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*Original Article*

# Developmental-dependent DNA methylation of the *IGF2* and *H19* promoters is correlated to the promoter activities in human liver development

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**ABSTRACT We have previously shown that the four promoters of the *IGF2* gene are under a tight but dynamic control during human liver development, whereby P3 and P1 are reciprocally active before and after birth respectively while the P2 and P4 promoters are constitutively active at a relatively lower level. In this study, we investigated the methylation status of the promoters P1 and P3 of *IGF2* and the promoter region of the *H19* gene in developing human livers ranging from fetal to late adult. A region of about 300bp immediately upstream of the *IGF2* exon 5 was found to be subjected to a developmental-specific methylation and this may correlate to the P3 promoter activity. The P1 domain of *IGF2* was also found to be methylated in a developmentally-specific pattern. The promoter region of the *H19* gene displayed different methylation patterns in different development stages showing decreased general methylation with increase of age. Therefore, regional- and developmental-specific DNA methylation is displayed in the promoter regions of the *IGF2* and *H19* genes. This may be an important factor involved in gene regulation in the developing human liver.**

KEY WORDS: *CpG, transcription, regulation, epigenetics*

## Introduction

DNA methylation is an epigenetic modification representing another kind of information involved in gene regulation in addition to the primary genetic information encoded by the four bases. Although both 6-methyladenine and 5-methylcytosine can be found in bacteria and some lower eukaryotes, only cytosine can be subjected to the activity of the DNA methyltransferase in mammals resulting in 5-methylcytosine occurring mostly in CpG doublets (Bird, 1992).

The role of DNA methylation during development has been intensively studied. Oncogenically transformed mouse cell lines can differentiate into muscle and other cell types after treatment with the methylation inhibitor, 5-azacytidine (Taylor and Jones, 1979). Although the question remains if 5-azacytidine can mimic a real *in vivo* mechanism, recent evidence has shown that DNA methylation is important for embryo development since mice with a methyltransferase mutation fail to develop beyond the early embryonic stage (Li *et al.*, 1993).

DNA methylation may regulate gene expression in different ways. It may inhibit gene transcription through binding of proteins to methylated sites, thus preventing the binding of transcription factors and/or altering chromatin structures. Suppression of gene transcription by DNA methylation is well illustrated by the fact that the inactivated X chromosome is highly methylated in CpG islands while the active one is not (Monk, 1990). DNA methylation may also promote transcription by suppressing the binding of repressors (Stöger *et al.*, 1993). The mouse *Igf2r* gene is a good example in support of this, since in order to be expressed, the active maternal allele has to be methylated in an intron region and unmethylated in its 5´-region (Wutz *et al.*, 1997).

Plenty of data have shown a correlation between DNA methylation and *Igf2* expression. In a mouse cell line, the *Igf2* gene can be reactivated by treatment with the methylation inhibitor, 5-aza-2´-deoxycytidine (Eversole-Cire *et al.*, 1993). Studies from human cell lines also show that the *IGF2* P3 promoter is highly methylated

*Abbreviations used in this paper: IGF2,* human insulin-like growth factor-2 gene; *Igf2,* mouse insulin-like growth factor-2 gene; RPA, RNase protection assay; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; *Igf2r,* mouse insulinlike growth factor 2 receptor gene.

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when it is inactive (Raizis *et al.*, 1993). In normal adult human leukocyte DNA, the *IGF2* gene was found to be allele-specifically methylated by showing paternal allele-specific hypermethylation (Schneid *et al.*, 1993).

Current data has shown that the four promoters of *IGF2* may function in different ways. The mRNA from the promoter P3 was found to be translated only in exponentially growing cells, while the mRNA from promoter P4 was suggested to be responsible for the constitutive IGF-II production (Nielsen *et al.*, 1995). The promoter P1 is different from the other three since it escapes the imprinting process and is only expressed at a relatively high level in human liver after birth (Vu and Hoffman, 1994; Ekström *et al.*, 1995; Taniguchi *et al.*, 1995a; Li *et al.*, 1996). In human liver, which may be the major site for IGF-II production and where *IGF2* is expressed in a developmental- and promoter-specific way (Li *et al.*, 1996), the involvement of DNA methylation in the regulation of the gene is still unclear. To our knowledge, no information regarding the methylation status specifically in the *IGF2* P3 or P1 promoters in human liver has been published. We therefore found it important to gain more insight into the promoter-specific methylation status of *IGF2*.

The *H19* gene has been shown to be expressed in a developmental-specific way in that it is highly expressed in fetal tissues and significantly downregulated shortly after birth. In human placenta, the effect of DNA methylation on *H19* expression is shown by the progressive methylation of the 3′ portion of the gene at the paternal allele after gestation, and which occurs in parallel with the silencing of this allele (Jinno *et al.*, 1995). However, the regulatory relationship between DNA methylation and *H19* expression is not yet clear although allele-specific methylation is considered to be an imprinting marker for this gene (Forne *et al.*, 1997).

In this study, we investigated the methylation status of the promoters P1 and P3 of *IGF2* and the promoter region of the *H19* gene in human normal livers from different developmental stages. The results suggest that developmental- and region-specific DNA methylation in the promoter domains of these two genes may be correlated to the gene expressions.

## Results

### *Methylation pattern at the IGF2 P3 region is developmentally-specific in human livers*

The promoter P3 of the *IGF2* gene produces about 70% of the total *IGF2* transcripts in fetal and neonatal liver, and becomes silenced shortly after birth in most cases (Li *et al.*, 1996). To find out more about the regulatory mechanisms of this phenomenon, we used a 1.37 kb fragment spanning almost the whole intron region between exons 4 and 5, and 130bp of the 5′ end of the exon 5 (Figs. 1A and 2A, probe A) as a probe to investigate the methylation pattern of this region of the *IGF2* gene in nine normal human livers ranging from perinatal to late adult and the liver tumor cell line, HepG2. The results show that the P3 region was hypermethylated shortly after birth and maintained a relatively stable pattern throughout different ages in adult (Fig. 2B). In the two perinatal liver samples, which displayed high P3 activity according to previous studies (Li *et al.*, 1996), the P3 region was hypomethylated. From the age of two-months and onwards when P3 starts to be silenced (Li *et al.*, 1996), the promoter region also starts to become methylated as shown by the extra bands in the *HpaII* cut lanes (indicated by the left arrows in Fig. 2B). The human hepatoblastoma cell line, HepG2, showed a similar hypomethylated pattern as observed in livers from prematurely born individuals, although the cell line may be slightly more methylated displayed by a weak band not present in the perinatal livers. The hypermethylation in this region after birth may thus be involved in the silencing of the P3 promoter activity.

### *Mapping of the developmentally-specific methylated region of P3*

The entire P3 region of *IGF2* is about 1.4 kb. It contains two Sp1-like sequences, two Egr-1 consensus sequences, a potential CCAAT-box and a TATA box within the 300bp region immediately upstream of exon 5. In order to find out if this specific region is subjected to the developmental-specific methylation, a 430bp fragment spanning 130bp of exon 5 and its upstream region (Fig. 2A, probe B) was subcloned and used as a probe to hybridize the same membranes analyzed with the 1.37 kb probe described above. This probe revealed that the region methylated after birth is located within or around this 430bp region (Fig. 2C). The picture shows that the common bands present in all the samples from different developmental stages disappeared (indicated by the arrow between Fig. 2B and C) as compared with using the 1.37 kb probe, while the bands only existing in the samples after birth remain (indicated by the left arrows in Fig. 2B). We conclude that CpG sites within or around the 430bp region of *IGF2* P3 are a target for developmental-specific methylation which may affect the promoter activity.

### *Methylation of the promoter P1 domain of IGF2 is decreased with age in normal human livers*

The promoter P1 of the *IGF2* gene is silent in fetal tissues and activated shortly after birth in human liver. The function and the mechanism of its activation are poorly understood. Seven normal human livers from 5-week fetal to 65-year old adult were investigated for their methylation status around the *IGF2* promoter P1 region. A genomic fragment of about 8 kb including the *INS* gene and the first intron of *IGF2* (Fig. 3A) was used as a probe. Figure 3B shows that in the samples of the fetal, perinatal, two-month and

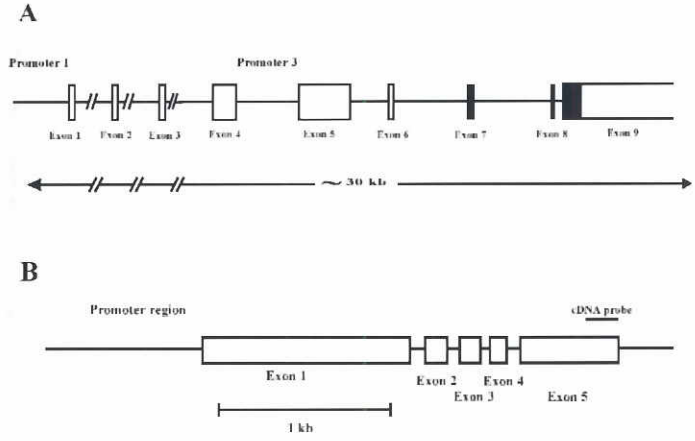


**Fig. 1. Gene structures of *IGF2* and *H19*. (A)** *IGF2 gene. The open boxes are the nine exons of IGF2, and the black boxes are the common coding region. Promoter P1 and P3 regions are investigated for DNA methylation status.* **(B)** *H19 gene. The open boxes are the five exons of the gene. The indicated promoter region is investigated for methylation status in this study. The cDNA probe is used to investigate the H19 expression level.*

one-year old individuals which displayed no or low transcripts from P1 (Li *et al.*, 1996), this region is highly methylated (indicated by the left arrows in Fig. 3B). After one-year of age, when P1 becomes more activated as reported previously (Li *et al.*, 1996), the region is significantly demethylated. We have not yet mapped the precise region of the demethylated sites. However, the region around P1 may be subjected to a developmental-specific methylation and the specific methylation pattern may be related to the developmental-specific regulation of the promoter P1 activity.

### *The H19 promoter is developmentally-specific methylated in human liver*

Although plenty of data have shown that the regulation of the *H19* gene is developmental-specific, the molecular basis of this regulation is not yet completely illustrated. We have previously shown that the *H19* gene is highly expressed in fetal liver and downregulated after birth. In liver from the older adult, the expression is very low or undetectable (Ekström *et al.*, 1995). To continue this study, seven normal human livers ranging from 5-week fetal to 65-year old were investigated for their methylation patterns within the *H19* promoter region. A fragment spanning 0.8 kb immediately upstream of the first exon of the gene was used as a probe (Fig. 4A). The results are illustrated in Figure 4B. In the livers from individuals younger than one year, which expressed high levels of *H19* mRNA (Fig. 5), the promoter was highly methylated. The left, upper arrow in Figure 4B indicates the fully methylated 2.1 kb *RsaI* band covering the promoter region as represented in Figure 4A. In the adult samples, the intensity of the 2.1 kb fragment is decreased while the intensity of the 0.54 kb band (left, lower arrow in Fig. 4B) is increased, suggesting that demethylation occurred within the 2.1 kb region. The intensities of the 2.1 kb and the 0.54 kb bands were measured by densitometry, and the ratios of the 2.1 kb signal/0.54 kb signal are shown in Figure 4B for each sample. In adult, this value is about 4-fold decreased (average) compared to that of the young individuals. However, additional bands (the right arrow in Fig. 4B) coincidentally appear with the demethylation of the 2.1 kb bands in the adult as well as in the 2 months samples. These additional bands are not likely due to be incomplete digestion, since when another probe, the 8 kb P1 fragment, was used with the same filters, these samples displayed obvious demethylation (Fig. 3B). We therefore suggest that retention, or even re-establishment of methylation in specific sites occur synchronously with the demethylation of other sites within the 2.1 kb region covering the *H19* promoter, displaying a developmental-specific methylation of this region.

To further characterize the relationship of the methylation pattern observed in Figure 3B to *H19* gene expression, in particular to examine if the small extra band (the right arrow in Fig. 4B) is associated with the suppression of *H19* expression, RPA (RNase protection analysis) was performed to investigate the expression level of *H19* in those samples using an *H19* cDNA probe (Fig. 1B). The results were quantified by phosphor imager using GAPDH as an internal control. The values of *H19* expression shown in Figure 5 are arbitrary numbers normalized to GAPDH. As shown in Figure 5, *H19* expression is decreasing with age in adult samples in agreement with previous reports. However, this decrease does not seem to be correlated with the observed additional band (the right arrow in Fig. 4B) since samples with or without this band can display high (2 months) or very low (34 years) *H19* expression respectively.

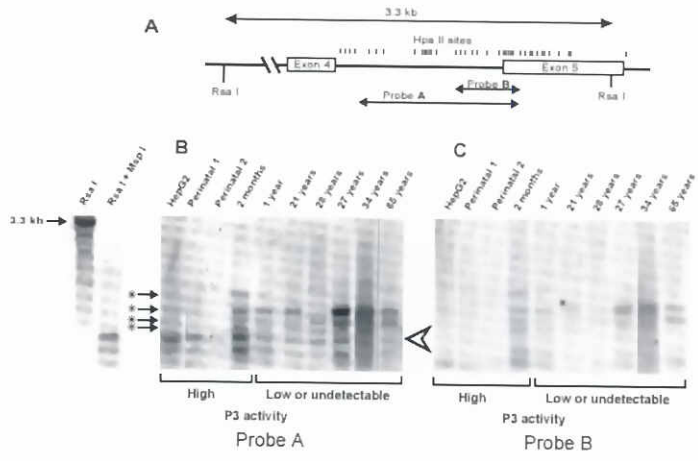


**Fig. 2. Developmental-specific methylation of the P3 region of *IGF2*.** **(A)** *Probes used in this study. The 3.3 kb fragment is generated by the RsaI digestion. The open boxes are the exons 4 and 5 of IGF2. Vertical bars on the top indicate the HpaII sites in P3 and exon 5 region.* **(B)** *Methylation status of P3 region in human developing liver using probe A. Arrows with asterisks to the left indicate the bands in the livers after birth representing a developmental-specific methylation. P3 expression status is indicated under each sample according to previous reports. Control analysis of genomic DNA using digestion with RsaI alone and with RsaI + MspI were performed to assess the pattern for total digestion as shown in the first two lanes to the left.* **(C)** *Methylation status of P3 region using probe B with the same membrane as in Figure 2B. The arrow between Figures 2B and C indicates the disappearance of the common bands when probe B was used, while the developmental-specific bands are maintained.*

## Discussion

### *Methylation of the P3 domain of IGF2*

We and others have previously shown that DNA methylation of the *IGF2* P3 region and the promoter activity are inversely correlated in both human hepatoblastoma (Li *et al.*, 1997) and liver cell lines (Raizis *et al.*, 1993). During human liver development, the expression status of this promoter has been intensively studied but little is known about the regulatory mechanism behind the developmental-specific control of this promoter.

In this study, the two perinatal liver samples which expressed high levels of P3 transcripts display hypomethylation of the promoter region while the samples coming from patients of two months and older display hypermethylation. This may suggest a potential methylation switch after birth in the P3 region. Interestingly, this potential switch matches well with the time point of the activation of the promoter P1 of *IGF2*. We do not yet know if these simultaneous events are correlated or just coincidental. A larger number of samples is needed for further investigation to confirm this. However, the possibility exists that region-specific methylation might affect common factors involved in the regulation of the P1 and P3 promoters. Our previous data also support the fact that the P1 and P3 promoters of the *IGF2* gene are always oppositely regulated in normal liver and liver tumors (Li *et al.*, 1995,1996), but additional investigations are needed to examine the possible regulatory relationship of the two promoters.

In the present study, the P3 region was found to be hypermethylated after birth in parallel with the disappearance of the P3 transcript. Thus, this hypermethylation may be correlated

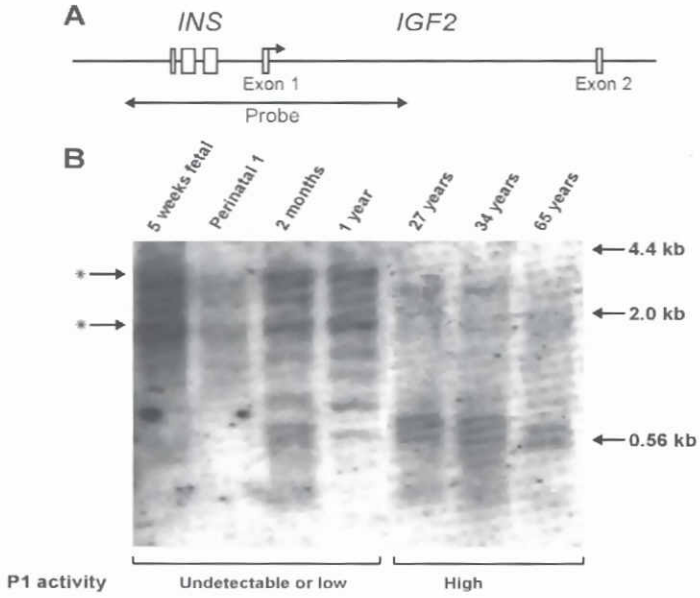


**Fig. 3. Developmental-specific methylation of the P1 domain in human developing livers. (A)** *The probe used in the study. Open boxes are the exons of the IGF2 and INS genes. The arrow indicates the start of the transcription site of the IGF2 gene. The RsaI digestion pattern is not indicated because of the unknown sequence of the IGF2 gene.* **(B)** *Methylation status of the IGF2 P1 domain in human developing liver. The two arrows with asterisks to the left indicate the developmental-specific methylation that is decreased in samples from individuals older than one year. The respective P1 activity of each sample is indicated below according to previous reports.*

with the silencing of the P3 promoter in adult liver. Similar results reported previously showed that the P3 region was hypermethylated in the HeLa cell line which does not express any P3 transcript (Raizis *et al.*, 1993). However, DNA methylation is not likely to be the only factor responsible for the suppression of P3 since small amounts of P3 transcripts can still be seen within the first half year after birth in human liver (Li *et al.*, 1996) irrespective of the hypermethylation of this region.

Previous data have shown that the immediate upstream region of *IGF2* exon 5 plays an important role for the activity of the P3 promoter (van Dijk *et al.*, 1991). Our finding is consistent with the previous reports. This region may participate in the control of the P3 activity by means of a developmental-specific DNA methylation pattern. It is possible that hypomethylation of the P3 region may be a prerequisite for the promoter activity, and region-specific methylation may be involved in the slow silencing of the promoter in human liver after birth. At this point one can not exclude however, that the developmental-specific methylation pattern is established as a consequence of a reduced promoter P3 activity rather than being the cause of silencing. The complexity of this regulation however, may be reflected by the fact that at the branchpoint of P3 deregulation, in the 2 months individual when the promoter still has high activity, it has already become hypermethylated.

### *Methylation of the P1 domain of IGF2*

Although a low level of the *IGF2* P1 expression can be seen in fetal choroid plexus/leptomeninges (Ekström *et al.*, 1995), levels of P1 transcript detectable by RNase protection analysis, can only be seen in human liver after birth, and transcript levels further increase with age (Li *et al.*, 1996). C/EBP, the CCAAT/enhancer binding protein which is expressed predominantly in adult liver, adipose tissue and lung has been shown to be involved in the activation of P1 (van Dijk *et al.*, 1992). Recent data have also shown that the Sp1 sites within the P1 region are important for the promoter activity (Rodenburg, 1997). This fact also ties methylation as a possible factor to the regulation of P1 since the Sp1 site has been shown to be involved in methylation directed regulation (Clark *et al.*, 1997). The function of this transcript and the regulatory mechanism of P1 are still far from clear.

In this study, the P1 domain is shown to be highly methylated in normal human liver of fetal and young children but becomes demethylated after one-year of age. This may suggest that a methylation-free state of some specific sequences is needed for full P1 expression, which is consistent with the classical hypothesis in which methylation at the promoter region is commonly considered to be a suppressive factor for gene expression. However, DNA methylation is clearly not the only factor involved in P1 activity. The methylation patterns in the livers of the two months and the one year old patients where P1 activation is initiated, do not change much compared to that of the fetal sample in which P1 transcripts can not be detected at all. It is thus possible that a demethylated state in the P1 domain may be a requirement for allowing the full activity of the P1 promoter. It will also be necessary to map the developmentally-specific methylated sequences in more detail in order to understand the functional relevance of the DNA methylation status. Shorter probes should give more accurate information since subtle changes could be overlooked when an 8 kb long

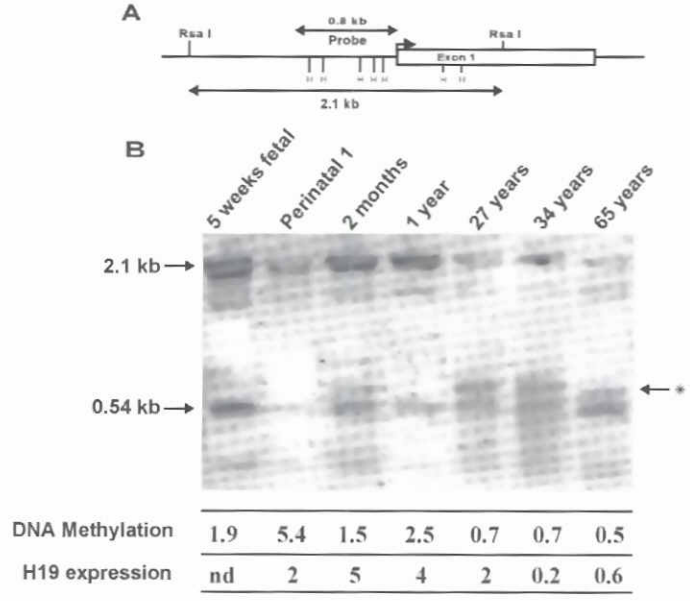


**Fig. 3. Developmental-specific methylation of the P1 domain in human developing livers. (A)** *The probe used in the study. Open boxes are the exons of the IGF2 and INS genes. The arrow indicates the start of the transcription site of the IGF2 gene. The RsaI digestion pattern is not indicated because of the unknown sequence of the IGF2 gene.* **(B)** *Methylation status of the IGF2 P1 domain in human developing liver. The two arrows with asterisks to the left indicate the developmental-specific methylation that is decreased in samples from individuals older than one year. The respective P1 activity of each sample is indicated in below according to previous reports.*

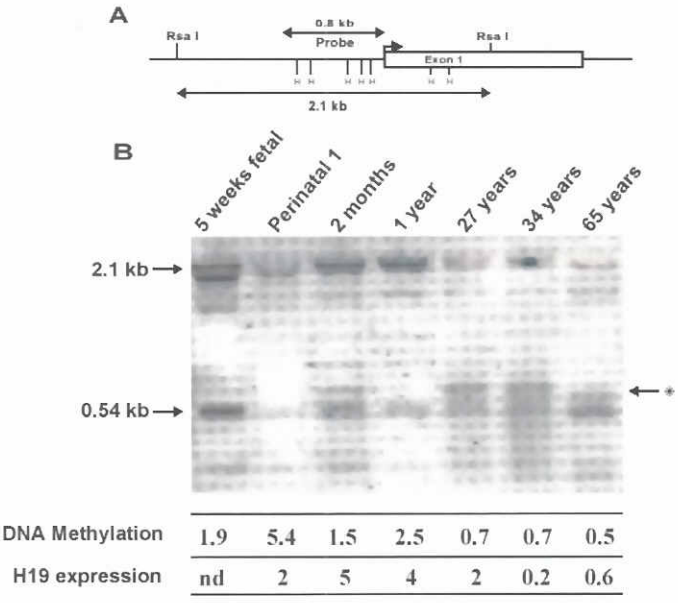


**Fig. 4. Developmental-specific methylation of the *H19* promoter region. (A)** *The 0.8 kb probe used in this study. The 2.1 kb fragment is generated by the RsaI digestion. The open box and the single arrow indicate the exon 1 of the H19 gene.* **(B)** *Methylation status of the H19 promoter region in normal livers. The upper arrow indicates the fully methylated 2.1 kb fragment. The lower left arrow indicates the fully digested band in all the samples. The right arrow indicates the extra bands in the adult samples as well as in the sample of the 2 month-old individual. The methylation degree of each sample is presented in arbitrary numbers according to the 2.1 kb/0.54 kb values from densitometric signals, The H19 expression status in arbitrary numbers normalized to GAPDH is also shown (also see Fig. 5). nd, not determined.*

fragment is used as a probe. Another possibility which can not be completely excluded is that the demethylation is due to the *INS* gene which is also covered by the probe. However, since the *INS* gene is not transcribed in the liver throughout development, this possibility is not likely.

### *DNA Methylation and the H19 gene*

Several lines of evidence confirm that methylation at the upstream region of the *H19* gene is established during spermatogenesis and preserved in the development of the embryo in both human and mouse (Tremblay *et al.*, 1995; Jinno *et al.*, 1996). Hypermethylation and correlated downregulation of the gene expression have been observed for several kinds of diseases, including Wilms´ tumor (Steenman *et al.* 1994; Taniguchi *et al.*, 1995b) and hepatoblastoma (Li *et al.*, 1997). In this study, the methylation of the promoter region of the *H19* gene is regulated in a developmental-specific way. The 2.1 kb region is highly methylated in liver from individuals younger than one year old. However, in samples from older individuals, demethylation occurs in this region with concomitant remethylation or retention of methylation in specific sites as displayed by extra small bands in our assay. As explained in the Results section, these bands are not likely due to incomplete digestion, since on the same membrane, when hybridized with the 8 kb P1 fragment, those samples with the extra bands displayed obvious demethylation. However, the relationship of the *H19* expression and the extra bands, which suggest specific methylation, are not conclusive based on the limited number of samples investigated and this prompts for additional more comprehensive studies.

Recently, new methods have been established regarding DNA methylation studies. One of these is the bisulphite sequencing method whereby single methylated cytosines can be detected by chemical conversion of unmethylated C:s into U:s, followed by PCR and sequencing. This method is more informative but is mainly appropriate for use in cell lines or tissues where a high cell type homogeneity can be obtained. Another technique is the methylation-sensitive PCR method in which genomic DNA is cut by DNA methylation-sensitive restriction enzymes followed by PCR amplification. This method is more accurate when a single restriction site is investigated. However, when the investigated region contains a cluster of methylation sensitive sites, like the *IGF2* P3 region, this method is not optimal because of the possibility of losing information in methylated sites when there is an unmethylated site within the investigated region, or when there is a partial methylation/demethylation for a specific site. In this study, the investigated regions (P3 promoter and *H19* promoter) are CpG islands in a mixed cell population (approximately, 70% hepatocytes, 15% kupffer cells and 15% others). This heterogeneity may also be the reason for the partial demethylation shown here in the *H19* promoter in some of the samples. Therefore, a better technique needs to be established to study the methylation status in detail in the *IGF2* and *H19* promoters in human liver.

The mechanism and biological significance of this developmental- and sequence- specific demethylation and methylation of the promoter region of *H19* is unknown. However, despite the demethylation of the *H19* promoter occurring in adult liver, a downregulation is still evident. Since the observed demethylation most likely occurs on the paternal allele (the 2.1 kb band in Fig 4), the investigated region can not involve methylated sites responsible for maintaining the imprinting status, since *H19* has been shown to be mono-allelelically expressed in these tissues (Zhang *et al.*, 1993).

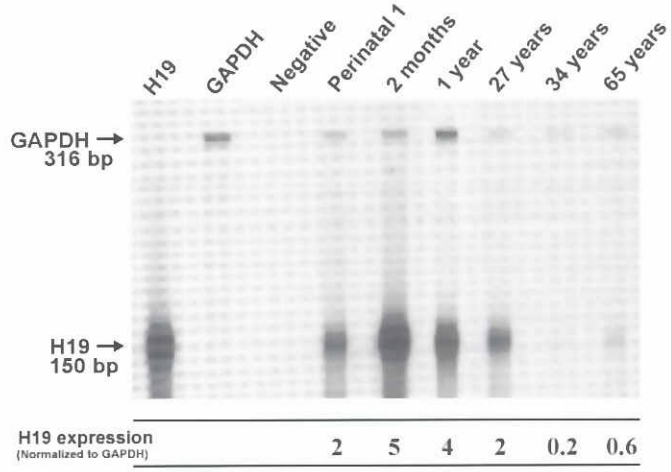


**Fig. 5. *H19* expression status in normal human livers**. *An H19 cDNA probe of 150bp (Fig. 1B) and the GAPDH probe were used simultaneously in the RPA assay with total cellular RNA. The first two lanes to the left show the individual probes hybridized with human liver RNA. The negative control is GAPDH and H19 probes hybridized to yeast RNA. The results were quantified by a phosphor imager. The H19 expression data are in arbitrary numbers and in relation to GAPDH.*

Although the reason and the consequence of the demethylation or remethylation in the *H19* promoter region after birth is unclear, a dynamic methylation pattern which might be necessary for the developmental-specific regulation observed for the *H19* gene, suggests that further study is necessary to characterize the precise relationship between gene expression and DNA methylation during human liver development.

## Materials and Methods

### *Tissue samples*

Normal liver tissues were obtained from operational material on autopsy from Huddinge Hospital, Huddinge, Sweden; St Göran Children´s Hospital, Stockholm, Sweden. The tissues were snap frozen in liquid nitrogen and stored at -70°C. All tissues were examined histologically and morphologically at the respective pathology department and were found to be normal.

### *Nucleic acid extraction*

DNA was extracted from tissues by incubation with Proteinase K in the presence of SDS and EDTA, followed by phenol:chloroform extraction and alcohol precipitation as described (Li *et al.* 1995). DNA quality was examined by gel electrophoresis.

### *Analysis of methylation status*

Fifteen µg of DNA were taken for each digestion with 10x unit excess of *Rsa*I. Complete digestion was checked for the expected *Rsa*I digestion pattern by Southern blot hybridization with specific probes. 15 µg from the samples were subsequently digested with 10x unit excess of each *Hpa*II or *Msp*I and subjected to electrophoresis using 1.6% agarose gels and transferred to Hybond Nfp membrane (Amersham). Hybridizations were carried out according to the manufacturers protocol (Amersham). Before membranes were reprobed, complete stripping was ascertained by overnight exposure on phosphor imaging screens.

### *Probes used in this study*

For the *IGF2* promoter P3 region, a 1.3 kb fragment spanning part of exon 5 and its upstream region (Fig. 2A, probe A), cloned into the *BamH*I-*Hind*III sites of a bluescript SK II vector (Stratagene) was used as the probe for the methylation investigation. In order to map the developmental-specific methylation sites more accurately, a 450bp fragment was subcloned into the *EcoR*I-*Sal*I sites of the same vector, containing a small part of exon 5 and its upstream region (Fig. 2A, probe B).

For the methylation study of the *IGF2* P1 domain, an 8 kb fragment (Fig. 3A) including the insulin gene and part of the first intron of the *IGF2* gene was used.

A 0.8 kb probe for the methylation study of the *H19* promoter region (Fig. 4A) was generated by PCR amplification encompassing five *Hpa*II sites using primers 5´-AACAACCCTCACCAAAGGCC-3´ (upstream) and 5'-CCTGCTCCTCGGTCCTAGCCCGG-3' (downstream).

All probe clones were labeled using a random primer labeling kit (Amersham) and $^{32}$P-dCTP at a specific activity of 6000 Ci/mmol, according to the manufacturers instructions (Amersham).

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## References

BIRD, A. (1992). The essentials of DNA methylation. *Cell. 70:* 5-8.

CLARK, S.J, HARRISON, J. and MOLLOY, P.L. (1997). Sp1 binding is inhibited by $^{m}$Cp$^{m}$CpG methylation. *Gene 195:* 67-71.

EKSTRÖM, T.J, CUI, H., LI, X. and OHLSSON, R. (1995). Promoter-specific IGF2 imprinting status and its plasticity during human liver development. *Development 121:* 309-316.

EVERSOLE-CIRE, P., FERGUSON-SMITH, A.C., SASAKI, H., BROWN, K.D., CATTANACH, B.M, GONZALES, F.A, SURANI, M.A. and JONES, P.A (1993). Activation of an imprinted Igf 2 gene in mouse somatic cell cultures. *Mol. Cell. Biol. 13:* 4928-4938.

FORNE, T., OSWALD, J., DEAN, W., SAAM, J.R., BAILLEUL, B., DANDOLO, L. TILGHMAN, S.M., WALTER, J. and REIK, W. (1997). Loss of the maternal *H19* gene induces changes in *IGF2* methylation in both ces and trans. *Proc. Natl. Acad. Sci. USA 94:* 10243-10248.

JINNO, Y., IKEDA, Y., YUN, K., MAW, M., MASUZAKI, H., FUKUDA, H., INUZUKA, K., FUJISHITA, A., OHTANI, Y., OKIMOTO, T., ISHIMARU, T. and NIIKAWA, N. (1995). Establishment of functional imprinting of H19 gene in human developing placentae. *Nature Genet. 10:* 318-324.

JINNO, Y., SENGOKU, K., NAKAO, M., TAMATE, K., MIYAMOTO, T., MATSUZAKA, T., SUTCLIFFE, J.S., ANAN, T., TAKUMA, N., NISHIWAKI, K., IKEDA, Y., ISHIMARU, T., ISHIKAWA, M. and NIIKAWA, N.. (1996). Mouse/Human Sequence Divergence In a Region With a Paternal-Specific Methylation Imprint At the Human H19 Locus. *Hum. Mol. Genet. 5:* 1155-1161.

LI, E., BEARD, C. and JAENISCH, R. (1993). Role for DNA methylation in genomic imprinting. *Nature 366:* 362-365.

LI, X., ADAM, G., CUI, H., SANDSTEDT, B., OHLSSON, R. and EKSTRÖM, T.J. (1995). Expression,promoter usage and parental imprinting status of IGF2 and H19 status in human hepatoblastoma. *Oncogene 11:* 221-229.

LI, X., CUI, H., SANDSTEDT, B., NORDLINDER, H., LARSSON, E. and EKSTRÖM, T.J. (1996). Expression level of IGF2 in the human liver: Developmental relationships of the four promoters. *J. Endocrinol. 149:* 117-124.

LI, X., KOGNER, P., SANDSTEDT, B., HAAS, O.A. and EKSTRÖM, T.J. (1997). Promoter-specific methylation and expression alterations involved in human hepatoblastoma. *Int. J. Cancer. 75:* 1-5.

MONK, M. (1990). Changes in DNA methylation during mouse embryonic development in relation to X-chromosome activity and imprinting. *Philos. Trans. R. Soc. Lond. [Biol.] 326:* 299-321.

NIELSEN, F.C., OSTERGAARD, L., NIELSEN, J. and CHRISTIANSEN, J. (1995). Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. *Nature 377:* 358-362.

RAIZIS, A.M., ECCLES, M.R. and REEVE, A.E. (1993). Structural analysis of the human insulin-like growth factor-II P3 promoter. *Biochem. J. 289:* 133-139.

RODENBURG, R.J.T., HOLTHUIZEN, P.E. and SUSSENBACH, J.S. (1997). A functional Sp1 binding site is essential for the activity of the adult liver-specific human insulin-like growth factor II promoter. *Mol. Endocrinol. 11:* 237-250.

SCHNEID, H., SEURIN, D., VAZQUEZ, M-P., GOURMELEN, M., CABROL, S. and BOUC, Y.L. (1993). Parental allele specific methylation of the human insulin-like growth factor II gene and Beckwith-Wiedemann syndrome. *J. Med. Genet. 30:* 353-362.

STEENMAN, M.J.C., RAINIER, S., DOBRY, C.J., GRUNDY, P., HORON, I.L. and FEINBERG, A.P. (1994). Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumor. *Nature Genet. 7:* 433-439.

STÖGER, R., KUBICKA, P., LIU, C.G., KAFRI, T., RAZIN, A., CEDAR, H. and BARLOW, D.P. (1993). Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. *Cell 73:* 61-71.

TANIGUCHI, T., SCHOFIELD, A.E., SCARLET, J.L., MORISON, I.M., SULLIVAN, M.J. and REEVE, A.E. (1995a). Altered specificity of IGF2 promoter imprinting during fetal development and onset of Wilms tumor. *Oncogene 11:* 751-756.

TANIGUCHI, T., SULLIVAN, M.J., OGAWA, O. and REEVE, A. (1995b). Epigenetic changes encompassing the IGF2/H19 locus associated with relaxation of IGF2 imprinting and silencing of H19 in Wilms tumor. *Proc. Natl. Acad. Sci. USA. 92:* 2159-2163.

TAYLOR, S.M. and JONES, P.A. (1979). Multiple New Phenotypes Induced in 10T1/2 and 3T3 Cells Treated with 5-azacytidine. *Cell 17:* 771-779.

TREMBLAY, K.D., SAAM, J.R., INGRAM, R.S., TILGHMAN, S.M. and

BARTOLOMEI, M.S. (1995). A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. *Nature Genet. 9:* 407-413.

VAN DIJK, M.A., RODENBURG, R.J., HOLTHUIZEN, P. and SUSSENBACH, J.S. (1992). The liver-specific promoter of the human insulin-like growth factor II gene is activated by CCAAT/enhancer binding protein (C/EBP). *Nucleic Acids Res. 20:* 3099-3104.

VAN DIJK, M.A., VAN SCHAIK, F.M., BOOTSMA, H.J., HOLTHUIZEN, P. and SUSSENBACH, J.S. (1991). Initial characterization of the four promoters of the human insulin-like growth factor II gene. *Mol. Cell. Endocrinol. 81:* 81-94.

VU, T.H. and HOFFMAN, A.R. (1994). Promoter-specific imprinting of the human insulin-like growth factor-II gene. *Nature 371:* 714-717.

WUTZ, A., SMRZKA, O.W., SCHWEIFER, N., SCHELLANDER, K., WAGNER, E.F. and BARLOW, D.P. (1997). Imprinted expression of the Igf2r gene depends on an intronic CpG island. *Nature 389:* 745-749.

ZHANG, Y., SHIELDS, T., CRENSHAW, T., HAO, Y., MOULTON, T. and TYCKO, B. (1993). Imprinting of human *H19*: allele-specific CpG methylation, loss of the active allele in Wilms' tumor, and potential for somatic allele switching. *Am. J. Hum. Genet 53:* 113-124.

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