Vol. 192, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1993 Pages 111-118

TRANSACTIVATION OF CELLULAR PROMOTERS BY AN INTEGRATED HEPATITIS B VIRUS DNA

Shuji Yamamoto, Hiroshi Nakatake, Shoko Kawamoto, Masato Takimoto, Rajen Koshy* , and Kenichi Matsubara

Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita 565, Japan

Received February 18, 1993

A new Hepatitis B virus(HBV) DNA integrant clone DA2-6, isolated from a human hepatocellular carcinoma(HCC) genomic library, was tested for its ability to transactivate expression of other genes. DA2-6 consists of 3.7 kb flanking cellular sequences and an integrated 2.8 kb HBV DNA which covers the region of preS, S, and the 3' truncated X. Using a chloramphenicol acetyltransferase(CAT) assay, a number of cellular and viral promoters were transactivated by DA2-6, and the spectrum of transactivational effect was the same as that by the wild type X gene of the virus. Deletion mutant analyses indicated that the transactivation function of DA2-6 is expressed by the region that encodes a truncated X-cell fusion product.

Hepatitis B virus(HBV) is a causative agent of human hepatitis that affects some 200 million people in the world. Epidemiological evidence has linked chronic HBV infection and the development of hepatocellular carcinoma(HCC)(1). Although the mechanism by which HBV infection is linked to HCC formation is not clear, a significant fraction of such HCCs carry HBV DNA integrated in cellular DNA. This finding has created discussions on correlation of the integration with cancer development. Such discussions regard the integration event or the integrant as; i) an insertional mutagen; ii) a causative agent for inducing secondary chromosomal rearrangements; iii) a portable promoter that invokes altered expression of cellular genes; and iv) a transactivator for cellular genes(2). The fourth possibility has attracted much interest in the light of recent findings that the HBV X gene product can transactivate some cellular genes(3, 4, 5, 6). However, as shown previously(7), none of several

* Present address: Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, U.K.

0006-291X/93 $4.00

HBV integrants are intact, and none of them can code for an intact X; rather, most of them code for a truncated X with or without being fused in-frame to cellular or viral sequences. In this study, we focused on one HBV integrant, DA2-6(7), that allows transcription of a gene, encoding a 3' truncated X protein fused to ten amino acid sequences from cellular DNA.

Materials and Methods

THE EFFECTOR PLASMIDS AND THE HBV INTEGRANT CLONE DA2-6.
The integrant HBV DNA clone DA2-6 was obtained from an HCC(7). This clone carries a 2.8kb contiguous stretch of a single HBV genome with no deletion or rearrangment. The viral genome covers the region of preS, S and a 3' truncated X, with 464bp around the cohesive end region being deleted(see Fig. 1). This integrated HBV DNA with its flanking cellular sequences was cleaved from a λclone by EcoR I, inserted into the EcoR I site of plasmid pBR325, and used as an effector for the transactivation assay. In addition, two other effector plasmids were used as references. They were pRSVX that expresses the intact X protein under control of the RSV promoter, and pRSVXm that carries TAG codons in places of the 1st, 79th and 103rd ATG codons in the X open reading frame, and therefore unable to allow transactivation of any portion of the X polypeptide. The construction of these plasmids is described elsewhere(8).
CELLS AND TRANSFECTION.
HepG2 cells(9) were grown at 37℃ in Dulbecco modified Eagle medium(GIBCO) supplemented with 10% fetal calf serum. Cells were plated at a density of 5×10^5 cells per 60mm plate, incubated for 24 hours, and the medium was renewed two hours prior to the DNA transfection. Transfection was performed by the calcium phosphate method using a mixture of the effector plasmid(2.5μg) and a reporter plasmid (0.5μg). After incubation for 10 hours, the cells were rinsed with serum-free medium, treated with 15%(v/v) glycerol for 1 min at room temperature, and then incubated with 3mℓ of fresh medium.
CAT ASSAYS.
Cells were harvested at forty-eight hours after the transfection, and CAT assays were performed essentially as described by Gorman et al(10). The cells were washed in the plate with isotonic phosphate saline, removed by scraping, and pelleted by centrifugation. After being resuspended in 0.25M Tris-HCl(pH 7.5). They were disrupted by two cycles of freezing and thawing. Fifty μg of total protein(determined by using a Bio-Rad protein assay kit) from the lysates were added to a standard CAT reaction mix(10), and CAT activity was assayed using [^{14}C]-labeled chloramphenicol by excising spots of non acetylated and acetylated forms from thin layer chromatography plates and counting in a liquid scintilation counter.
NORTHERN BLOT ANALYSES.
Total RNA of HepG2 cells at 48 hours after transfection was extracted by standard methods and polyadenylated mRNA was selected with oligo dT cellulose. Afer electrophoresis in a formaldehyde/agarose gel, the RNA was transferred to a Hybond-N filter(Amersham). The [^{32}P]-labeled hybridization probes from the HBV DNA fragment and the DA2-6 DNA were made by using a multiprime labeling system(Amersham).

Results

The structure of the DA2-6 clone.

The plasmid DA2-6 carries a 6.5 kb EcoR I fragment of genomic DNA from an HCC(7). It consists of an integrated 2.8 kb HBV DNA which is a continuous

stretch from 2255 through 3215/1 to 1791, along with 3.7 kb of flanking cellular sequences. The viral DNA has a 464 bp deletion(between 1791 and 2255), that covers the preC and the N terminal portions of the gene C, and the C terminal portion of the X gene, along with the DR1(Fig. 1). The coding regions of the Pol, preS/S genes are intact. Although the major viral promoter that is responsible for the synthesis of the pregenomic RNA is absent, the three viral promoters that can encode 2.4kb, 2.1kb, 0.8kb RNAs(11) are present. In addition, the deletion has eliminated 47 bases at the 3'-terminal region of the X gene and fused it in frame to flanking cellular DNA such that a truncated X protein fused to ten amino acids of cellular origin can be made. The viral transcription termination sequence

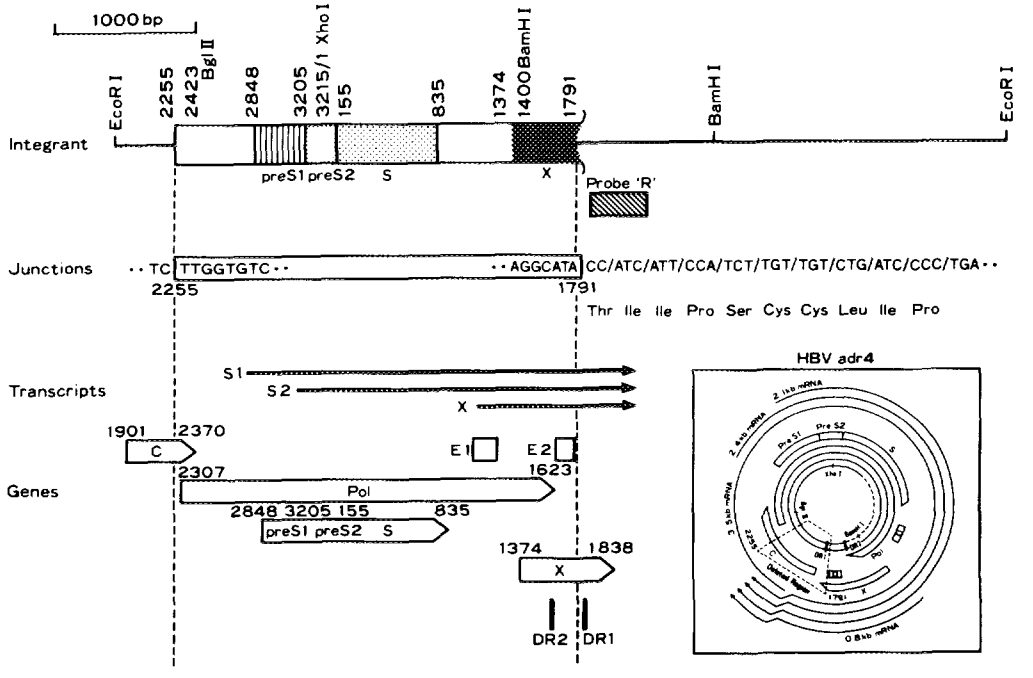

Fig. 1. The structure of the DA2-6 clone.
The box with 2255 and 1791 at either end represents the integrated HBV DNA. The horizontal lines represent the flanking cellular DNA. The HBV DNA is a 2.8 kb contiguous stretch of the viral genome, with a 464 bp deletion of the region shown in the inset. "probe R" represents the region of a 0.4 kb flanking cellular DNA in the "right arm". This sequence is unique and was used as a probe for monitoring read-through transcripts. The nucleotide sequences around the junctions are provided. The amino acid sequence represents the peptide coded by the cellular sequence fused to the carboxy terminal portion of the truncated X gene. 'Transcripts' represent three RNAs encoded by this integrant. Some of the relevant DNA structure and genes are displayed. The numbers represent the positions, in nucleotides, along the intact HBV adr4 genome. This numbering system was chosen to coordinate the map with others(1). The insert represents the intact HBV adr4 genome in circular form. For explanations of the genes, structure and transcripts, see references(1, 11).

that is located near to the DR1 is also missing. Thus, the three possible transcriptions should terminate at some place in the cellular sequence located downstream of the fused X gene.

We examined RNA by Northern blot analyses from HepG2 cells transfected with the DA2-6 DNA. The results in Fig. 2 show that transcripts of 2.6kb, 2.3kb, 1.0kb are made, each of which hybridizes with the cellular DNA probe "R", as well as with the HBV DNA probe. The 2.6kb, 2.3kb and 1.0kb RNAs must be the transcripts from the S1, S2 and X promoters, respectively. Though we have not determined the initiation sites for these transcripts, comparison with the published data(11) on intact HBV mRNA, viz. 2.4kb, 2.1kb, 0.8kb for the transcripts from PreS1, PreS2 and X promoters, respectively, makes it very likely that they originated from the same promoters, thus implying that the termination site must be located within the cellular sequences, at approximately 200 bp downstream of the junction. The presence of an AT-rich stretch at around 200 bp downstream of the junction is consistent with this notion.

Transactivation of cellular promoters by the DA2-6.

The DA2-6 was used as an effector to see whether it can transactivate cellular or viral promoters. For this purpose, we used several reporter plasmids, containing various promoter regions of cellular or viral genes fused to the CAT gene. Upon cotransfection of these reporter plasmids with the DA2-6, an augmented expression of the CAT gene was expected if the DA2-6 were transactivating. As controls, we used two other effector plasmids, pRSVX that codes for the intact X protein under the control of the RSV LTR, and pRSVXm1 which is a null-mutant of the pRSVX in which all

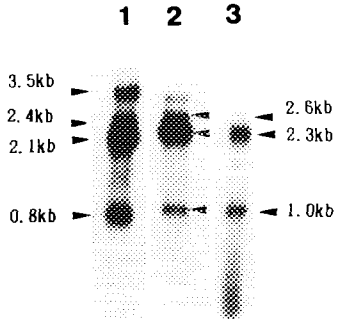

Fig. 2. RNA synthesis in transfected HepG2 cells.
Northern blot analyses of poly(A) RNA were done as described in the Methods section. Lane 1, control RNA from cells transfected by dimeric HBV DNA, and probed by total HBV DNA. Lane 2, RNA from DA2-6 transfected cells, probed by total HBV DNA. Lane 3, the same RNA as in Lane 2, probed by the R probe(see Fig. 1) from the cellular DNA. Arrowheads represent the HBV-specific transcripts.

the ATG codons in the X gene have been substituted by TAG codons(8). The results of the cotransfection experiments displayed in Table 1 demonstrate the broad transactivation potential of pRSVX on various cellular and viral promoters. The results obtained with DA2-6 as an effector were very similar to those obtained with pRSVX(Table 1.). Of particular interest is that in several cases the activity of DA2-6 is better than pRSVX.

Deletion mutants of the DA2-6.

To see if the region of the DA2-6 responsible for the transactivation can be narrowed down, a set of subfragments of DA2-6 were cloned for testing their transactivational effect. The results in Fig. 3 demonstrate that 3' deletions extending into the X and/or its flanking cellular sequences abrogated the transactivation activities. On the other hand, m4, that covers the region between Xho I and BamH I, having the enhancer I, II and the promoter for the X gene, but not PreS1 or PreS2, showed transactivation. This DNA covers the coding region for the fused X gene, with the transcription termination site in the downstream cellular sequence. Although not proved directly, these observations can only be explained by assuming that the transactivation function observed with the m4 fragment is due to the fused X gene product. The coding sequences for the S gene and the carboxy terminal portion of the Pol gene are not likely to be relevant, because their promoters are missing in this fragment.

Discussion

In this study, we showed that the HBV integrant clone DA2-6, which contains only a partial X gene, exhibited trans-activation on a number of cellular genes including oncogenes like c-fos, c-Ha-ras. The spectrum of the stimulatory effect was essentially the same as that by the intact X protein. The X protein has been shown to modify the specificity of transcription factors, which may account for the promiscuity of X transactivation(12). In this study, we showed that both X protein and DA2-6 stimulate NF-κB dependent gene expression, using the three tandemly arranged sequence(GGGAAATTCC), which is the putative NF-κB binding sequence. It is interesting that DA2-6 exhibited trans-activation on a glutathione S-transferase P(GST-P) gene, because this gene is known to be induced specifically at an early stage of chemical hepatocarcinogenesis in rat.

Analyses using deletion mutants have indicated that the trans-activation function of the DA2-6 is expressed by the region that encodes the

Table 1 Transactivation of cellular or viral promoters by DA2-6

Reporter Plasmids (Cellular or Viral Promoter)		Effectors	
		pRSVX	DA2-6
Elongation Factor	pEF321CAT	9.1	11.0
GST-P	E-CAT	4.5	10.0
Interferon β	p-105CAT	3.6	9.9
NF κ B	p-55A2CAT	2.6	4.9
IRF-1	pIRF-CAT	2.1	4.5
Interleukin2	pIL2-319CAT	1.0	1.1
Actin	pActin-CAT	3.3	8.6
c-myc	mycCAT	1.0	1.1
c-fos	fc-1	4.0	4.6
c-Ha-ras	p-rasCAT1	2.5	3.0
HIV-1 LTR	pBennCAT	3.5	5.4
HTLV-1 LTR	LTR-CAT	2.1	N.T.

HepG2 cells were cotransfected by the calcium phosphate method with one of the reporter plasmids carrying the CAT gene hooked to the promoter under analysis, and an effector plasmid, pRSVX, DA2-6 or pRSVXm(8). Cells were harvested, lysed and used for CAT activity assays as described in the Methods section. pRSVXm is a null-mutant of pRSVX, that does not code for any part of the X gene polypeptide. The observed CAT activities with DA2-6 or pRSVX were divided by the corresponding CAT activity from the sample with pRSVXm. The source and properties of reporter plasmids are as follows: pActin-CAT contains the promoter of the chick-cytoplasmic β-actin gene(Kost et al. Nucleic Acids Res. 11, 8287-8301, 1983). The plsmid pEF321CAT contains the 2.5 kb DNA carrying the promoter for the human elongation factor-1 α (Uetsuki et al. J. Biol. Chem. 264, 3448-3453, 1989). The plasmid p-105CAT contains the 105 bp DNA carrying the immediate upstream region of the interferon β gene(Fujita et al. Cell 49, 357-367, 1987). The plasmid p-55A2CAT contains three tandemly arranged sequence(GGGAAATTCC), which is the putative NF κ B binding sequence, and which is placed upstream of the interferon β gene(Fujita et al. Nucleic Acids Res. 17, 3335-3346, 1989). The plsmid pIRF-CAT contains the 3.5 kb of upstream region of the IRF-1 gene. The plsmid pBennCAT contains the human immunodeficiency virus long terminal repeat sequence(Gandelman et al. Proc. Natl. Acad. Sci. USA. 83, 9759-9763, 1986). pIL2-319CAT contains the 5' flanking region of the IL-2 gene(Fujita et al. Cell 46, 401-407, 1986). These seven plasmids were the gifts from Dr. T. Taniguchi(Institute for Molecular and Cellular Biology, Osaka University). The plasmid E-CAT obtained from Dr. Muramatsu(The University of Tokyo Faculty of Medicine) contains a 3.0 kb DNA fragment covering the cap site of the glutathione S-alkyltransferase(GST-P) gene(Sakai et al. Proc. Natl. Acad. Sci. USA. 85, 9456-9460, 1988). The plasmid fc-1 contains the upstream sequence and the promoter of the fos gene(Deschamps et al. Science 230, 1174-1176, 1985). The plasmid mycCAT contains the 2.8kb DNA carrying the promoter of human myc gene. The plasmid p-rasCAT1 contains the DNA region for the c-Ha-ras promoter(Isii et al. Science 230, 1378-1381). These three plasmids were gifts from Dr. Ishii (The Riken Institute, Tsukuba City). The plasmid LTR-CAT obtained from Dr. Yoshida(Institute for Medical Sciences, The University of Tokyo) contains the HTLV-1 long terminal repeat sequence(Fujisawa et al. Proc. Natl. Acad. Sci. USA. 82, 2277-2281).

truncated X-cell fusion product. No other regions of the DA2-6 genome showed similar activities. Wollersheim et al(13). have observed transactivation by a truncated X gene product that has lost 28 bases in the carboxy-terminal region, and is fused to a 228 bp sequence from

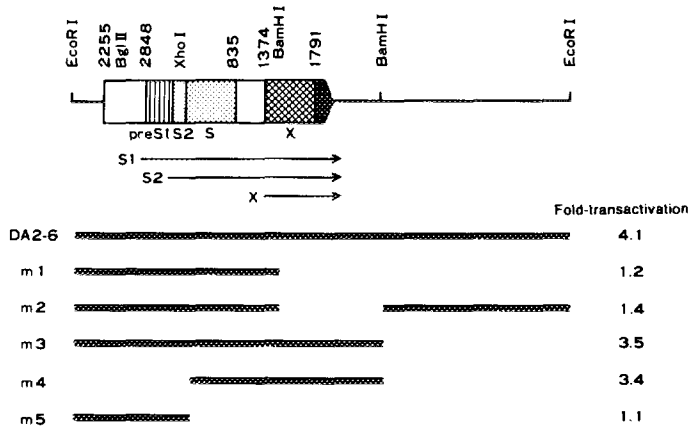

Fig. 3. Transactivation by the deletion mutants of DA2-6.
The structure of DA2-6 and its deletion derivatives are schematically shown. For symbols and explanations, see Fig. 1. Each one of these DNA constructs (2.5 μg) was used as an effector in co-transfection experiments using fc-1 plasmid(see Table 1) as the reporter. The level of transactivation was assayed and normalized, using pRSVXm, as described in Table 1. The shaded area of the box shows the truncated X gene region fused with an extra ten amino acid-coding sequence from cellular DNA.

cellular DNA. Takada et al(14). have also found integrants from chronic hepatitis tissues to encode 3' truncated X-cell fusion products with trans-activation activity. In another study, an X deletion mutant lacking 37 amino-acids has been shown to exhibit transactivation(15). In this study, the deletion in the X gene of DA2-6 is 47 bases. It is interesting that in several cases the activity of DA2-6 was better than the wild type X (Table 1). At this stage we cannot speculate how the truncated X and fused transcripts retain the function.

The finding that the integrated HBV DNA can potentially express X-mediated trans-activation attracts our special interest, since the X sequence is often observed in the HBV integrants as a truncated fused ORF(7). If many of such X-fusion genes are active, a significant fraction of the HCCs may be under the influence of the complex transactivation effect of the integrants. Some of these effects may be correlated to hepatocarcinogenesis.

References

1. Tiollais, P., Pourcel, C., and Dejean, A. (1985). The hepatitis B viruses. Nature 317, 489-495.
2. Matsubara, K. (1991). Chromosomal changes associated with Hepatitis B Viruses DNA integration and hepatocarcinogenesis. In Molecular Biology of the Hepatitis B Viruses (McLachlan, A. ed., CRC Press, Florida) pp245-261.

3. Twu, J. S., and Schloemer, R. (1987). Transcriptional transactivating function of hepatitis B virus. J. Virol. 61, 3448-3453.
4. Zahm, P., Hofschneider, P. H., and Koshy, R. (1988). The HBV X-ORF encodes a transactivator: a potential factor in hepatocarcinogenesis. Oncogene 3 169-177.
5. Koshy, R., and Hofschneider, P. H. (1989). Transactivation by hepatitis B virus may contribute to hepatocarcinogesis. Current topics in microbiology and immunology 265-281.
6. Seto, E., Mitchel, P. J., and Yen T. S. B. (1990). Transactivation by hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature 344, 72-74.
7. Nagaya, T., Nakamura, T., Tokino, T., Imai, T., Mayumi, K., Kamino, K., Yamamura, K., and Matsubara, K. (1987). The mode of Hepatitis B virus DNA integration in chromosome of human hepatocellular carcinoma. Genes Dev. 1, 773-782.
8. Nakatake, H., Chisaka, O., Yamamoto, S., Matsubara, K, and Koshy, R. Effect of X protein on transactivation of Hepatitis B virus promoter and on viral replication. (Virology, in press).
9. Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282, 615-617.
10. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2, 1044-1051.
11. Ganem, D., and Varmus, H. (1987). The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. 56, 651-693.
12. Maguire, H. F., Hoeffler, J. P., and Siddiqui, A. (1991). HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science. 252, 842-844.
13. Wollersheim, M., Debelka, U., and Hofschneider, P. H. (1988). A transactivating function encoded in the hepatitis B virus X gene is conserved in the integrated state. Oncogene 3, 545-552.
14. Takada, S., and Koike, K. (1990). Transactivation function of a 3' truncated X -cell fusion product from integrated Hepatitis B virus DNA in chronic hepatitis tissues. Proc. Natl. Acad. Sci. U. S. A. 87, 5628-5632.
15. Balsano, C., Avantaggiati, M. L., Natoli, G., Marzio, E., Will, H., Perricaudet, M., and Levrero, M. (1991). Full length and truncated versions of the hepatitis B virus X protein transactivate the c-myc proto-oncogene at the transcriptional level. Biochem. Biophys. Res. Communi. 176, 985-992.