting program SCTFIT (Lohse et al. 1984).

Results

Expression of β_2-AR in *H. salinarium*

In order to attempt expression of β_2-AR in halobacteria we adapted strategies that have recently been developed for homologous high level expression of BR. We constructed a halobacterial expression vector based on the one used for the expression of BO (Ferrando et al. 1993). The major parts of the BO coding region were replaced by the corresponding β_2-AR sequences. Flanking BO sequences 438 bp upstream and about 200 bp downstream from the coding region were retained, because they are thought to contain putative control elements for transcription and translation of BO. Transcription of the BO gene starts only two nucleotides 5′ to the start codon, suggesting that coding RNA sequences may be important for translation. Therefore, the cDNA coding for the first 15 amino acids of BO was fused to the β_2-AR cDNA in the β_2-AR expression vector.

Transformation of the *H. salinarium* strain Flx37 (which lacks endogenous BR) with this expression vector gave rise to a large number of selectable clones, which were identified by colony screening. Analysis of these clones by PCR with specific primers indicated homologous recombination of the BO/β_2-AR sequences of the vector into the corresponding BO sequences of the halobacterial genome (Fig. 3).

The transcription of the vector sequences into mRNA was analyzed in nine individual clones by Northern blot (Fig. 4). When a labelled probe for the β_2-AR was used, a transcript of about 1400 nucleotides was detected, which corresponds to the calculated length of the β_2-AR mRNA as transcribed from this vector. No such transcript was detected with the β_2-AR probe in a control clone which had been transformed with a vector encoding BO. The different clones produced varying amounts of β_2-AR mRNA, which is due to the switch to microaerobic conditions during the induction of BR expression. Probing the same Northern blot with a BO probe revealed the BO mRNA of about 800 nucleotides in the clone transformed with the BO vector, but no such signal in the clones transformed with the β_2-AR construct. The BO mRNA was 8 ± 2-times (n = 9) more abundant than that of the β_2-AR (Fig. 4). These data suggest that the levels of β_2-AR mRNA transcribed from the integrated vector sequences were in the range of those that could be obtained for BO.

Production of the receptor protein was monitored in experiments using a myc-tagged β_2-AR construct. Fusion of the 10 amino-acid myc-tag sequence (EQKLISEEDL,

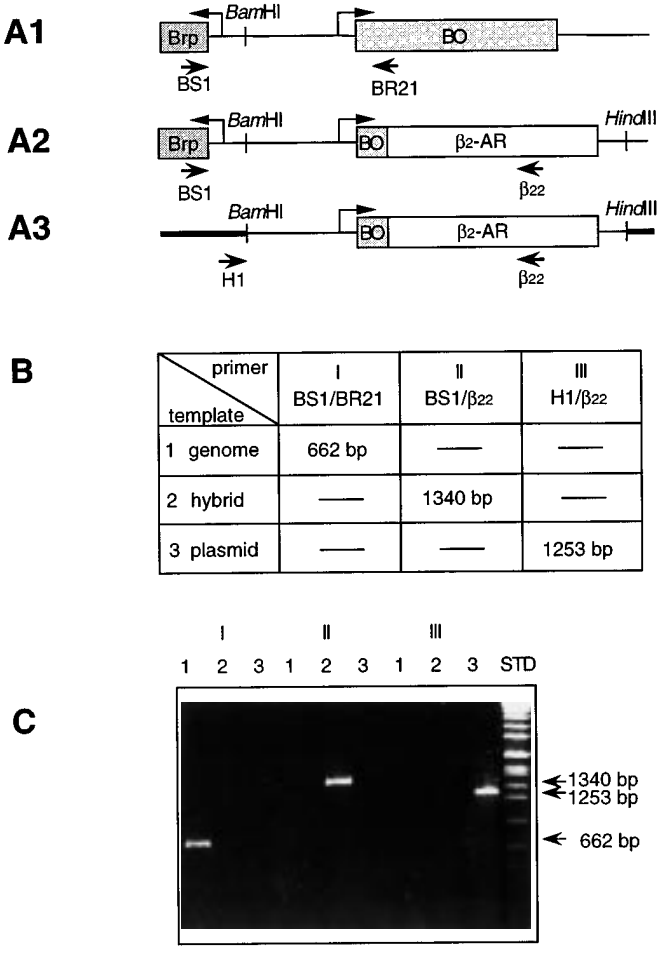

Fig. 3 A–C Analysis of site-specific vector integration into the halobacterial genome. **A** Diagram showing the localization of the BO sequences in the halobacterial genome (**A1**), of the β_2-AR cDNA integrated into the halobacterial genome (**A2**), and of the β_2-AR cDNA on the expression vector (**A3**). *The arrows below the diagrams* represent the localization of PCR primers, other symbols are used as in Fig. 2. Brp indicates the coding region for the BO related protein. **B** Table of the calculated length of PCR products that should result from different primer combinations for the three templates shown in part A. The primer BS1 is specific for the genomic situation, H1 for the vector, BR21 for the BO gene and β22 for the β_2-AR cDNA. **C** PCR products obtained with the primer combinations (I, II, III) and templates (1,2,3) as listed in part B. The size is indicated by *arrows*, the molecular weight standards (Boehringer Mannheim, SPP1 DNA cut with *Eco*RI) are shown on the *right side*. 10 ng of total DNA from *H. salinarium* strain Flx37 (1), or DNA from an individual *H. salinarium* clone transformed with the β_2-AR expression vector (2), or 1 ng from the β_2-AR expression vector (3) were used as templates for the PCR reaction

Evan et al. 1985; Munro and Pelham 1986) to the C-terminus of the receptor did not alter the functional properties of the receptors when expressed in eukaryotic HEK-293 cells (see below). Transformed halobacterial clones were grown in the presence of 10 μM of the β_2-AR antagonist alprenolol which had been shown to stabilize the receptor during overexpression in eukaryotic cells (Lohse 1992). Western blots of these cells developed with an anti-myc antibody revealed a protein band of about 45 kDa in cells

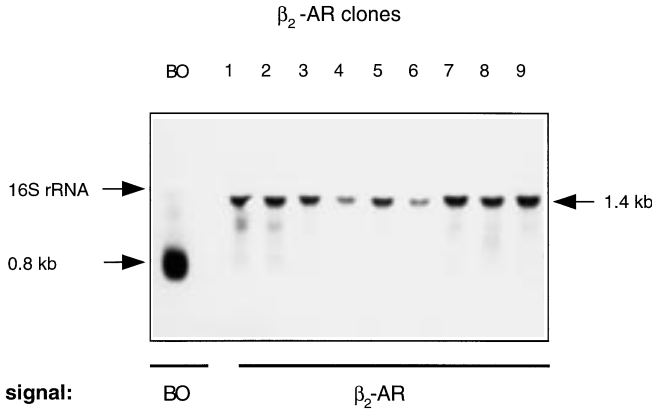

Fig. 4 Analysis of β_2-AR and BO transcripts. 15 µg of glyoxylated RNA of individual halobacterial transformants selected by colony screening (*lanes 1–9*) or of a selected halobacterial clone transformed with an expression vector for BO (*lane BO*) were separated on a 1.2% agarose gel, blotted onto a nylon membrane and hybridized with a probe specific for the β_2-AR. After detection of the β_2-AR mRNA, the blot was developed a second time using a probe specific for BO. The size of the transcripts and the position of the 16S rRNA are indicated by an *arrow*

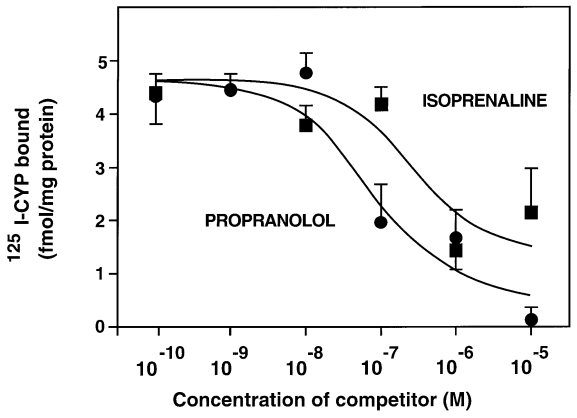

Fig. 6 Competition for ^{125}I-CYP binding to β_2-AR expressed in *H. salinarium*. The cells from an individual selected clone were grown as described in Fig. 5. After washing the cells, 10 µg of total cellular protein and 35 pM ^{125}I-CYP were used in the binding assay containing basal salt for *H. salinarium*. Varying concentrations of (-)-isoprenaline (■) or (-)-propranolol (●) were present as indicated in the figure. Non-specific binding was subtracted as determined from non-transfected control cells. Analysis by non-linear curve-fitting gave IC_{50}-values of ≈100 nM (isoprenaline) and ≈30 nM (propranolol)

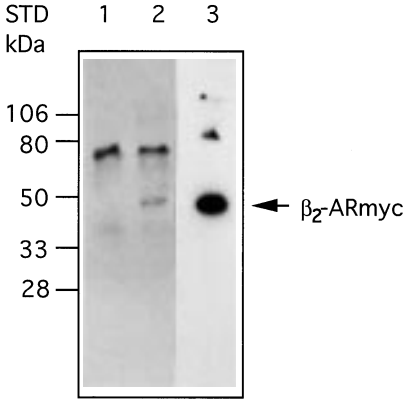

Fig. 5 Immunological detection of the myc-tagged β_2-AR expressed in halobacteria or Sf9 cells. Halobacterial cells were harvested after five days of incubation in the presence of 25 µM mevinolin and 10 µM alprenolol. 100 µg of protein from non-transformed halobacteria (*lane 1*) or halobacteria transformed with the vector encoding the myc-tagged β_2-AR (*lane 2*) were subjected to SDS-PAGE and blotted onto polyvinylidenedifluoride membranes. 1 µg of protein from Sf9 insect cells infected with recombinant baculoviruses coding for the myc-tagged β_2-AR were used as a control (*lane 3*). The blot was probed with the anti-myc antibody Ab-1 and developed using enhanced chemiluminescence. The β_2-ARmyc is indicated by an *arrow* and the sizes of the molecular weight standards (STD) are indicated *on the left*

transformed with the β_2-ARmyc vector, whereas this band was not detected in control cells (Fig. 5). For comparison, the myc-tagged β_2-AR was expressed in Sf9 insect cells infected with a recombinant baculovirus. This receptor showed a similar electrophoretic mobility because of only primitive glycosylation of the β_2-AR in insect cells (Fig. 5). Immunological screening by filter assays of about 200 halobacterial clones expressing the myc-tagged receptor did not reveal any clones expressing significantly enhanced amounts of the receptor (not shown).

Next we sought to determine the functionality of the recombinant receptors in radioligand binding experiments using the high affinity antagonist ^{125}I-CYP. The binding assays were performed in high salt buffers since the purple membranes lyse under low salt conditions. The stability of receptors expressed in eukaryotic cells was examined under the same conditions. Human A431 cells endogenously expressing the β_2-AR did not reveal major differences in ^{125}I-CYP binding when higher salt concentrations (5 M NaCl in 50 mM Tris pH 7.4) were used as described for halobacterial binding assays (not shown).

Thus, binding assays were performed in basal salt solution for *H. salinarium*. The halobacterial clones transformed with the β_2-AR expression vector showed binding of ^{125}I-CYP which could be blocked by β_2-AR antagonists like alprenolol or propranolol, whereas control cells did not reveal significant binding. The levels of β_2-AR expression as determined at 200 pM ^{125}I-CYP were ≈5 mol/mg total cell protein (not shown). At 35 pM ^{125}I-CYP non-specific binding varied from 40–60%, precluding an accurate saturation analysis. Competition for 35 pM ^{125}I-CYP binding by the β_2-AR agonist isoprenaline or the antagonist propranolol revealed IC_{50}-values of ≈100 nM and ≈30 nM, respectively (Fig. 6). The affinity of isoprenaline for the β_2-AR in eukaryotic cells (when uncoupled from its G-protein) is in the range of 400 nM (Lohse et al. 1990) and thus similar to the value determined here. However, the affinity of propranolol measured here is more than 10-fold lower than expected, indicating that the receptors expressed in *H. salinarium* did not show native binding characteristics.

We also transformed wild-type *H. salinarium* (strain NRL) which expresses endogenous BR, but co-expression

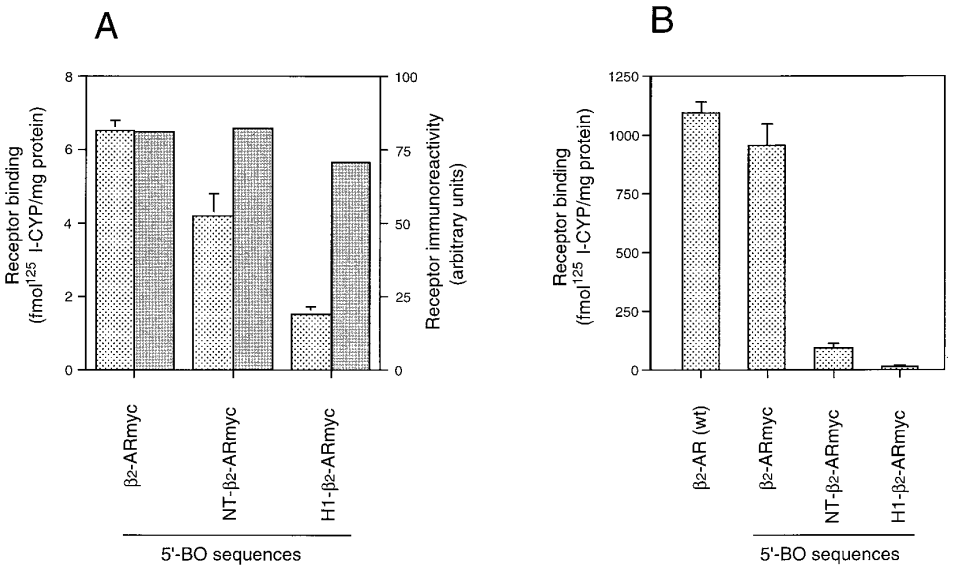

Fig. 7 Determination of chimeric BO/β_2-AR receptor expression in halobacteria (**A**) or in eukaryotic HEK-293 cells (**B**). Receptor expression was determined functionally by ^{125}I-CYP binding (*light bars*, **A** and **B**) and in Western blots using the anti-myc antibody Ab-1 (*dark bars*, **A**). Selected halobacterial clones (50 µg of protein, each) expressing the β_2-ARmyc, NT-β_2-ARmyc, or H1-β_2-ARmyc, respectively, were grown and analyzed as described in Fig. 6. Immunological detection of the expressed myc-tagged receptors was done in Western blots as described in Fig. 5 followed by quantitation by densitometric scanning. HEK-293 cells were transiently transfected with plasmids coding for the same receptor proteins and 10 µg of protein were assayed for ^{125}I-CYP binding in basal salt for *H. salinarium* (see Methods). Specific binding of 35 pM ^{125}I-CYP was defined with 10 µM (-)-alprenolol. The *bar* indicates chimeric β_2-ARs containing 15 amino acids of BO at the N-terminus. Results are means ± SEM (n = 4)

of the β_2-AR with endogenous BR did not affect the levels of β_2-AR expression (data not shown).

Expression of chimeric BO/β_2-AR-constructs

In an attempt to increase the expression of functional receptors, chimeric receptors were constructed to overcome potential problems of translation initiation or insertion of proteins into the halobacterial membrane. In the first chimeric receptor (NT-β_2-ARmyc, see Fig. 1C), the complete extracellular amino terminus of the β_2-AR was replaced by the corresponding part of BO. In the second chimeric receptor (H1-β_2-ARmyc, Fig. 1D) the BO part also included the first transmembrane helix. The two vectors for the chimeric receptors were transformed into *H. salinarium* as described above. The amounts of chimeric receptor protein present in the selected clones were determined in Western blots and were found to be very similar to the levels of β_2-ARmyc (Fig. 7A). However, the binding of ^{125}I-CYP was lower for NT-β_2-ARmyc as compared to β_2-AR or β_2-ARmyc and was lowest for H1-β_2-ARmyc, the chimeric receptor with the most extended BO portion (Fig. 7A).

Ligand binding experiments were also performed with the chimeric receptors transiently expressed in eukaryotic HEK-293 cells (Fig. 7B). This was achieved by the use of a standard expression vector containing a CMV promoter (Lohse 1992) with the coding sequences of wild-type β_2-AR and the various β_2-AR constructs. The ligand binding activity was similar for the wild-type β_2-AR and the β_2-AR containing the first 15 amino acids of BO at its N-terminus and the myc-tag at its C-terminus. In contrast, replacement of the entire β_2-AR amino terminus by that of BO (NT-β_2-ARmyc) resulted in a marked loss of ^{125}I-CYP binding, and binding was lowest when the first transmembrane helix was also replaced (H1-β_2-ARmyc). Thus, the observed loss of ligand binding activity of chimeric receptors with extended amino terminal BO portions was paralleled in eukaryotic cells.

Expression of β_2-AR in *Haloferax volcanii*

β_2-AR mRNA and immunoreactivity could be readily obtained in *H. salinarium*, but β_2-AR ligand binding was lower than predicted. This suggested that expression in *H. salinarium* interferes with proper folding of the β_2-AR. Therefore, we next attempted the expression of β_2-AR in *Hf. volcanii* which is closely related to *H. salinarium*. In contrast to *H. salinarium*, *Hf. volcanii* has not been reported to contain halobacterial retinal proteins. It requires lower salt concentrations (3.2 M NaCl in basal salt), grows faster than *H. salinarium*, and thus colonies appear earlier following transformation.

Transformation of *Hf. volcanii* with a vector for the β_2-AR yielded selectable clones. In contrast to *H. salinarium*, the vectors usually were not integrated into the genome, but replicated autonomously. Therefore, vectors of transformed clones were re-isolated and restriction analysis revealed identity with original plasmids (not shown). Total cell protein prepared from *Hf. volcanii* clones was further

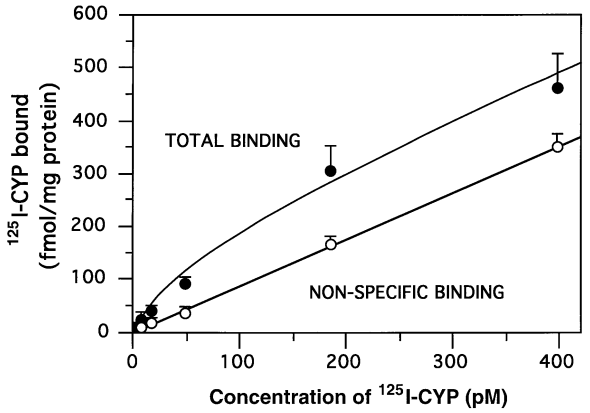

Fig. 8 Saturation analysis with ^{125}I-CYP of β_2-AR expressed in *Hf. volcanii.* Cells transformed with the β_2-AR expression vector described in Fig. 2B were grown as described under Methods. 34 µg of protein and varying concentrations of ^{125}I-CYP were used in the binding assay containing spharoblasting solution for *Hf. volcanii.* Non-specific binding was determined by blocking with 10 µM (-)-propranolol. Analysis by non-linear curve-fitting gave K_D value for ^{125}I-CYP of 59 ± 44 pM and a B_{max} of 177 ± 49 fmol/mg protein. Results are means ± SEM (n = 6)

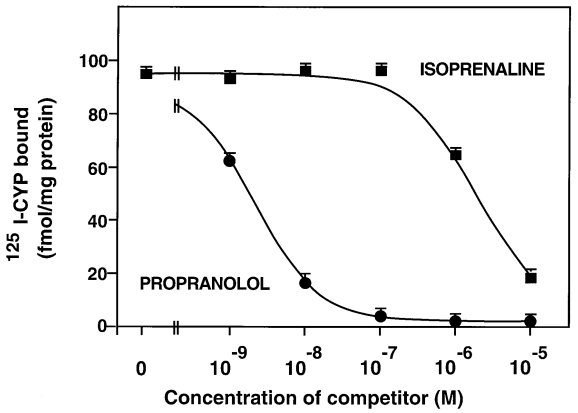

Fig. 9 Competition for ^{125}I-CYP binding to β_2-AR expressed in *Hf. volcanii.* The conditions were as described in Fig. 8 and 35 pM ^{125}I-CYP was used in the binding assay containing spharoblasting solution for *Hf. volcanii.* Varying concentrations of (-)-isoprenaline (■) or (–)-propranolol (●) were present as indicated in the figure. Analysis by non-linear curve-fitting gave K_i-values of 704 ± 180 nM (isoprenaline) and 0.52 ± 0.05 nM (propranolol), respectively. Results are means ± SEM (n = 6)

analyzed by saturation of ^{125}I-CYP binding (Fig. 8). These experiments yielded a K_D value of 59 ± 44 pM and a B_{max} of 177 ± 49 fmol/mg protein. Non-specific binding was found to be 20% at 35 pM ^{125}I-CYP. In CHO cells containing the β_2-AR, the K_D-value for ^{125}I-CYP was 41 ± 2 pM (data not shown). Competition experiments were done with the β_2-AR agonist isoprenaline and the antagonist propranolol (Fig. 9). From these competition curves K_i-values of 704 ± 180 nM and 0.52 ± 0.05 nM were calculated for isoprenaline and propranolol, respectively (Fig. 9). These values correspond to those expected for native functional β_2-AR, indicating that the human β_2-AR can be expressed in *Hf. volcanii* at reasonably high levels and with intact ligand binding properties.

Discussion

Three transmembrane proteins have recently been homologously expressed in *H. salinarium.* This was achieved by site-specific vector integration using the regulatory elements of BO. Homologous expression of recombinant BR reached the very high wild-type levels (Ferrando et al. 1993), whereas sensory rhodopsin I could be overexpressed 40- to 50-fold (Ferrando-May et al. 1993) and halorhodopsin 6-fold as compared to endogenous levels (Heymann et al. 1993). Cells overexpressing halorhodopsin contained two-dimensional crystals on the membrane surface, which were large enough to analyze the projection structure by cryo-electron microscopy (Havelka et al. 1993). In the present work, we investigated whether the high tolerance of *H. salinarium* for its own seven-transmembrane-helix proteins could be utilized for the heterologous expression of the human β_2-AR, a member of the family of G protein-coupled receptors, which is also a seven-transmembrane-helix protein.

The initial strategy was to exchange the BO cDNA for that of the β_2-AR, but to retain the putative regulatory elements of BO on the shuttle vector. A similar strategy had been used for the homologous expression of halorhodopsin and sensory rhodopsin I (Heymann et al. 1993, Ferrando-May et al. 1993). In wild-type cells transcription starts only two bp upstream of the BO start codon (DasSarma et al. 1984). The requirements for translation initiation in halobacteria are not yet clear, but it seems reasonable to assume that coding sequences must play a role, since the two 5′-non-coding bases are very unlikely to be sufficient for translation initiation. Therefore, the cDNA for the first 15 amino acids of BO (Fig. 1) were fused in-frame to the coding cDNA of the β_2-AR in order to retain the sequences upstream and downstream of the BO start codon.

Methods for transformation of *H. salinarium* and propagation of vectors have recently been developed. These transformation procedures have resulted in integration of the vector sequences in the halobacterial genome. We have analyzed this integration of the BO/β_2-AR cDNA-constructs by PCR using a set of specific primers (Fig. 3). We found that homologous recombination had occurred in the DNA regions upstream of the β_2-AR coding sequence, resulting in alignment of the BO putative cis-active elements upstream of the β_2-AR start codon. Thereby, it was ensured that the gene dosage, the location in the chromosome and the regulation of expression was identical to that of the BO gene in *H. salinarium.*

This integrated cDNA resulted in the appearance of clones with specific transcripts of the expected length (Fig. 4) which might be due to the similarity of archaebacterial and eukaryotic transcription system components. The amounts of mRNA were about one tenth of BO mRNA.

Translation of the β_2-AR transcript was shown by immunological detection of the myc-tagged β_2-AR which showed the appropriate electrophoretic mobility (Fig. 5). Western blots allowed an estimate of the levels of β_2-AR protein of several ng receptor protein per mg protein, which is less than 0.01% of the protein. Radio ligand binding gave levels of functional β_2-AR in the range of only 5 fmol/mg protein. BR in similarily transformed halobacteria is expressed at levels of about 50% of the membrane protein. Thus, if we compare the expression of BR and of β_2-AR, we found that the levels of β_2-AR mRNA are about one order of magnitude lower, those of the protein (Western blot) four orders of magnitude, and those of functional protein (ligand binding) six orders of magnitude. These estimates indicate that a substantial part of the receptor protein is non-functional, i.e. probably misfolded. Possible reasons for improper folding might be special growth conditions or factors of the expression machinery of *H. salinarium* that are not capable of correctly processing the newly synthesized β_2-AR protein.

In order to explore the possibility that the β_2-AR failed to utilize the BO synthesis machinery we created BO/β_2-AR-chimeras containing larger BO-N-termini. We reasoned that this might improve synthesis and processing of the receptor protein. However, we found no enhancement of the amounts of receptor protein expressed in *H. salinarium* (as assessed in Western blots), and at the same time we even observed a reduction in radioligand binding, suggesting that the chimeras were dysfunctional in their ligand binding properties (Fig. 7A). This was confirmed upon expression of the chimeras in eukaryotic HEK-293 cells, which showed in a very similar manner reductions in ^{125}I-CYP binding. The reasons for this decrease in ligand binding were not further investigated, but might involve disruption of the overall receptor arrangement induced by mal-fitting of the first transmembrane helix (Schertler et al. 1993; Ungerer and Schertler 1995; Suryanarayana et al. 1992; Pittel and Wess 1994).

We also examined the expression of the receptor in *Hf. volcanii*, which grows at lower salt concentrations. While the lipid composition of this halobacterium is very similar to that of *H. salinarium*, it does not synthesize halobacterial retinal proteins and therefore presumably lacks an efficient machinery for the synthesis of such proteins. Expression of β_2-AR in *Hf. volcanii* revealed ^{125}I-CYP binding which could be displaced by the agonist isoprenaline or the antagonist propranolol. Saturation analysis with ^{125}I-CYP gave a K_D of 59 ± 44 pM (Fig. 8) that was very similar to the K_D of 41 ± 2 pM obtained for β_2-AR expressed in CHO cells. The levels of functional β_2-AR as assessed by saturation analysis were 177 ± 49 fmol/mg protein and therefore were much higher than the levels achieved in *H. salinarium*. Furthermore, the affinities of the antagonist propranolol and the agonist isoprenaline were in the range expected for the native β_2-AR (Fig. 9), indicating that the receptor expressed in *Hf. volcanii* has normal ligand binding properties. Expression of the β_2-AR is in the range that is usual for several eukaryotic cells, or *E. coli* (Marullo et al. 1989; Chapot et al. 1990).

Recently, Archaea have been grouped in a separate domain. Some features of the Archaea resemble those of Bacteria, but others are closer to Eukarya (and are different from Bacteria). The membrane lipids of Archaea differ from both, Bacteria and Eukarya. They predominantly consist of isoprenoid glycerol diethers or diglycerol tetraethers. In contrast, diacyl glycerol diesters predominantly are found in eubacterial membranes and glycerol fatty acyl diesters in eukaryotic cells (reviewed in Woese et al. 1990). The morphology of archaebacteria, the lack of nuclei, the single circular chromosome, polycistronic operons, 70S-type ribosomes, and the organization of RNA polymerase subunit gene clusters are features similar to Bacteria. The features in common with Eukarya include the components of RNA polymerase, the RNA polymerase II-type promoter, transcription factors, sequences of ribosomal proteins, and the presence of tRNA introns (Brown et al. 1989; for review see Baumann et al. 1995). These similarities of Eukarya with Archaea might provide advantages for heterologous expression, but the differences could also hide disadvantages that are not considered so far.

In summary, we show here that heterologous expression is possible in halobacteria. This capacity can be used for the synthesis of the human β_2-AR, a member of the family of G protein-coupled receptors. However, in spite of the similar transmembrane topology of the β_2-AR and BR the levels of functional β_2-AR obtained in *H. salinarium* were modest compared to the high level synthesis of BR. Therefore we conclude that the efficient machinery for translation, insertion into the membrane and proper folding of BR cannot be transferred directly to heterologous expression of fully active β_2-ARs. In addition, other factors might be limiting for ligand binding activity in *H. salinarium*. Higher levels of active β_2-AR were expressed in *Hf. volcanii*. Therefore we believe that in *Hf. volcanii* other factors of the expression machinery are responsible for β_2-AR expression than in *H. salinarium* and that these factors dominate over the inability in *Hf. volcanii* to synthesize retinal proteins.

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