ISOLATION, CLONING, AND SEQUENCING OF A PORCINE MELANOCORTIN-1 RECEPTOR LIKE GENE

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ABSTRACT

Initially we started out to isolate, clone, and sequence the porcine Melanocortin-1 Receptor (MC1R) in swine (*Sus scrofa*). Using human MC1R primers we used PCR to amplify the swine DNA to isolate the MC1R gene. However, we have isolated what may be a melanocortin-1 receptor-like gene in pigs but not the actual MC1R. BLAST searches indicate that our data sequence is statistically similar to the human melanocortin-1 receptor. However other computer analysis indicate that our sequence is roughly 40% similar to various melanocortin-1 receptors of related species. We attempted to isolate 18 base pairs on the 3`end of this gene. Initial analysis of the 3` sequence indicates that we have not isolated the same gene sequence.

Keywords

Melanocortin-1 Receptor, Agouti protein, Pig, Alpha-Melanocyte Stimulating Hormone (a-MSH).

INTRODUCTION

The Melanocortin Receptor-1 (MC1R) in humans and other mammals is coupled to a G-protein complex that is partially responsible for pigmentation in mammals At least two different gene products competitively bind to this receptor. One of the gene products is alpha-Melanocyte Stimulating Hormone (a-MSH), and it causes normal pigmentation pattern of the mammal when it binds to MC1R. The other gene product is the agouti protein. Agouti protein as well as other melanocortins act through G-protein receptors to modify metabolism in a wide variety of cells.(Cone et al. 1996). Agouti protein, a competitive inhibitor of a-MSH, acts on Melanocortin receptors one and four (MC1R and MC4R, Lu et al., 1994.)

There are five distinct melanocortin receptors (MC1R-MC5R) which exhibit wide tissue distribution in mammals. The lethal yellow allele (A^y) causes an

aberrant overproduction of agouti protein in virtually all tissues. A^y may promote the lethal yellow syndrome (i.e. obesity, yellow coat, diabetes, infertility, compromised immunity, and greater body size) by virtue of its competitive inhibition of the melanocortins. The agouti gene is thought to be highly conserved in mammals (Searle, 1968; Silvers, 1979). Both murine (Bultman et al., 1992; Miller et al., 1993) and human (Kwon et al., 1994; Wilson et al., 1995) agouti genes have been isolated, cloned, and sequenced (Wang et al. 1998).

Alpha-Melanocyte Stimulating Hormone on the other hand does not cause the lethal yellow syndrome problems when it binds to the melanocortin-1 receptor. In this situation the mammal exhibits normal pigmentation, body weight, fat/protein body composition, normal metabolism, and fertility. The purpose of this study is to identify and characterize genes that encode one or more members of the melanocortin receptor family. We have cloned and identified a sequence which may be a melanocortin-1 receptor like gene.

METHODS

Because of their fundamental role in metabolism, we want to characterize the melanocortin-1 receptor (MC1R) in pigs. Since agouti protein and a-MSH ligands competitively bind to MC1R, we were interested in isolating, cloning, and sequencing the MC1R. However, about six months into our research we discovered that Lief Andersson's group in Sweden had isolated, cloned, and sequenced the porcine MC1R from swine. At the same time though through Polymerase Chain Reaction (PCR) we were consistently amplifying an 800 base pair fragment that we believed to be the porcine MC1R. We cloned this 800 base pair DNA into competent *E. coli* cells via the pCRII vector. The transformant E. coli cells were grown in LB-Kanamycin medium and the plasmid DNA was isolated from the cells using the *Power prep minikit* which is used to isolate plasmid DNA from chromosomal DNA and other cellular debris. A restriction enzyme digest was performed using (EcoR1) to determine that the 800 base pair insert was actually in the vector. Analysis of the restriction enzyme digestion by agarose gel electrophoresis indicated that the insert was in fact present. Once we verified presence of the insert DNA in the cells, we then isolated the plasmid DNA that contained the insert DNA and sent it to Iowa State Sequencing Facility. In order to sequence the DNA, they employed a technique called Primer Walking to complete the sequencing. Our next step was to analyze the data from the sequencing facility (Fig. 1). In order to determine what gene or genes we had isolated we employed three computer programs 1) Gene Jockey, 2) Gene Inspector, and 3) BLAST (Basic Local Alignment Tool).

RESULTS

Gene Jockey Analysis

Gene Jockey is used to align two different sequences and determine the percent similarity between the two. Since the PCR primers that we designed were from the human melanocortin-1 receptor it seemed logical to align our

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          10          20          30          40          50
           |           |           |           |           |
ATGAGTCCCT GCCCTGAGAC TGTGGTGTG TGAGGGTCCT GGCATGAGTC
          60          70          80          90         100
           |           |           |           |           |
CTGCTTGGCA AGAGACTTGG AGACGGGGGC GGGGAGCAAG CCCCCCTGGG
         110         120         130         140         150
           |           |           |           |           |
CTAGAACTGT ACGTCACCAC TGATAGCCAT GACTTCAGCC CACCCCACCC
         160         170         180         190         200
           |           |           |           |           |
ACCCCCCACA TGCTGTGGGA TGGAGGTCCC TGTCACCAGG CTGCCCCCAC
         210         220         230         240         250
           |           |           |           |           |
CTGGCTGCTG GAGCCCCTTG AGGAACAGAC CCTTGTCTCA GGCTCAGCAT
         260         270         280         290         300
           |           |           |           |           |
CAGTCTCGGC TGGCCGGTTG GACACTCAGC CACATGGCCC TGGGAGCCGG
         310         320         330
           |           |           |
AGCTGATGAC GCGCGGTGGC CAGAGAGAGA TGCTGA
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Figure 1: Nucleotide Sequence of Porcine MC1R-like Candidate Gene

data with the human MC1R. In fact we aligned our data with human, bovine, goat, dog, fox, and horse melanocortin-1 receptor sequences. This analysis demonstrated that our sequence was roughly 40% similar with each of the different melanocortin-1 receptors.

Gene Inspector Analysis

Gene Inspector is an alternate program that can be used to align different sequences, determine percent GC content, and derive the amino acid translation from the nucleotides (Fig. 2). This program also yielded results showing our sequence is about 40% similar to the other melanocortin receptors. An interesting aspect of this program was, however, that it was able to determine the possible open reading frame (ORF) in which the isolated gene is read. Our sequence is read in ORF 2.

BLAST Search Analysis

Basic Local Alignment Search Tool is used to compare our sequence data to every entry submitted to and contained in GenBank. This search tool gave encouraging results. It displayed a statistically significant match with the Human Melanocortin-1 Receptor DNA and mRNA. However, these results also conflict with Gene Jockey and Gene Inspector. Both of those programs indi-

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          10         20         30         40         50
          |          |          |          |          |
MSPCPETVVC EGPGMSPAWQ ETWRRGRGAS PPGLELYVTT DSHDFSPPHP
          60         70         80         90         100
          |          |          |          |          |
PPTCCGMEVP VTRLPPPGCW SPLRNRPLSQ AQHQSRLAGW TLSHMALGAG
          110
          |
ADDAVARERC
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Figure 2. Deduced Amino Acid Sequence of Porcine MC1R-like Candidate Gene

cated that our nucleotide and amino acid sequence did not match up with the other Melanocortin-1 Receptors any better than 40% similarity. The BLAST search gave a 4e-05 "hit", with any hit smaller than 0.1 being considered significant (Cheesbrough, 1999).

While we believe we have isolated an MC1R-like candidate gene we are still are not sure regarding our ability to define it as an MC1R-like sequence. Therefore we have isolated and sequenced a small segment of the MC1R gene that our initial PCR primers did not cover. This small piece was 18 base pairs at the 3` end of the gene. When we designed these primers, we overlapped our original sequence with roughly 450 base pairs. The purpose of this was two-fold. First, it is not possible to amplify an eighteen base pair segment through PCR because of its size. Second, this overlap would tell us if we had isolated the original gene. This DNA was amplified
through PCR, the DNA was cloned via the pCRII vector into *E. coli* and sequenced at Iowa State as described above.

CONCLUSION

Upon preliminary examination of the most recent results it does not appear that the original and the latter genes are identical. We believe we may have a melanocortin like gene because repeated BLAST searches have indicated the high statistical similarity between the human MC1R and both of our data sets. The computer software used for gene analysis has given some conflicting results that we are still trying to sort through. The BLAST search indicates that both sets of our data are similar to the human MC1R which is what one would expect since our PCR primers were designed from the human MC1R. However, when we line up the sequences with Gene Jockey or Gene Inspector, the percent similarities are only about 40%. Therefore we are still in the midst of determining what our data actually means.

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