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Demethylation by 5-Azacytidine Results in the Expression of Hepatitis B Virus Surface Antigen in Transgenic Mice

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In 14p3HB transgenic mice, which carry three tandem copies of hepatitis B virus (HBV) DNA, the HBV DNA was significantly methylated and no viral proteins were produced. To analyze the causal relationship between hypermethylation and gene inactivity, 5-azacytidine was injected into the mice to demethylate HBV DNA. When postnatal 14p3HB mice were treated with the drug, hepatitis virus surface antigen was produced in these mice by 3 weeks of age, and the integrated HBV DNA of the liver was less heavily methylated. Our results suggest that injection of 5-azacytidine can be used to efficiently activate a silent transgene such as HBV DNA in transgenic mice.

Key words: Hepatitis B virus — Transgenic mice — 5-Azacytidine — Methylation

Hepatitis B virus (HBV) infection is a serious worldwide health problem that is pathologically linked to viral hepatitis and hepatocellular carcinoma (HCC).[1,2] Beasly *et al.*[2] showed from prospective epidemiological studies that the relative risk to hepatitis B surface antigen (HBsAg) carriers of developing HCC was 217 times as compared with noncarriers. Despite the crucial role of HBV in human health problems, there is only limited knowledge of the molecular mechanism of HCC development because the virus multiplies only in human and chimpanzee livers. Although cell culture systems have been established that allow expression and replication of the HBV genome following transfection with cloned HBV DNA,[3-6] they are not suitable for studies on the development of hepatitis and HCC.

One approach to overcoming this problem is to make a transgenic animal carrying HBV DNA. So far, three groups have demonstrated successful expression of HBsAg in transgenic mice after introduction of HBV DNA.[7-9] In a previous study,[10] we produced transgenic mice by injecting HBV DNA containing three tandem repeats (p3HB). In no. 14 founder mouse, 14p3HB, three copies of p3HB were integrated into the mouse Y chromosome in a head-to-tail array (Fig. 1). However, the HBV DNA was heavily methylated, aberrant RNA was produced in various tissues except liver, and no HBV-related antigens were detected in the sera.[10]

To analyze whether the methylation of HBV DNA is the cause of the absence of expression, we attempted to demethylate the HBV DNA by treating the transgenic mice with 5-azacytidine (5AZ), which is a specific inhibitor of eukaryotic methylases.[11] The 14p3HB transgenic mice were treated with 5AZ according to the method of Jaenisch *et al.*,[12] who showed that the treatment of mice with 5AZ resulted in transcriptional activation of silent proviruses. Postnatal mice were treated with three drug injections, the first injection being administered at 5 days of age. Surviving animals were reinjected at weekly intervals. The amount of 5AZ was 25 μg for the first and 50 μg for subsequent injections. HBsAg and HBeAg in sera were assayed by an enzyme immunoassay kit (Abbot). The 14p3HB mice that were injected three times become HBsAg-positive in their sera by 3 weeks of age (Table I). The amount of HBsAg ranged 3 to 6 ng/ml.

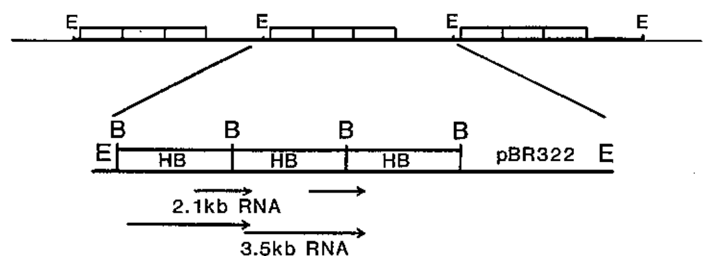

Fig. 1. Integration pattern of HBV-DNA fragment in 14p3HB transgenic mice. Three p3HB fragments (*Eco*RI fragment) were integrated in a head-to-tail array. Thin arrows represent the transcripts of HBV, 2.1-kb and 3.5-kb RNA.[15] Open boxes marked HB represent one copy of the HBV genome. B, *Bam*HI; E, *Eco*RI.

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Table I. Titers for HBsAg and HBeAg in the Sera of 14p3HB F_3 Mice Treated with 5AZ Three Times

Mice	1	3	4	Normal
HBsAg (A_{420})	0.688	1.044	1.372	0.040
HBeAg (A_{420})	not done	0.196	0.188	0.156

Titers were assayed by enzyme immunoassay kit (Abbot). Values above 0.05 and 0.21 were considered positive for HBsAg and HBeAg, respectively.

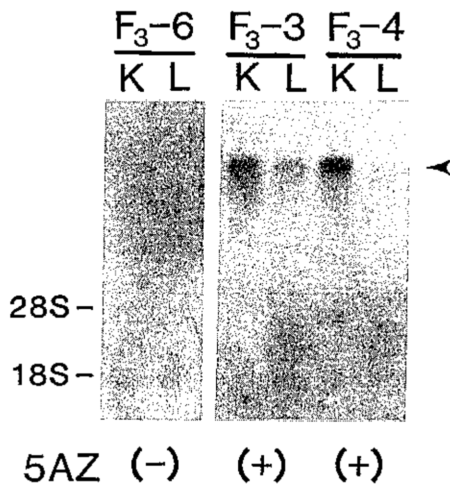

Fig. 2. RNA blot analysis. Total RNA (10 μg) prepared from 5AZ-treated (+) or non-treated (−) 14p3HB F_3 mice was subjected to electrophoresis followed by transfer to a nylon membrane and hybridization with ^{32}P-labeled HBV DNA probe. L, liver; K, kidney.

To analyze the transcripts of HBV DNA, total RNAs of the livers and kidneys of two 5AZ-treated 14p3HB F_3 mice (F_3-3 and F_3-4) and a non-treated 14p3HB F_3 mouse (F_3-6) were prepared as described.[13] RNAs were subjected to electrophoresis in a 1% agarose gel containing 6.6% formaldehyde and then transferred to nylon membranes (GeneScreen Plus). Hybridizations were done under stringent conditions with a random-primed ^{32}P-labeled whole HBV DNA probe.[14] As shown in Fig. 2, about 10-kb RNAs were detected in the tissues of 5AZ-treated mice. As the authentic HBV transcripts are 2.1 kb and 3.5 kb in size[15] (see Fig. 1), this 10-kb RNA is fairly long. As only HBsAg is detected in sera, this unusually long RNA can be translated to produce HBsAg but not HBeAg. These results suggest that these RNAs are transcribed from the initiation site of 2.1-kb RNA, but did not terminate at the right position.

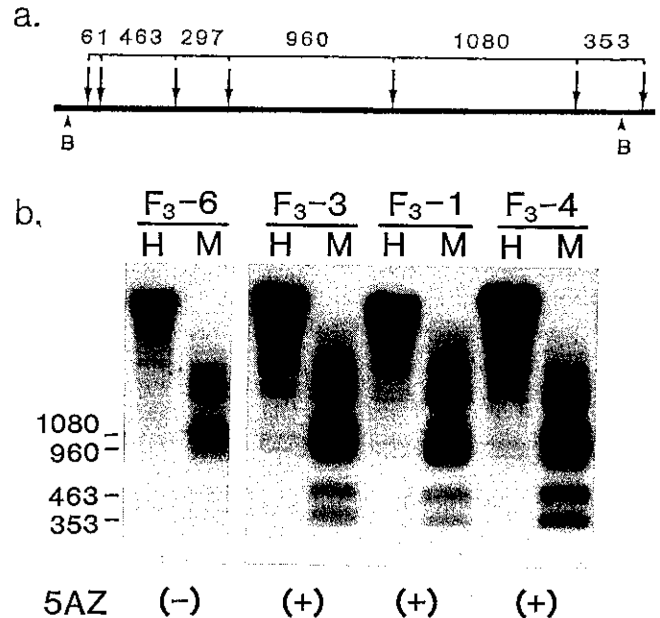

Fig. 3. Methylation of HBV DNA in 5AZ-treated (+) or non-treated (−) 14p3HB F_3 mice. a, The map of HBV DNA. Arrows represent the action sites of restriction endonucleases *Hpa*II and *Msp*I. b, Southern blot analysis[16] of the liver DNAs. M, *Msp*I; H, *Hpa*II; B, *Bam*HI.

To study the degree of methylation of HBV DNA in 14p3HB mice after treatment with 5AZ, the liver DNAs of the mice were digested with *Msp*I or *Hpa*II (recognizing CCGG sequence) followed by Southern blot analysis.[16] Six CCGG sequences were recognized in HBV DNA (Fig. 3a), leading to the production of six fragments when p3HB was digested with appropriate restriction enzymes such as *Msp*I. When the DNAs were digested with *Msp*I, the expected fragments (1080 bp, 960 bp, 463 bp and 353 bp) were detected (Fig. 3b). Then, the DNAs were digested with methylation-sensitive restriction enzyme, *Hpa*II. Although most of the HBV DNA was not digested, some digested fragments could be detected, whereas no digested fragments could be detected before 5AZ treatment (Fig. 3b). This result suggests that the HBV DNA integrated in chromosomes had been partially demethylated by 5AZ.

Our results clearly demonstrated that the treatment with 5AZ inhibited the methylation of HBV DNA, leading to the production of HBsAg in transgenic mice.

The importance of DNA methylation for gene expression is still controversial. Restriction enzyme analyses have demonstrated that the methylation pattern of specific genes changes during development and that hypomethylation correlates with activation of some[17-20] but not other[21-24] genes. However recent findings on genomic

imprinting using transgenic mouse systems have provided strong evidence that DNA methylation is the cause of heritable molecular differences between maternally and paternally derived alleles on mouse chromosome.[25–28]

De novo methylation of an introduced gene in transgenic mice is often observed in the cases of HBV DNA[3] and retrovirus,[29] but is less frequent in introduced mammalian genes. Actually in another of our experiments in which HBV DNA was also introduced, only one out of eight transgenic mice expressed HBV antigens efficiently.[30] On the other hand, in other transgenic mouse lines in which mammalian gene was introduced, about 80% of them usually expressed at high efficiency. These results suggest that HBV DNA has a DNA sequence which is easily methylated after integration.

Jaenisch *et al.*[12] showed that 5AZ treatment can demethylate and activate retroviral genomes in mice infected at an early stage of development. We also observed the expression of HBV DNA after demethylation by 5AZ, treatment suggesting that methylation of the introduced gene is one of the main causes of inhibition of expression in transgenic mice. As a matter of fact, Townes *et al.* clearly demonstrated that the removal of a plasmid sequence which could be a target for methylation resulted in high and appropriate expression of an introduced gene.[31]

Our transgenic mice treated with 5AZ produce HBsAg after birth. This situation is similar to the case of human HBsAg carriers. Thus, an immune response would be provoked in these transgenic mice during later development.

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