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Clonal heterogeneity at allelic methylation sites diagnostic for Prader–Willi and Angelman syndromes

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ABSTRACT **Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are developmental disorders resulting from the absence of the paternal or maternal contribution to the 15q11–13 region, respectively. Allele-specific methylation at D15S63 (PW71) has routinely been used as a diagnostic indicator of PWS and AS in DNA samples derived from peripheral blood. Extensive variation in allele-specific methylation patterns, however, has been observed at this site in different tissues, but the frequency or mechanism of this variation has remained uncharacterized. Herein, we have investigated the cellular basis of variation in methylation patterns at four sites of allelic methylation near *SNRPN* by using DNA samples derived from a panel of primary T lymphocyte clones. Interclonal variability was observed at three of these sites, including the diagnostic PW71 site. Changes in allele-specific methylation patterns occurred at a frequency of about one change in 50% of the cells every 22–25 doublings. In contrast, stable allele-specific methylation was observed in these clonal populations at exon 1 of *SNRPN* and the androgen receptor locus on the inactive X chromosome, suggesting that methylation at some CpG sites is more faithfully maintained than others. Clonal heterogeneity at PW71 was not an artifact of cell culture because the absence of allelic methylation was also observed in about 20% of the alleles in unstimulated peripheral blood. These results demonstrate that variation in allele-specific methylation at PW71 and other sites in the PWS/AS region appear to depend on the clonal complexity of the particular tissue and on the lack of strict maintenance of methylation within clones.**

Methylation of CpG sites in the mammalian genome is an essential process for development, cellular differentiation, X chromosome dosage compensation, and parental imprinting (1–3). Methylation has been associated with diverse functions, such as transcriptional silencing, maintenance of chromatin organization, and inactivation of foreign DNA sequences (2, 4–6). The role of methylation as an epigenetic marker of transcriptional states has been most clearly defined for alleles that display both methylated and unmethylated states, such as the two X chromosomes in females or the maternal and paternal alleles of imprinted regions.

Human chromosomal subregion 15q11–13 is subject to parental imprinting, as is evident from the opposite parental origin inheritance of the distinct genetic diseases Prader–Willi syndrome (PWS) and Angelman syndrome (AS) (7–9). Sites of allele-specific methylation are well-characterized near the 5′ end of *SNRPN*, a gene that demonstrates exclusive paternal expression in the 15q11–13 region (10–12). In addition to *SNRPN* exon 1, several sites at D15S63 (PW71) have also been used extensively for molecular diagnosis of all classes of AS and PWS, demonstrating absence of allelic methylation patterns (12–17). The PW71 and *SNRPN* exon 1 sites are unmethylated on the paternal allele, consistent with the paternal expression pattern of *SNRPN*. A few CpG sites are methylated on the expressed paternal allele, however, including a site within an intron of *SNRPN* (11) and one within the recently described BD exons (BD1A, BD1B, BD1B′, BD2, and BD3), which are alternative transcripts at the 5′ untranslated region of *SNRPN* (12, 18).

Methylation patterns have been reported to be strictly clonally maintained on the basis of extensive investigation of methylation on the inactive X chromosome in mammalian females (3, 19). Furthermore, because the substrate for DNA methyltransferase is hemimethylated DNA, a mechanism involving this enzyme can explain how nascent DNA is methylated after DNA replication to ensure the maintenance of epigenetic states during cell proliferation (5). In imprinted regions, however, patterns of DNA methylation are quite dynamic and change frequently during different stages of development (20). Tissue-specific differences in methylation patterns have often been found in sites of allelic methylation (15, 17). In addition, several CpG sites near D15S63 and the intron of *SNRPN* are described as partially methylated from mixed patterns on Southern blots of total genomic DNA (11–13, 17, 18, 21).

Our hypothesis is that tissue-to-tissue variation in the allelic methylation of sites near *SNRPN* may reflect intercellular heterogeneity in methylation patterns rather than true tissue specificity. In other words, the variation of allelic methylation in a particular tissue may depend on the complexity of its cellular composition. The possibility was excluded that allelic methylation patterns vary during different stages of the cell cycle or in different functional populations of T lymphocytes (R.J.R., unpublished data). Thus, we decided to address the question of whether random variations at allelic methylation sites may exist between individual cells within a population.

It has recently been demonstrated that the sites of allelic methylation at D15S63 are more variable than those in the 5′ end of *SNRPN* exon 1 (22). To understand the cellular basis underlying this variability, we directly examined the degree of intercellular heterogeneity of allele-specific methylation in peripheral blood lymphocytes (PBLs) by using a novel single cell cloning approach. Clonal populations of T lymphocytes from two normal individuals were randomly selected, cultured, and examined for allele-specific methylation at several sites within the 5′ region of *SNRPN*. Three of these methylation sites displayed a high degree of variability between different T cell clones. In contrast, one site at the 5′ end of exon 1 of *SNRPN* demonstrated an allele-specific methylation pattern that was homogeneous and clonally maintained, similar to methylation patterns on the inactive X chromosome. These

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Abbreviations: PWS, Prader–Willi syndrome; AS, Angelman syndrome; AR, androgen receptor; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell.

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results suggest different mechanisms for maintenance of allelic methylation at sites within a 100-kb region around *SNRPN*.

MATERIALS AND METHODS

T Lymphocyte Single Cell Cloning. Peripheral blood mononuclear cells (PBMCs) were isolated from a normal female (NS1) by density sedimentation with Ficoll/Hypaque (Pharmacia). PBMCs were cultured at limiting dilution (30, 3, and 0.3 cells per well) in 96-well V-bottom plates containing irradiated (5,000 rad; 1 rad = 0.01 Gy) autologous PBMCs at 10^5 per well. RPMI culture medium 1640 contained 20% pooled human serum (Sigma), glutamine, antibiotics, and phytohemagglutinin PHA.P (Welcome Diagnostics; 1 μg/ml). After a 48-h culture, interleukin 2 (Human T Stim, Collaborative Research) was added at a 5% final concentration, and this complete medium, excluding PHA.P was changed in half the cultures every 4 days. After 12 days of primary culture, wells were scored for positive growth, and clonal populations derived from lowest dilution were restimulated at 5×10^3 cells per well as described for primary culture except with allogeneic irradiated PBMCs. After an additional 8 days of culture, T cell clones were counted, and DNA was isolated by using the Puregene kit (Gentra Systems) for clonal cultures of greater than 10^6 total cells. Subcloning of NS1 was performed as above with one round of stimulation at limiting dilution and a second restimulation after 10 days. The stimulation and restimulation of the total lymphocyte population (NS1.PHA and NS2.PHA) and DNA isolation were performed as above except primary culture was at 10^5 cells per well without irradiated feeders and with PHA.

Southern Blot Analysis. Genomic DNA digestion, Southern transfer, and probe hybridization were performed as described (11, 15, 23). Probe PW71B (gift from B. Horsthemke, University of Essen, Germany) was hybridized to *Hin*dIII/*Hpa*II-digested DNA, probe Y48.5 (gift from B. Horsthemke) was hybridized to *Sac*I/*Hpa*II digests, and probe pNO.9 (American Type Culture Collection) was hybridized to *Xba*I/*Not*I digests. A reverse transcription-coupled PCR product between *SNRPN* exons 2 and 3 described previously (11) was hybridized to *Hin*dIII/*Hpa*II-digested DNA on blots. Images of autoradiographs were captured by using a UVP gel documentation system and quantitative analysis was performed on a Macintosh computer using the public domain NIH IMAGE program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Androgen-Receptor (AR) Allele-Specific Methylation Assay. DNA digestion and PCR were performed as described (24) except that 28 cycles were performed without radioactivity and samples were electrophoresed on 10% nondenaturing polyacrilamide gels and detected by staining in ethidium bromide (1 μg/ml). Gel images were captured by using a UVP gel documentation system and analyzed with NIH IMAGE.

PW71 Allele-Specific Methylation Assay. Approximately 400 ng of genomic DNA from a normal individual polymorphic for the *Nci*I site detected by PW71B (23) was digested overnight at 37°C with or without *Hha*I (*Cfo*I). One-half of the digestion was amplified (primers 5′-TCTGAAGAAAGCTACCCATCCAATGAAGAAG and 5′-GCAGCAGGACGACCGGATTC) in a 50-μl reaction containing 0.5 μM primers, all four dNTPs (each at 125 μM), 2.5% dimethyl sulfoxide, *Taq* polymerase, and salts as described by the manufacturer (Boehringer Mannheim) in a GeneAmp PCR system 2400 (Perkin–Elmer). PCR conditions were as follows: 1 cycle (94°C, 3 min), 5 cycles (94°C, 30 sec; 72°C, 2.5 min), 10 cycles (94°C, 30 sec; 71°C, 30 sec; 72°C, 2 min), 10 cycles (94°C, 30 sec; 70°C, 30 sec; 72°C, 2 min). PCR products were purified with M_r 30,000 Ultra-free-MC filter unit (Millipore) to remove salts and then resuspended in *Nci*I digestion buffer with or without *Nci*I for at least 3 h at 37°C. Resulting digested products were separated on 1% agarose gels. Gel images were captured by using a UVP gel documentation system and analyzed with NIH IMAGE. As a control for completion of *Cfo*I digestion, the remaining half of the predigested DNA was amplified with primers spanning a 105-bp sequence containing six *Cfo*I sites from the *BRCA2* locus. PCR conditions were as described for PW71 except MT1 amplification was carried out in a 25-μl reaction with 5% dimethyl sulfoxide for 32 cycles (94°C, 30 sec; 70°C, 30 sec) with primers 5′-CCAAGCCTGAGCCTGGCAGC and 5′-GCTCCCTCCTCTCCGTGCCC.

RESULTS

Isolation of T Cell Clones from Human Peripheral Blood. PBLs were isolated from a normal female and cultured at limiting dilution in the presence of irradiated allogeneic PBLs and interleukin 2. After 2 weeks, wells displaying growth were selected and restimulated for further growth. One week after the second stimulation, genomic DNA was isolated from T cell clones grown for 19–25 generations (5×10^5 to 5×10^7 cells). The frequency of clonal growth was 39%, sufficient to ensure a random selection of the original cell population.

Determination of Methylation of SNRPN Sites in Normal T Cell Clones. Four sites of previously described allelic methylation at *SNRPN* were examined in the panel of T cell clones (Fig. 1*E*). Probe PW71B detects an allelically methylated *Hpa*II site in the long terminal repeat sequence 5′ of exon BD2, which has been routinely used for clinical diagnosis of AS and PWS in blood (23). Probe Y48.5 detects two *Hpa*II sites between BD2 and BD3, one of which shows an allelic pattern of methylation (12, 18). Probe pNO.9 detects an allelic *Not*I site at the 5′ end of *SNRPN* exon 1 (10), and the reverse transcription-coupled PCR product between *SNRPN* exons 4 and 5 detects a site of partial allelic methylation at a *Hpa*II site within the intron (10, 11, 21).

Genomic DNA from T cell clones (NS1.1–7) and from PHA-stimulated total lymphocytes (NS1.PHA) was digested with *Hin*dIII and *Hpa*II and analyzed by Southern blot hybridization with PW71B (Fig. 1*A*). In the total population, NS1.PHA, both the 6.6-kb methylated allele and the 4.0-kb unmethylated allele are about equally represented. In contrast, the pattern of methylation of the different T cell clones was quite variable. In four of the seven T cell clones, one predominant pattern was observed, either methylated (NS1.2–4) or unmethylated (NS1.1). The two additional bands at 2.7 and 2.5 kb, which are often observed with the PW71B probe (15), also were variably detected in different T cell clones.

An even more striking variability in methylation patterns was observed in a Southern blot analysis with Y48.5 on *Sac*I/*Hpa*II- digested DNA from the same panel of T cell clones (Fig. 1*B*). In total blood DNA, allelic methylation is represented by the 2.5- and 1.0-kb bands of equal intensity. Only one of the seven T cell clones (NS1.4), however, showed an equal intensity of these two bands as measured by image analysis densitometry. The analysis of each of the T cell clones revealed variations in the intensity of all four bands, suggesting several different combinations of methylation patterns at this site.

In contrast to the variable methylation patterns observed in T cell clones in Fig. 1 *A* and *B*, allelic methylation at the 5′ end of *SNRPN* exon 1 detected by probe pNO.9 was equivalent between T cell clones and total lymphocytes (Fig. 1*C*). In unstimulated blood, both the 4.3-kb methylated and 0.9-kb unmethylated sites are observed at approximately equal intensities (10). The equal intensity of these two bands was consistently observed in both stimulated lymphocytes (NS1.PHA) and all T cell clones (NS1.1–7).

The site of allelic methylation within the intron of *SNRPN* has been previously reported to be a site of partial methylation with the unmethylated 3.3-kb band representing about 20–

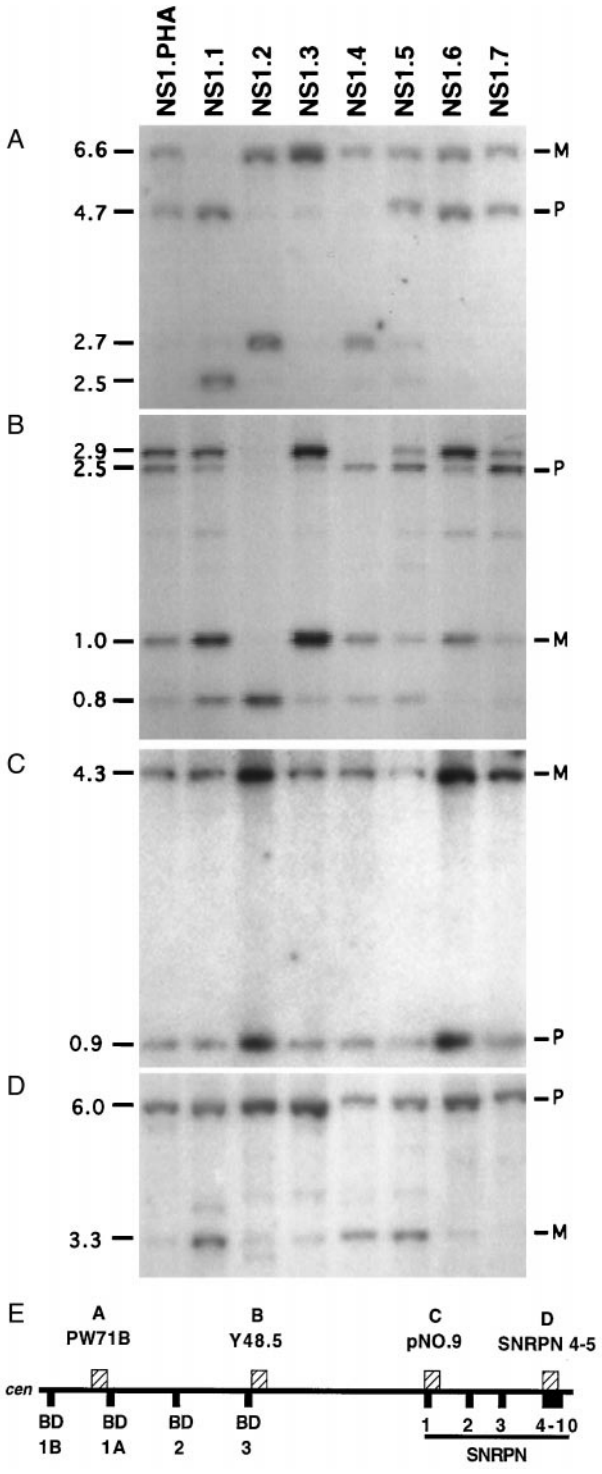

FIG. 1. Allele-specific methylation patterns in DNA isolated from a panel of T cell clones (NS1.1–7) or peripheral blood (NS1.PHA). (*A*) *Hin*dIII/*Hpa*II-digested DNA was analyzed by a Southern blot detected with probe PW71B. The 6.6-kb and 4.7-kb bands are the expected sizes for the maternal (M) and paternal (P) alleles, respectively. T cell clones vary in the proportion of these allelic bands and the nonallelic bands at 2.7 and 2.5 kb (15). (*B*) DNA digested with *Sac*I and *Hpa*II and probed with Y48.5 revealed four bands that vary in intensity in different T cell clones, two of which are allelic in peripheral blood (bands P and M as indicated) (13). (*C*) *Xba*I/*Not*I-digested DNA probed with pNO.9 from exon 1 of *SNRPN* reveals bands of 4.3 kb (M) and 0.9 kb (P) that do not vary between different T cell clones. (*D*) *Hin*dIII/*Hpa*II-digested DNA was detected with an reverse transcription-coupled PCR product that spans exons 4 and 5 of *SNRPN*, revealing a partial allelic methylation site within the intron (11, 20). Variability in the presence of the 3.3-kb band (M) was observed between different T cell clones. (*E*) Relative genomic locations of probes used to detect methylation by Southern blot analysis in relationship to *SNRPN* (11, 12, 18). BD1A, 1B, 2, and 3 are alternative 5′ exons of *SNRPN* (18).

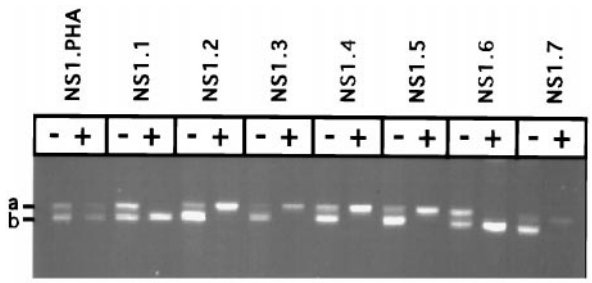

FIG. 2. Assay for allelic methylation status of AR on the X chromosome in female reveals stable clonal maintenance of methylation. Genomic DNA from the same panel of T cell clones used in Fig. 1 was predigested with *Alu*I and *Hpa*II (+) or *Alu*I alone (−), amplified with ARA-specific primers, and separated by PAGE on 10% gels. Two alleles of the polymorphic $(CAG)_n$ are observed in this individual (bands a and b). In peripheral blood (NS1.PHA), both alleles are observed in both *Hpa*II- undigested and -digested, but in T cell clones only one of the two alleles are amplified in *Hpa*II-digested DNA. The methylated allele on the inactive X chromosome is both randomly determined and clonally maintained (23, 24).

35% of the total hybridization signal. Because each allele must be either methylated or unmethylated in each cell, this pattern has been hypothesized to reflect different cell populations (10, 11, 21). This hypothesis is directly tested in Fig. 1*D*. Total lymphocyte culture NS1.PHA exhibit the pattern predicted from blood, with 20% of the unmethylated 3.3-kb band present, but T cell clones exhibited variable hybridization intensity of this band. Clones NS1.1, 4, and 5 exhibit roughly equal intensity of the 6.0- and 3.3-kb bands, clones NS1.2 and 7 almost completely lack the 3.3-kb band, and the patterns of NS1.3 and 6 are similar to the total population. This experiment demonstrates that although there are cell variations in the methylation patterns, each clone does not display a single pattern with a single allele methylated and suggests that the methylation patterns are not clonally maintained.

Clonality of Allele-Specific Methylation on the X Chromosome. Methylation patterns on the inactive X chromosomes in females are strictly maintained in an assay for clonality and random X chromosome inactivation that uses a polymorphic CAG repeat at the 5′ end of the AR gene (24, 25). This site is heavily methylated on the inactive but not the active X chromosome in females so that both parental alleles can be detected in the inactive methylated state in a mixed population of cells in an adult female (24, 25). In clonal populations, however, only one allele will be amplified, representing the methylated allele on the inactive X chromosome in that clone. We therefore used the AR clonality assay to test the maintenance of methylation at this site on DNA from the panel of T cell clones (Fig. 2). For the total lymphocyte culture (NS1.PHA), both alleles of the AR CAG repeat are amplified in DNA with or without *Hpa*II predigestion, although the intensity is reduced in the *Hpa*II-predigested DNA, due to the loss of the unmethylated fraction from both alleles. Amplification of the AR site in DNA from the T cell clones, however, resulted in only one of the two alleles being amplified from *Hpa*II-predigested DNA. The upper allele was the methylated inactive allele in five of the clones, and the lower allele was methylated in the remaining two T cell clones.

Subcloning of a T Cell Clone to Assess Maintenance of Clonal Methylation Patterns. The variable methylation patterns observed in Fig. 1 could have arisen in early development

and later be clonally maintained, as occurs on the X chromosome. Alternatively, variations in these methylation patterns may arise during somatic cell division. To directly address whether methylation is clonally maintained at the variable PW71 site, a subcloning experiment was performed on T cell clone NS1.3. Cells from NS1.3 T cell clone taken from the same generation as the DNA isolation in Fig. 1 (25 doublings) were subcloned at limiting dilution and cultured for two subsequent PHA stimulations. Four new subclones were cultured to an additional 22–23 generations (47–48 total doublings from original clone) and DNA was isolated from each subclone (NS1.3A-D). Although a methylated pattern was observed at the PW71 site of the original clone NS1.3, only two of the four subclones retained this original pattern (Fig. 3, NS1.3A and 3B). Two other subclones, NS1.3C and 3D, exhibited a methylation pattern that has equal intensities of both methylated (6.6 kb) and unmethylated (4.7 kb) bands, like the original peripheral blood sample. These results demonstrate that the methylation pattern is not stably maintained during cell division but instead changes, with roughly a 50% chance of a single clone switching the methylation pattern of a single site observed every 22–25 generations.

Variation of Allelic Methylation of PW71B Locus in Normal Unstimulated Peripheral Lymphocytes vs. T Cell Clones. Analysis of methylation at the PW71 (D15S63) locus is a reliable diagnostic indicator of most cases of PWS and AS (13, 14, 17, 21, 26). The fully methylated 6.6-kb band is predominant in genomic DNA from PWS peripheral blood, whereas the unmethylated 4.7-kb band is predominant in AS blood. The 6.6-kb band is referred to as the maternal allele because it is absent in AS, and the 4.7-kb band is assumed to be paternal because it is absent in PWS (13, 14). Analysis of allele-specific methylation in normal blood has not been reported, however, by using a combined methylation and polymorphism analyses. In light of our results in Fig. 1*A*, in which some normal T cell clones have the PWS pattern of PW71B methylation (NS1.2–4) and another displays the AS pattern (NS1.1), we wished to confirm that allelic methylation occurs in normal unstimulated peripheral blood and to determine whether the pattern of allelic methylation changes in culture.

A polymorphic *Nci*I site has been described that can be detected by the PW71B probe by Southern blot analysis (23). Genomic DNA from peripheral blood of several normal individuals was screened for the *Nci*I polymorphism and a heterozygous individual was selected (NS2). Primers were designed that flanked both the polymorphic *Nci*I site and three *Cfo*I (*Hha*I) sites (Fig. 4*A*). Two *Cfo*I sites are methylated, and

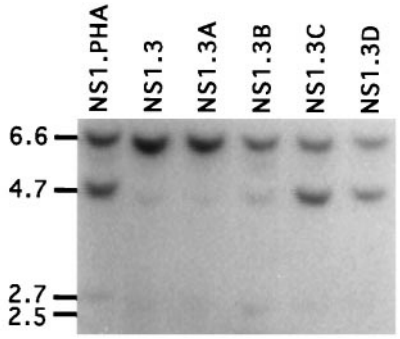

FIG. 3. Methylation pattern at allelic PW71 *Hpa*II site is not clonally maintained in T cell subclones. T cell clone NS1.3, which exhibits a totally methylated pattern at PW71, was subcloned at limiting dilution and cultured for an additional 22–23 generations. Genomic DNA isolated from the subclones (NS1.3A–D) was analyzed as in Fig. 1*A*. Two of the subclones (NS1.A and B) exhibited a methylation pattern similar to the original clone NS1.3, but the remaining two subclones (NS1.3C and D) demonstrated the presence of the 4.7-kb band observed in peripheral blood (NS1.PHA).

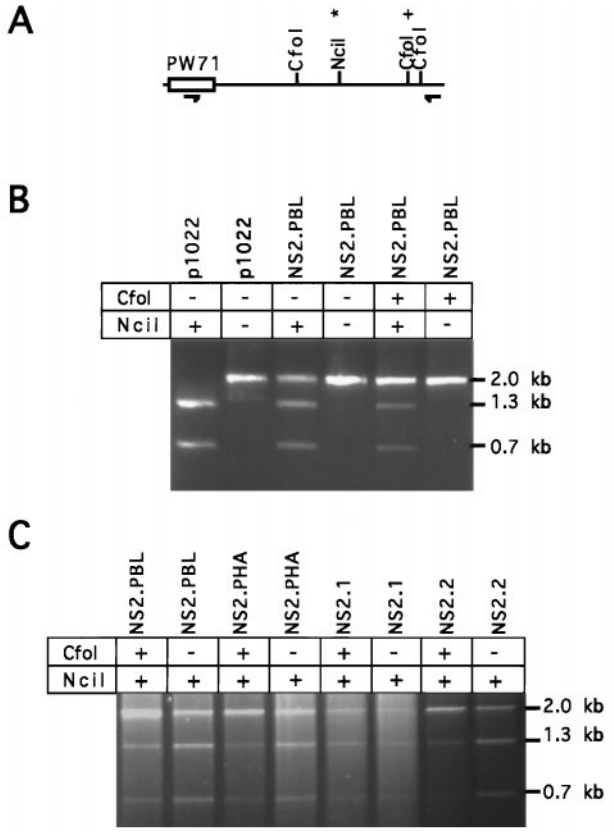

FIG. 4. Assay for both *Nci*I polymorphism and *Cfo*I methylation at PW71 reveals loss of allelic methylation in peripheral blood and T cell clones. (*A*) Primers were designed to span both a polymorphic *Nci*I site (*) and a diagnostic site of allelic methylation (+). (*B*) Genomic DNA from a plasmid clone of the region (p1022) and peripheral blood from a normal individual heterozygous for the *Nci*I site (NS2.PBL) were predigested with (+) or without (−) *Cfo*I prior to PCR amplification. After amplification, PCR products were then digested with (+) or without (−) *Nci*I to determine methylation status of each allele. NS2.PBL DNA predigested with *Cfo*I showed a predominance of the *Nci*I− allele, demonstrating partial allelic methylation. (*C*) Genomic DNA from peripheral blood (NS2.PBL), PBLs stimulated and cultured for around 25 generations with PHA (NS2.PHA), and two T cell clones (NS2.1 and 2) was assayed for allelic methylation as described in *B*. NS2.PHA and NS2.PBL exhibited the same preference for the methylated allele, but the two T cell clones varied in the degree of methylation of the *Nci*I+ allele. *Cfo*I predigestion was complete, as determined by the lack of amplification of a sequence containing unmethylated *Cfo*I sites from the *BRCA2* locus (data not shown).

the third has been shown to be diagnostic for AS and PWS (15). Similar to the AR PCR assay, DNA from unstimulated peripheral blood was predigested with or without *Cfo*I prior to amplification with flanking primers in Fig. 4*A*. After PCR amplification, samples were digested with *Nci*I. In Fig. 4*B*, both *Nci*I alleles are detected in non-*Cfo*I-digested DNA, but in *Cfo*I-predigested DNA, the allele without the *Nci*I site is predominant. Quantitation of relative band intensity revealed the relative intensities of the 2.0-, 1.3-, and 0.7-kb bands to be 45%, 31%, and 24% for non-*Cfo*I-treated DNA and 80%, 11%, and 9% for *Cfo*I-digested DNA, respectively. These results are representative of four experiments in which the ratios did not vary more than 10%. These results demonstrate that one parental allele is preferentially methylated in normal unstimulated blood cells, but variation exists in at least 20% of the cells on the opposite allele.

With this same approach to detect allelic methylation at PW71B, we then directly addressed whether the allelic methylation changes after stimulation in culture in the total vs. clonal populations. In Fig. 4*C*, T cells that had been PHA-

stimulated in a bulk population for 20–25 cell divisions (NS2.PHA) exhibited roughly the same pattern of allelic methylation as unstimulated blood (NS2.PBL) with a 70% preferential methylation of the *Nci*I-allele. In the two T cell clones that were derived from NS2, one (NS2.1) had no evidence for allelic methylation and the other (NS2.2) was predominantly methylated on the *Nci*I-allele (87% *Nci*I-allele). These results demonstrate that clonal populations vary in the maintenance of the allelic methylation pattern. The predominance of one methylated allele, however, does not change within the total population after two rounds of PHA stimulation (20–25 generations).

DISCUSSION

The analysis of allelic methylation by Southern blot analysis at the PW71 (D15S63) region has proven to be a reliable molecular diagnostic test for PWS and AS. Despite its usefulness, several reports have demonstrated variability of allelic methylation patterns in tissues other than peripheral blood (26). This variability has also been observed in single alleles cloned from peripheral blood DNA as presented in a recent report using the bisulfite protocol of genomic sequencing (22). Although these previous reports have suggested that intercellular variability may explain the variation, this hypothesis has not been previously tested. The purpose of this report is to investigate the cellular basis of methylation variability by a direct examination of allelic methylation in individual clonal cell populations. The pattern of allelic methylation at four regions within the 5′ end of the imprinted gene *SNRPN* was examined in a panel of clonally derived T lymphocytes, revealing clone to clone variation in the allelic methylation pattern at several of these sites. In addition, variation between sites was observed within a single clonal population. For instance, no clone was either completely methylated or unmethylated at all sites within the *SNRPN* region, indicating that the variations in each site occur as independent events. The variation appears to occur as a result of somatic cell division and is not stably maintained in a clonal population. We have calculated the frequency of variation at these sites to be roughly one change in methylation pattern observed in 50% of the cells every 22–25 generations.

Several lines of evidence argue against the variation in allelic methylation patterns observed in T cell clones being simply an artifact of cell culture *in vitro*. (*i*) The variability in allelic methylation is also observed in DNA derived from uncultured peripheral blood in around 20% of the alleles by both our PW71/*Nci*I methylation-sensitive PCR assay (Fig. 4) and the genomic sequencing method (22). (*ii*) Variation in allelic methylation was not observed on the inactive X chromosome at the AR locus (Fig. 2 and refs. 3, 24, and 25) or at exon 1 of *SNRPN* (Fig. 1*C* and ref. 22) even after 50 doublings in culture (data not shown). The lack of an overall change in methylation pattern is consistent with the observation by restriction landmark genomic scanning analysis that the overall methylation pattern of primary T lymphocytes does not change after several rounds of *in vitro* stimulation (27). (*iii*) The pattern of allelic methylation does not change in the total population during a comparable number of doublings as it does in the clonal populations, demonstrating that although allelic methylation is dominant in a mixed population, the absence of allelic methylation patterns are observed in some clones (Figs. 1 and 4*C*).

Variation in methylation patterns have been previously observed at several sites including the promoter regions of genes in clonal fibroblast populations (28–32) and *PGK1* in somatic cell hybrids (19). Moreover, little evidence for faithful clonal maintenance of methylation patterns was observed in lymphocytes at the *FMR1* locus on X chromosome (33). The variable methylation at *PGK1* and *FMR1* is in contrast to the apparently more stable methylation in T cell clones at the AR locus (Fig. 2). The reason for the differences in variability between these X chromosome loci is not clear, although it should be noted that several sites at *FMR1* were examined but only one AR site was analyzed herein. Analysis of additional sites in the AR region might reveal a degree of variation similar to that at *FMR1*. The unstable methylation at *FMR1* (33) and at PW71, as shown herein, appears to occur randomly as a result of somatic cell division, suggesting that a low fidelity in the maintenance of methylation during DNA replication plays a role in the generation of variability (33). The random variation in methylation patterns could be beneficial by ensuring a degree of cellular heterogeneity within a continually dividing cell population. Perhaps epigenetic heterogeneity of clonally derived cells is advantageous in some regions of the genome to prevent integrated viral or other harmful sequences from being expressed in all cells (5, 34).

The observation of clonal heterogeneity in allelic methylation patterns in an imprinted region, however, brings up the issue of how faithfully parental imprints are maintained during somatic cell division. We have demonstrated that in peripheral blood, methylation appears on the opposite allele in 20–30% of the total DNA. Interclonal variation in methylation does not occur, however, at one site in exon 1 of *SNRPN* and at the AR locus on the inactive X chromosome. The difference suggests that variation at these two sites is selected against in a dividing population because of a growth disadvantage or that a different mechanism of methylation maintenance exists at some sites of allelic methylation. One possibility is that binding of transcription factors to certain CpG sites actually protects the methylation state from variation. DNA from a panel of T cell clones, such as those investigated in this study, could provide a useful resource for testing the maintenance of methylation at specific sites in the human genome and further investigating the mechanism of methylation maintenance.

Methylation patterns of imprinted regions are dynamic during early developmental stages in diploid or tetraploid but not haploid parthenogenic embryos, suggesting that a mechanism of methylation exchange may exist for imprinted regions (20). In the fungus *Ascobolus immersus*, methylation can be tranferred between alleles of homologous chromosomes during meiotic pairing (35). Homologous association between oppositely imprinted chromosomal domains is observed in human lymphocytes during late S phase of mitotic cell division (36), suggesting that methylation transfer between paired alleles may also occur during somatic cell division. In support of this model, a lower frequency of variation in the PW71 methylation pattern was observed in T cell clones from an AS patient whose lymphocytes are deficient in homologous association of 15q11–13 alleles (J.M.L., unpublished results). Furthermore, trans effects on methylation of the *Igf2* gene were recently observed in *H19* heterozygous deletions in mouse, providing additional evidence for transfer of methylation between parental alleles (37).

Our results may have implications concerning the use of allele-specific methylation as a diagnostic test for PWS and AS. Previous reports have demonstrated that PW71 is an inaccurate diagnostic indicator in DNA derived from fibroblast and lymphoblast cell lines as well as cells cultured from amniotic fluid and chorionic villus (17). Our results would suggest that the irreproducibility of PW71 allelic methylation pattern in these tissues reflects more of their clonal complexity after growth in culture rather than any true tissue-specific difference or artifact of cell culture as suggested (17). The most likely reason that peripheral blood is the best tissue source for the PW71 methylation assay is that this tissue is clonally complex due to the generation of diversity for its immune function. Consistent with recent reports (17, 26), our results indicate that the site at exon 1 of *SNRPN* may be the most suitable site for

molecular diagnosis if genomic DNA is derived from sources other than peripheral blood.

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