Physical Mapping of Important Trait Loci in the Pig

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Abstract

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The main driving force for gene mapping in farm animals is to understand the underlying genetics of traits, such as growth, reproduction, disease resistance and production. This study has been focused on physical mapping of important trait loci in the pig. The different physical mapping techniques are powerful to construct restriction maps, reveal syntenic groups of genes and to determine the chromosomal location of genes and markers. The aim has also been to expand the number of genes mapped in the pig and to compare the results with available human data.

The major histocompability complex (Mhc), has a key role in the immune system. A fibre-FISH (fluorescence *in situ* hybridization) analysis was performed using clones from the Mhc class III region in pig. The objective was to fine map the region and to estimate the size of a region using three different approaches, and compare the results with available pulsed field gel electrophoresis (PFGE) data. The results showed that all three methods can be useful and they were in fairly good agreement with the PFGE data.

The *RN* gene has a major effect on muscle glycogen content. The mutant *RN*$^-$ allele is dominant and result in a lower technological yield of cooked ham and reduced meat quality. A FISH analysis of six YAC clones containing five microsatellites from the *RN* region were ordered and assigned to the distal half of band q25 on pig chromosome 15. The results provided a more precise localization of the *RN* gene which facilitated the subsequent positional cloning of this gene.

Chromosome 13 (SSC13) harbours the *K88acR/abR* locus, which encodes a receptor that allows the adherence of enterotoxic *E.coli* bacteria. This makes newborn piglets more susceptible to lethal diharrea. SSC13 also contain QTLs (quantitative trait locus) affecting carcass quality and early growth. In order to expand the comparative map between pig and human, eight genes residing on the human homologue (chromosome 3) were chosen and FISH mapped. The results revealed conservation of gene content but also a number of intra chromosomal rearrangements between pig and human.

In a FISH analysis the *IGF2* gene was assigned to the distal tip of chromosome 2p. QTL analysis using markers from the *IGF2* region revealed a paternally expressed locus with large effect on muscle development. *IGF2*, which also is paternally imprinted became a strong candidate gene. The results further confirms the conserved terminal location of this gene through evolution. The QTL can have practical use, since males having the favourable *IGF2* allele can be selected for breeding.

The *Dominant White* locus in pig is one of the major coat colour loci. Four alleles have hitherto been described, one of them was found in this study. The dominant *Belt* gene causes a white belt across the shoulders and front legs in Hampshire pigs. A genome scan assigned *Belt* to the centromeric region of chromosome 8 (SSC8). *EDNRA*, which was an alternative candidate gene, was FISH mapped to the q-arm of SSC8 and thus excluded. Complete cosegregation in 105 informative meiosis and its phenotypic effect strongly suggested that *Belt* is a fourth allele at the *KIT* locus. A quantitative PCR analysis showed that *Belt* is not associated with a duplication. PFGE analysis was carried out and a BAC contig was constructed to characterize the duplication of *KIT* in white pigs. The size of the duplication was estimated at about 450 kb. The results showed that the entire coding sequence of *KIT* was duplicated but not the flanking genes *PDGFRA* and *KDR*.

Keywords: Pig, Genome Analysis, Physical Mapping, FISH, trait loci
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Contents

Introduction 7
Background 7
Mammalian Genomes 7

Genome Analysis 8
Linkage mapping 9
Physical mapping 10
Restriction site mapping 10
Somatic cell hybrid mapping 11
Radiation hybrid mapping 11
Clone contigs 12
In situ hybridization 12

The pig genome 14
Status of the pig gene map 15

Comparative mapping 15
Chromosome specific libraries 15
Zoo-FISH 16

Aims of this thesis 17

Results and Discussion 18
Fibre-FISH analysis of the porcine Mhc class III region (Paper I) 18
Physical ordering of six YACs from the *RN* region (Paper II) 19
Comparative physical mapping of HSA3 and SSC13 (Paper III) 20
Mapping of the *IGF2* locus (Paper IV) 22
Molecular coat colour genetics (Papers V and VI) 23

Future prospects 28

References 30

Acknowledgements 38

Appendix I-VI

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. A. Sjöberg, L.J. Peelman, B.P. Chowdhary (1997). Application of three different methods to analyze fiber-FISH results obtained using four lambda clones from the porcine MHC III region Chrom. Res. 5, 247-253.

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Introduction

Background

Domestication of animals started about 10,000 years ago. Without genetic knowledge, the early farmers were actually genetically modifying animals by selecting the best and most fit individuals. Genetics as we know it today began with Gregor Mendel, an Austrian monk and botanist, who in 1866 published the results from his experiments with breeding of garden peas. He formulated the laws of heredity (Mendel's laws). The word gene, which is the basic functional unit of heredity and focal point of genetics, was proposed in the beginning of last century.

W.S Sutton observed microscopic structures in the cell and called them chromosomes, they were believed to contain the genes. For various reasons, the importance of Mendel's ideas was not recognized until after his death. Eventually, in 1900, three botanists, De Vries, Correns and Tschermak, each independently rediscovered and confirmed Mendel's theories. About ten years later Thomas Hunt Morgan initiated gene mapping studies. He used *Drosophila melanogaster* (the fruit fly) in his experiments. It was a suitable model organism for building a gene map, because of the short generation time and large number of offspring. Already at this time several genes causing inherited morphological characteristics were positioned on the four pairs of chromosomes of *Drosophila.* Another milestone in the history of genetics was 1953 when Watson and Crick described the structure of DNA, as the molecular carrier of the hereditary material.

Mammalian Genomes

The nuclear DNA in mitotic cells is highly condensed and can be visualized in light microscope as chromosomes. DNA molecules are long chains, the backbone being composed of deoxyribose phosphate units joined to each other through phosphodiester bonds, attached to each deoxyribose is one of four nitrogenous bases (adenine, guanine, cytosine or thymine). Two complementary DNA chains wind along a common axis and form a double helix. At regular intervals the DNA helix is wound around an octamer of two molecules each of the four different histone molecules. This complex is called a nucleosome, and occurs approximately every 145 bp. The chromosome contains two defined regions known as the centromere and the telomere. The centromere is a constriction at a fixed position for each chromosome. During mitosis, the microtubules of the spindle fibres attach to the centomere and separates the two sister chromatides. The telomeres are the terminal regions of a chromosome. The chromatin exists in two forms: the condensed heterochromatin and the euchromatin that is less condensed. Both centromeres and telomeres consist of heterochromatin.

The number and morphology of chromosomes in a species is called the karyotype. The chromosomes are classified on the basis of their size, shape and on the position of the centrome. There are two types of chromosomes the

autosomes and the sex chromosomes. There is an enormous diversity of chromosome number and structure between mammalian species, spanning from the indian muntjak with 2n=6/7, pig (2n=38), cattle (2n=60), horse (2n=64), dog (2n=78) to a South American rodent with 2n=102 (Qumsiyeh 1994). In each species there is a homogametic sex and a heterogametic sex. In mammals, the homogametic sex is the female, bearing two X chromosomes. In birds the male is the homogametic, having two Z chromosomes.

The genomes of mammals are complex and the haploid genome consists of approximately 3 billion basepairs and about 100,000 genes. A minor part of the genome, (5-10%) is coding sequences (Hochgeschwender and Brennan 1991) and the remaining part is non-coding and repetitive DNA. Comparison of the genome size of different eukaryotes shows that the amount of non-coding and repetitive DNA is extremely variable and constitute anything from less than 30 % to about 99 % of the genome (Cavalier-Smith 1985). This DNA probably remain in the genome because of weak selection on the amount of non coding and repetitive DNA.

Genome Analysis

The sequence of the human genome will in the near future be completed, earlier this year it was announced that approximately 80% of the sequence is known. One of the main driving forces for the human genome research is to understand the genetic background of inherited diseases. An important goal is also to develop new and more specific pharmaceuticals with less side effects. The drug industry can utilize the genome information in order to find candidate drug targets. It will also be possible to create individual treatments, since patients suffering from the same disease react differently on medication, due to genetic differences. When the sequence of the human genome is known, the most time consuming and difficult work remains, to interpret the relationship between genes and function.

Cross-species chromosome painting studies with flow sorted or microdissected chromosomes have revealed remarkably high chromosomal homologies among mammals (reviewed in Chowdhary et al. 1998a). The use of this technique has allowed the transfer of gene mapping information from map rich species as human and mouse to other species. The main target for conducting genome analysis in domestic animals is to characterize genes controlling important traits. There is not yet any initiative to determine the complete genome sequence of domestic animals. Nevertheless, sometime in the future the genomes of these species will be sequenced and it will thus be much easier to find genes responsible for a certain disease or a trait. Gene maps are an important source of information on the genetic organization of individual species, and the use of gene mapping for breeding healthy animals with improved productivity is increasing. Today, there are several gene maps established in all farm animals, like pig, cattle, sheep, horse and chicken (see review by Georges and Andersson 1996).

Gene mapping is a composite approach that needs confirmation by the use of complementary techniques. Basically, there are two kinds of genome maps: physical and genetic. The genetic map is based on recombination frequencies and is obtained by linkage analysis of polymorphic markers. The relative order and distance between markers are deduced using the frequency of recombination between markers. Physical mapping determines the chromosomal position of a marker or a gene.

Markers used for gene mapping have been named type I, type II (O'Brien et al. 1991) and type III (Andersson et al. 1994b). The type I markers are coding sequences, that are evolutionary conserved and suitable for comparative gene mapping. Type II markers are highly polymorphic, for example microsatellites and thus suitable for linkage analysis. Type III markers have been mapped both by linkage and physical mapping and are used to connect the linkage and physical map.

A trait can be mapped to a region by linkage analysis, and the final goal is to clone the gene and identify the causative mutation. Monogenic traits are controlled by a single gene, while multfactorial traits are controlled by an unknown number of genes together with environmental factors. A locus influencing a quantitative trait like litter size or carcass quality, is referred to as a QTL (quantitative trait locus). A QTL is thus a chromosomal region containing one or several genes responsible for the observed genetic variation.

Linkage mapping

A collection of polymorphic markers and a suitable pedigree are required when constructing a linkage map. The most commonly used markers for linkage analysis in mammals are currently microsatellites, which are short tandem repeats of 1-5 basepairs and the number of repeat copies varies between individuals. They are analysed with the polymerase chain reaction (PCR) technique (Saiki et al.1985; Mullis et al. 1986) in combination with a gel electrophoresis step. The pig genome is estimated to contain 65,000-100,000 microsatellites (Archibald et al. 1995). A particularly powerful approach for linkage mapping is to generate a resource pedigree by crossing two divergent breeds to get a high heterozygosity in the F1 generation. The F1 animals, are either mated to each other to get an F2 generation, or to one of the parental populations, thus creating a backcross.

Genetic linkage between two loci occurs when they are cosegregating during meiosis. The further they are located from each other the possibility decreases that they show linkage. Markers on different chromosomes segregate independently. The recombination frequency, which is a measure of the distance between the two markers range from 0% to 50%. In case of genetic linkage, the recombination frequency will be lower than 50%. The distance is expressed in centimorgans (cM); one cM corresponds to 1% recombination.

Meiotic recombination is a fundamental process that increases the genetic diversity in the gametes. The frequency of recombination varies along the chromosome as well as between the sexes and over age. Regions close to telomeres tend to recombine more frequently, and the heterogametic sex generally

show less recombination than the homogametic sex (Dunn and Bennett 1967). It has been reported that recombination in females decreases over age (Tanzi et al. 1992). In addition, there are certain areas called "hot spots" (Steinmetz et al. 1986), where recombination events are frequent. QTL mapping involves a statistical analysis testing for cosegregation at marker loci and loci controlling the quantitative trait (Haley et al. 1994). The analysis is repeated at every cM along the chromosome and the results are plotted against the chromosome map. The highest peak on the plot indicates the most likely position of a QTL. However, the relationship between genotype and phenotype is not direct, because of the environmental factors involved, meaning that the QTL will not be able to explain 100% of the phenotypic variation observed in the pedigree. The cloning of a QTL is a very difficult task due the poor precision in QTL mapping and because causative mutations may have larger effects than those causing obvious disorders.

Physical mapping

The positioning of genetic markers and genes is made through physical mapping approaches (Chowdhary 1998b). There are cytogenetically based methods and molecularly based methods. Somatic cell hybrid mapping reveals syntenic groups of genes, meaning that they are located on the same chromosome. To further find out the order of genes and markers, mapping using radiation hybrid panels is the method of choice. The FISH technique (fluorescence *in situ* hybridization) makes it possible to assign cloned DNA fragments to specific chromosome regions. A molecular method such as restriction site mapping has been widely used. Pulsed field gel electrophoresis (PFGE) is used to determine the size and order of large fragments obtained from digesting genomic DNA with rare-cutting enzymes. Construction of contigs of large genomic insert clones are useful for STS mapping and candidate gene cloning. I will in more detail go through a number of physical mapping techniques and discuss resolution, utility, advantages, and disadvantages.

Restriction site mapping

A long-range restriction map contains the relative position of the restriction sites for restriction endonucleases on genomic DNA. The standard strategy for mapping restriction sites in genomic DNA involves digestion of high molecular weight DNA with rare cutting enzymes, gel electrophoresis, blotting and hybridization (Brown and Bird 1986). The fragments will be too large for separation by conventional electrophoresis and therefore pulsed field gel electrophoresis (PFGE) is used. The technique was developed by Schwartz and Cantor (1984) and is based on electrophoresis with alternately pulsed electrical fields. In the present study a CHEF mapper (contour-clamped homogenous electrical field) has been used (Chu et al. 1986; Cantor et al. 1988) where a hexagonal array of fixed electrodes is used and this creates a homogenous electrical field resulting in enhanced resolution of large DNA fragments. The gel obtained is blotted to a nylon membrane and used for hybridizations to labelled probes.

Somatic cell hybrid mapping

The technique for producing somatic cell hybrids was developed in 1975 by Goss and Harris. It involves a fusion between cells from the species the map will be made for (donor cells) and cells from a second species (recipient cells). Rodent cell lines, such as hamster are often used as the recipient line. After fusion, there is a progressive and preferential loss of the chromosomes derived from the donor species. By using a selective medium only the hybrid cells will survive. The hybrid clones may be characterized for their foreign chromosomal content by cytogenetic methods. Banding techniques are time consuming, but if a species specific SINE (short interspersed elements) (Frengen et al. 1991) is used as FISH probe the chromosomal content can be revealed (Zijlstra et al. 1994)

The presence of a particular locus in a cell line can for instance be detected with electrophoretic analysis of isozymes or by PCR analysis. The result will be either positive or negative meaning that the marker is present in the cell line or not. The approach will reveal syntenic loci (present on the same chromosome), but not the order of the genes/marker. A marker does not have to be polymorphic, to be mapped by this technique.

The number of chromosomes from the donor cells will vary greatly from one hybrid cell line to another. This heterogenity leads to the fact that some chromosomes occur more frequently than others, therefore classification based on the frequency with which a chromosome is found in the cells of a hybrid cell line is essential (Zijlstra et al. 1994, 1996). Somatic cell hybrid panels have been developed for pig (Rettenberger et al. 1994b; Yerle et al. 1996; Zijlstra et al. 1996), horse (Shiue et al. 1999), cattle (Ma et al. 1998) and sheep (Burkin et al. 1998).

Radiation hybrid mapping

A radiation hybrid panel (RH) differs from a somatic cell hybrid in the sense that the donor cells are irradiated with high doses of X-ray before fusion (reviewed in McCarthy 1996). The chromosomes of the donor cells will thus be fragmented, but will later be recovered by fusion to recipient chromosomes. The analysis is carried out in a similar way as for somatic cell hybrid mapping. The distance between genes/markers is expressed in centiRays (cR) and the order along the chromosome can be determined. A distance of one cR between two markers corresponds to 1% frequency of chromosomal breakage between these two markers after exposure of a particular X-ray dose (Walter and Goodfellow 1993). This value in cR can be changed to a physical distance with the help of PFGE data or YAC cloning experiments. The relationship between the distance in cR and the actual physical distance depends on the radiation dose. In 1990, Cox et al. used a modified version of the method for constructing a high-resolution map of human chromosome 21. RH panels exist for several farm animals such as pig (Yerle et al.1998), cattle (Womack et al. 1997) and horse (Kiguwa et al. 2000).

Clone contigs

The construction of large insert libraries and clone contigs covering a particular chromosome is a very important step in detailed genome analysis. The overlapping clones can be searched for candidate genes. Until 1987, when Burke and coworkers described the development of YAC (Yeast Artificial Chromosome) vectors, it was only possible to clone fragments up to 40 kb in cosmid vectors (Anand 1992). YACs can harbour inserts up to 1 Mb, but they are instable and have a high tendency of being chimearic. Several porcine YAC libraries have been constructed (Leeb et al. 1995; Alexander et al. 1997; Rogel-Gaillard et al. 1997). YAC libraries are available for other farm animals, for example cattle (Smith et al. 1996).

BAC (Bacterial Artificial Chromosomes) is another type of large insert vector that can carry up to 300 kb inserts (Shizuya et al. 1992), however the range of normal insert sizes in a BAC is 100-200 kb. The advantage with BAC clones over YAC clones is that they are less chimeric and easier to work with. There are several BAC libraries constructed for the pig genome (Rogel-Gaillard et al. 1999; Suzuki et al. 2000; Anderson et al. 2000). The procedure for chromosome walking and contig construction is by selecting BAC or YAC clones from known STSs (sequenced tagged sites) and develop and map new STS markers. An STS is a piece of DNA, around 200 bp that is uniquely defined by a primer pair. Two clones that contain the same STS must therefore overlap.

In situ hybridization

ISH (*in situ* hybridization) is an effective and rapid method to determine the chromosomal location of specific sequences, to identify chromosomes or detect chromosomal abnormalities. The technique was introduced by Gall and Pardue in 1969. The method involves a direct hybridization of probes to chromosomes fixed and denatured on glass slides. In the beginning, the probes were radioactively labeled with Tritium (^{3}H). The technique had many drawbacks, like poor resolution, long exposure times and high background making it difficult to distinguish between true signals and background noise. The problems were solved when nonisotopic fluorescence ISH (FISH) was developed (Langer et al. 1981; Pinkel et al. 1986; Lichter et al. 1991 and Trask 1991). It became possible to label the DNA with nucleotides coupled to molecules like biotin or digoxigenin. When the DNA is biotinylated, the hybridization signal is detected with indirect immunofluorescence, which means that avidin coupled to fluorescein isothiocyanate (FITC) is added to the slide. To increase the signal intensity an anti-avidin antibody is added. By using different fluorochromes such as Rhodamin, FITC and Texas red it is possible to hybridize several probes in the same experiment (paper I and II). The labelling can also be direct, which means that the probe is directly coupled to a fluoroscent dye. Direct labelling can be advantagous because of decreased background noise. However, the use of indirect labelling is the most commonly used method.

The resolution of a FISH experiment depends on the degree of condensation of the target DNA. During the mitotic cell cycle the chromatin in the cells condense into well-defined chromosomes (metaphase stage) and as the degree of condensation increases the resolution decreases. FISH on metaphase chromosomes has a resolution down to 1 Mb, and the resolution can be increased if the chromosomes are subjected to cytocentrifugation, which involves mechanical extension of the chromosomes. When conducting FISH analysis on such target DNA, it is possible to separate probes only 200 kb apart (Laan et al. 1995).

Mapping in interphase cells was first introduced by Lawrence et al. (1988) and the technique is based on the assumption that the chromatin behaves like a random polymer over distances of 1-2 Mb (Van den Engh et al. 1992; Trask et al. 1993). Thus, with mathematical models it is possible to calculate the distance between two probes located 50-1000 kb apart. A multicolour interphase FISH analysis was able to resolve the order of three genes (Chowdhary et al. 1995).

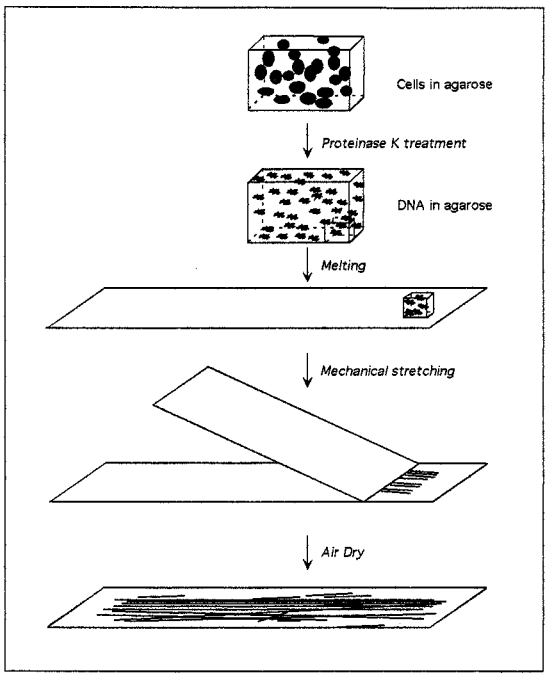


Figure 1. Schematic drawing showing the principle of preparing DNA fibres for a FISH analysis (from Heiskanen et al. 1994).

High resolution FISH techniques in which released, less condensed, linearized chromatin or DNA fibres are used as target DNA, are called fibre-FISH (Florijn et al. 1995). The target DNA, can be prepared in several ways. One way is to release the chromatin from fixed cells with sodium hydroxide or formamide (Fidlerova et al. 1994). Another way is to mechanically extend genomic DNA embedded in low melting agarose described in Fig.1 (Heiskanen et al. 1994, 1996). The latter technique has been used in this thesis (paper I). Theoretically the resolution of the technique is down to 1 kb and probes separated up to 400 kb can be detected. The limitations are the length of the fibres and the available microscope equipment. The technique has demonstrated its usefulness in a study of genomic rearrangements in human small cell lung cancer (Heiskanen et al. 1995). Fibre-FISH is powerful when it comes to order clones/genes (relative to each other), measure distances between genes, find out if the genes are overlapping or detect deletions and duplications.

The pig genome

Cytogenetic investigations of the domestic pig (*Sus scrofa*) started in the beginning of last century. Various studies during several years reported a diploid number of 40 but the correct number of 2n=38 pig chromosomes was finally determined by Gimenez-Martin et al. (1962) and Mc Connell et al. (1963). However, some European wild boars have only 17 autosomal pairs (2n=36) because of a Robertsonian fusion between SSC15 and 17. A standard for the pig karyotype has been established (Committee for the standardized karyotype of the domestic pig 1988).

Compared to several other farm animals, the pig chromosomes are easier to recognize without any particular staining method because of their small number and very variable size and shape. They consist of 5 metacentric-, 7 submetacentric- and 6 acrocentric chromosome pairs plus the X and Y. The size of the pig genome has been estimated to 2.7×10^9 bp (Schmitz et al. 1992). The pig is suitable for genome analysis for several reasons. The short gestation time (114 days), large litter size and the short generation time (approximately one year) make it feasible to establish resource pedigrees. It is also an important animal in many other aspects. It is a fast growing animal used for meat production, it can function as an animal model for human diseases because of similar physiological properties, and it is a possible organ donator in future xenotransplantations. Several linkage maps for the porcine genome have been described (Archibald et al. 1995; Marklund et al. 1996; Rohrer et al. 1996).

In the pig genome several QTLs have been detected. A QTL having large effects on growth, length of the small intestine and fat deposition was found on pig chromosome 4 (Andersson et al. 1994a; Knott et al. 1998). The finding of the fat QTL was confirmed in Marklund et al. (1999). Moreover, a QTL affecting early growth rate (Andersson et al. 1994; Knott et al. 1998) and carcass quality

(Yu et al. 1995) was located on pig chromosome 13. A QTL on the p-arm of chromosome 2 with moderate effect on muscle mass was reported in Andersson-Eklund et al (1998). It was demonstrated in paper IV that IGF2 is a strong candidate gene for that QTL (Jeon et al. 1999). In Rohrer et al. (2000) several genomic regions affecting birth characters and accretion of weight and backfat in a Meishan-White backcross were found on different pig chromosomes.

Status of the pig gene map

Summaries of the information stored in databases of gene mapping projects in different farm animals can be found at:
http://www.ri.bbsrc.ac.uk/cgi-bin/arkdb/browsers/browser.sh?species.
Today, the number of assigned loci in the pig are and 2097 of which 631 are designated genes. Among them, 499 of them have been mapped by *in situ* hybridization and 1292 are microsatellites.

Comparative mapping

Comparative mapping demonstrates that genome organization is well conserved in mammals, much more than expected from the variety of karyotypes. Comparative maps display the chromosomal location of homologous genes in different species and conserved genetic segments in evolution. The gene mapping information available for human and mouse can be transferred to other species only with well-developed comparative maps. One way to achieve this task is by comparative chromosome painting. However, there are limitations of the techniques, the probe has to be at least 5 Mb in order to detect a hybridization signal (Schertan et al. 1994). At the same time, it gives only partial information about intrachromosomal rearrangements. In this case the technique should be complemented with available linkage or RH mapping data.

Chromosome specific libraries

Chromosomes can be separated and isolated using two different approaches, either by flow-sorting or by chromosome microdissection. For isolation by flow sorting, the chromosomes are fluorescently stained and separated using a FACS (fluorescence activated cell sorter system). The amount of fluorescence detected is proportional to the DNA content in each individual chromosome (Lebo 1982). The technique of microdissection involves removal of the desired chromosomal material from a metaphase spread with either a dissecting needle or a laser beam (Lüdecke et al. 1989; Senger et al. 1990).

Physical dissection of an entire chromosome or a chromosomal band and recovery of DNA fragments have a great advantage in aquiring a large number of clones from a defined region, referred to as chromosome specific libraries (CSL). Flow sorting of chromosomes was the first technique used for constructing CSL (Collins et al. 1991; Voojis et al. 1993). With chromosome microdissection, a library from only one arm, a region or even a single band of a chromosome can be

obtained (reviewed in Cannizzaro 1996; Saitoh and Ikeda 1997). The collected material is treated with topoisomerase (Guan et al. 1993), amplified by PCR using degenerated oligonucleotide primers (Telenius et al. 1992) and cloned. The amplified DNA can be labelled with for example biotin and used for chromosome painting in a FISH analysis. There have been some reports of chromosome microdissection involving pig (Ambady et al. 1997; Chaudhary et al. 1998) and cattle (Schmutz et al. 1994). It is possible to isolate new markers from such a library, but the technique is time consuming and a more efficient and approach is to use BAC or YAC clones for the construction of contigs covering the region of interest.

Chromosome libraries obtained from flow sorted human chromosomes have been used for chromosome painting in evolutionary studies between human and several different species. The term chromosome painting was used for the first time by Pinkel et al. (1988). A CSL can be useful in cytogenetic studies, as a diagnostic tool for detection of numerical and structural chromosomal aberrations, (translocations, inversions, deletions, amplifications), marker chromosomes (Viersbach et al. 1994) and double minutes (Rajcan-Separovic et al. 1995). These applications also include prenatal diagnostics.

Zoo-FISH

The concepts of evolution implies that any pair of two species share a common ancestor carrying an ancestral genome. Comparative chromosome painting (Zoo-FISH) is a powerful method for detecting evolutionary conserved chromosomal regions (see review Chowdhary et al.1998a). It involves hybridization of whole or parts of chromosomes derived from one species to metaphase chromosomes of a second species. Prior to cross-species hybridization, the probe has to be tested for purity and origin. The Zoo-FISH can be either monodirectional e.g pig to human or bidirectional pig to human and human to pig. Human CSL have been hybridized to pig (Frönicke et al. 1996, Rettenberger et al. 1995a), horse (Raudsepp et al. 1996), cat (Rettenberger et al. 1995b) and cattle chromosomes (Solinas-Toldo et al. 1995; Chowdhary et al. 1996). Bidirectional heterologous painting allows to detect segment to segment homologies between the two species and may reveal new homologies combined with comparative gene mapping data. Bidirectional chromosome painting has been done between pig and human (Goreau et al. 1996) and cat and human (Wienberg et al. 1997).

Aims of this thesis

The objectives of this study have been:

- to use fibre-FISH to study the organization of the porcine Mhc class III region
- to use FISH analysis to increase the number of mapped loci on pig chromosome 13 and to precisely map the *RN* gene
- to map *IGF2* and evaluate it as a candidate gene for growth and development
- to study the molecular basis for the *Belt* and *Dominant White* coat colours in the domestic pig

Results and Discussion

Fibre-FISH analysis of the porcine Mhc class III region (Paper I)

The Major Histocompatibility Complex region (Mhc) is one of the most genetically variable regions in mammalian genomes. The diversity depends mainly on two things, presence of multiple isoforms and allelic polymorphism (Andersson 1996). The Mhc consists of three different classes of genes. Class I and II genes encode antigen presenting molecules that are present on the cell surface. They bind and present peptides to T-cells of the immune system. The Mhc class I molecules present endogenously derived peptides to cytotoxic T-cells while Mhc class II molecules present exogenously derived peptides to T-helper cells. Class III genes encode a variety of proteins, some that are directly or indirectly related to the immune system but are not structurally related to class I or class II genes. It is therefore the most poorly studied class of Mhc genes. Several of the class III loci in human and mouse are associated with specific immune parameters (Schook 1996).

The Mhc in pigs also denoted as the SLA (swine leukocyte antigen) complex plays an important role in the immune system. It has been proposed that some SLA haplotypes influence physiological traits, such as carcass composition and growth rate performance, which might be related to genetic variation in the class III region (Peelman et al. 1996). The association between a specific haplotype and a phenotype is very much dependent on several parameters such as the pig breed, the age of the animals and the type of analysis used (Schook 1996; Mallard et al. 1991). It can not be excluded that the observed differences are secondary effects, and the primary effect involves resistance to infectious diseases. It is notable that SLA class I and class III genes are assigned to the p-arm of chromosome 7, whereas class II genes are on the q-arm with the centromere located in between (Smith et al. 1995).

A fibre-FISH experiment was carried out with four different lambda clones derived from the region (λG11, λG14, λG17 and λC4) in order to fine map the SLA class III region and to compare the results with available PFGE data (Peelman et al. 1996). The chromosomal location of individual probes was confirmed by hybridization to porcine metaphases. The probes were then cohybridized in two different combinations to mechanically stretched porcine DNA fibres. Three different methods were used to convert the measurements from fibre-FISH to physical distances in the same stretch of DNA. This was the first fibre-FISH experiment carried out in farm animals. The method has previously been used in humans (Heiskanen et al. 1995; Klockars et al. 1997).

The WCS (Watson-Crick standard) method converts the measurements on the basis that one micrometer of DNA corresponds to 2.9 kb. In the PSS method (probe size standard) the values are calibrated using the known size of one probe included in the study. The RL (relative length) method is expressing the relative percentage values of the distances.

There are advantages and drawbacks associated whith the different methods. The WCS assumes that all fibres are uniformly streched on the slide, this may not be the case due to incomplete digestion of extranuclear material or by variation in the mechanical stretching of the fibres. The method gives a fairly unbiased quantification of the distances. For the PSS, the calibration can be misleading if the probe contains a lot of repetitive sequences at one or two ends. The repetitive sequences are blocked during prehybridization annealing, allowing unique sequences to produce signals. This means that the signal will appear shorter than expected. On the contrary, if the probe contains repetitive sequences in the middle, the length of the obtained hybridization signal will not be affected. The RL method had not been used until this study was conducted. It is a suitable method if no specific software is available to convert the measurements of DNA fibres into physical distances. In this study, the relative values in percent were converted into kilobases based on the size of the DNA stretch derived from PFGE data. This makes the estimations fairly biased towards the PFGE results.

The estimates obtained from the three different approaches were compared and divided into two categories, namely probe size and gap size. For the probe size, the RL method gave larger estimates and they were also in close agreement with PFGE data. Compared to PGFE the size of the two probes, λG11 and λG17 obtained from WCS and PSS were in fairly good agreement and the values for λG14 and λC4 were considerably lower. The size of the gap between λG17 and λG14 were similar between the three methods and smaller compared to PFGE data. For the gap λG14-λC4 the RL and WCS were close but slightly smaller than the PFGE data while the PSS values were larger.

For the gap between λC4 and λG11, WCS and PFGE data were in good agreement while RL and PSS gave smaller and larger estimates, respectively. For the two gaps λG11-λG14 and λG11-λG17 the PFGE, RL and PSS were in good agreement but the WCS gave smaller estimates.

The differences observed for the size of the λC4 probe using RL and WCS/PSS methods may be due to genetic variation or polymorphism. For the RL method DNA from the backcross between Large White and Wild Pig were used and for the two other methods, DNA from a Hampshire animal was used. The λC4 contains the genes *C4*, *CYP21* and part of *G12*. The region is known to be evolutionary unstable and has undergone tandem duplication in several mammalian species, like human, mouse and some ruminants (Kawaguchi et al. 1991). So far there have not been any indications of a duplication of these genes in the pig.

The estimates for different parameters obtained for the same stretch of DNA, using these three different methods, provided a comparative overview of applicability and suitability of the individual approaches.

Physical ordering of six YACs from the *RN* region (Paper II)

The *RN* (Rendement Napole) has a major effect on muscle glycogen content. The mutant allele, *RN* , occurs at a high frequency in the Hampshire breed. *RN* is dominant and results in a lower technological yield of cooked ham and reduced

meat quality (Naveau et al. 1985; Le Roy et al. 1990; Estrade et al. 1993a, b). The frequency of the mutant allele has most likely increased because *RN*⁻ carriers show higher growth rate and produce carcasses with larger lean meat content compared to animals carrying the normal allele (Enfält et al. 1997). The *RN* locus had previously been mapped by linkage analysis to the distal part of chromosome 15 (SSC15) (Mariani et al. 1996).

This study was carried out to fine map the *RN* region. Six YACs representing five microsatellite markers (Sw120, Sw1683, Sw2083, Sw1309 and Sw936) were physically mapped with dual colour FISH analysis in ten different combinations. The assignments of them to the distal half of SSC15q25 were in agreement with previously reported physical mapping (Milan et al. 1996), but our study revealed a more precise localization. The genetic distance covered by the markers in the present study is about 12 cM (Rohrer et al. 1996). The physical size of the region is approximately 15 Mb (estimated on the basis of the fractional length of the distal half of SSC15q25 band (Lin et al. 1980) and the proposed size of the distal half of the same band (Schmitz et al. 1992). The clones were pairwise cohybridized and the order along the chromosome was determined.

The results of the hybridizations were divided into three different categories viz., favoured (the order predominantly observed), reversed order (the order reversed as to compared to the latter) and overlap (where the signals overlapped to the extent that it was not possible to assess the order of the clones). A χ^2 test was carried out to statistically analyze the results. The order of microsatellites Sw120 and Sw1683 was deduced and in addition the order of Sw2083 and Sw1309 was found to be reversed as compared to the USDA-MARC.2 linkage map. The position of Sw1309 on the USDA-MARC.2 map was based on only 26 informative meioses. When markers are closely linked to each other it is important to have as many meioses as possible to make the analysis reliable. The study gave a more precise physical localization of the *RN* gene and revealed the uncertain order of the microsatelleites mapped to the region.

Today, the *RN* gene has been positionally cloned and identified as being in the *PRKAG3* gene, encoding a muscle specific isoform of the regulatory γ subunit of AMP-activated protein kinase (*AMPK*) (Milan et al. 2000). A non-conservative substitution in the gene was found as the causative mutation.

Comparative physical mapping of HSA3 and SSC13 (Paper III)

The middle part of the longest acrocentric pig chromosome, number 13 (SSC13), harbours the *K88acR/abR* locus (Edfors-Lilja et al. 1995). It encodes a receptor that allows the adherence of enterotoxic *E.coli* bacteria, which makes the neonatal piglets more susceptible to lethal diharrea. QTLs affecting carcass quality (Yu et al. 1995) and early growth (Knott et al. 1998) have also been mapped to SSC13. The major part of SSC13 has demonstated homologies with human chromosome 3 (HSA3), except for the three terminal bands that correspond to human chromosome 21 (Rettenberger et al. 1995; Frönicke et al. 1996; Goreau et al. 1996).

The objective of this study was to expand the comparative map between pig and human, eight genes residing on HSA3 were chosen and physically mapped to pig chromosomes by FISH analysis. In this and our previous study (Van Poucke et al. 1997) ten type I markers residing on HSA3 were FISH mapped to porcine chromosomes (Fig. 2). The two chromosomes were divided into four blocks and possible rearrangements of these blocks were deduced. It appeared that the order of blocks and the order of genes within each block were inverted. Despite the inversions, the blocks on the HSA3 p-arm tend to keep together in the proximal part of SSC13, the same was the case for the blocks on the human q-arm, they were found inverted on the distal half of the pig chromosome (Figure 3). Jørgenssen et al. (2000) presented the mapping of eigtheen genes to SSC13 that were found on HSA3. The authors revealed possible chromosomal breakpoints and confirmed our results (van Poucke at al. 1997; this study). Mapping of these coding sequences to SSC13 contributed to, and confirmed the existence of conserved synteny between SSC13 and HSA3.

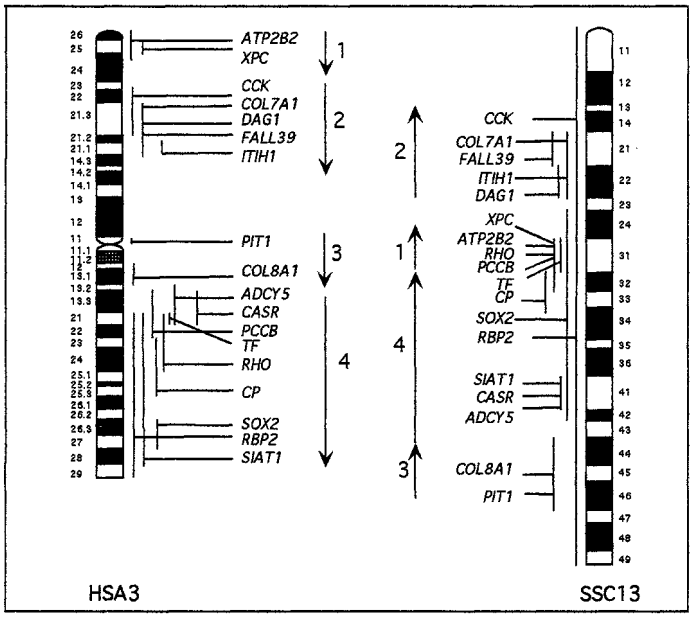


Figure 2. A schematic drawing of the human chromosome 3 (HSA3) and pig chromosome 13 (SSC13). In addition to the ten genes mapped by us (paper III and Van Poucke at al. 1997) eight other genes mapped to this region was included for comparative purposes. The genes are divided into blocks and the orientation of each block is indicated with arrowheads. (http://www.ri.bbsrc.ac.uk/pigmap/pigbase/pigbase.html; http://gdbwww.gdb.org/gdb-bin/fg/genes)

Mapping of the *IGF2* locus (Paper IV)

Parental imprinting is an important genetic mechanism, by which some genes are exclusively expressed depending on whether they are inherited from the mother or the father. The reason for imprinting is still unknown, but DNA methylations play an important role. Insulin like growth factor 2 (*IGF2*), has shown to be paternally imprinted in mouse and humans (de Chiara et al. 1991; Ohlsson et al. 1993). Another imprinted gene, *H19,* which has been shown to be maternally expressed is located downstream of *IGF2* (Bartolomei et al. 1991).

The aim with the study was to map the *IGF2* gene in pig and evaluate it as a candidate gene for growth and development. A BAC clone containing *IGF2* was used in a FISH analysis and the gene was assigned to the terminal end of the p-arm of pig chromosome 2 (SSC2pter) (Fig 3). Previously, *IGF2* has been physically assigned in several species including human (Henry et al. 1985), sheep (Ansari et al. 1994), cattle (Schmutz et al. 1996), horse and donkey (Raudsepp et al. 1997). In these species *IGF2* has retained a terminal chromosomal position. Our study further confirms the terminal location of this gene during mammalian evolution.

The intercross between Euopean Wild Boar and Large White domestic pigs were genotyped for a polymorphic microsatellite (*Swc9*) located 800 bp downstream of the *IGF2* stop codon. This made it possible to determine the breed and parent of origin for each allele in F2 animals. A quantitative trait locus (QTL) on SSC2p with a moderate effect on muscle mass had previously been reported (Andersson-Eklund et al. 1998). In this study, QTL analyses was carried out using a statistical model (Knott et al. 1998), testing for the presence of an imprinting effect. The QTL at the distal tip of SSC2p had large effects on lean meat content, heart weight and backfat thickness. Noteable is that it did not have any effect on abdominal fat in contrast to the *FAT1* QTL located on pig chromosome 4 (Andersson et al. 1994a; Knott et al. 1998; Marklund et al. 1999). The Large White allele at the *IGF2*-linked QTL was associated with larger muscle mass and reduced backfat thickness. The results demonstrated that a paternally expressed QTL maps to the same position as *IGF2*. This together with the fact that both the gene and the QTL are imprinted makes *IGF2* a possible candidate gene for the QTL effect.

This study suggests that *IGF2* not only has an important role during prenatal development, but also in postnatal development. The findings that a single locus with a large effect is controlling the trait differ from the classical model that a large number of genes, each having a minor effect, are responsible for QTL variation. The QTL can have practical implications for the pig industry. Breeding of individual males with many females should favour the selection of alleles at paternally expressed QTLs. In order to find polymorphisms and look for association to the QTL, comparison of the sequence of the region from several pig breeds including the Wild Boar is currently in progress.

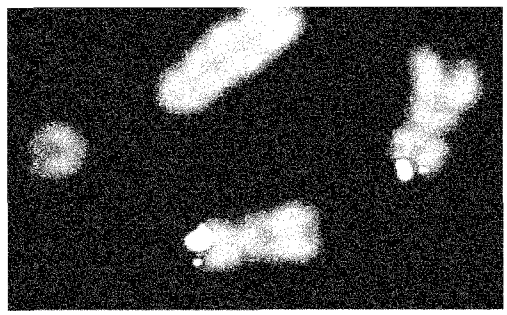


Figure 3. A partial porcine metaphase spread showing hybridization of the BAC clone containing *IGF2* on the distal tip of chromosome 2p.

Molecular coat colour genetics (Papers V and VI)

Pigmentation involves a large number of genes that interact with each other (Hearing and Tsukamoto 1991; Jackson 1994). The phenomenon has been well studied in mouse and almost 100 genes and 150 mutations have been found (see reviews by Silvers 1979; Jackson 1991; Jackson 1997). Coat colour genetics has contributed to our general understanding of gene action and gene interaction. In farm animals diagnostic tests for causative mutations may be developed and used in breeding programmes in order to ensure that the animals will have the desired coat colour.

The melanocytes are the cells producing pigment in skin and hair, they originate and migrate from the neural crest and reside in the epidermis and in the hairbulbs. Pigment is the substance that gives colour to skin and hair and protects the skin against harmful UV radiation. It can exist as granules or be dissolved in the melanosomes, which are organelles within the melanocytes. There are two variants of pigment; eumelanin (brown/black) and phaeomelanin (red/yellow).

In mouse, several genes influencing the pigmentation have been described. There are genes influencing the migration and development of melanocytes, the gene expression in melanocytes, the morphology of the melanocytes, the structure and function of melanoytes, the enzymes in melanin synthesis and genes controlling the type of pigment produced. Loci influencing melanocyte migration, proliferation and differentiation include *dominant white spotting* (*W*) and *steel* (*Sl*). The development of three unrelated cell types is affected by these loci.

W is an allele of the *KIT* gene encoding the mast/stem cell growth factor receptor, which belongs to the tyrosine kinase transmembrane receptor family. The receptor consists of a ligand-binding extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain. *Sl* encodes the ligand known as mast/stem cell factor or the Steel factor. The binding of the ligand to the receptor leads to an intracellular signal transduction affecting survival and migration of primordial germ cells, melanoblasts and hematopoietic cells (Vandenbark et al. 1992). Loss of function mutations at the *W* locus has mild effects on the pigmentation in the heterozygous state, but a more dramatic effect in homozygous individuals. Homozygotes for *W* mutations often show

pigmentation disorders, anemia or sterility and they are often lethal (Pawson and Bernstein 1990). The mutations *Dominant white spotting (W)* in mouse and *Piebald* in humans display similar patches of white hair and skin in heterozygous individuals (Fleischman et al. 1991). The pigmented cells in the eye (pigmented retinal epithelium, PRE), is not influenced by these type of mutations because these melanocytes differentiate from the optic cup.

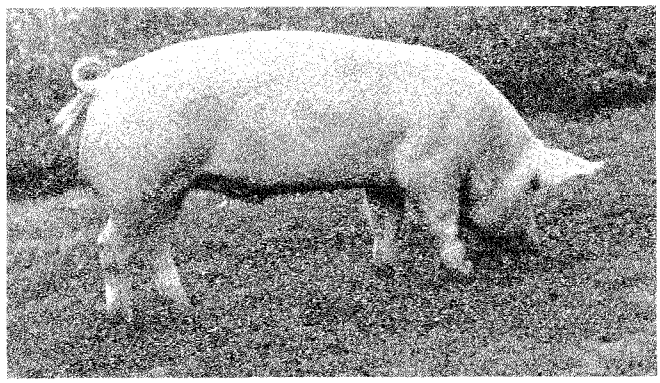
Another mutation at *W* locus is the *Rump White* (*Rw)* mutation. In mouse it is caused by an inversion in the dipeptidyl aminopeptidase-like protein 6 (*Dpp6*), leading to a dysregulation of *Kit* (Hough et al. 1998). *Ph* is a mutation which is caused by a deletion of not more than 400 kb including the closely linked *Pdgfra* gene (platelet derived growth factor α) (Nagle et al. 1994). A study by Duttlinger and associates (1995) demonstrated that the 3' deletion endpoint is located between *Pdgfra* and *Kit* and therefore, negative 5' upstream elements controlling *Kit* expression are most likely affected.

The *KIT* gene is located on mouse chromosome 5 and human chromosome 4q12 (Spritz et al. 1994). In pig, the gene is assigned to chromosome 8p12 (Johansson-Moller et al. 1996). These findings are in agreement with previous comparative mapping data (Frönicke et al. 1996; Goreau et al. 1996). Both *KIT* and *PDGFRA* belong to the tyrosinase kinase receptor family (RTK). An additional gene, *KDR* (kinase insert domain receptor) or *FLK1*, which also is a member of the RTKs is physically mapped to chromosome 4q12 in humans.

For pig breeders, the white coat colour is economically important since pork is marketed with the skin attached and remains from hair rots and pigmented skin can be mistaken as mould. In the USA the skin is removed from pigs with pigmented spots. Prior to this study, three alleles had been identified at the *I/ Dominant White* locus: *I*, I^P (*Patch*), and *i* (*wild type*). Both the *I* and I^P alleles carry a large duplication of the entire *KIT* gene (Johansson-Moller et al. 1996). The molecular difference between *I* and I^P is that one copy of the gene in the *I* allele contains a splice mutation leading to the skipping of exon 17 (Marklund et al. 1998). It is possible to detect this with a PCR-RFLP test, since the first nucleotide in intron 17 is changed from G to A, creating a *Nla*I restriction site. The Patch phenotype has a white coat with fully coloured patches (both skin and hair) with sharp borders. The *Dominant White* mutation in pigs has a more drastic effect on pigmentation than the mouse *dominant white spotting* and human *piebald* mutations, since the pigs are entirely white (Fig 4a). The gene product of *KIT* functions as a dimer and a receptor lacking the part encoded by exon 17, is most likely non-functional. The corresponding part of the protein is an essential and conserved region of the intracellular domain in the tyrosine kinases (Hanks et al. 1988; Hubbard et al. 1994). In mouse, the *W/Kit* mutations are of two kinds: regulatory and structural (Klüppel et al. 1997). The regulatory mutations alter *Kit* expression and the structural mutations are resulting in a receptor with impaired or absent activity. It was suggested that the duplication in white pigs acts as a regulatory mutation leading to a dysregulated expression of one or both copies of *KIT* (Marklund et al. 1998). Dysregulation of *KIT* expression and impaired tyrosinase kinase activity may cause a severe defect in survival and migration of

melanocytes. One normal copy of the gene is expressed per chromosome and that is apparently sufficient for the pig to avoid serious defects on hematopoiesis or fertility.

4a



4b

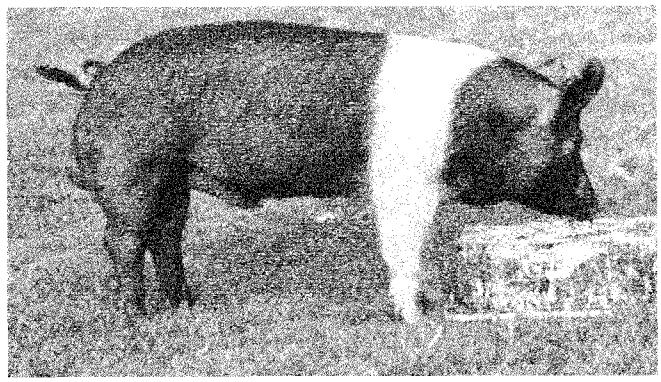


Figure 4a and b. Showing the phenotypic characteristics for two animals having different alleles at the *I/Dominant White* locus a) *I/I* and b) I^{Be} / I^{Be}, respectively.

In paper V the objective was to find the mutation causing the Belt phenotype. *Belt* gives a white belt across the shoulders and front legs, which is the characteristic phenotype of the Hampshire breed (Fig 4b) and was described already in 1907 by Spillman. In this study, a genome scan was performed with 67 offspring from a Hampshire/Pietrain backcross segregating for the white Belt trait. In the genome scan 65 microsatellite markers chosen from the available pig linkage map (Marklund et al. 1996; Rohrer et al. 1996) were used. A two point linkage analysis assigned the *Belt* locus to the centromeric region of chromosome 8. This was based on significant lod score against microsatellite S0086.

The results revealed two potential positional candidate genes, *KIT* and *EDNRA*. *EDNRA* has a role in early development, including the proliferation of neural crest derivatives *EDNRA* role in early development, including the proliferation of neural crest derivatives (Hirata 1996; Reid et al. 1996). Mutations in the related *EDNRB* gene cause pigmentation disorders in mouse, humans and horse. Human

EDNRA homologue is located on human chromosome 4, which indicates that the porcine corresponding gene should map to pig chromosome 8. To test the *EDNRA* as a candidate gene for *Belt* a clone containing *EDNRA* was isolated from the pig BAC library described in Rogel-Gaillard et al. (1999). The BAC clone was FISH mapped to porcine metaphase chromosomes and assigned to the q-arm of chromosome 8 (SSC8q21), thereby excluded as a candidate gene for *Belt*.
A single nucleotide polymorphism at position 2678 of exon 19 in the *KIT* gene (Marklund et al. 1998) was used to analyze the segregation at the *KIT* locus in the pedigree No recombinants with the Belt trait and *KIT* were observed among 105 informative meiosis. This finding suggest that *Belt* is an allele at the *Dominant White* locus. A quantitative PCR analysis clearly showed that *Belt* was not associated with a duplication of the *KIT* gene.

The aim of the study in paper VI was to build a BAC contig over the *Dominant White/KIT* locus with clones derived from the INRA porcine BAC library (Rogel-Gaillard et al. 1999). Two BAC clones were selected from the *PDGFRA* region using primer sequences derived from the human gene, whereas BACs containing the *KIT* gene were selected using porcine *KIT* primers. The contig was expanded on both sides by chromosome walking using STSs developed by BAC end-sequencing. From an end-sequence of one of the *PDGFRA* BACs homology to the human promoter sequence was found. This made it possible to orient the contig and to continue walking from the 3' end of the gene, assuming that the two genes are located head-to tail as is the case in humans (Spritz et al. 1994). Finally, a STS containing the upstream region of *KIT* was positive for one *PDGFRA* BAC, which then closed the contig, between the two genes. A restriction map was constructed with complete and partial digestion with *Sma*I and *Not*I, overlap and distances between clones could be revealed. PFGE data with genomic DNA from different pig breeds, including the Large White, showed that the genes *PDGFRA* and *KDR*, which are located upstream and downstream of *KIT*, respectively are not duplicated. Further by hybridizations of two STSs located between *PDGFRA* and *KIT* made it possible to locate the duplication breakpoint. One of the STSs (211E125') detected two fragments in the Large White breed and only one fragment in coloured breeds, whereas the other STS detected the same fragment in all breeds. These results indicated that one duplication breakpoint is located between *KIT* and *PDGFRA* and another between *KIT* and *KDR* (Fig 5).

The BAC library was screened with the 211E125' STS to identify BACs from the 5'end of the duplication breakpoint and from the 3'-5' duplication break-point. Three new BACs were isolated, end-sequenced and two of the STSs were not able to detect any other clone in the region between *KIT* and *PDGFRA*. The corresponding clones were therefore thought to contain the 3'-5' duplication break-point. However, a FISH analysis revealed that one of them was chimaeric, giving signal on chromosome 5 and on chromosome 8. This clone was not subjected to any further analysis. The second clone only hybridized to chromosome 8 and the BAC library was screened with the STS derived from the 3'end of that BAC clone (953F11) and four new clones were selected.

Strong homology to the human *KDR* gene was detected with the sequence obtained from the 3' end of one of the new BACs. The *KDR* gene belongs to the same receptor-tyrosine kinases gene family as the other two. In human the gene is located downstream of *KIT* (Spritz et al. 1994). This finding together with the PFGE data showing that *KDR* is not duplicated was a confirmation of that 953F11 contained the 3'-5' duplication break-point, having parts homologous to the the three duplication breakpoints *KIT5'*, 3'-5' and 3'. The contig comprised of 25 BAC clones, spanning approximately 1.3 Mb and will be a powerful resource for further characterization of the duplication breakpoint. The results showed that the orientation of the three genes are conserved between pig and human that the duplication involves the entire coding sequence of *KIT* and that it must be a tandem duplication. The next step is to clone restriction fragments from the three duplication breakpoints for comparative sequence analysis.

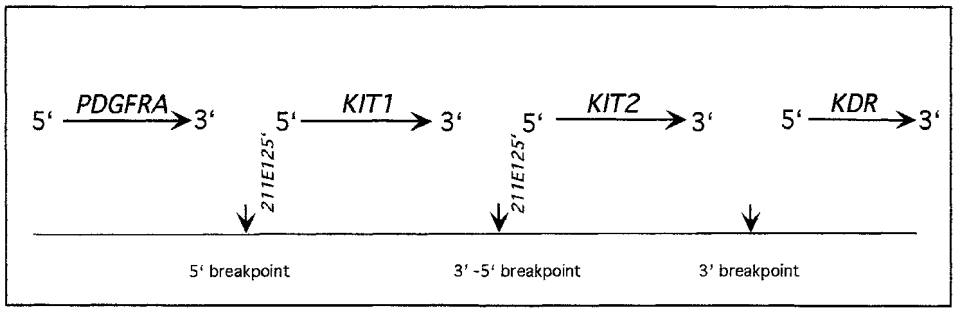


Figure 5. A schematic drawing of the BAC contig spanning the three genes *PDGFRA, KIT* and *KDR*. The relative order of *KIT1* and *KIT 2* has not yet been determined. The gene copy containing the splice mutation has earlier been denoted as *KIT2* (Marklund et al. 1998). *KIT1* is the normal copy.

Future Prospects

The ultimate goal for dissecting the genomes of farm animals is to unravel the genetic control underlying important traits like meat production, growth, reproduction and disease reistance. So far no attempts have been made to sequence the entire genomes of the major farm animals, such as cattle, chicken, sheep and pig. However, it is just a matter of time before such initiatives will be taken.

Physical mapping is not only a complement to genetic linkage analysis, but it is an important part of genome analysis. In this study, the FISH technique has been extensively used. The resolution of a FISH analysis depends on the DNA used as target for the hybridization. FISH on metaphase chromosomes is an important tool for detecting chimaeric YAC and BAC clones. This is clearly demonstrated in this study (paper VI). In addition, the technique will still be number one for the diagnosis of various chromosomal aberrations. Fibre-FISH, which is a high-resolution FISH technique may be used to reveal duplications or deletions of genes (Klockars et al. 1997).

When a trait has been localized to a chromosomal region, it is important to have a high-resolution map of coding sequences to be able to identify possible candidate genes. In paper IV both *IGF2* and a QTL affecting skeletal and cardiac muscle mass was mapped to the terminal end of chromosome 2p. This finding together with the fact that the QTL and the gene are paternally expressed made *IGF2* a strong candidate gene for this QTL effect.

Comparative mapping is an important task and the use of chromosome specific libraries (CSL) in cross-species chromosome painting analyses only reveals gross chromosomal homologies. Using CSL to screen for markers is a time consuming approach and the use of BAC and YAC clones is much more efficient. Contigs of clones spanning chromosomal regions of interest are efficient tools to find polymorphic markers and new genes. The next step is to understand the correlation between the genes and function.

The white phenotype is important in the pig breeding industry and the white coat is caused by mutations in the *KIT* gene. Hitherto, four alleles have been described at this particular locus and two of them namely *I* and I^{P} are associated with a duplication of the entire coding sequence of the *KIT* gene. The I^{Be} allele causes the belt phenotype, characteristic for the Hampshire breed, was described in this thesis as an allele at the *Dominant White* locus. The latter is not duplicated and it is thought to be a regulatory mutation, since the homozygous animals are fully viable. Information from the restriction map of the BAC contig covering 1.3 Mb including the genes *PDGFRA-KIT-KDR* will be an important tool for the molecular characterization the different *KIT* mutations (structural and regulatory). When the fragments containing the three different duplication breakpoints, (described in paper VI) are cloned, comparative sequencing of breeds having different alleles at the *I* locus will answer questions like: What kind of mutation is causing the Belt phenotype? Are I^{P} and *I* associated with the same duplication event?

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