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Chromatin Modification of the Human Imprinted *NDN* (Necdin) Gene Detected by In Vivo Footprinting

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Abstract Allele-specific transcription is a characteristic feature of imprinted genes. Many imprinted genes are also transcribed in a tissue- or cell type-specific manner. Overlapping epigenetic signals must, therefore, modulate allele-specific and tissue-specific expression at imprinted loci. In addition, long-range interactions with an Imprinting Center (IC) may influence transcription, in an allele-specific or cell-type specific manner. The IC on human chromosome 15q11 controls parent-of-origin specific allelic identity of a set of genes located in *cis* configuration within 2 Mb. We have now examined the chromatin accessibility of the promoter region of one of the Imprinting Centre-controlled genes, *NDN* encoding necdin, using in vivo DNA footprinting to identify sites of DNA–protein interaction and altered chromatin configuration. We identified sites of modified chromatin that mark the parental alleles in *NDN*-expressing cells, and in cells in which *NDN* is not expressed. Our results suggest that long-lasting allele-specific marks and more labile tissue-specific marks layer epigenetic information that can be discriminated using DNA footprinting methodologies. Sites of modified chromatin mark the parental alleles in *NDN*-expressing cells, and in cells in which *NDN* is not expressed. Our results suggest that a layering of epigenetic information controls allele- and tissue-specific gene expression of this imprinted gene. J. Cell. Biochem. 94: 1046–1057, 2005.

Key words: promoter; tissue-specific gene regulation; imprinting; in vivo footprinting

Individuals with either Prader–Willi syndrome (PWS) (OMIM 176270) or Angelman syndrome (AS) (OMIM 105830) typically carry deletions of approximately 3.5 Mb on human chromosome 15q11-q13 [Nicholls et al., 1998]. The parental origin of the deletion distinguishes the two syndromes, whereby PWS individuals have deletions of paternal origin while deletions in AS occur on the maternally inherited allele. A *cis*-acting Imprinting Center (IC) located within the deletion, acts in an intergenerational fashion to specify the parent-of-origin identity for a set of nearby genes. *NDN*, the gene encoding necdin, is one of this set of genes, and is imprinted and expressed only from the paternal allele. *NDN* is also expressed in a cell-type specific manner. Thus a mechanism must exist that coordinately controls temporal, spatial, and allelic expression of *NDN* and other imprinted, developmentally regulated genes. These mechanisms must operate epigenetically, as the gene sequence is identical in all cells and on both alleles.

The promoter contains sequences essential for RNA polymerase II binding and initiation, and additional transcription factor binding sites that mediate gene-specific transcription. The accessibility of these elements to transcription factors is governed by epigenetic modifications to the core DNA sequence. These modifications include DNA methylation and nucleosome spacing, which is governed by the presence of post-translational modifications to core histones [Turner, 1993; Rice and Allis, 2001]. Regions of DNase I hypersensitivity often coincide with disrupted nucleosome positioning in regulatory regions of genes [Bresnick et al., 1992]. The hypersensitivity correlates with cooperative binding of regulatory proteins to the DNA, in a

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manner dependent on nucleosomal structure [Boyes and Felsenfeld, 1996; Khosla et al., 1999]. In addition to nuclease hypersensitive sites, some genes contain extended regions of generalized increased sensitivity [Feil and Kelsey, 1997].

Allele-specificity is conferred to imprinted genes in the gametes, while other epigenetic modifications related to cell-type specificity are likely conferred at the time of lineage restriction. While the initial imprint is established by the time of implantation, subsequent allele-specific epigenetic modifications can be established as a consequence of the initial imprint. DNA methylation has been linked to allelic inactivation [Szabo et al., 1998; Birger et al., 1999; Hori et al., 2002]. In imprinted genes, nuclease hypersensitive sites are often found on the expressed allele in the equivalent region that is methylated on the non-expressed allele [Bartolomei et al., 1993; Hark and Tilghman, 1998; Khosla et al., 1999; Schweizer et al., 1999]. A region in the *H19-Igf2* IC is methylated on the paternal allele and is bound by the methylation sensitive insulator, CTCF, on the unmethylated maternal allele [Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000]. Parent-of-origin specific epigenetic modifications mark the *H19* alleles in embryonic stem cells, suggesting that protein-DNA binding could serve as an imprint [Szabo et al., 1998].

NDN expression is tissue- and allele-specific, allowing dissection of epigenetic marks that are related to gene expression from those that stably discriminate alleles [Hanel and Wevrick, 2001]. We previously demonstrated developmentally dynamic patterns of maternal hypermethylation and paternal hypomethylation of the promoter CpG island in mouse *Ndn*, by sodium bisulfite sequencing [Hanel and Wevrick, 2001]. We then characterized DNA methylation in a similar promoter CpG island and a second downstream CpG island in human *NDN* by sodium bisulfite sequencing, and mapped regions of histone modifications in the *NDN* promoter and surrounding region [Lau et al., 2004]. To search for allele-specific sites of DNA binding proteins and differences in chromatin structure in the 5′-region of *NDN*, we have now performed high-resolution in vivo footprinting with ligation-mediated PCR (LMPCR) on human cell lines containing only the paternal or maternal copies of chromosome 15. We identified a set of allele-specific and cell type-specific differences in chromatin structure that we propose may be important in maintaining allelic identity in different cell types. Finally, we performed fine scale chromatin mapping that revealed distinct sites that strongly differentiate alleles in expressing cell types, while other epigenetic modifications differentiate alleles even in cell types in which both alleles are repressed.

MATERIALS AND METHODS

Cell Lines

Control fibroblasts from the NIGMS Human Genetic Cell repository (GM00650), PWS fibroblasts (University of Miami Brain and Tissue Bank for Developmental Disorders #1889), and AS fibroblasts (15q11-q13 deletion cell line, from Dr. A. Beaudet, Baylor College of Medicine) were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Control lymphoblasts (our laboratory number LCL10), PWS lymphoblasts (GM09024B, GM09133), and AS lymphoblasts (GM11515) were grown in RPMI supplemented with 15% FBS. Genomic DNA was extracted by proteinase K/SDS digestion, phenol/chloroform extraction and ethanol precipitation [Ausubel et al., 1993].

In Vivo DNA Footprint Analysis

In vivo DNA footprinting analyses were performed essentially as described [Drouin et al., 2001]. For each treatment, about 7.5×10^7 lymphoblast cells or five confluent 150-mm tissue culture plates of fibroblasts were used. For DMS treatment of lymphoblasts, the cells were sedimented by centrifugation and diluted in 50 ml Hank's balanced salt solution (HBSS) (1.5×10^6 cells/ml) before treatment. For fibroblasts, cells were treated with DMS as an attached monolayer. For UVC irradiation of lymphoblasts, cells were sedimented, then resuspended at a concentration of 1×10^6 cells/ml with cold 0.9% NaCl and placed in thin layers in 150 mm Petri dishes. Fibroblasts were treated in their culture dishes. For DNase I treatment, lymphoblasts were sedimented and resuspended before treatment. Attached fibroblasts were treated directly.

Ligation-Mediated PCR

A detailed LMPCR procedure has been reported elsewhere [Angers et al., 2001; Drouin

et al., 2001]. For the extension step, 0.5–2 μg of DNA processed from one of the treatments was mixed with 3 μl of 10× Pfu buffer (200 mM Tris-HCl, pH 8.8, 20 mM $MgSO_4$, 100 mM NaCl, 100 mM $(NH_4)SO_4$, 1% (v/v) Triton X-100, 1 mg/ml nuclease-free BSA), 1 pmol of the extension primer (see below), 5 μl of cloned Pfu mix (1.5 mM of each dNTP and 1.5 U cloned Pfu DNA polymerase) and H_2O to a final volume of 30 μl. Reactions were denatured at 98°C for 5 min, incubated at T_m of primer-1°C for 4 min, then at 75°C for 20 min. Primers NEC1.1, NEC1.1B, NEC3.1 and NEC5.1 were used for extension of the bottom (transcribed) strand DNA fragments and primers NEC2.1, NEC4.1, and NEC6.1 were used for extension of the top (non-transcribed) strand DNA fragments (see Table I for sequence, position and T_m of primers).

For the ligation step, linkers were prepared by slow annealing of the 25-mer, L25 5′-GCGGTGACCCGGGAGATCTGAATTC with the 11-mer, L11 5′-GAATTCAGATC. Ligation was performed by adding 45 μl of ligation mix (30 mM DTT, 1 mM ATP, 80 ng/ml BSA, 2 pmol/μl linker, 3U T_4 ligase) to the extension reaction, followed by overnight incubation at 18°C. DNA was ethanol precipitated with the addition of 20 μg/ml glycogen and the pellet was resuspended in 50 μl H_2O.

Primers NEC1.2, NEC1.2B NEC3.2, and NEC5.2 each in conjunction with the linker L25 oligonucleotide were used to amplify off of the bottom (transcribed) strand (Table I). Primers NEC2.2, NEC4.2, and NEC6.2 each in conjunction with the linker L25 oligonucleotide were used to amplify off the top (non-transcribed) strand (Table I). Since the L25 oligonucleotide is overhanging and does not have a complete complementary strand, one round of linear amplification takes place before L25 can anneal. For primers NEC1.2, NEC2.2, NEC3.2, and NEC4.2, PCR was done with Pfu as follows. To the ligated samples, 50 μl of cloned Pfu DNA polymerase mix was added (2× cloned Pfu buffer, 0.5 mM of each dNTP, 10 pmol LP25 (linker primer), 10 pmol of gene-specific primer, 3.5 U cloned Pfu DNA polymerase). A final concentration of 7.5% DMSO was added to the reaction mix for primers NEC2.2, NEC3.2 and NEC4.2. For primers NEC5.2 and NEC6.2, PCR was done with Taq as follows: To the above ligated samples, 50 μl of Taq polymerase mix (4 mM $MgCl_2$, 2× Taq buffer, 0.5 mM dNTP, 20 pmol LP25, 20 pmol gene-specific primer, 6 U Taq polymerase) was added.

LMPCR samples were electrophoresed on denaturing acrylamide gels. Sequencing ladders were prepared by chemical cleavage as described [Drouin et al., 2001]. The gel was electroblotted to a positively charged nylon membrane (Roche Molecular Biochemicals) and hybridized. The single-stranded probes were generated by PCR amplification with a single primer on a purified PCR fragment amplified with NEC1.2 and NEC2.2 or NEC5.2 and NEC6.2. ^{33}P dCTP was incorporated in the PCR amplification. Blots were hybridized and exposed to film for 3 h to 3 days. Recognition sites for transcription factors were identified using MatInspector (transfac.gbf.de/cgi-bin/matsearch/matSearch.pl).

TABLE I. Synthetic Oligonucleotide Primers for LMPCR Analysis of the Human *NDN* Gene

Primer	Sequence (5′–3′)	Position[a]	T_m (°C)[b]
Non-transcribed (upper) strand			
NEC1.1	TCACTGGTTCGCATAAAGC	−402 to −384	55.4
NEC1.2	GCTCATGTTTACAAAGCCGCCCAGACC	−385 to −359	66.5
NEC1.1B	TGGATATACCCAGGTAAGCG	−326 to −307	55.4
NEC1.2B	GCGTTTCCCAAGAAACTTGACCCCAACATCC	−309 to −279	66.5
NEC3.1	CCAGTACGCATCCATCTC	−238 to −221	57.2
NEC3.2	ACTTCTCTCCTGCCCTAGATCTTCTCAGCC	−220 to −191	67.5
NEC5.1	TCCCGCCGCCGCCC	−57 to −44	64.6
NEC5.2	GCCCTGCCCGTCGCTGCGGAAGGC	−42 to −19	73.9
Transcribed (bottom) strand			
NEC2.1	CGGCACTGCGCCTGCG	−60 to −75	64.3
NEC2.2	CGCGGCCTTGGCCAGCGGCTGG	−80 to −101	73.1
NEC4.1	AGATCCTTACTTTGTTCTGACATG	110 to 87	56.8
NEC4.2	TCTGCGCCGTCTGGCAAGGGCAGG	86 to 63	70.5
NEC6.1	CTGGGGAGGCGGCG	264 to 251	61.7
NEC6.2	GCCTGCGGAGCGGCCGTCGGGC	248 to 227	74.9

[a]Primer positions are given relative to the transcription start site.
[b]T_m determined by the GeneJockey software program.

RESULTS

In Vivo Footprinting in the *NDN* Promoter Region

We used the highly sensitive technique of in vivo footprinting analysis on PWS and AS fibroblast and lymphoblast cell lines to investigate specific aspects of allele-specific chromatin structure, and to identify possible binding sites for regulatory proteins. These cell lines carry only the paternally derived (AS), or only the maternally derived (PWS) 15q11-q13 region, because of a constitutional chromosomal deletion in the individuals from whom the cell lines were derived. The murine promoter region was previously defined using a reporter assay in neurally differentiated P19 cells [Nakada et al., 1998]. The minimal postmitotic neuron-restrictive promoter elements are located between −177 and −33 from the transcription start site. We focused on a 688 bp region that includes the upstream part of the 5′-CpG island, part of the human promoter region as defined by transfections of promoter constructs into murine P19 cells [Nakada et al., 1998], and 198 bp of exonic sequence and (Fig. 1). Notably, the region from the transcription start site upstream to position −118 is about 65% conserved between human and mouse and likely defines the minimal promoter, while the region upstream of this shows no sequence similarity with the similarly placed murine region. We focused on a 688 bp region in the human promoter region and upstream part of the 5′-CpG island as defined by transfections of promoter constructs into murine P19 cells. Murine *Ndn* is primarily expressed in the nervous system and in muscle. In contrast, human *NDN* is widely expressed, although importantly both alleles are silent in lymphocytes and lymphoblasts.

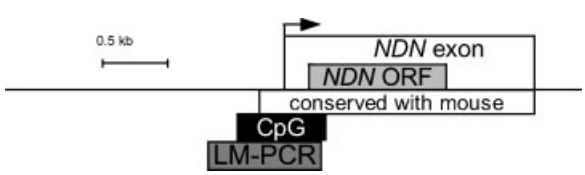

Fig. 1. *NDN* and surrounding regions. The white box indicates the single exon of the *NDN* gene with its open reading frame (ORF), the arrow indicates transcription start site and CpG island is indicated in black. The region with significant sequence similarity to murine *Ndn* is indicated. The region surveyed by LMPCR primers is indicated as a shaded box, and is shown in more detail in Figure 2.

The in vivo footprinting analysis included DNase I, UV, and DMS treatments on PWS and AS fibroblasts and lymphoblasts [Drouin et al., 2001]. The activity of DNase I is generally not sequence-specific, and therefore, this treatment can detect most regions bound by protein. It can also identify regional changes in chromatin structure, and detect the nucleotide positions of protein–DNA contact points. DMS and UVC can detect specific nucleotide bases involved in DNA–protein interactions, but can only detect footprints at guanine residues and at pyrimidine dimers, respectively, that are protected by the protein. However, DNase I treatment is more disruptive to the cells than DMS and UVC because activity of the enzyme requires permeabilization of the cells.

Treatment samples consisted of cells from each of the four cell lines (in vivo, *v*) or DNA extracted from the AS lymphoblast line (in vitro, *t*) for comparison. Cells or DNA were treated with each of the three agents, and then the DNA was extracted and amplified by LMPCR. Multiple sites of chromatin accessibility differing from the in vitro DNA sample were found. Most of these were found in all four cell lines, and are, therefore, neither allele-nor tissue-specific. Four types of footprints of interest to imprinting were identified, based on their correlation with expression and parent-of-origin of the allele. The first subset of allele-specific footprints is seen only on the expressed, paternal allele in fibroblasts. A second subset is seen on both alleles in lymphoblasts and the maternal allele in fibroblasts, where the gene is not expressed. The third footprint type is allele-specific regardless of expression state, reflecting only parent-of-origin. The final type of footprint is cell-type specific, but not parent-of-origin specific. A 688 bp region was analyzed, and footprints were found in the region from −283 to +196, relative to the transcription start site. This region is displayed in the form of the DNA sequence overlaid symbols indicating each type of modification identified.

DNA–Protein Interactions Strongly Mark the Paternal Promoter in Fibroblasts

The first footprint type includes sites within the *NDN* promoter region that may correspond to transcription-related factors used by the expressing allele. A striking paternal allele-specific DNase I footprint on the bottom strand is located in the promoter between −51 and

−81 bp upstream of the transcription start site (Fig. 2A, the fibroblast paternal (FB) cell line lane 15 compared to equivalently treated DNA, lane 19). The occupancy of this site was supported by the presence of UVC and DMS footprints (Fig. 2A, lane 10 compared to lane 14 and lane 5 compared to lane 1), and hyposensitivity of the complementary region on the top strand

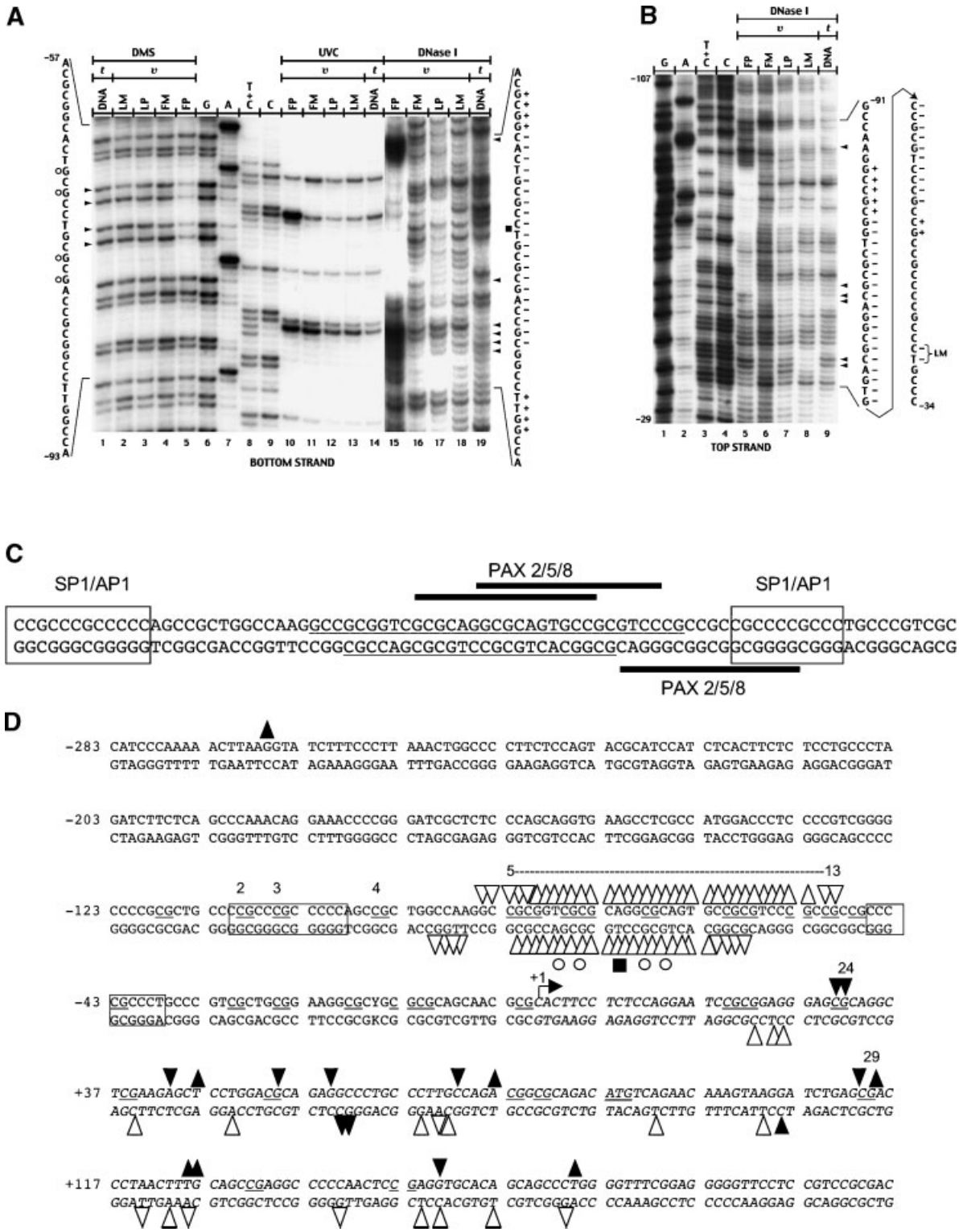

Fig. 2.

(Fig. 2B, lane 5). This indicates that a protein or protein complex is bound to the active allele, covering both strands over a region of about 30 bp with high-GC content. We then analyzed the protected sequence for predicted transcription factor binding sites. Predicted SP1 and AP1 binding sites, which occur frequently in GC-rich DNA, as well as partially degenerate binding sites for the transcription factors CPBP, Egr-1, and ZNF278 were identified bioinformatically within the footprint. We identified three good consensus binding sites for the PAX subfamily that includes the PAX2, PAX5, and PAX8 proteins (Fig. 2C). In humans, these proteins are normally expressed postnatally only in retina and kidney (PAX2), B-cell lineages (PAX5), and thyroid (PAX8). We performed reverse transcription-PCR for these three PAX genes in the fibroblast cell lines and found no evidence for expression of *PAX2*, *PAX5*, or *PAX8* (data not shown). This large footprint may thus identify a binding site for an as yet unidentified human transcription factor, which may be related in binding specificity to proteins of the PAX family. The large DNase hyposensitive region is flanked by 10–12 nucleotide CG-rich regions containing consensus binding sites for SP-1-like and AP-1-like proteins. Notably, these CG-rich flank the large footprint are not themselves differentially footprinted across the different cell lines. A set of expressed allele DNaseI hypo- and hyper-sensitive sites was evident on the bottom strand, within the 5′-untranslated region (UTR) and into the beginning of the open reading frame (ORF). These did not cluster like the large promoter footprint, but may be indicative of a general open conformation (Fig. 2D).

Altered Chromatin Structure Marks the Silent Alleles

A chromatin structure specific to non-expressed (NE) alleles was detected on the top strand in the 5′-UTR, between positions +32 and +168 (Fig. 3A, lanes 6–8, sites marked NE). A set of DNase I hypersensitive sites (upward, closed arrowheads in Fig. 2D) are present and intermingled with a comparable number of hyposensitive sites (downward, closed arrowheads in Fig. 2D), on both alleles in lymphoblasts and on the non-expressed, maternal allele in fibroblasts. Although, the hyper/hyposensitive sites show a tendency towards periodicity of about 12 nucleotides, which would suggest an ordered structure, the pattern is not as striking as that seen when nucleosomes are rotationally positioned, as they are in the promoter of the *HPRT* gene on the inactive X chromosome [Chen and Yang, 2001]. The lack of these sites on the expressing, paternal allele in fibroblasts may reflect an accessible chromatin configuration throughout the 5′-UTR and promoter region on this allele. Finally, a single site of DNase I hypersensitivity specific to non-expressed (NE) alleles was detected on the top strand at position −266 (Fig. 3B).

Parental Alleles Are Epigenetically Distinct Independent of Expression State

Footprints found in only one or the other lymphoblast line, each representing a single *NDN* allele, could identify epigenetic marks identifying the alleles in the absence of expression from either allele. As both alleles are silent in lymphoblasts, such allele-specific modifications may represent remnants of an imprint

Fig. 2. In vivo footprinting distinguishes expressing and non-expressing alleles. **A**: In vitro treated (*t*) DNA was compared with in vivo (*v*) treated samples (DMS, UVC, or DNase I) from maternal allele only and paternal only lymphoblastoid cell lines (LM or LP) and maternal allele only and paternal only fibroblast cell lines (FM or FP). The position in the DNA sequence was determined by chemical cleavage (G, A, T + C, C). All differences among cell lines are also indicated by an arrow at the side of the sequencing gel. LMPCR analysis of the bottom strand between positions −57 and −93 relative to the transcription start site is shown. Open circles on the left represent DMS-sites, the black square on the right a UVC + site, and DNase I hyper- ''+'' or hypo-sensitive sites ''−'' are also indicated on the right. All these sites were found in FP cells only. **B**: A region overlapping that in (A), but showing the top strand with DNase I treatment alone. All sites were found in FP cells only, except for the pair of DNase I hypersensitive sites indicated by LM and found only in the maternal lymphoblast allele. **C**: Predicted transcription factor binding sites near the −57 to −93 FP footprinted region. **D**: Sequence of the region examined by LMPCR. CpG dinucleotides are underlined and those showing differential chromatin accessibility are numbered according to Figure 6. The transcribed region is italicized, with the transcription start site as +1. The ATG start codon is doubly underlined. Differences from the in vitro treated sample are noted as follows. Upward arrowheads indicate DNase I hypersensitive sites; downward arrowheads indicate DNase I hyposensitive sites. Open arrowheads are DNase I hyper/hyposensitive sites found only on the expressed allele (the paternal allele in fibroblasts). Filled arrowheads represent DNase I hyper/hyposensitive sites found on non-expressed alleles. The white circles are expressed paternal fibroblast (FP) DMS-(hyporeactive or protected) sites. The black square is a UVC+ (increased cyclobutane pyrimidine dimer formation) FP site.

needed for allele-specific expression in a precursor cell in the lymphocyte lineage, or may be a mark of a continuing influence from the *cis*-acting IC. Four maternal allele DNase I hyposensitive sites were identified in lymphoblasts, as summarized in Figure 4. These sites are located upstream of the transcription initiation site at top strand positions −39 and −38 (Fig. 2B, lane 8 compared to lane 9, marked LM) and also positions −266 and −264 (Fig. 3B, lane 8 compared to lane 9, marked LM). Neither position contains a CpG dinucleotide, although the second pair of hyposensitive sites is within a dense region of CpG dinucleotides. These are the only allele-specific modifications that are unrelated to expression that are on the top strand, and upstream of the transcription start site. Most of the allele-specific modifications are on the bottom strand, within the transcription unit, and found on either allele. We identified additional allele-specific DNase I sites on the bottom strand in the 5′-UTR and the beginning of the ORF. Notably, most of the maternal allele-specific sites are DNase I hyposensitive (downward arrows), whereas more of the paternal allele-specific sites are DNase I hypersensitive (upward arrowheads) (Fig. 4), consistent with a more open chromatin conformation on the paternal allele, even in the absence of expression. Finally, overlaid on the epigenetic modifications distinguishing alleles and expression state, cell-type specific footprints were seen with UVC and DNase I on the bottom strand, throughout the region analyzed (Fig. 5).

DISCUSSION

The initial chromatin state of each allele within the one-cell embryo represents a parental imprint. This state is altered during development through epigenetic modifications, some of which are detectable as conformational changes to chromatin. In particular, the chromatin domain encompassing an imprinted transcription unit and its regulatory sequences is remodeled to impart allele-specific expression while concurrently ensuring tissue-specificity of each gene as appropriate for cellular function in fully differentiated cell types. A transcriptional activator that specifically binds unmethylated CpG sites without sequence specificity could be a candidate for a role in remodeling [Voo et al., 2000]. For example, two closely positioned dyad Oct-binding sequences maintain an unmethylated state on the maternal allele of the *H19-Igf2* IC in a cell type that is equivalent to an early post-implantation stage [Hori et al., 2002]. We had previously shown that regional epigenetic characteristics, such as differential histone acetylation and histone methylation distinguish alleles in *NDN*. We now demonstrate differences at the nucleotide level that likely determine the transcriptional state of each allele, in each of two cell types (Fig. 6).

The developmentally regulated protection of the promoter region from DNA methylation and consequent open chromatin structure on the paternal allele may make it possible for sequence-specific transcription factors to selectively bind the paternal allele in terminally differentiated cells. This model is consistent with the paternal allele-specific protein binding found at the *NDN* promoter, where we have detected a set of chromatin modifications intrinsic to the expressed, paternal allele in fibroblasts. The large footprinted site on the paternal allele in the fibroblast cell line likely represents the binding of a protein or protein complex that may be a transcriptional activator and that is blocked from binding to the maternal allele by DNA methylation or other epigenetic factors. Our prediction that these are sites of protein/DNA contacts with the transcription machinery is based on their position upstream of the transcription start site, the size of the footprinted region, and occupancy of both strands. Alternatively, protein binding at this site could have a role in the establishment of the imprint on the non-expressed paternal allele by binding in the sperm or early embryo, and could protect this region from de novo DNA methylation during embryogenesis thus facilitating the activity of this allele through local conformational changes. Identifying the proteins bound at this site, the developmental timing of the appearance of the binding site and its dependence on the *cis*-acting IC would assist in the discrimination of these alternative hypotheses. Further analysis of this region and the proteins associated with it, in the in vivo context, will also elucidate the role of specific transcription factors in *NDN* expression.

The imprinted *SNRPN* and *H19* genes have stable regions of nuclease hypersensitivity on the unmethylated paternal allele in the differentially methylated region [Khosla et al., 1999; Schweizer et al., 1999]. Similarly, the

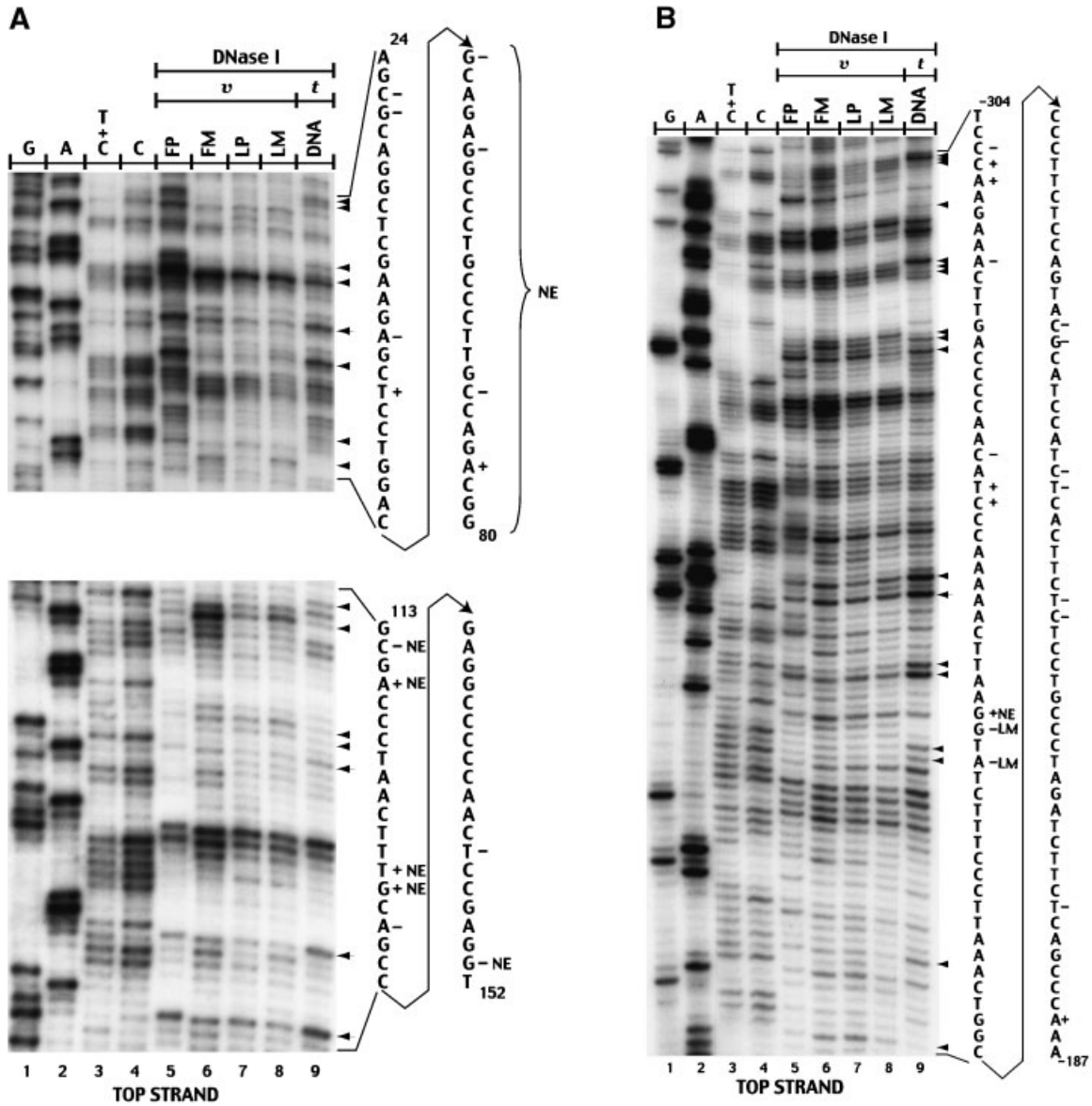

Fig. 3. DNAse I hyper/hypo-sensitive sites identified on non-expressed alleles. **A**: Symbols are the same as in Figure 2A,B. LMPCR analysis of the top strand 5′-untranslated region (UTR) and ORF, between positions +24 and +152 reveals periodic DNase I hyper- and hypo-sensitive sites on non-expressed (NE) alleles. A portion of the middle of the gel was removed from the figure as it contained no cell line specific modifications. **B**: LMPCR analysis of the top strand far upstream region, between positions −304 and −187.

NDN/*Ndn* promoter is significantly hypomethylated in sperm in human and mouse [El-Maarri et al., 2001; Hanel and Wevrick, 2001]. The lack of DNA methylation on the paternal allele may allow an epigenetic mark such as chromatin conformation or specific protein–DNA interaction to carry the allelic imprint from the male germ line. The patterns of DNA methylation of each CpG dinucleotide within the CpG island had previously been determined by bisulfite sequencing [Lau et al., 2004]. We can now compare the methylation patterns with the chromatin modifications identified by in vivo footprinting. The large fibroblast paternal allele-specific footprint contains CpG sites 5–13 (Fig. 2D), which are moderately methylated on both alleles in lymphoblasts and on the maternal allele in fibroblasts. The exception is at site 12, which falls at the edge of the footprint and is relatively hypomethylated in both alleles in fibroblasts. DNA methylation of two cytosines within *Hpa* II restriction enzyme sites

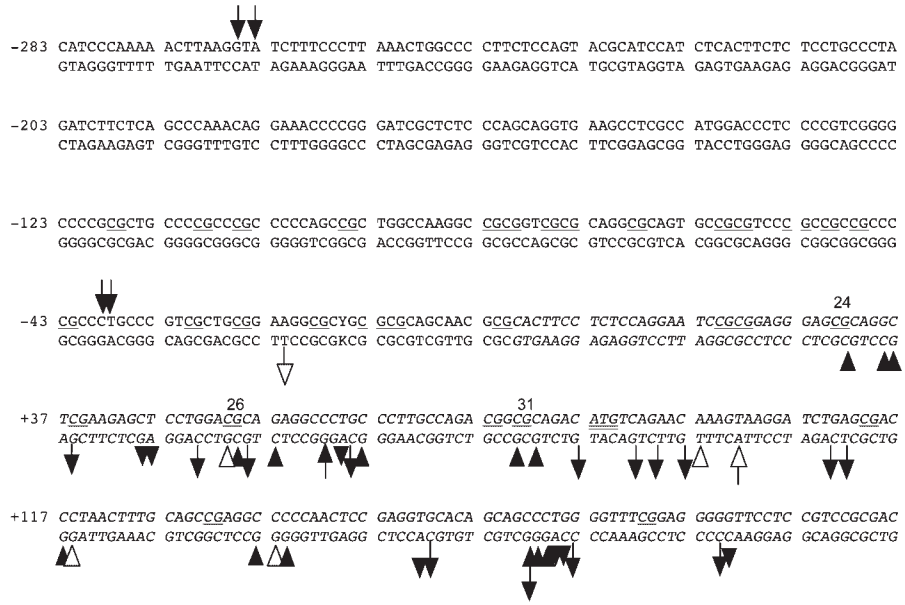

Fig. 4. Allele-specific DNAse I hyper/hypo-sensitive sites unrelated to gene expression. Upward arrow(head)s indicate DNase I hypersensitive sites; downward arrow(head)s indicate DNase I hyposensitive sites. Open arrow(head)s are DNase I hyper/hyposensitive sites that are allele-specific in lymphoblasts and fibroblasts, whereas the filled arrow(head)s represent DNase I hyper/hyposensitive sites that are allele-specific only in lymphoblasts. Arrows represent DNase I sites on the maternal allele, whereas arrowheads represent DNase I sites on the paternal allele. CpG dinucleotide sites are underlined, with key sites numbered.

(CpG sites 8 and 9) on reporter constructs caused transcriptional repression [Nakada et al., 1998]. The DNase I footprint is flanked on both sides by pairs of GC-boxes, which contain CpG sites 2–3 and 14–15, that are hypomethylated on both alleles in fibroblasts, but are not themselves differentially footprinted. Together these data suggest that the large footprint represents methylation-sensitive binding of a transcription factor important for tissue-specific expression and allele-specific expression. Alternatively, allele-specific bind-

Fig. 5. Cell-type-specific DNAse I hyper/hyposensitive sites. Upward arrowheads indicate DNase I hypersensitive sites; downward arrowheads indicate DNase I hyposensitive sites. Open arrowheads represent DNase I hyper/hyposensitive sites that are only in lymphoblasts and filled arrowheads represent the sites that are only in fibroblasts. The open squares are UVC-lymphoblast-specific sites and the filled square is a UVC+ fibroblast-specific site.

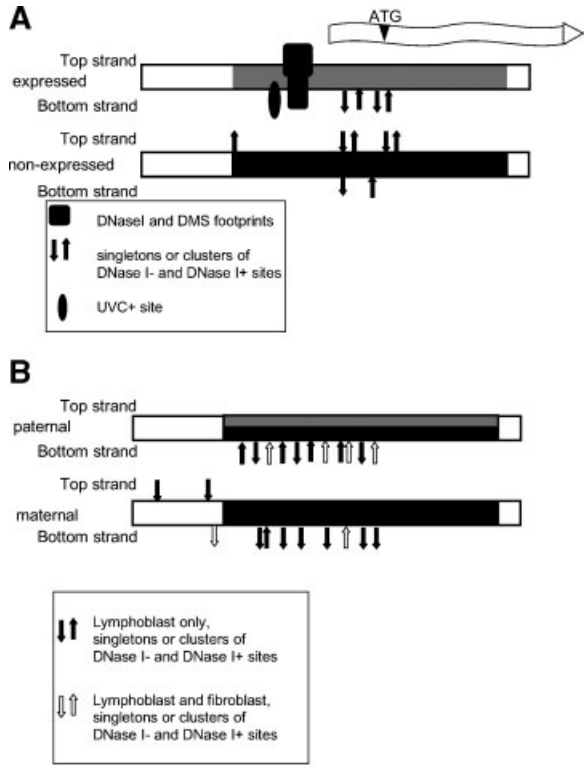

Fig. 6. Summary of epigenetic marks. **A**: Gene expression related epigenetic marks. The two bars represent the 688 bp region analyzed on the expressed (FP) or non-expressed (FM, LP, and LM) alleles. The two bars represent the expressed (FP) or non-expressed (FM, LP, and LM) The white wavy arrow line above the expressed allele (white bar) represents the transcription from that allele only. The black (mostly methylated) or gray (partially methylated) bars represent the CpG islands. Epigenetic marks are symbolically represented as described in the figure key. ''Multiple footprints'' includes all sites represented in Figure 2A,B. **B**: Parent-of-origin specific marks. The two bars represent the 688 bp region analyzed on the paternal or maternal alleles. The black (mostly methylated, FM, LP, and LM) or gray (partly methylated, FP) bars represent the CpG islands for each allele. Epigenetic marks are symbolically represented as described in the figure key.

ing in fibroblasts may be modulated by local chromatin configuration, and only indirectly correlated with differential methylation. Two other expressed allele DNase I hyposensitive sites fall on CpG dinucleotides (sites 24 and 29, Fig. 2). These two sites have low to moderate levels of methylation on both alleles in fibroblasts, suggesting that this differential sensitivity is likely due to differential protein interactions or local chromatin structure rather than being strictly methylation dependent.

In fibroblasts, the domain encompassing *NDN* is in an open conformation on the expressed paternal allele, assayed by increased histone acetylation and a broad region of paternal allele-specific DNase I sensitivity detected by limited DNase I digestion and Southern blot analysis (data not shown). In cells that do not express *NDN* the paternal allele was in a closed chromatin conformation that was indistinguishable from the closed chromatin on the maternal allele when assayed by histone acetylation and by DNase I sensitivity. Consistent with this differential sensitivity, we identified altered DNase I sensitivity on non-expressed alleles, and localized within the start of the transcribed region. Also within the 5′-transcribed region, the paternal allele tended towards hypersensitivity and the maternal allele towards hyposensitivity, suggesting that the alleles are distinguishable even when neither is expressed. Imprinted domains can occupy different subnuclear domains that reflect their parent-of-origin [Gribnau et al., 2003]. The subnuclear localization of each imprinted allele likely influences its chromatin state, and this localization may also be influenced by the transcriptional state of nearby genes and the IC itself. In summary, the final state of the imprinted alleles as detected by in vivo footprinting is dependent on its original post-conception state, developmental remodeling at a local level, and the higher-order chromatin and subnuclear domain in which it resides.

Allelic differences in chromatin structure were also noted in lymphoblastoid cell lines, on either parental allele. As neither allele is expressed in these cell lines, these epigenetic allelic differences are presumed to reflect either remnants of an original imprint or ongoing maintenance by the *cis*-acting IC, but in either case these differences are no longer needed for allele-specific transcriptional activity or competence in this terminally differentiated cell type. That some of these allelic differences were also found in fibroblasts suggests that these sites may be specific targets of the imprinting maintenance system, through recognition of epigenetic state emerging from the initial gametic imprint, or by differential subnuclear localization. This system may continuously label the parental origin of the allele regardless of the more labile tissue-specific, and expression-related epigenetic modifications [Bielinska et al., 2000]. We also noted that all of the paternal allele-specific marks are in the transcription unit, suggesting that if these are related to allelic discrimination they do not act

directly on the promoter. The pattern emerging from the analysis of epigenetic marks at the *NDN* locus has revealed a complex layering of epigenetic modifications. Further studies will elucidate the dependence of chromatin modifications on the presence of the IC and the role of tissue-specific factors in maintaining allele-specific expression.

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