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• VIRAL LIVER DISEASES •

Seek protein which can interact with hepatitis B virus X protein from human liver cDNA library by yeast two-hybrid system

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Abstract

AIM: **To seek the X associated protein (XAP) with the constructed bait vector pAS2-1X from normal human liver cDNA library.**

METHODS: **The X region of the HBV gene was amplied by PCR and cloned into the eukaryotic expression vector pAS2-1.The reconstituted plasmid pAS2-1X was transformed into the yeast cells and the expression of X protein (pX) was confirmed by Western blot analysis. Yeast cells were cotransformed with pAS2-1X and the normal human liver cDNA library and were grown in selective SC/-trp-leu-his-ade medium, the second screen was performed with the LacZ report gene. Furthermore, segregation analysis and mating experiment were performed to eliminate the false positive and the true positive clones were selected for PCR and sequencing.**

RESULTS: **Reconstituted plasmid pAS2-1X including the anticipated fragment of X gene was proved by auto-sequencing assay. Western blot analysis showed that reconstituted plasmid pAS2-1X expressed BD:X fusion protein in yeast cells. Of 5×10^6 transformed colonies screened, 65 grew in the selective SC/-trp-leu-his-ade medium, 5 scored positive for β-gal activity, and only 2 remaining clones passed through the segregation analysis and mating experiment. Sequence analysis identified that two clones contained similar cDNA fragment: GAACTTGCG.**

CONCLUSION: **The short peptide (glutacid-leucine-alanine)is a possible required site for XAP binding to pX. Normal human liver cDNA library has difficulties in expressing the integrated XAP on yeast cells.**

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INTRODUCTION

Modern biological investigations indicate many proteins have relationship with the development of hepatocellular carcinoma[1-8]. More and more evidences have demonstrated hepatitis B virus (HBV) X protein which encoded by the smallest open reading frame (ORF) of the HBV genome plays an important role in the carcinogenesis[9-13]. At a molecular level, several endogenous genes critical for cell proliferation or apoptosis and the inflammatory response seemed interacted with HBx, such as c-Fos, c-Jun, CREB, CD44,TNF-α,p21 and p53[14-21]. In addition, DNA transfection approaches have clearly demonstrated that pX is a transactivator of a wide variety of viral and cellar promoters[22-24],but the underlying mechanism of transactivation is currently obsure. Since HBx has no ability to bind DNA, protein- protein interaction seems to be crucial for HBx transactivation[25-28]. One most direct way to identify the mechanism of HBx transactivation is to find out host proteins that interact specifically with HBx. We use the yeast two -hybrid system, a genetic approach to search the clone genes that interact with a protein of interest by *in vivo* complementation in yeast cells, to seek XAP from normal human liver cDNA library.

MATERIALS AND METHODS

Plasmid Construction

To make the bait plasmid, the X region of the HBV gene was amplified by PCR using as forward and reverse primers XF and XR, which contain an EcoRI and Pst I site respectively for ease of cloning.The sequence of the primers, with the restriction enzyme site underlined are: XF: ACGGAATTCATGGCTGCTAGGCTGTG, XR: ATCCTGCAGAGGTGAAAAAGTTGCAT. These bind to nucletide positions 1374 and 1838 on HBV genome. The templates for this reaction were sera of HBV DNA posistive patients. The 464bp product was cloned into the respective restriction enzyme sites of plasmid pAS2-1 (Clontech) and this plamid was subsequently named pAS2-1X.

Westem Blot analysis

Saccharomyces cerevisiae AH109(Clontech) was grown in YPD medium($10g\cdot L^{-1}$ yeast extract, $20\ g\cdot L^{-1}$ peptone, $20\ g\cdot L^{-1}$dextrose). This yeast strain carries LacZ. HIS3 and ADE2 reporter genes under the control of Gal4-binding sites was used to screen the liver cDNA library. Yeast cells were transformed with pAS2-1X using lithium acetate method previsously published by Gietz *et al*[29] and were grown in selective SC/-trp medium. Cells were collected by centrifugation and lysates were prepared according to Urea/SDS method. A part of protein exact were resolved on a $120g\cdot L^{-1}$ SDS-polyacrylamide gel and transferred onto polyvinylidene difluride membrane. After blocking with nonfat dried milk, the membrane was treated with 1:3000 diluted Gal4 DNA-BD monoclonal antibody(Clontech) followed by 1:1000 dilured alkaline phosphatase-conjugated goat anti-mouse IgG. Subsequently the blot was developed by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. The untransformed yeast cells were used for negative control.

Screening of the liver cell cDNA library by the yeast two-hybrid system

The screening procedure used was a modification of the method

described by Gietz *et al*[29]. Yeast cells AH109 were transformed with pAS2-1X and pACT2-cDNA library (Clotech) by Liac-mediated transformation and were grown in selective SC/-trp-leu-his-ade medium for 7 days. After about 3 days at 30℃, the growing colonies were assayed for α-gal activity by replica plating the yeast transformants onto Whatman filter papers, the filters were snap frozen in liquid nitrogen for 10s twice and incubated for 1-8h at 30℃ in a buffer containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside(X-gal) solution. Positive interactions were detected by the appearance of blue colonies. Segregation analysis and mating experiment were done to exclude the type I ,II, III false positive and true positive colonies were obtained.

Sequence analysis of pACT2-cDNA

The pACT2-cDNA plasmid genome was isolated following the standard protocol. Briefly, the true positive clones were grown in SC/-leu medium, cells were collected by centrifugation and resuspended in lysis buffer (20g·L^{-1} Triton 100, 10g·L^{-1}SDS, 10mmol L^{-1} NaCl, 10mmol·L^{-1} Tris-HCl pH8.0, 1mmol·L^{-1} Na_2EDTA) and phenol, chloroform and isoamyl alcohol(volume fraction 25:24:1). After addition of acid-washed glass beads (Sigma), samples were centrifugated and plamid DNA recovered. The pACT2-cDNA plasmid DNA was purified by CsCl gradient centrifugation to permit PCR using the Matchmaker AD LD-Insert Screening Amplimers (Clotech) which anneals to GAL4-AD. Auto-sequencing assay was performed in Shanghai Shenggong Biological Corporation.

RESULTS

Plasmid construction

The HBV X fragment was successfulily generated by PCR(figure 1) and cloned into plamsid pAS2-1. Reconstituted plasmid pAS2-1X including the anticipated fragment of X gene was proved by digesting with resticted endonuclease and auto-sequencing assay as follows:
GACTGTATCGCCGGTATTGCAATACCCAGCTTTGACTCATATGG
CCATGGAGGCCGAATTCATGGCTGCTAGGCTGTGCTGCCAACT
GGATCCTGCGCGGGACGTCCTTTGTTTACGTCCCGTCAGCGCTG
AATCCCGCGGACGACCCCTCCCGGGGCCGCTTGGGGCTCTACCG
CCCGCTTCTCCGCCTGTTGTACCGACCGACCACGGGGCGCACCT
CTCTTTACGCGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGAC
CGTGTGCACTTCGCTTCACCTCTGCACGTCGCATGGAAACCACC
GTGAACGCCCACCGGAACCTGCCCAAGGCCTTGCATAAGAGGAC
TCTTGGACTTGCAGCAATGTCAACGACCGACCTTGAGGCATACT
TCAAAGACTGTGTGTTTAACGAGTGGGAGGAGTTGGGGGAGGAG
ATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATT
GGTGTGTTCACCAGCACCATGCAACTTTTTCACCTCTGCAGCCA
AGCTAATTCCGGGCGAATTTCTTATGATTTATGATTTTTATTATT
AAATAAGTTATAAAAAAAATAAGTGTATACAAATTTTAAAGGTG
ACTTTTANGTTTTAAAACGAAAATTNTTATTNTTGAGTAACTNTT
TCCTGGAGGTCAAGGTTGCTT (underlined fractions are restriction enzyme site).

Westem Blot analysis

Westem Blot Analysis proved that yeast cells transformed with pAS2-1X have positive signal which can not be seen in the control, pAS2-1X can express BD:X fusion protein yeast cells(figure 2). Besides, The colony-lift assay showed that the reconstruted plasmid could not active LacZ reporter gene in yeast. pAS2-1X can be used as bait vector in yeast two-hybrid system.

Screening of the liver cell cDNA library

Of 5×10^6 transformed colonies screened, 65 grew in the selective SC/-trp-leu-his-ade medium. Out of these HIS+ ADE+ prototrophs, 5 scored positive for β-gal activity, only 2 remaining clones passed through the segregation analysis and mating experiment.

Sequence analysis

PCR (Figure 3) and sequence analysis identified that two clones contained the same cDNA sequence: GAACTTGCG, which encodes glutacid-leucine -alanine.

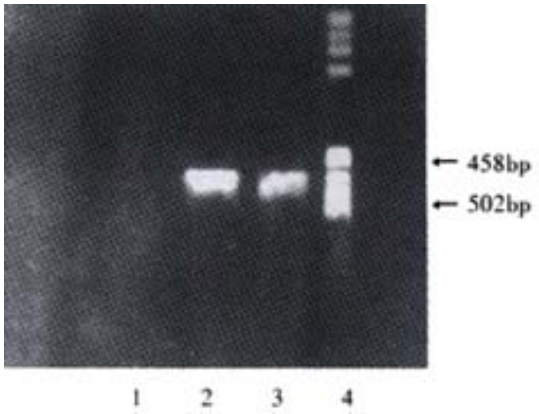

Figure 1 Electrophoresis of X gene PCR products
1: Negative control; 2:Positive control; 3:Sample; 4: Marker

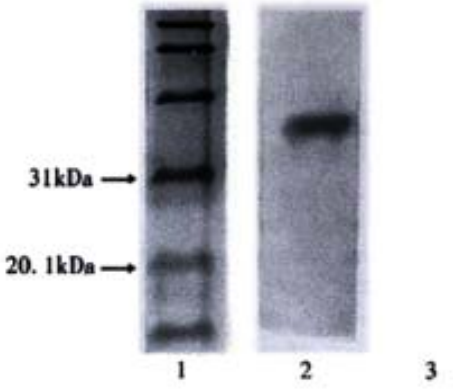

Figure 2 Western blot of yeast after being transformed with pAS2-1X
1:Marker; 2: pX; 3: Negative control

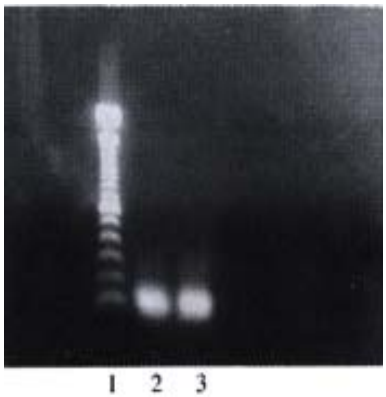

Figure 3 Library cDNA amplified from positive yeast clones
1:100bp DNA ladder; 2. 3: positive cloned

DISSCUSION

Persistent Hepatitis B virus infection is strongly associated with the development of hepatocellular carcinoma(HCC). The viral X gene encodes a 17-kd protein, termed pX, fuctions a transcriptional activator of a variety of viral and cellular genes, it is capable of interacting with a wide variety of cellular protein, including cell-cycle control and apoptosis protein. There are well documented that pX acts through apoptosis pathway involving Fas and caspase 3[30-33], it also

interacts with p53, a wellknow tumor suppressor gene[34-36] and inhibits nucleotide excision repair[37-39]. However, ample evidences showed that pX may function by additional profound mechanisms in HCC[40-43].

Identification and characterization of proteins in a cell with which a given protein interacts is often helpful for understanding the function and mechanisms of action of that protein.The yeast two-hybrid system is a molecular genetic test for protein interaction, which is firstly established by Fileds *et al* in 1989[44].It's a powerful and sensitive technique for the identification of genes that code for proteins which interact in a biologically significant fashion with a protein of interest. The assay is performed in the yeast cells so as to reflect the real situations *in vivo* and has the potential to identify the weak and transient interaction between two protein. In addition, since the cDNA libiary can be constructed according to various type of tissues,organs and cells, many proteins interact with transcription factors, protein kinases, phosphatases, receptors, cytoskeletal proteins as well as proteins involved in cell cycle regulation and apoptosis can be study by this elegant approach. Although the yeast two-hybrid system has become a standard procedure for molecular biologists, it remains some deficiency. The most important problem is different types of false positives, fortunately, they can be eliminated by other method such as segregation analysis and mating experiment[29,45].

Our study successfully constructed the bait vector pAS2-1X, HIS and ADE independent growth and blue -colony formation in the α-gal assay by yeast cells harboring both pAS2-1X and pACT2-cDNA recombinant plasmids and the behaviors of cells in false-positive elimination tests suggested the isolated clones can specifically interact with pX and the results were reliable.

It should be greatly concerned that XAPs studied by the yeast two-hybrid system in the past were different in size, structure and biological functions[46]: Lee *et al*[47] indentified an XAP1 that is a human homolog of the monkey UV-damaged DNA-binding protein in 1995; Kuzhandaivelu *et al*[48] discovered an XAP2 which is known as the p38 subunit of the aryl hydrocarbon receptor complex (ARA9) in 1996; Huang *et al*[49] reported an XAPC7 contained a polypeptide with high sequence homology to the PROS-28.1 subunit of proteasome of Drosophila melanogaster and the α proteasome subunit of Arabidopsis thalinna in 1996; Cong *et al*[50] isolated an XAP3 which is a human homolog of the rat protein kinase C-binding protein in 1997; Melegari *et al*[51] proved an XIP incluing two consensus phosphorylation sites for protein kinase C and Casein kinase II in 1998; Sun *et al*[52]demonstrared a p55sen appeared to be related to the family of EGF-like protein in gengeral in 1998; Rahmani *et al*[53,54] identified an HVDVC3 which is a third member of the family of human genes that encode the voltage-dependent anion channel. The reasonable explantation for these distinct results has not been obtained yet. Whether there exists different kinds of XAP or funtional fragment interacting with pX or the repetitive results depend on the high transformation efficiency remains obscure. One of the possible mechanism may be a specific fragment can interact with pX specially and the proteins containing this fragment thus can bind to pX. Sequence analysis shown that both two true positive clones we isolated contained the sequence encodes glutacid-leucine -alanine, therefore, it's rational to deduce this short peptide is a required site for XAP binding to pX.

The interaction of proteins shall locate at nucleus in yeast two-hybrid system. However, some proteins require modification such as glycosylation outside the necleus after the expression, and others are only correctly folded and actived in the cooperation of some particular proteins which don't exist in yeast cells, so not all proteins can obtain normal structure and biological function in yeast cells. The cDNA library used for seeking XAP in the past research including Epstein-Barr virus transformed human peripheral lymphocyte cDNA library, Hela λ gt11 cDNA library or senescent human liver cDNA library, were all library consititued from abnormal cells.Our study had not isolated integrated cDNA sequence of XAP partly owing to the difference between normal human liver cDNA library and abnormal cells cDNA library in protein's expression, modification and activation.

In conclusion, the short peptide(glutacid-leucine-alanine) is a possibly required site for XAP binding to pX. Normal human liver cDNA library has difficulties in expressing the integrated XAP on yeast cells.

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