Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5628–5632, August 1990
Medical Sciences

Trans-activation function of a 3′ truncated *X* gene–cell fusion product from integrated hepatitis B virus DNA in chronic hepatitis tissues

(chronic infection/integration/virus–cell fusion transcript/X protein/hepatocarcinogenesis)

SHINAKO TAKADA AND KATSURO KOIKE*

Department of Gene Research, Cancer Institute, Kami-Ikebukuro Toshima-ku, Tokyo 170, Japan

Communicated by Susumu Ohno, April 30, 1990

ABSTRACT To investigate the expression and trans-activation function of the *X* gene in integrated hepatitis B virus (HBV) DNA from chronic hepatitis tissues, a series of transfectants containing cloned integrated HBV DNAs was made and analyzed for X mRNA expression and trans-activation activity by using a chloramphenicol acetyltransferase assay. Most of the integrated HBV DNAs expressed X mRNA and encoded a product with trans-activation activity in spite of the loss of the 3′ end region of the *X* gene due to integration. From cDNA cloning and sequence analysis of X mRNA transcribed from native or integrated HBV DNA, the X protein was found to be translated from the *X* open reading frame without splicing. For integrated HBV DNA, transcription was extended to a cellular flanking DNA and an *X* gene–cell fusion transcript was terminated by using a cellular poly(A) signal. The amino acid sequence deduced from an X–cell fusion transcript indicated truncation of the carboxyl-terminal five amino acids, but the upstream region of seven amino acids conserved among hepadnaviruses was retained in the integrated HBV DNA, suggesting that this conserved region is essential for the trans-activation function of the X protein. These findings support the following explanation for hepatocarcinogenesis by HBV DNA integration: the expression of a cellular oncogene(s) is trans-activated at the time of chronic infection by the increasing amounts of the integrated HBV gene product(s), such as the X–cell fusion product.

Integration of hepatitis B virus (HBV) DNA into the cellular DNA of hepatocellular carcinoma and chronic hepatitis tissues is frequently found (1–3) and may be essential to HBV-related hepatocarcinogenesis. A detailed examination of chronic hepatitis samples (3) indicated that random integration and rearrangement of HBV DNA and cellular flanking DNA occurred early in viral infection. This rearrangement apparently depends on the individual characteristics or conditions of the host or viral replication, irrespective of hepatocellular carcinoma or chronic hepatitis. In fact, some hepatocellular carcinomas exclusively contain one or more colinear integrants in which rearrangement has not occurred (4, 5). Thus structural rearrangement would not appear to be essential to hepatocarcinogenesis in many cases. Integration of HBV DNA into particular sites of cellular DNA has also been found to result in activation of a cellular gene (6). However, such site-specific integration is not applicable to most cases, since HBV DNA integrates into cellular DNA at random (2, 3, 7).

One mechanism for hepatocarcinogenesis that appears particularly reasonable is trans-activation or trans-repression of cellular gene(s) or factor(s) by HBV-related gene product(s) encoded by the integrated HBV DNA. The *X* gene product was found to possess the ability to trans-activate homologous and heterologous transcriptional regulatory sequences of viruses and various cellular genes (8–11). This trans-activation of cellular gene expression may be essential to hepatocarcinogenesis. Most integrated HBV DNAs retain the major part of the *X* gene as well as the surface antigen (*S*) gene (2, 3, 7), and thus whether integrated HBV DNAs encode products that have a trans-activation function is a matter of considerable importance. Wollersheim *et al.* (12) found that the integrant from hepatocellular carcinoma tissue encoded a product with trans-activation activity. To assess whether integrated HBV DNAs not only from hepatocellular carcinoma but also from chronic hepatitis tissues possess this activity, many integrated HBV DNAs from chronic hepatitis tissues were examined for this feature as well as for RNA expression. A DNA transfection assay indicated many integrants expressed the *X* gene and had trans-activation activity. Moreover, cDNA cloning and X mRNA sequence analysis provided strong evidence that *X* gene expression terminated at a cellular poly(A) signal so that the trans-activation function was preserved in the integrated HBV DNA in spite of truncation of the carboxyl-terminal region of the *X* open reading frame (ORF). A general mechanism for HBV-related hepatocarcinogenesis by HBV integration is discussed based on the trans-activation function conserved in the X–cell fusion product.

MATERIALS AND METHODS

Cell Lines and DNA Transfection. The HuH-7 cell line was derived from a human hepatocellular carcinoma (13) and the HepG2 cell line was from a hepatoblastoma (14). Both cell lines are negative for HBV DNA. The cells were maintained in DM-160AU medium (Kyokuto) supplemented with 10% (vol/vol) fetal calf serum and kanamycin (60 μg/ml).

For the transient expression assay, DNA transfection was carried out by calcium phosphate precipitation (15). The cells were plated at 4–7 × 10^6 per 10-cm dish, cultured for 24 hr before transfection, and then incubated with DNA precipitates for 6 hr at 37°C. Cloned integrated HBV DNA (30–40 μg per 10-cm dish) digested with *Hin*dIII or *Sal* I, to separate it from λ phage vector DNA, was used for transfection. Integrated HBV DNA clones N2-1 to N2-11, N1-42, and T4-171 to T4-182 for transfection were from chronic active hepatitis tissue N2, chronic active hepatitis tissue N1, and chronic persistent hepatitis tissue T4, respectively (3).

Northern Blot Analysis. Total RNA of HuH-7 cells 48 hr after transfection was extracted by the guanidinium/cesium chloride method (16). After electrophoresis in a formaldehyde/agarose gel, RNA was transferred to a nitrocellulose

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; CAT, chloramphenicol acetyltransferase; ORF, open reading frame; nt, nucleotide(s).
*To whom reprint requests should be addressed.

filter (17). The ^{32}P-labeled hybridization probe from the HBV DNA fragment was made by nick-translation (18).

Chloramphenicol Acetyltransferase (CAT) Assay. HepG2 cells were cotransfected with the cloned integrated forms of HBV DNA (35 μg) and pSV2CAT DNA (5 μg). After 48 hr, cell extracts were prepared as described by Gorman *et al.* (19) and subjected to the CAT assay using [^{14}C]chloramphenicol as the substrate (DuPont/NEN). Chloramphenicol and its acetylated derivatives were separated by ascending thin-layer chromatography on a silica-gel plate [$CHCl_3$/methanol, 95:5 (vol/vol)] and visualized by autoradiography.

cDNA Cloning of Native X mRNA and X–Cell Fusion Transcript. To obtain the cDNA clone of native X mRNA, pHBVX-1 DNA containing a 0.87-kilobase (kb) *Stu* I–*Bgl* II [nucleotides (nt) 988–1860] fragment (10) was transfected into HuH-7 cells. For cDNA cloning of the X-cell fusion transcript expressed from the integrated form of HBV DNA, clone N2-7 was selected from 14 integrated HBV DNAs and digested with *Xba* I to inactivate the *S* gene prior to transfection. After 48 hr, total RNA was extracted and poly(A) RNA was isolated by oligo(dT)-cellulose column chromatography. cDNA was synthesized by the procedure of Gubler and Hoffman (20). The resultant double-stranded DNA was ligated with an *Eco*RI adaptor and then with λgt10 arms. After *in vitro* packaging, the recombinant library was screened with the X probe.

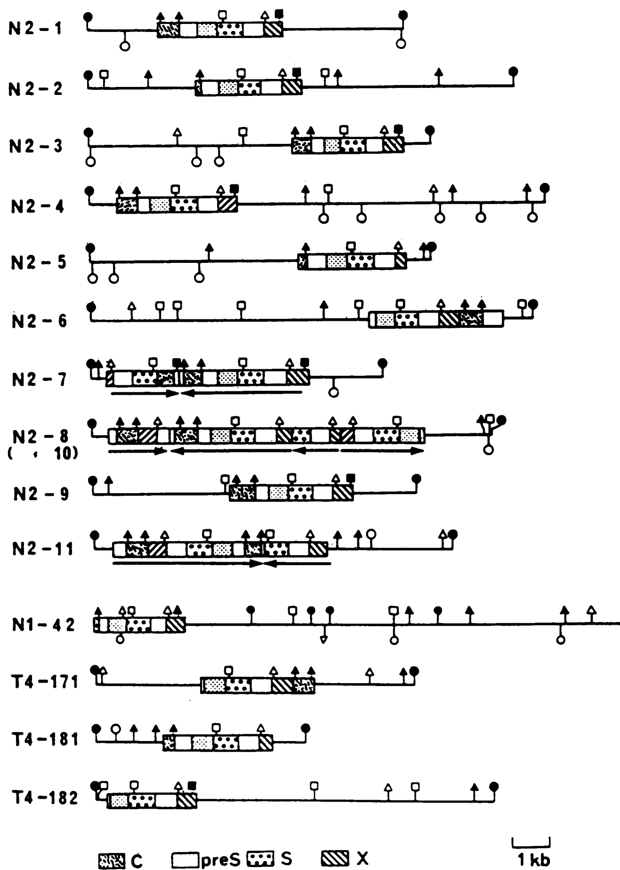

FIG. 1. Restriction map and genetic organization of integrated HBV DNA cloned from chronic hepatitis tissues. N2-1 to N2-11 were cloned from chronic active hepatitis tissue N2. N1-42 was from chronic active hepatitis tissue N1. T4-171 to T4-182 were from chronic persistent hepatitis tissue T4 (3). C, preS, S, and X represent the *C* (hepatitis B core antigen) gene, preS region, *S* (hepatitis B surface antigen) gene, and *X* gene, respectively. Boxed region, integrated HBV DNA; solid line, cellular flanking DNA; arrows, rearrangement of HBV DNA according to the direction of the viral minus strand.

DNA sequencing was conducted either by the chain-termination method (21) or by the chemical-modification method (22).

RESULTS

Expression of Integrated HBV DNA Cloned from Chronic Hepatitis Tissues. To assess the expression of chromosomally integrated HBV DNAs, 14 integrated HBV DNAs cloned from chronic hepatitis tissues (clones N2-1 to 11, N1-42, T4-171 to 182; Fig. 1), as described (3), were studied by a transient expression assay using the human hepatoma cell line HuH-7 as host (23). Total RNA was extracted 48 hr after DNA transfection and analyzed by Northern blot hybridization. Since the 3′ end region of the S mRNA overlaps that of the X mRNA, S and X mRNAs were distinguished by using two probes, the 0.58-kb *Bam*HI–*Bgl* II (nt 1276–1860) *X* gene region fragment for X mRNA and the 0.82-kb *Taq* I (nt 3–823) *S* gene region fragment for S mRNA. S mRNA was detected with both the X and S probes, but X mRNA was detected only with the X probe.

As shown in Fig. 2 and Table 1, all 14 integrated HBV DNAs expressed a significant amount of S mRNA, but only 12 integrated HBV DNAs expressed detectable levels of X mRNA. The level of expression in different integrants was different. mRNA size was somewhat variable; clones with a viral poly(A) signal (for example, N2-6) produced a 0.9-kb X mRNA and a 2.2-kb S mRNA, and clones without the viral poly(A) signal (such as N2-7) produced HBV mRNA with at least the native size. The size difference between S and X

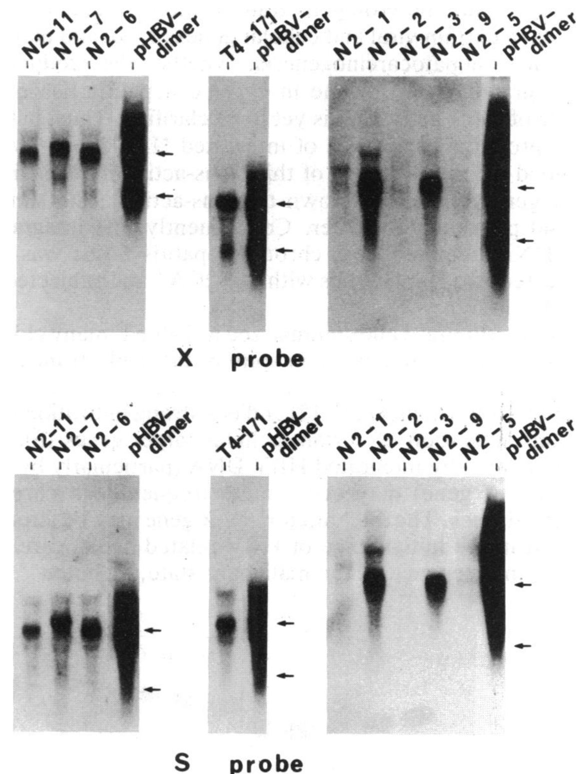

FIG. 2. Northern blot analysis of transcripts from integrated HBV DNA. Total RNA (20 μg) extracted from HuH-7 cells transfected with various integrated HBV DNAs was subjected to Northern blot analysis. (*Upper* and *Lower*) Hybridization patterns with the X probe (0.58-kb *Bam*HI–*Bgl* II fragment) and S probe (0.82-kb *Taq* I fragment), respectively. RNA from pHBV-dimer-transfected HuH-7 cells was used as a size marker and control for hybridization. The upper and lower arrows indicate the positions of 2.2-kb S mRNA and 0.9-kb X mRNA, respectively. The clones of integrated HBV DNA used in this experiment are summarized in Fig. 1.

Table 1. Expression and trans-activation function of integrated HBV DNAs from chronic hepatitis tissues

Integrated HBV DNA	Expression		Trans-activation
	S mRNA	X mRNA	
N2-1	+	vw	+
N2-2	+	+	+
N2-3	vw	–	–
N2-4	+	vw	+
N2-5	vw	–	–
N2-6	+	+	+
N2-7	+	+	+
N2-8(10)	+	+	+
N2-9	+	+	+
N2-11	+	+	–
N1-42	+	+	–
T4-171	+	+	+
T4-181	+	vw	+
T4-182	+	+	+

vw, Very weak; +, clearly detected; –, not detected.

mRNAs was constant at about 1.3 kb, suggesting that both S and X mRNAs start at different but fixed sites and terminate at the same poly(A) signal on the viral or cellular sequence despite structural differences among the 12 integrated forms. Thus the structural rearrangements of HBV DNA or cellular flanking DNA are not directly correlated with the expression of integrated HBV DNA.

Trans-Activation Function of Integrated HBV DNA. The *X* gene product possesses trans-activating activities toward homologous and heterologous transcriptional regulatory sequences of certain viral and cellular genes (8–10) and may be essential to hepatocarcinogenesis. Whether the product of the integrated HBV *X* gene in chronic hepatitis tissues is capable of trans-activation is yet to be clarified. Thus, in this study, products of a series of integrated HBV DNAs were examined for the presence of this trans-activation function. The *X* gene product is known to trans-activate the simian virus 40 promoter/enhancer. Consequently, the integrated HBV DNA obtained from chronic hepatitis tissue was cotransfected into HepG2 cells with pSV2CAT and subjected to the CAT assay.

As shown in Fig. 3 and summarized in Table 1, many clones had trans-activation activity in spite of the slight truncation at the 3′ end of the *X* ORF. This is consistent with observations by Wollersheim *et al.* (12) and is a strong indication that the trans-activation of certain cellular target genes (for instance, c-*myc*) by integrated HBV DNA (particularly by the integrated *X* gene) may occur quite frequently in chronic hepatitis tissues. The integrated HBV *X* gene may be directly involved in the initial stage of HBV-related hepatocarcinogenesis, maintenance of the malignant state, or both.

Structure of X mRNA Expressed from Native or Integrated HBV DNA. Due to the loss of the intrinsic viral poly(A) signal by integration, the generation of HBV-related mRNA must depend on a poly(A) signal from the cellular flanking DNA for its termination. In contrast to the *S* gene product, the *X* gene product may possibly be replaced by an X–cell fusion protein due to absence of the carboxyl-terminal end of the *X* ORF and its termination codon in the integrated form. For confirmation, cDNAs of X mRNA expressed from the native or integrated form of HBV DNA were cloned. A significant structural difference between cDNAs for native and X–cell fusion proteins with trans-activation activity in HBV-infected cells was found.

cDNA cloning of native X mRNA obtained by the transient expression of pHBVX-1 in the human hepatoma cell line HuH-7 was conducted by the λgt10 host–vector system, and cDNA clones were sequenced. The 3′ and 5′ ends of the X mRNA were found to be situated at about nt 1809 [14 base pairs (bp) downstream from the poly(A) signal] and nt 1149, respectively (Fig. 4*A*). Their coding sequences corresponded exactly to the *X* gene coding region in the HBV genome. X protein was thus a direct translation product from the *X* ORF. The present data also clearly indicate that the viral poly(A) signal TATAAA at nt 1790 was used.

cDNA cloning of the X mRNA from the integrated HBV DNA was performed using a representative clone, N2-7. This clone contains an inversely repeated structure of HBV DNA and expresses a 1.3-kb X mRNA and a 2.6-kb S mRNA in the transient expression system (see Figs. 2 and 4*B*). To efficiently clone X mRNA, N2-7 DNA was first digested with *Xba* I, which cuts the inside of the *S* ORF, and then introduced into HuH-7 cells. The structural features of some of the cDNA clones obtained are shown in Fig. 4*B*. The X mRNA produced from N2-7 DNA was clearly an X–cell fusion transcript. The transcription was terminated by a cellular poly(A) signal AATAAA located 500 bp downstream from the virus–cell DNA junction. The 3′ end of the mRNA was localized about 10 bp downstream from the poly(A) signal. No splicing was observed. The 5′ ends of X mRNA from N2-7 DNA and native X mRNA were at the same site. The distance between the putative mRNA start site and the poly(A) addition signal in the N2-7 X mRNA was about 400 bp longer than the corresponding distance in native X mRNA. This difference corresponds exactly to the size difference between native X mRNA (0.9 kb) and the X–cell fusion transcript from N2-7 (1.3 kb). The integrated form of the *X* gene is thus concluded to have lost its 3′ end and poly(A) signal during integration but to be capable of producing X mRNA as an X–cell fusion transcript by using a cellular poly(A) signal (Fig. 4 *A* and *B*).

Amino Acid Sequence of X–Cell Fusion Protein from the N2-7 Integrant. The *X* gene is encoded between nt 1248 and

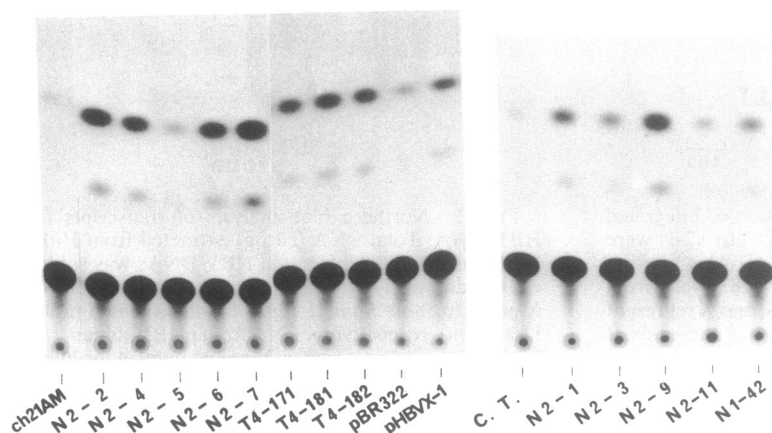

FIG. 3. CAT assay for the trans-activation function of various integrated HBV DNAs. HepG2 cells were cotransfected with 30–40 μg of integrated HBV DNA cloned in the λ phage ch21AM vector and 5 μg of pSV2CAT DNA as the reporter plasmid. ch21AM, C. T. (calf thymus DNA), and pBR322 DNAs were used as controls. Plasmid pHBVX-1 was used for X mRNA expression (see Fig. 4*A*).

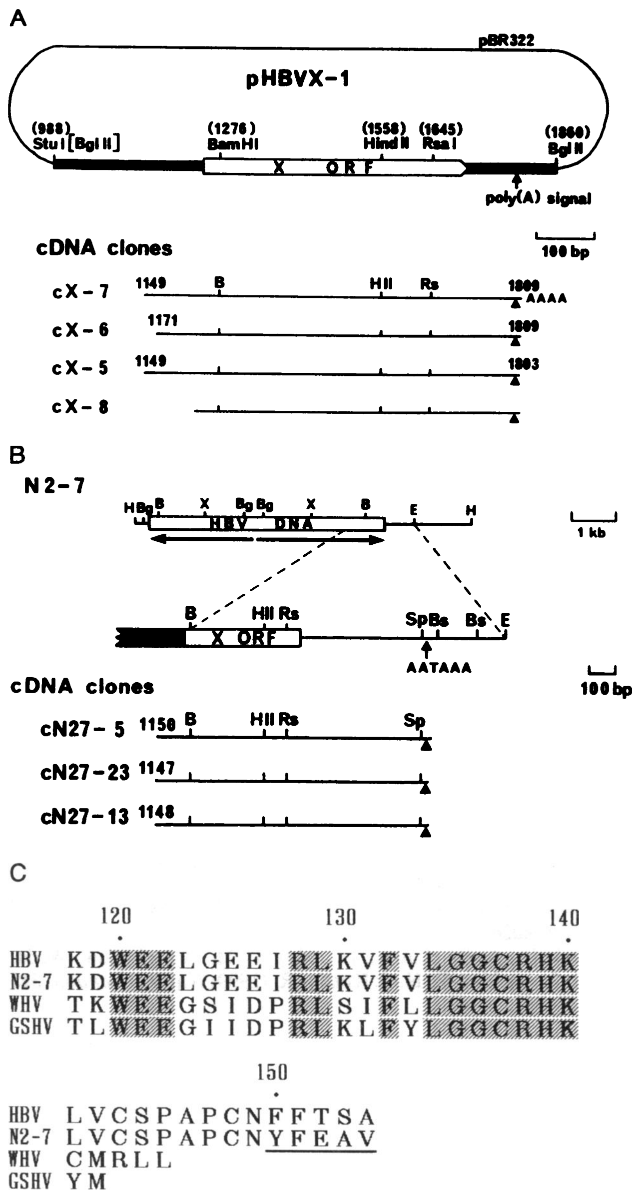

FIG. 4. Structures of X mRNAs expressed from native and integrated HBV DNAs and amino acid sequences in the carboxyl-terminal region of native X protein and X–cell fusion protein. (*A*) The *Stu* I–*Bgl* II HBV DNA fragment of the X expression plasmid pHBVX-1 contains promoter/enhancer sequences, X ORF, and poly(A) addition signal for expressing native X mRNA. The structures of cDNA clones (cX-5 to cX-8) are also shown. B, HII, and Rs are cleavage sites for *Bam*HI, *Hind*II, and *Rsa* I, respectively. Nucleotide numbers of the 5′ and 3′ ends of cDNAs are indicated according to Kobayashi and Koike (24), where nt 1 corresponds to nt 127 of the system for numbering by Ono *et al.* (25). The solid triangles indicate the sites of the poly(A) addition signal. (*B*) Entire structure of integrated HBV DNA N2-7 and detailed map at the X–cell junction region are shown. Beneath the map, structures of the cDNA clones from the N2-7 DNA are shown. Nucleotide numbers of the 5′ ends are indicated. Abbreviations are given in *A*. (*C*) Amino acid sequences in the carboxyl-terminal region of native X protein and X–cell fusion protein as deduced from nucleotide sequence of clone N2-7. Amino acids translated from the cellular nucleotide sequence are underlined. Amino acid sequences in the same carboxyl-terminal regions of woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) (26) are also arranged. The amino acid sequences conserved among hepadnavirus family are enclosed by the hatched boxes. The single-letter amino acid code is used.

1709, has a coding capacity of 154 amino acids, and is essentially conserved in the other hepadnaviruses (26). The amino acid sequence of X–cell fusion protein was deduced from nucleotide sequences (Fig. 4*C*). The HBV DNA sequence is connected to cellular DNA at nt 1695, the amino acid sequence of X protein ends at residue 149 near the carboxyl-terminal end, and cellular flanking DNA provides an additional five amino acids for this truncated X polypeptide. By comparing the X–cell fusion protein with other hepadnaviral X proteins (Fig. 4*C*), the mutation at the carboxyl-terminal region of the N2-7 X protein appeared not to have a deleterious effect on its structure or function. The deletion of the last 22 amino acids resulted in virtually the complete loss of the trans-activation function (K.K., S.T., and M. Arii, unpublished data). The last 14 amino acids are not conserved among hepadnaviruses (Fig. 4*C*). Thus, the seven conserved amino acids (Leu-Gly-Gly-Cys-Arg-His-Lys) are probably essential to the trans-activation function of the X protein.

DISCUSSION

In the present study, integrated HBV DNAs from chronic hepatitis tissues were examined in close detail for RNA expression and trans-activation activity. Many integrated HBV DNAs could express trans-activation activity under the conditions used. Integrated HBV DNA could thus function as a trans-activator of cellular genes, such as c-*myc* (10), in chronic hepatitis tissues and hepatoma tissues as well. This trans-activation mechanism requires neither a unique integrated site nor specific rearrangement of HBV DNA or cellular flanking DNA. Consequently, the general mechanism of hepatocarcinogenesis can perhaps be explained by HBV DNA integration. Based on the previous data indicating that there are no significant differences in the structure of integrated HBV DNA between chronic hepatitis and hepatoma tissues (3), the present observations provide evidence that chronic hepatitis with or without cirrhosis is a premalignant stage of hepatocellular carcinoma. However, this would not mean that chronic hepatitis results in most cases in hepatocellular carcinoma development, since other changes in cellular conditions or genes are probably required for hepatocytes to become fully malignant. Genetic factor(s) may perhaps influence the conditions for this. For example, woodchuck hepatitis virus causes hepatocellular carcinoma at a 100% frequency in carrier animals (27), whereas HBV causes human hepatocellular carcinoma in only 3% of HBV carriers (28).

Based on accumulating evidence that many hepatocellular carcinomas possess the integrated HBV DNA even after the disappearance of free viruses, the integrated *X* gene may also be essential for maintaining the tumor phenotype that develops at the early stage of carcinogenesis. For confirmation, detailed research on X mRNA and X protein produced from integrated HBV DNA in chronic hepatitis and hepatoma tissues should be done.

The present study on the cDNA structure of X mRNA from integrated HBV DNA indicated that X–cell fusion mRNA and also truncated X protein can be produced. Although the reason why many 3′ truncated *X* gene products retain the trans-activation function is not fully understood, possibly truncation at the carboxyl-terminal end is sufficiently small so that this function is maintained. In ground squirrel hepatitis virus, X protein has the trans-activation function (29) in spite of missing the carboxyl-terminal region corresponding to the last 12 amino acids of HBV X protein (Fig. 4*C*). The present results on the 5-amino acid truncation also support this possibility. On the other hand, the deletion of the last 22 amino acids caused almost the complete loss of the trans-activation function, indicating that the 7 conserved amino acids (Leu-Gly-Gly-Cys-Arg-His-Lys) are important for this trans-activation function.

The promoter and enhancer elements of the HBV *X* gene were found to be uniformly conserved in most integrated HBV DNAs, but the transcriptional level of X mRNA showed considerable variation from one integrant to another. If the integration site is controlled by some negative regulatory element or if the poly(A) signal is not situated at an appropriate position, the integrated HBV DNA may be transcribed to a lesser extent. It is also possible that some molecular clones of integrated HBV DNA obtained may have lost transcriptional activity because cellular flanking DNA for poly(A) signal and other necessary sequences were not included in the clones. In addition to the trans-activation function of the *X* gene product, a trans-activation function has been identified in the product of the pre*S*/*S* region (30).

This work was supported in part by a grant-in-aid from the Ministry of Health and Welfare, Japan, for Comprehensive 10-year Strategy for Cancer Control and by a grant-in-aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, to K.K.

1. Tiollais, P., Pourcel, C. & Dejean, A. (1985) *Nature (London)* **317**, 489–495.
2. Koike, K., Kobayashi, M., Yaginuma, K. & Shirakata, Y. (1987) in *Structure and Function of Integrated HBV DNA: Hepadna Viruses*, eds. Robinson, W. S., Koike, K. & Will, H. (Liss, New York), pp. 267–286.
3. Takada, S., Gotoh, Y., Hayashi, S., Yoshida, M. & Koike, K. (1990) *J. Virol.* **64**, 822–828.
4. Yaginuma, K., Kobayashi, M., Yoshida, E. & Koike, K. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4458–4462.
5. Yaginuma, K., Kobayashi, H., Kobayashi, M., Morishima, T., Matsuyama, K. & Koike, K. (1987) *J. Virol.* **61**, 1808–1813.
6. Dejean, A., Bougueleret, L., Grzeschik, K.-H. & Tiollais, P. (1986) *Nature (London)* **322**, 70–72.
7. Nagaya, T., Nakamura, T., Tokino, T., Tsurimoto, T., Imai, M., Mayumi, T., Kamino, K., Yamamura, K. & Matsubara, K. (1987) *Genes Dev.* **1**, 773–782.
8. Spandau, D. F. & Lee, C. H. (1988) *J. Virol.* **62**, 427–434.
9. Seto, E., Yen, T. S. B., Perterlin, B. M. & Ou, J.-H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8286–8290.
10. Koike, K., Shirakata, Y., Yaginuma, K., Arii, M., Takada, S., Nakamura, I., Hayashi, Y., Kawada, M. & Kobayashi, M. (1989) *Mol. Biol. Med.* **6**, 151–160.
11. Twu, J.-S. & Schloemer, R. H. (1987) *J. Virol.* **61**, 3448–3453.
12. Wollersheim, M., Debelka, U. & Hofschneider, P. H. (1988) *Oncogene* **3**, 545–552.
13. Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. & Sato, J. (1982) *Cancer Res.* **42**, 3858–3863.
14. Knowles, B. B., Howe, C. C. & Aden, D. P. (1980) *Science* **209**, 497–499.
15. Graham, F. L. & Van der Eb, A. J. (1973) *J. Virol.* **52**, 456–467.
16. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
17. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
18. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
19. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
20. Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–269.
21. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
22. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
23. Yaginuma, K., Shirakata, Y., Kobayashi, M. & Koike, K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2678–2682.
24. Kobayashi, M. & Koike, K. (1984) *Gene* **30**, 227–232.
25. Ono, Y., Onda, H., Sasada, R., Igarashi, K., Sugino, Y. & Nishioka, K. (1983) *Nucleic Acids Res.* **11**, 1747–1757.
26. Kodama, K., Ogasawara, N., Yoshikawa, H. & Murakami, S. (1985) *J. Virol.* **56**, 978–986.
27. Popper, H., Roth, L., Purcell, R. H., Tennant, B. C. & Gerin, J. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 866–870.
28. Beasley, R. P., Huang, L. Y., Lin, C. C. & Chien, C. S. (1981) *Lancet* **ii**, 1129–1133.
29. Colgrove, R., Simon, G. & Ganem, D. (1989) *J. Virol.* **63**, 4019–4026.
30. Kekulé, A. S., Lauer, U., Meyer, M., Caselmann, W. H., Hofschneider, P. H. & Koshy, R. (1990) *Nature (London)* **343**, 457–461.