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Correlation of Tissue-specific Methylation with Gene Inactivity in Hepatitis B Virus Transgenic Mice

Kimi Araki,[1] Kiwamu Akagi,[1] Jun-ichi Miyazaki,[1] Kenichi Matsubara[2] and Ken-ichi Yamamura[1]

[1]*Institute for Medical Genetics, Kumamoto University Medical School, Kuhonji, Kumamoto 862 and*
[2]*Institute for Molecular and Cellular Biology, Osaka University, Yamadaoka, Suita 565*

We produced transgenic mice using two constructs, HB-GII and 1.2HB-BS, of hepatitis B virus (HBV) DNA. The former has been designed to express mRNAs for HBV surface antigen (HBsAg), and the later to express all mRNAs of HBV. Several lines of the transgenic mice carrying each construct were examined for the tissue-specificity and level of HBV DNA expression, and for the relationship between expression and methylation of the transgenes. Only one out of ten for HB-GII and one out of eight for 1.2HB-BS were high producers of viral antigens. In high producers, transgenes were expressed in the liver and the kidneys. But in low producers, transgenes were usually expressed only in the kidneys. There is a reciprocal relationship between the level of expression and the degree of methylation, that is, the higher the level of expression, the less the degree of methylation. We also observed that the expression of the integrated HBV-DNA was repressed by methylation following its passage through the female germline in one line. Thus, in addition to transacting factors that can control the gene expression positively or negatively, this tissue-specific methylation may also be involved in the regulation of HBV gene expression.

Key words: Hepatitis B virus — Transgenic mouse — Methylation

Eucaryotic DNA contains 5-methylcytosine, which occurs almost exclusively in the dinucleotide CpG.[1-3] Considerable evidence suggests that methylated cytosine residues in the regulatory region of both viral and eukaryotic genes reduce the gene transcription,[2,4,5] and that the methylation is a key developmental process, often invoked in models of cell differentiation,[6,7] X-dosage compensation,[8,9] and parental imprinting.[10-12] In addition, DNA methylation influences chromatin structure *in vivo*, suggesting that methylation may regulate transcription by altering protein-DNA interactions.[13]

Recently, Reik and Surani[14,15] suggested that imprinting could at times be involved in situations where this balance is altered, resulting in tumors, and that the genes that control imprinting in mammals may be intricately involved in crucial genetic processes such as reduced penetrance, variable expressivity, and the genetic predisposition to cancer. Therefore, investigations into the genetics of imprinting and methylation represent one of the major challenges of research.

So far, however, the molecular mechanisms of the methylation-dependent imprinting and regulation of gene expression are poorly understood. We can use random DNA insertions in transgenic mice to probe the genome for modified regions.[10-12] Transgenes integrated at different sites in the genome can show how the degree or pattern of methylation of the transgene is influenced by the genomic regions flanking the site of integration.

We produced several transgenic mouse lines using two constructs of hepatitis B virus (HBV) DNA. HBV DNA is a useful tool for the analysis of methylation because it tends to be methylated upon integration into mouse genome.[16-18] We previously showed that the methylation of HBV DNA is the main cause of gene inactivity by demonstrating that the demethylation by 5-azacytidine induced the expression of HBV surface antigen (HBsAg) gene.[19] In this paper, we further analyzed for the relationship between the level of expression and the degree of methylation of HBV transgene in seven lines of HBV transgenic mice. We here report that the tissue specificity and level of HBV expression were under the influence of the degree of methylation, and that in one line HBsAg gene was repressed following its passage through the female germ line.

MATERIALS AND METHODS

DNA The HBV genome used in our studies was derived from the plasmid pBRHBadr4.[20] The plasmid HB-GII was constructed by inserting a 2.6-kb *Bgl*II fragment from 3HB-neo[21] into the *Bam*HI site of pBR322. This fragment retains the putative promoter region for the 2.1-kb RNA, thus allowing the translation of HBsAg (see Fig. 1). The plasmid 1.2HB-BS[18] carries one full length of HBV (*Bam*HI fragment) plus a 619 bp overlapping region (*Bam*HI/*Stu*I fragment) (Fig. 1). This HBV fragment contains the minimum region necessary for transcription of all kinds of mRNA including 2.1-kb and 3.5-kb RNA[18,22] (Fig. 1). The plasmid HB-GII and 1.2HB-BS were digested with *Hin*dIII and *Sal*I and with

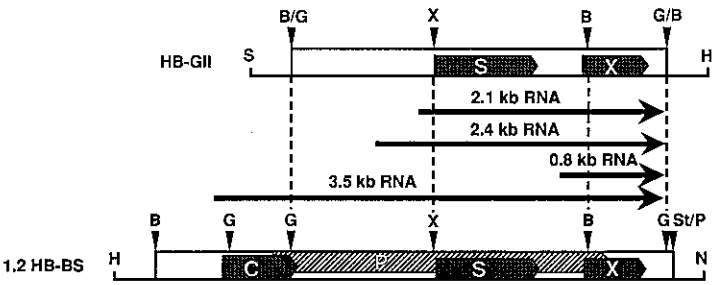

Fig. 1. The structures of HB-GII and 1.2HB-BS. The coding regions of HBcAg, HBsAg, HBx and polymerase are displayed with hatched arrows marked C, S and X, and a striped arrow marked P, respectively. The expected transcripts from these HBV fragments are represented by thin arrows marked 2.1-kb RNA, 2.4-kb RNA, 0.8-kb RNA and 3.5-kb RNA. B, G, H, N, S, St, P and X represent the sites of restriction endonucleases *Bam*HI, *Bgl*II, *Hin*dIII, *Nde*I, *Sal*I, *Stu*I, *Pvu*II and *Xho*I, respectively.

*Hin*dIII and *Nde*I, and the resulting 3.4-kb and 4.4-kb fragments, respectively, were isolated and used for microinjection into fertilized mouse eggs.

DNA injection C57BL/6 mice were used for production of transgenic mice. Several hundred molecules of each construct were microinjected into the pronucleus of fertilized eggs according to the method as described.[23]

Isolation of DNA and RNA Tissues were lysed with $NaDodSO_4$/Pronase E (Kaken Kagaku, Tokyo), and RNase A (Sigma). They were treated twice with phenol/chloroform (1:1 v/v), precipitated with isopropyl alcohol and dissolved in TE (10 m*M* Tris-HCl, pH 7.5/1 m*M* EDTA). Total RNA was prepared as described.[24]

Hybridization studies DNAs digested with appropriate restriction enzymes were subjected to electrophoresis in 1% agarose gel and transferred to nylon membranes (GeneScreen *Plus*) according to the manufacturer's recommendations. RNAs were subjected to electrophoresis in a 1% agarose gel containing 6.6% formaldehyde, then transferred to nylon membranes (GeneScreen *Plus*). Hybridizations were done under stringent conditions with a random-primed ^{32}P-labeled whole HBV DNA probe[25] prepared from pBRHBadr4.

Preparation of extract of tissues Samples were homogenized in extraction buffer (0.2% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride in phosphate-buffered saline) and centrifuged. The supernatants were used for enzyme immunoassay (Abbott).

RESULTS

Establishment of the transgenic mouse lines Ten and eight founder mice were obtained by the introduction of HB-GII and 1.2HB-BS, respectively. Table I shows the titers of HBsAg and HBV e antigen (HBeAg) in their sera. Among HB-GII lines, no. 26 was a high producer of HBsAg, nos. 5, 27 and 30 were low producers, and the rest did not have any detectable amount of HBsAg. Among 1.2HB-BS lines, no. 10 was a high producer for both HBsAg and HBeAg, and the others had no or very low titers of these antigens. We chose 7 lines, HB-GII 25, 26 and 27, and 1.2HB-BS 1, 2, 10 and 15, for the

Table I. Titers of HBsAg and HBeAg in the Sera of HB-GII and 1.2HB-BS Founder Mice and the Copy Number of HBV Transgene of These Mice

	HB-GII									
No.	5	6	25	26	27	30	37	40	42	45
sAg titer	28	<1	<1	754	17	15	<1	<1	<1	<1
Copy number	2–3	3–5	>10	2–3	2–3	3–5	>10	>20	>10	3–5
	1.2HB-BS									
No.	1	2	6	10	13	15	17	19		
sAg titer	<1	<1	<1	45	<1	<1	4.4	<1		
eAg titer	<1	<1	<1	20	<1	<1	1.8	<1		
Copy number	3–5	>10	7–8	1	>10	>10	3–5	5–7		

Titers were assayed by using an enzyme immunoassay kit (Abbott) and are expressed as the ratios against cut-off values (=A492 of negative control+0.050 for HBsAg and+0.06 for HBeAg). Values under 1.0 were considered negative, and are represented by <1. One ng of HBsAg per ml in a particle form is roughly equivalent to the value of 6. Quantitation between the titer and concentration of HBeAg has not been done. Values above 2.1 were considered positive. Copy number of HBV transgene was estimated from the result of Southern blot analysis.

subsequent studies. From the result of Southern blot analysis it was found that 1.2HB-BS 10 carried 1 copy of the transgene, HB-GII 26 and 27 carried 2–3 copies, 1.2-HB-BS 1 carried 3–5 copies, HB-GII 25, 1.2HB-BS 2 and 15 carried more than 10 copies (Table I), and that these multiple copies of transgene are in tandem array without any gross deletion or rearrangement (data not shown).

Parental-specific expression of HBV DNA in the HB-GII 26 line The integrated HBV DNAs of these 7 transgenic mice lines were transmitted to their offspring. In contrast to other transgenic lines, in HB-GII 26 line, the offspring from transgenic females did not express HBsAg in their sera (Fig. 2). In other words, HBsAg expression in this line was repressed following its passage through the female germ line. Such parental-specific expression had been reported by other groups,[10-12] and it is thought that methylation is involved in parental imprinting. Since it was expected that this repression was due to methylation of HBV DNA in this line, these repressed HB-GII 26 mice, HB-GII 26(−), were also used for further analyses.

Tissue specificity of HBV expression in transgenic mice Total RNAs prepared from liver, kidney, testis and brain of these transgenic mice were subjected to RNA blot analysis (Fig. 3). In the case of HB-GII construct, three kinds of mRNA, that is, 2.4-kb, 2.1-kb and 0.8-kb mRNAs can be transcribed from HB-GII fragment (see Fig. 1). In the HB-GII 26 mouse, which was a high producer of HBsAg, the 2.1-kb RNA was detected in the liver and the kidneys, and the 0.8-kb RNA that may be the transcript for X protein[18] was detected in the testis. We also examine for HBsAg titer of the tissue extracts. As shown in Fig. 3, the liver and the kidneys, in which the 2.1-kb RNA was detected, showed high HBsAg titer. These results represent liver- and kidney-specific expression in HB-GII 26 line. In the case of HB-GII 27 mouse, which was a low HBsAg producer, no HBV-RNA was detected in the liver, and instead two RNAs of about 3.5 kb and 2.1 kb in size were detected in the kidneys and the brain. The latter may be the transcript for HBsAg, because its translation product, HBsAg, was detected in the serum. But the identity of the former is not clear, and the transcription may start at flanking cellular DNA or an abnormal site of HBV DNA. In the testis, there was

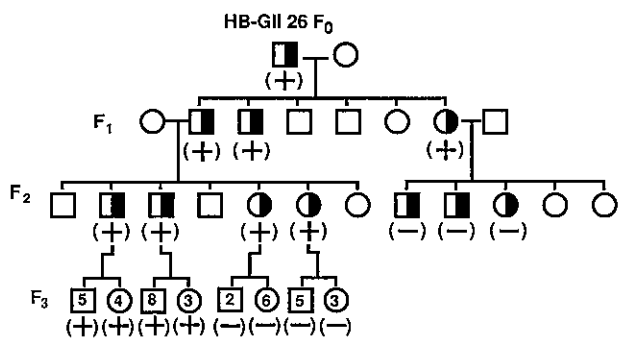

Fig. 2. Pedigree chart of strain HB-GII 26. The founder male was mated with a C57BL/6 female to produce offspring. The mice were tested for HBV DNA by Southern blot hybridization. Open symbols indicate non-transgenic individuals, and half-filled symbols indicate individuals that are hemizygous for the transgene. Serum HBsAg was measured with the AUSZYME II (Abbott), and the mice which had a high titer for HBsAg are represented as (+). Four F_2 mice were mated with normal C57BL/6 mice, and obtained transgenic offspring were tested for serum HBsAg. The numbers in the F_3 generation represent the number of offspring carrying the transgene. The offspring from males were all positive for HBsAg, but the offspring from females were all negative.

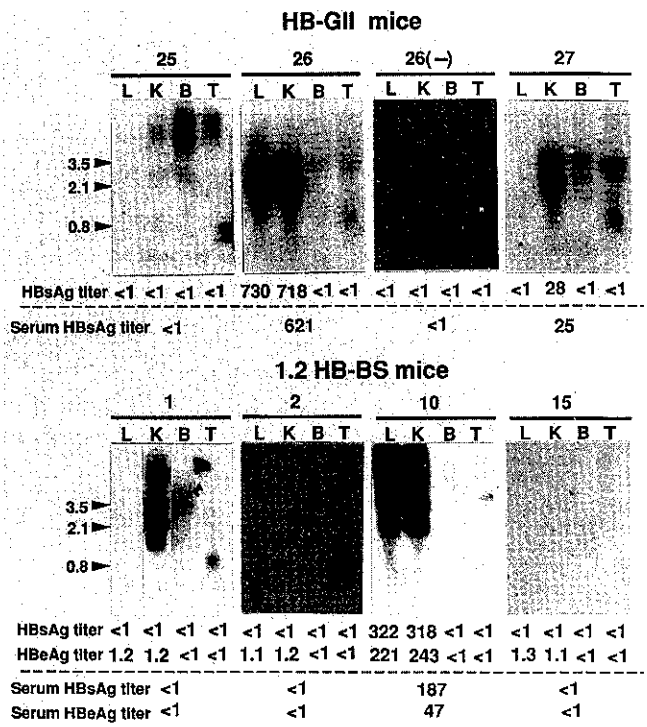

Fig. 3. RNA blot analysis and titers for HBsAg and HBeAg in the tissues. Total RNA (10 μg) prepared from the mice of HB-GII 25, 26, 26(−), 27, 1.2HB-BS 1, 2, 10 and 15 was subjected to electrophoresis followed by transfer to a nylon membrane and hybridization with ^{32}P-labeled HBV DNA probe. Extracts of tissues of transgenic mice were prepared and assayed for HBsAg and HBeAg titer by using an enzyme immunoassay kit (Abbott). Titers were expressed in the same way as in Table I, and are shown under each lane. Serum HBsAg and HBeAg were also assayed, and the results are indicated below tissue titer. L, liver; K, kidney; B, brain; T, testis.

an additional unknown RNA of about 3 kb in size beside the 0.8-kb RNA for X protein. When the HBsAg titers of these tissue extracts were examined, the kidneys showed only very low titer, and the other tissues, including the brain and the testis, had no titer. Compared with the band intensity of RNA blot analysis, this HBsAg expression level in the kidney was very low, indicating that only a small fraction of these RNAs are correct transcripts. In the HB-GII 25, only smear RNAs were observed, suggesting that most of these RNAs are read-through. This pattern of RNA production is quite similar to that of p3HB transgenic mice, which we reported previously.[16] No RNAs were detected in any tissues in the HB-GII 26(−). The 2.4-kb RNA was not clear in this analysis.

The pattern of RNA production in 1.2HB-BS mice was similar to that of HB-GII mice. In 1.2HB-BS 10, the expected RNAs, 3.5-kb and 2.1 kb in size (see Fig. 1), were expressed in the liver and the kidneys, and the 0.8-kb RNA was detected in these tissues as well as in the testis. High titers of HBsAg and HBeAg were detected in tissue extracts of the liver and the kidneys, in the same way as with HB-GII 26. In 1.2HB-BS 1 mice, HBV RNAs of unusual sizes were detected but without any production of HBV-related antigens in their sera. Interestingly, HBV RNAs of various sizes were observed in the kidneys, brain and testis, as with HB-GII 27. As no HBV-related protein was detected in extracts of these tissues, all these RNAs except for the 0.8-kb RNA in the testis were aberrant ones. The 1.2HB-BS 2 mouse expressed only 0.8-kb RNA in the testis, and in the 1.2-HB-BS 15 mouse, no RNA was detected in any of these tissues. We could not detect any HBV RNAs in other tissues including spleen, heart, lung, intestine, thymus and muscle in any of the mice (data not shown).

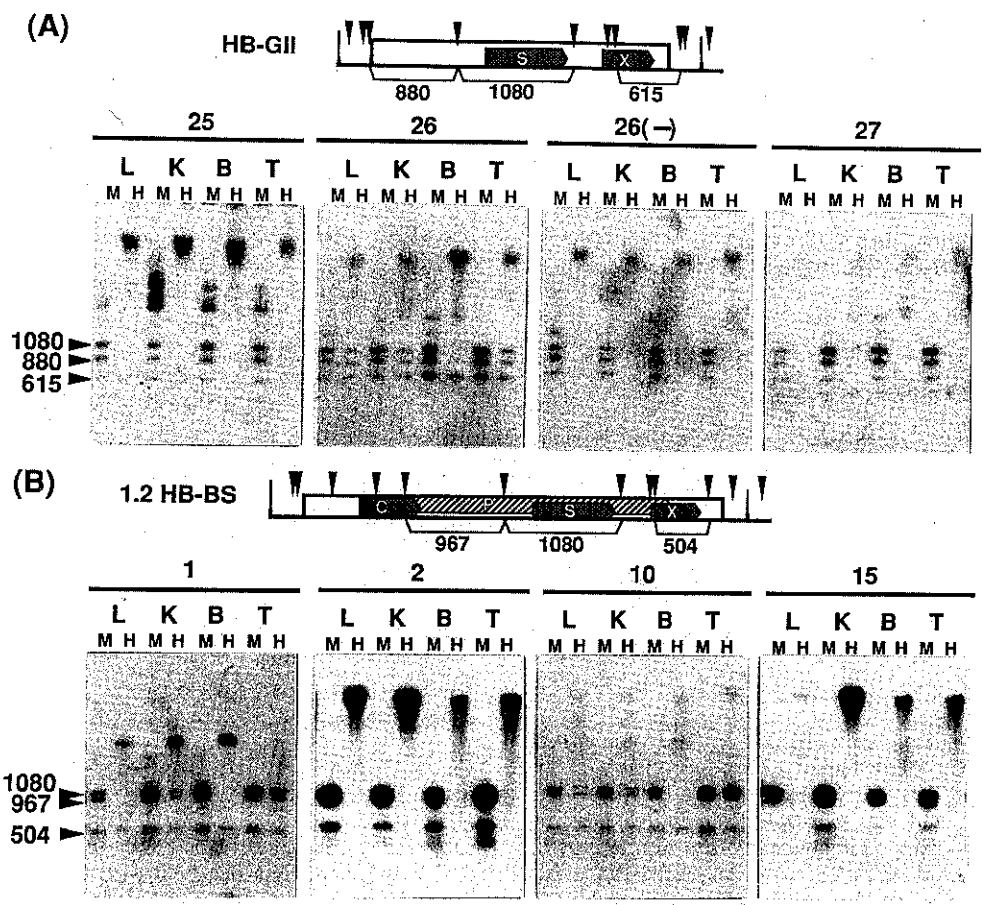

Fig. 4. Methylation of HBV DNA in the HB-GII mice (A) and the 1.2HB-BS mice (B). The thick lines on the maps indicate HBV DNA. The arrows represent the action sites of restriction endonucleases *Hpa*II and *Msp*I (CCGG). In HB-GII, the three major expected fragments were 880, 1080 and 615 bp. In 1.2HB-BS, they were 967, 1080 and 504 bp. The DNAs from liver (L), kidneys (K), brain (B) and testis (T) were digested with *Msp*I (M) or *Hpa*II (H) followed by Southern blot analysis with ^{32}P-labeled HBV DNA probe.

All these results suggest that the expression of HBV authentic RNAs is liver- and kidney-specific. This expression pattern seems to represent HBV tissue-specificity.

Methylation of HBV DNA To examine whether the tissue specificity and the level of expression are correlated with the degree of methylation, we prepared DNAs from liver, kidney, brain and testis. These DNAs were digested with *Hpa*II or *Msp*I, followed by Southern blot analysis.

As shown in Fig. 4(A), when HB-GII were digested with *Msp*I, three main fragments (1080 bp, 880 bp and 615 bp) were detected in all DNA samples (lane M). Then, the DNAs were digested with methylation-sensitive restriction enzyme, *Hpa*II. Only the DNAs of the liver, the kidneys and the testis from HB-GII 26 were digested to some extent to produce 1080 bp, 880 bp and 615 bp bands, and these undermethylated tissues corresponded to the tissues in which HBV RNAs are expressed. In HB-GII 26(−), all DNAs were heavily methylated. From these results, we could conclude that the maternal inhibition of this line is accompanied by the methylation of the integrated HBV DNA, leading to the complete inhibition of HBV DNA expression. In HB-GII 25, all the DNAs were methylated. This may be the reason for the production of smear RNA in the kidneys, the brain and the testis. In HB-GII 27, most DNAs were methylated. However, faint bands of 615 bp or smaller were detected in the kidneys and the brain, suggesting that HBV DNA is not methylated in a small number of cells, leading to the production of HBsAg in the kidneys.

Fig. 4(B) shows the results for 1.2HB-BS line. By digestion of 1.2HB-BS with *Msp*I (lane M), three fragments (1080, 967 and 504 bp) were mainly generated. In 1.2HB-BS 10 DNAs from the liver, the kidneys and the testis were digested with *Hpa*II to a considerable degree as judged from the hybridizing intensity of the three main fragments (1080, 967 and 504 bp). However, DNA from the brain was not digested with *Hpa*II except the 504 bp band. In 1.2HB-BS 1, DNAs were also digested with *Hpa*II but to a lesser extent as compared with 1.2HB-BS 10. The degree of methylation is less in the kidneys and the testis, where clear but abnormal RNA bands are present, than in the brain and the liver. In spite of no expression, the DNAs from the brains of HB-GII 26, 1.2HB-BS 1 and 1.2HB-BS 10 showed one band 615 bp or 504 bp. These fragments come from the 3′ region of these transgenes (see Fig. 4), so it is expected that the undermethylation of this region does not affect the expression. In contrast to 1.2HB-BS 1 and 10, DNAs from 1.2HB-BS 2 and 15 were methylated almost completely. As described before, no expression was observed in any of the tissues from these two lines except the testis of 1.2-HB-BS 2, where the 0.8-kb RNA was detected. We also examined for the methylation of HBV DNA from spleen, heart, lung, muscle and ovary in 1.2HB-BS 10. All these HBV DNAs were methylated (data not shown). These results suggest that there is a reciprocal relationship between the level of correct expression and the degree of methylation, that is, the higher the level of expression, the less the degree of methylation. It is also clear that other factors are involved in the tissue-specific expression of HBV DNA, because no other type of RNA except 0.8-kb RNA is expressed in the testis. Taken together, the results suggest that the tissue-specificity of HBV expression may be partly regulated by the methylation of DNA.

DISCUSSION

Recent molecular biological studies have shown that tissue specificity and developmental specificity of gene expression can be directed, in principle, by two factors. One is the *cis*-acting DNA element(s) which is usually located in the 5′ region of the gene, but may be either in the intron or the 3′ region. The other is the transacting factor(s) which can bind to specific DNA sequences to activate or suppress the transcription of the gene. The third mechanism involved in the control of gene expression may be the methylation of DNA. Although accumulating data indicate the involvement of methylation in gene regulation, it is still controversial whether the methylation plays an important role or not.

On the other hand, the transgenic mouse has been shown to be a useful system for the analysis of tissue- and development-specific gene expression, because the introduced gene is located at the same chromosomal site of all types of cell. Furthermore, in our previous studies[16] we showed that HBV DNA is easily methylated upon integration into mouse chromosome, leading to the suppression of gene expression. This was confirmed in this experiment by showing that only two out of 18 mice were high producers of HBV-related antigens. Based on these results, we consider that the transgenic mouse is a unique system for evaluating the role of DNA methylation in the control of gene expression. In this work, we examined the correlation between the degree of methylation and the control of transgene expression, such as tissue-specificity and level of expression.

Tissue-specific expression of HBV-DNA has been shown by Burk *et al.*[26] and ourselves[16] using the transgenic mouse system and was considered to be due to the presence of specific sequences such as the enhancer element and the glucocorticoid-responsive element. In this paper, we confirmed that the HBV DNA was expessed in a liver- and kidney-specific manner, and that in the high-producer mice the transgene was undermethylated in the tissues in which HBV RNAs were expressed. The levels of expression were better associated with undermethylation in the 5′ region of HBV DNA, core coding region, S promoter region and enhancer sequence[27, 28]

(880 or 967 bp and 1080 bp fragment; see Fig. 4) than with that in the 3′ end of the transgene. Taken together, the results suggest that the level of expression is reciprocally related with the degree of methylation in the 5′ part of HBV DNA although not all cells were undermethylated even in liver and kidney. We expect that the level of expression depends on the number of cells in which HBV DNA is undermethylated. All these results indicate that tissue-specific methylation of HBV DNA is one of the factors which control the tissue-specificity of HBV expression. The same mechanism may be applicable to other DNAs, such as retrovirus or vector sequences, because similar *de novo* methylation was reported in these DNAs.[29, 30] Further study will be needed for the elucidation of the mechanism of tissue-specific methylation.

In the mouse, both maternal and paternal sets of chromosomes are necessary for complete and undisturbed development, because of differential genome imprinting during male and female gametogenesis.[31–35] A genetic experiment using chromosomally balanced mice carrying a pair of homologous chromosomes derived from only one parent, i.e. maternal or paternal disomy, has allowed the assignment of imprinted and non-imprinted regions of the mouse genome.[36] Furthermore, several groups have observed in transgenic mice that DNA methylation and expression of transgenes depends on transmission from mother or father.[10-12] This suggests that methylation could provide a molecular mechanism by which homologous chromosomes can be imprinted according to their parental origin.

In HB-GII 26 line, methylation of the transgene was acquired by its passage through the female parent. It is interesting that the methylation by imprinting covered all regions of the transgene, whereas tissue-specific methylation was mainly located on the 5′-region. This suggests that the mechanism of methylation is different between imprinting and regulation of gene expression. The former seems to involve a wider region.

Although our data only concern HBV DNA in transgenic mice, the same event, that is, methylation, may occur in HBV DNA which was integrated into the chromosome of human liver cells[37] during the course of infection. Then, the HBV sequence could cause the adjacent DNA to be methylated irreversibly, as demonstrated by Jähner *et al.*,[29] who found that insertion of a provirus sequence resulted in methylation of the flanking genomic sequence to as far as 1 kb from the integration site. The pathophysiological consequences of DNA methylation are not known yet, but it is possible that the methylation of integrated HBV DNA together with the flanking genomic sequence results in a change of local structure, such as the inactivation of an anti-oncogene, leading to an increase of the risk of developing tumors.

The elucidation of the mechanism and effects of the methylation of inserted HBV DNA will provide useful information about HBV-related liver diseases, such as hepatocellular carcinoma.

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