Construction of a BAC/PAC contig of SSC 6q1.2 and comparative analysis of this genome region

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Dedicated to my parents

and to Carsten

Abstract

The *RYR1* gene region on the porcine chromosome 6q1.2 is known to harbour the locus for malignant hyperthermia (MH) or stress susceptibility. Porcine stress syndrome (PSS) has been responsible for economic losses in stress-susceptible pigs. These animals have increased muscling and lean meat content. So far it is unknown whether only the *RYR1* mutation is responsible for the positive carcass traits in stress-susceptible pigs or if these complex growth traits are influenced by other closely linked genes on SSC 6q1.2.

The purpose of this study was to construct a detailed physical map of the *RYR1* gene region as an important resource for the DNA sequencing of the coding regions on the porcine chromosome 6q1.2. The data generated here are a prerequisite for a mutation analysis necessary to find a functional polymorphism responsible for the observed phenotypes. Another reason for the construction of the contig is integrate the physical and genetic maps. The completion of the human genome project facilitates the identification and the localization of genes in the physical map, leading to a comparative analysis between different species.

To generate a detailed physical map between the genes *CLIPR-59* and *LIPE* on SSC 6q1.2, a porcine bacterial artificial chromosome (BAC) and a P1 derived artificial chromosome (PAC) library were screened, resulting in a sequence-ready 4.62 Mb BAC/PAC contig. Several heterologous probes from the extensively characterised human syntenic region on HSA 19q13 were used to screen both libraries. Gaps between clones were closed using a chromosome walking strategy, so that a single contig of 258 BAC and PAC clones was obtained. During the construction of the contig 111 new sequence-tagged site (STS) markers were generated. The clone-based physical map was validated and confirmed by radiation hybrid mapping. Detailed physical mapping of this gene-rich region made it possible to assign 49 porcine genes orthologous to known human chromosome 19 genes to this contig.

Comparative analysis of the porcine BAC/PAC contig with the human HSA 19q13 map revealed complete conservation of the gene order of this segment between the pig and human genomes. In contrast, the rodent genomes of mouse and rat show

two blocks of conserved synteny with human/pig. In the rodent species the segment between the genes *ARHGEF1* and *LIPE* is inverted with respect to the segment *BCKDH*A and *POLR2I* indicating a chromosomal rearrangement.

Key words: *RYR1*, SSC 6, HSA 19, contig, comparative mapping, RH map, physical map, pig

Zusammenfassung

Die chromosomale Region SSC 6q1.2 beherbergt beim Schwein das *RYR1* Gen und den Genort für die maligne Hyperthermie (MH), die zu Stressanfälligkeit bei den betroffenen Tieren führt. Die maligne Hyperthermie verursacht das sogenannte porcine Stress-Syndrom (PSS) und war in der Vergangenheit für erhebliche wirtschaftliche Verluste in der Schweineproduktion verantwortlich. Eng gekoppelt mit der Stressanfälligkeit werden auch günstige Merkmale wie starke Bemuskelung und hoher Magerfleischanteil vererbt. Trotz weltweiter, intensiver Forschungsaktivitäten ist bis heute nicht klar, ob die ursächliche Mutation für die Stressanfälligkeit im *RYR1* Gen gleichzeitig auch für den positiven Effekt auf die Fleischleistung verantwortlich ist, oder ob dieser Effekt von einer zweiten Mutation in einem eng benachbarten Gen verursacht wird.

Die Klonierung des porcinen Chromosoms 6q1.2 als BAC/PAC-Contig ist ein wichtiger Schritt auf dem Weg zur DNA-Sequenz der codierenden Bereiche dieser chromosomalen Region. Diese Daten sind eine Grundvoraussetzung für die systematische Mutationsanalyse, welche nötig ist, um tatsächlich die funktionellen Polymorphismen aufzuspüren, die für die beobachteten phänotypischen Effekte auf die Fleischleistung verantwortlich sind. Weiterhin ermöglicht ein solcher BAC/PAC-Contig einen wesentlich genaueren Zusammenschluss der existierenden physikalischen und genetischen Karten. Die Daten des menschlichen Genomprojektes erleichtern die Lokalisierung von Genen auf dem Contig. Durch die detaillierte physikalische Karte ist es nun möglich, die Genreihenfolge mit anderen Spezies zu vergleichen.

Um eine detaillierte physikalische Karte von SSC 6q1.2 zwischen den Genen *CLIPR-59* und *LIPE* zu erzeugen, wurde ein BAC/PAC-Contig dieser Region erstellt.

Hierfür wurde eine porcine PAC-Genbank (TAIGP714) mit 3.2 haploiden Genomäquivalenten und durchschnittlicher Insertgröße von 120 kb und eine BAC-Genbank (RPCI-44) mit 10 haploiden Genomäquivalenten und einer durchschnittlichen Insertgröße von 165 kb durchmustert.

Für die Durchmusterung der PAC- und der BAC-Genbank wurden insgesamt 49 STS Marker aus den orthologen Genen der syntänischen Region auf HSA 19q13 und 111

sequence tagged site (STS) Marker aus den nicht-repetitiven Randsequenzen isolierter Contig-Klone verwendet.

Der Contig umfasst 4,62 Mb und besteht aus 258 BAC- und PAC-Klonen. Die klonbasierte physikalischen Kartierung wurde durch Radiation Hybrid (RH) Kartierung von ausgewählten STS-Markern auf zwei verschiedenen RH-Panels überprüft und bestätigt.

Die Genreihenfolge beim Schwein in der Region auf SSC 6q1.2 stimmt vollkommen mit der Genreihenfolge beim Menschen auf HSA 19q13 überein. Die Syntänie zwischen den Genomen des Schweins, der Maus (MMU 7) und der Ratte (RNO 1) ist in dem untersuchten durch zwei konservierte Blöcke Bereich charakterisiert. Jeweils zwischen den Genen *BCKDHA* und *POLR2I* sowie *ARHGEF1* und *LIPE* herrscht völlige Übereinstimmung in der Genreihenfolge zwischen Mensch/Schwein und Maus/Ratte, allerdings ist der Block *ARHGEF1* – *LIPE* bei den Nagern im Vergleich zu Mensch und Schwein invertiert.

Schlagwörter: *RYR1*, SSC 6, HSA 19, Contig, Vergleichende Genomanalyse, RH Karte, physikalische Karte, Schwein

Contents

Chapter 1 Introduction

1.1 The porcine genome

The main reason for conducting genome analysis in domestic animals is to characterise the genes that control important traits. Molecular genetics offers new opportunities in this field. Genetic information can be used to develop new genetic markers which allow selection with respect to economically important traits. There are two types of genetic markers (O'Brien, 1991):

- Type I markers are coding sequences that are evolutionarily conserved and suitable for comparative gene mapping.
- Type II markers, such as microsatellites, are highly polymorphic, and therefore suitable for linkage analysis.

1.1.1 Genetic map

The genetic map is obtained by linkage analysis of polymorphic markers. It is based on recombination frequencies between these markers, which leads to the determination of their relative order and distance. Genetic distance, which is expressed in centimorgans (cM), is not the same as the physical distance. By definition, two markers that show 1% recombination are 1 cM apart from each other on the genetic map.

The relation between physical and genetic distances is not constant across the whole genome. In the centromeric region, for example, less recombination takes place than toward the telomere of a chromosome. However, 1 cM = 1 Mb is a useful approximation.

Single nucleotide polymorphisms (SNPs) and microsatellites are among the most extensively used markers for species genotyping and linkage studies. SNPs denote any polymorphic variation at a single nucleotide; within a gene, a SNP can cause a functional mutation affecting the trait under investigation. Polymorphic microsatellites, on the other hand, rarely alter a gene function. They consist of tandem repeats of one to six base pairs and can be found mostly in introns or intergenic regions. The density of microsatellites in mammals is limited to about one every 10-100 kb, whereas SNPs

can be found more frequently (approximately one SNP per kb; Strachman and Read, 1999).

In linkage analysis genotyping of SNPs or microsatellites is followed by statistical procedures that determine whether the marker alleles and certain traits cosegregate or not.

Many studies have been carried out to demonstrate and make use of the linkage between microsatellites and trait loci (e.g. Spötter et al., 2002; Chang et al., 2003).

1.1.2 Physical maps

Unlike the genetic map, physical mapping determines the chromosomal position of a marker or a gene. These maps are based on direct assignment of genes or markers to chromosome regions, bands or base pairs. The different methods that can be used to construct physical maps are described below.

1.1.2.1 Contig

In order to increase the resources available for the identification of economically important genes in any chromosomal region, a detailed characterisation of this region can be undertaken by the construction of a contig. A clone contig describes a set of overlapping clones detected by a variety of experimental techniques. The assembled DNA fragments of the contig collectively provide full representation of the genomic segment.

At present, contigs are built using bacterial artificial chromosomes (BACs; Shizuya et al., 1992) or P1 derived artificial chromosomes (PACs; Ioannou et al., 1994). The major advantage of BACs and PACs is ease of handling the DNA, which is very stable. Deletions and chimerism rarely occur. The cloning segment of BAC vectors includes several restriction enzyme sites and T7 and SP6 primer binding sites, which can be used for the end sequencing of the insert. BAC and PAC cloning systems have a capacity of up to 300 kb, high cloning efficiency, and insert stability. Depending on the nature of the library, it can be screened using PCR and/or radioactive hybridisation.

Currently, there is only one porcine PAC library available to the scientific community (Al-Bayati et al., 1999), whereas there are five porcine BAC libraries (Rogel-Gaillard

et al., 1999; Anderson et al., 2000; Suzuki et al., 2000; Fahrenkrug et al., 2001; http://bacpac.chori.org/porcine242.htm).

The TAIGP714-PAC library (Al-Bayati et al., 1999) consists of 90,240 clones sorted in 235 384-well plates (www.rzpd.de). This library covers 3.24 genome equivalents, with an average clone size of 120 kb. The porcine RPCI-44 BAC library (Fahrenkrug et al., 2001) has 10.2-fold genomic coverage and comprises about 185,500 clones with an average insert size of 165 kb.

As an alternative to BACs and PACs several swine yeast artificial chromosome (YAC) libraries corresponding to about three genome equivalents have already been reported (Zehetner and Lehrach, 1994; Leeb et al., 1995; Rogel-Gaillard et al., 1997; Alexander et al., 1997). Although YAC clones have inserts up to 2,000 kb in length, YAC libraries have some negative aspects, such as frequent chimerism, clone instability, and time-consuming procedures.

The screening of filters of YAC libraries by radioactive hybridisation is described by Ragoussis and co-authors (1991). The same technique can be used to screen the RPCI-44 BAC library. By using cDNA probes or PCR product as probes, it is possible to build a contig of a specific part of the genome. The secondary screening of the positive clones is used to confirm the first hybridisation. This step can be carried out by hybridisation on a Southern blot membrane or on a colony lift membrane (Register et al., 1995) as well as by PCR on the isolated DNA or colony, or by restriction fingerprinting. In addition to cDNA and PCR product as probes, oligonucleotide-based hybridisation (“overgo”) probes can also be used to screen high density filters (Han et al., 2000; Thomas et al., 2002).

A genome-wide physical map can be constructed by restriction fingerprint analysis, by which the DNA is cleaved using restriction enzymes. The restriction fragments are then separated by gel electrophoresis and assembled to a contig by application of computer programmes. The overlap of different clones is determined according to the fragmentation pattern, also with a computer programme. This method requires the DNA isolation of the complete library, and the result does not always cover the entire genome, as in the example of the genomes of rice (Tao et al., 2001) and human (McPherson et al., 2001), for which 300,000 BAC clones with a 15-fold genome coverage have been fingerprinted.

1.1.2.2 Radiation hybrid map

Under specific experimental conditions, cultured cells from different species can be induced to fuse together to generate somatic cell hybrids. During this process, parts of the donor chromosome fuse with chromosomes of the recipient cell. The cell fusion is initiated using polyethylene glycol.

The construction of a human radiation hybrid (RH) panel has been described (Cox et al., 1990) on the basis of studies by Goss and Harris (1975). Chromosomes are broken into numerous fragments by a high dose of x-rays. The number of chromosome breaks is a function of the x-ray doses (Goss and Harris, 1977). Radiation hybrids emerge by fusing the lethally irradiated donor cells with recipient cells, commonly from the hamster. In this way the arisen hybrids contain a full complement of the host's chromosomes and pieces of the fragmented donor chromosomes (Figure 1). The presence of a particular locus in a cell line can be detected by performing a PCR and subsequent gel electrophoresis.

Similar results are obtained for the somatic cell hybrid and the radiation hybrid panels by both experimental methods (PCR and gel electrophoresis).

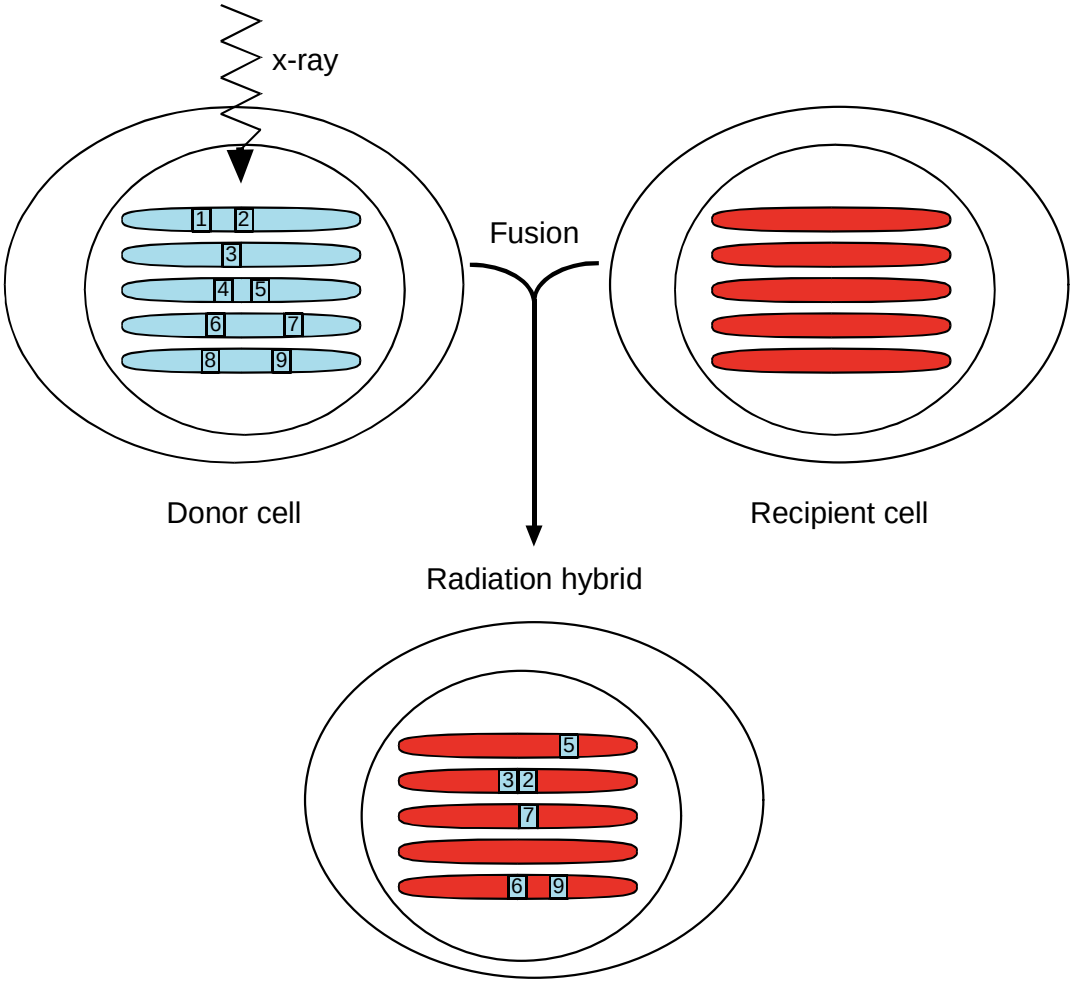

Figure 1: The construction of a radiation hybrid: irradiation and fusion

RH maps are built up by linkage analysis and reflect the relative order and distance of markers along a chromosome. The distance between two markers can be estimated by the frequency of breakage, since the closer two markers are on a chromosome, the lower the probability is that the x-rays will separate them. The breakage frequency between more than two markers allows the determination of the order of these markers on the chromosome.

The frequency of breakage underestimates the distance between two more distant markers and can be converted into an additive distance which takes the x-ray dose into consideration. The additive distance is expressed in centi Ray (cR). As described by Cox et al. (1990), 1 cR_{8000} corresponds to a 1% breakage frequency between two markers after an exposure to 8,000 rad of x-rays. This unit is analogous to the centi Morgan (cM).

Three porcine radiation hybrid panels are available to the research community: the IMpRH (7,000 rad), the IMNpRH2 (12,000 rad), and the Goodfellow T43 (3,000 rad) panels (http://compgen.rutgers.edu/rhmap/). The whole-genome 12,000 radIMNpRH2 panel complements the 7,000 rad IMpRH panel and allows the construction of higher resolution maps (Yerle et al. 1998 and 2002). Figure 2 shows the current radiation hybrid map on the IMpRH panel and the genetic map of the porcine chromosome 6.

Many efforts have been made to assign microsatellites and genomic sequences to the swine RH map (Hawken et al., 1999; Kiuchi et al., 2002). More than 4,500 markers, ESTs and genes have been mapped on the IMpRH panel, resulting in a second generation map (Milan et al., 2002). Hamasima et al. (2003) report the compilation of a whole-genome framework map comprising 589 microsatellite markers on a 5,000 rad radiation hybrid panel.

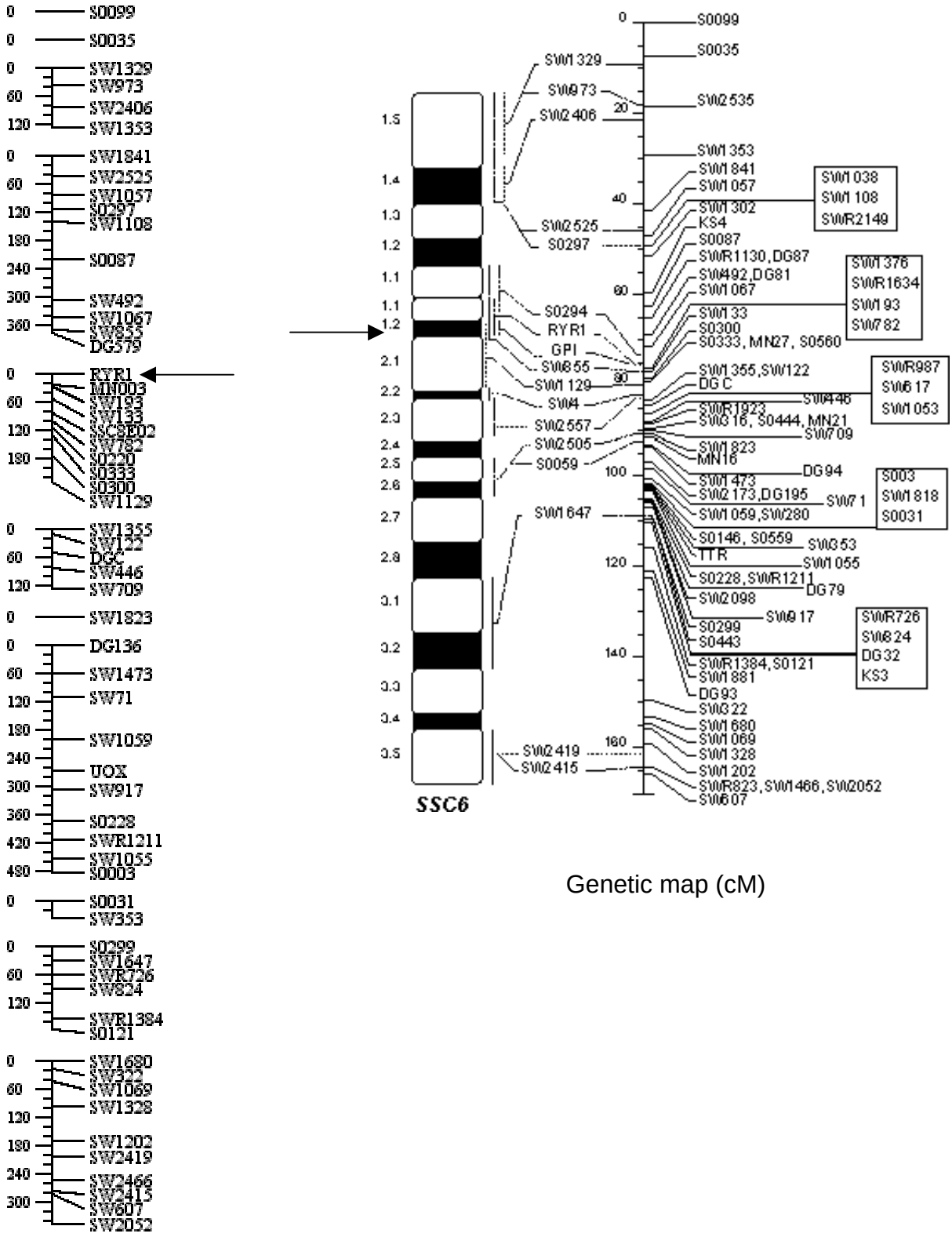

Figure 2: RH map and genetic map of porcine chromosome 6 (http://abcenter.coafes.umn.edu/RHmaps/chromosome/chromosome6.html), the *RYR1* gene is indicated with an arrow.

1.1.2.3 Fluorescence *in situ* hybridisation (FISH)

Another powerful tool for physical mapping is fluorescence in situ hybridisation, as genes can be mapped on metaphase chromosomes of the different species. For the determination of the physical location of genes and DNA segments, FISH is an alternative method to the radiation hybrid panel, if this is not available or if the known RH data are not sufficient for satisfactory mapping.

Probes for the in situ hybridisation are labelled with fluorescent tags which hybridise to the complementary DNA (John et al., 1969; Figure 3).

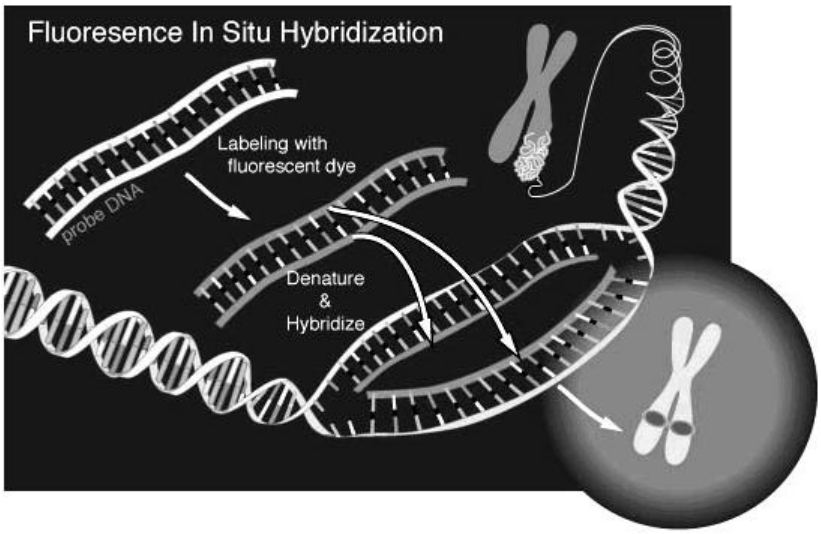

Figure 3: Fluorescence in situ hybridization (http://www.accessexcellence.org/AB/GG/fish.html)

The resolution power of metaphase FISH averages 1 Mb and that of the interphase FISH, 50 kb (Heng et al., 1992), although the resolution of interphase FISH decreases with the increasing distance between two probes. Probes between 5 and 200 kb are normally used for FISH, but Lemieux and co-authors (1992) report the successful use of probes as short as 0.5 kb. The possibilities of fluorescence *in situ* hybridization can be greatly increased by the simultaneous use of multiple fluorescent colours, so that many probes can be analysed at the same time (Ried et al., 1992).

1.1.3 Comparative analysis

Comparative analysis in a poorly characterised species can only be conducted if detailed gene maps of different species are available (e.g. human-pig). Comparative gene mapping is an important tool for the extrapolation of information between genomes from different species and makes use of the conserved synteny between them. The conserved synteny describes equality in gene order between two species and can be determined for example by cross-species chromosome painting experiments.

Cross-species chromosome painting, also referred to as Zoo-FISH, has contributed to our understanding of the relationship between the genomes from different species. The probe used for comparative chromosome painting experiments contains not just a region of a chromosome but usually the whole chromosome, which originates from another species (Chowdhary et al., 2001). If the result of the chromosome painting shows the homology of the chromosome of one species to two or more chromosomes of the other species, a reverse chromosome painting experiment has to be carried out to find out which part of the chromosome corresponds to the chromosome originally tested.

The pig was the first farm animal to be used for comparative chromosome painting experiments with human probes (Rettenberger et al., 1995; Chowdhary et al., 2001). Frönicke et al. (1996) reported the identification of 47 segments on pig metaphase chromosomes homologous to human segments. That study showed that porcine chromosome 6 has homology to the human chromosomes 1, 18 and 19. According to Chaudhary et al. (1998), the q arm of human chromosome 19 paints with porcine chromosome 6cen-q21.

The construction of comparative maps between humans, pigs, sheep and cattle has been described (Frönicke et al., 2001). Both the porcine genome and the comparative maps between pigs, goats and humans were improved when 113 genes originating from goat BAC clones were tested by Zoo-FISH on porcine chromosomes (Pinton et al., 2000).

Jeon and co-authors (2001) reported the comparative analysis between a porcine contig in the RN gene region to human RH data. Except for one minor discrepancy in the order of two genes, the gene order on the porcine data did not differ from the

human RH data. On the other hand, major disagreements were detected when the data were compared with the data on the mouse genome available at that time. Over the past few years, the human and mouse genomes have been periodically updated; differences between the different assemblies are common.

Van Poucke et al. (2001) observed the synteny between human chromosome 3 and porcine chromosome 13cen-q46. The study showed that there was no complete gene order conservation in the region. The localisation of 18 loci on the cytogenetic map of porcine chromosome 13 confirmed some intrachromosomal rearrangements, but the gene order in the major blocks seemed to be conserved.

Another study dealt with the synteny between the pig and human genomes using radiation hybrid mapping. As indicated by Shi and co-authors (2001), the gene order between porcine chromosome 12 and human chromosome 17 seemed to be the same, although the orientation on the chromosomes was inverted. This result was confirmed by a cross-species FISH experiment with goat BAC clones onto pig chromosomes (Pinton et al., 2000).

The homology between porcine chromosome 2 (SSC 2) and human chromosome 11 (HSA 11) has already been studied (Rattink et al., 2001). Two conserved segments with identical gene order were identified between HSA 11pter-q13 and SSC 2. This conclusion is based on 20 genes that were mapped on both the human and porcine radiation hybrid panels. In addition, sample sequencing of porcine BAC clones located on this area revealed homology to human chromosome 11.

The only available study that compares both human and porcine genomes not only through RH data but also through the construction of a contig was that reported by Robic and co-authors (2001). Ten genes could be identified along the 2.4 Mb-long BAC contig, and the gene order between both species seemed to be the same.

Another study reported the conserved synteny between the porcine and human genomes in the region of the major histocompatibility complex (MHC) genes (Genet et al., 2001). The well characterised swine leukocyte antigens (SLA) region on porcine chromosome 7 has been mapped using the IMpRH panel, which improved the existing map. Altogether, 23 new markers were typed by Genet et al. (2001) on the IMpRH panel.

A whole genome radiation hybrid map has been constructed to complete and to refine the comparative map between the porcine and the human genomes, which were previously developed by cross-species chromosome painting. In this way, new syntenic breakpoints and rearrangements could be identified, and the resolution of the comparative map was improved. That study was carried out by testing 1058 EST markers on the IMpRH-panel (Goureau et al., 1996; Rink et al., 2002).

Detailed comparative analyses have also been performed between humans and mice, humans and rats, and humans and other mammals or vertebrates:

- A PAC based physical map of a ca. 1.5 Mb region on rat chromosome 4 encompassing the *Cftr* gene was constructed using cross-species overgo probes (Summers et al., 2001). The study showed that the most gene/EST sequences were similar enough between the mouse and the rat to be used as overgo probes for hybridisation-based rat PAC library screening. In comparison to the human and mouse genomes, the rat gene order and content of the *Cftr* gene region is well conserved (Summers et al., 2001 and Ellsworth et al., 2000).

- A 2 Mb rat PAC contig on the region homologous to human chromosome 7q21 containing the *CDK6* gene revealed differences in gene order between both species (Summers et al., 2001). This region was of special interest due to the presence of an evolutionary breakpoint between the corresponding human and mouse genomic regions (Thomas et al., 1999).

- Fahrenkrug and co-authors (2000) reported the construction of a radiation hybrid map in the region around the ovine *CLPG* gene on chromosome 18. They used a bovine radiation hybrid panel, as the high conservation in sequence content and marker order between both species was known (Cockett et al., 1994). That study also showed the complete homology and synteny in the region surrounded by the genes *BDKRB2* and *AKT* between human, ovine and bovine genomes. The *CLPG* gene region has also been physically mapped using an ovine BAC library. For this purpose, bovine STSs were used for the PCR based screening of the ovine BAC library (Segers et al., 2000).

- A result similar to the one obtained in the present work was reported by Puttagunta et al. (2000). That report showed the conserved gene order between

human chromosome 19p13.3 and murine chromosome 10, although, there was an inversion of about 1.2 Mb surrounded by the conserved linkage group. The gene order within this inversion seemed to be identical between both species.

- Smith and co-authors (2002) detected the synteny between human chromosome 19 and chicken chromosomes using a combination of PCR-based screening of chicken BAC and cosmid libraries, sequencing, genetic mapping, and FISH experiments. The authors observed that there is synteny between parts of human chromosome 19 and chicken chromosome E25, but that the gene order between the genes *CAPN4* to *TGFB1* is inverted.

Figure 4 illustrates the synteny between the porcine chromosome 6 and the human chromosomes 1, 16,18 and 19.

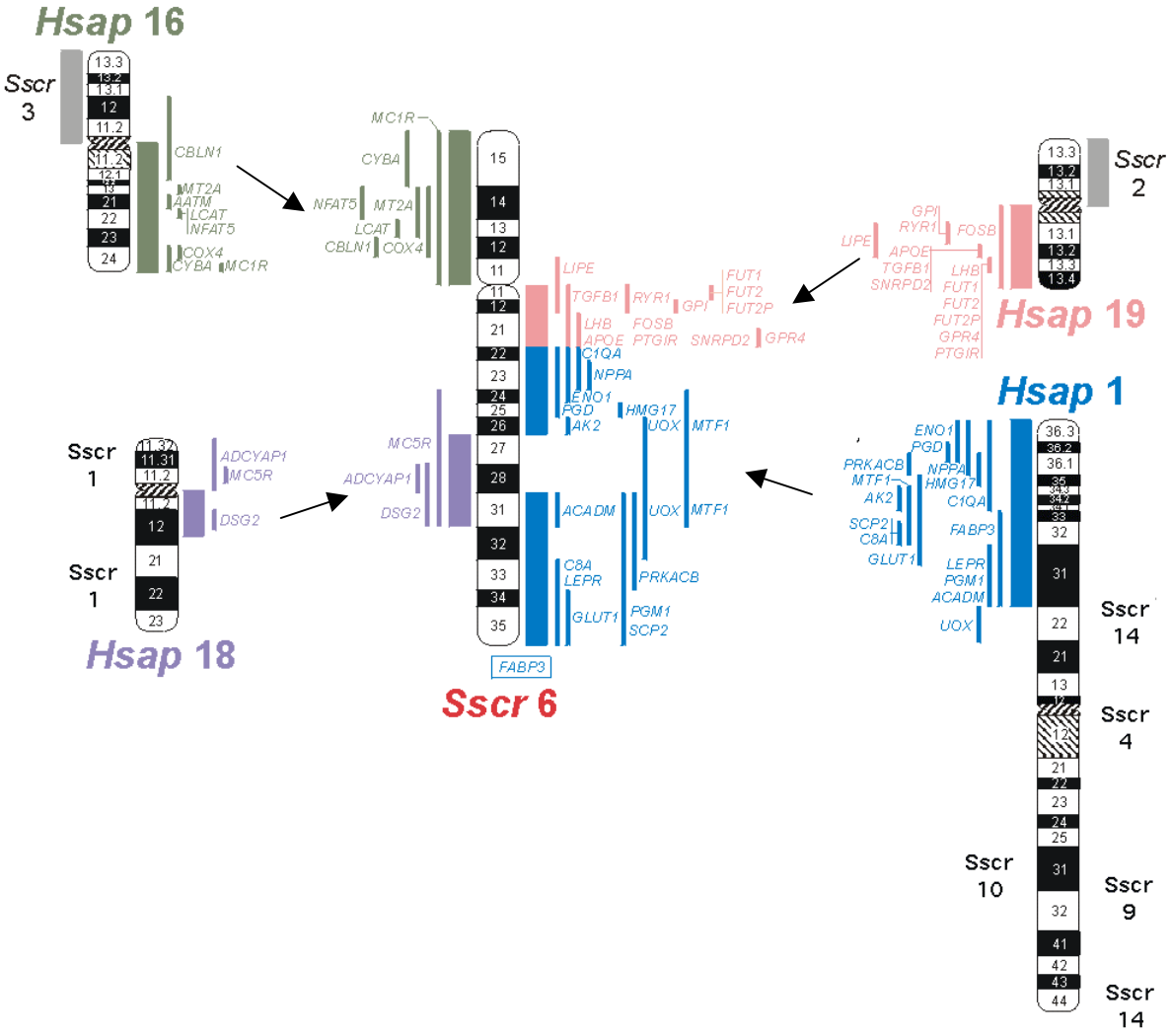

Figure 4: Comparative mapping based on porcine chromosome 6 (Sscr6) and the human chromosomes (Hsap) (http://www.toulouse.inra.fr/lgc/pig/compare/SSCHTML/SSC6S.HTM)

1.2 Malignant hyperthermia and the *RYR1* gene

Porcine chromosome 6q1.2 harbours the locus that causes malignant hyperthermia (MH), but there are only limited physical and genetic mapping resources available. MH is a disorder of skeletal muscle tissue and originates from a mutation in the ryanodine receptor 1 gene (*RYR1*). The mutation is located in exon 17 of the *RYR1* gene at nucleotide position 1843. A C/T transition results in an Arg615Cys substitution (Fujii et al., 1991; Leeb and Brenig, 1998).

The skeletal muscle ryanodine receptor is a calcium release channel (Figure 5) that mediates the efflux of calcium ions from the sarcoplasmic reticulum into the myoplasm during excitation-contraction coupling. For patients with malignant hyperthermia, the mutation in the *RYR1* gene leads to the permanent opening of the calcium release channel. The disease is clinically characterised by spasm, hypermetabolism, muscle rigidity, rapid increase in body temperature (which can climb from the normal 37°C to a fatal 43°C), tachycardia, and acidosis and if untreated progresses to severe damage or to death during or after general anaesthesia (Mickelson and Louis, 1996; McCarthy et al., 2000; Melzer et al., 2001). MH can be initiated by commonly used volatile halogenated anaesthetic agents and muscle relaxants such as succinylcholine.

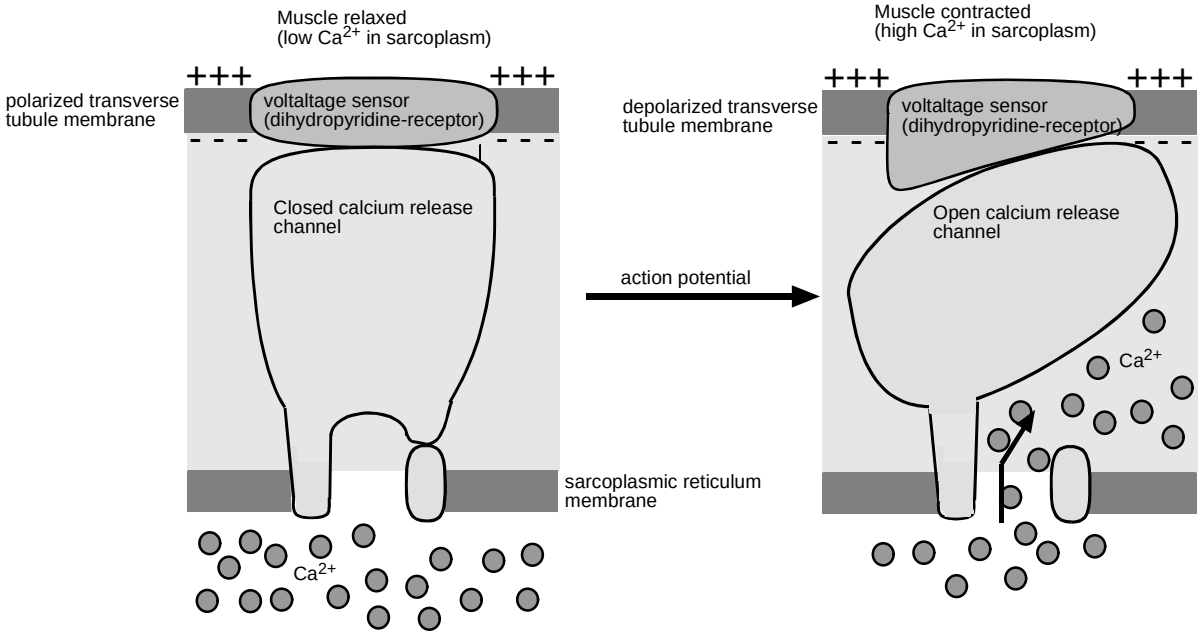

Figure 5: A calcium release channel in the sarcoplasmic reticulum membrane (from http://www.faseb.org/opar/mh/muscle.html)

MH syndrome in pigs is very similar to that in humans and has comparable physiological and clinical effects. MH in pigs is triggered by stress and excitement associated with exercise, fighting and transport. The halothane challenge test can be performed to make a diagnosis of MH in pigs. In this test, animals inhale halothane through a face mask. MHS-positive animals exhibit muscle rigidity, tachycardia and increased body temperature (Mickelson and Louis, 1996).

1.2.1 Carcass Traits

Malignant hyperthermia is triggered by stress and is therefore often referred to as porcine stress syndrome (PSS) (Leeb and Brenig, 1998). The three *RYR1*-genotypes, also known as halothane genotypes, are:

- NN (homozygous stress resistant, C/C),
- NP (heterozygous stress resistant, C/T) and
- PP (homozygous stress susceptible, T/T).

A leak in the calcium channel is caused by the mutation of the *RYR1* gene. This contributes to elevated myoplasmic calcium concentrations and increased skeletal muscle metabolism at slaughter, often resulting in the development of pale, soft and exudative (PSE) meat (Byrem et al., 1999). De Smet et al. (1996) report the correlation between the different halothane genotypes and the meat quality traits related to the PSE condition. This condition can be observed in carcasses with a rapid pH drop and a low final pH value resulting from protein denaturation. In that study, the PP pigs were always significantly different from NP and NN pigs. For example, the amount of lean meat in carcass is increased in PP pigs, as is the risk of PSE meat. That study also shows that there was no significant difference in lean meat content between NN and NP pigs. As reported by Monin et al. (1999), the dominance of the N allele is trait dependent.

There have been several reports of the relationship between the halothane genotypes and feed efficiency, carcass yield, and carcass lean content (Leach et al., 1996; Pommier et al., 1998; Tam et al., 1998). According to these studies, PP animals have poorer meat quality than homozygous stress resistant (NN) pigs. Hamilton et al. (2000) give a summary of the correlations between the *RYR1* genotypes, meat quality and carcass quality: heterozygous stress resistant (NP) animals have significantly shorter carcasses than homozygous stress resistant animals (NN), whereas there were no differences in backfat thickness measurements between the genotypes.

Molecular genetic studies of humans and pigs have established the ryanodine receptor on chromosomes 19 and 6, respectively, as the primary MHS locus (MacLennan et al., 1990; McCarthy et al., 1990). De Smet et al. (1996) came to the

conclusion that the inheritance of the P allele with respect to meat quality is not consistently recessive or partly recessive, but may vary according to the specific trait. This conclusion supports the presumption that not only the *RYR1* mutation is responsible for positive carcass traits in stress-susceptible pigs but that these complex growth traits may be influenced by other closely linked genes on SSC 6q1.2.

1.2.1.1 QTL studies for carcass traits

A quantitative trait locus (QTL) denotes a locus that influences a quantitative trait such as carcass quality and consequently a chromosomal region containing one or several genes partially responsible for the observed genetic variation.

A QTL study concerning meat quality traits in a cross consisting of individuals from the Duroc, Norwegian Landrace and Yorkshire breeds was conducted by Grindflek et al. (2001), who found evidence for a QTL affecting intramuscular fat (IMF) content on porcine chromosome 6. That study predicted that the highest probability of the QTL position is between the markers SW1823 and S0003, located between 74 and 79 cM from the first marker on the chromosome (see Figure 2). According to Grindflek et al. (2001), this QTL may also influence meat tenderness, but has no impact on the backfat thickness.

As indicated by de Koning and co-authors (1999), a QTL for intramuscular fat content is expected on the long arm of SSC 6. Their study was based on a F2 cross between the Chinese Meishan pig breed and commercial Dutch pig lines. For the QTL for backfat thickness, the authors suggested an overdominance where there is no certainty whether these are true effects of single genes or the influence of other, closely linked genes. The QTL detected on the SSC 6 map lies about 70 cM away from the *RYR1* gene between markers Sw1057 and S0220 (see Figure 2).

No evidence for QTL was found in studies in which the *RYR1* mutation is not present in the population (Rohrer et al., 1998).

Figure 6 shows the QTL curve for backfat and intramuscular fat on the porcine chromosome 6.

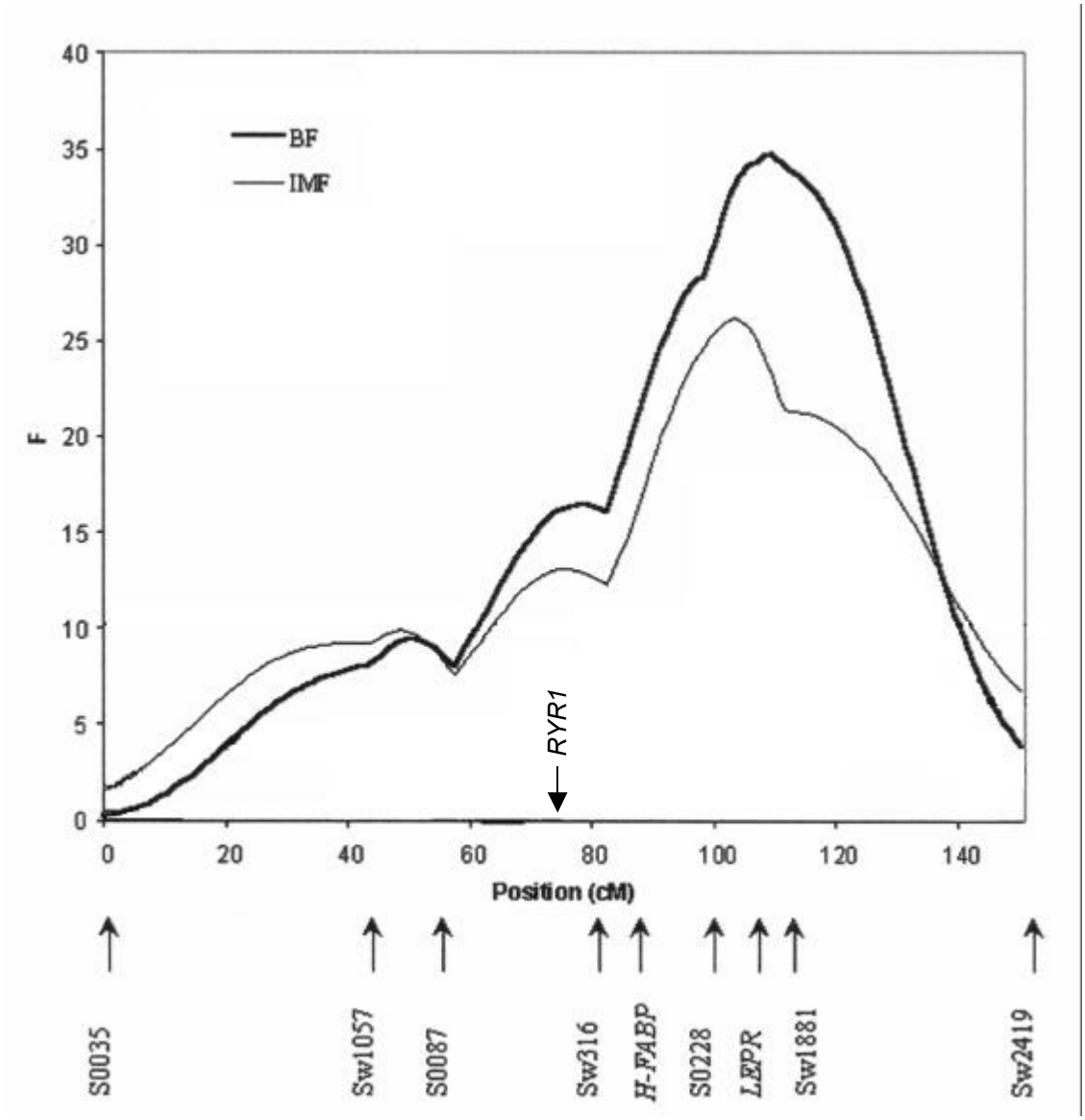

Figure 6: QTL curve across the porcine chromosome 6 for backfat (BF) and intramuscular fat (IMF) (OVilo et al., 2002; modified)

1.3 Aims of the thesis

- Construction of a BAC/PAC contig in the *RYR1* gene region on porcine chromosome 6.
- Analysis of the contig using different methods – pulsed field gel electrophoresis, clone end sequencing and radiation hybrid mapping.
- Comparative analysis of gene order and distance with the data available for the human, murine and rat genomes.
- Establishment of the connection between physical, RH and genetic maps.

1.4 Overview/Survey of the contents

Chapter 2 deals with the initial work done on the project and describes the methods used during the project.

Chapter III presents the use of the IMpRH and IMNpRH2 panels. Here, the differences between these RH panels are explained. The results complete the existing data on the IMNpRH2 panel.

Chapter 4 contains a summary of the results of this thesis in addition to the integration of the physical map into the present genetic map on porcine chromosome 6.

Chapter 4 introduces a high-throughput method for pulsed field gel electrophoresis that was developed during the project.

Chapter 6 provides a comprehensive discussion to Chapters 1-5.

The Appendix includes additional information:

- primer table
- detailed contig map

Chapter 2 Construction of a 1.2 Mb BAC/PAC contig of the porcine *RYR1* gene region on SSC 6q1.2 and comparative analysis with HSA 19q13.13

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Construction of a 1.2-Mb BAC/PAC Contig of the Porcine Gene *RYR1* Region on SSC 6q1.2 and Comparative Analysis with HSA 19q13.13

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We screened a porcine bacterial artificial chromosome (BAC) and a P1 derived artificial chromosome (PAC) library to construct a sequence-ready ~ 1.2-Mb BAC/PAC contig of the ryanodine receptor-1 gene (*RYR1*) region on porcine chromosome (SSC) 6q1.2. This genomic segment is of special interest because it harbors the locus for stress susceptibility in pigs and a putative quantitative trait locus for muscle growth. Detailed physical mapping of this gene-rich region allowed us to assign to this contig 17 porcine genes orthologous to known human chromosome 19 genes. Apart from the relatively well-characterized porcine gene *RYR1*, the other 16 genes represent novel chromosomal assignments and 14 genes have been cloned for the first time in pig. Comparative analysis of the porcine BAC/PAC contig with the human chromosome (HSA) 19q13.13 map revealed a completely conserved gene order of this segment between pig and human. A detailed porcine–human–mouse comparative map of this region was constructed.

Key Words: SSC 6, HSA 19, MMU 7, *RYR1*, contig, comparative mapping, pig, malignant hyperthermia, stress susceptibility, QTL

INTRODUCTION

Since the initial completion of the human genome sequence, the creation of detailed physical maps in other mammalian species has been greatly facilitated through comparative mapping approaches [1]. High-resolution physical maps of specific genome regions (that is, genomic contigs of cloned DNA) are key elements used in the localization, isolation, and characterization of genes, including those involved in the development of specific diseases. The conservation of chromosome fragments between human and pig has been determined with low resolution by chromosome painting experiments [2], but detailed comparative maps of specific pig genome regions have been rare. One example is the comparative analysis of the porcine *RN* gene region on porcine chromosome (SSC) 15q25 versus its syntenic counterpart on human chromosome (HSA) 2q35 [3].

The porcine *RYR1* gene region is of special interest due to its economic importance. Mutations in human *RYR1* have been detected in about 50% of patients suffering from malignant hyperthermia (MH). This disease is also known in pigs, in which it is triggered by stress and therefore often referred to as porcine stress syndrome (PSS) [4]. PSS, which in pigs is caused by a single *RYR1* point mutation, is known to be associated with positive characteristics like increased muscling and increased lean meat content [5]. So far, it is not clear whether the *RYR1* mutation is also responsible for the positive carcass traits in stress-susceptible pigs or whether these complex growth traits are influenced by other closely linked genes on SSC 6q1.2. Here we report the construction of a bacterial artificial chromosome (BAC)/P1 derived artificial chromosome (PAC) contig of this region together with a high-resolution physical map. This map represents a resource for large-scale sequencing efforts and it may facilitate further efforts to dissect the complex genetics of muscle growth.

RESULTS

Construction of the BAC and PAC Contig

To construct a BAC/PAC contig of the porcine *RYR1* gene region, we screened the porcine TAIGP714 PAC library with four different PCR amplicons from the ~ 160-kb porcine *RYR1* gene (Table 1). Further primer pairs and hybridization probes

0888-7543/02 $35.00

TABLE 1: Primer sequences for library screening and STS content analysis

Primer[a]	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	T_M	PCR product
714_G22_81_T7	ACT GTC CTT CTT GCT CC	GTC CAA AAA ACC AAG AGG	52°C	98 bp
714_E17_112_Sp6	GTG ACT TGT AGA CCA CAG	CCT ACA GAG GGA GAA TCC	55°C	346 bp
714_L20_221_Sp6	TGG AAG TCT TGA GCA CAG	AGA ACA CAG CCT GAT GTG	58°C	218 bp
714_G22_81_Sp6	ATC TTC TGG GCC TCT CTG	GGG ACA GGA ATG AGG AAG	56°C	446 bp
PPP1R14[b]	GCT GAG CAA GCT GCA GTC	GGT ACA GCT CCT CCA AGC	58°C	164 bp
714_J03_209_T7	ACT GCC TTT TAA CAT TGG	GAC AGC AAA CAA AAT GAG	50°C	364 bp
714_L20_221_T7	CCA AAA TCC ACC CAG AAG C	TGC TTC CTG TGG GGA CAG	58°C	113 bp
714_A03_39_T7	TAG AGC ACC AAC CAT GTG	GTT CTA TTG GCA CCA TGG	54°C	462 bp
714_G13_89_T7	GGC CAG GGG AAT CAA GTG	GAG AAC CTC CAG CCT GTG	58°C	466 bp
714_M08_6_SP6	GGA TTG TGT ACC TGG TAC	GAC CAC CAG GAA TAC TTC	54°C	496 bp
714_I17_138_T7	AAC AAA CAA CCT AGG GCC	CTC CCA CTT CCA ATC CTC	55°C	110 bp
714_J03_209_SP6	GCC CCT GAG GAC ATT ACC	ACC TAG GGC CCC TAA GTC	58°C	473 bp
714_E17_112_T7	GGC AGA TGA AAA GCA AGG	TCA TGA AGA CCT CCA TGC	54°C	517 bp
PSMD8	CGG AGT CAG CCT ATA TGC	GAC ACC GGA TGC TTG ATG	56°C	145 bp
714_I17_37_T7	AGG CTG AGC ACA CCC ATC	ACC AGA CCC CTG CAC TTG	58°C	412 bp
RYR103/104 (RYR_Promoter)[b,c]	TTC GTT TCT GCT TCG CC	CTC TCT CCT CCC ATT TC	48°C	162 bp
714_G13_89_Sp6	GTT GGC AGG ACT GCT CTG	AAA GCC CTG TGC AGA AGG	57°C	128 bp
RYR115/116	GGT AAA AGG GTA TAT GG	GTG CAA AAT TTC AGA GG	48°C	164 bp
RP44-187_C16_Sp6	TCA CCC AAG ACC TGT TCC	TGC TGC AAA ATG CCT GTG	55°C	412 bp
RYR101/102	CAG ACA GGG AAC ACA AC	TGG ATG TAT TCT GTG AGG	52°C	309 bp
714_M08_6_T7	CGG GGT CCC TAT TTG TAT	TCT AGT TCC TTT CCC CTG	54°C	126 bp
714_M08_41_T7	ATC TAG TCT GAA GAG CTC CC	GTT CGC AGA CGG GAC CTC	60°C	125 bp
RYR111/112	AGG AGA AGG TAA GGG TG	GGA AGA GTC AGT TGG TC	52°C	235 bp
MAP4K1	CCT ACC CAC GCC TAT GC	CCA GCC AGC AGG AAA GC	56°C	136 bp
714_N01_50_T7	GAG TTT CTG CAT CTG TTC	TCA ACG TTT TAT ATA ACC ATG	53°C	333 bp
RP44-162_H07_Sp6	ACA CCT TCT TGG CCA TGC	TGA GGA CGC CAC CAT CAC	57°C	116 bp
714_I05_215_SP6	GGA AAC TCC CTG TAA AGG	CCT CCA GCT ATT TCT AGG	54°C	150 bp
RP44-235_B21_Sp6	ACA GCA TGG TTG CGA CTC	GCC CGA GAC ATA GGT GAC	57°C	366 bp
714_F04_202_T7	CCC CTT TCA CTG GCT CTG	GCC CAG GAG CTC AAG TAC	58°C	513 bp
ACTN4	GAT GCC GAG TTC AAC CGC ATC	TTG TCA CCT GCC AGG ACC TTG	66°C	155 bp
714_N01_50_Sp6	ACT GAT CTT CGG CCC TTG	CAA GGA TGC ACC CAA TCG	56°C	517 bp
714_K13_61_Sp6 (ITZ001)[b,c]	TAG CCT TTC CTG TGG AGG	ACA CAA AAG CAC ACA CCG	56°C	423 bp
RP44-172_J01_T7	ACA ATG AGC CTT GGA GTC	GAA GCC AGT GTC GTG TTG	55°C	318 bp
714_M08_41_SP6	TCC AAG ACA GAG CTT GAG	GGC AGT GAA TCA TGA GTC	54°C	135 bp
LGALS4[b]	GAT GTC GCC TTC CAC TTC	TGA TGA CCA GCT CGA AGG	56°C	142 bp
ECH1[b]	ACC AGG AGG TCC TGC TTG	ATG TAG TTG AGG CCC TCT G	58°C	136 bp
RP44-162_H07_T7	CTC CAC AGG CAG AAT GTG	AGA GAA GCC CTG CAA GAG	56°C	443 bp
714_D22_199_T7	TGG GGA CAA ACG CTT AGG	CCG ATT AAG AAG CCA CTC	55°C	514 bp
714_H13_41_T7	TGA GCG CCC AGA TCC ATG	GCA GGG GAT GCA GAG ATG	58°C	506 bp
NFKBIB	CTG CAC CTG GCA GCC ATC	GCT GGA GCA GCA CGC AAG	60°C	154 bp
RP44-528_O23_Sp6	GTA TCC TCC CTG AAA GTC	CCA GGA AGA ACC TGG AAC	55°C	148 bp

Table 1 continued on next page

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TABLE 1: (continued)

Primer[a]	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	T_M	PCR product
714_C02_171_Sp6	GGT GGC ATG TGG CTT TGC	GGG TCA CAG TGT GGT CTG	58°C	418 bp
714_H13_41_Sp6	CAC CTG ACC CCA CAA ATG AAC	TTT TCT TCG GAC GCT CCA GAG	64°C	334 bp
RP44-172_J01_Sp6	AAT TCG CCT ATC GGA GGT	TGC ACC TCC AAC AGG CAA	55°C	110 bp
714_I11_51_Sp6	ATT GCT TGC CAG GCA GAG	CCC CCT TAT TGG GAC TTC	56°C	119 bp
714_H16_159_T7	AAG TGA TTC CAG CCA CTG	ATT GAT GGG TGG ATA GAG	53°C	127 bp
RP44-299_D01_T7	GAA TCA ACA ATA GTA GGT	AGA GCA AAT CTT TAT ACG	48°C	144 bp
RP44-338_B22_T7	AGC TGA GAC CAA TGC CAT	ATA ATT GGG AGT TCT CGC	53°C	122 bp
PAK4[b]	CAG CGA GTG TCC CAT GAG	CAT GGG TCA GCA GGA TGG	58°C	~ 1600 bp
714_H06_5_T7	GTA ACC CTC ATG GGG GAG AC	CCT GGT CCA GGT GGC ACC T	64°C	131 bp
RP44-338_B22_Sp6	AGT GGG GGG GTC TTC TAC	CTC TTC ACG ACC TCT CTG	57°C	254 bp
RP44-299_D01_Sp6	GGG GAA AGA AAA GGC TTC	CCT CAC TCC TGG AAA TCC	55°C	156 bp
RP44-477_B07_T7	ACA TAC AAC CCC CAA CTC	TGG TTC AGG AGA AGA TGG	54°C	517 bp
RP44-528_O23_T7	CTG CTC CTC ATT CCC ATG	CCG TCT TAT GCT TGA GTC	55°C	187 bp

[a]Primer names of the format 714_xxx denote primer pairs that were generated from PAC end sequences (Fig. 1). Primer names of the format RP-44_xxx denote primers pairs that were generated from BAC end sequences. All other primer names denote gene-derived PCR primer pairs that were generated from publicly available porcine EST sequences.
[b]The RH results of these markers have been submitted to the IMpRH database.
[c]The RH results of these markers have been submitted to the IMpRH database under the names in brackets.

of 16 human genes closely linked to human *RYR1* were used to screen the TAIGP714 PAC library and the porcine RPCI-44 BAC library. For 8 of these 16 human genes orthologous porcine expressed sequence tags (ESTs) were available that allowed the design of porcine PCR primer pairs for the library screening (Table 1). Clones for the other eight genes were screened by hybridization of high-density colony filters with human cDNA probes (Table 2). We determined the insert size and end sequences from every isolated BAC or PAC clone. Non-repetitive end sequences were used to develop new sequence tagged site (STS) markers that were employed for chromosome walking in order to join the individual emerging contigs into one large contig. In total, we generated 42 new STS markers in addition to the 12 gene-specific STSs that were used during the initial library screenings. We tested every STS marker either on a somatic cell hybrid panel or on the IMpRH panel for the correct chromosomal localization on SSC 6 to avoid any problems due to chimeric clones during the chromosome walking steps. In this way we detected one chimeric clone whose PCR results were discarded. The complete BAC/PAC contig consisted of 59 clones (23 BACs and 36 PACs; Fig. 1). We determined overlaps between clones by STS content analysis and *Eco*RI fingerprinting. The entire contig spans approximately 1.2 Mb and can be covered with a minimal tiling path of nine clones.

Physical Mapping and Gene Order

We subjected all BAC and PAC end sequences generated during this study to BLAST searches against the public nucleotide databases. Several significant matches (e-value < 10^{-5}) against genomic sequences of HSA 19q13.13 were obtained. These matches corresponded well with the overall clone order in the porcine BAC/PAC contig and confirmed the correct assembly. During construction of the porcine contig we designed heterologous primers from the human *PAK4*, *NFKBIB*, *ECH1*, *LGALS4*, *ACTN4*, *MAP4K1*, *PSMD8*, and *PPP1R14A* sequences. PCR analysis of all contig clones with the gene-specific primer pairs revealed the localization of these genes on the contig. RH results of six selected STS markers (PAK4, ECH1, LGALS4, 714_K13_61_Sp6 (ITZ001), RYR1_Promoter, PPP1R14A) were also submitted to the IMpRH porcine RH mapping database (http://www.toulouse.inra.fr/lgc/pig/RH/IMpRH.htm). Eight human genes, which are located close to the *RYR1* gene region, were tested by hybridization of IMAGE cDNA clones on a membrane with *Eco*RI digested DNA of the porcine BAC and PAC clones. These genes could also be precisely localized on the contig. In total, we assigned 17 genes to the contig on SSC6q1.2 (Table 2). Previously, only the porcine genes *RYR1*, *LGALS4*, and *PPP1R14A* had been partially cloned [6–8] and only *RYR1* had been chromosomally assigned [9]. Therefore, the construction of this contig provides 16 new chromosomal assignments for porcine genes.

Comparative Analysis

The gene order in the pig genome corresponds exactly to the gene order of the NCBI HSA 19 map (http://www.ncbi.nlm.nih.gov, as of April 28, 2002; Fig. 2). It is also highly similar to the Ensembl HSA 19 map; however, in the Ensembl HSA 19 map the gene *LGALS4* is currently not annotated (http://www.ensembl.org, as of April 28, 2002). The physical distance between the investigated genes is much smaller in pig than in both human maps. Although the distance between

porcine *NEUD4* and *GMFG* is roughly 1 Mb, the orthologous genes in human are separated by 2.4 Mb. A certain degree of gene order conservation can also be observed with respect to the mouse. The current Ensembl map of mouse chromosome 7 (as of April 28, 2002) lists 12 of the 17 analyzed genes in a similar order as in pig or human. The only discrepancy within these 12 genes is an inversion of the five genes *LGALS7–LGALS4–ECH1–HNRPL–SIRT2*. On the other hand, in mouse some of the remaining genes are currently assigned to other chromosomes, for example, the gene *Gmfg* is assigned to MMU 6 and *Actn4* is assigned to MMU 12. At the time of this writing, *Kcnk8* and *Kcnk6* are considered to be synonyms for the same mouse gene on MMU 19 (http://www.informatics.jax.org/). The comparative data indicate that there might be a true *KCNK6* ortholog on MMU 7, whereas the gene on MMU 19 is probably only the related paralog *Kcnk8*. So far, neither mouse ortholog for human and porcine *PAK4*, encoding the p21(CDKN1A)-activated kinase-4, has been described. A BLAST search with the human *PAK4* cDNA did not reveal any significant match to the mouse genome draft sequence.

TABLE 2: Genes and cDNA hybridization probes within the BAC/PAC contig

Human gene symbol	Mouse gene symbol[a]	IMAGE-ID[b]	RZPD clone ID
NEUD4	*Neud4* (*Reqn*)	4397074	IMAGp 998 A1110097
PPP1R14A	*Ppp1r14a*		
SPINT2	*Spint2* (*Spt2*)	3857277	IRAKp 961 B2321
KCNK6	*Kcnk8*	3639657	IRALp 962 L1413
PSMD8	*Psmd8*		
RYR1	*Ryr1*		
MAP4K1	*Map4k1* (*Hpk1*)		
ACTN4	*Actn4*		
LGALS7	*Lgals7* (*Leg7*)	2251428	IMAGp 998 G135572
LGALS4	*Lgals4*		
ECH1	*Ech1*		
HNRPL	*Hnrpl*	3903111	IMAGp 998 K169706
SIRT2	*Sirt2*	4179027	IMAGp 998 D049488
NFKBIB	*Nfkbib* (*Ikbb*)		
MRPS12	*Mrps12* (*Rt12*)	2958861	IMAGp 958 K2249
PAK4	–		
GMFG	*Gmfg*	785347	IMAGp 998 H201935

[a]In some cases commonly used aliases, which differ from the official mouse gene names are given in parentheses. No ortholog of human *PAK4* has been identified in the mouse.
[b]For those genes where IMAGE IDs are given, the localization of the porcine orthologs on the contig was performed by hybridization with the IMAGE clone inserts. The other genes were localized on the contig by PCR with primers given in Table 1.

DISCUSSION

The construction of BAC/PAC contigs of farm animals still represents a challenging task because there are only a restricted number of sequences for the design of PCR primer pairs or hybridization probes available. Currently, the method of choice to overcome these difficulties is the use of a comparative approach, that is, using appropriate human or mouse heterologous screening probes for the construction of region-specific contigs in farm animals. Our results demonstrate that in a gene-rich region like SSC 6q1.2 or HSA 19q13.13, enough conserved sequences are present to allow the successful cloning of BAC/PAC contigs. The parallel use of many conserved genes as probes for the library screenings limits the number of time-consuming chromosome walking steps and simultaneously provides valuable informations on the positions of genes within a given clone contig.

The use of two different libraries for the contig construction facilitated gap closure between initially isolated smaller contigs. Two genomic regions were not represented in the smaller TAIGP714 PAC library, which had to be expected as this library contains only three genome equivalents. On the other hand, we found several positive clones for each probe in the larger 10 genome equivalent RPCI-44 BAC library. Although the coverage of the TAIGP714 PAC library is not as good as in the RPCI-44 BAC library, it was also used because DNA pools for PCR-based library screenings were available for this library.

The analysis of gene content of the investigated genomic region on SSC 6q1.2 revealed a perfectly conserved gene order between pig and human (NCBI map). In other studies different gene orders within conserved synteny groups were observed across mammalian species [10,11]. One possible reason for the strong conservation observed here may be that the extremely high gene content of SSC 6q1.2 interfered with major chromosome rearrangements during mammalian evolution. Another reason might be that this region is located near the centromere on SSC 6 as well as on HSA 19. Meiotic recombinations, as one possible trigger mechanism for a chromosomal rearrangement, generally occur very rarely in such regions close to the centromere. For SSC 6q1.2 the low recombination fraction was demonstrated during the construction of the porcine linkage map [12].

The compilation of a clone contig and detailed physical map of the genomic region of porcine *RYR1* provides an important prerequisite to the answer of the question whether the well-known MH mutation in porcine *RYR1* is responsible for both the stress susceptibility and the increased muscle growth in pigs, or whether the increased muscle growth that has been observed in stress susceptible pigs is caused by an independent mutation in a closely linked gene. The low recombination frequency of this genomic region suggests that,

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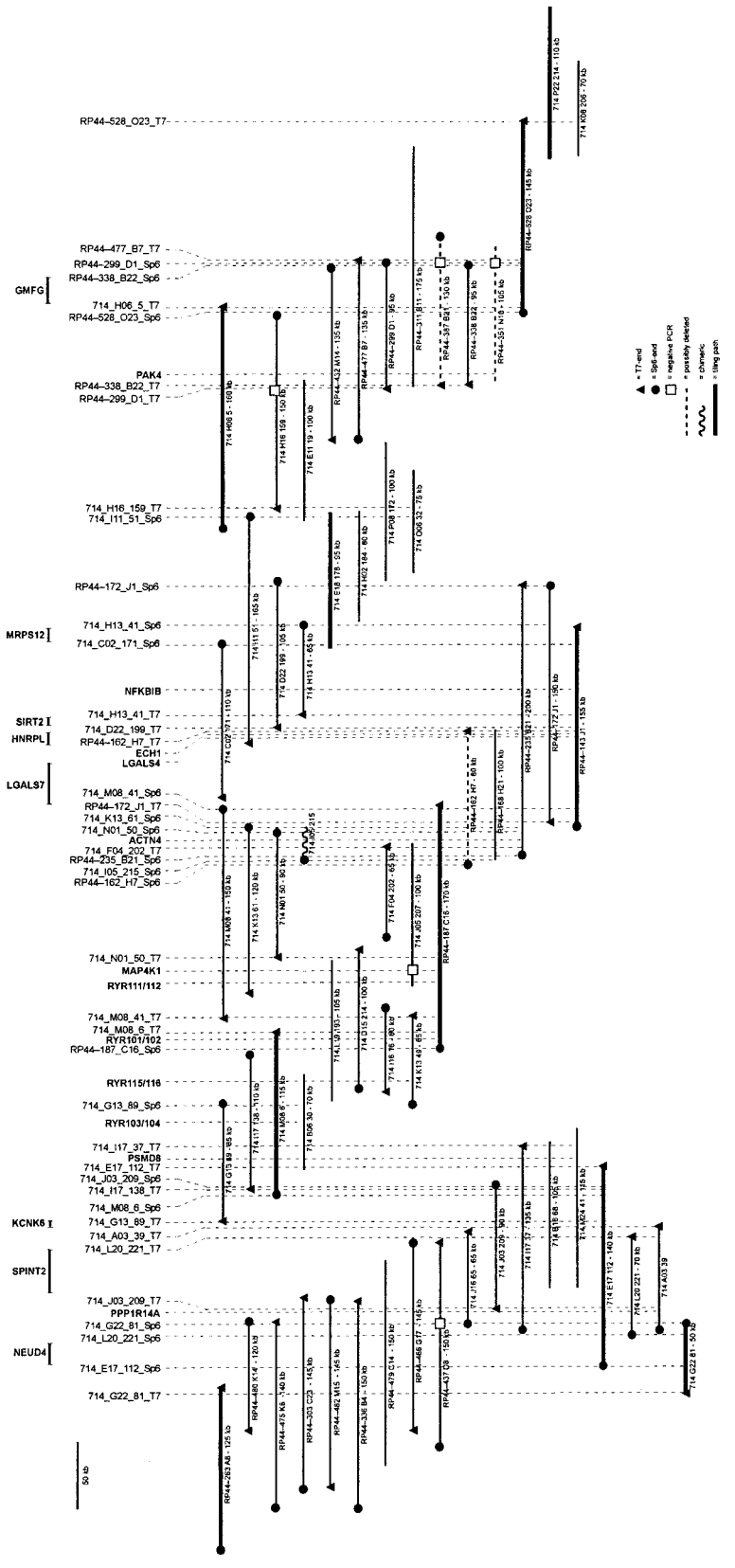

FIG. 1. Physical map of the porcine *RYR1* region and the isolated BAC/PAC contig. STS markers are indicated vertically on the top, cDNA hybridization probes are represented as horizontal lines at the top, and markers that are associated with genes are denoted in bold. The physical sizes covered by the different hybridization probes depend on the intron sizes of their respective genomic targets. BACs and PACs are indicated below the markers with their corresponding clone names and insert sizes. A minimal tiling path of nine clones is indicated.

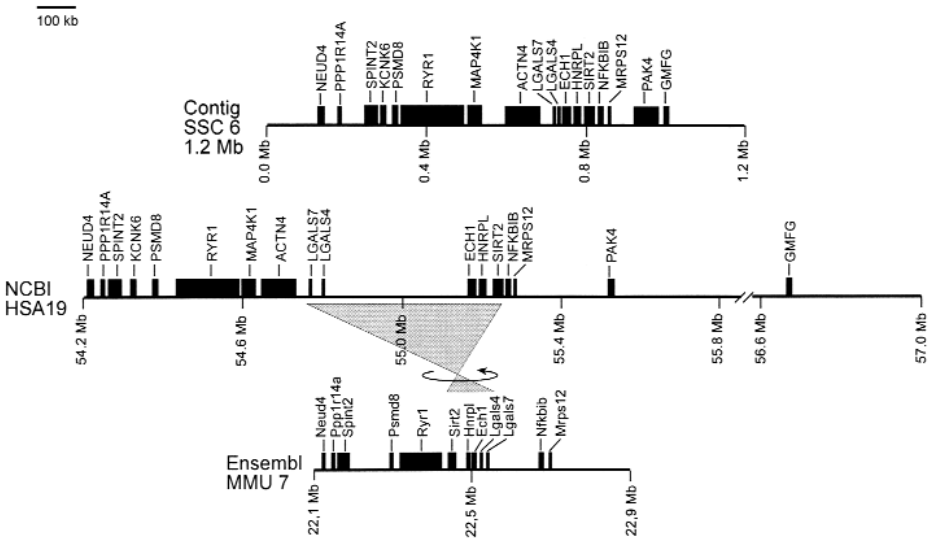

FIG. 2. Pig–human–mouse comparative map. Physical mapping of the *RYR1* region in *Sus scrofa* (results from this study), *Homo sapiens* (NCBI from April 28, 2002), and *Mus musculus* (Ensembl from April 28, 2002) is shown. Note the identical gene order between porcine and human maps. The Ensembl map of HSA 19 in the analyzed region is virtually identical to the NCBI HSA 19 map, with the only difference being that in the Ensembl map the gene *LGALS4* is not annotated. The mouse genes *Kcnk6* (*Kcnk8*), *Actn4*, and *Gmfg* are annotated on other chromosomes. Mouse *Map4k1* is currently unassigned and for human/porcine *PAK4*, no mouse ortholog is known.

within breeds, strong linkage disequilibrium over distances covering several other genes in addition to *RYR1* may be expected. The availability of the BAC/PAC contig will thus allow the sequencing and subsequent mutation analysis of the genes that are in the immediate neighborhood of *RYR1*. Furthermore, this extensively characterized region in the pig genome might provide valuable comparative information to improve the current human and mouse genome assemblies in the corresponding syntenic regions.

MATERIALS AND METHODS

DNA library screening. We performed standard molecular biology methods as described [13]. PAC clones were isolated by three-dimensional PCR-based screening of the TAIGP714 PAC library (http://www.rzpd.de) [14]. Primer sequences and annealing temperatures are given in Table 1. PCR was carried out in a 16 μl reaction volume containing 100 μM of each dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer, and 0.35 U *Taq* DNA polymerase in the reaction buffer supplied by the manufacturer (Qiagen, Hilden, Germany). After a 5-minute initial denaturation at 94°C, 35 cycles of 45 seconds at 94°C, 45 seconds at the annealing temperature of the specific primer pair, and 45 seconds at 72°C were performed in an MJ Research thermocycler (Biozym, Hess. Oldendorf, Germany). Finally an extension step at 72°C for 5 minutes was performed.

For the isolation of BAC clones, we screened high-density clone filters of the porcine genomic BAC library RPCI-44 constructed in pTARBAC2 [15] according to the RPCI protocols (http://www.chori.org/bacpac/). PCR-amplified DNA fragments were labeled with ^{32}P and used as probes. Positive clones were provided by the Children's Hospital Oakland Research Institute, BAC/PAC Resources.

We prepared BAC and PAC DNA from 100 ml overnight cultures using the Qiagen Midi plasmid kit according to the modified protocol for BACs (Qiagen, Hilden, Germany). To determine the insert sizes, ~ 100 ng BAC or PAC DNA was digested with 5 U *Not*I and then fractionated on 1% agarose pulsed-field gels with linear ramped pulse times of 1–30 seconds.

BAC/PAC ends sequencing. We sequenced isolated BAC and PAC DNA with the thermosequenase kit (Amersham Biosciences, Freiburg, Germany) and a LICOR 4200L automated sequencer using IRD-labeled T7 and Sp6 sequencing primers. Sequence data were analyzed with Sequencher 4.0.5 (GeneCodes, Ann Arbor, MI). Further analyses were performed with the online tools of the European Bioinformatics Institute (http://www.ebi.ac.uk/), BLAST database searches in the GenBank database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), and the RepeatMasker searching tool for repetitive elements (A. F. A. Smit and P. Green, http://repeatmasker.genome.washington.edu/). We used single-copy sequences to design primer pairs for the chromosome walking using the program GeneFisher (http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/gf_submit?mode=START).

Somatic cell hybrid and RH mapping. We tested STS markers on a somatic cell hybrid panel [16] or mapped them on the IMpRH panel [17] according to the INRA protocols (http://www.toulouse.inra.fr/lgc/lgc.htm). After genotyping the IMpRH panel the chromosomal assignments were carried out with software available on the INRA World-Wide Web server and submitted to the IMpRH database (http://www.toulouse.inra.fr/lgc/pig/RH/IMpRH.htm).

BAC/PAC fingerprinting and physical mapping. BAC/PAC DNA of the 59 isolated clones was *Eco*RI digested and separated by gel-electrophoresis on a single 0.8% agarose gel. We visually inspected the band patterns and estimated the overlap of two given clones from the number and size of sheared bands. We transferred the DNA from the gel to a nylon membrane, which we then hybridized with different inserts of IMAGE cDNA clones (Table 2) using the ECL direct labeling and detection kit (Amersham Biosciences, Freiburg, Germany). IMAGE clones [18] were provided by the Resource Center/Primary Database of the German Human Genome Project (http://www.rzpd.de/).

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Chapter 3 A high resolution physical and RH map of pig chromosome 6q1.2 and comparative analysis with human chromosome 19q13.1

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A high resolution physical and RH map of pig chromosome 6q1.2 and comparative analysis with human chromosome 19q13.1

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Abstract

Background: The generation of BAC/PAC contigs in targeted genome regions is a powerful method to establish high-resolution physical maps. In domestic animal species the generation of such contigs is typically initiated with the screening of libraries with probes derived from human genes that are expected to be located in the region of interest by comparative mapping. However, in many instances the available gene-derived probes are too far apart to allow the cloning of BAC/PAC contigs larger than a few hundred kb. High resolution physical mapping allows to estimate the sizes of gaps and to control the orientation of the individual sub-contigs, which helps to avoid errors during the assembly of smaller contigs into final Mb-sized contigs. The recently constructed porcine IMNpRH2 panel allowed us to use this approach for the construction of high-resolution physical maps of SSC 6q1.2.

Results: Two sequence-ready BAC/PAC contigs of the gene-rich region on porcine chromosome 6q1.2 (SSC 6q1.2) containing the *RYRI* gene were constructed. The two contigs spanned about 1.2 Mb and 2.0 Mb respectively. The construction of these contigs was monitored by the results provided by the mapping of 15 markers on the $IMpRH_{7000rad}$ and 35 markers on the $IMNpRH2_{12000rad}$ radiation hybrid panels. Analyses on the IMpRH panel allowed us to globally link and orientate preliminary smaller contigs, whereas analyses on the high resolution IMNpRH2 panel allowed us to finally identify the order of genes and markers.

Conclusions: A framework map of 523 cR_{12000} was established covering the whole studied region. The order of markers on the framework 1000:1 RH map was found totally consistent with the data deduced from the contig map. The kb/cR ratio was very constant in the whole region, with an average value of 6.6 kb/cR. We estimate that the size of the remaining gap between the two contigs is of about 300 kb. The integrated physical and RH map of the investigated region on SSC 6q1.2 was used for a comparative analysis with respect to the syntenic regions on HSA 19q13.1 and MMU 7 and revealed a perfectly conserved gene order across the entire studied interval.

Background

Comparative genome analysis increases the knowledge of genome evolution and is especially important in livestock species where the currently available sequence information is very limited as compared to the vast amount of information available from the human and mouse genomes. Radiation hybrid mapping is seen as an efficient technique for the generation of high-resolution gene maps in different species and RH maps can be integrated in comparative mapping approaches to reveal the degree of synteny conservation between species [1].

Two RH panels have been reported for the pig: the 7 000 rad IMpRH panel [2] that provides medium-resolution global mapping information, and the 12 000 rad IMNpRH2 [3], that can be used to construct high-resolution local RH maps. Panels developed after a high level of cell irradiation (10 000 to 50 000 rads) are very useful for high resolution regional mapping studies but they require a characterization with a very large number of markers to be useful for genome-wide mapping studies [4].

The porcine *RYRl* gene region on SSC 6q1.2 is of special interest due to its economical importance. The porcine stress syndrome (PSS), which in pigs is caused by a single *RYRl* point mutation, is known to be associated with positive characteristics like increased muscling and increased lean meat content. Until now, it is not clear whether the *RYRl* mutation is also responsible for the positive carcass traits in stress susceptible pigs or whether these complex growth traits are influenced by other closely linked genes on SSC 6q1.2 [5–7]. Furthermore, this genomic region is also of special interest as it represents a GC-rich genomic region with a very high gene content. To investigate this genomic region we have previously reported the construction and analysis of a 1.2 Mb BAC/PAC contig [8].

In the present study, we report the construction of high-resolution framework and comprehensive RH maps of the *RYRl* gene region on the porcine chromosome 6q1.2 using the porcine IMpRH and IMNpRH2 panels as well as the comparison of the RH maps to an extended clone-based physical map of this region.

Results and Discussion

Construction of the BAC and PAC contig and analysis of end sequences

We previously reported the construction of a 1.2 Mb BAC/PAC contig on SSC 6q1.2 [8]. To extend the existing contig the porcine TAIGP714 PAC and RPCI-44 BAC libraries were screened with new probes either derived from end fragments of previously isolated porcine genomic clones or from human HSA 19q13.1 genes. Assembly of all 171 isolated BAC and PAC clones according to STS content, insert sizes and fingerprinting data resulted in the expansion of the existing 1.2 Mb contig [8] to 2.0 Mb and the generation of a new 1.2 Mb contig (Fig. 1). End sequences from all clones of the contig were generated and submitted to the EMBL database under accessions AJ514457-AJ514832. In total 292 end sequences from SSC 6q1.2 with an average read length of 708 bp totaling 207 kb of genomic survey sequences were generated. Thus, the BAC/PAC end sequences cover approximately 6 % of the studied genomic region. The end sequences contain an average GC content of 47 % exceeding the value of 41 % that is generally accepted as the average GC content in mammalian genomes [9]. The GC content analysis further confirms that SSC 6q1.2 is indeed closely related to HSA 19q13.1, which has a GC content of 46 % in the corresponding 4 Mb region. An analysis of repetitive elements revealed that 39.8 % of the end sequences consisted of repetitive DNA. Of the 39.8 % repetitive DNA, 20.5 % were SINE, 13.3 % were LINE, 2.4 % were of retroviral origin (LTRs), and 2.0 % represented DNA transposons. The predominance of SINEs is another typical hallmark of GC-rich and gene-rich genome segments [10]. The analysis of the end sequences also revealed three dinucleotide and one tetranucleotide microsatellite (AJ514594, AJ514613, AJ514706, AJ514795).

The availability of the end sequences allowed the continuous verification of the contig assembly by comparative mapping. In BLAST searches against the human draft genome sequence, approximately 15 % of the BAC/PAC end sequences showed significant ($E < 10^{-5}$) matches to HSA 19q13.1, which allowed the precise comparative mapping of 27 % of the tested BAC/PAC clones. Of the investigated clones, 73 % had no match in the human genome sequence, 23 % had matches with one end sequence, and 4 % had matches with both end sequences.

Physical mapping and comparative analysis

During the contig construction many gene-specific STSs were used, which allowed the unequivocal assignment of genes to individual clones. Further genes were localized by hybridization of heterologous cDNA probes to the individual BAC/PAC clones and BLAST analysis of the clone end sequences. Using these approaches, 33 genes in total were localized. Furthermore, the microsatellite SW193 was also localized by STS content analysis thus anchor in the physical clone-based map to the linkage map of this region [11].

The gene assignments were compared with human and mouse maps and a comparative map for SSC 6q1.2, HSA 19q13.1 and MMU 7 was developed (Fig. 2). The gene order in this region of the pig genome corresponds exactly to the gene order of the NCBI HSA 19 map (http://www.ncbi.nlm.nih.gov build 31). The gene order of MMU 7 (http://www.ncbi.nlm.nih.gov MGSCv3) also

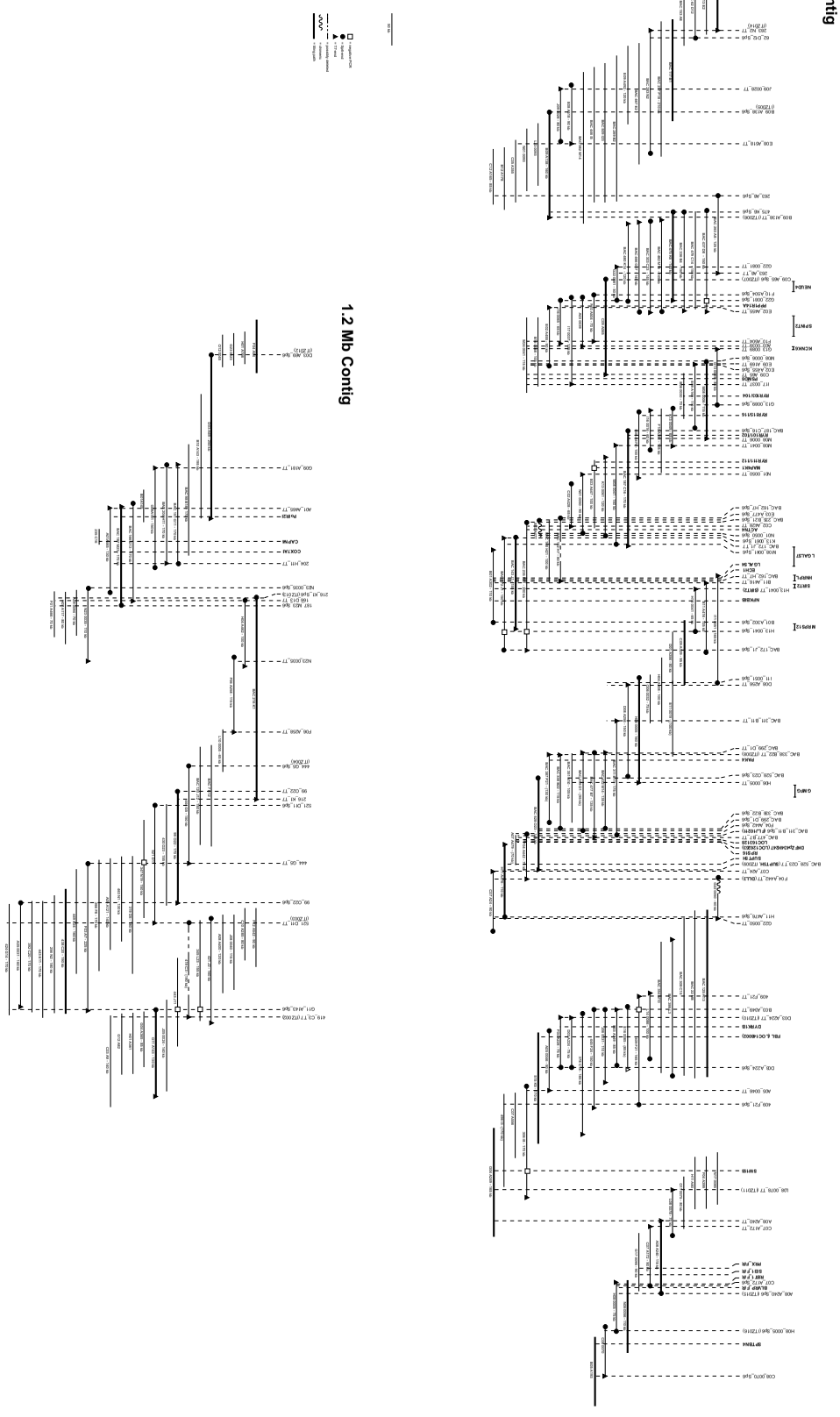

Figure 1
Physical map of the isolated BAC/PAC contigs. STS markers are represented by vertical dotted lines, cDNA hybridization probes are represented as horizontal lines at the top, markers that are associated with genes are denoted in bold. The physical sizes covered by the different hybridization probes depend on the intron sizes of their respective genomic targets. BACs and PACs are indicated below the markers with their corresponding clone names, positions, and insert sizes.

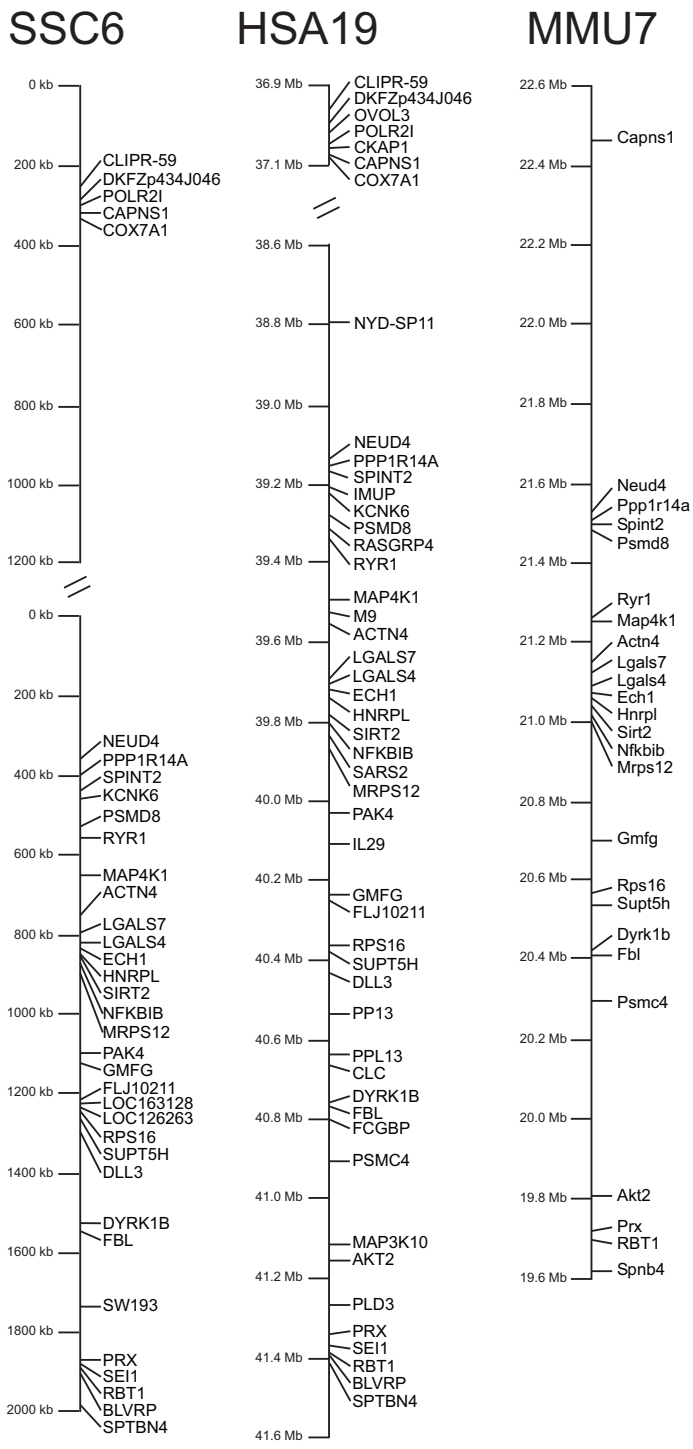

Figure 2

Comparative maps of Sus scrofa chromosome 6q1.2, Homo sapiens chromosome 19q13 and Mus musculus chromosome 7. Comparative maps of *Sus scrofa* chromosome 6q1.2 (results from this study), *Homo sapiens* chromosome 19q13 (http://www.ncbi.nlm.nih.gov build 31), and *Mus musculus* chromosome 7 (http://www.ncbi.nlm.nih.gov MGSCv3). Gene order is perfectly conserved between the three species, however the gene order is inverted in the mouse with respect to the other two species. In the human map all known genes without hypothetical gene predictions are listed, while in the murine map only those genes are listed that have also been mapped in the pig. In the porcine map the position of the microsatellite SW193 is also indicated.

corresponds exactly to the gene order of SSC 6 and HSA 19 but the orientation is inverted. The perfect synteny conservation between mouse and the two other species can only be observed since the latest update of the mouse maps as in the previous mouse genome assembly a major rearrangement of the gene order in this genome region was observed [8].

Whereas the gene order is perfectly conserved between human, mouse and pig, the physical distances between genes vary somewhat between the three species. Within the investigated region the gene-poor stretch between COX7A1 and NEUD4 accounts for the biggest part of these size deviations. The cloned region has a very uneven gene density. At the top and at the bottom of the map (Fig. 2) genes are clustered extremely dense with very short intergenic regions, while in the middle of the map, between the *COX7A1* and the *NEUD4* gene the gene content is actually very low.

RH mapping

In this study, we were able to build two comprehensive RH maps for SSC 6q1.2. On the 7000 rad IMpRH panel 15 STS markers were genotyped, while on the 12 000 rad IMNpRH2 35 STS markers were analyzed. Retention frequencies of markers ranged from 18.1 % to 32.8 % (average 22.9 %) on the IMpRH panel and from 27.8 % to 44.3 % with an average retention frequency of 37.5 % on the porcine IMNpRH2 panel.

During the building of these two contigs, we simultaneously analyzed data obtained on both IMpRH and IMNpRH2 panels using the Carthagene program. Intermediate rough analyses of RH data allowed us to monitor the construction of the contig. In particular it allowed us to orient a subcontig in the gene poor region from ITZ002 to ITZ004 as well as to estimate the size of remainmg gaps.

When the full RH data set was available for both panels, it appeared that at the scale of 10–100 kb, the degree of resolution of the IMpRH panel is not high enough, and furthermore the order of genes that could be determined on this panel is very sensitive to some small genotyping errors. To produce a final reference map we thus computed a 1000:1 framework map using only the 35 vectors produced on IMNpRH2 panel. The framework status of the map was tested by calculation of likelihood of maps produced after all local permutations in a slipping window of 6 markers, and by global local inversions. We confirmed that no altemate order could be identified with a difference of log likelihood of less than 3 compared to the proposed order. The framework map contained 24 of the 35 IMNpRH2 markers. Using this framework map comprehensive maps were produced on each panel. In order to avoid inflation of the map size, we chose to project additional markers at their most likely location, without altering the multipoint distance between framework markers (Fig. 3).

As shown in figure 3, the gene orders on the RH and physical maps are generally in good agreement. This agreement is perfect between the physical map and the 1000:1 framework RH map produced on IMNpRH2 panel. It demonstrates that at the 50–100 kb scale, fully accurate maps can be produced on this panel provided that 1000:1 framework maps are drawn.

Some minor discrepancies can be found when comprehensive maps are drawn. For instance, the location of SPTBN4 on the IMNpRH2 map seems incorrect. However a difference of log likelihood of only 1.57 is found between the maps constructed under the most likely order and the expected order. We thus think that our RH data do not sufficiently support the hypothesis of a very small rearrangement of this region. It should be pointed out, that even if additional markers are added at their most likely location on this kind of comprehensive map, their mapping does not affect the distance calculated between framework markers.

We also compared the resolution of both panels on the framework map established between COX7A1 and BLVRP. On IMpRH the distance is 146 cR_{7000}, whereas the same fragment is 438 cR_{12000} long on IMNpRH2. In this region the ratio between the resolutions is thus 3.01, which is slightly higher than the value of 2.77 observed in the PRKAG3-RN region [3] and of 2.43 observed in a QTL region close to the centromere of SSC 7 [12]. In the gene rich region between RYRl and BLVRP, which is precisely mapped on the reported clone contig, a ratio of 6.6 kb / cR_{12000} (1370 kb / 207 cR_{12000}) is observed on the IMNpRH2 panel.

The RH map allowed us to confirm the close link between the two contigs we produced. The distance between the extremity markers of the contigs (ITZ002 and ITZ014) was estimated at 43.2 cR_{12000}. Considering a ratio of 6.6 kb/ cR_{12000} in this region, we can estimate that the physical distance between both contigs could be around 285 kb, which is roughly similar to the 360 kb distance that would be estimated from the human-pig comparative map.

Conclusion

The IMNpRH2 panel allowed a highly accurate resolution of closely spaced markers and was very useful in evaluating the assembly of a clone contig. In most instances not only the order of markers but also the physical distances between markers could be very accurately estimated from the RH_{12000} map. During the contig building it helped us to orientate small sub-contigs, which were originally un-

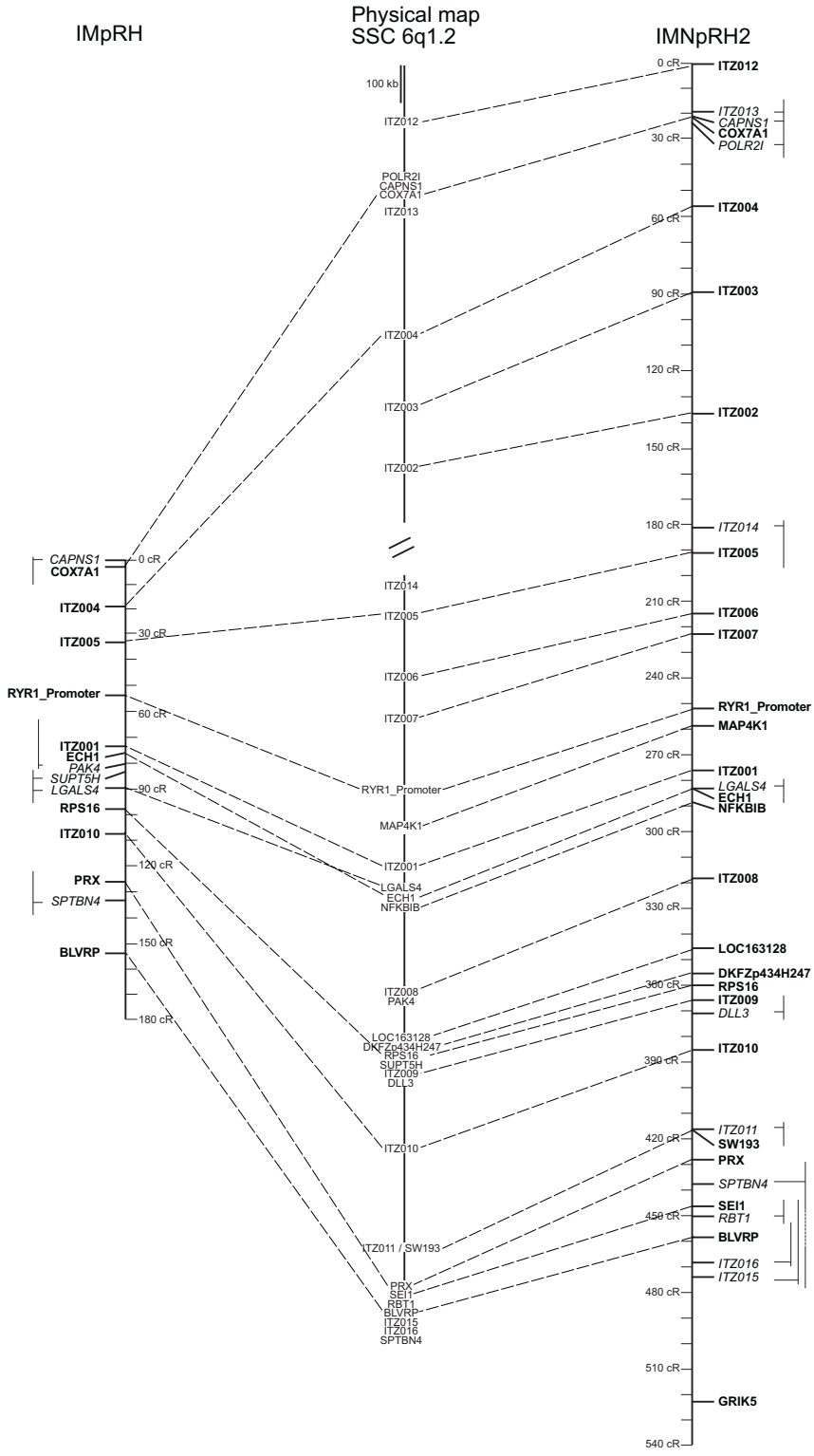

Figure 3

Comparison of the clone-based physical map, RH7000 map and RH12000 mapofSSC 6q1.2. The RH maps represent comprehensive maps, where the markers of the 1000:1 framework map are highlighted in bold. Note that there is perfect agreement in the order of framework markers between the IMNpRH2 RH_{12000} map and the clone-based physical map. For the non-framework markers, altemate positions are indicated by thin vertical lines.

Table 1: Primer sequences for RH mapping

Primer	Forward sequence (5 '-3')	Reverse sequence (5 '-3')	Tm (°C)	PCR-Product (bp)	Retention IMpRH	Retention IMNpRH2
BLVRB	GGT TAT GAG GTG ACA GTG	CCA AGA GCA CGA TGA TGG	55	148	0.14	0.38
CAPNSI	TGT ATT CCT GAA CGG GAG	TGC AAG AGA GGG CTA ATG	54	272	0.31	0.38
COX7A1	CAC CTA CTG GAC GAA TCC	AGG TCC CGA GGT ATT ACA G	59	388	0.29	0.38
DLL3	ACC CCA GAA TCC TCG TAC	AGA GCA GGA CAT AGC ATC	55	395		0.39
ECHI	ACC AGG AGG TCC TGC TTG	ATG TAG TTG AGG CCC TCT G	58	136	0.18	0.34
GRIK5	CAA CTT CCA GGC CCT GTC	TTG ATG AGC TCG CCA ACC	60	174		0.31
ITZ001	TAG CCT TTC CTG TGG AGG	ACA CAA AAG CAC ACA CCG	56	423	0.19	0.32
ITZ002	AGC CTT CAC CCA GAA GTC	GAT GCA CAA GGA GCT GAG	59	287		0.36
ITZ003	CAT AGG GCT TTT CAC CAG	TTC TTG CCG AAT ATC AGA G	54	297		0.28
ITZ004	TTC ACT TTG GGT CTG CTG	ACC TAC CCA CTG CTA TGC	55	311	0.24	0.26
ITZ005	TCC AGA CAA GTG AGA AAC AA	TTA ATT TAT AAT GCC TGG TCA	55	320	0.24	0.37
ITZ006	ATC TGC GGA GAG GAA AAG	TCG TGT TTG TTG GAA TGT C	54	201		0.35
ITZ007	GTG ACT TGT AGA CCA CAG	CCT ACA GAG GGA GAA TCC	55	346		0.35
ITZ008	AGC TGA GAC CAA TGC CAT	ATA ATT GGG AGT TCT CGC	53	122		0.36
ITZ009	CTG CTC CTC ATT CCC ATG	CCG TCT TAT GCT TGA GTC	55	187		0.40
ITZ010	CAG ATT GGG ATG AAG CTC	TTA TCA ATC CCA ACA CAC C	54	114	0.22	0.37
ITZ011	AGA CGG GAA ATT GAG ACC	TCC CTG TGG CAG TAA ATG	58	345		0.37
ITZ012	GTG GGG CCC TAT AAA GAC	CCG AGG GTC AAA TGT CTG	56	223		0.38
ITZ013	TTG GAG GGT TCA ACT ACG	GTA AAC CCG TTC ACG TTG	57	197		0.38
ITZ014	TGC CTG TTC ACG AAC CAC	TCC TTG TGT GGG CTA CAG	59	100		0.32
ITZ015	AAG AAA AGG AAA AGG TTT GG	TGT TCT CGC AAA CAG TGA G	58	304		0.33
ITZ016	AGA CAG GCT CCG ATG AAG	AGA AAG GCT TCC CTG CTG	59	209		0.35
LGALS4	GAT GTC GCC TTC CAC TTC	TGA TGA CCA GCT CGA AGG	56	142	0.18	0.35
LOC 163128	TCC AAA GAG AAG GTG GTG	ATG AGG GCA TAG GAG AGC	55	170		0.36
DKFZp434H247	TTC TGC AGC TTC TTC TGC	ATC GAA GTC CTG TTG CTG	54	145		0.41
MAP4K1	CCT ACC CAC GCC TAT GC	CCA GCC AGC AGG AAA GC	56	136		0.33
NFKBIB	CTG CAC CTG GCA GCC ATC	GCT GGA GCA GCA CGC AAG	60	154		0.32
PAK4	CAG CGA GTG TCC CAT GAG	CAT GGG TCA GCA GGA TGG	58	~1600	0.19	
POLR2I	ACC ACC CGT GCC AAA AG	CCG CGC ACT GTG TGA CT	55	172		0.37
PPP1R14	GCT GAG CAA GCT GCA GTC	GGT ACA GCT CCT CCA AGC	58	164	0.32	
PRX	GCC TCA GGT GAC CTT GTC	CCC ACA TCC AGC TCA AGC	58	117	0.21	0.38
RBTI	CCA TGG ATG AGA CTG AGC	GGC ACA GAA GAG GTT GTG	56	218		0.33
RPS16	CGC TGA TCA TCA CGA TGG	GCT TTT GGG CAA GGA ACG	56	294	0.24	0.41
RYR103/104 (RYR_Promoter)	TTC GTT TCT GCT TCG CC	CTC TCT CCT CCC ATT TC	48	162	0.20	0.34
SEII	GAG CTG GAT GAT GCT GAG	GCT GTG ATG GAG CTT GAG	56	184		0.32
SPTBN4	ATA TCC TGC CCC AAG AAG	GAG GAG GTC GAC GTT TTG	58	282	0.20	0.38
SUPT5H	AGG AGC TTC CCC AGG AAG	TGG GTG AGG ATC GGG AAG	58	121	0.18	
SW193	TGC CAT CCT TTC TTT CAT TAC G	TCA CTC TGA GGG GTC CTG AC	62	101		0.37

linked, and to estimate the size of remaining gaps. Combining analyses on both IMpRH and IMNpRH2 panels provides both the possibility to detect significant linkage between relatively distant markers on the IMpRH panel as well as to determine the accurate gene order on the higher resolving IMNpRH2 panel.

Methods

DNA library screening and chromosome walking

Library screenings were done as described [8]. Briefly, the TAIGP714 PAC library [13], http://www.rzpd.de was screened by PCR of hierarchical DNA pools. The porcine genomic BAC library RPCI-44 was screened by radioactive hybridization according to the RPCI protocols http://www.bacpac.chori.org.

DNA sequence analysis

End sequences of isolated BAC and PAC DNA were generated with a LICOR 4200L automated sequencer system. Further analyses were performed with the online tools of the European Bioinformatics Institute http://www.ebi.ac.uk/, BLAST database searches in the GenBank database of the National Center for Biotechnology Information NCBI and the RepeatMasker searching tool for repetitive elements (Smit, A.F.A. and Green, P. http://repeatmasker.genome.washington.edu/). Single copy sequences were used to design primer pairs for the chromosome walking using the programs GeneFisher and Primer3 http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/gf_submit?mode=START, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.

RH mapping

Prior to RH mapping of swine genomic inserts, each designed primer pair was tested for correct localization on SSC 6 on a somatic cell hybrid panel [14]. PCR was performed in a 22.5 μl reaction volume containing 25 ng of template DNA, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer and 1 U Taq polymerase in the reaction buffer supplied by the manufacturer (QBiogene, Heidelberg, Germany). After a 5 min initial denaturation at 94°C, 38 cycles of 45 s at 94°C, 45 s at the annealing temperature of the specific primer pair, and 45 s at 72°C were performed in a MJ Research thermocycler (Biozym, Hess. Oldendorf, Germany). Finally an extension step at 72°C for 5 min was performed. Primer sequences and annealing temperatures are given in table 1. PCR products were separated on 1–2 % agarose gels. Gels were scored twice by independent investigators.

To construct the RH map, 15 markers were mapped on the IMpRH panel [2] and 35 on the IMNpRH2 panel [3] according to the INRA protocols http://www.toulouse.inra.fr/lgc/pig/RH/IMpRH.htm. After genotyping the IMpRH panel the chromosomal assignments were performed with software available on the INRA WWW server and submitted to the IMpRH database http://imprh.toulouse.inra.fr/.

Statistical analysis of RH results

Vectors obtained on IMpRH and IMNpRH2 panels were analyzed with Carthagene software [15]. A framework map was built using buildfw option, which constructs a 1000:1 framework map by a stepwise locus adding strategy under the haploid model of fragment retention. The framework map was tested using a flips algorithm, which checks all local permutations in a window of 6 markers, and a greedy algorithm, which tries to improve the map by inversion of parts of the reference map. When the most likely order did not fit the expected order based on the human-pig comparative map, the likelihood of the two possible orders were calculated to determine the strength of the indication of a possible modification of gene order between both species. The final framework map was recomputed under a diploid model. Additional markers were mapped relatively to the framework map at their most likely location, projecting the markers on the map using the following formula (using the diploid model).

$$Loc(M) = Loc(Fwk_n) + \left[Dmltpt(Fwk_n, Fwk_{n+1}) * \frac{D2pt(Fwk_n, M)}{D2pt(Fwk_n, M) + D2pt(M, Fwk_{n+1})} \right]$$

where Loc (M) is the location on the framework map of marker M mapped between the n^{th} and $n + 1^{th}$ markers of the framework (respectively named Fwk_n and Fwk_{n+1}), Dmltpt(X,Y) and D2pt(X,Y) are the multipoint and two point distances between markers X and Y.

List of abbreviations

BAC bacterial artificial chromosome; cR centi Ray; HSA human chromosome; PAC Pl derived artificial chromosome; RH radiation hybrid; SSC porcine chromosome; STS sequence tagged site; MMU murine chromosome; IMpRH INRA Minnesota porcine Radiation Hybrid panel, IMNpRH2 INRA Minnesota Nevada porcine Radiation Hybrid panel 2

Authors' contributions

FM constructed most of the BAC/PAC contig, did the genotypings of the RH panels and drafted parts of the manuscript. DM analyzed the RH data and wrote the radiation hybrid mapping related parts of the manuscript. CD provided some initial help with the RH analysis. RV isolated several BACs and PACs of the final contigs. BB provided the TAIGP714 PAC library. AR and MY provided the IMpRH and IMNpRH2 panels and helped with analysis of RH data. TL conceptualized the investigation, coordinated the planning of the experiments and finalized the manuscript. All authors have read and approved the final manuscript.

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Chapter 4 Generation of a 5.5 Mb BAC/PAC contig of pig chromosome 6q1.2 and its integration with existing RH, genetic and comparative maps

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Generation of a 5.5 Mb BAC/PAC contig of pig chromosome 6q1.2 and its integration with existing RH, genetic and comparative maps

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4.1 Abstract

We generated a sequence-ready BAC/PAC contig spanning approximately 5.5 Mb on the porcine chromosome 6q1.2, which represents a very gene-rich genome region. STS content mapping was used as the main strategy for the assembly of the contig and a total of 6 microsatellite markers, 51 gene-related STS and 117 STS corresponding to BAC and PAC end sequences were analyzed. The contig comprises 316 BAC and PAC clones covering the region between the genes *GPI* and *LIPE*. The correct contig assembly was verified by RH-mapping of STS markers and comparative mapping of BAC/PAC end sequences using BLAST searches. The use of microsatellite primer pairs allowed the integration of the physical maps with the genetic map of this region. Comparative mapping of the porcine BAC/PAC contig with respect to the gene-rich region on the human chromosome 19q13.1 map revealed a completely conserved gene order of this segment, however, physical distances differ somewhat between HSA 19q13.1 and SSC 6q1.2. Three major differences in DNA content between human and pig are found in two large intergenic regions and in one region of a clustered gene family, respectively. While there is a complete conservation of gene order between pig and human, the comparative analysis with respect to the rodent species mouse and rat shows one breakpoint where a genome segment is inverted.

4.2 Introduction

The pig has recently joined the organisms that are considered high scientific priority for genome sequencing by the NHGRI (http://www.genome.gov/10002154; Rohrer et al., 2002). For the correct assembly of whole genome shotgun sequences highly accurate physical maps will be indispensable. High-resolution physical maps of specific genome regions are also required for the localization, isolation and characterization of genes, including those involved in the development of specific diseases or QTLs. Since the human genome project is nearly finished, comparative mapping approaches using the human information greatly facilitate the construction of physical maps in other mammalian species. In the pig, the first comparative maps have been determined at low resolution by chromosome painting experiments that demonstrated conservation of large chromosome fragments between human and pig (Goureau et al., 1996). Increased resolution of the comparative maps was achieved with the use of defined anchor probes in FISH experiments (Pinton et al., 2000). Recently, medium-resolution global comparative maps were provided by RH-mapping (Rink et al., 2002). Detailed and high-resolution comparative maps of specific pig genome regions require clone-based approaches and have rarely been reported in the past, e.g. the comparative analysis of the porcine *RN* gene region on SSC 15q25 with its syntenic counterpart on HSA 2q35 (Jeon et al., 2001; Robic et al., 2001). Another very recent comparative analysis of several genome regions in multiple mammalian species also included the pig and described methods for increasing the throughput of the time-consuming clone-based mapping strategies (Thomas et al., 2002).

We chose the gene-rich region on porcine chromosome 6q1.2 to generate high-resolution clone-based physical maps that are integrated with RH and genetic maps as this region contains at least one gene of large economic importance to swine production (*RYR1*) and possibly other QTLs related to muscle growth (Fujii et al., 1991; Bidanel et al., 2001). We have previously reported the initial construction and analysis of a 1.2 Mb BAC/PAC contig in this region (Martins-Wess et al., 2002) and the use of different resolution RH-panels in the analysis and quality control of this contig (Martins-Wess et al., 2003).

In the present study, we report the extension of this BAC/PAC contig to 5.5 Mb and its analysis and integration with other existing maps. The resulting high-resolution integrated map should facilitate the assembly of large-scale sequencing data of this region and help to define positional candidate genes for QTLs in this region.

4.3 Materials and methods

4.3.1 DNA library screening and chromosome walking

Library screenings were done as described (Martins-Wess et al., 2002). Briefly, the TAIGP714 PAC library (Al-Bayati et al., 1999; http://www.rzpd.de/) was screened by PCR of hierarchical DNA pools. The porcine genomic BAC library RPCI-44 (Fahrenkrug et al., 2001) was screened by radioactive hybridization according to the RPCI protocol (http://www.chori.org/bacpac/).

BAC and PAC DNA was prepared from 100 ml overnight cultures using the Qiagen Midi plasmid kit according to the modified protocol for BACs (Qiagen, Hilden, Germany). Insert sizes were determined as described (Martins-Wess and Leeb, 2003). A detailed list of the primer sequences used for library screenings can be found in the supplementary information.

4.3.2 DNA sequence analysis

BAC and PAC end sequences were determined using T7 and SP6 primers and the thermosequenase kit (Amersham Biosciences, Freiburg, Germany) on a LICOR 4200L automated sequencer. Sequence data were analyzed with Sequencher 4.0.5 (GeneCodes, Ann Arbor, MI). The sequence analysis of one complete PAC clone of this contig has been described elsewhere (Drögemüller and Leeb, 2002). Further analyses were performed with the online tools of the European Bioinformatics Institute (http://www.ebi.ac.uk/), BLAST database searches against the human genome and nr sections of the GenBank database of the National Center for Biotechnology Information NCBI (http://www.ncbi.nlm.nih.gov/) and the RepeatMasker searching tool for repetitive elements (Smit, A.F.A. and Green, P. http://repeatmasker.genome.washington.edu/). Single copy sequences were used to design primer pairs for the chromosome walking using the programs GeneFisher and

Primer3 (http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/gf_submit?mode=START, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

4.3.3 Somatic cell hybrid and RH mapping

STS markers were tested on a somatic cell hybrid panel (Yerle et al., 1996) or mapped on the IMpRH (Yerle et al., 1998) and IMNpRH2 panels (Yerle et al., 2003) according to the INRA protocols (http://www.toulouse.inra.fr/lgc/lgc.htm). After genotyping the IMpRH panel the chromosomal assignments were performed with software available on the INRA WWW server and submitted to the IMpRH database (http://imprh.toulouse.inra.fr; Milan et al., 2000).

4.3.4 Genetic mapping of microsatellite markers

Microsatellite markers were genotyped across seven families of the USDA Meat Animal Research Center Swine Reference Population as described (Rohrer et al., 1994). Initial localization of each marker was based on TWOPOINT analyses (CRIMAP 2.4) against all markers previously mapped in the population. Specific locations within the linkage group were determined with the ALL option. FLIPS was then ran to determine if the current order was the most likely, and then the results from CHROMPIC were used to identify suspect genotypes. All suspect genotypes were evaluated and corrections were made.

4.4 Results and discussion

4.4.1 Construction of the BAC and PAC Contig

The construction of the BAC/PAC contig was initiated by screening the TAIGP714 PAC library or the RPCI-44 BAC library with probes derived from human HSA 19q13.1 genes. Further probes that allowed the gradual joining of the individual emerging contigs into one large contig were generated from the end sequences of isolated clones. In the process of the contig construction 172 region-specific STS markers of SSC 6q1.2 were generated including 51 gene-associated STS markers (see supplementary table). All STS markers were either tested on a somatic cell hybrid panel or on the 7000 rad IMpRH panel to confirm the correct chromosomal localization on SSC 6. In addition to this, the 12000 rad IMNpRH2 panel was used to verify the order and orientation of subcontigs prior to major assembly steps. The

complete BAC/PAC contig consisted of 316 clones (223 BACs and 93 PACs, see supplementary figure). The entire contig spans approximately 5.5 Mb and can be covered with a minimal tiling path of 45 clones.

4.4.2 Physical mapping and gene order

537 end sequences from the BAC/PAC clones of the contig were generated and submitted to the EMBL database under accessions AJ514457-AJ514832, AJ560805-AJ561041 and AJ561044-AJ561089. All these BAC and PAC end sequences were subjected to BLAST searches against the human genome sequences. Of the 537 sequences 52 (9.7%) had significant (e-value < 10^{-5}) and unique matches against genomic sequences of HSA 19q13.1. These matches corresponded well with the overall clone order in the porcine BAC/PAC contig and confirmed the correct assembly. The physical mapping information derived from the contig assembly was refined by taking into account BAC/PAC insert sizes and clone overlap sizes, which were determined by *Eco*RI fingerprinting. The clone-based physical map was anchored to the RH map of the pig genome by analyzing STS markers from the contig on the IMpRH panel. In total 20 evenly spaced RH results were submitted to the IMpRH porcine RH mapping database (http://imprh.toulouse.inra.fr/).

During construction of the porcine contig three techniques were used to localize porcine genes on the contig: (1) Heterologous primers were designed from the human gene sequences in order to localize the porcine orthologs by STS content mapping on the contig. In cases where orthologous porcine EST information was available for HSA 19q13.1 genes, the genomic structure of the porcine gene was inferred from the human ortholog and primers for genomic PCR were designed from the porcine EST sequence. (2) Alternatively, some genes were localized by hybridization of human cDNA probes to membranes with ordered sets of BAC/PAC DNAs. (3) Finally, in some cases the BLAST searches with BAC/PAC end sequences revealed the presence of exons within these end sequences. Thus the corresponding genes could be localized on the contig *in silico*. Using these three approaches 58 genes could be assigned to the BAC/PAC contig.

4.4.3 Integration of the physical map with the genetic map

STS content analysis of all clones of the BAC/PAC contig revealed that two previously described porcine microsatellites are located on the contig (HAL, SW193).

The precise physical assignment of these microsatellites will benefit future QTL analyses with these markers, as their position with respect to coding genes is now available.

Additionally, several novel microsatellites were found during this study in the BAC/PAC end sequences. Flanking PCR primers for four of these microsatellites (L105_MS, NEUD4_MS, PAK4_MS, CD79A_MS) were designed and the microsatellites were genotyped on seven litters (86 progeny) of the MARC swine reference population. The markers were included in the linkage map of SSC 6 (Figure 7). The genetic mapping of all six markers is consistent with the physical mapping. The recombination frequency can not be reliably estimated in the investigated region as there were only two recombinations observed between the two bins of markers with an average of 86 informative meioses for the six physically mapped microsatellites. A more reliable recombination frequency will be estimated if one takes the interval between the markers SW1067 and SW193 that are separated by 4.6 cM on the genetic map and by 255 cR_{7000} on the latest RH framework map (unpublished data). Considering a ratio of 20 kb/cR_{7000} (Martins-Wess et al., 2003) this would mean that SW1067 and SW193 are separated by roughly 5.1 Mb and that the recombination frequency would be 0.9 cM/Mb. This intermediate value for the recombination frequency seems reasonable considering on one hand that the investigated region is located close to the centromere, where low recombination frequencies have to be expected and on the other hand that the region is GC- and gene-rich, where elevated recombination frequencies are expected.

4.4.4 Comparative analysis

The gene order (Figure 7) of the 58 localized genes on SSC 6q1.2 corresponds exactly to the gene order of the NCBI HSA 19 map (http://www.ncbi.nlm.nih.gov; build 33), which lists 285 gene loci in the interval between *GPI* and *LIPE*. Of these 285 gene loci 136 represent computer predicted hypothetical genes while 149 have at least some experimental evidence. Interestingly, the physical size of the investigated region is much smaller in pig than in human (5.5 Mb vs. 8.1 Mb). Three major differences between pig and human account for a large fraction of this deviation in DNA content. Two of these three sites are located in regions, where there are extended non-coding regions in the human genome. In the human genome the distance between *GPI* and *USF2* is about 600 kb larger than in pig and the

distance in the gene-poor region between *COX7A1* and *NYD-SP11* is roughly 300 kb larger in human than in pig. Another significant difference of about 400 kb is found in the region flanked by the *BCKDHA* and *ARHGEF1* genes. In this region the human genome harbors a clustered family of six carcinoembryonic antigen-related cell adhesion molecule genes (*CECAM3* – *CECAM7*, *CECAMP3*). The reduced size of the pig genome could be an indication that this gene family is smaller in pig than in human.

High overall gene order conservation can also be observed with respect to the mouse and to the rat. The current NCBI map of murine chromosome 7 (build 30) shows an identical albeit inverted order of the genes *Gpi* – *Bckdha* with respect to pig or human. In contrast, in the mouse the segment between the genes *Arhgef1* – *Lipe* is parallel to the human and pig. This indicates an internal rearrangement within a block of conserved synteny. The same phenomenon can be observed on the rat chromosome 1, which shows complete conservation to MMU 7 in this region.

Recent RH data in cattle demonstrate that parts of BTA18 are homologous to the investigated region of this study (Goldammer et al., 2002). However, the proposed gene order in cattle (*UBA2 – GPI - COX6B - CAPN4 - POLR2I – GMFG – BLVRP – MIA - PAFAH1B3 – LIPE – BCKDHA – AXL*) is not in good agreement with the other mammalian species indicating several chromosomal rearrangements in cattle and/or inaccuracies of the bovine RH map.

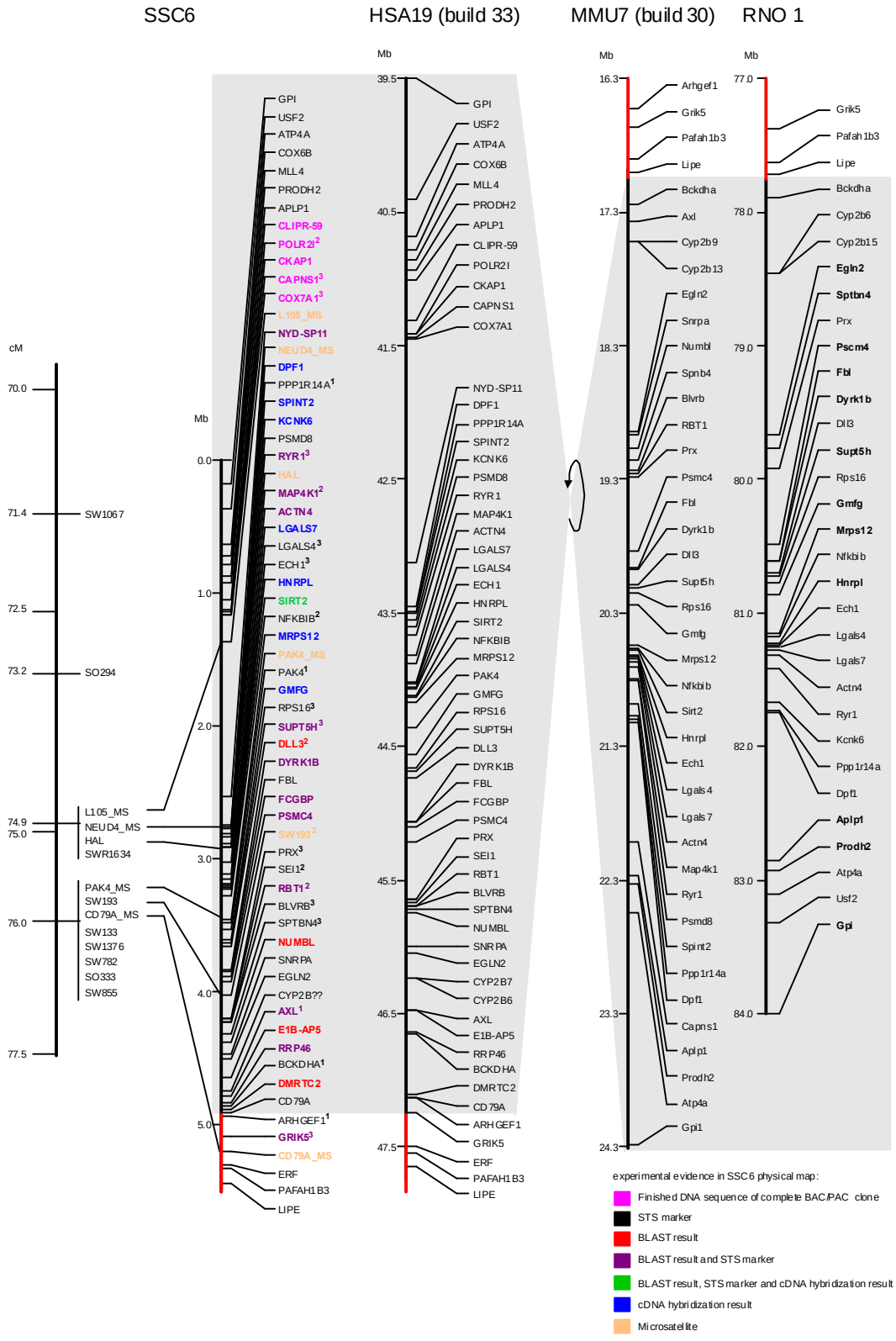

Figure 7: Comparative mapping of 58 gene-associated markers that were used in this study in *Sus scrofa* (results from this study), *Homo sapiens* (NCBI build 33), *Mus musculus* (NCBI build 30) and *Rattus norvegicus* (NCBI 15-April-2003). The experimental evidence that led to the assignment of the porcine genes is indicated by the color of the marker. For one porcine STS marker in the region of the CYP2B gene cluster the exact porcine orthologous gene name could not be determined, therefore this marker was termed “CYP2B??”. Note that there is complete conservation of the gene order across the entire 5.5 Mb interval between pig and human. The two rodent species show an inversion of the genes *Arhgef1* – *Lipe* (red line) with respect to the segment *Gpi* – *Bckdha*. The bold gene names in the rat map denote computer predicted genes, which are similar either to a murine or to a human gene.

The porcine physical mapping data about the gene order on SSC 6q1.2 were also integrated with the genetic map of this region. Physically assigned microsatellites are shown in orange next to the gene map. Detailed information on the microsatellite markers can be found at http://www.marc.usda.gov/. Superscripts denote gene-associated markers that have been used for RH mapping on 1 the IMpRH, 2 the IMNpRH2, 3 both the IMpRH and the IMNpRH2 panel, respectively.

4.5 Conclusion

Our report provides a detailed high-resolution map and clone-contig of a 5.5 Mb region on SSC 6q1.2, which represents one of the most gene-rich regions of the pig genome. The mapping information will facilitate the accurate assembly of whole genome shotgun DNA sequences of this region during the upcoming pig genome project. Furthermore, the integration of physical mapping information with the genetic map allows the rational selection of positional candidate genes for QTLs that have been mapped to this genome region.

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Chapter 5 Increased throughput of BAC/PAC insert size determinations by stacking gels during pulsed field gel electrophoresis

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Increased Throughput of BAC/PAC Insert Size Determinations by Stacking Gels during Pulsed-Field Gel Electrophoresis

BioTechniques 34:718-720 (April 2003)

We recently reported the construction of a BAC/PAC-contig on the porcine chromosome 6q1.2 (1). For the construction of high-resolution physical maps, the insert size of clones within clone contigs is usually determined by pulsed-field gel electrophoresis (2). The information about clone sizes is essential to extend contigs by a chromosome walking strategy, where end sequences of protruding clones are used to find neighboring clones.

We have developed a method to increase the throughput for determining the insert size of BAC and PAC clones using pulsed-field gel electrophoresis. The method consists of stacking up to four agarose gels on top of each other during electrophoresis. This way, four times as many samples can be analyzed in one experiment compared to standard pulsed-field gel electrophoresis.

BAC and PAC DNAs were isolated using a Plasmid Midi Kit from Qiagen (Hilden, Germany), according to the manufacturer's protocol. Approximately 80 ng BAC/PAC DNA were digested with 2 U *Not*I in a total volume of 15 µL for at least 1 h at 37°C. DNA was analyzed after digestion by using a CHEF-DR® III system (Bio-Rad Laboratories, Munich, Germany) with the following parameters: 1% agarose gel (210 × 139 × 4 mm) in 0.4× TBE (36 mM Tris, pH 8.3, 36 mM borate, 0.8 mM EDTA), 0.4× TBE running buffer, 6 V/cm with linear ramped pulse times of 1–30 s, angle 120°, 16°C buffer temperature, and 20 h running time. The insert sizes were determined using a BioDocAnalyzer video documentation system (Biometra, Göttingen, Germany). The MidRange PFG Marker II (New England Biolabs, Schwalbach/Taunus, Germany) was used as marker for DNA size determination.

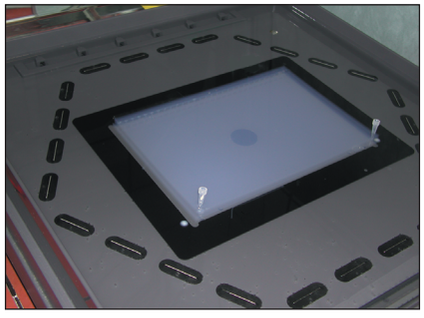

Figure 1. Assembly of the pulsed-field gel electrophoresis using two gels. After the samples of the bottom gel have been loaded, the top gel can be carefully placed on top of the bottom gel. To ensure that the gels cannot move during the run, they are fixed with two thin pipet tips at the front edges. Gels are completely covered with the running buffer 0.4× TBE. Up to four gels stacked on top of each other have been successfully run using these conditions.

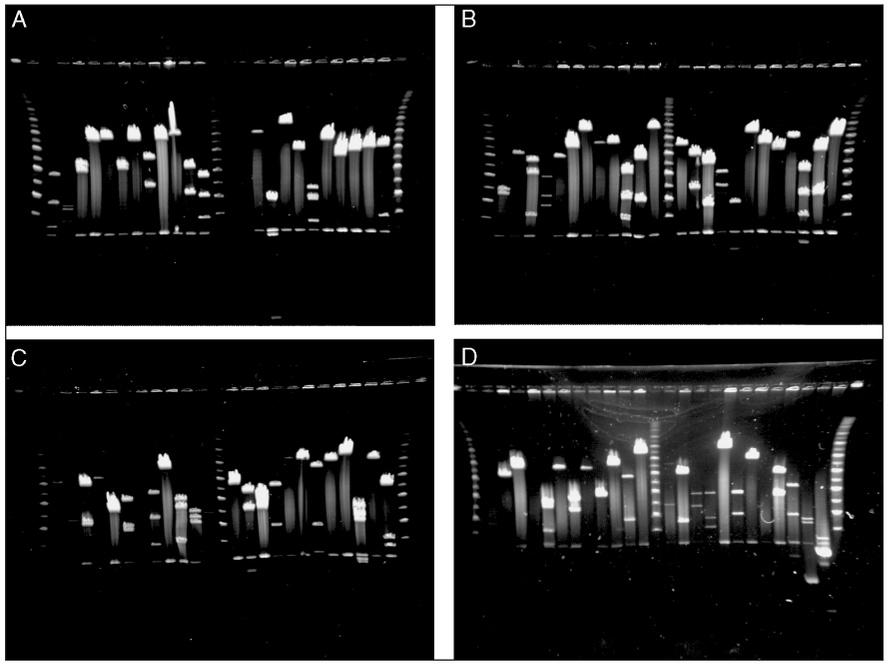

Figure 2. Results of a pulsed-field electrophoresis with four stacked gels. 96 BAC DNAs were digested with *Not*I and applied to the gels. The MidRange PFG Marker II was used as marker for BAC insert size determination. The marker was loaded in the middle and on both sides of the gels so that smiling effects could be corrected. Gels are shown from (A) bottom to (D) top.

To generate more data in a shorter time, we run up to four gels at a time. After the first agarose gel is loaded, subsequent gels are placed carefully on top of the first one. Gels are kept together with two thin pipet tips at the front edge, as shown in Figure 1. It is important to be aware that the gels should lie exactly above each other and that the gels are completely covered with buffer.

Once the gels are stacked and covered with enough buffer, there is no difference between results achieved with one single or with multiple stacked gels. Figure 2 shows the results of a run with four stacked gels and *Not*I-digested BAC DNA. Thus, 120 samples can be separated in parallel using the standard Bio-Rad gels and 30-well combs. Further increases in throughput may be achieved with custom-made combs that allow more samples per gel and with stacking even more gels on top of each other.

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Chapter 6 Discussion

6.1 Construction of the contig

A dense physical map with overlapping BAC and PAC clones was generated by screening the porcine TAIGP714 PAC (Al-Bayati et al., 1999) and RPCI-44 BAC (Fahrenkrug et al., 2001) libraries with heterologous probes and primer pairs from the extensively characterised human syntenic region on HSA 19q13 as starting points. Further, gaps between clones were closed to a single contig by a chromosome walking strategy using new STS markers derived from non-repetitive end sequences of BAC/PAC clones. The BAC/PAC end sequences were obtained by direct sequencing. The BAC/PAC-insert sizes were determined by pulse-field gel electrophoresis and comprised in the construction of the scaled contig map.

The screening of the PAC library was carried out with a three-dimensional hierarchical PCR. The DNA was pooled from each microtiter plate to a single plate pool. Twenty-four plate pools were then pooled to form a single superpool to a total of 28 superpools. Final identification of a positive PAC clone was made by joining the DNA of a microtiter plate to row and column pools, so that two positive PCR products were detected for each positive clone.

The BAC library was screened by radioactive hybridisation. Only positive clones confirmed both by hybridisation and subsequent PCR were used to construct the contig.

The contig spans the region between the genes *CLIPR-59* and *LIPE* and consists of 165 BAC and 93 PAC clones adding up to 258 clones in total. In the 4.62 Mb contig, 45 confirmed genes were identified either with heterologous STSs and probes or according to BLAST results of clone end sequences (http://www.ncbi.nlm.nih.gov/BLAST/). In addition, four computer-predicted genes (LOC, KIAA and FLJ) and five microsatellites were identified and localised.

The average size of the PAC clones is 105 kb; that of the BAC clones is 155 kb. Both average sizes are lower than that expected for TAIGP714 PACs (120 kb, Al-Bayati et al., 1999) or for RPCI-44 BACs (165 kb, Fahrenkrug et al., 2001).

The clone TAIGP714 M17141 was sequenced during this project and has been submitted to the public database under the accession number AJ410870. This clone contains the genes *COX7A1*, *CAPNS1*, *CKAP1*, *POLR2I* and *CLIPR-59* (partial) (Drögemüller et al., 2001; Drögemüller and Leeb, 2002).

A total of 417 sequences generated during the construction of the contig have been submitted to the public database and can be found among other sequences of the entire project under the accession numbers AJ514457-AJ514832, AJ560805-AJ561041, AJ561044-AJ561089. Table 1 summarises some parameters of the sequences that have been submitted to the public database. All sequences have been subjected to BLAST searches against the human genome. Only significant (e-value lower than e^{-5}) matches to human chromosome 19 were considered.

Table 1: Statistical results on the submitted sequences

Clones with end-sequences	223
Total end-sequences submitted	417
Clones with 2 end sequences	88%
Total bases submitted	296842 bp
GC content	47%
Repetitive sequence	38%
SINE	21%
LINE	12%
LTR elements	2%
DNA elements	2%
Match to HSA 19	16%
Match to other HSA chromosomes	8%

The average GC content in human genome is 41% (Lander et al., 2001), while the average GC content on the human chromosome 19 is 46%, which is very similar to that on the homologous porcine chromosome 6q1.2. The contents of the LINE, SINE, LTR and DNA elements differ from those of the human genome (LINE 21%, SINE 13%, LTR elements 8% and DNA elements 3%). The predominance of SINE is a typical hallmark of GC-rich regions such as porcine chromosome 6q1.2.

6.2 Isolation of BAC/PAC clones

Although the PCR based screening method of the PAC library was very simple and rapid, this library showed insufficient coverage of the genome, so that in some regions no clone could be found in the library. This problem has been mentioned before (Palazzolo et al., 1991). According to that study, some regions of the genome are not represented in libraries, either due to the difficulty of cloning certain regions or the fact that some regions are not cloned by chance. In addition, with its 3.24-fold genome coverage (Al-Bayati et al., 1999), the TAIGP714 PAC library is smaller than the RPCI-44 BAC library (10.2 genome equivalents, Fahrenkrug et al., 2001). As mentioned above clones with about 50 to 70 kb were detected during the construction of the contig, and they were of little benefit for the chromosome walking strategy.

In order to avoid incorrect contig assemblies due to chimeric clones, newly developed STSs from clone end sequences were checked for their correct chromosomal localization on a somatic cell hybrid panel.

The method for the construction of the contig and the comparative analysis described here is very accurate, and the relationship between the results achieved and the time spent is reasonable, when the region of interest is not too large (as with whole chromosomes or even an entire genome). The construction of a whole chromosome contig is impracticable using the screening methods presented here (PCR based screening of the PAC library and radioactive hybridisation of the BAC library). For the construction of whole genome contigs fingerprinting methods are favoured.

The construction of targeted BAC contigs in domestic animals has rarely been reported, e.g. the 2.4 Mb-long BAC contig of the porcine *RN* region (porcine chromosome 15q; Jeon et al., 2001). The data produced during the completion of the contig have been compared with the data of the human genome and a complete synteny has been found to human chromosome 2q35 and to murine chromosome 1.

6.3 Radiation hybrid maps

During the construction of the contig, all STS markers were tested on the somatic cell hybrid panel (Yerle et al., 1996) to avoid any problems with chimeric clones. The clone-based physical map was anchored to the RH map of the pig genome by analyzing STS markers from the contig on the IMpRH (Yerle et al., 1998) panel. Analyses on the high resolution IMNpRH2 (Yerle et al. 2002) panel allowed the identification of the gene order and the order of the markers.

The major advantage of the 7,000 rad IMpRH panel is the online supported data analysis (Milan et al., 2000). Data produced with the 12,000 rad IMNpRH2 panel have to be analysed locally with programmes such as the Carthagene software (Schiex and Gaspin, 1997). The resolution of the IMNpRH2 panel is remarkably higher than that obtained with the IMpRH panel (Demeure et al., 2003). In a 5 Mb region studied on porcine chromosome 7, the IMNpRH2 panel presented a 2.43-fold higher resolution than the IMpRH panel. In the work presented here (Chapter 3) the resolution of the IMNpRH2 map was increased by 3.01-fold relative to the IMpRH map on the cR/kb ratio.

The majority of the previously mentioned studies (see 1.1.3) used radiation hybrid mapping to build comparative maps. This technique is efficient when mapping whole or large parts of chromosomes, but RH mapping may have insufficient resolution to compare a relatively small part of a chromosome. Cox and co-authors (1990) emphasised the fact that RH mapping is a statistical procedure; even if the RH map is cautiously made, the marker order is not necessarily the correct one. This conclusion is confirmed by a study made on bovine chromosome 18 (Goldammer et al., 2002). According to their RH data, the gene order on bovine chromosome 18 differed markedly from that of the homologous human chromosome 19. As radiation hybrid mapping is a statistical method, the results are not as accurate as data derived from a cloned BAC contig. The RH map can only give an approximate position of the genes. Rearrangements between genes in a small part of a chromosome can only be determined precisely by cloning the region. A rearrangement in gene order within a syntenic block also cannot be detected by using Zoo-FISH experiments.

6.4 Comparative analysis

According to human genome data, a total of 213 genes are expected to be located between the *CLIPR-59* and *LIPE genes*. Included in these 213 genes are 103 computer-predicted genes (62 LOC, 7 KIAA, 22 FLJ, 8 MGC and 4 DKFZ clones). Out of these 213 genes, 49 genes were experimentally confirmed on the isolated contig. According to the physical map generated, the gene order on porcine chromosome 6q1.2 corresponds exactly to the gene order on the homologous human chromosome 19q13. Other previous studies also reported the homology between the pig and the human genomes (see 1.1.3; Jeon et al., 2001).

Many studies report rearrangements in some species (e.g. Goldammer et al., 2002). These rearrangements may be the result of incomplete data of the human genome project and the large distance between the markers used for the RH mapping experiments. In the past, rearrangements were thought to be common, but with the continued development of the data from the human genome project, these rearrangements have often turned out to be a result of incomplete human data (Drögemüller and Leeb, unpublished observation).

6.5 Gene content

A total of 213 genes are expected to be located in the studied region according to the human genome data. Table 2 summarises some of the genes localised in the present study with respect to their function.

Table 2: List of the genes localised in this study

Gene	Name / Function	Reference[1]	function experimentally confirmed	function computer predicted
CLIPR-59	CLIP-170-related protein / trans-Golgi/TGN cytoplasmic linker protein	Perez et al., 2002	yes	
FLJ32891	hypothetical protein FLJ32891 / unknown			
FLJ33298	hypothetical protein FLJ33298 / unknown			
DKFZP434J046	DKFZP434J046 protein / unknown			
OVOL3	ovo-like 3 (Drosophila) / unknown			
POLR2I	polymerase (RNA) II (DNA directed) polypeptide I, 14.5kDa / encodes a subunit of RNA polymerase II, the polymerase responsible for synthesising messenger RNA in eukaryotes		yes	
CKAP1	cytoskeleton-associated protein 1 / microtubule cytoskeleton		yes	
CAPNS1	calpain, small subunit 1 / catalyses limited proteolysis of substrates involved in cytoskeletal remodelling and signal transduction		yes	
COX7A1	cytochrome c oxidase subunit VIIa polypeptide 1 (muscle) / terminal component of the mitochondrial respiratory chain and catalyses the electron transfer from reduced cytochrome c to oxygen	Wolz et al., 1997; Drögemüller et al., 2001	yes	
FLJ36991	hypothetical protein FLJ36991 / unknown			
ZNF146	zinc finger protein 146 / unknown			
LOC339322	unknown			
LOC284405	unknown			
KIAA1559	KIAA1559 protein / may function as a transcription factor			yes
MGC45380	hypothetical protein MGC45380 / unknown			
LOC126162	similar to hypothetical protein FLJ31235 /unknown			
FLJ14779	hypothetical protein FLJ14779 / unknown			
LOC342890	unknown			
LOC350601	similar to C-terminal binding protein 2 isoform 2; ribeye/ unknown			
LOC339324	similar to zinc finger protein / unknown			
KIAA1615	KIAA1615 protein / unknown			
ZNF382	zinc finger protein 382 / unknown			
GIOT-1	gonadotropin inducible transcription repressor 1 / unknown			
MGC45586	hypothetical protein MGC45586 / unknown			
LOC342892	similar to zinc finger protein 91 (HPF7, HTF10) / unknown			
LOC284407	similar to OK/SW-CL.18 / unknown			
LOC284408	unknown			
ZNF345	zinc finger protein 345 / may function as a transcription factor			yes
LOC342893	unknown			
LOC126167	similar to Hypothetical zinc finger protein KIAA1559 / unknown			

[1] If the reference is not specified, the information comes from LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/) and from GeneCards™ (http://bioinformatics.weizmann.ac.il/cards/).

LOC284409	unknown			
LOC350602	unknown			
LOC284410	unknown			
FLJ32191	hypothetical protein FLJ32191 / unknown			
FLJ23765	hypothetical protein FLJ23765 / unknown			
SZFP41	zinc finger protein 41-like / unknown			
FLJ35863	hypothetical protein FLJ35863 / unknown			
LOC284411	unknown			
LOC284412	unknown			
LOC284459	hypothetical protein LOC284459 / may be involved in transcriptional regulation			yes
KIAA1829	KIAA1829 protein / unknown			
FLJ32053	hypothetical protein FLJ32053 / unknown			
FLJ30791	hypothetical protein FLJ30791 / unknown			
LOC199718	similar to Zinc finger protein 334 / unknown			
LOC284317	similar to hypothetical protein / unknown			
DKFZp547B0714	hypothetical protein DKFZp547B0714 / unknown			
HSPC059	HSPC059 protein / unknown			
KIAA0961	KIAA0961 protein / unknown			
FLJ37549	hypothetical protein FLJ37549 / unknown			
MGC13071	hypothetical protein MGC13071 / unknown			
LOC284318	similar to hypothetical protein FLJ31030 / unknown			
FLJ30921	hypothetical protein FLJ30921 / unknown			
LOC342894	similar to HSPC059 protein / unknown			
LOC147975	unknown			
LOC199719	similar to hypothetical protein / unknown			
NYD-SP11	NYD-SP11 protein / unknown			
LOC342895	unknown			
LOC342896	unknown			
KIAA0545	KIAA0545 protein / unknown			
DPF1	D4, zinc and double PHD fingers family 1 / participates in the regulation of cell survival	Ninkina et al., 2001	yes	
PPP1R14A	protein phosphatase 1, regulatory (inhibitor) subunit 14A / unknown			
SPINT2	serine protease inhibitor, Kunitz type, 2 / inhibitor of HGF activator		yes	
IMUP	immortalization-upregulated protein / unknown			
LOC90522	similar to putative transmembrane protein; homolog of yeast Golgi membrane protein Yif1p (Yip1p-interacting factor) / unknown			
KCNK6	potassium channel, subfamily K, member 6 / exhibits outward rectification in a physiological K(+) gradient		yes	
DKFZP434A1022	hypothetical protein DKFZp434A1022 / unknown			
PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 / 19S proteasome regulatory particle		yes	
FLJ35713	hypothetical protein FLJ35713 / encodes proteins that interact with proliferation of germ cells		yes	
LOC284319	similar to Spred-1/ unknown			
LOC147965	hypothetical protein LOC147965 / unknown			
RASGRP4	RAS guanyl releasing protein 4 / may play an important role in the final stages of mast cell development			yes
RYR1	ryanodine receptor 1 (skeletal) / involved in communication between transverse-tubules and sarcoplasmic reticulum		yes	
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1 / may play a role in the response to environmental stress			yes

M9	muscle specific gene / binds to the 40S ribosome and promotes the binding of methiohyl-tRNA and mRNA			yes
ACTN4	actinin, alpha 4/ thought to be involved in metastatic processes			yes
MGC20576	hypothetical protein MGC20576 / unknown			
LGALS7	lectin, galactoside-binding, soluble, 7 (galectin 7) / implicated in modulating cell-cell and cell-matrix interactions		yes	
LGALS4	lectin, galactoside-binding, soluble, 4 (galectin 4) / implicated in modulating cell-cell and cell-matrix interactions	Huflejt et al., 1997	yes	
ECH1	enoyl Coenzyme A hydratase 1, peroxisomal / involved in the auxiliary step of the fatty acid beta-oxidation pathway			yes
HNRPL	heterogeneous nuclear ribonucleoprotein L / plays a major role in the formation, packaging, processing, and function of mRNA		yes	
LOC126432	similar to RIKEN cDNA 5830482F20 gene / unknown			
SIRT2	sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae) / known to regulate epigenetic gene silencing and suppress recombination of rDNA			yes
NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta / transcription co-activator, signal transduction		yes	
SARS2	seryl-tRNA synthetase 2 / unknown			
MRPS12	mitochondrial ribosomal protein S12 / helps in protein synthesis within the mitochondrion		yes	
LOC284320	similar to envelope glycoprotein / unknown			
FBXO26	F-box only protein 26 / probably recognises and binds to some phosphorylated proteins and promotes their ubiquitination and degradation			yes
FBXO27	F-box only protein 27 / unknown			
LOC284321	unknown			
PAK4	p21(CDKN1A)-activated kinase 4 / induces localised actin polymerisation and the formation of filopodia	Abo et al., 1998; Lu et al., 2003	yes	
LOC342897	similar to F-box only protein 2 / unknown			
LOC342898	similar to syncollin / unknown			
IL28B	interleukin 28B (interferon, lambda 3) / belongs to a cytokine gene cluster			yes
LOC350603	unknown			
IL28A	interleukin 28A (interferon, lambda 2) / belongs to a cytokine gene cluster			yes
IL29	interleukin 29 (interferon, lambda 1) / belongs to a cytokine gene cluster			yes
KIAA1484	KIAA1484 protein / unknown			
GMFG	glia maturation factor, gamma / may play a fundamental role in cellular functions	Lim and Zaheer, 1996	yes	
LOC339325	similar to PRO0663 / unknown			
FLJ10211	hypothetical protein FLJ10211 / unknown			
PD2	hypothetical protein F23149_1 / unknown			
DKFZp434H247	hypothetical protein DKFZp434H247 / unknown			
ZFP36	zinc finger protein 36, C3H type, homolog (mouse) / probable regulatory protein with a novel zinc finger structure involved in regulating the response to growth factors			yes
CLG	likely ortholog of mouse common-site lymphoma/leukemia GEF / unknown			
RPS16	ribosomal protein S16 / encodes a ribosomal protein that is a component of the 40S subunit	Batra et al., 1991	yes	
SUPT5H	suppressor of Ty 5 homolog (S. cerevisiae) / putative modulator of the chromatin structure	Stachora et al., 1997	yes	
TIM50L	homolog of yeast Tim50 / unknown			

DLL3	delta-like 3 (Drosophila) / Mutations in the DLL3 genes cause abnormal vertebral segmentation in spondylocostal dysostosis.	Turnpenny et al., 2003	yes	
LOC348303	unknown			
FLJ38944	hypothetical protein FLJ38944 / unknown			
MGC20452	hypothetical protein MGC20452 / carboxypeptidase A		yes	
LOC348304	similar to ribosomal protein S29 / unknown			
PP13	placental protein 13 / lysophospholipase activity		yes	
LOC199725	unknown			
LOC148003	similar to placental protein 13; galectin-13 / unknown			
LOC350604	similar to Charcot-Leyden crystal protein ortholog / unknown			
PPL13	placental protein 13-like protein / unknown			
CLC	Charot-Leyden crystal protein / may have both lysophospholipase and carbohydrate-binding activities			yes
LOC342900	unknown			
LOC342901	unknown			
DYRK1B	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B / proposed involvement in the regulation of nuclear functions, presumably involved in brain development	Becker et al., 1998	yes	
FBL	fibrillarin / thought to participate in the first step in processing preribosomal RNA			yes
FCGBP	Fc fragment of IgG binding protein / unknown			
LOC255736	similar to DKFZp564J157 protein / unknown			
PSMC4	proteasome (prosome, macropain) 26S subunit, ATPase, 4 / involved in the ATP-dependent degradation of ubiquitinated proteins		yes	
LOC339327	similar to hypothetical protein FLJ32191 / unknown			
LOC342902	similar to hypothetical protein FLJ36874 / unknown			
LOC163131	similar to hypothetical protein FLJ90036 / unknown			
LOC348305	similar to hypothetical protein LOC284323 / unknown			
LOC284323	hypothetical protein LOC284323 / unknown			
LOC284324	similar to FKSG60 / unknown			
MAP3K10	mitogen-activated protein kinase kinase kinase 10 / involved in nerve growth factor (NGF) induced neuronal apoptosis		yes	
FLJ30373	hypothetical protein FLJ30373 / unknown			
FLJ13265	hypothetical protein FLJ13265 / unknown			
AKT2	v-akt murine thymoma viral oncogene homolog 2 / a general protein kinase capable of phophorylating several known proteins		yes	
FLJ36888	hypothetical protein FLJ36888 / unknown			
PLD3	phospholipase D3 / unknown			
FLJ32818	hypothetical protein FLJ32818 / unknown			
PRX	periaxin / apparently required for maintenance of peripheral nerve myelin sheath			yes
SEI1	CDK4-binding protein p34SEI1 / transcriptional regulator interacting with the PHD-bromodomain 1		yes	
RBT1	RPA-binding trans-activator / a strong transcriptional co-activator; its expression is significantly higher in cancer cell lines than in normal cell lines	Cho et al., 2000	yes	
BLVRB	biliverdin reductase B (flavin reductase (NADPH)) / possible role in protecting cells from oxidative damage or in regulating iron metabolism			yes
SPTBN4	spectrin, beta, non-erythrocytic 4 / structural constituent of cytoskeleton, carbohydrate metabolism		yes	
LOC92799	hypothetical protein BC007653 / voltage-gated potassium channel complex		yes	

LOC342903	unknown			
LTBP4	latent transforming growth factor beta binding protein 4 / unknown		yes	
NUMBL	numb homolog (Drosophila)-like / unknown			
FLJ12229	hypothetical protein FLJ12229 / unknown			
ITPKC	inositol 1,4,5-trisphosphate 3-kinase C / catalyses the phosphorylation of Ins(1,4,5)P3 to Ins(1,4,5)P4		yes	
LOC284325	similar to CG33108-PA / unknown			
SNRPA	small nuclear ribonucleoprotein polypeptide A / binds stem loop II of U1 SNRNA		yes	
MIA	melanoma inhibitory activity / elicits growth inhibition on melanoma cells in vitro as well as some other neuroectodermal tumors		yes	
RAB4B	RAB4B, member RAS oncogene family / probably involved in vesicular traffic			yes
EGLN2	egl nine homolog 2 (C. elegans) / involved in oxygen homeostasis		yes	
CYP2T2P	cytochrome P450, family 2, subfamily T, polypeptide 2 pseudogene / unknown			
CYP2F1P	cytochrome P450, family 2, subfamily F, polypeptide 1 pseudogene / unknown			
CYP2A6	cytochrome P450, family 2, subfamily A, polypeptide 6 / an enzyme known to hydroxylate coumarin,;also metabolises nicotine, aflatoxin B1, nitrosamines, and some pharmaceuticals		yes	
CYP2A7	cytochrome P450, family 2, subfamily A, polypeptide 7 / catalyses many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids		yes	
CYP2G1	cytochrome P450, family 2, subfamily G, polypeptide 1 pseudogene / catalyses many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids		yes	
CYP2A7P1	cytochrome P450, family 2, subfamily A, polypeptide 7 pseudogene 1 / catalyses many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids		yes	
CYP2B7	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene / catalyses many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids		yes	
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6 / known to metabolize some xenobiotics, such as the anti-cancer drugs cyclophosphamide and ifosphamide		yes	
CYP2G2	cytochrome P450, family 2, subfamily G, polypeptide 2 / catalyses many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids		yes	
CYP2A13	cytochrome P450, family 2, subfamily A, polypeptide 13 / known to metabolize 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a major nitrosamine specific to tobacco		yes	
CYP2F1	cytochrome P450, family 2, subfamily F, polypeptide 1 / known to dehydrogenate 3-methylindole, an endogenous toxin derived from the fermentation of tryptophan, as well as xenobiotic substrates such as naphthalene and ethoxycoumarin		yes	
CYP2T3P	cytochrome P450, family 2, subfamily T, polypeptide 3 pseudogene / catalyses many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids		yes	
CYP2S1	cytochrome P450, family 2, subfamily S, polypeptide 1 /metabolises certain carcinogens			yes
AXL	AXL receptor tyrosine kinase / a chronic myelogenous leukemia-associated oncogene also associated with colon cancer and melanoma		yes	

E1B-AP5	E1B-55kDa-associated protein 5 / protein with a regulatory role in adenoviral and host-cell mRNA processing and nuclear export, binds specifically to adenovirus E1B-55 kDa oncoprotein	Kzhyshkowska et al., 2001	yes	
TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease) / multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types		yes	
MGC20255	hypothetical protein MGC20255 / unknown			
MGC4093	hypothetical protein MGC4093 / unknown			
RRP46	exosome component Rrp46 / required for the 3' processing of the 7S pre-RNA to the mature 5.8S rRNA		yes	
BCKDHA	branched chain keto acid dehydrogenase E1, alpha polypeptide (maple syrup urine disease) / a protein complex that catalyses the oxidative decarboxylation of the a-keto acids resulting from the transamination of the branched chain amino acids leucine, isoleucine, and valine	Chuang et al., 1993	yes	
FLJ10241	hypothetical protein FLJ10241 / branched chain keto acid dehydrogenase E1, alpha polypeptide (maple syrup urine disease) / unknown			
LOC350606	unknown			
LOC125966	similar to Tpm3 protein / unknown			
PLEKHA3P	pleckstrin homology domain-containing, family A member 3 pseudogene 1 / unknown			
LOC284335	similar to pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 3; pleckstrin homology domain-containing, family A (phosphoinositide binding specific) member 3 / unknown			
R29124_1	hypothetical protein R29124_1 / unknown			
CEACAMP3	carcinoembryonic antigen-related cell adhesion molecule pseudogene 3 / unknown			
CEACAM4	carcinoembryonic antigen-related cell adhesion molecule 4 / unknown			
CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7 / unknown			
LOC350607	unknown			
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5 / unknown			
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen) / involved in intercellular adhesion activity and subdomains involved in inhibition of cell differentation		yes	
CEACAM3	carcinoembryonic antigen-related cell adhesion molecule 3 / has a role in internalising bacteria to epithelial cells		yes	
LOC342905	similar to Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing protein) (Single-strand binding protein) (hnRNP core protein A1) (HDP) / unknown			
MGC42718	hypothetical protein MGC42718 / unknown			
DMRTC2	DMRT-like family C2 / transcription factor, sex differentiation		yes	
RPS19	ribosomal protein S19 / encodes a ribosomal protein that is a component of the 40S subunit		yes	
CD79A	CD79A antigen (immunoglobulin-associated alpha) / a B lymphocyte antigen receptor; plays a role in signal transduction following antigen binding	Hashimoto et al., 2002	yes	
ARHGEF1	Rho guanine nucleotide exchange factor (GEF) 1 / plays a fundamental role in numerous cellular processes that are initiated by extracellular stimuli that work through G protein coupled receptors		yes	
RABAC1	Rab acceptor 1 (prenylated) / non-selective vesicle transport		yes	

ATP1A3	ATPase, Na+/K+ transporting, alpha 3 polypeptide / catalyses the hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane		yes	
GRIK5	glutamate receptor, ionotropic, kainate 5 / functions as the major excitatory neurotransmitter in the central nervous system	Szpirer et al., 1995	yes	
FLJ22059	hypothetical protein FLJ22059 / unknown			
LOC284336	unknown			
POU2F2	POU domain, class 2, transcription factor 2 / plays an important role in regulating transcription in a number of tissues in addition to activating immunoglobulin gene expression		yes	
LOC342906	unknown			
LOC284337	unknown			
DEDD2	death effector domain-containing DNA binding protein 2 / DNA binding, apoptosis regulator		yes	
KIAA1951	KIAA1951 protein / unknown			
GSK3A	glycogen synthase kinase 3 alpha / implicated in the hormonal control of several regulatory proteins			yes
ERF	Ets2 repressor factor / physically interacts with the cytomegalovirus major immediate-early promoter (MIEP)	Bain et al., 2003	yes	
CIC	capicua homolog (Drosophila) / implicated in granule cell development		yes	
PAFAH1B3	platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa / plays an important role during the development of the brain		yes	
LOC284338	unknown			
FLJ90805	hypothetical protein FLJ90805 / unknown			
EGFL4	EGF-like-domain, multiple 4 / structural molecule, calcium ion binding		yes	
SBP1	HBV pre-s2 binding protein 1 / unknown			
LOC84518	protein related with psoriasis / unknown			
LIPE	lipase, hormone-sensitive / a fatty acid hydrolase that cleaves fatty acyl esters of cholesterol, steroid hormones, and glycerol	Harbitz et al., 1999; Li et al., 2002	yes	

6.6 Conclusions

The refined comparative and clone-based physical maps developed here provide important insights into genome evolution. This study demonstrates how the publicly available human and mouse sequence data can be used to accelerate the comparative mapping of mammalian genomes.

The primary goal of the present study was to create a physical map around the *RYR1* gene and to prove that the synteny between the human and the porcine genomes in the region is conserved. These results will be useful for the identification of genes underlying QTL. The isolated clones of the contig can be used as templates for sequencing targeted genome regions. They can also be transfected into mammalian cells or used to generate transgenic animals to study the function and/or expression of the cloned genes.

Chapter 7 References

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Chapter 8 Appendix

8.1 PCR primers

Table 3: Primer sequences of all used STS markers

STS marker	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	T_M [°C]	PCR-Product
714_A01_212_T7	CTC TTG TTC AGG CAG AAG	GAG TCT CCT CTC TAA AGG	54	199 bp
714_A03_39_T7	TAG AGC ACC AAC CAT GTG	GTT CTA TTG GCA CCA TGG	54	462 bp
714_A05_46_T7	ACA AGC CCC TAG GTC AAC	GTA TTT GGG GCC TAC CTG	56	292 bp
714_B16_155_Sp6	AAG AAA AGG AAA AGG TTT GG	TGT TCT CGC AAA CAG TGA G	54	304 bp
714_B16_155_T7	CAT CAC AAC CCT CCT TTG	TGA GTC AGT GGG GTT AGC	54	259 bp
714_C02_171_Sp6	GGT GGC ATG TGG CTT TGC	GGG TCA CAG TGT GGT CTG	58	418 bp
714_C05_183_T7	CGG GTG GAC TGA TTA TTT C	CAC CTG AAT TCA TGG ACA G	56	123 bp
714_C08_70_Sp6	ATG GTA CCC GAG CCA CAG	CTT GCT GCT TCT TCA CAT ATC	58	172 bp
714_C18_130_Sp6 (ITZ005)	TCC AGA CAA GTG AGA AAC AA	TTA ATT TAT AAT GCC TGG TCA	56	320 bp
714_C18_130_T7 (ITZ006)	ATC TGC GGA GAG GAA AAG	TCG TGT TTG TTG GAA TGT C	54	201 bp
714_D22_199_T7	TGG GGA CAA ACG CTT AGG	CCG ATT AAG AAG CCA CTC	56	514 bp
714_E17_112_Sp6 (ITZ007)	GTG ACT TGT AGA CCA CAG	CCT ACA GAG GGA GAA TCC	54	346 bp
714_E17_112_T7	GGC AGA TGA AAA GCA AGG	TCA TGA AGA CCT CCA TGC	54	517 bp
714_F04_202_T7	CCC CTT TCA CTG GCT CTG	GCC CAG GAG CTC AAG TAC	58	513 bp
714_F14_101_T7	ACT GGA TCC CTC GGT TAG	CCC GAG AGA GAC AAA AGG	56	207 bp
714_F14_138_Sp6	AGC TGA GTG GAC AGT TGG	TCC AGC TGT GTG GAG AAG	56	304 bp
714_F14_138_T7	GTG GAA ATG GAA GTG CAG	CCA AGA CAG CTC AGA TGC	54	315 bp
714_G13_89_Sp6	GTT GGC AGG ACT GCT CTG	AAA GCC CTG TGC AGA AGG	58	128 bp
714_G13_89_T7	GGC CAG GGG AAT CAA GTG	GAG AAC CTC CAG CCT GTG	58	466 bp
714_G22_50_T7	TAA CCC CAA AGC GTA ACC	CTT TTG TGT CCC AGT TGC	54	154 bp
714_G22_81_Sp6	ATC TTC TGG GCC TCT CTG	GGG ACA GGA ATG AGG AAG	56	446 bp
714_G22_81_T7	ACT GTC CTT CTT GCT CC	GTC CAA AAA ACC AAG AGG	52	98 bp
714_H06_151_Sp6	CCT GAC AGC TGT GTC CTC	TCT GTT CCC CAG ACA CAC	58	214 bp
714_H06_151_T7 (ITZ010)	CAG ATT GGG ATG AAG CTC	TTA TCA ATC CCA ACA CAC C	54	114 bp
714_H06_5_T7	GTA ACC CTC ATG GGG GAG AC	CCT GGT CCA GGT GGC ACC T	64	131 bp
714_H08_5_Sp6	AGA CAG GCT CCG ATG AAG	AGA AAG GCT TCC CTG CTG	56	209 bp
714_H13_41_Sp6	CAC CTG ACC CCA CAA ATG AAC	TTT TCT TCG GAC GCT CCA GAG	64	334 bp
714_H13_41_T7	TGA GCG CCC AGA TCC ATG	GCA GGG GAT GCA GAG ATG	58	506 bp
714_H16_159_T7	AAG TGA TTC CAG CCA CTG	ATT GAT GGG TGG ATA GAG	54	127 bp
714_I05_215_SP6	GGA AAC TCC CTG TAA AGG	CCT CCA GCT ATT TCT AGG	54	150 bp
714_I11_51_Sp6	ATT GCT TGC CAG GCA GAG	CCC CCT TAT TGG GAC TTC	56	119 bp
714_I16_225_T7	GCC ATC AAC TTC CAA ATG	AAT TTG ACC GAG GAA AGG	52	278 bp
714_I17_138_T7	AAC AAA CAA CCT AGG GCC	CTC CCA CTT CCA ATC CTC	55	110 bp
714_I17_37_T7	AGG CTG AGC ACA CCC ATC	ACC AGA CCC CTG CAC TTG	58	412 bp
714_J03_209_SP6	GCC CCT GAG GAC ATT ACC	ACC TAG GGC CCC TAA GTC	58	473 bp
714_J03_209_T7	ACT GCC TTT TAA CAT TGG	GAC AGC AAA CAA AAT GAG	50	364 bp
714_J09_28_T7	TAT TGC CAC CAT CCA ATG	TCA GGA TTG GCT TCT TTG	52	137 bp
714_K08_206_Sp6	CCA GGG CTT TGC TTC TAG	CGG GGG AAC ATT TGT GAC	56	222 bp

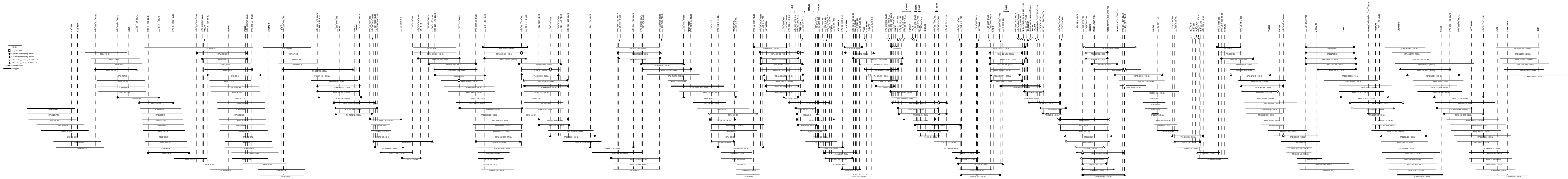
714_K08_206_T7	ACC CCA GAA TCC TCG TAC	AGA GCA GGA CAT AGC ATC	56	395 bp
714_K12_160_T7	TGG TGA CCA TTT CTC TGC	TGA ACA GGG CTG TTG AAG	54	291 bp
714_K13_61_Sp6 (ITZ001)	TAG CCT TTC CTG TGG AGG	ACA CAA AAG CAC ACA CCG	56	423 bp
714_L08_78_T7 (ITZ011)	AGA CGG GAA ATT GAG ACC	TCC CTG TGG CAG TAA ATG	54	345 bp
714_L20_221_Sp6	TGG AAG TCT TGA GCA CAG	AGA ACA CAG CCT GAT GTG	54	218 bp
714_L20_221_T7	CCA AAA TCC ACC CAG AAG C	TGC TTC CTG TGG GGA CAG	58	113 bp
714_M08_41_Sp6	TCC AAG ACA GAG CTT GAG	GGC AGT GAA TCA TGA GTC	54	135 bp
714_M08_41_T7	ATC TAG TCT GAA GAG CTC CC	GTT CGC AGA CGG GAC CTC	60	125 bp
714_M08_6_Sp6	GGA TTG TGT ACC TGG TAC	GAC CAC CAG GAA TAC TTC	54	496 bp
714_M08_6_T7	CGG GGT CCC TAT TTG TAT	TCT AGT TCC TTT CCC CTG	54	126 bp
714_M17_141_T7	AGA CTC TGC TGA GTG TTG	CCT CTC TGA AGC CTC AAG	54	458 bp
714_N01_50_Sp6	ACT GAT CTT CGG CCC TTG	CAA GGA TGC ACC CAA TCG	56	517 bp
714_N01_50_T7	GAG TTT CTG CAT CTG TTC	TCA ACG TTT TAT ATA ACC ATG	52	333 bp
714_N21_131_Sp6	GCA TGA GGT ATG GTT TAT CC	CCA TAT TCT GTG GGT AGG G	58	236 bp
714_N21_131_T7	AAC CTC AAA CTC CCA GTC C	GCT GCA GTT TGC GGA TTG T	58	80 bp
714_N23_35_Sp6	CGG CAT TGA GAT AAC GTC	ATC CTA GAA CCC CAG ACC	54	414 bp
714_N23_35_T7	AAA GCT CCA CCT GGC TAC	CCT CCC ATT GAT GAG TTG	56	290 bp
714_P22_214_Sp6	GAA TCT GAC TGC CTT AGC	AGT TGA GAC ACA GCC TAG	54	218 bp
ACTN4	GAT GCC GAG TTC AAC CGC ATC	TTG TCA CCT GCC AGG ACC TTG	66	155 bp
ARHGEF1	CAG AGG AGC AAA GCA ACC	AAC TGC ATG GCC ACA TAC	56	94 bp
AXL	TGG ACG CTA TGT CCT CTG	TAG GAA GAG GGG GCT ATG	56	209 bp
BCKDHA	AGC AGC CCT GGA CAA CAC	GTA GTG GAC GGG CAT CTG	58	151 bp
BLVRB	GGT TAT GAG GTG ACA GTG	CCA AGA GCA CGA TGA TGG	54	148 bp
CAPN4	TGT ATT CCT GAA CGG GAG	TGC AAG AGA GGG CTA ATG	54	272 bp
CD79A_MS	CAG ATG GAG GTG CTG CTA TG	CCC AAA GGC ACC TCA AAA TC	62	130 bp
COX7A1	AAC AGA AGC TCT TCC AGG	TCG GTA CAG GAT GTT GTC	54	199 bp
CYP2B??	CAT TTG TCT TGG GGA AGG	CTT CCC TCA GCC TCT TTG	56	191 bp
DKFZp434H247	TTC TGC AGC TTC TTC TGC	ATC GAA GTC CTG TTG CTG	54	145 bp
DYRK1B	CCA ACC TAG CCA CCA ACC	CAG AGT GGC AGG GTC ATC	58	174 bp
ECH1	ACC AGG AGG TCC TGC TTG	ATG TAG TTG AGG CCC TCT G	58	136 bp
EGLN2	GCC TGC TGC AGA TAT TCC	GTG GCA TAG GCT GGT TTC	56	141 bp
ERF	GGT ACC GTT TCA TGT CCT C	CTG TGT CGC CTC TAG CTG	58	195 bp
FBL	CTG GAA CCC CTT CCG TTC	GTC AGA GAC GTG GGA GAC	58	130 bp
GRIK5	CAA CTT CCA GGC CCT GTC	TTG ATG AGC TCG CCA ACC	58	174 bp
HAL	GGG CAG CTA AGG TGA GGA G	CTT GCG GAA GAA GTT GAA GG	55	270 bp
L105_MS	TCT GTT TTG ATC CGA AGA AGC	AGC AGG TTT GGC ACA GGA G	60	112 bp
LGALS4	GAT GTC GCC TTC CAC TTC	TGA TGA CCA GCT CGA AGG	56	142 bp
LIPE	CTG GAC CCC ATG CTG GAC	CCG CTA GGG TCA GGA AGC	60	115 bp
LOC163128	TCC AAA GAG AAG GTG GTG	ATG AGG GCA TAG GAG AGC	54	170 bp
MAP4K1	CCT ACC CAC GCC TAT GC	CCA GCC AGC AGG AAA GC	56	136 bp
NEUD4_MS	CAG CAT GGC TGT TGG AAA G	GCC ATG ACT TAC ACA GTG C	58	127 bp
NFKBIB	CTG CAC CTG GCA GCC ATC	GCT GGA GCA GCA CGC AAG	60	154 bp
PAFAH1B3	GGC TTC TTA GTT CCA CCA C	GTT CTC CTC TTC GCT CAT C	58	230 bp
PAK4	CAG CGA GTG TCC CAT GAG	CAT GGG TCA GCA GGA TGG	58	463 bp
PAK4_MS	TGG GAA CCT CCA TAT GAC AC	ACC ACA AGG GAA CTC CAA AG	60	199 bp
POLR2I	ACC ACC CGT GCC AAA AG	CCG CGC ACT GTG TGA CT	54	172 bp

PPP1R14A	GCT GAG CAA GCT GCA GTC	GGT ACA GCT CCT CCA AGC	58	164 bp
PRX	GCC TCA GGT GAC CTT GTC	CCC ACA TCC AGC TCA AGC	58	117 bp
PSMD8	CGG AGT CAG CCT ATA TGC	GAC ACC GGA TGC TTG ATG	56	145 bp
RBT1	CCA TGG ATG AGA CTG AGC	GGC ACA GAA GAG GTT GTG	56	218 bp
RP44_162_H7_Sp6	ACA CCT TCT TGG CCA TGC	TGA GGA CGC CAC CAT CAC	56	116 bp
RP44_162_H7_T7	CTC CAC AGG CAG AAT GTG	AGA GAA GCC CTG CAA GAG	56	443 bp
RP44_166_D13_T7	GCA GAA GTT CCC AGG TTA G	TCT ATA CTT GGG GGT CTG G	58	183 bp
RP44_172_J1_Sp6	AAT TCG CCT ATC GGA GGT	TGC ACC TCC AAC AGG CAA	54	110 bp
RP44_172_J1_T7	ACA ATG AGC CTT GGA GTC	GAA GCC AGT GTC GTG TTG	54	318 bp
RP44_184_B20_Sp6	TTT GCT CTC AGG GTG AAG	AGT GGG AGA GGG AAT CTG	54	198 bp
RP44_187_C16_Sp6	TCA CCC AAG ACC TGT TCC	TGC TGC AAA ATG CCT GTG	56	412 bp
RP44_187_M23_Sp6	TGG AGC TAC TCC CAA AAG	TTT GTG CCA GAT CTC CTC	54	217 bp
RP44_204_H11_Sp6	CAC CAC CTC CGT GCC TAC	CAC CAG CCA GAG CAG AGG	60	209 bp
RP44_204_H11_T7	GTA AGG AAT GTG GCA AGG	AAT GAG CTG TGA GCC AAG	54	464 bp
RP44_211_B19_T7	GCA TTC AAA GGG CTT TTC	TGC CCA CAT TTC TTC AAC	52	242 bp
RP44_216_K1_Sp6 (ITZ013)	TTG GAG GGT TCA ACT ACG	GTA AAC CCG TTC ACG TTG	54	197 bp
RP44_216_K1_T7	GCA GTA TCT AGG GCA TAA AAC	TTA CAT TCC ACC TCC ATA TTC	54	170 bp
RP44_231_J20_T7	TCT GCA TCT CAC CCT CAG	AGC ACA GCC TGC TTC TTC	56	177 bp
RP44_235_B21_Sp6	ACA GCA TGG TTG CGA CTC	GCC CGA GAC ATA GGT GAC	56	366 bp
RP44_263_A8_Sp6	TTG CCG CTT TGT CGG TTG	TGG CCC CTA ATA CGA AGC	56	324 bp
RP44_263_A8_T7	GCG TGT GCA TCT CTT ACC	CAT GGC AAA TGC ACG TTC	56	512 bp
RP44_283_N2_T7 (ITZ014)	TGC CTG TTC ACG AAC CAC	TCC TTG TGT GGG CTA CAG	56	100 bp
RP44_292_D4_Sp6	CAC AAC CTC AGG CAT TTG	TTT GTG TTC CTG GGT GAG	54	232 bp
RP44_298_C10_Sp6	AAG GCT TGA GGA GAC AGC	CCT CTT TGC CCT AGT TGG	56	189 bp
RP44_299_D1_Sp6	GGG GAA AGA AAA GGC TTC	CCT CAC TCC TGG AAA TCC	54	156 bp
RP44_299_D1_T7	GAA TCA ACA ATA GTA GGT	AGA GCA AAT CTT TAT ACG	48	144 bp
RP44_311_B11_Sp6	TCC TTT CCA GCT CTG AAG	CAA CAG TGC TGG TGA GTC	54	381 bp
RP44_311_B11_T7	GTC TCC ATT ACA TTA AAA ATG	CTG GAA GAC CAC CAG AAA	54	124 bp
RP44_316_A16_Sp6	GTA AGG GCT ATG CGT CTG	ATG TGG GAA GGC CTT TAG	56	204 bp
RP44_316_A16_T7	TGG TCC CAA AAT TTC TCT C	CTC CAA AGA AAC CCC ATC	54	178 bp
RP44_32_N2_Sp6	TCA CAC CTA GAT CCA CAG TC	CCC TTT ATC ACA TTC TAC CAG	60	168 bp
RP44_338_B22_Sp6	AGT GGG GGG GTC TTC TAC	CTC TTC ACG ACC TCT CTG	58	254 bp
RP44_338_B22_T7 (ITZ008)	AGC TGA GAC CAA TGC CAT	ATA ATT GGG AGT TCT CGC	54	122 bp
RP44_373_B21_Sp6	TTG GTT ATT TTT CTG CAT TC	TCC TTT TCC ATA TTT TCT GC	52	95 bp
RP44_373_B21_T7	GCT GCT GGA ATC TTT TTG	AAC ACC AAG GCT AAT TGT G	52	134 bp
RP44_409_F21_Sp6	GAA TTC TGC GAT GCT GAG	ACA CCA GCG AAT TCA CAC	54	182 bp
RP44_409_F21_T7	CAT GTT TGT AAA AAT GCT CA	TTG CCC AAT TGT TCT CTC	52	126 bp
RP44_419_C3_T7 (ITZ002)	AGC CTT CAC CCA GAA GTC	GAT GCA CAA GGA GCT GAG	56	287 bp
RP44_437_P18_Sp6	GCA GGA AGG CCT TTA ATC	CCA AAA CTG AAT GTC ATG G	54	210 bp
RP44_437_P18_T7	TAA GGG CTG AGC TTT GAG	TGG GGA GAA ACC TTA TGC	54	230 bp
RP44_444_G5_Sp6 (ITZ004)	TTC ACT TTG GGT CTG CTG	ACC TAC CCA CTG CTA TGC	54	311 bp
RP44_444_G5_T7	CTT CGG TCA CAC AAG AGG	TGG GCA TCA CTG AGA GAC	56	324 bp
RP44_445_F24_T7	CAC TTT CCC TCA CCA AGG	TGA CTG GGT CAC TAT GCT G	56	189 bp
RP44_475_K6_Sp6	TGA ATC TGC CCC ATA TCC	AGG GTC CAA AGA CCT GAC	54	108 bp
RP44_477_B7_T7	ACA TAC AAC CCC CAA CTC	TGG TTC AGG AGA AGA TGG	54	517 bp
RP44_490_D6_Sp6	CCT TGC CAC ATT CCC TAC	TGG GAA GGC TTT TAG TTT G	56	176 bp

RP44_490_D6_T7	AGG GAG CCA CGA TTA AAG	TGC AGC ATC AGA GGA TTC	54	247 bp
RP44_521_D11_Sp6	ATG TGG GAA GGC CTT TAG	AAC CCC GAG TCA ACA GAG	54	293 bp
RP44_521_D11_T7 (ITZ003)	CAT AGG GCT TTT CAC CAG	TTC TTG CCG AAT ATC AGA G	54	297 bp
RP44_521_D20_Sp6	AGG GCC TCT TAT CAG GTG	CTG AGA TTG TGG CTG AGG	56	208 bp
RP44_521_D20_T7	CTT GCA AAG CTG GGA TTG	TTC AGT TGG CAC CTC ATC	54	158 bp
RP44_528_O23_Sp6	GTA TCC TCC CTG AAA GTC	CCA GGA AGA ACC TGG AAC	54	148 bp
RP44_528_O23_T7 (ITZ009)	CTG CTC CTC ATT CCC ATG	CCG TCT TAT GCT TGA GTC	56	187 bp
RP44_58_D7_Sp6	ATC TGG GAA GGA AAG GAA C	ATG TCA CTT CCC AAA GAG G	56	100 bp
RP44_62_D12_Sp6	TGA GAA AGC CAT CTA CGT G	AGA GCT TCT TTC TGG GAT G	56	209 bp
RP44_62_D12_T7	TTT ACC AGC CAT CCC TAT C	CTC CTG CAA TCC TTT GTT C	56	193 bp
RP44_77_L18_Sp6	GGA GGT GAT AGG CCT GAG	AAG CCA GCC TGA GTT GAC	58	138 bp
RP44_84_K5_T7	AAC GTG AAG ATG CTC CTG	CGG AAA CTA CGC AAA GTC	54	187 bp
RP44_99_O22_Sp6	TCC CTC TGT TCC AAG TTC	GCC AGA GTA TGC AAG AGG	54	309 bp
RP44_99_O22_T7	CAT GAG CTT CAG GCT GTG	CCT CAT CTG CAC CTG AAC	56	300 bp
RPS16	CGC TGA TCA TCA CGA TGG	GCT TTT GGG CAA GGA ACG	56	294 bp
RYR101/102	CAG ACA GGG AAC ACA AC	TGG ATG TAT TCT GTG AGG	52	309 bp
RYR103/104 (RYR_Promoter)	TTC GTT TCT GCT TCG CC	CTC TCT CCT CCC ATT TC	48	162 bp
RYR111/112	AGG AGA AGG TAA GGG TG	GGA AGA GTC AGT TGG TC	52	235 bp
RYR115/116	GGT AAA AGG GTA TAT GG	GTG CAA AAT TTC AGA GG	48	164 bp
SEI1	GAG CTG GAT GAT GCT GAG	GCT GTG ATG GAG CTT GAG	56	184 bp
SNRPA	AAG TCC CTG TAC GCC ATC	GTC GTA GAA GGG GAA ACC	56	156 bp
SPTBN4	ATA TCC TGC CCC AAG AAG	GAG GAG GTC GAC GTT TTG	54	282 bp
SUPT5H	AGG AGC TTC CCC AGG AAG	TGG GTG AGG ATC GGG AAG	58	121 bp
SW193	TGC CAT CCT TTC TTT CAT TAC G	TCA CTC TGA GGG GTC CTG AC	62	101 bp

8.2 Contig

Physical map of the isolated BAC/PAC contig on SSC 6q1.2. STS markers are indicated vertically on the top and by dotted vertical lines, cDNA hybridization probes are represented as horizontal lines at the top, markers that are associated with genes are denoted in bold. The physical sizes of the hybridization probes depend on the intron sizes of their respective genomic targets. BACs and PACs are indicated as horizontal lines below the markers with their corresponding clone names and insert sizes. For the RPCI-44 BAC clones the full names are given while for the TAIGP714 PAC abbreviated clone names are given. For example the official clone designation (http://www.rzpd.de/) TAIGP714G05113 was abbreviated to 714G05113. A minimal tiling path of 37 clones is indicated in bold.



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