Short title:

DNA polymorphisms in the bovine ODC and GH genes

ABSTRACT

Ph.D. Jianbo Yao Animal Science

The ornithine decarboxylase (ODC) and growth hormone (GH) genes are candidate genes for milk production traits in dairy cattle. In this study, the bovine ODC gene was cloned and characterized, followed by studies of genetic variations of the ODC and GH genes in Holsteins. A cDNA coding for bovine ODC was first isolated and analyzed. The clone (1758 bp) consists of 5'- and 3'-untranslated regions of 185 and 187 nucleotides, respectively, and an open reading frame of 1383 nucleotides encoding an ODC protein of 461 amino acids. Cloning and sequence analysis of the ODC genomic gene (9452 bp) revealed that the gene consists of 12 exons separated by 11 introns. The structure and nucleotide sequence of the gene were shown to be highly conserved among several mammalian species. Southern blot analysis identified 4 restriction fragment length polymorphisms (RFLPs) at the ODC locus. Two of them, a *Taq*I and a *Msp*I, were mapped to the ODC gene and PCR-based methods for rapid detection of the 2 RFLPs were developed. These polymorphisms were analyzed in 127 Holstein bulls and 3 of the 4 possible combinations from the 2 polymorphisms were observed indicating the presence of 3 ODC alleles (designated A1, A2 and A4). There were significant changes (X^2 test: $P<0.005$) in allele frequency between bulls born in 1950-1970 and bulls born in the 1980s. The frequency of the A2 allele increased, whereas, that of the A4 allele decreased in the bulls born in the 1980s. In addition, significant effect of ODC genotypes on milk protein content was observed. To search for sequence variations in the bovine GH gene, 7 gene fragments covering almost the entire length of the

gene (2.7 kb) were PCR amplified and subjected to single strand conformation polymorphism (SSCP) analysis. SSCPs were detected in 4 of these fragments and a total of 6 diallelic polymorphisms were found in a sample of 128 Holstein bulls. Two of the polymorphisms, a T to C transition in the third intron and an A to C transversion in the fifth exon, were shown to have significant effects on either milk yield ($P<0.0006$ and 0.0004, respectively), fat content ($P<0.0225$ and 0.0217, respectively) or protein content ($P<0.0038$ and 0.0037, respectively). The average effects of the gene substitution for the two polymorphisms were similar, with ± 300 kg for milk yield, ± 8 kg for fat content and ±7 kg for protein content per lactation. The results of this study indicated that both ODC and GH genes are highly polymorphic and some polymorphisms are trait-associated. The use of these polymorphisms as genetic markers may have potential in the future for improving milk performance in dairy cattle.

RÉSUMÉ

Les gènes de l'ornithine décarboxylase (ODC) et de l'hormone de croissance (GH) sont deux gènes intéressants pour des traits de production laitière chez les bovins. Dans cette étude, le gène de l'ODC a été cloné et caractérisé, puis les variations génétiques des gènes de l'ODC et de la GH ont été analysées chez la Holstein. Le clone (1758 pb) comprend des régions non-traduites en 5' et en 3' de 185 et 187 nucléotides respectivement, ainsi qu'un cadre de lecture de 1383 nucléotides qui code pour une protéine ODC de 461 acides aminés. Le clonage et l'analyse de séquence du gène de l'ODC (9452 pb) a révélé que le gène est composé de 12 exons séparés par 11 introns. La structure et la séquence de nucléotides du gène ont démontré être hautement conservées au sein des mammiferès. Quatre polymorphismes de longueur des fragments de restriction (RFLPs) ont été identifiés sur le locus de l'ODC par Southern blot. Deux d'entre eux, un *Taq*I et un *Msp*I, ont été localisés au niveau du gène. Deux méthodes de détection par la réaction de polymérisation en chaîne (PCR) ont été élaborées pour déterminer rapidement le génotype sur ces deux sites chez 127 taureaux de race Holstein. Trois des quatre combinaisons possibles à partir de ces deux sites de restriction ont été observées donnant lieu à trois allèles différents pour le gène de l'ODC (désignés A1, A2, et A4). Des changements significatifs (X^2 test: $P<0.005$) dans la fréquence des allèles entre les taureaux nés entre 1950 et 1970 et les taureaux nés entre 1970 et 1980 ont été observés. La fréquence de l'allèle A2 a augmentée alors que celle de l'allèle A4 a diminuée chez les taureaux nés entre 1980 et 1990. De plus, un effet significatif du

génotype de l'ODC sur la teneur en protéines du lait a été observé. Dans le but de chercher des variations dans la séquence de l'hormone de croissance, sept fragments du gène qui couvrent presque la totalité du gène, (2.7 kbp) ont été amplifiés par PCR et analysés par la technique de conformation polymorphique de simple brins (SSCP). Six diallelique polymorphismes ont été détectés dans quatre de ces fragments à l'intérieur d'un échantillon de 128 taureaux de race Holstein. Deux de ces fragments avaient deux polymorphismes chacun alors que les deux autres fragments en avaient chacun un seul. Pour deux de ces polymorphismes, une transition de T à C dans le troisième intron et une transversion de A à C dans le cinquième exon ont été observées. Ces deux polymorphismes avaient des effets significatifs sur la production de lait ($P<0.0225$ et 0.0217, respectivement) ou sur le contenu des protéines ($P<0.0038$ et 0.0037, respectivement). La moyenne des effets de la substitution génétique pour les deux polymorphismes était similaire, avec ± 300 kg pour la production de lait, ± 8 kg pour le contenu en gras, et ± 7 kg pour le contenu en protéines par lactation. Les résultats de cette étude montrent que les gènes de l'ODC et de la GH sont fortement polymorphiques et que certains de ces polymorphismes sont associes à des traits de production. L'utilisation de ces polymorphismes comme marqueurs génétiques pourrait avoir du potentiel pour améliorer la performance de la production laitière chez les bovins dans le futur.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor, Dr. D. Zadworny for his invaluable advice, guidance, encouragement and generous support throughout the course of this study. I also would like to extend my great thanks to Dr. U. Kühnlein for his invaluable advice, kindness and tremendous assistance.

I am greatly indebted to Dr. J. F. Hayes for his kindness, helpful suggestions and his input in the quantitative analysis of the study. Special thanks are due to Dr. S.E. Aggrey for his assistance in the statistical analysis of the data.

I also would like to thank Ms. L. Volkov for her expert technical assistance and all the fellow graduate students for their help and friendship. My special thanks go to Nicola for the French translation of the abstract.

I am grateful to the Department of Animal Science for providing the necessary facilities for my study. Special thanks are due to the secretaries of the department, Barbara, Lisa, Joyce and Sharon for their help and friendship.

I wish to express my deep appreciation to CEMARP for granting me a fellowship, the Canadian Association of Animal Breeders and the Natural Science and Engineering Research Council (NSERC) of Canada for providing financial support for the project. My appreciation is also extended to the Centre d'insemination artificielle du Quebec, the Western Ontario Breeders Inc. and the United Breeder Inc. for providing the bovine semen samples.

Finally, my deepest gratitude goes to my dear wife, Wei Wang for her love, patience, and support, and my little son, Raymon for giving me joy always.

CONTRIBUTIONS TO KNOWLEDGE

1) A cDNA coding for bovine ODC was cloned and characterized for the first time. It is also the first reported ODC cDNA in domestic animals. The nucleotide sequence of the cDNA has been submitted to GenBank (Accession number: M92441) and the amino acid sequence of the enzyme has been deduced from the cDNA sequence.

2) Four RFLPs in the bovine ODC gene were detected using the cloned cDNA as a probe in Southern hybridization. All 4 polymorphisms were reported for the first time and it is the first study showing that the bovine ODC gene is highly polymorphic.

3) A genomic clone containing the bovine ODC gene was isolated and the nucleotide sequence of the bovine ODC genomic gene (9452 bp) was determined. The structure of the gene was elucidated and several regulatory elements were found in the promoter region of the gene. The nucleotide sequence of the bovine ODC gene has been submitted to GenBank (Accession number: U36394).

4) Two of the polymorphic sites, namely a *Msp*I and a *Taq*I restriction site, were mapped on the ODC gene and both polymorphisms were characterized by sequence analysis. PCR-based methods for rapid detection of the 2 polymorphisms were developed and some ODC alleles were shown to be associated with selection for milk production.

6) Almost the entire length of the bovine GH gene was screened for the first time for sequence variations by using SSCP analysis. Six diallelic polymorphisms were

identified and characterized. Four of them were new and were reported for the first time. Two GH polymorphisms were found to be strongly associated with milk production traits in dairy cattle.

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MANUSCRIPTS AND AUTHORSHIP

The following text, from the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research, must be "cited in full in the introductory sections of any thesis to which it applies" :

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to the accuracy of such claims at the Ph.D. Oral Defence. Since the task of the examiners is made more difficult in thesis cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers".

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

The main body of this thesis consists of 4 manuscripts corresponding to chapters 3, 4, 5 and 6. Manuscript I (Chapter 3) has been published in *Genome* (38: 325-331, 1995). Manuscript II (Chapter 4) has been submitted to *DNA Sequence* for publication. Manuscript III (Chapter 5) is expanded from a paper which has been published in *Animal Genetics* (27: 283-284, 1996). Manuscript IV (Chapter 6) has been published in *Genetics* (144: 1809-1816, 1996). Professors Dr. D. Zadworny, Dr. U. Kuhnlein and Dr. J.F. Hayes are co-authors on all manuscripts. Dr. D. Zadworny, my supervisor, was responsible for the overall design and supervision of the research project. Dr. U. Kuhnlein, a co-investigator of the project, helped supervising and correcting the manuscripts. Dr. J.F. Hayes helped in obtaining the bovine semen samples, provided bull's breeding value data and gave advice in statistical analysis. Dr. S.E. Aggrey is a co-author on manuscripts II, III and IV. He made major contributions to the statistical analysis of the data.

CHAPTER 1. INTRODUCTION

Studies in theoretical quantitative genetics have suggested that genetic gain can be achieved using genetic markers that are closely linked to genes that affect quantitative traits (Soller and Beckmann, 1983; Simth and Simpson, 1986). The direct genotyping of an animal for the marker loci linked to quantitative trait loci (QTL) would increase the accuracy of selection by avoiding the confounding effects of environmental factors which are inherent in the present selection methods. Two main approaches have been proposed to the identification of genetic markers linked to QTL (Routman and Cheverud, 1994). One approach is to saturate the genome with markers and evaluate the effect of each marker on the trait by crossing lines and testing for co-segregation of markers and traits. The other approach, or "candidate" gene approach, is to evaluate the effect of genes of known physiological function on a trait of interest. For large species with a long generation interval which produce few offspring such as dairy cattle, this approach is particularly practicable as the development of specific lines with large population size is not economically feasible. Successful applications of this approach for many traits in different species have been described recently (Sneyers *et al.*, 1994; Casas-Carrillo *et al.*, 1994; Rothschild *et al.*, 1996; Lagziel *et al.*, 1996). In dairy cattle, genes associated with mammary growth, development and function are excellent candidate genes for linkage studies with milk related traits. Genes coding for ornithine decarboxylase (ODC) and growth hormone (GH) are two such candidate genes.

ODC is the first and key enzyme in the biosynthesis of polyamines that are

essential for DNA replication, cell proliferation, development and growth (Pegg, 1986). It has been shown that trophic hormones such as prolactin (PRL) and GH that are associated with lactation are required to induce differentiation of bovine mammary epithelial cells (Huynh *et al.*, 1991; Flint and Gardner, 1994), and both mitogenic and lactogenic effects of PRL, insulin and hydrocortisone appear to be mediated through the polyamine pathway (Rillema *et al.*, 1977; 1986; Bedford and Zadworny, 1990). In addition, ODC is highly expressed in the lactating mammary gland and a dramatic decrease in expression immediately precedes the onset of mammary involution (Strange *et al.*, 1992). Furthermore, genetic variants of ODC have been identified in a number of species that have been divergently selected for growth related traits (Bulfield *et al.*, 1988; Gray and Tait, 1993). Thus, it is possible that particular variants of ODC could be associated with increased mammary gland function and thus influence milking performance.

GH is the central hormone of the somatotropic axis, which in conjunction with insulin-like growth factors (IGFs), is essential for postnatal growth in animals (Scanes and Daughaday, 1995). Numerous studies have shown that treatment with exogenous GH affects many growth-related traits such as rate of gain, feed efficiency and lean meat deposition (Scanes, 1995). GH also affects both the growth and function of the bovine mammary gland (Knight *et al.*, 1982). When administered to young, peri-pubertal heifers, GH stimulates a significant increase in mammary gland development (Sejrsen *et al.*, 1986). Similarly, administration of GH to lactating dairy cows induces a well-documented rise in milk production (Bauman, 1992; Burton *et al.*, 1994). Some studies have also indicated that selection for milk yield is associated with increased

plasma levels of GH (Peel and Bauman, 1987; Bronczek *et al.*, 1988; Lukes *et al.*, 1989) and GH variants can influence the circulating concentrations of GH (Schlee *et al.*, 1994). Therefore, the GH gene is a promising candidate gene for studying gene-specific DNA polymorphisms in association with milk performance and growth.

The main objectives of this study were to clone and characterize the bovine ODC gene, to identify DNA polymorphisms in both the ODC and GH genes, and to further investigate into the association of the polymorphisms with milk production traits in Holstein dairy cattle. Such knowledge should provide the basis for characterizing the molecular nature of the allelic variation which affects quantitative traits and the development of marker-assisted selection for milk production in dairy cattle.

CHAPTER 2. LITERATURE REVIEW

2.1 Molecular methods for detecting DNA sequence polymorphisms

2.1.1 Restriction fragment length polymorphism (RFLP)

RFLP was the first DNA-level polymorphism described by Botstein *et al.* (1980). The discovery of RFLP was based on the finding of a group of so-called restriction enzymes, each of which is able to cut the DNA molecule at a large number of specific sites. Base changes can alter the DNA sequences that are recognized by these enzymes, abolishing sites or creating new sites for particular enzymes. Deletion or transpositions of large elements will make simultaneous changes in the restriction patterns of a number of enzymes. As a result, a given restriction enzyme will not always cleave a given DNA molecule at the same point in different individuals. Consequently, fragments of different length will be formed when the DNA of the different individuals is digested. The unequal-sized fragments will travel at different rates through the gel and the band formed, following hybridization (with radiolabelled DNA fragment of a homologous sequence) and autoradiography, will be located at different positions on the film. In this way, a RFLP will have been demonstrated.

The main advantages of RFLPs are that they are inherited codominantly, available in all tissues and organs, at all ages, in both sexes and independent of gene activity or gene products. However, because the production of a new allele depends on a point mutation, the number of alleles at a given locus is limited and so is the level of polymorphism.

2.1.2 Variable number of tandem repeats (VNTR)

Following the discovery of RFLP, another type of RFLP, based on minisatellites (Jeffreys *et al.*, 1985a) or variable number of tandem repeats (VNTR, Nakumura *et al.*, 1987) has been developed. Minisatellites are regions of DNA that contain a number of repeated sequences in tandem, and the length of the restriction fragment is determined by the number of copies of the repeats within the minisatellites. There is considerable variability among individuals as to the number of repeats of a sequence at a particular locus. When restriction digests of total cellular DNA are probed with radiolabled repeats, an autoradiograph with large numbers of bands will be observed that cannot easily be assigned to specific loci. Because molecular size and intensity of bands are unique, no two individuals from an outbred population would have identical patterns. This characteristic justifies the term 'DNA fingerprint' (Jeffreys *et al.*, 1985b). The advantage of using DNA fingerprints is that polymorphism is abundant, and differences at many loci can be revealed in a single assay. On the other hand, it is difficult or impossible to follow the segregation of individual loci.

2.1.3 Random amplified polymorphic DNA (RAPD)

The RAPD technique is a PCR-based method which was developed by Williams *et al.* (1990) and Welsh and McClelland (1990). It differs from standard PCR in that the normal pair of 20- to 30-base primers specific for a particular gene region is replaced by a single 10-base primer. This primer, by chance alone, may be complementary to the sequence at a variety of sites in the genome. Occasionally, the

primer will hybridize at sites close together and in opposite orientation so that the PCR reaction can proceed, and a fragment will be amplified. Each 10-base primer usually produces a number of bands, and by using an array of different primers a large number of markers can by developed in a very short time.

Because the sequences of the 10-base primers are arbitrary, nothing need be known about the sequence of the template DNA. This eliminates an enormous amount of labour associated with cloning and sequencing to obtain specific primers. However, most RAPD markers show dominant inheritance. This feature can be a disadvantage for applications where it is important to distinguish homozygotes from heterozygotes.

2.1.4 Single strand conformation polymorphism (SSCP)

SSCP is another PCR-based technique which was first described by Orita *et al.* (1989a.b). In SSCP analysis, a DNA sequence of interest (or a cDNA that has been reverse transcribed from mRNA) is first amplified by PCR. The PCR product is then heated to dissociate the strands, and analyzed by non-denaturing polyacrylamide gel electrophoresis. Under nondenaturing conditions, single stranded DNA fragments will fold into unique conformation determined by their primary sequence because the structure is stabilized by intramolecular interactions. This secondary structure is difficult to predict theoretically and is highly dependent on variables such as temperature and ionic concentrations. Experience in many laboratories has confirmed that even a single base substitution in a PCR fragment several hundred nucleotides in length can induce a conformational change that is

detectable as altered mobility in the gel. The original method required radioactivity, but nonradioactive methods using ethidium bromide or silver staining for DNA detection were later developed (Hongyo *et al.*, 1993; Oto *et al.*, 1993).

Like RFLPs, SSCP variants are inherited in Mendelian fashion, and therefore they should be useful genetic markers. Moreover, SSCP analysis has the advantage over RFLP analysis in that it can detect DNA polymorphisms and point mutations at a variety of positions in DNA fragments.

2.2 Approaches for identifying quantitative trait loci

2.2.1 Candidate gene approach

The candidate gene approach is the method most suitable for use on natural populations. In this approach, genotypic values are measured at loci with known physiological and biochemical relationships to the phenotype of interest. To carry out a candidate gene study, one first identifies a gene of interest based on prior knowledge of trait development and physiology. Molecular variants are then identified and they are often RFLPs. Once molecular variants are identified, the phenotype of interest is measured and the genotypes identified in a sample drawn from a defined population. The phenotypic mean of each genotypic class is obtained and tested statistically for significant differences using analysis of variance or regression methods. If the gene has an effect on phenotype, then differences among genotypic means are expected. An excellent example of a candidate gene study is the work of Rothschild and colleagues (1996) on a gene affecting litter size in pigs. They identified a *PvuII* RFLP in the estrogen receptor (ESR) gene, a candidate gene involved in

reproduction, and demonstrated that one ESR allele, originating from the Meishan breed, is significantly associated with larger litter size.

The major advantages of this approach, other than the ease of use in natural populations, are that the results are physiologically interpretable and yield a direct estimate of the genotypic effect at the QTL itself. However, the method does have some disadvantages. It requires a considerable knowledge of the physiology and biochemistry of the phenotype. Otherwise, the probability of success for the candidate gene chosen is very small. Most importantly, no new genes are found.

2.2.2 *Marker locus approach*

As in the candidate gene approach, the marker locus approach involves correlating genetic variants with a phenotype of interest. However, in this case the measured loci are not expected to be the actual loci affecting a trait, but instead are chosen as markers for various areas of the genome. Ideally, the marker loci are spread across the entire genome so that variable markers are available every 10 to 20 centiMorgans or less apart. With such a high density of marker loci, it is likely that marker loci will be in linkage disequilibrium with QTL resulting in a correlation between quantitative trait values and marker genotypes.

As a first step in carrying out a marker locus study, one needs to compare an appropriate pair of genetically and phenotypically divergent populations, such as two inbred strains (Edwards *et al.*, 1987, 1992). High levels of genetic divergence assure that a large number of markers will differ between populations. The populations are then crossed to produce an F1 generation which is either intercrossed to produce an

F2 generation or backcrossed to one parent population or the other. The phenotypes of interest and marker genotypes are measured in the backcross or F2 generation and the phenotypes are then correlated with marker genotypes. The crossing design assures intermediate allele frequencies and maximal samples for each genotype. Several studies have used the marker locus approach and have typically detected many likely QTLs of moderate phenotypic effect (Weller *et al.*, 1988; Edwards *et al.*, 1987, 1992; Horvat and Medrano, 1994; Lakshmanan *et al.*, 1994).

To take the fullest advantage of linkage maps for quantitative studies, Lander and Botstein (1989) developed a method called interval mapping. This method uses the linkage map to "scan" the intervals between adjacent markers. When a QTL is present the flanking markers form a two locus genotype that contains information about the location and effect of the QTL. By using linked markers for analysis, it is possible to compensate for recombination between the markers and the QTL, increasing the probability of statistically detecting the QTL and also providing an unbiased estimate of the QTL effect on the character. Interval mapping, using a molecular linkage map of an entire genome, was first demonstrated on an interspecific backcross of tomato (Paterson *et al.*, 1988) and has subsequently been used successfully for several quantitative trait linkage studies (Stuber *et al.*, 1992; Jacob *et al.*, 1991; Paterson *et al.*, 1991).

The advantages of the marker locus approach are that it surveys the entire genome and allows mapping and quantitative assessment of unknown quantitative trait loci. However, it has several disadvantages relative to the candidate gene approach. Only indirect measures of QTL genotypic values are available through the

interval mapping method. The measures of genetic effect obtained may be difficult to interpret in specific physiological and biochemical terms because the loci discovered are of unknown function. Finally, since the approach relies on divergent populations, crosses, and backcrosses, it is not well suited to the study of quantitative trait loci in natural populations.

2.2.3 Selective genotyping

Despite technological improvements in the speed and accuracy with which molecular markers can be assayed, it can still be time consuming and expensive to assay large populations. When the time and expense of assaying molecular markers is significantly greater than measuring the quantitative trait of interest on each individual, it is possible to use a modified approach to detect QTL. The approach, also proposed by Lander and Botstein (1989), starts with a large segregating population (F2 or backcross). A quantitative measure of the trait of interest is taken on each individual in the population. Marker analysis is performed only on individuals in the extreme tails of the distribution (*i.e.* those with the lowest and highest values for the trait). If the allele frequency at any molecular marker locus differs significantly between the two tails, it is inferred that a QTL controlling the trait of interest is located near the marker.

The benefit of this approach is in the saving of time and resources in assaying molecular markers. Given the same number of individuals assayed for molecular markers in total population analysis versus selective genotyping, the statistical power of detecting QTL will be greater for the latter (Lander and Botstein, 1989). However,

it should be noted that with this approach, more segregating individuals must be analyzed for the quantitative phenotype to collect enough individuals in both tails. And also, it is often impractical to use this method to map more than one trait, since the individuals with extreme phenotypes for one trait are not likely to represent the extremes for other traits.

2.2.4 Granddaughter designs

To detect QTL with molecular markers normally requires analysis of fairly large segregation populations. Although most plants and some animals readily produce offspring in such large numbers, not all species do. The granddaughter design developed by Weller *et al.* (1990) is particularly useful for mapping QTLs in dairy cattle where offspring numbers are limiting. In this design, sons rather than daughters, of a heterozygous elite sire are scored for markers and divided into two groups on the basis of the marker allele transmitted from their sire. Each of the sons is then progeny tested to estimate his quantitative trait value. This method allows a very large number of phenotypes to be measured without the expense and time of surveying a large number of genotypes. Using this approach, Georges *et al.* (1995) successfully identified QTL underlying the genetic variation of milk production in an elite dairy cattle population.

2.3 Ornithine decarboxylase (ODC)

2.3.1 Polyamine metabolism

Polyamine biosynthesis begins with two decarboxylation reactions working in

parallel (Figure 2.1). Ornithine is decarboxylated to putrescine by ODC, and S-adenosylmethionine (SAM) is transformed to decarboxylated SAM by SAM decarboxylase. Decarboxylated SAM is used to make spermidine by transfer of its aminopropyl moiety to putrescine and to make spermine by another aminopropyl transfer to spermidine. Putrescine is an obligatory activator of SAM decarboxylase in mammals and fungi. Therefore, ODC is a dominant controlling factor of the entire pathway (Kashiwagi *et al.*, 1986). Spermidine and spermine are both prominent in mammals as end products of the pathway, and the putrescine pool is usually low (Kashiwagi *et al.*, 1986). In fungi, spermidine is the major polyamine (Stevens and Winther, 1979). Putrescine and spermine are usually present at 1/10 or less the level of the spermidine pool.

2.3.2 Roles of polyamines

Polyamines are essential for normal growth, as many studies with mutants and pathway inhibitors have shown (Marton and Morris, 1987; Steglich and Scheffler, 1982; Tabor *et al.*, 1980). Upon severe polyamine deprivation, protein and nucleic acid elongation rates diminish, the fidelity of translations is impaired (Marton and Morris, 1987), and chromosomes may disintegrate in later stages of starvation (Pohjanpelto and Knuutila, 1982). Precise molecular mechanisms underlying these effects have not been defined.

Spermidine and spermine, bearing three and four net positive charges, respectively, are the most cationic small molecules of the cell. They therefore bind polyanionic macromolecules such as DNA, RNA, and phospholipids (Igarashi *et al.*,

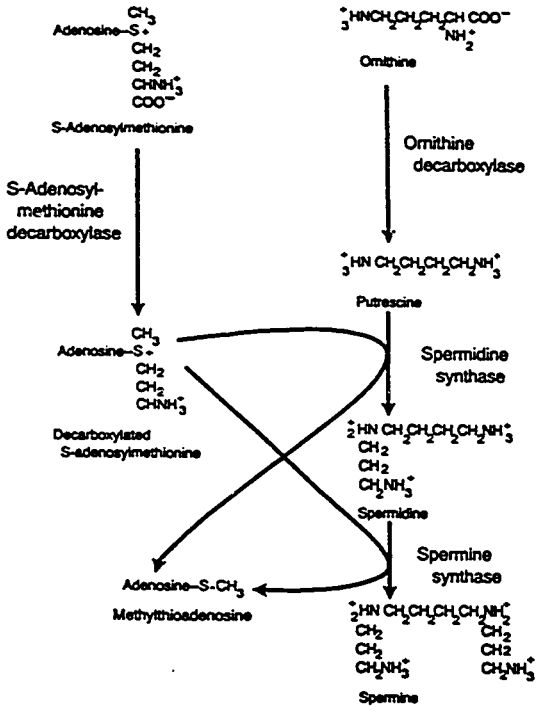

Figure 2.1 The polyamine synthetic pathway

1982). The polyamines are different from other multivalent cations such as Mg2+ in having a distributed charge, whose spacing may allow them to interact more flexibly with the phosphates of DNA and RNA (Marton and Morris, 1987). *In vitro*, spermidine and spermine have profound and beneficial effects upon macromolecular transactions in DNA replication (Krasnow and Cozzarelli, 1982), transcription, and translation. In many cases polyamines simply stabilize interactions between macromolecules rather than occupy specific binding sites. One of the few specific roles that is known for a polyamine in macromolecular synthesis is in the synthesis of hypusine, a post-translationally modified lysyl residue in eucaryotic initiation factor 5A (Park, 1989). This modification arises by the oxidation of the aminobutyl group of spermidine after its transfer to a specific lysine residue (Schnier *et al.*, 1991).

2.3.3 ODC protein

ODC proteins have been isolated from a considerable array of species (Tabor and Tabor, 1984) and in all cases, the enzyme has been found to be dependent on pyridoxal 5'-phosphate for activity. The complete amino acid sequences of mouse, rat and human ODC have been deduced from the nucleotide sequences of cDNAs containing the full coding region of the ODC mRNA (Gupta and Coffino, 1985; Kahana and Nathans, 1985; Hickok *et al.*, 1987; Wen *et al.*, 1989). The encoded proteins contain 461 amino acid residues with a molecular mass of about 51 KDa and represent the subunit of the homodimeric native enzyme. The overall identity of the amino acid sequences of mouse, rat and human ODC proteins is greater than 90 %.

The mouse ODC, which is virtually identical to the enzyme from human and

rat, is the best studied ODC. The lysine residue responsible for binding with pyridoxal 5'-phosphate is Lys-69 (Poulin *et al.*, 1992). This lysine is present within a highly conserved region of mammalian ODCs. Mutation of this residue to alanine or arginine drastically reduces the ODC activity (Tsirka and Coffino, 1992; Coleman *et al.*, 1993; Tobias and Kahana, 1993; Coleman *et al.*, 1994). In addition, there are several other highly conserved regions in the amino acid sequence of ODC including residues 164-171, 193-201 and 357-361. Mutation of residues within these regions namely Lys-169, His-197 and Cys-360 to alanines abolishes or greatly reduces activity indicating that these sequences contribute towards the catalytic site (Lu *et al.*, 1991; Coleman *et al.*, 1993).

A point mutation of glycine to aspartic acid at position 381 which abolishes ODC activity in an ODC deficient mutant CHO cell line was reported by Pilz *et al.* (1990). The equivalent glycine in mouse ODC is Gly-387, and this residue appears to be essential for dimerization as its mutation to many other amino acids, including aspartic acid, prevented the formation of dimer (Tobias *et al.*, 1993).

ODC has a very short half-life in mammalian cells. Deletion of the C-terminal region of the mouse ODC from residue 423 onwards results in the stabilization of the protein (Lu *et al.*, 1991; Ghoda *et al.*, 1989; Rosenberg-Hasson *et al.*, 1991; Ghoda *et al.*, 1992). Furthermore, addition of the 37 C-terminal residues from ODC to dihydrofolate reductase increased the rate of degradation of this hybrid protein (Loetscher *et al.*, 1991). This suggests that this region of the mammalian ODC protein contains a recognition signal for rapid degradation and the region does include the critical part of residues 423-449, a PEST (proline-glutamate-serine-threonine) region

that was postulated to be such a recognition signal (Rogers *et al.*, 1986). These results are entirely consistent with comparison with the *Trypanosoma* ODC that lacks this carboxyl sequence and is not rapidly degraded (Ghoda *et al.*, 1990). However, the exact role of the PEST sequence is unclear. Removal of five or more of the residues from the extreme C-terminus or deletion of some internal portions of the C-terminal domain also stabilize mouse ODC (Ghoda *et al.*, 1992).

2.3.4 ODC gene

Sequences of recombinant genomic clones containing the yeast (Fonzi and Sypherd, 1987), trypanosome (Phillips *et al.*, 1987), mouse (Katz and Kahana, 1988), rat (Wen *et al.*, 1989) and human (van Steeg *et al.*, 1989, Fitzgerald and Flanagan, 1989) ODC gene have been described. The genomic sequences of yeast and trypanosome lack introns, whereas 11 introns interrupt those of mammals. The positions of the 12 exons in the mammalian genes were inferred by comparing the nucleotide sequences of ODC cDNA and genomic DNA. The first two exons make up most of the long 5'-UTR, and the last exon makes up part of coding region as well as the long 3'-UTR. A comparison of the mouse, rat, and human genes reveals a striking conservation of genomic organization. The splice sites utilized are identical in the protein-coding region of all three genes, yet the introns of the human gene are generally larger than those of the mouse and rat. The nucleotide sequence in the coding region of exons is highly conserved, as well as an 82 % identity within the first 148 bp of the 5'-flanking regions of the mouse, rat and human ODC genes (Fitzgerald and Flanagan, 1989). The well conserved 5'-flanking region contains

several promoter/enhancer elements such as a TATA box, a putative CAAT box, a cAMP response element (CRE) and several putative SP1 transcription factor binding sites (GC box) (Heby and Persson, 1990). The 5'-flanking sequence and the promoter of the mouse ODC gene, when placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene and introduced into mouse cells, directs the expression of the bacterial gene and is comparable in strength to the Rous sarcoma virus long terminal repeat (Brabant *et al.*, 1988; Katz and Kahana, 1988).

A single transcript of 2.2 kb ODC mRNA has been observed in human (Hickok *et al.*, 1990). However, two transcripts of ODC mRNA, 2.0-2.4 and 2.6-2.7 kb in length, have been detected in the mouse and rat (Berger *et al.*, 1984; Kahana and Nathans, 1984; Wen *et al.*, 1989). The size heterogeneity is mainly due to different lengths of the 3'-UTR, consistent with the presence of two polyadenylation signals in the gene (Hickok *et al.*, 1986).

2.3.5 ODC regulation

ODC is one of the most highly regulated enzymes known. Early observations on ODC showed that regenerating tissues (Russell and Snyder, 1968), hormonally stimulated tissues, and mitogenically activated cells in culture displayed rapid, 10- to 100-fold augmentation of ODC activity (Abrahamsen and Morris, 1991), followed by a decline even before the cells initiate DNA synthesis (Degen and Morris, 1980). The transient expression of ODC activity suggested a dynamic balance between synthesis and inactivation, consistent with the short half-life of the enzyme (Russell and Snyder, 1968).

The DNA sequences surrounding the promoter of the mammalian ODC gene are highly conserved and contain multiple regulatory elements (Fitzgerald and Flanagan, 1989; Moshier *et al.*, 1992). These elements may regulate the responsiveness of the gene to a variety of growth stimuli (Abrahamsen and Morris, 1991). One of the most studied elements is a CRE element on the promoter of the mouse ODC gene (Abrahamsen *et al.*, 1992). The stimuli which are capable of activating second messenger cAMP elevate cAMP-dependent protein kinase (PKA) through a signal transduction pathway. PKA regulates specific CRE site binding factors which induce ODC gene transcription. Site mutation of the CRE element diminishes greatly the stimulation by cAMP-related stimuli.

The ODC cDNA cloned from a variety of species all have a long, highly conserved 5'-UTR of 275 to 310 nt. In all cases, this 5'-UTR is predicted to have extensive secondary structure and contains a small open reading frame located about 150 nt 5' to the initiator codon AUG. Both of these features are well known to reduce translational efficiency in other mRNAs (Hershey, 1991; Kozak, 1992). The rate of translation of the full-length ODC mRNA is very poor in reticulocyte lysates and is improved by deleting large sections of the 5'-UTR (Ito *et al.*, 1990; Manzella and Blackshear, 1990; van Steeg *et al.*, 1991; Kashiwagi *et al.*, 1991). Attachment of the 5'-UTR of ODC mRNA to a reporter mRNA reduces synthesis in cells and in reticulocyte lysates (Ito *et al.*, 1990; Manzella and Blackshear, 1990; Grens and Scheffler, 1990; Shantz and Pegg, 1994). All these experiments provided evidence suggesting the importance of the 5'-UTR of ODC mRNA in translational regulation.

In many experiment systems, the rate of ODC synthesis does not correlate with the level of ODC mRNA and the cellular polyamine content has been proposed to influence the translational efficiency of ODC mRNA (Hayashi, 1989; Heby and Persson, 1990; Davis *et al.*, 1992). Polyamines are needed for general protein synthesis but excess polyamine levels inhibit synthesis from all mRNAs. In the case of ODC mRNA, it has been suggested that polyamines at low concentration promote translation by binding to the GC-rich region of ODC 5'-UTR and changing its conformation (Kashiwagi *et al.*, 1991). However, an increase in the content of polyamines reduces ODC synthesis both in reticulocyte lysates (Kameji and Pegg, 1987; Ito *et al.*, 1990; Kashiwagi *et al.*, 1991) and in cells (Davis *et al.*, 1992; Lövkvist *et al.*, 1993). Exposure to polyamine analogues has a similar effect in repressing ODC synthesis without affecting the mRNA content (Pegg *et al.*, 1988). Removing or truncating the 5'UTR from the ODC mRNA abolishes the effects (Lövkvist *et al.*, 1993; Ito *et al.*, 1990; Kashiwagi *et al.*, 1991).

The degradation of ODC at the posttranslational level is subject to control by the levels of cellular polyamines. Exogenously added polyamines not only suppress ODC induction in response to various stimuli, but also elicit a rapid decay of preinduced ODC activity (Seely and Pegg, 1983). The molecular mechanism for this polyamine-mediated degradation has not been clarified. Nevertheless, polyamines have been shown to induce the synthesis or release of a 26.5 KDa ODC-inhibitory protein, named antizyme (Murakami and Hayashi, 1985), which binds non-covalently to ODC. The binding of antizyme to ODC is extremely strong and promotes the degradation of ODC by exposing the domain at the C-terminus (Li and Coffino,

1993). Although the site of binding to antizyme is not known precisely, it has been identified by deletion analysis as lying between residues 117 and 140 (Li and Coffino, 1992). It can be speculated that the rapid dissociation of the ODC subunits permits antizyme binding. The presence of antizyme would then prevent dimerization and expose the regions needed for proteolysis.

2.3.6 ODC as a candidate gene for growth-related traits

As discussed above, polyamine metabolism plays a central role in cellular growth and proliferation and ODC is the first rate-limiting enzyme in the pathway of polyamine synthesis. Therefore, change of ODC activity resulting from genetic variations of the gene could alter the biosynthesis of polyamine thus affecting cell growth and differentiation.

Alterations in the ODC gene, such as amplification of ODC gene copy number (Thomas, *et al.*, 1991) and decreased methylation of ODC DNA sequence (Wahlfors, 1991) have been reported to result in up-regulated ODC gene expression in neoplastic growth. Sequence variations in the ODC mRNA between two murine species, *Mus domesticus* and *Mus pahari*, which have different levels of ODC protein in their kidneys have been reported (Johannes and Berger, 1992). In the 5'-UTR, which is likely to play an important role in controlling translation, the *Mus pahari* transcript contains several single-base changes, as well as a 12-base deletion. These changes confer a distinct predicted secondary structure to this region of the mRNA and therefore have been suggested to be responsible for decreased efficiency of ODC mRNA translation in *Mus pahari* (Johannes and Berger, 1992). Comparison of the

nucleotide sequence of ODC cDNA obtained from human hepatoma tissue with that of ODC cDNA obtained from nontumorous tissue revealed three point mutations (Tamori *et al.*, 1995). The mutations are accompanied by replacements of amino acids in hepatoma tissue with other amino acids or a stop codon and it was suggested that the formation of truncated and stabilized ODC protein due to the mutations was responsible for higher ODC activity in the hepatoma tissue (Tamori *et al.*, 1995).

Studies of lines of mice selected for lean body mass (Bishop and Hill, 1985; Sharp *et al.*, 1984) have shown that there is a significant genetic component affecting this trait. Using replicated lines of mice differing in lean body mass, Gray and Tait (1993) have demonstrated that significantly higher levels of ODC mRNA and ODC activity are present in the high lean body mass line than the low lean body mass line when measured between days 10 and 13 of gestation. RFLP analysis of the expressed ODC gene has shown that a *HaeIII* polymorphism has been selected in the high lean body mass line (Gray and Tait, 1993). This suggests that selection for variation in ODC may be a component contributing to high lean mass. A recent study showed that the *HaeIII* polymorphism associated with the 5'end of the ODC structure gene in the high lean mass line is associated with increased transcription of the gene (Gray *et al.*, 1995).

In chickens, it was reported that there were significant differences among levels of ODC in broiler lines selected for growth, the same broiler line in which selection had been relaxed and a White Leghorn egg layer strain (Bulfield *et al.*, 1988). The study showed that at one week of age, the selected line of broiler chickens had about 20-fold higher ODC activity than in layer chickens and that in broiler lines

where selection had been relaxed, ODC levels were intermediate. These differences occurred at an age when body weight was similar and prior to a major difference in growth rate. RFLP analysis of genomic DNA from two divergently selected broiler chicken lines with the 3'end of the ODC gene revealed five *MspI* polymorphic bands and two of them were found to be present at a higher frequency in individuals of the lean line compared to the fat line (Zhang, 1995). Recently, a *Hind*III polymorphism was found in the chicken ODC gene. Analysis of the polymorphism in a closed random mating White Leghorn chicken population revealed that the polymorphism was associated with some egg production traits (Aggrey *et al.*, 1996).

2.4 Growth hormone (GH)

2.4.1 GH protein

GH, also known as somatotropin, is a protein hormone produced and secreted into the circulation mainly by the pituitary gland. It is structurally and functionally related to a family of polypeptide hormones which includes prolactin and placenta lactogen (Miller and Eberhardt, 1983).

Characterization in various species revealed that GH is a single-chain polypeptide of about 22 KDa made up of 190 to 199 amino acid residues (Miller and Eberhardt, 1983; Scanes and Campbell, 1995). The biologically active conformation of GH is maintained by two disulfide bridges that link, respectively, large and small peptide loops (Charrier and Martal, 1988). Based on the study of the crystal structure of porcine GH (Abdel-Meguid *et al.*, 1987), it was demonstrated that the GH molecule consists of a bundle of four α-helices in an antiparallel arrangement which

brings the parts that interact with the GH-binding sites of the GH-receptor into proximity.

In addition to the 22 KDa GH which accounts for about 70-75% of human pituitary GH, there is a second abundant GH which has a molecular weight of 20 KDa and constitutes about 10% of the pituitary GH (Lewis *et al.*, 1978). This GH variant lacks 15 internal amino acids (residues 32-46) compared with the 22-KDa form. Besides, glycosylated GH variants have been detected in the murine pituitary gland (Sinha and Jacobsen, 1987) and similar variants have been suggested to be present in human (Ray *et al*, 1989), porcine (Sinha *et al.*, 1990) and rat (Bollenger *et al.*, 1989) pituitary. Some naturally occurring fragments of GH in pituitary extracts have also been observed in several species. Another source of GH heterogeneity comes from the oligomerization between the same or different GH monomers, which occurs either covalently or non-covalently to form di-, tri-, tetra, or even pentameric forms (Baumann, 1991).

2.4.2 GH gene

In humans, pituitary GH is expressed by the hGH-N gene (N for normal), a member of the GH-chorionic somatomammotropin (hCS) gene family (Parks, 1989). This family consists of five similar genes (hGH-N, hCS-L, hCS-A, hGH-V and hCS-B) aligned on the long arm of chromosome 17, of which only the hGH-N gene is expressed in somatotroph cells. The hGH-N gene, which is contained within a 2.6-kb DNA fragment (Fiddes *et al.*, 1979), is composed of five exons and four introns. This gene is similar in structure to other mammalian GH genes (Miller and Eberhardt,

1983) but is smaller than the chicken GH gene (3.5 kb), which contains expanded intron sequences (Tanaka *et al.*, 1992). The hGH-V gene (V for variant) is another GH gene which encodes a protein with structural homologies to pituitary GH but is expressed only in the placenta. The hCS genes express placental lactogens, which have close homology (85%) to hGH. Two GH genes are only known to be present in the human genome but not in the other mammalian genomes.

Primary transcripts of the hGH-N gene are subjected to alternative splicing to produce mRNAs that code for the 22- and 20-KDa GH respectively, whereas the hGH-V gene only produces a 22-KDa GH which differs by 13 amino acid from the 22-KDa hGH-N gene product. The promoter region which contains the binding sites for transcription factors and RNA polymerase II extends about 500 bp 5' to the start site for gene transcription and is highly conserved among mammals. There are more distant enhancer sequences which assist the tissue specific expression of the gene (Parks, 1989).

2.4.3 GH and growth

There is abundant evidence that GH is required for growth in all species examined. Growth is reduced or completely suppressed in the absence of GH. For instance, in rats hypophysectomized after 25 days of age, there is a complete cessation of growth irrespective of whether body weight gain or tail growth is considered and growth is restored by GH administration. (Gluckman *et al.*, 1981; Groesbeck *et al.*, 1987). Administration of polyclonal antisera against GH has been demonstrated to decrease growth in a number of species (Sinha and Vanderlaan,

1982; Gardner and Flint, 1990; Flint and Gardner, 1993). Lesions in pituitary gland or hGH-N gene in children have been shown to cause severe retarded growth (Holder *et al.*, 1980; Parks, 1989) and mutations in the GH gene is known to be responsible for causing dwarfism in the Snell-smith mice (Holder *et al.*, 1980). Dramatically increased growth rate have been observed in mice carrying GH transgenes and expressing high levels of GH (Palmiter *et al.*, 1982; 1983), while transgenic mice expressing a mutant form of bovine GH have been shown to display reduced growth (Chen *et al.*, 1990; 1991). In agricultural animals, exogenous GH enhances growth performance. The effects of chronic administration of GH on growth rate as well as feed efficiency and carcass quality in many meat producing animals have been recently summarized (Scanes, 1995)

GH regulates growth through hypertrophy, hyperplasia, or both, as a result of tissue differentiation, cell proliferation, and protein synthesis. GH can exert its effects either directly or indirectly through the actions of a mediator, insulin-like growth factor-I (IGF-I, Holly and Wass, 1989). IGF-I is produced in many tissues (primarily in liver) in response to GH and enhances the proliferation and maturation of many tissues, including bone, cartilage, and skeletal muscle. IGF-I is suppressed or undetectable in the serum and tissues of GH-deficient individuals but can be stimulated by GH administration (Scanes and Daughaday, 1995). GH stimulation of IGF-I expression can either occur in a paracrine manner at the target tissue or through a distal endocrine manner by IGF-I production at tissues such as liver (Scanes and Daughaday, 1995).

2.4.4 GH and lactation

It is well established that GH stimulates milk production (galactopoiesis) in dairy cattle. As early as in the 1930s, extracts of anterior pituitary glands were shown to increase the amount of milk produced by cows (Asimov and Krouze, 1937). These findings were confirmed by Folley and Young (1940), who also showed that the galactopoietic effect was particularly dramatic during declining lactation. Shortly after, the galactopoietic component of the extracts was identified as anterior pituitary GH (Young, 1947; Cotes *et al.*, 1949). The galactopoietic effect of GH in dairy cows was repeatedly confirmed thereafter (Brumby and Hancock, 1955; Machlin, 1973; Bines *et al.*, 1980). With the advent and sophistication of recombinant DNA techniques in the 1980s, sufficient quantities of recombinant bovine GH (rbGH) were made available allowing further studies of the galactopoietic effects of GH. Bauman *et al.* (1985) demonstrated significant increase (23-41%) in milk yield from dairy cows that were treated with varying daily doses of rbGH (13.5, 27 or 40.5 mg/cow), which were higher than the increase (16%) observed when 27 mg pituitary-derived GH was injected. Since then, numerous trials have confirmed the galactopoietic effects of rbGH when administered during single lactation trials (Bauman and Vernon, 1993; Bauman *et al.*, 1994). In the majority of these trials, rbGH administration increased milk yield within 2-3 days of initiating treatment and prolonged the persistency of lactation. Average gains in milk yield of 3-5 kg/day are reported for dairy cows of different breeds, genetic potential, parity, and geographical location, and translate into lactational increases of 10-15% (Peel and Bauman, 1987; Thomas *et al.*, 1991; Muller, 1992). These milk yield responses to rbGH have been confirmed for multiple

lactations of its use (Annexstad *et al.*, 1990; Leonard *et al.*, 1990; McBride *et al.*, 1990; Gibson *et al.*, 1992). The effect of GH on increasing milk production was observed whether bGH is administered as a daily injection, as an intravenous pulse every 2 h, or as a continuous subcutaneous infusion (Fronk *et al.*, 1983).

The galactopoietic effect of GH may be mediated or potentiated by peripheral or locally produced IGF-I. The roles of IGF-I in the galactopoietic effect of GH are not fully established. Administration of bGH is followed by increases in the concentration of IGF-I (Davis *et al.*, 1987; Vicini *et al.*, 1991) in the circulation. It seems likely that these changes in circulating concentrations of IGF-I contribute to the galactopoietic effect of GH. Indeed, IGF-I has been found to increase mammary DNA synthesis *in vitro* (Baumrucker and Stemberger, 1989) and thus is involved, presumably, in stimulating mammary cell proliferation and tissue growth. Moreover, IGF-I infused directly into one mammary gland of goats has recently been found to rapidly increase milk production (Prosser *et al.*, 1994). Whether GH also exerts its effect directly on mammary gland is not confirmed. Evidence against a direct effect of GH on the mammary gland includes the failure of bGH to stimulate milk production in sheep and goats when GH is infused into the mammary artery (McDowell *et al.*, 1987). Conversely, demonstration of the presence of GH receptors in bovine mammary tissue would support a direct galactopoietic effect of GH (Hauser *et al.*, 1990; Glimm *et al.*, 1990).

2.4.5 GH gene polymorphisms

Molecular genetic studies have shown that considerable polymorphisms exist

in the GH gene of various species. Deletions, frameshift mutations and nonsense mutations in the human GH gene have been identified to be responsible for Familial Isolated GH Deficiency (Phillips III and Cogan, 1994). In mice, polymorphism in the GH gene was detected with the restriction enzyme *Hind*III and the polymorphism was found to be correlated with 42-day weight (Winkelman *et al.*, 1990; Winkelman and Hodgetts, 1992). Polymorphisms of the porcine GH gene was reported by Nielsona and Larsen (1991) who identified a *Dra*I- and a *TaqI*-RFLP. In addition, Kirkpatrick (1991) reported a *Hae*II and a *Msp*I polymorphism in the second intron of the gene. However, both studies made no attempt to correlate the RFLPs with production traits. In chickens, four RFLPs in the GH gene were revealed by Southern analysis; one at a *Sac*I site and three at *Msp*I sites (Fotouhi *et al.*, 1993). These RFLPs were shown to respond to selection for size of the abdominal fat-pad in meat-type birds (Fotouhi *et al.*, 1993) and egg production traits in a series of egg layers (Kuhnlein and Zadworny, 1994). The search for the bovine GH polymorphisms was initiated by Beckmann *et al.* (1986). So far several RFLPs have been identified in the bovine GH gene. These include a *Msp*I polymorphic site at the third intron (Cowan *et al.*, 1989; Hillert *et al.*, 1989), an *Alu*I polymorphism at the fifth exon which is associated with a codon substitution that results in either valine or leucine being present in the GH protein at position 127 (Lucy *et al.*, 1993; Zhang *et al.*, 1993) and an insertion/deletion of ±1 kb DNA fragment in the 3'region of the gene (Hallerman *et al.*, 1987; Cowan *et al.*, 1989; Hilbert *et al.*, 1989). Recent studies suggested that there could be some associations between these polymorphisms and milk production as well as growth traits in different breeds of cattle (Rocha *et al.*, 1992; Lucy *et al.*,

1993; Lee *et al.*, 1993; Hoj *et al.*, 1993; Sneyers *et al.*, 1994).

2.5 Conclusions

The development of recombinant DNA methodologies allowed for detection of genetic polymorphisms at the level of DNA molecule. A number of different types of DNA polymorphisms have been described, and each has its advantages and disadvantages.

Several apprcaches have been developed for identifying QTL. The candidate gene and the marker locus approaches are two major approaches, each having its own advantages and disadvantages. For the most part, the advantages of one approach are the limitations of the other, making them complementary rather than mutually exclusive. For large species with long generation interval and which produce few offspring such as dairy cattle, the candidate gene approach is the most suitable method for QTL studies.

Candidate genes are those with known physiological and biochemical effects on the traits of interest. ODC is an enzyme which is associated with DNA synthesis and cell proliferation, and the gene is strongly expressed in lactating mammary gland. GH is well known to have profound effects on growth, lactation and mammary gland development. Therefore, both genes are excellent candidate genes for milk production traits.

So far, no studies have been conducted to characterize the bovine ODC gene and whether genetic variations exist in the gene nas not been revealed. The gene coding for GH has been cloned and characterized and several RFLPs in the gene

have been identified. However, RFLP analysis limits the detection of those sequence variations that change restriction enzyme recognition sites. Additional DNA polymorphisms may be present that can only be detected by more sophisticated techniques such as SSCP.

The following study was undertaken to clone and characterize the bovine ODC gene as a first step for further studying genetic variability of the gene. In addition, the SSCP technique was exploited to search for additional sequence variations in the bovine GH gene. DNA polymorphisms identified in both genes were further analyzed for their association with milk production traits in dairy cattle.

CHAPTER 3

MOLECULAR CLONING OF A BOVINE ORNITHINE DECARBOXYLASE (ODC) CDNA AND ITS USE IN THE DETECTION OF RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN HOLSTEINS

Jianbo Yao, David Zadworny, Urs Kühnlein and J.Flan Hayes

Dept. of Animal Science, McGill University, Macdonald Campus

Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Published in *Genome*, 38:325-331 (1995)

3.1 Abstract

A cDNA coding for ornithine decarboxylase (ODC) was isolated from a bovine liver cDNA library. The clone (1758 bp) consisted of 5'- and 3'-untranslated regions of 185 and 187 nucleotides, respectively, and an open reading frame of 1383 nucleotides encoding an ODC protein (Mr. 51,342 daltons) of 461 amino acids. Comparison of the nucleotide and the predicted amino acid of the cDNA to other mammalian ODCs showed a very high degree of homology both at the DNA and protein levels. The bovine ODC mRNA was identified by Northern blot to be a single species with a molecular size of 2.35 kb. Primer extension analysis indicated that the 5'-untranslated region of the bovine ODC mRNA was 312 nucleotides long. Southern blot analysis of bovine genomic DNA revealed restriction fragment length polymorphisms when cleaved with restriction enzymes *Pst*I, *Msp*I, *Taq*I and *Bgl*I.

3.2 Introduction

The polyamines, putrescine, spermidine, and spermine, are low molecular weight polycations that have been shown to exert an extraordinary degree of control over the growth, development and division of all cells (Pegg, 1986). Ornithine decarboxylase (ODC: EC 4.1.1.17) catalyses the conversion of ornithine to putrescine, the first and rate limiting step in polyamine biosynthesis. The level of ODC activity is close to zero in quiescent, nonproliferating cells, but is readily induced by a wide variety of trophic stimuli, such as hormone, growth factors and drugs (Pegg and McCann, 1982; Pegg, 1986). Numerous studies have shown that regulation of ODC can occur at multiple levels, including gene transcription; mRNA translation; protein turnover and post-translational modifications (Tabor and Tabor, 1984; Pegg, 1986).

In dairy cattle, animal selection has resulted in an approximate doubling of milk production over the last 40 years (Fallert and Liebrand, 1991), however, very little is known about the biochemical or genetic changes which have accompanied this increase in milk production. Genetic variants of ODC have been identified in a number of species which have been divergently selected for growth related traits (Bulfield *et al.*, 1988; Grey and Tait, 1993) and it is possible that particular variants of ODC could be associated with increased mammary gland function. Indeed, trophic hormones such as prolactin (PRL) which are associated with lactation have been shown to be required to induce differentiation of bovine mammary epithelial cells (Huynh *et al.*, 1991) and both the mitogenic and lactogenic effects of PRL appear to be mediated through the polyamine pathway (Rillema *et al.*, 1986; Bedford and Zadworny, 1990). As a first step in characterizing ODC at the molecular level, we

have cloned a bovine ODC cDNA and shown that the ODC gene is highly polymorphic in Holsteins.

3.3 Materials and Methods

3.3.1 cDNA cloning and nucleotide sequence analysis

A cDNA library was constructed from mRNA isolated from the liver of a Holstein cow using the lambda ZAP II cloning kit according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Approximately, 5 x 10^5 plaques were screened with chicken ODC cDNA as a probe (pODZ3: 2068bp, Zhang *et al.*, 1992) which had been radioactively labelled by using a T7 QuikPrime™ kit (Pharmacia, Baie d'Urfe, P.Q., Canada) and [α-^{32}P]-dCTP (ICN, Irvine, CA, USA). Four positive plaques were isolated. Following phagemid recovery, strands were partially sequenced using a T7 sequencing kit (Pharmacia, Baie d'Urfe, P.Q., Canada). Preliminary sequence analysis of the 5' and 3' ends of these clones revealed that three of them were truncated cDNA products but otherwise identical to the largest clone (pY11). To facilitate sequence analysis, the cDNA insert (1758 bp) from pY11 was subcloned into pUC18 following digestion with *Hind*III, *Hinc*II, *Ava*I or *Mbo*II to generate overlapping fragments and the sequence for both + and - strands was completely determined. DNA sequence data were processed and analyzed using the computer program DNASIS (HIBIO, Brisbane, CA, USA).

3.3.2 Northern analysis

Total RNA was isolated from cultured bovine mammary epithelial cells

(Huynh *et al.*, 1991) by acid guanidine thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) using RNAzol^{TM}B (TEL-TEST, INC. Friendswood, TX, USA). Twenty μg of the RNA was fractionated on a 1 % agarose gel containing 2.2 M formaldehyde and transferred to a Zeta-probe membrane (BIO-RAD, Richmond, CA, USA) in 20x SSC. The bovine ODC cDNA (pY11) was labelled with [α-^{32}P]dCTP (ICN) and hybridized to the filter in 10 % dextran sulphate, 40 % deionised formamide, 5x Denhardt's solution, 4x SSPE, 1 % SDS and 100 μg/ml of Herring sperm DNA at 42^{0}C overnight. The membrane was washed twice with 2xSSC, 0.1 % SDS at 42^{0}C for 15 min followed by washing with 0.5 x SSC, 0.1 % SDS at 42^{0}C for 15 min and with 0.1xSSC, 0.1% SDS at 50^{0}C for 20 min prior to autoradiography.

3.3.3 Primer extension

A synthetic oligonucleotide primer (21-mers; 5'-AAACACACAATCTGCTGTCTC-3') complementary to nucleotide position -178 to -158 (the A of the AUG translation initiation codon is designated number 1) of the ODC mRNA was end-labelled using polynucleotide kinase and [γ-^{32}P]ATP. The labelled primer (10^6 cpm) was hybridized to 12 μg of total RNA at 30^{0}C overnight is a reaction mixture of 30 μl containing 200 mM Tris.HCl (pH 8.5), 40mM $MgCl_2$ and 400 mM KCl. The nucleic acids were precipitated with ethanol and redissolved in 20 μl of 50 mM Tris.HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol containing 0.5 mM each of dNTPs, RNase inhibitor (1 unit/μl, RNAguard, Pharmacia) and Moloney Murine Leukemia Virus Reverse Transcriptase

(2 units/μl, Pharmacia) and incubated for 2 hrs at 37°C. The extended products were fractionated on a 7 M urea/8 % polyacrylamide sequencing gel and visualized by autoradiography.

3.3.4 Southern blot analysis of bovine genomic DNA

High molecular weight genomic DNA was isolated from bovine semen from 20 unrelated Holstein sires as previously described (Zadworny and Kühnlein, 1990). These sires represented the extremes (10 highest and 10 lowest) of breed class average (BCA) values for bulls which were progeny tested for milk production traits at the Centre d'insemination artificielle du Quebec (Saint-Hyacinthe, Quebec, Canada) in 1992. The averages were +48.6 $\pm$ 3.8 and -37.5 $\pm$ 4.1 for the top and low ranked sires, respectively. Five μg of DNA was digested overnight with 5 units/μg of various restriction enzymes (Pharmacia). The digested DNA was separated by electrophoresis in a 1 % agarose gel at 29 V for 18 hrs and transferred overnight to Zeta-probe membrane (BIO-RAD). The cDNA insert from the bovine ODC cDNA clone (pY11) was ^{32}P-labelled by random prime extension and hybridized to filter bound DNA in 4x SSPE, 10 % dextran sulphate, 50 % deionized formamide, 1 % SDS and 5 mg/ml skim milk powder at 42°C overnight. The filters were washed as for Northern analysis and subjected to autoradiography.

3.4 Results and discussion

3.4.1 Characterization of the bovine ODC cDNA

The cDNA and the structural gene for ODC have been cloned from several

mammalian species including human (Hickok *et al.*, 1987; 1990) rat (van Kranen *et al.*, 1987; van Steeg *et al.*, 1988; Wen *et al.*, 1989) and mouse (Kahana and Nathans 1985; Brabant *et al.*, 1988; Katz and Kahana, 1988). As revealed by sequence analysis, the coding sequence is highly conserved, as is the general arrangement of introns and exons in the gene. Similarly, bovine ODC cloned in this study had substantial homology to other mammals. Due to the presence of substantial secondary structure located in the mRNA at 5' end in other animals which may contribute to cloning artefacts (Coffino, 1988; Widegren *et al.*, 1991), a genomic clone was partially sequenced (1634 bp) to confirm the cDNA sequence. This clone contained exons 2 (143 bp) and 3 (134 bp) which had 100 % homology with the cDNA clone. Exon 1 was not present in the genomic clone although 1144 bp of intron 1 was also sequenced.

The sequence of pY11 (GenBank and EMBL Accession number: M92441) was 1758 nucleotides and consisted of 5'- and 3'-untranslated regions (UTR) of 185 and 187 nucleotides, repectively, and a coding region of 1383 nucleotides. The protein predicted from the open reading frame contained 461 amino acids (51,342 daltons) which was identical to that reported for other mammalian ODC cDNAs. Comparison of bovine with other mammalian ODC cDNAs showed that the amino acid sequence was highly conserved and had an overall homology of 93 % with the human (Hickok *et al.*, 1987), 91 % with the rat (van Kranen *et al.*, 1987) and 90 % with the mouse (Kahana and Nathans, 1985), respectively. About 40 % of the differences in amino acid residues occurred in the 50 residues at the carboxyl terminus. However, these differences did not substantially change the PEST regions (stretches enriched in

proline, glutamic acid, serine, and threonine) which have been shown to modulate protein turnover (Ghoda *et al.*, 1990; Loetscher *et al.*, 1991).

The synthesis of ODC is, in part, regulated at the translational level. All the mammalian ODC cDNAs reported contain an unusually long 5'-UTR of about 300 nucleotides which markedly affects the rate of translation (Grens and Scheffler, 1990; Manzella and Blackshear, 1990). The general arrangement of the 5'-UTRs is well conserved. It consists of a GC-rich region of about 150 nucleotides at the 5' end and a short open reading frame located about 150 to 170 nucleotides upstream of the true initiation codon. The 5'-UTR of the bovine ODC clone was 185 nucleotides and did not represent the full length. Nevertheless, a high degree of secondary structure with GC stem loops was observed with a free energy of formation of -59.5 kcal/mol (Zuker and Steigler, 1981). The comparable region in human, rat and mouse UTRs has a free energy of formation between -76.1 and -84.7 kcal/mol. Since secondary structures with predicted free energies greater than -42 kcal/mol have been shown to inhibit *in vitro* translation of rat ODC transcripts by more than 50 % (Manzella and Blackshear, 1990), it is likely that the 5'-UTR of the bovine ODC is an important regulatory element of translation as has been suggested for other species.

In all other mammalian ODC cDNAs, a short open reading frame is observed about 150 to 170 nucleotides upstream of the initiation codon. The function of this potential leader peptide has not as yet been resolved (van Steeg *et al.*, 1988; Manzella and Blackshear, 1990). In the bovine, an open reading frame in this region was not observed. We are uncertain as to the significance of this difference between the bovine and other mammals but do exclude the possibility of a cloning artefact

since a genomic clone containing exons 2 and 3 region was sequenced. It is possible, however, that a similar open reading frame may be located further upstream in the 5' UTR. Further analysis is required to clarify this issue.

There is a large difference in homology among species between the 5'- and the 3'-UTRs, respectively (Figure 3.1). Homology at the 5'-UTR amongst the bovine, human, and mouse was low (less than 60 % in 185 nucleotides upstream of the initiation codon), whereas homology at the 3'-UTR was high (greater than 80 % in 187 nucleotides downstream of the stop codon). The 5'-UTR is considered to have a major role in the control of translation of ODC yet is relatively poorly conserved amongst mammalian species. The extraordinary degree of nucleotide conservation which occurs in the coding region (more than 86 %) is indicative of a very slow rate of evolutionary divergence and reflects the importance of this enzyme in animal growth and differentiation. That the 3'-UTR should also be conserved to the same extent may imply that it plays a key role in regulation. What this role may be is at this time not clear. Deletion of this region has been shown to decrease the rate of translation (Grens and Scheffler, 1990; Manzella and Blackshear, 1990) and an interaction between the 5'- and 3'-UTRs has been proposed as a mechanism for reducing the inhibition of transcription imposed by the 5'-UTR secondary structure *in vitro* (Grens and Scheffler, 1990). However, computer analysis of complementarity between the 3'-UTR sequence and sequence in both the 5'-UTR and the coding region did not reveal any obvious interacting domains.

3.4.2 Determination of the size and the transcription start site of bovine ODC mRNA

To determine the size of the bovine ODC transcript, the bovine cDNA was used to probe RNA isolated from bovine mammary epithelial cells by Northern blotting. As shown in Figure 3.2, hybridization of the blot to the probe revealed a single mRNA species of about 2.35 kb. This result is in agreement with that reported in human (Hickok *et al.*, 1987) in which only a single transcript of about 2.25 kb was detected, but is different from that in the mouse (Kahana and Nathans, 1985) and rat (Wen *et al.*, 1989) where two mRNA species of 2.2 and 2.6 kb were always present, presumably due to the alternative usage of the polyadenylation signals (Hickok *et al.*, 1986; Wen *et al.*, 1989).

To determine the transcription start site of the bovine ODC mRNA, we examined the bovine RNA by primer extension. The extension product was shown by electrophoresis to be a single band with a size of 155 bp (Figure 3.3) as determined by comparison to a known sequence fragment. This indicates that the 5'-UTR of the bovine ODC mRNA is 312 nucleotides long which is 127 bp longer than that present in the cDNA we have isolated. This size is very close to the reported length of 5'-UTR in humans (335 nt; Hickok *et al.*, 1987) and murine mRNA (330 nt; Gupta and Coffino, 1985).

3.4.3 Genetic variability of the ODC gene in Holsteins

Southern blot analysis of bovine genomic DNA revealed the ODC locus to be highly polymorphic. Restriction fragment length polymorphisms (RFLPs) were shown when DNA from 20 unrelated individual bulls was cleaved with restriction enzymes *Pst*I, *Msp*I, *Taq*I and *Bgl*I (Figure 3.4) but not with *Eco*RI, *Sac*I, *Hind*III, *BamH*I,

*Rsa*I, and *Kpn*I. The overall frequencies of the various polymorphic bands are shown in Table 3.1. With the exception of RFLPs associated with BglI digestion, there were no significant differences between the animals which had been ranked for high (+48 BCA) or low (-37 BCA) lactational performance. However, in the case of BglI, the 2.1 kb band was present in heterozygous form in only 1 individual (frequency = .05) which was ranked in the high performance group, whereas, in the low performance group this band was present in 5 individuals in heterozygous form and in 1 individual in homozygous form (frequency = .35). Whether or not the presence of this RFLP has any physiological relevance is not known. However, in murine species an *Hae*III RFLP has been shown to be associated with increased ODC bioactivity during embryogenesis in mice lines selected for high lean body mass (Grey and Tait, 1993). Similarly, genetic variants which affect the efficiency of translation of ODC mRNA have also been identified (Johannes and Berger, 1992). Since ODC is the first and key regulatory enzyme in the biosynthesis of polyamines, which are essential for protein biosynthesis, DNA replication and cellular differentiation (Pegg, 1986), it is possible that genetic variants would affect mammary gland growth and/or involution or interact differently with trophic hormones which modulate lactation. The DNA polymorphisms at the ODC gene identified in the present study may serve as genetic markers for correlating ODC genetic variants with milk production traits, however, further investigation is required.

Table 3.1 Frequency of RFLP alleles at the ODC locus in Holsteins

Enzyme	n	Band size (kb)	Allele frequencies
*Pst*I	17	5.0	0.735
		5.5	0.265
*Msp*I	20	3.5	0.925
		4.8	0.075
*Taq*I	20	6.3	0.375
		6.9	0.625
*Bgl*I	20	2.1	0.200
		2.7	0.800

Comparison Among Nucleotide Sequences 185 bp Upstream of the Initiation Codon (+1)

```
Bovine  -185 CCGCTCTGAG ACAGCAGATT GTGTGTTTAT TGAACATTTC CACTGCTGCA   50
Human        AG****C*GC GTCTGC*C** CCCCA*GGGG CTGG*C*GCG GCGCCTG*GC  165
Rat          *G*G*GC*CC TTG*GGTT*A ***GCGGCT* CTCCATGGGT *CAGC*A**C  286

Bovine  -135 CAGAGAGCAC ACGCATCTTC GGTGGACTTG GAATTCCTGG GGAATTGCCT  100
Human        GCTCTGAG*T TGT**CTGCT *T*CC*AGG* C*CA*G*A*A **G***TGGA  215
Rat          GCTTCCCTGT G*TGTGAG*G TT*CC**CAC TCCAGGAGAC A*C***CAGA  336

Bovine  -85  TTGTGAAAAG TTGGCATAAT CCCTTTAAAT TCCATCTCTT TTACGTTTTC  150
Human        A*TCCTGG** AGTTGCCTT* GTGAGA*GC* GGA*ATAT** C*TTCAG***  265
Rat          G*TGACCTT* *GA*AGCTGG **A*AATTTA ATTCCA***C *AGGT***CT  386
                                                  +1
Bovine  -35  TATTTTGTTG TGTCTCAAAA AGACGTCAAG AAACCATG.. ..........  188
Human        C**C*CT*A* *T*TC**T*G GA**A***** ***T****.. ..........  303
Rat          GTC**AT*GT *TCAGAGGC* CAT**AG**C C******.. ..........  424
```

Comparison Among Nucleotide Sequences 187 Downstream of the Stop Codon (underlined)

```
Bovine     1 TAGATACCAC TCTTGTAGCT GTTAACTGCG AGTTTAGCTT GATTTAAGGG  1618
Human        ******G*** ***G****** *********A ********** **A*******  1735
Rat          *****G***T ********** C***C****A ********** **G**C*C**  1592

Bovine    51 -TTTGGGGGG GACCATTTAA CTTAATTACT GCTAGTTCTG AGATGTCTAT  1668
Human        -A**T***** ******G*** ********** *******T** *A******T*  1785
Rat          CA**T***** ********** ********** *******-** GA******T*  1642

Bovine   101 GTGAG--TAG GGTTGGCACA GGTGCACCAA TGTGGAA-GA CTGGGAGATG  1718
Human        **A**AG*** ***C*C**-T *A****G*C* *A*****-** **A***T***  1835
Rat          **A**AG*** *********C AA****G--- *A*****A** **A*******  1692

Bovine   151 GGG-TCACAC TTATCTGTGT TCCTATGGAA ACTATTTGAA TATTT.....  1763
Human        -**-****** ********** ********** ********** *****.....  1880
Rat          ***G****** ***-****** ********** **--****** *****.....  1742
```

Figure 3.1 Comparison of 5' and 3'- untranslated regions of bovine, human and rat ODCs 185 and 187 nucleotides upstream and downstream of the true initiation codon and stop codon, respectively. Potential initiation and stop codons in the leader sequence are underlined. Asterisks indicate nucleotide identity.

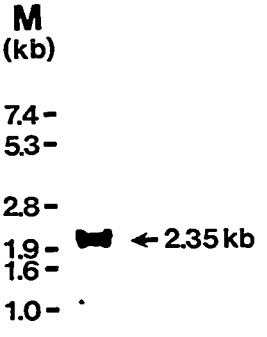

Figure 3.2 Northern analysis of total RNA from bovine mammary epithelial cells using pY11 as the hybridization probe. RNA size was determined by comparison to an ethidium bromide-stained RNA molecular weight marker (Boehringer Mannheim, Laval, P.Q. Canada)

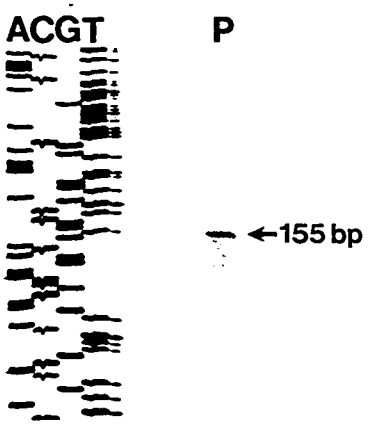

Figure 3.3 Mapping of the 5' end of bovine ODC mRNA by primer extension. Lanes A, C, G, T are a known DNA sequence used for size determination. Lane P, the extended product.

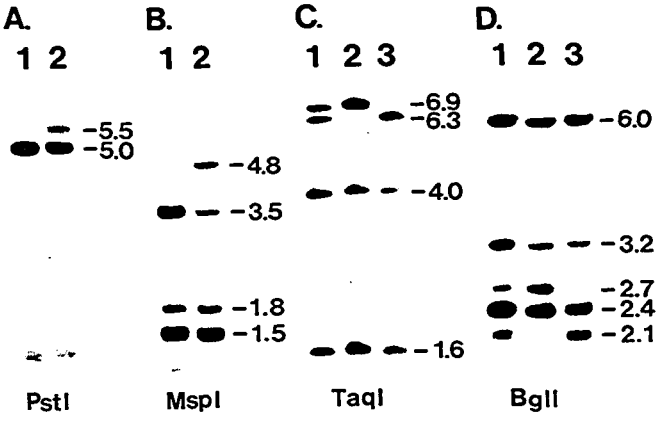

Figure 3.4 Southern blot analysis of bovine genomic DNA showing restriction fragment length polymorphisms when cleaved with *PstI* (A), *MspI* (B), *TaqI* (C) and *BglI* (D). The sizes (kb) of the restriction fragments are shown on the right of the respective blots. Cleavage with *PstI* revealed 2 hybridization patterns (A). The individual shown in lane 1 is homozygous for the 5.0 kb PstI fragment, while the individual in lane 2 is heterozygous for the 5.5 and 5.0 kb bands. No animals homozygous for the 5.5 kb fragment were observed. DNA samples digested with *MspI* also revealed 2 patterns (B). The majority of animals appeared homozygous for the 3.5 kb fragment (lane 1). Animals showing the other pattern demonstrated two bands of 3.5 and 4.8 kb (lane 2). Again, no individuals homozygous for the 4.8 kb fragment were observed in the samples investigated. Digestion with the restriction enzyme *TaqI* exhibited 3 distinct patterns (C), with one heterozygous for the 6.9 kb and 6.3 kb fragments (lane 1) and two homozygous for 6.9 kb and 6.3 kb fragments, respectively (Lane 2 and 3). With *BglI*, two allelic polymorphic bands of 2.1 kb and 2.7 kb and 3 non-polymorphic bands of 6.0, 3.2 and 2.4 kb were detected (D). Lane 1 shows a heterozygous individual, while lane 2 and 3 represent homozygous animals for the 2.7 kb and 2.1 kb fragments, respectively.

Connecting statement I

In chapter 3, we have described the cloning of a cDNA coding for bovine ODC and the use of the cDNA as a probe for detection of RFLPs in the ODC gene. However, neither the 5'end nor the 3'end of the cDNA is complete and the strong secondary structure in the 5'-UTR of the ODC mRNA may lead to cDNA cloning artefact in the region. To further characterize the bovine ODC gene at the molecular level, the following research was undertaken to study the bovine ODC genomic gene. Cloning and sequence analysis of the bovine ODC genomic gene elucidated detailed structure of the ODC gene, confirmed the cDNA sequence and provided information for mapping the previously identified polymorphisms.

CHAPTER 4

BOVINE ORNITHINE DECARBOXYLASE GENE: CLONING, STRUCTURE AND POLYMORPHISMS

Jianbo Yao, David Zadworny, Samuel E. Aggrey, Urs Kühnlein and J.Flan Hayes

Dept. of Animal Science, McGill University, Macdonald Campus

Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Submitted to *DNA Sequence* for publication (1996)

The nucleotide sequence data reported in this manuscript have been submitted to GenBank and have been assigned the accession number: U36394

4.1 Abstract

Bovine ornithine decarboxylase (ODC) genomic clones were isolated from a bacteriophage lambda DASH genomic library. A total of 9452 bp sequence was determined which covers the entire sequence of the bovine ODC gene. Sequence analysis showed that the bovine ODC gene consisted of 12 exons which encode a protein identical to that inferred from a bovine ODC cDNA. Comparison of the structure and nucleotide sequence of the bovine, human and mouse ODC genes revealed that the gene was highly conserved. Primer extension analysis demonstrated that the transcription start point of bovine ODC mRNA was located 378 bp upstream from the A residue in the translation initiation codon. The 5'-untranslated region (UTR) of ODC mRNA was highly G+C rich, particularly in its 5'-most portion, and computer predictions suggested a very stable secondary structure for this region, with an overall free energy of formation of -134.4 kcal/mol. Conserved sequences and potential promoter elements including a TATA box, a possible CCAAT element, SP1 transcription factor binding sites (GC boxes) and cAMP response elements (CRE) were identified in the 5'-flanking region of the gene. Two polymorphic restriction sites, a *Taq*I and a *Msp*I, were mapped to the ODC gene and PCR-based methods for detection of the 2 polymorphisms were developed.

4.2 Introduction

The polyamines putrescine, spermidine and spermine are present in all prokaryotic and eukaryotic cells and appear to be indispensable for cellular growth and differentiation (Tabor and Tabor, 1984; Pegg, 1986). Ornithine decarboxylase (ODC: EC 4.1.1.17), which catalyses the conversion of ornithine to putrescine, is the major site of regulation of polyamine biosynthesis in mammalian tissues. The enzyme is characterized by a very short half-life and by rapid and dramatic changes in its activity in response to a wide variety of trophic stimuli, such as hormones, growth factors and drugs (Pegg, 1986; Abrahamsen and Morris, 1991). Numerous studies have shown that the enzyme activity is highly regulated by multiple processes including gene transcription, mRNA translation, protein turnover and post-translational modifications of the enzyme protein (Tabor and Tabor, 1984; Pegg, 1986).

Our laboratory is interested in studying the genetic variants of candidate genes which are associated with economically important traits in animals. Since ODC is tightly associated with DNA synthesis and cell proliferation, it could be an excellent candidate gene to study for its relationship to traits such as growth rate and milk production. In some species, ODC has been implicated as a candidate gene which affects the growth rate (Bulfield *et al.*, 1988; Gray and Tait, 1993). In addition, whereas, ODC is highly expressed in the lactating mammary gland of mice, its levels fall precipitously in association with the expression of sulfated glycoprotein-2 (SGP-2) at the onset of involution of the mammary gland (Strange *et al.*, 1992). The SGP-2 gene products is associated with the apoptotic pathway and thus ODC is postulated

to be a major component of tissue remodelling pathways (Strange *et al.*, 1992; Packham and Cleveland, 1994). In dairy cattle, genetic variants of ODC could be associated with mammary gland activity or interact with trophic hormones during lactation, thus affecting milk performance. We have previously reported the cloning of a bovine ODC cDNA and the identification of several restriction fragment length polymorphisms (RFLPs) in the bovine ODC gene using the cloned cDNA as a probe (Yao *et al.*, 1995). To further characterize the bovine ODC at the molecular level, we have cloned the bovine ODC genomic gene and determined its entire nucleotide sequence. Using the sequence information, we mapped a polymorphic *MspI* and a *TaqI* site to the ODC gene and developed PCR-based methods for detection of the 2 polymorphisms in dairy cattle.

4.3 Materials and methods

4.3.1 Genomic gene cloning

A genomic library was constructed from DNA isolated from the liver of a Holstein cow using a lambda DASH cloning kit according to the manufacturer's protocol (Stratagene, La Jolla, Calif., U.S.A.). The library were screened by plaque hybridization with bovine ODC cDNA as a probe (pY11: 1758 bp, Yao *et al.*, 1995). Plaques were plated at a density of $5x10^4$ plaque forming units/dish (150x15 mm) and transferred in duplicate onto Hybond-N (Amersham, Oakville, Ont., Canada) filters. Phage DNA on the filters was denatured in 1.5M NaCl, 0.5M NaOH for 2 minutes, neutralized in 1.5M NaCl, 0.5M Tris.HCl (pH 8.0) for 5 minutes. The filters were then baked at 80^0C for 2 hours. The cDNA insert from pY11 was ^{32}P-radiolabelled

by random primer extension using a T7 oligolabelling kit (Pharmacia, Baie d'Urfe, P.Q., Canada) and hybridized to the replicate filters for 16-20 hrs at 42^{0}C in a solution containing 5x SSC, 0.5x Denhard's solution, 20mM Tris (pH 7.6), 8% dextran sulphate, 24% deionized formamide, 0.1% SDS and 100 μg/ml of Herring sperm DNA. The filters were washed twice with 2x SSC, 0.1% SDS at 42^{0}C, 3 times with 0.5x SSC, 0.1% SDS and once with 0.1 x SSC, 0.1 % SDS at 50^{0}C and subjected to autoradiography. Plaques yielding positive hybridization signals were replated for secondary and tertiary rounds of screening with densities of 50 to 200 plaques per dish (100x15 mm). Four positive phage plaques, pODC2-1, pODC2-2, pODC2-4 and pODC2-7 were selected for further characterization.

4.3.2 DNA sequencing

Phage DNA from positive clones was purified by polyethylene glycol precipitation and analyzed by Southern blotting. Three *Pst*I fragments (4.4, 0.8 and 1.2 kb) and one *Hind*III fragment (2.6 kb) from pODC2-2 and one *Acc*I fragment (4.2 kb) from pODC2-7 were subcloned into pUC18 plasmid for sequence analysis (Figure 1). Nested deletions of the subclones were constructed by unidirectional digestion with exonuclease III using a double-strand nested deletion kit (Pharmacia) according to the manufacturer's protocol. Sequence was determined by the dideoxy-chain termination method of Sanger (1977) using a T7 sequencing kit (Pharmacia) with [^{35}S]dATP as the labelled nucleotide. The GC-rich regions were sequenced using Deaza G/A T7 sequencing mixes (Pharmacia) to eliminate compressions. DNA sequence data were processed and analyzed using the computer program DNASIS

(HIBIO, Brisbane, Calif., U.S.A.).

4.3.3 Primer extension analysis

A synthetic oligonucleotide primer (20-mers; 5'-GCCTCGCTCCCTCCCGCGGA-3') complementary to nucleotide 67 to 86 of exon 1, corresponding to the 5'-UTR of ODC mRNA, was end-labelled using polynucleotide kinase and [γ-^{32}P]ATP. The hybridization and extension conditions were as described (Yao *et al.*, 1995). The extended products were fractionated on a 7 M urea/8 % polyacrylamide sequencing gel. A sequence of the DNA which was obtained using the same synthetic oligonucleotide as sequencing primer was included for determination of the transcription start point (tsp).

4.3.4 PCR-RFLP analysis

The locations of a *Taq*I and a *Msp*I RFLP previously identified by Southern blot analysis (Yao *et al.*, 1995) were predicted, based on the bovine ODC genomic sequence information. The PCR was used to amplify 2 gene fragments covering the 2 polymorphic sites, respectively. One fragment is 1393 bp in length (position 2342 to 3734) which contains the polymorphic *Taq*I site. The other one is 796 bp long enclosing sequence from position 5413 to 6208 which harbours the polymorphic *Msp*I site. The sequences of the forward and reverse strand primers, respectively, were 5'-GTCAGGAAGATTCTCTAGAGA-3' and 5'-TGGATTTGCATAGATAATCC-3' for the 1393 bp fragment, and 5'-ACCACAGGATATGCAGACTGG-3' and 5'-GCACCCATGTTCTCAAAGAGC-3' for the 796 bp fragment. PCR was performed

in a reaction volume of 25 μl using 100 ng of DNA, 0.5 μM of each primer, 1x PCR buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$ and 50 mM KCl), 5% deionized formamide, 200 μM dNTP and 0.625 units of *Thermus thermophilus* (Tth) DNA polymerase (Pharmacia). DNA was denatured at 94°C for 5 min prior to the start of temperature cycle and subsequently the amplification was carried out for 35 cycles at 92°C x 30 sec, 50°C x 80 sec and 72°C x 120 sec for the 1393 bp fragment, and at 92°C x 30 sec, 61°C x 80 sec and 72°C x 90 sec for the 796 bp fragment using a DNA thermal cycler (Perkin Elmer Cetus Corp., New Jersey, U.S.A.). Seven μl of the amplified DNA were digested with 5 units of *TaqI* (for the 1393 bp fragment) or *MspI* (for the 796 bp fragment) at 37°C for 2 hours. The digested DNA fragments were then separated by electrophoresis in a 1.5% agarose gel in 1x TPE (90mM Tris-phosphate, 2mM EDTA). The gel was stained with ethidium bromide and visualized under UV light.

4.4 Results and discussion

4.4.1 Isolation of the bovine ODC gene

Approximately 500,000 plaques from a bovine genomic library were screened with bovine ODC cDNA as the hybridization probe. Four positive plaques were detected. Phage from these plaques were purified by two more rounds of screening. DNA was then isolated from purified phage and digested with *NotI* which excises the inserts from lambda vector. Analysis of the digested DNA by agarose gel electrophoresis demonstrated that pODC2-1, pODC2-2 and pODC2-7 contained inserts of about 15 kb, and pODC2-4 contained a bovine genomic fragment of about

17 kb. Transfer of the electrophoretically separated DNA to a membrane followed by hybridization with either end fragment of the bovine ODC cDNA showed that pODC2-1 and pODC2-4 only hybridized to the 3'end fragment, and pODC2-7 only hybridized to the 5'end fragment, while pODC2-2 hybridized to both fragments. The insert of the clone pODC2-2 was therefore subcloned into pUC18 plasmid. Three *Pst*I subclones (4.4, 0.8 and 1.2 kb) and one 2.6 kb *Hind*III subclone which overlaps the 3 *Pst*I fragments were generated. In addition, a 4.2 kb *Acc*I fragment from pODC2-7 was cloned which covers the 5'-flanking region and the exon 1 of the gene (Figure 4.1).

4.4.2 Structure and sequence of the bovine ODC gene

Unidirectional nested deletions were prepared for each of the overlapping subclones by limited digestion with exonuclease III and S1 nuclease and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). A total of 9452 bp sequence (GenBank and EMBL accession number: U36394) was determined by connecting the overlapping fragments which cover the entire sequence of the bovine ODC gene. Schematic representation of the structure of the bovine ODC gene is presented in Figure 4.1. As shown, the gene consists of 12 exons separated by 11 introns. The exon-intron junctions were deduced by comparison of the genomic sequence with the ODC cDNA sequence (Yao *et al.*, 1995) and all the introns are bordered by GT/AG sequences which conform to the gt-ag splice rule (Aebi *et al.*, 1986). The first two exons plus 32 bp of the 3rd exon represent the 5'-untranslated region (UTR) of the bovine ODC mRNA. The remaining part of the 3rd exon, the

4th through 11th exons and the first 142 bp of the 12th exon encode the ODC protein (461 amino acids). The rest of the 12th exon from the termination codon (TAG) to the polyadenylation signal (AATAAA) corresponds to the 3'-UTR of the ODC mRNA. There is only one nucleotide difference (G/A) between the bovine ODC genomic and cDNA sequences. The difference is at position 5654 of the genomic sequence (corresponding to position 1010 of the cDNA sequence) which was later identified to be a spontaneous mutation giving rise to a *Msp*I polymorphism.

Comparison of the nucleotide sequence of the bovine ODC gene with those of human (van Steeg *et al.*, 1989; Hickok *et al.*, 1990) and mouse (Katz and Kahana, 1988) shows that the genomic organization of the gene is highly conserved. Both the sizes and positions of protein-coding exons are completely conserved among the three species. The non-coding exons of bovine and other mammalian ODCs have a lower sequence homology (72-80% between bovine and human, 39-69% between bovine and mouse) compared to the coding exons (82-94% between bovine and human, 80-93% between bovine and mouse). The introns are, as expected, less conserved and display size heterogeneity among the species. In general, the introns of the bovine gene are shorter than those of human but longer than those of mouse (Table 4.1).

Unlike human and mouse ODC genes which contain 2 AATAAA polyadenylation signals separated from each other by about 400 nucleotides (Katz and Kahana, 1988; Hickok *et al.*, 1990), we observed in the present study that the bovine ODC gene has only one AATAAA polyadenylation signal which corresponds to the 1st AATAAA in human and mouse, and no additional AATAAA sequences were found in 715 nucleotides downstream from this signal. The alternative utilization

of the 2 signals has been suggested to cause the two ODC mRNA species detected in mouse (Berger *et al.*, 1984; Kahana and Nathans, 1984; Hickok *et al.*, 1986). However, the use of the 2nd signal in human was not observed as longer ODC mRNA in human cells and tissues have never been detected (Hickok *et al.*, 1987). Our observation is consistent to our previous finding that only one ODC mRNA species was detected in bovine cells (Yao *et al.*, 1995).

The transcription start point (tsp) of the bovine ODC gene was determined by primer extension analysis using a 5'-labelled oligonucleotide complementary to nucleotide +67 to +86 of the gene. The length of the extended products was determined on a denaturing polyacrylamide gel by comparison with the sequence ladder obtained from the gene fragment using the same primer. Clearly a single band of 86 bp is discernable which corresponds to a G residue (a C in normal sequence, Figure 4.2). This position is numbered +1 in the genomic gene sequence.

4.4.3 Sequence analysis of the promoter region of the bovine ODC gene

The nucleotide sequence (position -300 to +240) around the promoter region of the bovine ODC gene is shown in Figure 4.3. A number of sequence motifs that are identical or similar to consensus sequences for transcription regulatory elements were identified in the 5'flanking region of the gene. These putative regulatory elements include a TATA box (-40), a potential CCAAT (CCGAT) element (-92), 2 cAMP response elements (CRE, -180 and -58) and 2 GC boxes (CCGCCC or inverted form GGGCGG, -208 and -125) that represent potential binding sites for SP1 transcription factors. In addition, there are 4 GC boxes (+22, +90, +115, and

+123) present in exon 1 and another one at the beginning of intron 1 (+222). The GC box at position -125 is identical to the full consensus sequence GCCCCGCCCC (Dynan and Tjian, 1985) for SP1 binding sites.

The sequence in the promoter region of the bovine ODC gene is highly GC rich, particularly between -1 to -300 (75.3 %). This is a characteristic typical of housekeeping genes (Kim *et al.*, 1986; Bird, 1986). Alignment of the 300 bp 5'flanking region of the bovine ODC gene with the corresponding regions of human and mouse genes revealed a highly conserved region of approximately 120 bp (Figure 4.4). Sequence identities among the three species in this portion of the 5'flanking region are 84.6% to 87.8%. The conserved region contains the TATA box, the potential CCAAT element (CCAGT) and the completely matched GC box (-125) that are all fully conserved among the three species. Such highly conserved sequences are most likely to have a role in regulating ODC gene transcription. Indeed, both the GC box and the CCAGT sequence have been shown to be functional in a study (van Steeg *et al.*, 1990) using a bacterial reporter gene encoding CAT.

4.4.4 The 5'-UTR of the bovine ODC mRNA

Primer extension of the present study indicated that ODC mRNA transcription is initiated at a C residing 378 nucleotides upstream of the translation initiation site. This result does not agree with our previous estimate of the length (312 bp) for the 5'-UTR of the ODC mRNA (Yao *et al.*, 1995). The oligonucleotide primer (178 to 158 nucleotides upstream of the translation start site) used in the previous primer extension study was designed on the basis of available 185-bp 5'-UTR of the bovine

ODC cDNA. Due to this limitation, the distance between the primer annealing site and the tsp is long. This may cause reverse transcriptase to stop or pause in certain regions, particularly in regions with extensive secondary structure. Therefore, the primer extension product (155 bp) observed in the previous experiment may have resulted from incomplete transcription.

The 378 bp long 5'-UTR of the bovine ODC mRNA is longer compared to those of the other mammalian species (*e.g.* 334 bp for human, 310 bp for mouse) and it is the larger exons 2 and 3 which mainly account for this difference (see Figure 4.2). The significance of the considerably longer exons 2 and 3 in the bovine ODC gene is unknown.

Like the human and mouse ODC, the 5'-UTR of the bovine ODC mRNA is very rich in GC content, particularly in the region of exon 1 (203 bp) where GC content reaches 82%. Computer analysis (Zuker and Stiegler, 1981) of the sequence of the whole 5'-UTR of the bovine ODC mRNA predicted a highly stable secondary structure with an overall free energy of formation of -134.4 kcal/mol (Figure 4.5). Almost the entire 5'-UTR is involved in these structures. This predicted structure appears to be more complex than those found in the mouse (Katz and Kahana, 1988) and human (Pelletier and Kozak *et al.*, 1990) mRNA. The formation of extensive secondary structure in the 5'end of mRNA is well known to reduce translational efficiency in other mRNAs (Hershey, 1991; Kozak, 1992). Studies with rodent ODC have shown that the rate of translation of the full-length ODC mRNA is very poor in reticulocyte lysates and is improved by deleting large sections of the 5'-UTR (Ito *et al.*, 1990; Manzella and Blackshear, 1990; van Steeg *et al.*, 1991). It is likely that the secondary

structure predicted for the bovine ODC mRNA could affect its translation.

A short open reading frame (ORF) which encodes a peptide sequence of Met-Gly-Leu-Ala-Arg-Arg-Ala-Pro-Val-Leu was found at the 3'end of bovine exon 1 (position +170 to +202). Such small ORFs have also been found in the corresponding regions of human and mouse genes. The peptide sequences encoded by the human and mouse ORFs are Met-Gly-Leu-Ala-Cys-Gly-Ala-Trp-Ala-Leu and Met-Gly-Glu-Ala-Ser-Arg-Ala-Thr-Val-Leu, respectively. The bovine peptide sequence is 60% identical to the human and 70% to the mouse sequences. Such potential leader peptides have been suggested in other systems to affect the translation of the downstream major ORF (Werner *et al.*, 1987). Manzella and Blackshear (1990) reported that when the 3'-most 160 bp sequence (containing the small ORF) of the rat ODC 5'-UTR was attached to the growth hormone reporter mRNA, translation was repressed and this effect was abolished by the mutation of the AUG initiation codon of the small ORF. In contrast, several other studies showed that the small ORF present in the 5'-UTR of ODC mRNA had little or no effect on translation (van Steeg *et al.*, 1991; Kashiwagi *et al.*, 1991; Grens and Scheffler, 1990).

4.4.5 PCR-RFLP analysis of the bovine ODC gene

Based on the restriction map of the bovine ODC gene and our previous Southern blot analysis (Yao *et al.*, 1995), we mapped two polymorphic restriction sites, a *Taq*I and a *Msp*I, to the bovine ODC gene. The polymorphic *Taq*I site was found to be in the first intron and the *Msp*I site in the middle of the ninth exon. The regions covering either site were amplified by PCR and analyzed by digestion with

respective restriction enzymes. The polymorphic *Taq*I site is the only *Taq*I site present in the amplified 1393 bp fragment which is restricted into a 1224 bp and a 169 bp fragment when the *Taq*I site is present. Similarly, The 796 bp fragment containing the *Msp*I site is cleaved to a 240 bp and a 556 bp fragment when the *Msp*I site is present. Sequence analysis of respective PCR fragments revealed that the absence of the *Taq*I site (*Taq*I [-]) was due to a G to T change at position 2512 and the absence of the *Msp*I site (*Msp*I[-]) was due to a G to A change at position 5654 (Figure 4.6). The G to A transition changes the codon CCG to CCA. However, it is a silent mutation as both triplets code for the same amino acid, proline.

Implementation of marker-assisted selection programs will first require identification of candidate genes or anonymous genetic markers associated with the traits of interest. In dairy cattle, genes associated with mammary gland growth and lactation are excellent candidate genes for milk production. ODC is believed to play an essential role in the growth and differentiation of cells by regulating the biosynthesis of polyamines. It has been shown that ongoing polyamine synthesis is essential for prolactin to stimulate the synthesis of milk products (Golden and Rillema, 1993) and the expression of the ODC gene in lactating mammary gland is extremely high and drops dramatically after weaning, suggesting that the ODC gene is tightly associated with lactation (Strange *et al.*, 1992). Genetic variants of ODC in mice have been shown to affect both transcription and translation of the gene (Johannes and Berger, 1992; Gray and Tait, 1993; Gray *et al.*, 1995). It is possible that in dairy cattle, genetic variants of ODC would affect mammary gland growth and (or) involution or interact differently with trophic hormones that modulate lactation.

The 2 polymorphisms could be further studied for their correlations to milk production traits in dairy cattle. The PCR-based methods for detection of the 2 polymorphisms should make genotyping a large number of animals for quantitative analysis feasible.

Table 1. Structural homology between the bovine and other mammalians ODC genes

Exon	Length (bp)			Homology (%)			Intron	Length (bp)			Homology	
	Bovine[a]	Human[b]	Mouse[c]	B/H[d]	B/M[d]			Bovine[a]	Human[b]	Mouse[c]	B/H	B/M
1	203	207	196	72	69		1	2502	2856	1928	52	51
2	143	110	98	80	39		2	85	105	89	66	46
3	134	119	118	87	80		3	268	283	232	59	50
4	174	174	174	90	87		4	162	206	143	56	41
5	173	173	173	93	88		5	224	258	186	63	48
6	135	135	135	91	93		6	122	130	101	63	48
7	82	82	82	90	84		7	180	176	160	66	54
8	84	84	84	94	89		8	908	1055	544	58	45
9	163	163	163	92	88		9	128	82	81	67	57
10	113	113	113	82	85		10	88	93	82	61	40
11	215	215	215	91	89		11	489	640	517	43	42
12	469[e]	467[e]	459[e]	82	79							

a) The length of the exons and introns of the bovine ODC gene was determinied by comparing the ODC genomic sequence (present study) with the ODC cDNA sequence (Yao *et al.*, 1995).
b) Information obtained from van Steeg *et al.* (1989).
c) Information obtained from Katz and Kahana (1988).
d) B/H: bovine vs. human; B/M: bovine vs mouse.
e) Length up to the end of the polyadenylation signal (the first signal in cases of human and mouse).

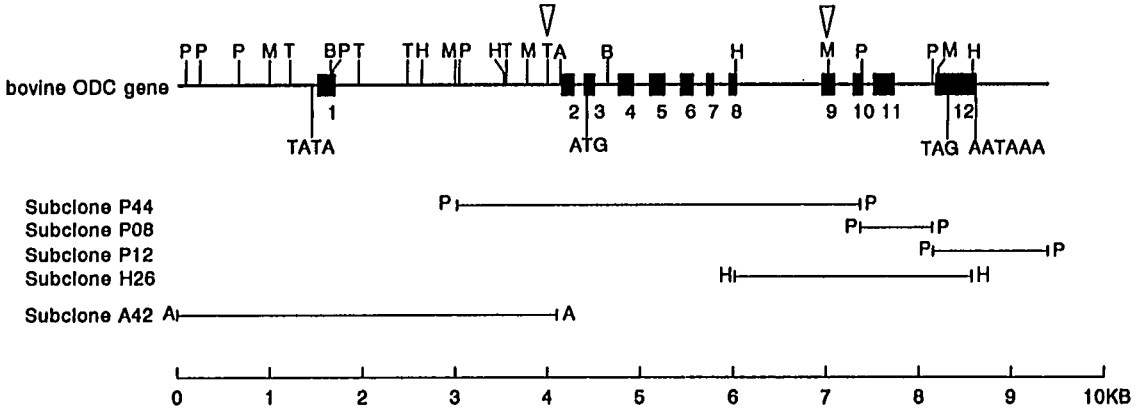

Figure 4.1 Schematic representation of the structure of the bovine ODC gene. The solid boxes represent exons which are numbered underneath. The position of the TATA box, ATG initiation codon, TAG termination codon and AATAAA polyadenylation signal are indicated. The restriction endonuclease sites shown are as follows: A, *Acc*I; B, *Bam*HI; H, *Hind*III; M, *Msp*I; P, *Pst*I; T, *Taq*I. The two vertical arrowheads indicate the polymorphic TaqI and MspI sites. The extent of the overlapping subclones are displayed under the structural map. The numbers below the bottom line indicate length in kilobase pairs (kb).

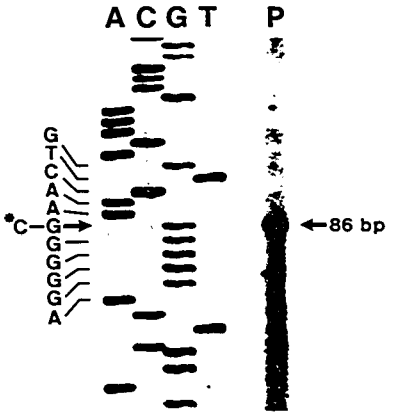

Figure 4.2 Determination of the transcription start point by primer extension analysis. A synthetic oligonucleotide primer complementary to nucleotide 67 to 86 in exon 1 was end-labelled and hybridized to 12 μg of total RNA from bovine mammary gland epithelial cells (Huynh *et al.*, 1991). The extended products were resolved in a 7 M urea-8% polyacrylamide sequencing gel. Lanes A, C, G, T show a sequencing reaction ladder obtained from the gene fragment using the same primer. Lane P is the extended product. The deduced nucleotide sequence (complementary) around the tsp is displayed on the left with the tsp marked by an asterisk.

Figure 4.3 Nucleotide sequence (position -300 to +240) around the promoter region of the bovine ODC gene. Putative regulatory sequences are underlined. They include 1 TATA box, 1 CCAAT element, 2 cAMP response elements (CRE) and 7 GC boxes. The sequence is part of the bovine ODC genomic sequence (9452 bp), which has been submitted to the GenBank with the accession number: U36394.

```
          GC box                                        CCAAT
Bovine    CCCCGCCCCC TCCCTGGGAG GACGCCCGGA CGGAACCGAT CGCGGCTGGT TTGAGCTGGT
Human     *********- ****CC*GCC *TGT*****C ********** **T******* **********
Mouse     *********- ****CC*-CC *--T*****C ********** **C******* **********

                     CRE                  TATA
Bovine    GCGTCTCCAT GACGACACGC GCGGCGCTAT AAGTAGCGGC GCGGCGGCGC GCCGGGCTTT
Human     ********** *G****CC** -***T*C*** ******-**A ***G**TGC* *TG*******
Mouse     ********** *A****GT** T***C*-*** ******C**C ***T**CAC* *TC*******
```

Figure 4.4 Comparison of an about 120 bp conserved region in the 5'flanking sequence of bovine, human and mouse ODC genes. The nucleotide sequence for positions -127 to -8 of the bovine ODC gene (this report), -117 to -1 of the human ODC gene (van Steeg *et al.*, 1989) and -115 to -1 of the mouse ODC gene (Kutz and Kahana, 1988) are shown. [-] represent gaps inserted to enhance alignment of conserved nucleotides. Asterisks indicate nucleotide identity.

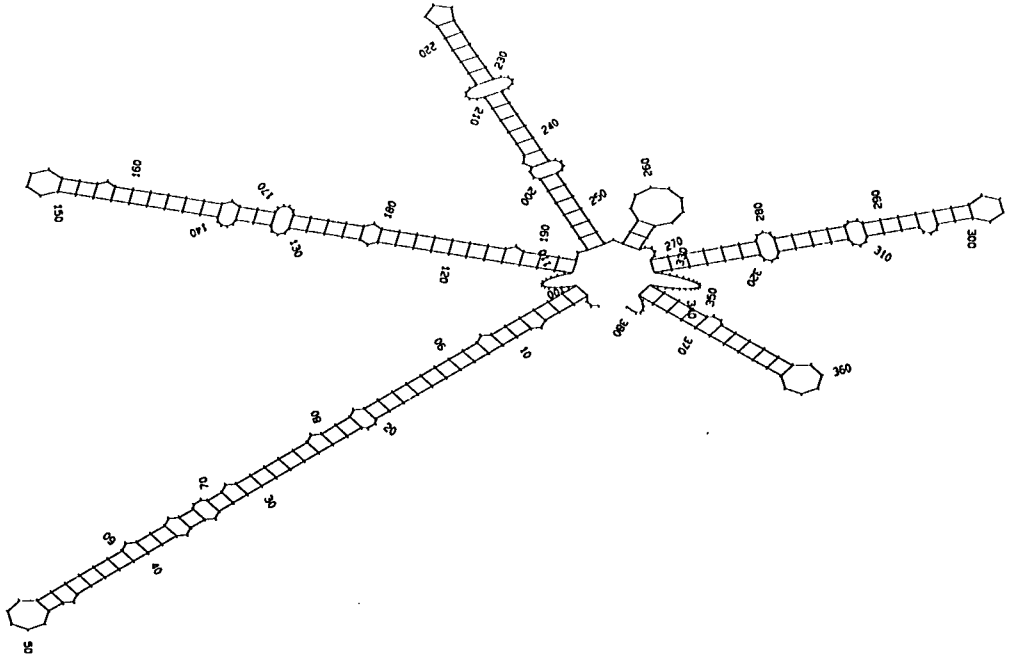

Figure 4.5 Potential secondary structure in the 5'-UTR of bovine ODC mRNA. The entire 5'-UTR of the bovine ODC mRNA was subjected to secondary structure analysis using the mfold program (Wisconsin GCG computer package) based on the method of Zucker and Steigler (1981). The calculated free energy of the entire structure is -134.2 kcal/mol.

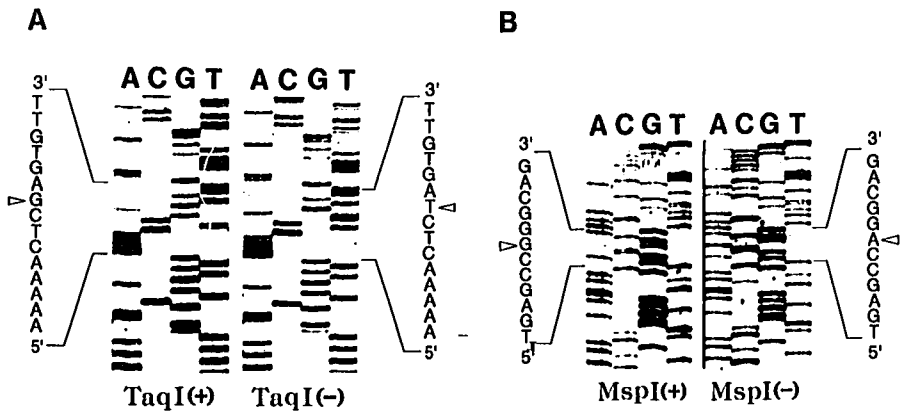

Figure 4.6 Sequence analysis of the mutations causing the *Taq*I and *Msp*I polymorphisms. (A) Partial sequence of the 1393 bp fragment of the ODC gene. the absence of the *Taq*I restriction site was due to a G to T change at position 2512 (indicated by arrows). (B) Partial sequence of the 796 bp fragment of the ODC gene. The absence of the *Msp*I restriction site was due to a G to A change at position 5654 (indicated by arrows).

Connecting statement II

In the previous 2 chapters, we have described the cloning and characterization of the bovine ODC gene and the development of PCR-based methods for detection of a *Msp*I and a *Taq*I polymorphism in the ODC gene. The following chapter describes the analysis of the 2 polymorphisms in a total of 127 Holstein bulls using the PCR-based methods and the identification of ODC alleles that are associated with selection for milk production in Holstein bulls.

CHAPTER 5

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS AT THE ORNITHINE DECARBOXYLASE LOCUS ASSOCIATED WITH MILK PROTEIN IN HOLSTEINS

Jianbo Yao, Samuel E. Aggrey, David Zadworny, J. Flan Hayes and Urs Kühnlein

Dept. of Animal Science, McGill University, Macdonald Campus

Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

The manuscript is expanded from a paper published in *Animal Genetics* (27: 283-284, 1996)

5.1 Abstract

Restriction fragment length polmorphisms (RFLPs) for ornithine decarboxylase (ODC) gene were tested for associations with milk related traits in Holsteins. Analysis of 127 bulls revealed the presence of two RFLPs, one at a *Msp*I site and another at a *Taq*I site. Three of the possible four RFLP combinations were observed indicating the presence of three ODC alleles (A1, A2 and A4) in the bull population sampled. There were significant changes (X^2 test: $P \leq 0.005$) in allele frequencies between bulls born in 1950-1970 and bulls born in the 1980s. The frequency of the A2 allele increased, whereas, that of the A4 allele decreased in the 1980s. Milk protein yield among the daughters of bulls with A1A2 genotype was 10 kg higher ($P \leq 0.007$) than bulls with A1A4 genotype per lactation. These results suggest that selection for milk production may have resulted in the change of frequencies of ODC alleles and different ODC genotypes may have different effects on milk protein yield.

5.2 Introduction

Marker-assisted selection may provide the opportunity to make significant genetic gains in the improvement of economically important traits in livestock (Soller and Beckmann, 1983; Smith and Simpson, 1986). Implementation of this approach will first require identification of candidate genes or anonymous gene markers associated with the traits of interest. Candidate genes are those with a known relationship between physiological or biochemical processes and an economically important trait. In dairy cattle, genes associated with mammary gland growth, development and function are excellent candidate genes for milk production traits.

The polyamines are low molecular weight polycations that influence cell proliferation and growth (Tabor and Tabor, 1984; Pegg, 1986). Ornithine decarboxylase (ODC, 4.1.1.17) catalyses the conversion of ornithine to putrescine, the rate limiting step in polyamine biosynthesis (Pegg, 1986). The level of ODC is induced in quiescent cells exposed to a wide variety of stimuli such as growth hormone (GH), corticosteroids, testosterone and growth factors (Tabor and Tabor, 1984). Evidence is accumulating that growth factors influence morphogenesis and differentiation of the mammary gland (Imagawa *et al.*, 1994). In particular, it has been shown that trophic hormones such as prolactin (PRL) and GH that are associated with lactation are also required to induce differentiation of bovine mammary epithelial cells (Huynh *et al.*, 1991; Flint and Gardner, 1994), and both mitogenic and lactogenic effects of PRL, insulin and hydrocortisone appear to be mediated through the polyamine pathway (Rillema *et al.*, 1977, 1986; Bedford and Zadworny, 1990). In mice, ODC gene is highly expressed in lactating mammary gland

and drops dramatically after weaning (Strange *et al.*, 1992). Genetic variants of ODC have been identified in a number of species that have been divergently selected for growth related traits (Bulfield *et al.*, 1988; Gray and Tait, 1993) and it is therefore possible that particular variants of ODC could be associated with increased mammary gland function and thus influence milk related traits.

The objectives of the present study were to estimate allele frequencies of ODC polymorphisms in Holstein bulls, and to further evaluate the genotypic effects of ODC variants on milk production traits.

5.3 Materials and methods

5.3.1 Amplification of DNA by polymerase chain reaction (PCR)

High molecular weight genomic DNA was isolated from 127 proven Holstein bulls, as described (Zadworny and Kühnlein, 1990). Pairs of primers were designed according to the sequence of the bovine ODC gene (GenBank accession number: U36394, Yao *et al.*, unpublished data) to amplify 2 fragments in the bovine ODC gene by PCR. One fragment was 1393 bp in length (position 2342 to 3734) which contained a polymorphic *TaqI* site. The other fragment was 796 bp in length (position 5413 to 6208) which harbored a polymorphic *MspI* site. The sequence of the forward and reverse strand primers, respectively, were 5'GTCAGGAAGATTCTCTAGAGA-3' and 5' TGGATTTGCATAGATAATCC-3' for the 1393 bp fragment, and 5'ACCACAGGATATGCAGACTGG-3' and 5'-GCACCCATGTTCTCAAAAGAGC-3' for the 796 bp fragment. The PCRs for both fragments were performed in a reaction volume of 25 μl using 100 ng of DNA, 0.5 μM of each primer, 1x PCR

buffer, 5% deionized formamide, 200 μM dNTP and 0.625 units of *Thermus thermophilus* (Tth) DNA polymerase (Pharmacia, Baie d'Urfe, Québec, Canada). The amplification was carried out for 35 cycles at 92°C x 30 s, 50°C x 80 s and 72°C x 120 s for the 1393 bp fragment, and at 92°C x 30 s, 61°C x 80 s and 72°C x 90 s for the 796 bp fragment using a DNA thermal cycler (Perkin Elmer Cetus Corp., New Jersey, USA).

5.3.2 RFLP analysis

For RFLP analysis of the 2 fragments, 7 μl of each amplified DNA were digested with 5 units of *TaqI* (for the 1393 bp fragment), or *MspI* (for the 796 bp fragment) at 37°C for 2 hours. The digested DNA fragments were then separated by electrophoresis in a 1.5% agarose gel in 1x TPE (90mM Tris-phosphate, 2mM EDTA). The gel was stained with ethidium bromide and visualized under UV light.

5.3.3 Production traits

All estimated breeding values were provided by the Canadian Holstein Breeders Association. The bull's breeding values for milk related traits (kg milk, kg fat and kg protein) were estimated with the Best Linear Unbiased Procedure (BLUP) based on an animal model with a relationship matrix. Preadjustments were made for effects of age and month of calving of daughters. The model included fixed effects of herd-year-season and age group of sires. The breeding values were expressed as deviations from the mean.

5.3.4 Statistical analysis

Differences in allele frequencies between bulls born between 1950-1970 and bulls of the 1980s were tested with Chi square. The effects of ODC genotypes on the bull's estimated breeding values for kg milk, kg fat and kg protein were analyzed according to least squares methods. As the breeding values are the best available estimates of the additive genotype of the bulls, no environmental effects were included in the model. The effect of birth-year of the bulls were included in the model to account for genetic trend in the bulls from the 1950s to the 1980s. The model used was as follows:

$$Y_{ijk} = \mu + Year_i + \alpha_j + e_{ijk}$$

where Y_{ijk} is the breeding value (kg milk, kg fat or kg protein) of the k^{th} bull; μ is the least square mean of the trait, $Year_i$ is the effect of the i^{th} birth-year of the bull; α_j is the effect of the j^{th} genotype; and e_{ijk} is the random residual effect. Due to small numbers, bulls with genotypes A1A1 (n=7) and A4A4 (n=4) were removed from the analysis. Difference in milk related traits values exhibited by the genotypes were compared by least square means.

5.4 Results

Restriction fragment length polymorphisms at the ODC locus were recognized following digestion of the PCR products with *Msp*I and *Taq*I restriction enzymes. When the *Taq*I site is present, the 1393 bp fragment is cleaved into a 1224 bp and

a 169 bp fragment (Figure 5.1A). The *Taq*I polymorphic site is located at the end of intron 1 and it occurs as a result of a G to T base substitution. When the *Msp*I site is present, the amplified 796 bp fragment is cleaved into a 556 bp and a 240 bp fragment (Figure 5.1B). The *Msp*I polymorphic site is located in the middle of exon 9 and results from a G to A base substitution. From the *Msp*I and *Taq*I polymorphic sites, 4 possible alleles (A1, A2, A3, and A4) were expected, however, the A3 allele was not observed. Table 5.1 shows the allele frequencies of A1, A2 and A4 in the 1950-1970 and 1980-1987 groups, respectively. The difference in allele frequencies between the two groups was significant ($X^2_{df=2}$=12.18; $P \leq 0.005$).

Estimates of milk related traits for ODC genotypes are presented in Table 5.2. Bulls with A1A2 genotype had greater kg milk protein ($P \leq 0.007$) than bulls with A1A4 genotype. The difference in kg milk protein between bulls with A1A2 and A2A4 genotypes was also significant ($P \leq 0.05$). There were no differences ($P \geq 0.05$) in kg milk and kg fat between the ODC genotypes.

5.5 Discussion

This study reports on the identification of a candidate gene for quantitative traits based on a combined genetic and biochemical approach. This type of study provides biological data to augment statistical insight into the nature of genes affecting quantitative variation. Both *Msp*I and *Taq*I ODC polymorphisms have been previously reported (Yao *et al.*, 1995). RFLP analysis in the Holstein population investigated revealed three ODC alleles. Comparing the allele frequencies in bulls born between 1950-1970 and bulls of the 1980s indicated that the A2 allele had

increased, whereas, the A4 allele had decreased. Over the past 40 years, dairy improvement has resulted in an approximate doubling of milk production (Fallert and Liebrand, 1991). Therefore, this result suggests that selection for milk production may be associated with the changes in the ODC allele frequencies. The result could also be due solely to genetic drift. However, estimates of genotypic effects of the ODC variants in Table 5.2 indicate significant difference between different ODC genotypes for milk protein yield.

There is a large body of literature suggesting that polyamines can influence protein synthesis in a variety of ways affecting both the rate and the fidelity of translation (McCann, 1982; Takemoto *et al.*, 1983; Tabor and Tabor, 1984). ODC is the key regulatory enzyme in the biosynthesis of polyamines. Thus a particular variant of the ODC gene could alter the rate of polyamine synthesis. It has been shown that the rate of casein synthesis is enhanced when spermidine is combined with the agents which stimulate the rate of RNA synthesis (Rillema *et al.*, 1977). It is therefore possible that the regulatory mechanism of ODC directly or indirectly affects milk protein biosynthesis. This could imply that allelic variation in the ODC gene would have an effect on milk protein yield or composition.

Table 5.1 Allele frequency of ODC polymorphisms at *Msp*I and *Taq*I restriction sites in two groups of Holstein bulls

Allele[1]	1950 - 1970 (N = 66)	1980 - 1987 (N = 61)
A1	0.28	0.25
A2	0.49	0.67
A4	0.23	0.08

[1]:A1, Presence of *Msp*I and *Taq*I sites;

A2, Presence of *Msp*I site only;

A4, Absence of *Msp*I and *Taq*I sites.

Alleles where only the *Taq*I site was present were not observed.

Table 5.2 Least square means (± standard error) of breeding value for milk related traits in Holstein bulls with different ODC genotypes

		Trait (kg)		
Genotype	No. of Bulls	Milk	Fat	Protein
A1A2	41	-300±82	-11±3	-11±2[a]
A1A4	13	-307±142	-20±5	-21±3[b]
A2A2	44	-446±91	-15±3	-15±2
A2A4	18	-398±125	-15±4	-12±3[a]

[a,b] means within columns with different superscripts differ significantly ($P<0.05$).

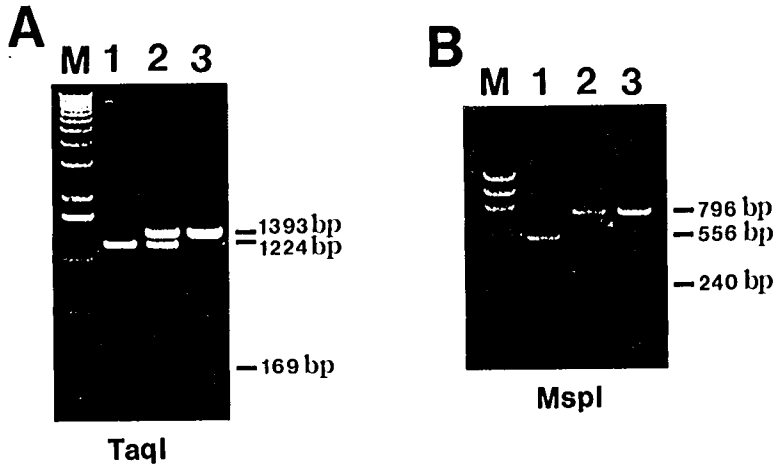

Figure 5.1. Separation of PCR products in 1.5 % agarose gel stained with ethidium bromide following digestion with *Taq*I (A) and *Msp*I (B). (A) *Taq*I-RFLP pattern. Lane M: 1 kb DNA molecular weight marker. Lanes 1 to 3 represent individuals with genotypes *Taq*I(+/+), *Taq*I(+/-) and *Taq*I(-/-), respectively. (B) *Msp*I-RFLP pattern. Lane M: *Hae*III digest of PhiX 174 DNA as molecular weight marker. Lanes 1 to 3 represent individuals with genotypes *Msp*I(+/+), *Msp*I(+/-) and *Msp*I(-/-), respectively.

Connecting statement III

In the previous three chapters, we have studied one of the candidate genes, ODC, by cloning and RFLP analysis of the gene. GH gene is another attractive candidate gene for milk production in cattle. Although DNA polymorphisms of the gene have been previously studied by several groups, most of the work were based on Southern blot analysis that limits the detection of single base changes which do not alter any recognition sequences of restriction enzymes. The following chapter decribes a comprehensive screening of sequence variations in the bovine GH gene by using SSCP analysis and the detection of polymorphisms that are strongly associated with milk production traits.

CHAPTER 6

SEQUENCE VARIATIONS IN THE BOVINE GROWTH HORMONE GENE CHARACTERIZED BY SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS AND THEIR ASSOCIATION WITH MILK PRODUCTION TRAITS IN HOLSTEINS

Jianbo Yao, Samuel E. Aggrey, David Zadworny, J. Flan Hayes and Urs Kühnlein

Dept. of Animal Science, McGill University, Macdonald Campus

Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Published in *Genetics* (144: 1809-1816, 1996)

6.1 Abstract

Sequence variations in the bovine growth hormone (GH) gene were investigated by single strand conformation polymorphism (SSCP) analysis of 7 amplified fragments covering almost the entire gene (2.7 kb). SSCPs were detected in 4 of these fragments and a total of 6 diallelic polymorphisms were found in various regions of the gene in a sample of 128 Holstein bulls. Two of the polymorphisms, a T to C transition in the third intron (designated GH4.1) and an A to C transversion in the fifth exon (designated GH6.2), were shown to be associated with milk production traits. Bulls with $GH4.1^{c}/GH4.1^{c}$ genotype had higher milk yield compared to $GH4.1^{c}/GH4.1^{t}$ ($P \leq 0.005$) and $GH4.1^{t}/GH4.1^{t}$ ($P \leq 0.0022$) bulls, respectively. Bulls with $GH4.1^{c}/GH4.1^{c}$ genotype had higher kg fat ($P \leq 0.0076$) and protein ($P \leq 0.0018$) compared to bulls with $GH4.1^{c}/GH4.1^{t}$ genotype. Similar effects were observed with the GH6.2 polymorphism on milk production traits with the $GH6.2^{a}$ allele being the favourable allele. The average effects of the gene substitution for GH4.1 and GH6.2 are similar, with ± 300 kg for milk yield, ± 8 kg for fat content and ±7 kg for protein content per lactation. The positive association of the $GH4.1^{c}$ and $GH6.2^{a}$ alleles with milk production traits may be useful for improving milk performance in dairy cattle by selection at the DNA level.

6.2 Introduction

In dairy cattle, the primary focus of selection, at least in North America, has been to improve milk yield and tremendous gains have been achieved using classical quantitative genetics. Although the genes which affect a polygenic trait such as milk production traits are unknown, a number of potential candidate genes have been recognized. Candidate genes are selected on the basis of known relationship between physiological or biochemical processes and a trait, and are tested as putative quantitative trait loci (QTL). Current knowledge in dairy biology indicates that genetically superior animals differ from lesser animals mainly in their regulation of nutrient utilization and that growth hormone (GH) exerts a key control in nutrient use (Baumann, 1992), mammary development (Sejrsen *et al.*, 1986), growth (Brier *et al.*, 1991), and also modulates intermediary metabolism and other physiological processes e.g. aging (Copras *et al.*, 1993) and immune responsiveness (Blalock, 1994). Thus the GH gene is a promising candidate gene worth studying for its effects on milk and growth related and immune response traits. Selection for milk yield has been shown to be associated with increased blood levels of GH (Peel and Baumann, 1987; Bronczek *et al.*, 1988; Lukes *et al.*, 1989). This suggests that variations in the levels of GH could be used as an indicator for potential milk yield and thus be incorporated into a selection index. However, blood levels of GH are dependent on physiological states of the animal and vary with the stage of lactation or even the time of day. For this reason, estimating breeding value on the basis of GH levels would require multiple measurements over the course of several lactations.

There is evidence for an association of genetic variants of the GH gene with

plasma levels of GH (Schlee *et al.*, 1994), suggesting that at least some variations of the GH levels are caused by mutations in the GH gene itself. Identification of such mutations would permit selection at the DNA level without necessitating measurement of GH levels. In order to detect such mutations, we have used single strand conformation polymorphism (SSCP) analysis (Orita *et al.*, 1989a,b), a technique based on the principle that single-stranded DNA molecules form specific sequence-based secondary structures under nondenaturing conditions. Our objective was to screen the entire length of the bovine GH gene for sequence variations and study the associations of changes in potential GH sequence variants with changes in milk production traits in Holsteins.

6.3 Materials and methods

6.3.1 DNA samples

Semen samples from 128 Holstein bulls representing bulls used for artificial insemination from 1950 to 1987 were obtained from the Centre d'insemination artificielle du Quebec (Saint-Hyacinthe, Quebec, Canada), the Western Ontario Breeders Inc. (Woodstock, Ontaria, Canada) and the United Breeder Inc. (Guelph, Ontario, Canada). Genomic DNA was extracted as previously described (Zadworny and Kuhnlein, 1990).

6.3.2 DNA amplification with polymerase chain reaction (PCR):

Based on the published nucleotide sequence information of the bovine GH gene (Gordon *et al.*, 1983), pairs of oligonucleotide primers were synthesized to

amplify 7 GH fragments (designated GH1, GH2, GH3, GH4, GH5, GH6 and GH7). The gene from -641 bp upstream of the first exon to 411 bp downstream of the last exon, except a 155 bp fragment from position 1225 to 1379 were analyzed for each bull. The primer sequences, location and size of the amplified fragments are shown (Table 6.1). PCR was performed in a reaction volume of 25 μl using 100 ng of DNA, 0.5 μM of each primer, 1x PCR buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$ and 50 mM KCl), 5% deionized formamide, 200 μM dNTP and 0.625 units of *Thermus thermophilus* (Tth) DNA polymerase (Pharmacia). The amplification was carried out for 35 cycles at 92°C x 30 sec, 59°C x 80 sec and 72°C x 90 sec using a DNA thermal cycler (Perkin Elmer Cetus Corp.).

6.3.3 SSCP analysis

SSCP was carried out with a Bio-Rad "Mini-Protein II" (Bio-Rad) vertical gel. One μl of the PCR product was diluted with 15 μl of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The mixture was then denatured at 100°C for 5 min, cooled in ice for 5 min and loaded on a nondenaturing 12-20% acrylamide:bis-acrylamide (49:1) gel. Electrophoresis was performed in 1x Tris borate (pH 8.3)-EDTA buffer at 10-12.5 volts/cm for 6-24 hours at room temperature. DNA was detected by silver staining.

6.3.4 Sequence analysis

DNA fragments which displayed a modified electrophoretic pattern were selected for sequencing. For blunt-end ligation into plasmid vectors, the amplified

PCR products were concentrated by ethanol precipitation and subjected to electrophoresis in a 1% agarose gel. The DNA was isolated from the gel using DEAE cellulose membrane (Sambrook *et al.*, 1989), phosphorylated at the 5'-terminus by T4 polynucleotide kinase (Pharmacia) and ligated into the *Sma*I site of pUC18 plasmid. DNA sequences were determined by the dideoxy-chain termination method of Sanger *et al.* (1977) with [^{35}S]dATP using a T7 sequencing kit (Pharmacia).

6.3.5 Production traits

All estimated breeding values were provided by the Canadian Holstein Breeders Association. The bull's breeding values for milk related traits (kg milk, kg fat and kg protein) were estimated with the Best Linear Unbiased Procedure (BLUP) based on an animal model with a relationship matrix. Preadjustments were made for effects of age and month of calving of daughters. The model included fixed effects of herd-year-season and age group of sires.

6.3.6 Statistical analysis

The effect of GH genotypes on the bull's estimated breeding values for kg milk, kg fat and kg protein for each polymorphic site were analyzed using least squares methods. As the breeding values are the best available estimates of the additive genotype of the bulls, no environmental effects were included in the model. The effect of birth-year of the bulls were included in the model to account for genetic progress made in the bulls from the 1950s to the 1980s. The model used was as follows:

$$Y_{ijk} = \mu + Year_i + \alpha_j + e_{ijk}$$

where Y_{ijk} is the breeding value (kg milk, kg fat or kg protein) of the k^{th} bull; μ is the least square mean of the trait; $Year_i$ is the effect of the i^{th} birth-year of the bull (genetic trend); α_j is the effect of the j^{th} genotype (j=1,2,3); and e_{ijk} is the random residual effect. Type III sum of squares were used to evaluate the effect of GH polymorphisms. Difference in milk related traits values exhibited by the genotypes were compared by least square means. Average additive gene substitution effects of the milk production traits were calculated for the alleles of the GH polymorphisms which have significant effects on the traits according to Falconer (1989).

6.4 Results

Seven GH fragments (GH1-GH7) which cover almost the entire length of the bovine GH gene were amplified. The position and length of the fragments are shown in Figure 6.1. No SSCPs were detected in the GH2, GH3 and GH7 regions under various electrophoretic conditions (data not shown). However, analysis of the GH1, GH4, GH5 and GH6 fragments did reveal polymorphisms. The optimal conditions of SSCP analysis for the 4 polymorphic fragments are listed in Table 6.2.

Analysis of the 464 bp GH1 fragment revealed 2 SSCP alleles. The faster migrating band was designated as A_1, and the slower one as B_1 (Figure 6.2A). The heterozygous individual (A_1B_1) presented 3 distinct bands indicating that only one strand of an allele had a novel conformation with a different electrophoretic mobility.

The other strand, possessing the complementary sequence change, did not have a new conformation to cause a mobility shift and continued to migrate with the corresponding strand of the other allele. Sequence analysis revealed that the difference in mobility was due to a deletion (A_1) or an insertion (B_1) of a TGC repeat at position between 125 and 142 (Figure 6.1), resulting in 5 TGC repeats for the A_1 allele and 6 TGC repeats for the B_1 allele (Figure 6.2B).

Figure 6.3A shows the SSCP pattern of the GH4 fragment of the gene. Three alleles were observed and designated as A_4, B_4 and C_4, respectively, on the basis of increasing distances in the gel. All possible genotypes, A_4A_4, B_4B_4, C_4C_4, A_4B_4, A_4C_4 and B_4C_4 were observed in the samples examined. For homozygous individuals (lanes 1, 2 and 3), three bands were observed under the SSCP conditions used. The two slowest migrating bands may represent two different conformations of the same strand. Sequence analysis of the region revealed 2 mutations in the third intron of the gene (Figure 6.1). Both mutations are T to C transitions with one at position 1547 and the other one at 1692 (Figure 6.3B). Three of the four possible combinations from the two mutations, $C^{1547}...T^{1692}$, $T^{1547}...T^{1692}$ and $C^{1547}...C^{1692}$, were observed, representing SSCP alleles A_4, B_4 and C_4, respectively. The polymorphisms in the GH fragment at the 1547 and 1692 mutation sites were designated GH4.1 and GH4.2, respectively.

The 366 bp GH5 region harboured one mutation at position 2017 as determined by sequence analysis. The mutation was a T to C transition which gave rise to 2 alleles (designated A_5 and B_5) detected by SSCP (Figure 6.4). As in the case of GH1, only three bands appeared in the heterozygous samples due to the same

electrophoretic mobility of two of the four strands.

Figure 6.5A shows the SSCP results for the GH6 fragment. Three different alleles, A_6, B_6 and C_6, were identified and all the possible genotypes were observed. Sequence determination of the entire length of the fragment revealed 2 polymorphic sites at positions 2141 and 2291, respectively (Figure 6.5B). Both were base substitutions and located in the fifth exon (Figure 6.1). The mutation at position 2141 was a C to G transversion, while the mutation at 2291 was an A to C transversion. SSCP analysis detected 3 (A_6: G^{2141}...A^{2291}; B_6: C^{2141}...A^{2291} and C_6: C^{2141}...C^{2291}) of the 4 possible combinations from the 2 mutations. The other possible combination (G^{2141}...C^{2291}) either does not produce unique band migration or does not exist in the sample population investigated. The polymorphisms in the GH6 fragment at positions 2141 and 2291 were designated GH6.1 and GH6.2, respectively. The sequence changes, nucleotide positions and allelic designation of the 6 polymorphisms in the bovine GH gene are presented in Table 6.3. In this table, alternative alleles of each polymorphism were assigned a superscript on the basis of the nucleotide present.

The effects of the genotypes of the 6 GH polymorphisms on the breeding values for milk yield, fat content and protein content per lactation were examined in 128 Holstein bulls using least square methods. Least square means of the three GH genotypic classes of the 6 polymorphisms are presented in Table 6.4. Bulls with *GH4.1*^{c}/*GH4.1*^{c} genotype had higher milk yield compared to *GH4.1*^{c}/*GH4.1*^{t} ($P \leq 0.005$) and *GH4.1*^{t}/*GH4.1*^{t} ($P \leq 0.0022$) bulls, respectively. Bulls with *GH4.1*^{c}/*GH4.1*^{c} genotype had higher kg fat ($P \leq 0.0076$) and kg protein ($P \leq 0.0018$) compared to bulls with *GH4.1*^{c}/*GH4.1*^{t} genotype. Similar effects were observed with the GH6.2 polymorphism

on milk production traits with the *GH6.2*^{a} allele being the favourable allele (Table 6.4). No associations ($P \geq 0.05$) were found between the genotypes of the other GH polymorphisms (GH1, GH4.2, GH5 and GH6.1) and milk production traits. The average effects of alleles of GH4.1 and GH6.2 polymorphisms are given in Table 6.5. The average effects of the gene substitution for GH4.1 and GH6.2 were similar and amounted to ± 300 kg for milk yield, ± 8 kg for fat content and ±7 kg for protein content per lactation.

6.5 Discussion

DNA sequence variations have been conventionally identified as restriction fragment length polymorphism (RFLP). However, RFLP can only be detected when DNA polymorphisms are present in the recognition sequences for the corresponding restriction enzymes or when a deletion/insertion is present in the region detected by a specified probe. SSCP analysis overcomes this limitation and allows the detection of any sequence changes which lead to mobility differences of single-stranded DNA molecules. Using this technique, we detected a total of 6 polymorphic sites in the GH gene, indicating that SSCP is a useful tool to identify DNA polymorphisms.

The 5′ region of the GH gene contains regulatory sequences which control the expression of GH and interact with a large number of cis-acting (Crone *et al.*, 1990) and trans-acting factors (Courtois *et al.*, 1990). Modulation of the affinity of binding of any of these factors by minor sequence changes in the region may affect GH transcription and thus the concentration of GH measured in the blood. Therefore,

the GH1 SSCP involving a deletion/insertion of a TGC repeat in the 5′ flanking region (506 to 524 bp upstream from the transcription initiation site) of the gene becomes of interest for further study. However, no significant associations between the GH1 polymorphism and milk production traits were observed, suggesting that the repetitive TGC sequence may not be involved in the regulation of the gene.

Using Southern hybridization with bovine GH cDNA as a probe, Cowan *et al.* (1989) and Hilbert *et al.* (1989) detected a *Msp*I-RFLP in the GH gene and mapped it to the third intron. Restriction fragment analysis of the GH4 fragment which covers the third intron confirmed the presence of this polymorphic site. Our analysis revealed that the 345 bp fragment (GH4) habours a non-polymorphic *Msp*I site (position 1438-1439) which cleaves into two fragments of 59 and 286 bp upon digestion with *Msp*I. The 286 bp fragment is further cleaved to yield a 109 and a 177 bp fragments when the polymorphic *Msp*I site is present. Using the sequence information of Gordon *et al.* (1983), we mapped the polymorphic *Msp*I site to position 1547-1548. Sequence analysis of the GH4 fragment showed that this polymorphism was caused by a C to T transition at position 1547. Our sequence did not agree with that of Høj *et al.* (1993) who reported that the loss of the *Msp*I site resulted from the insertion of a T at position +837 (relative to translation start codon) and a C to G change at position +838. However, the sequences flanking the *Msp*I site in our analysis agreed with the GH gene sequence reported by Woychik *et al.* (1982) but did not match the sequence reported by Gordon *et al.* (1983) where a T between 1540-1541 and a G between 1549-1550 were absent. *Msp*I-RFLP analysis of all the samples showed that the A_4 and C_4 SSCP alleles were all *Msp*I (+), while

the B_4 allele was *Msp*I (-), showing the consistency of the two methods.

Estimates of genotypic effects of the GH4.1 variants in Table 4 indicate an association with milk production traits. In our study of the GH4.1 polymorphism, the $GH4.1^c$ allele (*Msp*I+) was the favourable allele with a substition effect of 300 kg for milk yield, 8 kg for fat content, and 7 kg for protein per lactation. Our results contradict the studies of Høj *et al.* (1993) who reported a positive association of of *Msp*I(-) with milk fat content in Red Danish and Norwegian dairy cattle, and Lee *et al.* (1994) who also observed a positive association of *Msp*I(-) with milk fat content in Holstein cows. Based on a 9 fragment-allelic combination, Lagziel *et al.* (1996) recently reported a GH haplotype of *Bos indicus* origin which was positively associated with milk protein percentage.

The GH6.1 polymorphism is also known as *Alu*I-RFLP which was previously characterized by Zhang *et al.* (1992; 1993) and Lucy *et al.* (1993). It is caused by a C to G nucleotide change in the fifth exon of the GH gene which gives rise to 2 alleles that are responsible for alternative forms of bovine GH with a leucine or valine amino acid residue at position 127. Restriction analysis of all 128 bulls revealed that the *Alu*I restriction recognition site was present in the B_6 and C_6 (*Alu*I+) but not in the A_6 allele (*Alu*I-). Eppard *et al.* (1992) demonstrated that lactating Holstein cows injected with valine variant recombinant-derived bovine GH, had greater milk yield than cows that received leucine variant GH. However, Lee *et al.* (1993) and Lucy *et al.* (1993) reported a decreased milk yield associated with valine variant bovine GH in Holstein cows. Further studies by Schlee *et al.* (1994) in German Black and White bulls revealed that animals homozygous for the leucine variant had higher

plasma levels of GH than their heterozygous counterparts. In our sample the frequency of the *GH6.1*^{g} allele (equivalent to the valine variant) was low and only one *GH6.1*^{g}/*GH6.1*^{g} homozygote carrier could be identified. Differences of least square means between the homozygote *GH6.1*^{c}/*GH6.1*^{c} (n=106) and the heterozygote *GH6.1*^{c}/*GH6.1*^{g} (n=21) for milk yield, fat content and protein yield were not significant (P≥0.05).

The GH6.2 polymorphism is due to an A to C transversion which changes the codon AGG to CGG in the fifth exon of the gene. However, either triplet codes for the same amino acid, arginine. This mutation changes the recognition sequence (C↓TNAG) of the restriction enzyme *Dde*I. Therefore, this polymorphism could also be identified as *Dde*I-RFLP. Assessment of the 128 individuals investigated indicated that all 95 individuals homozygous for *GH4.1*^{c} were also homozygous for *GH6.2*^{a}, all 29 heterozygotes *GH4.1*^{c}/*GH4.1*^{t} were heterozygotes *GH6.2*^{a}/*GH6.2*^{c}, and of the 4 homozygous *GH4.1*^{t}/*GH4.1*^{t} individuals, 2 were *GH6.2*^{c}/*GH6.2*^{c} homozygotes and 2 were *GH6.2*^{a}/*GH6.2*^{c} heterozygotes. This observation indicates extensive linkage disequilibrium between the 2 polymorphisms and may explain why significant associations were found for both polymorphisms. The effects of GH4.1 and GH6.2 on milk production traits could be attributed to variations in the expression of the bovine GH or due to tight linkage disequilibruim with another mutation.

Previous studies using Southern analysis revealed RFLPs in the bovine GH gene with *Bgl*I, *BamH*I, *EcoR*I, *Pst*I, *Pvu*II and *Taq*I, all of which were caused by an insertion/deletion event of 1kb DNA fragment in the 3′region of the gene (Hallerman *et al.*, 1987; Cowan *et al.*, 1989; Hilbert *et al.*, 1989). The exact location

of the deletion/insertion is unknown but was proposed to involve a segment 300 bp to 1300 bp downstream from the polyadenylation site (Hilbert *et al.*, 1989). Analysis of the PCR products of the GH7 fragment (454 bp) that covers the polyadenylation site and 429 bp 3′ from the site revealed that in some individuals, a single major band of 454 bp (expected) was present, while in others, beside the same major band with approximately half of the intensity, 2 additional minor bands of 500 and 550 bp, respectively, were present (data not shown). Assessment of 108 bulls revealed that those individuals with a single major band were all homozygous for *Msp*I (+), while the ones showing one major band with less intensity plus 2 minor bands were all *Msp*I (+/-) animals with few exceptions. Based on the observation by Høj *et al.* (1993) that the insertion allele was linked to the *Msp*I (+) allele and the deletion allele was linked to the *Msp*I (-) allele, we therefore conclude that those animals with a single amplification are homozygous for the insertion of the 1 kb fragment, and those with 2 additional bands are heterozygous for the deletion and insertion. We further conclude that the GH sequence reported by (Gordon *et al.*, 1983) contains part of the 1 kb insertion at the 3′ end as hypothesized by (Hallerman *et al.*, 1987) and that the P14 primer (reverse primer for GH7, position 2829-2850) is located in this region. The presence of the 2 minor bands in heterozygous animals could be explained as non-specific amplifications due to the elimination of the P14 primer binding site by deletion of the 1 kb fragment.

Associations between single genes or putative QTL and quantitative traits without taking into account the breeding structure and gene flow may be spurious because of non random association of gametes (Kennedy *et al.*, 1992). In this study,

the estimated breeding values are based on an animal model which accounts for the relationships between all animals and the bias of selection. The birth-year of the bulls was fitted to account for genetic trend in improvements over the past 40 years. However, a definitive conclusion requires segregation studies.

In summary our study revealed 6 different polymorphisms in the bovine GH gene, four of which have not been reported previously. The association of two of these polymorphisms GH4.1 and GH6.2 with milk production traits suggests that these markers may be useful for selection at the DNA level.

Table 6.1 Sequence and position of oligonucleotide primers for PCR analysis of the bovine GH gene[a]

Fragment	Primer	Primer sequence	Location	Size(bp)
GH1	P1	5′-GGTGGGTTGCCTTTCTCTTCT-3′	8-471	464
	P2	5′-TGTCATCATCCCGTCTCCACT-3′		
GH2	P3	5′-TCTCAAGCTGAGACCCTGTGT-3′	408-860	453
	P4	5′-GGCCAAATGTCTGGGTGTAGA-3′		
GH3	P5	5′-TTGGGCTTTAGGGCTTCCGAA-3′	805-1224	420
	P6	5′-TGAACTCCTCAGTTTCCTCCC-3′		
GH4	P7	5′-GGACAGAGATACTCCATCCAG-3′	1380-1724	345
	P8	5′-AGATGCGAAGCAGCTCCAAGT-3′		
GH5	P9	5′-TTGGAGCTGCTTCGCATCTCA-3′	1706-2071	366
	P10	5′-ATTTTCCACCCTCCCCTACAG-3′		
GH6	P11	5′-TAGGGGAGGGTGGAAAATGGA-3′	2054-2457	404
	P12	5′-GACACCTACTCAGACAATGCG-3′		
GH7	P13	5′-CACTCCCACTGTCCTTTCCTA-3′	2396-2850	455
	P14	5′-ACTTCCTCACATGTTGGAGGC-3′		

[a]Based on the nucleotide sequence of bovine GH gene from GORDON *et al.* (1983).

Table 6.2 Optimal conditions for SSCP analysis of GH1, GH4, GH5 and GH6 fragments of the bovine GH gene

GH fragment	Gel percentage(%)	Voltage (v)	Running time (h)
GH1	20	100	20
GH4	15	80	24
GH5	20	100	24
GH6	12	100	6

Table 6.3 Six polymorphisms in the bovine GH gene identified by SSCP analysis

Polymorphisms	Sequence change	Nucleotide position	Allele designation
GH1	del→ins TGC	125-140	$GH1^{d} \rightarrow GH1^{i}$
GH4.1	C→T	1547	$GH4.1^{c} \rightarrow GH4.1^{t}$
GH4.2	T→C	1692	$GH4.2^{t} \rightarrow GH4.2^{c}$
GH5	T→C	2017	$GH5^{t} \rightarrow GH5^{c}$
GH6.1	C→G	2141	$GH6.1^{c} \rightarrow GH6.1^{g}$
GH6.2	A→C	2291	$GH6.2^{a} \rightarrow GH6.2^{c}$

Table 6.4 Least square means of breeding values (kg) for milk yield, fat content and protein content in Holstein bulls with different GH genotypes

Polymorphism	GH Genotypes			P values
GH1	$GH1^d/GH1^d$ (N=21)	$GH1^d/GH1^i$ (N=68)	$GH1^i/GH1^i$ (N=39)	
Milk	-372.5±106.7	-419.7±66.1	-458.4±81.1	NS
Fat	-12.3±3.6	-13.2±2.2	-16.2±2.7	NS
Protein	-12.4±2.7	-14.6±1.6	-14.2±2.0	NS
GH4.1	$GH4.1^c/GH4.1^c$ (N=95)	$GH4.1^c/GH4.1^t$ (N=29)	$GH4.1^t/GH4.1^t$ (N=4)	
Milk	-319.2±56.3	-591.0±82.3	-1033.7±222.1	0.0006
Fat	-11.1±2.0	-20.1±2.9	-19.3±7.8	0.0225
Protein	-11.5±1.4	-19.2±2.1	-21.0±5.6	0.0038
GH4.2	$GH4.2^t/GH4.2^t$ (N=86)	$GH4.2^t/GH4.2^c$ (N=37)	$GH4.2^c/GH4.2^c$ (N=5)	
Milk	-477.6±59.3	-316.1±82.1	-223.3±197.1	NS
Fat	-14.6±2.0	-12.8±2.8	-9.8±6.8	NS
Protein	-14.8±1.5	-12.8±2.1	-7.2±4.9	NS
GH5	$GH5^t/GH5^t$ (N=57)	$GH5^t/GH5^c$ (N=55)	$GH5^c/GH5^c$ (N=16)	
Milk	-476.1±72.9	-385.2±68.7	-360.7±125.5	NS
Fat	-15.1±2.5	-12.9±2.3	-15.0±4.2	NS
Protein	-14.2±1.8	-14.3±1.7	-11.8±3.1	NS
GH6.1	$GH6.1^c/GH6.1^c$ (N=106)	$GH6.1^c/GH6.1^g$ (N=21)	$GH6.1^g/GH6.1^g$ (N=1)	
Milk	-458.4±55.6	-324.0±108.5	578.9±485.1	NS
Fat	-13.8±1.9	-15.1±3.8	-8.0±16.8	NS
Protein	-14.5±1.4	-12.9±2.7	3.4±12.3	NS
GH6.2	$GH6.2^a/GH6.2^a$ (N=95)	$GH6.2^a/GH6.2^c$ (N=31)	$GH6.2^c/GH6.2^c$ (N=2)	
Milk	-324.5±56.1	-595.4±80.4	-1281.8±308.4	0.0004
Fat	-11.1±2.0	-19.8±2.8	-23.0±10.9	0.0217
Protein	-11.5±1.4	-19.2±2.1	-22.6±7.9	0.0037

Values are means ± SE expressed in kg.

Table 6.5 Average additive gene substitution effects of alleles of GH4.1 and GH6.2 polymorphisms on milk yield, fat content and protein content of milk

GH alleles	Traits		
	Milk (kg)	Fat (kg)	Protein (kg)
GH4.1^{c}	43.0	1.1	1.0
GH4.1^{t}	-253.6	-6.5	-5.9
GH6.2^{a}	44.9	1.1	1.0
GH6.2^{c}	-282.9	-6.8	-6.1

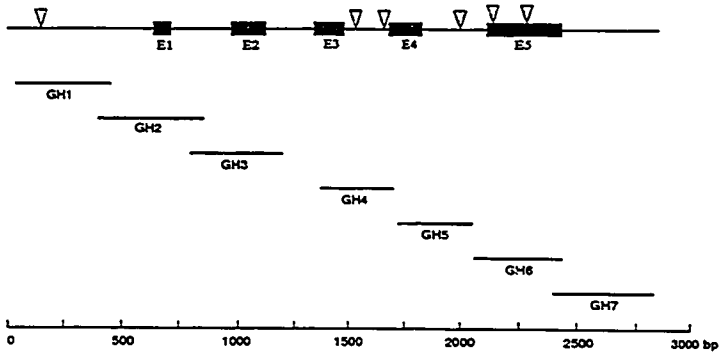

Figure 6.1 Map of the bovine GH gene with the position and length of the GH fragments. Open arrowheads indicate the locations of the polymorphic sites.

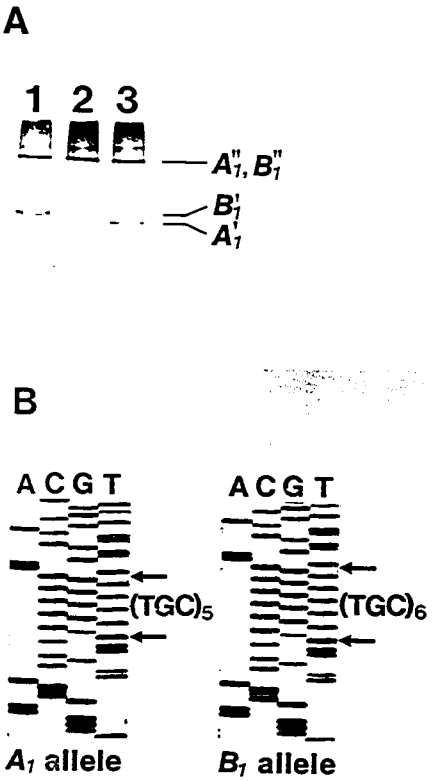

Figure 6.2 Detection of a 2-allele SSCP in the GH1 fragment of the bovine GH gene. (A) Banding pattern of GH1 SSCP. Lanes 1-3, individuals with genotypes B_1B_1, A_1B_1 and A_1A_1, respectively. [′] and ["] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH1 fragment showing the deletion/insertion of a TGC repeat at position between 125 and 142. A_1 allele: deletion of TGC; B_1 allele: insertion of TGC.

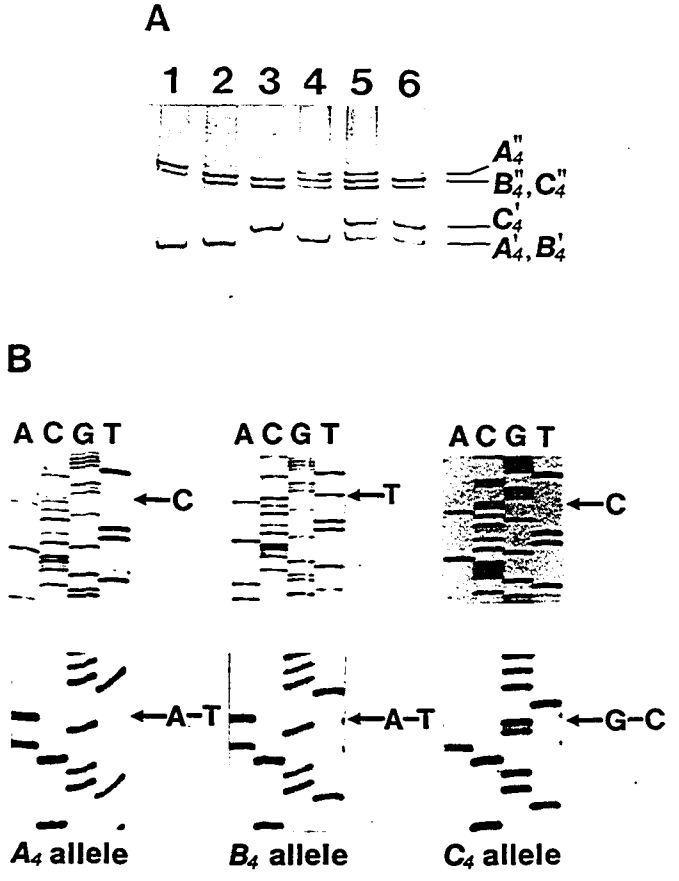

Figure 6.3 Detection of a 3-allele SSCP in the GH4 fragment of the bovine GH gene. (A) Banding pattern of GH4 SSCP. Lanes 1-6, individuals with genotypes A_4A_4, B_4B_4, C_4C_4, A_4B_4, A_4C_4 and B_4C_4, respectively. [$'$] and ["] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH4 fragment showing the 2 mutations at positions 1547 (upper portion) and 1692 (lower portion, sequences shown are antisence strands). A_4: $C^{1547}...T^{1692}$; B_4: $T^{1547}...T^{1692}$; C_4: $C^{1574}...C^{1692}$.

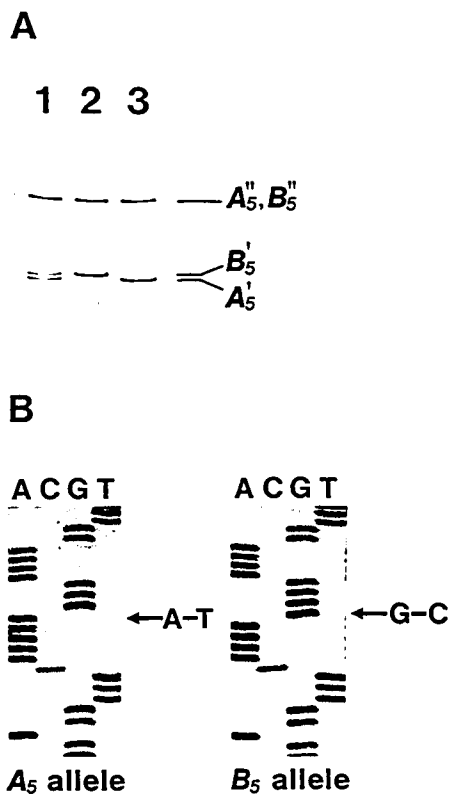

Figure 6.4 Detection of a 2-allele SSCP in the GH5 fragment of the bovine GH gene. (A) Banding pattern of GH5 SSCP. Lanes 1-3, individuals with genotypes A_5B_5, B_5B_5 and A_5A_5, respectively. [′] and ["] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH5 fragment showing the mutation at position 2017 (sequences shown are antisence strands). A_5: T^{2017}; B_5: C^{2017}.

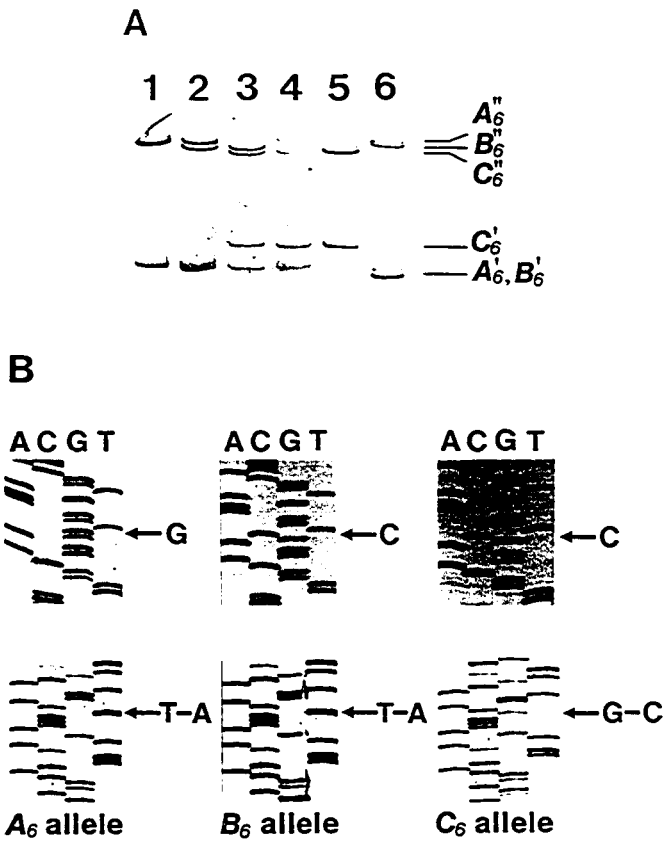

Figure 6.5 Detection of a 3-allele SSCP in the GH6 fragment of the bovine GH gene. (A) Banding pattern of GH6 SSCP. Lanes 1-6, individuals with genotypes A_6A_6, A_6B_6, A_6C_6, B_6B_6, B_6C_6 and C_6C_6, respectively. [′] and ["] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH6 fragment showing the 2 mutations at positions 2141 (upper portion) and 2291 (lower portion, sequences shown are antisence strands). A_6: $G^{2141}...A^{2291}$; B_6: $C^{2141}...A^{2291}$; C_6: $C^{2141}...C^{2291}$.

CHAPTER 7. GENERAL CONCLUSIONS

The identification of genetic markers associated with milk production performance could have a great economic impact on the dairy industry. Such genetic polymorphisms could be employed in marker-assisted selection programs for genetic improvement of dairy cattle. Considering the importance of ODC and GH in mammary gland development and functions, we have chosen these two genes to search for genetic variants which are linked to milk production traits.

Cloning of the bovine ODC cDNA revealed detailed information of ODC at the molecular level and provided a valuable tool for studying genetic variability of the gene in cattle. Using the cDNA as a hybridization probe in Southern blot analysis, we have shown that the ODC gene is highly polymorphic in Holsteins.

The structure of the bovine ODC gene was revealed by cloning and sequence analysis of an ODC genomic clone. The gene was shown to be highly conserved in mammals. Mapping of the *Msp*I and the *Taq*I polymorphisms on the ODC gene with the genomic sequence information facilitated development of PCR-based methods for rapid genotyping the 2 RFLPs in a relatively large sample of Holstein bulls. Of the 3 available ODC alleles (combinations of the 2 RFLPs), significant changes in allele frequencies were observed between bulls born in 1950-1970 and bulls born in the 1980s. The changes are possibly associated with selection for milk production. However, genetic drift could not be excluded.

Cloning of the ODC genomic gene also provided sequence information in the 5'-flanking region of the gene and a number of potential promoter elements were

identified in this region. Further studies may focus on searching for sequence variations in this region. Sequence changes in this region are likely to modulate the binding of the regulatory factors and thus affect ODC gene expression.

We have successfully applied the SSCP technique to detect DNA polymorphisms and SSCPs were detected in 4 of the 7 amplified GH fragments. A total of 6 polymorphisms were found in various regions of the GH gene. Two of the polymorphisms, a T to C transition in intron 3 (GH4.1) and an A to C transversion in exon 5 (GH6.2), were shown to be strongly associated with milk production traits. Whether the effects of the 2 polymorphisms on milk production reflect an altered expression of GH associated with the particular alleles or whether it is due to linkage to a neighbouring gene requires further studies.

Based on our observations, we found that the 2 polymorphisms are likely linked. The GH4.1 polymorphism (equivalent to a *Msp*I-RFLP) has been previously reported to be linked to an insertion/deletion of 1 kb DNA fragment in the 3'region of the gene and our study also confirmed this linkage relationship. Therefore, the effects of the 2 polymorphisms could be due to their linkage to this 1 kb fragment downstream of the gene. The insertion/deletion event in the 3'region may play a role in determining the rate of transcription of the gene, however, further studies at the transcriptional level are still required.

The association of the 2 GH polymorphisms with milk production traits suggests that these markers may be useful for selection at the DNA level. However, further segregation studies using a "granddaughter" design should be carried out to draw a definitive conclusion.

APPENDICES

Appendix 1. Nucleotide and deduced amino acid sequence of bovine ODC cDNA (GenBank and EMBL accession number: M92441). Nucleotide 1 is the start of the open reading frame.

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-185       CC GCT CTG AGA CAG CAG ATT GTG TGT TTA TTG AAC ATT TCC ACT
-141  GCT GCA CAG AGA GCA CAC GCA TCT TCG GTG GAC TTG GAA TTC CTG GGG
 -93  AAT TGC CTT TGT GAA AAG TTG GCA TAA TCC CTT TAA ATT CCA TCT CTT

   1                                                              M
 -45  TTA CGT TTT CTA TTT TGT TGT GTC TCA AAA AGA CGT CAA GAA ACC ATG

   2   N   S   F   S   N   E   E   F   D   C   H   F   L   D   E   G
   4  AAC AGC TTT AGC AAT GAA GAG TTT GAC TGC CAT TTC TTG GAT GAA GGC

  18   F   T   A   K   D   I   L   D   Q   K   I   N   E   V   S   Y
  52  TTT ACT GCC AAG GAT ATT CTG GAC CAA AAA ATT AAT GAA GTT TCT TAT

  34   S   D   D   K   D   A   F   Y   V   A   D   L   G   D   I   L
 100  TCT GAT GAT AAG GAT GCC TTC TAT GTT GCG GAC CTG GGA GAC ATT CTG

  50   K   K   H   L   R   W   L   K   A   L   P   R   V   T   P   F
 148  AAG AAA CAT CTG AGA TGG TTG AAA GCT CTT CCT CGG GTC ACC CCC TTT

  66   Y   A   V   K   C   N   D   S   R   T   I   V   K   T   L   A
 196  TAT GCC GTC AAA TGC AAT GAT AGC AGA ACC ATA GTG AAG ACA CTC GCT

  82   A   I   G   T   G   F   D   C   A   S   K   T   E   I   Q   L
 244  GCC ATT GGG ACA GGA TTT GAC TGT GCC AGC AAG ACT GAA ATA CAA TTG

  98   V   Q   S   L   G   V   P   P   E   R   I   I   Y   A   N   P
 292  GTG CAG AGT CTC GGG GTG CCC CCA GAG AGG ATT ATC TAT GCA AAT CCA

 114   C   K   Q   V   S   Q   I   K   Y   A   A   N   N   G   V   Q
 340  TGT AAA CAA GTG TCT CAG ATT AAG TAT GCT GCC AAT AAC GGA GTC CAG

 130   M   M   T   F   D   S   E   V   E   L   M   K   V   A   R   A
 388  ATG ATG ACT TTT GAT AGT GAA GTT GAG CTG ATG AAA GTT GCC AGG GCA

 146   H   P   K   A   K   L   V   L   R   I   A   T   D   D   S   K
 436  CAT CCA AAG GCC AAG TTG GTT TTA CGG ATC GCC ACT GAT GAT TCC AAA

 162   A   V   C   R   L   S   V   K   F   G   A   T   L   K   T   S
 484  GCA GTC TGT CGC CTC AGT GTC AAA TTT GGT GCC ACA CTC AAA ACC AGC

 178   R   L   L   L   E   R   A   K   E   L   D   I   D   V   I   G
 532  AGG CTT CTT TTG GAA CGG GCG AAA GAG CTA GAT ATT GAT GTC ATT GGT

 194   V   S   F   H   V   G   S   G   C   T   D   P   E   T   F   V
 580  GTC AGC TTC CAC GTG GGA AGT GGC TGT ACT GAT CCT GAG ACC TTT GTG

 210   Q   A   I   S   D   A   R   C   V   F   D   M   G   A   E   V
 628  CAG GCC ATC TCT GAT GCC CGC TGT GTC TTT GAC ATG GGC GCT GAG GTT

 226   G   F   N   M   Y   L   L   D   I   G   G   G   F   P   G   S
 676  GGT TTC AAC ATG TAT CTG CTT GAT ATT GGT GGT GGC TTT CCT GGA TCA
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 242   E   D   V   K   L   K   F   E   E   I   T   S   V   I   N   P
 724  GAG GAT GTA AAG CTT AAA TTT GAA GAG ATC ACC AGT GTA ATC AAC CCA

 258   A   L   D   K   Y   F   P   S   D   S   G   V   R   I   I   A
 772  GCA CTG GAC AAG TAT TTT CCA TCA GAC TCT GGA GTG AGA ATC ATA GCT

 274   E   P   G   R   Y   Y   V   A   S   A   F   T   L   A   V   N
 820  GAG CCA GGC AGA TAC TAT GTT GCA TCA GCT TTC ACG CTC GCA GTT AAT

 290   I   I   A   K   K   L   V   L   K   E   Q   T   G   S   D   D
 868  ATT ATT GCC AAA AAA CTT GTA TTA AAG GAA CAG ACA GGC TCT GAT GAT

 306   E   E   E   S   T   D   R   T   F   M   Y   Y   V   N   D   G
 916  GAA GAG GAG TCA ACT GAT CGG ACA TTT ATG TAT TAT GTG AAC GAT GGA

 322   V   Y   G   S   F   N   C   I   L   Y   D   H   A   H   V   K
 964  GTA TAT GGG TCA TTC AAC TGC ATC CTT TAT GAT CAC GCA CAC GTG AAG

 338   P   L   L   Q   K   R   P   K   P   D   E   K   Y   Y   S   S
1012  CCT CTT CTG CAG AAG AGA CCC AAA CCA GAT GAG AAG TAT TAT TCA TCC

 354   S   I   W   G   P   T   C   D   G   L   D   R   I   V   E   R
1060  AGC ATC TGG GGA CCG ACC TGT GAC GGC CTG GAC CGC ATT GTT GAG CGC

 370   C   N   L   P   E   M   H   V   G   D   W   M   L   F   E   N
1108  TGT AAC CTG CCT GAG ATG CAT GTG GGT GAT TGG ATG CTC TTT GAG AAC

 386   M   G   A   Y   T   V   A   A   A   S   T   F   N   G   F   Q
1156  ATG GGT GCT TAC ACT GTT GCT GCT GCT TCT ACC TTC AAT GGA TTC CAG

 402   R   P   T   I   Y   Y   V   M   S   G   P   T   W   Q   L   M
1204  AGA CCC ACC ATC TAC TAT GTG ATG TCA GGG CCA ACG TGG CAA CTG ATG

 418   Q   Q   I   R   T   Q   D   F   P   P   G   V   E   E   P   D
1252  CAG CAG ATC CGG ACC CAG GAC TTC CCG CCT GGA GTG GAG GAG CCG GAC

 434   V   G   P   L   P   V   S   C   A   W   E   S   G   M   K   R
1300  GTC GGT CCC CTG CCC GTG TCC TGT GCC TGG GAG AGC GGC ATG AAG CGG

 450   H   S   A   A   C   A   S   T   R   I   N   V   *
1348  CAC TCG GCA GCC TGC GCT TCC ACG CGT ATT AAC GTG TAG ATA CCA CTC

1396  TTG TAG CTG TTA ACT GCG AGT TTA GCT TGA TTT AAG GGT TTG GGG GGG
1444  ACC ATT TAA CTT AAT TAC TGC TAG TTC TGA GAT GTC TAT GTG AGT AGG
1492  GTT GGC ACA GGT GCA CCA ATG TGG AAG ACT GGG AGA TGG GGT CAC ACT
1540  TAT CTG TGT TCC TAT GGA AAC TAT TTG AAT ATT T
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Appendix 2. Nucleotide sequence of the bovine ODC gene (GenBank and EMBL accession number: U36394). Flanking and intronic sequences are indicated by lowercase letters, whereas, the exons are indicated by uppercase letter. Nucleotide 1 is the transcription start point.

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-1493       agacactggtgctgtgttgggactcacataacctcccaaagcaagccaatcat
-1440  agctgccctcagagaagtcattgataagcgccactcaatacagtaacagactttattatc
-1380  ttaggctccaaaatcactgcagatggtgactgcagccatgaaattaaaagatgcttgctc
-1320  cttggaagaaaagctatgacaaacctagacagcatattaaaaagtggagacataactttg
-1260  ccaacaaaggtctgaatagccaaagctatggtcttcccagtagtcatatatggatgttag
-1200  agttggaccataaagaaagctgagcacggaagaattgatgctttcgaactgtggtgttgg
-1140  agaaatctcttgagagtcccttggactgtaaggagatcaaaccagtccatcctaaaggaa
-1080  ataaatcctgaatattcactggaaggactgaggctaaagctccaatactttggccaccgg
-1020  atttgataagcccactgactagaaaaagaccccgatgctgggaaagattgaaggcaggag
 -960  gagaagggatgacagaggatgagatggctggatggcatcaccgactcaaccaacaggag
 -900  tctgatcaagctccaggaggagatggtgacggacagggacgcctggcgtgctgcagtccc
 -840  tggggtcgcaatgtcggaaacgactgagcgactgaacaatacgaaggacagtggatcgcc
 -780  cggtggcttgcagacctgttacctcccacccctgccctaatgcccttagaagagtctct
 -720  ctggcttaacaagcgcaacgtgcctcttcgcgtgccctctaccttctaggaagacacccg
 -660  gacatgtcagacgcggttcaatacgctcagcggctcgagagctgcccagagctgtgcctc
 -600  ttaatttgcccgctgaccgctgccccttctgcctcctgccccccttcatcgtgcgctgga
 -540  gctgctccaggatcacctccaggtcctcccaccggcatatacagacgccccgcagaaaga
 -480  aaggggcccgggtccgccaggtagggtccacgtggcccgtcgcgccaagcgatttccgtt
 -420  ctctcctgagggcacgcggcatttgatgccaggtggaaggagagcgctggcttggcgcac
 -360  acgctgccatccggcgggcgttcagaggcaggttttgcaatccttcggctggcgtgccag
 -300  aggagagcagccttaggcggccggccgggcgcgcggagccccgggcagtccgcggctggt
 -240  gcgcatgcgcgggctggcggcgggccagggaggggcggggttggcggggctgggaggtgc
 -180  tgacgtcgatcagccggcgaccccccacccctccctctccccatcccgggttggccccgcc
 -120  ccctccctgggaggacgcccggacggaaccgatcgcggctggtttgagctggtgcgtctc
  -60  catgacgacacgcgcggcgctataagtagcggcgcggcggcgcgccgggctttgtcagtt
    1  CCCCCTGAGCCTCCGCCGCCCGCCGCCCCTCTGCCAGCGCTCCGGCGCCACCTCGGGCCGG
   61  CGGTCCTCCGCGGGAGGGAGCGAGGCGGCGGGCGGCCGGGCGAGCGGCTGACGGGGCGG
  121  CGGGGCGGCCGGCGGATCCTGCAGCGCTCGGCGGCGGCGCGTTGTTTCCATGGGGTTGGC
  181  CCGCCGCGCCCCTGTGCTCTGAGgtaaaaggggtcgcgtccgggcggtagggaggccggc
  241  gggcgggcgggcgggcgcgggtgaggggcaggggggtgccggccacgtgtcccggaggccg
  301  gagccgaccgggcgcctggagctgcgaggacacgtggtcgcagagcccggctcgtcggcc
  361  ctcggggtcccgagtggagccgggccccgaccccctcccggctcaagggcagcctggcgct
  421  ggaacggcccggggttcacctttttctgaaccacgtcagtgagggttttctgagccggaag
  481  ctctttgaggagctcgatcattcagccgctgttttttttttaaacgcatgtggtggtccg
  541  gtagcaggtgcaggagaggcaggaagcgagcccggcacaggtctcggggtgccgcctctc
  601  gtgcacgtgccgttccgccgtgttgccctggaatttccttttttttttttttttaatttt
  661  aggttgtttttttttttaccttgctgtgtgtacagagcgaatggcccaatatagaaagc
  721  cactcagatcgctagagctgagataacccgtgtgtaactgatactgctcggccgggctta
  781  gaaatgttgacggaatctttgaatgacttgatgatttaagaaaagtccttagattattaa
  841  gatcaggagaacgaccaagacgtaggagccagtcccaagattttccccaaacggcctact
  901  gtttgagaatccgaaataatgaggcaagatgtggggagcagaacctcttaggagtgtcta
  961  gtagggaggacagaatgtgctaatttcattcaaacataaaatttaatcgatttgtaaatt
 1021  ttcagttgcggtttgaattggtacctgtctaacgtttcccaattaccagagaaggaccac
 1081  gttttgctaatccatgtcattttaagtaacgtagccataacagaaaacacccaaaggtgt
 1141  tctagggtgaactttctctgtatcaaaagttactgaagtaaagctttgaaagatacaagg
 1201  aagcaaaatacttaatagtggtgtgtttcagagaaacacacacttgctaagtttaagcct
 1261  gaatgttgtctttttttttttttttaaagaccctaaacggcccagtctatcactggtgacc
 1321  ctatttaaatgttacttatgtaaacagaatgtgtgtgctggctgttgatctgttcccttc
 1381  tcctctgggcgaattaggaaactaggaggttgacaaagccagcaagtcacattagatcaa
 1441  gtctcgcaagttctcccatgcatgggtttggatttatggcaggctcgtccccctgggcct
 1501  ctcatagtgtcccatgccagagtaaatccggcccgaaccattgcctgcctctggcagtag
 1561  gctgcaggactgaagtgggttgcagagtggaaaagatgccctccctatcagattaaatag
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1621  attaaaataattattttaaggtaactcattgacctatgtcacgtttcatgaacgccaagg
1681  acagtggctctgatggttaaatgagctttatttttgtttattcataaatcattgtttgct
1741  aatttttgtaattcatagttgagaatgactcccaggtgattatcattcatagccaagcat
1801  gtaagttagagtgcctgtgattatagttgggattcccagaatcccaagtagttaagtagg
1861  cagcactggttttagaatattgattgccattcagatacagcagctgtaatacattctgtt
1921  gatttttttagattgcatttttaaaaaaacacatgcctagcatgaaaattagctcatt
1981  tctcaggaaaatttagagcactcagtaaattgataacaatgcctgttgttcactaaaagt
2041  tcttctgtataagacttagcaaatgtcagaaatagcaaggtgtttatatggcagctttgc
2101  taaattgctatcatggtgttttaagcttcgagagaaaaacaggtctgaaataaagtaagc
2161  taactgtcatgaagccttttccctctcagtggactaagtgcaggaaagtttctgaaacct
2221  gtattctgcattttagcaggaatgtttggagtgccttttttgaggtggacaaaactatat
2281  gggcttccttggtagctgagctggtaaagaatccacctgcaatgctgggttccatccccg
2341  ggtcaggaagattctctagagaagggcatggcaacccactccagtattcttgcctggaga
2401  atcccatggacagaggagcctggcgggctacagtccatggggtcacaaagagtcagacac
2461  gactgagtgactaagtacagtactatattggaagctgagtgtgaaaaactcgagtgttga
2521  atttgtgaggttctcattccattgagatttatagaatcatggtacttaaacctaggaata
2581  ataggatttgcaaacccagggagtgagctgtctggagggtggtttagtagccctgcttct
2641  tgtgtgtgtgtgtctgtgtgctgtcaatacctgatcttccaatttaatgtgttctgaa
2701  ggtagACAGCAGATTGTGTGTTTATTGAACATTTCCACTGCTGCACAGAGAGCACACGCA
2761  TCTTCGGTGGACTTGGAATTCCTGGGGAATTGCCTTTGTGAAAAGTTGGCATAATCCCTT
2821  TAAATTCCATCTCTTTTACGTTTTCTATgtaagtatttgtgattgttacaagtttatctt
2881  aggagttactaagtattaagacttctgagtagatttgtctgccctatttgcagTTTGTTG
2941  TGTCTCAAAAAGACGTCAAGAAACCATGAACAGCTTTAGCAATGAAGAGTTTGACTGCCA
3001  TTTCTTGGATGAAGGCTTTACTGCCAAGGATATTCTGGACCAAAAAATTAATGAAGTTTC
3061  TTATTCTgtaagtatatatgaagtctatgctggtagtgcagctttgagaatgttaggcag
3121  atgaatgggaaacttgagaggggtcttatggaaatgtcttgttagggaattgggagttta
3181  attggctgtggatcctccagtaacaatagtgattatcatgtccatgattgactagagtag
3241  ggttcatgagaatgatgggcaagagaagggaaggctgtgccttggtcacatctgttaaat
3301  tgctgatgggtctcctgtaaccactcttattgcagGATGATAAGGATGCCTTCTATGTTG
3361  CGGACCTGGGAGACATTCTGAAGAAACATCTGAGATGGTTGAAAGCTCTTCCTCGGGTCA
3421  CCCCCTTTTATGCCGTCAAATGCAATGATAGCAGAACCATAGTGAAGACACTCGCTGCCA
3481  TTGGGACAGGATTTGACTGTGCCAGCAAGgtgagcaaaagcagcagaactcaaatgatgt
3541  ctgtaatggccctggtgctcccagcagggcagatcagaattactttgcttgagcagagga
3601  tgctaaagagagtgggcatgggccagattttctatatattaactacagtgtaaatacctc
3661  ttctttttcagACTGAAATACAATTGGTGCAGAGTCTCGGGGTGCCCCCAGAGAGGATTA
3721  TCTATGCAAATCCATGTAAACAAGTGTCTCAGATTAAGTATGCTGCCAATAACGGAGTCC
3781  AGATGATGACTTTTGATAGTGAAGTTGAGCTGATGAAAGTTGCCAGGGCACATCCAAAGG
3841  CCAAgtgagttactccatttgagggcggatcagatatgggacgtgtataatgtgaacaaa
3901  ctcaaatttccagttgttagatttccatactagtaaattatctgtgtactgagttaaacc
3961  aaattagactcagaccacagggtatcagagccaaggagtcttatttttaaccacggctgta
4021  gatgatcttgtgtcggtgggaaatttgatatacttttcttgcttctagGTTGGTTTTACG
4081  GATCGCCACTGATGATTCCAAAGCAGTCTGTCGCCTCAGTGTCAAATTTGGTGCCACACT
4141  CAAAACCAGCAGGCTTCTTTTGGAACGGGCGAAAGAGCTAGATATTGATGTCATTGGTGT
4201  CAGgtgagatcttggtgatggcagagatggagatgaagtattagacaatgcaagttttct
4261  cccaccatgaatagttatttccagaaatagtaagaaatagtgtatttttgtgttttgac
4321  accagCTTCCACGTGGGAAGTGGCTGTACTGATCCTGAGACCTTTGTGCAGGCCATCTCT
4381  GATGCCCGCTGTGTCTTTGACATGGGCgtgagtatctgtgagccccatgtgtgaggaggg
4441  gggcagggatggcattaacaactagtcccagttctagaatttacctttgtaaaagtcatc
4501  tcatattacatatttaaccttaacctgctgcatacatttttcatgacttgttaatctggc
4561  tgattgacttaatttttcttgactctagGCTGAGGTTGGTTTCAACATGTATCTGCTTGAT
4621  ATTGGTGGTGGCTTTCCTGGATCAGAGGATGTAAAGCTTAAATTTGAAGAGgtaatttat
4681  aacaaaattacagtctgtagctcttaggttccttttttgaaatgtttcaaaacttatatgt
4741  tacaactaataaccaaaagggactgtacaaacatgaaagtaaatgtcttgtgtggtattt
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4801 tgaatgacttctgtgatatttaaaattgtattacgttaattaattagtctctcagttgtg
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4921 ccaggcagaaatcctggagtgggtagccagttccttctctgcggatcatcttgagccagg
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5641 TCATAGCTGAGCCGGGCAGATACTATGTTGCATCAGCTTTCACGCTCGCAGTTAATATTA
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5761 tcattacatatatatactgaaagttcgtatctaaaatggttaagtggtaattgagactat
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5941 CATCCTTTATGATCACGCACACGTGAAGCCTCTTCTGCAGAAGgtaccttccaaatgtgt
6001 tgtatacaccagttgaaagatgactggtcagaacaagtgtaagcaggtgtgtttctctgt
6061 ctcaaatgtagAGACCCAAACCAGATGAGAAGTATTATTCATCCAGCATCTGGGGACCGA
6121 CCTGTGACGGCCTGGACCGCATTGTTGAGCGCTGTAACCTGCCTGAGATGCATGTGGGTG
6181 ATTGGATGCTCTTTGAGAACATGGGTGCTTACACTGTTGCTGCTGCTTCTACCTTCAATG
6241 GATTCCAGAGACCCACCATCTACTATGTGATGTCAGGGCCAACGTGgttagtgacagtgt
6301 gtgtttggttctgataagaattaggtactttccagggttgattatgcttgttcttgtcca
6361 gtagaattaagaaattgcttagaattaaataataacaattatttttttgtttttctcccca
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6481 gtaatagtgatgtatatagttgcagaattttaaggcaaagttgatttagaaaagactggt
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6841 CCCTGCCCGTGTCCTGTGCCTGGGAGAGCGGCATGAAGCGGCACTCGGCAGCCTGCGCTT
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7141 TGCTACTAAAGGCTTCAACTGCTGAGCAAGCGTTTGTGGCTTGTGTATCAGCAGGATAGG
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7261 tggcactgggaagtttttacggtgtgttcatttcaggcggctcacctgagcgtgtgttat
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7621 tggtctttgcctgttacagcggctgcctaggcggggtacacagggccctctgagccctgc
7681 ctccagagaccagtctcatatttgcaccttgacttcacacacaggaaaaggtacagttac
7741 aaactagcttgatacagagttgtagctcttcatctgaccttggccttttgtattctctta
7801 ctcaacttggaccccacacctcagaaaaagggtggcaggctcgcatttaaatttaaagac
7861 aggaattgtgaatccttgccgtagctctggacaggagggcaggccacgtaagagccagag
7921 tcacttgagtaagtctctcactggccccagtccctgcag

REFERENCES

Abdel-Meguid SS, Sheih H-S, Smith WW, Dayringer HE, Violand BN and Bentle LA (1987) Three-dimensional structure of a genetically engineered variant of porcine growth hormone. Proc Natl Acad Sci USA 84:6434-6437.

Abrahamsen MS and Morris DR (1991) Regulation of expression of the ornithine decarboxylase gene by intracellular signal transduction pathways. pp. 107-119. In: *Perspectives on Cellular Regulation: From Bacteria to Cancer.* Edited by M. Inouye, J. Campisi, D.D. Cunningham and M. Riley. John Wiley and Sons, New York.

Abrahamsen MS, Li R, Dietrich-Goetz W and Morris DR (1992) Multiple DNA elements responsible for transcriptional regulation of ornithine decarboxylase gene by protein kinase A. J Biol Chem 267:18866-18873.

Aebi M, Horning H, Padgett RA, Reiser J and Weissmann C (1986) Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell 47:555-565.

Aggrey SE, Kuhnlein U, Fairfull RW and Zadworny D (1996) Association of ornithine decarboxylase polymorphism with quantitative traits in poultry. Proc XXVth Internatl Conf Anim Genet Inra, France. pp.169.

Annexstad RJ, Otterby DE, Linn JG, Hansen WP, Soderholm CG and Eggert RG (1990) Somatotropin treatment for a second consecutive lactation. J Dairy Sci 73:2423-2436.

Asimov GJ and Krouze (1937) The lactogenic preparation from the anterior pituitary and the increase of milk yield in cows. J Dairy Sci 20:289-306.

Bauman DE (1992) Bovine somatotropin: review of an emerging animal technology. J Dairy Sci 75:3432-3451.

Bauman DE, McBride BW, Burton JL and Sejrsen K (1994) Somatotropin (BST): International Dairy Federation Technical Report. Bulletin of the IDF. 293:2 (Feb. 1994). (Internation Dairy Federation Group A22).

Bauman DE and Vernon RG (1993) Effects of exogenous somatotropin on lactation. Ann Rev Nutr 13:437-461.

Bauman DE, Eppard PJ, DeGeeter MJ and Lanza GM (1985) Responses of high-producing dairy cows to long-term treatment with pituitary somatotropin and recombinant somatotropin. J Dairy Sci 68:1352-1362.

Baumann G (1991) Growth Hormone heterogeneity: genes, isohormones, variants,

and binding proteins. Endocrine Rev 12:424-449.

Baumrucker CR and Stemberger BH (1989) Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue in vitro. J Anim Sci 67:3503-3514.

Beckmann JS, Kashi Y, Hallerman EM, Nave A and Soller M (1986) Restriction fragment length polymorphism among Israeli Holstein-Friesian dairy bulls. Anim Genet 17:25-38.

Bedford MR and Zadworny D (1990) Effect of PRL, ornithine, and DFMO on spermidine in bovine mammary epithelial cells. Cell Biol Int 14: 143.

Berger F, Szymanski P, Read E and Watson G (1984) Androgen-regulated ornithine decarboxylase mRNA of mouse kidney. J Biol Chem 259:7941-7946.

Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209-213.

Bines JA, Hart IC and Morant SV (1980) Endocrine control of energy metabolism in the cow: the effect on milk yield and levels of some blood constituents of injecting growth hormone and growth hormone fragments. Br J Nutr 43:179-188.

Bishop S and Hill WG (1985) Effects of selection on growth, body composition and food intake in mice. Genetical Res 46:57-74.

Blalock JE (1994) The syntax of immuno-neuroendocrine communication. Immun Today 15:504-511.

Bollengier F, Velkeniers B, Hooghe-Peters E, Mahler A and Vanhaelst L (1989) Multiple forms of rat prolactin and growth hormone in pituitary cell subpopulations separated using a Percoll gradient system: disulphide-bridged dimers and glycosylated variants. J Endocrinol 120:201-206.

Botstein D, White RL, Skolnick M and Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Human Genet 32:314-319.

Brabant M, McConlogue L, van Daalen T and Coffino P (1988) Mouse ornithine decarboxylase gene: cloning, structure and expression. Proc Natl Acad Sci USA 85:2200-2204.

Breier BH and Gluckman PD (1991) The regulation of postnatal growth: nutritional influences on endocrine pathways and function of the somatotrophic axis. Livst Prod Sci 27:77-94.

Bronczek RR, Young CW, Wheaton JE and Miller KP (1988) Response of somatotropin,insulin, prolactin, and thyroxine to selection for milk yield in Holsteins. J Dairy Sci 71:2470-2479.

Brumby PJ and Hancock JH (1955) The galactopoietic role of growth hormone in dairy cattle. New Zealand J Sci Tech 36:417-436.

Bulfield G, Isaacson JH and Middleton RJ (1988) Biochemical correlates of selection for weight-for-age in chickens: twenty-fold higher muscle ornithine decarboxylase levels in modern broilers. Theor Appl Genet 75:432-437.

Burton JL, McBride BW, Block E, Glimm DR and Kennelly JJ (1994) A review of bovine growth hormone. Can J Anim Sci 74:167-201.

Casas-Carrillo E, Prill-Adams A, Price SG and Kirkpatrick BW (1994) Association of growth hormone and insulin-like growth factor-1 genotypes with growth and carcass traits in offspring of purebred swine. Proc 5th World Congr Gen Appl to Livest Prod 21:272-275.

Charrier J and Martal J (1988) Growth hormones. 1. Polymorphism (miniview). Reprod Nutr Dev 28:857-887.

Chen WY, Wright DC, Wagner TE and Kopchick JJ (1990) Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice. Proc Natl Acad Sci USA 87:5061-5065.

Chen WY, Wright DC, Chen NY, Coleman TA, Wagner TE and Kopchick JJ (1991) Mutations in the third alpha-helix of bovine growth hormone dramatically affect its intracellular distribution in vitro and growth enhancement in transgenic mice. J Biol Chem 266:2252-2258.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 162:156-159.

Coffino P (1988) Probable cloning artefact previously interpreted as unusual leader sequence of rodent ornithine decarboxylase mRNAs - a cautionary tale. Gene 69:365-368.

Coleman CS, Stanley BA, Viswanath R and Pegg AE (1994) Rapid exchange of subunits of mammalian ornithine decarboxylase. J Biol Chem 269:3155-3158.

Coleman CS, Stanley BA and Pegg AE (1993) Effect of mutations at active site residues on the activity of ornithine decarboxylase and its inhibition by active site-directed irreversible inhibitors. J Biol Chem 268:24572-24579.

Copras ES, Harman SM and Blackman MR (1993) Human growth hormone and human aging. Endocrine Rev 14:20-39.

Cotes PM, Crichton JA, Folley SJ and Young FG (1949) Galactopoietic activity of purified anterior pituitary growth hormone. Nature 164:992-993.

Courtois SJ, Lafontaine DA, Lemaigre FP, Durviaux SM and Rousseau GG (1990) Nuclear factor-I and activator protein-2 bind in a mutually exclusive way to overlapping promoter sequences and trans-activate the human growth hormone gene. Nucleic Acids Res. 18:57-64.

Cowan CM, Dentine MR, Ax RL and Schuler LA (1989) Restriction fragment length polymorphisms associated with growth hormone and prolactin genes in Holstein bulls: evidence for a novel growth hormone allele. Anim Genet 20:157-165.

Crone DE, Kim H and Spindler SR (1990) α and ß thyroid hormone receptors bind immediately adjacent to the rat growth hormone gene TATA box in a negatively hormone-responsive promoter region. J Biol Chem 265:10851-10856.

Davis RH, Morris DR and Coffino P (1992) Sequestered end products and enzyme regulation: the case of ornithine decarboxylase. Microbiol Rev 56:280-290.

Davis SR, Gluckman PD, Hart IC and Henderson HV (1987) Effects of injecting growth hormone or thyroxine on milk production and blood plasma concentrations of insulin-like growth factors I and II in dairy cows. J Endocrinol 114:17-24.

Degen JL and Morris DR (1980). Activation of early enzyme production in small lymphocytes in response to high, nonmitogenic concentrations of concanavalin A. Proc Natl Acad Sci USA 77:3479-3483.

Dynan WS and Tjian R (1985) Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature 316:774-778.

Edwards M, Stuber C and Wendel J (1987) Molecular-marker-facilitated investigations of quantitative-trait loci in maize. I. Numbers, genomic distribution and types of gene action. Genetics 116:113-125.

Edwards M, Helentjaris T, Wright S and Stuber C (1992) Molecular-marker-facilitated investigations of quantitative trait loci in maize. 4. Analysis based on genome saturation with isozyme and restriction fragment length polymorphism markers. Theor Appl Genet 83:765-774.

Eppard PJ, Bentle LA, Violand BN, Ganguli S, Hintz RL, Kung LJr, Krivi GG and Lanza GM (1992) Comparison of the galactopoietic response to pituitary-derived and recombinant-derived variants of bovine growth hormone. J Endocrinol 132:47-56.

Falconer, D.S., 1989 *Introduction to quantitative genetics*. 3th edition, Longman Scientific & Technical, England.

Fallert RF and Liebrand CB (1991) Economic implications of bovine somatotropin for the United States dairy industry. J Dairy Sci 74(Suppl. 2):12-19.

Fiddes JC, Seeburg PH, DeNoto FM, Hallewel RA, Baxter JD and Goodman HM (1979) Structure of genes for human growth hormone and chorionic somatomammotropin. Proc Natl Acad Sci USA 76:4294-4298.

Fitzgerald MC and Flanagan MA (1989) Characterization and sequence analysis of the human ornithine decarboxylase gene. DNA 8:623-634.

Flint DJ and Gardner MJ (1993) Influence of growth hormone deficiency on growth and body composition in rats: site-specific effects upon adipose tissue development. J Endocrinol 137:203-211.

Flint DJ and Gardner M (1994) Evidence that growth hormone stimulates milk synthesis by direct action on the mammary gland and that prolactin exerts effects on milk secretion by maintenance of mammary deoxyribonucleic acid content and tight junction status. Endocrinol 135:1119-1124.

Folley SJ and Young FG (1940) Further experiments on the continued treatment of lactating cows with anterior pituitary extracts. J Endocrinol 2:226-...

Fonzi WA and Sypherd PS (1987) The gene and the primary structure of ornithine decarboxylase from *Saccharomyces cerevisiae*. J Biol Chem 262:10127-10133.

Fotouli N, Karatzas CN, Kuhnlein U and Zadworny D (1993) Identification of growth hormone DNA polymorphisms which respond to divergent selection for abdominal fat content in chickens. Theor Appl Genet 85:931-936.

Fronk TJ, Peel CJ and Bauman DE (1983) Comparison of different patterns of exogenous growth hormone administration of milk production in Holstein cows. J Anim Sci 57:699-705.

Gardner MJ and Flint DJ (1990) Long-term reductions in GH, insulin-like growth factor-I and body weight gain in rats treated neonatally with antibodies to rat GH. J Endocrinol 124:381-386.

Georges M, Nielsen D, Mackinnon M, Mishra A, Okimoto R, Pasquino AT, Sargeant LS, Sorensen A, Steele MR, Zhao X, Womack JE and Hoeschele I (1995) Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. Genetics 139: 907-920.

Ghoda L, Phillips MA, Bass KE, Wang CC and Coffino P (1990) Trypanosome ornithine decarboxylase is stable because it lacks sequences found in the carboxyl terminus of the mouse enzyme which target the later for intracellular degradation. J Biol Chem 265:11823-11826.

Ghoda L, Sidney D, Macrae M and Coffino P (1992) Structural elements of ornithine decarboxylase required for intracellular degradation and polyamine-dependent regulation. Mol Cell Biol 12:2178-2185.

Ghoda L, van Daalen Wetters T, Macrae M, Ascherman D and Coffino P (1989) Prevention of rapid intracellular degradation of ODC by carboxyl-terminal truncation. Science 243:1493-1495.

Gibson JP, McBride BW, Burton JH, Politis I and Zhao X (1992) Effect of production traits of bovine somatotropin for up to three consecutive lactations. J Dairy Sci 75:837-846.

Glimm DR, Baracos VS and Kennelly JJ (1990) Molecular evidence for the presence of growth hormone receptors in the bovine mammary gland. J Endocrinol 126:R5-8.

Gluckman PD, Grumbach MM and Kaplan SL (1981) The neuroendocrine regulation and function of growth hormone and prolactin in the mammalian fetus. Endocrine Rev 2:363-395.

Golden K and Rillema JA (1993) Studies on the Mechanism by which prolactin stimulates α-aminoisobutyric acid uptake into cultured mouse mammary tissues. Horm metab Res 25: 466-469.

Gordon DF, Quick DP, Erwin CR, Donelson JE and Maurer RA (1983) Nucleotide sequence of the bovine growth hormone chromosomal gene. Mol Cell Endocrinol 33:81-95.

Gray A, Tait A and Bulfield G (1995) Differential transcription and expression of ornithine decarboxylase in embryos of replicated mouse lines divergently selected for lean body mass. Biochem J 308:161-166.

Gray A and Tait A (1993) Identification of ornithine decarboxylase as a trait gene for growth in replicated mouse lines divergently selected for lean body mass. Genetical Res 62:31-37.

Grens A and Scheffler IE (1990) The 5'- and 3'- untranslated region of ornithine decarboxylase mRNA affected the translational efficiency. J Biol Chem 265:11810-11816.

Groesbeck MD, Parlow AF and Daughaday WH (1987) Stimulation of supranormal

growth in prepubertal, adult plateaued, and hypophysectomized female rats by large doses of rat growth hormone: physiological effects and adverse consequences. Endocrinol 120:1963-1975.

Gupta M and Coffino P (1985) Mouse ornithine decarboxylase: complete amino acid sequence deduced from cDNA. J Biol Chem 260:2941-2944.

Hallerman EM, Nave A, Kashi Y, Holzer Z, Soller M, Beckmann JS (1987) Restriction fragment length polymorphisms in dairy and beef cattle at the growth hormone and prolactin loci. Anim Genet 18:213-222.

Hauser SD, McGrath MF, Collier RJ and Krivi GG (1990) Cloning and *in vivo* expression of bovine growth hormone receptor mRNA. Mol Cell Endocrinol 72:187-200.

Hayashi S (1989) *Ornithine decarboxylase: biology, enzymology and molecular genetics*, pp. 1-147, Pergamon Press, New York.

Heby O and Persson L (1990) Molecular genetics of polyamine synthesis in eukaryotic cells. Trends Biochem Sci 15:153-158.

Hershey JW (1991) Translational control in mammalian cells. Annu Rev Biochem 60:717-755.

Hickok NJ, Seppänen PJ, Gunsalus GL and Jänne OA (1987) Complete amino acid sequence of human ornithine decarboxylase deduced from complementary DNA. DNA 6:179-187.

Hickok NJ, Seppänen PJ, Kontula KK, Jänne PA, Bardin CW and Jänne OA (1986) Two ornithine decarboxylase mRNA species in mouse kidney arise from size heterogeneity at their 3' termini. Proc Natl Acad Sci USA 83:594-598.

Hickok NJ, Wahlfors J, Crozat A, Halmekyto M, Alhonen L, Jänne J and Jänne OA (1990) Human ornithine decarboxylase-encoding loci: nucleotide sequence of the expressed gene and characterization of a pseudogene. Gene 93:257-263.

Hilbert P, Marcotte A, Schwers A, Hanset R, Vassart G and Georges M (1989) Analysis of genetic variation in the Belgian Blue Cattle breed using DNA sequence polymorphism at the growth hormone, low density lipoprotein receptor, α-subunit of glycoprotein hormones and thyroglobulin loci. Anim Genet 20:383-394.

Høj S, Fredholm M, Larsen NJ and Nielsen VH (1993) Growth hormone gene polymorphism associated with selection for milk fat production in lines of cattle. Anim Genet 24:91-96.

Holder AT, Wallis M, Biggs P and Preece MA (1980) Effects of growth hormone, prolactin and thyroxine on body weight, somatomedin-like activity and in vivo sulphation of cartilage in hypopituitary dwarf mice. J Endocrinol 85:35-47.

Holly JM and Wass JA (1989) Insulin-like growth factors; autocrine, paracrine, or endocrine? New perspectives of the somatomedin hypothesis in the light of recent developments. J Endocrinol. 122:611-618.

Hongyo T, Buzard GS, Calvert RJ and Weghorst CM (1993) 'Cold SSCP': a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. Nucleic Acids Res 21:3637-3642.

Horvat S and Medrano JF (1994). Targeting a specific genomic interval to identify RAPD markers linked to the high growth (GH) locus in mice. Proc 5th World Congr Gen Appl to Livest Prod 21:71-74.

Huynh HT, Robitaille G and Turner JD (1991) Establishment of bovine mammary epithelial cells (MAC-T): an *in vitro* model for bovine lactation. Exp Cell Res 197:191-199.

Igarashi K, Sakamoto I, Goto N, Kashiwagi K, Honma R and Hirose S (1982) Interaction between polyamines and nucleic acids or phospholipids. Arch Biochem Biophys 219:438-443.

Imagawa W, Yang J, Guzman R and Nandi S (1994) Control of mammary gland development. In: *The Physiology of Reproduction*, 2nd Ed. Edited by E Knobil and JD Neil. pp 1033-1063. Raven Press, New York.

Ito K, Kashiwagi K, Watanabe S, Kameji T, Hayashi S and Igarashi K (1990) Influence of the 5'-untranslated region of ornithine decarboxylase mRNA and spermidine on ornithine decarboxylase synthesis. J Biol Chem 265:13036-13041.

Jacob HJ, Lindpaintner K, Lincoln SE, Kusumi K, Bunker RK, Mao YP, Ganten D, Dzau VJ and Lander ES (1991) Genetic mapping of a gene causing hypertesion in the stroke prone spontaneously hypertensive rat. Cell 67:213-224.

Jeffreys AJ, Wilson V and Thein SL (1985a) hypervariable 'minisatellite' regions in human DNA. Nature 314:67.

Jeffreys AJ, Wilson V and Thein SL (1985b) Individual-specific 'fingerprint' of human DNA. Nature 316:76-79.

Johannes G and Berger FG (1992) Alteration in mRNA translation as a mechanism for the modification of enzyme synthesis during evolution. J Biol Chem 267:10108-10115.

Kahana C and Nathans D (1985) Nucleotide sequence of murine ornithine decarboxylase mRNA. Proc Nalt Acad Sci USA 82:1673-1677.

Kahana C and Nathans D (1984) Isolation of cloned cDNA encoding mammalian ornithine decarboxylase. Proc Natl Acad Sci USA 81:3645-3649.

Kameji T and Pegg AE (1987) Inhibition of translation of mRNAs for ornithine decarboxylase and S-adenosylmethionine decarboxylase by polyamines. J Biol Chem 262:2427-2430.

Kashiwagi K, Ito K and Igarashi K (1991) Spermidine regulation of ornithine decarboxylase synthesis by a GC-rich sequence of the 5' untranslated region. Biochem Biophys Res Communications 178:815-822.

Kashiwagi K, Kobayashi H and Igarashi K (1986) Apparently unidierctional polyamine transport by proton motive force in polyamine-deficient *Escherichia coli*. J Bacteriol 165:972-977.

Katz A and Kahana C (1988) Isolation and characterization of the mouse ornithine decarboxylase gene. J Biol Chem 263:7604-7609.

Kennedy BW, Quinton M and Van Arendonk JAM (1992) Estimation of single genes on quantitative traits. J Anim Sci 70:2000-2012.

Kim SH, Moores JC, David D, Respess JG, Jolly DJ and Friedmann T (1986) The organization of the human HPRT gene. Nucleic Acids Res 14:3103-3118.

Kirkpatrick BW (1991) HaeII and MspI polymorphisms are detected in the second intron of the porcine growth hormone gene. Anim Genet 22:180-181.

Knight CH, Peaker M (1982) Development of the mammary gland. J Reprod Fertil 65:521-536.

Kozak M (1992) Regulation of translation in eukaryotic systems. Annu Rev Cell Biology 8:197-225.

Krasnow MA and Cozzarelli NR (1982) Catenation of DNA rings by topoisomerases. Mechanism of control by spermidine. J Biol Chem 257:2687-2693.

Kuhnlein U and Zadworny D (1994) Disease resistance genetics: selection at the DNA level. Proc 5th World Congr Gen Appl Livestock Prod 21:249-256.

Lagziel A, Lipkin E and Soller M (1996) Association between SSCP haplotypes at the bovine growth hormone gene and milk protein percentage. Genetics 142:945-951.

Lakshmanan N, Lamont SJ, Kaiser MG, Arhtur JA and O'Sullivan NP (1994) Genetic markers linked with quantitative traits in chickens. Proc 5th World Congr Gen Appl to Livest Prod 21:205-208.

Lander ES and Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185-199.

Lee BK, Lin GF, Crooker BA, Murtaugh MP, Hansen LB and Chester-Jones H (1993) Association of somatotropin (bST) gene polymorphism with selection for milk yield in Holstein cows. J Dairy Sci 76: suppl(1) 149.

Lee BK, Crooker BA, Hansen LB and Chester-Jones H (1994) Polymorphism in the third intron of somatotropin (bST) gene and its association with selection for milk yield in Holstein cows. J Anim Sci 72:316.

Leonard M, Gallo M, Gallo G and Block E (1990) Effects of a 28-day sustained-release formulation of recombinant bovine somatotropin (rbST) administered to cows over two consecutive lactations. Can J Anim Sci 70:795-809.

Lewis UJ, Dunn JT, Bonewald LF, Seavey BK and Vanderlaan WP (1978) A naturally occurring variant of human growth hormone. J Biol Chem 253:2679-2687.

Li X and Coffino P (1992) Regulated degradation of ornithine decarboxylase requires interaction with the polyamine-inducible protein antizyme. Mol Cell Biol 12:3556-3562.

Li X and Coffino P (1993) Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitory protein. Mol Cell Biol 13:2377-2383.

Loetscher P, Pratt G and Rechsteiner M (1991) The C terminus of mouse ornithine decarboxylase confers rapid degradation on dihydrofolated reductase: support for the PEST hypothesis. J Biol Chem 266:11213-11220.

LÖvkvist E, Stjernborg L and Persson L (1993) Feedback regulation of mammalian ornithine decarboxylase - studies using a transient expression system. Eur J Biochem 215:753-759.

Lu L, Stanley BA and Pegg AE (1991) Identification of residues in ornithine decarboxylase essential for enzymic activity and for rapid protein turnover. Biochem J 277:671-675.

Lucy MC, Hauser SD, Eppard PJ, Krivi GG, Clark JH, Bauman DE and Collier RJ (1993) Variants of somatotropin in cattle: gene frequencies in major dairy breeds and associated milk production. Dom Anim Endocrinol 10:325-333.

Lukes AJ, Barnes MA and Pearson RE (1989) Response to selection for milk yield and metabolic challenges in primiparous dairy cows. Dom Anim Endocrinol 6:287-293.

Machlin LJ (1973) Effect of growth hormone on milk production and feed utilization in dairy cows. J Dairy Sci 56:575-580.

Manzella JM and Blackshear PJ (1990) Regulation of rat ornithine decarboxylase mRNA translation by its 5'-untranslated region. J Biol Chem 265:11817-11822.

Marton LJ and Morris DR (1987) Molecular and cellular functions of the polyamines. pp. 79-105. In: *Inhibition of polyamine biosynthesis: biological significance and basis for new therapies*. Edited by P.P McCann, A.E. Pegg and A. Sjoerdsma. Academic Press, Inc., New York.

McBride BW, Burton JL, Gibson JP, Burton JH and Eggert RG (1990) Use of recombinant bovine somatotropin for up to two consecutive lactations on dairy production traits. J Dairy Sci 73:3248-3257.

McDowell GH, Hart IC and Kirby AC (1987) Local intra-arterial infusion of growth hormone into the mammary glands of sheep and goats: effects on milk yield and composition, plasma hormones and metabolites. Aust J Biol Sci 40:181-189.

Miller WL and Eberhardt NL (1983) Structure and evolution of the growth hormone gene family. Endocrine Rev 4:97-130.

Moshier JA, Osborne DL, Skunca M, Dosescu J, Gilbert JD, Fitzgerald MC, Polidori G, Wagner RL, Friezner Degen SJ, Luk GD, and Flanagan MA (1992) Multiple promoter elements govern expression of the human ornithine decarboxylase gene in colon carcinoma cells. Nucleic Acids Res 20:2581-2590.

Muller LD (1992) BST and dairy cow performance. pp.53. In: *Bovine somatotropin and emerging issues: An assesssment*. Edited by M.C. Hallberg. Westview Press, Boulder, CO.

Murakami Y and Hayashi S (1985) Role of antizyme in degradation of ornithine decarboxylase in HTC cells. Biochem J 226:893-896.

Nakamuna Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E and White R (1987) Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235:1616-1622.

Niclsen VH and Larsen NJ (1991) Restriction fragment length polymorphisms at the growth hormone gene in pigs. Anim Genet 22:291-294.

Orita M, Iwahana H, Kanazawa H and Sekiya T (1989a) Detection of polymorphism of human DNA by gel electrophoresis as single strand conformation polymorphism. Proc Natl Acad Sci USA 86:2766-2770.

Orita M, Suzuki Y, Sekiya T and Hayashi K (1989b) A rapid and sensitive detection of point mutations and genetic polymorphism using polymorphism chain reaction. Genomics 5:874-879.

Oto M, Miyake S and Yuasa Y (1993) Optimization of nonradioisotopic single strand conformation polymorphism analysis with a conventional minislab gel electrophoresis apparatus. Anal Biochem 213:19-22.

Packman G and Cleveland JL (1994) Ornithine decarboxylase is a mediator of c-myc-induced apoptosis. Mol Cell Biol 14: 5741-5747.

Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC and Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611-615.

Palmiter RD, Norstedt FM, Gelinas RE, Hammer RE and Brinster RL (1983) Metallothionein-human GH fusion genes stimulate growth of mice. Science 222:809-14.

Park MH (1989) The essential role of hypusine in eukaryotic translation initiation factor 4D (eIF-4D). J Biol Chem 264:18531-18535.

Parks JS (1989) Molecular biology of growth hormone. Acta Paediatr Scand (suppl) 349:127-135.

Paterson AH, Damon S, Hewitt JD, Zamir SD, Rabinowitch HD, Lincoln SE, Lander ES and Tanksley SD (1991) Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments. Genetics 127:181-197.

Paterson AH, Lander ES, Hewitt JD, Peterson S, Lincoln SE and Tanksley SD (1988) Resolution of quantitative traits into Mendelian factors by using a complete RFLP linkage map. Nature 335:721-726.

Peel CJ and Bauman DE (1987) Somatotropin and lactation. J Dairy Sci 70:474-486.

Pegg AE and McCann PP (1982) Polyamine metabolism and function. Am J Physiol 243:212-221.

Pegg AE (1986) Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234:249-262.

Pegg AE, Madhubala R, Kameji T and Bergeron RJ (1988) Control of ornithine decarboxylase activity in alpha-difluoromethylornithine-resistant L1210 cells by polyamines and synthetic analogues. J Biol Chem 263:11008-11014.

Phillips MA, Coffino P and Wang CC (1987) Cloning and sequencing of the ornithine decarboxylase gene from *Trypanpspma brucei*. J Biol Chem 262:8721-8727.

Phillips III JA and Cogan JD (1994) Genetic basis of endocrine disease 6 molecular basis of Familial Human Growth Hormone Deficiency. J Clin Endocrinol Metab 78:11-16.

Pilz RB, Steglich C and Scheffler IE (1990) Molecular and genetic characterization of an ornithine decarboxylase-deficient Chinese hamster cell line. J Biol Chem 265:8880-8886.

Pohjanpelto P and Knuutila S (1982) Polyamine deprivation causes major chromosome aberrations in a polyamine-dependent Chinese hamster cell line. Exp Cell Res 141:333-339.

Poulin R, Lu L, Ackermann B, Bey P and Pegg AE (1992) Mechanism of the irreversible inactivation of mouse ornithine decarboxylase by α-difluoromethylornithine. J Biol Chem 267:150-158.

Prosser CG, Davis SR, Farr VC, Moore LG and Gluckman PD (1994) Effects of close-arterial (external pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in lactating goats. J Endocrinol 142:93-99.

Ray J, Jones BK, Liebhaber SA and Cooke NE (1989) Glycosylated human growth hormone variant. Endocrinology 125:566-568.

Rillema JA, Linebaugh BE and Mulder JA (1977) Regulation of casein synthesis by polyamine in mammary gland explants of mice. Endocrinol 100: 529-336.

Rillema JA, Etindi RN, Ofenstein JP and Waters SB (1986). *In:* Actions of PRL on molecular processes. Edited by J.A. Rillema. CRC Press, Boca Raton, FL.

Rocha JL, Baker JF, Womack JE, Sanders JO and Taylor JF (1992) Statistical association between restriction fragment length polymorphisms and quantitative traits in beef cattle. J Anim Sci 70:3360-3370.

Rogers S, Wells R and Rechsteiner M (1986) Amino acid sequences common to rapidly degrade proteins: the PSET hypothesis. Science 234:364-368.

Routman E and Cheverud JM (1994) Individual genes underlying quantitative traits: Molecular and analytical methods. *In:* Molecular Ecology and Evolution Approaches

and Applications. pp. 593-606. Edited by B. Schierwater, B. Streit, G.P. Wagner and R. DeSalle. EXS. Birkhauser Verlag, Basel, Switzerland.

Rosenberg-Hasson Y, Bercovich Z and Kahana C (1991) Characterization of sequences involved in mediating degradation of ornithine decarboxylase in cells and in reticulocyte lysate. Eur J Biochem 196:647-651.

Rothschild M, Jacobson C, Vaske D, Tuggle C, Wang L, Short T, Eckardt G, Sasaki S, Vincent A, McLaren D, Southwood O, van der Steen H, Mileham A and Plastow G (1996) The estrogen receptor locus is associated with a major gene influencing litter size in pigs. Proc Natl Acad Sci USA 93: 201-205.

Russell D and Snyder SH (1968) Amine synthesis in rapidly growing tissues: ornithine decarboxylase activity in regenerating rat liver, chick embryo, and various tumors. Proc Natl Acad Sci USA 60:1420-1427.

Sambrook J, Fritsch EF and Maniatis T (1989) *Molecular cloning, a laboratory manual*. Second edition. Cold Spring Habor Laboratory Press.

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain terminating inhibitor. Proc Natl Acad Sci USA 74:5463-5467.

Scanes CG and Campbell RM (1995) Growth hormone: chemistry. pp. 1-24. In: *Growth Hormone*. Edited by S. Harvey, C.G. Scanes and W.H. Daughaday. CRC press. Boca Raton, FL.

Scanes CG (1995) Growth hormone action: agriculture significance. pp. 451-474. In: *Growth Hormone*. Edited by S. Harvey, C.G. Scanes and W.H. Daughaday. CRC press. Boca Raton, FL.

Scanes CG and Daughaday WH (1995) Growth hormone action: growth. pp. 351-369. In: *Growth Hormone*. Edited by S. Harvey, C.G. Scanes and W.H. Daughaday. CRC press. Boca Raton, FL.

Schlee P, Graml R, Schallenberger E, Schams D, Rottmann O, Olbrich-Bludau A and Pirchner F (1994) Growth hormone and insulin-like growth factor I concentrations in bulls of various growth hormone genotypes. Theor Appl Genet 88:497-500.

Schnier J, Schwelberger HG, Smit-McBride Z, Kang HA and Hershey JB (1991) Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast Saccharomyces cerevisiae. Mol Cell Biol 11:3105-3114.

Seely JE and Pegg AE (1983) Effect of 1,3-diaminopropane on ornithine decarboxylase enzyme protein in thioacetamide-treated rat liver. Biochem J 216:701-707.

Sejrsen K, Foldager J, Sorensen MT, Akers RM and Bauman DE (1986) Effect of exogenous bovine somatotropin on pubertal mammary development in heifers. J Dairy Sci 69:1528-1535.

Shantz LM and Pegg AE (1994) Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. Cancer Res 54:2313-2316.

Sharp GL, Hill WG and Robertson A (1984) Effects of selection on growth, body composition and food intake in mice. I. Responses in selected traits. Genetical Res 42:75-92.

Sinha YN and Vanderlaan WP (1982) Effect on growth of prolactin deficiency induced in infant mice. Endocrinol 110:1871-1878.

Sinha YN, Klemcke HG, Maurer RR and Jacobsen BP (1990) Changes in the glycosylated and nonglycosylated forms of prolactin and growth hormone in lean and obese pigs during pregnancy. Endocrinol 127:410-418.

Sinha YN and Jacobsen BP (1987) Glycosylated growth hormone: detection in murine pituitary gland and evidence of physiological fluctuations. Biochem Biophys Res Commun 145:1382-1395.

Smith C and Simpson SP (1986) The use of genetic polymorphisms in livestock improvement. J Anim Breed Genet 103:205-217.

Sneyers M, Renaville R, Falaki M, Massart S, Devolder A, Boonen F, Marchand E, Prandi A, Burny A and Portetelle D (1994) TaqI restriction fragment length polymorphisms for growth hormone in bovine breeds and their association with quantitative traits. Growth Reg 4:108-112.

Soller M and Beckmann JS (1983) Genetic polymorphism in varietal identification and genetic improvement. Theor Appl Genet 67:25-32.

Steglich C and Scheffler IE (1982) An ornithine-decarboxylase-deficient mutant of Chinese hamster ovary cells. J Biol Chem 257:4603-4609.

Stevens L and Winther MD (1979) Spermine, spermidine and putrescine in fungi development. Adv Microb Physiol 19:63-148.

Strange R, Li F, Saurer S, Burkhardt A and Friis RR (1992) Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 115: 49-58.

Stuber CW, Lincoln SE, Wolff DW, Helentjaris T, Lander ES (1992) Identification of genetic factors contributing to heterosis in a hybrid from two elite maize inbred

lines using molecular markers. Genetics 132:823-839.

Tabor CW and Tabor H (1984) Polyamines. Annu Rev Biochem 53:747-790.

Tabor H, Hafner EW and Tabor CW (1980) Construction of an Escherichia coli strain unable to synthesize putrescine, spermidine, or cadaverine: characterization of two genes controlling lysine decarboxylase. J Bacteriol 144:952-956.

Takemoto T, Nagamatsu Y and Oka T (1983) The study of spermidine stimulated polypeptide synthesis in cell-free translation of mRNA from lactating mouse mammary gland. Biochem Biophys Acta 740:73-79.

Tamori A, Nishiguchi S, Kuroki T, Koh N, Kobayashi K, Yano Y and Otani S (1995) Point mutation of ornithine decarboxylase gene in human hepatocellular carcinoma. Cancer Res 55:3500-3503.

Tanaka M, Hosokawa Y, Watahiki M and Nakashima K (1992) Structure of the chicken growth hormone-encoding gene and its promoter region. Gene 112:235-239.

Thomas T, Kiang DT, Jänne OA and Thomas TJ (1991) Variations in amplification and expression of the ornithine decarboxylase gene in human breast cancer cells. Breast Cancer Res Treat 19:257-267.

Thomas JW, Erdman RA, Galton DM, Lamb RC, Arambel MJ, Olson JD, Madsen KS, Samuels WA, Peel CJ and Green GA (1991) Responses by lactating cows in commercial dairy herds to recombinant bovine somatotropin. J Dairy Sci 74:945-964.

Tobias KE and Kahana C (1993) Intersubunit location of the active site of mammalian ornithine decarboxylase as determined by hybridization of site-directed mutants. Biochem 32:5842-5847.

Tobias KE, Mamroud-Kidron E and Kahana C (1993) Gly387 of murine ornithine decarboxylase is essential for the formation of stable homodimers. Eur J Biochem 218:245-250.

Tsirka S and Coffino P (1992) Dominant negative mutants of ornithine decarboxylase. J Biol Chem 267:23057-23062.

van Steeg H, van Oostrom CTM, Hodemaekers HM, Peters L and Thomas AAM (1991) The translation *in vitro* of rat ornithine decarboxylase mRNA is blocked its 5' untranslated region in a polyamine-independent way. Biochem J 274:521-526.

van Steeg H, van Oostom CTM, Martens JWM, van Kreyl CF, Schepens J and Wieringa B (1989) Nucleotide sequence of the human ornithine decarboxylase gene. Nucleic Acids Res 17: 8855-8856.

van Steeg H, van Oostom CTM, Hodemaekers HM and van Kreyl CF (1990) Cloning and function analysis of the rat ornithine decarboxylase encoding gene. Gene 93:249-256.

van Steeg H, van Oostom, ThM, van Kranen HJ and van Kreyl CF (1988) Nucleotide sequence of the rat ornithine decarboxylase gene. Nucleic Acids Res 16:8173.

van Kranen HJ, ven de Zande L, van Kreyl CF, Bisschop A and Wieringa B (1987) Cloning and nucleotide sequence of rat ornithine decarboxylase cDNA. Gene 60:145-155.

Vicini JL, Buonomo FC, Veenhuizen JJ, Miller MA, Clemmons DR and Collier RJ (1991) Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. J Nutr 121:1656-1664.

Wahlfors J (1991) Certain changes in ornithine decarboxylase gene methylation accompany gene amplification. Biochem J 279:435-440.

Weller J, Soller M and Brody T (1988) Linkage analysis of quantitative traits in an interspecific cross of tomato (*Lycopersicon esculentum x Lycopericon pimpinellifolium*) by means of genetic markers. Genetics 118:329-339.

Weller JI, Kashi Y and Soller M (1990) Power of daughter and granddaughter disigns for mapping of quantitative traits in dairy cattle using genetic markers. J Dairy Sci 73:2525-2537.

Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213-7218.

Wen L, Huang JK and Blackshear PJ (1989) Rat ornithine decarboxylase gene: nucleotide sequence, potential regulatory elements and comparison to the mouse gene. J Biol Chem 264:9016-9021.

Werner M, Feller A, Messenguy F and Pierard A (1987) The leader peptide of yeast gene CPA1 is essential for the translational repression of its expression. Cell 49:805-813.

Widegren B, Blomberg J and Arnason U (1991) The long leader sequence of the mouse ornithine decarboxylase mRNA, previously suspected to be a cloning artefact, is probably a product of recombination with a MuLV-like retrovirus. Gene 109:303-305.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic

Acids Res 18:6531-6535.

Winkelman DC, Querengesser LD and Hodgetts R (1990) Growth hormone restriction fragment length polymorphisms that segregate with 42-day live weight in mice. Genome 33:235-239.

Winkelman DC and Hodgetts R (1992) RFLPs for somatotropic genes identify quantitative trait loci for growth in mice. Genetics 131:929-937.

Woychik RP, Camper SA, Lyons RH, Horowitz S, Goodmin EC and Rottman FM (1982) Cloning and nucleotide sequencing of the bovine growth hormone gene. Nucleic Acids Res. 10:7197-7210.

Yao J, Zadworny D, Kühnlein U, Hayes JF (1995) Molecular cloning of a bovine ornithine decarboxylase cDNA and its use in the detection of restriction fragment length polymorphisms in Holsteins. Genome 38:325-331.

Young FG (1947) Experimental stimulation (galactopoiesis) of lactation. Br Med Bull 5:155-160.

Zadworny D and Kühnlein U (1990) The identification of the kappa-casein genotype in Holstein dairy cattle using the polymerase chain reaction. Theor Appl Genet 80:631-634.

Zhang HM, Maddock KC, Brown DR, DeNise SK and Ax RL (1993) Bovine growth hormone gene frequencies in samples of U.S. AI bulls. J Anim Sci 71:93.

Zhang HM, Brown DR, DeNise SK and Ax RL (1992) Nucleotide sequence determination of a bovine somatotropin allele. Anim Genet 23:578.

Zhang L (1995) Molecular cloning and characterization of the chicken ornithine decarboxylase gene. MSc thesis, McGill University, Montreal, Canada.

Zhang L, Zadworny D, Kühnlein U and Sun ZT (1992) Sequence analysis of the chicken ornithine decarboxylase (ODC) gene. Proc 19th World's Poult Congr Vol. 1:496-500.

Zuker M and Steigler P (1981) RNA secondary structure prediction. Nucleic Acids Res 9:133-148.