Chromatin structure based gene activation analysis in imprinted gene *Neuronatin*

Thesis submitted to

Manipal University

For the Degree of

Doctor of Philosophy

By

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July 2008

DECLARATION

The research work presented in this thesis entitled **"Chromatin structure based gene activation analysis in imprinted gene *Neuronatin*"**, has been carried out by me at Centre for DNA fingerprinting and Diagnostics, Hyderabad, under the guidance of Dr. Sanjeev Khosla. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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CERTIFICATE

This is to certify that this thesis entitled **"Chromatin structure based gene activation analysis in imprinted gene *Neuronatin*"**, submitted by Mr. Sudhish Sharma for the degree of Doctor of Philosophy to Manipal University is based on the work carried out by him at the Centre for DNA fingerprinting and Diagnostics, Hyderabad. This work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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Acknowledgments

This thesis is the compilation of results of the work that has been accomplished in the last five and half years, whereby and before that as well, I have been accompanied and supported by many people. It is the most pleasant opportunity to extend my sincere gratitude to all of them.

First and foremost I would like to express my heartfelt gratitude to my supervisor Dr. Sanjeev Khosla who taught me all the good science I know. He has been a guide, a teacher, my mentor, an advisor and a friend as well. I thank him for his immense support, outstanding guidance and encouragement during my stay here. Words fail to express my deepest regards towards him. I am indeed fortunate to learn science under his outstanding guidance and thank him for personally teaching me the experiments, for helping me in making phrases from the words and the most important thing he has taught me is to be patient and analytical. The freedom of thought, amiable environment and motivation extended by him was of immense help which provided me confidence in analyzing my research problems. Dr. Sanjeev has been a wonderful person, I feel privileged to be associated with him and will always be in indebt to him for the wonderful time in and outside the lab and for his infinite patience with a difficult student like me. Last but the least, I would like to thank him for conducting the most interactive course work classes. It was an amazing experience and completely changed the concept of a class for me.

I am grateful to the former director, Centre for DNA fingerprinting and Diagnostics, Dr. Syed E. Hasnain for allowing me to work in this esteemed institute. I express my thanks to present director, CDFD, Dr. J. Gowrishankar for providing facilities and encouragement to carry out research work and also for various inputs and helpful discussions during 'Genetics Club' meetings. I would like to acknowledge the financial support provided by University Grant Commission for my Junior and Senior research fellowships and CDFD during the last year of my work. I would like to thank Dr. Shanker for kindly supporting my trips to attend and present posters in national and international conferences, especially 'Keystone symposia ' on epignetics held in Breckenridge, Colorado, USA.

I am thankful to the Vice Chancellor, Registrar, and the Head, Dept. of Biotechnology at Manipal University for permitting me to register as a PhD graduate student. I thank Dr. Rakesh Mishra for allowing me to conduct experiments in his lab.

I would like to acknowledge Dr. Hans Rotheneder for providing Sp1 protein expressing clone.

I express my appreciation to the CDFD family for providing scienctific envirionment. I thank Dr. Shekhar C Mande for his fantastic support. Dr. Shekhar has always been very supportive and I thank him for allowing me to use his laboratory facilities in an unrestrictive way. He has a special respectful place in my heart and I will always remember the time spend with him (specially the one on Manipal trip). I thank Drs Ranjen Sen, Gayatari Ramakrishna, Mahalingam and Manna for helpful discussions during various meetings and excellent teaching during course work. The work would have been incomplete without the help extended from all support staff from different sections like Bioinformatics, NGTF, Administaration, Accounts, EMPC, Transport and Canteen. I am thankful to my present and past colleagues including Yashin, Sreenu, Radha, Naresh, Abira, Rajendra, KV, Prachee, Santosh, Sailu, Vartul, Kshama and Abhijit. I would like to greatly acknowledge the big help provided by Nanci Shariff, Hasan and Gowresh. No experiment was feasible without their help.

Traditional wisdom conventionally held does not allow otherwise I would love to put the name of all my friends on the cover page of my thesis. I have been blessed with many friends who have extended their unconditional love and support during all the times. I would like to acknowledge all of them for their help.

I would like to thank Mr. Shailesh Gupta for introducing me to the subject of Biotechnology during my under graduation days. Many thanks to my M.Sc classmates Neeta, Savitri, Debarti, late Ashish, Sanjay, Dr. Vinod, Sandeep Singh, and Pallavi Gupta for making my life in Shimla the best part of my life till now. The patience they have shown towards my Violin skills and other eccentrics in the department deserves to be greatly acknowledged. I thank Pallavi for encouraging me to study Molecular biology and for all the help during M.Sc and after that. My seniors Khalid, Mrigank, Irfan Ghazi, Aditya Basu, Alok has helped me a lot all throughout my M.SC especially during the initial days in Shimla. I thank my teachers Drs. T.C. Bhalla, S. S. Kanwar, Reena Gupta, Dunichand, Arvind Bhatt and Wamik Azmi for their wonderful teachings.

Many thanks are due to the present and past scholars of CDFD. I really enjoyed table tennis matches with Senthil, Sharmistha and Sailu, thanks to KV and Prachee for always offering a helping hand. I enjoyed a lot the lively company of Rohini and Anoop, my heartfelt thanks to Rohini for organizing all the funfilled festivals and

always lending an ear for my grievances. My batchmates Arvind, Akif, Anoop, Aisha, Archana, Prabhat, Vaibhav and late showkat has always been a source of inspiration. I shared a special relationship with Vaibhav, Anoop, Jayendra and Gokul. I thank them for allowing me to be the part of the home we made in Hyderabad and bearing my presence despite all my craziness, this alone deserves an acknowledgment. Anoop, Vaibhav Gokul and Rachana has always been a source of inspiration and inspired me to always better my performances.

I profoundly appreciate the camaraderie I shared with my labmates. My heartiest thanks to all the present and past members of the lab and various trainees who has helped me in my research. I thank Mallikarjuna for his help in Yeast one hybrid assay and the work regarding Phf7 protein and for all the deligent efforts he has put in while working with me during different points of time. I thank Devi for helping me in ChIP assays and critical analysis of my experiments. I greatly appreciate the vivacious and humorous company of Gokul and Tej, it's very difficult to imagine a day without them. I heartily thank Tej for helping me in learning English (listening it as well) and inspiring me for the game of badminton. I am really out of words to express my deepest sense of gratitude towards Gokul. I thank him for all the small things and the big ones. My profoundest thank to my most beautiful wife, Rachana for her care and love and especially her faith in me. I thank her from the core of my heart for bearing my long absences (sometimes my presence as well), her help during thesis writing needs to be especially acknowledged.

I thank the creators of Google, Microsoft word, Adobe, without which I can't think of writing nowadays and this thesis would not have been in this form without it.

I thank my family for always staying so close despite the distance. Finally but really before anyone else I thank my 'tauji' who was always there for me to support and encourage. Although he is no more in this world, but I always feel him close to my heart. This thesis is dedicated to him.

Sudhish

List of ABBREVATIONS

3-AT	3 amino -1,2,4-trizol
ATP	Adenosine tri Phospahte
APS	Ammonium per sulphate
bp	Basepair
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
C-terminus	Carboxy terminus
DTT	Di thioThreitol
DMR	Differentially methylated regions
Dnmt	DNA methyl Transferase
DMSO	Dimethyl Sulphoxide
DNA	Deoxy ribonucleic acid
dNTP	Deoxy Ribonucleotide Tri Phosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EtBr	Ethidium Bromide
E.Coli	*Escherichia Coli*
EMSA	Electrophoretic mobility shift assay
GST	Glutathione S transferase
HEPES	N-(2-Hydroxy ethyl) Peiperazine-N′-(2-ethanesulfonic acid)
IPTG	Isopropyl β-D thiogalactopyranoside
ICR	Imprinting control region
Kb	Kilo basepair
KDa	Kilo Dalton
KCl	Potassium Chloride

L	Litre
LB	Luria Broth
M	
min	Minute
M	Molar
MALDI-TOF	Matrix assisted laser desorption/ionization – Time of Flight
$MgCl_2$	Magnesium Chloride
ml	Millilitre
mM	Millimolar
N_2	Nitrogen
NaCl	Sodium Chloride
OD	Optical Density
OD_{600}	Optical Density at 600 nm
PAGE	Poly Acrylamide Gel Electrophoresis
PMSF	Phenyl methyl sulphonyl fluoride
PCR	Polymerase Chain Reaction
pmole	Picomole
PVDF	Polyvinylidene fluoride
PEG	Poly ethylene glycol
Peg	Paternally expressed gene
Meg	Maternally expressed gene
dCTP	Deoxy cytosine triphosphate
SDS	Sodium Dodecyl Sulphate
SSC	Sodium Saline Citrate
SSPE	Saline Sodium Phosphate –EDTA
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction

TBE	Tris-borate EDTA
TE	Tris-EDTA
Tm	melting temperature
Tris	Tris-hydroxymethyl amino methane
TEMED	N,N,N'N' Tetramethylethylenediamine
UV	Ultra Violet
ºC	degree centigrade
µg	microgram
µl	microlitre
µM	micromolar

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Synopsis

Genomic imprinting in mammals defines parent-of-origin-specific functional differences between two alleles of a gene. It has been described as a mode of transcriptional regulation in which the expression of a specific set of mammalian genes is dictated by epigenetic modifications and is believed to be established in the male or female germ line. Even two decades after the discovery of first imprinted gene, molecular mechanisms governing genomic imprinting are not completely understood. The regulation of genomic imprinting is complex with several layers of control involving multiple cis and trans acting elements. The presence of tandem repeats, differential methylation of CpG islands, boundary elements, nontranslated RNA and temporal differences in DNA replication have all been associated with imprinting (Delaval and Lewis 2004). Most of the molecular mechanisms put forth to elucidate the phenomenon of genomic imprinting by DNA methylation, chromatin organization or by non-coding RNA explain the silencing of one parental allele, but fail to describe the mechanisms that prevent the silencing of transcriptionally active allele.

This thesis aims to probe the mechanism and to identify the factors responsible for the imprinted status of *Neuronatin* gene in mouse. *Neuronatin*, a paternally expressed gene on chromosome 2 in mouse (chromosome 20 in human), is expressed in the central nervous system from midgestation through early postnatal development, correlating with the onset and termination of brain development in mice and humans (Joseph *et al* 1994 and Wijnholds *et al* 1995). It has also been shown to be expressed in all cells of the developing

pancreas in mice, becoming restricted to beta cells of pancreas in adulthood (Chu and Tsai 2005). The *Neuronatin* gene is located within the 8.5kb long first intron of the non-imprinted gene, Bladder Cancer Associated Protein (*Bc10/Blcap*) (John *et al* 2001) and unlike the imprinted genes in clusters, is the only imprinted gene within this locus. The small imprinted domain in this region indicates that the cis-acting sequences, important for maintaining the differential allelic expression of *Neuronatin* and for preventing the spread of imprinting to *Bc10/Blcap*, should be present within a relatively short distance of the *Neuronatin* gene.

The methylation studies of mouse *Neuronatin* domain showed the maternal allele to be methylated from its promoter to the last exon (Kagatini *et al* 1997, Kikyo *et al* 1997) while paternal allele remains unmethylated. Preliminary nuclease sensitivity assays done in the lab had identified differential chromatin organization on two alleles within the differentially methylated domain of *Neuronatin*. In other imprinted genes like *H19/Igf2* (Tremblay *et al* 1997, Thorvaldsen *et al* 1998, Hark *et al* 1998), *U2af1-rs1* (Shibata *et al* 1997, Feil *et al* 1997), *Snrpn/Snurf* (Schweizer *et al* 1999), *Kcnq1* (Yatshuki *et al* 2003), *Peg3* (Kim *et al* 2003) and *Gnas* (Coombes *et al* 2003) differential chromatin organisation within their differentially methylated regions have been correlated with their imprinted mechanism. The work presented in this thesis encompasses detailed biochemical characterization of the chromatin organisation at the *Neuronatin* locus in mice.

Chapter I reviews the various facets of genomic imprinting and the recent advancements in this field. Along with discussing the role of epigenetic modifications like DNA methylation and chromatin organisation in relation to the various mechanisms put forth to describe the phenomenon of genomic imprinting, the chapter also collates the various

features that have been described about imprinted genes. In addition, a table compiling all the known imprinted genes in mouse and human with information about their location, expressed allele, known differentially methylated domains, imprinting controlling regions and their function has also been provided. The chapter ends by discussing the imprinted mouse *Neuronatin* gene in context of the aim of this thesis.

Chapter II describes nuclease sensitivity analysis of chromatin organisation at the *Neuronatin* locus in mouse. Nuclease sensitivity assay performed to analyze the chromatin organisation within the *Neuronatin* locus demonstrated that the two parental alleles of *Neuronatin* are organised into different chromatin conformation within the known differentially methylated domain of *Neuronatin* gene. DNase I sensitivity assay revealed two DNase I hypersensitive sites present exclusively on the paternal unmethylated allele. One of the hypersentive sites (HS-I) was found to be independent of the transcription of *Neuronatin* gene and was mapped to the second intron of *Neuronatin* gene whereas the second hypersensitive site (HS-P) was mapped to its promoter region. Analysis of chromatin from various tissues showed the hypersensitive site HS-I to be transcription independent. It was also shown by MNase digestion that the paternal allele had phased nucleosomes in contrast to maternal allele on which nucleosomes were organised randomly.

Previous studies on the imprinted genes like *H19/Igf2, Peg3* and *Gnas* locus have shown the differential binding of proteins like CTCF (Bell *et al* 2000, Szabo *et al* 2004, Kim 2008) and YY1 (Kim 2008) to the two alleles within the region of differential chromatin organization. The differential chromatin organization, observed between the two alleles of *Neuronatin*, could be due to the exclusive binding of protein (s) to the paternal allele,

leading to non-nucleosomal chromatin organization and resulting in DNase I hypersensitive sites. Phylogenetic comparison of second intron of *Neuronatin* gene showed this region to be conserved among all the eutherian mammals analyzed. By Electrophoretic Mobility Shift Assay (EMSA) it was found that a 'GC' rich region within the second intron interacts with a protein or protein complex. The interaction of protein (s) with 'GC' DNA was found in all the tissues analyzed, suggesting the possibility that the interacting protein is present ubiquitously and its interaction with 'GC' in not related to the transcription of *Neuronatin* gene. Experiments including point mutations, methylation modifications and deletions of nucleotides done on the 'GC' fragment, identified a 25 bp region to be the minimum binding site. Using methylated 'GC' fragment as probe in EMSA, we also showed this binding to be methylation-restricted, with only unmethylated 'GC' showing protein binding.

The role of CTCF and YY1 (reviewed by Kim 2008) has been well documented in the mechanism of genomic imprinting at the *H19/Igf2, Peg3 and Gnas* loci. Bioinformatic scanning of *Neuronatin* gene and its flanking region did not reveal the presence of binding sites for these proteins, suggesting that some other factor or protein might be involved in the imprinting mechanism of *Neuronatin*. Chapter IV describes three different approaches to identify 'GC' binding protein (s). Yeast mono hybrid assay and MALDI-TOF analysis of DNA affinity purified nuclear proteins suggested a list of the probable proteins that can bind to 'GC' fragment and play a role in chromatin organization. In addition, as bioinformatic analysis of 'GC' fragment revealed the presence of Sp1 binding sites, the interaction of Sp1 protein with 'GC' DNA was analyzed by both EMSA and Chromatin

Immunoprecipitation (ChIP) analysis. The results indicated that Sp1 can bind with different affinities to the ‘GC’ and meth‘GC’ fragment.

In summary, in this thesis, analysis of chromatin organization revealed the presence of a ubiquitous, transcription-independent DNase I hypersensitive site HS-I, within the second intron of *Neuronatin* gene only on unmethylated paternal allele. The significance of this differential chromatin organization with the imprinted mouse *Neuronatin* gene in context of identification of non-histone protein binding within the second intron has been discussed in the final chapter.

Chapter I

Introduction: Imprinted genes in Mammals

Genomic imprinting refers to gamete-specific differential epigenetic modifications of certain genes leading to their expression in a parent-of-origin-specific manner. In diploid organisms, somatic cells possess two copies of an autosomal gene, one copy contributed by each parent. While most of the autosomal genes can be expressed from both the alleles, a subset of autosomal genes referred to as 'imprinted genes' show monoallelic expression. Imprinted genes are, therefore, functionally hemizygous and their mono-allelic expression is determined by which parent the expressing allele was inherited from (Kelsey 2007). Imprinted genes are known to play important roles in the development of embryo (Keifer 2007), development of different lineages (Mikkelsen *et al* 2007), growth (Charalambous *et al* 2007), development of brain (Wilkinson *et al* 2007) and behavior (Wood *et al* 2006, Keverne *et al* 2008). The importance of genomic imprinting can also be judged by several growth syndromes and diseases that the misregulation of imprinted genes causes in humans. In fact, the studies of human development diseases like Beckwith-Wiedemann syndrome, Prader-Willi and Angleman syndrome and Silver Russel syndrome caused by uniparental disomy for chromosome 11, 15 and 7 respectively, have enlightened researchers on various aspects of genomic imprinting (Ubeda *et al* 2008, Feinberg *et al* 2007).

Though genomic imprinting as a phenomenon has been known in insects since early 1960's through the work of H. V. Crouse (Crouse 1960, Rieffel *et al* 1966) in Sciara and in coccids insects through the work of S. W. Brown, W. A. Nelson-Rees, H. S. Chandra (Nelson-Rees 1961, Chandra *et al* 1967, Brown 1969, Chandra 1971, Brown *et al* 1973), it was discovered experimentally in mammals only in 1980's. It was a series of elegant and

novel experiments in the laboratories of Solter and Surani, (McGrath and Solter 1984, Surani *et al* 1984) that laid the foundation of what we know of genomic imprinting in mammals. Nuclear transplantation experiments performed in Solter and Surani laboratories (Mcgrath and Solter 1984, Surani *et al* 1984) showed that androgenetic (containing two male pronulei) and gynogenetic (containing two female nuclei) failed to develop normally and died very early during development. These experiments suggested that maternal and paternal contributions to the embryonic genome in mammals are not equivalent and that a diploid genome derived from only one of the parent is incapable of supporting complete embryogenesis. At around the same time Cattanach *et al* (1985) through experiments on the maternal duplication/paternal deficiency and its reciprocal for chromosome regions in mice reported that not all of the genome is involved in the parental effects. Mice with uniparental disomies (UPD; inheritance of both copies of a chromosome or a chromosomal region from one parent only) for a specific chromosome or subchromosomal region permitted the identification and localization of chromosomal regions subjected to genomic imprinting. It was only in 1990 that the first endogenous imprinted gene was identified (DeChiara *et al* 1991, Ferguson-Smith *et al* 1991). The first imprinted genes to be identified was Insulin growth factor 2 (*Igf2*) (DeChiara *et al* 1991) and *H19* (Bartolomei *et al* 1991) in mice. Since then, several different approaches have been used to identify imprinted genes including screening of cDNA libraries from parthenogenic and androgenic embryos (Mann *et al* 1995), genome-wide search for differentially methylated regions which are a landmark of imprinted genes (Strichman-Almashanu *et al* 2002) and analysis of genes in a chromosomal region associated with an imprinted phenotype (Ozcelik *et al*

1992). At present, nearly 100 imprinted genes have been identified in both human and mice (Beechey et al 2008, Morrison *et al* 2005).

The different facets of imprinted genes in mammals, which have emerged since their discovery in 1980s has been elucidated below:

I. Imprinted genes are organized in clusters:

Most of the imprinted genes are found in clusters and are regulated coordinately with other genes in the same cluster. The clustered organization of imprinted genes suggests a co-ordinate regulation of the imprinted genes in a chromosomal domain by sharing of cis regulatory elements suggesting that the mechanisms of genomic imprinting are not local or gene specific but can work over long distances (Edwards and Ferguson-Smith 2007). One of the largest clusters of autosomal imprinted genes is found at the distal end of mouse chromosome 7 and proximal end of human chromosome 11p15.5. The most studied example of this is *H19/Igf2* locus on chromosome 7 (Thorvaldsen *et al* 2006, Kaffer *et al* 2001). In fact, chromosome 7 of mouse has three clusters of imprinted genes: a) *H19/Igf2* locus containing *H19, Igf2, Ins2,* b) *Kcnq1* locus containing *Kcnq1 Osbp15, Tnfrsf23, Tnfrsf22, Tnfrsf26, Cars, Nap1l4, Phlda2, Slc22a18, Cdkn1c, Trpm5, Tssc4, Cd81, Ascl2,* c) *Snrpn*/*Snurf* locus containing *Snrpn/Snurf, Frat3, Magel2, Mkrn3, Ndn, Sno RNAs, Ube3a, Atp10a, Ube3a-as* (Table 1a). Other well studied and characterized clusters are *Gnas* locus on chromosome 2 containing imprinted genes *Nespas, Nesp, Gnasxl1, Gnas, Exon1a; Igf2*r locus on chromosome 17 containing *Igf2r, Slc22a1, Slc22a2, Slc22a3* and *Gtl2* locus on chromosome 12 containing *Gtl2, Dlk1, Rtl1, Mirg, Dio3* (Table 1a). The clusters of imprinted genes have almost the equal distribution of maternally and paternally expressed genes suggesting that genomic imprinting affect maternal and paternal genome

equally. The numbers of imprinted genes found in isolation are very less. Only nine genes out of 100 imprinted genes are present in isolation.

II. Epigenetic Regulation of imprinted genes:

The term 'Epigenetic' is used to describe all the modifications of the chromatin and DNA that form part of the transcriptional memory of a cell. The epigenetic information complements the genetic information to determine which genes are transcribed and at what level (Wu and Morris 2001, Jaenisch and Bird 2003). The importance of epigenetic events in gene regulation has gained prominence because even though epigenetic modifications can be stably transmitted through cell mitosis and meiosis, they are dynamic and reversible during development (Reik *et al* 2001, Ahmad and Henikoff 2002, Oxford *et al* 2008, Kiefer 2007) and cell differentiation (Thomas *et al* 2006). Epigenetic modifications have also been shown to play a crucial role in genomic imprinting (Lewis *et al* 2006, Kato *et al* 2005, Delaval and Reik 2004) and described below is the role played by various epigenetic modifications like DNA methylation, histone modifications and the interaction of non-histone proteins with DNA in regulation of imprinted genes (Wozniak *et al* 2008, Choi *et al* 2008).

DNA methylation:

Even before endogenous imprinted genes were identified in 1991, transgenic studies had suggested DNA methylation as a possible mark or imprint (Reik *et al* 1987, Sapienza *et al* 1987, Swain *et al* 1987, Hadchouel *et al* 1987, Sasaki *et al* 1990) by which two parental alleles could be distinguished. Over the years, the role of DNA methylation has been firmly established in regulation of imprinted genes and most imprinted genes have been shown to have allele specific DNA methylation. Table 1a, shows details of differentially

methylated regions (DMRs) for most imprinted genes and as can be seen the DMRs for most of the genes can stretch across the whole gene. In mammalian genomes, the CpG dinucleotide is greatly underrepresented due to the increased spontaneous deamination rate of 5-methylcytosine into thymine (Antequera *et al* 1993). Of the CpGs present, approximately 70% are methylated whereas the majority of unmethylated CpGs occur in small clusters known as CpG islands, which are ordinarily found within or near promoters or first exons of genes (Suzuki *et al* 2008). DNA methylation satisfies the criterion required for an imprint: it can be stably inherited and can be removed passively during replication (Tada *et al* 1997, Tada 1998, Ramchandani *et al* 1999). Active demethylation has been reported but has not been conclusively proved (Weiss *et al* 1996, Bhattacharya *et al* 1999, Zhu *et al* 2001, Jost *et al* 2001). Importantly, DNA methylation can silence genes (Macleod *et al* 1999, Esteller 2007).

Methylation of cytosines in mammals is achieved by a set of DNA methytransferases. The enzymes responsible for DNA methylation can be grouped as *de novo* methyl transferases as *Dnmt3a* and *Dnmt3b* which can act on unmethylated DNA and add methyl group to cytosine on both DNA strands in CpG context whereas maintenance methyl transferase as *Dnmt1*, *Dnmt1*o (oocytes specific isoform of *Dnmt1*) methylate hemimethylated DNA after each replication cycle. DNA methylation by *Dnmt1* plays a central role and is very critical in the mechanism of genomic imprinting (Li *et al* 1993).

DNA methylation can affect the genomic imprinting mechanism in following ways:

A) As DNA methylation on the promoter regions of a number of imprinted genes like *H19* and *Snrpn* directly interferes with the binding of transcription factors. It can indirectly

affect transcription by recruiting the methyl-binding protein MeCP2 and its associated histone deacetylases (Jones *et al* 1998b, Nan *et al* 1998).

B) As Boundary elements: A DNA element that prevents interaction between regulatory elements (for example between an enhancer and a promoter) is referred to as a boundary element. The observation that enhancers can be shared between the paternally expressed *Igf2* and the maternally expressed *H19* genes suggested the possibility that chromatin boundaries might be involved in the mechanism of genomic imprinting (Bell *et al* 2000). The region upstream of *H19* carries the paternal germline methylation imprint, which when deleted; leads to the expression of *Igf2* gene on the maternal allele (Thorvaldsen *et al* 1998). This led to the model that the *H19* DMR is a chromatin boundary that is 'closed' when unmethylated, and 'open' when methylated.

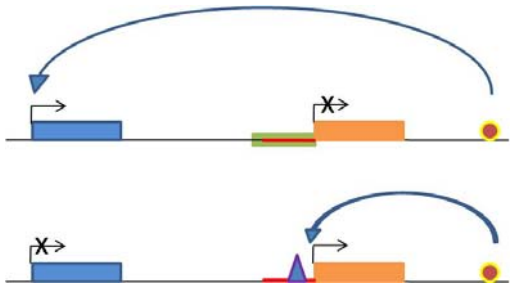


Figure 1A. Enhancer competition. The methylated gametic mark is indicated by green shadow box, an enhancer by a red circle and a boundary element by red line. Raised arrows depicts the transcription of the gene, blue arrows the interaction of enhancer, triangle represents the interacting transcription factor (Bell *et al* 2000, Reik and Walter 2003).

C) As Silencer: *Igf2* DMR1 is methylated on the active transcribed allele. This has led to the proposal that these sequences contain silencers that are inactivated by methylation, perhaps by excluding repressor factors (Sasaki *et al* 1992, Stoger *et al* 1993, Constância *et al* 2000). *Igf2* is paternally expressed in various fetal tissues and DMR1 functions as a maternal silencer in a subset of those tissues (Murell *et al* 2001). This is supported by the

knockout of DMR1 that caused the derepression of maternal allele of *Igf2* suggesting that DMR1 acts as a silencer (Constância *et al* 2000).

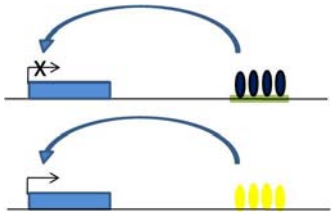


Figure 1B. Differential methylation results in differential binding of silencing factors. Blue box; gene, raised arrows; the transcription of the gene, green shadow box; CpG methylation; bend arrows shows the interaction of silencer with the gene (Constância *et al* 2000).

Chromatin organization and Histone Modifications

In the nucleus of eukaryotes, genomic DNA is tightly packed with histone proteins in a series of hierarchical compact structures to form chromatin. Chromatin represents a scaffold for many genetic events and shows varying degrees of condensation, including a relatively open form (euchromatin) and a highly condensed form (heterochromatin). It was initially thought that the role of chromatin was limited to compacting genomic DNA into the nucleus. However, over the last few years evidence has accumulated showing that the three dimensional chromatin structures at particular loci actively participates in regulating nuclear functions like transcription, DNA repair (Green and Almouzni 2002) and other nuclear organization functions. In the chromatin, genomic DNA is associated with histones to form nucleosomes. This structure can be inhibitory for the transcription process (Narlikar *et al* 2002). Activation or repression of a gene is usually associated with nucleosome reorganization at its promoter and the subsequent changes in chromatin structure makes the DNA accessible or inaccessible for the transcription factors. The change in nucleosomal organization is the effect of the interplay of many factors (Schulze

et al 2007). The network of interacting proteins is thought to control the degree of chromatin condensation or its organization for the availability or unavailability of DNA for genomic processes. DNA methylation, the interplay of DNA and histone protein modifications and the interaction of non-histone proteins; all are known to have their effect on the canonical nucleosomal structure of chromatin.

Chromatin organization is usually judged by assays that measure the accessibility of compacted DNA to nucleases. The transcriptionally active chromatin is less compact or more open (active or euchromatic state). On the contrary transcriptionally repressed chromatin tends to be more compact or closed (inactive or heterochromatic state). Additionally, regions hypersensitive to nuclease digestion have been found associated with regions in DNA that function in cis as regulatory regions (Elgin 1988). These nuclease hypersensitive sites (NHS) may reflect the disruption of chromatin at the nucleosome level with the possibility of DNA-non-histone proteins interaction (Hark *et al* 1998, Hark *et al* 2000, Kim *et al* 2003, Yaragatti *et al* 2008, Kim 2008).

Several studies document chromatin organization in relation to imprinted genes. In imprinted genes like *Nesp* (Coombes *et al* 2003)*, Igf2r* (Pauler *et al* 2005*), Snrpn/ Snurf (*Schweizer *et al* 1999*)*, *H19* (Bartolomei *et al* 1993, Hark *et al* 1998, Szabo P *et al* 1998 and Khosla *et al* 1999) and *U2af-rs1* (Shibata *et al* 1996, Feil *et al* 1997) have shown nuclease hypersensitive sites within imprinted loci. Interestingly, the presence of DNase I hypersensitive sites (DHS) was found in all the above mentioned cases exclusively on the unmethylated allele. DHS in most instances arise due to the interaction of non-histone proteins with DNA which results in the disruption of canonical nucleosomal structure (Gross and Garrard, 1988). Two non-histone proteins CTCF and YY1 are well known for

their role in genomic imprinting and have been found to be associated exclusively with unmethylated allele (Rituparna *et al* 2004) of the imprinted gene with the regions of allele-specific DHS in *H19/Igf2* (Bell *et al* 2000) and *Peg3* and *Gnas* (Reviewed by Kim 2008).

The dynamic nature of chromatin structure is made possible by modifications of N-termini of the various histones. Histone tails are the target of several posttranslational modifications including acetylation (Turner *et al* 2000), methylation (Rea *et al* 2000), phosphorylation (Clayton and Mahadevan 2003) and ubiquitination (Zhang *et al* 2003). Recent evidence suggests that these histone modifications may result in altered chromatin structure which, in turn, may lead to changes in the transcriptional status of a gene (Jenuwein and Allis 2001, Berger 2007). Histone modifications, either sequentially or in combination have been proposed to represent 'histone code' (Strahl and Allis 2000, Fischle *et al* 2003). The histone code can be recognized by specific chromatin associated factors adding another layer of regulation of gene expression. Recent reports have also linked differential histone modifications with genomic imprinting. The study of allele-specific histone lysine methylation marks in the regulatory regions at imprinted mouse genes (*Snrpn*, *U2af1-rs1* and *Igf2r*) has shown histone H3 on the active allele has lysine 4 methylation and is acetylated, while the inactive allele is marked by hypermethylation on lysine 9 of H3 (Fournier *et al* 2002). Analysis of *Snurf/Snrpn* 5' region revealed preferential association of hyperacetylated H3 and H4 and H3-K4 dimethylation with the paternally-inherited allele (Xin *et al* 2001). Conversely, on the maternally-inherited allele of *Snurf*/*Snrpn*, Histone 3 and Histone 4 were found to be hypoacetylated (Gregory *et al* 2001, Fulmer-Smentek and Francke 2001, Saitoh and Wada 2000) and H3 was found to be dimethylated at lysine 9 (Xin *et al* 2001). According to Xin *et al* methylation of H3 [H3-

K4-dimethylation (H3K4me2) and H3-K9-dimethylation (H3K4me2)] is restricted to a small well defined region flanking *Snurf/Snrpn* exon 1. However, similar studies in mouse, showed H3-K9 hypermethylation (combination of di- and tri- methylation) associated with a region further downstream in intron 1 in the maternally-inherited allele (Fournier *et al* 2002). Methylation of H3-K9 is also thought to be related to DNA methylation (Tamaru and Selker 2001). Accordingly, it has been shown in mouse ES cells that DNA methylation patterns in the PWS-IC maternally-inherited allele are dependent on the G9a histone H3 Lys9/Lys27 methyltransferase (Xin *et al* 2003). G9a -/- ES cells also showed the loss of *Snurf*/*Snrpn* mono-alleleic expression. The histone modification studies on *Kcnq1* gene locus comprising *Kcnq1ot1, Osbp15, Phlda2, Cdkn1c, Kcnq1, Cd81, Tssc4* and *Ascl2* have also shown allele-specific differential histone modifications in ES (Embryonic stem) cells (Lewis *et al* 2006) and the establishment of these marks during differentiation of extra-embryonic lineages. At the *KvDMR1* (overlapping the *Kcnq1ot1* promoter region), an enrichment of activating histone marks (acetylation and H3K4me2) on the paternal allele, and enrichment of repressive modification (H3K9me2) on the maternal allele in both cell types was found (Verona *et al* 2008). Imprinted genes *Phlda2* and *Cdkn1c* were found to be enriched for acetylation and H3K4me2 on the maternal chromosome and for H3K27me3 on the paternal chromosome both in TS (trophectoderm stem) and ES cells (Verona *et al* 2008). These findings of allele-specific histone modifications and the establishment of epigenetic mark suggests histone modifications, like DNA methylation is indeed a major player and determinant for the imprinting status of a gene.

III. Develpmental epigenetic reprogramming of imprinted genes:

As the imprints that distinguish the two alleles are parent-of-origin-specific, they need to be reestablished in each generation as the progeny of one generation would be the parent for the next generation. Therefore, three distinct phases of genomic imprinting has been envisaged. These are:

Establishment: In mammalian embryos there are two major cycles of epigenetic reprogramming of the genome during pre-implantation development and during germ cell development. Reprogramming in germ cells is necessary for resetting the imprints. The establishment of parent specific imprints is initiated as *de novo* methylation begins in both germ lines at late fetal stages, and continues after birth. Oocytes are in meiotic arrest and methylation occurs during their growth, whereas during spermatogenesis, methylation occurs before meiosis by *Dnmt1* (DNA methyltransferase 1) and its germ-cell specific isoforms, but it is also possible that *Dnmt3a* or *Dnmt3b*, which are required for *de novo* methylation in post-implantation embryo carry out this function in germ cells. The *Dnmt* involved in the acquisition of methylation imprints in the male germ line are currently unknown; however, *Dnmt3a* and *Dnmt3l* are postulated to be involved since male mice with targeted knockouts of the genes encoding these enzymes have abnormalities in spermatogenesis (Bourc'his *et al* 2001a, Hata *et al* 2002). The fact that methylation imprints are normal in mouse oocytes deficient in the only form of *Dnmt1*, i.e. *Dnmt1o*, that is present in growing oocytes, indicates that *Dnmt1* is not required for the acquisition of imprints in female germ cells (Howell *et al* 2001). In contrast, mouse knockout studies of *Dnmt3l* suggest a critical role for this protein in the acquisition of maternal methylation imprints in the oocytes (Bourc'his *et al* 2001a, Hata *et al* 2002). Interestingly, *Dnmt3l* does not share any of the conserved *Dnmt* catalytic motifs responsible for enzymatic activity.

The involvement of *Dnmt3a* and *Dnmt3b* in the acquisition of imprints is suggested by experiments done by Hata *et al* (2002). Other than methylation, other imprint marks are also known to play a role, as *BORIS* (Brother of the regulator of imprinting sites), a paralog of *CTCF* functions in testis to control parental specific expression (Loukinov *et al* 2002, Hong *et al* 2005). *BORIS* is present only in the testis and expressed in a mutually exclusive manner with *CTCF* during male germ cell development. The erasure of methylation marks during male germ-line development is associated with dramatic up-regulation of *BORIS* and down-regulation of *CTCF* expression (Vatolin *et al* 2005, Hong *et al* 2005). Because BORIS bears the same DNA-binding domain that CTCF employs for recognition of methylation marks in soma, BORIS is a candidate protein for the elusive epigenetic reprogramming factor acting in the male germ line.

Maintenance: Once the imprints in the form of CpG methylation and chromatin conformation are established in the germ cells, they are maintained thorugh DNA replication during growth and development. *Dnmt1o* is produced in mouse oocytes during their postnatal growth phase and is present throughout preimplantation development when genome epigenetic reprogramming occurs. Gene targeting experiments have shown that *Dnmt1o* is essential for the maintenance of methylation on imprinted genes at the 8-cell stage, the only time-point when this normally cytoplasmic isoform translocates to the nucleus; all embryos developing in the absence of *Dnmt1o* during the preimplantation period implant successfully but then die, prior to birth (Dean and Ferguson-Smith 2001, Howell *et al* 2001). The presence of *Dnmt1o* to faithfully propagate imprints at this stage of preimplantation development suggests that maintaining imprints is a crucial and highly regulated process. Although other *Dnmt*s are postulated to maintain gametic methylation

imprints at stages of preimplantation development other than the 8-cell stage, the *Dnmt1* helps in maintenance as it recognizes hemi-methylated CpG sites at replication foci and transfers methyl group to cytosine on the nascent DNA strand to replicate the methylation pattern of parental DNA strand (Yoder *et al* 1997, 1998, Hirasawa *et al* 2008). A combination of DNA methylation, modified histones (Carr *et al* 2007, Yamasaki-Ishizaki *et al* 2007), histone variants (Ooi *et al* 2007) and the various non-histone sequences specific interaction are required to maintain the imprints. Non-coding RNA also plays a role in the maintenance; regulation and functioning of imprints in clustural or long distance imprint gene control (see below). As can be seen in figure 1C, where reprogramming of DNA methylation at imprinted loci has been represented as line diagram (figure. 1C), once the imprints are rearranged according to the sex of the parent, after fertilization the global demethylation and remethylation wave does not affect these imprints (Reik *et al* 2001, Yagamata 2008).

Erasure: Before new imprints which reflect the sex of the progeny are put on for next generation, the parent-specific imprints need to be removed. This process requires passage of genome through gametogenesis. The germ line has the role of resetting imprints (Reik and Walter 2001a, 2001b, Lucefero *et al* 2002). During erasure there is marked and apparently genome-wide demethylation on germ cells, which is completed by embryonic day 12-13 in mice for both the sexes (Lee *et al* 2002). Recent evidence from mouse studies indicates that erasure may take place over a very short time, in as little as 24 h, at about the time when the germ cells initially enter the gonad (Walsh and Bestor 1999, Reik *et al* 2001). This observation suggests an active erasure process, although the identity of the

enzymes or the molecular complex that is responsible for this demethylation is unknown (Hajkova *et al* 2002). All methylated imprints probably get erased at this stage.

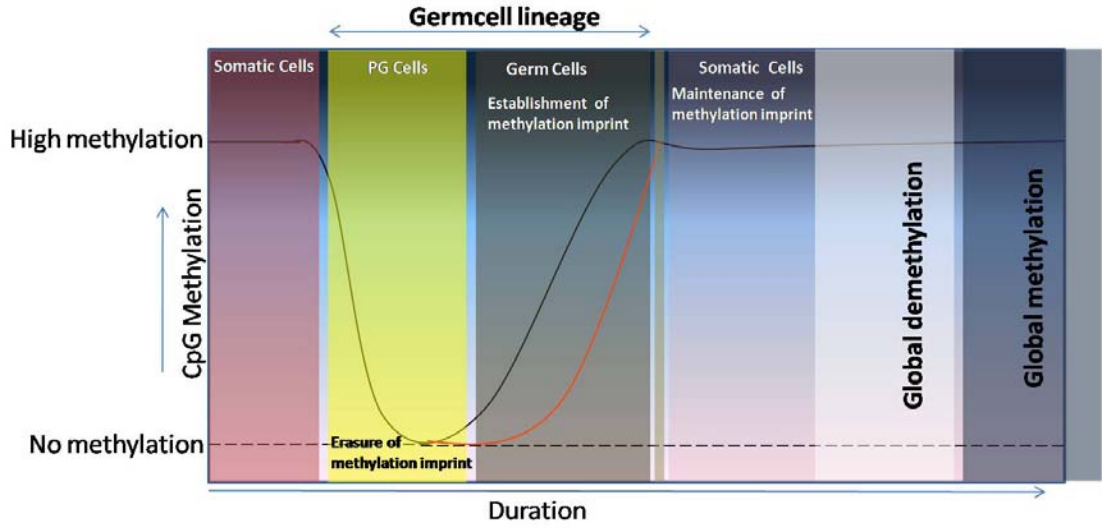


Figure 1C. Methylation reprogramming of imprinted genes in the germ line and adult organism**.** The methylation dynamics of maternally and paternally methylated imprinted genes are shown by the red and black lines respectively. The imprints are maintained in somatic cells. In primordial germ cells, the imprint in the form of methylation (represented by brown line) gets erased, while the unmethylated DNA remains unaffected (represented by dashed lines). During gametogenesis DNA methylation imprinted gets established on male (represented by black line) and female germ cells (red line). After fertilization the DNA methylation status of the maternal and paternal allele of imprinted genes does not get affected by the global wave of DNA demethylation and methylation (Reik and Walter 2001).

IV. Imprinting control region:

Imprinting control regions (ICRs) or imprinting centres (ICs) are domains within imprinted loci that are essential for establishing and maintaining the imprinted status of genes within the locus (Delavel and Feil 2004, Lewis and Reik 2006) and have been identified for several imprinted loci like *Igf2/H19*, *Snrpn*, the *Gnas* cluster and the *Kcnq1* locus, by genetic studies (Sutcliffe *et al* 1994, Thorvaldsen *et al* 1998, Fitzpatrick *et al* 2002, Williamson *et al* 2006). Each imprinting cluster is thought to be under the control of a single major cis-acting element, the imprinting control region (ICR), though other elements

may modulate the function of the ICR itself and of individual gene expression. ICRs acquire differential methylation between the two copies during germ cell development and can be classified as: ICR that are methylated during oogenesis on the maternally inherited chromosome, and ICR that are methylated during spermatogenesis upon paternal inheritance (Edwards and Ferguson-Smith 2007). Discussed below are few examples of Imprinting control regions controlled by a maternally inherited methylation mark. One of the well characterized imprinted domains is the *Igf2*r/*Air* region which contains three maternally expressed imprinted genes - *Igf2*r, *Slc22a2* and *Slc22a3* and the paternally expressed non-coding RNA, *Air* (Zwart *et al* 2001). The ICR for this cluster (intron 2 of *Igf2*r) is methylated on the maternal allele and contains the *Air* promoter. When the *Air* transcript is truncated, the expression of all the imprinted genes of the cluster becomes biallelic suggesting this region to be the controlling region for this cluster (Sleutels *et al* 2002). The *Kcnq1* imprinted gene cluster has a number of maternally expressed genes and one paternally expressed *Kcnq1ot1*, which is antisense to *Kcnq1* (Smilinich *et al* 1999). Truncation of the *Kcnq1ot1* transcript perturbs imprinting of all the genes (*Kcnq1*, *Osbp15, Tnfrsf23, Tnfrsf22, Tnfrsf26, Cars, Nap1l4, Phlda2, Slc22a18, Cdkn1c, Trpm5, Tssc4, Cd81, and Ascl2*) in the domain (Mancini-Dinardo *et al* 2006). Similarly, in *Gnas* locus the methylation of the promoter region of *Nesp-as* controls the expression of *Nesp, GnasXl, Exon1A, and Gnas* (Williamson *et al* 2006). Three paternally methylated germline-derived DMRs have also been identified. These are the *Igf2*/*H19* ICR (Thorvaldsen *et al* 1998), the *Dlk1-Dio3* ICR (Lin *et al* 2003), and the ICR regulating *RasGrf1* (Yoon *et al* 2005). Expression of *Igf2* and *H19* is dependent on shared endodermal and mesodermal enhancers that are located 3' to the *H19* gene (Leighton *et al* 1995). There are various DMRs in this

locus (see table 1a) but the DMR located 2kb upstream of *H19* acts as ICR and paternal inheritance of a *H19*-DMD deletion leads to the up-regulation of the normally repressed *H19* gene and a reduction in *Igf2* transcription on the paternal chromosome. The reciprocal effect is seen upon maternal transmission (Thorvaldsen *et al* 1998).

V. Non- coding RNAs:

Another interesting fact about the imprinted genes is that several of them codes for non-coding RNA. The imprinted locus to provide the first example of a spliced, poly-adenylated non-coding RNA was *H19* (Bartolomie *et al* 1991). This locus features reciprocal imprinted expression of *H19* (non-coding RNA) and *Igf2* (protein coding) genes. Non-coding RNAs lacks an open reading frame and often are transcribed antisense to bona fide protein coding genes. In mammals, these types of transcripts are highly coincident with allele-specific silencing of imprinted genes and have a proven role in dosage compensation via X-inactivation (reviewed by Huynh and Leev 2005, Pauler *et al* 2007, Wutz 2007). Almost every imprinted genes cluster has several non-coding RNA genes. The most studied of these non-coding RNAs are *H19* (Brannan *et al* 1990), *Air* (Oka *et al* 1985, Sleutels *et al* 2002), *Gnas* (Li *et al* 2000, Wroe *et al* 2000), and *Kcnq1*ot1 (Smilinich *et al* 1999). Recently several miRNAs like *anti-Rtl1*, *Rtl1-AS, miR-380,miR-376b, miR-376, miR-134, miR-154, miR-410, miR-431, miR-433, miR-127, miR-434, miR-432, miR-136, miR-370*, and snoRNA, coded by (*MbII-78*) *Meg*8 (*MbII-19*) (*MbII-48*) (*MbII-49*) (*MbII-426*) (*MbII-343*) (*Mirg*) have been shown to be imprinted (reviewed by O'Neill, 2005). Though, genes like *H19, Igf2r, Kcnq1ot1, Nespas, Gtl2, Snrpn/Snurf* codes for RNA, which is not translated they play very crucial role in the expression of other imprinted genes in the imprinted cluster. The function of *H19* RNA is still not clear but

maternally expressed non-coding RNA of *Gtl2* silences genes like *Dio3, MiRNAs, SnoRNAs*, which are found in its vicinity (Da-Rocha *et al* 2007). Paternally expressed non-coding RNAs like *Air* silences the genes as *Igf2r, Slc22a2* and *Slc22a3* (Zwart *et al* 2001), non-coding RNA from *Kcnq10t1* silences *Osbp15, Tnfrs23, Nap14, PhlDa2, Slc22a18, TSSC4, Cd81, Ascl2* and *Kcnq* (Smilinich *et al* 1999). Non-coding RNA product from *Ube3a-as* targets *SnoRNAs* and *Ube3a* on *Snrpn*/*Snurf* locus (landers *et al* 2004, 2005) and RNA from *Nesp-as* silences the product and controls the expression of *Gnas, GnasXl1, Exon1a* and *Nesp* (Wroe *et al* 2000). There are two mechanisms proposed for the imprinted genes regulation by non-coding RNAs. Firstly, transcription of the anti-sense RNA could directly interfere with the transcription of the sense promoter; secondly, non-coding RNAs can degrade the sense transcript by anit-sense RNA mechanism (reviewed by O'Neill 2005).

VI. Genomic Imprinting and Evolution:

As a result of the mono-alleleic expression of imprinted genes, these genes are in functional haploidy. The functional haploidy could have a detrimental effect on the fitness and well being of a species/organism in evolutionary terms. Why has then genomic imprinting evolved as a means of transcription regulation? What benefit do imprinted genes provide to affect the harm that their presence causes to the organisms? Based on the function of parent-specific gene expression, several schools of thought have emerged to answer the evolution of genomic imprinting (Wilkins and Haig, 2003).

A) Evolvability theory Beaudet and Jiang in 2002 suggested that functional haploidy (imprinting) evolved to confer increased evolvability on a population as the silent alleles at a locus can gather many mutations to increase the rate of adaptive evolution and is shielded

from temporary deleterious effects of the fluctuating environment. This model allows for a rapid and reversible adaptability to fluctuating conditions and thus a selective advantage, but this theory does not explain origin, benefits and patchy phylogenetic distribution of imprinted genes.

B) The ovarian time bomb theory (OTB) This model (Varmuza and Mann, 1994) proposes that imprinting arose as a way to protect females from invasive trophoblast disease. OTB theory explains about the inactivation of genes that are responsible for trophoblast development in the oocytes providing an explanation for the silencing of maternally derived allele coding for enhancers of trophoblast growth but OTB is silent about the imprinting of genes in marsupials and the silencing of growth enhancers in female germ lines.

C) The parent-of-spring conflict Model or the kinship theory of genomic imprinting: This model by Haig and Westoby (1989) proposed a conflict between maternal and paternal interests with respect to the genes that affect the resources that the offspring acquire from the mother. This model predicts that paternally expressed genes will promote growth and maternal genes will inhibit growth. Kinship theory claims that fitness effects on asymmetric kin have been the principal factors responsible for the evolution and maintenance of parent-specific expression at imprinted loci. The Kinship theory has been supported by the known physiological function of many of the known imprinting genes.

D) Evolution of genomic imprinting on the basis of gene duplication (Walter and Paulsen 2003) deals with mechanistic aspects and with the molecular basis for imprinting. It suggests that like host defense mechanisms, repeated sequences and retrotransposons might be marked and silenced by allele-specific epigenetic modifications (Neumann,

Kubicka and Barlow 1995). Imprinted retrotransposon like genes: *Peg10*/*Rtl*, *Nap1l5*/*Nap1l4*, *Mkrn3*/*Mkrn1* and *Mas1*/*Mrgg*/*Mrge* support this theory. The evolution of genomic imprinting by gene duplication can be summarized as a process of duplication and translocation events of imprinted domains, hence, suggesting evolution of imprinting as an on-going process in mammals (Toyco and Morisson 2005).

VII. Genomic imprinting and diseases:

Equivalent to the uniparental disomic mice, the mainstay of experiments done by Cattanach *et al* (1985 and 1986) are the sporadic or familial cases of UPD human patients. Infact studies of these patients have tremendously enriched the knowledge of genomic imprinting. The best studied of these imprinting defects are Prader-Willi (PWS) and Angelman Syndrome (AS) and Beckwith-Weidemann syndrome (BWS).

Prader-Willi and Angelman Syndrome

Prader-Willi syndrome (PWS) and Angelman Syndrome (AS) are neurogenetic syndromes caused by chromosome 15q13 UPD. Angelman syndrome is caused by a loss of function of the maternal allele or duplication of the paternal allele within a region that spans *UBE3A* on chromosome 15q13. AS is characterized by ataxia, hypotonia, severe mental and motor retardation, epilepsy and absence of speech (Horsler *et al* 2006). The methylation defect associated with AS involves loss of methylation within the *SNRPN* imprinting centre (IC) (Shemer *et al* 2000, Runte *et al* 2001). Prader-Willi syndrome is associated with a loss of function of the paternal allele or maternal duplication at the *SNRPN* locus (Shemer *et al* 2000). Patients with PWS are generally obese, mentally retarded, of short stature, suffer from muscular hypotonia, hypogonadotropic hypogonadism and have characteristic reduced fetal activity in the womb (Cassidy 1997). In a subset of PWS patients, a

methylation defect within the *SNRPN* imprinting centre has been described (Horsthemke *et al* 2006, Runte *et al* 2001). This defect results in a gain of methylation at the IC resulting in the paternal allele assuming a maternal (methylated) profile and loss of function of the paternally expressed genes within this region (*ZNF127, NDN, MAGEL2, SNRPN and IPW)*

Beckwith-Wiedemann syndrome (BWS)

Beckwith-Wiedemann syndrome (BWS) maps to 11p15 and is characterized by general overgrowth with symptoms including hemihypertrophy, macroglossia, and visceromegaly (Viljoen *et al* 1992). This disorder is linked to a loss of function of the maternal allele at 11p15, where the imprinting cluster that includes *H19, IGF2, CDKN1C, KCNQ1* and *KCNQ1OT1* resides. Children with BWS are predisposed to developing embryonic and childhood cancers. The most common molecular event occurring in BWS patients that do not have cytogenetic abnormalities is the biallelic expression of *IGF2* due to loss of imprinting (LOI) (Weksberg *et al* 1993). Two methylation defects have been described in BWS patients. BWS imprinting centre 1 (BWSIC1) defect results in a gain of methylation within the *H19* DMD on the maternal allele such that it assumes a paternal (methylated) profile. As a result the normally expressed *H19* maternal allele is silenced and *IGF2* is biallelicly expressed. The second methylation defect occurs at BWSIC2 and involves loss of maternal methylation at *KCNQ1*OT1, the *KCNQ1* antisense transcript. Expression of *KCNQ1*OT1 becomes biallelic while both *CDKN1C* and *KCNQ1* are silenced. Five to 10% of sporadic BWS cases have a BWSIC1 defect, while 40% manifest a BWSIC2 defect (Maher and Reik 2000).

Silver-Russell syndrome

Silver-Russell syndrome or Silver-Russell dwarfism is a disorder characterized by low birth weight, dwarfism and lateral asymmetry and has been linked to the loss of function of gene(s) on chromosome 7 (Preece 2002). About 7-10% of patients with Silver-Russell syndrome show maternal uniparental disomy for a region on chromosome 7, while patients with paternal uniparental disomy of the same region are unaffected; these findings implicate an imprinted gene(s) in the aetiology of the disease in a subset of patients. Although *PEG1* was proposed to be a likely candidate, the gene affected in this disease remains unclear (Nishita *et al* 1996, Riesewijk *et al* 1998).

Cancer

Apart from uniparental disomies, imprinted genes have been implicated in the ontogeny of other diseases like cancer, neuronal and behavioural disorders (see table 1a.) Involvement of imprinted genes in cancer results from either the activation of imprinted genes on the normally silenced allele (loss of imprinting or 'LOI') or the loss of expression from the normally active allele (loss of heterozygosity, 'LOH') by UPDs. *IGF2* is involved in the progression of certain cancers (Cui 2007, Tycko and Morison 2002). *H19* is known to be associated with tumors as Wilm's, Bladder, cervical, Choriocarcinoma, esophageal, Hepatocellular, lung, meduloblastoma and testicular germ cells (Falls 1999). Several researchers have indicated a correlation of loss of imprinting with cancer in hepatoblastoma, neuroblastoma, sporadic osteosarcoma, rhabdomyosarcoma and choriocarcinoma (Farrell 2005, Robertson 2005, Feinberg *et al* 2006 and Weidman *et al* 2007).

Neurological Disorders

Many imprinted genes are expressed in central nervous system (Davies *et al* 2005). Studies of *Peg1, Peg3, Ube3a, Grf1* and *Gabrb3* knockout mice, as well as mice carrying a uniparental disomy at chromosome 2, suggested a functional role of imprinted genes in cognition and behavior. Mouse knockouts of some imprinted genes show significant neurological defects ranging from abnormal maternal behavior *(Peg3* and *Peg1*) and impaired memory (*Grf1* and Gabrb3) to motor dysfunction with seizures (*Ube3a*) (Devies *et al* 2008, Ubeda *et al* 2008). In addition, several of the human imprinting diseases show neuro-developmental impairment. Several other neurological disorders also appear to be inherited in a parent-of-origin-dependent manner. Some examples include bipolar affective disorder (paternal chromosome 18), autism (maternal chromosome 15 and paternal chromosome 7), epilepsy, schizophrenia (paternal Chromosome 22), Tourette syndrome (maternal location unknown), Turner's syndrome and late onset of Alzheimer's disease (reviewed by Isles and Wilkinson 2000, Nicholls 2000). Imprinted genes as *PEG1/MEST, PEG3, and PEG5/NNAT* are suggested to contribute to the ability of the female mice to nurture their pups. Non-functional paternal allele of *PEG1*/*Mest* and *PEG3*, in females results in apathy towards nest building, lack of retrieval, inefficient pub nursing (see table 1a).

VIII. Other important features

1. Most of the imprinted genes are unusually rich in CpG islands and around 88% of mouse imprinted genes have CpG islands, compared with the average figure of 47%. These CpG islands are commonly flanked with direct repeats. These repeats have been proposed to be involved in conferring or maintaining differential methylation (Neumann *et al* 1995).

2. Although some genes like *H19* and *Snrpn* are imprinted all the times and in all stages of development, there are genes like *Igf2* (paternally expressed in most except choroid plexus and leptomeninges of the brain), *Ins2* (imprinted only in extra-embryonic tissue but biallelically expressed in the islet cells of pancreas), *KvLQT1* (maternally expressed in most tissues but heart). There are other examples like *Igf2*r and *Mash2* genes which are initially expressed in a biallelic fashion in mouse embryo but in the later embryogenesis assume maternal-specific expression. Promoter specific expression has also been observed for *IGF2* in human as during development the gene is expressed from three separate promoters in a paternal specific manner, while in adult liver a distal promoter gets activated and the gene is biallelically expressed.

3. It has been noted for a few imprinted genes like *Igf2*, *Igf2*r, *H19* and *Snrpn* that the two parental alleles replicated asynchronously, with the paternal allele replicating early. Replication timing in the cell cycle is often correlated with the level of gene expression (Kitsberg *et al* 1993, Kagotani *et al* 2002), with early replication associated with gene activity. However in the case of imprinted genes this is not true as the inactive paternal allele of *H19* and *Igf2*r has been observed to replicate early (LaSalle and Lalande 1995). These findings suggest that asynchronous DNA replication might be a control mechanism to preserve the imprinted marks of source parent on the alleles of imprinted gene. Thus, preserving the parental mark of one allele and establishing it without being influenced or influencing the mark of other allele during the DNA replication.

Aim of the thesis

Neuronatin

Neuronatin was identified by Wijnholds et al (1995), in a subtractive hybridization screening for regulatory genes expressed in the hind brain. The expression of *Neuronatin* was reported in Rathke's pouch; the lamina terminalis; tissues (partially) derived from neural crest such as dorsal root ganglia, the cranial ganglia, and branchial arches; the somites; the splachnic mesoderm; the mesenchyma of the head and limb; and the floor of the foregut pocket and in dividing neuro epithelial cells. At later stages a very high levels of expression were observed in the retina and throughout the central nervous system, it was stronger in post mitotic than in mitotically active regions. It was suggested that *Neuronatin* may play an important role in signal transduction, cell to cell communication, cell adhesion processes in the maturation of the nervous system and in the development or the differentiation of the hind brain. On the molecular level *Neuronatin* (*Peg5/Nnat*) encodes a small putative transmembrane protein having basic hydrophilic carboxylic end.

Neuronatin was found to be imprinted in two separate studies (Kikyo *et al* 1997 and Kagitani *et al* 1997) using differential display and subtractive hybridization methods, respectively. Using chr.2 disomic mice, Kikyo *et al* (1997) also mapped the location of *Neuronatin* to a position proximal to the T2Wa breakpoint and outside the previously imprinted region on Chr.2 in mouse. *Neuronatin* is present on chromosome 20q11.2 in humans (Evans *et al* 2001) and has been found to be conserved in rat, mouse and human. While investigating the genomic organization of *Neuronatin* in mice, John *et al* (2001) observed that *Neuronatin* was present within the intron of another gene *Bc10/Blcap*. Interestingly, *Bc10/Blcap* was found to be expressed biallelically and hence was a non-imprinted gene (Figure 1D).

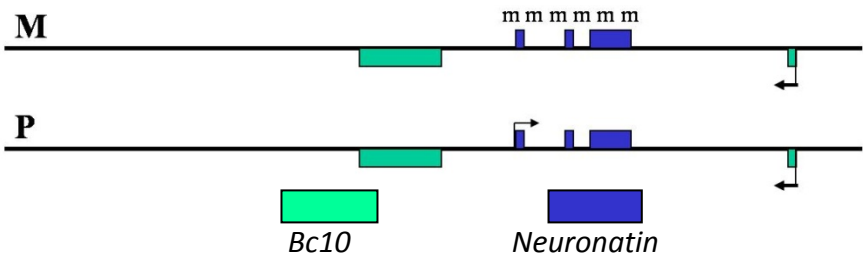


Figure 1D. Genomic organization of mouse *Neuronatin* gene. Raised arrows indicate the transcription direction of genes and boxes represents the exons of the genes. The space between the exons represents the intronic regions. *Neuronatin* has two introns while bc10 has one intron. 'M' - Maternal allele, 'P'- Paternal allele, 'm m m m'- DNA methylation.

Similar observations were made for human *Neuronatin* gene (Evans *et al* 2001). As a 30kb transgene containing the *Neuronatin* gene but not containing *Bc10* promoter and its first exon, was able to show imprinted expression of *Neuronatin*, it ruled out the possibility that *Bc10*, a non-coding RNA, and expressed antisense to *Neuronatin* had a role to play in the imprinting of *Neuronatin* (John *et al* 2001). Therefore, unlike most other imprinted loci as *Igf2*/*H19*, *Gtl2/Dlk*, *Igf2r* and *Snrpn* regions where the domain of imprinting is spread over hundreds of kilobases and affects several genes (Lewis and Reik 2006) the imprinted domain within the *Neuronatin* locus is small and may reside within the 8.5kb long intron of *Bc10/Blcap*. Only nine imprinted genes (including *Neuronatin*) out of around 100 known till date (Beechey *et al* 2005) have been found to be present outside a cluster and of these 9 genes 4 genes (*Arh1, Nap115, Tceb3c, U2af1-rs1*) were found within an intron of other protein encoding genes like *Neuronatin* (Morrison *et al* 2005). In the light of these interesting features of *Neuronatin*, it was decided to probe the imprinting mechanism for this gene in mice.

Kikyo *et al* (1997) and Kagatini *et al* (1997) showed that transcriptionally silent maternal allele of *Neuronatin* was methylated and the differentially methylated region (DMR)

within this locus extended from the promoter to the end of *Neuronatin* gene (John *et al* 2001). This is similar to other imprinted genes where the differentially methylated regions (DMRs) for the imprinted loci extent over several kilobases and can encompass the whole length of the gene (Thorvaldsen *et al* 1998, Yatshuki *et al* 2002, Williamson *et al* 2006). In contrast to DMRs, ICR is the region responsible for controlling the imprinting status of imprinted genes are limited only to a small region within their respective DMRs. Interestingly, these Imprinting control regions (ICRs) are the only regions within an imprinted loci to show differential chromatin organization (Feil *et al* 1999, Kato *et al* 2005), as judged by chromatin assays like DNaseI hypersensitivity and histone modifications (Lewis *et al* 2006, Delawal *et al* 2004). For example, in the *H19/Igf2* locus, the *H19* DMR extends from 4kb upstream of *H19* transcription start site (TSS) (see Table 1a) extends to the 3' end of *H19*. Similarly for *Igf2*, three large DMRs have been identified which span across the whole gene (Table 1a). However the differential chromatin region as judged by nuclease sensitivity for the *H19/Igf2* region is limited to only the 2kb ICR present -2kb to -4kb upstream of *H19* transcription start site (see Table 1a). Importantly for all imprinted loci where differential chromatin organization has been demonstrated, the DNaseI hypersensitive sites have been found only on the unmethylated allele (Hark *et al* 1998, Khosla *et al* 1999, Coombes *et al* 2003), suggesting a mutual exclusiveness between DNA methylation and nuclease hypersensitivity on the two alleles (Feil and Khosla 1999). This mutual exclusiveness either indicates a mechanism by which the unmethylated allele is protected from getting methylated or suggests the presence of an alternate imprint (DNA methylation).

The goal of this project was to determine the imprinting mechanism at the *Neuronatin* locus by examining the correlation of chromatin organization and imprinting. In this thesis we have defined chromatin organization of the *Neuronatin* locus and components that differentiate chromatin organization on the two alleles of the *Neuronatin* gene.

Chapter II

Chromatin organization within the imprinted *Neuronatin* locus

Introduction

The mouse *Neuronatin* gene present on distal part of chromosome 2 spans approximately to 2.5kb and contains three exons and two introns (Figure 1D). As mentioned in chapter I, this gene is expressed exclusively from the paternal allele, is methylated only on the maternal allele (Figure. 1D, and Kikyo *et al* 1997, John *et al* 2001) and present within the intron of *Bc10/Blcap*, which is approximately 10kb in length.

Analysis of chromatin organization within this locus was previously initiated to identify chromatin conformation unique within this genomic region. There are several ways in which one can probe chromatin organization. The most well established technique adopted for examining chromatin conformation is the use of nucleases like DNase I and Micrococcal Nuclease (Keene *et al* 1981, McGhee *et al* 1981, Elgin 1984). Sensitivity of chromatin to DNase I reflects chromatin accessibility or "openness". In the nucleus, the DNA is wrapped around histone proteins in repeating units of nucleosomes to form chromatin. DNase I can cut DNA that is organized into nucleosomes, only at 10bp intervals due to stearic hindrance that binding of histone creates. Therefore, chromatin regions, where local modifications to this chromatin structure displaces canonical nucleosomal structure, could allow for better accessibility and easier digestion by DNase I and such regions of preferential digestion by DNase I are referred to as DNase I hypersensitive site. Similarly, Micrococcal Nuclease, which can digest DNA that is organized into chromatin, only between two nucleosomes provides information about canonical and non-canonical organization of chromatin.

Preliminary DNase I sensitivity assays were carried out on *Neuronatin/Bc*10 locus using PatDp (dist2) and MatDp (dist2) mouse embryos to analyze chromatin organisation on the maternal and paternal alleles separately (Sowpati *et al* 2008). 19kb locus of *Neuronatin* was subdivided using *Bgl*II after DNase I digestion and the DNase I sensitivity within each *Bgl*II fragment was analyzed by indirect end-labelling using small 300-500 bp end probes. Difference in sensitivity to DNase I between the paternal and maternal alleles was observed only in the *Bgl*II fragment containing the *Neuronatin* gene (Figure. 2B).

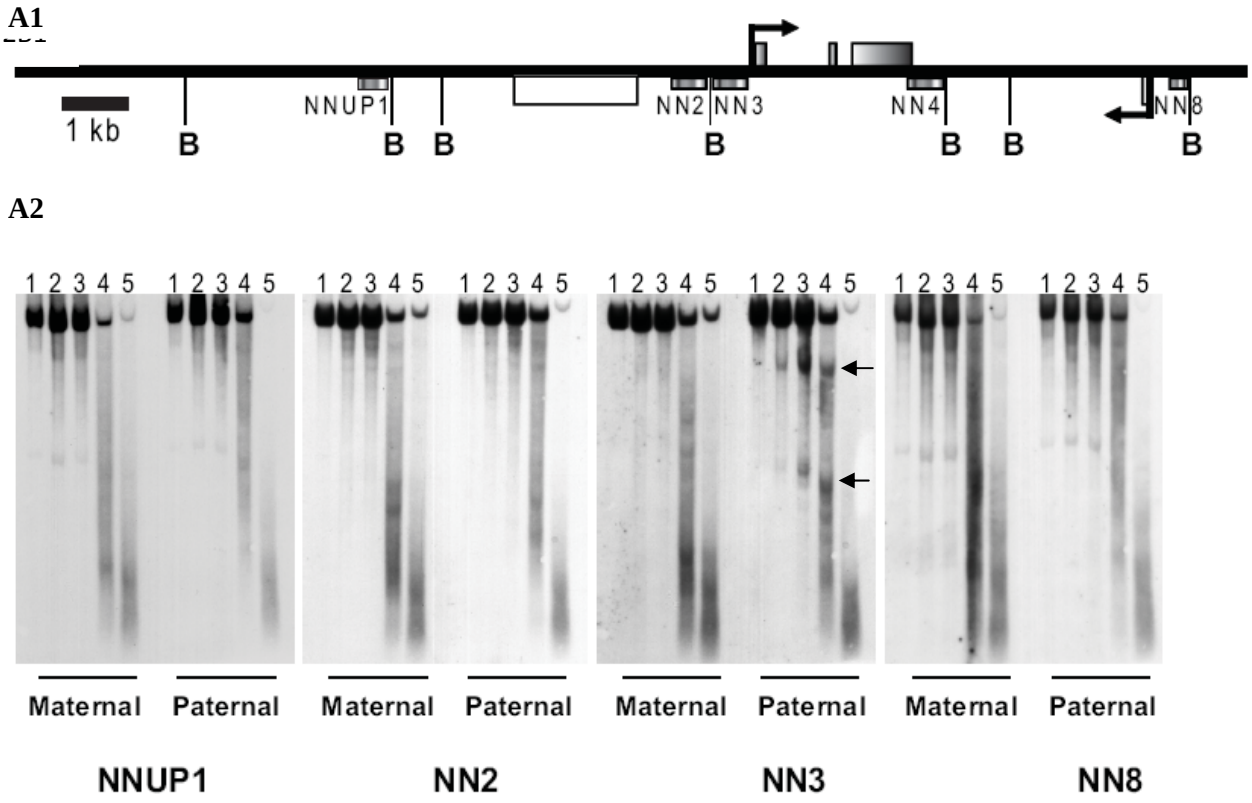


Figure 2A. Analysis of chromatin organization on maternal and paternal alleles in the *Neuronatin* locus. 2A1). Line diagram of mouse *Neuronatin* locus. The filled rectangles above the line shows three exons of the mouse *Neuronatin* gene while, the two exons of *Bc10* gene are shown below the line as open boxes, raised arrows shows the transcription of both genes. 'B' indicates *Bgl*II sites within the 19kb *Neuronatin* gene locus (GenBank Accession no. AF303656). Radiolabbled probes used to analyze the chromatin structure are shown as shaded boxes below the line abutting the ends of *Bgl*II fragments. 2A2). Nuclei were isolated from maternally and paternally disomic (for distal part of chromosome 2) mouse embryos (E14.5), treated with DNase I and further subdivided using *Bgl*II. DNA was electrophoresed on a 1.1 % agarose gel and southern blotted. Lane

1-5 corresponds to 0, 5, 10, 20, 40 units of DNase I/ ml treated nuclei. The blot was sequentially probed with the endprobes (abutting the *Bgl* II ends) indicated in the line diagram below the panel of autoradiograms. Maternal refers to nuclei MatDi(Chr.2) mouse embryos (E14.5) whereas paternal refers to nuclei from PatDi(Chr.2) mouse embryos (E14.5). Arrows represent the specific DNase I hypersensitive sites observed only on paternal allele of *Neuronatin* (Sowpati *et al* 2008).

Further examination of chromatin organization within *Neuronatin* gene using NN3 and NN4 end probes suggested the presence of two regions of DNase I hypersensitivity that are specific to the unmethylated paternal chromosome. The strong hypersensitive sites were mapped to a region within the promoter (HS-P) of *Neuronatin* and within the second intron (HS-I) of *Neuronatin* (Figure. 2B).

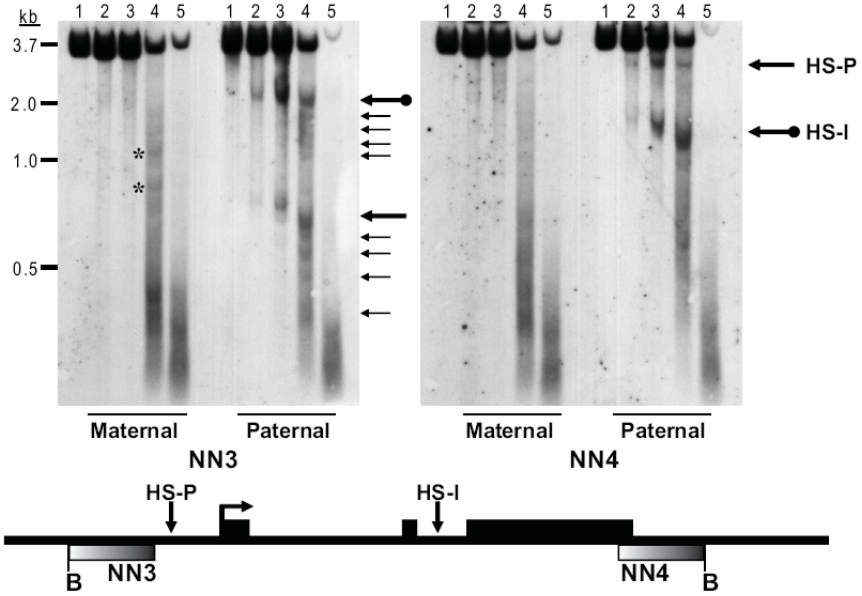


Figure 2B. Mapping of DNase I hypersensitive sites in *Neuronatin* gene. DNA was isolated from maternally and paternally disomic (for distal part of chromosome 2) mouse embryos (E14.5) after incubated with increasing concentration of DNase I and subdivided with *Bgl*II. DNA was southern blotted and probed with the end-probes NN3 and NN4 (the positions of the end probe are indicated in the line diagram below the autoradiograms. Lane 1-5 corresponds to 0, 5, 10, 20, 40 units of DNase I/ml Maternal refers to DNase I incubation of nuclei from chromosome 2 maternally mouse embryos (E14.5) whereas paternal refers to DNase I digestion of nuclei from paternally disomic mouse embryos (E14.5) for chromosome 2. Verticle arrows indicate the position of hypersensitive sites present on the paternal allele. '*' indicate minor hypersensitive sites present on the

maternal allele of *Neuronatin*. HS-P corresponds to DNase I hypersensitive site mapped to the promoter region and HS-I indicates the hypersensitive site mapped to within the second intron of *Neuronatin* (Sowpati *et al* 2008).

Of the two hypersensitive sites observed on paternal allele, one (HS-P) was mapped to promoter region of *Neuronatin,* and the other (HS-I) to its intronic region. Hypersensitive site HS-P can be related to the transcription status of the gene (Elgin 1988) but the role of HS-I needs to be established. In this chapter we report further characterization of nuclease sensitivity within the mouse *Neuronatin* locus.

Materials and Methods

Isolation of nuclei:

All the steps were carried out at 4^0C unless otherwise mentioned. The tissues were homogenized in buffer A [50mM Tris-Cl (pH 7.5), 15mM NaCl, 5mM $MgCl_2$, 25mM KCl, 0.34mM Sucrose, 0.5mM EDTA (pH 8.0)] with 1X protease inhibitor cocktail (Roche Applied Sciences, Cat# 1 836 170) in Dounce homogenizer (Sigma-Aldrich, Cat # T0691) till the suspension became homogenous (no visible clumps). The resulting suspension was filtered through 1mm strainer. Cells suspension was transferred to round bottom tubes and cells were collected through centrifugation at 1500g in swing bucket rotor. Pellet was washed once with buffer A. Cells were suspended in cell lysis buffer [50mM Tris-Cl (pH 7.5), 15mM NaCl, 5mM $MgCl_2$, 25mM KCl, 0.34mM Sucrose, 0.5mM EDTA (pH 8.0), 0.4% NP-40] and incubated for 5 minutes. The suspension was carefully overlaid on buffer B [50mM Tris-Cl (pH 7.5), 15mM NaCl, 5mM $MgCl_2$, 25mM KCl, 1.0M Sucrose, and 0.5mM EDTA (pH 8.0)] and centrifugation was carried out for 15 minutes at 4500g in a swing bucket rotor. Nuclei thus obtained were washed twice with DNase I Digestion buffer

[50mM Tris-Cl (pH 7.5), 15mM NaCl, 5mM $MgCl_2$, 25mM KCl], suspended to the final volume of 2ml in DNase I Digestion buffer and kept on ice till further use.

DNase I hypersensitivity assay:

400µl aliquots were taken from the 2ml nuclei suspension and treated with increasing concentration of DNase I (Roche Cat # 4536282). The increasing concentration of DNase I used for nuclei treatment are 0, 5, 10, 20, 40 units of DNase I/ml. DNase I digestion was carried out at 25^0C for 10 minutes. The reaction was stopped with the addition of 100µl of 5x stop solution (2.5% SDS, 10mM EDTA and 20µg proteinase K). Nuclease treated samples were incubated at 55^oC for 3 hours in stop solution and DNA was isolated using standard phenol/chloroform protocol. The DNA recovered after precipitation with ethanol was digested with *Bgl*II (NEB, Cat # R0144L) enzyme. To differentiate between the methylated (maternal) and unmethylated (paternal) allele, the DNase I treated and *Bgl*II digested DNA was redigested with DNA methylation sensitive enzyme *Hpa*II (NEB Cat # R0171L).

MNase hypersensitivity assay

The 2ml nuclei suspension was supplemented with $CaCl_2$ to the final concentration of 1mM. The suspension was incubated at 37^oC for 1 minute. 10 units of MNase (Roche Cat # 107921)) were added to the nuclei suspension and digestion was carried out for increasing periods of time. The reaction was stopped by withdrawing 400µl aliquots after 30, 60, 120 seconds and adding them to 100µl of stop solution. MNase treated samples were incubated at 55^oC for 3 hours in stop solution. DNA was isolated using standard phenol/chloroform/ethanol precipitation protocol and digested with *Bgl*II enzyme.

Southern blotting

DNA after nuclease and *Bgl*II digestion was electrophoresed on a 1.1 % agarose gel and southern blotted using alkaline transfer method (Lichtenstein *et al* 1990). Briefly, the agarose gel was incubated in 0.4N sodium hydroxide solution to denature the double-stranded DNA. Three sheets of 3mm Whatman paper wet with 0.4N NaOH were placed on the top of a stack of paper towels followed by wet (0.4N NaOH) Hybond N^+ membrane (GE Healthcare lifesciences Cat # RPN203B). Denatured agarose gel was placed on top of the membrane. Good and even contact between gel and membrane was ensured by removing bubbles with a glass rod and free ends were sealed with saran wrap to prevent short circuiting of buffer movement. The gel was further covered with sheets of 3mm Whatman paper. A wick of 3mm whatman paper was used to transfer buffer (0.4N NaOH) from the reservoir and transfer was done overnight. The membrane was neutralized by incubating with 2X SSC [Stock Solution 20X SSC (175.3g Sodium Chloride (3.0M) and 88.2g Sodium Citrate (0.3M) dissolved in 800ml water, pH adjusted to 7.0 with 10N NaOH. Volume was adjusted to 1000ml and sterilized by autoclaving.)] for 2 minutes and excess buffer was removed. DNA was covalently crosslinked to the nylon membrane by UV crosslinker (UVP Inc. Cat. # CL 1000 Ultraviolent Crosslinker).

Generation of radiolabel Probe:

For analysis of DNase I hypersensitive sites and nucleosomal positioning, ~ 500 base pairs end-probes were generated by PCR amplifications and radio-labelled by indirect end-labeling. NN3 and NN4 DNA (abutting the *Bgl* II ends) were generated by PCR amplification on mouse genomic DNA. NN3 DNA used as probe corresponding to

nucleotide 10927-11470 (GeneBank accession no. AF303656) was amplified using following primers set (Sigma Genosys or MWG biosciences):

NN3fr- 5'CGCTAACAGAGGACACAAGCTTTAC 3'

NN3bk- 5' ATTCTACATGCCGCGTAACCAGTGC 3'.

NN4 DNA corresponding to nucleotide 13981 to 14526 (GeneBank accession no. AF303656) was amplified using following primers set (Sigma Genosys or MWG biosciences):

NN4fr- 5'TCTTGCTTATCCCTGGTCTCACGC 3'

NN4bk- 5'TAGAGGGCCAGTCCCAGTTATTTTC 3'.

NN3 and NN4 DNA was amplified using the following PCR conditions: Initial Denaturation 95^0C for 5 minutes, denaturation at 95^0C for 45 seconds, annealing at 60^0C for 1 minute, elongation at 72^0C for 1 minute (30 cycles) and final elongation at 72^0C for 5 minutes. The DNA after PCR was electrophoresed on 1.5% agarose gel and DNA was eluted using Gel Elution Kit (Eppendorf Inc, Perfectprep™ Gel Cleanup, Cat. # 955152051). Amersham Megaprime™ DNA Labelling kit (Cat. # RPN 1607) was used for radiolabeling of DNA. 25ng of the PCR amplified DNA (NN3 or NN4) was used for labeling with αP^{32}dCTP. After labeling, the purification of labeled DNA from unincorporated labeled nucleotides was done using G-50 spin column (GE lifesciences Cat. # 27 5335 01). Probe was denatured at 95^0C for 5 minutes before hybridization.

Southern Hybridisation:

The blot was sequentially probed with the end-probes NN3 and NN4. The Hybond N^+ membrane membrane was transferred to hybridization bottle with 10ml of pre-hybridization buffer [6X SSPE (Stock solution 20X SSPE contain 175.32 g NaCl (3M),

24.00 g NaH_2PO_4 (0.2M), 7.44 g disodium EDTA $2H_2O$ (0.02M), dissolved in 1000ml of water, pH adjusted to 7.4 with 10N NaOH), 5X Dendhardt's Reagent (Stock solution 50X Dendhardt's solution contains 1% Ficoll (type 400), 1% polyvinylpyrrolidone, and 1% bovine serum albumin), 0.5%SDS, 1mg/ml sheared Salmon-sperm, 10% Dextran Sulphate]. Incubation was carried out at 65^0C for 2 hours in hybridization chamber. After 2 hours radio-labeled probe was added to the pre-hybridization buffer and incubation was carried out at 65^0C. The membrane was washed once with wash buffer 1 (1X SSPE, 0.5% SDS) at 65^0C for 15 minutes and twice with wash buffer 2 (0.5X SSPE, 0.5% SDS) at 37^0C for 15 minutes in hybridization chamber. The membrane was taken out of the hybridization bottle and excess buffer was removed. Wet membrane was wrapped in saran wrap and radioactive signals were read on Typhoon 9200 (GE Healthcare Lifesciences) or using X-Ray films (Kodak BioMax MS Film Cat. # 822 2648).

Results

Mapping of hypersensitive site HS-I

It was already established from previous DNase I sensitive assays that the hypersensitive site, HS-I was present within the *Neuronatin* gene. To further map the hypersensitive site HS-I within the *Neuronatin* locus, DNase I assay was performed on maternal and paternal Chr.2 disomic mice embryos. Nuclei were incubated with increasing concentrations of DNase I. DNA from DNase I treated samples was further digested with *Bgl*II and *Sac*I (figure. 2C1). The DNase I hypersensitive site with the *Bgl*II fragment containing *Neuronatin* gene was analyzed by southern blotting using NN4 probe (Figure 2C2).

C1

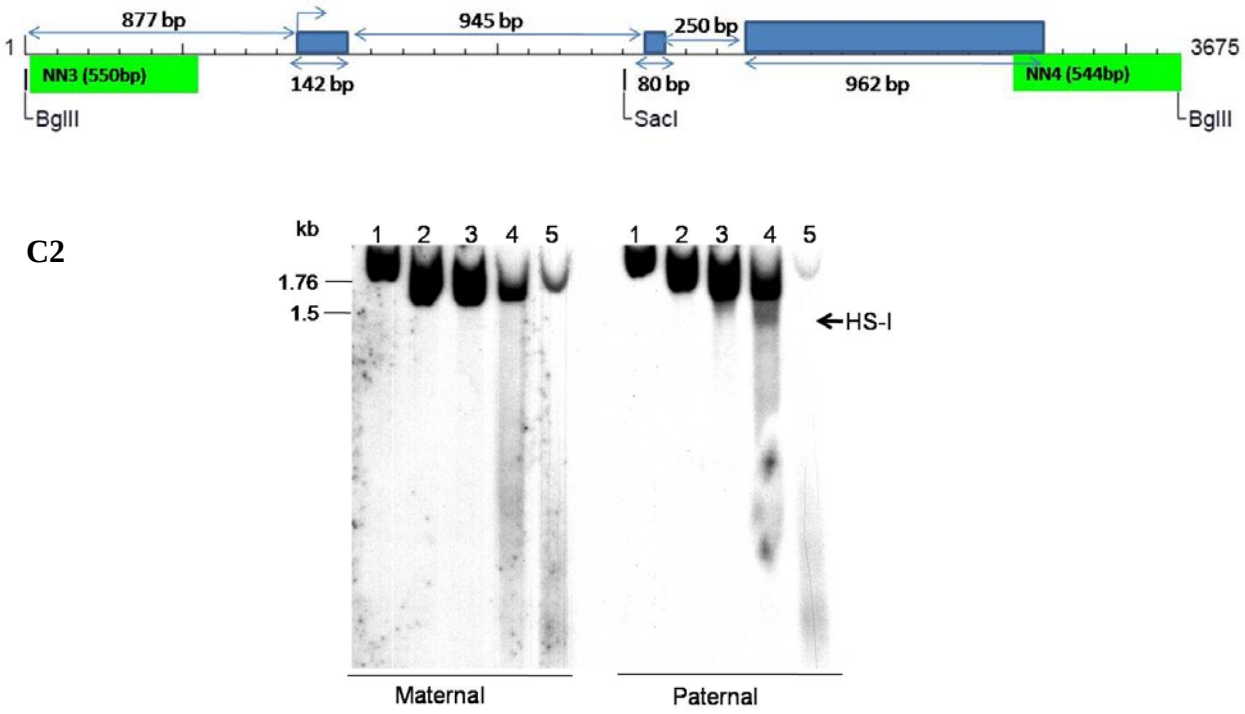


Figure 2C. Mapping of hypersensitive site HS-I. 2D1). Restriction map of mouse *Neuronatin* gene locus (GeneBank Accession no. AF303656). *Neuronatin* locus is shown as horizontal line. *Bgl*II and *Sac*II below the line shows the restriction sites of *Bgl*II and *Sac*II sites. Boxes above the line represent the exons of *Neuronatin* gene and boxes below the line represent the end probes NN3 and NN4. 2D2). Adult liver nuclei of wild type (MF1) mouse were incubated with increasing concentration of DNase I (Lane 1-5 corresponds to 0, 5, 10, 20, 40 units of DNase I/ml). DNA isolated from DNase I digests was re-digested with *Bgl*II and *Sac*I, southern blotted and probed with the end-probe NN4. Arrow indicates the position of hypersensitive site, HS-I indicates the hypersensitive site mapped to within the second intron of *Neuronatin.*

Hypersenstive site HS-I is independent of the transcription of *Neuronatin* gene

The analysis of chromatin organisation of *Neuronatin* gene using DNase I hypersensitive assay showed two hypersensitive sites, HS-I and HS-P (Figure. 2C) to be present exclusively on the unmethylated paternal allele. As DNase I hypersensitive sites are usually associated with the transcription of a gene (Elgin, 1988, Boyle *et al* 2008), and since the DNase I hypersensitive sites in *Neuronatin* gene were observed on the

transcriptionally active paternal allele, we wanted to test whether the hypersensitive sites correlated with transcription of the gene or are associated with imprinting of the gene as seen for other imprinted genes (Feil *et al* 1995, Khosla *et al* 1999, Schweizer *et al* 1999, Coombes *et al* 2003). To analyze the association of HS-P and HS-I with the transcription of *Neuronatin* gene, DNase I hypersensitive assay was performed on adult mice liver tissue, where *Neuronatin* is not expressed (Wijnholds *et al* 1995). DNA from DNase I treated chromatin was redigested *Bgl* II restriction enzyme, fractionated on 1.1% agarose gel and southern blotted. As can be seen in Figure 2D, hypersensitive site HS-I, which maps to the second intron of *Neuronatin* was present, while HS-P was absent. This result suggests that the paternal-allele-specific hypersensitive site HS-I is not related to the transcription of *Neuronatin* and could possibly be related to the epigenetic control of the imprinted gene *Neuronatin*.

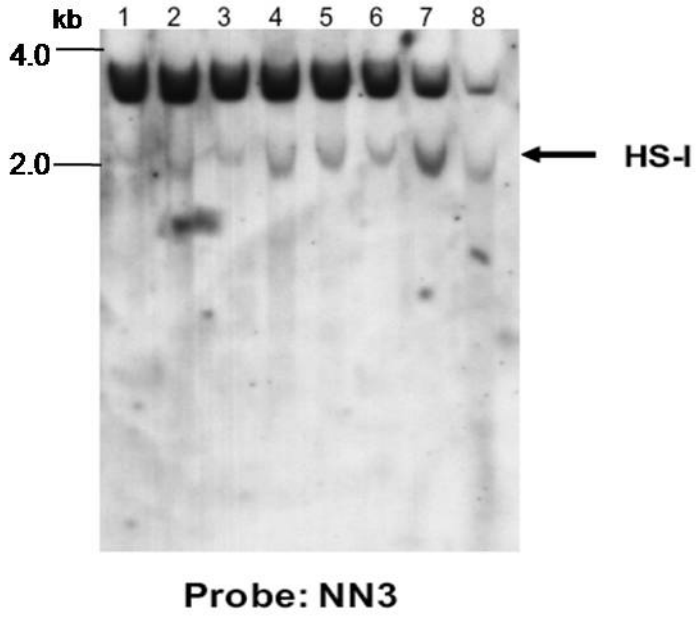
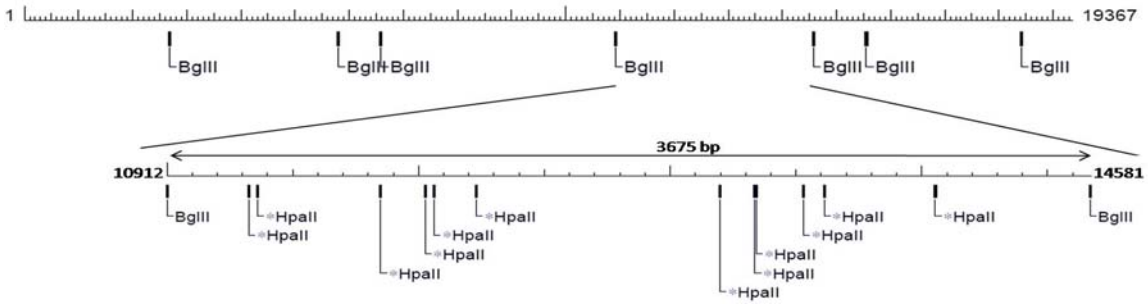


Figure 2D. Hypersensitive site HS-I is not correlated to the transcription status of *Neuronatin***.** Adult liver nuclei of wild type (MF1) mouse were incubated with increasing concentration of DNase I (Lane 1-8 corresponds to 0, 5, 10, 15, 20, 25, 30, 40 units of DNase I/ml). DNA isolated from DNase I digests was re-digested with *Bgl*II, southern

blotted and probed with the end-probe NN3 (see Fig. 2 for position of NN3). Arrow indicates the position of hypersensitive site. HS-I indicates the hypersensitive site mapped to within the second intron of *Neuronatin.*

To confirm the presence of HS-I on unmethylated paternal allele of *Neuronatin* and its transcription independence, DNase I assay coupled with methylation restriction analysis, was done on adult mouse liver tissue. The DNA from DNase I treated nuclei was either digested with *Bgl*II alone or *Bgl*II plus *Hpa*II. *Hpa*II digests unmethylated DNA and has 13 restriction sites within mouse *Neuronatin* gene (Figure. 2E1). Digested DNA was electrophoresed on 1.1% agarose gel and southern blotted. The analysis of chromatin organization (HS-I) was done using end probes, NN3 and NN4 (Figure.2D1). As observed in figure 2E2, HS-I was detected in *Bgl*II digested DNA but not in *Bgl*II plus *Hpa*II digested DNA, suggesting that the hypersensitive site is present only on unmethylated paternal allele.

E1



E2

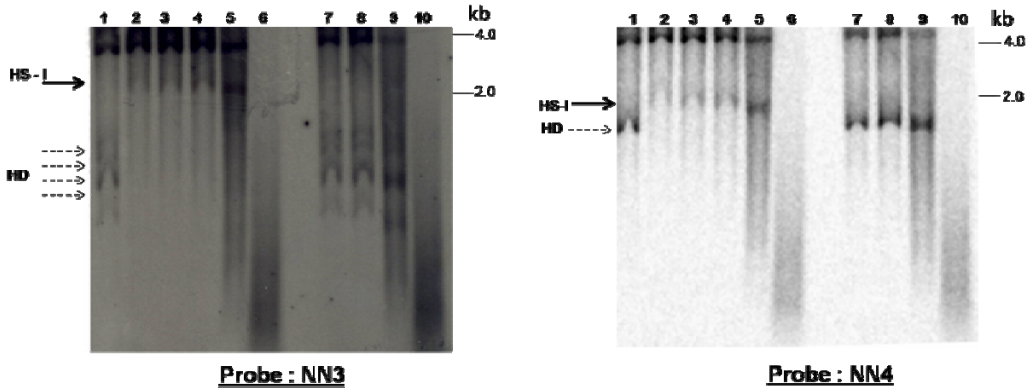


Figure 2E. HS-I is present on unmethylated paternal allele. E1). Restriction map of mouse *Neuronatin* gene locus (GeneBank Accession no. AF303656). *Neuronatin* locus is shown as horizontal line. *Bgl* II below the line shows the restriction sites of *Bgl*II. 3675 base pair region between two restriction sites of *Bgl* II (10912 to 14581) has been enlarged and *Hpa*II restriction sites has been shown. E2). DNase I hypersensitive assay performed on nuclei isolated from liver tissue of adult mouse. Tissue nuclei were incubated with the increasing concentration of DnaseI (0, 5, 10, 20 and 40 units of DNase I). DNA isolated from DNaseI digests was re-digested with *Bgl*II (lanes 2, 3, 4, 5, 6,) and *Bgl*II +*Hpa*II (lanes 1, 7, 8, 9, 10) respectively. HS-I indicates the presence of hypresensitive site in DNaseI treated and *Bgl*II re-digested DNA while no hypersensitive site was observed in DNaseI treated and *Bgl*II+*Hpa*II re-digested DNA. Bands denoted by HD represent *Bgl*II/*Hpa*II fragments.

To further establish the transcription independence of HS-I on the unmethylated paternal allele of *Neuronatin*, the same experiment was performed on nuclei from kidney (*Neuronatin* is not expressed), as can be seen from figure 2F, HS-I was present on the unmethylated allele.

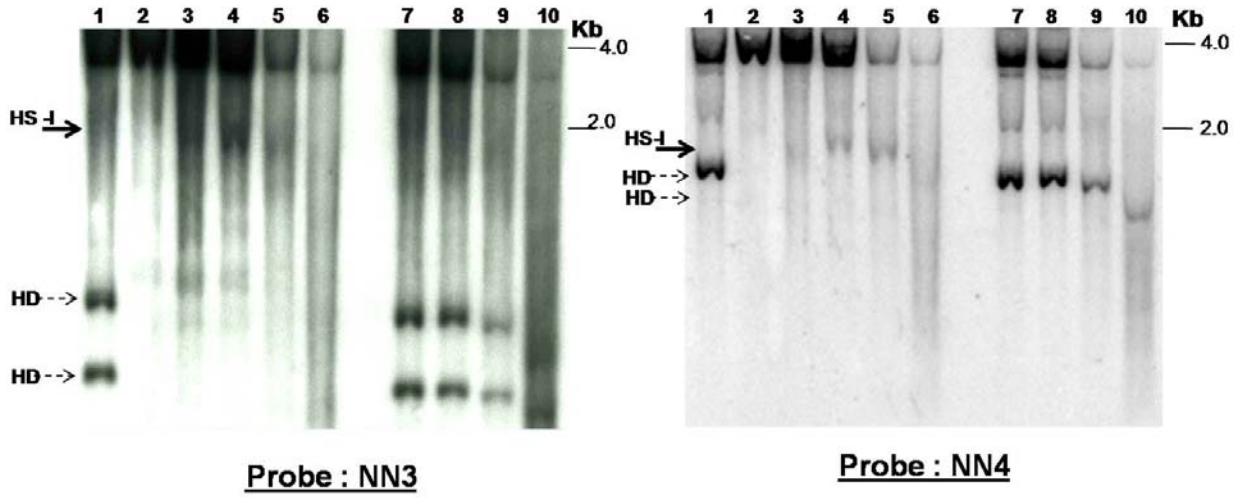


Figure 2F. DNase I hypersensitive assay performed on nuclei isolated from kidney of adult mouse. Tissue nuclei were incubated with the increasing concentration of DNaseI (0, 5, 10, 20 and 40 units of DNase I). DNA isolated from DNase I digests was re-digested with *Bgl*II (lanes 2, 3, 4, 5, 6,) or *Bgl*II +*Hpa*II (lanes 1, 7, 8, 9, 10) respectively. HS-I indicates the presence of hypresensitive site in DNaseI treated and *Bgl*II re-digested DNA while no hypersensitive site was observed in DNaseI treated and *Bgl*II+*Hpa*II re-digested DNA. Bands denoted by HD represent *Bgl*II/*Hpa*II fragments.

Analysis of nucleosomal conformations within the *Neuronatin* locus

Presence of hypersensitive site usually reflects associations of non-histone DNA binding protein(s) within the chromatin context disrupt the canonical arrangement of nucleosomes. As DNase I hypersensitive sites were observed within the *Neuronatin* gene, it was decided to analyze the nucleosomal organization within *Neuronatin* locus by MNase nuclease sensitivity assay. As mentioned earlier, MNase can digest DNA only in the inter-nucleosomal region, there by generating a ladder of bands corresponding to different number of nucleosomal units. Any disruption in nucleosomal organization would disrupt this ladder of bands. Initially, MNase digestion was performed on liver from mice disomic for chromosome 2 (Figure 2G). As was done for DNase I assay, the nucleosomal organisation within the *Neuronatin* gene was analysed using the end-probes NN3 and NN4. With the end-probe NN4, both alleles showed similar profiles for approximately 1000 bp (corresponding to DNA wound around approximately four to five nucleosomes)

from the 3' *Bgl*II end. However, in the region corresponding to the second intron (beyond 1000 bp from the 3' *Bgl* II end) the pattern of MNase digestion was very different on the two alleles. On the paternal unmethylated allele there were two prominent bands (indicated by thicker arrows). In contrast, the maternal profile appeared as a smear. Using the probe NN3, the difference between the two alleles was more discernible. The paternal allele, in addition to showing a prominent band (thick arrow) around the second intron also showed very regularly spaced nucleosomal ladder (thin arrows). However, the MNase profile for the maternal allele was smeary. There is a nucleosomal ladder but with a lot of background suggesting that the maternal allele is nucleosomally organised but the nucleosomes are randomly present. As the MNase digestion profile obtained is the sum total for several cells, the composite profile appears as a smear.

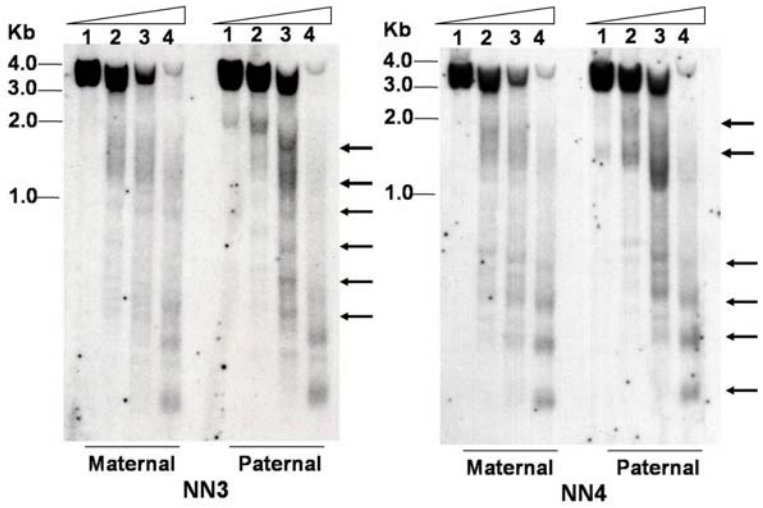
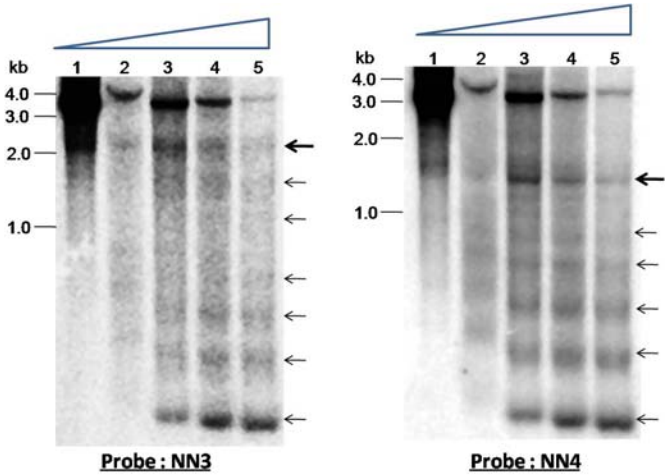


Figure 2G. MNase profile for *Neuronatin* in liver of Matdi2 and Patdi2 mice. Liver nuclei from paternally (Patdi2) and maternally (Matdi2) disomic mice for distal chromosome 2 were incubated with MNase for increasing periods of time (lane 1 to 4 correspond to 0, 30, 60, and 120 seconds of incubation with MNase at 37^0C). DNA samples were digested with *Bgl*II, and analyzed by southern hybridisation with probes NN3 (left panel) and NN4 (right panel) respectively. Arrows indicate the bands that are present only in paternal MNase profile.

To further analyse the nucleosomal organization within mouse *Neuronatin* locus in other tissues, MNase digestion was done on wild type MF1 mice. We observed prominent sharp bands in the region corresponding to second intron (beyond 1000 bp from 3'end of *Bgl*II digest, corresponding to DNA wound around approximately four or five nucleosomes) using NN3 and NN4 probe (figure 2H). A smeary background was also observed in this region. This probably is a result due to combination of differential nucleosomal organization on the maternal and paternal alleles.

H1



H2

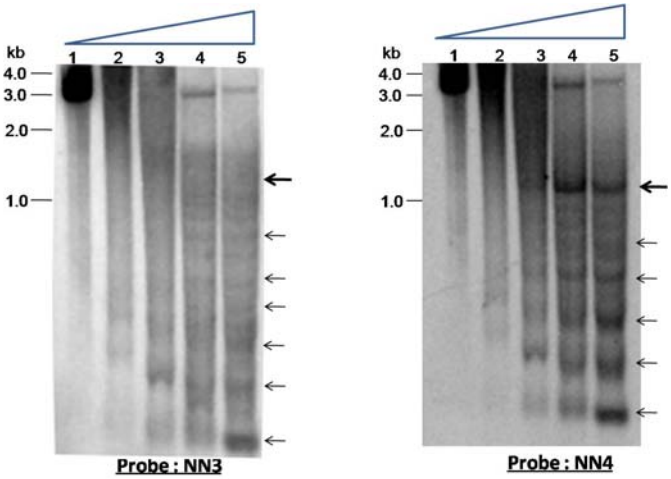


Figure 2H. MNase analysis on different tissues of adult mice. Nuclei were incubated with increasing period of time (lane 1,2,3,4,5 corresponds to 0,25,50,75,and 100 seconds

incubation with 15 units of MNase (Roche). Southern hybridization was done with the probes NN3 and NN4. Arrows shows nucleosomal bands. Thick arrow shows the prominent of ~1.5 kb in NN4 probed blot. A smeary background can also be observed after four nucleosomes (~1000bp) in NN3 as well as NN4 probed southern hybridization. 2G1). MNase assay done for adult mouse kidney, 2G2). MNase assay done for adult mice brain.

Conclusions

Experiments presented in this chapter examined the allele-specific chromatin organization and chromatin conformation at the mouse *Neuronatin* locus. Nuclease sensitivity assay (DNase I and MNase) on various mouse tissues indicated the presence of a constitutive, transcription- independent and methylation dependent DNase I hypersensitive site approximately 1.3-1.5 kb 5' to the NN4 probe and approximately 2.2-2.4 kb 3' to the NN3 probe location (figure 2H). Presence of nuclease hypersensitive sites (HS-I) irrespective of the transcription of the gene, suggested the possibility that the hypersensitive site in the second intron of *Neuronatin* could be linked to the mechanism correlated with imprinting of *Neuronatin*. DNase I hypersensitivity (DHS) is associated with several types of cis-acting transcriptional regulatory elements (Elgin 1988). For instances, DHS in CFTR gene are associated with its transcription regulation (Marios *et al* 2002); DHS in the 5' region of human plasminogen activator inhibitor type 2 gene (PAI-2) are associated with TNF-α induced transcription (Mahony *et al* 1998); the DHS II in 5' region of murine γ1 heavy contains binding sites for NF-κB and STAT6 and shown to regulate the transcription of this gene (Cunningham *et al* 1998). DHS usually represent non- nucleosome organization of the chromatin fiber (Gross and Garrard 1988, Erkine *et al* 1995, Felsenfeld and Gourdine 2003). MNase profile for the two alleles of the mouse *Neuronatin* gene, shown in this chapter confirmed a non-canonical chromatin organization within the second intron of *Neuronatin*. It is possible that the DNase I hypersensitive region (HS-I) indicates the

presence of a regulatory element within the second intron of *Neuronatin* gene (figure 2I). It has been previously reported that proteins like CTCF and YY1 binds within the differentially nuclease sensitive regions of *H19* (Bell *et al* 2000, Hark *et al* 2000), *Peg3* and *Gnas* imprinted genes (Kim *et al* 2007, 2008) locus. Whether similar proteins and similar mechanism operate for the differentially nuclease sensitivity region of *Neuronatin*, need to be examined.

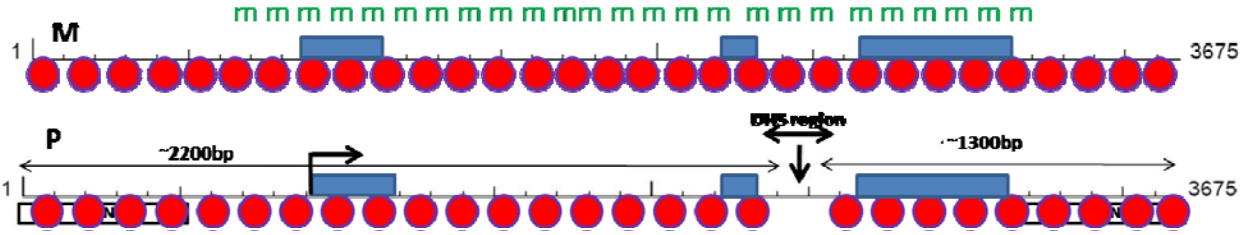


Figure 2I. The proposed model of the chromatin organization in *Neuronatin* locus. 'M' and 'P' denotes maternal and paternal alleles of *Neuronatin* respectively. 'm m m' denotes the methylation on maternal allele. Circles represents the nucleosomes and boxes represent exons of the *Neuronatin* gene. Raised black arrow represents transcription site and direction of *Neuronatin* gene. Maternal allele is shown to be in canonical nucleosomal structure, while in the second intronic region disturbance was found in nucleosomal organization. Star represents the factor which might be responsible to give rise to a specialized chromatin organization on paternal allele. Double headed arrows represent the distance of second intron from probes and DHS represents the proposed ICR region.

Therefore, to characterize this intronic region with respect to specialized chromatin conformation observed in this region, studies were carried out to identify cis as well as trans factors interacting within intron and are present in chapter III chapter IV.

Chapter III

Identification of DNA motifs within second intron of mouse *Neuronatin* that show methylation-restricted protein binding

Introduction

In chapter II chromatin analysis of the mouse *Neuronatin* locus identified differential chromatin organization on the two paternal alleles. Results showed the presence of DNase I hypersensitive sites and disruption of the canonical nucleosomal structure within the second intronic region of the gene only on the paternal unmethylated allele. The constitutive presence of DNase I hypersensitive sites within the second intron of *Neuronatin* in all tissues analyzed suggested it to be independent of the transcription status of the gene and possibly associated with its imprinting status. In the well studied imprinted genes like *H19/Igf2*, *Peg3*, and *Gnas* locus the regions with differential chromatin organization have been shown to be the binding sites of proteins like CTCF (Bell *et al* 2000, Schoenherr *et al* 2004, Szabo *et al* 2004, Kim 2008) and YY1 (Kim 2008) that play a important role in the imprinting control of these genes. The differential chromatin organization observed in the second intronic region between the two alleles of *Neuronatin* could also be due to the binding of a protein (s) to one of the allele which could lead to non-nucleosomal chromatin organisation, resulting in DNase I hypersensitive sites. The comparison of cDNA sequence of mouse *Neuronatin* gene showed 97% homology with human and chimpanzee cDNA, while 100% homology has been found with rat, dog and hamster, suggesting *Neuronatin* gene is highly conserved among the eutherian mammals. This observation also suggests that the mode of mechanism for the maintenance of *Neuronatin* imprinted status might be similar in all eutherian mammals.

This chapter deals with the identification of the minimum DNA sequence within the second intron of mouse *Neuronatin* gene which could be involved in possible protein

binding and in organizing the two alleles of *Neuronatin* into different chromatin conformation.

Materials and methods

Generation of DNA corresponding to Second intron of *Neuronatin*

DNA corresponding to second intron of *Neuronatin* was generated by polymerase chain reaction (PCR) on mouse genomic DNA using 50 pico moles of the primers (Sigma Genosys Inc.):

IT2fr 5'TGAGGTATACTTAAGTTGTGGGTCC3'

IT2bk 5'CACCTGCGGTGAGAGACCCAGGAC 3'.

PCR was done using recombinant Taq DNA polymerase (Fermentas Life Sciences, GeneTAQ, Cat. # EP0407, Initial denaturation at 95^0C for 5 minutes, For amplification; denaturation at 95^0C for 45 seconds, annealing of primers at 60^0C for 1 minute and elongation at 72^0C for 1 minute, final elongation step at 72^0C for 5 minutes.). The 250 base pair PCR product thus obtained was run on 1.5% agarose gel in 0.5X TBE (Stock solution 5X TBE- 54g Tris + 27.5g Boric Acid + 3.82 g EDTA.H_2O, dissolved in deionized water and autoclaved). DNA was eluted from the gel (Figure. 3A) using Gel Elution Kit (Eppendorf Inc, Perfectprep^{TM} Gel Cleanup, Cat. # 955152051).

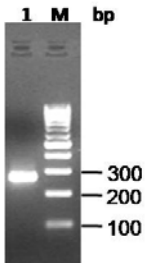


Figure 3A. 2% Agarose gel showing PCR product corresponding to 250bp second intron of *Neuronatin*. 1: PCR product; M: 100bp DNA ladder (MBI fermentas Cat. # SM0321).

Concentration of the PCR product was measured by spectrophotometer at 260nm wavelength using NanoDrop (Thermo Scientific Inc. Cat.# ND1000). For end labeling, DNA was heated to 60^0C and snapped chilled. 50 pico moles of the DNA were used for labeling by T4 Polynucleotide Kinase (NEB, Cat. # M0201S). The reaction (50 pico moles DNA, 1X T4 PNK buffer (NEB, Cat. # B0201S), 75 pico moles γ P^{32}ATP (GE Healthcare Life Sciences, Cat. # PBK 9860), 5% Polyethylene Glycol-8000, 10 units T4 PNK enzyme) mixture was incubated at 37^0C for one hour and stopped by heat inactivation at 80^0C for 20 minutes. After labeling of DNA, the purification of labeled DNA from unincorporated labeled nucleotides was done using G-50 spin column (GE Lifesciences Cat. # 27533501).

Generation of sub-fragments of Second intron of *Neuronatin*

For the identification of the minimum DNA sequence required for the protein-DNA interaction, the second intron of *Neuronatin* was divided into four sub-fragments; 5'IT, M'IT, GCIT, 3'IT (figure 3B).

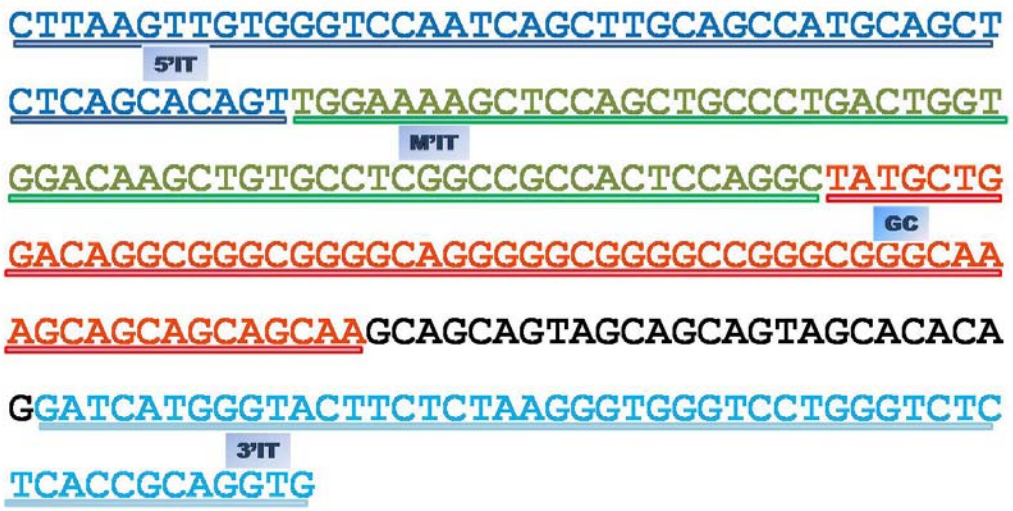


Figure 3B. DNA sequence for second intron of mouse *Neuronatin* gene. The 4 sub-fragments of second intron used for EMSA analysis were named as 5'IT, M'IT, GC and 3'IT (indicated below each fragment).

The sub-fragments of second intron of *Neuronatin* were generated by annealing equimolar concentrations of overlapping oligonucleotides corresponding to various regions of second intron. 500pmoles of sense strand and 500pmoles of antisense strand the oligos for

5'IT:
sense strand 5' CTTAAGTTGTGGGTCCAATCAGCTTGCAGCC 3'

antisense strand 5' ACTGTGCTGAGAGCTGCATGGCTGCAAGCTGATTGG 3',

M'IT:

sense strand 5' TGGAAAAGCTCCAGCTGCCCTGACTGGTGGACAAGC 3',

antisense strand 5' GCCTGGAGTGGCGGCCGAGGCACAGCTTGTCCACCAGTCA 3',

GC:

sense strand 5'TATGCTGGACAGGCGGGCGGGGCAGGGGGCGGGGCCGGGC3',

antisense strand 5' TTGCTGCTGCTGCTTTGCCCGCCCGGCCCCGCCCCCTGCC 3',

3'IT:

sense strand 5' GATCATGGGTACTTCTCTAAGGGTGGGTCC 3',

antisense strand 5'CACCTGCGGTGAGAGACCCAGGACCCACCCTTAGAG3'

were annealed in 50µl TEN (10mM Tris-HCl, pH 8.0, 0.5mM EDTA, 50mM NaCl) buffer by incubating in boiling water for 5 minutes followed by slow cooling to room temperature (over approximately 1 hour). The ends were filled using DNA polymerase (Klenow fragment) in a labeling reaction (50 pico moles DNA, 1X NEB Buffer 2 (NEB Cat. # B7002S), 75 pico moles α P^{32}dCTP (GE Healthcare Life Sciences, Cat # PB10475) 5 units of Klenow enzyme (NEB Cat. # M0210L), 1mM dNTPs-dCTP) carried out at room temperature for 30 minutes and stopped by addition of EDTA to the final concentration of

10mM. Purification of labeled DNA from unincorporated labeled nucleotides was done using G-50 spin column (GE Lifesciences Cat. # 27 5335 01).

Radiolabelling of Complementary Oligonucleotides

The sense and anti-sense strand Complementary oligos were annealed in equimolar concentration in TEN buffer. 5' End-labeling was done by by T4 Polynucleotide Kinase (T4 PNK) as described above.

Preparation of nuclear protein extract

Nuclear extract was prepared essentially as described by Lewis and Konradi (1996). All the steps were carried out on ice unless otherwise mentioned. Tissues were homogenized in Dounce homogenizer (Sigma Inc. Cat # T0691) in 3ml tissue homogenization buffer (Buffer A) (10mM HEPES (pH 7.9), 10mM KCl, 0.1mM EDTA, 1.5mM $MgCl_2$, 0.34M Sucrose, 10% Glycerol, 1mM DTT) in the presence of 1X Protease Inhibitors Cocktail (Roche Applied Sciences Cat. # 1836170) and suspended in 15ml buffer A. The cell suspension was incubated for 5-10 minutes on ice and filtered through 1mm strainer to remove cell debris and particulate material. Cells were collected by centrifugation at 1500g for 5 minutes. Cells were washed twice in buffer A, and suspended in 5ml of buffer A with 0.2% NP-40. Cell suspension was incubated for 5 minutes on ice. The suspension was carefully overlaid on 1M sucrose buffer (10mM HEPES (pH 7.9), 10mM KCl, 0.1mM EDTA, 1.5mM $MgCl_2$, 1.0M Sucrose, 10% Glycerol, 1mM DTT) and the centrifugation was carried out for 15 minutes at 4500g in swing bucket rotor. Supernatant was discarded and the pellet containing nuclei was retained. Nuclei thus obtained were washed twice with buffer A to remove the trace amount of NP-40 and suspended in 2 ml of nuclear protein extraction buffer B (20mM HEPES (pH 7.9), 500mM KCl, 0.4mM EDTA, 1.5mM $MgCl_2$,

25% Glycerol, 1mM DTT) with 1X protease inhibitors cocktail and incubated for 30 minutes on ice. The suspension was centrifuged for 15 minutes at 21,000g at 4^0C and supernatant containing nuclear proteins was collected. The concentration of total nuclear protein was determined by spectrophometric analysis.

Electrophoretic Mobility Shift Assay

Stock solution of 5X EMSA binding buffer was prepared with 50mM HEPES, 50% Glycerol and 0.5mM EDTA and stored at -20^0C. Working solution of 2.5 X EMSA binding buffer [2.5X binding buffer, 2.5mM Spermidine, 5mM $MgCl_2$, 25ng/µl Poly dI-dC , 1mM DTT] was prepared during the experiment. 5% native PAGE used for electrophoresing EMSA samples was prepared by mixing of 150 µl of 20% APS and 30 µl of TEMED with 5%Acrylamide-Bis solution (37.5 : 1), 3.5% Glycerol, 0.5X TBE. The gel was pre-run for 2 hours until the amperage had dropped to 12mA. After electrophoresis, gels were vacuum dried at 80^0C and phosphorimaged using Molecular dynamics software of Typhoon-9200 system from Amersham Biosciences or exposed to X-Ray films (Kodak BioMax MS Film Cat. # 822 2648).

Cloning of GCs in pBluescript SK (+)

Complementary oligonucleotides corresponding to GCs region were annealed. The vector was linearized with *Sma*I enzyme and ligation reaction of *Sma*I linearized pBluescript with GCs was set according to manufacturer's direction in the buffer containing 5% PEG-8000 using T4 DNA ligase (NEB) enzyme. The ligation mix was transformed into DH10β *E.coli* strain and insertion was confirmed by sequencing.

Results

As our preliminary experiments and the data shown in Chapter II showed the presence of allele specific and constitutive DNase I hypersensitive sites within the second intron of *Neuronatin*, it was decided to identify the *cis* DNA element present within this intron which might be involved in conferring allele-specific chromatin organization.

The second intron of *Neuronatin* is conserved among eutherian mammals

The phylogenetic study of *Neuronatin* has shown this gene to be conserved and imprinted in all the eutherian mammals (Evans *et al*, 2005). To delineate phylogentic importance of sequence within the second intron, a comparison of this region was done across mouse, rat, human, chimpanzee, dog, and cow using BLAST (www.ncbi.nlm.gov/BLAST)(figure 3C).

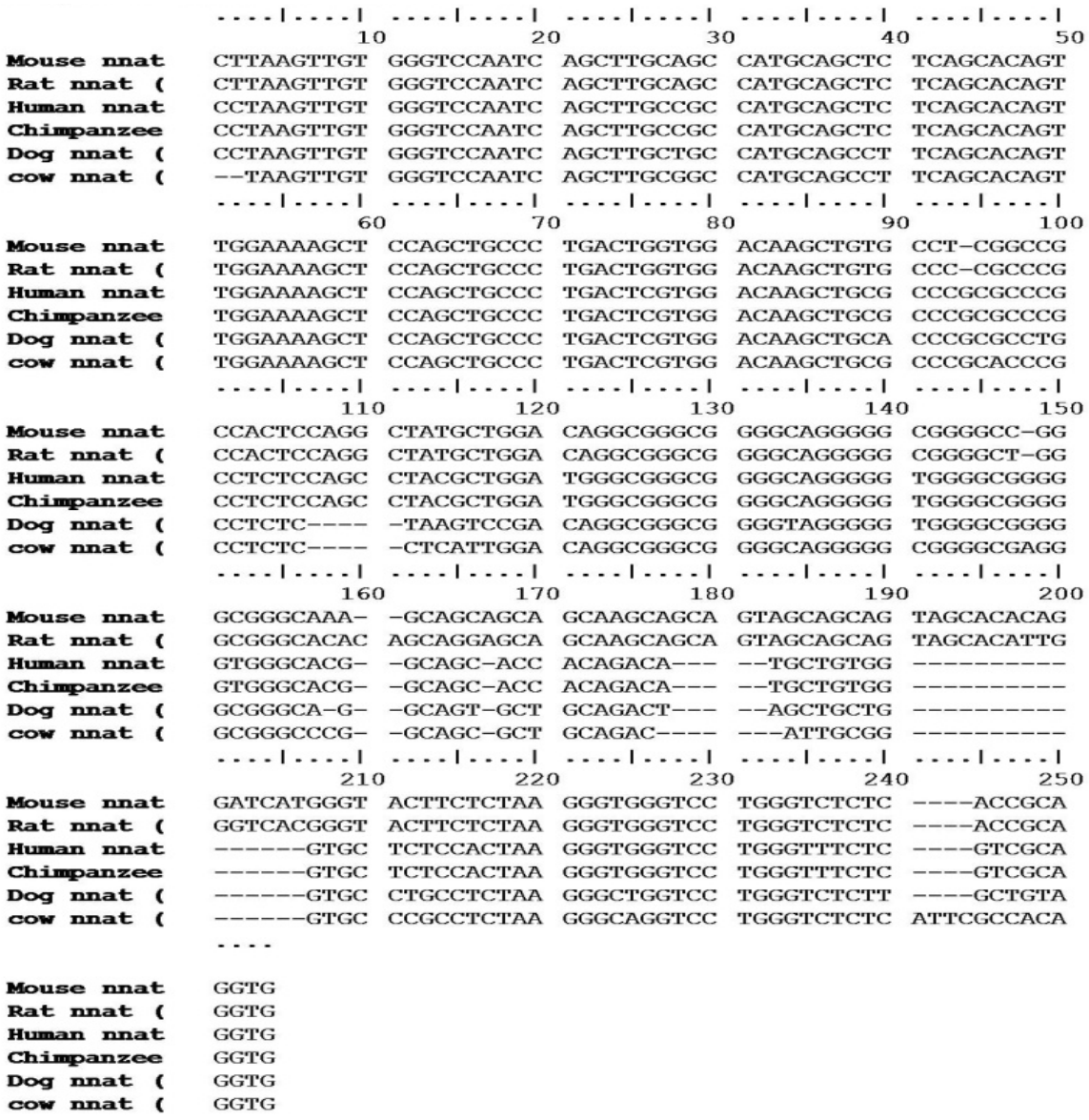


Figure 3C. Phylogenetic comparison of second intron of *Neuronatin* among various mammalian species.

Being an intronic region the conservation of this region was found to be very remarkable. The only difference found among the various mammalian species was the presence of CAG repeats in mouse and rat. These repeats were absent in human, chimpanzee, cow and dog. The second intronic region of mouse (gi:149338249) and rat (gi:62750360) was found to be 99% similar, while human (gi:51511747) and chimpanzee (gi:114795211) second

intron also showed 99% similarity. In comparison to mouse, the second intron of human, chimpanzee, cow (gi:119947340) and dog (gi:74034289) showed 83%, 85%, 78%, 78% similarity, respectively.

Identification of DNA sequence within II intron of *nnat* for protein binding

EMSA analysis showing interaction of liver nuclear proteins with sub-fragments of second intron of *Neuronatin*

To analyze whether any protein(s) binds to a region within the second intron of *Neuronatin*, **E**lectrophoresis **M**obility **S**hift **A**ssay (**EMSA**) was performed. The 250bp intronic region was sub-divided into four fragments; 5'IT, M'IT, GC and 3'IT (Figure. 3A) and EMSA was done with these fragments in the presence of specific (self) and non-specific competitor; (poly dI-dC and NN3, a 500bp fragment flanking the 5' region of *Neuronatin* gene). As can be seen in figure 3D, specific binding was observed only with the "GC" fragment. In comparison to 'GC' very weak and non-specific protein binding was observed in 5'IT and 3'IT region, while no protein binding was observed in the M'IT region. Moreover the 'GC' fragment could compete out the binding to 5'IT and 3'IT region.

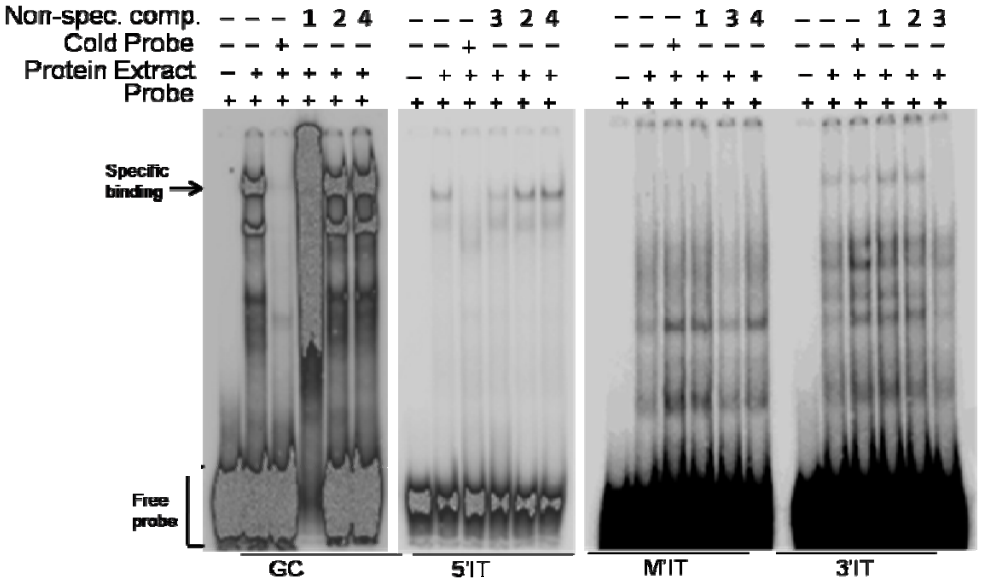


Figure 3D. EMSA analysis showing protein interaction with different fragments of 250 bp second intron of *Neuronatin***.** '1'- 5'IT; 2- M'IT; 3- 'GC'; '4'-3'IT. GC region of 250 bp intron shows the protein binding to DNA.Non-specific protein binding was observed with 5'IT, M'IT, 3'IT fragments of the intron.

<u>EMSA analysis showing interaction of liver nuclear proteins with second intron of *Neuronatin*</u>

To ensure that the binding of protein (s) to 'GC' DNA was specific, an EMSA analysis was carried out using the 250bp long second intron as a probe. As the result in figure 3E shows, the protein binding to the second intronic region could be competed out only with 60bp 'GC' DNA.

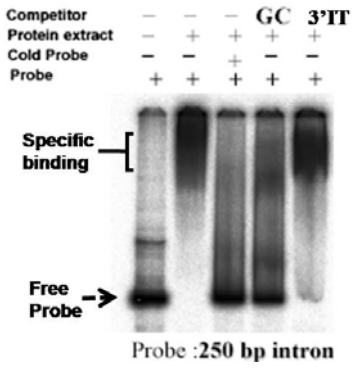


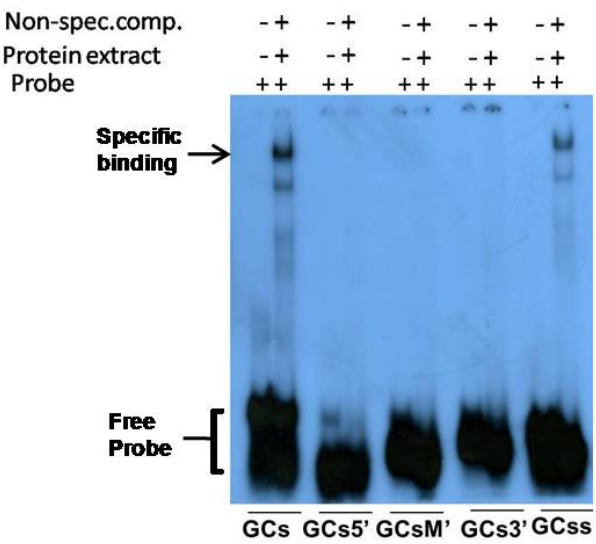
Figure 3E. EMSA analysis showing protein interaction with the DNA sequence of IInd intron of *Neuronatin.* 'GC' DNA could compete out the DNA sequence specific protein binding to 250bp Intronic region. The protein binding to second intron remains unaffected by 3'IT DNA. Dashed arrow represents free probe.

EMSA analysis showing interaction of nuclear proteins with sub-fragments of 'GC' region of second intron of *Neuronatin*

Furthermore, the possibility that instead of the whole 'GC' fragment only a part of the 'GC' fragment is responsible for the DNA-protein interaction, was also analyzed. The 60bp 'GC' fragment was divided into different overlapping fragments namely GCs, GCs5', GCsM', GCs3' and GCss (figure 3F1). With both liver and brain nuclear extracts, the sub-fragments; 38bp DNA of 'GCs' and 25 bp DNA of 'GCss' showed protein-DNA interaction (figure 3F2 and 3F3). Other fragments did not show any binding.

F1

F2



F3

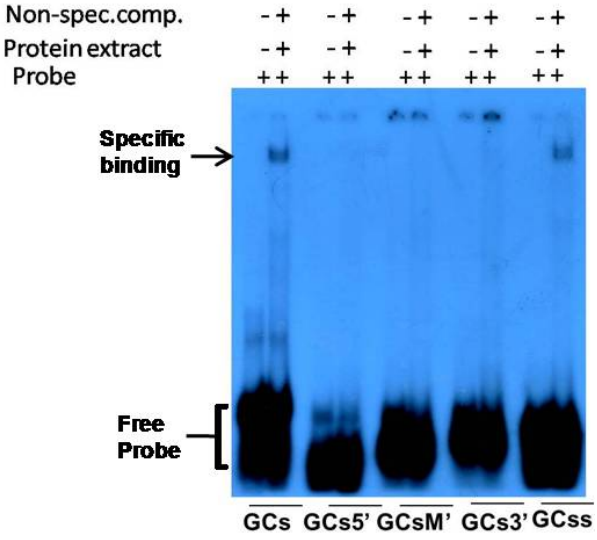


Figure 3F. EMSA analysis with various 'GC' fragments**.** F1). 60bp GC fragment was sub-divided into various overlapping fragments as indicated. F2). EMSA analysis with adult mice liver nuclear protein extract. F3) EMSA analysis with adult brain nuclear extract.

<u>EMSA analysis showing the protein interaction with 'GCss'and 'GCssm'</u>

Since GC3' differs from GCss only in the presence of two nucleotides at the 5' end we wanted to explore whether these nucleotides form the part of the binding site. Oligonucleotides for GCssm fragment where CAGG were replaced by AAAA at the 5' end of GCss, were synthesized and annealed (Figure. 3G.1). The results showed similar binding with GCss and GCssm (figure. 3G2) suggesting that CAGG does not form the part of the binding site, but the extra nucleotides at the 5'end probably are required for stable binding with GCss.

G1

GCss CAGGGGGCGGGGCCGGGCGGGCAA

GCssm AAAAGGGCGGGGCCGGGCGGGCAA

G2

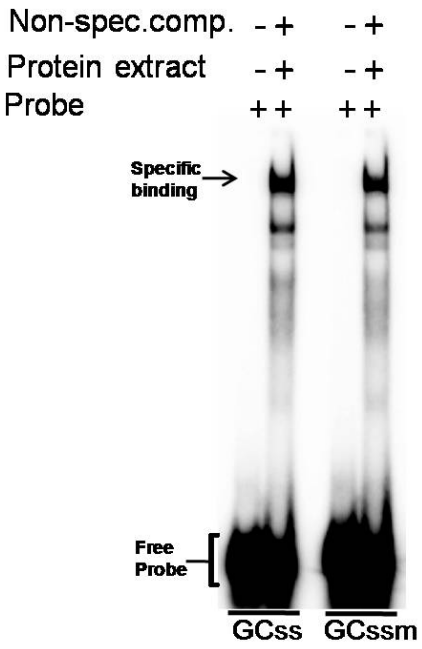


Figure 3G. EMSA analysis showing the protein interaction with GCss and GCssm. G1). the sequence of GCss and the modified sequence GCssm. GCssm consists of AAAA in place of CAGG of GCss and two additional AA more than GCs3'. G2). EMSA analysis showing the protein-DNA interaction with GCss and GCssm using liver nuclear extract.

In addition, EMSA analysis (figure 3H) also suggested that probably the same protein binds to GCs and GCss as both fragments were able to complete each other in the binding reaction.

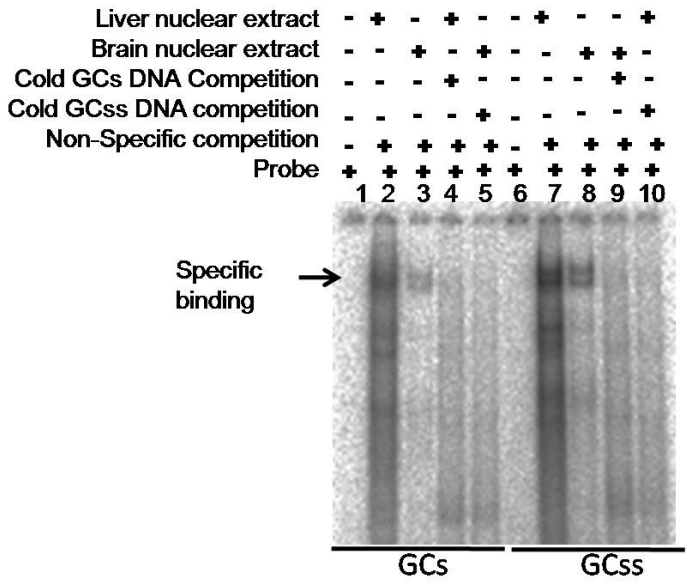


Figure 3H. EMSA analysis showing the protein interaction with 'GCs' and 'GCss'. 'GCss' fragment could compete out the protein binding with 'GCs' from liver (lane 10) and brain (lane 5) nuclear extract.

Thus, these results suggest that the minimum binding motif of DNA for the protein-DNA interaction in the second intronic region is 'NNNNGGGCGGGCCGGGCGGGAAA'.

Interaction of protein with 'GC' is sequence specific

To further ascertain the sequence specific protein(s) interaction with GCs, it was cloned in the *Sma*I site of pBluescript II SK (+) vector. The vector was digested with *Xho*I and *Xba*I to obtain a 133bp DNA fragment containing 'GC' (60bp) with part of the multiple cloning site (MCS) (73bp) acting as flanking region. Similar fragment from control vector was also obtained after digestion with *Xba*I and *Xho*I containing only MCS (73bp) and not 'GC'. The result, (figure 3I) showed that protein interacts with GCsMCS while no protein interaction was observed with MCS.

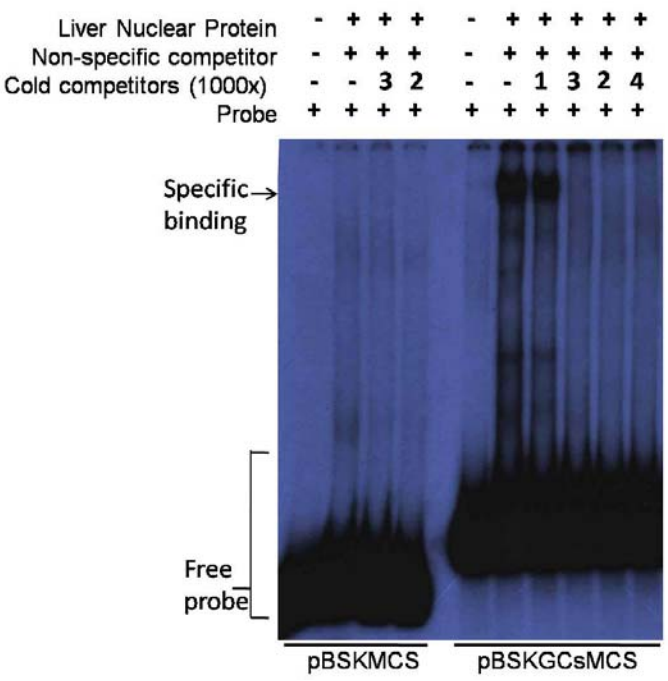


Figure 3I. EMSA analysis showing the interaction of protein with pBSKGCMCS. Cold competitors 1- pBSKMCS; 2- 'GCs'; 3- pBSKGCMCS; 4- meth'GC'. No protein DNA interaction was observed using pBSKMCS as radio-labeled probe. pBSKGCsMCS shows protein binding. The protein-pBSKGCsMCS DNA interaction remains unaffected by addition of cold pBSKMCS The protein-pBSKGCsMCS DNAinteraction was competed out by the addition of cold pBSKGCsMCS, GCs and meth GCs.

The 'GC' fragment of *Neuronatin* is conserved among eutherian mammals

A phylogenetic analysis (figure 3J) of GC fragment in mouse, rat, human, cow, dog and pig revealed that similar to mouse 'GCs', all the analyzed species have at least two regions of GGGCGGG.

Mouse nnat	CAGGCGGG CGGGGCAGGG GGCGGGGCC- GGGCGGGCAA A-
Rat nnat	CAGGCGGG CGGGGCAGGG GGCGGGGCT- GGGCGGGCAC A-
cow nnat	CAGGCGGG CGGGGCAGGG GGCGGGGCGA GGGCGGGCCC G-
Human nnat	TGGGCGGG CGGGGCAGGG GGTGGGGCGG GGGTGGGCAC G-
Chimpanzee	TGGGCGGG CGGGGCAGGG GGTGGGGCGG GGGTGGGCAC G-
Dog nnat	CAGGCGGG CGGGGTAGGG GGTGGGGCGG GGGCGGGCAG --

Figure 3J. Phylogenetic analysis of GCs fragment within various eutherian mammals species. The bar below the sequence shows the conserved 'GGGCGGG' motif in various eutherian mammalian species.

Ubiquitous presence of 'GC' fragment binding protein

The possibility that binding protein is present ubiquitously was analyzed by performing EMSA with nuclear protein extract from various tissues using the 38bp 'GCs'. As can be seen from figure 3K, specific binding was observed in all the tissues analyzed.

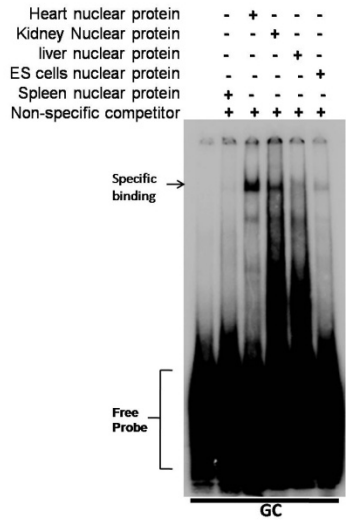


Figure 3K. EMSA analysis showing protein binding with 'GCs' DNA in various tissues**.**

Protein (s) interaction with 'GCs' DNA is influenced by CpG methylation

As shown above nuclear protein (s) bind to 'GCs' DNA in a sequence specific manner. The experiments described in chapter two showed the presence of DNase I hypersensitive sites exclusively on the unmethylated paternal allele. Therefore, the aim of our experiments was not just to identify protein (s) interaction to 'GC' but also to analyze whether protein (s) binds to 'GC' in a methylation restricted manner. In addition, previous studies on *H19/Igf2* (Hark *et al* 2000, Bell *et al* 2000) and *Peg3* (Kim *et al* 2003) loci have shown that the interaction of proteins like CTCF and YY1 binds in a methylation restricted

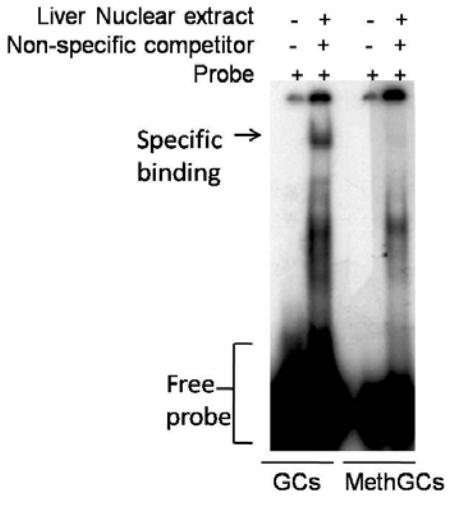
manner. As 'GC' fragment contains 5 CpG dinucleotides within the 38bp sequence, oligonucleotides corresponding to the 38bp 'GCs' having methylated cytosines incorporated at CpG locations were synthesized (figure 3L1). To generate double stranded CpG methylated 'GCs' DNA (henceforth called as methGCs), complementary oligonucleotides were annealed in equimolar concentration and 5' end-labelled using $\gamma P^{32}ATP$ by T4 PNK. EMSA analysis was done with nuclear extract using GCs and methGCs as probe. The result (figure 3L2 and 3L3) suggested that the protein interaction with GCs is indeed influenced by CpG methylation and only the unmethylated 'GC' fragment shows the protein binding.

L1

GC = CAGGCGGGCGGGGCAGGGGGCGGGGCCGGGCGGGCAAA

methGC = CAGG^{m}CGGG^{m}CGGGGCAGGGGG^{m}CGGGGC^{m}CGGG^{m}CGGGCAAA

L2



L3

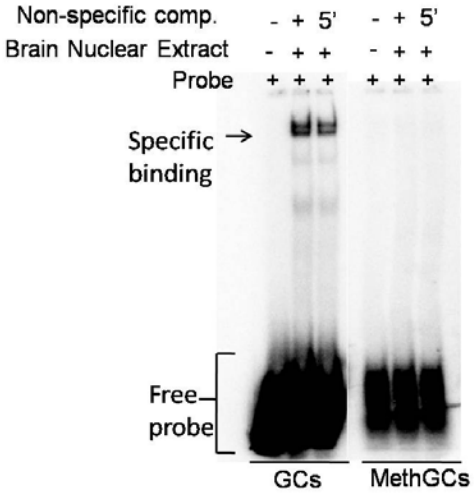


Figure 3L. Protein interaction with GCs DNA is influenced by CpG methylation. L1)The DNA sequence of GCs and methGCs. L2) EMSA analysis for protein interaction with GCs

and meth GCs using liver nuclear extract, L3) EMSA analysis for protein interaction with GCs and meth GCs using liver nuclear extract.

Protein interaction with GCs DNA is dependent on cytosine of CpG nucleotides.

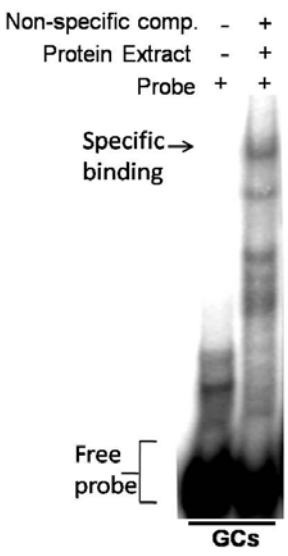
To further confirm the role of CpG nucleotides in the protein binding, oligonucleotides corresponding to GCs fragment, where C in context of CpG were replaced with thymine (henceforth called as MutGCs) were synthesized (figure. 3M1). EMSA was done using complementary oligonucleotides for MutGCs and with liver nuclear extract. The result showed that the replacement of cytosine with thymine in relation to CpG nucleotides, completely impairs the specific interaction of protein with GCs (Figure. 3M2 and 3M3).

M1

GCs = CAGGCGGGCGGGGCAGGGGGCGGGGCCGGGCGGGCAAA

MutGCs = CAGGTGGGTGGGGCAGGGGGTGGGGCTGGGTGGGCAAA

M2



M3

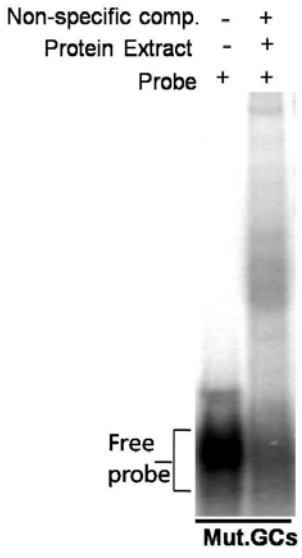


Figure 3M. Protein interaction with 'GCs' DNA is influenced by Cytosine of CpG nucleotides. M1). The DNA sequence of 'GCs' and 'mutGCs'.M2) The DNA sequence of

'GCs' and 'mutGCs'. 3K2a) EMSA analysis for Protein interaction with GCs, M3) EMSA analysis for protein interaction with 'mutGCs'.

Thus, the protein (s) interaction with GCs DNA is sequence specific and not affected by the sequence of the flanking region. The protein interacts with double stranded GCs DNA and the methylation of CpG nucleotides has a negative effect on the interaction of protein (s) with GCs.

Conclusions

Hypersensitive sites are usually regions of non-nucleosomal organization within chromatin (Elgin 1984) and in most examples of DNase hypersensitivity, the HS sites are a result of non-histone protein binding (Gross and Garrard 1988, Erkine *et al* 1995, Boyle *et al* 2007). In this chapter, results from our studies on identifying protein binding sites corresponding to HS-I within the second intron of *Neuronatin* locus are presented. Being an intron, the phylogenetic similarity of this region among various mammalian species was found to be remarkably high and was suggestive of a role for the *cis*-elements within this intron in regulation of *Neuronatin* gene.

Electrophoretic mobility shift assay performed on various fragments of the second intron, chosen based on similarity across various mammalian species, showed that only a GC-rich region within the intron can bind specifically to proteins. It was also found that the interaction of protein (s) with GCs DNA sequence was influenced by DNA methylation as specific proteins interacted with only unmethylated 'GC'. In addition the minimum 'GC' fragment showing this methylation-restricted binding was found to be NNNNGGGCGGGCCGGGCGGGAAA.

Chapter IV

Identification and characterization of the protein(s) binding to 'GC' within the second intron of *Neuronatin* gene

Introduction

In the previous chapter, the analysis of *cis* elements within the second intron of *Neuronatin* had identified a 25bp DNA sequence 'GCss' to which protein (s) binding was been observed. EMSA analysis had further suggested the interaction to be DNA methylation-restricted. Examples of such DNA methylation restricted protein-DNA interaction have been shown for other imprinted genes like KvDMR1 of *Kcnq1* (Fitzpatrick *et al* 2007) *H19/Igf* ICR (Hark *et al* 1998, 2000), *Gnas Peg3 (K*im *et al* 2008*)*. CTCF (Han *et al* 2008) and YY1 (Kim 2008) proteins have been identified in these studies to be the protein showing DNA methylation restricted binding. Analysis of the sequence in and around the mouse *Neuronatin* gene using bioinformatic approaches did not identify any CTCF or Gli-type transcription factor YY1 binding site. This raised the possibility of protein (s) other than CTCF and YY1 binding within the intronic region. To identify and characterize protein (s), binding in a methylation restricted manner to the 'GC' fragment, Yeast Mono Hybrid and affinity chromatography assays were performed. The results from these assays and Chromatin Immuno-Precipitation with antibodies against transcription factor Sp1 are presented in this chapter.

Materials and Methods

Yeast mono-hybrid assay

Yeast Mono Hybrid assay (YMH) was performed to identify and characterize protein(s)-DNA ('GC') interaction using BD MATCHMAKER^{TM} Library construction and screening kit (Cat. # K1615-1 K1617-1), purchased from BD biosciences. YMH assay was performed as described in figure 4A.

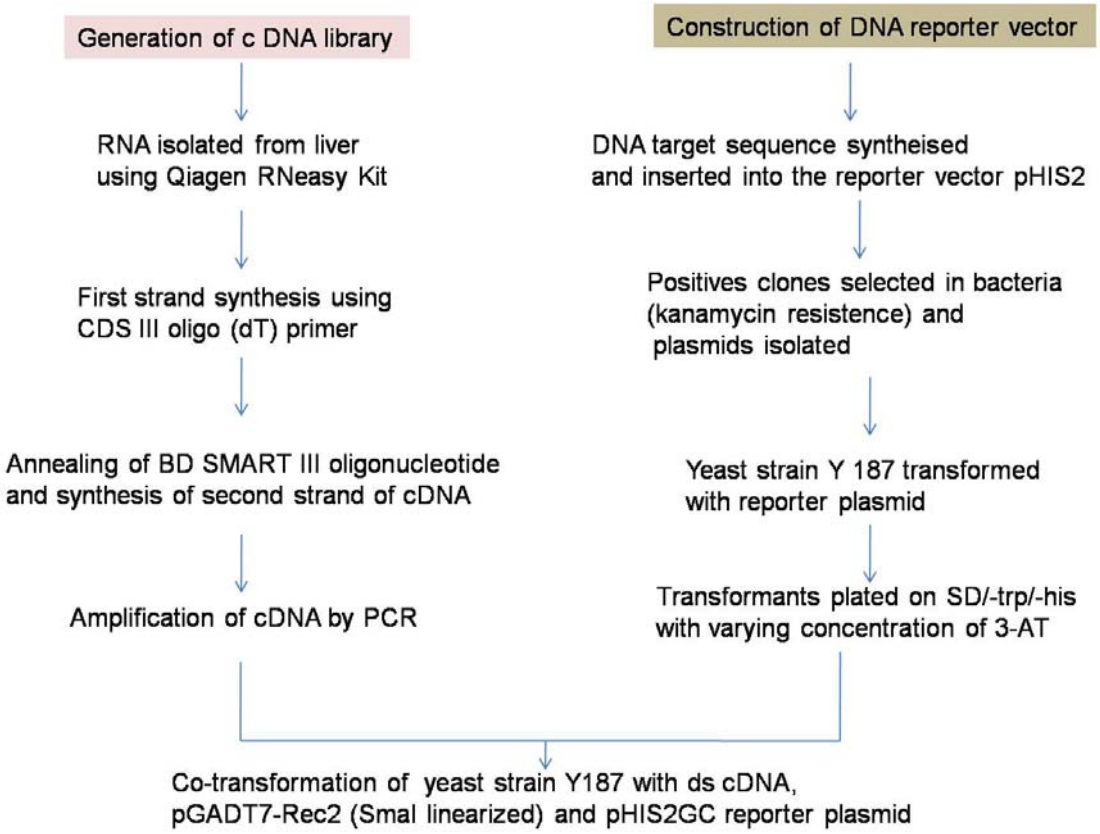


Figure 4A. Schematic representation of yeast mono hybrid assay protocol.

Construction of DNA reporter vector

Double stranded DNA corresponding to 'GC' was obtained by annealing 300 pmoles of sense and antisense (see chapter three, material and methods) in 15µl TEN (10mM Tris-HCl, pH 8.0, 0.5mM EDTA, 50mM NaCl) buffer, by keeping it in boiling water for 5 minutes and then slowly cooling to room temperature over 1 hour. 5' phosphorylated 'GC' was self ligated [Reaction components: 300 pmoles DNA, 4% PEG-4000, 1 X T4 DNA ligase buffer (NEB Cat. # B0202) and 20 units of T4 DNA ligase ((NEB Cat. # M0202S)] in 10µl reaction volume at 16^0C overnight and desalted using sephadex G-50 columns to get multiple copies of 'GC'. The 'GC' fragment was ligated in the reporter vector, pHis2 with HIS3 the *Sma*I site within the MCS and transformed in *E.coli* (DH10β) to get a stable

reporter vector. The ligation and cloning were done as per standard protocols described in Sambrook and Russel (2001). Positive clones (pHis2GC) were selected by colony PCR and confirmed by sequencing.

Transformation of pHis2'GC' in Yeast Y187

Transformation of pHis2GC in yeast strain Y187 (*S. cerevisiae* strain Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met^{-}, gal80Δ, URA3 :: GAL1UAS-GAL1TATA-lacZ, MEL1) was done essentially as per the instruction in YEASTMAKER YEAST TRANSFORMATION SYSTEM 2 (Cat. # K1606-1, PT1172-1). The incubations were carried out at 30^0C and the liquid cultures of yeast were grown with shaking (220-250 rpm). A single yeast colony (Y187) was inoculated into 3 ml of YPDA [1% yeast extract, 2% peptone, 2% dextrose, 0.003% Adenine Hemisulphate (stock solution 0.2%)] medium and incubated with shaking for 8 hours. 5µl of this yeast culture was transferred to 50ml YPDA in 250ml flask and Incubation was carried out at 30^0C with shaking for 16–20 hours till the OD_{600} reached 0.15–0.3. The cells were collected by centrifugation (700g) for 5 minutes at room temperature, resuspended in 100ml of YPDA and further incubated at 30^0C with shaking for 4-5 hours till the OD_{600} reached 0.4–0.5. Cells were harvested by centrifugation (700g) for 5 minutes at room temperature, washed twice with 60 ml of sterile deionized water and suspended in 3ml of 1.1x TE/Li-Ac [stock solution 1.0M Lithium Acetate 10X, 10 X TE Buffer (pH 8.0) (100mM Tris-HCl, 10mM EDTA)] solution. The cell suspension was transferred to 1.5 ml tubes and centrifuged at high speed for 15 seconds. Supernatant was discarded and cells were resuspended in 600µl of 1.1x TE/Li-Ac. For transformation, 50µg of carrier DNA (denatured Herring testis DNA), 500ng reporter vector (pHis2'GC') was gently mixed with 50µl of the cell suspension and

500µl of PEG/LiAc solution [40 % Polyethylene Glycol-4000 (stock solution 50 % Polyethylene Glycol -4000), 1X TE, 1X LiAc] was added to it. The suspension was incubated for 30 minutes at 30^0C with intermitted mixing, 20µl of DMSO was added and cells were transferred to 42^0C water bath for 15 minutes with intermitted mixing. After heat treatment, cells were centrifuged at 13000g for 15 seconds, supernatant was removed and YPD plus liquid media was added to the cells and further incubated for 90 minutes in YPDA at 30^0C. Cells were harvested and spread on SD/-His-trp [6.67g/L Yeast nitrogen base + ammonium sulphate + 100ml of required 10X amino acid drop out solution (As described in YEAST PROTOCOL HANDBOOK (PT3024-1), BD Biosciences, Cat. # K1612-1)] medium containing varying concentration of 3-AT.

Generation of cDNA library from liver tissue of mouse using BD SMART™ technology

Messanger RNA was reverse transcribed into dsDNA using BD SMART™ (**S**witching **M**echanism **a**t 5' end of **R**NA **T**ranscript) as per the manufacturer's directions (BD Biosciences PT 3529-1). Briefly, total RNA was isolated from murine liver tissue using Qiagen RNeasy Kit (Cat. # 74104) and visualized by ethidium bromide staining to verify the quality of RNA samples (figure 4B). The concentration of RNA was measured using NanoDrop™ 1000 spectrophotometer (Thermo Scientific Inc.) at 260nm.

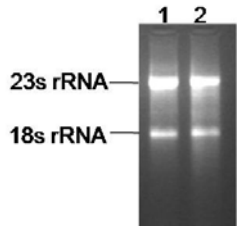


Figure 4B. Agarose formaldehyde gel scan showing the total RNA from different tissues of mice. Lane 1 represents the total RNA isolated from liver; lane 2 represents total RNA isolated from Kidney of mice.

To synthesize first strand cDNA, 2.0 µg of total RNA was mixed to 10µM of CD III/6 (5'ATTCTAGAG'GC'CGAG'GC'G'GC'CGACATG-NNNNNN-3') primers in the final volume of 4µl and denatured at 72^0C for 5 minutes. The tubes were snap chilled in ice and spun briefly. 2.0 µl of 5X First-Strand Buffer (250 mM Tris (pH 8.3); 30 mM $MgCl_2$; 375 mM KCl), 1.0 µl DTT (20 mM), 1.0 µl dNTP Mix (10 mM) and 5 units of MMLV Reverse Transcriptase (1.0 µl) were added. Components were mixed and incubated at 30^0C for 10minutes and 42^0C for another 20 minutes. 1.0 µl (10µM) of BD SMART^{TM}III Oligonucleotide (5'-AA'GC'AGTGGTATCAAC'GC'AGAGTG'GC'CATTATG'GC'CGGG-3') was added to the reaction and further incubated at 42^0C for 1 hr. First strand DNA synthesis was terminated by incubating the reaction at 75^0C for 10 minutes. The removal of mRNA from DNA-RNA hybrid after first strand synthesis was done by addition of 2 units of RNase H (Invitrogen Inc., Cat. # 18021-014) and incubation at 37^0C for 20 minutes. After first strand cDNA generation, two 100 µl PCR reactions were set up [2 µl First-Strand cDNA (RNase H treated), 70 µl Deionized H_2O, 10 µl 10X BD Advantage 2 PCR Buffer, 2 µl 50X dNTP mix, 2 µl 5' PCR Primer (5'-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3'), 2 µl 3' PCR Primer (5'-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA-3'), 10 µl 10X 'GC'-Melt Solution and 2 µl 50X BD Advantage 2 Polymerase Mix]. PCR parameters were as follows: 95^0C for 5 minutes, (95^0C for 30 seconds, 68^0C for 1 minute, 68^0C for 5 minutes) x 30 cycles, at 72^0C for 5 minutes. The thermocycler was programmed to increase the extension time by 5 seconds with each successive cycle. The PCR products were purified through gel fractionation in a spin column (BD CHROMA SPIN^{TM}+400 Colums) with a

cut-off limit of 200 base pair DNA. DNA of the two PCRs was mixed and precipitated using 1/10 volumes of sodium acetate (pH 5.4) and 3 volumes of 100% ethanol. The pellet containing cDNA having BD SMART III sequence on one end and CDS III/6 sequence on the other end was suspended in 20 µl of deionized water.

Co-transformation of cDNA, reporter and cloning vector in Yeast transformation

pHis2GC and BD SMART cDNA (as prepared and described above) and *Sma*I linearized pGADT7-Rec2 containing Gal4 activation domain (figure 4C) were transformed into yeast as per the manufacturer's suggestions.

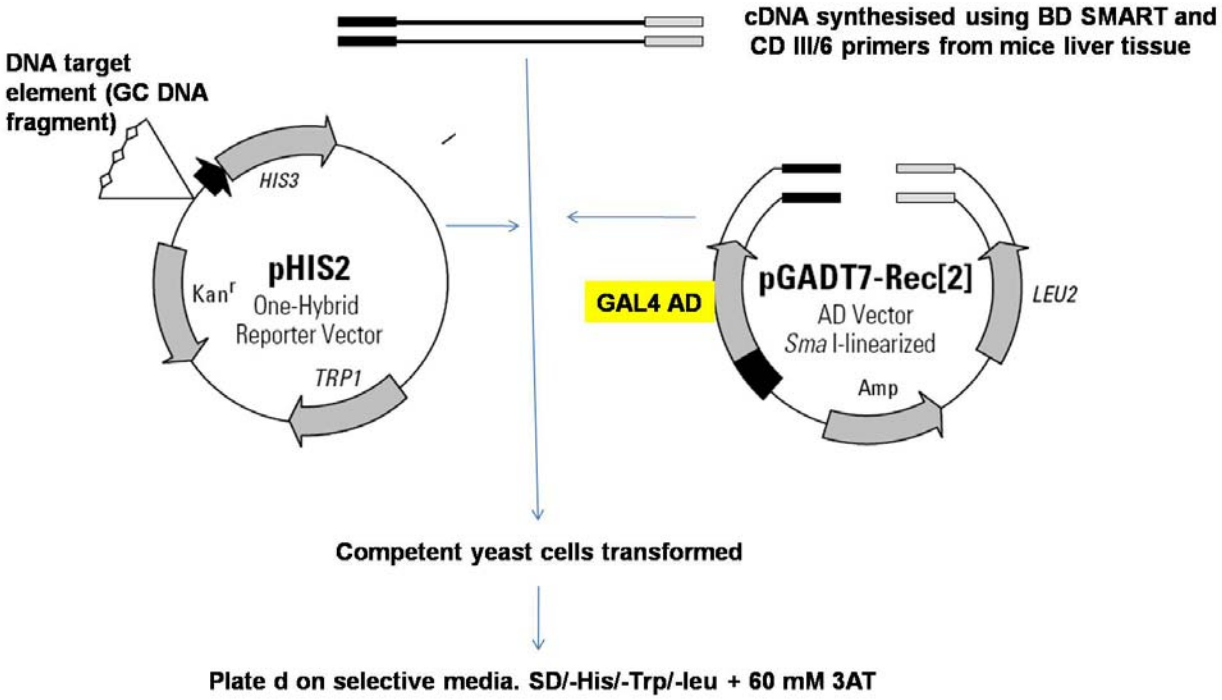


Figure 4C. Diagrammatic representation of yeast transformation**.** DNA element vector pHIS2, cloning vector pGADT7 Rec2 and cDNA are cotransformed in competent yeast cells. cDNA library is constructed by homologous recombination of cDNA with pGAD T7 Rec2, mediated by BD SMART nucleotides on 5' end and CD III oligonucleotides on 3' end of cDNA and *Sma*I linearized pGADT7Rec-2 vector. Cloning of cDNA in pGAD T7 Rec-2 results in the expression of fusion protein (cDNA library protein with GAL 4 **A**ctivation **D**omain).

Briefly, 20 µl BD SMART ds cDNA, 3.0 µg pGADT7-Rec2 vector, 5 µg pHIS2/target DNA, 200µg denatured Herring Testes Carrier DNA were mixed together with 600 µl of yeast competent cells (as decribed above) gently, 2.5ml of PEG/LiCl solution was added and incubated at 30^0C for 45 minutes with intermitant shaking. 160 µl DMSO was added and reaction was incubated for 20 minutes at 42^0C. The cells were collected by centrifugation at 700g for 5 minutes and supernatant was discarded. YPD Plus liquid medium was added to the cells and incubation was carried out at 30^0C for 90 minutes. The cells were washed twice with 0.9% NaCl and plated on SD/-His/-trp/-leu plates.

Isolation of plasmids from yeast for the screening of library cDNA insert

Single colonies were picked from the SD/-His/-trp/-leu plate and inoculated in 2ml SD/-His/-trp/-leu liquid medium. The cells were grown overnight at 30^0C and harvested by centrifugation at 18,000g for 1 minute. Yeast cells were suspended in 0.2 ml of cell lysis buffer [10mM Tris-HCl (pH 8.0), 1mM EDTA, 2% triton X-100, 100mM NaCl and 1% SDS), 0.2 ml of Phenol/Chloroform and 0.3g of acid washed glass beads]. The mixture was vortexed for 2 minutes and cemtrifuged at 21,000g for 10 minutes. Supernatant was collected and precipitated using 2.5 volumes of 100% ethanol. The DNA precipitate was washed with 70% ethanol, air dried and suspended in 20 µl of deionized distilled water. 2 µl of DNA was used for electroporation in *E.coli* strain DH10β and selected on LB-agar-ampicillin (100µg/ml) plates. Plasmids was isolated from bacteria using alkaline lysis method, the positive clones were screened by restriction digestion using *Bgl*II and *Xho*I enzymes for cDNA insert and the identification of c-DNA was done by sequencing with BD SMART and CDS III/6 oligonucleotides.

Preparation of Yeast Nuclear protein extract

A single colony of yeast was grown till late log phase (O. D_{600} reached 0.8 – 1.0) and cells were harvested by centrifugation at 1500g for 10 minutes at 4^0C. The cells were suspended in 10ml of pre-incubation buffer (10mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 1mM EDTA, 50mM DTT, 10mM KCl, 1M Sorbitol) and incubated at 30^0C for 15 minutes with gentle shaking followed by centrifugation at 1500g for 5 minutes at 4^0C and washed twice with 10ml of spheroplasting buffer (10mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 1mM EDTA, 10mM KCl, 1M Sorbitol) and finally suspended in 1ml spheroplasting buffer. 100 units of lyticase (Sigma Inc.) were added to the cell suspension and incubation was carried out for 30 minutes at 30^0C. The spheroplasts were washed twice with wash buffer (10mM Sodium Cocodylate, pH 6.0, 5mM $MgCl_2$, 1mM EDTA, 10mM KCl) and suspended in 10ml of lysis buffer (10mM Sodium Cacodylate, pH 6.0, 18% Ficoll-400) and further incubated at 4^0C for 30 minutes with gentle shaking. The suspension was centrifuged at 1500g for 10 minutes at 4^0C and pelleted nuclei were washed with Buffer A (0.34M sucrose, 20mM HEPES pH 7.9, 60mM KCl, 30mM NaCl, 1.5mM $MgCl_2$, 1mM DTT) to remove the traces of Ficoll and Sodium Cacodylate. Proteins were extracted from nuclei by suspending the nuclei in nuclear protein extraction buffer (25mM HEPES (pH-7.9), 500mM KCl, 15mM NaCl, 1mM EDTA, 1mM DTT, 25% glycerol, 5mM $MgCl_2$) for 30 minutes on ice and centrifugation for 15 minutes at 21, 000g.

Cloning, over-expression and purification of proteins in *Picha pastoris*

For the construction of full length cDNA corresponding to the proteins, total RNA was isolated from murine liver tissue using Qiagen RNeasy Kit. 2µg of total RNA was reverse transcribed using 10 picomoles of oligo dT_{20} primer (Sigma Genosys) and 200 units of

MMLV RT SuperScript III (Invitrogen Cat. # 18080-93). PCR was performed using Accutaq^{TM} LA DNA Polymerase (Sigma Inc, Cat. # 8045). The proteins were expressed in the yeast *Pichia pastoris* using Easyselect^{TM}Pichia expression Kit (Invitrogen Inc. Cat. # K1740-01). cDNA was ligated with linearized pPICZαA vector. Ligation mixture was transformed into competent *E. coli* (DH10β) and selected on low Salt LB medium [1% Tryptone, 0.5% yeast extract, 0.5% Nacl (pH 7.5)] with antibiotic Zeocin^{TM}. Plasmid was isolated using standard alkaline lysis method and positive clones were verified by sequencing using 5′ *AOX1* (5′-GACTGGTTCCAATTGACAAGC-3′), 3′ *AOX1* (5′-GCAAATGGCATTCTGACATCC-3′) and α-Factor (5′-TACTATTGCCAGCAT-TGCTGC-3′). The verified positive clones were transformed to yeast *Pichia pastoris* using 100mM Lithium chloride instead of 100mM Lithium acteate as the cell wall of pichia is sensensitive to lithium acetate. Positive clones were selected on Zeocin (100µg/ml) containing YPD (1% yeast extract, 2% peptone, 2% dextrose) Agar plates. To screen for Mut^{+} (Methanol Utilization plus) phenotype (the promoter regulating the production of alchohal oxidase is the one used to drive hetrologous protein expression in *pichia*). the selected colonies on YPD zeocin plates were streaked on Minimal Dextrose with histidine (MDH) [1.34% Yeast Nitrogen base (stock solution 10X prepared with 134 g of yeast nitrogen base (YNB) with ammonium sulphate in 1000ml water), 4 x 10^{-5} % biotin (500X stock solution was prepared with 20 mg biotin in 100 ml of water and filter sterilized Store at +4°C), 2% dextrose (Stock solution 10X, prepared with 20g dextrose dissolved in 100ml water,filter sterilized and stored at 4^{0}C) and 0.004 % Histidine (stock solution 100X, prepared by dissolving 400 mg of L-histidine in 100 ml of water, filter sterilized and stored

at +4°C)] agar plates and Minimal Methanol with histidine (MMH) [1.34% YNB, 4 x 10-5% biotin, 0.5% methanol 0.004 % Histidine] agar plates. Clones obtained on MMH plate were selected for over-expression of protein.

A single colony from MMH plate was selected and grown in 1 liter Buffered Glycerol Complex Medium (BMGY) [1% yeast extract, 2% peptone, 100 mM potassium (10X stock solution 1.0M Potassium phosphate buffer; prepared by dissolving 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 pH 6.0 (adjusted by KOH)] medium at 28-30°C in a shaking incubator (250-300 rpm) until the culture reached an OD600 of 2-6. The cells were harvested by centrifuging at 1500g-3000g for 5 minutes at room temperature. Supernatant was discarded and to induce expression, cell pellet was suspended in 100 ml of Buffered Methanol Complex Medium (BMMY) [1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10-5% biotin] medium. 100% methanol was added to it at the final concentration of 0.5%. The cells were further grown for 24 hours. Cells were pelleted down at 1500g for 10 minutes and supernatant was collected and concentrated using 60% ammonium sulphate. The pelleted protein was suspended in DNA-protein binding (10mM HEPES, pH 7.9, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 10% Glycerol, 1mM DTT) buffer. Homogeneity of the purified protein was analyzed on 10% SDS-PAGE (Laemmli 1970) [10% Acrylamide/Bis {Stock solution 30% Acrylamide (29.2% acrylamide + 0.8% bis-acrylamide)}, 1X Separation buffer {4X Stock Solution (1.5M Tris-HCl (pH 8.8)+ 0.4% SDS)}, 1X Stacking buffer {4XStock Solution (1.0M Tris-HCl, pH 6.8 + 0.4% SDS)} 1X Laemmli sample buffer (10% Glycerol + 1% merceptoethanol + 2% SDS + 0.1% Bromophenol blue in 1X separating buffer). SDS-PAGE was run in 1X Running buffer (3g Tris-HCl + 14.4g Glycine + 1g SDS per litre),

stained by (Staining Solution 0.25g/litre of Coomassie Brilliant blue R-250 in Methanol: Acetic acid:Water:: 4:1:5) and destained by destaining Solution (Methanol: Acetic acid:Water:: 4:1:5)

Affnity purification of protein from nuclear extract

Ion Exchange Chromatography

DEAE- sepharose (anion-exchanger) pre-packed 1ml column (GE Lifesciences Cat. # 17-5055-01) was regenerated with 10 column volume (CV) of Equilibration buffer (25mM HEPES (pH 7.9), 50mM KCl, 5mM $MgCl_2$, 10mM NaCl, 15% Glycerol, 0.5mM EDTA, 1mM DTT). The concentration of KCl in nuclear extract (500mM) was reduced to 100mM using protein suspension buffer (25mM HEPES (pH 7.9), 30mM KCl, 5mM $MgCl_2$, 10mM NaCl, 15% Glycerol, 0.5mM EDTA, 1mM DTT). Diluted nuclear extract was applied to regenerated DEAE-Sepharose (anion exchnager) column at the rate of 1ml/minute. The column was washed with 5 CV of wash buffer (25mM HEPES (pH 7.9), 150mM KCl, 5mM $MgCl_2$, 10mM NaCl, 15% Glycerol, 0.5mM EDTA, 1mM DTT). The bound proteins to DEAE were eluted with elution buffer (25mM HEPES (pH-7.9), 500mM KCl, 15mM NaCl, 1mM EDTA, 1mM DTT, 15% glycerol, 5mM $MgCl_2$). The proteins in the eluate and the flow through were checked by EMSA for binding with 'GC'.

DEAE sepharose purified fractions of proteins, which showed binding, were applied to regenerated and equilibrated SP-FF (cation exchanger) (GE Lifesciences Cat. # 17-5054-01) column at the rate of 1ml/minute. The column was washed with 5 CV of wash buffer. Step gradient of 100-500mM KCl elution buffer (5 column volume each step) was used to elute the proteins from the column. All the fractions were desalted to get a final concentration of 30mM KCl using dilution buffer (25mM HEPES (pH-7.9), 30mM KCl,

15mM NaCl, 0.4mM EDTA, 1mM DTT, 15% glycerol and 5mM $MgCl_2$) and a final volume of 1ml using Amicon^{TM} (10kDa) filtration unit (Millipore Inc. Cat. # UFC801024).

To enrich DNA-binding proteins, the eluants from SP-FF column which showed binding with 'GC' in EMSA analysis were pooled together and applied to an equilibrated Heparin column (GE Lifesciences Cat. # 17-0407-01) at the rate of 1ml/minute. The column was washed with 5 CV of wash buffer. Step gradient of 100-500mM KCl elution buffer (5 column volume each step) was used to elute the proteins from the column. All the fractions were desalted to get a final concentration of 30mM KCl using dilution buffer (25mM HEPES (pH-7.9), 30mM KCl, 15mM NaCl, 0.4mM EDTA, 1mM DTT, 15% glycerol and 5mM $MgCl_2$) and a final volume of 1ml using Amicon^{TM} (10kDa) filtration unit.

DNA affinity chromatography

Double stranded 'GC' fragment was prepared by annealing biotinylated sense strand Bio'G'Fr-

5'TATGCTGGACAGGCGGGCGGGGCAGGGGGCGGGGCCGGGCGGGCAAAG3'

and antisense-strand 'C'bk-

5'TTGCTGCTGCTGCTTTGCCCGCCCGGCCCCGCCCCCTGC3'

and end-filling by DNA polymerase (Klenow fragment) as described in chapter three. 0.5ml of Streptavidin-Agarose (Sigma-Aldrich Inc. Cat. # 1638) was poured into 1ml empty column and regenerated with 10 column volumes of equilibration buffer. 100µl of biotinylated 'GC' (500 pico moles) was suspended in 400 µl of equalibiration buffer and applied to the column at the flow rate of 0.2ml/minute twice. The column was washed with

10ml of equalibration buffer and Heparin column purified protein fractions, were applied at the rate of 0.2 ml/minute in the presence of non-specific competitor (10µg poly (dI-dC)). This step was performed thrice. The column was washed with 5 ml of wash buffer. 'GC' bound proteins were eluted with 5 ml of elution buffer. Eluted proteins were concentrated using Amicon^{TM} 10 kDa filtration unit.

Silver staining of SDS-PAGE

The SDS-PAGE was silver stained using FOCUS-FASTsilver^{TM} protein gels staining kit (G Biosciences Cat.# 789-240). Briefly, the gel was fixed in 30% ethanol and 10% aetic acid solution in water for 30 minutes. The gel was washed twice, 5-10 minutes each in 10% ethanol and thrice with water. The gel was stained with FOCUS-Silver Stain with 65µl of Sensitizer-I for 30 minutes with gentle rocking. The gel was rinsed with water and developed with DEVELOPER-SENSITIZER-I and II solution [5g FOCUS-DEVELOPER ($NaHCO_3$) with 65µl of Sensitizer-I and 65µl of Sensitizer-II in 100 ml water] till the bands appear. The reaction was stopped by the addition of 2% acetic acid and gel was washed thrice with water.

MALDI-TOF analysis

The bands from gel were cut and prepared for MALDI-TOF. Briefly, the cut bands from gel were transferred to siliconised 1.5 ml eppendorf tubes and destained with destaining solution (30mM Potassium ferricynaide and 100mM Sodium thiosulphate solution) till the gel became colourless. The gel was washed with water to remove the reagents and the gel was washed four times with digestion buffer (100mM ammonium bicarbonate). The gel was dehydrated with two changes of 50% acetonitrile and dried in SpeedVac. The gel was rehydrated in 10µl Trypsin digestion buffer (stock solution 20 ug of Porcine trypsin

(Promega, Cat. # V5111) dissolved in 200 ul water) and incubated overnight at 37^0C. Peptides were extracted twice with 100µl of extraction solution (50% Acetonitrile, 0.1% trifloroacetic acid in water) and dried in SpeedVac. The peptides were cleaned through ZipTip (Millipore Cat.# ZTC18S008) and analyzed by mass spectrometry (performed at TCGA, Delhi).

Overexpression and Purification of Sp1

Purification of GST-Sp1

pGEX-2TKMCS-Sp1, a kind gift from Hans Rotheneder (Karlseder *et al* 1996) plasmid was transformed in *E.coli* Bl21(DE3) and *E.coli* BL21(pLys) hetrologous expression system and tranformants were selected on ampicillin (100 µg/ml)- Chloremphenicol (12.5 µg/ml) LB Agar plates. Single colonies were picked and grown overnight in 2ml LB medium with 100g/ml ampicillin. 10ml LB medium was inoculated with 0.1% of overnight grown culture and grown till OD_{600} reached 0.4 - 0.5. Induation was done in 0.5mM IPTG or 1mM IPTG containing medium by further incubation for upto 6 hours. 1ml sample was collected at the interval of 1 hour for the analysis of protein over-expression and electrophoresed on 10% SDS-PAGE. Once conditions were standardized, 200 ml culture was inoculated with 500 µl of 2ml overnight culture and grown at 37^0C till OD_{600} reached 0.5 (mid log phase). After induction for 5-6 hours, cells were were harvested by centrifugation and lysed in 10 ml of lysis buffer (50mM Tris-HCl pH 7.4, 200mM NaCl, 1% Triton X-100, 2.0 mM EDTA, 10% Glycerol, 1mM DTT, 0.1 mM PMSF, 100ug lysozyme and protease inhibitor cocktail) by incubation for 30 minutes on ice and finally disrupted by sonication. Cell lysate was centrifuged at 18,000g for 15 minutes in fixed angle rotor to remove particulate matters and cell debris. The resulting supernatant was

carefully collected and applied to pre-equilibrated with lysis buffer (- lysozyme) GST syringe column (GE Lifesciences 17-5130-01) at the rate of 0.5ml per minute. The column was washed with 10 bed volumes of wash buffer (same as lysis buffer), followed by elution with elution buffer (50mM Tris-HCl pH 8.0, 1mM DTT, 10mM GSH, 1.0mM EDTA, 10% Glycerol) with 1X protease inhibitor cocktail. Eluted proteins were concentrated with buffer exchange (Heparin column binding buffer: 20mM HEPES pH 7.9, 50mM KCl, 1.5mM $MgCl_2$, 10mM NaCl, 0.5mM EDTA, 10% Glycerol, and 1mM DTT) using Amicon concentrator with 10kDa cut off membrane. Concentrated GST column eluate was applied to pre-packed Heparin Syringe column at the rate of 0.5 ml/ minute. Heparin column was washed with 10 bed volumes of wash buffer (Heparin column binding buffer with 200mM KCl). The protein was eluted with elution buffer (Heparin column binding buffer with 500mM KCl) and the homogeneity of the protein was checked on SDS-PAGE followed by silver staining.

Western blotting

Western blotting was preformed as described by Towbin *et al* (1979). Briefly, the protein samples were electrophoresed on 10% SDS-PAGE and the gel was equilibirated in transfer buffer (25mM glycine, 192mM Tris and 20% methanol). Prewet Sponges, filter papers (slightly bigger than gel), gel and membrane (Amersham Hybond^{TM} – P, GE Lifesciences Inc. Cat. # RPN303F) were arranged in transfer apparatus [Mighty Small Transphor (GE Lifesciences Inc. Cat. # 80 6204 26)] and transfer was done according to manufacturer's instructions. Detection of protein was done using Amersham ECL Plus Western Blotting Detection System (GE Lifesciences Cat. # RPN2132). Briefly, after transfer the membrane was washed with 1X PBST (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3mM KCl, 135mM

NaCl, 0.05% Tween 20, pH 7.4) and incubated in blocking buffer (5 % non-fat dried milk powder in 1X PBST) for 4 hours at room temperature. Diluted primary antibody was added to the membrane and further incubated for 2 hours. Membrane was washed four times (10 minutes each) on a rocking platform. Diluted secondary antibody was added and protein detection as per standard protocol (Sambrook and Russel 2001).

Chromatin Immunoprecipitation Assay

Crosslinking and Sonication of chromatin

ChIP was done essentially as described by Antigone Kouskouti & Irene Kyrmizi (2005). All the steps were carried out at 4^0C unless otherwise mentioned. Nuclei were isolated as described in Chapter II. Chromatin crosslinking was carried out by drop-wise addition of 37% formaldehyde (final concentration of 0.75%) to the nuclei suspension and incubation at room temperature for 10 minutes. Fixation was stopped with the addition of glycine to the final concentration of 0.125M. Nuclei were centrifuged in round bottom tube and washed twice with tissue homogenization buffer for the complete removal of excess formaldehyde. Resulting nuclei were suspended at the concentration of 2 x 10^6 / 300µl of sonication buffer (50mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, and 0.5 mM PMSF with 1X Protease inhibitor coctail (Roche)). Suspension was incubated for 10 minutes on ice and sonication was carried out on ice in Bioruptur^{TM}200 (Diagenode Inc) (high power setting with 30s on and 1 minute off for 15 minutes) to get the chromatin size between the range of 200- 500bps (Figure. 4F). Sonicated sample was centrifuged at 21000g and supernatant containing chromatin (lysate) was collected. The lysate was pre-cleared by incubating at constant rotation with 60µl of 50% Protein-A Sepharose slurry (Upstate Inc. Cat.# 16-157) for 2 hours at 4^0C.

Supernatant (precleared chromatin) was collected after centrifugation at 2000g. 50µl ($1/20^{th}$ of the sample) of sample was saved (input DNA) and 4µg of Sp1 antibody (Abcam Cat. # 13370) was added to 1ml of pre-cleared chromatin and incubated overnight at constant rotation at 4^0C followed by addition of 60µl of 50% Protein-A Sepharose slurry to the samples and further incubation of 2 hours at constant rotation. Agarose beads were collected by centrifugation at 3000g for 1 minute. Washing of the beads was done twice with 1 ml Sonication buffer, twice with 1 ml Wash buffer A (50 mM Hepes (pH 7.9), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1X Protease inhibitor coctail (Roche), two times with 1 ml Wash buffer B (20mM Tris, (pH 8.0), 1mM EDTA, 250mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 0.5 mM PMSF, 1X Protease inhibitor coctail (Roche)) and twice with 1 ml TE buffer. Immuno-chromatin complex was eluted from the beads with the addition of 400 µl of elution buffer (50mM Tris, pH 8.0, 1mM EDTA, 1% SDS and 50mM $NaHCO_3$) (200µl twice) and incubation at 65^0C for 10 minutes followed by centrifugation at 18,000g for 1 minute. As can be seen in figure 4D, the fragmentation of DNA was achieved in the range of 200-700bp.

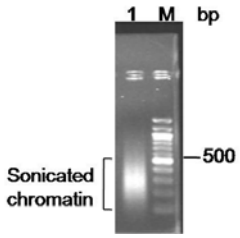


Figure 4D. Agarose gel showing sonication of chromatin. Lane 1 represents the sonicated chromatin after crosslinking from liver tissue, lane 2 represnts the 100bp DNA marker.

Reverse crosslinking of immuno-chromatin complex (supernatant) was done at 65^0C for 5 hours. 10µg of RNase A and 4µl of EDTA (500mM stock) was added to the samples after

reverse crosslinking and samples were incubated at 42^0C for 2 hours. Samples were extracted with phenol/chlorophorm and precipitated with 1 µl glycogen (from 20 mg/ml stock), 40 µl Na-acetate (from 3M stock pH 5.4) and 1 ml 100% ethanol.

Bisulphite sequencing

Bisulphite treatment was done as described elsewhere (Gokul *et al* 2007). Briefly, DNA was denatured by boiling in a water bath for 6 min and immediately snap chilled for 2-3 min.The denatured sample was incubated with 4ul of 2M NaOH (final concentration 0.3M) for 15 minutes at 50^oC. Meanwhile a 2% Low melting point agarose (BMA) was prepared in water. The denatured DNA was mixed with two volumes of low melting point agarose. The bisulfite modification solution was prepared by mixing Sodium bisulfite (1.9g in 2.5ml water and 750ul of 2M NaOH) with hydroquinone (0.055 grams in 500µl of water). 1ml of this modification solution was aliquoted into a 2ml eppendorf and overlayed with 750ul of cold mineral oil. 10 µl aliquots of the DNA-agarose mix were pipetted into the cold mineral oil from top to form the beads. The beads are allowed to firm up for some time and pushed into bisulfite solution. The tubes were incubated in ice for 30 min and at 50^0C for 3.5 hours. The solutions were removed from the beads and the beads were washed 4 times (15 min each) with 1ml of 1X TE (pH 8.0) at room temperature. The beads were incubated with 500ul of 0.2M NaOH for 15 minutes at 30 degrees, and washed (three times) with 1ml 1X TE (pH 8.0) for 10 min. The beads were washed with sterile water twice for 15 min each at 1000 rpm in thermomixer. To melt the agarose, beads were incubated at 60-65 degrees and 5 ul was taken for a 25ul PCR. The primers for PCR on bisluphite treated DNA for second intronic region of *Neuronatin* were designed using MethPrimer software (Li and Dahiya 2002). PCR were done for 30 cycles each as follows:

95^0C for 5 minutes, 95^0C for 45 seconds, 55^0C for 1 minute, 72^0C for 1 minute and final elongation at 72^0C for 5 minutes. PCR products were run on 1.5% agarose gel in 0.5X TBE. DNA was eluted from the gel using Gel extraction kit. Extracted DNA was ligated to pBSK+ vector compatible of TA cloning. The ligated mixture was transformed into competent DH10β cells. Plasmids were isolated using standard methods (Sambrook 1989) and sequenced.

Results

I. Yeast Mono hybrid Assay

In Yeast mono hybrid assay DNA-binding proteins are expressed as fusions to the GAL4 vector and the reporter vector containing DNA binding elements are co-transformed in yeast and selected for the auxotrophy. Interaction between a DNA-binding protein and target sequence stimulates transcription of HIS3, enabling yeast (His auxotroph) to grow on minimal media lacking histidine.

Cloning of 'GC' and 3-Amino-1, 2, 4-triazole concentration optimization

Even though, self ligation of 'GC' generated multiple copies of 'GC' (figure 4E), we were able to clone only single and two copies of 'GC' into pHIS2 vector in *E. Coli* (DH10β). The minimal HIS promoter in pHIS2 vector has a propensity for the leaky expression of *His3* gene in yeast strain, Y187 and the DNA target element inserted upstream of this minimal promoter can alter the expression level of *His3* gene, thus making GCpHis2 transformant derived from yeast (Y187) capable of growing on SD medium lacking histidine. 3-amino-1, 2, 4-triazole (3-AT) is a competitive inhibitor of the yeast His3 protein (His3p) and is used to inhibit low levels of His3p expression. Therefore,

tranformants GCpHis2 and $(GC)_2$pHis2 were plated on SD/-His/-Trp plates containing different concentrations (10mM to 100mM) of 3-AT to optimize the *His3* gene expression.

Although a small amount of 3-AT (5mM to 45 mM) is generally sufficient to suppress the background growth of transformants on SD/-His medium. The results shown in figure 4F indicate that transformants, pHis2GC and pHis2GC_2 were able to survive even on 70mM 3-AT.

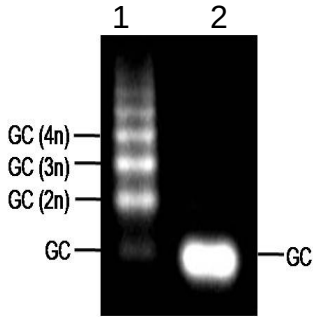


Figure 4E. Cloning of 'GC' upstream to the minimal promoter of HIS3 gene. Agarose gel showing Self ligation of 'GC'. Lane 1 self ligation of 'GC', lane 2 'GC'.

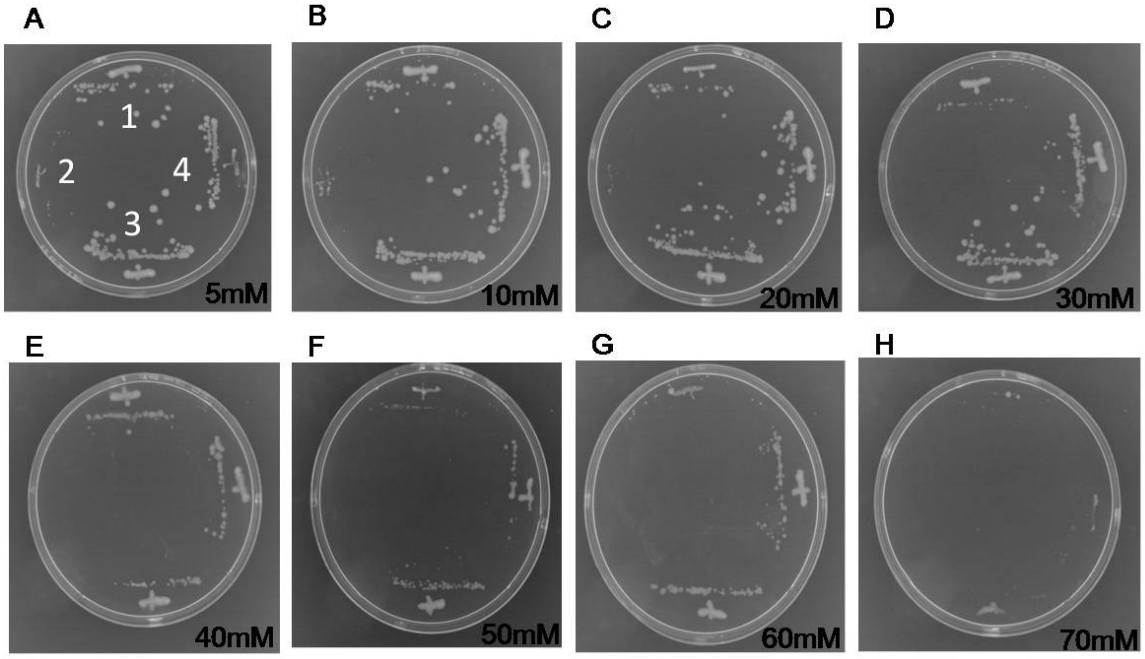


Figure 4F. Optimisation of 3AT concentration to check leaky expression of HIS3 protein. Concentration of 3-AT used is shown at the bottom right hand corner of each plate. 1, 2, 3, 4 represents yeast transformants of pHis2, p53His2, GCpHis2, $(GC)_2$pHis2, respectively grown on SD/-His-Trp.

The ability of 'GC'pHis2 transformants to grow on SD/-His/-Trp medium containing very high concentrations of 3-AT suggested yeast proteins might have binding affinity for 'GC' DNA and their interaction with 'GC' could be the factor responsible for the leaky transcription shown by HIS3 reporter gene. To analyze whether this was the cause, an EMSA was done in the presence of 1000 times more non-specific DNA (poly dI-dC) with liver nuclear extract and yeast nuclear protein extract as protein source. The EMSA results in figure 4G showed that one or more yeast nuclear proteins can interact with 'GC' DNA.

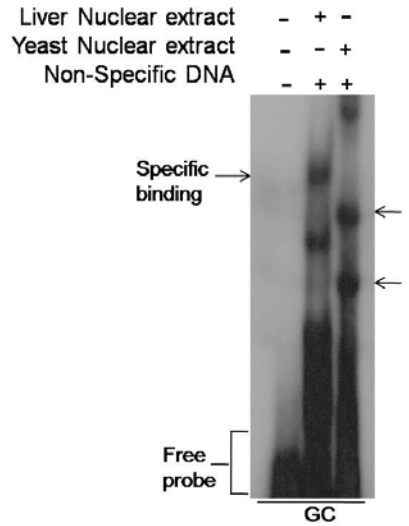


Figure 4G. EMSA showing the binding of yeast nuclear proteins to 'GC' DNA**.** 38 base pair fragment of 'GC' was end-labeled using γP^{32}ATP. Arrows indicate the specific binding of protein with 'GC' probe.

Identification of 'GC' binding proteins

Even though 70mM 3-AT concentration was found to be optimal for the screening one hybrid using pHis2GC construct, this concentration was higher than what is recommended for yeast transformation, as 3-AT above 45mM concentration can lyse freshly prepared

spheroplast yeast cells. To avoid the lysing of the cells, the yeast cells were transformed with pHis2GC, cDNA and *Sma*I linearized pGADT7Rec2 by spheroplasting method and transformed cells were plated on SD/-His-Trp-Leu with 45mM 3-AT. Transformed colonies were re-streaked on 60mM 3-AT plates to rule out the false positives. 110 colonies were obtained on selection plate and were streaked on SD/-His-Trp-Leu with 45mM 3-AT. Finally, 60 yeast colonies were obtained on SD/-His-Trp-Leu plates with 60mM 3-AT. Total DNA was isolated from these colonies and transformed in *E.coli* (DH10β) and selected on LB-ampicillin plates using the selection marker of pGAD-T7Rec2 plasmid (cDNA cloning Vector). The plasmid, pGADT7Rec2 was isolated from the bacteria and cDNA insert was checked by *Bgl*II and *Xho*I enzyme digestion (figure 4H).

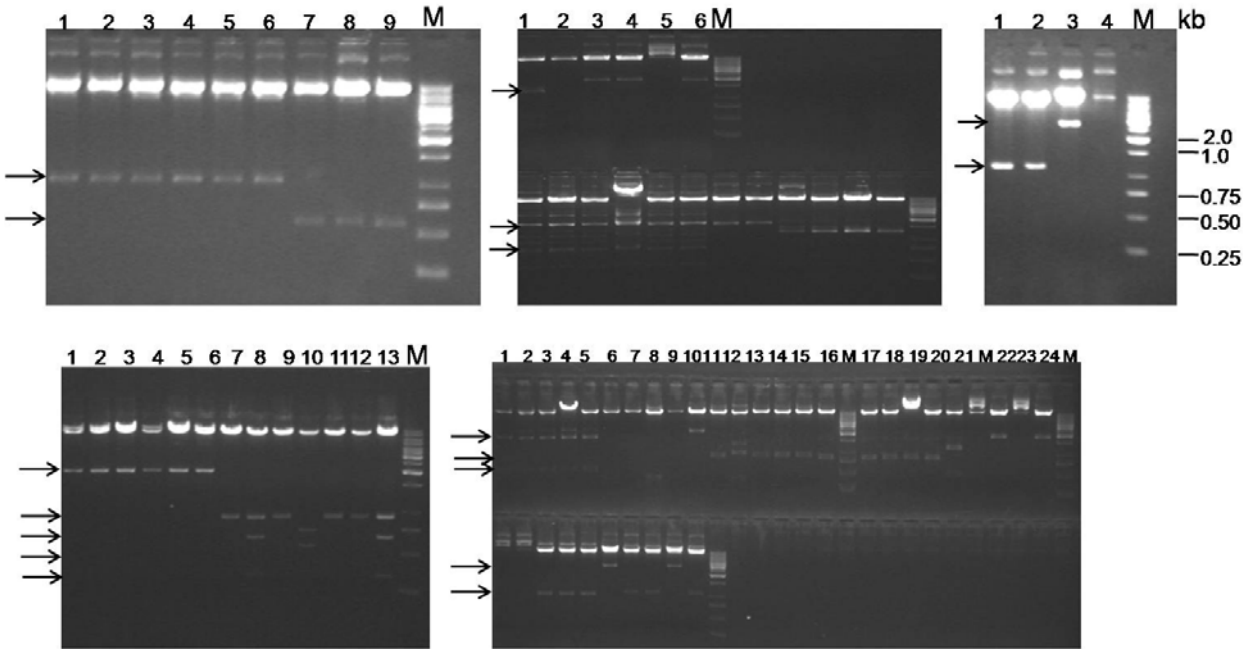


Figure 4H. Agarose gels showing the screening of positive clones for interaction of library protein with 'GC' element in the reporter vector. pGADT7-Rec2- cDNA plasmids were isolated from the yeast Y187 colonies grown on SD/-his/-trp/-leu. Plasmids were digested by Bgl II and Xho I. 'M' represents 1kb DNA ladder, arrow represent cDNA insert.

All the 60 clones of pGADT7-Rec2 containing cDNA from mouse library were sequenced using BD SMART^{TM} and CD III primers. The sequences of cDNA were searched for the match on www.ncbi.nlm.gov using BLAST. Table 4A tabulates some of the identified proteins.

Clone no.	**Gene ID and Name**	**Function**	**Motifs**
YMH8-2	16818 & Lck	Signal Transduction	Tyrosine Kinase family
YMH9-1	73412 & Txndc3	Redox Reactions	Thioredoxin domain
YMH10-1	73412 & Txndc3	Redox Reactions	Thioredoxin domain
YMH16-2	217578 & Baz1a	Acetylation of Lysine residues on histone tails	Bromodomain & PHD
YMH18-2	66865 & Pmpca	Unknown	
YMH35-1	234967 & Slc36a4	Solute carrier family	Solute carrier family
YMH40-2	30054 & Rnf17	Spermatogenesis	Tutor domain
YMH2-3	16783 & Lamp1	Unknown	Lamp domain
YMH4-1	83962 & Btbd1	Mediates transcriptional repression and interacts with Histone deacetylase corepressor	Btb domain
YMH4-2	70144 & Lrch3	Unkown	Leucine rich protein
YMH5-1	57784 & Bin3	Unknown	Bridging integrator domain
YMH12-1	76088 & Dock8	Potential guanine nucleotide exchange factor	Dedicator of cytokinesis family
YMH12-3	107569 & Nt5c3	Unknown	Uridine

			monophospahte hydrolase1 (UMPH-1)
YMH13-4	69339 & Ccdc54	Unknown	Unknown
YMH17-1	226982 & Eif5b	Translation	Eukaryotic Translation initiation factor
YMH22-1	71838 & *Phf7*	Unknown	Plant homeodomain
YMH25-2	8459 & Pabpc2	Regulation of alternative splicing	RNA recognition motif
YMH28-2	12314 & Calm2	Signal transduction	Calcium binding motif
YMH28-1	665155 & Signal Recognition particle	Recognizes N-terminal signal sequences of newly synthesized polypeptide at the ribosome	Signal recognisation particle
YMH24-4	232223 & Txnrd3	Post translational modifications	Thioredoxin reductase
YMH34-1a	382867 & Zfp488	Unknown	Zinc finger
YMH34-1b	235907 & Zfp71-rs1	Unknown	Kruppel associated box (KRAB)
YMH34-1C	15493 & Hsd3b2	Unknown	Unknown
YMH-37-3	15369 & Hmox2	Heme oxygenase	Heme Homeostatis and cellular signaling in mammals
YMH38-1	12419 & Cbx5	Organization of chromatin structure	Chromodomain
YMH39-1	98432 & Phlpp	Unknown	PH domain
YMH52-1	237465 &	Unknown	Unknown

	Ccdc38		
YMH52-2	22187 & Ubb	Unknown	An1
YMH53-1	18646 & Pfr1	Unknown	Membrane attack complex/ Perforin
YMH53-2	17709 & Cox2	Cytochrome C oxidase	Cytochrome C oxidase
YMH54-1	66480 & Rpl15	Unknown	Ribosmal L 15
YMH41-2	52348 & Vps37a	Modifier of Rudimentary protein	Modifier of Rudimentary protein
YMH42-1	83563 & USp26	Unknown	Peptidase C 19
YMH43-1	50492 & Thop1	Unknown	Peptidase Family M3
YMH47-1	72674 & Adipor1	Unknown	Uncharacterised protein family
YMH48-1	216618 & Ccdc104	Unknown	Unknown
YMh11M5	Phf20	Unknown	Bromodomain, PHD

Table 4A. List of the Proteins interaction with 'GC' in yeast mono hybrid assay.

Seven proteins with various domains known to play a role in chromatin organization were found to be interacting with 'GC' in this screen and are shown in (Table. 4B).

Clone Name	**Gene Name**	**Motif**
YMH 16-2	*Baz1a*	Bromodomain, PHD
YMH 11M5	*Phf20*	PHD
YMH 17-1	*Phf7*	PHD
YMH 25-2	*Pabpc2*	RRM domain
YMH 34-1	*Zfp488*	Zn finger protein
YMH 38-1	*Cbx5*	Chromodomain

YMH 39-1	*Phlpp*	PHD

Table 4B. List of the proteins identified by yeast one hybrid assay with a possible role in chromatin organisation

Overexpression of *Phf7* and its interaction with 'GC' DNA

To examine whether *Phf7* protein, identified as a 'GC' interactant in yeast mono hybrid screen, could bind to 'GC' in a methylation dependent manner, *Phf7* cDNA (figure 4I) was prepared using the primers (*PHF7*Fp-5'-GAAGAATTCATGAAGACTTTAAAAGAAAA3') and *PHF7*BK (5'-TCTTCTAGACTAACTCGTGGTTGAA'GC'AG3') and cloned into pCDNA 3.1 vector. It was later sub-cloned into *Pichia* vector pPICZαA and checked by sequencing for correct sequence and frame with respect to its α-signal DNA. pPICZαA-*Phf7* was transformed into *Pichia pastoris*. The protein was over-expressed and purified as mentioned above from the yeast *Pichia pastoris*. The purified protein (Figure 4J) was used for EMSA analysis to examine its interaction with 'GC' DNA (Figure. 4K).

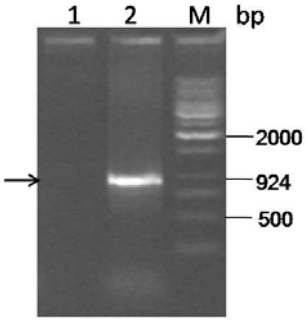


Figure 4I. Agarose gel showing the synthesis of cDNA of PHF 7 gene. Lane 1 PCR negative control, Lane 2 synthesis of cDNA of *Phf7* gene, 'M' 1 Kb DNA marker.

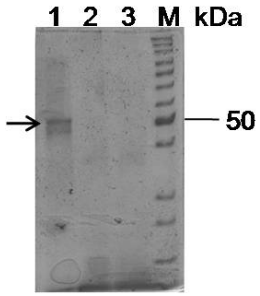


Figure 4J. 10% SDS PAGE scan showing the expression of *Phf7* in *Pichia pastoris*. Lane 1 represents the expression of *Phf7* in BMMY media. No expression of *Phf7* was observed in BMGY and BMMH medium (Lane 2 and 3). 'M' denotes protein marker (MBI fermentas Cat. # SM0661).

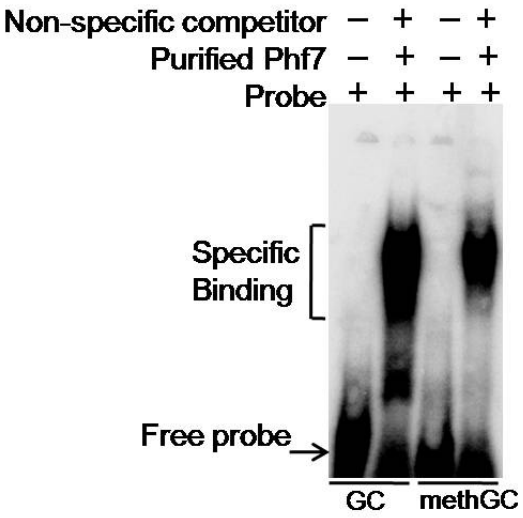


Figure 4K. EMSA analysis for the interaction of *Phf7* with 'GC' DNA. Binding was observed with 'GC' as well as with 'methGC'

EMSA shows that even though *Phf7* interacted with 'GC' DNA, it also interacted with methylated 'GC' DNA. The role of *Phf7* with respect to the imprinting mechanism of *Neuronatin* could not be established, as the requirement was for a protein with an ability to differentiate between the methylated and unmethylated 'GC'.

Similar experiment to test other chromatin proteins for 'GC' binding is being taken by in the lab.

II. Identification of ‘‘GC’’ binding proteins by DNA-affinity chromatography

DNA affinity chromatography has been used extensively to purify DNA sequence-specific binding proteins (Gadgil *et al* 2001) and is based on the fact that a DNA binding protein recognizes a specific consensus sequence with affinities in the picomolar range and have a 10^3-10^5 higher affinity for this specific DNA sequence than for any other DNA sequences. However, due to multitude of DNA binding proteins present in the nuclear extract, it is usually difficult to resort to DNA affinity chromatography directly on a nuclear extract. Therefore, to isolate proteins which interact with ‘GC’, purification was done through several chromatography steps viz.. Ion exchange chromatography, Heparin chromatography followed by DNA-affinity chromatography. Nuclear extract was prepared essentially as described previously (see Materials and Methods, Chapter II). The purification of the protein was done as described elsewhere (Coligan, *et al* 2000, Briggs and Kadonaga 1986, Jones and Kadonaga 1987, Gadgil *et al* 2001) and outlined in figure 4L.

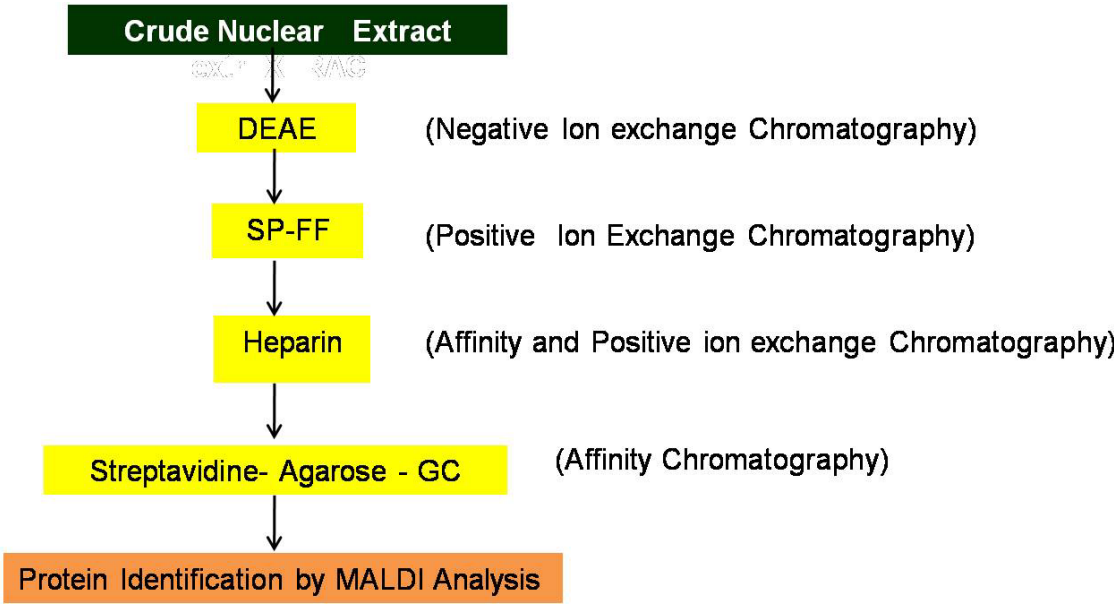


Figure 4L. Schematic flow chart for purification of ‘GC’binding protein(s) from nuclear extract.

EMSA analysis showing the binding of DEAE-sepharose purified nuclear protein extract with ‘GC’ DNA

Since DNA-binding proteins are expected to be positively charged, it was decided to perform two step ion-exchange chromatography. In the first step negatively charged proteins were depleted by anion exchange chromatography (DEAE Sepharose column), followed by enrichment of positively charged proteins by cation exchanger (SP-FF Sepharose column). Nuclear protein extract from liver tissue with low salt concentration (150mM) was applied to 1ml prepacked DEAE anion exchanger syringe column. The flow through fraction and eluted fractions were checked for ‘GC’ fragment binding ability. As expected EMSA results (figure. 4M) showed ‘GC’ binding acivity was present in the flow through from the column.

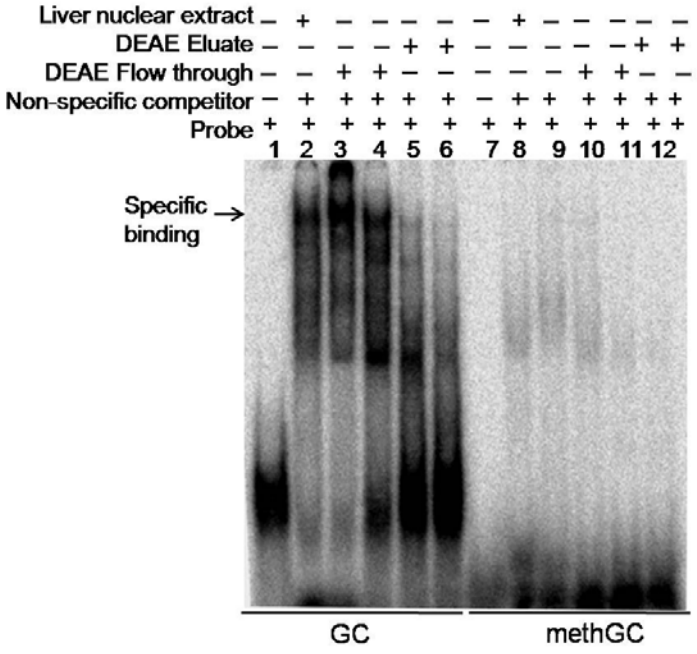


Figure 4M**.** EMSA analysis showing the binding of DEAE-sepharose purified nuclear protein extract with ‘GC’ DNA**.** Lane 1 and 7 represents radiolabeled ‘GC’ and ‘methGC’

respectively. The binding has been observed with the flow through of DEAE sepahrose column. Neglible binding was observed with methylated 'GC' probe (lane 7-12)

EMSA analysis showing the binding of SP-FF sepharose purified nuclear protein extract with 'GC' DNA

Flow through fractions from DEAE sepharose column (anion exchanger), were desalted (material and methods) and applied to SP-FF column (cation exchanger). Again all the fractions including the flow through and the salt eluted fractions were desalted and analyzed for 'GC' binding capability by EMSA. As shown in figure 4N, 'GC' binding protein activity was recovered from SP-FF column at 300mM KCl concentration.

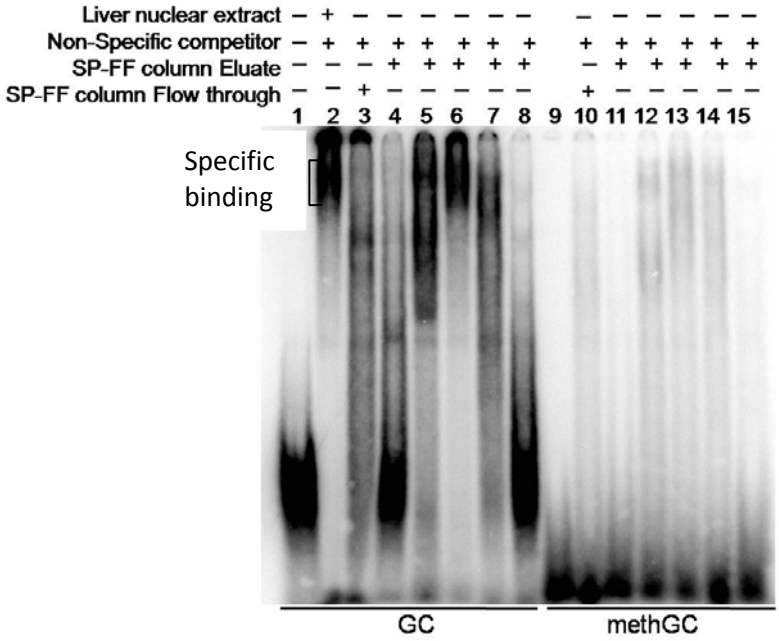


Figure 4N**.** EMSA analysis showing the binding of SP-FF sepharose purified nuclear protein extract with 'GC' DNA**.** Lane 4 and 11 represents elution with 100mM KCl salt, 5 and 12 represents elution with 200mM KCl salt, 6 and 13 represents elution with 300mM KCl salt, 7 and 14 represents elution with 400mM KCl salt, and lane 8 and 15 represents elution with 500mM KCl salt. 300mM KCl eluation showed the maximum interaction of protein with 'GC' DNA. Methylated 'GC' showed negligible binding in all the elution fractions.

EMSA analysis showing binding of DEAE-sepharose, SP-FF sepharose, and heparin agarose column purified, nuclear protein extract with 'GC' DNA

As heparin binding is known to enrich DNA binding proteins (Kessavetis *et al* 1989, Gadgil *et al* 1999), for the enrichment of the DNA binding proteins (Kovelman *et al* 1992, Malhotra *et al* 1993), the desalted protein fraction eluted from the cation exchanger SP-FF, was applied to a Heparin column. Protein fractions obtained after step gradient salt elution were desalted and analyzed by EMSA for 'GC' binding protein. EMSA showed 'GC' bindng activity was in 300mM KCl eluate fraction from the Heparin column (figure 4O).

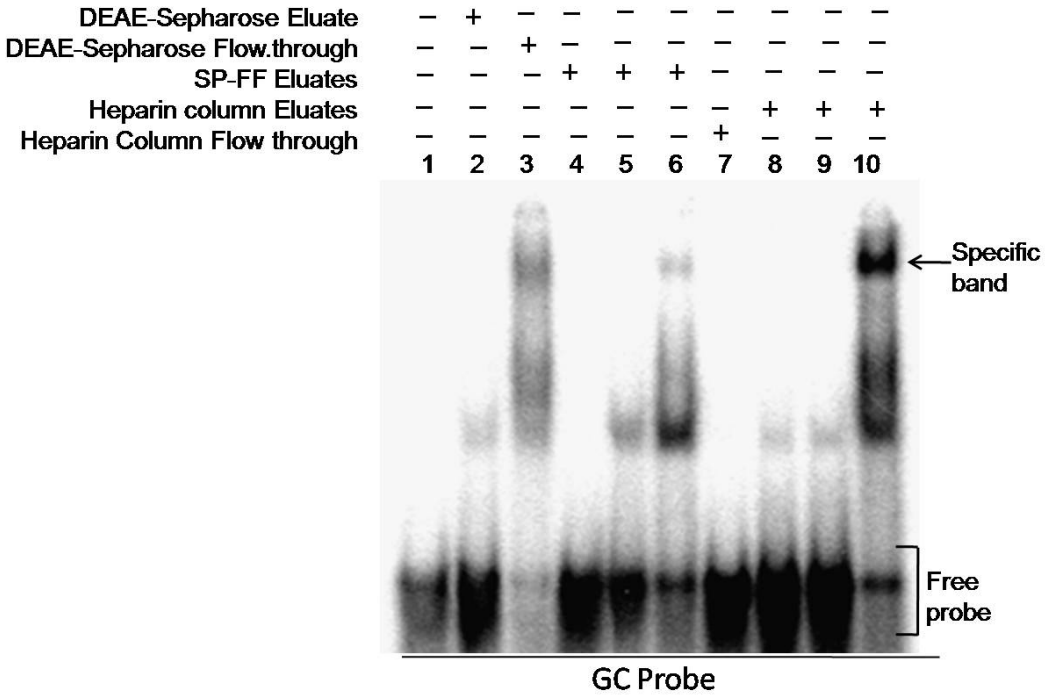


Figure 4O. EMSA analysis showing binding of DEAE-sepharose, SP-FF sepharose, and heparin agarose column purified, nuclear protein extract with 'GC' DNA. Protein binding to DNA was observed in lane no. 3 (DEAE-sepharose flow through), lane no. 6 (300mM eluant of SP-FF column) and lane no. 10 (300mM eluant of heparin column). Arrow represents specific binding while bracket represents free probe.

EMSA analysis showing interaction of DNA affinity chromatography purified proteins with 'GC' DNA

The proteins partially purified through a combination of chromatographic steps including DEAE Sepharose (anion exchanger), SP-FF sepharose (cation exchanger), and Heparin column were finally loaded onto 'GC'agarose column for affinity chromatography (see Material and Methods). The proteins were eluted using a KCl gradient (100-1000mM). 'GC' binding proteins were eluted at 300mM KCl as can be seen in figure 4O. Since, the main property that was required for 'GC' binding protein was its ability to distinguish between unmethylated and methylated 'GC', the proteins eluted after 'GC'affinity chromatography were subjected to EMSA with both methylated and unmethylated 'GC' fragment (see chapter 3 for EMSA conditions. As can be seen in figure 4P, strong binding was observed with unmethylated 'GC' but negligible binding was observed with methylated 'GC' probe.

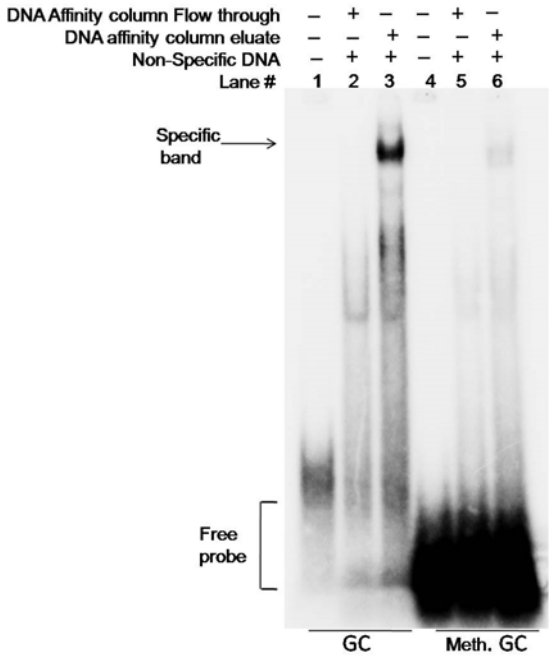


Figure 4P. EMSA analysis showing interaction of DNA affinity chromatography purified proteins with 'GC' DNA**.** The protein purified through DNA affinity column

chromatography showed negligible binding with methylated 'GC' probe (compare lane 3 and lane 6).

The summary of the various purification steps and the results obtained are shown in figure 4Q.

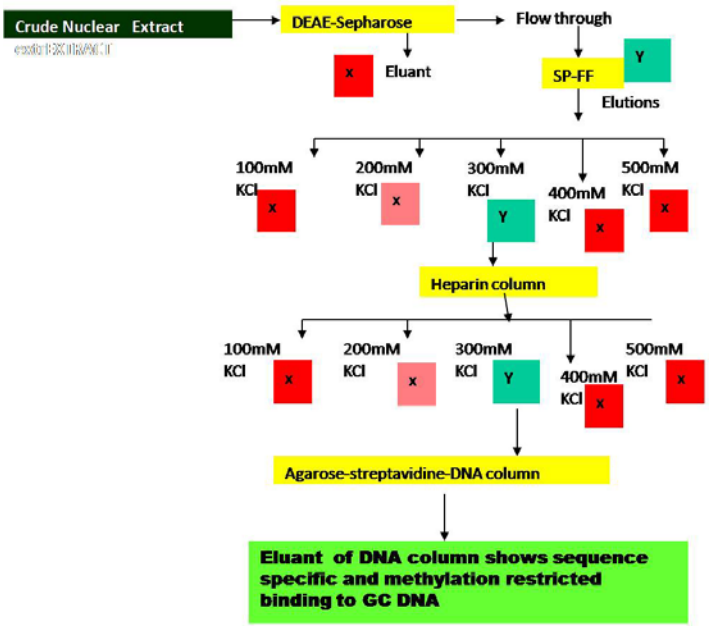


Figure 4Q. Schematic diagram for purification of sequence specific DNA binding protein(s) from Nuclear extract.

Identification of the 'GC' binding protein by Mass Spectroscopy

For the identification of the protein (s) which interacts with 'GC' DNA in a methylation dependent manner, the proteins purified through DNA-affinity chromatography were fractionated on 10% SDS PAGE (figure 4R), the protein bands cut out from the gel and send for peptide fingerprinting by MALDI-TOF at The Centre for Genomic Excellence (TCGA), Delhi.

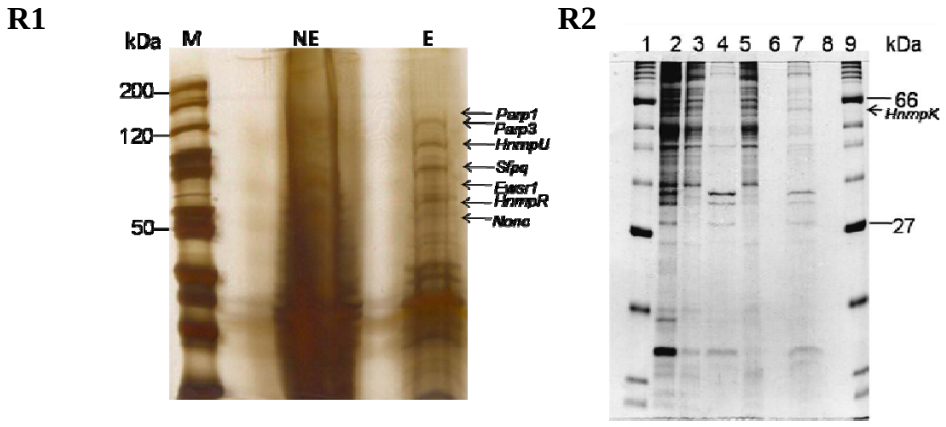


Figure 4R. Protein purification using DNA affinity chromatography. R1) Protein was purified from brain tissue. M= Broad range marker (MBI fermentas Cat. # SM0661), NE= nuclear extract, E=Eluate of DNA affinity column. R2). Protein purification from brain tissue. Lane1,9=Marker, 2=Brain nuclear extract, 3&4=Unbound of only beads and anchored DNA respectively, Lane4= 500mM KCl eluate of beads only, lane 6,8 blank, 7= 500mMKCl eluate of anchored GC to beads. A band of ~60kDa(lane# 7) is present only with the eluate of anchored GC which is not present in the control(lane # 5) .

MALDI-TOF analysis of the protein bands identified the following proteins (Table 4C):

S. No.	**Gene symbol**	**Molecular Weight(kDa)**	**Protein Name**	**Motifs**
1.	*Sfpq*	*75.5*	*Spilicing factor, proline/glutamine rich (polypyrimidine tract binding protein associated)*	RRM, RRM1, NOPS
2.	*HnrnpR*	*60.1*	*Heterogenous nuclear ribonucleoprotein- R*	RRM
3.	*Nono*	*54.54*		RRM, RRM1, NOPS
4.	*Ewsr1*	*68.418*	*Ewing sarcoma breakpoint region 1*	RRM, zf-RanBP
5.	*Parp1*	*113.1*	*poly (ADP-ribose) polymerase family, member 1*	Parp_like, ligA, zF-PARP, WGR, PADR1

6.	*Parp3*	*91.53*	*poly (ADP-ribose) polymerase family, member 1*	Parp_like, WGR
7.	*HnrnpU*	*87.9*	*Heterogenous nuclear ribonucleoprotein U*	SAP, SPRY, ATPase
8.	*HnrnpK*	*66.0*	*Interacts with transcription factors and regulate transcription, binds to DNA and RNA*	RNA-binding domain, contains KH-I domain

Table 4C. DNA affinity purified proteins, identified by MALDI-TOF analysis.

The identified proteins need to be tested for their ability to bind 'GC' fragment in a methylation restricted manner.

Sp1 protein binding to 'GC' fragment

In our bioinformatic analysis of the 'GC' fragment ['GCss' (NNNNGGGCGGGCCGGGCGGGAAA)], we found two binding sites for the transcription factor Sp1 (see chapter three). Though there are some studies showing Sp1 binding to only unmethylated DNA (Macleod *et al* 1994, Brandis *et al* 1994, Douet *et al* 2006, Luca *et al* 2007), there are equal number of studies which have showed otherwise (Jane *et al* 1993, Holler *et al* 1993). To analyze, if Sp1 can bind to 'GC' in a methylation restricted manner, the following experiments were performed.

Supershift EMSA

In EMSA while the unbound DNA fragment migrates faster during electrophoresis through a polyacryamide gel, proteins binding to the fragment significantly slow its migration. One simple way to characterize proteins that comprise protein–DNA complexes identified by EMSA is to use antibodies to known transcription factors (Kristie *et al* 1986). Incubation

of the antibody with protein in binding buffer before adding the labeled probe can affect the assay in one of three ways. If the protein recognized by the antibody is not a component of the protein–DNA complex, the antibody will have no effect on complex mobility. If the protein recognized by the antibody is a part of the complex, the antibody can bind to the protein-DNA complex, forming a ternary complex with further reduced mobility (supershift complex), or it can bind the protein in a way that disrupts the protein-DNA complex, resulting in the disappearance of the low mobility band, without the appearance of a supershifted band. To analyze if Sp1 protein forms a part of the protein-'GC' DNA complex, an EMSA analysis was done using liver nuclear extract (Figure 4G1a) and DNA affinity column purified protein (Figure 4G1b) with Sp1 antibody. Antibodies against various other DNA binding proteins were also used. As the results showed a supershift was observed only with Sp1 antibody.

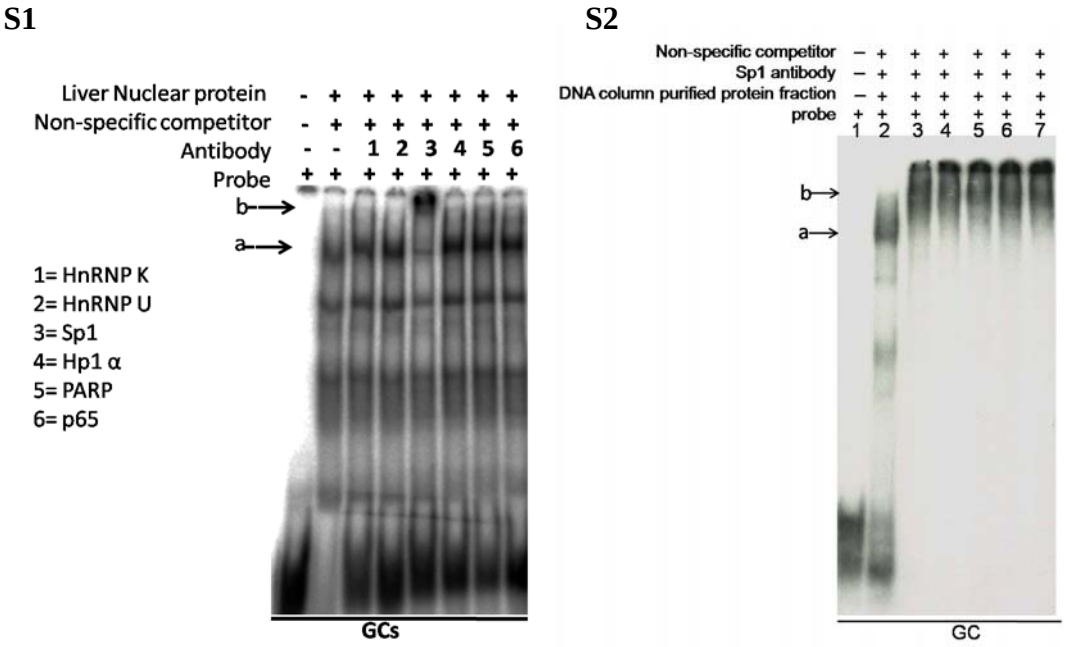


Figure 4S. Supershift assay using various antibodies. S1). Along with Sp1 antibody, antibodies against the proteins which are known to play a role in chromatin organization were used. Arrow 'a' represents the band shift position, while arrow 'b' represents the super shifted position using Sp1 antibody. S2). EMSA analysis showing supershift assay with DNA affinity purified protein. Antibody was added in the increasing concentration Lane 3, 4, 5, 6, 7, contains 125ng, 250ng, 500ng, 1µg, 2µg of anti Sp1 antibodies.

Supershift using nuclear protein extract from various tissues

To further analyze the interaction of Sp1 with ''GC'' DNA, nuclear extracts from various tissues were prepared and supershift assay was done using Sp1 antibody. As the results (figure 4T) showed Sp1 was found to be interacting with 'GC' in all the tissues analyzed.

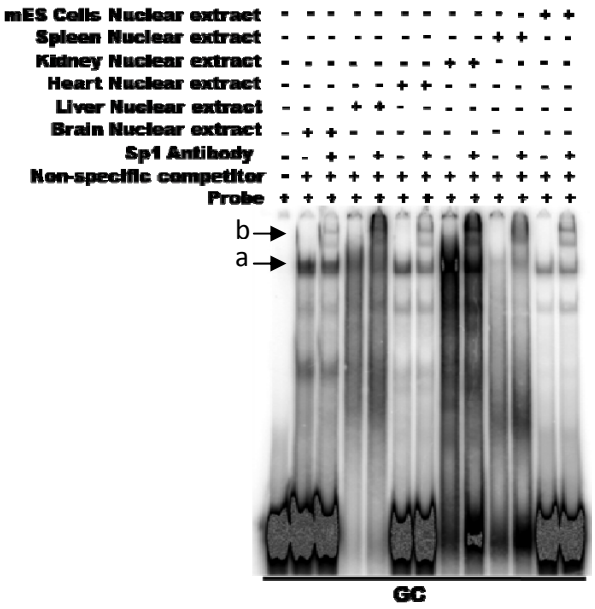


Figure 4T. Supershift using nuclear protein extract from various tissues**.** The probe used is 'GC'and NN3 (200ng) and polydI-dc(100ng) was used as non-specific competitor in each lane. Sp1 antibody was used at the concentration of 125ng/lane. 'a'- the band shift position;'b'- the super shifted position using Sp1 antibody.

EMSA with Sp1 protein immunodepleted nuclear extract

To further prove the results of supershift assay, nuclear extract was depleted of Sp1 protein using Sp1 antibodies and EMSA analysis was done using Sp1 protein depleted nuclear extract. As the result in figure 4U shows, the mobility shift was not observed in the Sp1 depleted nuclear extract, while the depletion of other DNA binding protein showed no effect.

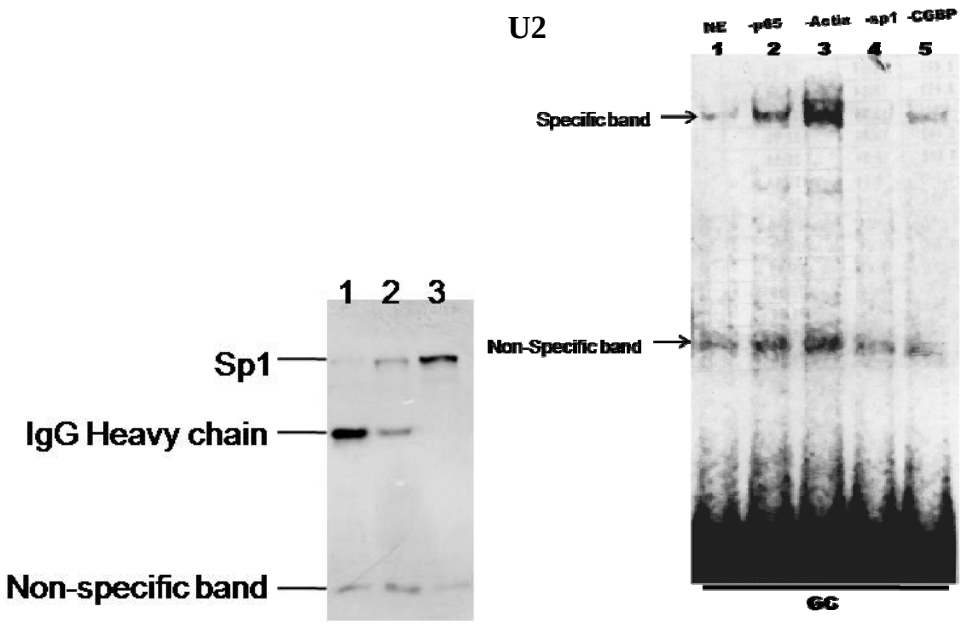


Figure 4U. EMSA with nuclear extract after Sp1 protein immunodepletion. U1) western blotting analysis showing the depletion of SP1 from the nuclear extract. Lane 1 represents Sp1 protein depletion from nuclear extract after two rounds of depletion, lane 2 represents Sp1 protein depletion from nuclear extract after one round of depletion, lane 3 represents nuclear protein extract. U2) EMSA analysis showing the interaction of 'GC' probe with nuclear extract. Lane 1; nuclear extract, lanes; 2-5 represents nuclear protein extract depletion with p65, actin, Sp1, and CGBP antibodies respectively.

III. Expression and purification of Sp1 protein in bacteria

To test whether Sp1 protein could bind specifically to ''GC'' fragment, it was decided to overexpress Sp1 protein to be used for EMSA. The optimization of Sp1 protein expression was done in two strains of *E.coli BL-21 (DE3)* and *BL-21 (Plys*). The maximum expression of the protein was found in BL-21 (Plys) strain (figure 4V1) after 4 hours of induction in 0.5mM IPTG. Even though majority of the fusion protein was found in the inclusion bodies (figure 4V2), only the soluble protein present in the supernatant was taken up for purification.

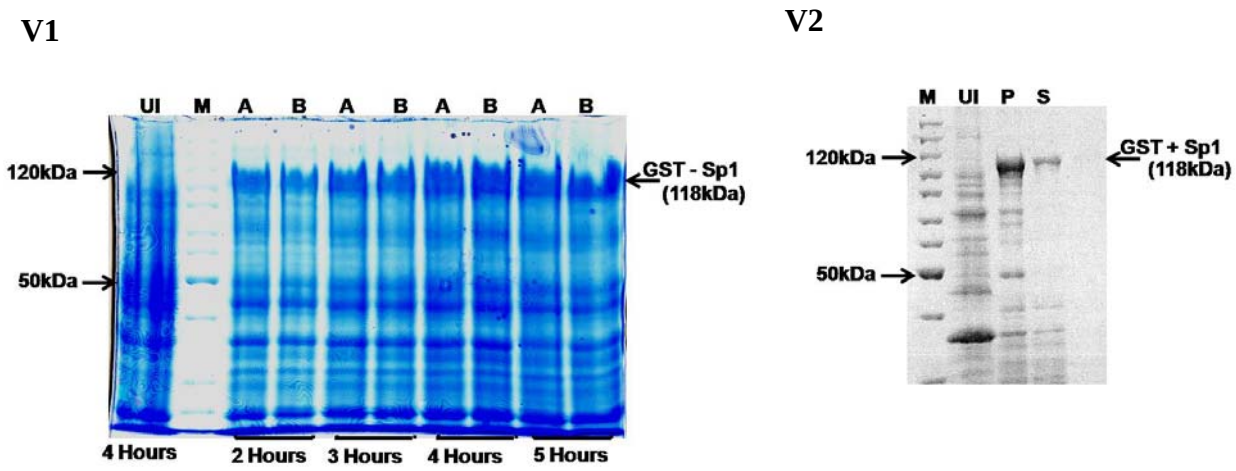


Figure. 4V. SDS-PAGE showing the induction of pGEX-2TKMCS-SP1 vector and purification of GST-SP1 protein**.** 4H1) Maximum expression of Sp1 protein was observed in BL-21 Plys after 4 hours of induction upon with IPTG. UI = bacterial cell lysate before induction, 'M' Marker. 'A' and 'B' represents 0.5mM and 1.0mM IPTG concentration, respectively. 4H2) Protein was observed to form inclusion bodies. 'P'= pellet of cell lysis, 'S'= supernatant of cell lysate.

The cell lysate was purified through Glutathione syringe column (see Material and Methods) and the homogeneity of the purified protein was observed on 10% SDS-PAGE (figure 4W1). GST-column purified GST-SP1 fusion protein was purified using heparin column to more than 95% homogeneity (figure 4W2).

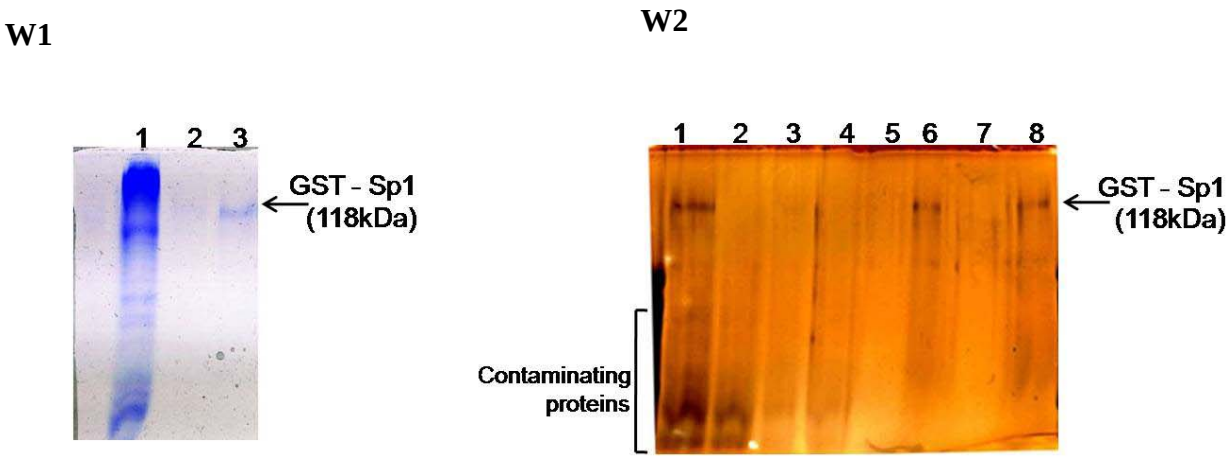


Figure. 4W. SDS-PAGE scan of the purification of Sp1 protein using GST column and Heparin column. W1) lane 1; pooled GST-column eluate, lane 2; wash, lane 3; eluate from

GST-Column. W2) Silver stained SDS-PAGE scan showing heparin purification of SP1 protein. Lane 1; the GST-Column purified Sp1 protein, Lane 2; unbound fraction of heparin column, lanes 3-5; various, Lane 6; the Heparin eluted protein. The protein was desalted and concentrated through Amicon 10kDa filter. Lane 7; the filtrate of Amicon 10kDa filtration unit and Lane 8 the retainate of the filtration unit.

The expression of protein was also analyzed by western blotting (figure 4X). The proteins were transferred to PVDF membrane and probed for GST-tag by immunoblotting with Anti-GST antibody (Abcam, Inc. Cat. # ab6613).

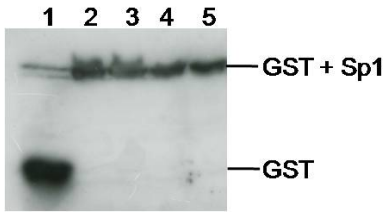


Figure 4X. Western blot scan showing the expression of fusion protein. Lane 1; purified GST. Lanes 2-5; bacterial whole cell lysate, GST-column eluate, Heparin column eluate and the desalted concentrated purified SP1-GST protein, respectively.

EMSA analysis using recombinant Sp1 protein with 'GC' DNA

The purified GST-Sp1 protein was analyzed for its interaction with 'GC' in EMSA. The protein was found to be interacting only with 'GC', while some binding was observed with 'methGC' (figure 4Y).

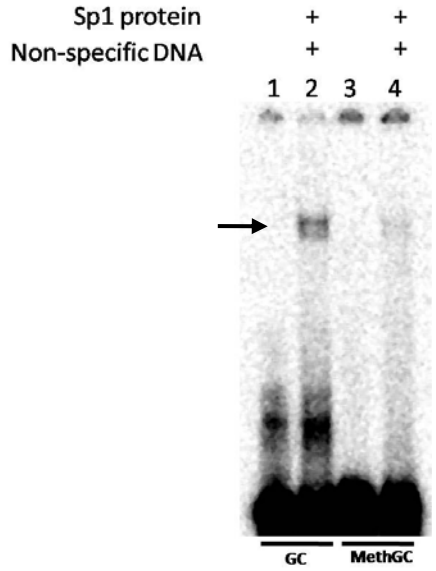
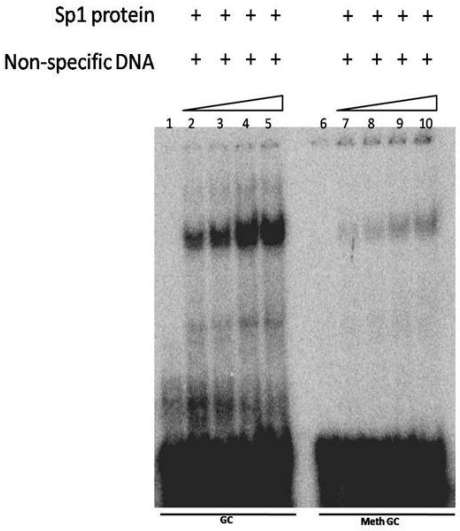


Figure 4Y. EMSA analysis showing interaction of Sp1 protein with 'GC' DNA**.** Lane 1 and 3 represents the negative control (only labeled DNA), lane 2 and 4 represents the interaction of the protein with labeled DNA. Arrow represents the shift indicating the intraction of Sp1 with 'GC' DNA

To confirm the interaction of Sp1 with 'GC', EMSA analysis was done with different concentrations of Sp1 protein. Results (figure 4Z1) showed that Sp1 binds with 'GC' and to some extend with methylated 'GC'. Analysis using Image Quant software of the intensity of band corresponding to DNA/Protein complex (figure 4Z2) showed that Sp1 binding to 'GC' was 2.481 times more than 'methGC' with a regression coefficient (r^2) value of 0.899.

Z1



Z2

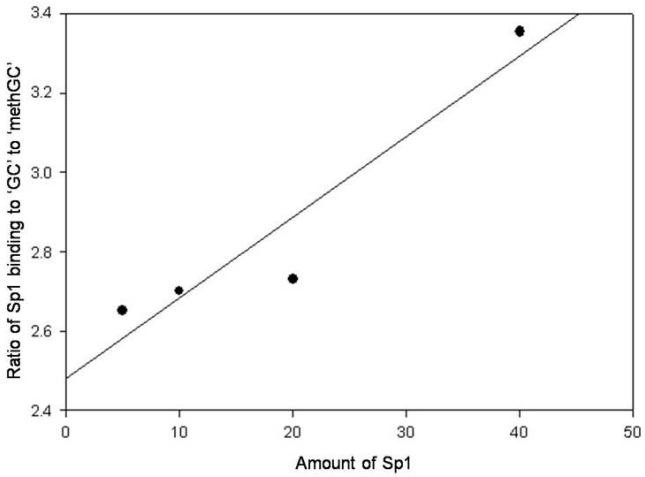


Figure 4Z. EMSA with increasing concentration of Sp1 with the DNA probe of 'GC' and Meth 'GC'. Z1). Lane 1-5 shows binding of Sp1 with 'GC' and 6-10 shows binding of Sp1 with 'methGC'. Sp1 was used at increasing concentration of 5, 10, 20, 40 ng. 4H6b). Graph showing comparison of Sp1 binding with 'GC' and meth'GC'. Radioactivity distribution per lane in EMSA was calculated using ImageQuant software.

IV. **Chromatin Immuno-precipitation (ChIP) assay using Sp1 antibodies**.

To analyze whether Sp1 protein binds to 'GC' fragment within the second intron of *Neuronatin*, *in vivo* conditions, Chromatin Immunoprecipitation (ChIP) assay was performed with Sp1 antibody on several tissues including brain (where *Neuronatin* is expressed), liver and kidney (where *Neuronatin* is not expressed) from wild type MF1 mice. ChIP is a powerful tool to study the DNA-protein interaction *in vivo*, involves isolation and fragmentation of chromatin followed by immunoprecipitation using a protein-specific antibody.

PCR performed on Sp1 antibody immunoprecipitated DNA

To examine whether Sp1 binds to 'GC' fragment within *Neuronatin*'s second intron, PCR using primers [IT2fr and IT2bk (Chapter three, see material and methods)] specific for this region was performed. As can be seen in figure 4AA, PCR product corresponding to *Neuronatin* second intron was detected in Sp1 immunoprecipitated fraction.

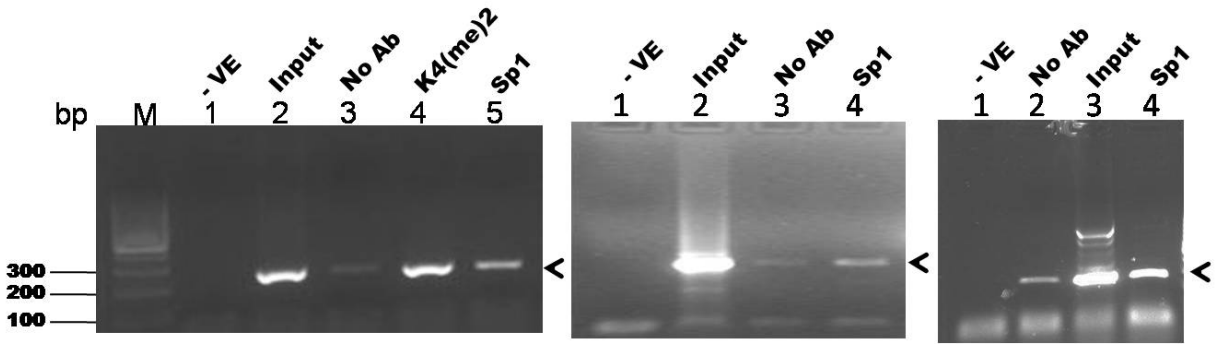


Figure 4AA. PCR performed on Sp1 antibody immunoprecipitated DNA**.** Antibodies used were $K4(me)_2$ - Histone 3 Lysine 9 dimethylation; Ab-Sp1 - Sp1 antibody. M= 100bp DNA marker (MBI fermentas Cat. # S0301), INP- Input DNA, -VE- PCR reaction with no template, -Ab- no antibody added for ChIP assay. Arrow head indicates the 250bp DNA band corresponding to the second intron of *Neuronatin*. 1, 2, 3, 4 represents different lanes. 4I1a). ChIP analysis on liver tissue, 4I1b). ChIP analysis on kidney tissue, 4I1c). ChIP analysis on brain tissue.

To probe the specificity of this binding PCR was also done for the Neuronatin promoter region using the specific primers:

Forward: 5' CATCACCCCTCCTTCTCAAC 3',

Reverse: 5' AGCCGATGATGAGCAGTTCT 3'.

As the result in figure 4AB shows, Sp1 antibodies immnoprecipitated DNA did not show any enrichment corresponding to the promoter region (Compare lane # 3 with lane # 7).

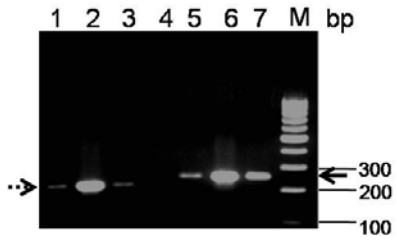


Figure 4AB. PCR for promoter region and intronic region of *Neuronatin*. Dashed arrow indicates the bands corresponding to promoter region DNA, while arrow indicates the band corresponding to second intronic region of Neuronatin. Lanes 1 & 5; no antibody added, lane 2 & 6; Input, lane 3 & 7; Sp1 immunoprecipitated DNA.

Bisulphite sequencing of Sp1 immunoprecipitated DNA

Furthermore, to check whether Sp1 binds in a methylation restricted manner within this intron, it was decided to find the DNA methylation status of the bound DNA fraction. This was done to take advantage of the fact that the maternal allele of mouse *Neuronatin* gene is methylated whereas the paternal is unmethylated (Kikyo *et al* 1997, John *et al* 2001). Therefore, by analyzing the DNA methylation profile of the bound fraction it was possible to distinguish the maternal and paternal alleles. Bisulfite sequencing was performed on the Sp1 immunoprecipitated DNA (Bis-on-ChIP). Sp1 immunoprecipitated DNA fraction was collected and along with Input treated with sodium bisulfite as described previously (Gokul *et al* 2007). Two rounds of PCR amplification was done for 30 cycles, each in a 25µl reaction containing 1X PCR Buffer, 1.5 mM $MgCl_2$ and 200 µM dNTPs along with 10 pmol of following primers:

Forward: 5’ TTGATTGGTGGATAAGTTGTGTTT 3’

Reverse: 5’CCACCCTTAAAAAAATACCCATAAT3’.

5 µl of first round PCR product was used as template for the second round PCR. As can be seen from figure 4I3, bisulphite sequencing of Sp1 immunoprecipitated DNA from brain tissue of MF1 mice showed predominantly unmethylated profile for the second intron.

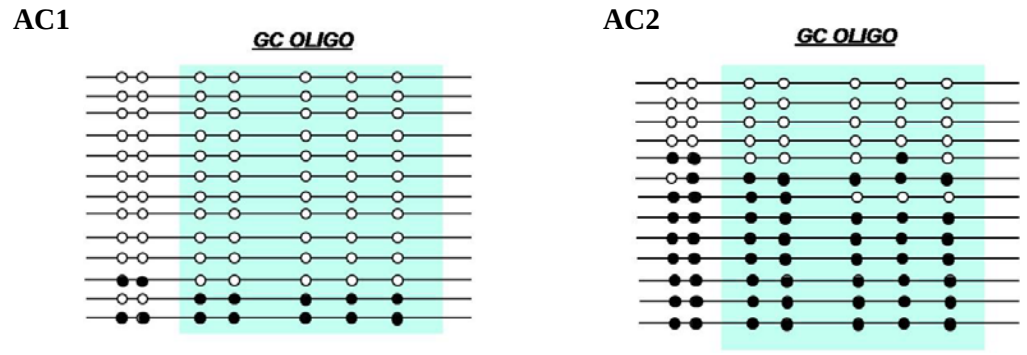


Figure 4AC. CpG methylation profile of Sp1 immunoprecipitated DNA in the second intronic region of *Neuronatin* in brain tissue**.** Bisulphite analysis was carried out on Sp1 immunoprecipitated DNA. Each horizontal line represents a single clone for the respective PCR product after bisulfite treatment. Open circles indicate no methylation. Filled circles refer to methylated cytosines. The region of second intron corresponding to ‘‘GC’’ has been represented with shaded box. AC1). Bis-on-ChIP analysis done on brain tissue of adult MF1 mice, AC2). Bis-on-ChIP analysis of input (Brain tissue).

To confirm the above mentioned result, Bis-on-ChIP was performed on liver and kidney tissues, as can be seen in figure 4AD, bisulphite sequencing of Sp1 immunoprecipitated DNA from both of the tissues of MF1 mice showed predominantly unmethylated profile for the second intron.

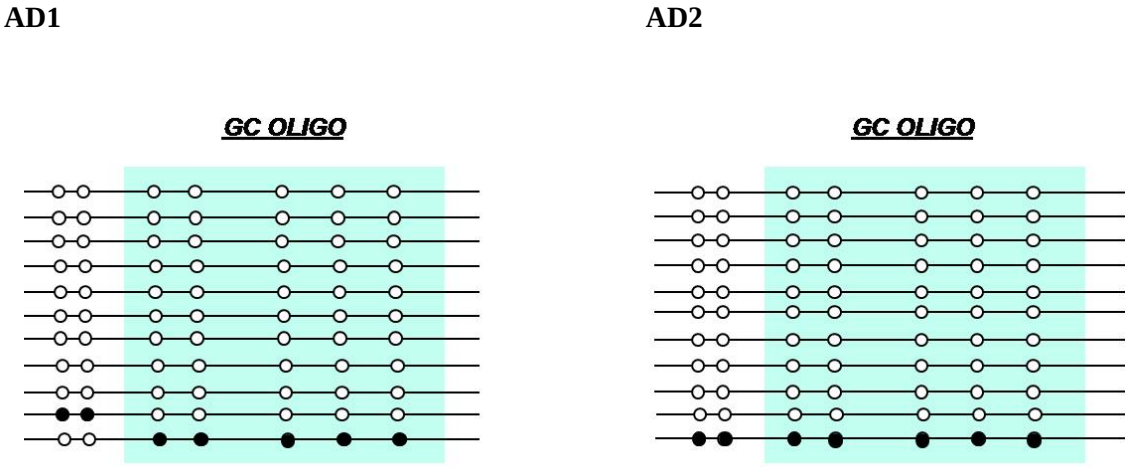


Figure 4AD. CpG methylation profile of Sp1 immunoprecipitated DNA in the second intronic region of *Neuronatin* in liver and kidney tissue**.** AD1). Bis-on-ChIP analysis done on kidney tissue of adult MF1 mice, AD2). Bis-on-ChIP analysis done on brain tissue of adult MF1 mice.

Conclusions

The role of CTCF and YY1 has been documented (Bell *et al* 2000, Hark *et al* 2000, Kim *et al* 2003, 2008) in the mechaism of genomic imprinting. Both of these proteins have been shown to be involved in the silencing of the one allele (reviewed by Kim 2008). As mentioned earlier, we were not able to find any CTCF or YY1 protein binding site within the *Neuronatin*/*Bc10* locus. Having defined the minimum binding site for the methylation-restricted protein binding, in this chapter, it was sought to identify the factors which interact with this binding site.

Two types of approaches were followed in identification of 'GC' binding proteins. The first was a top-down approach where, by various elimination steps of chromatography or yeast mono hybrid assay, the binding proteins were identified. In the other method, bioinformatics tools were used to find candidate binding proteins and the results were validated by experimental approaches. In the first approach using two different techniques we were able to identify several proteins, which are being tested for their binding ability to 'GC'. In the latter approach it was found that the motif present within the 'GC' fragment, 'GGGCGGG' was the binding site for the transcription factor Sp1 (Dyan and Tjian, 1983a, 1983b, Gidoni *et al* 1984, 1985). EMSA analysis using Sp1 antibody could supershift the GC-protein complex. Furthermore, EMSA analysis using purified Sp1 protein from bacterial source showed the interaction of Sp1 with 'GC' was with more affinity than with methylated 'GC'. The binding of Sp1 to intronic region of *Neuronatin* was also confirmed by the Chromatin Immuno-precipitation assay using Sp1 antibody. Whether Sp1 alone or in combination with other proteins bind to *Neuronatin* second intron and is involved in the regulation of its transcription, is yet to be tested.

Chapter V

Discussion

Various mechanisms have been put forth to explain the phenomenon of genomic imprinting. For instance, the Imprinting control centers for *H19*/*Igf2*, *Peg3* and *Rasgrf1*loci have been found to act as an insulator on the methylated allele preventing the interaction of promoter with its enhancers (Sutcliffe *et al* 1994, Thorvaldsen *et al* 1998, Fitzpatrick *et al* 2002, Kim *et al* 2003, Yoon *et al* 2005, Williamson *et al* 2006). For *Gnas* (Stephanie *et al* 2000, Williamson *et al* 2006), *Kcnq1*(Smilinich *et al* 1999, Fitzpatrick *et al* 2002, Mancini-DeNardo *et al* 2006), *Gtl2/Dlk1*(Lin *et al* 2003), *Igf2*r/*Air* (Wutz *et al* 1997, Birger *et al* 1999, Zwart *et al* 2001) locus and imprinted genes like *Ube3a4* (Chamberlain and Brannan 2001, Landers *et al* 2004, 2005), *Rtl1* (*Peg11*) (Davis *et al* 2005, Tierling *et al* 2006) silencing of an allele by anti sense non-coding RNA has been proposed. However, in all cases, the factors identified and the mechanisms put forth suggested, the repression of an allele of imprinted genes. The mechanisms which prevent the silencing of the transcriptionally active allele are still unknown. In addition, most of the studies to dissect the mechanism of genomic imprinting have been on the loci where imprinted genes are organised in clusters. The study presented in this thesis has tried to dissect out the factors correlated with active allele of mouse imprinted *Neuronatin* gene.

Imprinting control regions are domains within imprinted loci that are essential for establishment and maintaining the imprinted status of the genes within the locus (Delavel and Feil 2004, Lewis and Reik 2006). An important biochemical characteristic of known ICR is the mutual exclusiveness of DNA methylation and specialized chromatin conformation on the two alleles (Khosla *et al* 1999, Schweizer *et al* 1999, Bell and Felsenfeld 2000, Hark *et al* 2000, Coombes *et al* 2003, Mancini-DiNardo *et al* 2003, reviewed by Feil and Khosla 1999). As the two alleles of the mouse *Neuronatin* gene

showed similar mutual exclusiveness of DNA methylation on the maternal allele and DNase I hypersensitivity on the paternal allele within its second intron, this region has been proposed to be a putative Imprinting Controlling Region (ICR) for this locus (this thesis and Sowpati *et al* 2008). That the second intron indeed functions as an ICR for the *Neuronatin* locus, needs to be tested by genetic deletion experiments in mice.

Differential chromatin organization on the two parental alleles of an imprinted gene indicates association of different epigenetic modifications with each allele. Maternal allele of *Neuronatin* has been shown to be methylated (Kikyo *et al* 1997, Kagitani *et al* 1997, John *et al* 2001). DNase I hypersensitivity on the paternal allele would suggest its interaction with non-histone protein(s). As the hypersensitive site HS-I was detected only on the unmethylated allele, the binding of the candidate non-histone protein should also be methylation-restricted. In this study, we have identified methylation-restricted protein binding activity of a 'GC' rich region from within the second intron of *Neuronatin*. A 25bp fragment (NNNNGGGCGGGCCGGGCGGAAA) was able to bind to a protein complex only when it was unmethylated. Even though the identity of the protein complex has not been unrevealed, one of the proteins in this complex could be the transcription factor Sp1as the 25bp 'GC' fragment contains two Sp1 binding sites and the results presented in chapter IV indicate that Sp1 can to some extent discriminate between methylated and unmethylated 'GC' fragment. Moreover, it was found to be bound *in vivo* within the second intron of *Neuronatin*. Several other proteins have been identified which can bind to 'GC' by Yeast Mono hybrid and affinity chromatography. However, whether the identified proteins or Sp1 alone could be the part of the differential chromatin organization within the *Neuronatin* gene remains to be examined.

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