ORIGINAL ARTICLE
IZVORNI ZNANSTVENI RAD

Libri Oncol., Vol 21 (1992), No 3, 153–160

DIFFERENT TRANSFORMING POTENTIAL OF NORMAL AND MUTATED HEPATITIS B VIRUS X GENE

ZORAN TADIĆ

Zavod za animalnu fiziologiju PMF-a, Rooseveltov trg 6, 41000 Zagreb

SUMMARY

Hepatitis B virus is etiologic agent of acute hepatitis and is considered to be a major factor in hepatocellular carcinoma initiation. Here, the transforming capability of normal and mutated HBV X gene was investigated. NIH 3T3 cells were transfected with plasmids carrying normal X gene and a frameshift mutant in which only small part is translated correctly. Both constructs were driven by Rous sarcoma virus LTH promoter. The transformed phenotype was tested in focus-forming assay and ability to grow in semisolid medium (soft agar). In both tests mutated HBV X gene showed significantly greater transforming capability than its normal counterpart. The possible mechanisms of these observations are discussed with the special emphasis on additional open reading frames within the X-ORF.

KEY WORDS: hepatitis B virus, X gene, transformation

RAZLIČITA TRANSFORMACIJSKA SPOSOBNOST
NORMALNOG I MUTIRANOG GENA X VIRUSA HEPATITISA B

SAŽETAK

U ovome je radu ispitana transformacijska sposobnost gena X virusa hepatitisa koji bi mogao biti odgovoran za indukciju primarnog hepatocelularnog karcinoma u kroničnih nositelja virusa. U NIH 3T3 stanice uneseni su plazmidi s normalnim i mutiranim genom X i nakon toga je praćena sposobnost rasta stanica u mekom agaru te njihova sposobnost da stvore fokuse rasta na konfluentnim staničnim slojevima. Pokazalo se da mutirani gen X pokazuje statistički značajnu povećanu sposobnost transformacije stanica u odnosu na normalni gen. Isti su pokusi obavljeni i s virusnim integratom kloniranim iz HuH-7 stanica, koji, kao i normalni gen X, nije pokazao značajnije povećanu sposobnost transformacije u odnosu na kontrolnu skupinu. Raspravom je obuhvaćena ovakva razlika s obzirom na postojanje dodatnih okvira čitanja unutar gena X.

KLJUČNE RIJEČI: hepatitis b virus, hepatocelularni karcinom, gen X, transformacija

Hepatitis B virus (Fig. 1) is an etiologic agent of hepatitis B (20). The prodromal phase of the disease, in more than a half of the patients, is characterized by malaise, myalgia, anorexia, nausea and, in some cases, slight fever. The icteric phase, whic follows prodromal phase, is ushered by appearance of dark urine, pale stools and yellowing of the mucous membranes, conjunctivae, sclerae and skin (jaundice). In 90 - 95% of patients recovery is complete and no virus can be isolated from blood after the disease. However, 5 - 10% of patients develop chronic hepatitis, a state in which HBV continues to persist in the liver throughout the life (35). It is estimated that there are over 200 millions of chronic HBV carriers in the world, mainly in southern and southeastern Asia and they present a continous reservoir of the virus.

Hepatitis B virus is a DNA virus which replicates via RNA intermediate (14). The HBV genome is approximately 3200 bp long, the smallest of any DNA virus yet encountered. HBV DNA displays two remarkable features. First, there is an asymmetry in length of the strands. One strand is a unit length while the other is less than the unit length. The long strand is designated to be of minus polarity and is complementary to viral

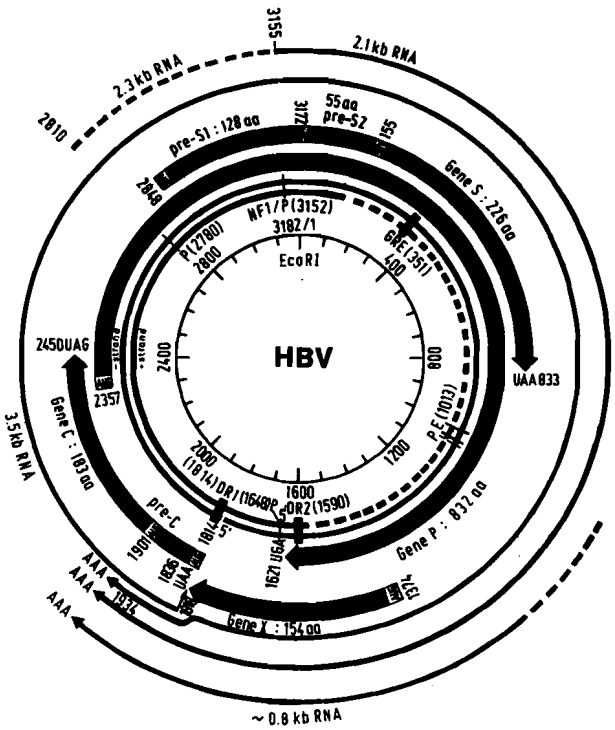

Figure 1. Genome map of the hepatitis B virus

mRNAs. The shorter strand is, therefore, of minus polarity. Thus, molecule possesses a single-stranded gap of variable extent (20 - 80%) of the unit length). Second, the minus strand contains a protein covalently linked to its 5' end (16) whereas there is a short strech of oligoribonucleotides attached to the 5' of plus strand. These asymmetries are direct consequences of replicative mechanism of the HBV (37).

Viral DNA contains four open reading frames - S, C, P and X (39). The smallest of three polypeptides which are derived from S region was discovered as the major component of hepatitis B virus surface antigen (HBsAg) particles in blood of virus carriers. This protein is derived from the S region and exists in both unglycosylated ($P24^S$) and glycosylated ($GP27^S$) form (34). It is also the main constituent of 22 nm HBsAg particles and filamentous HBsAg particles which are together with complete virions found in blood.

The middle S protein is encoded by pre S-2 and S region (19). This protein is a glycoprotein and is present in two forms, $GP33^S$ and $GP36^S$; the molecular weight being the direct consequence of the extent of glycosylation. The pre S-2 region can bind polymerized human serum albumin (pHSA) (26) and hence the speculation that pHSA may be a mediator of hepatitis B virus attachment to hepatocytes. This has been supported by the fact that hepatocytes also have receptors for pHSA.

The large protein is encoded by pre S-1, pre S-2 and S region (26) and is present in glycosylated ($GP42^S$) and unglycosylated ($P39^S$) form. This protein may also be involved in HBV binding to hepatocytes since it has been known that the pre S-1 region can bind to hepatocytes (32).

The principal viral protein forming viral nucleocapsid is referred to as core antigen (HBcAg) and is derived from C region of the genome (33). However, another protein, similar to the HBcAg is usually present in serum of infected individuals. This protein is called HBeAg and is immunologically cross-reactive with HBcAg (27). It is translated from pre C and C region; the pre C region is believed to act as a signal peptide (15) which targets HBeAg of secretory pathway and causes its secretion (7,33).

Region P encodes for endogenous polymerase/reverse transcriptase (2). The protein is unusually rich in histidine and bears tracts of homology with *pol* region of retroviruses (13).

The X coding region encodes a small protein (cca. 154 amino acids) whose function in the HBV life cycle is unknown.

Epidemiological studies show strong positive correlation between chronic HBV infection and development of primary hepatocellular carcinoma (PHC) (3). The risk of PHC development in chronic HBV carriers is over 100-fold that of non-carriers and PHC usually develops after 20 - 30 years of chronic disease. Liver failure is also a common cause of death of chronic carriers. Indeed, the most of the mortality from the HBV results from chronic rather than from acute disease. Molecular analysis of the DNA isolated from most PHCs reveales that HBV DNA is stably integrated into the host genoma (8,9,11,29). Sequence analysis of HBV- host DNA junctions and S1 nuclease mapping of mRNAs derived from such junctions suggests two models of HBV DNA integration. The first (25) model is based on the fact that integration takes place in the single-

stranded region of the virus, close to the 5' end of the plus strand. During replication, the DNA polymerase could switch from host DNA to HBV DNA. A recombination effect then joins minus strand with cellular DNA. Then, a cellular gap in the plus strand is filled and ligated to cellular DNA. The second model (12) is based on the assumption that there is a specific integration mechanism mediated through two 11-base pairs repeats present in HBV DNA. Both repeats can be the target of replication upon which the first two bases of the repeats are deleted.

Integration of HBV DNA is a random event. Extensive studies have been undertaken to determine whether HBV integrates preferentially in the vicinity of oncogens or other cellular genes which may be relevant to hepatoma initiation but all have failed to detect common place of integration. Only in one case, an integration near *c-erba*, has been reported (11). In this respect HBV is similar to retroviruses (41) which also integrate randomly throughout cellular genoma. Also, neither of HBV genes has been assigned with any specific function which may contribute to hepatocarcinogenesis. The situation is complicated further by the fact that all HBV tumor integrates examined to date are greatly rearranged (31). The rearrangements include substantial deletions of both viral and cellular DNA at the site of junction, rearrangements of viral and cellular DNA and readtrough transcription from viral down to adjacent cellular DNA. Recent finding that open reading frame X (ORF X) encodes a transcriptional transactivator (36,40,43) and supports the notion that HBV integration into the cellular DNA may contribute to liver carcinogenesis by activating cellular genes "in trans". However, there is no experimental evidence that ORF X contributes to cellular transformation both "in vitro" and "in vivo". Therefore, a comparative study was done to investigate the transforming potential of intact and mutated ORF X and to compare it with transforming potential of HBV DNA from tumor integrate.

MATERIAL AND METHODS

Plasmids (Fig. 2) pRSVX (4.2 kb) contains NcoI-BglII HBV X fragment cloned in HindII-BamHI larger fragment of pRSVcat (17). pRSVXfs is a frameshift mutant of pRSVX in which mutation is introduced by cutting pRSVX with BamHI (28 pb downstream from ORF X ATG codon), filling the sticky ends using T4 DNA polymerase and blunt-end ligation.

pHuc (9.44 kb) contains EcoRI-PstI 6.75 kb HBV DNA integrate together with adjacent cellular DNA from HuH-7 hepatoma cell line cloned in EcoRI-PstI-cut pUC 19.

pAG60 (10) contains PvuI-BglII fragment with herpes simplex virus (HSV) thymidine kinase (tk) promoter followed by BglII-SmaI fragment

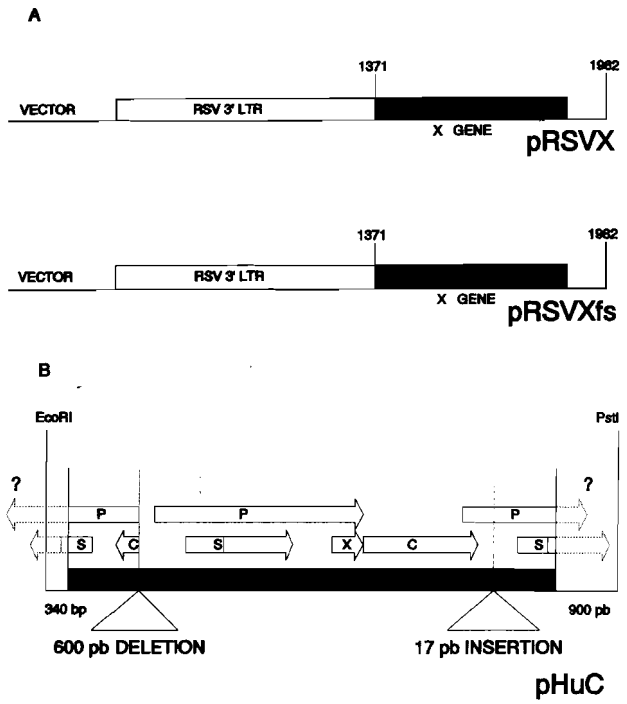

Figure 2. Plasmids. A: pRSVx and pRSVxfs. Thick lines represent cloned promoter and viral gene sequences, thin horizontal lines represent vector sequences. Numbers indicate the numbers of corresponding nucleotides in the HBV genome. B: pHuc. Thick horizontal line represents integrated HBV, thin lines are adjacent human DNA. Thick empty arrows denote the orientation of the HBV genes in the integrate. Dashed arrows indicate possible direction of transcription of viral - host gene junctions.

with *neo* gene from transposon Tn 5 and SmaI-PvuI fragment containing HSV tk polyadenylation signal.

Small scale plasmid isolations were done by alkaline lysis (4). Plasmids used for transfections were prepared on large scale by cesium chloride equilibrium buoyant density centrifugation.

Cell culture and gene transfer. All cell lines were grown on Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (FCS). 1.5×10^6 cells were plated on 100 mm^2 tissue culture dishes the day before transfection. For transfection, the appropriate amounts of test plasmid and plasmid carrying the marker gene were mixed together and applied to the culture medium using calcium phosphate precipitation procedure (18). The precipitate was washed after 16 hours and cells supplemented with fresh medium with 10% FCS. After 24 hours, a new medium containing G418 (600 μg/ml) was applied. This medium was changed every three days until colonies appeared. These colonies were subcloned in larger dishes and were grown until enough cells were present for DNA and RNA isolation. Then, the cells were scraped off with rubber policeman and used for RNA and DNA preparation. Anchorage independence was tested as described (1). Briefly, the cells were seeded in DMEM + 10% FCS + 0.3% Noble Agar which was poured over 1% agarose in DMEM. The cells were fed every 3 days until colonies appeared.

RNA preparation and Northern hybridization. Cytoplasmic RNA was extracted by lysis with NP-40 in ten volumes of 140 mM NaCl; 10 mM Tris-HCl ph 8.0; 1.5 mM $MgCl_2$; 5 mM DTT in presence of 200 units of RNAsin. After pelleting nuclei and debris, supernatant was digested with proteinase K (200 μg/ml) in 100 mM Tris-HCl ph 7.5; 150 mM NaCl; 12.5 mM EDTA and 1% SDS. The mixture was phenol-extracted and RNA was precipitated in 3M NaCl with 2.5 volumes of ethanol at -70°C. mRNA was isolated on oligo-dT cellulose columns (28). Electrophoresis of RNA was done in 1% agarose gels with 20 mM MOPS; 5 mM NaAc pH 5.0; 0.1 mM EDTA and 2.2 M formaldehyde running buffer. Approximately, 20 μg of RNA were applied to the gel. After electrophoresis, RNA was transferred to nylon membrane by capillary blot. Prehybridization and hybridization were done in 5×SSC; 1% Sarkosyl (Na-laurylsarcosine); 50% formamide at 40°C for two hours and 24 hours, respectively. For hybridization, 10^6 cpm/ml of probe was used. The probes were radiolabelled by nick translation and their specific activity was 3×10^8 cpm/μg. After hybridization the filters were sequentially washed twice in 2×SSC; 0.5% Sarkosyl at 60°C, dried and exposed to films at -70°C.

DNA hybridization. High molecular weight DNA (hmwDNA) was isolated by method of Blin and Stafford (5). 10 μg of hmwDNA was digested with appropriate enzyme. After digestion, electrophoresis was done in 1% agarose gels. DNA was then transferred to nylon filters using capillary blot. Filter was hybridized with probe labelled by nick translation to high specific activity (10^9 cpm/μg). All other hybridization procedures were done as described for Northern hybridization.

RESULTS

In order to see if the X gene has a transforming potential, the plasmids pRSVX and pRSVXfs were introduced into NIH 3T3 mouse fibroblasts (ATCC CCL 92) together with pAG60 allowing selection of posetively transfected cells. DNA was isolated from the two best-growing clones (CX and CXfs), digested with ScaI and hybridized with BamHI-BglII 580 bp probe derived from pRSVX. After autoradiogrphy, a band of approximately 4.2 kb was seen in both clones indicating plasmid DNA was stably integrated in transfected cells. (Fig. 3, lanes 1 and 2). To determine functionality of transfected genes, mRNA was isolated from the cell clones and hybridized to the X probe. In both cases, a major band of 0.8 appeared (Fig. 4, lanes 2 and 3). This band corresponds to the 0.8 kb X mRNA from which X protein is translated (24). This suggests that cells have transcriptionally active introduced X gene. However, together with this band three additional bands are visible.

It is known that transformed cells have the ability to form colonies in semisolid media and to form foci on confluent monolayer. To determine whether CX and CXfs clones have such properties

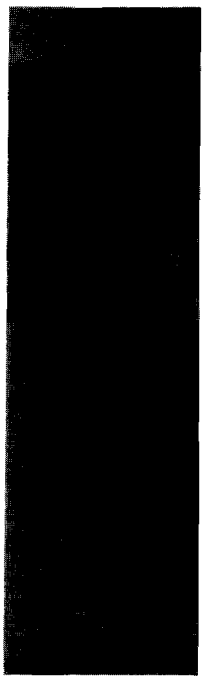

Figure 3. Southern blot of NIH 3T3 cellular DNA digested with ScaI and hybridized to the X probe. Lane 1: pRSVX, Lane 2: pRSVXfs, Lane 3: pUC 19

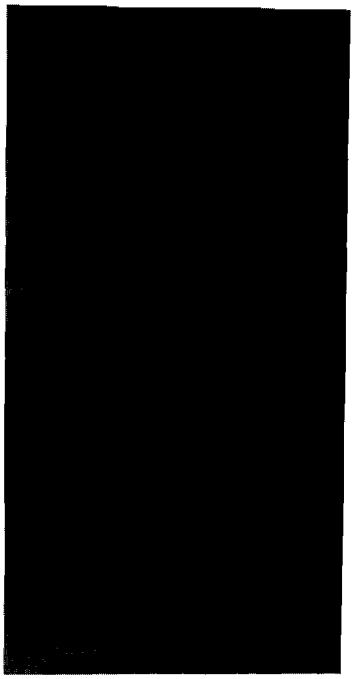

Figure 4. Hybridization of cytoplasmic mRNA isolated from NIH 3T3 cells with the X probe. Lane 1: pUC 19, Lane 2: pRSVXfs, Lane 3: pRSVX. Arrow denot X mRNA.

they were plated in 0.3% Noble Agar. Results were summarized in Table 1. Surprisingly, CXfs clone formed five time more colonies on confluent monolayer and in soft agar than the CX clone.

The X genes used in this experiment were cloned from HBV DNA isolated directly from the virus. However, HBV DNA from all the tumor integrates examined up to now is mutated and rearranged. These aberrations accumulate over a long period of time (20 - 40 years) and it may be that mutations in HBV DNA, which may somehow alter the expression of the X gene, are responsible for transformation. To stimulate this situation, I took plasmid pHuc containing HBV DNA integrate from HuH-7 cell line together with adjacent human DNA. The integrate consists of two HBV DNAs joined together in such a way that transcription is going in opposite directions and terminates in human DNA sequences (Fig. 2c). Since HBV exhibits extremely strong hepatotropism, efficient expression necessitates the use

Table 1. Focus – forming assay and induction of growth in soft agar. The results are mean of the five experiments. All the results were tested in Student t – test ($P<0.05$).

Cells	Plasmids	No of *neo*^{r} colonies/ 10^6 cells	% of transformants/ 10^6 cells	No. of colonies in soft agar/10^6 cells
NIH 3T3	pRSVX+ pAG60	130±18.3	10±2.8	15±2.2
NIH 3T3	pRSVXfs+ pAG60	220±16.8	55±6.2	120±6.0
NIH 3T3	pAG60	1140±23.3	0	0
BNL CV2	pHuc+ pAG60	170±32.1	14±4.8	7±2.2

of differentiated liver cell lines for monitoring of the transcription. Therefore, I used BNL CV 2 (ATCC TIB 73) mouse embryo liver cell line. This line was derived from Balb/c embryo liver and was adapted to grow in DMEM. This cell line was transfected with pHuc together with pAG60. Surprisingly, compared with CX and CXfs clones the clones grew poorly. Only after 40 days visible clones appeared. After 60 days the best-growing was picked. The clone was designated CHuc and was grown until enough cells were obtained to isolate DNA and RNA. Isolated cellular DNA was cut with ScaI and hybridized with BamHI- BglII X probe derived from pRSVX. The presence of 9.4 kb band (Fig. 5, lane 2) confirmed the presence of integrated copy of pHuc in the cells. Northern hybridization revealed that three species of X mRNA were present. Apart from 0.8 kb band, two additional bands were present (Fig. 6, lane 2).

To test anchorage independence the cells were plated in 0.3% Noble Agar. However, the number of colonies was not significantly different from number of CX colonies (results not shown).

DISCUSSION

HBV-mediated hepatocarcinogenesis is an ill-defined process in which products of the hepatitis B virus may interact with cellular processes; the consequence of this being hepatocellular carcinoma. In this study, a different transforming

Figure 5. Hybridization of DNA from BNL CV 2 cells with the X probe. Lane 1: pUC 19, Lane 2: pHuc.

Figure 6. Hybridization of the cytoplasmic mRNA from BNL CV 2 cells with the X probe. Lane 1: pUC 19, Lane 2: pHuc.

potential of normal and mutated HBV X gene was shown. Efficient transcription of the X gene was achieved by putting it under control of the Rous Sarcoma Virus long terminal repeat promoter since all HBV promoters were shown to be silent in non-hepatocytes. The presence of 0.8 kb mRNA the activity of introduced genes. However, mRNAs of 1.5 and 2.3 kb were also present. Their presence may be due to "head-to-tail" integration of transfected DNA. The best guess is that, during transcription of the transfected genes, RNA polymerase sometimes runs through the poly-A addition site without termination of transcription. Because of this, higher multimers of 0.8 may be produced together with transcripts which contain both promoter and coding region. Moreover, when transfected cells were harvested 24 hours after transfection, just before the onset of the DNA integration only 0.8 kn mRNA transcripts could be detected (results not shown).

The colony-formation in soft agar and focus--forming assay on confluent monolayers showed that the transforming potential of mutated HBV X gene was significantly higher than that of its normal counterpart. This is surprising since the frameshift mutation was placed in the coding region of the gene and this may be crucial for the viability of its product. However, recent computer analysis of the HBV genome revealed a novel open reading frame situated within the ORF X. This ORF, called ORF 5, is between 80 - 100 codons long (depending on the subtype of the virus) and is found in both human and animal hepatitis B viruses. Since its sequence is conserved in various hepadnaviruses, it is tempting to speculate it may have some important role in HBV life cycle. Interestingly, the start codon of ORF 5 is ACG rather than ATG. Apart from this ORF, the only viruses which use this start codon are adenoviruses.

In the X gene, ORF 5 is placed in +1 regarding frame in respect to normal start codon and, probably, it is not very efficiently expressed. However, during translation, the ribosomal frameshifting may occur. The ribosome slippage into the +1 reading frame before ACG may put the ORF 5 in favorable position for the translation. Ribosomal frameshifting is a common process in the generation of some retroviral proteins; the *gag-pol* polyprotein of RSV (21,22) and MMTV (23) are being thus created. Since retroviruses and hepadnaviruses share common evolutionary origin, it may be that ribosomal frameshifting is also used by hepatitis viruses to generate proteins which could not otherwise be expressed from monocistronic mRNA. In pRSVXfs, a +1 frameshift mutation places ORF 5 in favorable position for translation. The product is then efficiently produced and is probably responsible for tumorigenic conversion of NIH 3T3 cells. Contrary, the larger product of ORF X is not as tumorigenic as the smaller protein. The small number of colonies on plates with CX clone can be ascribed to occasional frameshifting during translation.

Generation of three species of mRNAs in cells transfected with pHuc to activity of promoters other than X promoter. Three transcripts containing ORF X were generated. The first transcript is 0.7 kb long and probably starts from S promoter. This transcript contains the Sas well as the X region which is not translated to the X protein. The origin of the third transcript is not clear. It may be that it originates from S promoter and is later somehow truncated or this may be the degradation product of 3.2 kb pregenome or C mRNA.

Sequence analysis of pHuc plasmid showed that X gene is not mutated (results not shown). The small number of colonies of transformed CHuc clones may be the consequence of ribosomal frameshifting.

It is conceivable that HBV X gene may play a role in hepatocarcinogenesis. It is known that it can activate transcription of *c-myc* gene (43) - a gene which is involved in the regulation of the cell cycle. Computer sequence analysis of ORF X revealed two "zinc-finger"-like regions (38), but preliminary experiments excluded any direct binding of the X product either to the *c-myc* promoter or any other promoter tested (42). It is possible that the X gene product directly binds to *c-myc* protein or some other regulatory protein(s) altering their function. These results suggest ORF X produces two proteins with different functions. The first, more abundant protein, may be a transactivator while the other may be responsible for cell transformation. Thus, a model can be proposed in which integrated DNA, during the time course, accumulates mutations. In the start, the X gene is intact and produces a non-tumorigenic product but it somehow alters the cell cycle and predisposes the cells for further changes. As the time passes, the HBV DNA accumulates mutations and rearrangements which may alter the structure of ORF X. The consequence of this mutation may be the activation of ORF 5 which may be put in the translation frame and, thus, efficiently expressed. Once ORF 5 is in the favorable position for transcription, its product may accumulate which then may induce increased proliferation and ultimately lead to hepatocellular carcinoma formation.

REFERENCES

1. Barret J.C., Tso P.O.P.: Evidence for the progressive nature of neoplastic transformation "in vitro". Proc. Natl. Acad. Sci. USA 75: 3761-3764, 1975.
2. Bavand M., Feitelson M., Laub O.: The hepatitis B virus-associated reverse transcriptase is encoded by viral *pol* gene. J. Virol. 63: 1019-1021, 1989.
3. Beasley R.P.: Hepatitis B virus as etiologic agent in hepatocellular carcinoma - epidemiologic considerations. Hepatology 2: 21-26, 1982.
4. Birnboim H.C., Doly J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523, 1979.
5. Blin N., Stafford D.W.: A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. 3: 2303-2304, 1976.
6. Brechot C., Pourcel C., Louise A., Rain B., Tiollais P.: Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature 286: 533-535, 1980.
7. Bruss V., Gerlich W.: Formation of transmembraneous hepatitis B *e* antigen by cotranslational "in vitro" processing of the viral precore protein. Virology 63: 268- 275, 1988.
8. Chakraborty P.R., Ruiz-Opazo N., Shouval D., Shafritz D.A.: Identification of integrated hepatitis B virus DNA and expression of viral DNA in an HBsAg-producing human hepatocellular carcinoma cell line. Nature 286: 531-533, 1980.
9. Chen J.Y., Harrison T.J., Lee C.S., Chen D.S., Zuckermann A.J.: Detection of hepatitis B virus DNA in hepatocellular carcinoma: analysis by hybridization with subgenomic DNa fragments. Hepatology 8: 518-523, 1988.
10. Colbere-Garapin F., Horodniccau F., Kourilsky P., Garapin A.C.: A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. 150: 1-14, 1981.
11. Dejean A., Bouqueleret L., Grzeschnik K.H., Tiollais P.: Hepatitis B virus DNA integration in a sequence homologous to *v-erbA* and steroid receptor genes in a hepatocellular carcinoma. Nature 322: 70-72, 1986.
12. Dejean A., Sonigo P., Wain-Hobson S., Tiollais P.: Specific hepatitis B virus DNA integration in a hepatocellular carcinoma through an 11 base-pair direct repeat. Proc. Natl. Acad. Sci. USA 81: 5350-5354, 1984.
13. Dickson C., Eisenmann R., Fan H., Hunter E., Teich N.: Protein biosynthesis and assembly. In: RNA tumor viruses, R. Weiss, N. Teich, H. Varmus, J. Coffin eds., Cold Spring Harbor Laboratory Press, 1984, 513-684.

14. Ganem D., Varmus H.: The molecular biology of hepatitis B viruses. Ann. Rev. Biochem. 56: 651-93, 1987.
15. Garcia P.D., Ou J.H., Rutter W.J., Walter P.: Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage, translocation can be aborted and product released in cytoplasm. J. Cell. Biol. 106: 1093-2004, 1988.
16. Gerlich W., Robinson W.S.: Hepatitis B virus contains protein covalently attached to the 5'-terminus of its complete DNA strand. Cell 21: 801-809, 1980.
17. Gorman C.M., Merlino T., Willingham M.C., Pastan I., Howard B.H.: The Rous Sarcoma Virus long terminal repeat is a strong promoter when introduced in into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA 79: 6777-6781, 1982.
18. Graham F.L., Van der Eb A.J.: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456- 467, 1972.
19. Heerman K.H., Goldman U., Schwartz W., Seyfarth T., Baumgarten H., Gerlich W.: Large surface proteins of hepatitis B virus containing the pre-S sequence. J. Virol. 52: 396-402, 1984.
20. Hollinger B.L., Melnick J.L.: Viral Hepatitis In: Virology. B.H. Vields ed., Raven Press, 1373-1377, 1985.
21. Huges S.H., Payvar F., Spector R., Schimke T., Robinson H.L., Payne G.S., Bishop J.M., Varmus H.E.: Heterogenity of genetic loci in chickens: analysis of endogenous viral and nonviral genes by cleavage of DNA with restriction endonuclease. Cell 18: 347-359, 1979.
22. Jacks T., Varmus H.D.: Expression of Rous Sarcoma Virus *pol* gene by ribosomal frameshifting. Science 230: 1237-1242, 1985.
23. Jacks T., Townsley K., Varmus H.E., Majors J.: Two efficient ribosomal frameshifting events are required for synthesis of Mouse Mammary Tumor Virus *gag*-related polyproteins. Proc. Natl. Acad. Sci. USA 84: 4298-4302, 1987.
24. Kaneko S., Miller R.H.: X region - specific transcript in mammalian hepatitis B virus-infected liver. J. Virol. 62: 3979-3984, 1988.
25. Koshy R., Koch S., Freytag fon Loringhoven A., Kahman R., Murray K., Hofschneider P.H.: Integration of hepatitis B virus: evidence for integration in the single-stranded gap. Cell 34: 215-223, 1983.
26. Machida A., Kishimoto S., Ohnuma H., Miyamoto H., Baba K., Itoh Y.: A polypeptide containing 55 amino acids residues coded by the pre-S region of hepatitis B virus deoxyribonucleic acid bears the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology 86: 910-918, 1984.
27. Magnius L.O., Espmark J.A.: New specificities in Australia antigen positive sera distinct from LeBouvier determinants. J. Immunolol. 109: 1017-1021, 1972.
28. Maniatis T., Fritsch E.F., Sambrook J.: Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, 1982.
29. Marion P.L., Salazar F.H., Alexander J.J., Robinson W.S.: State of hepatitis B virus DNA in human hepatoma cell line. J. Virol. 33: 795-806, 1980.
30. Miller R.H., Kaneko S., Chung C.T., Girones R., Purcell R.H.: Compact organization of hepatitis B virus genome. Hepatology 9: 322-327, 1989.
31. Nagaya T., Nakamura T., Tokino T., Tsurimoto T., Imai M., Mayumi T,. Kamino K., Yamamura K., Matsubara K.: The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. Genes Develop. 1: 773-782, 1987.
32. Neurath A.R., Kent S.B.H., Strick N., Parker K.: Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 429-436, 1986.
33. Ou J.H., Laub O., Rutter W.J.: Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the *e* antigen. Proc. Natl. Acad. Sci. USA 83: 1578-1582, 1986.
34. Peterson D.L., Nath N., Gavillanes F.: Structure of hepatitis B virus surface antigen J. Biol. Dhem. 257: 10414- -10420, 1982.
35. Redeker A.G.: Viral hepatitis: clinical aspets. Am. J. Med. Sci. 270: 9-16, 1975.
36. Spandau D.F., Lee C.H.: Transactivation of viral enhancers by the hepatitis B virus X protein. J. Virol. 62: 427-434, 1988.
37. Summers J., Mason W.S.: Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29: 403-415, 1982.
38. Szecheng J.L., Chien M.L., Lee Y.H.W.: Characteristics of the X gene of hepatitis B virus. Virology 167: 289-292, 1988.
39. Tiollais P., Pourcel C., Dejean A.: The hepatitis B virus. Nature 317: 489-495, 1985.
40. Twu J.S., Schloemer R.H.: Transcriptional transactivating function of hepatitis B virus. J. Virol. 61: 3448-3453, 1987.
41. Varmus H.E., Swanstrom R.: Replication of retroviruses. In: RNA tumor viruses. R. Weiss, N. Teich, H. Varmus, J. Coffin, eds. Cold Spring Harbor Laboratory Press, 369-512, 1984.
42. Wells J. Unpublished results
43. Zahm P., Hofschneider P.H., Koshy R.: The HBV x - ORF encodes a transactivator: a potential factor in viral hepatocarcinogenesis. Oncogene 3: 169-177, 1988.

Author's address: Zoran Tadić, Department of Animal Physiology, Rooseveltov trg 6, 41000 Zagreb, Croatia

Received for publication: June 15, 1992