

Structural and functional genomics in farm animals a laboratory view point

Habilitation Thesis

Author(s): Neuenschwander, Stefan

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Eidgenössische Technische Hochschule Zürich

STRUCTURAL AND FUNCTIONAL GENOMICS IN FARM ANIMALS: A LABORATORY VIEW POINT

Habilitationsschrift

von

Stefan Neuenschwander

Departement der Agrar- und Lebensmittelwissenschaften Institut für Nutztierwissenschaften, Gruppe Züchtungsbiologie

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Vorwort

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Zusammenfassung

Die rasante Entwicklung in der Genomforschung der Nutztierzucht und –haltung der letzten Jahre war nur möglich dank der Zusammenarbeit von Spezialisten aus unterschiedlichen Fachrichtungen wie der Molekularbiologie, Veterinärmedizin, Biochemie und der qualitativen und quantitativen Genetik. Die fast unbegrenzten Möglichkeiten im Labor stellen dabei hohe Anforderungen an die Wissenschaftler, das Laborpersonal und die finanziellen Ressourcen. Diese Arbeit gibt eine Übersicht über die neuesten Entwicklungen in der strukturellen und funktionellen Genomik und zeigt die zukünftigen Möglichkeiten eines universitären Labors auf, das sich mit Nutztieren beschäftigt. Die wichtigsten Erkenntnisse der Arbeit sind wie folgt:

- Der Aufbau einer Versuchsherde mit segregierenden Phänotypen ist eine der wichtigsten Grundlagen zur erfolgreichen Genomanalyse.
- Obwohl die Mikrosatelliten immer noch das Rückgrat der genetischen Charakterisierung von Nutztieren darstellen, werden sie durch die immer zahlreicheren SNPs (single nucleotide polymorphisms) ergänzt und langfristig vielleicht sogar ersetzt werden.
- Das Potential der SNPs liegt in der Möglichkeit der Automatisation der Diagnose, darüber hinaus können SNPs auch eine funktionelle Bedeutung haben.
- Aus ökonomischen Gründen wird sich die Suche nach genetischen Variationen auf eng begrenzte chromosomale Regionen beschränken, wo ein QTL (quantitative trait locus) oder ETL (economic trait locus) vermutet wird (z.B. in einem Contig von genomischen Klonen). Screening-Methoden, die unabhängig vom Sequenzieren sind, werden vermehrt zur Identifikation von SNPs zum Einsatz kommen.
- Die Nutztiergenomik wird sich auf exprimierte Sequenzen fokussieren. Funktionelle Untersuchungen auf der Stufe der mRNA und der Proteine werden an Bedeutung gewinnen.
- EST-Programmen (expressed sequence tags) kommt bei der Entwicklung von SNPs und bei der Identifikation von positionellen Kandidatengenen eine besondere Bedeutung zu. Sie bilden auch die Grundlage zur Entwicklung von DNA Chips oder Arrays von landwirtschaftlichen Nutztieren, die zu "erweiterten Phänotypen" führen (Expressionsprofile).
- Leider stehen für die Nutztiere keine Genchips mit hoher Dichte zur Verfügung und es ist fraglich, ob sie überhaupt entwickelt werden. Weiter ist unklar, ob sich die Methode

des "Differential Display" mit Probenmaterial aus Geweben oder ganzen Organen für das Auffinden von Kandidatengenen eignet. Gute Chancen hat das Verfahren, wenn sich primäre Zellkulturen von unterschiedlichen Geweben verschiedener Phänotypen anlegen und untersuchen lassen.

 Nicht mendelistische Vererbung quantitativer Merkmale scheint häufiger aufzutreten als bisher angenommen (Imprinting, Allel-spezifische Expression). Ihre Bedeutung in der Nutztierzucht ist abzuklären.

In dieser Arbeit werden Beispiele für Erfolge der molekularen Tierzucht beschrieben und die molekularen Grundlagen der zugrundeliegenden monogenetisch vererbten Eigenschaften dargestellt. Die Krankheitsmodelle, auf die wir uns während meiner Oberassistenzzeit hauptsächlich konzentriert haben, waren die Hämophilie beim Schaf, die Vitamin C-Defizienz, die congenitale progressive Ataxie und die Resistenz gegenüber der Besiedelung mit pathogenen *E. coli* Bakterien beim Schwein. Dabei wird die Verwendung von landwirtschaftlichen Nutztieren als biomedizinische Tiermodelle diskutiert.

Summary

The rapid development that has occurred in the field of genome analysis over the past several years has required the cooperation of specialists from a diverse array of fields. Scientists with expertise in molecular biology, veterinary medicine, biochemistry, and qualitative and quantitative genetics are collaborating to solve various problems in agricultural breeding and husbandry. The almost unlimited possibilities in the laboratory make high demands on scientists, research technicians, and on the financial resources. This study gives an overview of the newest developments in structural and functional genomics and discusses future research possibilities for a university laboratory that studies farm animals. The most important findings of this survey are as follows:

- The formation of an experimental herd with segregating phenotypes is one of the most critical components for a successful genome analysis.
- Although microsatellites still form the backbone of genetic characterization of farm animals, they are complemented by the growing number of single nucleotide polymorphisms (SNPs). In the future, SNPs may even replace microsatellites.
- The greatest potential of SNPs lies in the possibility to automate the diagnosis process. In addition, certain SNPs are also associated with physiological functions.
- For economic reasons, the search for genetic variants will be restricted to clearly defined chromosomal regions where a quantitative trait locus (QTL) or an economic trait locus (ETL) is predicted (*e.g.*, in a contig of genomic clones). Sequencing-independent screening methods will be most frequently used for the identification of SNP's.
- Farm animal genomic research will focus on expressed sequences. As a result, functional studies on the level of mRNA and the proteins will become more important.
- Expressed sequence tags (ESTs) have a special importance in the development of SNPs and in the identification of positional candidate genes. ESTs also form the basis for the development of DNA chips or arrays that are specific for farm animals. These, in turn, can be used to develop "extended phenotypes" (expression profiles).
- Unfortunately, there are currently no high density gene chips available for farm animals, and it is uncertain whether such tools will be developed at all. Furthermore, it is not clear whether the "differential display" method, using probe material derived from tissues or whole organs is suitable for the identification of candidate genes. If primary cell cultures can be developed from different tissues and phenotypes, the prospects for success with the differential display procedure are good.
- Non-mendelian inheritance of quantitative traits seems to be found more often than expected (*e.g.*, imprinting, allele specific expression). The importance of this in breeding programs has to be investigated.

I will describe several examples of successful molecular animal breeding to characterize monogenetic inherited traits. The diseases that we have focused on during my stay as a senior research scientist include hemophilia in sheep, as well as vitamin C deficiency, congenital progressive ataxia, and resistance to the adhesion of pathogen *E. coli* bacteria, in pigs. The suitability of farm animals as useful models in biomedical research is discussed.

1. Introduction

Domesticated farm animals have been genetically isolated from the wild members of their species for approximately 10'000 years. Occasional mutations and forced selection towards characteristics suitable for the production of food, clothing, leather, and animals' strength dramatically changed the phenotype and the behavior of these animals. Approximately 100 years ago, the coalescence of biometrics and Mendelian genetics gave rise to the field of quantitative genetics. The understanding that quantitative traits are influenced by both an individual's genotype and by environmental factors has led to a spectacular increase in the productivity of all major livestock species during the last few decades. This success is largely due to extensive phenotypic record keeping (milk yield, growth, number of piglets per litter, etc.) and to the implementation of specific breeding schemes. However, for traits with low heritability, such as quality of products for human consumption, reproductive traits, and resistance to diseases, there is much need for improvement.

Scientists have developed tools designed to characterize an individual at the level of its genomic DNA. These DNA polymorphic markers are independent of age, gender, and environmental factors, and are evenly distributed across the whole genome. These markers have proven to be especially useful in localizing monogenetic traits, such as inherited disease genes. The majority of quantitative traits in livestock, however, are typically multifactorial. Consequently, it is very difficult to use genomic strategies to identify chromosomal regions or genes that are involved in these economically important traits. Quantitative trait loci (QTL) mapping and establishment of markers which could be used in marker assisted selection (MAS) breeding programs represent important first steps in this direction.

The term "genomics" was proposed in 1986 by Thomas Roderick (McKusick & Ruddle, 1987; McKusick, 1997) to describe the study of a genome by molecular means distinct from traditional genetic approaches. Genomics evolved from a much older word, "genome", and represents a fusion of <u>gene</u> and chromos<u>ome</u>. A genome is the complete collection of genes possessed by an organism. Living creatures result from the delicate interplay between a "functional" genome and various environmental factors.

The complete genetic sequence information is now available for many organisms, including *E.coli* (Blattner et al., 1997), Sacharomyces cerevisiae (Mewes et al., 1997), Caenorhabditis elegans (Genome sequence of the nematode C. elegans: a platform for investigating biology. The C. elegans Sequencing Consortium, Science <u>282</u>: 2012-2018), Drosophila melano-



Figure 1a. Genotype / phenotype interactions. Details see text

gaster (Adams et al., 2000) and human (Adams et al., 1995; Lander et al., 2001; Venter et al., 2001). With the complete sequences of complex genomes now established, the scientific focus is shifting towards the development of methods that effectively use this wealth of structural genomic information. For an updated overview see

www.tigr.org/tdb; www.ncbi.nlm.nih.gov; www.cbs.dtu.dk/services/GenomeAtlas.

The most important question facing us today is: How does the genome build, maintain, and operate an organism? Multiple strategies at the levels of DNA, RNA, protein, cells, and the whole organism will help to answer this question. Genomic DNA effectively serves as the "blueprint" of an organism. Figure 1 (a and b) demonstrates the interplay between genomic DNA, which is condensed into the chromosomes of each cell nucleus, and the phenotype. Single genes are transcribed into RNA which is, in case of structural genes, further modified (spliced, polyadenylated, capped) and transported into the cytosol. Ribosomes bind to the cap structure (including the terminal 7-methyl guanosine triphosphate) and the information encoded by the mRNA is translated into a polypeptide. Finally, posttranslational modifications convert the premature peptides into functional proteins. Regulation of gene expression takes place at many levels. The 5'-flanking region of a gene, which may include promoter sequences, determines the rate of transcription.



Figure 1b. Molecular basis of the phenotype

The binding of specific proteins to the promoter or enhancer / silencer sequences (transcription factors, polymerases) may accelerate or stop gene transcription. Finally, a protein can be stored as an inactive precursor and can be activated as needed by enzymatic cleavage, in the absence of mRNA.

The ultimate goal in genome analysis is to identify the allelic variants of major genes and to understand why such variants have a great influence on the phenotypic appearance. The chances of success in farm animals, as compared to laboratory animals, are hindered by long generation times, limited numbers of offspring per female, and high genetic variance. New strategies to identify such allelic variants are being developed at all levels of gene expression (DNA sequence, mRNA and proteins), and these strategies need to be adapted for application in livestock.

In this habilitation thesis, I will review these strategies and the potential impact of structural and functional genomics in farm animal research. Such approaches will increase our understanding of the molecular basis of agriculturally important traits and may be advantageous for all, including the animals, the breeders and the farmers.

I also include an overview of projects I have been in charge of during the last years. They clearly demonstrate the switch from the descriptive genome analysis towards an extended analysis including gene expression and activity studies of allele specific gene products.

2. Structural genomics in farm animals

Although the genomic sequence of an organism is the ultimate structural characterization of a genome, it is available only for small genomes and for humans. However, new methods have been developed to detect genetic variants (type 1 and type 2 markers), which are suitable to use in linkage analysis or association studies.

The use of single nucleotide polymorphisms (SNPs) for characterizing genomes is becoming more and more important. As increasing amounts of sequence data become available, SNPs are more rapidly being detected. In addition, the use of SNPs will increase greatly as more high throughput methods for detecting and typing them are developed.

2.1. Microsatellites

Microsatellites are stretches of sequence of about 100 to 200 nucleotides consisting of tandemly repeated short nucleotide motifs. These are evenly distributed throughout the genome and are highly polymorphic. In combination with the powerful technique of the polymerase chain reaction (PCR), the use of microsatellites has become the method of choice in genotyping farm animals (for review see (Beattie, 1994)). Individual as well as internationally coordinated programs have led to the generation of a number of linkage maps in various species. These efforts resulted in a new database (Arkdb) for genome mapping data (http://roslin.thearkdb.org) that includes, cat, cattle, chicken, deer, horse, pig, salmon, sheep, tilapia and turkey (Hu et al., 2001) and in the BOVMAP database

http://locus.jouy.inra.fr/cgi-bin/bovmap/Bovmap/intro.pl

Most of the linkage groups have been anchored and oriented to specific chromosomes, at least in cattle and pig, by mapping with fluorescence in situ hybridization (FISH). With the availability of somatic cell hybrid and radiation hybrid panels, the resolution of mapping new marker loci is considerably faster, easier and has dramatically increased the cytogenic resolution.

As of November 2001, the pig map included 2344 loci. Of these, 829 are designated as genes and 1371 are designated as microsatellites. In cattle, over 1200 microsatellites are available, 1739 in sheep, and 1277 in chicken. Thus, with the available genetic maps, the marker resources are not the limiting factor when attempting to map trait loci by exploiting family linkage disequilibrium (resolution around 2.5 cM). For the purposes of fine mapping, however, a higher density of marker coverage may be required. Alternative approaches, such as the development of SNPs or contigs of genomic clones are discussed below.

2.2. Type 1 markers and comparative mapping

Although microsatellites form the backbone of all livestock linkage maps, a sufficient number of evolutionarily conserved Type 1 markers (expressed genes) are interspersed in these maps. These markers confirm the extensive conservation of synteny among distantly related mammals that was predicted from early mapping data (Womack & Moll, 1986). Among mammals, cattle (Eggen & Fries, 1995; Womack & Kata, 1995; Yang & Womack, 1998) and pig (Robic et al., 1996; Yerle et al., 1996; Yerle et al., 1997; Yerle et al., 1998; Yerle et al., 1999) have well-developed syntheny and linkage maps. Most of these genes have been mapped by physical methods, such as somatic cell hybrid analysis and in situ hybridization, leading to the identification of conserved synteny among a diverse spectrum of vertebrate genomes.

Comparative chromosomal painting (Zoo-FISH) using human chromosome specific libraries has further defined the boundaries of conserved chromosomal segments between the genomes of humans, pigs (Rettenberger et al., 1995; Goureau et al., 1996), and cattle (Solinas-Toldo et al., 1995). However, there is currently insufficient comparative map information available to determine whether the gene order and content are identical or only closely similar within the conserved syntenic blocks in different species.

2.2.1. EST mapping by somatic cell hybrids or radiation hybrids

Expressed sequence tags (ESTs) are nucleotide sequences generated from the ends of (randomly) selected clones from unmodified or normalized cDNA libraries. EST projects are being conducted on a diverse collection of organisms and tissues. As of November 2001, there were over 9,3 millions entries reported in the dbEST database, the vast majority from human, mouse and rat genes. However, increasing numbers of entries from farm animals and plants are being identified (Table 1).

ESTs are primarily used for gene identification in large sequencing projects. While sequencing genomic DNA is the only definitive way to access all the genes of an organism, this process is both expensive and slow. By accumulating EST sequences, it is possible to identify important genes in livestock by leaping across taxonomic boundaries, from genes identified in "model organisms", such as human, mouse and rat. Moreover, ESTs are important in the construction of gene-based physical maps. PCR or hybridization assays developed from ESTs can be used to identify large-insert clones, from which genome physical maps are constructed (*e.g.*, by constructing a contig covering a quantitative trait locus (QTL) or an economically important locus (ETL, economic trait locus)).

(http://www.ncbi.nlm.nih.gov/dbEST/dbE	ST_summary.html)
Homo sapiens	3'800'000
Mus musculus	2'300'000
Ratus sp.	317'000
Bos taurus	193'000
Sus scrofa	98'000
Gallus gallus	33'000
Oryctolagus cuniculus	2'113
Capra hircus	245
Ovis aries	8

Table 1. ESTs from selected species in the NCBI database

The GenetPig consortium has mapped over 700 ESTs from liver, muscle, small intestine, granulosa cells, and fat cDNAs by using a somatic cell hybrid panel and a radiation hybrid panel (Gellin, 2001, personal communication). The experimental procedure used was as follows:

- 1. selection of clones;
- 2. single pass sequencing from 5'- and 3'-region of the unknown sequence (200-500 bp);
- 3. identification of homologous genes by a BLAST search of the Genbank database with the edited 5'-sequences;
- 4. designing a primer pair from the 3'-end of the gene resulting in a PCR fragment of 100 to 500 bp;
- 5. testing the primers with genomic DNA from pig (positive control), mouse, and hamster (parental cell lines of the hybrid panel as negative control);
- 6. testing the primers using DNA from the somatic cell hybrid panel (Yerle et al., 1996) or the radiation hybrid panel (Yerle et al., 1998);
- 7. assignment of EST to chromosomal regions based on likelihood calculations and Bayes' theorem (Chevalet et al., 1997).

Other EST projects in pig are currently underway (Fahrenkrug et al., 2000; Smith et al., 2001), and alternative mapping strategies have been used to improve the efficiency of the labor-intensive comparative mapping process and to take full advantage of the vast information generated by the human genome sequencing effort. A research program has been implemented to directly integrate variability detected in swine expressed sequence tags (ESTs) into the existing genetic map. The program objectives are to map porcine ESTs orthologous to genes with known human map positions using SNPs. The SNP discovery phase has identified

polymorphic positions within amplicons for automated design of genotyping assays. Observed single nucleotide polymorphisms (SNPs) in the MARC swine reference population (US Meat Animal Research Center) were genotyped via microsequencing and MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry). Map positions of ESTs were determined by linkage analysis. Genotypic data from SNP assays within amplicons developed from EST sequences are being used to develop an integrated, high density, type-I marker map of the porcine genome (Ferking et al., 2000).

2.2.2. Microdissection

Despite dramatic advances in the identification and mapping of expressed sequence tags (ESTs), new techniques that facilitate the isolation of chromosome or chromosome bandspecific ESTs would be of considerable value. Chen-Liu and colleagues (1995) have established a cytogenetic approach for preparing chromosome-specific cDNA sublibraries, called selection of hybrids by affinity capture (SHAC). This procedure consists of two stages. In the first stage, a particular chromosome region was microdissected, then amplified by PCR with a universal degenerate primer and biotinylated nucleotides. In the second stage, a cDNA library with unique linker-adapter ends was hybridized to the DNA prepared during the first stage. The resulting DNA duplexes were captured on streptavidin-coated magnetic beads. The cDNAs were then released from their biotin-labeled target homologs and recovered by PCR amplification.

A reverse approach of this method may also be successful. For example, Gracia and colleagues (1997) first hybridized a tagged cDNA library to metaphase chromosomes. Microdissection was then performed and locus-specific cDNA was amplified by PCR using tag primers. Isolated sequences may be tested by the methods described above to verify the correct localization.

2.2.3. "In silico" mapping

Comparative mapping by annotation and sequence similarity (COMPASS) has been shown to be an effective approach for predicting the chromosomes location of ESTs on the basis of comparative mapping information. Rebeiz and Lewin (2000) developed a computer program to identify orthologs and predict map locations of 47'787 cattle ESTs. Among these, approximately 30'000 had significant matches with sequences in the human UniGene database and around 21' 000 were annotated with human GB4 radiation hybrid mapping data. Based

on previous experiences (Band et al., 2000), the authors claimed that these predictions of annotation and in "*in silico*" mapping are accurate in 95% of the cases. A new web-based version of the COMPASS predictive gene mapping tool for cattle is available at:

http://keck1.biotec.uiuc.edu:8887/examples/servlets/mainpage.html

The program works by first subjecting the query sequence to BLASTN against the human UniGene collection. Significant hits are retrieved and relevant information about the sequence matches, including human map locations, are stored in memory. The cattle chromosome and bin location are then predicted on the basis of the map (Band et al., 2000) and the results are presented in downloadable tabular form.

In human, when a non-redundant collection of over 900 ESTs was used to evaluate the draft genome sequence coverage and mapping fidelity, a considerable amount of discrepancies (~20%) were detected in both the reported coverage of the human genome and the accuracy of mapping of genomic clones (Katsanis et al., 2001). This high error rate suggests that there are some limitations of the draft genome sequence in providing accurate positional and detailed characterization of chromosomal subregions. As the comparative maps are rather coarse, this should have only a negligible impact on the predicted loci using the COMPASS approach.

2.3. Single nucleotide polymorphisms (SNPs)

The most common type of genetic variation is single nucleotide polymorphisms (SNPs). In the human, around 1.4 million SNPs have been mapped by the SNP consortium (Sachidanandam et al., 2001) and these are publicly accessible (<u>http://snp.cshl.org</u>). This provides an average density of one SNP per every 1.9 kb, which is close to the expected density of one per every 1.3 kb, based on the analysis of nucleotide diversity studies.

In farm animals, there are relatively few SNP markers available, as compared to the numbers of known microsatellites. However, in the ongoing EST mapping experiments, we expect to find an increasing number of SNPs that are expressed (cSNPs), and may therefore have functional significance.

Bi-allelic SNPs are comprised of four distinct subtypes. Using the abbreviation $P \Leftrightarrow Q$ ($P_1 \Leftrightarrow Q_1$) to represent allelic nucleotides P and Q of a SNP on one strand, with their base pairing P_1 and Q_1 of the second strand shown in parentheses, the four SNP alternatives include one transition $C \Leftrightarrow T (G \Leftrightarrow A)$ and three transversions, $C \Leftrightarrow A (G \Leftrightarrow T)$, $C \Leftrightarrow G (G \Leftrightarrow C)$ and $T \Leftrightarrow A$ $(A \Leftrightarrow T)$. If one considers each DNA strand to be equivalent, then $C \Leftrightarrow T$ ($G \Leftrightarrow A$) is an identical mirror image or sequence complement of $G \Leftrightarrow A$ ($C \Leftrightarrow T$). In the case where the two DNA strands have to be distinguished (*e.g.*, during transcription) $C \Leftrightarrow T$ ($G \Leftrightarrow A$) and $C \Leftrightarrow A$ ($G \Leftrightarrow T$) represent two separate subtypes of SNPs. The most abundant SNP is the $C \Leftrightarrow T$ ($G \Leftrightarrow A$) variant, which may be due to the 5-methylcytosine deamination reaction, which occurs particularly at CpG dinucleotides (Holliday & Grigg, 1993). The occurrence of SNPs is about 1/1000 bp and is not equally distributed over the genome. In general, expressed sequences contain fewer SNPs than do non-coding regions. In the non-coding HLA region (human lymphocyte system A), nucleotide diversity levels are between 5% and 10% (Guillaudeux et al., 1998; Horton et al., 1998)

2.3.1. Detection and diagnosis

The number of methods that are currently used to screen or detect DNA variations is overwhelming (Table 2). This diversity also reflects the fact that none of these methods is strikingly superior to the others and that the best method may not yet be developed. The ideal high-throughput mutation screening method should be cheap, and not require expensive equipment with high running and maintenance costs. It should be simple, no more complex than electrophoresis, for example. Finally, such a method must be fast. The method of choice also depends on the basic equipment available in a laboratory, such as an automated sequencer, quantitative PCR, fluorometry, HPLC or mass spectroscopy. A rate-limiting step in high-throughput genotyping is amplification of DNA. Most current assays include a step that produces many copies of a short segment of DNA that spans each target SNP. This amplification is necessary because either only small amounts of DNA can be harvested from single samples or a large number of SNPs has to be genotyped. Also, the amplification step improves the signal to background ratio of the assay, thereby increasing the reliability of the detection. Most genotyping systems are based on the polymerase chain reaction (PCR). Although widely used and highly efficient in DNA amplification, PCR is expensive and slow. Amplifying more than 10 targets in parallel is extremely difficult and requires extensive optimization of the reactions (Nechtelberger et al., 2001; Glowatzki, 2001, personal communication). Therefore, some researchers are amplifying and genotyping the same SNP from many individuals simultaneously (e.g., 100 individuals (Wolford et al., 2000; Buetow et al., 2001)). Although pooling obscures the presence of rare alleles and results in the loss of information on individual haplotypes, it greatly accelerates the genotyping process. This approach is especially well-suited for association studies using extreme pools (e.g., healthy or diseased individuals, high *versus* low responders, etc.), where causative mutations and associated markers should occur more frequently. In contrast, using microsatellites on pooled DNA samples is unreliable, as the interpretation of alleles is extremely difficult. The Taq DNA polymerase tends to slip along highly repetitive DNA motifs, thereby resulting in artifact bands.

Assay	Templates	Principle	Detection	Comments	References
Enzyme based					
Endonuclease type II RFLP restriction fragment length polymorphism	Genomic DNA, PCR fragments	Existence / absence of the allele specific endonucle- ase type II recognition site	Gelelectro- phoresis, length of fragments	Slow, multi- step, reliable, not for all SNPs	(Southern, 1975)
Cleavase CFLP cleavase fragment length polymorphism	End labeled PCR frag- ments	Sequence dependent sec- ondary structures of single stranded DNA are recog- nized and cleaved	Gelelectro- phoresis, length of fragments	Slow, multi- step, not all SNPs are detected	(Oldenburg & Siebert, 2000)
Resolvase ECM enzyme mismatch cleav- age; EMD en- zyme mutation detection	Heteroduplex DNA, PCR fragments	Resolvase (T4 or T7 en- donuclease I) recognizes and cleaves DNA forming a heteroduplex at the mismatch site	Gelelectro- phoresis, length of fragments, kit for fluores- cence detec- tion available	Slow, multi- step, reliable	(Mashal et al., 1995; Youil et al., 1996)
Flap endonucle- ase (FEN) Invader assay	Genomic DNA	FENs are structure spe- cific 5'endonucleases that recognize and cleave a structure formed when two overlapping oligonu- cleotides hybridize to a target DNA strand	Fluorescence	Fast, reliable, single reac- tion	(Lyamichev et al., 1999; Hall et al., 2000; Lyami- chev et al., 2000)
DNA polymerase PASA PCR am- plification of spe- cific alleles; Bi-PASA bi- directional PASA	Genomic DNA	Heat stable DNA poly- merase amplifies genomic DNA using allele specific primers Bi-PASA: two inner (al- lele specific primers) and two outer primers are used in one reaction	Gel electro- phoresis	Slow, Bi- PASA in- cludes a con- trol fragment, optimization required	(Liu et al., 1997; Jiang & Gibson, 1999).
DNA polymerase ASPE allele spe- cific primer ex- tension; GBA genetic bit analysis; SBE single base extension; TAG-SBE minis- equencing; TDI template directed dye terminator incorporation	PCR frag- ments	A sequencing primer is annealed to template DNA and a one base ex- tension is performed (dideoxinucleotide)	Gel electro- phoresis, fluorescence, mass spec- troscopy, flow cytome- try	Slow (fast if using TAG- arrays, micro- spheres or DNA pool- ing), multi- step, reliable. Purification of fragments needed as PCR primers may interfere with assay. TDI see also figure 2D	(Nikiforov et al., 1994; Cai et al., 2000; Fan et al., 2000; Taylor et al., 2001)

Table 2. Methods to detect SNPs

Assay	Templates	Principle	Detection	Comments	References
DNA ligase LCR ligase chain reaction; OLA oligo liga- tion assay; DOLA dye la- beled OLA	PCR frag- ments	Two contiguous oligonu- cleotides are hybridized to target DNA fragments and ligated by DNA ligase only in case of complete match	ELISA, fluo- rescence	Fast, reliable, microtiter- plate format. DOLA see also figure 2C	(Landegren et al., 1988; Nickerson et al., 1990; Tobe et al., 1996; Chen et al., 1998; Chen &
					Kwok, 1999b)
DNA ligase / DNA polymerase RCR rolling circle replication; RCA rolling cir- cle assay; HRCA hyper- branched RCA	Genomic DNA	Allele specific open circle oligonucleotides are hy- bridized to genomic DNA and circularized by ligase. Generic primers are used for isothermal signal am- plification by a DNA polymerase	Fluorescence	Fast, reliable, microtiter- plate format. RCA see also figure 2F	(Banér et al., 1998; Lizardi et al., 1998; Clark & Pickering, 2000)
DNA ligase / type two endonucle- ase/ /DNA poly- merase AFLP amplified fragment length polymorphism	Genomic DNA	Double digestion of ge- nomic DNA with a rare and a frequent cutter re- striction enzyme. Ligation of adaptors and amplifica- tion with adaptor specific primers	Gel electro- phoresis, fluorescence detection	Fast, anony- mous markers	(Myburg et al., 2001)
Chemical cleava	ge				
CCM chemical cleavage of mismatch	Labeled PCR fragments	OsO_4 (KmnO ₄) modifies mismatched Ts and Cs and hydroxylamine mis- matched Cs. Incubation with piperidine cleaves the DNA at the modified mismatched base	Gel electro- phoresis, fluorescence detection	Fast, reliable, toxic chemi- cals are re- quired	(Cotton et al., 1988; Verpy et al., 1994; Roberts et al., 1997)
Electrophoretic	discriminatio	n of allelic variants			
SSCP single-strand con- formation polymorphism; DOVAM-S detec- tion of virtually all mutation- SSCP	PCR frag- ments	Radiolabeled PCR frag- ments are denatured and electrophoresed through a non denaturating poly- acrylamide gel	PAGE (poly- acryl gel elelectropho- resis)	Cheap, sim- ple, need of optimization (<i>e.g.</i> , up to 5 different gel conditions). Homozygous samples are indistinguis- hable	(Orita et al., 1989; Buzin et al., 2000)
CSGE Conformation sensitive gel elec- trophoresis; hete- roduplex analysis	PCR frag- ments	Migration of heteroduplex DNA fragments in a semi denaturating polyacryla- mide gels is different than of homoduplex fragments	PAGE	Cheap, sim- ple, sensitive, need of opti- mization, homozygous samples are indistinguish- able	(Leung et al., 2001)

Table 2. Methods to detect SNPs cont.

Assay	Templates	Principle	Detection	Comments	References	
DGGE	PCR frag-	Migration of double		Difficult to	(Fischer &	
denaturing gradi-	ments	stranded DNA is depend-		perform,	Lerman,	
ent gel electro-		ent on its conformation.		fragments of	1983)	
phoresis		Melting domains within		up to 600 bp		
		the DNA fragment occur		can be		
		depending on temperature		screened for		
		and the denaturant con-		multiple		
		centration during gel elec-		SNPs		
		trophoresis				
Other physical methods						
Melting curves	(Tagged)	Gel free PASA or RFLP-	Temperature	Simple, a	(Germer &	
McSNP melting	PCR frag-	PCR using dsDNA spe-	and confor-	temperature-	Higuchi,	
curve analysis of	ments	cific dyes (eg. SYBR	mation de-	controlled	1999; Akey et	
SNPs		Green I, SYBR Gold)	pendent on	fluorometer is	al., 2001)	
			fluorescence	required		
Solid-phase det	ermination					
Including high density oligonucleotide arrays for hybridization analysis or primer extension analysis (see						
above)						
In silico methods						
such as high-throug	ghput analysis o	of expressed sequence data				

Table 2. Methods to detect SNPs cont.

2.3.2. FRET based assays

Although SNPs can be analyzed by traditional methods (Table 2), the use of fluorescencebased detection systems is becoming increasingly popular, as one can develop "walk away" assays that are simple and fast. Although such assays do not approach the degree of parallelism or multiplexing offered by high density DNA arrays, where thousands of SNPs per reaction can be analyzed simultaneously, fluorescence-based detection systems are much more flexible. New SNPs can be diagnosed without having to remanufacture the DNA chip, because the specific probes are easy to synthesize. In most cases, all reagents are initially combined and changes in fluorescence are monitored in real time during the course of the assay without having to process the samples further and without the need of expensive imaging equipment.

Fluorescence resonance energy transfer (FRET) is observed when two fluorescent dyes are in close proximity and the emission spectrum of the donor dye overlaps with the excitation spectrum of the acceptor dye. When the donor is excited, the donor's specific emission decreases (quenching), with a concomitant increase in the acceptor's specific emission.

TaqMan assay

The TaqMan assay (Livak et al., 1995; Livak et al., 1995) is based on FRET between a fluorescent donor (D) and a fluorescent acceptor (A), which are at the 5'- and the 3'-ends of an oligonucleotide. The fluorescence of the 5'-donor fluorophore is quenched by the 3'-acceptor when the allele discriminating TaqMan probe molecules hybridize to their targets. If there is a perfect match between the probe and the target, the probe degrades via the 5'-exonuclease activity of the Taq DNA polymerase. This releases the 5'-reporter fluorophore from the 3'-acceptor, thereby decreasing FRET and restoring fluorescence during the extension step of the PCR reaction (Figure 2A).

Molecular beacon assay

Molecular beacons are hairpin-shaped oligonucleotide probes that report the presence of specific nucleic acids (*e.g.*, alleles) in homogenous solutions (Tyagi & Kramer, 1996). The loop portion of the molecule is a probe sequence that is complementary to a target sequence in the nucleic acid to be detected, and the stem is formed by the annealing of complementary arm sequences that are on the ends of the probe sequence (Figure 2B). The probe carries 5'fluorescent reporter molecules (D) and 3'-quencher molecules (Q). In unhybridized probe molecules, the stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Since the quencher moiety is a non-fluorescent chromophore and emits the energy that it receives from the fluorophore as heat, the probe is unable to fluoresce. When molecular beacons are allowed to hybridize to their targets (*e.g.*, during the annealing step of the PCR), they undergo a conformational change that restores the fluorescence of the internally quenched fluorophore, which can be recorded (Figure 2B).

In SNP analysis, one mismatched nucleotide is sufficient to discriminate between the alleles. As molecular beacons can possess a wide variety of differently colored fluorophores, multiple targets can be distinguished within the same solution (Tyagi et al., 1998; Marras et al., 1999).

Molecular beacons are as efficient to detect $C \Leftrightarrow T$ transitions as linear TaqMan probes and tend to discriminate better between homozygous and heterozygous genotypes of a $C \Leftrightarrow G$ transversion (Täpp et al., 2000). The higher specificity of molecular beacons may be a consequence of their hairpin structure, which does not base pair with the target sequence and destabilizes the hybridization of mismatches (Tyagi et al., 1998).



Figure 2. Diagnosis of SNPs by FRET based assays: A: Taq-Man assay, B: molecular beacon assay, C: dye labeled oligoligase assay (DOLA), D: template directed dye-terminator incorporation (TDI), E: Serial invasive signal amplification reaction (SISAR, invader assay), F: Rolling circle assay (RCA). A, acceptor. D, donor. Q, quencher. Taq, Taq-DNA polymerase. Lig, DNA ligase. Pol, DNA polymerase. FEN, flap endonuclease enzyme. Details of the assays are given in the text.

"Weavelength shifting" molecular beacons contain three labels: a quencher moiety (3'-arm), a harvester fluorophore (5'-arm) and an emitter fluorophore at the distal part of its 5'-arm. The harvester fluorophore is excited by a monochromatic light source, and in the absence of target sequences, this energy is transferred to the quencher and lost as heat. In the presence of target, however, molecular beacons undergo conformational change which separates the 5'- and the 3'-arms. The energy absorbed by the harvester is transferred to the emitter by FRET, resulting in fluorescent light of its own characteristic color. These molecular beacons are highly sensitive and improve multiplex genetic analysis (Tyagi et al., 2000).

Dye labeled oligoligase assay (DOLA)

A ligase-based assay to detect polymorphisms was originally described by Landegren et al. (1988). This approach combines the ability of oligonucleotides to hybridize to the sequence of interest and the potential of DNA ligase to distinguish mismatched nucleotides in a DNA double stranded helix (Figure 2C). A mismatch at the junction between two contiguous oligonucleotides (one labeled with a fluorescence donor (D) the other with an acceptor (A)) prevents DNA ligase from joining the two fragments together, thereby preventing the transfer of energy between D and A (Figure 2C, left). A perfect match allows DNA ligase to link the oligonucleotides, resulting in FRET (Chen et al., 1998). The ability to detect intramolecular FRET against the background of unquenched donor emission provides a detection system that requires no separation or purification of the product.

Template directed dye-terminator incorporation (TDI)

In the TDI assay, the target DNA is amplified by PCR. Excess primers are degraded by exonuclease I and alkaline phosphatase. After these enzymes have been heat inactivated, the TDI reaction mix containing the donor dye-labeled TDI primer, allele-specific acceptor dyelabeled nucleotide terminators (ddNTPs), and a heat stable DNA polymerase are added (Figure 2D). Fluorescence intensities of donor and acceptor dyes are monitored during thermal cycling of the primer extension reaction (Chen & Kwok, 1999b). This assay requires almost no optimization, but three separate steps that include adding reagents twice after the initial reaction setup.

Invader assay

Flap endonucleases (FENs) isolated from *Arachae* are structure specific 5'-endonucleases and have been shown to recognize and cleave a structure formed when two overlapping oligonu-

cleotides hybridize to a target DNA strand (Lyamichev et al., 1999). The downstream oligonucleotide probe is cleaved and the site of cleavage is determined by the extent to which the upstream oligonucleotide (the invader oligo) overlaps the 5'-region of the downstream oligonucleotide. Cleavage by these enzymes is dependent on this invaded structure and is sensitive to single-base mismatches, if positioned immediately upstream of the cleavage site (Figure 2E).

Using thermostable FENs, the reactions are performed near the melting temperature of the signal probe. Under these conditions, individual signal probes occupy their complementary DNA target only briefly, thereby allowing frequent probe exchange. When a probe hybridizes and is cleaved, the molar excess of signal probe ensures that it will be replaced by an uncut copy, and permitting amplification without temperature cycling. The amount of cleaved product, however is still too small for direct detection. Only the development of the serial invasive signal amplification reaction (SISAR) enables the detection of 20 ng quantities of genomic DNA without the need for preamplification of the target DNA (Hall et al., 2000; Ly-amichev et al., 2000).

In this assay (Figure 2E), the probe contains two regions, an analyte specific region that forms a duplex with the target and a non-complementary 5'-arm region (flap). The flap is not required for enzyme activity, but serves as a reporter molecule precursor. Cleavage of the probe at the diagnostic site occurs only when the probe and the invader oligonucleotide overlap. The cleaved 5'-arm of the target-specific primary reaction serves as an invader oligo and is used to drive a secondary generic invasive reaction. To simplify detection, the secondary probe is labeled with a FRET dye pair (FRET cassette), in which a donor dye is quenched by an acceptor dye. After cleavage, the dyes are separated and the increased fluorescence of the donor dye is directly detected with a conventional fluorescence plate reader.

In the case of mismatch, the probe and the invader oligo do not overlap and the 5'-arm is only very rarely cleaved at the diagnostic site. Occasionally, and depending on the FENs used in the assay, the 5'-arm may be cleaved at position +1, resulting in a bigger reporter, which does not lead to a functional invader oligo in the secondary signal amplification reaction (Lyamichev et al., 2000). Including two FRET cassettes with two different dyes, both alleles may be simultaneously examined in the same reaction tube (Lyamichev, 2001, personal communication).

Rolling circle amplification (RCA)

Circularizing oligonucleotide probes (padlock probes) and amplification by the rolling circle approach is a promising alternative to PCR based genetic analysis systems. The 5'- and 3'end regions of these linear oligonucleotides (open circle probe, OCP) are designed to base pair next to each other on a target strand. If properly hybridized, the ends can be joined by enzymatic ligation, thereby converting the probes to circularly closed molecules that are topologically linked to the target molecules (Figure 2F). A mismatch between the OCP and the SNP prevents ligation and circularization. In this manner, single base selectivity is achieved not only by the specific hybridization of the OCP ends to the target sequence adjacent to the SNP, but also by the highly discriminative nick closure activity of the thermostable DNA ligase towards a perfectly matched substrate (Nilsson et al., 1994; Lizardi et al., 1998). For high throughput SNP detection, two different OCP backbones are used. Signal amplification is performed using two non-crossreacting primers for each allele specific probe (directed to the two backbone regions of the circularized oligonucleotides). This reaction is extended by an exonuclease-deficient DNA polymerase. The extending probe eventually displaces itself and its 5'-end once every revolution of the circularized probe is complemented. Continued polymerization and displacement generate single-stranded complementary concatamers. The second primer is the same sense as the probe. It binds to each tandem repeat of the first strand product at sites corresponding to a second region of the backbone, where it primes DNA synthesis at each site. As these multiple binding events elongate, strand displacing activity of the DNA polymerase causes nascent strands to be displaced and a multitude of new recognition sites for the first primer is exposed in a process called branching. The second primers of each backbone are labeled with two different 5'-hairpin loop-containing FRET dyes, enabling discrete detection of both alleles of a SNP within a single reaction (Clark & Pickering, 2000; Faruqi et al., 2001).

2.4. Applications

Access to a large patient population brings commensurate benefits in the ability to map a disease locus as precisely as possible. Clearly, animals with gross, cytogenetically detectable chromosome abnormalities can be instrumental in pinpointing a specific chromosome region. However, these models are rare except for laboratory animals (Yang et al., 1992). In addition, mutagenesis programs in farm animals with natural occurring mutations or chemically-induced (particularly N-ethyl-N-nitrosourea) mutations or radiation-induced mutations (Fries & Stranzinger, 1982) have a low acceptance in our current society. Linkage analysis is becoming very popular and can be performed on families with multiple affected cases in several generations.

Alternatively, in other organisms such as the mouse or the rat, the availability of homologous traits or mutations may persuade investigators that these model systems may be more accessible for isolating disease-causing genes. In any event, the availability of an animal model is clearly a bonus for investigators following up the function of the disease gene and the biology of the pathways involved.

2.4.1. Identification of monogenetic traits

It has been estimated that in mammals there are about 1 to 5 gene mutations per individual and per generation. New mutations largely have a negative influence on "output / performance / efficiency characteristics", if they have an influence at all. However, mutations can also lead to useful gene variations and keep evolution running.

To identify gene defects and to prevent them from spreading in the population is an important concern of responsible breeders. In the case of a so-called recessive inheritance the heterozygous animals with a normal and a defect copy of the hereditary disease are not affected and thus, make selection on phenotyp against this disease insufficient.

An efficient method to find an (un)desired allele in a family with the help of genetic methods is described below:

- 1. Unambiguous identification of the phenotype which has a hereditary component (*e.g.*, segregation in a selected family).
- 2. Have comparable phenotypes already been described in another species and, if so, are the corresponding functional candidate genes known? If the gene is unknown, proceed with point 3 below. If a candidate gene is known, proceed with point 8 below.

- 3. Formation of an experimental family by mating of informative parents. Alternatively, unrelated affected individuals are genotyped and allelic frequencies are compared to unaffected animals (association studies).
- 4. Typing of all animals with 3 to 5 informative markers per chromosome (depending on the size of the chromosome).
- 5. Linkage / association studies: On which chromosome is the unknown gene located?
- 6. Saturation of the chromosomal region with more informative markers: How close is a certain marker linked to phenotype? Define the haplotypes of affected animals.
- 7. Searching for positional candidate genes with the help of comparative gene maps. Are genes of other species in the homologous region described? Do these have altered gene products that could be causally connected to the observed phenomenon?
- 8. Cloning and sequencing of candidate genes (genomic DNA, cDNA) and identifying of sequence differences which lead to new intragenous markers (*e.g.*, SNPs).
- 9. Demonstrating that intragenous markers are closely linked to the phenotype.
- 10. Showing in independent experiments that the genetic differences found are causally responsible for the observed phenotype.
- 11. Development of a genetic test.

For monogenous inherited characteristics, there are good chances of identifying at least one suitable marker for the trait which can be used to type the animals independently of age, sex and environmental influences. However, it is not easy to prove that the found (genetic) differences are really causal for the trait. At the level of genome analysis, polygenous characteristics show a significant linkage disequilibrium between a phenotype and several marker alleles, which can be localized on different chromosomes. For a closer analysis, subfamilies that only differ in one marker locus must be examined.

For a number of inherited traits, especially those that are genetically complex, an appropriate pedigree that includes multiple informative offspring simply might not exist. The breeding of informative pedigrees *de novo* is a very time consuming and expensive approach. Alternatively, "identity by descent mapping" or "homozygosity mapping" strategies have been proposed for situations in which ideal families are lacking (Charlier et al., 1996). All of these approaches seek to identify shared marker haplotypes among individuals expressing the trait of interest. This is especially advantageous in mapping bovine traits, where the number of offspring per female is low, the generation time is relatively long, and the effective population size is small, due to the systematic use of artificial insemination.

The same identity by descent status may apply for "between breed" comparisons, where the conserved haplotypes have been obviously formed in a common ancestor, before different breeds were established. The Robertsonian 1/29 translocation, for example, is found in nearly all cattle breeds (Popescu, 1990). The expensive and time consuming cytogenetic examination of chromosome spreads can be partially replaced by haplotyping with chromosome 1 and 29 markers, which are close to the centromere region (Jörg et al., 1999).

In the pig, the same C \rightarrow T transition in the calcium channel gene (*CRC*, *RYR1*) is found in all populations in which porcine malignant hyperthermia (MH) has been described. This mutation has been propagated because of its association with muscularity, better feed conversion and favorable meat to fat ratio. Another example of conserved haplotype across different breeds is the G \rightarrow A transition in the fucosyltransferase 1 gene (*FUT1*), which belongs to the same synteny group as is MH on chromosome 6q. This phenotype is associated with resistance to the colonisation of pathogenic F18 fibriated *E. coli*. Ironically, in populations were pigs susceptible to MH still exist, the favorable FUT1 allele is associated with the MH-causing allele of the CRC gene (Meijerink et al., 1997; Vögeli et al., 1997; Meijerink et al., 2000).

On the other hand, the "double-muscling" trait in several European cattle breeds is based on phenocopies. At least 5 different mutations in the myostatin gene (*MSTN*) have been described that cause a functional knock out and lead to the same phenotype (Grobet et al., 1997; Grobet et al., 1998).

Alternative approaches for the identification of hereditary defects are limited to the analysis of expressed sequences at the protein level (2D-gel electrophoresis, mass spectrometry and amino acid analysis) or at the mRNA level (differential display, gene chips etc., see below). With these techniques, tissues of affected and healthy animals can be investigated simultaneously and differences in the expression levels of individual genes can directly or indirectly be connected to the syndrome. Because of the high parallelism that can be achieved, the use of so-called gene chips has taken on a special importance. In these experiments, the expression levels of thousands of genes can be simultaneously examined in a single experiment. It is important to note that a major limitation of this approach is that specific gene chips for farm animals do not yet exist, and mutations that do not cause a change in the expression rate can not be directly recognized.

A syndrome survey of known hereditary diseases of pet and farm animals shows that in only a few cases the molecular cause is known (Nicholas, 1996; Herzog, 2001). Table 3 shows a selection of monogenetic traits which can be diagnosed in farm animals directly or indirectly by gene or chromosome tests.

Species	Phenotype	Molecular and phenotypic characteris- tics, occurrence	Gene, Chromo- some	Literature
Cattle	α-Glucosi- dase defi- ciency, Pompe's dis- ease	Various deletions in the α -glucosidase gene lead to a frame-shift and an inactive enzyme. Impaired glycogen breakdown. Progressive muscular weakness, death within the first year. Occurrence: Shorthorn, Brahman (Aus- tralia).	GAA Chr. 19	(Dennis et al., 2000; Tammen et al., 2000)
	Bovine leuko- cyte adhesion deficiency	An A \rightarrow G transition in the gene encoding CD18 leads to an Asp \rightarrow Gly amino acid sub- stitution. As a consequence there is a lack of β_2 -integrins on the surface of the leukocytes (heterodimers consisting of one of the three α subunits, CD11a, CD11b, or CD11c, and a β subunit, CD18). There is no phagocytosis and endothelial adhesion of the leukocytes. Oc- currence: Holstein Friesian.	CD18 (ITGB2) Chr. 1	(Threadgill et al., 1991; Shus- ter et al., 1992)
	Citrullinemia	A C \rightarrow T transition in the argininosuccinate synthetase gene leads to a premature stop and an inactive enzyme. The disruption in the urea cycle leads to an accumulation of citrul- line in the blood and urine. Occurrence: Hol- stein Friesian (Australia).	ASS Chr. 11	(Dennis et al., 1989; Threadgill & Womack, 1990)
	Coat color	Deletion in the MSHR gene.	MSHR	(Jörg et al., 1996)
	Double- muscling	Five different mutations in the myostatin gene resulting in an inactive protein, cause muscular hypertrophy and hyperplasia (hind- quarters, buttock, thigh, loin). Occurrence: Belgian Blue Cattle Breed, Charolais, Main Anjou, Gasconne, Parthen- aise, Piedmontese, Asturiana de los Valles, Rubia Gallega, South Devon.	MSTN (GDF8) Chr. 2q14-15	(Charlier et al., 1995; Grobet et al., 1997; Kambadur et al., 1997; McPherron & Lee, 1997; Smith et al., 1997; Grobet et al., 1998; Smith et al., 2000)
	Spinal dys- myelination	Congenital neurological disorder with demye- lination, characterized by congenital recum- bency, opisthotonus and extension of the limbs. Diagnosis: indirect using microsatel- lites BP38 (0% recombination), BMS1953 (3%) and BMS 2569 (4%). Occurrence: Brown Swiss.	 Chr. 11	(Nissen et al., 2001)
	Syndactyly, mulefoot di- sease	Fusion of peripheral parts of the foot bones, phalanx of the 3. and 4. digit of the foot or deficient differentiation of the fetal pha- langes. One or more extremities are affected. Diagnosis (Holstein Friesian): indirect using microsatellites BM848 (0% recombination), TGLA75 (6%).	 Chr. 15	(Charlier et al., 1996)

Table 3. Characterized monogenetic traits in farm animals

Cattle cont. Uridine monophos-phate synthase deficiency, DUMPS A C→T transition in the uridine monophos-phate synthase gene leads to a premature stop and a reduced enzyme activity (catalyzing the chr. final two steps of the de novo pyrimidine synthesis). This causes elevated levels of orotic acid in the milk, the urine and the erythrocytes. Occurrence: Holstein Friesian. Som Weaven dia Programsing degenerative multiplication Programsing degenerative multiplication DDM	PS (Schwenger et al., 1993; Fried 1q34-36 & Rottmann, 1994; Schwen- ger et al., 1994); ME (Georges et al. 1993)	t dl
cont. Uridine mo- nophos-phate synthase defi- ciency, A C→T transition in the uridine monophos- phate synthase gene leads to a premature stop and a reduced enzyme activity (catalyzing the final two steps of the de novo pyrimidine synthesis). This causes elevated levels of orotic acid in the milk, the urine and the erythrocytes. Occurrence: Holstein Friesian. UMI	PS (Schwenger et al., 1993; Fried 1q34-36 & Rottmann, 1994; Schwen- ger et al., 1994); <u>ME</u> (Georges et al. 1993)	t dl
synthase deficiency, and a reduced enzyme activity (catalyzing the Chr. final two steps of the de novo pyrimidine synthesis). This causes elevated levels of orotic acid in the milk, the urine and the erythrocytes. Occurrence: Holstein Friesian. Weaven dia Proprosive desenantive multiple multin multiple multiple multiple multiple multiple multiple multiple	1q34-36 & Rottmann, 1994; Schwen- ger et al., 1994); ME (Georges et al. 1993)	-
Waavan die Drogragive degenerative mysleeneenhele DDM	ME (Georges et al. 1993)	
ease pathy characterized by progressive signs of	1,2,2,	.,
pelvic limb paresis, ataxia and proprioceptive Chr. placing deficits. Linkage to microsatellite TGLA116 (indirect diagnosis of the gene by haplotyping is possible). Occurrence: Brown Swiss, Danish red cattle.	4	
Horse		
Coat colors An 11 bp deletion in the agouti signaling ASIF protein (ASIP) is responsible for the recessive Chr. black (A ^a) phenotype.	P (Rieder et al., 22q16 2001)	
Chestnut, a C \rightarrow T transition in the melano- cortin-1-receptor leads to a 83Ser \rightarrow Phe sub- stitution. (Extension locus)MC1 (MS) Chr.	1R (Marklund et HR) al., 1996) 3p13	
Lethal white foal syndrome (LWFS)A TC→AG mutation of the endothelin-B receptor gene changes isoleucine to lysine. This congenital anomaly is characterized by a white coat color and aganglionosis of the bowels.EDN Chr.	NRB (Metallinos et al., 1998; Yang 17q23-q4 et al., 1998; Godard et al., 2000)	g
Occurrence: result from mating overo x overo paint horses ("frame" overo).		
Hyper- kalaemic peri- odic paralysis (HYPP), $C \rightarrow G$ transversion leads to a phenylalanin to leucin substitution in the 4 th transmembrane domain of the sodium channel of the muscle.HYP HYP Chr."Impressiveity accross the skeletal muscle membrane	PP (Rudolph et al. 1992; Rudolph 11 et al., 1992; Bowling et al., 1996)	h,
syndrome" Affected animals suffer from periodic weak- ness or paralysis accompanied by increased serum potassium concentrations. Inheritance: autosomal dominant. Occurrence: Quarter Horse (Stallion Impressive), Paint, Appaloo- sa.		
Ρία		
Acidic meat, RN mutationThe fully dominant RN^- allele is associated with 70% higher glycogen content of the skeletal muscle. This leads to low pH 24 hours after slaughtering, reduced water hold- ing capacity and reduced yield of cured cooked ham. An A \rightarrow G transition leads to a nonconservative Arg200Gln substitution of the γ subunit of the adenosine monophos- phate-activated protein kinase (AMPK).PRK (RN)	XAG3 (Milan et al.,) 1995; Mariani et al., 1996; 15q23-26 Milan et al., 1996; Robic et al., 1999; Mila et al., 2000)	t

Table 3. Characterized monogenetic traits in farm animals cont.

Table 3.	Characterized	monogenetic traits in farm animals cont		
Species	Phenotype	Molecular and phenotypic characteris-	Gene, Chro-	Literature
Pia			mooonio	
cont.	Coat color	Dominant white. These pigs show no mela- nocytes in their skin, due to a duplication of	KIT	(Johansson Moller et al.,
		at least a part of the proto-oncogene c-kit	Chr. 8p12	1996)
	Congenital progressive ataxia (CPA)	Neuropathic disorder with unknown etiology characterized by spastic gait, incoordination and rapidly progressive ataxia in the hind	- Chr 3	(Kratzsch et al., 1999; Kratzsch et al. 2000)
		limbs. Diagnosis: indirect by microsatellites Sw902 (189 bp-allele, 0% recombination) and Sw1066 (5%). Occurrence: Large White.		or u., 2000)
	Hypercho- lesterolemia	Missense mutation in the the low density lipoprotein receptor gene leads to an amino acid substitution 84Arg→84Cys and hyper- lipidemia.	LDLR Chr. 2 cen.	(Hasler-Rapacz et al., 1998; Grunwald et al., 1999)
	Malignant hyperthermia, stress myopa- thy	A C→T transition in the skeletal muscle ry- anodine receptor (<i>RYR1</i>) gene leads to an alteration in amino acid sequence from an arginine at position 615 to a cysteine. As a consequence the calcium metabolism in the skeletal muscle is disturbed. Stress (transport, exposure to inhalational anaesthetics, most notably halothane) leads to an increased deg- radation of glycogen (lactate production, acidosis) and to a muscular hypermetabolism and contraction, which can cause the death of the animal. Occurrence: various Landrace breeds, Piétrain, Duroc.	RYR1 (CRC) Chr. 6q11-q21	(Vögeli et al., 1984; Vögeli et al., 1988; Fujii et al., 1991; Yang et al., 1992)
	Oedema dis- ease, post- weaning diar- rhoea	A ${}^{307}\text{G} \rightarrow \text{A}$ transition in the <i>Fut1</i> gene leads to an amino acid substitution at position 103 (Ala \rightarrow Thr). As a consequence the fucosyl- transferase 1 is less active and receptors me- diating the binding to the <i>E. coli</i> fimbriae F18, are not expressed in the small intestine (resistant animals). Occurrence: various Lan- drace breeds, Large White, Hampshire, Pié- train, Duroc.	FUT1 Chr. 6q11	(Meijerink et al., 1997; Vö- geli et al., 1997; Meije- rink et al., 2000)
	Vitamin C deficiency	Deletion of exon 8 (77 bp) of the <i>L-gulono-gamma-lactone oxidase</i> gene leads to a frame shift after 236 amino acids followed by 120 altered amino acids. The peptide is truncated and inactive. Homozygote animals suffer from scurvy, if they are not supplemented	GULO Chr. 14	(Hasan et al., 1999; Hasan et al., 2000)

Tab

	uenciency	shift after 236 amino acids followed by 120 altered amino acids. The peptide is truncated and inactive. Homozygote animals suffer from scurvy, if they are not supplemented with 30 mg vitamin C /kg of body weight. Occurrence: Danish Landrace-Yorkshire crossbreed, Duroc.	Chr. 14	al., 2000)
Sheep	Callipyge	Calli = beautiful, pyge = buttocks. Muscular hypertrophy is associated with extensive muscling of the loin and hindquarters. Affec- ted muscles: m. longisimus dorsi, m semiten- dinosus, m. gluteus medius. Mode of inheri- tance: polar overdominace and paternally imprinted. Only heterocygous animals which inherited the <i>CPLG</i> allele from the father express the callipyge phenotype. These ani- mals overexpress <i>DLK1</i> and <i>PEG11</i> (cis, paternally expressed). Expression of <i>GTL2</i> , <i>MEG8</i> (maternally expressed).	CLPG Chr. 18	(Carpenter et al., 1996; Coc- kett et al., 1996; Shay et al., 2001; Char- lier et al., 2001a)

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Species	Phenotype	Molecular and phenotypic characteris- tics, occurrence	Gene, Chro- mosome	Literature
Sheen		,		
cont.	Haemonhilia	Blood clotting factor VIII (F-VIII) coagulopa-	HEMA (F8C)	(Neu-
	A	thy with extensive subcutaneous and intramus-		enschwander &
		cular hematomas especially in the muscle of	Chr. Xq24-q33	Pliska, 1990;
		the hind legs and in <i>m. psoas minor</i> or <i>m. iliop</i> -	1 1	Neuenschwan-
		soas and spontaneous hemarthrosis, mainly in		der et al., 1992;
		the weight bearing joints.		Backfisch et
		Diagnosis: indirect, Msp I RFLP (restriction		al., 1994)
		fragment length polymorphism) in the <i>F</i> -VIII		
		gene is linked to the syndrome (no recombina-		
	<u> </u>	tion events detected).	D N	(D : + 1
	Inverdale	Inverdale ($FecX^{i}$): C \rightarrow T transition leads to a	FecX ¹	(Davis et al.,
	and Hanna-	Val31Asp substitution of the mature BMP15	FecX	1991; Davis et
	type of in-	peptide (identical to growth differentiation	Chr V	al., 1992; Gal-
	creased pro-	factor 9B (GDF9B)), impairing the ability to		2000
	infortility	form dimers.		2000)
	micrunty	Hallia ($FecA$). A \rightarrow 1 transversion leads to a		
		$\operatorname{Fec} X^{\mathrm{I}} / \operatorname{Fec} X^{+}$ and $\operatorname{Fec} X^{\mathrm{H}} / \operatorname{Fec} X^{+}$ show in-		
		creased prolificacy, whereas $Fec X^{I} / Fec X^{I}$		
		$Fec X^{H} / Fec X^{I} / Fec X^{I} / Fec X^{H}$ animals are infer-		
		tile		
		Occurrence: Romney sheep.		
	Booroola-	An $A \rightarrow G$ transition leads to a nonconservative	FecB ^B ,	(Mulsant et al.,
	type of	Gln249Arg amino acid substitution in the bone	(BMPR-IB)	2001; Souza et
	increased	morphogenic protein receptor IB (Mulsant et		al., 2001; Wil-
	prolificacy	al. 2000). This may lead to a partial inactivated	Chr. 6q23-31	son et al.,
		receptor (less responsive to the natural ligands		2001)
		GDF-5 and BMP-4) and advanced differentia-		
		tion of granulosa cells and advanced matura-		
		tion of ovulatory follicles. Occurence: origi-		
		nally described in the Booroola strain of Me-		
	Suiday Lamb	Conceptible chandrady analogie with diamonar	CI C	(Coolcott at al
	Syndrome	tionately long "spider-like" legs curvature of	515	(Cockett et al., 1999)
	(SLS)	the spine deformed ribs and sterna facial de-	Chr. 6	1777)
	(515)	formities lack of body fat and muscular atro-	Ciii: U	
		phy.		
		Diagnosis: a single-strand conformational		
		polymorphism (SSCP) in the fibroblast growth		
		factor receptor 3 is 100% linked to the syn-		
_		drome.		
Diffe-				
rent	Chromo-	Increase / reduction of the number of chromo-		(Braun et al.,
species	some muta-	somes, translocations und chromosome aberra-		1988; Stranzin-
	tions	tions have been described for various farm		ger, 1989; Jörg
		animals. Phenotypically the animals can be		et al., 1999)
		Often a reduced fartility can be found. Co		
		tologic tests (karvotyping, fluorescent in situ		
		hybridization) help to identify affected animals		
		and to exclude them from breeding		

Table 3. Characterized monogenetic traits in farm animals cont.

2.4.2. Parentage control and individual identification

As SNPs are diallelic, their polymorphic information content (PIC, (Botstein et al., 1980)) is quite low, as compared to microsatellites with up to 16 alleles or more in a given population. In verifying the identity and parentage control of livestock, the number of markers that need to be tested is therefore considerably higher. Fries and Durstewitz proposed to select 40 SNPs per individual, with an allele frequency of the minor allele of \geq 20% (Durstewitz et al., 2000; Fries & Durstewitz, 2001). For genotyping purposes, each SNP position is queried for the presence or absence of a particular base, resulting in a binary string such as 10, homozygous for allele 1; 01, homozygous for allele 2; 11, heterozygous and 00 for assay failure. These strings may be used as a digital DNA signature to identify individuals or for parentage control.

The formula of exclusion probability with codominant loci is given by (Weir, 1996),

$$Q_{l} = \sum_{u} p_{u}(1-p_{u})^{2} - \frac{1}{2} \sum_{u} \sum_{v \neq u} p_{u}^{2} p_{v}^{2} (4-3p_{u}-3p_{v})$$

where Q_1 is the exclusion probability at locus 1. The over all probability of exclusion Q is one minus the probability that non of the loci allows exclusion (if Q_1 are independent).

$$Q = 1 - \prod_{l} (1 - Q_l)$$

Under the proposed conditions the exclusion power is 99.9968% and the probability of two individual probes showing the same pattern being identical is 2.66×10^{-12} .

As an international standard for parentage control in cattle, nine microsatellite markers (MS) are required and the combined exclusion probability (CEP) was estimated to be around 99.91 %. In Switzerland, the parentage control in cattle is performed in a single PCR reaction, using 11 to 13 MS markers with an exclusion probability of > 99.97% (Glowatzki, 2001, personal communication).

Traditionally, the parentage control in the pig has been done by blood group typing (*e.g.*, A, S, H, K, G, E), by the determination of polymorphic enzyme systems of the erythrocytes (*e.g.*, PHI, 6PGD, PGM), and with serum proteins (*e.g.*, PO1A, PO2). The advantage of these tests is that they are cheap, they require only minimal technical equipment, and a considerable number of animals can be analyzed by one person in a single day. In Switzerland, the CEP is >90% using this system.
Nechtelberger and colleagues developed a 10-plex and a 5-plex microsatellite-based parentage test (Nechtelberger et al., 2001). In Austria, the CEP for the 10-plex was >99.1% in the Landrace, the Piétrain and the Large White breed. The 5-plex test is only used for special cases. The allele frequencies of the proposed markers for Swiss breeds are under investigation at our institute.

2.4.3. Genetic diversity

The conservation of natural genetic variation is important not only for ethical and aesthetic reasons, but also to ensure that we use the earth's living resources as efficiently as possible to sustain agriculture, forestry, food production and other industries. More information is needed about existing genetic variation and how it can be conserved and accessed. The food and agriculture organization of the united nations (FAO) maintains a virtual electronic library related to animal genetic resources (AnGR). <u>http://dad.fao.org/en/Home.htm</u>

Several methods can be used to estimate genetic variation. For a review, see Weir (1996)

- Heterozygosity: A simple measure of genetic variation in a population is the number of heterozygous individuals observed. This can be reported for a single locus or as an average over several loci.
- Gene diversity: Gene diversity is formed from the sum of squares of allelic frequencies. This is a more appropriate measure of variability for inbred populations, where there are relatively few heterozygotes, but there may be several different types of homozygotes. For randomly mating populations, genetic diversity will be close to the heterozygosity.
- Genetic distances: When haplotypes are determined from several populations, it is possible to estimate measures of population structure. Generally, genetic distance is related to the time that has elapsed since two or more populations diverged from a single ancestral population. The forces that drive populations towards divergence include genetic drift, mutations, and, in farm animals, inbreeding and selection.

In conservation genetic studies, microsatellites, SNPs, sequence variation in mitochondrial DNA, variation in blood type, and enzymatic variants are often used to genetically characterize populations and sub-populations. The greatest challenge in the laboratory is often related to sampling and isolation of high quality genetic material. However, we currently have no objective indicators available to help determine why one particular breed should be preserved, when another population is sacrificed. Thus, it is difficult to make such a decision based on genetic information alone.

2.4.4. QTL and ETL analysis

Quantitative variation of traits is ubiquitous in biology and almost always has an important genetic component. The term quantitative trait loci (QTL) was first introduced by Geldermann (1975) and is also designated as economic trait loci (ETL). Finding and characterizing the molecular basis of QTL should yield new insights into the biology of a large number of phenotypes. Until recently, it was believed that quantitative traits are influenced by a large number of genes, each having only a small effect on the phenotype. After the development of high-density genetic maps and polymorphic markers, it became possible to dissect genetically quantitative traits. Fortunately, the data from the first genome scans revealed a much simpler genetic architecture than had been expected. Genetic mapping of any trait, whether simple or complex, boils down to finding those chromosomal regions that tend to be shared among affected relatives and tend to differ between affected and unaffected individuals. Conceptually, this leads to a three-step protocol:

- scan the entire genome with a dense collection of genetic markers;
- calculate appropriate linkage statistics S(x) at each position x along the genome;
- identify the regions in which the statistics S show a significant deviation from what is expected from random assortment.

However, since the statistic S(x) fluctuates substantially merely by chance across an entire genome scan, Lander and Kruglyak developed guidelines for what constitutes a significant deviation and what standard should be required for declaring linkage (Lander & Kruglyak, 1995).

To date, QTL detection has only been consistently successful in species that can be inbred. Typically, two inbred strains (A, B) are crossed to form F1 progeny, which can either be intercrossed to form an F2 generation or backcrossed to one parental strain. The animals are then genotyped with markers covering all chromosomes. A statistical test is performed to detect an association on the phenotypes with the three possible genotypes in the F2 generation (aa, ab, bb). The power of the method is based on simple genetic principles: only two alleles segregate at each locus, the parental origin of each allele is unambiguously established, and all offspring effectively have the same parent.

The situation in farm animals is more complicated, as this involves outbred populations with only minor inbreeding. To make such analysis tractable, structured populations have been used, such as the large half-sib families found in dairy cattle (grand-daughter design) (Georges et al., 1995). Alternatively, the lack of inbred lines has been partially overcome by using exotic crosses between breeds of commercial interest and unselected breeds or wild animals. For example, in cattle, *bos indicus* and *bos taurus* have been crossed, and in pigs, Chinese breeds or Wild Boar have been crossed with Large White. Linkage analysis may be performed using standard software packages (*e.g.*, CRIMAP, (Green et al., 1990)). For QTL mapping, a least square method was developed for the analysis of crosses between outbred lines (Haley et al., 1994), where the lines may be segregating at marker loci, but can be assumed to be fixed for alternative alleles at the QTL that affect the particular traits being analyzed.

Alternatively, the F2 population may be considered to be a mixture of full- and half-sib families. In this approach, no assumptions are made about the number of QTL alleles and the allelic frequencies within the founder populations (Bidanel et al., 2001).

It has been suggested that threshold models, or the nearly equivalent logistic regression models in the generalized linear model (GLM) framework, can be used to identify and map QTLs for polygenic binary traits, where phenotypes fall into two or more categories (*e.g.*, healthy *versus* diseased, calving ease, single, twin or triplet offspring, etc.). However, in most cases, only minor differences are found between generalized and regression interval mapping approaches (Kadarmideen et al., 2000).

In many cases, the QTLs detected in crosses of divergent lines are not of immediate practical interest, because the exotic breeds often exhibit poor performance with respect to several traits of importance to modern breeding. However, loci that do respond to selection certainly may be identified. On the other hand, resource families have been established in the pig to identify chromosomal regions responsible for breed differences in a comprehensive set of growth, body composition, muscle and meat quality traits (Nezer et al., 1999; Malek et al., 2001a; Malek et al., 2001b). Table 4 summarizes the QTL studies that have been performed in pig over the past few years. QTLs with $p \le 0.05$ (genome wide), the corresponding map positions, and the percentage of phenotypic variance in the F2 population, which may be explained by the segregating QTL are indicated.

T_1-1-1		OTI	aturdiaa	:	
I ad	le 4.	VIL	studies	m	pig

	Body composition and quality traits			
Chromo- some	Study- Design	Position, Effect, Phenotypic Variance	Literature	
SSC1	BC1 ME x WC	138 cM; average backfat 136 cM; last-lumbar backfat 150 cM; last-rib backfat	(Rohrer & Keele 1998a)	
	F2, ME x LW	175 cM; back fat thickness at 13,17,22 weeks and back fat thickness at 40, 60 kg live weight; PV=4-6%	(Bidanel et al., 2001)	
	F2, BE x LW	29 cM; 10 th rib back fat thickness	(Malek et al., 2001a)	
	F2, BE x LW	48 cM; marbeling	(Malek et al., 2001b)	
SSC2	F2, WB x LW	7 cM; bone/lean meat in back	(Andersson-Eklund et al., 1998)	
	F2, ME x Dutch pig	36cM; back fat thickness, paternal imprinted	(de Koning et al., 2000)	
	F2, ME x Dutch pig	26cM, 56cM; backfat thicknes (dependent on the statistical model)	(Rattink et al., 2000)	
	F2, LWxPI	0-1 cM; IGF II paternal imprinted, % lean cuts, % ham, % loin, back fat thickness, PV=50%	(Nezer et al., 1999)	
SSC3	F2, ME x Dutch pig	41 cM; meat color (color-A)	(de Koning et al., 2001)	
SSC4	F2, WB x LW	64 (m), 68 (f) cM (39-89cM); back fat thickness	(Andersson et al., 1994; Knott et al., 1998)	
	F2, WB x LW	62 (m), 64 (f) cM; % abdominal fat, PV=17%	(Andersson et al., 1994; Knott et al., 1998)	
	F2, WB x LW	88 cM; bone/lean meat in ham	(Andersson-Eklund et al., 1998)	
	F2, WB x LW	49 cM; bone/lean meat in back	(Andersson-Eklund et al., 1998)	
	F2, BC2 ¹⁾	60 cM; abdominal fat, PV=15%, back fat thick- ness, PV=7%	(Marklund et al., 1999)	
	F2; IB x LR^{3}	80 cM; back fat weight, PV=11% 81 cM; linoleic acid content in subcutanous fat, PV=25%	(Perez-Enciso et al., 2000)	
	F2, ME x	83 cM; back fat thickness, $PV=1/\%$ 95-98 cM; intramuscular fat (indication of im-	(Rattink et al., 2000)	
	Loint analysis ²⁾	86 cM: back fat thickness	(Walling et al. 2000)	
	F2, ME x LW	62 cM; back fat thickness at 40 kg live weight 72 cM; back fat thickness at 60 kg live weight; PV=3-4%	(Bidanel et al., 2001)	
	F2, ME x Dutch pig	71 cM; meat color (color-B)	(de Koning et al., 2001)	
	F1: DU x LR F2: F1 x (LW x LR)	46 cM; subacid smell 65 cM; intensity taste 84 cM subacid smell 94 cM; fatty acid C17:0 97 cM; back fat thickness	(Grindflek et al., 2001)	
SSC5		>, own in anomioso		
5500	F2, ME x LW	41 cM; back fat thickness at 17 weeks 37 cM; back fat thickness at 40 kg live weight 42 cM; back fat thickness at 60 kg live weight; PV=2-5%	(Bidanel et al., 2001)	
	F2, BE x LW	113 cM; last rib back fat thickness	(Malek et al., 2001a)	

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Table 4 ()		studies	1n	$n_{1\sigma}$	cont
	11	studios	111	pis	cont.

		Body composition and quality traits			
Chromo- Study- some Design		Position, Effect, Phenotypic Variance	Literature		
SSC5	F2, BE x LW	113 cM; Hormel loin pH	(Malek et al., 2001b)		
SSC6	<i>.</i>	· · ·			
	I:F2, WB x PI	(<i>RYR1</i>), carcass composition up to 19 traits, PV< 7% for (I) and PV< 18% for (II), meat	(Geldermann et al., 1996)		
	II:F2, ME x PI	quality (up to 10 traits), $PV= 28-57\%$ (1) and $PV=3-38\%$ (II)			
	F2, ME x Dutch nig	23 cM; intramuscular fat content (maternal im- printed)	(de Koning et al., 2000)		
	Duton pig	117cM [·] paternal imprinted			
	F2, IB x LR ³⁾	97 cM; intramuscular fat, PV=16% 98 cM; back fat thickness, PV=26%	(Ovilo et al., 2000b)		
	F2, ME x LW	60-63 cM; back fat thickness at 13,17 weeks; back fat thickness at 40 kg live weight	(Bidanel et al., 2001)		
	F1: DU x LR	63 cM; meat %	(Grindflek et al., 2001)		
	F2: F1 x (LW	74-79 cM; intramuscular fat			
0007	x LK)	92 cM; intensity smell			
SSC7	F2. ME or MI	24 cM ² back fat thickness last rib	(Wang et al 1998a)		
	x American	27 cM; average back fat thickness	(() ung et un, 1990u)		
	breed (DU,	58 cM; back fat thickness 10 th rib			
	HA, LR,)	(pooled data)			
	BC1	40 cM; last-lumbar backfat	(Rohrer & Keele 1998a)		
	ME x WC	58 cM; average backfat	``````````````````````````````````````		
		62 cM; last-rib backfat			
	F2, ME x	73-75 cM; backfat thickness	(de Koning et al., 1999)		
	Dutch pig				
	F2, ME x Dutch nig	55-57 cM; back fat thickness, maternal im-	(Rattink et al., 2000; de Koning et al., 2000)		
	F2 ME x LW	57-62 cM ² back fat thickness 13 17 22 weeks	(Bidanel et al 2001)		
	12, 111 A D W	and back fat thickness at 40, 60 kg live weight;PV=5-14%	(Brailer et al., 2001)		
	F2, BE x LW	58 cM; average back fat thickness 72 cM; lumbar back fat thickness	(Malek et al., 2001a)		
	F1: DU x LR	40 cM; intensity smell-fat	(Grindflek et al., 2001)		
	F2: F1 x (LW				
~~~~~	x LR)				
SSC8	F2, WB x LW	10 cM; bone/lean meat in ham	(Andersson-Eklund et al., 1998)		
	F2, ME x LW	33 cM; back fat thickness at 40kg live weight	(Bidanel et al., 2001)		
SSC12					
	F2, BE x LW	73 cM; color score	(Malek et al., 2001b)		
SSC13					
	F2, ME x Dutch pig	55 cM; meat color (color-L)	(de Koning et al., 2001)		
SSC15		45 aM: lab lain pH	(Malak at al. 2001b)		
	12, DE ALW	65 cM: glycogen content	(whatek et al., 20010)		
		72 cM: Hormel lab nH			
		76 cM: Hormel loin pH			
SSC17		· · · · · · · · · · · · · · · · · · ·			
55017	F2, BE x LW	82 cM; color score, lab loin Hunter, lab loin Minolta	(Malek et al., 2001b)		

# . . . .

	Body composition and quality traits				
Chromo- some	Study- Design	Position, Effect, Phenotypic Variance	Literature		
SSCX	BC1 ME x WC	59 cM; last-lumbar backfat 61 cM; average backfat 63 last-rib backfat 68 cM; tenth-rib backfat	(Rohrer & Keele 1998a)		
	F2, ME x LW	73-74 cM; back fat thickness 13,17,22 weeks and back fat thickness at 40, 60 kg live weight (only in males, except at 22 weeks); PV8-40%	(Bidanel et al., 2001)		
		Fertility			
Chromo- some	Study- Design	Position, Effect, Phenotypic Variance	Literature		
SSC8					
	F2, Line I x Line C ⁴⁾	105 cM ("Fec ^B in sheep"); ovulation rate and embryonic survival, PV=11%	(Rathje et al., 1997)		
	F4, ME, $WC^{5}$	5 cM; ovulation rate (suggestive QTL at 71 cM for uterine capacity and at 122 cM for weight of ovary)	(Rohrer et al., 1999)		
	F2, ME x LW	99 cM; corpora lutea (ovulation rate)	(Wilkie et al., 1999; Braun- schweig et al., 2001)		
		Growth			
Chromo- some	Study- Design	Position, Effect, Phenotypic Variance	Literature		
SSC1	BC1 ME x WC	134 cM; loin eye area 136 cM; trimmed whole sale product / live weight ⁶⁾	(Rohrer & Keele, 1998b)		
	F2, ME x LW	209-214 cM; average daily gain from weaning to 56 kg, $PV=25\%$ , from birth to 56 kg, $PV=18\%$ , and from 35-56 kg, $PV=15\%$	(Paszek et al., 1999)		
	F2, ME x LW	175 cM; average daily gain 0-10 weeks, weight at 10 and 13 weeks; PV< 3%	(Bidanel et al., 2001)		
	F2, BE x LW	29 cM; loin area	(Malek et al., 2001a)		
SSC2	F2, WB x LW	0-1cM; (IGF II) longissimus muscle area and	(Jeon et al., 1999)		
	F2, LWxPI	0-1 cM (IGF II), % lean cuts, % ham, % loin, paternal imprinted, PV= 50%	(Nezer et al., 1999)		
SSC3					
	F2, WB x LW	79 cM; longissimus muscle area	(Andersson-Eklund et al., 1998)		
SSC4	F2, WB x LW	86 (m), 94 (f) cM; growth rate birth to 70 kg, PV=12%	(Andersson et al., 1994; Knott et al., 1998)		
	F2, WB x LW	66 (m), 76 (f) cM; length of small intestine	(Knott et al., 1998)		
	F2, ME x LW	33 cM; birth weight, PV=16%	(Paszek et al., 1999)		
	F2, ME x LW	65-/0 cM; average daily gain 3-10 weeks and 10-22 weeks; weight at 10,13,17,22 weeks; PV=9%	(Bidanel et al., 2001)		

# Table 4. QTL studies in pig cont.

Growth				
Chromo- some	Study- Design	Position, Effect, Phenotypic Variance	Literature	
SSC4	F2, BE x LW	97 cM; average daily gain 123 cM; carcass weight	(Malek et al., 2001a)	
	F2; IB x $LR^{3}$	83 cM; longissimus muscle area	(Perez-Enciso et al., 2000)	
	Joint analysis ²⁾	81 cM growth rate birth to slaughter 85 cM; birth weight	(Walling et al., 2000)	
SSC6				
	F2, ME x PI F2, IB x LR ³⁾	( <i>RYR1</i> ) fattening parameters, $PV=4-6\%$ 113 cM; eye muscle area, $PV=14\%$	(Geldermann et al., 1996) (Ovilo et al., 2000b)	
SSC7	·			
	BC1 ME x WC	60 cM; hot carcass weight 55 cM; carcass lenght	(Rohrer & Keele, 1998b)	
	F2, ME x LW	65-69 cM; average daily gain 3-10 weeks and 10-22 weeks; weight at 10,13,17,22 weeks; PV=11%	(Bidanel et al., 2001)	
SSC8	F2, WB x LW	9 cM; carcass length	(Andersson-Eklund et al., 1998)	
SSC10				
	F2, WB x LW	34 (m), 74 (f) cM; growth rate 30kg to 70kg	(Knott et al., 1998)	
SSC13				
	F2, WB x LW F2, ME x LW	132 (m), 104(f) cM; growth rate birth to 30 kg 6 cM; weight at 22 weeks	(Knott et al., 1998) (Bidanel et al., 2001)	
SSCX	BC1 ME x WC	50 cM; trimmed whole sale product / carcass weight ⁶⁾	(Rohrer & Keele, 1998b)	

#### Table 4. QTL studies in pig cont.

Table 4 legend:

1) BC1: F2(WBxLW)xLW. BC2: BC1x(LWxLR)

2) joint analysis from six different countries. All populations are based on F2 crosses between genetically divergent breeds (~3000 F2 animals)

3) F1 also called IBMAP cross

4) Line I had undergone mass selection 10 generations for increased ovulation rate and embryonal survival. Line C had undergone 10 generations for random selection
5) F1: reziprocal matings (MexWC). F2(ME-BC): F1xME; F2(WC-BC): F1xWC; F3: F2(ME-BC)xF2(WC-BC). F4: F3xF3
6) trimmed ham, loin, picnic and Boston butt

*BC: Back Cross. cM: centi Morgan. (m) male, (f) female. PV: % of phenotypic variance explained by QTL. BE: Berkshire. DU: Duroc. HA: Hampshire. IB: Iberian Boar. LR: Landrace. LW: Large White (also called Yorkshire in some countries). ME: Chinese Meishan. MI: Chinese Minzhu. PI: Piétrain. WB: Wild Boar. WC: White Composite Swine.* 

It is important to note that QTL studies have potential pitfalls, which have been previously addressed (Lander & Kruglyak, 1995; Flint & Mott, 2001). Avoiding false positive and false negative QTLs seems to be the keystone issue. This has led researchers in this field to ask for confirmation of reported QTLs, preferably by independent studies. Some of the potential pitfalls in QTL studies include:

- QTL detection experiments do not necessarily map a single gene, but rather map a genetic effect in a chromosomal region that might consist of many linked genes. A single, large-effect locus might therefore contain several small-effect genes or genes that have opposing effects on a phenotype and the linkage may break up in subsequent generations.
- QTL detection experiments do not detect epistatic interaction between loci
- the original observation may be a type I error
- a particular effect may be overestimated (seriously inflated) when the power of QTL detection is low
- segregation at the QTL in the recipient population may yield some back-crossed animals that are homozygous at the QTL or make the phenotypic difference between QTL alleles too small
- an overly cautious approach risks missing true hints of linkage
- a QTL may be imprinted and therefore may not be detected by assuming Mendelian inheritance
- accuracy of genotyping

The last two points are worth some additional commentary. In general, imprinting is regarded as a rare phenomenon and is consequently ignored in most QTL studies. The phenomenon of genomic imprinting, as a form of epigenic gene regulation, has been shown to influence several subchromosomal areas in mammals (in the mouse see

http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html).

Parental genomes undergo modifications during gametogenesis, resulting in parent-of-originspecific expression of some genes in the offspring. Thus, although offspring inherit two alleles, one from the father and one from the mother, only a single allele is effectively expressed: either the paternal or the maternal variant of the gene. In farm animals, only the callipyge gene in the sheep (Cockett et al., 1996) and the IGF II gene in the pig have been identified as being imprinted (Jeon et al., 1999; Nezer et al., 1999). More recently, in a QTL study at Wageningen University, four of five QTLs have been predicted to be imprinted, by using a statistical model that separates the expression of paternally and maternally inherited alleles (de Koning et al., 2000; Rattink et al., 2000). By including "imprinting" in the model, the information content could be increased on all chromosomes and the confidence interval of the QTL could be narrowed. The QTL on SSC6 for intramuscular fat content was found only in the imprinting model. Imprinting may only be detected when the parental origin of the marker alleles can be derived for the offspring. This requirement excludes studies based on F2 crosses or a single back cross between inbred lines, such as those that are commonly used in mice or rats. However, a systematic screen for imprinted regions in farm animals, based on the comparative map of the mouse and human genomes, may help to improve the identification of QTL regions and to avoid reporting of false negatives. The occurrence of imprinting has important practical implications for the pig breeding industry. The practice of breeding individual males with many females may, in fact, favor the selection of alleles at paternally expressed QTLs.

As genotyping adds substantially to the cost of a QTL study, there have been various suggestions as how to limit the number of typings. In the "selective genotyping" approach, only individuals from the high and low phenotypic tails of the entire sample population are genotyped (*e.g.*, < 25%) (Darvasi & Soller, 1992; Darvasi & Soller, 1994; Lipkin et al., 1998; Dekkers, 2000; Mosig et al., 2001). In addition DNA pooling is performed (*e.g.*, 50 animals per pool) to reduce the number of individuals that need to be genotyped. This approach may be economically beneficial, but the cost savings comes with the risk of losing accuracy, particularly when using microsatellites. A technical problem may arise when different genotyping systems are involved. Considerable differences in allelic size may occur, depending on whether genotyping is performed using radioactive nucleotides, flat bed gel electrophoresis, fluorescence-based vertical gel electrophoresis or capillary gel electrophoresis (Mansfield et al., 1996; Haberl & Tautz, 1999; Delmotte et al., 2001).

In summary, many of the QTLs for body composition, quality traits, fertility and growth could be verified by different independent studies; however this has often occurred at markedly different positions. The high expectations that arose 7 years ago, when the first QTL study was published in farm animals, have not been fulfilled. So far, only few, if any markers have been published from QTL studies, which may be used in marker assisted selection programs. Also, the candidate gene approach, which uses QTL location and comparative map information, has been of limited success, as the role of the candidate genes in different study designs either gave contradicting results or their effect was much smaller than expected (Gerbens et al., 2000; Linville et al., 2001). These findings show that genetic information alone is not sufficient to map complex traits or genes within a QTL region with suitable precision. We may have to learn more about the physiology and the molecular aspects that influence the trait of interest (*e.g.*, gene expression, biochemical pathways, protein interactions). In this respect, mapped ESTs will help to annotate genes within chromosomal regions, where a QTL has been previously found.

### 2.4.5. Outlook

In the immediate future, structural genomics in farm animals will be restricted to the development of even better genome maps, including linkage maps, radiation hybrid maps, contig maps and transcript maps. As the human project could be finished much earlier than predicted, there are many sequencing facilities with free capacity. Within the next 5 years, the chicken genome will most likely be sequenced first, as its size is estimated to be only a third of that of the mammalian genome and it represents a distinctive evolutionary time point. The genomes of cattle, pig and sheep will be characterized soon afterwards. Once we have this information at our disposition, the next challenge will be to identify single genes within QTL regions and dissect their contribution to the traits of interest.

# 3. Functional genomics in farm animals

Unfortunately, the billions of bases of DNA sequence do not tell us what all the genes do, how cells work, how organisms form, what is going wrong in disease states, or how an organism ages. This is where functional genomics comes into play. The overall goal is not simply to provide a catalog of all the genes and the information about their functions, but to understand how the components work together to comprise functioning cells and organisms. Gene expression can be analyzed at the RNA or protein level or, in those cases in which expression is affected by alterations in the copy number of genes, also at the level of DNA. Although in most cases protein production is the ultimate output of the gene, protein analysis techniques are presently more difficult to perform, less sensitive, and have a lower throughput than RNA-based ones. Currently, tools for studying gene expression at the level of transcription can be divided into three major groups:

- hybridization-based techniques (northern blotting, RNase protection assays, subtraction cloning, RNA invader assay, DNA arrays),
- PCR-based techniques (reverse transcription PCR (RT-PCR), differential display (DD, DDRT-PCR), representational difference analysis (RDA) and sequence based techniques (expressed sequence tags (ESTs)),
- serial analysis of gene expression (SAGE), DNA sequencing chip, massive parallel signature sequencing (MPSS), mass spectroscopy sequencing.

Two different questions may be addressed when investigating gene expression at the level of transcription: First, what is the expression level of a particular gene in different samples (individuals) and second, which genes (gene families) are differentially expressed under different conditions such as healthy *versus* diseased states, normal tissue *versus* cancer tissue, high productive *versus* low productive tissue, etc.

## 3.1. Analysis of single gene expression

The availability of the mRNA source, knowledge of the species-specific cDNA sequence and the amount of expected transcripts, determines the possible methods of analysis.

## 3.1.1. Northern blotting

Northern blotting, the most widely used method in the analysis of gene expression was developed in the seventies (Alwine et al., 1977; Alwine et al., 1979). With this technique, labeled cDNA or antisense RNA probes are hybridized to mRNA that has been electrophoresed and transferred to a filter. Although this technique is relatively insensitive and cumbersome, requiring micrograms of RNA or purified poly(A)+ RNA, differences in transcript expression are almost always confirmed by northern blot analysis. One important advantage of this technique is that nucleic acid probes derived from other species may be used and alternatively spliced transcripts or the expression of related sequences can be detected. Northern blots and related slot blots and dot blots are semiquantitative, and as the signal is not amplified, they are reliable and linear over a broad range, especially if the signal is detected by phospho-imager or direct imager.

#### 3.1.2. RNase protection assay (RPA)

The ribonuclease protection assay is an extremely sensitive technique for the quantification of specific mRNAs in solution (Krieg & Melton, 1984; Melton et al., 1984). A radiolabeled antisense RNA probe (riboprobe) is synthesized in vitro using a linearized DNA fragment containing the probe adjacent to a bacteriophage RNA polymerase promoter as a template (SP6-, T7- or T3-promotor). The riboprobe is then hybridized to the sample mRNA, after which the mixture is treated with ribonuclease (RNase A and T1), to degrade non-hybridized or mismatched probe molecules. Double-stranded RNA is resistant to RNases and can be visualized by electrophoresis and autoradiography, as shown in Figure 3. Signals can be quantitated by either densitometry, phospho-imager or by direct imager. Several genes can be simultaneously analyzed by PAGE. Relative expression levels may be calculated, in order to compare samples between different gels. As the measured signal is dependent on the specific activity of each riboprobe, inter-assay comparisons are hardly possible, unless one includes control samples that are determined in each assay (Werner et al., in prep; Hasan et al., in prep.).

#### 3.1.3. Reverse transcription PCR (RT-PCR)

The tremendous amplification rate of DNA fragments by the polymerase chain reaction (PCR) technique (Saiki et al. 1985) provides another method to quantify mRNA levels. This PCR-based method has been termed RNA-PCR, reverse transcriptase PCR (RT-PCR) or RNA phenotyping and message amplification phenotyping (MAPPing). The distinct advantage of this approach is that low-copy mRNAs or mRNAs derived from a small number of cells can be readily detected.



Figure 3. RNase protection assay. Left: in vitro labeling and transcription of antisense riboprobe. After DNase treatment, the riboprobe is hybridized to sample RNA. Only double stranded RNA molecules are resistant to treatment with RNases. These protected molecules are visualized by gel electrophoresis and autoradiography (right). Lane A: Undigested full length transcript. Lane B: Control lane without template RNA. Lanes C and D: protected fragments of different samples and different intensities.

In a first step, total RNA or mRNA is reverse transcribed using reverse transcriptase (*e.g.*, Moloney murine leukemia virus (MMLV), avian myoblastosis virus (AMV), Tth polymerase) and reverse DNA primers (poly(dT) primers (5'-dT₁₇VX-3'; V = all nucleotides except T, X all nucleotides), random primers (dN₆ or dN₉) or gene specific reverse primers). An aliquot of the single stranded RT product is used in a PCR reaction using gene-specific upstream and downstream primers. In the first cycle, only the forward primers hybridize to the RT products and the second strand is synthesized. In the subsequent cycles, an exponential amplification of the target sequence takes place. The advantage of using generic primers (dT, dN) in the RT reaction is that any expressed gene may be amplified in the PCR, and cross-species primers or degenerate primers may be used to amplify conserved cDNA fragments (Neuenschwander et al., 1996).

# Quantitative RT-PCR

Homemade semi-quantitative RT-PCR approaches are laborious and difficult to perform, as many parameters need to be optimized. Two general methods have been reported for quantification of mRNA. One approach compares the RT-PCR signal from a target mRNA to that of a housekeeping mRNA. This method controls for RNA loading, degradation and efficiencies of RT. Although control and target sequences do not compete for the same primers, a shortcoming of this approach is that the two primer sets might not amplify with the same efficiency. These restrictions also apply to the commercially available TaqMan assay, where the quantification of mRNA transcripts is estimated indirectly, by determining the accumulation of PCR products in real time.

The second approach uses an exogenous RNA (*e.g., in vitro* transcribed) or DNA that also contains the same primer targets as the mRNA under examination, but the fragment differs slightly in size or by the presence of a diagnostic restriction site (Becker-Andre & Hahlbrock, 1989). In multiple RT-PCR reactions, the amount of standard mRNA is titrated against a constant amount of experimental mRNA. The resulting DNA fragments are electrophoresed and the intensities of the bands are determined. The concentration of the sample mRNA corresponds to the standard concentration, if the fragments show the same intensities. This approach provides an absolute quantification of the experimental mRNA, but does not correct for differences in RNA loading or degradation. The multiplex competitive RT-PCR titration assay (MPTA) combines the strengths of these two approaches to produce a very sensitive and accurate method to measure expression levels of single genes (Dostal et al., 1994; Dostal et al., 1998).

# 3.1.4. RNA invader assay

A direct quantitation of specific transcripts is achieved by using an invasive cleavage approach (Eis et al., 2001, see Figure 2E for a summary of the principles of this assay). In the primary reaction, an invasive oligonucleotide and a DNA probe containing non-complementary generic 5'-flaps are hybridized to RNA. The generic flap is released through 5'-nuclease cleavage. At the optimal reaction temperature, the invasive oligonucleotides remain annealed to their RNA targets, while the probes, which are present in a large excess, undergo rapid dissociation and reassociation with the RNA targets. The generic flap serves in the secondary reaction (FRET cassette) as an invasive oligonucleotide, thereby leading to signal amplification. Using different FRET cassettes, multiple genes may be analyzed simultaneously. In contrast to PCR-based assays, only the signal is amplified, whereas the number of targets does not change during the assay in the invader approach.

### 3.2. Expression profiling

An expression profile refers to a molecular fingerprint of cells or tissue in which relative levels of numerous known mRNAs of interest are determined. For a given tissue and under defined conditions, the expression pattern may vary substantially, *i.e.*, depending on the environment, or the individual's genotype or health status. High throughput methods have been developed to study gene expression in parallel and to identify unknown genes. This will facilitate our understanding of the mechanisms of complex biological processes in response to various stimuli, treatments or conditions. Differentially expressed genes may be causal for quantitative or qualitative differences in economically important traits, and therefore represent good candidates for quantitative or economic trait loci (QTL or ETL).

### 3.2.1. Differential display (DD)

The classical, differential display (DDRT-PCR differential display reverse transcription PCR) is a multiplex RT-PCR technique first described by Liang and Pardee (1992). The process begins with reverse transcription using one of three primers that anchor to the poly(A) tail at the 3'-end of mRNA (*e.g.*,  $T_nG$ ,  $T_nC$ ,  $T_n$  A, which define the three possible junctions of the poly(A) tail). Multiplex PCR is then carried out using a defined arbitrary oligomer as the upstream primer. Each RT-PCR amplifies a set of fragments corresponding to the 3'-ends of various mRNA species. Resolving the fragments on a high resolution gel (usually polyacryl-amide gels) gives a "fingerprint" of gene expression (or expression profile) and allows a side-by-side comparison of different mRNA populations. The procedure requires only small amounts of RNA and is therefore a perfect method for studying changes in gene expression from scarce biological materials.

Because of a high number of false positive results, many changes to the original procedure have been proposed. As outlined in Figure 4, there are three major steps that are critical for obtaining reproducible results: the RT reaction, the PCR amplification, and the separation and excision of DNA fragments from gel matrices.

- Choice of RNA: Either total RNA or mRNA is successfully used in DD. However it is crucial to isolate samples that are not degraded and are free of genomic DNA.
- RT enzyme: Most protocols suggest using either RNase H-negative Moloney murine leukemia virus (MMLV) reverse transcriptase or the thermally stable Tth polymerase, which can be used in the RT reaction and the PCR.
- Choice of reverse primer: In the original protocols, primers that were degenerated at the two last positions were used, resulting in 12 different RT pools. Alternatively, degenerated primers at the penultimate position (G,C,A) can be successfully used, which reduces the number of RT pools to 4



# **Options:**

# **Reverse Transcription**

**RNA:** total RNA, mRNA **Reverse Transcriptase:** MMLV, AMV, Tth **Primer:** 12 RTs ( $dT_{11}VX$  (two base anchored), 4 RTs (penultimate nucleotide degenerated)

# **PCR** amplification

**Tag:** none, ³³P, ³²P, ³⁵S, fluorescent, biotin labeling: end labeled, incorporated labeled nucleotides Polymerase: Taq, Vent, Pfu, Tth **Primer:** random, domain tagged

# **Separation**

Gel type: polyacrylamide (denaturing, non denaturing), Poly(NAT), Clearose BG, visualization: CYBR gold, autoradiography, excision: from wet/dried gels, reconfirmation: reverse Northern, quantitative

Figure 4: Differential Display: Reverse transcription, PCR amplification, fragment separation. Critical steps and potential modifications.

- Choice of 5'-primers: In most cases, primers of 10 to 12 bases, that have similar melting temperature as the reverse primers, are used (Bauer et al., 1993; Ikonomov & Jacob, 1996). Several investigators have increased the size of their 3'- and 5'-primers in order to use higher annealing temperatures and to thereby increase specificity. Others used inosine at different positions with success (Rohrwild et al., 1995).
- Separation and excision: The standard procedure uses denaturing and non-denaturing • PAGE. However, these gels suffer from several drawbacks. DNA is difficult to extract from polyacrylamide, resulting in poor yield and reproducibility. Therefore, a PCR amplification step is usually carried out prior to cloning or sequencing the excised fragments. The extra PCR step is often inhibited by contaminants from the residual acrylamide (Etokebe & Spurkland, 2000) and increases the risk of amplifying unwanted DNA (Callard et al., 1994; Miele et al., 1998).

We therefore tested alternative gel matrices with good resolution and developed a disposable Band Pick[™] device in order to minimize cross contamination. The excised gel pieces which included the DNA fragments could be used directly for subcloning and *in situ* sequencing (Meijerink et al., 2001).

The most significant source of artifacts, however, might be inherent to the design of the differential display method. The combination of short primers and low annealing temperatures during PCR results in nonspecific and inefficient amplification. To minimize the redundancy and to increase the specificity in the amplified cDNAs, a kind of cDNA-AFLP (amplified length polymorphism) was introduced to analyze 3'-end restriction fragments of differentially expressed genes (GEF Gene Expression Fingerprinting (Ivanova & Belyavsky, 1995), READS <u>Restriction Enzyme Analysis of Differentially Expressed Sequences</u> (Prashar & Weissman, 1996)). In the GEF approach, RT was performed with a biotinylated anchored dT primer, the double-stranded cDNA was digested with a restriction enzyme and 3'-ends were captured by streptavidin coated beads. Then, a linker was added to the 5'-end of the cDNA fragments to allow high stringency PCR (forward linker primer and reverse anchor primer) for the generation of radiolabeled cDNA fragments of very low redundancy. After amplification, the fragments are again captured, and treated sequentially with a set of restriction endonucle-ases. The products of individual reactions are then resolved by PAGE and analyzed.

A slightly modified approach, called RAGE (<u>Rapid Analysis of Gene Expression</u>), which uses two frequent cutting restriction enzymes and bitags was introduced by Wang and coworkers (1999). After RT using a biotinylated dTVX primer, the double-stranded cDNA was digested with *DpaII*. The mRNA was then captured using streptavidin coated magnetic particles and tagged *DpaII* linkers were added to the 5'-ends. Digestion with *NlaIII* and ligation of a second tag linker resulted in bitaged fragments which share a *NlaIII* site between the last *DpaII* site and the poly(A) tail. PCR was then carried out using 3'-degenerated tag-specific primers leading to over 32'000 primer combinations, with comparable annealing temperatures of about 60°C.

Another source of false positive differentially expressed sequences is related to the basic approach. The (random) use of different polyadenylation sites within the same gene may lead to different alternative 3'-UTRs that falsely portray different expression levels of the same gene or pretending the expression of two independent genes. Differential display at the 5'-end may theoretically circumvent these drawbacks, however there is no reproducible method for this technique currently available. The classical DD technique generates fragments of 150 to 300 bp upstream of the poly(A) tail, which usually represent the 3'-UTR. These sequences

may be difficult to identify when querying sequence databases, particularly in organisms whose genomes have not yet been fully sequenced.

Although many researchers have addressed this problem, the search for full length cDNA sequences remains tedious and difficult. While library construction and screening is always a possibility, this process is time-consuming and does not promise isolation of complete cDNA sequences. Alternative methods can be summarized under the name RACE (rapid amplification of cDNA ends).

<u>5'-tailing, anchored PCR:</u> In this approach, the template independent DNA polymerase activity of the terminal deoxynucleotidyltransferase (TdT) is used to add a homopolymer tail to the 3'-end of single stranded cDNA derived from reverse transcribed mRNA. In subsequent PCR, single full length sequences may be amplified using a gene specific reverse primer and tagged / untagged oligonucleotides complementary to the homopolymer tail (Frohman et al., 1988; Loh et al., 1989).

<u>SMART PCR:</u> MMLV reverse transcriptase possesses intrinsic template independent transferase activity and template switching activity in the presence of oligonucleotides containing ribonucleotides at their 3'-ends (preferentially r(GGG)). The template switching oligonucleotide contains a unique tag, which serves as a target for the forward primer in subsequent PCR.

Ligation-mediated approaches use the ability of T4 RNA-ligase to join single stranded RNA or single stranded DNA (ssDNA) molecules (such as ligation-anchored PCR (LA-PCR), single-strand ligation to cDNA ends (SLIC), RNA ligase-mediated RACE (RLM-RACE), also called reverse ligation mediated PCR (RLPCR)). After the isolation and purification of ssDNA, an anchor oligonucleotide (which is 5'-phosphorylated and blocked at the 3'-end to prevent the formation of oligo concatomers ) is covalently attached to the 3'-hydroxyl end. 5'-ends are then amplified in the subsequent PCR using an anchor primer and a reverse gene specific primer (Edwards et al., 1991; Troutt et al., 1992; Apte & Siebert, 1993; Chen, 1996). These strategies eliminate the use of homopolymer primers in the PCR, which may lead to mispriming. However, the T4-RNA ligase is not very efficient in ligating ssDNA, and incomplete reverse transcribed molecules are also ligated and amplified, which may increase background.

In contrast, the RLM-RACE can circumvent all mechanisms by which truncated 5'-ends are generated (Fromont-Racine et al., 1993; Liu & Gorovsky, 1993; Schaefer, 1997). Degraded and non mRNA molecules are first dephosphorylated using alkaline phosphatase (*e.g.*, CIP). The mRNA is then decapped by removing the 7-methyl guanosine triphosphate by tobacco acid pyrophosphatase (TAP), leaving one reactive 5'-phosphate. An RNA primer (either chemically synthesized or transcribed *in vitro* from linearized plasmid containing an appropriate promoter) is ligated to the decapped mRNA using T4-RNA ligase. Finally RT-PCR is performed for the amplification of the cDNA ends.

Compared to double strand specific T4-DNA ligase, the single strand specific T4-RNA ligase is not very efficient. We therefore modified the RLM protocol using the ligation of a double stranded anchor to an RNA/cDNA hybrid (Neuenschwander et al., in prep.). Besides the increased efficiency of the ligation reaction, this approach leads to "true" full length cDNAs that lack artifact sequences at the 5'-end, which may be introduced by the non template dependent transferase activity of reverse transcriptases (Swanstrom et al., 1981; Clark, 1988; Chenchik et al., 1998; Chen & Patton, 2001).

# 3.2.2. Representational difference analysis (RDA) and suppression subtractive hybridization (SSH)

RDA was originally described for cloning the differences between two complex genomes (Lisitsyn & Wigler, 1993). The protocol was subsequently adapted for use with cDNA by Hubank and Schatz (1994) and was further improved by O'Neill and Sinclair (1997). This assay merges the advantages of subtractive hybridization with the power of PCR. Double stranded cDNA of a tester and a driver sample are digested with a restriction enzyme and different adapters are ligated. If an amplifiable target restriction fragment exists in the tester and is absent in the driver, a kinetic enrichment of the target can be achieved by subtractive hybridization of the tester in the presence of excess driver. All driver / tester hybrid fragments are excluded from amplification, thereby leading to an enrichment of targets that are overexpressed in the tester.

In the suppression subtractive hybridization (SSH) approach, normalization and subtraction are combined, thereby equalizing abundant cDNA (Diatchenko et al., 1996). Suppression of unwanted fragments is achieved by long terminal inverted repeats, which was originally described by Siebert et al. (1995), Figure 5.

## Spinal muscular atrophy in cattle

Spinal muscular atrophy (SMA) in Brown Swiss Calves is a disorder of the nervous system characterized clinically by skeletal muscle atrophy, decreased spinal reflexes and motor



Figure 5. Suppression subtractive hybridization (SSH). Double stranded cDNA is synthesized from mRNA of Tester and Driver samples and digested with a restriction enzyme. Two different adapters are ligated in separated reactions to the tester cDNA and then hybridized to an excess of Driver cDNA (no adapters) resulting in the products A-D. A second hybridisation is performed without denaturation. The newly formed products E represent differentially expressed cDNA in the Tester and Driver samples.

weakness. The main neuropathological signs exhibited by affected animals include degeneration and loss of neurons of the ventral horns and the axons of the spinal cord, as well as degeneration of nerve axons in the extremities (El-Hamidi et al., 1989; Stocker et al., 1992). All methods used in an attempt to identify the molecular defects in these animals have failed. Neither physiological investigations, genetic analysis of affected families, nor comparative genetic approaches have been successful to date. Indeed, we excluded the SMN gene as being involved in cattle SMA, which is mutated in human SMA.

We therefore performed SSH (Figure 5) on spinal cord mRNA samples from calves suffering from SMA and healthy control animals. We were able to isolate over 100 unique cDNAs which are presumably differentially expressed. We performed a reverse northern, by spotting these fragments onto nylon membranes and hybridizing the radiolabeled cDNA of healthy and (in a separate experiment) diseased spinal cord mRNA. Fourteen clones show differences in expression levels that are greater than 2-fold (Meijerink et al., in prep.). These promising results show that complex mechanisms may indeed be resolved by investigating the differences in gene expression by performing differential display on tissues derived from healthy and diseased animals.

### 3.2.3. DNA arrays

In order to profit from the flood of new sequences arriving in the databases on a daily basis, it has become necessary to develop methods to analyze gene expression in parallel. Nucleic acid arrays work by hybridization of labeled RNA or DNA molecules in solution (complex target) to DNA molecules attached at specific localizations on the surface of a glass slide or nylon membrane (probes). The probes may consist of either cDNA (inserts of a cDNA library, PCR products) or oligonucleotides. The target is produced by reverse transcription of mRNA (or total RNA using  $dT_{17}VX$  primer) and radioactive or fluorescent labeled nucleotides. The complexity of the probe is determined by many different sequences, which are present in various amounts in the hybridization solution, and should reflect the numbers of copies of the original mRNA species extracted from the sample.

The hybridization of a sample requires a highly parallel search by each molecule for a matching partner on an "affinity matrix". The conditions of hybridization using DNA arrays are quite different from the well-established conditions for saturation hybridization of northern blots, in which the target is a single molecular species, present in high concentrations and is present in great excess over its probes. Under these conditions, all probe sequences are completely covered by the labeled target and the signals are only dependent on the amount of probe and the specific activity of the labeled target. In contrast, in hybridization to DNA arrays, only a small fraction of the labeled probe actually hybridizes to the target, which is present in huge excess. Signals are therefore proportional to the relative concentration of the labeled target, the specific activity, the duration of hybridization and the amount of probe material in each spot. Due to incomplete probe coverage, the signal intensities are quite small and the detection and quantification require high instrumental sensitivity, as well as suitable controls in order to estimate nonspecific or background hybridization.

Bertucci and colleagues demonstrated that actual sensitivity, defined as the amount of sample necessary for detection of a given mRNA species, is in fact similar for all three sys-

tems (a nylon cDNA macroarray with radioactive detection; a glass cDNA microarray, and oligonucleotide chips, which are each detected by fluorescence labeling) (Bertucci et al., 1999a). However, the numbers of probe sequences differ substantially in these experiments. Densities of 250'000 different oligonucleotide targets or 10'000 cDNAs per square centimeter may be achieved on glass slides, whereas on nylon membranes, not more than about 5000 spots per membrane may be attached.

However, to use microarrays, the sequences to be analyzed must be either known or cloned and individually processed beforehand, typically with the aid of complex robotics systems. This makes it difficult to isolate and monitor important genes that are differentially expressed at relatively low levels against the background of more abundantly expressed genes. Consequently, arrays from human and mice can not be readily applied to other organisms of interest. Therefore, I anticipate that PCR-based and sequence-based methods will remain invaluable for the analysis for other species for many years to come.

#### 3.3. Outlook

The revolution has just begun, in terms of using high throughput methods in functional genomic analysis. It is still unclear in which areas small university laboratories may compete with large industrial laboratories in these approaches. There is no question that protein- and RNA-based methods are complementary, and that protein-based methods are important, as they measure observable traits that are not readily detected in other ways.

One of the unsolved problems in genome analysis is that no fast and reliable methods of getting from a genetic marker to the gene of interest are currently available. On one hand, a recombination rate of 1 percent between a marker and the unknown gene indicates very close linkage. For economic reasons, among others, this interval may not be substantially narrowed by increasing the amount of informative meiosis when working with farm animals. On the other hand, a 1 percent recombination rate corresponds to an interval of about 2.4 megabases of genomic DNA surrounding the marker. This may be covered by a contig of around 40 genomic PAC clones. Assuming the genome consists of 30'000 genes, we may expect to find up to 24 genes, each of which may correspond to the unknown gene.

This illustrates the importance of EST mapping programs and the need for introducing new methods in a "traditional genetics" laboratory, in order to efficiently pinpoint the molecular basis of various phenotypes. Reverse northern, exon trapping, direct cDNA selection from genomic sequences and proteomics are only some of the key skills.

# 4. Farm animals as models in biomedical research: Examples at our institute

Animal experimentation has always been a subject of considerable public and scientific controversy. Significant questions concerning its humanistic side and its methodological problems (*e.g.*, adequacy, suitability of extrapolations to different species) have been raised in the past and we are still struggling with these issues today. At the time when the Swiss Society of Genetics organized a symposium on *Genetic Variants of Farm animals as Biomedical Models* (Pliska & Stranzinger, 1990), we believed that animals with an inborn (genetically determined) defect were preferable to an artificially-treated laboratory animal. In some cases, these models mimic the genetic disease as it occurs in humans, and these animals may therefore be better adapted to the pathologic condition than an artificially treated animal. However, this may no longer be true, as homologous recombination and "timed knock out" techniques have been developed in mice. With these approaches, models can be generated such that virtually any genetic disease may be mimicked in mice. Still, the size and the closer physiological relation of farm animals (*e.g.*, the pig) to the human counterpart, makes farm animals still very valuable.

Animal experiments are also required when trying to characterize a genetic defect independent of the suitability of the animal as a model for a human disease. In the case of vitamin C deficiency, this is not a problem at all, as supplementing the animals with ascorbic acid prevents them from suffering scurvy. However, in hemophilic animals, bleeding incidences could not always be avoided and adequately treated. The breeding of CPA (congenital progressive ataxia) animals in an experimental family is justified by ethical and economic considerations: the identification of heterozygous animals and their exclusion from further breeding may prevent the birth of many sick animals that would be destined to suffer. In addition, farmers will lose fewer animals.

In the next few paragraphs, I will describe some of the animal models we have explored at our institute over the last few years. Because the traits are monogenetic and show a simple Mendelian inheritance pattern, we have been successful in typing and characterizing the molecular background of the observed phenotypes (see also paragraph 2.4.1).

## 4.1. Hemophilia

Bleeding incidents (hemorrhage) are widespread in both humans and in animals. They occur either after injuries or as a consequence of pathogenic circumstances, such as rupture of scle-



Figure 6. Coagulation cascade and global coagulation tests. F: coagulation factor. PTT: partial thromboplastin time tests the intrinsic coagulation pathway and the common end of the cascade. Quick (prothrombin time, thromboplastin time) tests the extrinsic pathway and the common end. TT: thrombin time tests the fibrinogen (F I) to form a fibrin clot.

rotic blood vessels, or dysfunction of the coagulation system. Blood coagulation requires a cascade of various enzymatic processes, whereby inactive proenzymes (coagulation factors) are activated initiating the next step (Davie & Ratnoff, 1964; MacFarlane, 1964). Three principle steps in the formation of a blood clot may be distinguished: 1) formation of activated factor X (FXa), 2) formation of thrombin, 3) formation of fibrin, as shown in Figure 6. Hemophiliacs suffer from a lack of functionally active blood coagulation factors VIII or IX (hemophilia A or B, respectively), which causes intermittent bleeding (spontaneous or after small injuries), mostly in extremities and subserosal tissue. The severity of hemophilia A is closely related to the *in vitro* estimated plasma F VIII clotting activity. Forms with less than 5% F VIII activity are classified as severe and may lead to spontaneous life threatening bleeding incidents.

Although it has been long established that there is an X-chromosome related defect in hemophilia A, the complete characterization and cloning of the human *F VIII* gene and the protein structure was not reported until 1984 (Gitschier et al., 1984; Vehar et al., 1984; Wood et al., 1984). The molecular background of hemophilia in human has been collected and is

accessible at the HAMSTeR web site (<u>H</u>emophilia <u>A</u> <u>M</u>utation <u>S</u>tructure, <u>T</u>est, <u>R</u>esource Site (Wacey et al., 1996; Kemball-Cook et al., 1998)

http://europium.csc.mrc.ac.uk/usr/WWW/WebPages/main.dir/main.htm.

To date, 629 unique mutations have been described in the F VIII gene that can cause hemophilia. The vast majority of these are missense and nonsense (stop) mutations. Several splice sites are also affected, and various deletions, insertions and inversions have been reported that cause this disease.

#### 4.1.1. Hemophilia in animals

Detailed reviews of bleeding disorders in animals have been previously published (Grün, 1985; Fogh & Fogh, 1988b). Naturally occurring hemophilia A (F VIII / vWF) and hemophilia B (F IX) have been reported in dog, cat, horse, cattle and sheep. With the availability of the gene targeting method, artificial hemophilic mice have also been developed (Bi et al., 1995; Wang et al., 1997). Hemophilic animals suffer from the same symptoms as their human counterparts and the inheritance pattern of the disease is also X-linked and dominant in the male.

### Hemophilia in sheep

Several male offspring of a single ewe in the sheep flock (White Alpine sheep) at one of our experimental stations (ETH) were born with a severe bleeding disorder. They all died within a few days due to bleeding from the umbilical cord (Pliska et al., 1982). Daughters and grand-daughters of this ewe also gave birth to male lambs exhibiting the same syndrome. Autopsies confirmed that the afflicted males suffered from extensive subcutaneous and intramuscular hematomas, especially in the muscle of the hind legs and in *m. psoas minor* or *m. iliopsoas*. There was also a frequent occurrence of spontaneous hemarthrosis, mainly in the weight bearing joints (Neuenschwander & Pliska, 1990). Consequently, the animals showed signs of pain during standing up for nursing and walking.

The activated partial thromboplastin time (PTT) was prolonged by a factor of 2 to 3 in the affected lambs. Furthermore, a normal Quick-test and thromboplastin time (TT) indicated that these animals had a disorder of the intrinsic pathway of the coagulation cascade (see Figure 6). Detailed examination of the activities of single coagulation factors showed that the pathologic PTT was indeed due to the very low F VIII activity (about 2% of the normal level), thus indicating a severe hemophilia A. Heterozygous animals (female carriers) could not be

distinguished from normal animals using plasma clotting tests (Neuenschwander et al., 1992). However, based on the analysis of clotting time-plasma dilution curves, we came to the conclusion that hemophilia A in sheep is caused by the production of a less potent coagulation factor, rather than by a complete lack of this factor (Neuenschwander & Pliska, 1994).

In order to identify female carriers, a F VIII-RFLP was developed using a human F VIIIcDNA probe. The 5,8 kb *Msp*I fragment of the *F VIII* gene was in linkage phase with the mutated allele responsible for hemophilia A. No recombination event was detected in our hemophilic sheep family (Backfisch et al., 1994). As the same polymorphism was detected in other families, without the occurrence of bleeders, the altered *Msp*I site is not responsible for the disease. The same human F VIII cDNA probe was used to hybridize to metaphase chromosoms prepared from peripheral blood lymphocytes of a normal ram. The predicted X chromosomal localization could be narrowed to the Xq24-q33 region of the sheep.

### 4.1.2. The use of animals in hemophilia research

We have attempted to establish normal, not affected sheep as a possible animal model for the screening of antihemophilic drugs (Heiniger et al., 1988; Neuenschwander & Pliska, 1997). When using F VIII activities as an estimate of antihemophilic potencies, it is vital to withdraw the blood without extensive injury to the punctured vein, and to avoid stress and anesthesia. These precautions are necessary to prevent activation of the coagulation cascade and to obtain accurate and reproducible activity measurements. These conditions preclude the use of small laboratory animals. However, when using hemophilic animals with very low or non F VIII / IX activities that exhibit the typical signs of severe hemophilia when not treated, this restriction may not apply.

To date, replacement therapy with blood-derived F VIII / IX concentrates or recombinant coagulation factors are the only way to manage severe hemophilia in humans. Fortunately, the risk of viral contamination with agents such as hepatitis and AIDS has decreased substantially in the therapy of hemophiliacs. However, neither of these treatments are curative. Therefore, somatic gene therapy, which allows sustained coagulation factor synthesis in the patient, may be the only way to maintain coagulation factors at a therapeutically effective level. Hemophilic animals are particularly critical for developing new vectors and new strategies for somatic gene therapy. For review see (Lozier & Brinkhous, 1994; Greengard & Jolly, 1999; Thompson, 2000; Chuah et al., 2001).

#### 4.2. Vitamin C deficiency

Scurvy (vitamin C deficiency) was probably already known to the people of ancient Egypt, Greece and Rome. In the middle ages, when progress in ship design made it possible to cross the oceans, scurvy became epidemic among sailors, before it was finally recognized as a nutritional deficiency. Because vitamins A and B had already been detected, Drummond suggested designating the antiscorbutic factor "C", which became later vitamin C. The chemical isolation of vitamin C was first achieved in 1932 by Albert Sczent-Györgyi, who isolated a reducing factor from adrenal tissue that he called "hexuronic acid". Later, he suggested that the name should be changed to L-ascorbic acid, to underline its antiscorbutic properties. In 1937, Haworth (chemistry) and Sczent-Györgyi (medicine) were awarded the Nobel Prize for their work with vitamin C (Moser, 1990).

Most mammals are able to synthesize L-ascorbic acid (AsA, vitamin C) in their livers and therefore do not require a dietary source of vitamin C to prevent scurvy. For these animals, AsA is not a vitamin in the narrower sense. However, guinea pigs and primates (including man) are unable to synthesize AsA, due to their inability to convert L-gulonolactone to Lascorbic acid. Thus, these species lack an active L-gulonolacton oxidase (GULO, EC 1.1.3.8), which catalyzes the last step in the synthesis of vitamin C (Burns et al., 1956; Burns, 1957), as shown in Figure 7. Molecular biological studies showed that the human and guinea pig genomes contains DNA that cross hybridizes to the cDNA for rat GULO. These findings suggest that the GULO locus has not been completely lost. Indeed, sequence analysis of the human GULO gene revealed that the isolated 3'-part of the gene corresponds to the exons VII, IX, X and XII of the rat GULO gene. Various nucleotide changes, such as deletions, insertions and the loss of consensus splice sites were found in the exons of GULO identified in humans and in guinea pigs. In summary, the nonfunctional human GULO pseudogene (GULOP) has accumulated a large number of mutations, presumably due to the lack of selective pressure since it ceased to function during evolution (Nishikimi et al., 1994). A GULO pseudogene containing various mutations, as compared to the rat gene, was also characterized in guinea pig (Nishikimi et al., 1992).

#### 4.2.1. Vitamin C deficiency in the pig

Pigs will normally produce sufficient levels of vitamin C to meet their physiological needs. Some years ago, however, a Danish mutant strain of pigs was discovered (Landrace breed) that lack the ability to produce AsA (Jensen et al., 1983). The clinical symptoms, such as



Figure 7. Vitamin C synthesis. With relationship to the D-glucuronic acid pathway . X: a conjugating molecule for glucuronidation. UDPGT: uridine-di-phospho glucuronosyl transferase (according to Nishikimi and Yagi (1996) with modifications)

lethargy and swelling around the joints began 2 to 3 weeks after weaning. The vitamin Cdeficient pig, when fed a diet lacking AsA, exhibits deformity of the legs, multiple fractures, osteoporosis, growth retardation and hemorrhagic tendencies. This trait was found to be controlled by a single autosomal recessive allele, designated *OD* (osteogenic disorder). The affected pigs suffer from GULO deficiency, as liver microsomes from these animals were unable to synthesize AsA in *vitro* from the L-gulonolactone substrate (Jensen et al., 1983).

#### 4.2.2. Molecular aspects of pig vitamin C deficiency

In order to study the molecular basis of the disease and the regulation of GULO (i.e., vitamin C production) in the pig, we started generating an experimental family. To do this, a heterozygote boar (OD/od) was mated to three deficient sows (od/od). The pigs were provided by Dr. J. Schulze, Centre de Recherche en Nutrition Animale, Société Chimique Roche, Village Neuve, France. Vitamin C levels were determined in the plasma of piglets one week after weaning. AsA plasma levels < 0.8 mg/l were considered to be deficient and AsA > 1.6 mg/l were classified as nondeficient (heterozygous in this mating scheme).

The experimental herd was screened with SSC4- and SSC14-specific microsatellites, as the nonfunctional human *GULOP* gene was mapped to human chromosome 8p21 (Nishikimi

et al., 1994), a region which corresponds to an evolutionarily conserved segment on either porcine chromosome 4 (SSC4) or 14 (SSC14) (Rettenberger et al., 1995). We detected significant linkage between *od* and microsatellites *SW857* and *S0089*, located in the subcentromeric region of SSC14. Reverse transcription (RT) ( $T_{17}VX$  primer) and PCR was performed using cross species primers derived from the rat and mouse *GULO* cDNA sequence. We amplified a porcine-specific cDNA fragment (accession # AF136938), which was used to isolate *GULO* clones from a porcine genomic cosmid library. *GULO* was then physically mapped to the same region as *od* (SSC14q14) by hybridizing the labeled *GULO* cosmid to porcine metaphase chromosomes (Hasan et al., 1999). Thus, the porcine *GULO* gene was considered to be a good functional and positional candidate gene for vitamin C deficiency in this family.

Using the same porcine *GULO* fragment, we screened a porcine liver cDNA library. A full-length clone was isolated, sequenced and queried against the GenBank database. The 1320 bp putative open reading frame of the porcine cDNA was 87% homologous to the rat sequence (at the nucleotide level) and 92.3% homologous at the amino acid level.

Sequence analyses from RT-PCR products of deficient animals revealed the molecular basis of porcine vitamin C deficiency: a whole exon was missing (exon VIII, according to the numbering used in rat). Further analyses, at the genomic level, revealed that parts of the adjacent introns VII and VIII were also deleted (approximately 3.4 kb). Based on this information, we established a diagnostic test in order to detect normal, deficient and carrier animals (Figure 8 a, b) (Hasan et al., 2000, Hasan in prep.).

### 4.2.3. The deficient pig as an animal model to study vitamin C effects

While the dramatic effects of AsA deficiency (*i.e.*, scurvy) have been well-documented, the ascorbic acid levels that are necessary for optimal growth and health remain a matter of debate. Various studies have shown that AsA is a cofactor required in collagen synthesis and in bone metabolism (for review see (Geesin et al., 1993)). Like other antioxidants, AsA reduces the damage caused by metabolic reactive oxygen intermediates (ROI). AsA may also prevent degenerative diseases, enhance immune function, and thereby improve the overall health status (reviewed in Ames et al. (1993)). The use of deficient animals as models is particularly useful, since systemic AsA levels can be changed in a controlled and predictable way. In addition, access to a strain of pigs that are unable to synthesize AsA has made it possible to evaluate the role of vitamin C in female reproduction, bone development, and immunological



Figure 8a. Genomic structure from exon VII to exon IX for normal (OD) and deficient (od) alleles of the porcine GULO. The nucleotide sequence (in black) of the last 398 bp of intron VII, the complete exon VIII (77 bp) and of the first 3.7 kbp of intron VIII is deleted in the mutated allele.



Figure 8b. Genetic test for pig GULO variants. Four primers were included in the genetic test discriminating between normal and deficient alleles. The normal allele revealed a 1020bp and a 168bp fragment using primers P1/P2 and P3/P2. Fragment P1/P4 was normally not amplified to detectable amounts of DNA during the PCR, due to its size. The deficient allele resulted in a 543bp fragment with primers P1/P4. Heterozygous animals showed all three fragments.

responses in a species that, in many biochemical and physiological respects, more closely resembles humans than do guinea pigs and other rodents.

Wegger and Palludan (1994) showed that in vitamin C deficient (*od/od*) sows, even a transient lack of vitamin C supplementation during pregnancy severely impaired fetal development and viability. The detrimental effects are most pronounced in the development of the

skeleton and the vascular system. Collagen matrix and osteoid formation was impaired and the fetus exhibited marked subcutaneous hemorrhages and hematomas.

Schwager and Schulze, (1998a) investigated the role of vitamin C in the young *od/od* pig. Depletion of AsA for longer than three weeks caused growth retardation, hypothermia and decreased levels of bone alkaline phosphatase with a time delay of one week. Activation of polymorphonuclear leucocytes (PMNL, which represent about 50% of the leucocyte population) plays an important role in host defenses. Activation of these cells is accompanied by an increase in oxygen consumption (oxidative burst) and the production of ROI, which are toxic for pathogens, but might also be deleterious to normal cell function. AsA acts as a scavenger of ROI, as demonstrated by a quenching effect on chemiluminescence, when PMNL were activated by phorbol myristate acetate *in vitro*. In addition, in peripheral blood mononuclear cells (PBMC) AsA decreased ROI dependent IL-2 expression (Schwager & Schulze, 1998b)

To our knowledge, there is no data available on the regulation of GULO activity and/or GULO gene expression in the pig. Among all animals (deficient animals (od/od, suplemented with 1500 mg AsA /kg feed), normal (OD/OD) and heterozygous (OD/od) animals given a standard finishing feed), even those within the same genotypes, we observed large differences in GULO expression levels (as determined by northern analysis). The amount of transcripts in deficient animals was lower than that in the phenotypically normal animals. In order to determine whether a loss of function or a quantitative problem with the GULO transcripts and /or protein may cause the vitamin C deficiency in the pig, we also measured GULO enzyme activity. We developed a modified GULO assay, where liver microsomal preparations, rather than crude homogenates, were used. AsA was first oxidized to dehydroascorbic acid by 2,6dichlorophenolindophenol which spontaneously reacts to 2,3-diketo-L-gulonic acid. Finally, a colored dinitrophenylhydrazone derivative is formed by adding 2,4-dinitrophenylhydrazine. GULO activity was expressed as the amount of newly synthesized vitamin C per hour per mg of microsomal protein, by calculating the difference in vitamin C levels in the presence and absence of gulonolactone substrate. Deficient animals showed no GULO activity, whereas heterozygous animals showed a tendency to have lower GULO activities than normal animals.

As *GULO* is expressed at only moderate levels, we developed a RNase protection assay using an antisense riboprobe derived from a *PstI/Bgl*II fragment of *GULO* cDNA including part of exon 6, exons 7 and 8, and part of exon 9. This resulted in a 333 bp protected fragment in normal animals, two fragments (163 and 93 bp) in deficient animals, and all three of these bands in heterozygous animals. Protection assays confirmed the results of the northern

analysis. Over all experiments, the normal animals expressed significantly higher levels of *GULO* mRNA than did the vitamin C-deficient animals (corrected for allele number and U content of the protected fragments). However, we also found that in heterozygous animals, the deficient allele was expressed at a lower level than the normal allele. So far, we do not have an explanation for this. The full length riboprobe may hybridize inefficiently to mRNA molecules that lack the central portion (exon VIII). Alternatively, differences in RNA stability may explain these findings.

Although farm animals are able to produce their own AsA, in some cases, supplementation with vitamin C has been reported to be advantageous, as outlined during two conferences on "Ascorbic acid in domestic animals" (Wegger et al., 1984; Wenk et al., 1990). In view of this, we designed a study to characterize the influence of nutritional AsA on *GULO* expression and GULO activity. In short term experiments (14 days), when animals were either deprived of AsA or supplemented with AsA (1.5 g / kg of feed), there was no significant change in liver GULO activity (*OD/od* and *OD/OD* animals) nor were there changes in GULO mRNA levels (all genotypes). However, when the experiment was extended to four weeks, GULO activity in *OD/OD* and *OD/od* animals was markedly reduced, by roughly half, comparing the activity in supplemented animals to that in animals receiving no AsA additives.

The mechanism by which AsA inhibits GULO activity remains unknown. GULO mRNA levels were not influenced by dietary vitamin C supplementation in any of the animals. In contrast to our observations, several other studies have reported that AsA can regulate expression of various genes, including collagen type I, procollagen, acetylcholine receptor  $\alpha$ -subunit, lipid binding protein, lipoprotein lipase, myosin light chain 2 and myoglobin (reviewed by (Hitomi & Tsukagoshi, 1996). We have excluded the possibility that AsA has a direct effect on the activity of the GULO enzyme, as the presence of super-physiological concentrations of AsA did not influence the *de novo* synthesis of vitamin C *in vitro*, in microsomal preparations using glucuronolactone as a substrate. Tsao and Young (1989) also found a negative correlation between GULO activity and AsA content in the hepatic portal blood of mice. The control mechanism for the conversion of glucuronolactone to ascorbic acid is not stereospecific because large amounts of dietary erythorbic acid, a stereoisomer of ascorbic acid, inhibited the rate of ascorbic acid formation when glucuronolactone was used as substrate (Tsao & Young, 1990).

High levels of oxidative stress would be expected in non-supplemented *od/od* animals. Therefore, we investigated the activity of the antioxidant enzyme superoxide dismutase (SOD) in various tissues and thiobarbituric acid reactive substances (TBARS) in fresh erythrocytes. However, we did not detect any significant differences in SOD or TBARS activity between normal and deficient animals, nor was there a difference between AsA-suplemented or AsA-deprived animals. This suggests that there are vitamin C-independent mechanisms that protect the animals from oxidative injuries. Alternatively, it is possible that the vitamin C dosage was not high enough to show an additional benefit. However, in preliminary studies using SOD-induced relaxation of pre-contracted aorta, it was found that  $O_2^-$  content was enhanced in porcine coronary arteries of non-supplemented AsA-deficient pigs, as compared to normal animals (Christine Barandier, personal communication.). These results are in agreement with the studies of Maeda and co-workers (2000) using GULO knock out mice (see below).

### 4.2.4. Other animal models for vitamin C deficiency

#### ODS rats

When osteogenetic disorder Shionogi (ODS) rats are fed an AsA-deficient diet, they show symptoms similar to those of infantile scurvy in humans. Mizushima et al. (1984) have shown that this defect is controlled by a single autosomal recessive gene, and that the liver of ODS rats lacks an active L-gulonolactone oxidase (GULO), comparable to the defects previously observed in human, guinea pig and *od* pigs. Using a more sensitive assay, Nishikimi and coworkers (1989) were able to detect trace amounts of GULO activity in these rats. ODS rat expressed the same amount of GULO mRNA as normal litter mates, whereas expression of the GULO protein was markedly reduced in an *in vitro* translation system, as compared to normal or heterozygous rats. The molecular defect of the *GULO* gene was finally characterized as an  $A \rightarrow G$  transition, which alters 61Cys to 61Tyr in the rat GULO protein (Kawai et al., 1992).

#### GULO knockout mouse

Maeda and colleagues (2000) used gene targeting to disrupt the mouse *GULO* gene. These mice were then dependent on dietary AsA to prevent scurvy. The most striking effects in these mice were that they were more susceptible to coronary heart disease. Some of the smooth muscle cells in these mice were morphologically altered, such that prominent breaks and fragmentation of the elastic lamina were located in the superficial and deep media. These

breaks were most likely caused by defects in the cross-linking of collagen and elastin, due to the need for vitamin C to generate hydroxylysine and hydoxyproline.

Despite a reduction in plasma antioxidative capacities, the levels of lipid peroxidation were below the limits of detection in both wild type mice and knockout mice. Furthermore, there was no difference in accumulation of lipid peroxides in tissues, as measured by free malonaldehyde and 4-hydroxyalkenals.

## 4.3. Resistance to enteropathogenic E.coli

Attachment is a prerequisite for bacterial survival on epithelial surfaces and, accordingly, colonizing bacteria have developed specialized adhesion molecules that allow them to efficiently adhere to eukaryotic cell surfaces. Bacteria that lack appropriate adherence properties will be removed by local clearance factors, such as peristalsis, turnover of epithelial cell populations, mucus layer and ciliary activity. There are two categories of appendages on the bacterial cell surface: first, 5- to 8-nm thick rigid, rod like filaments designated pili (type 1 pili, P-pili), and second, 2- to 5-nm-thick, flexible, hair like filaments designated fimbriae (*e.g.*, K88, K99, F18) (Smyth et al., 1994; Mol & Oudega, 1996). The F18 are 1 to 2  $\mu$ m long filaments that are built from FedA, a major structural 15.1 kD protein (Imberechts et al., 1992). Two associated minor subunits, FedE and FedF, with molecular masses of 15.9 and 30.1 kDa, respectively, are important for fimbrial adhesion (Imberechts et al., 1996). These structures have recently been investigated by electron microscopy. A resolution of better than 2 nm reveals that the axially repeating units alternate in a one-start helix to give rise to the striking zigzag pattern. The axial rise was determined to be 2.2 nm, thereby forming a helical repeat of 4.3 nm (Hahn et al., 2000).

F18 fimbriated *E.coli* strains produce enterotoxins, which can cause diarrhea, and Shigatoxins, which cause edema disease in newly weaned pigs (Bertschinger & Gyles, 1994). These diseases are of considerable economic significance in the pig breeding industry (Bertschinger et al., 1992). Susceptibility to adhesion, meaning expression of receptors that mediate the binding to the bacterial fimbriae, has been shown to be genetically controlled by the host and is inherited as a dominant trait (Bertschinger et al., 1993). The genetic locus for this *E. coli* F18 receptor (*ECF18R*) has two allelic variants: susceptible animals are either of the *ECF18R^{BB}* or *ECF18R^{Bb}* genotype, whereas resistant animals are *ECF18R^{bb}*. *ECF18R* has been mapped to porcine chromosome 6 (SSC 6), based on its close linkage to the halothane



Figure 9. Homologous chromosomes of pig and human. Chromosome 6 of the pig (SSC6) contains homologous regions which are located on human chromosomes (HSA) 1, 16, 18 and 19. RYR1 (HAL): Ryanodin receptor 1, (halothane-locus), GPI: glucose-phosphat isomerase, APOE: Apolipoprotein E, TGF $\beta$ 1: Transforming growth factor  $\beta$ 1, S: Blood group S, ECF18R: E. coli-F18-receptor, FUT1:  $\alpha$ -(1,2)-fucosyltransferase 1, FUT2:  $\alpha$ -(1,2)-fucosyltransferase 2.

(*HAL*) linkage group, which includes the ryanodin receptor (*RYR1*, *HAL*), glucose phosphate isomerase (*GPI*), the blood group A0 inhibitor locus *S*, and the erythrocyte antigen H (*EAH*) (Vögeli et al., 1996). This region is evolutionarily conserved and corresponds to the human chromosome HSA19q13.1-13.3 (Figure 9). The human blood group *H* and *Se* loci have been mapped in the same linkage group (Oriol et al., 1984).

Carbohydrate structures of blood group antigens have been shown to mediate the adhesion of some pathogenic microorganisms to host tissues. For example, *Helicobacter pylori* adheres to Lewis^b blood group antigens (Borén et al., 1993), and *E.coli* strains that cause urinary tract infections adhere to P blood group substances (glycosphingolipids P1 and Pk) (Svenson et al., 1983). Thus, genes encoding glycosyltransferases that are responsible for the formation of the blood group-specific carbohydrate structures represent candidate genes that hosts may use to control bacterial colonization. The adhesion of bacteria can be determined either by monitoring fecal excretion of weaned pigs orally inoculated with the *E. coli* strain O139:K12(B):H1:F18ab serotype or by a microscopic *in vitro* adhesion assay, using an F18ab positive E.coli strain and small intestinal enterocyte preparations (Vögeli et al., 1996). In pig, the recombination rate of the *ECF18R* locus was lowest, with 0.5% for the *S* locus. Since susceptibility to adhesion segregates with expression of the blood group A precursor 0 structure, we hypothesized that *S* may be directly involved in the binding of ECF18 bacteria. From the perspectives of comparative genetics and functional aspects, fucosyltransferases 1 or 2 (*FUT1, FUT2*) may be identical to *S*. Therefore, we decided to clone these genes. Further studies, however, showed that *S* is closely linked, but a distinct locus from *FUT1* and *FUT2*.

#### 4.3.1. Porcine FUT1 and FUT2

A genomic cosmid library from the pig and a cDNA library from small intestinal mucosal scrapings were screened using a radiolabeled cross-species PCR fragment based on the sequences of human FUT1 and FUT2. Sequencing of positive clones revealed the full length cDNA sequence and the genomic organization of both genes (Meijerink et al., 2000). Four allelic variants of FUT1 were identified, but so far no sequence differences have been detected in the FUT2 gene. The FUT1 polymorphisms result in amino acid substitutions at position 70 (Leu $\rightarrow$ Phe), 103 (Ala $\rightarrow$ Thr) and 286 (Arg $\rightarrow$ Glu), wherease the T714C transition was silent. A genetic test was developed (Figure 10), and used to genotype 34 Swiss Landrace families with 221 progeny. As expected, the locus controlling resistance and susceptibility to E. coli F18 adhesion and colonization in the small intestine (ECF18R) was closely linked to the locus of the blood group inhibitor S. Moreover, there was a high linkage disequilibrium of  $FUT1^{103Thr}$  and  $ECF18R^{bb}$ , making this allele a good marker for marker-assisted selection of E. coli F18 adhesion-resistant animals (Meijerink et al., 1997). Extended studies yielded preliminary evidence that no more than 5 to 10% of Swiss Landrace, Large White and Duroc pigs are resistant to infection with highly toxigenic E. coli F18. The availability of a diagnostic test for the FUT1^{103Thr} variant provides breeders with the opportunity to eliminate the  $ECF18R^{B}$  allele from their herds, thereby removing a prerequisite for E. coli F18 bacterial adhesion, which causes edema and post-weaning diarrhea. In such a selection program, identification of the RYR1 variants must be applied in order to prevent an increase in the RYR1^{615Cys} susceptibility variants within the population. Preliminary studies in Switzerland did not show any negative correlation between FUT1^{103Thr} to other performances, such as growth rate, meat quality or reproduction traits.


Figure 10. Genetic test for resistency / susceptibility of E. coli F18 adhesion to enterocytes of the small intestine. FUT1-spezific PCR-fragments, containing the diagnostic nucleotide 307, were digested with the restriction enzyme Cfo I (restriction site: GCGC) and separated by gel electrophoresis. (1) FUT1-307^{AA} homozygous resistant, (2) FUT1-307^{GG} homozygous susceptible, (3) FUT1-307^{AG} heterozygous susceptible.

## 4.3.2. FUT1^{103THR}: a good marker or the causative mutation?

From genetic studies alone, it is not possible to "prove" whether an allelic variant causes a particular phenotype or whether it is only linked to a yet unknown locus. The very low recombination frequency of 0.01 (in 221 progeny) that was observed between  $ECF18R^{bb}$  and  $FUT1^{103Thr}$  could be due to either phenotypic misclassification or to FUT1 being only a very closely linked marker. In order to clarify these possibilities, we decided to perform independent functional *in vitro* and *in vivo* tests.

Fucosyltransferases (EC 2.4.1.-) are membrane bound enzymes localized in the trans cisternae of the Golgi apparatus. They transfer a fucose molecule from a fucosyl donor (GDP-fucose) to an acceptor substrate in  $\alpha(1,2)$ ;  $\alpha(1,3)$ ;  $\alpha(1,6)$  linkage. To date, eight fucosyltransferases have been cloned, each of which exhibits its own acceptor preference and that may be developmentally regulated, tissue-specific, or even protein-specific.

FUT1 and FUT2 (EC 2.4.1.69) are  $\alpha(1,2)$  fucosyltransferases in humans that catalyze the penultimate step in the synthesis of the blood group ABO, with FUT1 preferentially using type 2 acceptor substrates and FUT2 preferentially using type 1 acceptor substrates. The product, the H determinant, is prerequisite for the action of  $\alpha(1,3)$ -N-acetyl-D-galactosaminyl

transferase (A-transferase) and  $\alpha(1,3)$ -D-galactosyltransferase (B-transferase) to form the blood group A and B antigens, respectively. Inactivating mutations in *FUT1* inhibit H determinant synthesis, leading to red blood cell phenotype h and a mutated *FUT2* allele (*se*). This further results in a nonfunctional protein, thereby preventing ABO antigen synthesis in secretory tissues (Kelly et al., 1994; Kelly et al., 1995).

The structures of blood group A and 0 glycolipids in pigs are similar to those in humans (Hanagata et al., 1990). However, in the pig, erythrocyte antigens A and 0 are derived from secretory tissues or cells that release antigens into the plasma. Expression of the A0 phenotypes (A,  $A^w$ , 0, –) depends first on the erythrocyte antigen A locus (*EAA*), which is identical to  $\alpha(1,3)$ N-acetylgalactosaminyltransferase (Meijerink et al., 2001) with the *EAA^A* allele enabling synthesis of A antigen, and the *EAA⁻* allele hindering A antigen expression. Secondly, synthesis of the A antigen depends on the epistatic *S* locus, which regulates the expression of precursor 0 (Rasmusen, 1964). The molecular genetics of the loci constituting the blood group A0 system, however, has not yet been clarified in the pig.

The conversion of amino acid 103 to threonine, a polar, uncharged amino acid, instead of alanine (a nonpolar, aliphatic amino acid) may have significant functional consequences. The alanine 103 is conserved in human, mouse and bovine FUT1 and the mutation occurs within the luminal, catalytic domain of the enzyme (Swissprot # Q29043, Domain # PD003541).

Thus, we decided to determine *FUT1* and *FUT2* expression and fucosyltransferase activity in extracts of small intestinal mucosa. Northern blots probed with a *FUT1*-specific sequence revealed no differences in FUT1 or FUT2 mRNA levels in the small intestine of susceptible and resistant animals, indicating that the absence of the ECF18 receptor was not due to an alteration in FUT1 expression. In contrast to the *FUT1* gene, the *FUT2* gene was not expressed in all intestinal samples. Not all ECF18-susceptible animals (ECF18R^{B•}) expressed *FUT2* in the intestine (Table 5, a-f) and *FUT2* was not expressed in all S^{S•} animals (Table 5, c,d). Nevertheless, all animals expressing abundant levels of FUT2 mRNA were S^{S•}, and S^{ss} animals did not express detectable levels of FUT2 mRNA.

In the progeny of the Large White and Landrace families, significant differences were found in total fucosyltransferase activity in small intestinal scrapings, depending on *FUT1* genotype, *FUT2* expression and ECF18R status (Table 5). Resistant animals (f) showed only minor total fucose transfer activities, while susceptible animals showed significantly higher activities (a-e). As we could not find FUT1-specific acceptors in order to distinguish between

Group	Genotype	Fucosyltransferase activity	Phenotype
	FUT1 ³⁰⁷ / FUT2 / S / ECF18R	(pmol/mg protein)	(adherance)
а	GG / + / S● / B●	$16400 \pm 2000$	susceptible
b	AG / + / S• / B•	$10600 \pm 2600$	susceptible
с	GG / − / S• / B•	$2460 \pm 1600$	susceptible
d	AG / – / S• / B•	2600 ± 700	susceptible
е	AG / – / ss / B•	$1670 \pm 450$	susceptible
f	AA / - / ss / bb	58 ± 26	resistant

Table 5. GDP-fucose transfer activity of various genotypes

Table 5 legend: Dots (•) symbolize the second allele for the respective genotypes, which could not be determined, due to the dominance of S and B alleles.

FUT1 and FUT2 activity, we overexpressed FUT1^{103Thr} and FUT1^{103Ala} in chinese hamster ovary (CHO) cells. As expected, the fucose activity in cell extracts expressing FUT1^{103Thr} was significantly lower than that in extracts containing the FUT1^{103Ala} variant. Taken together, these studies clearly demonstrate that the FUT1 enzyme plays a critical role in the process of adhesion of ECF18 bacteria to the mucosa of the small intestine in pigs. This suggests that the *FUT1* gene is a potentially useful target to prevent edemic disease and postweaning diarrhea in piglets. Although these experiments do not constitute conclusive proof that the *FUT1* gene is the causative mutation in these animals, so far we have not found any experimental situations in which our hypothesis could be rejected. Another experimental model that could be used to test our hypothesis could be developed by generating timed FUT1 knockout animals. As F18 fimbriated *E. coli* are host-specific, this could not be generated in a mouse model. Unfortunately, such an animal is be very difficult to achieve as, to date, there has been only one report of a sheep knockout, and this animal only survived for a few days (Denning et al., 2001).

### 4.4. Congenital progressive ataxia (CPA) in swine

Thanks to the astute attention of a breeder in Switzerland, the molecular basis for congenital progressive ataxia (CPA) was recently identified in pig (Kratzsch et al., 1999). CPA is a recessive inherited disease with unknown etiology, as it was originally described by (Rimaila-Parnanen, 1982). Within two days after birth affected pigs show a neuropathic disorder characterized by spastic gait, incoordination, and rapidly progressive ataxia in the hind limbs.

These piglets seldom suck and are often crushed by the mother, making it difficult to identify the disease in practice. Histological examination of the central nervous system and muscular tissue gave no clue to the etiology of this disorder.

To map the CPA phenotype, 60 microsatellites (Rohrer et al., 1994) distributed evenly over the whole genome were examined in an informative family with more than 150 offspring. The linkage analysis showed that *CPA* is located on chromosome 3q1-q14 in the interval *IL1-B* to *ACTG2*. Recombination was estimated to be 0.05 between *SS1066¹⁹⁴* (the 194 base pair allele of microsatellite *SW1066*) and *CPA* (lod score = 11.6), while no recombination occurred between *SW902¹⁸⁹* and *CPA* (lod score = 16.9) (Kratzsch et al., 1999). Looking at the comparative gene map of pig, human and mouse, conserved synteny groups have been described on chromosome 2q1-2 (human) and chromosome 2 (mouse). Based on the comparative gene map, several candidate genes could be involved in this genetic disorder in pig (sodium channel genes *SCN1*, *SCN2A*, *SCN3A*, *SCN6A*; *CACNB4* (β4-subunit of a voltage dependent calcium channel); *mdm* (muscular dystrophy with myositis); *rh* (rachiterata)). *CACNB4* of the pig has been cloned and sequenced (unpublished data). However, until now there has been no evidence that the original mutation for *CPA* is located in the structural part of this gene.

If there is no recombination between a marker and an unknown gene locus in more than 150 informative descendants, it makes no sense to type the pigs with new markers for the localization of the original mutation. Alternatively, the coverage of this region with genomic clones is another possibility to look for unknown genes. This procedure is also called positional cloning or "fine mapping". We are working on covering a chromosomal region of about 1–5 cM on chromosome 3 of the pig with genomic clones. We expect to identify 10 to 50 genes in this interval that could be responsible for the genetic disorder, because of their position.

In the meantime, CPA was identified on a number of farms in Switzerland. The defect can be traced back to a sire imported to Switzerland in 1978. Pigs which are related to this sire and show the microsatellite allele  $SW902^{189}$  associated with the disease must be excluded from breeding.

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# **Curriculum vitae**

Name in full	Stefan Neuenschwander
Address	Birchstrasse 424
	CH-8052 Zürich, Switzerland
Nationality	Swiss
Date of birth	August 10, 1959
Marital status	Married, one child

# Education

since July 1995	Group leader of the Molecular Genetic Group at the Institute of Animal Science of the Swiss Federal Institute of Technology (ETH).
1992-1995	3 years post-doc at the Diabetes Branch of the National Institutes of Health under supervision of Dr. D. LeRoith. Involved in the projects: a) Regulation of IGF-I gene expression in normal and diabetic state. b) IGF-I I system in breast development and breast cancer.
1991-1992	8 months post-doc at the Zoological Institute of the University Zurich (Prof. E. Kubli, Dr. R. Graf). Involved in the project: "Identification of peptide hormone receptors in the yellow fever mosquito <i>Aedes Aegyti</i> : membrane bound tyrosine kinases.
1986 - 1991:	Ph.D. with dissertation: "Hemophilia in sheep and the use of sheep in blood coagulation research" under the supervision of Prof. Dr. V. Pliška and Prof. Dr. G. Stranzinger.
1985 - 1986	Scientific co-worker at the Institute of Animal Science (ETH) involved in the project "Stress in the domestic pig".
1980-1985:	Agriculture Engineering at the Swiss Federal Institute of Technology (ETH), Zürich, Switzerland. Specialized in animal science (animal physiology, breeding, nutrition, animal management and health). Dipl. Ing. Agr. ETH (Zürich, Switzerland), corresponds to a M.Sc. degree.
1966-1980	School education in Zürich, High School Diploma in Bern, Switzerland.

## Publications (reviewed articles and book chapters)

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- 2. Pliška V, Backfisch W, <u>Neuenschwander S</u> (1989): Hämophilie beim Schaf: Modell der menschlichen Bluterkrankheit? Landwirtschaft Schweiz **2** (12), 737-741.
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### Fellowships and awards

1986	Travel stipend from Stiftung F.V.S., Kiel, F.R.G.	
1986-1989	Stipend for graduate degree from Swiss Federal Institute of Technology (ET)	
	Zürch)	
1991	Silver medal and money award from ETH Zürich for Ph.D. thesis.	
1992-1993	Post-Doc fellowship (Swiss National Science Foundation)	
1993-1995	Post-Doc fellowship (Fogarty International Center, NIH visiting program)	
1995	Poster-Award at the Gordon Conference on Mammary Biology and Develop-	
	ment (Colby Sawyer College, New London, NH, USA).	