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The Functional Significance and Chromatin Organisation of the Imprinting Control Regions of the *H19* and *Kcnq1* Genes

BY

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Abstract
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Genomic imprinting is a phenomenon through which a subset of genes are epigenetically marked during gemtogenisis. This mark is maintained in the soma to often manifest parent of origin-specific monoalleleic expresson patterns. Genetics evidence suggests that gene expression patterns in mprinted genes, which are frequently organised in clusters, are regulated by the imprinting control regions (ICR). This thesis is mainly focused on the mechanisms through which the ICRs control the imprinting in the cluster, containing the *Kcnq1, Igf2* and *H19* genes, located at the distal end of mouse chromosome 7.

The *H19* ICR, located in the 5' flank of the *H19* gene represses paternal *H19* and maternal *Igf2* expression, respectively, but has no effect on *Kcnq1* expression, which is controlled by another ICR located at the intron 10 of the *Kcnq1* gene. This thesis demonstrates that the maternal *H19* ICR allele contains several DNase I hypersensitive sites, which map to target sites for the chromatin insulator protein CTCF at the linker regions between the positioned nucleosomes. The thesis demonstrates that the *H19* ICR acts as a unidirectional insulator and that this property invovles three nucleosome positioning sites facilitating interaction between the *H19* ICR and CTCF. The *Kcnq1* ICR function is much more complex, since it horbours both lineage-specific silencing functions and a methylation sensitive unidirectional chromatin insulator function. Importantly, the thesis demonstrates that the *Kcnq1* ICR spreads DNA methylation into flanking region only when it is itself unmethylated. Both the methylation spreading and silencing functions map to the same regions.

In conclusion, the thesis has unraveled and unrivalled complexity of the epigenetic control and function of short strtches of sequences. The epigenetic status of these cis elements conspires to control long-range silencing and insulation. The manner these imprinting control regions can cause havoc in expresson domains in human diseases is hence emerging.

Keywords: DNA methylation, Imprinting control region, Chromatin, Insulator, Nucleosome positioning

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Contents

Abbreviations

DMR	Differentially Methylated Region
ICR	Imprinting Control Region
NHSSs	Nuclease Hypersensitive sites
NPSs	Nucleosome Positioning Sequences
CTCF	CCCTC-binding protein
MNase	Micrococcal Nucleases
DNase	Deoxy ribo Nucleases
Dnmt	DNA methyltransferase
MeCP	Methyl CpG binding protein
MBD	Methyl DNA binding domain
HDAC	Histone de-acetylase complex
HMTs	Histone Methyl Transferases
BWS	Beckwith Wiedemann Syndrome
ChIP	Chromatin Immuno Purification assay
CpG	Cytosine paired with Guanine dinucleotides
Kb	Kilo base
Bp	Base Pair

Introduction

The genome encodes all the genes required for the development and function of the organism. According to the recent estimates the human genome carries 30,000 to 35,000 genes, out of which the functions of around 50% of the genes remain unknown. It is essential that specific genes are expressed in the right cell type during the different phases of development and that the cells can respond to external and internal stimuli by altering the expression of specific genes. Even though we know much information about the genes invovled in developmental regulation, but how these tens of thousands of genes and proteins work together in interconnected networks to orchestrate the chemistry of life, is still poorly understood and still an outstanding challenge. It is crucial therefore, that the expression of the genome is strictly controlled, both during development and as a function of diverse stimuli in the adult organism. In this process of regulating gene expression, the packaging of DNA into chromatin plays a very central role.

Chromatin organisation and gene expression

The genetic information provides the framework for the manufacture of all the proteins necessary to create a living cell and chromatin structure controls how, where, and when the genetic information should be used. DNA in the eukaryotic nucleus exists in a highly compacted chromatin structure, where the 2m DNA threads were packaged into a microscopic unit. The control of chromatin packaging, into condensed or decondensed fibres plays a major role in the regulation of gene expression. Most DNA sequences in the compact chromatin organisation are structurally inaccessible and functionally inactive. Within this mass the actively transcribed genes are in minority. The fundamental subunit of chromatin is the nucleosome, which has the same type of design in all eukaryotes. Eukaryotic organisms package their genomes with a nucleoprotein complex known as histones. The non-covalent association of histones with DNA results in considerable compaction and allows the organisation of the genome into the cell nucleus. The nucleosome is arranged as follows: an octomeric core of histones, two each of H2A, H2B, H3, and H4, is encompassed by 146 base-pairs of DNA, which winds almost two left-handed super helical turns around it. This unit is called the nucleosome core particle. The remaining DNA, whose length is

both tissue and species specific, is referred to as linker DNA. It joins the adjacent nucleosome core particles and is associated with histones of H1-type. Nucleosomes can be randomly or precisely positioned on a DNA molecule.

The specific location of the DNA on the nucleosome is characterised by its translational positioning and rotational positioning**.** The translational positioning describes the position of DNA with regard to the boundaries of the nucleosome. In particular it determines which sequences are found in the linker regions that connect nucleosomes and therefore more sensitive to degradation by micrococcal nucleases, presumably reflecting increased accessibility. Depending upon its positioning with regard to the nucleosome, a site in DNA that must be recognised by a regulatory protein could be inaccessible or available. The exact position of the histone octomer with respect to DNA sequence may therefore be important. The rotational positioning describes the orientation of the double helix with regard to the octomer surface. The side of the helix that is bound to the protein is inaccessible, whereas the side that is exposed is more accessible. Rotational phasing could be a significant parameter in allowing accessibility for the factors that bind to DNA.

Genes that are being transcribed contain nucleosomes at the same frequency as non-transcribed sequences. Generally, genes do not necessarily enter an alternative form of organisation in order to be transcribed. But since the average transcribed gene probably only has a single RNA polymerase at any given moment, this does not reveal what is happening at sites actually engaged by the enzyme. Perhaps they retain nucleosomes; more likely the nucleosomes are temporarily displaced as RNA polymerase passes through, but reform immediately afterwards. The mechanism doubtless entails unpeeling of nucleosomal DNA in the course of transcription, and it results in the complete displacement of the histone octomer from the DNA. If the DNA is of sufficient size, it can recapture the octomer at the original location. In addition to this capacity for read through, polymerases can also receive help from other factors to diminish pausing or stalling and speed up transcription of nucleosomal DNA.

Nucleosome positioning

The location of nucleosome beads in chromatin could effect gene expression and other processes by interfering with the DNA binding of other proteins. Some regions of chromatin might be expected to show a non-random positioning of nucleosomes, leaving relatively exposed those DNA sequences that must be recognized by other proteins in the cell. Because the spacing between the nucleosomes is more or less regular, the exact placement of one nucleosome on the DNA will also effect the position of its neighbours. Thus, the whole repeating nucleosomal structure in a region may

have to be positioned uniquely, so as to leave a particular DNA sequence either free of nucleosomes, or in a region of linker DNA. The sequence specific placing of nucleosomes is known as nucleosome phasing or nucleosome positioning. Although there is evidence for the occurrence of nucleosome positioning, its role in the gene expression is still poorly understood. The evidence comes from the experiments in which nuclei are treated with micrococcal nuclease or with a higher dose of DNase I, which preferentially cuts the linker DNA between adjacent nucleosome beads. Such experiments have shown that nucleosomes are precisely placed with regard to specific DNA sequences in some regions of chromatin. Most of the studies are now mainly concentrated on what causes nucleosomes to become so precisely positioned or phased, or what exactly phasing means for the chromatin function. Sequences have been identified that direct the deposition of histone octomers to specific locations. The locational preference is usually considered in terms of translational and rotational positioning of the DNA. Variation in the length of linker DNA (1) is important for the diversity of gene regulation. Despite this variation, a chain of nucleosomes can still coil or fold in a regular manner to form a chromatin fiber. The length of linker DNA varies in many cases almost at random, but the locations of nucleosomes are sometimes constrained by barriers, such as sequence-specific DNA-binding proteins on the DNA, as well as by more subtle effects, such as sequence dependence of the energy of bending DNA. As a consequence of this, nucleosome positioning is proving to be an integral part of the organization of nucleoprotein complexes at the promoters, enhancers and other control sequences in the genome. Consequently, these elements have received considerable attention as potential regulators of genome function. It has been already shown that positioned nucleosomes can regulate both transcription and replication by controlling the accessibility of regulatory proteins to DNA (2,3,4). For example, some regulatory factors bind to their target sites with high affinity only if the sequence is located along the DNA separating two adjoining nucleosomes. Other regulatory factors bind to DNA only if this sequence is presented in a particular rotational orientation. The translational position of a DNA sequence within a nucleosome can influence its accessibility for protein factor binding. Thus, a complete understanding of genomic control mechanisms in eukaryotes will require a more thorough description of the determinants of nucleosome positioning than is currently available. An understanding of mechanisms that lead to nucleosome positioning, which control the site of nucleosome residency *in vivo*, may permit the development of new approaches to regulate gene activity and further clarify relationships between nucleosome positioning and genome function in the cell.

Mechanism of nucleosome positioning

Nucleosome positioning can be maintained by different mechanisms. In some cases, such as for genes encoding *Xenopus* 5S rRNA (5,6) and vitellogenin B1 (7), yeast PH05 (8), *Drosophila* adh (9) and the mouse mammary tumor virus long terminal repeat (10), DNA sequences possess the inherent ability to position nucleosomes precisely. In the above cases, nucleosomes can appear to occupy a single position. In other cases, such as at the *yeast* STE6 (11) and GAL1/GAL10 (12) and the *Drosophila* hsp26 (13) promoters, as well as at the mouse serum albumin enhancer (14), nucleosomes are positioned *in vivo* only as a consequence of specific factor binding. However, in these latter cases it is not clear whether the regulatory factors binding to their cognate sequences are sufficient to determine nucleosome position, or whether the underlying DNA sequence plays a role. These findings are used to develop the hypothesis that DNA factors position nucleosomes in combination with the positioning ability of the underlying DNA, and that such factors might stabilize different nucleosome configurations, causing different states of gene activity. Resolution of this issue is important to understand how such nucleoprotein complexes are assembled.

Nucleosome positioning sequences and their significance

DNA sequences that determine their own packaging in nucleosome through preferential positioning of histone octomer are called as nucleosome positioning sequences (NPSs). These NPSs are of increasing interest because of their relationship to gene regulation *in vivo* and their utility in physical studies of nucleosome structure and function *in vitro*. Most of the studies are focused on analyzing sets of positioning sequences for the sequence rules that are responsible for their nucleosome-positioning ability. It is interesting to compare these rules to actual genomic DNA sequences to examine whether the requirements of the chromatin organization of genomic DNA place evolutionary constraints on the sequences (15). Also the study of the properties of the NPSs potentially provides a route to the discovery of DNA sequence elements or motifs having novel structures and mechanical properties. This thesis is mainly focused on examining the main role of nucleosome positioning in the imprinting control regions that are responsible for the long range regulation of imprinted genes.

Genomic Imprinting

Genomic imprinting can be defined as the expression of the genes in a parent-of-origin specific manner. This means that the phenotype expressed from a locus is dependent on the sex of the transmitting parent. This process involves a reversible gamete-of-origin specific marking of the genome that ultimately produces a functional difference between the genetic information contributed by each parent.

Imprinting phenomena have been observed in a wide range of phyla from both the plant and animal kingdoms. Some manifestations of this gamete-of-origin dependent modification includes: paternal dominance in hybrid plants (16); paternal chromosome elimination in *Sciara* (17,18); inactivation of the paternal genome by heterochromatization in the scale insects (19); parent-of-origin specific modification of position effect variegation in *Drosophila* (20); phenotypic differences in progeny produced from interspecific crosses in fish (21); preferential inactivation of the paternally derived X-chromosome in marsupial and rodent extra-embryonic tissues (22); and parent-of-origin dependent switching of yeast mating types (23). Probably the most extensively studied and best-understood examples of genomic imprinting are the gamete-of-origin dependent modifications of transgene methylation and expression in mice (24,25,26). It is not yet clear whether all the above phenomena are occurring via similar molecular mechanisms. The occurrence of parent-of-origin effects in some species in a wide range of organisms suggests that it arose independently a number of times.

Genomic imprinting is counter to classical Mendelian genetic theory, which states that there is equal inheritance of parental traits. An imprinted mutant allele would appear to be recessive when inherited from one sex, because it would be inactive, whereas it would be active when inherited from the other sex and therefore, appear to be dominant. The pronuclear transplantation experiments (27,28,29) using mice have unequivocally demonstrated that there is an absolute requirement for the genetic contribution from both sexes in order for development to proceed normally; i.e. maternal and paternal contributions are not equivalent. More specifically androgenetic embryos show poorly developed embryos and excessive trophectoderm (extra embryonic supportive tissue), whereas gynogenetic embryos have well-developed embryos and poorly developed trophectoderm.

An understanding of the role of genomic imprinting in development has been hampered by the lack of a precise role for the presently known imprinted genes in development. Although genomic imprinting has been intensively studied in the past few years, it is still a new field. Our present knowledge of imprinting is limited to the identification of imprinted genes and the fundamental factors that contribute to the process. Studying epigenetic mechanisms offers a model for understanding the role of DNA

modifications and the chromatin structure in maintaining appropriate spatiotemporal patterns of expression. Present research is focusing on identifying the trans-acting modifiers of imprinting (i.e., "imprintor" genes), understanding the role of DNA conformation and chromatin structure surrounding imprinted loci (i.e., cis-elements) and on determining the role of imprinting in development (establishing of the imprint) and human disease. Several endogenous genes as well as regions of many mouse chromosomes (30) have been shown to be imprinted. Estimates of the number of imprinted genes in the mammalian genome ranges from less than 100 to greater than 200.

The recent discoveries in the study of genomic imprinting show that imprinted genes are grouped in large multigene domains (31,32,33). Several imprinted genes exist within a mega base of DNA or less. However not every gene in the cluster is imprinted. Thus, it is likely that both local and regional *cis*-acting elements are involved in the regulation of genomic imprinting. The exact mechanism of the imprinting is still not yet clearly understood, but the DNA methylation is showed to be one of the main mechanisms (26,35). The other mechanisms involves antisense transcripts (36), short repeat elements (37), DNA replication and *trans*-acting binding proteins that may interact with one or more of these sequences (38,39,40).

DNA methylation and genomic imprinting

The most important epigenetic modification involved in imprinting in mammals is the methylation of CpG dinucleotides. DNA methylation is an enzymatic modification performed by methyltransferases, which involves methylation of cytosine residues at the 5-C position. In eukaryotes, two different types of DNA methyltransferases have been characterized: *de novo* methyltransferases, e.g. Dnmt3a and Dnmt3b (41), which use non-methylated DNA as a substrate and a maintenance methyltransferase, (Dnmt1) (42), which methylates hemimethylated DNA that is generated during replication (43). Maintenance methylation implies the copying of the existing pattern of the old DNA strand onto the new one. Thus DNA methylation can be heritable and serve as an epigenetic mark that is transmitted by mitotic or meiotic cell division to the progeny. Using mouse as a model system for the role of DNA methylation in mammals, it has been shown that primordial germ cells and embryonic stem cells seem to develop normally with genome wide reductions in DNA methylation levels (44,45). However as stem cells start to differentiate, DNA methylation becomes essential for proper development. Imprinted genes are characterized by methylation patterns specific to each of the parental alleles. During early mammalian development, DNA methylation profiles undergo drastic changes, reaching the final methylation levels around gastrulation (45). Afterwards, the overall level of methylation is thought to be constant.

Although specific for each lineage, it is poorly understood how the imprinting marks set in the germ line are transmitted during the early stages of development until they become fixed at the onset of differentiation. During gametogenesis, the genome wide methylation patterns of the parental genomes are erased and new methylation patterns are set by *de novo* methylation. The methylation pattern is germ line-specific: sperm DNA is more methylated compared with oocyte DNA (46). These differences might reflect the parent of origin specific differences in methylation levels in the gametes as well as compact chromatin that must be condensed during spermatogenesis. The methylation marks on the imprinted genes are established during early gametogenesis and these methylation marks survives the demethylation and *de novo* methylation events after fertilization.

The importance of DNA methylation in the imprinted genes was first observed in the mutant Dnmt1-/- mice, which leads to the loss of imprinting for many but not all imprinted genes: *P57Kip2, Igf2r,* and *H19* are activated, *Kcnq1* and *Igf2* silenced, but *Mash2* remains imprinted (34). This suggests that methylation can function to repress or activate some imprinted alleles and indeed does not have a function in the regulation of others. Dnmt1-/- embryonic stem cells that were differentiated and later rescued by introduction of Dnmt I transgene restored the overall DNA methylation to normal levels but failed to restore the allele-specific methylation of the imprinted *Igf2* and *H19* genes (47,48). The normal mono-allelic methylation and expression patterns of these genes was re-established only by passage through the germ line suggesting that epigenetic marking of the parental alleles during gametogenesis is crucial for the correct parental specific expression patterns (47).

In general, methylation of the promoter regions leads to the repression of gene transcription in two different manners (49). The first mode of repression involves direct interference of the methyl group in binding of a protein to its DNA target site. Many of the factors, which bind to the CpG - containing sequences fail to bind when the CpG is methylated. The added methyl group does not affect the base pairing itself, but the protruding of methyl groups into the major groove can effect DNA-protein interactions (50). For example: c- Myc, a novel murine "leucine zipper" protein, recognizes a sequence specific DNA binding site in the c-Myc onco protein. CpG methylation of this recognition site greatly inhibits DNA binding, suggesting that DNA methylation may regulate the c-Myc complex *in vivo* (51). The second mode of repression, involves, the binding of certain proteins with repressive properties that are specifically attracted to the methylated CpG. The methyl CpG binding proteins that are characterized to date include, MeCP1, MeCP2, and MBD1, 2 and 3. These proteins promote the formation of a transcriptionally repressive multi-protein complex. For example, MeCP2 has a transcriptional repressor domain and also binds to the

co repressor mSin3A, which in turn recruits several additional proteins to the complex, including the histone deacetylases HDAC 1 and 2. Deacetylation of lysine moieties on histones H3 and H4 unmasks the positive charge of the lysine, strengthening the interaction between the histones and DNA, and thus blocking access to the transcription machinery (52). The functional relationship between histone acetylation and transcriptional activity is reflected in the activation of transcription by agents that inhibits histone deacetylating enzymes, such as trichostatin A (TSA). In addition, the treatment of cell lines with the methyltransferase inhibitor 5-azadeoxycytidine resulted in demethylation and increased H4 acetylation at the maternal *SNRPN* promoter. This is accompanied by activation of usually silent maternal *SNRPN* allele, implying a role for both DNA methylation and histone deacetylation in imprinting. Several studies on histone deacetylation and DNA methylation have shown that DNA methyltransferases and histone deacetylases are able to interact either directly or indirectly through several pathways. Recently, it has been shown that DNMT1 (53,54) and DNMT3a (55) able to bind directly to HDAC and to HDAC2 to presumably facilitate the silencing process.

Although DNA methylation is usually involved in gene repression, certain imprinted genes like *Igf2r* (36) and *Kcnq1* (56,57) require methylation for their expression. For example, Igf2r, which is insulin growth factor 2 receptor gene, is a maternally expressed imprinted gene located on mouse chromosome 7. The monoalleleic expression of this gene is mainly dependent on the methylation of the CpG island of region2, also called DMR2, on the maternal allele, which is unmethylated and overlapped with an antisense transcript on the repressed paternal allele. The loss of methylation of the DMR2 results in the loss of the Igf2r expression.

Imprinted genes show remarkable differences in chromatin structure between the two alleles. Early experiments demonstrated that artificially methylated DNA adopts a distinctive chromatin structure upon integration into the genome (58). This conformation renders the DNA refractory to nuclease or restriction endonuclease digestion and also leads to the loss of DNase I hypersensitive sites. On the other hand, unmethylated CpG islands, which are associated with promoters of many genes, possess a nuclease sensitive chromatin structure that differs from the bulk of the methylated genome (59). In order to study the association of DNA methylation with inactive chromatin and its role in loss of transcriptional activity, several experiments were done by analyzing the kinetics of transcriptional arrest from methylated templates (60). Some experiments (61) showed clear evidence for a time dependent repression of methylated template following injection into *Xenopus* oocytes. Loss of transcriptional activity from the methylated template coincided with the appearance of a nucleosomal array in the vicinity of the promoter and the disappearance of engaged RNA

polymerases. These studies demonstrate that DNA methylation can lead to alteration of chromatin structure.

The methylation marks that are established during germ line development at DMRs play an important role in regulation of imprinted genes (62). At several of these DMRs, the allelic methylation originates from the female or male germ line and is maintained throughout development. DNA methylation cannot, however, be the only determinant of imprints. Allelic methylation at the imprinted loci must somehow be protected from the demethylation that occurs following fertilization and during preimplantation development and, at later stages, from the acquisition of *de novo* methylation (62). It has been shown that, HDAC activities and Histone methyltransferases (HMTs) can be linked to the DNA methylation, but the exact mechanism by which the unmethylated allele is protected by these DNA methyltransferases, HDACs and HMTs, still remains largely unknown.

Histone methylation and its link with the DNA methylation in epigenetic gene regulation

Research during last few years demonstrated that histones constitute a major target for covalent modifications and many reports have shown the correlation between defined histone modifications and particular gene expression states. The known covalent histone modifications at the N-terminal tails of histones that are directly involved in epigenetic regulation are acetylation and methylation. As mentioned earlier, histone acetylation correlates in general with active gene transcription. Histone methylation, like histone acetylation, is a dynamic process and appears to have diverse functions in the control of gene activity and has recently emerged as a central mechanism in epigenetic regulation.

Methylation of the histones is catalyzed by histone methyl transferases (HMTs), which use S-adenosylmethionine (SAM), as a cofactor. The main target of histone methylation is the N-terminal tail of the histone H3. It has been shown that the methylation of lysine 4 (H3-K4), is associated with gene activity and the methylation of the lysine 9 (H3-K4), is associated with the gene silencing. More interestingly, gene regulation by the Polycomb and Trithorax group of proteins also involves methylation at the lysine residues of histone H3 tails. We know from earlier studies that the Polycomb group of proteins suppresses gene expression and the Trithorax group of proteins activates gene expression by stably modulating the higher order chromatin structure (63). Specifically, the Polycomb group protein, enhancer of Zeste, methylates H3-K9 (64, 65), and the trithorax homologue, HTRX, methylates H3-K4 (66,67). The gene regulation of the Polycomb and the Trithorax proteins involves therefore, methylation of lysine residues of the histone tails. The fact that histone methylation plays a crucial role in higher order chromatin structure was supported by Grewal and colleagues, using the

mating Yeast type locus as model system (68). They documented that in fission yeast, H3-K9 methylation is strictly localized to a 20kb silent heterochromatin region at the mating type locus, whereas H3-K4 methylation is specific to the surrounding euchromatic regions. More importantly, two inverted repeats that flank the silent region serve as boundary elements for heterochromatin and euchromatin and deletion of these boundary elements lead to spreading of H3-K9 methylation into the neighboring sequences. These studies show that site specific methylation can serve as chromatin domain markers.

The fundamental role of methylation of histone H3 tails in gene regulation came into light with the demonstration that histone methylation is closely linked to DNA methylation. Several lines of evidence have shown that H3-K9 methylation is an early event in heterochromatin formation and that histone methylation occurs prior to DNA methylation. In humans, H3-K9 methylation marks the inactive X chromosome shortly after Xist RNA coating and prior to gene inactivation (69). This is followed much later by DNA methylation (70). In the filamentous fungus *Neurospora crassa*, the H3-K9 methyl transferase dim-5 is necessary for the normal genomic DNA methylation (71), when they replaced the lysine 9 of H3 with non-methylatable amino acids the DNA methylation is lost. This shows that the DNA methylation is dependent on the H3-K9 methylation, and also suggests that methylation of H3-K9 occurs prior to the DNA methylation. In *Arabidopsis,* it has been shown that the loss of DNA methylation, on which gene function was deleted, resulted in the reduction of the H3-K9 methylation, suggesting that histone methylation can be controlled by DNA methylation (72). In *Drosophila*, DNA hypermethylation caused elevated H3-K9 methylation (73). All these examples suggest a strong relation between the histone methylation and the DNA methylation and that these two epigenetic marks may signal to one another to ensure propagation of the silenced state (74). However, it is still not known that whether DNA methylation can actually direct H3-K9 methylation or whether DNA methylation can act independently to silence gene expression in the absence of H3-K9 methylation.

Enhancers and chromatin insulators

In eukaryotes, enhancer-mediated activation is a fundamental mechanism of gene regulation. Enhancers are identified as cis-acting DNA sequences that increase transcription in a manner that is independent of their orientation and distance relative to the transcription start site. Enhancers, apart from selecting the correct promoter over long distances, can also act promiscuously to activate transcription of heterologous promoters. Since most, if not all, genes are clustered, there is a need to restrict enhancer actions without impeding the action of the enhancer upon its native locus. A

DNA element able to function in this way in effect constitutes a boundary to the action of an enhancer, preventing it from acting across the boundary, while otherwise leaving the enhancer unimpeded. This property is one of the defining characteristics of a kind of regulatory element only recently recognized as the chromatin insulator (75,76,77).

Insulators can be defined as evolutionary conserved neutral boundary elements, therefore, that block the communication of the enhancers with promoters in a position-dependent manner (78). An insulator is not by itself an activator or an enhancer or a repressor. The known insulators include the *scs* and *scs*' regulated induction of hsp70 gene (79) and gypsy elements in *Drosophila* (80,81). Insulator elements are also observed in the vertebrates, such as the constitutive DNase 1 hypersensitive site, 5'HS4 with strong enhancer blocking activity at the 5'end of the chicken ß-globin locus (82,83), and an enhancer blocking element between alpha and delta gene segments within the human T-cell receptor locus (84).

A number of models have been proposed for the mode of action of insulators. These models suggest that they might work by preventing "tracking" of a distinct enhancer complex along the chromatin, by serving as a signal for sequestering the region in the nuclear matrix so as to physically isolate the region, or by interacting with a second such insulator to form an isolated loop. In order to establish proper enhancer blocking activity function, insulator sequences have been shown to bind specific proteins that are likely to establish the insulator function. Most of the known insulators contain strong DNase 1 hypersensitive sites, which indicate that they interact with some proteins. So far all of the known vertebrate insulators interact with the CTCF protein (85).

The imprinted cluster located on the human chromosome 11 and the mouse chromosome 7

Normal mammalian development requires the correct parental contribution of imprinted genes. Lack of this biparental contribution leads to a variety of developmental abnormalities and diseases in the mouse and humans, affecting cell growth, development and behaviour. Loss of Imprinting (LOI) is one of the most frequent genetic alterations in cancer and by definition can involve both abnormal activation of the normally silent allele of a growth promoting gene, such as *IGF2* or silencing of a normally expressed allele of a growth inhibitory gene such as *P57kip2* (86).

Beckwith-Wiedemann Syndrome (BWS) is a complex genetic disease that is most frequently sporadic and characterized by somatic overgrowth, macroglossia, abdominal wall defects and a variety of secondary signs, including predisposition to embryonal tumors (87). Recent evidence suggest that the BWS is caused by the desregulation of one or more members of the cluster of imprinted genes located on mouse distal chromosome 7, and the

orthologous gene cluster on the human chromosome 11p15.5. The 800 kb region on the distal end of mouse chromosome 7 contains a cluster of four maternally expressed genes, *H19, Mash2, KvlqtI* and P^{57KIP2}, as well as two paternally expressed genes, *Igf2* and *Ins2* (88). Atleast ten imprinted genes on human chromosome 11p15.5 have been identified and characterized, including four paternally (*IGF2, LITI, IGF2AS* and *MTR1*) and six maternally (*IPL, IMPT,* $P^{57KIP2,}$ *KVLQTI, ASCL1* and *H19*) transcribed genes. In humans, this imprinted domain, can be divided into two separate sub domains, the more centromeric domain includes *KvLQT1, P57KIP2, TSSC5* and *TSSC3* and spans 500kb. The telomeric domain includes *IGF2, H19* and *Ascc2* and spans 200kb (32,47,88). These two domains are separated by~300kb. The imprinting status of the genes in this domain is mainly controlled by the Imprinting control regions (ICRs), which control gamete-specific imprints that regulate long range repression. The molecular genetic studies of BWS patients suggests that 10 to 20% of BWS cases can be due to the paternal Uni-Parental disomy, (UPD) of the chromosome 11p15.5, 40% cases are due to the maternally inherited germ line mutations in the coding region of *CDNK1* gene, 2 to 3% of the cases are due to the methylation abnormalities in the H19 gene and finally 50 to 60% of the cases are mainly due to the methylation abnormalities or the translocation break-points in the *KCNQ1* gene. This indicates that BWS mainly occurs due to the over expression of the paternally expressed genes and / or the deficiency of maternally expressed genes. Most of the sporadic patients exhibit abnormal biallelic expression of *IGF2*, a polypeptide growth factor encoding gene that is normally transcribed only from the paternal allele (89,90). It has been shown that the over expression of *Igf2* in mouse embryos also leads to the dosage dependent appearance of some, but not all of the BWS phenotypes (91,92).

We and others have shown that the biallelic expression of *Igf2* is mainly controlled by the ICR located 5' to the H19 gene. The targeted deletion experiments of the *H19* ICR showed that it affects only the *H19* and the *Igf2* imprinted genes, while other imprinted genes in this domain are not affected (47). Also many of the BWS patients showed biallelic expression of *IGF2*, but normal methylation status of the *H19* ICR, which indicates that imprinting of *IGF2* is also controlled by the presence of some other DMRs or ICRs in this domain (91,93,94). Several studies have shown that the chromosomal breakpoints and the translocations map to intron 10 of the *KVLQT1* gene, which is also a differentially methylated region, called *KCNQ1* ICR, in BWS patients (57). The *Kcnq1* ICR is methylated on the maternal allele and unmethylated on the paternal allele, which is associated with an antisense transcript. It has been also shown that targeted deletion of the *Kcnq1* ICR when paternally inherited results in the disruption of six imprinted genes in *cis* in this imprinted domain (95,96). Unlike the *H19* ICR,

which effects only the *H19* and the *Igf2* genes, the *Kcnq1* ICR affects the imprinting status of most of the neighboring genes throughout the domain.

My thesis mainly deals with the molecular mechanisms and the functional role behind the regulation of the imprinted genes in this domain by these two differentially methylated regions, namely *H19* ICR and *Kcnq1* ICR. I will focus therefore on the *H19* and *Kcnq1* ICRs.

The *H19* ICR

The first solid molecular basis for understanding imprinted gene expression has come from studying the linked and reciprocally imprinted mouse *Igf2* and *H19* genes. *H19,* which produces a non-coding RNA and *Igf2,* encoding, Insulin growth factor 2, are approximately 90kb apart from each other. A connection between the imprinting of *H19* and *Igf2* was first raised by the observation that the genes are co-expressed in endoderm and mesoderm. While *H19* is expressed only from the maternal chromosome, Igf2 expressed solely from the paternal chromosome. It was proposed that the imprinting of *H19* and *Igf2* is dependent upon competition of the endodermal specific enhancers that are located +9 and +11kb relative to the start of *H19* transcription (97). This model first gained support when it was shown that both genes require for their expression two endodermal specific enhancers that lie 3' of the *H19* gene (98). This was demonstrated by targeted deletion of these enhancers, which resulted in loss of *H19* and *Igf2* expression. Later on, insertion of an extra set of enhancers between the two genes resulted in the activation of the unmethylated maternal *Igf2* gene (99). The strong preference for *Igf2* expression on the paternal chromosome is thought to arise from the paternal-specific methylation of critical CpG residues in the 5' flank of the *H19* gene. This methylation has all the properties of a parental specific gametic mark, as it is established during spermatogenesis, but not oogenesis, is resistant to the global demethylation that occurs during preimplantation development and is maintained in all somatic cells of the organism (100,101,102,103). Further, it was shown that the expression of *Igf2* does not require its own DNA methylation, but depends on the methylation or deletion of the *H19* 5' flanking region (104). Furthermore, a study with transgenic mice has suggested that the *H19* upstream region is necessary for the imprinted expression of *H19* (105). A targeted deletion of the *H19* gene that included 10kb of upstream sequences resulted in biallelic expression of the *Igf2*. This clearly demonstrated that sequences upstream of or within the *H19* gene are necessary for the monoallelic expression of these two genes (91), while the targeted deletions of the *H19* gene itself had only minor effects on *Igf2* expression (104,106). An important outcome of the targeted deletion approaches was the demonstration that a differentially methylated region called DMR or ICR (Imprinting Control Region), located at -2kb to -4kb relative to the transcription start site of *H19* gene is crucial

for the manifestation of imprinted expression of both the *H19* and *Igf2* genes (102,103). The epigenetic mark on the *H19* ICR dictates whether it will act as a silencer for the paternal *H19* allele or an insulator for the maternal *Igf2* allele. The first experimental suggestion that the ICR could function as a silencer came from transgenic experiments in *Drosophila* (107). These data, however could not explain why the unmethylated ICR on the maternal allele, in addition to facilitate the *H19* transcription, could simultaneously exert a negative effect on *Igf2* in *cis*. These observations were influential for the realization of an insulator boundary model (39,108,110 and paper I).

On the maternal allele, the ICR acts as a unidirectional insulator. According to the insulator boundary model, ICR prevents the communication of the endodermal enhancers towards the *Igf2* promoter. When methylated on the paternal allele, the insulator complex is not formed, allowing free access of the *Igf2* promoter to the enhancers (paper I). Chromatin analysis of the *H19* ICR has revealed maternal specific nuclease hypersensitive sites (NHSSs) (102,103). This shows the possibility of some DNA-protein interactions specifically on the maternal allele. The 11 zinc finger protein CTCF (110,111) interacts with the core sequences of all currently known vertebrate insulators. CTCF binds through combinatorial use of its 11 zinc fingers binds to the target sites of about 50 bp that have remarkable sequence variations. Later on, it was shown that CTCF interacts with the unmethylated maternal ICR (39,108), in a methylation sensitive parent-of-origin specific manner (112). The first experimental support that the *H19* insulator function is regulated by CpG methylation was shown in (113). The detailed analysis of the chromatin organization of the ICR showed the presence of positioned nucleosomes, with strong NHSs (CTCF binding sites), mapped in the linker regions between the positioned nucleosomes (paper I). The study of the role of positioned nucleosomes in the *H19* 1CR resulted in the identification of nucleosome positioning sequences (NPSs), specifically in the 3'end of the ICR. These NPSs maintain the unique nucleosome positioning pattern on the maternal allele by constitutively maintaining the linker regions available for trans activating factors such as CTCF (paper II).

The *Kcnq1* ICR

The *KCNQ1* gene covers most of the (around one-third) of the 1Mb imprinted domain of human chromosome 11 and the mouse chromosome 7. *KCNQ1* encodes a protein which dimerizes with mink to produce a functional potassium channel (31,114). Human KCNQ1 was isolated in a search for genes associated with BWS. As mentioned above, most of the chromosomal translocation breakpoints in BWS patients map to this *KCNQ1* gene. Most of the fetal tissues, except heart, show monoallelic maternal expression of the *KCNQ1* gene, suggesting that this gene is imprinted (31).

In mouse, the *Kcnq1* gene is maternally expressed during the early stages of development and then the paternal allele progressively becomes active, so that in late juvenile and adult mice, *Kcnq1* is expressed biallelicaly (115). The mechanism behind the lack of manifestation of the imprint is still not yet clear, although it appears to involve a genetic background effect.

In intron 10 of the *Kcnq1* gene, which spans around 350 kb, a differentially methylated region known as the *Kcnq1* ICR was identified. The *Kcnq1* ICR is methylated on the actively expressed maternal allele. Conversely, the paternal allele is unmethylated and associated with a non-coding antisense transcript in both humans (*KNCQ1OT1* or LIT1) (116,117) and mice (*Kcnq1ot1* or Lit1) (57).

Horike et al (96) generated modified human chromosome carrying a deletion of the *Kcnq1* ICR. In these cells the *KCNQ1OT1* was abolished and the *KCNQI* and *CDNKI* genes are over expressed from the paternal chromosome bearing the deletion. These results suggest that the *Kcnq1* ICR functions as an imprinting control element that regulates the centromeric 11p15.5 imprinted domain. Paternal inheritance of the targeted deletion of *Kcnq1* ICR in mice (95) resulted in the derepression of six imprinted genes located between *Ipl* and *Ascl2* of the centromeric domain. This observation documents that the unmethylated paternal *Kcnq1* ICR allele regulates imprinted expression of the *Kvlqt1* gene, and other genes by long range silencing.

While the molecular mechanism behind this regulation of imprinted gene expression is still not yet known, we have mainly concentrated on understanding the properties of the *Kcnq1* ICR, using an episomal system. In paper III we have shown that the *Kcnq1* ICR acts as a unidirectional chromatin insulator in both Hep3B and the Jurket cell lines, and the insulator function is regulated in a methylation dependent manner, which is similar to the *H19* ICR. In Paper IV we have shown that the *Kcnq1* ICR bidirectionally silences reporter genes in an episomal based system propagated in the JEG-3 cell line. We have also observed that methylation spreading over the reporter gene occurs in a methylation sensitive manner. Fine mapping the regions responsible for the methylation spreading and the silencing activity leads to the same regions, R1 and R2 indicating that the silencing factors associated with these regions represses genes by spreading DNA methylation to the neighboring sequences. We have also shown that the loss of either of these two regions results in the complete loss of bidirectional silencing and the DNA methylation spreading property, suggesting that R1 and R2 are connected to each other.

Aims of the present study

The present investigation is mainly focused on the functional characterization and the detailed chromatin organization of the two imprinting control regions, the *H19* and the *Kcnq1* ICRs, which are responsible for the regulation of most of the imprinted genes, located several hundred Kb from each other, on the distal portions of human chromosome 11 and the mouse chromosome 7. The specific aims of this study are listed in the following:

1. What is the function of the *H19* ICR?
2. What is the chromatin organization of the *H19* ICR that could explain its insulator function?
3. Will the silencing functions of the *H19* and *Kcnq1* ICRs reflect similar mechanisms?

Results and discussion

Paper I

H19 and *Igf2* are oppositely imprinted genes, located 90kb apart from each other on mouse chromosome 7. Genetic evidence indicates that the *H19* differentially methylated imprinting control region (ICR), which is located -2 to -4 kb from the transcription start site of the *H19* promoter, is mainly responsible for maintaining the mono allelic expression of these imprinted genes. Deletion of the *H19* ICR region results in the biallelic expression of both the genes, but the manner in which the ICR suppresses the paternal *H19* and the maternal Igf2 genes was poorly understood. Hence it is of obvious interest to study in detail the mechanism behind the ICR function in regulating the gene expression of the *H19* and *Igf2* genes. This paper mainly deals with the functional characterization and the detailed chromatin analysis of the ICR.

The analysis of the chromatin organization of the paternal and the maternal H19 ICR alleles were facilitated by intra-specific mouse embryo hybrid crosses. The two strains that were used *M.m.musculus* and *M.m.domesticus*, displayed a strategically positioned *Bbs I* polymorphic restriction site, specific for *M.m.musculus*. An indirect end-labeling assay with the MNase digested DNA samples from the mouse fetal liver born to the off springs of the intra hybrid crosses showed the presence of three nuclease hypersensitive sites (NHSSs) that appear only when they are maternally inherited. These NHSSs were observed on the maternal allele from the MNase digested DNA samples of both the H19 expressed (liver) and the H19 unexpressed (brain) tissues. To rule out the possibility that the maternal specific hypersensitive sites could have appeared due to differential nuclease digestions, the blots were rehybridised with human PDGF probe and the results indicated that both paternal and maternal alleles were exposed to some amount of nuclease. To check that the NHSSs were derived due to specific preferences for certain DNA sequences, in methylated or unmethylated form, the DNase and MNase digestions were carried on unmethylated and *in vitro* methylated naked DNA samples. No specific preferences for MNase and DNase on the naked DNA were observed as the manner of digestion pattern were very similar between the methylated and mock methylated samples. Increasing amounts of MNase digestions showed the presence of the positioned nucleosomes in both the maternal and the

paternal alleles. This result indicates that the nucleosome positioning of the ICR is identical in both alleles, even though hypersensitive sites are restricted to the maternal allele. The presence of positioned nucleosomes in the H19 ICR region contradicts previously published data, which claims that the ICR is devoid of nucleosomes and that the NHSSs are due to interactions between some non- histone proteins, as they bind efficiently in the absence of the nucleosomes. But some papers support the presence of the positioned nucleosomes: the chromatin organization of the H19 domain contains a unique structural periodicity (~450bp) in humans (118), and (~425bp) in mouse spermatozoa, through the DNase1 digested samples (119) and identifying the nucleosome positioning signals in the DNA sequence of the mouse and human H19 imprinting control regions (120). To gain more insight into this issue, we have developed an episomal based assay system to recapitulate the endogenous H19 nucleosome positioning and also NHSSs formation. The episomal system was chosen because as it has advantages over the integrated plasmid system, whose expression undergo position-dependent effects. Indirect end-labeling of the stably maintained Hep3b (Hepato cellular carcinoma) cell lines, transfected with the episomal plasmid containing the H19 mini gene, which has both 5' and 3' UTR, showed that the nucleosome positioning and the NHSSs formation were the same as that of the endogenous H19 ICR. Fine mapping of the NHSSs, by using the different probes in the vicinity of the NHSSs, showed that they are located in the linker regions between the positioned nucleosomes.

Due to the presence of the unusual chromatin organization, with positioned nucleosomes and NHSSs, we were interested in characterizing role of the H19 ICR in H19 and Igf2 regulation. The mechanism involved by the H19 ICR in the repression of the maternal Igf2 allele was poorly understood. To test the possibility that the H19 ICR operates as a position-dependent or independent silencer, we generated a range of episomal constructs.

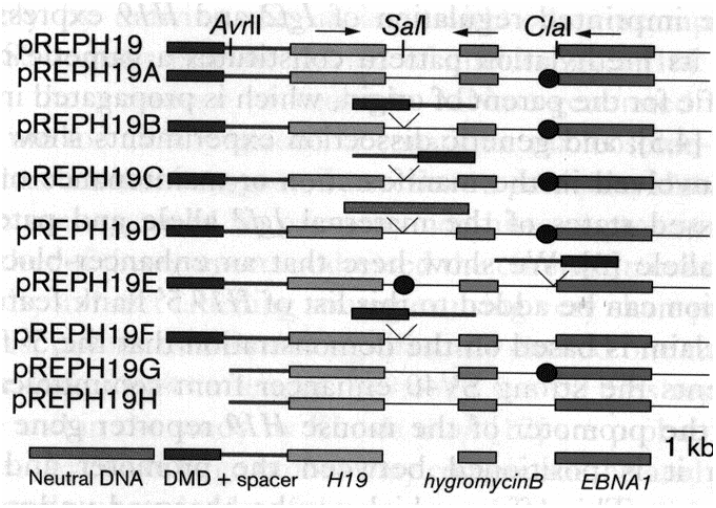

Fig: 1

Figure legend: Schematic map of the various constructs used in this study. The maps, which are to scale, do not show the entire pREP vector. The circle depicts the position of Sv40 enhancer. All the other symbols are explained in the panel.

To address a potential insulator function, we positioned the H19 ICR fragment between the enhancer and promoter (PREP4H19B), since insulators function only in a position-dependent manner. To characterize the silencer function, we placed the H19 ICR fragment upstream of the enhancer, as silencer interferes with promoter in a position independent manner (PREPH19C) and also the H19 ICR fragment was deleted to check whether any up regulation of H19 promoter (PREP4H19H). The construct containing only Sv40 enhancer (PREP4H19A) induced 200 fold increase of the H19 promoter activity. The H19 promoter activity, however, reduced several folds when H19ICR was inserted between the H19 promoter and the SV40 enhancer in one orientation but not in other orientation suggesting that the H19 ICR unidirectionally blocks enhancer- promoter communication. The H19 ICR, however did not interfere with enhancer promoter communication when it was inserted upstream of the SV40 enhancer. The latter observation rules out the possible silencer function for the H19 ICR. This was further supported by the fact that deletion of the H19 ICR fragment did not up regulate the H19 promoter activity, suggesting that the H19 ICR does not harbor any silencer activity, as claimed in the *Drosophila* transgene system (85).

MNase cuts preferentially in the linker regions, and the resultant NHSSs can be due to the interaction between some non-histone proteins and the DNA of the linker regions. The identical nucleosome positioning patterns in both the maternal and the paternal alleles indicates that there is no role for these proteins in maintaining the nucleosome positioning within the ICR.

These NHSSs are, however, always maintained in the linker regions, which are easily available for the proteins to bind, it can be possible that there can be a role of nucleosome positioning in the formation of these NHSSs, constitutively allowing factors to interact with pivotal *cis* elements. These proteins can be methylation-sensitive, as they are absent from the ICR of the paternal allele. Interestingly, linker-specific NHSSs contains 21bp repeat sequences, which might have a significant role, as these are the only evolutionarily conserved sequences between mouse and human. These highly conserved sequences were also shown to be present in the chicken ß-globin insulator, which binds to the transcription factor CTCF. Later on, we showed that the two strong hypersensitive sites in the linker regions of the H19 ICR, named NHSSI and NHSSII, were due to the methylation and parent of origin dependent binding of CTCF.

Hence the main role of the H19 ICR is to act as an insulator that isolates the maternal *Igf2* promoter from the downstream enhancers. On the paternal allele, the ICR and the H19 promoter is methylated and hence no hypersensitive sites formation is observed. The presence and the significance of the evolutionarily conserved regions in the ICR, suggests its main role is in the regulation of imprinted genes.

Paper II

The results of the first paper indicate that the H19 ICR acts as an insulator, by blocking the communication between the enhancers and the Igf2 promoter. The maternal-specific NHSSs contain 21 bp repeats which are evolutionary conserved between mouse and human, and these sequences were shown to bind to the CTCF protein (95). CTCF is a ubiquitously expressed protein, which contains 11 zinc fingers, and binds to highly variable target sequences, by using these fingers in various combinations. By using band shift assays we have earlier shown that CTCF binds strongly to two NHSSs in the H19 ICR, which are named NHSSIII and NHSSIV. Mutational analysis showed that these two CTCF sites are necessary and sufficient for the insulator activity. By using a chromatin immunopurification assay (ChIP assay), it was shown that CTCF binds to the maternal allele in a parent of origin specific manner.

We have earlier mapped CTCF binding sites to the linker regions between positioned nucleosomes. Could there be a possible role for nucleosome positioning in maintaining the CTCF target sites always in the linker regions? To address this question in detail, we have used an insertional mutagenesis of the episomal H19 ICR plasmids. The episomal-based system used in this study is same as that which has been used in our earlier studies, but lacks the 5' portion of the ICR. The wild type and mutated ICR episomal plasmids were transfected into the Hep3B cell line, and then subjected to

hygromycin selection. These transfected cell lines were propagated for two months, and then analyzed for chromatin organization using the indirect end-labeling technique. The functional analysis was carried out by analyzing the insulating activity of the ICR by the strong SV40 enhancer towards the expression of the H19 reporter gene. The chromatin organization of the endogenous H19 ICR, obtained from mouse fetal liver, has shown four hypersensitive sites which bind to CTCF. Two sites, NHSSI and NHSSII, were located in the 5' region of the ICR and the other two strong sites the NHSSIII and NHSSIV, were located at the 3'end of the H19 ICR. The indirect end-labeling assay using MNase digested samples of the PREP4H195'ICR, obtained from the stable transfected Hep3B cell lines, showed that there were no positioned nucleosomes, as there was a smear instead of the nucleosome ladder. However, when the same blot was hybridized with the full-length probe the nucleosome ladder was observed, which indicates that the even though the nucleosomes were present, they were not positioned. This also reveals the absence of nucleosomes positioning signals from the vector and highlights the possibility that the positioning signals are restricted to the 3' ICR. To check this in more detail, we made several PREP4H19MutB clones, which contains a mutated ICR with an inserted neutral fragment of various sizes at different locations of the ICR, which can result in the displacement of nucleosome positioning. The sizes of the neutral fragments were 80 bp, 140 bp and 280 bp, which constitutes around one half, one full turn and two turns of the average length of nucleosomal DNA. The neutral fragment was derived from a multiple cloning site. To rule out that the neutral fragment positioned nucleosomes, it was placed between the two NHSS sites, in construct, PREP4H19mutB1. Only the nucleosomes present 3' of the insertion site were placed in a new location. This type of change in the nucleosome positioning can be due to the directional positioning of the neutral fragment. But this was not the case as the negative orientation of the same 80bp fragment (PREP4H19mutBinv) has also shown the same effect. Both the orientations of the neutral fragment showed similar results, indicating the presence some nucleosome positioning sequences upstream of the inserted fragment. The insertions of 140 bp and 280 bp (PREPH19B2 and PREP4H19B3 respectively) in the same location also showed the same type of the result, where the nucleosomes of only one side of the insertion are moved to the new locations. These results indicate that nucleosome positioning sequence located upstream of the insertion site maintain positioning irrespective of the size and orientation of the inserted neutral fragment.

Another interesting observation was that the change of the nucleosomes positions was restricted to only a few nucleosomes in all the cases of neutral fragment insertions. This region contains the G-repeat sequence, which has been shown to have high nucleosome positioning properties. We identified two nucleosome positioning sequences (NPSII and NPSIII), one upstream of

the insertion site and the other downstream of the NHSSIV. When the same 80 bp fragment was inserted upstream of NHSSIII (PREP4H19mutB4), there was no change in the nucleosome positioning at either side of the insertion. This result neutralizes the argument that nucleosome positioning is induced by the inserted neutral fragment. Our data revealed the presence of another nucleosome positioning sequence (NPSI) present near the insertion site of the PREP4H19mutB4, which is maintaining the nucleosomes in the same location. Taken together, our results identified three nucleosome positioning sequences in the 3'ICR. Generally the ability of the NPS sequences to position nucleosomes is due to high affinity towards the histone octomer. Analysis using software that can recognize, strong NPS potential based on the histone octomer affinity and the presence of the relative distance of the dinucleotides, confirmed that these fine mapped sequences have high nucleosomes positioning potential.

The PREPH19mutB1 clone that contains the inserted 80bp fragment between the two NHSSs has shifted the NHSSIV into the nucleosomes, while the NHSSIII was still in the linker sequence as it was 5' of the insertion site. To address the role of NPS and insulator function, NHSSIII was point mutated by changing two base pairs to generate the PREP4H19mutB1* plasmid. We have already shown that the point mutation of either of these two NHSSs (PREPH19S1 and PREPH19S2 respectively) resulted in maximal loss of insulator activity. This PREP4H19mutB1*, where the NHSSIII was point mutated in the linker positions, while the NHSSIV was shifted into the nucleosome, was analyzed for insulator activity. The PREP4H19mutB1* plasmid was transfected and propagated to form stable cell lines along with the control plasmids, PREP4H19A (containing the wild type ICR) and PREPH19S1 (containing the point mutated NHSSIII), and the DNA and RNA were extracted for analyzing the H19 reporter gene expression. The mutICR showed a strong reduction in insulator activity when compared to the wild type ICR and NHSSIII point mutated ICR. However when the same analysis was done for transiently transfected clones, this PREP4H19mutB1*, again showed strong insulator activity. Therefore in the stably propagated cell lines, the insulator activity was lost when the NHSSIII was point-mutated and NHSSIV was covered by the nucleosome, but in the transient transfections, the same PREP4H19mutB1*, showed potent insulating activity. These results indicate two things; first, the insulator activity is dependent on the availability of the CTCF target sites in the linker regions, which are maintained by positioned nucleosomes. Second, CTCF cannot position the nucleosome as the paternal allele also shows the same nucleosome positioning pattern, even though it does not bind to CTCF as it is methylated. Similarly, the point mutated NHSSIII clone also showed the same nucleosome positioning, while the 5' ICR contained two CTCF target sites, although there was no nucleosome positioning observed. In the evolutionary aspect the distribution of CTCF

target sites and nucleosomes positioning seem to have co-evolved to always maintain CTCF target sites in the linker regions, to presumably ensure repression of the maternal Igf2 allele.

Given that the results indicated that CTCF does not bind directly when the target sites were covered by the nucleosomes, we tested this possibility. Following *in vitro* chromatin reconstitution and addition of recombinant CTCF, a band shift analysis was performed. In this assay, a 178 bp fragment was amplified and used a template for *in vitro* reconstitution. Since the CTCF target site is in the middle of the fragment it should be always covered by the nucleosomes. When the band shift assay was performed using this reconstituted chromatin, it revealed that CTCF does not bind to its target site if it is covered by a nucleosome. This clearly explains the loss of NHSS formation and insulator function in the cell lines with a stably transfected PREPH19mutB1* construct.

In conclusion, this paper shows that the binding of the CTCF to its target is dependent on its nucleosome positioning and that there exist at least three NPS both within and outside the H19 ICR.

Paper III

BWS is caused by the disruption of the imprinting status of genes located in the imprinted domain of human chromosome 11. The chromosomal breakpoints and the abnormal methylation changes that are observed in the BWS syndrome patients are mapped to the differentially methylated region located in intron 10 of the *KCNQ1* gene, called the *Kcnq1* ICR . This *Kcnq1* ICR, which is a 2kb CpG island, is methylated on the maternal allele and unmethylated and associated with an antisense transcript on the paternal allele. The importance of the *Kcnq1* ICR has been shown *in vivo* by targeted deletion experiments in mouse and in modified human chromosomal cell lines. These studies have indicated that this *Kcnq1* ICR plays an important role in controlling the long range expression of the imprinted genes located in this domain. In the earlier papers we have shown that the *H19* ICR acts as a chromatin insulator and controls the expression of the *H19* and *Igf2* genes. We are interested to see if this chromatin insulator function is a common feature of other imprinting control regions like the *Kcnq1* ICR, and if so whether it is methylation sensitive like the *H19* ICR, using our episomal based analyses.

To test the insulator function of the *Kcnq1* ICR (or KvDMR), we used two different types of insulator assays, which can determine whether the *Kcnq1* ICR contains position-dependent insulators or displays position-independent gene silencing function. In the first insulator assay, the neomycin resistance gene was used as the reporter gene. This is regulated by the Vd1 promoter and an endogenous Ed4 enhancer. To prevent the position-

dependent effects on the Vd1 promoter, the known mammalian insulator, scs was placed on either side of the vector. This vector with the neo resistant reporter gene, promoter, enhancer and the scs insulator is termed the E-p-neo-scs vector. The number of neomycin resistant colonies was analyzed after the electroporation of this construct into the Jurket cell line. The p-neo-scs vector without the enhancer showed much reduced number of colonies, and this number of colonies is considered as the base line expression of the reporter gene. When the enhancer is included, the neomycin resistant colonies have increased 10 fold in number, and this level of expression is considered to be 100%; to this value all the other constructs were compared, which contains the *Kcnq1* ICR at different strategic positions. When the 3.6 kb *Kcnq1* ICR fragment was inserted between Vd1 promoter and the Ed4 enhancer there was strong reduction in the number of neo-resistant colonies. However, when *Kcnq1* ICR was inserted upstream of the enhancer there was no reduction in the colony number, suggesting that the *Kcnq1* ICR is a position dependent insulator. The vector that is used as the negative control in this assay containing a 2.7 kb anonymous DNA fragment instead of the *Kcnq1* ICR, did not show any reduction in the number of colonies, indicating the fact that the insulator activity is specifically due to the *Kcnq1* ICR. Next, we addressed whether the orientation of *Kcnq1* ICR affects the insulator activity. Indeed, the construct containing the *Kcnq1* ICR in opposite orientation showed colony number of 100%, inferring that the *Kcnq1* ICR insulator function is subjected to orientation dependent effects.

The second insulator assay, an episomal based system, was used to confirm the insulator activity and also to explore the role of DNA methylation on the insulator activity of the *Kcnq1* ICR. This assay, as has been described in the first paper, contains again mouse *H19* as the reporter gene and the SV40 enhancer. To distinguish between the possibilities that the *Kcnq1* ICR is an insulator or a silencer element, we placed the *Kcnq1* ICR fragment between the enhancer and the reporter gene promoter and upstream of the enhancer. The constructs were transfected into the Hep3b cell line and the stably transfectants were propagated for 30 days or more. RNA extracted from the cell lines was subjected to an RNase protection assay to analyze the activity of the *H*19 reporter gene. In paper I, we have already shown that the SV40 enhancer activates the *H*19 reporter gene 200 fold. The results from the analysis indicated that the *Kcnq1* ICR down regulated reporter gene activity when inserted between the promoter and enhancer, but not when it is placed upstream of the SV40 enhancer, confirming the previous observation that the *Kcnq1* ICR is an insulator element. Given the possibility that the unidirectional insulating activity could be due to the presence of enhancers in the 3.6 kb *Kcnq1 ICR* fragment itself, we have removed the SV40 enhancer from the PS4 constructs (- enh). Following transfection, hygromycin selection and colony count observations, this construct showed a much reduced number of colonies, which indicates

no detectable enhancement of hygromycin activity by the *Kcnq1* ICR. This observation rules out the possibility that the unidirectional insulator activity is due to the presence of the any enhancers located within the *Kcnq1* ICR. Taken together, these two independent insulator assays establish the fact that *Kcnq1* ICR functions as unidirectional, orientation-dependent insulator.

Next, we investigated the effect of CpG methylation on the insulator activity of the *Kcnq1* ICR. To address this point we have generated *in vitro* methylated 3.6 kb *Kcnq1* ICR fragments that were ligated to the unmethylated PREP4A episomal vectors. The ligation mix was transfected into Hep3b cells, following selection for 3 to 4 weeks using hygromycin. Approximately 20 clones from each of methylated and the mock methylated constructs were expanded for further studies. These 20 clones were first genotyped and epigenotyped as described in paper I. Based on Southern blot analysis we have selected three clones containing the methylated *Kcnq1* ICR and one clone containing the mock methylated *Kcnq1* ICR, which were maintained episomally. To test the methylation pattern of the *Kcnq1* ICR insert in the selected methylated and the mock methylated clones, the DNA extracted from these cell lines was digested with the methylation sensitive enzyme *Hha1*. The results indicate that out of the three methylated clones, in one, the *Kcnq1* ICR was completely methylated and the other two contain minor proportion of unmethylated *Kcnq1* ICR sequence.

These clones were selected to test the effect of CpG methylation on the chromatin conformation of the *Kcnq1* ICR. We have shown that in mouse fetal and adult liver, there are three DNase hypersensitive sites and these sites were recapitulated in the *in vitro* mock methylated clone, but not in the *in vitro* methylated clone. These results suggest that the DNA-protein interactions in these regions are methylation-sensitive. To show that DNase I digested the methylated and the mock-methylated DNA equally the blot was reprobed with the *Bgl II* fragment of the PDGF gene. The loss of the hypersentive sites in the methylated *Kcnq1* ICR implies the methylation induced chromatin compaction directly or indirectly via methylation-sensitive trans-acting factors. Next we were interested to examine the effect of DNA methylation on the unidirectional insulator activity of the *Kcnq1* ICR. The RNase protection assay analysis of these clones was done as described earlier in the insulator assay, by determining the percentage activity of the *H*19 reporter gene activity against the SV40 enhancer. The results revealed that the *Kcnq1* ICR acts as a methylation-sensitive chromatin insulator.

Based on all the above results we propose a model that explains the relaxation of imprinting of the *Kcnq1* gene in adult stages. We have shown that the *Kcnq1* ICR acts as a unidirectional insulator and also that there are no other endogenous enhancers present in the *Kcnq1* ICR itself. There are several lineage specific enhancers that have been identified in the intergenic region between *Kcnq1* and *Cdnk1* (16). Enhancers that are present 3' to the

Kcnq1 ICR are not insulated by the *Kcnq1* ICR and they can always access the *Kcnq1* promoter. Therefore we propose that these enhancers that are located downstream of the *Kcnq1* ICR are active in the adult stages and the enhancers that are present upstream are active in the fetal stages of development.

Paper IV

In the above paper, we have shown that the *Kcnq1* ICR acts as a methylation sensitive unidirectional insulator in the Hep3b and Jurket cell lines. Since manifestation of the *KCNQ1* imprint is tissue specific, we were interested in knowing whether the function of *Kcnq1* ICR is the same in all cell types, or has cell type specific functions. To test this possibility we have examined the function of the *Kcnq1* ICR in the trophectodermally-derived JEG3 cell line. First we wanted to analyze the insulating and silencing function of the *Kcnq1* ICR using episomal constructs, as mentioned in paper III, which contain *H19* as the reporter gene and the SV40 enhancer.

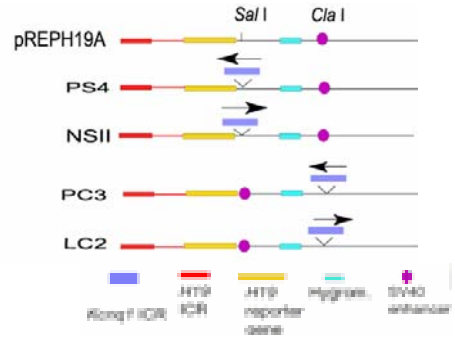

Fig. 2

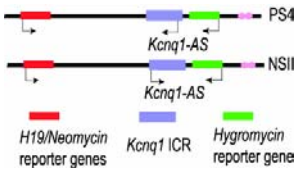

Fig. 3

Figure legend: Figure 2, shows the episomal plasmids containing the 3.6 kb fragment at the insulating position and silencing positions. Figure 3, shows the orientation of the antisense transcript of *Kcnq1* gene (*Kcnq1-AS*), with respect to the reporter genes, in the PS4 and NS11 plasmids.

The *Kcnq1* ICR was inserted in both orientations with respect to the *H19* promoter, in the insulating position between the *H19* reporter gene and the SV40 enhancer (PS4 & NS11) and the silencing position out of promoter and enhancer context (PC3 & LC2). The resulting plasmids were transfected in the JEG 3 cell line, RNA was extracted 9 days after transfection and an RNase protection assay was performed to test the activity of the reporter gene. The copy number was adjusted according to the procedure mentioned in Paper III. The results of this experiment showed that with the *Kcnq1* ICR in the insulating position at the PS4 orientation, H19 gene activity is reduced

10 fold, while in the NS11 orientation *H19* gene activity is reduced 7 fold. This shows that the *Kcnq1* ICR down regulates the *H19* reporter gene in an orientation-independent manner when it is placed in the insulating position, i.e. between the *H19* promoter and the SV40 enhancer.

However when the *Kcnq1* ICR was placed in the silencing position, it still reduced *H19* gene activity by more than 10 fold, but only in one orientation, i.e. PC3 orientation. The above results indicate that in the JEG3 cell line the *Kcnq1* ICR acts as a silencer rather then insulator, as the *H19* reporter gene activity is reduced in both insulating and silencing position. The orientation dependent silencing function of the *Kcnq1* ICR suggests, however, that it harbors both the insulating and the silencing elements.

In the *in vivo* context, it has been shown that the *Kcnq1* ICR represses the neighboring genes bidirectionally, and we wanted to test this scenario in our episomal based system. This was facilitated by the *H19* reporter gene and the hygromycin gene, on either side of the insulating position. *H19* gene activity was determined by the RNase protection analysis as above, while the hygromycin gene activity was determined by counting the number of resistant colonies upon hygromycin selection. The results clearly indicate that in both orientations (PS4 and the NS11), the *Kcnq1* ICR, represses bidirectionally in our episomal based system in the JEG 3 cell line, just like the *in vivo* situation.

Since we could recapitulate the bidirectional repression of the *Kcnq1* ICR in our episomal based analysis, we wanted further to fine map the regions that are responsible for the silencing function of the *Kcnq1* ICR. To address this issue we have made serial deletions in the 3.6 kb *Kcnq1* ICR fragment using the site-directed point mutagenesis method as described in Paper II.

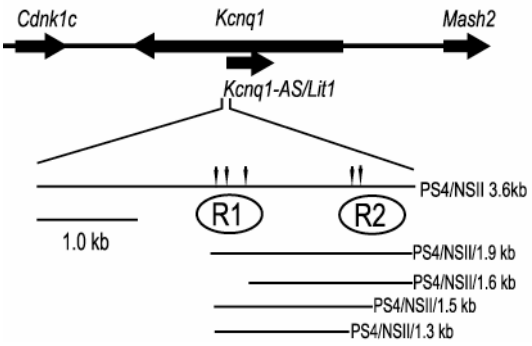

Fig. 4

Figure legend: The above figure shows the schematic map over the *Cdkn 1c, Kcnq1*, and *Mash2* region. The lower panel shows the 3.6 kb *Kcnq1* imprinting control region and the fine deletions within the 3.6 kb fragment.

We have analyzed *H19* reporter gene activity and hygromycin gene activity of the deletion fragments along with the full length 3.6 kb fragment of the *Kcnq1* ICR, by the same method as described above. The results show that the 1.9 kb fragment shows the same insulator activity as the 3.6 kb fragment while the 5' 300bp deletion fragment (1.6kb) resulted in complete loss of silencing activity of the *Kcnq1* ICR. Since the 3' 600bp deletion fragment (1.3kb) showed around 70% *H19* gene activity, it was suggested that these two regions, the 5' 300bp (containing R1) and the 3' 600bp (containing R2) are simultaneously required for the complete silencing, such that loss of either of these two fragments results in loss of the silencing function of the *Kcnq1* ICR. The bidirectional silencing activity of these *Kcnq1* ICR deletion fragments was analyzed by replacing *H19* gene with the neomycin gene as the reporter gene, so that the *Kcnq1* ICR is placed between two reporter genes, neomycin and hygromycin genes. The percentage activity of both the neomycin and the hygromycin genes was analyzed by counting the number of neomycin and hygromycin resistant colonies respectively. The results of this experiment indicate that the 1.9 kb fragment of the *Kcnq1* ICR represses both the reporter genes 10 fold, in both the directions, just like the full length *Kcnq1* ICR. The 1.6 kb and 1.3 kb deletion fragments of *Kcnq1* ICR did not show the silencing activity of either reporter genes, in both the orientations, suggesting that both the 3.6 kb and the 1.9 kb fragment of the *Kcnq1* ICR repress gene activity bidirectionally in an orientation-independent manner in the JEG 3 cell line at the insulating position. However, loss of either R1 or R2 results in loss of the bidirectional silencing activity.

Based on the above results we have shown that the *Kcnq1* ICR acts as a bidirectional silencer and fine-mapped the regions (R1 & R2) responsible for this silencing activity. Next, we wanted to examine the mechanism behind this bidirectional silencing activity. DNA methylation has been shown to be involved in gene silencing, so we expected that the *Kcnq1* ICR silenced the neighboring genes by spreading DNA methylation. Attempts to analyze the methylation status of the sequences that are flanking the *Kcnq1* ICR in the episomal constructs were made at various time points during the selection of stably transfected JEG3 cell lines, but we could not perform the long term methylation studied on the JEG3 cells. It was happened by our earlier demonstration that this cell line has strong *de novo* methylation activity, so we focused our efforts at this time, therefore on the Heb 3B cell line.

First, we analyzed the methylation spreading property of the 3.6 kb *Kcnq1* ICR in Hep3B cell were propagated for several days after transfection. DNA from the stably propagated cell line was collected every 14th day for a time period up to 42 days, followed by digestion with the methylation-sensitive *Hha1* enzyme. Southern hybridisation using a 2.4kb *BamH1* fragment that contains the *H*19 promoter region as a probe, shows that the *Kcnq1* ICR spreads methylation in the PS4 orientation, but not to that extent in the NS11

orientation in Hep3B cells. Similar data could be observed for JEG 3 cells propagated for a much shorter time period.

The methylation machinery spreading property could be due to the *de novo* methylation of the cell lines used or due to the *Kcnq1* ICR itself. To determine whether or not this methylation spreading property is unique to the *Kcnq1* ICR, we have performed the same methylation spreading assay with episomes containing the *H19* ICR, along with the *Kcnq1* ICR containing episomes. All these constructs were transfected into the JEG 3 cell line and analyzed after 4 days for the methylation spreading in the *H19* promoter region. The results showed that the *H19* ICR did not spread DNA methylation, and hence that this methylation spreading property is unique to the *Kcnq1* ICR. This result was reinforced by bisulphite sequencing analysis with the DNA extracted from Hep3B cells transfected with episomal constructs carrying the *Kcnq1* ICR in both orientation, PS4 and NS11, and selected for 42 days. The results of bisulphite sequencing analysis showed that the *Kcnq1* ICR is unmethylated in the PS4 orientation, which spreads methylation and *Kcnq1* ICR is methylated in the NS11 orientation, which does not spread methylation. We conclude therefore that the *Kcnq1* ICR methylation spreading property is methylation-sensitive, and that it spreads methylation to the neighboring sequences only when it is itself in the unmethylated state.

We then wanted to fine-map the regions that were responsible for the methylation-spreading property of the *Kcnq1* ICR in the PS4 orientation. We used the same deletion constructs of the *Kcnq1* ICR that we had used for fine mapping the bidirectional silencing activity, assuming that the same regions R1 & R2 are responsible for the methylation spreading property. The episomal constructs carrying the serial deletions of the *Kcnq1* ICR were transiently transfected in the JEG3 and Hep3B cell lines and were analyzed for the methylation-spreading property of the *Kcnq1* ICR in both orientations. The 1.9 kb *Kcnq1* ICR fragment showed methylation spreading in the PS4 orientation but not to that extent in the NS11 orientation, just like the 3.6 kb *Kcnq1* ICR. But when 400 bp were deleted in the 3' region of the 1.9 kb fragment containing R2, the 1.5 kb *Kcnq1* ICR spread the methylation in both orientations, documenting that the unidirectional methylation spreading property is maintained by this 400 bp fragment. The deletion of 600 bp from the same 3' region of the 1.9 kb *Kcnq1* ICR shows that the methylation spreading property is totally lost. In addition, the 300 bp 5' deletion of the 1.9 kb *Kcnq1* ICR containing R1, the methylation spreading property is lost. This indicates that the methylation spreading property of the *Kcnq1* ICR is maintained by the same R1 & R2 regions, which maintains the bidirectional silencing activity.

In conclusion, this paper highlights that the *Kcnq1* ICR contains cell type specific activity, since it acts as a bidirectional silencer in the JEG3 cell line and a unidirectional insulator in the Hep3B and Jurket cell lines. These

results provide a reason behind the tissue specific manifestation of the imprinted state. We have also shown that the *Kcnq1* ICR represses genes bidirectionally by spreading DNA methylation in an orientation and methylation-sensitive manner. This property might explain the effects of loss of methylation of the *Kcnq1* ICR in BWS patients, which results in spreading of repression to neighboring genes. This silencing proceeds well before DNA methylation of the reporter gene, indicating that the DNA methylation spreading is the consequence rather than the cause of silencing of the *Kcnq1* ICR. Finally, we have shown that the cis-elements that are responsible for both bidirectional silencing and the methylation spreading property of the *Kcnq1* ICR map to the same regions, R1 and R2. Loss of either one of the regions results in the loss of bidirectional silencing and methylation spreading properties. Furthermore, we have shown, by *in vivo* foot printing analysis that several factors and *cis* elements binds to R1 and R2. Moreover, the promoter of the antisense transcript maps to R1, indicating that the antisense transcript that originates from R1 may interact with R2 to form a repressive chromatin structure that spreads bidirectionally to inactivate the genes present on either side.

Conclusions

The imprinted genes have a major role in normal development, as their disruption leads to many disorders like cancer. Generally the imprinted genes are present in clusters, and the main reason behind this is still poorly understood. Even though the imprinted genes are clustered in domains, the differentially methylated imprinting control regions (ICRs) regulate the genes by maintaining different sub domains. The ICRs, located in the imprinted domains, control the imprinting status of many genes that are located several hundred base pairs away from each other. To unravel this complex mechanism of their precise gene regulation, several groups are studying different aspects. Our contribution has been the demonstration of methylation-sensitive chromatin insulators within the imprinting control regions. We have shown that the imprinted cluster located on human chromosome 11 and the mouse chromosome 7 is regulated by two different ICRs, the ICR of the *H*19 gene and the *Kcnq1* ICR, by acting as the methylation sensitive chromatin insulators. We have also shown that these ICRs contain unique chromatin conformations, and in the *H19* ICR, nucleosome positioning also plays an important function in its insulator activity. However, even though these ICRs have similar features and functions, their mechanism of controlling the expression of different genes in this cluster appears different as judged by the presence of lineage-specific silencing mechanism in the *Kcnq1* ICR, but not in the *H19* ICR.

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