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Retroviral Expression of the Hepatitis B Virus X Gene

Tadić Z, Bašić I.

Department of Animal Physiology,
41000 Zagreb, Croatia

Abstract

Using a retroviral vector, an efficient expression of the HBV X gene was achieved. Molecular analysis of isolated cellular DNA showed integrity of the provirus and Northern hybridization confirmed functionality of the integrated provirus. The tests of transformed phenotype revealed the mutated HBV X gene to transform NIH 3T3 cells more efficiently than the wild type gene.

Key Words:

Hepatitis B virus; DNA, viral; Genetic vectors; Retroviridae

Introduction

Hepatitis B virus (HBV) is an eveloped hepatotropic DNA virus which is an etiologic agent of acute hepatitis and is considered to be a major factor in hepatocyte injury and hepatoma formation (1). Nonetheless much is known about HBV structure, replication and molecular biology, the mechanisms responsible for malignant transformation are still unknown.

During recent years, much attention has been focused on the HBV X gene. This is due to the fact that its role in the viral life cycle is obscure and that it is capable of activating various viral and cellular promoters »in trans« (2-4). This supports the idea that the X gene is capable of transactivating various cellular genes which may be relevant to hepatoma initiation and formation.

We report here on expression of the HBV X gene cloned in a retroviral vector as well as on the transforming capability of such constructs.

Materials and Methods

Plasmids. pLJ (Figure 1) is a retroviral vector based on Moloney MLV. The vector contains neo gene which confers resistance to G418. This gene is driven by SV 40 early promoter (SV 40). The vector also contains the entire polyoma virus early region (not shown) which allows episomal replicaton in transfected cells, thus facilitating virus production.

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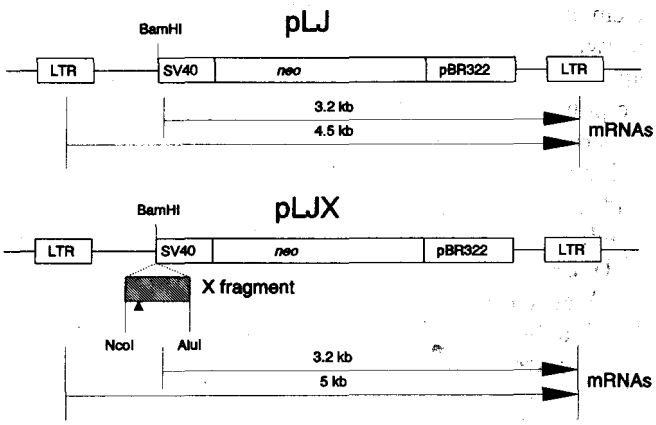
Figure 1. Plasmic pLJX. Legend: LTR – 5' Molonex MLV LTR, SV40 early promoter, neo-neomycin resistance gene, pBR322 – pBR322 origin of replication. Arrows denote direction of transcription and length of viral mRNAs. Small filled triangle denotes the position of frameshift mutation in pLJXfs. The plasmid is not drawn to scale.



pLJX (Figure 1) is a recombinant pLJ carrying the HBV X gene. This vector was constructed by cutting pSHH 2. 1 (5) with NcoI and Bg1II and isolating 611 bp fragment. This fragment was subsequently treated with AluI to eliminate poly A site and larger fragment was isolated. The recessed termini were filled with Klenow enzyme. pLJ vector was cut with BamHI and ends were filled using Klenow enzyme. The X fragment was inserted in cut vector by blunt-end ligation using T4 DNA ligase.

pLJXfs was made by cutting pLJX with BamHI (28 bp down-stream from the start codon of the ORF X), filling the sticky ends with Klenow enzyme and ligation using T4 DNA ligase. This created +1 frameshift mutation in the ORF X.

All plasmid manipulations and cloning procedures were done as described elsewhere (6). The plasmids used in cellular transfections were prepared by CsCl density gradient equilibrium centrifugation.

Cell culture and gene transfer. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS). For the production of the recombinant virus stocks 2 cell line was used (7). The cells were plated at the density of 4x10 cells into 6 cm dish the day before transfection. For the transfection, 15 μg of plasmid DNA was applied to the culture medium using calcium phosphate procedure of Graham and Van der Eb (8). After eight hours, the medium was changed and virus harvested 36 hours later. The optimal time for harvesting was determined empirically determining the titer by the »marker transduction« method.

Virus purification. The virus was harvested from cell culture medium by centrifugation at 5,000g/5 min at 4 °C to remove cell debris. Supernatant was then concentrated at 30,000g/14 hours at 4 °C, pellet reuspended in DMEM supplemented with 10% FCS and stored at –70 °C. Virus titer was determined by infecting NIH 3T3 cells with serial dilutions of the virus and calculating the frequency of neo marker transuction in medium supplemented with G418.

Infection of the host cells. The day before infection the cells were plated at density of $4x10^5$ cells into 6 cm dish. On the day of the infection, the medium was drawn off and replaced with 2 ml of medium containing appropriate quantity of the virus and 8 μg/ml Polybrene polycation. After two hours at 37 °C, the medium was replaced with 5 ml of DMEM + 10% FCS and left in incubator for 24 hours more. The cells were then split and a medium containing G418 (400 μg/ml) was applied.

DNA and RNA hybridizations and assays. All DNA and RNA analyses and isolations were done as previously described (6).

Transformation assays. For the focus-forming assay, the cells were trypsinized, washed twice in PBS and their number was set at 5×10^5 cells/ml. The cells were then seeded at dilutions $10^{-1} - 10^{-4}$ into 5 cm dishes in DMEM supplemented with 5% FCS. After formation of the colonies, the dishes were washed with PBS, fixed with 10% phormol and stained with Giemsa in water.

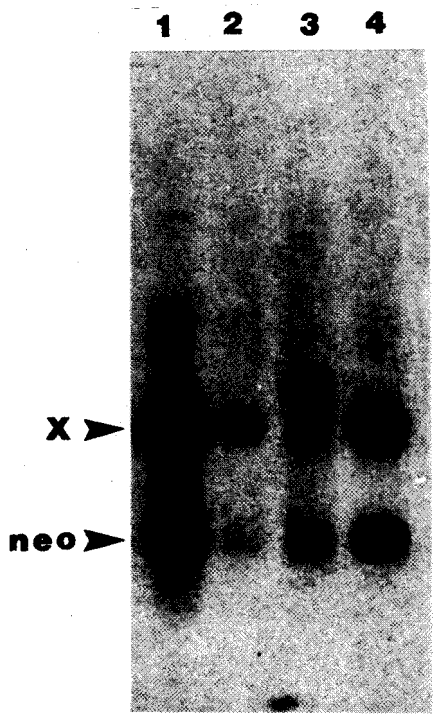
For testing the growth in semisolid medium, the cells were trypsinized, washed in PBS and diluted as described above. One ml of each suspension was mixed with soft agar and poured over 0.8% agar. The cells were fed every 2 days until colonies appeared.

Results

Infection of NIH 3T3 cells with recombinant retrovirus. Recombinant retrovirus was produced by transfection of pLJX and pLJXfs into 2 helper cell line. The virus concentration before infection was set at MOI = 10. After infection, the cells were grown in selective medium until resistant clones appeared. For further analysis, two best growing clones from each group were picked. The clones were designated C1 (pLJX), C2 (pLJX), C3 (pLJXfs) and C4 (pLJXfs).

For testing proviral integration, isolated cellular DNA was cut with XbaI and bybridized with neo probe (Figure 2, lanes 1 -4). The presence of 4.5 kb lanes shows that provirus is stably integrated in cellular DNA.

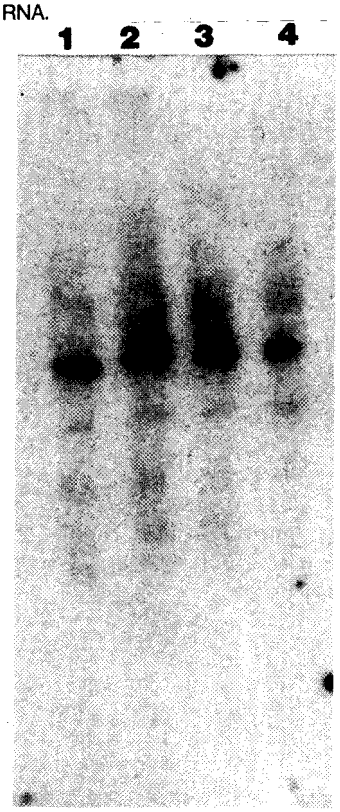
Figure 2. Integration of proviral DNA in cellular DNA. Cellular DNA was cut with XbaI, transferred to nitrocellulose filter and probed with BamHI – BglII X probe. Lanes 1 -2: Clones C1 and C2 (pLJX), Lanes 3 – 4: Clones C3 and C4 (pLJXfs).



Expression of integrated provirus. Isolated cytoplasmic mRNA was hybridized with neo probe to test the integrated provirus functionality. Two bands were present (Figure 3, lanes 1-4). The larger one (approx. 5 kb) is genomic mRNA which is probably the template for the translation of the X gene. The smaller one (approx. 3.2 kb) is neo mRNA and is transcribed from internal SV 40 early promoter. The presence of this two bands suggests that provirus is not rearranged and that it actively transcribes the inserted as well as the marker gene.

Transformation with the HBV X gene. The clones containing normal and mutated HBV X gene were tested on growth in semisolid media and on focus formation on confluent monolayers. Results are summa-

Figure 3. Expression of viral mRNAs in NIH 3T3 cells. Cytoplasmic mRNA was isolated, transferred to nitrocellulose filter and hybridized to radioactively labeled neo probe.
Lanes 3 – 4: Clones C3 – C4 (pLJXfs). X – XmRNA (viral genomic mRNA), neo – neo mRNA.



rized in Table 1. Surprisingly, the mutated gene with frameshift mutation showed greater transformation capability than its normal counterpart.

Table 1: Results of Infection NIH 3T3 Fibroblasts by Recombinant retrovirus containing Mutated HBV X Gene

Clone	Plasmid	% of Transform./106 Cells	No of colonies in soft agar/10^6 col.
1	pLJX	28± 6.3*	25± 6.9*
2	pLJX	16± 7.2	3± 0.8
3	pLJXfs	74± 6.4	62± 6.2
4	pLJXfs	102± 12.7	96± 4.3
5	pLJ	0	0

*Mean of 4 experiments ± SE P (t-test)

Discussion

In this series of experiments, we achieved efficient retroviral expression of the HBV X gene. The X gene was cloned in retroviral vector, recombinant retrovirus was produced and used to infect NIH 3T3 cells. Molecular analysis shows that cells carry proviral copies which express viral mRNAs. The differences in viral expression vary between the clones but this had no effect on cellular transformation within groups of clones carrying the same proviral construct.

The cells carrying mutant X gene formed more foci on confluent monolayers and in soft agar than cells with normal HBV X gene. However, there are several ATG codons as well as the ACG codon (9) within ORF-X whose functions are unknown. It may be that some of these ATG codons are used to generate the proteins with transforming properties. Currently, the experiments are being done to test this possibility.

It is conceivable that the HBV X gene may play a role in hepatocarcinogenesis. Molecular analyses have shown that the majority od isolated HBV integrates from human liver carry ORF-X. Such integrates are capable of transactivating viral and cellular promoters in vitro, even if truncated (10). Truncation of 5' end of the X gene may expose internal ATG to translation and production of proteins, which may be relevant to hepatoma formation and/or initiation.

Retroviral expresssion of the HBV X gene should allow us to introduce the X gene into other mouse cell lines and to construct amphotropic retroviruses with which we should be able to access the consequences of the X gene into other mouse cell lines and to construct amphotropic retroviruses with which we should be able to access the consequences of the X gene action in various non-mouse tissues.

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Sažetak

Kloniranjem gena X virusa hepatitisa B postigli smo njegovu ekspresiju u NIH 3T3 fibroblastima. Molekularnom analizom stanične DNA i citoplazmatske mRNA pokazali smo kako je provirus integriran u staničnu DNA te kako pravilno eksprimira vlastite mRNA. Istraživanjem sposobnosti stanica za stvaranje kolonija na staničnim jednoslojevima te stvaranje kolonija u mekom agaru pokazali smo kako stanice koje nose mutirani gen X stvaraju statistički značajno veći broj kolonija od stanica koje nose normalni gen X.

Ključne riječi:

hepatitis B virus; DNK, virusna; genetski vektor; Retroviridae

All corespondence should be addressed to
J. Bašić
Department of Animal Physiology
Rooseveltov trg 6
41000 Zagreb, Croatia