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An intronic DNA sequence within the mouse *Neuronatin* gene exhibits biochemical characteristics of an ICR and acts as a transcriptional activator in *Drosophila*

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ABSTRACT

Imprinting control regions (ICRs) are domains within imprinted loci that are essential for their establishment and maintenance. Imprinted loci can extend over several megabases, encompass both maternally and paternally-expressed genes and exhibit multiple and complex epigenetic modifications including large regions of allele-specific DNA methylation. Differential chromatin organisation has also been observed within imprinted loci but is restricted to the ICRs. In this study we report the identification of a novel imprinting control region for the mouse *Neuronatin* gene. This biochemically defined putative ICR, present within its 250 bp second intron, functions as transcriptional activator in *Drosophila*. This is unlike other known ICRs which have been shown to function as transcriptional silencers. Furthermore, at the endogenous locus, the activating signal from the ICR extends to the *Neuronatin* promoter via allele-specific unidirectional nucleosomal positioning. Our results support the proposal that the *Neuronatin* locus employs the most basic mechanism for establishing allele-specific gene expression and could provide the foundation for the multiplex arrangements reported at more complex loci.

1. Introduction

Neuronatin is a small imprinted gene that was identified in a screen for genes involved in neuronal differentiation and is present on the distal part of mouse chromosome 2 (Wijnholds et al., 1995) and chromosome 20q11.2 in humans (Evans et al., 2001). Like most other imprinted genes, *Neuronatin* is developmentally regulated and expressed at higher levels during early postnatal development (Wijnholds et al., 1995) but unlike most of them, *Neuronatin* is not present in a cluster of imprinted genes and is the only known imprinted gene within this locus (Evans et al., 2001; John et al., 2001). Interestingly, in both mice and humans this gene is present within the intron of a non-imprinted gene *Bc10/Blcap* (see Fig. 1A and Evans et al., 2001; John et al., 2001) and a 30 kb transgene spanning this locus is able to imprint at ectopic loci (John

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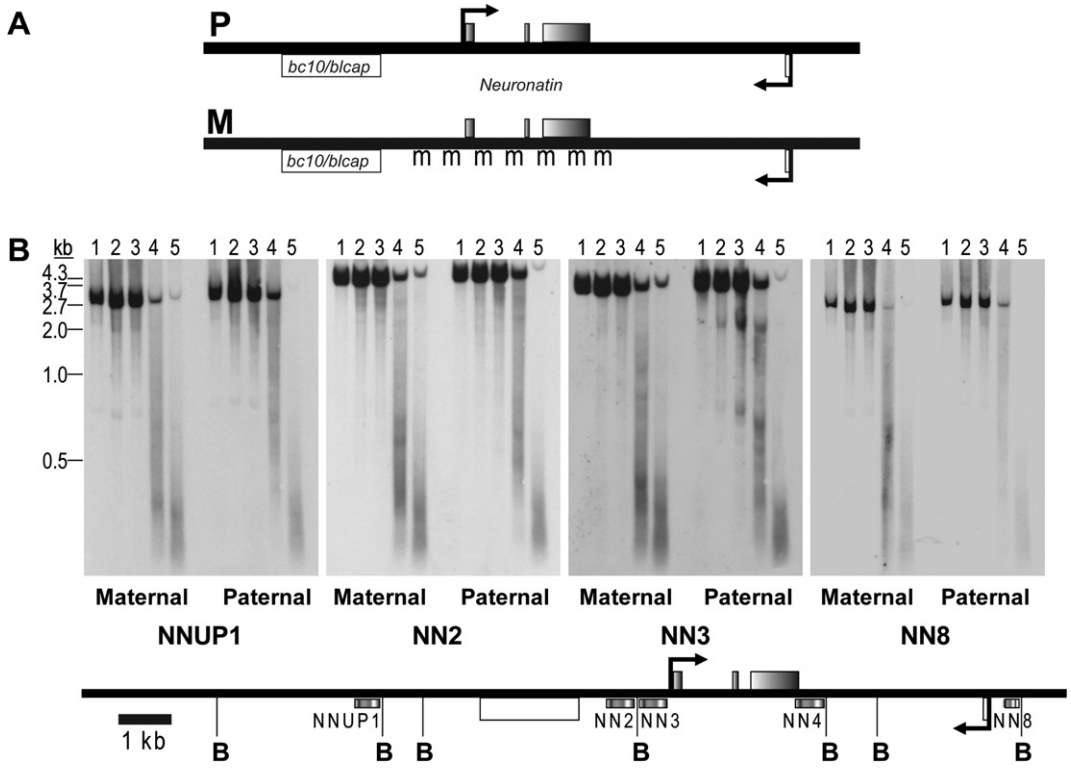

Fig. 1 – Allele-specific DNase I sensitivity in the *Neuronatin* locus. (A) Imprinted mouse *Neuronatin* locus. The three exons of the mouse *Neuronatin* gene are shown above the line as filled rectangles. The two exons of *Bc10* gene are shown below the line as open boxes. The direction and allele-specificity of transcription for *Neuronatin* and *Bc10* genes are shown by raised arrows above and below the thick horizontal line, respectively. P, paternal-allele; M, maternal allele. "mmmm" indicates methylation status. (B) Nuclei from maternally and paternally disomic (for distal part of chromosome 2) mouse embryos (E14.5) were incubated with increasing concentration of DNase I (lanes 1–5 corresponds to 0, 5, 10, 20, 40 U of DNase I/ml). DNA isolated from DNase I digests was re-digested with BglII, electrophoresed on a 1.1% agarose gel and Southern blotted. The blot was sequentially probed with the end-probes (abutting the BglII ends) indicated in the line diagram below the panel of autoradiograms. Maternal refers to nuclei from chr. 2 maternally disomic mouse embryos (E14.5) whereas paternal refers to nuclei from paternally disomic mouse embryos (E14.5) for chr. 2. The line diagram below the autoradiograms shows the mouse *Neuronatin* locus (GenBank Accession No. AF303656) as a thick line. 'B' indicates BglII sites within the locus. Shaded boxes below the line indicates probes abutting the ends of BglII fragments used in this study (see Section 4).

et al., 2001). This would indicate that the imprinted domain within the *Neuronatin* locus is quite small and may reside within the 8.5 kb long intron of *Bc10/Blcap*. This again is in contrast to most other imprinted loci like the *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). In fact, *Neuronatin* belongs to a group of only nine imprinted genes (out of the around 100 known till date Beechey et al., 2005) which have been found to be present outside a cluster. Five of these isolated imprinted genes, including *Neuronatin*, are present within the intron of other genes (Morison et al., 2005).

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 (Delaval 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). ICRs act by influencing both the gene expression and epigenetic status of imprinted genes and in all cases examined, result in the silencing of one of the alleles (Lewis and Reik, 2006). In the case of *H19/Igf2* locus the ICR manifests its silencing effect by acting as an insulator preventing interaction of the *Igf2* promoter with its enhancers (Bell and Felsenfeld, 2000; Hark et al., 2000; Lewis and Reik, 2006). Similar mechanisms have been proposed for the *Peg3* and *Rasgrf1* loci (Lewis and Reik, 2006). On the other hand the *Igf2r/Air* and *Kcnq1* loci ICR seems to involve non-coding RNAs (Lewis and Reik, 2006). However, most of the studies on imprinting control centres have been on loci where imprinting genes are present in clusters and there are very few studies (Delaval and Feil, 2004, review) that have tried to analyse the mechanism for imprinting of single genes which might be more straightforward.

In this study we set out to identify the imprinting control region within the mouse *Neuronatin* gene because of the relative simplicity of the locus. The aim was to use biochemical criteria of the known ICRs in identification of *Neuronatin* ICR and to analyse its function. As observed for the *H19/Igf2*, *Snrpn*, *Kcnq1* and the *Gnas* locus an important biochemical

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property of the known ICRs is the mutual exclusiveness of DNA methylation and specialised chromatin conformation on the two alleles, one allele being methylated whereas the other unmethylated allele shows specialised chromatin organisation as indicated by nuclease sensitivity assays and binding of non-histone proteins like CTCF and YY1 (Feil and Khosla, 1999; Khosla et al., 1999; Schweizer et al., 1999; Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2002; Coombes et al., 2003; Mancini-DiNardo et al., 2003). Previous analysis of the *Neuronatin* locus showed that the non-transcribed maternal allele is methylated whereas the paternal transcribed allele is unmethylated and the domain of differential methylation extends from the promoter to the last exon of *Neuronatin* (Fig. 1A and John et al., 2001). We now show differential chromatin organisation within the *Neuronatin* locus with the presence of transcription-independent DNase I hypersensitive site exclusively on the paternal unmethylated allele within the second intron of *Neuronatin*. This intronic region which fulfils the biochemical criterion for an ICR was analysed for its function using a transgene assay in *Drosophila melanogaster*. The implication of the transcriptional activation shown by this putative ICR in *Drosophila* is discussed with reference to mechanisms that might be involved in maintaining imprinting status of the mouse *Neuronatin* gene.

2. Results

2.1. *Paternal-allele-specific DNase I hypersensitive sites at the Neuronatin locus*

To analyse chromatin organisation within the *Neuronatin/Bc10* locus on the maternal and paternal alleles separately we performed DNase I assay on mouse embryos from T26H intercrosses (Kikyo et al., 1997) which were disomic for the distal part of chromosome 2. Nuclei from E14.5 chromosome 2 disomic embryos were incubated with different concentrations of DNase I. To subdivide the *Neuronatin* chromosomal locus, DNA isolated from the DNase I treated nuclei was digested with BglII (Fig. 1B, lower panel). The DNase I sensitivity within each BglII fragment was then analysed by indirect end-labelling using 300–500 bp end probes as described in Section 4. The maternal and paternal alleles showed a striking difference in sensitivity to DNase I in the BglII fragment containing the *Neuronatin* gene (using probe NN3, Fig. 1B). In contrast, no appreciable differences in DNase I sensitivity between the two parental alleles were observed for the regions outside the gene (with probes NNUP1, NN2 and NN8; Fig. 1B).

We used the probe NN4 in addition to NN3 to further analyse DNase I sensitivity within the *Neuronatin* gene from both ends. As can be seen in Fig. 2, several allele-specific DNase I hypersensitive sites were detected. Two weak DNase I hypersensitive sites on the maternal methylated allele (indicated by asterisks) were not detected on the paternal-allele. On the other hand, the paternal-allele, which is unmethylated, showed two strong and several weak DNase I hypersensitive sites (indicated by thick and thin arrows, respectively) that were absent on the methylated maternal allele. One of the two strong hypersensitive sites on the expressed unmethylated paternal-allele of the *Neuronatin* gene (HS-P) was mapped to a region within the *Neuronatin*'s promoter. The second and much stronger site was mapped to within the second intron of *Neuronatin* (HS-I).

2.2. *The hypersensitive site HS-I is independent of the transcription status of the Neuronatin gene*

Several reports previously have shown a correlation between DNase I hypersensitive sites and transcriptionally active regions in the genome (Elgin, 1988). To investigate whether the hypersensitive sites HS-I and HS-P, present only on the expressed paternal-allele of *Neuronatin*, are related to its transcriptional status, we assayed nuclei from liver (where *Neuronatin* is not expressed) for DNase I sensitivity. Since this assay was done on wild-type MF1 mice, the observed DNase I profile should comprise of hypersensitive sites present on both the alleles. As can be seen in Fig. 3, a hypersensitive site corresponding to the size of HS-I, the prominent paternal-specific hypersensitive site that maps to the second intron of *Neuronatin*, was observed in BglII re-digested DNase I samples (lanes 2–6). The promoter-specific hypersensitive site (HS-P) and all other minor DNase I sites were absent in the DNase I profile for liver chromatin. To confirm that the observed hypersensitive site was present on the unmethylated paternal-allele, DNase I treated samples were digested with methylation sensitive HpaII restriction enzyme along with BglII (lanes 7–10, Fig. 3). The hypersensitive site observed in the BglII only digests was not seen in the BglII + HpaII digests indicating that the observed hypersensitive site was present on the unmethylated allele. Thus, the results from this experiment suggested that the paternal-allele-specific hypersensitive site HS-I was not correlated to the transcriptional status of *Neuronatin*.

2.3. *Maternal and paternal alleles within the Neuronatin locus are organised into different nucleosomal conformations*

Do the factors responsible for DNase I hypersensitive site HS-I disrupt the canonical nucleosomal array on the unmethylated paternal-allele? To answer this, micrococcal nuclease (MNase) digestion was carried out on liver nuclei derived from neonatal mice disomic for chromosome 2. Any disruption in the regular arrangement of nucleosomes would be reflected by a change in the MNase digestion pattern. As was done for DNase I assay, the nucleosomal organisation within the *Neuronatin* gene was analysed using the end-probes NN3 and NN4 (see Fig. 2, lower panel for the position of these probes). 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′ BglII end (Fig. 4, panel 2). However, in the region corresponding to the second intron (beyond 1000 bp from the 3′ BglII end) the pattern of MNase digestion was very different on the two alleles and only on the paternal unmethylated allele two prominent bands were observed (see lane 2 in NN4 panel, indicated by thick arrows). In contrast, the maternal profile appeared as a smear (Fig. 4, NN4 panel). Using the probe NN3, the difference between the two alleles was more discernible (Fig. 4, NN3 panel). The paternal-allele, in addition to showing a prominent band (thick arrow) for the second intron, also showed very regularly spaced

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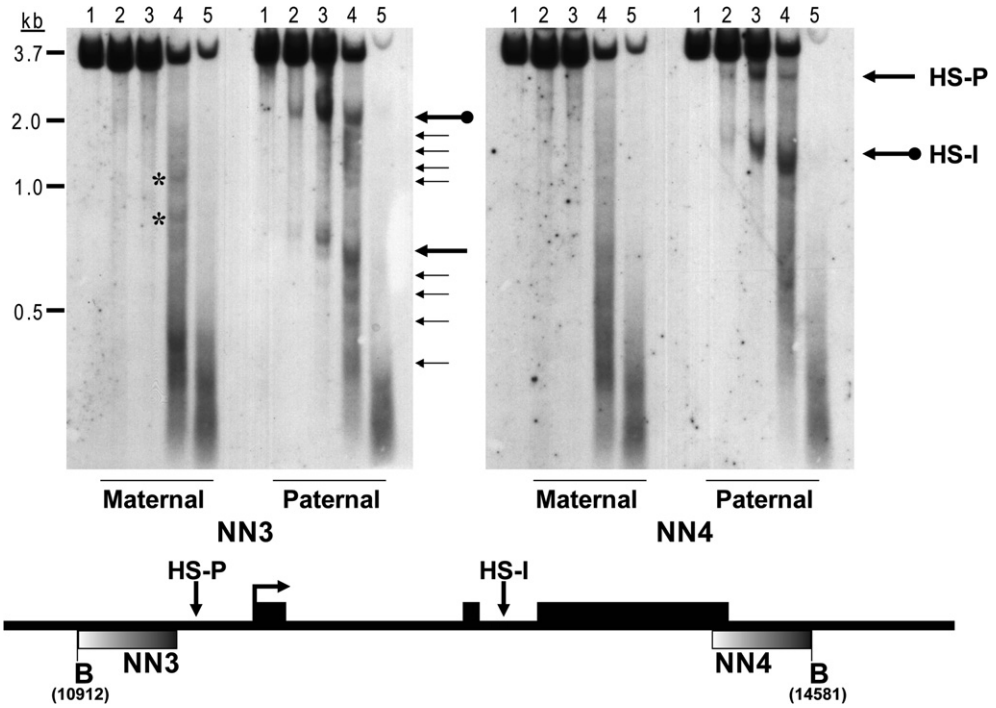

Fig. 2 – Paternal-allele-specific DNase I sensitive sites within the *Neuronatin* gene. Nuclei from maternally and paternally disomic (for distal part of chromosome 2) mouse embryos (E14.5) were incubated with increasing concentration of DNase I (lanes 1–5 corresponds to 0, 5, 10, 20, 40 U of DNase I/ml). DNA isolated from DNase I digests was re-digested with BglII, 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 panel of autoradiograms. Maternal refers to nuclei from chromosome 2 maternally mouse embryos (E14.5) whereas paternal refers nuclei from paternally disomic mouse embryos (E14.5) for chromosome 2. 'B' indicates BglII sites within the locus. The number given below the BglII site refers to their nucleotide position in the Genbank sequence AF303656. 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*.

nucleosomal ladder (thin arrows). However, the MNase profile for the maternal allele showed a nucleosomal ladder but with a lot of background suggesting that the maternal allele had randomly organised nucleosomes but as the MNase digestion profile obtained was the sum total for several cells, the composite profile appeared as a smear.

2.4. *Histone modifications associated with parental alleles of Neuronatin gene*

Previous studies have shown association of histone modifications for some imprinted genes in an allele-specific manner (Delaval et al., 2007; Feil and Berger, 2007; Mikkelsen et al., 2007). To examine whether the differential chromatin organisation with the *Neuronatin* locus was a result differential association of histone modifications, Chromatin immunoprecipitation (ChIP) analysis was undertaken using antibodies to the various histone H3 modifications. H3 lysine 9 (H3K9) acetylation and H3 lysine 4 (H3K4) dimethylation have previously been shown as marks for active chromatin. Similarly, H3 lysine 9 (H3K9) di- and trimethylation and H3 lysine 27 (H3K27) trimethylation have been correlated with inactive chromatin organisation (Peterson and Laniel, 2004; Bernstein et al., 2006). Therefore, ChIP analyses using antibodies to the above mentioned H3 modifications were performed on brain (where *Neuronatin* is expressed), liver and kidney (where *Neuronatin* is not expressed) tissues isolated from wild-type MF1 mice. DNA from the bound and unbound fractions for each antibody ChIP was isolated and analysed. The results shown in Fig. 5A and B are qualitative only. As shown in Fig. 5A and B, H3K9 acetylation and H3K9 dimethylation was neither associated with the promoter nor with the intronic region in any of the tissues examined whereas H3K4 dimethylation was found to be associated with both the promoter and intron in all the tissues analysed. H3K27 trimethylation was found to be associated with both promoter and intronic region in liver but only with the intron in kidney nuclei. Even though the PCR product band for the intronic region in kidney was faint, it was consistently observed in all our experiments. One of the possible explanations could be that the intronic region is associated with H3K27 containing nucleosomes in only a few cells of kidney. In brain nuclei, no association of H3K27 trimethylation was found either with promoter or ntron. H3K9 trimethylation was found to be associated with both promoter and intron in only kidney nuclei (Fig. 5A and B).

We further analysed the parental-allele specificity of these associations by taking advantage of the fact that the

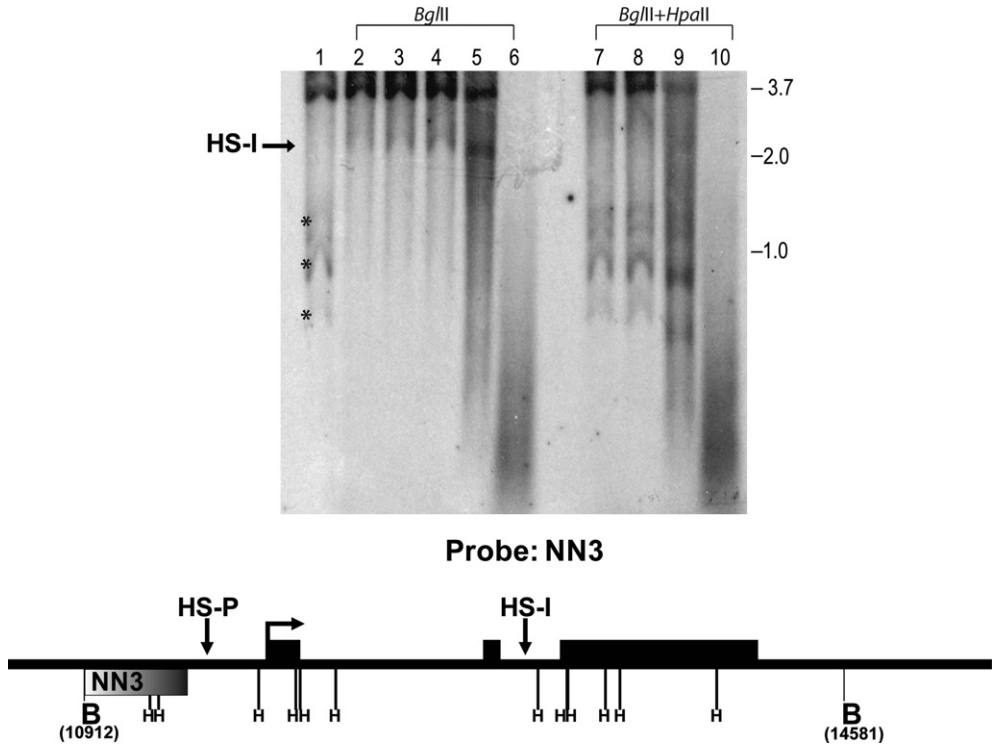

Fig. 3 – Hypersensitive site HS-I is not correlated to the transcription status of *Neuronatin*. Wild-type MF1 adult liver nuclei were incubated with increasing concentration of DNase I (lanes 3–6 and 7–8 corresponds to 5, 10, 20, 40 U of DNase I/ml, lanes 1 and 2 represent 0 U of DNase I/ml). DNA isolated from DNase I digests was re-digested with either BglII or BglII and HpaII, Southern blotted and probed with the end-probe NN3. Lanes 2–6, BglII digested DNA; lanes 1 and 7–10, BglII + HpaII digested DNA. '*' indicates HpaII fragments. See Fig. 2 for description of the line diagram. In addition 'H" indicates HpaII sites in the line diagram. Arrow indicates the position of hypersensitive site. HS-I indicates the hypersensitive site mapped to within the second intron of *Neuronatin* (compare with Fig. 2, NN3 panel).

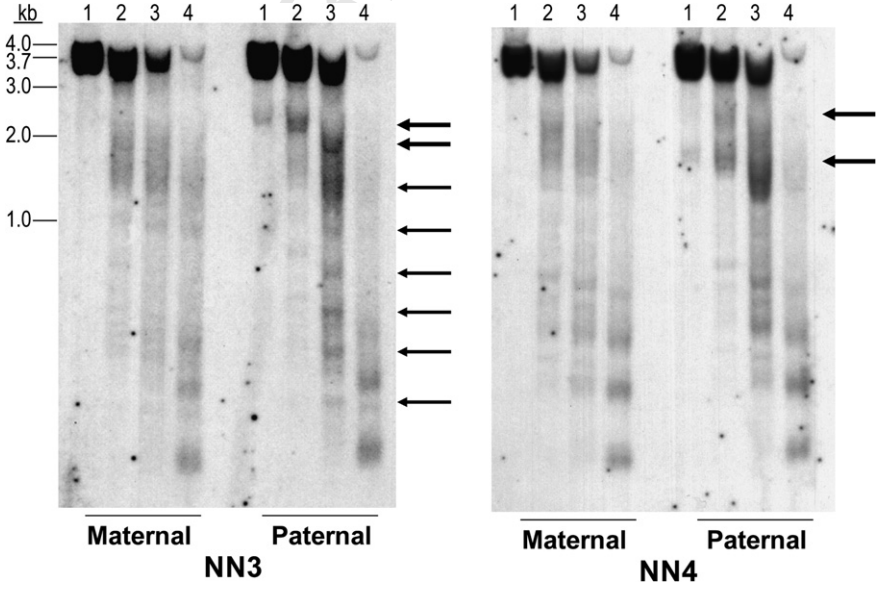

Fig. 4 – MNase digestion profiles in Matdi2 and Patdi2 mice. New born liver nuclei from paternally or maternally disomic mice for proximal chromosome 2 were incubated with MNase for increasing periods of time (lanes 1–4 correspond to 0, 30, 60 and 120 s of incubation with MNase at 37 °C). DNA samples were digested with BglII, and analysed by Southern hybridisation with probes NN3 (left panel) and NN4 (right panel), respectively (see Fig. 2 for position of the end-probes). Arrows indicate the bands that are present only in paternal MNase profile (see text for details).

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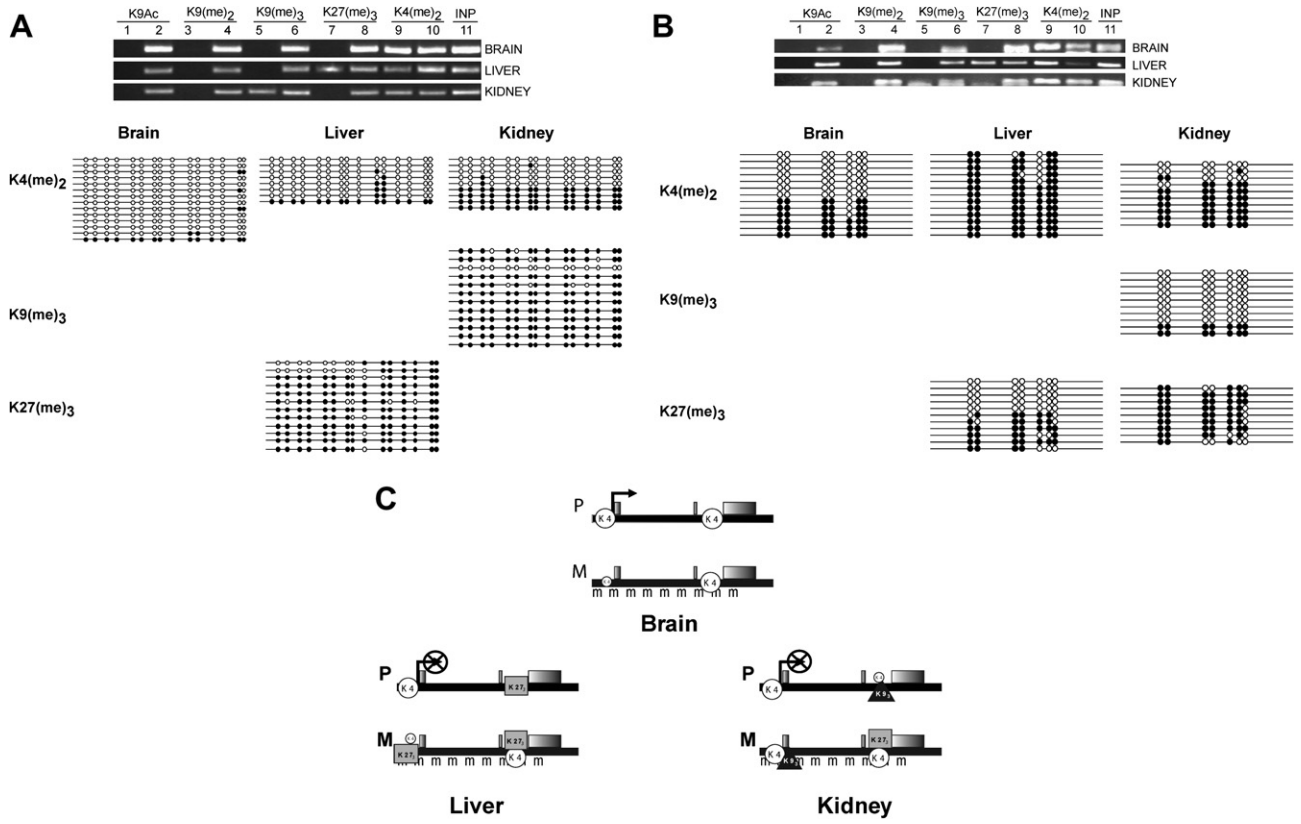

Fig. 5 – Histone modifications associated with parental alleles of *Neuronatin* gene. (A) Bis-on-ChIP analysis for the promoter region; (B) Bis-on-ChIP analysis for *Neuronatin*'s second intron. Chromatin immunoprecipitation was carried out with the indicated histone H3 modifications and qualitative PCR was performed. Antibodies used were K9ac-H3 lysine 9 acetylation; $K9(me)_2$-H3 lysine 9 dimethylation; $K9(me)_3$-H3 lysine 9 trimethylation; $K27(me)_3$-H3 lysine 27 trimethylation; $K4(me)_2$-H3 lysine 4 dimethylation. INP-input DNA. Lane 1, 3, 5, 7, 9 are antibody bound fractions whereas lanes 2, 4, 6, 8 and 10 are unbound fractions. Bisulfite analysis was carried out on immunoprecipitated DNA followed by PCR using specific primers. Input DNA showed approximately 50% methylation for promoter and intron (see Supplementary Fig. S1). The lower panel in A and B shows methylation profiles. 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. (C) Summary of the Bis-on-ChIP results. The three *Neuronatin* exons are represented by filled boxes above the horizontal line. Transcription is indicated by raised arrows. Crossed arrows indicates no transcription. Association of H3 lysine 9 trimethylation is represented by black triangles. Open circles refer to H3 lysine 4 dimethylation. Smaller open circles indicate that only a few clones showed association with H3 lysine 4 dimethylation. H3 lysine 27 trimethylation is indicated by grey squares.

maternal allele of mouse *Neuronatin* gene is methylated whereas the paternal is unmethylated for both the promoter and the second intronic region (Kagitani et al., 1997; Kikyo et al., 1997; John et al., 2001). To distinguish the two alleles, bisulfite sequencing was performed on the immunoprecipitated DNA in ChIP analyses (referred to as Bis-on-ChIP). The results are presented in Fig. 5A and B (lower panels) and a composite allele-specific representation of the histone associations are given in Fig. 5C. In brain where *Neuronatin* is transcribed, the methylated and unmethylated alleles showed association with only H3K4 dimethylation at both the promoter and intron. In contrast, in liver and kidney where *Neuronatin* is not transcribed, the parental alleles at the promoter and intron were found to be associated with different types of histone modifications. Although the unmethylated paternal-allele is not transcribed in liver and kidney the promoter region was found to be associated with only H3K4 dimethylation as was observed in brain nuclei. Importantly, the difference between the unmethylated paternal alleles in the three tissues was observed only at the intronic region. Unlike the brain, the intronic region on the unmethylated allele was associated with inactive chromatin histone marks in liver and kidney. Moreover, different inactive chromatin histone marks in liver (H3K27 trimethylation) and kidney (H3K9 trimethylation) were being utilised for transcriptional silencing of the paternal-allele in these tissues probably reflecting their developmental lineages. The methylated maternal allele in both liver and kidney were associated with inactive-chromatin histone marks in addition to H3K4 dimethylation. The intronic region on the methylated allele in both liver and kidney was associated with H3K27 trimethylation. In liver the promoter region was associated with H3K27 trimethylation whereas it was associated with H3K9 trimethylation in kidney. Thus, the difference in transcriptional status of the unmethylated paternal-allele of *Neuronatin* in the various tissues examined

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could be correlated to histone modifications associated with the intronic region but not of the promoter.

2.5. *Functional analysis of HS-I*

2.5.1. *Transgene reporter gene assay in Drosophila melanogaster*

To analyse the functional role of *cis* elements present within the second intron of *Neuronatin* in its unmethylated state we examined its effect on transcription of *mini-white* reporter transgene in *Drosophila* because DNA methylation is largely absent in *Drosophila* (Lyko et al., 2000). The 250 bp intron, flanked by *loxP* sites, was inserted in both orientations upstream of the *hsp70* promoter driven *mini-white* reporter gene containing P-element vector pCaSpeR (Fig. 6A). We generated 13 independent transgenic lines on different chromosomes, 8 of which had intron in the positive orientation construct with respect to the *hsp70* promoter and *mini-white* gene whereas 5 had it in the negative orientation. To investigate whether the observed effect on the expression of the *mini-white* gene was because of the presence of *Neuronatin* intron or due to the chromosomal location where the transgene was located, the intronic region was flipped out by crossing the transgenic lines to flies containing *cre* recombinase. Flipped out version of each transgenic line was established. The comparison of eye color for the transgenic lines with their respective flipped out versions indicated that the putative ICR functions as an activator of transcription. Five out of eight transgenic lines with the intronic region inserted in positive orientation (labelled as A lines) and 2 out of the 5 transgenic lines where intron is present in negative orientation ('B' transgenic lines) showed eye color that was darker than their respective flipped out counterparts (Fig. 6B), while remaining lines showed no detectable change. This was also confirmed by quantification of eye pigments. Results from the pigmentation assay for a few representative transgenic lines are shown in Fig. 6C. The observed difference in pigmentation was found to be statistically significant for all transgenic lines examined ($p < 0.05$, student's *t*-test). A39.4.3 and B88.7.2 did not show any difference in eye color. This suggested that the intronic region was behaving as an activator for the *mini-white* gene expression.

3. Discussion

In the present study we have shown the presence of two DNase I hypersensitive sites exclusively on the unmethylated paternal-allele of *Neuronatin*. The DNase I hypersensitive site present within the promoter region is associated with the transcriptional status of the *Neuronatin* gene as it was observed only in tissues where the gene is transcribed. On the other hand, HS-I, the hypersensitive site mapped to the second intron of *Neuronatin*, was found in all tissues examined, irrespective of transcriptional status of the gene. In the light of previous reports indicating correlation of constitutive nuclease hypersensitivity with genomic imprinting (Delaval and Feil, 2004; Feil and Khosla, 1999), our results suggest that the factor(s) responsible for this intronic hypersensitive site are potentially involved in mechanisms underlying genomic imprinting within the *Neuronatin* locus.

3.1. *Putative imprinting control region within the Neuronatin locus acts as a transcriptional activator*

Biochemical analysis of several imprinted loci, like the *H19/Igf2*, *Snrpn* and *Gnas* clusters, have shown that even though several kilobases within the locus is differentially methylated it is only within the respective ICRs that one allele is organised into specialised chromatin conformation (Khosla et al., 1999; Schweizer et al., 2001; Coombes et al., 2003). In the present study we have identified a similar mutual exclusiveness of DNA methylation on the maternal allele and specialised chromatin organisation (characterised by DNase I hypersensitive sites) on paternal-allele of the imprinted mouse *Neuronatin* gene. This allele-specific chromatin organisation and methylation was mapped within the second intron of the gene and since it fulfils the proposed biochemical criterion for an ICR we propose it to be the putative Imprinting Control Region for the *Neuronatin* locus. Our model would predict that deleting the second intron of *Neuronatin* from the endogenous locus would lead to loss of control on the imprinting status of the *Neuronatin* locus *in vivo*. Experiments to delete the intronic region from the endogenous *Neuronatin* locus in mice are underway in our laboratory. Meanwhile, functional analysis of the cis elements within this putative ICR using the reporter transgene assay in *Drosophila* showed that the 250 bp intron can act as a transcriptional activator. This was surprising, as in similar reporter gene experiments, the *H19* ICR had behaved as a silencer in *Drosophila* (Lyko et al., 1997). Moreover, all the ICRs examined till date have been shown to function only as silencers (Lewis and Reik, 2006; Delaval and Feil, 2004). These results in the context of the fact that CpG methylation is largely absent or present at very low levels in *Drosophila* (Lyko et al., 2000) would suggest that this putative ICR functions as a transcriptional activator in unmethylated state. This correlates with the status of *Neuronatin's* endogenous locus where only the unmethylated paternal-allele is transcriptionally active (Kagitani et al., 1997; Kikyo et al., 1997; John et al., 2001).

Evans et al. (2005) in their phylogenetic analysis had indicated that the *Neuronatin* gene may have been derived from a retrotransposition event. It is also known that retroelements and other parasitic DNA elements within the mammalian genomes are usually targets of *de novo* DNA methyltransferases (Yoder et al., 1997). In addition, it has been suggested that DNA inherited through the male germline, which is in many ways foreign DNA for the egg, has evolved mechanisms to prevent silencing of genetic loci (Morison et al., 2005). Therefore, by default, *Neuronatin* as a retroelement would be subjected to silencing through DNA methylation but makes use of the anti-silencing mechanisms in the male germline to prevent DNA methylation of its paternal-allele. Since a transcriptional activator is trapped within this locus, as indicated by our study, this would result in the transcription of *Neuronatin* gene only on the paternally (transmitted through the male germline) inherited allele. Whether the DNA elements within the activator themselves are part of the anti-silencing mechanism which prevent methylation and silencing of *Neuronatin's* paternal-allele needs to be tested. *Neuronatin* seems to be an isolated imprinted gene (John et al., 2001;

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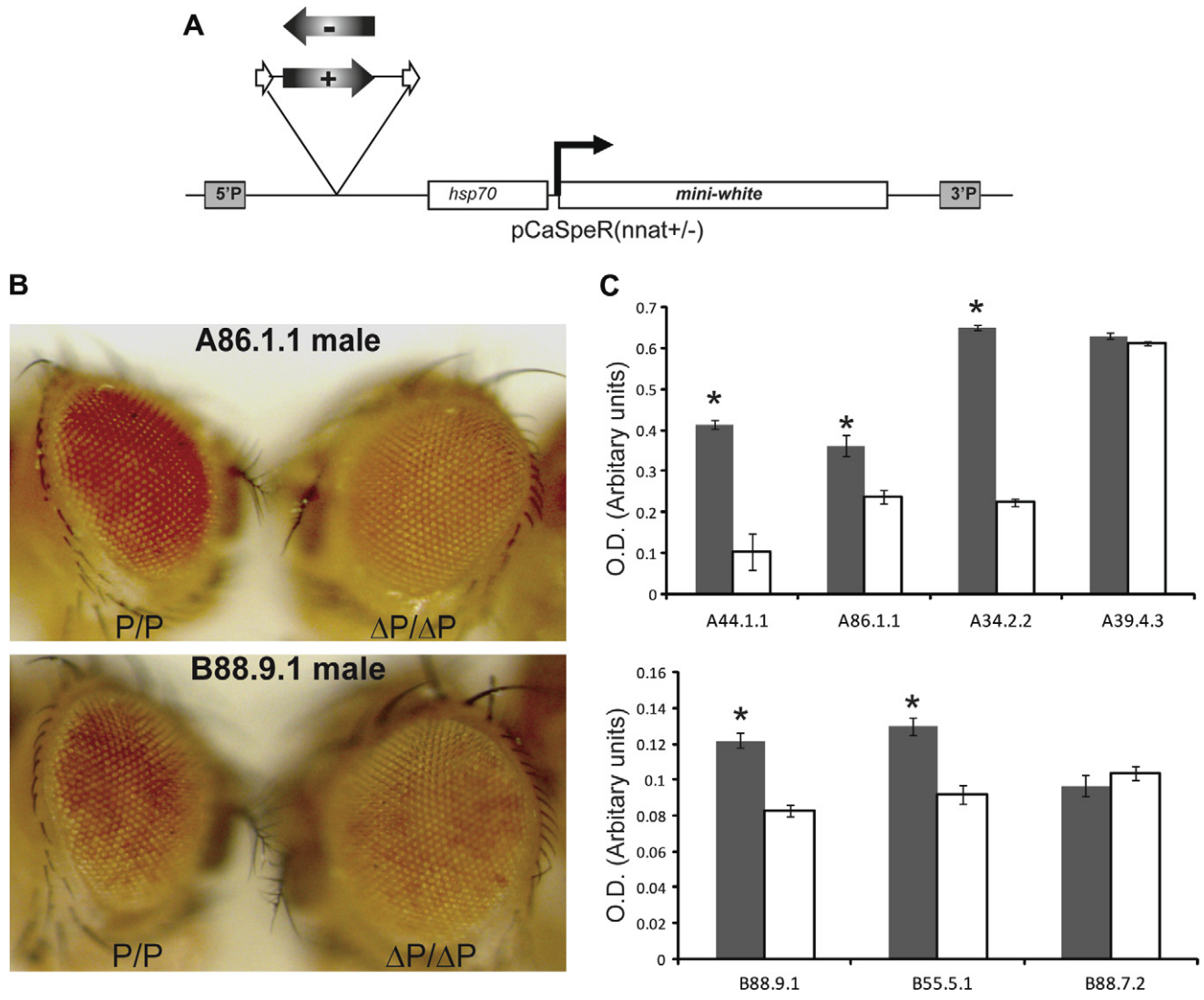

Fig. 6 – Functional analysis of *cis*-elements within the putative ICR in *Drosophila*: (A) Line diagram showing features of the reporter gene construct pCaSpeR(nnat±). The construct contains the *mini-white* reporter gene under the control of *hsp70* promoter (hsp70 pro). The intronic region from the *Neuronatin* gene (shown as filled arrows) was inserted upstream of the promoter in both orientations (indicated by + and −). 5′P and 3′P refer to P element present 5′ and 3′ to the reporter gene. The small unfilled arrows denote the *loxp* sites. (B) *Neuronatin*'s putative ICR is a transcriptional activator. The two panels show comparison of eye color phenotype between representative transgenic lines and their counterpart lines where the inserted intron had been flipped out using the surrounding *loxp* sites. A86.1.1 transgenic line had the intron in positive orientation whereas B88.9.1 had it in negative orientation. P/P, homozygous transgenic lines; ΔP/ΔP, their respective flipped-out counterparts. (C) Comparison of eye color pigmentation between representative transgenic lines and their flipped out counterparts. "A", transgenic lines: intron in the positive orientation; "B", intron in negative orientation. P/P lines, grey columns; ΔP/ΔP, white columns. The pigmentation assay was done on 20 flies for each line and the experiment was repeated at least thrice. O.D. measurements were done at 590 nm. Error bars represent standard deviation. '*' denotes significant difference in pigmentation ($p < 0.05$).

Morison et al., 2005) whereas to our knowledge, all the ICRs that have been examined (Lewis and Reik, 2006) are present within a cluster of imprinted genes. It would be interesting to test whether the above stated mechanism is also adopted by other isolated imprinted genes. It is possible that a similar mechanism involving transcriptional activators could provide the basis of imprinted regulation at more complex loci like *H19/Igf2* and *Snrpn*.

3.2. *Chromatin organisation within the putative ICR constitutively potentiates Neuronatin's paternal-allele into a transcriptionally active state*

The unmethylated paternal-allele of *Neuronatin* is transcriptionally active (Kagitani et al., 1997; Kikyo et al., 1997; John et al., 2001) and shows the presence of a constitutive DNase I hypersensitive site within its second intron (this study). It is

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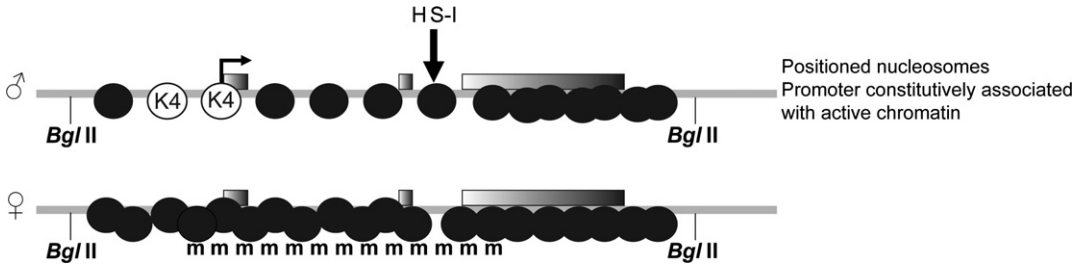

Fig. 7 – Specialised chromatin conformation within second intron on the paternal-allele maintains *Neuronatin* promoter in active chromatin state. Nucleosomes are shown as filled circles. Positioned nucleosomes are indicated by equally spaced circles. Circles containing the text K4 indicate H3K4 dimethylation within these nucleosomes.

possible that mechanisms that prevent DNA methylation of the unmethylated allele may help in avoiding the recruitment of DNA methylation-dependent DNA binding proteins like Mecp2, which can inhibit transcription. Another possibility is that the factors responsible for the specialised chromatin organisation (HS-I hypersensitive site) within the putative *Neuronatin* ICR keep the paternal unmethylated allele of *Neuronatin* in a constitutively active chromatin state. This possibility is based on our MNase analysis of the *Neuronatin* locus which indicated that on the paternal-allele the nucleosomes are always positioned from the second intron towards the promoter irrespective of whether the gene is being transcribed (in brain) or not (in liver) (Fig. 4 and data not shown). We suggest that this positioning of the nucleosomes somehow leaves the promoter region in an active chromatin conformation in which it is always accessible for transcription initiation. Whenever tissue-specific enhancers for *Neuronatin* (John et al., 2001) are available for interaction with the promoter, transcription is initiated (Fig. 7). This is also supported by our finding that the promoter region on the paternal unmethylated allele is always associated with active chromatin correlated H3K4 methylation irrespective of whether the gene is being transcribed or not (Figs. 7 and 5C). Importantly, our results suggest that the transcriptional status of the *Neuronatin* gene on the paternal-allele is correlated with the histone modifications associated with the second intron but not with those associated with the promoter (Fig. 5C). It was interesting to note that different inactive chromatin histone marks in liver (H3K27 trimethylation) and kidney (H3K9 trimethylation) were being utilised for transcriptional silencing of the paternal-allele probably reflecting their developmental lineages. In addition, according to our model (Fig. 7) since on the methylated maternal allele the nucleosomes are randomly positioned, even the tissue-specific enhancer availability does not ensure transcription. This situation on the maternal allele is also compounded by DNA methylation, which brings in DNA methylation-dependent binding proteins (e.g. MECP's) to make the allele even less accessible to transcription machinery.

4. Experimental procedures

4.1. *Mice disomic for chr2*

Newborn mice or embryos with maternal or paternal duplication for chromosome 2 generated by the standard method of inter-crossing reciprocal translocation heterozygotes (Cattanach, 1986; Searle and Beechey, 1978) and were a kind gift from Colin Beechey, Mammalian Genetics Unit, Harwell UK.

4.2. *Nuclease sensitivity assay and indirect end-labelling analysis*

DNase I (Roche) and MNase (S7 nuclease, Roche) digestion assays were done on isolated nuclei or cultured cells as previously described (Khosla et al., 1999). For analysis of DNase I hypersensitive sites and nucleosomal positioning, small 300–500 bp end-probes were generated by PCR amplifications. The following end probes were generated for the *Neuronatin* locus (GenBank Accession No.: AF303656): NN2 (nucleotide 10336–10890), NN3 (nucleotide 10927–11470), NN4 (nucleotide 13981–14526), NN8 (nucleotide 18133–18410), NNUP1 (nucleotide 2731–3066).

4.3. *Generation of transgenic Drosophila*

The 250 bp second intron of *Neuronatin* was PCR amplified and initially cloned into the smaI site (flanked by *lox*P sites) of pLML vector. The clone pLMLI2+ was taken for further cloning. The intronic insert flanked by the *lox*P sites was excised using XhoI restriction endonuclease and cloned into the XhoI site upstream of hsp70 promoter for *mini-white* reporter gene in pCasPer vector. Two clones, one with the intron in positive orientation (pCaSpeRI2+) and one in negative orientation (pCaSpeRI2−) were injected in W^{1118} *Drosophila* embryos following standard protocols to make transgenic lines (Voie and Cohen, 1997). The G1 progeny from crosses between G0 flies and W^{1118} flies were screened for the eye color and all the positive progeny were treated as individual lines.

Thirteen independent lines, 8 with the intron in positive orientation and five in negative orientation were established. Once the balanced stocks of all the lines were made a flipped out version for each line was generated. For this homozygous males of transgenic lines were crossed to virgins expressing *cre* recombinase. Stocks were balanced and the absence of the intronic region was confirmed by PCR using pLML vector-specific primers. Both homozygous and heterozygous transgenic lines were compared with their flipped out versions for differences in eye color. Quantitative assessment of the difference was done by pigment-extraction assay (Ashburner, 1989).

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4.4. Chromatin Immunoprecipitation and bisulfite sequencing

ChIP assay was performed according to the instructions of ChIP Assay Kit (Upstate, USA) with some modifications. Nuclei were isolated as described previously (Khosla et al., 1999). Nuclei obtained were suspended in lysis buffer (5 mM PIPES, pH 6.5, 85 mM KCl, 0.5% NP-40), incubated at 4 °C for 10 min and centrifuged at 1200*g* for 2 min. The pellet was re-suspended in SDS lysis buffer (Upstate) and incubated at 4 °C for 10 min. The sonication conditions were set so as to get average DNA fragments of around 400 bp. For chromatin immune-precipitation 2 μl of antibody (Upstate, USA) was added per reaction. The bound fractions were collected and both along with input were treated with sodium bisulfite as described previously (Gokul et al., 2007). PCR amplification was done for 30 cycles, each in a 25 μl reaction containing 1× PCR buffer, 1.5 mM $MgCl_2$ and 200 μM dNTPs along with 10 pmol of primers. The bisulfite primers were designed using Methprim software (Li and Dahiya, 2002).

Primers for Promoter region

- Forward: 5′TTTAGGTGGTAAGAGGGTATTTAAGGTA3′
- Reverse: 5′AATACATACTCACCTACAACA3′

Primers for Intronic region:

- Forward: 5′TTGATTGGTGGATAAGTTGTGTTT3′
- Reverse: 5′CCACCCTTAAAAAAATACCCATAAT3′.

The PCR products were electrophoresed on a 2% agarose gel. The bands were eluted and cloned into a TA cloning vector and 8–15 clones for each sample were sequenced.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2008.08.002.

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