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Changes in the allelic methylation patterns of c-H-*ras*-1, insulin and retinoblastoma genes in human development

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Summary

The methylation status of the c-H-*ras*-1, insulin and retinoblastoma genes was determined in human sperm, hydatidiform mole, fetal tissues, adult lymphocytes and adult kidney. Individual alleles of c-H-*ras*-1 and insulin were distinguishable due to presence of endogenous variable number of tandem repeat (VNTR) polymorphisms. Both alleles of the latter two genes were extensively methylated in sperm compared to the other tissues. Several sites within these genes were less methylated in fetal tissues and the two alleles were differentially methylated in some cases. The retinoblastoma gene was highly methylated in all tissues examined, with the exception of a single site that was undermethylated in sperm only. The sperm-specific methylation patterns in all three genes could represent imprinting of the parental chromosomes. Since 5-methylcytosine is inherently mutagenic, it is possible that methylation imprinting could alter the susceptibilities of human genes to point mutations.

Key words: DNA methylation, c-H-*ras*-1, insulin, retinoblastoma, human development.

Introduction

Nuclear transplantation studies in the mouse have shown that an embryo needs both parental genomes for development to term (McGrath and Solter, 1984; Surani *et al.* 1984; Solter, 1988). Gynogenones or parthenogenones, where both sets of chromosomes are maternal, and androgenones, where both sets of chromosomes are paternal, do not develop to term and the partially developed embryos are deficient in extraembryonic or fetal tissues respectively. These studies suggest that the two parental genomes, although homologous, are not functionally identical and that the genomes of sperm and egg are differentially marked or imprinted during gametogenesis (Surani *et al.* 1984).

Elegant genetic studies using reciprocal and Robertsonian translocations have narrowed the requirement for both parental genomes to specific regions of mouse chromosomes (Cattanach and Kirk, 1985; Searle and Beechey, 1985). A large part of the mouse genome is able to direct development independently of whether it is of paternal or maternal origin. However, certain regions result in defective complementation when they are solely maternal or paternal in origin. It is thought that genomic imprinting influences gene expression of these regions and hence the requirement for complementary pairs of parental sets for normal development (Surani *et al.* 1986).

There is evidence that imprinting might occur in humans since hydatidiform moles, which contain only paternal genomes, do not develop normally (Kajii and Ohama, 1977). There are also suggestions from analysis of human cancer genetics that parental genes are differentially susceptible to mutation and deletion (Table 1). For example, Wilms' tumors (Reeve *et al.* 1984; Schroeder *et al.* 1987; Mannens *et al.* 1988; Williams *et al.* 1989) and rhabdomyosarcomas (Scrable *et al.* 1989) show a strong preferential maintenance of the paternal chromosome 11p in tumors which have undergone reduction to homozygosity for this region. Similarly, osteogenic sarcomas nearly always maintain the paternal allele of chromosome 13q (Toguchida *et al.* 1989). The situation with retinoblastoma is not yet clear (Dryja *et al.* 1989; Zhu *et al.* 1989; Leach *et al.* 1990). In the sporadic bilateral form of the disease, there is a distinct preferential maintenance of the paternal allele in the 16 out of 17 tumors examined, implying that the initial mutation in the RB gene had been sustained on the paternal chromosome. On the other hand, only 11 of 18 unilateral tumors (61 %) show the presence of the paternal chromosome 13q which harbors the RB gene. The number of tumors is insufficient to determine whether there is a bias for mutations on the paternal RB gene in unilateral tumors. It is not therefore yet apparent whether the preferential mutations of the paternal RB gene in either form of the disease is a result of spermatogenesis or is due to imprinting (Ponder, 1989). Recently, Jadayel *et al.* (1990) have shown that the origin of new mutation in Von Recklinghausen neurofibromatosis (NF-1), located on chromosome 17q,

Table 1. *Paternal allele retention or mutation in cancer*

Tumor	Paternal allele maintained or mutated	Chromosome (Ref.)	
Wilms'	14/15	11p	(Reeve *et al.* 1984; Schroeder *et al.* 1987; Mannens *et al* 1988 and Williams *et al.* 1989)
Rhabdomyosarcoma	6/6	11p	(Scrable *et al.* 1989)
Retinoblastoma			
Unilateral	11/18	13q	(Dryja *et al* 1989 and Zhu *et al.* 1989
Bilateral	16/17	13q	Leach *et al.* 1990)
Osteosarcoma	12/13	13q	(Toguchida *et al.* 1989)
Von Recklinghausen Neurofibromatosis (NF-1)	12/14	17q	(Jadayel *et al.* 1990)

in 2 of 14 families studied were also of paternal origin. The data suggests that these human chromosome regions are somehow marked by their passage through the germline and might therefore be subject to imprinting (Reik, 1989).

The molecular mechanism(s) for genomic imprinting is not known. However, it is thought that DNA methylation might be involved in this process (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987). Studies with transgenic mice have shown that the methylation pattern of a transgene can be dependent on the sex of the parent from which it was inherited (Reik *et al.* 1987; Sapienza *et al.* 1987) and, at least in one case, this methylation imprinting influenced the expression of the transgene (Swain *et al.* 1987).

Because little is known of the potential role of methylation in the marking of human genes, we have examined the methylation status of individual parental alleles of certain loci in sperm, early fetal cells, hydatidiform moles and lymphocytes to determine whether human genes are marked by their passage through the germ line. We used polymorphic markers (VNTR regions) in the human c-H-*ras*-1 and insulin genes to track, as a function of development, the methylation status of a housekeeping and a tissue specific gene, respectively. The genes were selected because of their locations on chromosome 11p, a region thought to undergo imprinting (see Table 1). We also present preliminary results on the methylation status of the retinoblastoma gene (RB) since this gene is also a likely candidate for an imprinted gene.

Methodology

To study the methylation of the individual parental alleles in human cells, we took advantage of minisatellite polymorphic regions, known as VNTR (variable number of tandem repeats), which are present throughout the human genome (Bell *et al.* 1981; Nakamura *et al.* 1987). Specific restriction enzymes were used to generate fragments containing the VNTR regions together with their adjacent genomic sequences. Methylation-sensitive restriction enzymes were utilized to examine the methylation status of individual alleles which were identified by the different lengths of the VNTR-containing bands. The enzymes *Msp*I, which cuts at CCGG sites, and its methylation-sensitive isoschizomer *Hpa*II, which does not cut when the internal cytosine is methylated, were used in most studies. However, *Ava*I, which recognizes CPyCGPuG but does not cut sites where the internal cytosine residue is methylated, was employed for other analyses.

DNA from sperm, fetal tissues, lymphocytes, adult kidney and hydatidiform mole was used to represent different tissues and stages of human development. The methylation of three genes c-H-*ras*-1, insulin and retinoblastoma were studied. Both c-H-*ras*-1 and insulin are located on chromosome 11p 15.5 and have homologous loci on the mouse chromosome region 7 F1-F4 (Buckle *et al.* 1984), which is a region known to undergo genomic imprinting in the mouse (Searle and Beechey, 1985). The retinoblastoma gene is located on chromosome 13q and is involved in some childhood cancers including retinoblastoma and osteosarcoma (Dryja *et al.* 1989 and Toguchida *et al.* 1989).

Methylation of c-H-*ras*-1

c-H-*ras*-1 is a housekeeping gene with a high G+C content and a 5′ CpG island, which extends into part of the fourth intron (Gardiner-Garden and Frommer, 1987). The VNTR region of c-H-*ras*-1 is composed of a 28 bp repeat unit and is located at the 3′ end of the gene as illustrated in Fig. 1A. There are two *Msp*I sites flanking the VNTR region and digestion with this enzyme followed by Southern blot analysis using the VNTR region as a probe showed either one or two bands depending on whether the individual was homozygous or heterozygous for the VNTR region in c-H-*ras*-1 (Chandler *et al.* 1987). Examples of this are illustrated in Fig. 2, which is a Southern blot of *Msp*I and *Hpa*II digested DNA from human sperm, fetal cells, hydatidiform mole, adult kidney and lymphocytes probed with the c-H-*ras*-1 VNTR region. The lymphocytes and hydatidiform mole samples were homozygous for the c-H-*ras*-1 VNTR and a strongly hybridizing 1.0 kb band was seen in the *Msp*I digests (lanes 5 and 9). However, the sperm, fetus and kidney DNA were heterozygous for c-H-*ras*-1 and two major bands corresponding to the two parental alleles were seen in the *Msp*I digests in lanes 1 (1.2 and 1.5 kb), 3 (1.0 and 2.6 kb) and 7 (1.1 and 1.2 kb). Weakly hybridizing

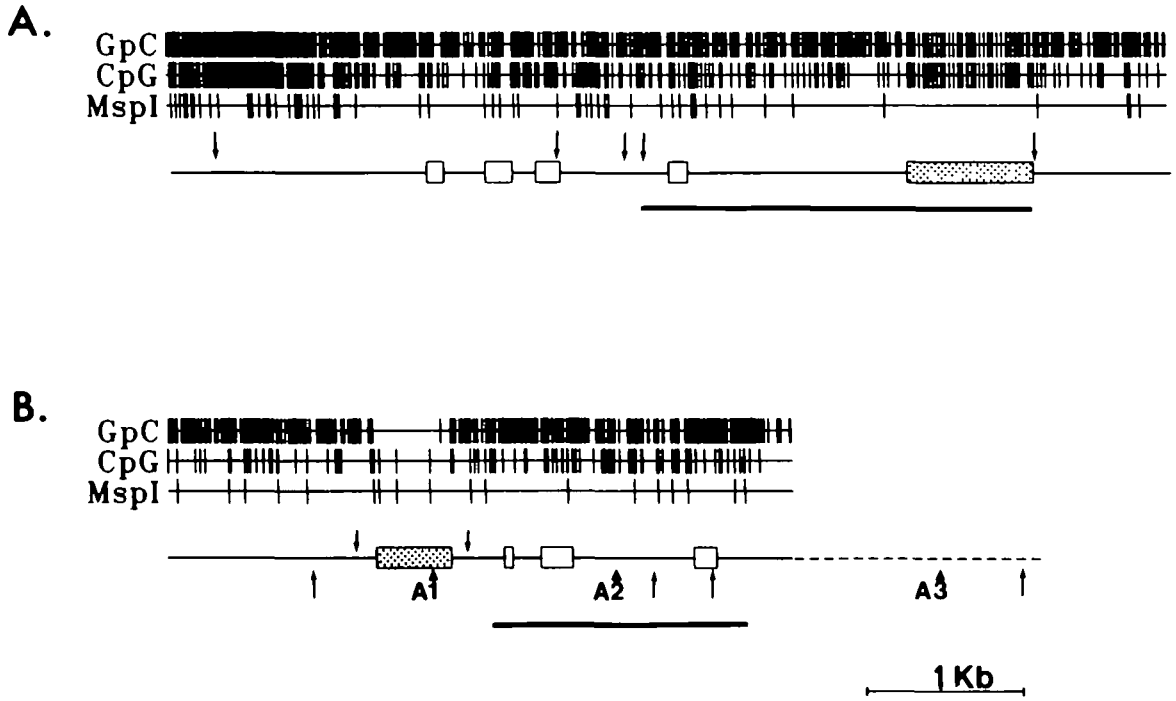

Fig. 1. Map of the human c-H-*ras*-1 (Capon *et al.* 1983) and insulin (Bell *et al.* 1981) genes. A schematic map of the c-H-*ras*-1 gene (A) and the insulin gene (B) with their exons (open boxes) VNTR regions (shaded boxes), *Msp*I sites and GpC and CpG doublet frequencies are shown. The *Taq*I sites in *c-H-ras*-1 are denoted by arrows and the line below the gene marks the probe used in Fig. 3. For the insulin gene, the top arrows represent the two *Pvu*II sites flanking the VNTR region and the bottom arrows represent the *Rsa*I sites. The *Ava*I sites are marked as A1–A3 and the line below the gene marks the probe used in Fig. 4.

bands were occasionally seen in *Msp*I digests (e.g. lanes 7 and 9) that were approximately 0.6 kb in size greater than the VNTR fragments. These bands correspond to a partial digestion of the *Msp*I sites flanking the VNTR which is sometimes seen in human DNA (Feinberg *et al.* 1983).

The methylation patterns of the c-H-*ras*-1 alleles are also shown in Fig. 2. The VNTR fragments in *Hpa*II digests shift to higher molecular weight bands when one or both of the CCGG sites flanking the VNTR region are methylated. In sperm, both alleles of c-H-*ras*-1 were extensively modified at these sites as well as the sites in surrounding areas since only very high molecular weight bands near the 20 kb region were seen in the *Hpa*II digest (lane 2). This sperm-specific methylation pattern was not completely retained through development. The extent of methylation for the hydatidiform mole, which contained a paternal genome only (Kovacs *et al.* 1990), was less than sperm and the range of bands in the *Hpa*II digest extended to about 7 kb (lane 10). However, lower molecular weight bands were seen in *Hpa*II digests of DNA obtained from a culture of fibroblasts homozygous for the 1 kb allele (data not shown). Thus the methylation levels at the c-H-*ras*-1 locus seen in sperm decreased during development independent of whether the conceptus was normal and had a complete set of parental genomes (fetus) or was abnormal with only a paternal genome (mole).

The fetal sample (albeit cultured cells) exhibited allele-specific methylation for c-H-*ras*-1 (Fig. 2, lanes 3 and 4). This sample was heterozygous for c-H-*ras*-1 with 1.0 and 2.6 kb *Msp*I bands corresponding to the two VNTR regions (lane 3). In the *Hpa*II digest, the 1.0 kb band was absent indicating that one or both of the CCGG sites flanking the VNTR region was methylated (lane 4). However, a fraction of the 2.6 kb band was present in the *Hpa*II digest suggesting that both sites were unmethylated in at least some molecules of DNA (lane 4). This implied that the two c-H-*ras*-1 alleles in this sample were differentially methylated at the CCGG sites flanking the VNTR region. Although we cannot rule out the possibility that the 2.6 kb band in the *Hpa*II digest in this case was generated from partial methylation around the 1.0 kb allele, we have previously obtained clear evidence for allele-specific methylation in fetal cells (Chandler *et al.* 1987). This allele-specific methylation may relate to a gamete-specific methylation pattern.

In adult kidney, an example of a somatic tissue, the two alleles with sizes of 1.1 and 1.2 kb (more clearly visible in under-exposed autoradiograms) were partially methylated at the CCGG sites flanking the VNTR region (lane 7) and the overall methylation levels of c-H-*ras*-1 were higher than those in fetal cultured cells, with bands ranging up to 8 kb in the *Hpa*II digest (lane 8). In adult lymphocytes, the c-H-*ras*-1 alleles were almost completely methylated at these sites and the methylation of the gene was more extensive than that in

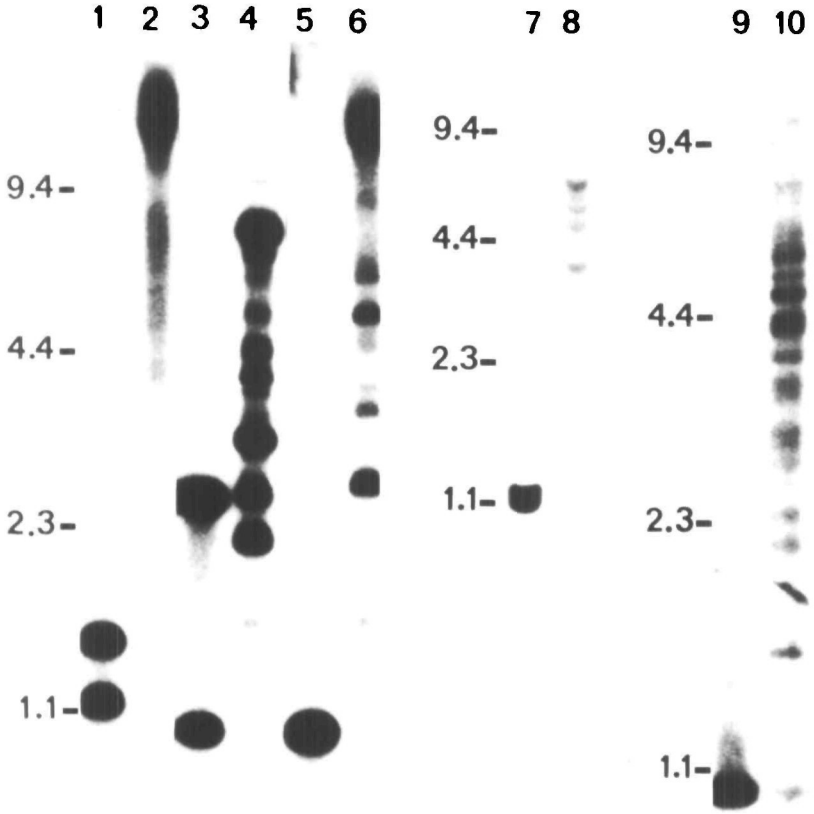

Fig. 2. Methylation of the c-H-*ras*-1 gene in human sperm (lanes 1,2), cultured fetal urothelial cells (lanes 3,4), adult lymphocytes (lanes 5,6), adult kidney (lanes 7,8) and hydatidiform mole (lanes 9,10). 5 μg of DNA were digested with *Msp*I (odd lanes) or *Hpa*II (even lanes), electrophoresed, blotted and probed with the VNTR region of the c-H-*ras*-1 gene. The size of the DNA markers are given in kb.

the kidney with bands ranging up to 20 kb (lane 6). The data showed that both alleles of c-H-*ras*-1 were methylated in adult lymphocytes or kidney, however, variations in methylation patterns of the gene were observed between different tissues. Since the sites flanking the VNTR regions were extensively methylated in sperm but less methylated in fetal cultured fibroblasts and relatively heavily modified in adult lymphocytes, the data are consistent with the idea that demethylation and *de novo* methylation events occur on the c-Ha-*ras*-1 gene during development. However, we did not examine the methylation status of the gene in lymphocyte precursor cells, during development and it is therefore possible that the pattern observed in adult cells reflects a partial demethylation from the sperm genome rather than the erasure of this pattern followed by re-establishment of new methylation. Although some of the data presented in Fig. 2 were obtained with cultured cells, we have previously shown that almost identical modification patterns of the c-H-*ras*-1 gene are seen in uncultured tissues (Ghazi *et al.* 1990).

The methylation analysis of the individual c-H-*ras*-1 alleles was extended to the area upstream of the VNTR region. There are five *Taq*I sites present in the gene and one of the fragments generated contains the VNTR region and encompasses 12 *Msp*I sites (Fig. 1A). Southern blots of *Taq*I digests probed with a *Taq*I fragment (see Fig. 1A) showed two bands corresponding to the two c-H-*ras*-1 alleles (Fig. 3, lanes 1, 4, 7 and 10) and double digests with *Taq*I and *Msp*I released the two VNTR fragments (lanes 3, 6, 9, and 12). Our previous studies have shown that the CCGG sites flanking the VNTR region were differentially methylated in two c-H-*ras*-1 alleles from a human keratinocyte cell line, HaCaT (Chandler *et al.* 1987). The two *Taq*I bands in this lane were 3.2 and 2.7 kb (lane 1) and the higher band was reduced to 1.5 kb in double digests with *Taq*I and *Hpa*II (lane 2). Thus, the CCGG site flanking the 5′ end of the VNTR region of the 3.2 kb band was not methylated. The other CCGG sites in the 3.2 kb *Taq*I fragment were found to be partially methylated in other experiments (not shown). However, all the *Hpa*II sites in the 2.7 kb *Taq*I fragment were completely methylated in a fraction of the sample (residual band at 2.7 kb) and sites were unmethylated (lower bands) in the remainder of the sample. These results show that the differential methylation of individual c-H-*ras*-1 alleles in the HaCaT cells (Chandler *et al.* 1987) extended into the area 5′ to the VNTR region.

Sperm DNA showed a different pattern of methylation by *Taq*I and *Hpa*II analysis (Fig. 3, lanes 7 and 8). Two bands of 2.9 kb and 3.3 kb were present in the *Taq*I digest of sperm DNA (lane 7). Double digests with *Taq*I and *Hpa*II did not change the size of the bands, indicating that this region of the gene was heavily methylated in both alleles (lane 8). Methylation of the 12 *Msp*I sites included in the *Taq*I fragment was not complete however, since there was some decrease in intensities of the *Taq*I bands after *Hpa*II digestion. The results were consistent with the previous observation of extensive methylation surrounding the VNTR region of both alleles in sperm (Fig. 2, lanes 1 and 2). About 0.4 kb of the 5′ end of the *Taq*I fragment is a part of the

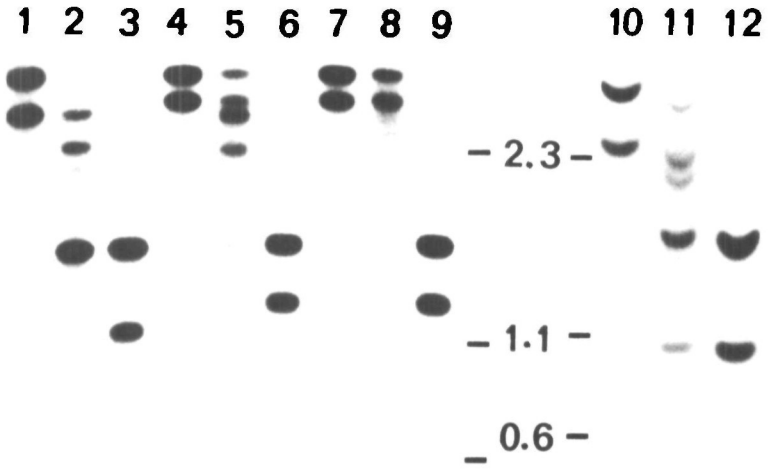

Fig. 3. Methylation of the *Taq*I fragments of the c-H-*ras*-1 gene in the HaCaT cell line (lanes 1–3), adult lymphocytes (lanes 4–6), sperm (lanes 7–9) and fetal muscle tissue (lanes 10–12). 20 μg of DNA were digested with *Taq*I, ethanol precipitated, and divided into 5 μg aliquots (lanes 1,4,7,10) and 7.5 μg aliquots which were then digested with *Hpa*II (lanes 2,5,8,11) or *Msp*I (lanes 3,6,9,12). A Southern blot using the c-H-*ras*-1 probe indicated in Fig. 1A, was performed. The size of DNA markers are given in kb.

5′ CpG island of the c-H-*ras*-1 gene (Gardiner-Garden and Frommer, 1987; see also Fig. 1) and the data clearly showed that the heavy methylation at the 3′end of the gene extends into parts of the 5′CpG island for both alleles in sperm. This result was unexpected since it has been suggested that CpG islands are never methylated unless they are present on an inactive X-chromosome (Gardiner-Garden and Frommer, 1987).

The two *Taq*I fragments in fetal DNA from uncultured tissue (lane 10) were digested with *Hpa*II (lane 11), indicating that the CCGG sites were partially methylated. There was a greater reduction in the methylation levels of this region in the fetal samples relative to sperm and lymphocytes, since smaller molecular weight bands were visualized (lane 11). The extent of methylation for both c-H-*ras*-1 alleles were similar in this sample. In the *Taq*I and *Hpa*II double digest of lymphocyte DNA (lane 5), four other bands in addition to the two *Taq*I bands (lane 4) were present, suggesting that the CCGG sites in a fraction of the *Taq*I fragments were methylated and, in the remainder of the sample, a specific site about 1.2 kb from the VNTR region was not methylated. The methylation status of both c-H-*ras*-1 alleles in the lymphocyte sample appeared to be the same, which was consistent with our previous observation that lymphocytes did not show differential methylation of c-H-*ras*-1 alleles.

In summary, these results confirm our previous observations (Chandler *et al.* 1987) that both alleles of c-H-*ras*-1 are extensively methylated in sperm and show that the methylation extends into part of the CpG island of the gene. The sperm-specific methylation sites are partially removed in fetal cells. In some fetal cells, one of the alleles of c-H-*ras*-1 was more methylated than the other allele; it is possible that the sperm-specific methylation is not completely lost in these samples. In somatic cells both alleles of c-H-*ras*-1 are methylated to similar extents and the methylation patterns are tissue specific. The sperm-specific methylation of c-H-*ras*-1 could potentially be involved in imprinting of the paternal genome and become partially removed in the fetal stage of development (summarized in Fig. 5).

Methylation of insulin

Insulin is a tissue-specific gene with a high G+C content which lacks a CpG island (Gardiner-Garden and Frommer, 1987). The VNTR region at this locus is composed of nonidentical repeat units located at the 5′ end of the gene as illustrated in Fig. 1B (Bell *et al.* 1981). Our previous studies showed that both alleles identified by different VNTR regions of the insulin gene in sperm were extensively methylated in the body of the gene as well as in the VNTR region. In fetal samples, *Hpa*II and *Ava*I sites within the VNTR region were partially methylated and in some samples the two insulin alleles showed differential methylation at these sites (Ghazi *et al.* 1990).

To extend these findings, the methylation status of the body of the individual alleles of the insulin gene and sequences downstream from them were examined using double digests with *Rsa*I and *Ava*I. *Rsa*I generated a polymorphic fragment containing the VNTR region plus flanking sequences (Fig. 1B). Two sites recognized by the methylation-sensitive restriction enzyme *Ava*I were present within this fragment. Site A1 was in the VNTR region and site A2 was in the body of the gene (Fig. 1B). Southern blots of *Rsa*I digests, probed with the coding region of insulin (see Fig. 1B), showed two constant bands of 0.4 and 4.7 kb molecular weights in addition to the polymorphic bands mentioned above (Fig. 4). The 0.4 kb band was generated by the two *Rsa*I sites within the gene near exon 3. The 4.7 kb fragment was generated by an *Rsa*I site in the third exon and a downstream *Rsa*I site flanking the 3′ end of the insulin gene (Fig. 1B). An additional *Ava*I site was present within the 4.7 kb *Rsa*I fragment (site A3).

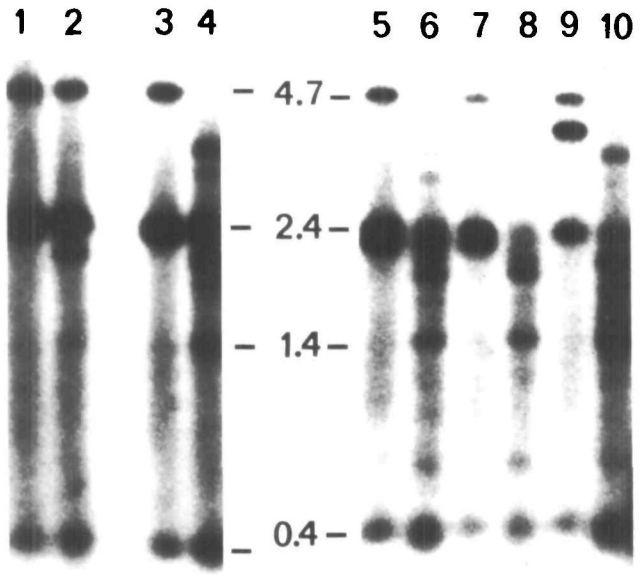

Fig. 4. Methylation of *Rsa*I fragments of the insulin gene in human sperm (lanes 1,2), adult lymphocytes (lanes 3,4), fetal pancreas tissue (lanes 5,6), fetal muscle tissue (lanes 7,8) and cultured fetal urothelial cells (lanes 9,10). 10 μg of DNA were digested with *Rsa*I (odd numbered lanes) or *Rsa*I and *Ava*I (even numbered lanes), electrophoresed, blotted, and probed with the insulin fragment indicated in Fig. 1B. The size of selected bands are marked in kb.

In the *Rsa*I digest of a sperm sample, homozygous for the insulin VNTR, three bands with molecular weights of 4.7, 2.5 and 0.4 kb were observed (Fig. 4, lane 1). The 4.7 kb band remained intact in double digests with *Rsa*I and *Ava*I, indicating that site A3 was methylated in sperm (lane 2). This site, however, was completely unmethylated in lymphocytes, fetal pancreas, fetal muscle and fetal urothelial cells (lanes 4, 6, 8 and 10, respectively). In these samples, digestion with *Ava*I resulted in the disappearance of the 4.7 kb band and generation of a new 3.7 kb band. Thus, site A3 showed sperm-specific methylation and was not methylated in any other tissue tested.

Sites A1 and A2 were also methylated in sperm (Fig. 4, lane 2). Most of the 2.5 kb fragment remained intact in *Ava*I and *Rsa*I double digests, but a small fraction of this band was reduced by 0.3 kb due to partial cutting by *Ava*I at site A2. The data therefore indicated that site A1 was completely methylated and that site A2 was partially methylated in sperm. Analysis of a sperm sample heterozygous for the VNTR showed that both alleles were extensively methylated at all the three *Ava*I sites (data not shown).

Sites A1 and A2 were partially methylated in fetal samples from different tissues (Fig. 4). *Rsa*I digests of DNA from pancreas and muscle tissues of a fetus heterozygous for the insulin VNTR, showed the constant bands of 4.7 kb and 0.4 kb and bands of 2.2 and 2.4 kb corresponding to the two VNTR alleles (lanes 5 and 7). These two bands were reduced in intensity when cut with *Ava*I (lanes 6 and 8) indicating that a small fraction of sites A1 and A2 were methylated. The presence of smaller fragments in the double digests clearly indicated that sites A1 and A2 were partially methylated in pancreas and muscle and that this pattern was not correlated with the potential for gene expression. *Rsa*I digests of a fetal urothelial sample (lane 9) showed 2.4 and 4.0 kb bands corresponding to the VNTR-containing bands. The 4.0 kb band was completely digested by *Ava*I whereas the 2.4 kb band was only partially digested (lane 10). In this sample, the two alleles were differentially methylated at sites A1 and A2. Therefore, in fetal samples both alleles of the insulin gene were partially methylated in the VNTR region as well as in the body of the gene and, in some samples, differential methylation of the two parental alleles was observed.

In adult lymphocytes (lanes 3 and 4), sites A1 and A2 were also partially methylated. *Rsa*I digests of a lymphocyte sample homozygous for the VNTR region showed a 2.4 kb band corresponding to the VNTR-containing fragment (lane 3). Digestion with *Ava*I resulted in the appearance of several smaller molecular weight bands with the majority of the 2.4 kb band remaining intact (lane 4). The data showed that in lymphocytes sites A1 and A2 were partially methylated.

Analysis of the methylation of CCGG sites within the VNTR region on the same set of samples was performed using double digests with *Pvu*II, which flanks the VNTR region, and *Hpa*II. In sperm and lymphocyte samples, these sites were heavily methylated in both alleles and the methylation in the fetal samples was partial in both alleles. The two VNTR regions in some fetal samples clearly had different methylation levels (Ghazi *et al.* 1990).

In summary (Fig. 5), our data indicate extensive methylation of both insulin alleles in sperm, most clearly at site A3, which is completely methylated in sperm but not methylated in any other tissue or cells examined. In fetal cells and lymphocytes, both alleles were partially methylated in the other two sites examined and differential methylation of the parental alleles was observed in some fetal samples.

Methylation of the retinoblastoma gene

The RB gene is located on chromosome 13q14 and its mutated form is involved in retinoblastoma and osteosarcoma (Dryja *et al.* 1986). The paternal copy of chromosome 13q was retained in 12 of 13 cases of osteosarcomas studied, therefore suggesting a potential for imprinting of this chromosome region in human cells (Toguchida *et al.* 1989). The methylation of the RB locus in sperm, fetal and lymphocyte DNAs were studied using double digests with *Hin*dIII and *Hpa*II probed with a cDNA fragment.

In sperm the RB locus was extensively methylated in all the *Hin*dIII fragments with the exception of a site in the region including exons 20–23. This region was completely unmethylated in sperm and was partially (50%) methylated in fetal cells. In lymphocytes, all the

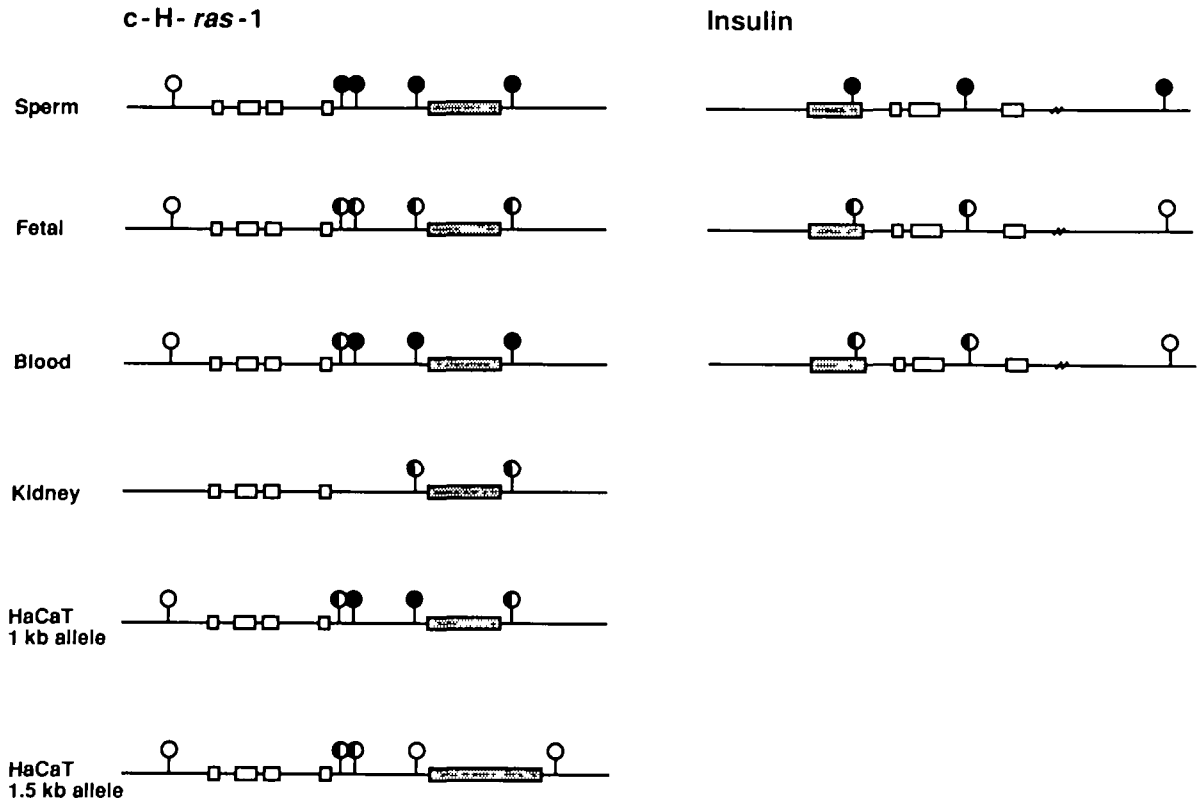

Fig. 5. Summary of the methylation studies of c-H-*ras*-1 and insulin genes. The open circles denote unmethylated sites, half filled circles denote partially methylated sites and filled circles denote methylated sites.

sites were completely methylated (data not shown). The data showed a sperm-specific methylation pattern at the RB locus and that despite extensive methylation of the gene, a specific site remained unmethylated in sperm. We are attempting to utilize the VNTR region associated with the RB locus, which is made up of 50–53 bp repeat units (Wiggs *et al.* 1988), to examine the methylation status of the individual RB alleles in fetal tissues.

Discussion

We have shown that the two parental alleles of c-H-*ras*-1 and insulin genes can be distinguished using the VNTR polymorphic regions at each locus. This allowed us to determine the methylation status of individual alleles of these two genes in human development. The existence of numerous VNTR-associated loci throughout the human genome (Nakamura *et al.* 1987) makes this a practical approach to study the methylation differences of homologous alleles in human cells (Chandler *et al.* 1987; Silva and White, 1988).

Both alleles of c-H-*ras*-1 and insulin genes were extensively methylated in human sperm and were partially demethylated in fetal cells. However, the insulin gene site in the 3′ flanking region was completely unmethylated in fetal cells as well as in lymphocytes. An opposite change in methylation pattern was observed at a site in the region including exons 20–23 of the RB locus. This site was not methylated in sperm but was methylated in fetal cells and in lymphocytes. Sperm-specific methylation differences have also been observed by Silva and White (1988) for the YNZ22 locus, which was not methylated in sperm, and the MCOC12 locus, which was completely methylated in sperm. In their study, six out of the eight loci examined were extensively methylated in sperm, suggesting that human sperm may contain many extensively methylated regions.

For DNA methylation to play a role in imprinting, there must presumably be differences in the methylation of genes in sperm and oocytes. Studies in the mouse have shown that the oocyte genome is strikingly under-methylated relative to sperm (Monk *et al.* 1987) and it was suggested that the differential methylation of the mouse gametes might be involved in genomic imprinting (Monk, 1988). Recently, Driscoll and Migeon (1990) have found that human male meiotic germ cells are extensively methylated at 25 CCGG sites outside of CpG islands. On the other hand, none of these same sites are methylated in germ cells obtained from fetal ovaries. This leads the authors to suggest that human oocytes might in fact be completely unmethylated. It is tempting to suggest that these marked differences in DNA modification may play a role in parental imprinting.

The extent of methylation of CCGG sites in non-CpG island regions of the genes examined in our study

was quite extensive, both in sperm and somatic tissues. In most cases, a decrease in the level of methylation of these sites occurred during development. If the human oocyte genome contains no methylation, as suggested by Driscoll and Migeon (1990), both demethylation and *de novo* methylation of the two alleles of a gene must occur during embryogenesis. The demethylation of the paternal genome does not require the participation of a maternal component because the hydatidiform mole examined showed that the c-H-*ras*-1 gene had a methylation pattern similar to that observed in normal fetal cells. The mechanisms by which individual alleles reach similar levels of methylation in the adult are completely unknown.

Analysis of the c-H-*ras*-1 gene methylation in both sperm and somatic tissues showed that cytosine methylation had occurred in a region normally classified as a CpG island (Gardiner-Garden and Frommer, 1987). This result is surprising because CpG islands are generally considered to be free of methylation unless tley are located on an inactive X-chromosome. The data may indicate that the criteria used to classify CpG islands are not sufficiently stringent. It was, however, significant that no methylation was observed in the 5′ region of the c-H-*ras*-1 gene which has the greatest concentration of CpG sites (Fig. 1). This may be particularly important since the strongest evidence linking cytosine methylation to gene suppression comes from studies on the methylation of CpG islands (Jones and Buckley, 1990). The question of whether the methylation we have examined has significance in differential gene utilization during human development therefore remains open.

The strikingly different levels of CpG methylation of genes in human sperm and oocytes may play a role in the increased potential for paternal genomes to sustain the initial mutations leading to the development of childhood cancers (Table 1). 5-Methylcytosine is inherently mutagenic and appears to function as a mutational hot spot in bacteria (Coulondre *et al.* 1978). It has also been realized that the CpG dinucleotide is involved in >35% of all point mutations in human genes (Cooper and Youssoufian, 1988). We have recently shown by direct genomic sequencing that cytosine residues known to have undergone a germ line mutation in the human LDL receptor or somatic mutation in the p53 tumor suppressor genes are methylated in all normal tissues analyzed (Rideout *et al.* 1990). Therefore, as we have pointed out previously (Jones and Buckley, 1990), differential methylation of parental genomes might explain differential mutation rates. The methylation imprint should therefore be considered for its ability to alter mutation frequencies on individual alleles in addition to its potential to alter gene utilization during development.

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References

Bell, G., Karam, J. H. and Rutter, W. J. (1981). Polymorphic DNA region adjacent to the 5′ end of the human insulin gene. *Proc. natn. Acad. Sci U.S.A.* **78**, 5759–5763.

Buckle, V. J., Edwards, J. H., Evans, E. P., Jonasson, J. A., Lyon, M. F., Peters, J., Searle, A. G. and Wedd, N. S. (1984) Chromosome maps of man and mouse II. *Clinical Genetics* **26**, 1–11

Capon, D. J., Chen, E Y., Levinson, A. D., Seeburg, P. H. and Goeddel, D. V (1983). Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature* **302**, 33–37

Cattanach, B. M. and Kirk, M. (1985). Differential activity of maternally and paternally derived chromosome regions in mice. *Nature* **315**, 496–498

Chandler, L. A., Ghazi, H., Jones, P. A., Boukamp, P. and Fusenig, N. E. (1987) Allele-specific methylation of the human c-Ha-*ras*-1 gene *Cell* **50**, 711–717.

Cooper, D. N. and Youssoufian, H. (1988). The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78**, 151–155

Coulondre, C., Miller, J. H., Farabaugh, P. J. and Gilbert, W. (1978). Molecular basis of base substitution hotspots in *Escherichia coli*. *Nature* **274**, 775–780

Driscoll, D. J. and Migeon, B. R. (1990). Sex difference in methylation of single-copy genes in human meiotic germ cells: implications for X-chromosome inactivation, parental imprinting and origin of CpG mutations. *Somat. cell. mol. Genet.* **16**, 267–283.

Dryja, T. P., Mukai, S., Petersen, R., Rapaport, J. M., Walton, D. and Yandell, D. W (1989). Parental origin of mutations of the retinoblastoma gene. *Nature* **339**, 556–558.

Dryja, T. P., Rapaport, J. M., Joyce, J. M. and Petersen, R A. (1986) Molecular detection of deletions involving band q14 of chromosome 13 in retinoblastoma. *Proc. natn. Acad. Sci. U.S.A* **83**, 7391–7394.

Feinberg, A P., Vogelstein, B., Droller, M. J., Baylin, S B and Nelkin, B. D. (1983). Mutation affecting the 12th amino acid of the c-Ha-*ras* oncogene product occurs infrequently in human cancer. *Science* **220**, 1175–1177

Gardiner-Garden, M and Frommer, M. (1987). CpG island in vertebrate genomes. *J. molec. Biol.* **196**, 261–282.

Ghazi, H., Erwin, A E., Leach, R. J. and Jones, P. A. (1990). Allele-specific methylation in human cells. In *UCLA Symposia on Molecular and Cellular Biology*, New Series, Vol. 128 (ed. G. A. Clawson, D B. Willis, A. Weissbach and P. A. Jones), pp. 381–394. New York: A R Liss, Inc.

Jadayel, D., Fain, P., Upadhyana, M., Ponder, M A., Huson, S. M., Carey, J. I., Fryer, A., Matthew, C. G. P., Barker, D. F and Ponder, B. A. J. (1990). Paternal origin of new mutations in Von Recklinghausen neurofibromatosis *Nature* **343**, 558–559.

Jones, P. A. and Buckley, J D (1990). The role of DNA methylation in cancer. *Ad Cancer Res* **54**, 1–23

Kajii, T. and Ohama, K. (1977). Androgenetic origin of hydatidiform mole. *Nature* **268**, 633–634.

Kovacs, B. W., Shahbahrami, B. and Curtin, J. P. (1990). Molecular genetic analysis of complete hydatidiform moles. *Am J. Obs Gyn.*, submitted.

Leach, R. J., Magewu, A. N., Buckley, J. D., Benedict, W. F., Rother, C., Murphree, A. L., Griegel, S., Rajewsky, M. and Jones, P A (1990) Preferential retention of paternal alleles in human retinoblastoma: evidence for genomic imprinting. *Cell Growth and Diff* **1**, 401–406.

Mannens, M., Slater, R M., Heyting, C., Bliek, J., de Kraker, J., Coad, N., de Pagter-Holthuizen, R. and Pearson, R. L. (1988). Molecular nature of genetic changes resulting in loss of heterozygosity of chromosome 11 in Wilms' tumor. *Hum Genet.* **81**, 41–48

McGrath, J. and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179–183.

MONK, M. (1988). Genomic imprinting. *Genes and Development* **2**, 921–925.

MONK, M., BOUBELIK, M. AND LEHNERT, S. (1987). Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99**, 371–382.

NAKAMURA, Y., LEPPERT, M., O'CONNELL, P., WOLFF, R., HOLM, T., CULVER, M., MARTIN, C., FUJIMOTO, E., HOFF, M., KUMLIN, E. AND WHITE, R. (1987). Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* **235**, 1616–1622.

PONDER, B. (1989). Is imprinting to blame? *Nature* **340**, 264.

REEVE, A. E., HOUSIAX, P. J., GARDNER, R. J. M., CHEWINGS, W. E., GRINDLEY, R. M. AND MILLOW, L. J. (1984). Loss of a Harvey *ras* allele in sporadic Wilms' tumor. *Nature* **309**, 174–176.

REIK, W. (1989). Genomic imprinting and genetic disorders in man. *Trends Genet.* **5**, 331–336.

REIK, W., COLLICK, A., NORRIS, M. L., BARTON, S. C. AND SURANI, M. A. (1987). Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature* **328**, 248–251.

RIDEOUT, W. M., COETZEE, G. A., OLUMI, A. F. AND JONES, P. A. (1990). 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* **249**, 1288–1290.

SAPIENZA, C., PETERSON, A. C., ROSSANT, J. AND BALLING, R. (1987). Degree of methylation of transgenes is dependent on gamete of origin. *Nature* **328**, 251–254.

SCHROEDER, W. T., CHAO, L. Y., DAO, D. D., STRONG, L. C., PATHNAK, S., RICCARDI, V., LEWIS, W. H. AND SAUNDERS, G. F. (1987). Nonrandom loss of maternal chromosome 11 alleles in Wilms' tumors. *Am. J. Genet.* **40**, 413–420.

SCRABLE, H., CAVENEE, W., GHAVIMI, F., LOVELL, M., MORGAN, K. AND SAPIENZA, C. (1989). A model for embryonal rhabdomyosarcoma tumorigenesis that involves genome imprinting. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7480–7484.

SEARLE, A. G. AND BEECHEY, C. V. (1985). Noncomplementation phenomena and their bearing on nondisjunction effects. In *Aneuploidy, Aetiology and Mechanisms* (ed. V. L. Dellarco, P. E. Voytek and A. Hollaender), pp. 363–376. New York: Plenum.

SILVA, A. J. AND WHITE, R. (1988). Inheritance of allelic blueprints for methylation patterns. *Cell* **54**, 145–152.

SOLTER, D. (1988). Differential imprinting and expression of maternal and paternal genomes. *Ann. Rev. Genet.* **22**, 127–146.

SURANI, M. A. H., BARTON, S. C. AND NORRIS, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548–550.

SURANI, M. A. H., BARTON, S. C. AND NORRIS, M. L. (1986). Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* **45**, 127–136.

SWAIN, J. L., STEWART, T. A. AND LEDER, P. (1987). Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. *Cell* **50**, 718–727.

TOGUCHIDA, J., ISHIZAKI, K., SASAKI, M. S., NAKAMURA, Y., IKENAGA, M., KEITO, M., SUGIMOTO, M., KOTOURA, Y. AND YAMAMURO, T. (1989). Preferential mutation of paternally derived RB gene as the initial event in sporadic osteosarcoma. *Nature* **338**, 156–158.

WIGGS, J., NORDENSKJÖLD, M., YANDELL, D., RAPAPORT, J., GRONDIN, V., JANSON, M., WERELIUS, B., PETERSEN, R., CRAFT, A., RIEDEL, K., LIBERFARB, R., WALTON, D., WILSON, W. AND DRYJA, T. P. (1988). Prediction of the risk of hereditary retinoblastoma, using DNA polymorphisms within the retinoblastoma gene. *New Engl. J Med.* **318**, 151–157.

WILLIAMS, J. C., BROWN, K. W., MOTT, M. G. AND MAITLAND, N. J. (1989). Maternal allele loss in Wilms' tumor. *The Lancet* **i**, 283–284.

ZHU, X., DUNN, J. M., PHILIPS, R. A., GODDARD, A. D., PATON, K. F., BECKER, A. AND GAILIE, B. L. (1989). Preferential germline mutation of the paternal allele in retinoblastoma. *Nature* **340**, 312–313.