The Journal of Natural Science,
Pai Chai University, Korea
Vol.8 No2: 35~41, 1996

Effects on the Arginine Transport of Mutant MCAT1, Mouse Cationic Aminoacid Transporter

Jung Woo Kim
Dept. of Biochemistry, Pai-Chai University

MCAT1의 돌연변이체가 Arginine 통과 능력에 미치는 영향

김정우
배재대학교 생화학과

To find the substrate interacting site of the MCAT1, charged amino acid residues in the transmembrane domain were changed to opposite charged amino acids and studied the arginine uptake, gp70 binding, efflux and protein expression using the Xenopus oocyte expression method. Among the five mutants of MCAT1, the D403K showed the most interesting characteristics, which had normal gp70 binding but low arginine uptake function, that means the normal expression on the membrane but decreased transport function. All mutants except K211E showed decreased arginine efflux, and kinetic study showed decreased Vmax. Together, Glu(403) residue of MCAT1 may show the possible substrate interacting site in the transmembrane domain of MCAT1.

MCAT1 의 기질과 결합하는 장소를 조사하기 위해 세포막내에 존재하는 부분의 극성 아미노산을 반대 극성의 아미노산으로 치환시킨 돌연변이체를 만들어 arginine 통과능력, gp70 결합능, efflux, Xenopus oocyte 를 이용한 단백질 발현 능력등을 조사하였다. 다섯개의 돌연변이체중 D403K 이 가장 흥미로운 성질을 나타냈는데, 그것은 정상적인 gp 70 결합능력과 낮은 arginine 통과능력을 나타냈다는 것으로 정상적인 단백질이 세포막에서 발현되고 있으나 arginine 통과능력이 감소했다는 사실을 나타내는 것이다. K211E 를 제외한 모든 돌연변이체가 감소된 arginine efflux 와 감소된 Vmax 값을 나타내었다. 이들 결과로부터 MCAT1 의 403 번째 아미노산인 glutamic acid 가 세포막내에서 기질과 상호작용하는 장소라는 가능성을 시사하였다.

Key words : arginine transporter, transmembrane, charged aminoacid, gp70 binding, site-directed mutagenesis

I. Introduction

The murine cationic amino acid transporter (MCAT1) is a hydrophobic membrane protein, which was firstly known as a murine ecotropic retrovirus receptor.[1] This MCAT1 can transport arginine, lysine and ornithine, sodium and pH independently, showing the property of system y+.[2] On the basis of hydrophobic transmembrane domains connected by hydrophilic loops and short hydrophilic C-terminus. We have found that the third outside loop between fifth and sixth transmembrane domains is important for virus envelope binding and virus infection,[3] also found that this loop contained two glycosylation sites and virus binding to this loop partially blocked the

glycosylation.[4] This MCAT1 has a dual function of amino acid transport and virus infecton. Mutations on the virus binding site which causes no binding and no infection, did not affect the amino acid transport.[4] These results may explain the virus binding site and amino acid transport site are different, although it was not known which site is important for amino acid transport.

I tried to find out the important site in MCAT1 for amino acid transport by mutating charged amino acids in transmembane domains because I thought there should be ionic interactions between substrate and transmembrane domains. These sites are all conserved in known cationic amino acid transporters except one site which is changed to similar amino acid. Actually, some charged amino acids in the transmembrane domains are important for their function of transporters,[5,6] permeases[7,8] and receptors[9,10,11,12] for neurotransmitters which have several transmembrane domains.

This paper shows some of charged amino acid residued in the transmembrane domains of MCAT1 are important for amino acid transport function.

II. Materials and Methods

1. Construction of plasmids encoding mutant MCAT-1 proteins

Mutations were introduced into the charged amino acid residues in the putative transmembrane domains shown in Figure 1 by using a two step PCR-based protocol.[13] Complementary oligonucleotides that alter this codon were used as primers in parallel PCR reactions to amplify the cDNA encodinjg MCAT-1. For D107K, 107 Glu(GAG) was changed to Lys(AAG) by using the oligonucleotide, 5′-GTGACGGTGGGGAAGCTTTGGGCC-3′, as the sense primer. For D165K, 165Asp(GAC) was changed to Lys(AAG) using 5′-CCCAAACCCCGAAGATATTTGCTGTG-3′. For K211E, 211Lys(AAA) were changed to Glu (GAA) using 5′-GTGTCCGGGTTCGTGGAAGGCTCC-3′ as the sense primer. For E397K, 397Glu (GAA) was changed to Lys (AAA) using 5′-GCCTTCCTCTTTAAACTGAAGGACCTG-3′. For D403K, 403Asp(GAC)was changed to Lys(AAG) using the sense primer 5′-GGACCTGGTGAAGCTCATGTCCATTG-3′.

For each amplification of MCAT-1 cDNA by PCR, the sense primer bearing the mutation and antisense primer, 5′-CGGGATCCGTCATTTGCACTGGTCC-3′ from the 3′ end of the MCAT1 cDNA were used in one reaction. In separate reactons, the complementary mutant primers and the sense primer, 5′-GCGGATCCTAATGGGCTGCAAAAACC-3′ , from the 5′-end of the MCAT-1 cDNA were used for PCR on the same MCAT-1 cDNA template. The products of two matching reactions were purified on agarose gels and combined to provide the template for an additional PCR using the flanking 5′ and 3′ primers. The amplified product was digested with BamH1 and the 2 Kb restriction fragment containing the mutant sequence was subcloned into the mammalian pJ3 or oocyte pSP64T expression vector.[2] The presence of the mutation was confirmed by determining the nucleotide sequence.

2. Amino acid uptake and gp70 binding assay using Xenopus oocytes

Xenopus laevis was purchased from Xenopus 1 (Ann Arbor, MI) . The protocol for injection of oocytes has been described previously.[2] Oocytes injected with mutant MCAT1 RNAs were incubated two days in 50% L-15 medium before uptake and efflux study. After preincubation in the uptake medium for one hour, the oocytes were transfered to 0.1 ml of 0.1 mM cold amino acid uptake solution containing 0.5 μCi ^{14}C-labeled amino acid for 30 minuites and washed three times, then counted the radioactivity of the oocytes. For efflux, 5 nCi ^{14}C-labeled amino acid in 100mM

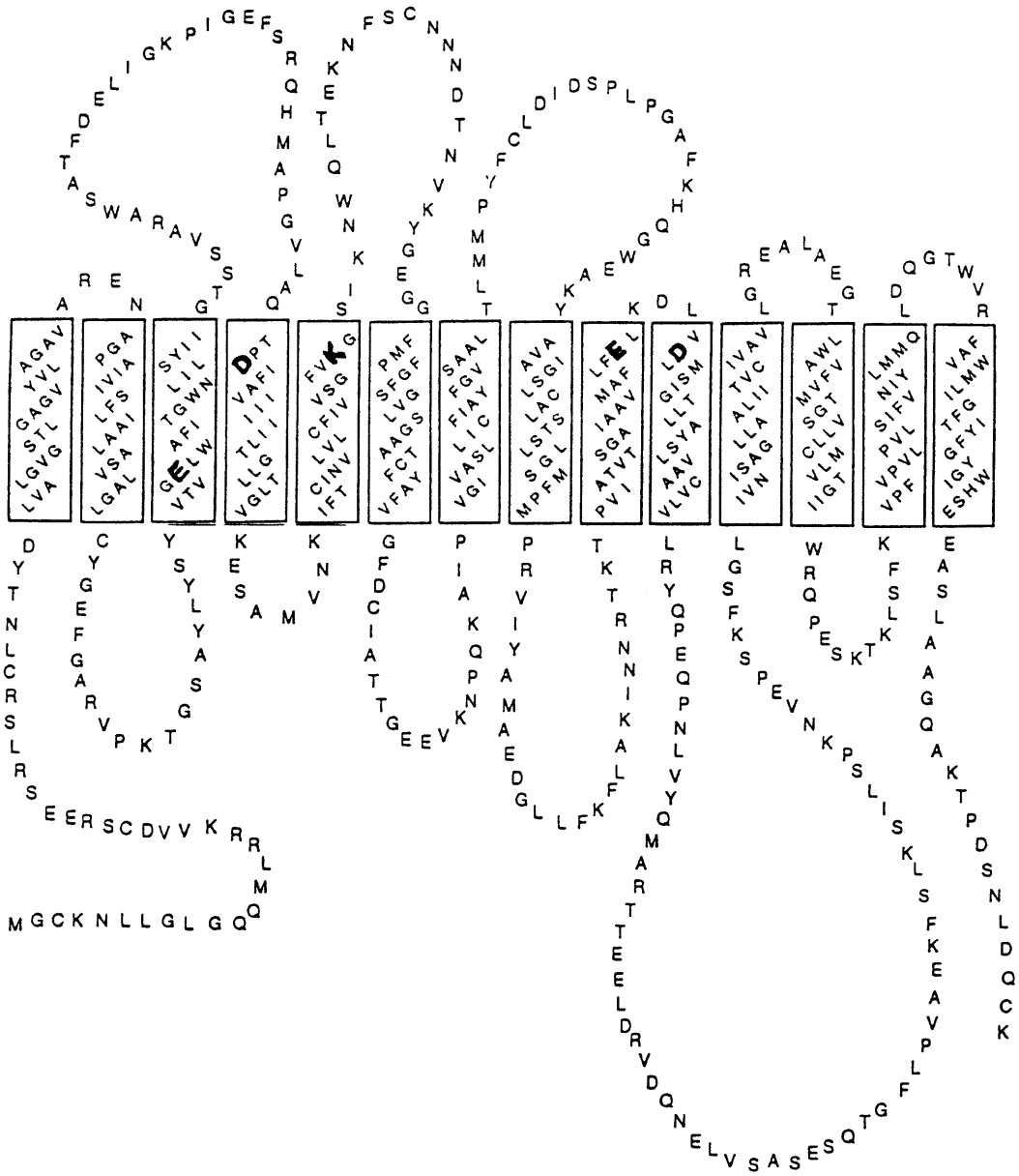

Figure 1. Amino acid sequence of putative fourteen transmembrane domains. The transmembrane domains were shown in boxes. Putative intramembrane charged amino acid residues (Glu107, Asp165, Lys211, Glu397, Asp403) that were converted to opposite charged amino acid, such as E107K, D165K, K211E, E397K, D403K which were shown in bold letters.

cold amino acid solution was injected to oocyte and quickly transfered to 0 and 5 mM cold amino acid solution for 30 minuites and counted the radioactivity of the medium. For

gp70 binding assay , the ^{125}I labelled gp 70 was added to the 5% BSA uptake solution (5 ng/0.1ml) after preincubation in the 1% BSA uptake solution.

The oocytes were washed three times with uptake solution followed by counting the radioactivity of oocytes.

3. Immunoblot

Ten oocytes injected three days previously with MCAT-1 or mutant RNA were

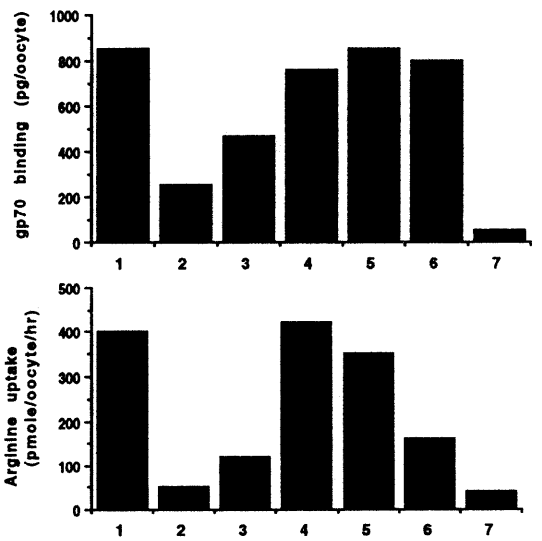

Figure 2. Functional expression of mutant MCAT1. Arginine uptake and gp70 binding assay were performed by using Xenopus oocytes injected with 10 ng RNAs of MCAT1 (lane 1), E107K (lane 2), D165K (lane 3), K211E (lane 4), E397K (lane 5), D403K (lane 6) and water (lane 7). Each value showed the mean of three experiments and three oocytes were used for one experiment.

extracted in RIPA buffer (50 μl) for 3 minutes and centrifuged at 10,000 x g for one minute. Supernatant was separated by SDS- PAGE (8 %) and transferred to nitrocellulose paper. After blocking the nitrocellulose paper with Blotto (50 mM Tris pH 8.0, 2 mM $CaCl_2$, 0.01% antifoam A, 0.05% Tween-20, 0.02% NaN_3), the paper was incubated in affinity-purified anti-MCAT-1 antibody (1:100, 1 hr, room temperature), washed three times in Blotto, and then incubated in ^{125}I goat anti-rabbit antibody for one hour at room temperature. The paper was then washed three times with TBST (10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween-20) and exposed to film

III. Results

There have been found several cationic amino acid transporters in mammals. The sequences of these genes showed very high homology in transmembrane domains. I looked into the five charged amino acid in transmembrane domains and found all sites were conserved. So I made an assuption that these sites are important for transporting the substrate as there should be an ionic

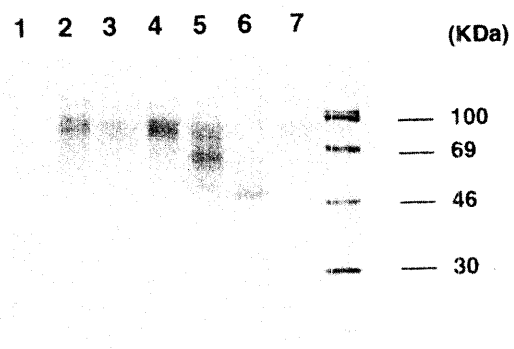

Figure 3. Western blot of mutant MCAT1. Mutant proteins were detected by rabbit anti-serum raised against MCAT1 C-terminal peptide. Protein extracts from 3 oocytes injected with water (lane 1), 10 ng RNAs of D403K (lane 2), E397K (lane 3), K211E (lane 4), D165K (lane 5), E107K (lane 6), and MCAT1 (lane 7), were seperated on the 8% SDS-PAGE and transfered to nitrocellulose paper. The paper was exposed for three days after treatment of anti-MCAT1 antibody and ^{125}I labelled secondary goat Ig G.

interaction between transporter and substrate, and I tried to make the mutant for those sites. As was shown in figure 1, there were five charged amino acid residues in transmembrane domains. These site were changed to opposite charged amino acid by using PCR site directed mutagenesis method. E107K was made by changing the 107Glu(GAG) in third transmembrane domain to Lys(AAG), D165K was made by changing the 165Asp(GAC) in the forth transmembrane domain to Lys(AAG), K211E was made by changing the 211Lys (AAA) in the fifth transmembrane domain to Glu(GAA), E397K was made by changing the 397Glu(GAA) in the ninth transmembrane domain to Lys(AAA), and D403K was made by changing the 403Asp(GAC) in the tenth transmembrane domain to Lys(AAG). These PCR products were inserted into pSP64T plasmid which has the Sp6 promoter region, and used to produce the RNAs in vitro. Following the microinjection method into the Xenopus oocytes, the amino acid uptake and gp70 binding assay were performed using oocytes injected with these mutant MCAT1 RNAs. Figure 2 showed the results of the arginine uptake and gp70 binding assay. Mutants showed over 70% gp70 binding ability except E107K which showed 20 % gp70 binding ability. But the arginine uptake showed quite different pattern. K211E and E397K was similar to wild type (over 60 %), E107K showed almost no arginine uptake, D165K and D403K showed 30 % arginine uptake compared to MCAT1. To know the expression in the oocytes, western blot study was performed using oocytes injected with mutant RNAs. As was shown in figure 3, E107K showed different patern in the mobility as low as the unglycosylated form of MCAT1 as previously

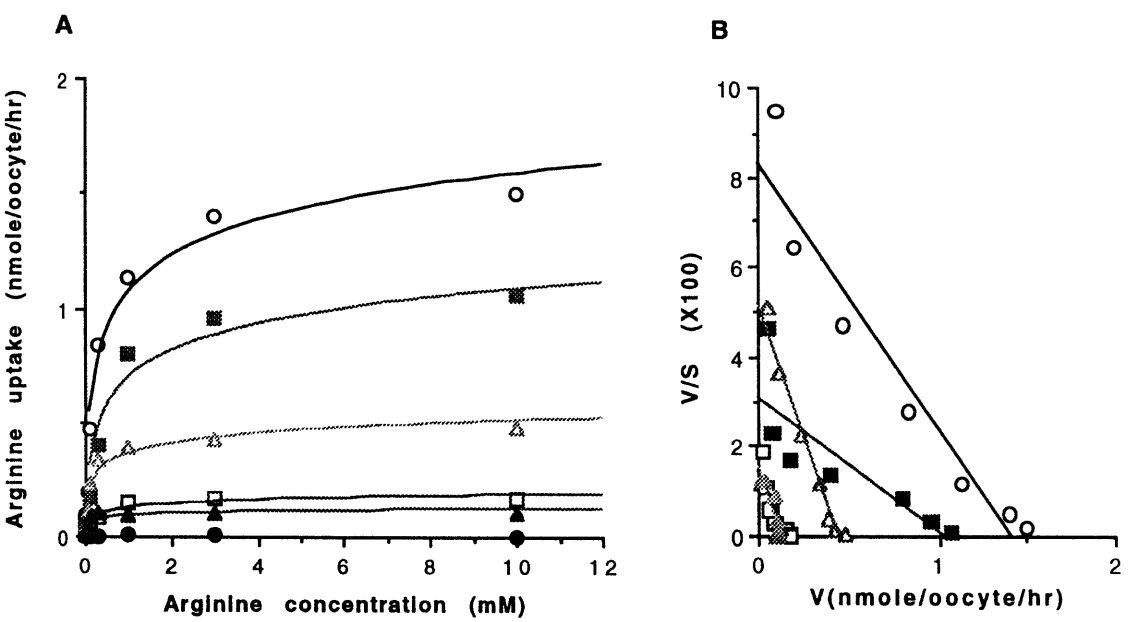

Figure 4. Kinetic study of mutant MCAT1. A) Arginine uptake was measured in various arginine concentrations by using oocytes injected with MCAT1 RNA (open circle), E107K RNA (closed circle), D165K RNA (open squire), K211E RNA (closed circle), E397K RNA (open triangle) and D403K RNA (closed triangle). The values obtained from water injected oocytes were subtracted from those mutant RNA injected oocytes. Each points are the mean of 3 oocytes. B) Arginine uptake was showed to Eadie-Hofstee plot.

reported.[4] D165K showed several bands in the gel including the same molecule as the MCAT1. K211E, E397K and D403K showed same pattern in the mobility as the MCAT1. These results correlated with the gp70 binding as D165K, K211E, E397K and D403K have similar gp70 binding capacity compared to MCAT1 and have fully glycosylated form, where E107K is not. Figure 4 illustrated the Km and Vmax of the arginine transport. The results showed that K211E and E397K have similar Km to MCAT1 but low Vmax, and E107K, D165K and D403K have much less arginine transport function. Also efflux study showed E107K, D165K and D403K have malfunction of efflux as illustrated in figure 5. This result showed that these amino acid sites are important for efflux not only for influx.

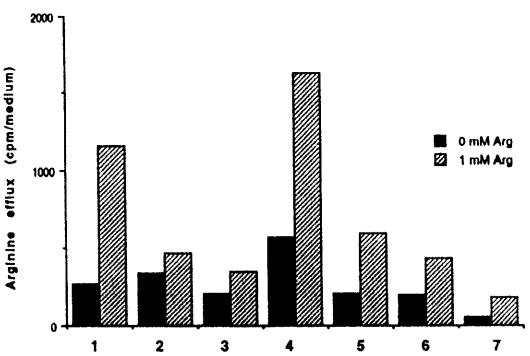

Figure 5. Efflux study of mutant MCAT1. Oocytes injected with 10 ng RNAs of MCAT1 (lane 1), E107K (lane 2), D165K (lane 3), K211E (lane 4), E397K (lane 5), D403K (lane 6), and water (lane 7) were reinjected 2 days later with 50 nl of ^{14}C arginine and 10 nmole of unlabelled arginine (cis arginine). Immediately after the second injection arginine efflux was measured by transferring oocytes (3 oocytes/0.1 ml) into isotonic solution containing 0 and 1 mM unlabelled arginine (trans arginine). Each point is the mean of data obtained from two experiments.

IV. Discussion

The conserved charged amino acid residues in the transmembrane domains seems to be very important not only for its function but also its proper localization or modification of the protein. As shown in some other proteins, the transmembrane domains are important for its golgi localization and transport to plasma membrane. In case of E107K, that one amino acid change causes dramatic effect on the amino acid transport function and the decreased molecular weight. Although it is not known to be in the plasma membrane, E107K has not been properly glycosylated and may not be properly folded so that the virus envelope protein can not bind to its binding site properly. D165K does not seemed to be properly processed to the plasma membrane, because the small molecular weight proteins are existed, although the fully processed molecules also exist. The existence of fully glycosylated form of D165K, K211E, E397K and D403K correlated with gp70 binding result, but not with the amino acid transport. K211E and E397K are quite a good transporter and also a virus receptor. D403K showed quite a good gp70 binding function, but not a good amino acid transport function. Also efflux study showed E107K, D165K, E397K and D403K have malfunction of efflux of arginine transport into medium. These data showed that these mutants clearly effect the amino acid transport function both influx and efflux. Among these mutants D403K has the most interesting function in amino acid transport as it transport arginine less than 40 % compared to MCAT1, and it showed normal gp70 binding which means that protein normally expressed in the plasma membrane. Again, amino acid 403 residue seems to be the one of the substrate interacting site of the MCAT1 protein.

Acknowledgements

I wish to thank Dr. J.M. Cunningham for his help to do an experiment in his laboratory. This study was financially supported by a Central Research Fund for the year of 1995 from Pai-Chai University.

References

1. Albritton, L.M., L. Tseng, D. Scadden, and J.M. Cunningham. (1989) A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57: 659-666.
2. Kim, J.W., E.I. Closs, L.M. Albritton, and J.M. Cunningham. (1991) Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature 352: 725-728.
3. Albritton, L.M., J.W. Kim, L. Tseng, and J.M. Cunningham. (1993) Envelope binding domain in the cationic amino acid transporter determines the host rangeof ecotropic murine retroviruses. J. Virol. 67: 2091-2096.
4. Kim, J.W. and J.M. Cunningham. (1993) N-linked glycosylation of the receptor for murine ecotropic retroviruses is altered in virus-infected cells. J. Biol. Chem. 268: 16316-16320.
5. Pantanowitz, S., A. Bendahan, and B. Kanner. (1993) Only one of the charged amino acids located in the transmembrane α-helices of the γ-aminobutyric acid transporter (subtype A) is essential for its activity. J. Biol. Chem. 268: 3222-3225
6. Kitayama, S., S. Shimada, H. Xu, L. Markham, D.M. Donovan, and U. G.R. (1992) Dopamine transporter site-directed mutations differentially alter substrate transport and cocain binding. Proc. Natl. Acad. Sci. USA 89: 7782-7785.
7. Sahin-Toth, M., R. Dunten, A. Gonzalez, and H.R. Kaback. (1992) Functional interactions between putative intramembrane charged residues in the lactose permease of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 89: 10547-10551.
8. Pourcher, T., M.L. Zani, and G. Leblanc. (1993) Mutagenesis of acidic residues in putative membrane-spanning segments of the melibiose permease of *Escherichia coli*-1. Effect on Na-dependent transport and binding properties. J. Biol. Chem. 268: 3209-3215.
9. Galzi, J.L., A. Devillers-Thiery, N. Hussy, S. Bertrand, J.P. Changgeux, and D. Bertrand. (1992) Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. Nature 359: 500-505.
10. Ji. I. and T.H. Ji. (1991) Asp383 in the second transmembrane domain of the lutropin receptor is important for high affinity hormone binding and cAMP production. J. Biol. Chem. 266: 14953-14957.
11. Revah, F., D. Bertrand, J.L. Galzi, A. Devillers-Thiery, C. Mulle, N. Hussy, S. Bertrand, M. Ballivet, and J.P. Changeux. (1991) Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. Nature 353: 846-849.
12. Suryanarayana, S., D.A. Daunt, M.V. Zastrow, and B.K. Kobilka. (1991) A point mutation in the seventh hydrophobic domain of the α2 adrenergic receptor increases its affinity for a family of β receptor antagonists. J. Biol. Chem. 266: 15488-15492.
13 Higuchi, R., B. Kurummel, and R.K. Saiki. (1988) Nucleic Acids Res. 16, 7351-7367.