Universidade Estadual de Campinas

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MUTAGÊNESE DA PROTEÍNA HBX DO VÍRUS DA HEPATITE B E ESTUDO DA INTERAÇÃO COM RNA E PROTEÍNAS HUMANAS

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Funcional e Molecular, na área de Bioquímica.

Orientador: Dr. Jörg Kobarg

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"Por uma coisa eu lutaria até o fim,

tanto em palavras como em atos se eu pudesse – que se nós acreditássemos que devemos tentar descobrir o que não é sabido, seríamos melhores e mais corajosos e menos prepotentes do que se acreditássemos

que aquilo que não sabemos é impossível de ser descoberto e

que não precisamos nem mesmo tentar conhecer."

Sócrates, em Mênon, de Platão

Dedico este trabalho...

aos meus pais,

José Eduardo Rui & Maria Inêz Defavare Rui

para minha esposa, companheira e amiga

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aos nossos queridos filhos

Luan & Victor

aos meus irmãos,

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...e a toda humanidade...

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Resumo

A hepatite B constitui um grave problema de saúde pública. Dados epidemiológicos estimam que aproximadamente 350 milhões de pessoas são portadores do vírus da hepatite B (HBV). Admite-se que a infecção evolui para a cura em média 95% dos casos, entretanto nos portadores crônicos a infecção pode evoluir para cirrose e carcinoma hepatocelular (HCC). Há um crescente acúmulo de evidências que relaciona a proteína HBx do HBV ao desenvolvimento do HCC. A maioria dos estudos sobre a função da proteína HBx sugere que ela é uma proteína reguladora de funções pleiotrópicas com a capacidade de induzir o crescimento tumoral. Isso é possivelmente devido à sua capacidade de interagir com uma vasta gama de proteínas celulares.

No presente estudo investigamos os aspectos moleculares da estrutura e função da proteína HBx e os resultados foram contextualizados no processo de transformação celular. Observamos pela primeira vez a capacidade da proteína HBx em se ligar ao RNA contendo seqüências ricas em adenina e uracila (AU), que são presentes em alguns proto-oncogenes como c-*myc* e c-*fos*. A geração de proteínas truncadas permitiu mapear a região de interação entre HBx e RNA. Realizamos ensaios de mutação sítio-dirigida em HBx e substituímos todas as cisteínas por serinas. Nossos resultados sugerem que as cisteínas na proteína HBx são de menor importância para a sua interação com o RNA e com as proteínas humanas p53 e RXR. Descobrimos ainda uma nova proteína humana que interage com HBx: E4F. Este fator de transcrição humano está relacionado com o controle do ciclo celular, com a segregação cromossômica e com a embriogênese. Ensaios em leveduras e *in vitro* mostraram que HBx selvagem, bem como as suas formas mutadas, foram capazes de interagir e regular a função de E4F, indicando um possível novo mecanismo para a transformação celular e a regulação da transcrição viral, uma vez que E4F exibiu uma capacidade de interagir com a região "*enhancer* II" do genoma do HBV.

Abstract

Viral hepatitis is an important global public health problem. Epidemiological data show that worldwide 350 million people are chronically infected with the hepatitis B virus. About 95% of the infections cure spontaneously, but the chronically infected patients may develop liver cirrhosis or even hepatocellular carcinoma (HCC). A large body of evidence points to the viral onco-protein HBx as the principal cause for the cellular transformation. The majority of studies on HBx function suggest that it is a regulatory protein with pleiotropic functions and its capacity to induce tumor growth may be due to its ability to interact with a diverse array of cellular proteins.

In the present study we investigated molecular and structural aspects of the function of the HBx protein in order to be able to shed light on the process of cellular transformation. We were able to demonstrate that HBx protein has the ability to bind to an AU-rich RNA sequences present in the mRNAs of certain proto-oncogenes such as c-*myc* and c-*fos*. The generation of truncated proteins allowed to map the region of interaction of HBx with the RNA. Furthermore, we performed site directed mutagenesis studies of HBx protein and substituted all of its cysteine residues with serines. Our data suggest that the cysteine residues in the HBx protein are of minor importance for its interaction with RNA, p53 and RXR proteins. Finally, we discovered in E4F a new human interacting protein partner for the HBx protein. The transcription factor E4F has been functionally implicated in the control of the cell cycle, the chromosomal segregation and embriogenesis. In studies using the yeast we further showed that wild-type HBx, as well as its mutated forms, can physically interact with the E4F protein and regulate its function. This indicates a possible new mechanism of cellular transformation and the regulation of the viral transcription, since the human protein E4F demonstrated the capacity to bind to the *enhancer II* region of the HBV genome.

Índice

I - Introdução

As hepatites virais são doenças provocadas por diferentes agentes etiológicos com tropismo pelo tecido hepático, e que apresentam diferentes características epidemiológicas, clínicas, imunológicas e laboratoriais. Podem ser agrupadas, segundo o modo de transmissão, em dois grupos: as de transmissão parenteral/sexual (hepatite B, C e D), e as de transmissão fecal-oral (hepatite A e E). As hepatites A e E são doenças autolimitadas, cuja morbimortalidade depende da faixa etária acometida e de outras condições. As hepatites B, C e D provocam infecções crônicas em percentual variado das pessoas infectadas, e podem evoluir para insuficiência hepática grave (cirrose) ou hepatocarcinoma.

As hepatites virais são um importante problema de saúde pública no mundo. A prevalência do antígeno-HBV é menor (menos que 1%) nos países desenvolvidos, como na Europa e nos EUA. Altas taxas (entre 5% a 20%) estão presentes em países como China, nos países da África, do Oriente Médio e da América do Sul. A Organização Mundial de Saúde estima que cerca de dois bilhões de pessoas já tiveram contato com o vírus da hepatite B (HBV). No mundo, são cerca de 350 milhões de portadores crônicos da hepatite B e 170 milhões da hepatite C. No Brasil, o Ministério da Saúde (MS) estima que pelo menos 15% da população já teve contato com o vírus da hepatite B e os casos crônicos de hepatite B e C devem corresponder a cerca de 1,0% e 1,5% da população brasileira, respectivamente (MS/Brasil, 2002).

A maioria das pessoas desconhece seu estado de portador e constituem elo importante na cadeia de transmissão do HBV e HCV, que perpetua as duas doenças. Os estudos epidemiológicos sobre a hepatite B na população brasileira são poucos e em populações restritas. Apesar da escassez de informações sobre a infecção pelo vírus da hepatite B no Brasil, sabe-se que a bacia amazônica é a região de mais alta prevalência desta infecção (Torres, J.R., 1996). Outras regiões brasileiras com alta prevalência do HBV são parte do Estado do Espírito Santo, o oeste dos Estados do Paraná e de Santa Catarina (Souto, F.J.D., 1999). Os bancos de sangue identificaram no ano de 2000, cerca de 12.600 portadores do HBV e 13.000 portadores do HCV entre dois milhões de doadores. Entre os 386 transplantes hepáticos realizados no ano de 2001, ao custo de R$ 23.096.373,00, as hepatites virais foram a principal indicação do procedimento. Inquestionavelmente, as hepatites virais, principalmente as formas crônicas da hepatite B e C, provocam grande morbimortalidade na população brasileira.

A hepatite B apresenta cura espontânea em até 95% dos casos, mas a taxa de cronificação varia de acordo com a idade da infecção, de 85% em recém-nascidos e entre 6% a 10% em adultos. Cerca de 50% dos doentes crônicos desenvolvem cirrose hepática ou carcinoma hepatocelular.

O genoma do HBV é constituído por um DNA circular de fita parcialmente dupla com 3,2 kb de comprimento. Filogeneticamente, o HBV está relacionado aos retrovírus e pertence à família hepadnaviridae, gênero orthohepadnavirus (Schaefer, S. et al, 2003). O genoma do HBV tem quatro ORFs, as quais são denominadas de S, C, P, e X. A região S codifica glicoproteínas da superfície do vírus, enquanto que C codifica a proteína do núcleocapsídeo e o gene P é a região codificadora para a transcriptase reversa. A ORF X codifica a proteína transativadora HBx, que pode aumentar a expressão de genes homólogos e heterólogos em ensaios de cultura de células (Hollinger, F.B., 1996).

A proteína HBx é sintetizada na célula durante o processo de infecção viral, e consiste em 154 resíduos de aminoácidos, com um peso molecular de 17 kDa. Evidências apontam que ela está relacionada com o desenvolvimento do HCC (Hohne, M. et al, 1990; Lo, S.J. et al, 1988). Em ratos transgênicos para HBx, há uma suscetibilidade extremamente alta para o desenvolvimento de tumores no fígado: 75% dos animais HBx-transgênicos desenvolvem HCC (Kim, C.M. et al, 1991). Esta descoberta está de acordo com os resultados epidemiológicos, confirmando a existência de uma forte relação entre a infecção pelo HBV e o aparecimento de tumores de fígado. Tais dados enfatizam uma função crucial da proteína HBx no estabelecimento do HCC.

Vários efeitos em diferentes níveis de ativação gênica têm sido propostos para o mecanismo de ação da proteína HBx, por diferentes pesquisadores. Hojima sugeriu que HBx pode desenvolver a formação de hepatocarcinoma celular por interferir com a atividade das telomerases durante o processo de proliferação dos hepatócitos (Kojima, H. et al, 2003). Forgues examinou o efeito de HBx sobre os centrossomos e descobriu que a proteína viral induz a formação de fusos multipolares, provenientes da ocorrência anormal de vários centrossomos e que tal fenômeno ocasionaria um aumento na freqüência de mitoses anômalas e a transmissão de erros aos cromossomos (Forgues, M. et al, 2003). HBx também está associada com o estresse oxidativo com significante dano ao fígado. Em células transfectadas com HBx, Yi observou a diminuição da expressão da selenoproteína e o aumento de TNF-α ocasionando um aumento de três vezes na peroxidação de lipídios (Yi, Y.S. et al, 2003). A angiogênese e o processo de metástase são importantes durante a hepatocarcinogênese. Yoo explorou a possibilidade de HIF-1a, um potente

indutor transcricional de fatores angiogênicos, ser alvo de HBx e seus estudos revelaram que a HIF-1a foi estabilizada e translocada para o núcleo da maioria das células analisadas (Yoo, Y.G. et al, 2003). Tal como a precisa origem do câncer de fígado, desencadeado por HBx ser um mistério, os processos pró-apoptóticos também são pouco compreendidos. Shirakata mostrou que a localização de HBx na mitocôndria fez com que as células transfectadas entrassem em apoptose devido a uma perda do potencial de membrana da mitocôndria, que foi desencadeada pela disfunção da permeabilidade do complexo da transição do poro (PTP) (Shirakata, Y. e Koike, K., 2003). Um outro trabalho demonstrou a interação direta entre HBx e a proteína inibitória FLICE, um regulador chave no complexo sinalizador indutor de morte celular (DISC). O seqüestro de FLICE por HBx tornou a célula hipersensível para o sinal apoptótico de TNF-α (Kim, K.H. e Seong, B.L., 2003).

Hoje é aceito que a proteína viral HBx é responsável pela transformação celular. Muitos estudos, com enfoque molecular, foram realizados com esta proteína na tentativa de elucidar o mecanismo pelo qual ela é capaz de transformar uma célula normal em uma célula tumoral. As pesquisas com a proteína HBx, até o momento, confirmam que sua a capacidade de induzir a tumorogenicidade, e outros efeitos pleiotrópicos, é devida à sua habilidade em interagir com uma diversidade de proteínas celulares, cuja função abrangem os fatores de transcrição: RXR (Kong, H.J. et al, 2000), TBP (Wang, H.D. et al, 1998), RPB5 (Cheong, J.H. et al, 1995), TFIIB (Haviv, I. et al, 1998); proteínas de reparo ao DNA UV-DDB1 (Bontron, S. et al, 2002), ERCC2 (Qadri, I. et al, 1996a) e ERCC3 (Wang, X.W. et al, 1994); proteínas classificadas como supressores de tumor p53 (Chung, T.W. et al, 2003), pRB (Choi, B.H. et al, 2001); na transdução da sinais celulares ERK (Noh, E.J. et al, 2004); no metabolismo de RNAs (Rui, E. et al, 2001); no controle do ciclo celular Ciclina A (Bouchard, M. et al, 2001) entre outros. A cada ano novos trabalhos geram mais informações sobre HBx, fornecendo importante contribuição para a compreensão da interferência da proteína viral nos processos celulares. Algumas destas publicações relaciona HBx com outras novas proteínas celulares como apolipoproteína-B (Kang, S.K. et al, 2004), metalloproteinase-9 (Chung, T.W. et al, 2004), HSP60 (Tanaka, Y. et al, 2004) e HIF-1alfa (Moon, E.J. et al, 2004). Assim, acumulam-se muitas evidências que sugerem um caráter multifuncional da proteína HBx nas células hospedeiras.

II - Objetivos

O objetivo geral deste trabalho foi contribuir com novas informações sobre como se processa a transformação celular desencadeada pela proteína HBx do vírus da hepatite B em células hepáticas. Analisamos a estrutura e a função desta proteína viral com o intuito de compreender sua multifuncionalidade nas células hospedeiras e entender como a proteína HBx tem a capacidade em interagir com várias proteínas humanas.

Objetivos específicos

1. Expressar e purificar a onco-proteína HBx do vírus da hepatite B em bactéria (*E. coli*.) para estudos estruturais e funcionais.

2. Desenvolver um teste de atividade biológica rápido e simples para estudar a funcionalidade da proteína recombinante HBx produzida em *E.coli*.

3. Gerar proteínas HBx truncadas para mapeamento de sítios de interação com RNA.

4. Realizar estudos estruturais com a proteína HBx por técnicas espectroscópicas como dicroísmo circular, a fluorescência e ressonância nuclear magnética.

5. Realizar ensaios de mutação sítio-dirigida para investigar a importância das cisteínas sobre a função e a estrutura da proteína HBx.

6. Investigar pela técnica de duplo-híbrido em levedura a interação com proteínas humanas relacionadas ao controle do ciclo celular ou com a transformação celular.

III - Materiais e métodos

Os reagentes químicos utilizados no preparo de soluções e nos experimentos de purificação de proteínas foram obtidos das empresas Aldrich, Fluka, Merck, Sigma ou Synth. No caso dos experimentos de biologia molecular, os reagentes específicos, as enzimas de restrição e os seus tampões adequados, foram obtidos das empresas Amersham Pharmacia Biotech, Fermentas, GIBCO-BRL, New England BioLabs, Promega, Roche ou Stratagene. Os "kits" apropriados foram obtidos das empresas Amersham Pharmacia Biotech, PE Applied Biosystems, Pharmingen, QIAgen, Roche, Santa Cruz Biotechnology ou Stratagene, e estão indicados no texto conforme a sua utilização. As seqüências dos oligonucleotideos usados neste projeto estão na tabela 1 e a composição das soluções, dos tampões e dos meios de cultura está descrita na tabela 2.

1 - Genes, vetores e células

Linhagens de bactérias (*Escherichia coli)*

Neste trabalho foram utilizadas duas linhagens de bactérias *Escherichia coli (E. coli)*: DH5α (Hanahan, 1983) para propagação de plasmídeos e BL21 (DE3) para expressão de proteínas (Studier e Moffat, 1986).

Linhagens de leveduras *(Saccharomyces cerevisiae)*

Linhagem W303 (genótipo *ade*2-1/*ade*2-1; *ura*3-1/*ura*3-1; *his*3-11,15/*his*3-11,15; *trp*1-1/*trp*1-1; *leu*2-3,112/*leu*2-3,112).

Linhagem L40 (genótipo *ade*2-1/*ade*2-1; *his*3-11,15/*his*3-11,15; *trp*1-1/*trp*1-1; *leu*2-3,112/*leu*2-3,112).

Obtenção do gene HBx

O plasmídeo pCMV-ad contendo o gene que codifica a proteína HBx selvagem (1-154 aminoácidos; subtipo *ayw*) foi cedido pelo Dr. Robert. J. Schneider da Universidade de Nova York (Estados Unidos). O plasmídeo pCMV-ad/HBx foi usado como "*template"* (molde) na reação de polimerização em cadeia (PCR) para amplificar o gene HBx, para gerar proteínas truncadas, para inserir mutações pontuais e para posteriores subclonagens nos vetores de expressão em *E.coli* e leveduras.

Obtenção do gene p53

O plasmídeo pGEX-2TK contendo o cDNA completo da proteína p53 humana em fusão com GST foi fornecido pelo Dr Gianni Del Sal (Laboratorio Nazionale CIB, Trieste, Itália). O cDNA de p53 foi usado como "*template"* em ensaios de PCR para amplificar o gene e para posterior subclonagem no vetor de expressão em leveduras pGAD424.

Obtenção do gene E4F

O plasmídeo pCITE/E4F2.5Kb contendo o cDNA completo de E4F, cedido pelo Dr Robert J. Rooney da *Duke University*/USA, foi usado como *template* em PCR para a amplificação do gene e para as subseqüentes subclonagens nos vetores pACT2 e pGex-5X2, para a geração das proteínas truncadas.

Obtenção dos genes RXR e UV-DDBI

O cDNA das duas proteínas foram isolados, em nosso laboratório, de clones de leveduras selecionadas em ensaios de duplo-hibrido, por sua capacidade em interagir com a proteína HBx.

Vetor de expressão em bactérias pET-28a +

No vetor pET 28a+, fornecido pela Novagen, a seqüência do cDNA de uma proteína pode ser inserida no pET28a+ sob o controle do promotor T7 (induzível por IPTG) e ser utilizado para transformar *E. coli* (BL21). Como resultado, a proteína recombinante possui um sítio de clivagem por trombina na parte N-terminal da proteína seguida por uma sequência que codifica consecutivamente seis resíduos de histidinas (His-*tag*) que foi utilizado em nossos trabalhos para os processos de purificação. Este plasmídeo possui 5369 pares de bases (pb) e um gene que confere resistência ao antibiótico kanamicina à bactéria. Os cDNAs inseridos neste vetor podem ser seqüenciados pelos oligonucleotídeos pET-S e pET-AS (tabela 1)

Vetor de expressão em bactérias pGEX-2TK

O vetor pGEX-2TK, da empresa Amersham Pharmacia Biotech, foi desenhado para induzir altos níveis de proteína intracelular em fusão com a proteína GST (*Glutathione S-Transferase*). O gene codificante de uma proteína pode ser inserido (via *Bam*HI e *Eco*RI), fusionado ao GST,

subclonado em *E. coli* (BL21) e, sob o controle do promotor *tac* pode-se induzir a expressão da proteína recombinate por IPTG. A proteína recombinante possui ainda um sítio de clivagem para trombina codificada pela região 918-935 do vetor, qual pode ser utilizada na clivagem entre a proteína de interesse e o GST. Este plasmídeo possui ~4,9 kb e contém um gene que confere resistência ao antibiótico ampicilina à bactéria.

Vetor de expressão em levedura pGAD424 e pACT2

Ambos os vetores de expressão em levedura são fornecidos pela Clontech. Estes vetores possibilitam a expressão de uma proteína híbrida (sob o controle do promotor ADHI) que possuí o domínio de ativação da transcrição de Gal4 na parte C-terminal. Os cDNAs inseridos nestes vetores, proveniente de uma biblioteca, podem ser seqüenciados pelos oligonucleotídeos Gal4 e ScrAmp-AS (tabela 1). Como marcador de seleção os vetores contém o gene de resistência a ampicilina para propagação em *E. coli*. Em leveduras, complementam a via metabólica para a biossíntese de leucina (LEU2).

Vetor de expressão em levedura pBTM116

Vetor de expressão em levedura fornecido pela Clontech. O vetor pBTM116 possibilita a expressão de uma proteína híbrida (sob o controle do promotor ADHI), que possuí o domínio de ligação ao DNA LexA na parte C-terminal e uma proteína de interesse como "isca" na parte N-terminal. Contém o gene de resistência a canamicina para propagação em *E. coli*. Em levedura, complementa a via metabólica para a biossíntese de triptofano (Trp1). Os cDNAs inseridos neste vetor podem ser seqüenciados pelos oligonucleotídeos pBTM-S e pBTM-AS (tabela 1).

Vetor de integração ao genoma de levedura pLacZi

O plasmídeo pLacZi foi desenhado pela Clontech para integrar-se ao genoma da levedura carregando; uma seqüência de DNA de interesse, o promotor mínimo *iso-1-cytochrome C* (PCYC1), o gene repórter LacZ e o gene URA3 de complementação para biossíntese da uracila como marcador de seleção em levedura. O gene URA3 é usado como um marcador de integração dentro do *lócus* não funcional de uracila, da linhagem *Saccharomyces cerevisiae* cepa W303. O DNA alvo é inserido no sito de clonagens múltiplas a *upstream* do PCYC1 e do repórter LacZ. O pLacZi não pode se replicar autonomamente em levedura pois não há no vetor o elemento de

origem em levedura. Este plasmídeo contém o gene de resistência à ampicilina para seleção e propagação em *E.coli.* Os DNAs inseridos neste vetor podem ser seqüenciados pelo oligonucleotídeo PLacZi-S (tabela 1)

A biblioteca de cDNA de cérebro fetal humano

Uma Biblioteca de cDNA de cérebro fetal humano foi adquirida da Clontech (Cat.n° HL4028AH). Os cDNAs provenientes deste tecido foram inseridos no vetor pACT2 em fusão com o gene que codifica o domínio de ativação da proteína GAL4 de levedura. O uso de uma biblioteca oriunda de um tecido em fase embrionária é recomendado pelo fato de que as células estão em plena atividade metabólica. Muitos dos genes quiescentes no tecido "maduro" são ativos na fase embrionária. Para a amplificação da biblioteca transformamos células de *E. coli* (cepa BNN132) para que se obtivesse um número aproximado de 0.5 x 10^6 clones. As células transformantes foram plaqueadas em placas de 150 mm de diâmetro e incubadas a 37 $^{\circ}$C por toda noite. As colônias foram coletadas acrescentando-se 5ml de meio LB-A/Glicerol 20% por placas e depois de homogeneizadas, as células foram aliquotadas em tubos de 50ml e estocados a –80 $^{\circ}$C. Duas alíquotas foram utilizadas para extração dos plasmídeos por maxi-preparações de acordo com as especificações do fabricante do *QIAgen Plasmid – Maxi-Purification* (QIAgen).

2 - Manipulação de ácidos nucléicos

Amplificação de DNA pela reação de PCR

A 1 µl do DNA *template* (cerca de 40 ng) foram adicionados 1 µl (10 pmol) dos oligonucleotídeos *sens*e e *anti-sense* (específicos para cada ensaio), 1 µl de dNTP a 10 mM, 2,5 µl do tampão Taq DNA polimerase 10X, 1 µl Taq DNA polimerase (0,1 U/µl), e 16,5 µl de água destilada para totalizar um volume de 25 µl de reação, realizadas sempre em triplicata.

Em média, ensaios de PCR consistiram de 30 ciclos de amplificações (desnaturação a 95 ^{o}C por 1 minuto; anelamento por 1 minuto, com temperatura variável entre 50 ^{o}C a 65 ^{o}C; extensão a 72 ^{o}C por 3 minutos) seguidos de uma extensão final de 72 ^{o}C por 5 minutos.

Purificação de fragmentos de DNA e de produtos de PCR

Os produtos de amplificação por PCR foram purificados com o *QIAquick spin – QIAquick PCR purification kit* (QIAgen) ou com o *QIAquick Extraction Gel Kit* (QIAgen), de acordo com os protocolos fornecidos pelo fabricante.

Digestão de DNA com endonucleases de restrição

Os plasmídeos e os produtos de amplificação por PCR previamente purificados foram digeridos com as endonucleases de restrição específicas para cada clonagem (tabela 1), de acordo com a metodologia descrita em Sambrook (Sambrook, J. et al, 1989). Para cada μg de DNA utilizou-se 1 U (unidade) de enzima para a digestão dos plasmídeos e dos produtos de amplificação por PCR, e as reações prosseguiram em banho termostatizado a 37 °C por um intervalo variável de 3 a 20 horas. As reações de digestão foram inativadas a 80 °C por 10 minutos.

Reações de ligação e transformação de bactérias competentes

As ligações dos vetores de clonagens aos fragmentos de DNA previamente digeridos e purificados foram realizadas de acordo com a metodologia descrita em Ausubel (Ausubel, F.M. et al, 1995). Os plasmídeos obtidos foram utilizados para a transformação de bactérias *Escherischia coli* cepa DH5α e BL21 de acordo com a metodologia descrita em Sambrook (Sambrook, J. et al, 1989). As colônias obtidas com a transformação de bactérias competentes (*E. coli* DH5α), e selecionadas através do PCR, foram incubadas em meio Luria-Bertani (LB) contendo 50 μg/ml de antibiótico específico (ampicilina ou canamicina) por 18h/200rpm/37^{o}C. O DNA plasmideal foi extraído com o *Miniprep Plasmid DNA kit*, *Midiprep Plasmid DNA kit* ou com o *Maxiprep Plasmid DNA kit* (QIAgen).

Para confirmar a presença dos insertos dos cDNAs, 4 μl (1,6 μg) de DNA plasmideal recombinante foram digeridos com as endonucleases de restrição específicas para cada subclonagem. Após 3 horas de incubação em banho-maria a 37^{o}C, as amostras de digestão foram submetidas a eletroforese em gel de agarose 1%. Aqueles plasmídeos contendo o inserto desejado foram seqüenciados e então transformados em bactérias competentes BL21, para posterior expressão da proteína recombinante, de acordo com a metodologia descrita em Sambrook (Sambrook, J. et al, 1989).

Seqüênciamento, análise e identificação dos clones.

Os DNAs foram seqüenciados com base na técnica de Sanger (Sanger, F. et al, 1977) utilizando-se um seqüenciador automático de DNA ABI PRISM 377 *Genetic Analyser* (Applied Biosystems). Reações de seqüênciamento foram feitas utilizando-se 400ng dos DNAs. Oligonucleotídeos foram utilizados na concentração de 3,2pmol. Todas as reações foram preparadas de acordo com a metodologia contida no manual de instruções do fabricante do *DNA Sequencing kit Big Dye (Terminator Cycle Sequencing Read Reaction* (*PE, Applied Biosystem*).

As seqüências geradas foram analisadas pelo *software* ORF Finder (*Open Reading Frame Finder*) quanto ao *frame* em relação ao vetor, início e término do inserto e presença ou ausência de código de término de tradução (*stop codon*) (http://www.ncbi.nlm.nih.gov/gorf/gorf.htm). Para a identificação dos cDNAs da biblioteca inseridos no plasmídeo pACT2 nós confrontamos o resultado do seqüenciamento com os dados depositados no banco de dados do NCBI (http://www.ncbi.nlm.nih.gov/) através *software* BLAST (www.ncbi.nlm.nih.gov/BLAST/) que compara a homologia entre seqüências de nucleotídeos.

Alinhamento

Depois do tratamento e análise das seqüências de DNA procedemos à decodificação, obedecendo o *frame* do vetor, para seqüências de aminoácidos. As seqüências foram alinhadas pelo *software* Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html). Checamos por alinhamento o tamanho, o início e o término do cDNA isolado em relação as seqüências das proteínas depositadas no NCBI.

Mutação sítio-dirigida

Os ensaios das mutações foram feitos por técnicas comuns de PCR, para as mutações C26S (lê-se cisteína 26 substituída por serina) e C137S, localizadas nas extremidades da molécula. Para as mutações localizadas na parte central da proteína foi utilizado uma segunda técnica desenvolvida pela empresa Stratagene, disponível no kit *"QuikChange™ Site-Directed Mutagenesis"*. Esta técnica permite a mutação sítio-específica em todos os plasmídeos dupla fita DNA (dsDNA, *double stranded DNA*), com mais de 80% de eficiência. O procedimento básico utiliza um vetor dsDNA com um inserto alvo e dois oligonucleotídeos sintéticos complementares,

contendo a mutação desejada (tabela 1). A enzima *Pfu* DNA polimerase estende e amplifica todo vetor e inserto por técnicas convencionais de PCR. O vetor selvagem foi depois digerido com a enzima Dpn I, que reconhece o DNA parental proveniente da bactéria por este estar metilado. Protocolos detalhados estão descritos no manual de instrução que acompanha o produto ("*QuikChange™ Site-Directed Mutagenesis*" – Catalog # 200518).

Eletroforese em gel de agarose e purificação de DNA

Os produtos de amplificação por PCR e os DNAs digeridos com enzima de restrição foram submetidos à eletroforese em gel de agarose a concentrações entre 0.8% e 1.2%. As eletroforeses foram corridas a 80 V (volts) em tampão TAE. Os géis foram corados em solução contendo brometo de etídio a concentração final de 1μg/ml e fotografados na câmera digital Kodak Digital Science, posteriormente as imagens foram analisadas qualitativa e quantitativamente pelo software 1D Image Analysis. Os fragmentos de interesse contidos nos géis foram purificados com o *QIAquick spin – QIAquick PCR purification kit* (QIAgen) ou com o *QIAquick Extraction Gel Kit* (QIAgen).

Sistema de duplo-híbrido em levedura

O sistema de duplo-híbrido em levedura (YTHS, *Yeast two-hybrid system*) é uma metodologia desenvolvida para identificar interações entre proteínas *in vivo* (Chien, C.T. et al, 1991; Fields, S. e Song, O., 1989; Fields, S. e Sternglanz, R., 1994). As interações proteína-proteína formam a base da ampla variedade de reações bioquímicas com funções regulatórias e a identificação de proteínas que interagem com a proteína de interesse é um aspecto essencial para a elucidação do funcionamento e da regulação desta proteína. O sistema de duplo-híbrido é um método muito sensível e de grande sucesso para identificar as interações protéicas, mas pode, além disso, ser também usado para definir domínios ou resíduos de aminoácidos que estão envolvidos, ou são necessários para a interação proteína-proteína. A técnica explora a estrutura dos fatores de transcrição que apresentam dois domínios fisicamente separados: o domínio de ligação ao DNA e o domínio de ativação da transcrição.

O domínio de ligação ao DNA se liga a uma seqüência promotora específica, que está situado no início do promotor de um gene repórter. O domínio de ativação atrai os componentes básicos do complexo de iniciação da transcrição. No nosso sistema duplo-híbrido, a proteína mini-HBx (isca)

foi fusionada ao domínio de ligação ao DNA, enquanto uma biblioteca de cDNAs – codificando outras proteínas (presas) – foi fundida ao domínio de ativação da transcrição. Caso haja alguma interação entre a proteína de interesse e qualquer outra proteína da biblioteca, um fator de transcrição completo e funcional será reconstituído e os "genes repórteres" His3 e LacZ, que estão sob seu controle, serão ativados, assim His3 complementará a via da biossíntese da histidina e LacZ expressará a "enzima repórter" β-galactosidase.

3 - Métodos de análise de proteínas

Transformação de levedura em pequena escala

Leveduras L40 foram inoculadas em 30ml de meio rico YPD e incubadas por 16h/200rpm/30^{o}C. As células foram recolhidas por centrifugação por 10min/1000rpm/25^{o}C, o sobrenadante foi retirado e as células lavadas em TE (1X) agitando-se em vórtex por 15s/ta (temperatura ambiente) e novamente coletadas por centrifugação. Ao *pellet*, foram adicionados na seguinte ordem: 640µl PEG MW 3350 (50%); 200µl acetato de lítio (1M); 100µl TE (10X), 100µl DTT (1M), 8µl DNA de esperma de salmão (2mg/ml, Sigma, cat No. L-1626) e 2µl do vetor para levedura (*miniprep*). Após a ressuspensão em vórtex, as células receberam choque térmico a 45^{o}C por 1h:30min. 200µl foram plaqueados em meio mínimo seletivo, em seguida as placas foram incubadas a 30^{o}C por 2-4 dias.

Transformação de levedura em larga escala

Leveduras L40 foram inoculadas em 30ml de meio seletivo SD-W (menos triptofano) e incubadas por 16h/200rpm/30^{o}C. As células foram recolhidas por centrifugação por 10min/1000rpm/25^{o}C e diluídas em 150ml de meio seletivo SD-W, após mais 16 horas as células foram novamente recolhidas e diluídas em 1l de meio seletivo, neste ponto o crescimento foi acompanhado até que atingisse uma absorbância de 0,6 (OD_{600}). Alcançada esta marca, as leveduras foram mais uma vez recolhidas por centrifugação, lavadas com 100ml de tampão TE, transferidas para um tubo de 50ml e centrifugadas, o sobrenadante foi descartado. Ao *pellet* celular foram acrescentados 8ml da solução TE/acetado de lítio, 100µl de DNA de esperma de salmão (2mg/ml, Sigma, cat No. L-1626) e 100µl da biblioteca (maxipreparação), seguido por agitação em vórtex por 1min/ta, depois foram adicionados 60ml de solução PEG/acetato de lítio. Toda a

mistura foi incubada por 30min/230rpm/30°C, adicionou-se mais 7ml de DMSO (100%) seguido por agitação em vórtex por 1min/ta, incubada por 15min/230rpm/45°C, recolhidas por centrifugação e ressupendida em 10ml de TE/acetado de lítio. Dessa solução usou-se 250µl para plaquear 40 placas de petri de 150mm de diâmetro com meio mínimo seletivo. As placas foram incubadas a 30°C por 2-4 dias.

Teste de auto-ativação do gene repórter por pBTM116/mini-HBx

As células transformantes L40 pBTM116/mini-HBx foram testadas quanto à sua capacidade de ativar os genes repórteres His3 e LacZ. Colônias crescidas em meio mínimo SD-W foram escolhidas e ressupendidas em 25µl tampão TE. Para averiguar se a construção LexA/HBx seria capaz de ativar o gene repórter His3, 2µl da solução contendo as células foram gotejadas em meio mínimo SD-WH (menos triptofano e histidina). 2µl das mesmas colônias foram gotejados em meio mínimo SD-W. Ambas as placas foram incubadas a 30°C por 2-4 dias. As colônias que esperadamente cresceram em SD-W foram testadas em relação a ativação do gene repórter LacZ pelo ensaio de β-galactosidase.

Teste de ativação do gene repórter" por pBTM116/mini-HBx com o domínio de ativação Gal4

As células transformantes L40 pBTM116/mini-HBx foram co-transformadas com o vetor pGAD vazio. Este vetor codifica o mesmo domínio de ativação Gal4 utilizado na biblioteca de cDNA de cérebro fetal humano fornecido pela Clontech. Colônias crescidas em meio mínimo SD-Wl foram escolhidas e ressupendidas em 250µl tampão TE. Para averiguar se a construção LexA/HBx teria a capacidade de interagir diretamente com a proteína do domínio de ativação Gal4 e assim ativar os genes repórteres, nós inoculamos vários clones em placas SD-WLH (menos triptofano, leucina e histidina) e SD-WL (menos triptofano e leucina) , e ambas as placas foram incubadas a 30°C por 2-4dias. As colônias que esperadamente cresceram em SD-WL foram testadas em relação à ativação do gene repórter LacZ pelo ensaio de β-galactosidase.

Extração dos plasmídeos de levedura

Clones transformados de L40 foram incubados em 10ml de meio mínimo seletivo a 30°C por 16 horas. 6ml do inóculo foram centrifugados por 30s/12000rpm/ta e ressuspendidos com 50 µl de

tampão TE, adicionou-se 20µl de liticase (1mg/ml), os microtubos foram vigorosamente homogeneizados por agitação em vórtex por 1min/ta seguido por 10 ciclos de choque térmico em nitrogênio líquido, o volume foi completado para 200µl com tampão TE para depois acrescentar 200µl de fenol:clorofórmio:álcool isoamílico (25:24:1); a mistura foi agitada novamente em vórtex por 1min/ta e centrifugada por 5min/14000rpm/ta. Ao sobrenadante foram acrescentados 600µl de etanol (100%) resfriado a –20^{o}C e 50µl de cloreto de lítio (8M). As amostras foram incubadas por 15min/-20^{o}C e centrifugadas por 5min/14000rpm/ta, o sobrenadante foi descartado e 500µl de álcool (70%) foram adicionados, depois de agitadas, centrifugadas e o sobrenadante descartado, o DNA precipitado foi seco por 1h/37^{o}C. Os plasmídeos foram ressuspendidos em 20µl de água e 10µl foram utilizados para a transformação de bactérias.

Ativação da transcrição baseada no sistema de mono híbrido em levedura.

O sistema de mono híbrido é baseado na descoberta de que muitos ativadores da transcrição em eucariotos são compostos de domínios funcionais fisicamente independentes, o domínio da ativação (AD) da transcrição e o domínio de ligação (BD) ao DNA. Esta característica permite construir várias fusões de genes que, quando expressos como proteínas de fusão em leveduras, podem simultaneamente ligar-se a uma seqüência de DNA alvo e ativar a transcrição de um gene repórter. Este sistema fornece uma ferramenta útil para realizar ensaios genéticos *in vivo* com a intenção de isolar novos genes codificantes de proteínas que se ligam a uma seqüência de DNA alvo. Basicamente, a técnica consiste em inserir no genoma da levedura um cassete contendo uma seqüência de DNA alvo posicionado a *upstream* de um promotor mínimo da levedura, o gene repórter LacZ e um marcador de seleção. A levedura transgênica é utilizada para a transformação com uma biblioteca de interesse, onde os genes estão fundidos com o domino de ativação da transcrição (AD) Gal4. Somente os clones que codificam proteínas híbridas que interagirem com o DNA alvo serão selecionados. Nós utilizamos o sistema de mono híbrido com o propósito de investigar se a proteína E4F seria capaz de reconhecer o *enhancer* II do HBV. Como controle usamos o promotor E4 do adenovirus, uma seqüência já descrita na literatura, que é reconhecida pela proteína humana. As transformações das leveduras e construções dos plasmídeos foram realizados conforme protocolo fornecido pela Clontech (*MATCHMAKER One-Hybrid System - User Manual*).

Ensaios com ONPG (*o-nitrophenyl-β-D-galactopyraniside*)

O ensaio com ONPG é usado para detectar a presença da β-galactosidase, uma enzima descoberta em leveduras que utiliza lactose como fonte de carbono. A utilização da lactose depende da presença de 2 enzimas: β-galactosidase permease, que catalisa o transporte da lactose para dentro da célula, e a β-galactosidase que cliva a lactose em galactose e glicose. A β-galactosidase não é específica somente para lactose, mas pode agir também em simples galactosideos como o ONPG (*o-nitrophenyl-β-D-galactopyranose*). A hidrólise do ONPG, pela β-galactosidase, resulta na liberação da galactose e do o-nitrofenol, um componente cromogênico amarelo cujo pico de absorção de luz é em 420nm. O gene LacZ, que codifica a β-galactosidase, quando ligado a certos promotores é empregado como repórter em ensaios de cunho quantitativo e qualitativo que almejam estudar a interação proteína-proteína ou a ativação da transcrição sob condições experimentais.

Expressão das proteínas recombinantes

As bactérias competentes *E. coli* (BL21) foram transformadas com os vetores de expressão contendo o cDNA de interesse. As células foram pré-inoculadas em meio LB contendo antibiótico específico (ampicilina ou canamicina) na concentração de 50 μg/ml, e incubadas por 18h/200rpm/37^{o}C. Após diluição 1:100 da pré-cultura em meio fresco LB+antibiótico, as bactérias foram incubadas sob as mesmas condições até atingirem a fase logarítmica (OD_{600} ~ 0,8).

A expressão das proteínas recombinantes foi induzida com a adição de IPTG na concentração final de 1 mM. Após incubação por 3h/37^{o}C ou 6h/25 ^{o}C, as bactérias foram coletadas por centrifugação por 20min/4000rpm/4^{o}C. Alíquotas de 1ml da cultura não-induzida e induzida com IPTG foram retiradas para posterior análise da indução das proteínas recombinantes através de géis de SDS-PAGE.

Purificação de proteínas

Foram utilizados dois sistemas de purificação de proteína. Cada qual possui características específicas que foram exploradas em nossos estudos.

Cromatografia de afinidade IMAC (*Immobilized Metal Affinity Chromatography*).

Protocolos detalhados são descritos no manual de instrução que acompanha o produto (*QIAexpress Ni-NTA Protein Purification System – The Qiaexpressionis*). Os seguintes tampões

foram utilizados na purificação em condições não desnaturantes: tampão PBS pH 7,4 para a manutenção das condições fisiológicas; tampão BS para a retirada de proteína que se ligam inespecificamente à coluna; tampão CS para a eluição da proteína de interesse. As soluções foram desgaseificadas em bomba de vácuo por um intervalo de 15 minutos. Concomitantemente foi aplicado 1ml de resina Ni-NTA em uma coluna BioLogic LP (Low Pressure) – BioRad, e após o empacotamento da resina, a coluna foi equilibrada com 20ml de tampão PBS pH 7,4. Em seguida a fração solúvel do lisado foi aplicada à coluna. A coluna foi lavada com 20ml do tampão BS. As proteínas recombinantes foram eluídas da coluna em 15 frações de 1ml com o tampão de CS.

Para proceder a purificação da proteína em situações em que esta se encontrava como corpos de inclusão (insolúveis), utilizamos soluções desnaturantes contendo uréia 8M. As soluções aplicadas para este processo devem possuir pHs diferenciados, pH 8.0 para que ocorra a ligação da proteína com a coluna, pH 6,3 para a remoção de compostos ligados inespecificamente e pH 4,5 para a recuperação da proteína interesse.

As soluções foram desgaseificadas em bomba de vácuo por um intervalo de 15 minutos. A fração insolúvel do lisado foi solubilizada com 20ml do tampão AI e centrifugada novamente por 20min/14000rpm/20^{o}C, o sobrenadante foi então filtrado a 0,22nm e, em seguida, o solubilizado foi aplicado em uma coluna (BioLogic LP) contendo 1ml de resina Ni-NTA previamente equilibrada com 20ml do tampão AI. A coluna foi então lavada com 20ml do tampão BI e, na seqüência, as proteínas recombinantes foram recuperadas da coluna em 15 frações de 1ml com o tampão de CI.

Cromatografia de afinidade por GST (*glutathione-S-transferase*).

Nesta purificação utilizou-se a fração solúvel do lisado e os tampões utilizados na purificação das proteínas em fusão com GST foram: tampão PBS pH 7,4 e tampão de eluição BG. As soluções foram desgaseificadas em bomba de vácuo por um intervalo de 15 minutos. 1ml de resina comercial *Glutatione-sepharose* 4B foi empacotada em uma coluna BioLogic LP e, após o empacotamento da resina, a coluna foi equilibrada com 20ml tampão PBS. Em seguida, a fração solúvel do lisado foi aplicada à coluna e, em seguida, lavada com 20ml do tampão PBS. A proteína em fusão com GST foi eluída em 15 frações de 1ml do tampão BG.

Marcação de sondas radioativas

Os oligonucleotídeos de RNA (tabela 1) foram marcados radioativamente nas suas extremidades 5' com [γ-^{32}P] dATP (Amersham Pharmacia Biotech) pela ação da *T4 polinucleotídeo kinase*, de acordo com a metodologia descrita em Sambrook e em Ausubel (Ausubel, F.M. et al, 1995; Sambrook, J. et al, 1989). Para um volume final de reação de 50 µl, adicionou-se 10 µl de [γ-^{32}P] dATP (cerca de 5 µCi), 1 µl de oligonucleotídeo de RNA na concentração de 30 pmol/µl, 1 µl de dATP não marcado na concentração final de 48,5 pmol/µl, 1 µl (20 unidades) de *T4 polinucleotídeo kinase* (Amersham Pharmacia Biotech), 5 µl de tampão OPA e 32 µl de água tratada com DEPC. A reação foi incubada a 37 ^{o}C por 1 hora quando foi interrompida pela adição de 2,5 µl de uma solução de EDTA 500 mM pH 8 (25 mM na reação).

Os oligonucleotídeos de RNA marcados radioativamente foram purificados numa coluna de gel filtração (colunas NAP-5, Amersham Pharmacia Biotech), de acordo com instruções do fornecedor, e alíquotas de 120 fmol de sondas marcadas foram utilizadas nas reações de interação com as proteínas recombinantes em fusão com a cauda de poli-histidina ou em fusão com a proteína GST.

Ensaios de retardamento de migração eletroforética gel (EMSA)

As proteínas HBx em fusão com GST foram utilizadas na análise das interações entre os oligonucleotídeos de RNA (tabela 1), marcados com o radioisótopo [γ-^{32}P] dATP, através de *EMSA*. Os oligonucleotídeos de RNA e as proteínas foram incubadas em gelo por 30 minutos no tampão de ligação EMSA, com um volume final de 25 µl.

Após a incubação em gelo, os complexos RNA-proteína foram imediatamente fracionados em um gel não-desnaturante a 6% (40:1 de acrilamida: bisacrilamida) como descrito em Wilson e Brewer (Wilson, G.M. e Brewer, G., 1999). As eletroforeses foram corridas a 200 V por cerca de 3 a 4 horas em tampão de corrida TBE. Os géis foram secos por aquecimento a 65 ^{o}C por 1 hora e 30 minutos, e submetidos à auto-radiografia a –80 ^{o}C por um período mínimo de 12 horas, utilizando o filme X-OMAT (Kodak, Rochester, NY).

UV-Cross linking RNA-proteína

A abordagem de indizir a formação de ligações covalentes (*Cross-Linking*) entre proteínas e ácidos nucléicos pode fornecer valiosas informações estruturais sobre a superfície de contato entre

estas duas moléculas. Apesar da existência de catalizadores químicos para a realização do *"cross-linking",* os métodos que envolvem a fotoestimulação são os preferidos quando se estuda a interação proteína-ácidos nucléicos, pois esta técnica possui a vantagem de provocar mínimas perturbações no sistema que se está estudando (Meisenheimer K.M. e Koch T.H., 1997)

As proteínas foram incubadas com os oligonucleotídeos de RNA marcados com o radioisótopo [γ-^{32}P] dATP no tampão de *Cross-Linking*, em um volume final de 25 µl. Após incubação à temperatura ambiente por 30 minutos, os tubos de reação com a tampa aberta foram expostos à luz ultravioleta (254 nm) por cinco minutos, numa distância de 100 mm, utilizando o aparelho *UV Stratalinker 2400* (Stratagene).

Os complexos RNA-proteína foram fracionados em gel de poliacrilamida a 12,5% sob condições desnaturantes, na presença de 5% de β-mercaptoetanol, e visualizados após uma exposição de 2-18 horas em placa BAS-IP MS2325 (Fujifilm), utilizando o aparelho *Phosporimager scan Bio-Imaging Analyzer* BAS-1800II (Fujifilm).

Western blot

As amostras de proteína foram separadas em géis de poliacrilamida a 10% ou a 12,5%. Após a eletroforese, o gel a ser transferido foi incubado na solução Ânodo II por cinco minutos. A membrana de PVDF (difluoreto de polivinila) *Immobilon^{TM}-P* (Millipore) foi pré-incubada em metanol e lavada com água MilliQ antes do uso.

Utilizou-se o sistema *Semi-Dry Blotting System* (W.E.P. Company) para a transferência das proteínas contidas no gel para a membrana de PVDF. Em contato com o ânodo do aparelho de transferência, foram colocados dois papéis (Whatman 3MM) embebidos na solução Ânodo I, e sobre estes um papel embebido na solução Ânodo II. Seguindo a montagem do "sanduíche", colocou-se a membrana (pré incubada em metanol e lavada com água MilliQ), o gel de poliacrilamida a ser transferido e três papéis embebidos no tampão Cátodo, que foram colocados em contato com o pólo negativo do aparelho de transferência. O tempo de transferência variou entre 1 hora e 1 hora e 30 minutos, a 50 mA por membrana.

Após a transferência, o "sanduíche" foi desmontado e a membrana foi bloqueada com a solução de bloqueio e incubada à temperatura ambiente por 3 horas ou a 4 ^{o}C por 18 horas. Depois da incubação em BSA, a membrana foi lavada três vezes com TBS 1X, antes da sua incubação, à

temperatura ambiente, com os anticorpos primários *anti-GST* ou *anti-penta His* (diluídos 1:3000 em TBS 1X) ou com o anti-soro anti-X (diluído 1:100 em TBS 1X).

Após duas horas de incubação, a membrana foi lavada três vezes com TBS 1X, e incubada por 2 horas com o anticorpo secundário *anti-mouse-IgG* (para os anticorpos primários *anti-GST* ou *anti-penta His*) ou com o anticorpo secundário *anti-rabbit-IgG* (para o anti-soro anti-X), diluídos 1:5000 em tampão TBS 1X.

Após a incubação com o anticorpo secundário, a membrana foi lavada três vezes com TBS 1X, antes da revelação utilizando o *Western Blotting Luminol Reagent* (Santa Cruz Biotechnology), de acordo com as especificações do fabricante. A exposição da membrana ao filme X-OMAT (Kodak) foi feita por intervalos de tempo de 30 segundos a 2 minutos, e depois os filmes foram revelados.

Absorbância no UV-Visível

A concentração das proteínas de fusão dialisadas contra diferentes tampões foi determinada através da medida de absorbância das mesmas a 280 nm (Edelhock, H., 1967). Desta forma, as proteínas foram previamente desnaturadas em solução de fosfato de sódio 15 mM pH 6,5 e guanidina-HCl 7,5 M. Os espectros de absorbância foram obtidos em um espectrofotômetro *JASCO V-530 UV/VIS* (JASCO), com varredura de comprimento de onda de 350-190 nm, utilizando-se uma cubeta de quartzo de 10 mm de caminho ótico.

Os espectros de absorbância das proteínas de fusão foram corrigidos com a subtração do espectro de absorbância do tampão correspondente ("branco"). A partir dos valores dos coeficientes de extinção molar (ε) obtidos através do programa *ProtParam* (http://ca.expasy.org) e dos valores de absorbância a 280 nm, determinou-se as concentrações molares das proteínas de fusão através da fórmula :

$$A_{280} = \varepsilon \times b \times c$$

onde $\mathbf{A_{280}}$ é a absorbância medida a 280 nm, **ε** é o coeficiente de extinção molar em 280 nm ($M^{-1}cm^{-1}$), **b** é o caminho ótico em centímetros (cm) e **c** é a concentração molar da amostra de proteína (M). As amostras de proteína foram centrifugadas a 2000 x g antes de serem utilizadas nos ensaios espectroscópicos de dicroísmo circular, fluorescência e de ressonância magnética nuclear.

Fluorescência

Os estudos de emissão intrínseca de fluorescência foram realizados no espectrômetro de luminescência *AMINCO-Bowman Series 2*, utilizando-se uma cubeta de quartzo de 10 mm de caminho ótico. A temperatura das amostras foi estabilizada a 25 ^{o}C ou a 37 ^{o}C através de um banho termostatizado com água circulante.

O comprimento de onda da excitação foi de 295 nm para as proteínas de fusão dialisadas contra diferentes tampões, e os espectros de emissão foram coletados entre 300 e 430 nm, com velocidade de varredura de 1 nm/segundo e tempo de resposta automático. Os espectros de emissão de fluorescência das amostras de proteína foram corrigidos com a subtração dos espectros de emissão dos tampões utilizados ("branco") e graficados utilizando-se o programa *ORIGIN 6.1*. Os valores de $\lambda_{máx}$ de emissão foram obtidos dos espectros de emissão de fluorescência.

Dicroísmo circular (CD)

Os experimentos de dicroísmo circular foram realizados no espectropolarímetro *JASCO J-810* (JASCO), com a temperatura controlada através de um sistema interno de controle de temperatura (*Peltier type control system* PFD 425S, JASCO). Os espectros de dicroísmo circular no UV distante (260-190 nm) foram adquiridos utilizando-se uma cubeta de quartzo de 1 mm de caminho ótico, com velocidade de varredura de 50 nm/minuto, tempo de resposta de 8 segundos e resolução de 0,5 nm. O espectro final foi obtido pela acumulação de 4 varreduras e corrigido com a subtração do espectro de dicroísmo circular do tampão correspondente.

Os dados gerados com a subtração dos espectros foram convertidos em elipticidade molar residual [θ], que é dada pela equação :

$$[\theta] = \theta / 10 \times l \times c \times n$$

onde **θ** é a elipticidade observada (degrees), **l** é o caminho ótico em centímetros (cm), **c** é a concentração molar (M) e **n** é o número de aminoácidos da proteína. Os dados convertidos foram graficados utilizando-se o programa *ORIGIN 6.1*.

As proteínas de fusão purificadas sob condições desnaturantes (uréia 8 M) foram renaturadas através de diálises diretas ou seqüenciais contra diferentes tampões (água ou PGDE), e utilizadas nos experimentos de dicroísmo circular. Para determinar a porcentagem de estrutura secundária para cada uma das três proteínas de fusão, utilizou-se o programa *CDNN deconvolution*

(http://bioinformatik.biochemtech.uni-halle.de/cdnn/), e uma base de dados contendo 33 espectros de CD de diferentes proteínas.

Ressonância Magnética Nuclear (RMN)

Para a proteína recombinante 6xHis-mini-HBx(-Cys) em água (pH 6,0), foi adquirido espectro de RMN ^{1}H monodimensionais no espectrômetro *Varian Inova 500* (campo magnético de 11,7 Telsa) operando à freqüência de ^{1}H de 499,730 MHz. Cerca de 500 µl da proteína 6xHis-mini-HBx(-Cys) em solução aquosa (pH 6,0), na concentração de ~400 µM, foi acrescida de 25 µl de D_2O (para uma concentração final de ~5%). A supressão da água foi feita pelas técnicas de pré-saturação, e os dados coletados foram processados utilizando-se o *software SpinWorks 2.0 beta*.

Os espectros das proteínas foram adquiridos em colaboração com a Dra Patrícia Ribeiro de Moura e a Dra. Thelma de Aguiar Pertinhez, do grupo de Ressonância Magnética Nuclear (RMN) do CEBIME/LNLS.

Artigo I

Expression of deletion mutants of the hepatitis B virus protein HBx in E. coli and characterization of their RNA binding activities.

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Expression of deletion mutants of the hepatitis B virus protein HBx in *E. coli* and characterization of their RNA binding activities

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Abstract

The hepatitis B virus protein HBx has been implicated in the development of liver cancer. It has been shown that the HBx protein is able to bind to single-stranded DNA in a specific manner. This DNA binding activity might be relevant for HBx oncogene character. To study the HBx interaction with nucleic acids in more detail we expressed full-length HBx as well as several N- and C-terminally truncated HBx proteins as 6xHis and GST-fusions in *E. coli*. Using a gel shift assay, we were able to demonstrate that all of the truncated HBx proteins have the ability to bind to an AU-rich RNA. The affinity of GST-HBx #3 (residues 80–142) was an order of magnitude higher than that of GST-HBx #2 (residues 5–79), indicating that a high affinity RNA binding site is located in HBx C-terminal half. AUF1 is the protein ligand that binds to AU-rich RNA regions present in certain proto-oncogene mRNAs and causes their rapid degradation. By a competitive binding experiment of AUF1 and HBx to the AU-rich RNA oligonucleotide, we show that HBx is able to displace AUF1 from its binding site on the RNA oligonucleotide. This new aspect of HBx function is discussed in the context of cellular transformation.

Keywords: Viral hepatitis; Hepatocellular carcinoma; Viral transactivator; Oncogene; mRNA stability

1. Introduction

Viral hepatitis, especially that caused by infection with hepatitis B virus (HBV), is a major worldwide public health problem (Hollinger, 1996). Worldwide over 250 million people are persistently infected with HBV. The prevalence of HBV-antigen is lowest (0.1–1%) in countries with the highest standards of living such as countries in Europe or the USA. Very high rates (5–20%) have been reported for China, Africa, the Middle East and South America including some parts of Brazil. Both epidemiological as well as experimental data indicate that chronic HBV infection is tightly associated with the liver cancer (hepatocel

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lular carcinoma, HCC) (Redeker, 1975; Robinson, 1994). The great majority of people that die from HCC have been infected with HBV in the past. HCC constitutes 90% of the primary malignant tumors of the liver and it is estimated that each year worldwide more than 500 000 people die from liver cancer (Hollinger, 1996).

HBV is a DNA virus with a small circular DNA genome of 3.2 kb length. It is phylogenetically related to the retroviruses in that it uses its own reverse transcriptase for DNA replication and belongs to the family of hepadnaviruses (for hepatotrophic DNA viruses) (Ganem, 1996). The HBV genome has at least four open reading frames (ORF) which are named S, C, P, and X. The C region encodes a protein of the HBV virus nucleoprotein, while the ORFs pre-S and S encode the viral surface glycoprotein and the P gene the viral reverse transcriptase. The ORF X encodes for the regulatory protein HBx which can enhance the expression of heterologous and homologous genes in trans in transient cell culture assays (Ganem, 1996; Hollinger, 1996). The HBx protein consists of 154 amino acids, possesses a molecular weight of $\sim$17 kDa and is translated in the course of the viral infection. HBx seems to have a central role in HBV mediated HCC (Lo et al., 1988; Hohne et al., 1990) since mice transgenic for HBx show an high susceptibility for the development of liver tumors: 75% of the HBx transgenic animals develop HCC (Kim et al., 1991). On the cellular and molecular levels, several functions have been attributed to the HBx protein, by different researchers. Some studies suggest that HBx might be involved in the induction of apoptosis (Chirillo et al., 1997; Su and Schneider, 1997). The most generally recognized function of HBx however is its ability to transactivate the transcription of a variety of cellular and viral promoters (Twu et al., 1990; Yen, 1996). Although HBx does not bind to double-stranded DNA directly it could be demonstrated that it binds in vitro to different transcription factors (Haviv et al., 1995; Maguire et al., 1991). It has recently been reported that HBx can interact with single-stranded DNA (ssDNA) in a sequence independent fashion (Qadri et al., 1996). Since the HBV DNA in the HBV virion consistently is partially single-stranded, HBx might also in vivo bind to the viral ssDNA and function in the replication of the virus DNA like it was reported for several other viral ssDNA binding proteins (Challberg and Kelly, 1989; Hang et al., 1995). It was also shown that HBx activates the reverse transcription of the viral pregenomic mRNA into genomic DNA (Klein et al., 1999). These studies showed that HBx can interact with ssDNA and suggested that this interaction might be of biological importance for HBx functions in the context of the HBV's life cycle. Since HBx can bind to ssDNA (Qadri et al. 1996), we speculated that it might also be able to interact with RNA. We further reasoned that if HBx can interact with RNA it might be able to compete with cellular mRNA binding factors like AUF1 (Zhang et al., 1993). To test these hypotheses we expressed full-length HBx protein as well as of N- and C-terminally truncated HBx mutants in *E. coli*. As the RNA test oligonucleotide we chose the AU-rich sequence to which AUF1 binds and which represents the putative destabilizing element found in the 3′-region of rapidly degraded mRNAs like those of certain proto-oncogenes and cytokines (Jones and Cole, 1987). In this paper we show that HBx binds to this AU-rich RNA oligonucleotide in an inhibitable manner, characterize HBx RNA binding domains and demonstrate that HBx can displace the cellular AU-rich mRNA binding factor AUF1 from the RNA oligonucleotide. The possibility that HBx displacement of cellular factors from proto-oncogene mRNAs in vivo might contribute to HBx oncogene character is discussed.

2. Materials and methods

2.1. Construction of recombinant HBx-psW 202 plasmids

DNA fragments encoding the full length or N- or C-terminally truncated HBx protein were amplified by PCR methodology and directionally cloned into the bacterial expression vector psW202 (W. Wels, Freiburg, Germany) (Klimka et al., 1996; Wels et al., 1995) via *Hind*III (sense

oligonucleotides) and *Xba*I (anti-sense oligonucleotides) restriction sites. The vector psW202 has *Hind*III and *Xba*I restriction sites, which allow the directional insertion of a DNA sequence to be expressed under the control of a tac-promotor. An omp A signal peptide encoding sequence and a six-histidine tag encoding sequence are located upstream of the insertion site. While the omp A signal sequence directs the recombinant fusion protein to the periplasmatic space of *E. coli* the 6xHis tag which will be located N-terminal of the recombinant protein partner allows the purification of the recombinant fusion protein via Ni-affinity chromatography. In all HBx construction sequences encoding a flexible linker region (GSGSG) and an enteropeptidase cleavage site (DDDDK) have been introduced via the sense oligonucleotide sequences. For the different HBx constructions the following oligonucleotides were used: HBx (1–154): sense 1 [5′GCA CTG AAG CTT GGT TCT GGT TCT GGT GAC GAC GAT GAC AAG ATG GCT GCT AGG TTG TGC TGC 3′] and anti-sense 1 [5′A GTC TCT AGA TTA TTA GGC AGA GGT GAA AAA GTT GCA TGG 3′]; HBx (1–142): sense 1 and anti-sense 2 [5′ TG GAA TTC TCT AGA TTA TTA GAC CAA TTT ATG CCT ACA GCC 3′]; HBx (1–77): sense 1 and anti-sense 3 [5′ TG GAA TTC TCT AGA TTA TTA GCG ACG TGC AGA GGT GAA GCG 3′]; HBx (48–154): sense 2 [5′ GCA CTG AAG CTT GGT TCT GGT TCT GGT GAC GAC GAT GAC AAG GAC CAC GGG GCG CAC CTC TCT 3′] and anti-sense 1; HBx (79–154): sense 3 [5′ GCA CTG AAG CTT GGT TCT GGT TCT GGT GAC GAC GAT GAC AAG GAG ACC ACC GTG AAC GCC CAC 3′] and anti-sense 1; HBx (48–142): sense 2 and anti-sense 2; HBx (79–142): sense 3 and anti-sense 2 (Fig. 4). Amino acid numbering refers to the 154 amino acid long full-length HBx protein of the *ayw* subtype (HBV genotype D, Gene bank accession no.: J02203) (Galibert et al., 1979). For the PCR amplification 30 cycles consisting of the following steps were used: 95°C (50 s), 50°C (1 min), 72°C (3 min, last cycle 8 min). HBx-DNA containing pCMV-Ad plasmid, kindly provided by Dr. R. Schneider, New York (New York University), served as the template DNA in the PCR reaction. Amplified PCR products were then digested with *Hind*III and *Xba*I and ligated into vector psW202. DH5α *E. coli* cells were transformed with the recombinant vectors. Recombinant plasmid DNA isolated from the DH5α cells was subsequently transformed in *E. coli* BL-21 cells, for the expression of the HBx proteins.

2.2. Construction of recombinant HBx-pGEX-2TK plasmids

To express selected HBx proteins as fusion proteins with the Glutathione *S*-transferase (GST) their DNAs were inserted into the bacterial expression vector pGEX-2TK (Amersham Pharmacia). Using the full-length HBx DNA inserted into pCMV-Ad as a template we amplified the three HBx constructs with the following primer combinations: GST-HBx-complete (5–154): X-GST-sense [GC GGA TCC TTG AGC TGC CAA CTG GAT CCT GCG CGG] and X-GST-anti-sense [GC GAA TTC TTA GGC AGA GGT GAA AAA GTT GC]; GST-X #2 (5–78): X-GST-sense and anti-sense 3; GST-X #3 (80–142): X-GST-sense 2 [GC GGA TCC GAG ACC GTG AAC GCC CAC], and anti-sense 2 (Fig. 5). An internal *Bam*HI restriction site was deleted by introducing a silent mutation via the oligonucleotide X-GST-sense. To keep this oligonucleotide at a reasonable size the first nucleotides encoding the first four amino acids were spared. The PCR products were digested with *Eco*RI and *Bam*HI and inserted into the vector pGEX-2TK in frame with GST.

2.3. Expression of HBx proteins

E. coli strain BL-21, transformed with the HBx DNA containing psW202 or pGEX-2TK vectors, was grown in LB medium containing ampicillin (50 μg/ml) to an $OD_{600} = 0.7$, followed by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and induced for the indicated times at 37°C. Cells were harvested by centrifugation and lysed by the addition of lysozyme (1 mg/ml), incubation for 20 min at room temperature (RT) and three subsequent

freeze-thaw cycles. Genomic DNA was digested by addition of DNAse (1 μg/ml) and incubation for 20 min at 37°C. The bacterial lysate was separated in soluble and insoluble fractions by centrifugation at 13 000 $\times g$ for 15 min at 4°C. After dissolving the protein samples from both fractions in reducing SDS-gel loading buffer for 2 min at 95°C the presence of recombinant protein was analyzed in a 12% SDS-PAGE gel, stained with Coomassie blue.

2.4. Purification of HBx-6His fusion proteins

A bacterial pellet derived from a 1 l culture induced as described above was lysed by addition of 1 mg/ml lysozyme and three cycles of passage through the French press. After centrifugation, the pellet containing the HBx inclusion bodies was solubilized in 20 ml of a solution of 6 M guanidinium–HCl, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8. The resulting solution was applied to a Ni-NTA column (Qiagen). The subsequent washing and elution steps were all carried out with buffer A (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl) but at different pHs. The column was washed first with buffer A, pH 8 and then with buffer A, pH 6.3. Bound protein was eluted with a linear pH gradient between 6.3 and 4.5 in buffer A. The column eluate was collected in 2-ml fractions and 10 μl aliquots were analyzed by SDS-PAGE. Peak fractions were pooled and dialyzed against renaturing buffer 1 (50 mM Pipes, pH 6, 0.5 mM PMSF) at RT with several changes of the buffer. Dialyzed protein was cleared by centrifugation at 2000 $\times g$ and used for the Electrophoretic mobility shift assays (EMSA).

2.5. Digestion with enteropeptidase

The full-length recombinant HBx-6xHis fusion protein from the peak fraction of the chromatography (in buffer A) was diluted with water to a final concentration of 3 M urea. Cleavage with enteropeptidase (Roche) was performed at a molar ratio of HBx-fusion to enteropeptidase of ~ 100:1 for the indicated periods of times at RT. Cleavage was analyzed by SDS-PAGE.

2.6. Purification of HBx-GST fusion proteins

The HBx-GST fusion proteins were prepared from the soluble protein fraction of the bacterial cell lysate. After cell disruption the bacterial lysate was centrifuged (13 000 $\times g$, 15 min, 4°C) and the supernatant was applied to a Glutathione Sepharose® 4B column (0.5 ml/min) that had been equilibrated with PBS. The column was washed with PBS and the bound GST-HBx fusion protein was eluted with 10 mM glutathione in 50 mM Tris–HCl, pH 8.0. Collected fractions were analyzed by SDS-PAGE in a 10% acrylamide gel. Concentrations of the recombinant proteins were determined by comparison of the Coomassie-staining of their bands to that of proteins of known concentration that were run in the same SDS-PAGE gel.

2.7. Electrophoretic mobility shift assay

An AU-rich RNA oligonucleotide (38-mer: GUGAUUAUUUAUUAUUUAUUUAUUAU-UUAUUUAUUUAG) was labeled with [γ-^{32}P]dATP in the presence of T4 polynucleotide kinase. The radiolabeled probe was purified on a gel filtration column, and aliquots of 240 pg (~ 10 000 cpm) were used in the binding reactions with GST, or the HBx-6xHis or HBx-GST fusion proteins. The radiolabeled probe and the proteins were incubated in a final reaction volume of 25 μl, containing 50 mM potassium acetate, 5 mM Tris (pH 7.5), 5 mM magnesium acetate, 4% glycerol, 10 μg/ml BSA, at 4°C for 30 min. The RNA-protein complexes were run out on a non-denaturating 6% polyacrylamid gel in 0.5 × TBE buffer at RT. Dried gels were exposed to X-ray films at − 80°C with an intensifying screen. For the competition EMSAs, the same amount of radioactive labeled RNA oligonucleotide as in the EMSA experiments above was used and different competing concentrations of homologous (AU-rich RNA oligonucleotide) or heterologous single-stranded DNA oligonucleotides (DNA oligo 1, GCA CTG AAG CTT GGT TCT GGT TCT GGT GAC GAC GAT GAC AAG ATG GCT GCT AGG TTG TGC TGC; DNA oligo 2, GCA CTG AAG CTT GGT TCT GGT TCT GGT GAC GAC

GAT GAC AAG ATG GCT GCT AGG TTG TGC TGC; oligo dT DNA, $d[T]_{18}$) were used at the molar excess indicated in the figure legends. Proteins were used at the concentration indicated in the figure legends. The recombinant purified 6His-AUF1 protein was a kind gift of Mrs. Karen C.M. de Moraes (CBME/LNLS, Campinas).

3. Results

3.1. Cloning and expression of full-length HBx fusion protein

In order to obtain sufficient quantities of recombinant HBx protein for functional and structural studies, we have used and developed an efficient expression system in *E. coli*. The full-length HBx protein was expressed in *E. coli* cells transformed with the plasmid psW202 that had the HBx gene of the *ayw* subtype (Lo et al., 1988) inserted in frame into its cloning site. This way the HBx protein was expressed as a fusion protein which contained N-terminal of the amino acids 1–154 of the HBx protein an omp-A signal peptide of 30 amino acids (MKKTA IAIAV ALAGF ATVAQ ADYKD DDDKL), a 6xHis tag and immediately before the first methionine of HBx an enteropeptidase cleavage site (DDDDK). The expression of this HBx-6xHis fusion protein resulted in a protein of $\sim$21 kDa that was exclusively found in the insoluble inclusion body fraction of the bacterial cell lysate (Fig. 1B). The production of the recombinant fusion protein was monitored with a time course experiment for the induction with IPTG. Although expression was already strong only after 30 min, it peaked at $\approx$4 h of induction. At none of the time points the HBx fusion could be detected in the soluble fraction (Fig. 1A). Induction of the expression at lower temperatures (27 and 23°C) did not result in a detectable expression of the fusion protein in the soluble fraction (data not shown).

The fact that the recombinant HBx protein was exclusively found in the form of inclusion bodies might be based on the lack of chaperones that could be required for the proper folding or might be due to the formation of abnormal inter- or intramolecular disulfide bonds (Yasukawa et al., 1995). Therefore, we assumed that a simultaneous overexpression of either chaperones or bacterial thioredoxin might result in the production of a more soluble protein. We co-transformed the recombinant psW202-HBx containing *E. coli* BL21

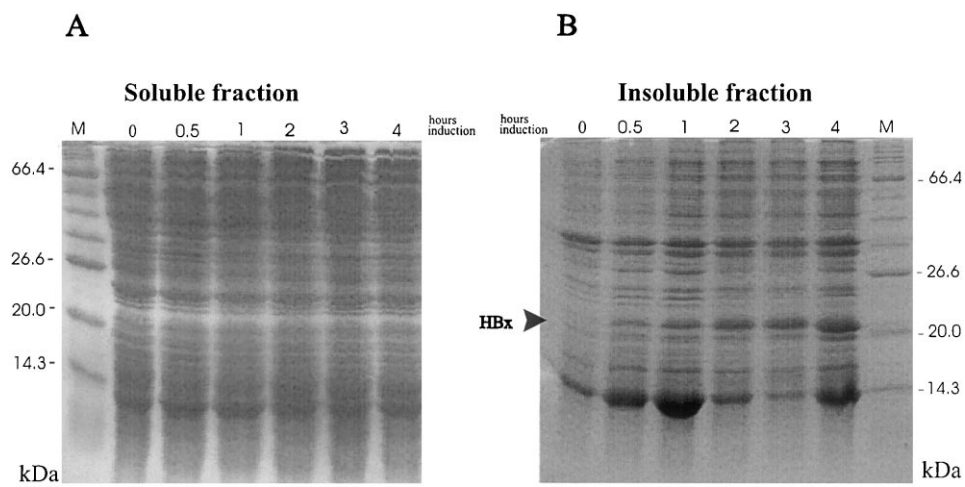

Fig. 1. Expression of the HBx-6xHis fusion protein. Time course of induction of the HBx protein expression in *E. coli*. Ten milliliters of bacterial culture in logarithmic growth phase were or were not induced for the times indicated at the top of the figure. Cells were harvested and lysed and the lysate was separated in soluble (A) and insoluble protein fraction (B) by centrifugation. Samples were dissolved in SDS-gel loading buffer and analyzed by SDS-PAGE. The molecular weights of the standard proteins are indicated and the HBx-fusion protein is marked with an arrow.

strain with plasmid pT-groE, encoding the chaperones GroEL and GroES, or pT-Trx containing the gene for thioredoxin (Yasukawa et al., 1995). After the co-induction of the HBx and either the chaperones or thioredoxin we found expression of both chaperon chains or the thioredoxin in the soluble fraction but still no expression of any soluble HBx protein (data not shown). Again, the expressed HBx was exclusively found in the insoluble lysate fraction, indicating that the lack of either chaperones or thioredoxin is not the cause for the accumulation of HBx in the inclusion bodies.

3.2. Purification of the HBx fusion proteins by Ni-NTA chromatography

Since the full-length HBx-6xHis fusion protein was only found in the inclusion bodies we solubilized them with 6 M guanidinium–HCl to purify the protein under denaturing conditions on a Ni-NTA column (Porath et al., 1975; Ljungquist et al., 1989). The column was washed and eluted in buffers containing 8 M urea and the collected fractions were analyzed by SDS-gel electrophoresis. The recombinant HBx-6xHis fusion protein elutes as a relatively broad peak (Fig. 2A). The analysis of the peak fractions by SDS-PAGE demonstrates the presence of two distinct protein species ~ 21 and ~ 20 kDa (Fig. 2B) that most likely represent the fusion protein with and without its N-terminal omp-A signal sequence. The fact that the majority of the protein is of the higher molecular weight, indicates that the cleavage of the leader sequence after translocation to the periplasmic space of *E. coli* is incomplete and that the majority of the recombinant protein ($\sim$ 90%) resides uncleaved (~ 21 kDa) in the cytoplasma of the bacteria. With the described expression and purification scheme we were able to obtain ~ 10 mg of purified HBx-6xHis fusion protein from 1 l of bacterial culture.

3.3. Digestion of the HBx fusion protein with enteropeptidase

Some of the downstream applications might require the removal of the N-terminal fusion partner consisting mainly of the omp-A signal sequence and the 6xHis tag/enteropeptidase domain. To demonstrate that the enteropeptidase cleavage site we introduced between HBx and the N-terminal fusion part is functionally active, we performed a test digestion with enteropeptidase in the presence of 3M urea (Fig. 3). The time course of the digestion demonstrates the disappearance of the $\sim 21/\sim 20$ kDa fusion protein bands and the appearance of a cleavage intermediate (~ 18.5 kDa) and cleavage end products (~ 16.5 kDa; < 5 kDa). The accumulation of free HBx protein is maximal after 4 h. Afterwards a gradual unspecific degradation of the HBx protein by the enteropeptidase seems to occur. The incompleteness of the digestion and the presence of small molecular weight cleavage products require additional chromatographic steps in order to obtain pure free HBx protein.

3.4. Cloning and expression of N- and C-terminal HBx deletion mutants

For the functional dissection of different domains in the HBx protein and to test if it is possible to improve HBx poor solubility by cutting of hydrophobic parts that might not be necessary for its function, we constructed DNA truncation mutants that lack different parts encoding for the N- and C-terminal regions of HBx (Fig. 4A). All of the constructions were inserted into the same expression vector psW202 that was used for the expression of the full-length HBx-6xHis fusion. The HBx constructs # 4, 5, and 6 could be expressed at levels comparable with the full-length HBx, whereas the HBx construct # 3 showed only weak expression under comparable conditions and the construct # 2 did not express at all (Fig. 4B and data not shown). The constructs # 2 and # 3 were the two smallest constructs and their low or missing expression might be due to their small molecular weight. The degradation or lack of expression of small proteins is frequently reported in *E. coli*. Because these two constructs # 2 and # 3 are especially important since they represent the most drastic truncations and span the N- and C-terminal extremes of the HBx protein, we decided to express them and the

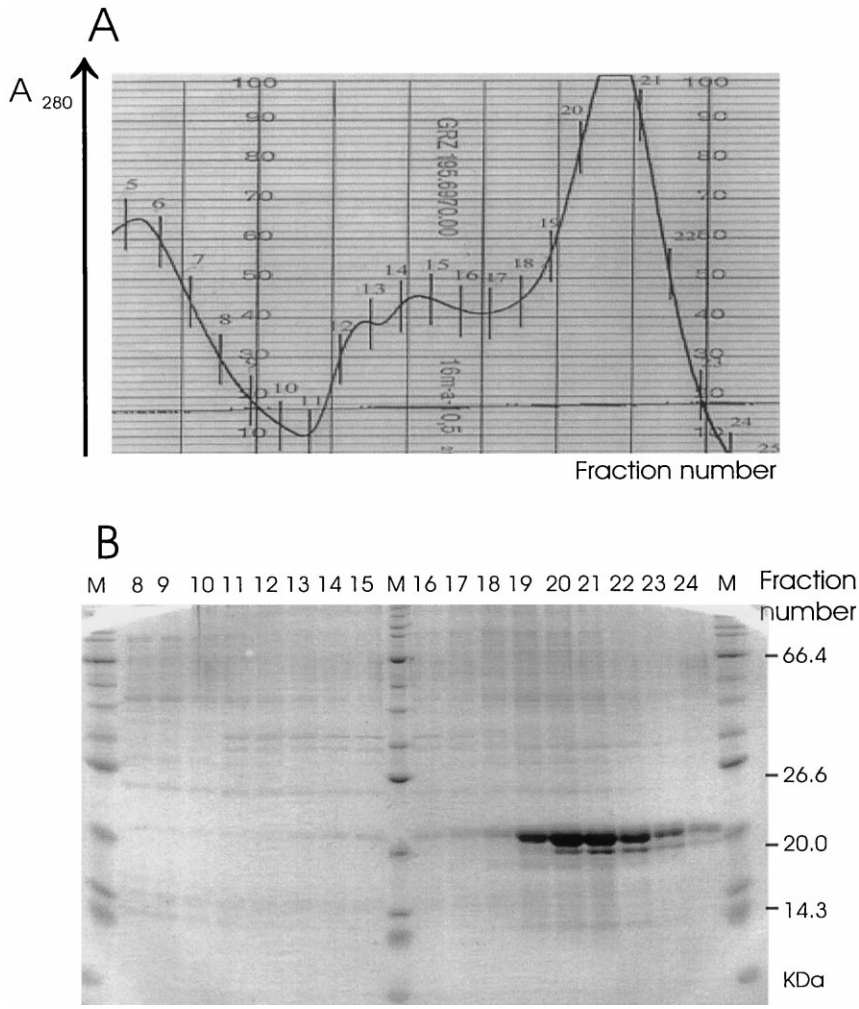

Fig. 2. Ni^{2+}-affinity chromatography of the recombinant HBx-6xHis fusion protein and analysis of the protein fractions by SDS-PAGE. Purification of the recombinant HBx-fusion protein from the insoluble fraction of *E. coli* by Ni-NTA affinity chromatography. One liter of bacterial culture was induced with 1 mM IPTG for 3 h at 25°C. Harvested cells were lysed and HBx-inclusion bodies were solubilized in 6 M guanidinium–HCl solution. The solubilisate was applied to a Ni-NTA superflow column which had been equilibrated with buffer A (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 8.0). The column was subsequently washed with buffer B (like buffer A but pH 6.3) and proteins were eluted in one step with buffer C (like buffer A but pH 4.5). (A) Elution profile of proteins and their fractioning in 2 ml fractions. (B) Aliquots of 10 μl of each of the fractions of (A) were analyzed by SDS-PAGE.

full-length HBx also as GST-fusion proteins (Fig. 5A). All of the constructs were inserted into the commercial GST-fusion expression vector pGEX-2TK. The expression level of the three GST-HBx fusion constructs in *E. coli* was high, although GST-HBx # 2 and # 3 were predominantly and GST-HBx-complete was exclusively found in the insoluble inclusion body fraction of the bacterial lysate (data not shown). Starting with 500 ml of bacterial culture induced with 1 mM IPTG for 4 h we were, however, able to purify >1 mg of each of the GST-HBx # 2 and # 3 fusion proteins from the soluble fraction of the cell lysate (Fig. 5B). Together with GST-HBx # 2 and # 3 fusion proteins we co-purified small amounts of free GST (~ 26 kDa) that must be either a product of

proteolytic cleavage or of a prematurely terminated transcript (Fig. 5B). It was not possible to purify GST-HBx-complete from the soluble fraction of the bacterial lysate. These experiments showed that the co-expression of the highly soluble GST in the form of a fusion partner was able to improve HBx poor solubility.

3.5. Mapping of the HBx RNA binding site by electrophoretic mobility shift assay

We decided to employ HBx ability to bind to single-stranded DNA in vitro as described by Qadri and coworkers (Qadri et al., 1996) as a marker for one potential biological activity of HBx. The binding of HBx to single-stranded nucleic acids can be reproducibly tested by an EMSA. Instead of ssDNA we used a radioactively labeled RNA oligonucleotide as the probe in the EMSA and showed that HBx protein binds also to RNA (Fig. 6A). Not only the full-length HBx-6xHis fusion protein but also all of the other five HBx deletion constructs were able to bind to the RNA oligonucleotide and caused its retardation in the non-denaturing gel. The complex formed between the AU-rich RNA oligonucleotide and the HBx-6xHis fusion proteins showed a greater shift than the complex formed between the RNA-oligonucleotide and the GST-HBx fusion proteins. The fact that both the GST-HBx # 2 and # 3 proteins bound to the RNA oligonucleotide indicated that the HBx protein might have two independent RNA binding sites which are located in its N-terminal (residues 5–78) and C-terminal (80–142) protein domains. Although four times more GST-HBx # 2 protein was loaded on the gel, the observed band was significantly weaker than that observed with the GST-HBx # 3 protein (Fig. 6a). This result suggested that the relative affinity of these two constructs might be different. Therefore, we compared the relative affinity of the GST-HBx constructs # 2 and # 3 in a separate experiment (Fig. 6b,c). Both proteins were gradually diluted and tested for their binding of the radioactively labeled RNA oligonucleotide. Whereas GST-HBx # 2 upon dilution rapidly lost its ability to bind to the RNAoligonucleotide the GST-HBx # 3 showed a

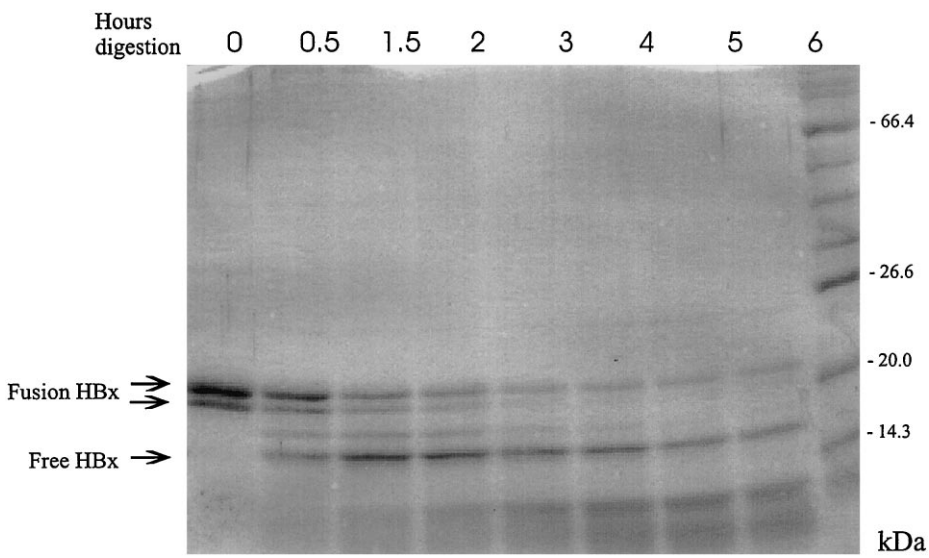

Fig. 3. Enteropeptidase digestion of the HBx-6xHis fusionprotein. Digestion of the HBx-fusion protein with bovine enteropeptidase. Recombinant HBx protein, purified under denaturing condition (in the presence of 8 M urea) by Ni-NTA metal affinity chromatography was digested with bovine enteropeptidase in the presence of 3 M urea for the times indicated at the top. The non-digested control and the digestion products were separated by SDS-PAGE. The molecular weights of the standard proteins are marked on the right side and the HBx-fusion protein and the free HBx are indicated with arrows on the left side.

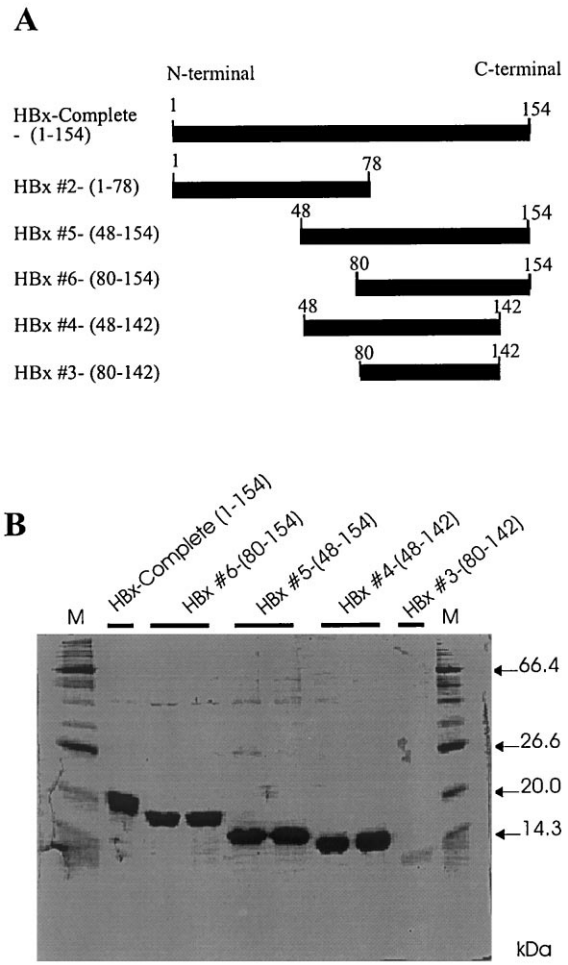

Fig. 4. Expression of the HBx-6xHis deletion constructs. (A) Schematic representation of the five HBx-6xHis deletion constructs. The DNA fragments encoding the depicted regions were amplified by PCR and cloned into the vector psW202. HBx constructs were expressed as 6xHis fusion proteins. The numbers indicate the residues of the 154 amino acid long HBx protein that are included in each deletion. (B) SDS-gel electrophoresis of the expressed and purified constructs. The constructs have been prepared and purified under denaturing conditions as described for the full-length HBx-6xHis fusion. Subsequently the peak fractions were pooled and an aliquot was run out on a SDS gel (12% acrylamide). The molecular weight of the marker proteins (M) are indicated and the according bands marked with arrows.

shift even at the highest dilutions tested. A faint shifted band could be observed for the GST-HBx #2 down to an amount of 1.5 μg. An equally intense band could be observed for GST-HBx #3 even at an amount of only 0.1 μg. These results indicate that the affinity of GST-HBx #3 for RNA is at least an order of magnitude higher than that of GST-HBx #2. Another interesting observation was that the GST-HBx #3 protein causes the shift of two distinct species of bands (Fig. 6c). The second more retarded band was only observed with GST-HBx #3 and not with GST-

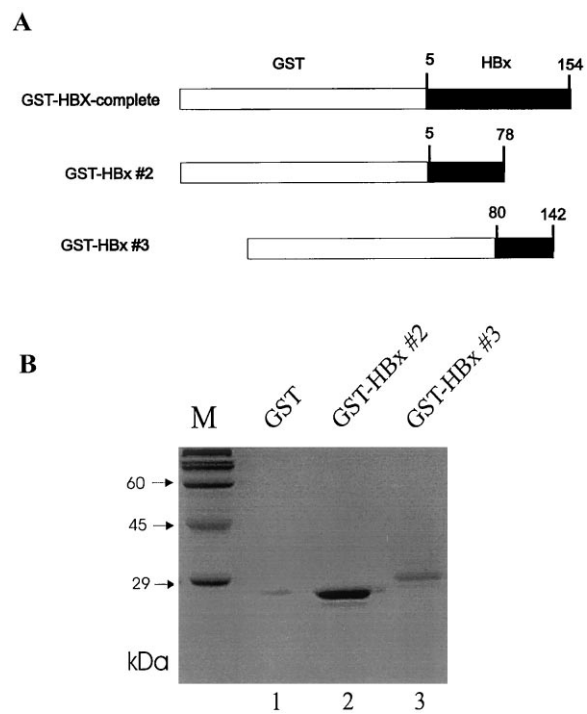

Fig. 5. Expression of the GST-HBx #2 and GST-HBx #3 deletion constructs. (A) Representation of the three different GST-HBx fusion constructs. (B) SDS-gel electrophoresis of the expressed and purified constructs. Bacteria were transformed with plasmids containing GST, GST-HBx #2 or #3 DNA constructs. Cells were induced for the expression of the GST fusion proteins, bacteria were lysed and the lysate was centrifuged at 13 000 $\times g$. The supernatant with the soluble proteins was applied to a glutathione sepharose column. Bound GST-HBx fusion proteins were eluted with glutahione. Peak fractions were pooled and analyzed by SDS-gel electrophoresis (10% acrlyamide gel). The molecular weights of the marker proteins (M) are indicated with arrows.

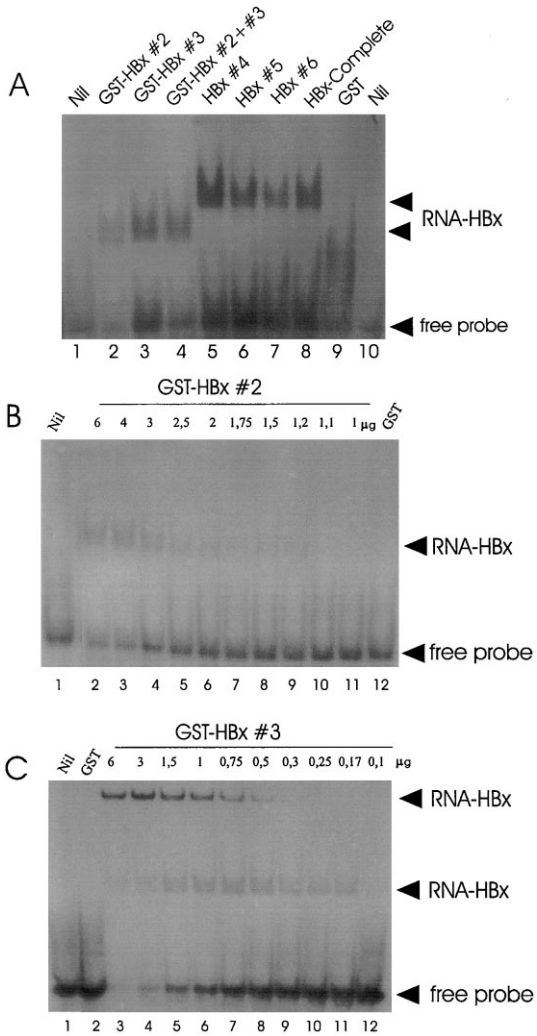

Fig. 6. EMSA of HBx-AU-rich RNA oligonucleotide interactions. (A) Analysis of the binding of the different GST-HBx fusion and HBx-6His fusion constructs to the AU-rich RNA oligonucleotide (240 pg per lane, all experiments). Lane 1, free oligo; lane 2, GST-HBx #2 (4 µg); lane 3, GST-HBx #3 (1 µg); lane 4, GST-HBx #2 (2 µg) and #3 (0.5 µg); lane 5, HBx-6xHis #4 (1 µg); lane 6 HBx-6xHis #5 (1 µg); lane 7, HBx-6xHis #6 (1 µg); lane 8, HBx-6xHis-complete (1 µg); lane 9, GST (1 µg); lane 10, free oligo. (B) Binding of GST-HBx #2 to the AU-rich RNA oligonucleotide. Lanes 1–12, radioactively labeled AU-rich RNA oligo; Proteins: lane 1, nil; lane 2–11 decreasing amounts of purified GST-HBx #2 protein as indicated on the top; lane 12, GST (2 µg). (C) Binding of GST-HBx #3 to the AU-rich RNA oligonucleotide. Lanes 1–12, radioactively labeled AU-rich RNA oligo; Proteins: lane 1, nil; lane 2, GST (2 µg), lanes 3–12, decreasing amounts of purified GST-HBx #3 protein as indicated on the top.

HBx #2 and might result from the formation of a GST-HBx #3 dimer on the AU-rich RNA oligonucleotide.

3.6. Competition experiments with unlabelled nucleic acids

Our finding that HBx binds an AU-rich RNA oligonucleotide prompted us to compare HBx binding to different single-stranded DNA substrates (Qadri et al., 1996). We tested the specificity of GST-HBx construct #3 to either the AU-rich RNA oligonucleotide or different unlabelled single-stranded DNA oligonucleotides by a competitive EMSA test (Fig. 7). This test revealed that a 10-fold molar excess of the unlabelled AU-rich RNA oligonucleotide competitor already completely abolished HBx binding to the radioactively labeled AU-rich RNA oligonucleotide. All three tested single-stranded DNA oligonucleotides (DNA oligo 1, DNA oligo 2 and oligo $d[T]_{18}$ DNA), however, showed no inhibition of HBx binding to the AU-rich RNA oligonucleotide when added in 10-fold or even 100-fold molar excess. These data indicate that GST-HBx #3 has a higher specificity for the AU-rich RNA oligonucleotide than for the two single-stranded DNA oligonucleotides of arbitrary sequence (DNA oligos 1 and 2, see Section 2 for sequences) or for the oligo $d[T]_{18}$ homopolymer.

3.7. Competitive binding experiments of GST-HBx #3 and AUF1 proteins to the AU-rich RNA oligonucleotide

The natural ligand of the AU-rich RNA oligonucleotide we used in our HBx-RNA binding studies is the protein AUF1 (Zhang et al., 1993). It had been shown that AUF1 binds specifically to the AU-rich domains present in the 3′-untranslated regions of many mRNAs with high turnover rates like those encoding certain proto-oncogene products (*c-myc*, *c-fos*) (Jones and Cole, 1987; Cleveland and Yen, 1989) and is involved in the rapid degradation of these mRNA species (Brewer, 1991). Since GST-HBx #3 bound with high specificity to the AU-rich RNA oligo that represents a natural substrate for the protein

AUF1 we were interested if the HBx protein would be able to displace AUF1 from its binding site on its RNA substrate. Descending amounts of AUF1 protein were incubated with the radioactively labeled AU-rich oligonucleotide in the presence of a constant amount of GST-HBx #3 protein (Fig. 8). When AUF1 protein alone is incubated with the AU-rich RNA oligonucleotide four distinct shifted bands could be observed (bands A–D, Fig. 8.). At very high amounts of added AUF1 protein (6–1.5 μg) these bands persist even in the presence of GST-HBx #3 (800 ng) protein. In the presence of equal or higher amounts of the GST-HBx #3 protein, however, the appearance of the typical two HBx #3–RNA bands (RNA-HBx 1 and 2, Fig. 8.) can be observed whereas the AUF1-RNA bands disappear in the order C/B → A → D. These results indicate that AUF1 and HBx #3 might occupy the same or overlapping binding sites on the AU-rich RNA oligonucleotide and that the HBx protein when present at equal or higher concentrations than AUF1 is able to displace AUF1 from its RNA binding site. The observation of an additional transient band neither observed with AUF1 nor with GST-HBx #3 alone seems to support this hypothesis (trimeric complex; Fig. 8). This band might represent some intermediate trimeric complex of the AU-rich RNA, AUF1 and GST-HBx #3.

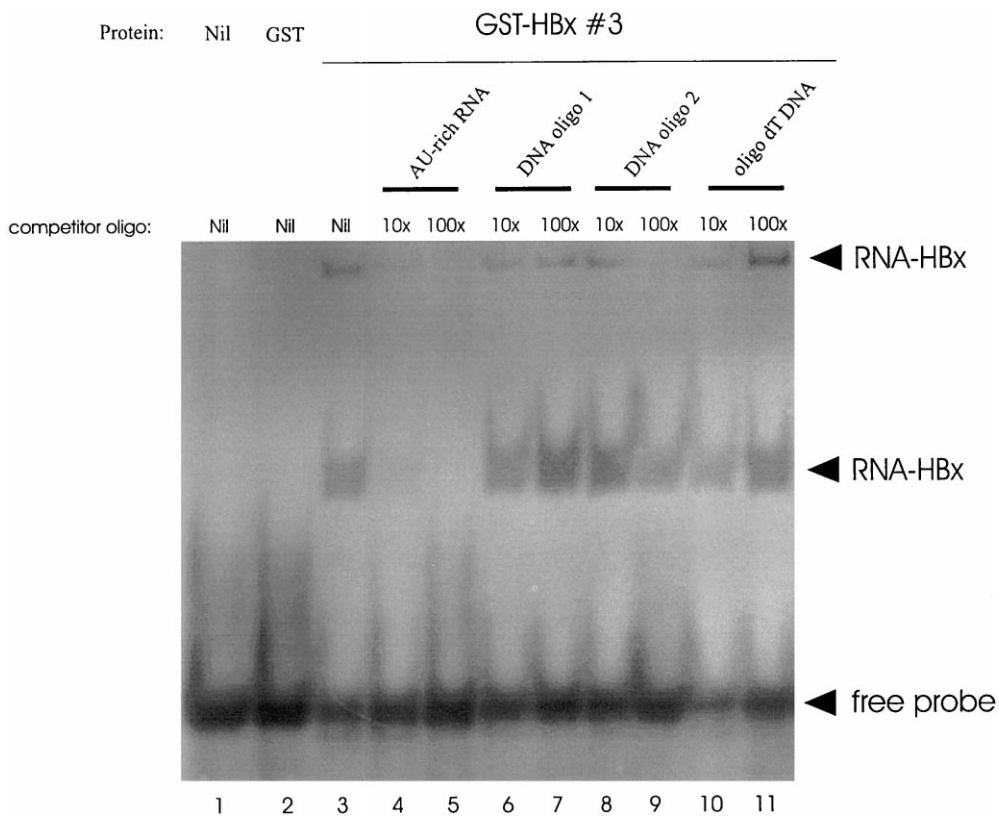

Fig. 7. EMSA of HBx #3–AU-RNA oligonucleotide interactions in the presence of oligonucleotide competitors. Analysis of the binding of GST-HBx #3 to the RNA oligonucleotide in the presence of 10 × or 100 × molar excess of unlabelled RNA or DNA oligonucleotides. Lanes 1–11, radioactively labeled AU-rich RNA oligonucleotide (240 pg per lane). Proteins: lane 1, nil; lane 2, GST (1 μg); lanes 3–11 GST-HBx #3 (1 μg). Competitors oligonucleotides: Lanes 1–3, no competitor; lane 4, 10 × molar excess of AU-rich RNA oligonucleotide; lane 5, 100 × AU-rich RNA oligo; lane 6, 10 × DNA oligo 1; lane 7, 100 × DNA oligo 1; lane 8, 10 × DNA oligo 2; lane 9, 100 × DNA oligo 2; lane 10, 10 × oligo $d(T)_{18}$ DNA; lane 11, 100 × oligo $d(T)_{18}$ DNA.

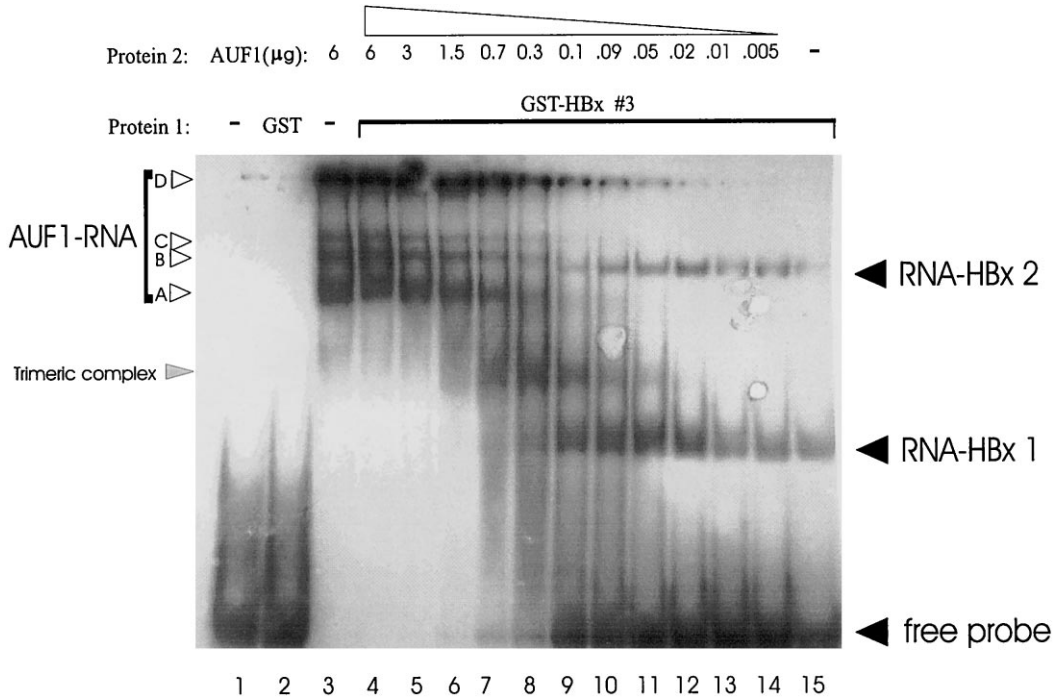

Fig. 8. Competitive binding of GST-HBx # 3 and 6His-AUF1 fusion proteins to the AU-RNA oligonucleotide as analyzed by EMSA. Analysis of the binding of decreasing amounts of 6His-AUF1 protein to the AU-rich RNA oligonucleotide in the presence of GST-HBx # 3 protein (800 ng per lane). Lanes 1–15, radioactively labeled AU-rich RNA oligo (240 pg per lane); Proteins: lane 1, nil; lane 2, GST (1 μg); lanes 4–15, GST-HBx # 3 (800 ng per lane); lane 3, 6His-AUF1 protein (6 μg); lanes 5–15, decreasing amounts of purified 6His-AUF1 protein as indicated on the top.

4. Discussion

Although a subject of intense research the function of the HBV protein HBx is still very controversial (Yen, 1996). A structural analysis of the HBx protein by crystallographic or NMR techniques would be of great value for the better understanding of its functions. Here, we present data that show the expression of HBx in *E. coli* and its purification in mg amounts as full length protein as well as in truncated form.

We found that the HBx protein is expressed in an insoluble form in *E. coli* like it was described previously by others (Jameel et al., 1990; Gupta et al., 1995; Qadri et al., 1996; Marczinovits et al., 1997). To obtain larger amounts of soluble HBx we tried several different approaches. The first was to co-express the HBx protein with either the chaperones GroEL and GroES or with the bacterial thioredoxin. (Yasukawa et al., 1995). The presence of these chaperones in increased concentration did however not result in any soluble HBx production. This might indicate that these specific chaperones are not involved in the folding pathway of the HBx protein. Likewise neither the co-expression of Trx also resulted in any detectable expression of soluble HBx, again indicating that additional factors are involved that contribute to the low solubility of the HBx protein.

The second approach to purify soluble HBx from *E. coli* was based on the presence of an N-terminal omp A signal sequence that directs the HBx protein to the bacterial periplasmatic space, an oxidizing environment that might help in the correct formation of disulfide bonds. The properly processed HBx fusion protein without the omp A signal sequence ($\sim$20 kDa band), however, made up only $\sim$10% of the total purified HBx fusion protein. Because our attempts to isolate soluble

HBx from the periplasmatic space (Koshland and Botstein, 1980) were unsuccessful (data not shown), we concluded that the HBx protein might also form inclusion bodies in the periplasmatic space.

The third approach to obtain soluble HBx protein expressed in *E. coli* was the systematic truncation of the N- and C-terminal regions of the protein. This approach was used with good success for other proteins expressed in *E. coli* or other expression systems. Two examples are the gp120 protein of HIV (Kwong et al., 1998) and the HIV integrase (Dyda et al., 1994). In both proteins both N- and C-terminal regions as well as internal extensive loop regions in the case of the gp120 were removed, since it had been shown in functional studies that these regions are not necessary. After these drastic modifications, both proteins were obtained in a more soluble form. Subsequently they could be crystallized and their structures were solved by X-ray crystallography (Dyda et al., 1994; Kwong et al., 1998). Using the expression vector psW202, we constructed five N- and C-terminal deletion mutants for the expression of truncated HBx-6xHis fusion proteins and two deletion mutants as well as full-length HBx for the expression as GST fusion proteins (Figs. 4 and 5). To assess the potential biological activity of both the full-length HBx-6xHis and the different deletion constructs, we next established a simple in vitro test system. It had been reported previously that HBx can bind to single-stranded DNA (ssDNA) (Qadri et al., 1996). We were interested to test if HBx can also bind to RNA and used an AU-rich RNA oligonucleotide as the test substrate in the EMSA.

We found that all of our HBx fusion constructs bound to the AU-rich RNA oligonucleotide, whereas the negative control GST was not able to cause the retardation of the oligonucleotide probe in the gel. Since the constructs GST-HBx # 2 and # 3 represent the N- and C-terminal halves of the HBx protein but both bound to the RNA oligonucleotide, we were interested to test their relative affinity to the RNA. According to Carey (Carey, 1991) the binding affinity can be estimated from the protein concentration required to bind half of the nucleic acid in the EMSA. From the data shown in Fig. 6 and other similar experiments (data not shown), we estimated that the relative affinity of GST-HBx # 3 to the RNA is ~ 10 times higher than that of GST-HBx # 2 (Fig. 6). This demonstrates that a domain with high affinity for RNA must be located in the region between residues 80–142 of HBx. These data seem to correlate with some other data from the literature that also functionally dissect the N-terminal and C-terminal halves of the HBx molecule (Kumar et al., 1996; Gottlob et al., 1998; Kwee et al., 1992). Interestingly HBx transactivating capacity is also attributed to the C-terminal half of the molecule (Kumar et al., 1996; Wentz et al., 2000).

Next we tested if the binding specificity of HBx to our RNA oligonucleotide probe differs of that observed for HBx binding to single-stranded DNA (Qadri et al., 1996). We started to address this question by testing the GST-HBx # 3 construct in a competition EMSA experiment where we added competing non-labeled oligonucleotides (Fig. 7). Different concentrations of homologous and heterologous single-stranded nucleic acids were used to try to inhibit the interaction. A $10\times$ molar excess of the unlabelled AU-rich oligonucleotide already showed complete inhibition of the binding whereas even a $100\times$ molar excess of none of the three tested DNA oligonucleotides showed inhibition of the binding (Fig. 7). These results indicate that the GST-HBx # 3 protein might have a higher specificity for the AU-rich RNA than for single-stranded DNA substrates.

It is tempting to speculate for a possible physiologic meaning of this affine binding to the AU-rich RNA substrate in the light of HBx role as an oncogene product. We chose the sequence of the RNA-oligonucleotide because it encodes the so-called AU-rich motif which is frequently found in the 3′-end of several rapidly degraded mRNAs like those of certain proto-oncogenes and cytokines (Jones and Cole, 1987; Cleveland and Yen, 1989). Since the HBx protein is expressed in the cytoplasma of the host liver cell, it is possible that HBx also binds in vivo in the liver cell to mRNA substrates that contain AU-rich motifs. The binding of HBx might compete with the binding of factors like the protein AUF1 which is bound to

the AU-rich motif in vivo and is involved in the rapid degradation of the mRNAs of certain proto-oncogenes like *c-myc* or *c-fos* (Brewer, 1991; Zhang et al., 1993). Such a displacement of AUF1 by HBx might cause the stabilization of AU-rich proto-oncogene mRNAs and thereby contribute to the transforming phenotype (Lee et al., 1988). The efficiency of this displacement in vivo depends on several unknown parameters like the steady-state concentrations of the proteins (AUF1, HBx) and mRNAs involved. Although the expression level of the HBx in vivo might be low, the contribution to the transformation of the liver cell via the described mechanism can be significant because the development of HCC from a chronic HBV infection is a very slow process and can take several years if not decades. It is therefore quite possible that the constant presence of the HBx protein in the chronically infected liver cells leads to the enduring stabilization of proto-oncogene mRNAs with AU-rich regions and thereby contributes to the malignant transformation of the liver cells. In future in vivo experiments we want to over express HBx in human cell lines to address HBx role in this possible mechanism of cellular transformation.

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Artigo II

The cysteine residues of the hepatitis B virus onco-protein HBx are not required for its interaction with RNA or with human p53.

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The cysteine residues of the hepatitis B virus onco-protein HBx are not required for its interaction with RNA or with human p53

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Abstract

The hepatitis B virus (HBV) protein HBx has been implicated to induce liver cancer in transgenic mice and transactivates a variety of viral and cellular promoters. The 17 kDa protein HBx consists of 154 amino acids, contains 10 cysteine residues and is translated during the viral infection. It has been shown previously that the HBx protein is able to bind to singlestranded DNA and RNA. This nucleic acid binding activity might be relevant for HBx oncogenic character. Furthermore, HBx has been reported to interact with a series of cellular proteins, especially with transcription factors, including the tumor suppressor protein p53. To evaluate the importance of the cysteine residues in HBx for its interaction with RNA and p53 we expressed full-length HBx-wt as well as several truncated mini-HBx(18–142) proteins with multiple cysteine to serine point mutations as 6xHis fusion proteins in *Escherichia coli*. Using UV cross-linking assays we demonstrate that all truncated mini-HBx proteins with cysteine/serine point mutations maintained the ability to bind to an AU-38 RNA oligonucleotide. Furthermore, we performed in vitro binding assays of selected HBx mutants with GST-p53, circular dichroism spectroscopic analysis of the mutant HBx protein secondary structure and a p53 based transcription activation assay in yeast cells. In summary, our data suggest that the cysteine residues in the HBx protein are of minor importance for its interaction with both RNA and the p53 protein.

Keywords: Viral hepatitis; Hepatocellular carcinoma; Viral transactivator; Oncogene

1. Introduction

Infection with hepatitis B virus (HBV) has been associated with development of liver cancer (HCC), one of the most prevalent malignant diseases worldwide (Hollinger, 1996). Of the 2 billion people who have been infected with HBV, more than 400 million are chronic carriers of HBV and these people have a 100-fold increased risk of developing HCC than non-carriers. It is estimated that each year worldwide more than 1 million people die from HCC. HBV is the smallest known DNA virus and it has a strict tissue tropism to the liver, causing acute or chronic hepatitis, leading to necrosis followed by multiple nodular hyperplasia and cirrhosis (Ganem, 1996). Although the induction of regenerative hyperplasia might be involved in the initiation of HCC, HBV may have a more direct role. The HBV genome is composed of a partially double stranded DNA of 3.2 kb that contains at least four open reading frames (ORF) which are named S, C, P, and X. The ORF X encodes a 17 kDa (154 amino acids) protein termed HBx, which is translated during viral infection. In animal models it has been suggested that HBx is essential for the viral replication in vivo (Zoulim et al., 1994; Chen et al., 1993). Aside this finding the HBx has been im-

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plicated in the development of HCC in transgenic mice (Kim et al., 1991). Due to its both cytoplasmatic and nuclear localization, HBx stimulates signal transduction pathways in the cytoplasm as well as interacts directly with transcription factors in the nucleus (Henkler et al., 2001). Morever, it has been shown that HBx is a potent transactivator of several viral and cellular promoters (Twu et al., 1990; Yen, 1996). It has recently been reported that HBx can interact with singlestranded DNA (ssDNA) in a sequence independent but base specific fashion (Qadri et al., 1996), although it does not bind to dsDNA. Since HBx can bind to ssDNA (Qadri et al., 1996), we speculated that it might also be able to interact with RNA. Our previous studies (Rui et al., 2001) have shown that HBx can displace the human protein AUF1 from its binding site on the RNA oligonucleotide in a competitive binding experiment of AUF1 and HBx to an AU-rich oligonucleotide (AU-38). The displacement of cellular factors from proto-oncogene mRNAs in vivo by HBx might contribute to its oncogenic character.

On the other hand HBx has not only been shown to interact with singlestranded DNA and RNA but with several different cellular proteins (Murakami, 1999). The interacting proteins include proteins from different functional groups, such as: gene specific transcription factors, including among others p53 (Feitelson et al., 1993, 1997), bZIP family proteins such as ATF (Barnabas et al., 1997) and NF-kB (Assogba et al., 2002), general transcription factors such as TBP (Wang et al., 1998) and TFIIB (Lin et al., 1997; Haviv et al., 1998), DNA repair proteins such as UVDDB1 (Sitterlin et al., 1997; Wentz et al., 2000), proteasome subunits XAPC7 (Fischer et al., 1995; Huang et al., 1996), and finally several proteins that are not yet fully characterized functionally such as XAP2 (Kuzhanaivelu et al., 1996), XIP (Melegari et al., 1998), to mention only two of them.

P53 is an important regulatory protein involved in safeguarding the genomic integrity (Lane, 1992), sensing of DNA damage (Lee et al., 1995), monitoring of the G1 checkpoint of the cell cycle (Kastan et al., 1992) and activation of the cellular apoptosis program to kill cells with damaged genomes (Shaw et al., 1992). The inactivation of p53 function through mutations or by interaction with viral proteins is a common event in human carcinogenesis and the majority of human cancers show mutations in the *p53* gene (Hollstein et al., 1991). The *p53* gene is in fact inactivated in 30–55% of hepatocellular carcinomas (Sohn et al., 2000). P53 is a cellular protein target for a series of viral onco-proteins, that are involved in neoplastic transformation and include the SV40 T antigen, E1A and B of the adenovirus, E6 and E7 of the HPV virus (Wang and Harris, 1996) and HBx of the hepatitis B virus (Elmore et al., 1997; Feitelson et al., 1993; Takada et al., 1995). The direct interaction of HBx with p53 can interfere with p53 sequence specific DNA binding and gene transactivation.

In this study, we expressed the full-length 6xHis-HBx protein and truncated 6xHis-mini-HBx proteins containing single or multiple cysteine to serine substitutions in *Escherichia coli*. We addressed the importance of Cysteines in the HBx protein for its binding to an AU-rich RNA oligonucleotide, for its interaction with the p53 protein and p53-dependent transactivation of a reporter gene. Using an UV cross-linking assay, we were able to demonstrate that all mini-HBx protein cysteine/serine point mutants have the ability to bind to the AU-rich oligonucleotide (AU-38). The mini-HBx protein without any cysteine 6xHis-mini-HBx(-Cys) was also able to interact with p53 protein in vitro just as full-length 6xHis-HBx with 10 Cys or 6xHis-mini-HBx with 5 Cys residues. CD spectroscopy studies demonstrated that the mini-HBx proteins with and without the Cysteines have almost identical secondary structure compositions thereby indicating that the Cysteines might not be relevant for HBx structure. Taken together, these results suggest that the Cys residues in the HBx protein are not important for its interaction with RNA nor with the p53 tumor suppressor protein.

2. Materials and methods

2.1. Construction of plasmids and yeast strains

The full-lenght HBx DNA (subtype *ayw*) (Galibert et al., 1979; Lo et al., 1988) inserted into pCMV-Ad (kindly provided by Dr. R. Schneider, New York) was used as a template DNA in the PCR reactions, in order to amplify the full-length *HBx* gene (510 bp) and a truncated mini *HBx* gene (372 bp). The full-length *HBx* gene was directionally cloned into the bacterial expression vector pET28a+ (Novagen) via *Nde*I (sense oligonucleotide: AC GAA TTC CAT ATG GCT GCT AGG TTG TGC TGC C) and *Xho*I (anti-sense oligonucleotide: GTT CTC GAG TTA GGC AGA GGT GAA AAA G) restriction sites, while the truncated *mini-HBx* gene was cloned into pET28a+ via *Nde*I (sense oligonucleotide: G GAA TTC CAT ATG CGT CCC GTC GGC GCT GAA TC) and *Bam*HI (anti-sense oligonucleotide: CG GGA TCC TTA GAC CAA TTT ATG CCT ACA GCC) restriction sites. pET28a+ encodes upstream of the insertion site a histidine tag (6xHis), which allows the purification of the recombinant fusion proteins via Ni-affinity chromatography. The recombinant plasmids HBx-pET28a+ and the mini-HBx-pET28a+ were transformed in *E. coli* BL-21 cells, for the expression of the 6xHis-HBx and the different 6xHis-mini-HBx proteins. The HBx(1–154), mini-HBx(18–142) and mini-HBx(18–142)-Cys (not coding any Cysteines) were amplified by PCR and subcloned in frame with the lexA DNA binding domain in the yeast expression vector pBTM116 via *Eco*RI and *Bam*HI restriction sites.

A pGEX plasmid containing the full-length human p53 in frame with GST was kindly provided by Dr. Gianni Del Sal (Laboratorio Nazionale CIB, Trieste, Italy). The gene coding human p53(1–393) protein was amplified from this vector with specific primers and inserted in frame with the Gal4 activation domain, via *Eco*RI and *Bam*HI sites, into the cloning site of the yeast expression vector pGAD424 (Clontech). The

correctness of all vector constructs was confirmed by DNA sequencing.

2.2. Site-directed mutagenesis

The 14 mini-HBx cysteine to serine point mutants were constructed through PCR methodology according to the *QuickChange*™*Site-Directed Mutagenesis* kit (Stratagene). All mutants were confirmed by DNA sequencing.

2.3. Expression of proteins and purification of the p53-GST fusion protein

E. coli strain BL-21 was transformed with the either HBx, or mutant mini-HBx DNA containing pET28a+ vector, with human p53 containing pGEX-2TK or empty pGEX-2TK vector. Cells were grown in LB medium containing kanamycin (pET) or ampicillin (pGEX) to an $OD_{600} = 0.7$, followed by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and induced for 3 h at 37 °C. Cells were harvested by centrifugation and HBx proteins were purified from inclusion bodies as described further.

GST and GST-p53 were expressed in a similar fashion but purified from the soluble protein fraction of the bacterial lysate using glutathione-sepharose 4B (Amersham) as described previously for other GST fusion proteins (Moraes et al., 2003).

2.4. Purification of 6xHis-HBx fusion proteins from inclusion bodies

A bacterial pellet derived from a 1 l culture was resuspended in lysis buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 5 mM DTT, 1 mM PMSF) containing lysozyme (1 mg/ml). After incubation on ice for 30 min, cells were lysed by three subsequent freeze–thaw cycles. Genomic DNA was digested by addition of DNAse (1 μg/ml) and incubation for 20 min at 37 °C. After incubation, the samples were sonicated and the pellet was separated by centrifugation at 18,600 × *g* for 15 min at 4 °C. The pellet was ressuspended in washing buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 5 mM DTT, 3 M urea, 1% Triton X-100) and then centrifuged at 9500 × *g* for 15 min at 4 °C for three times or until the supernatant was clear. The pellet containing the HBx inclusion bodies was solubilized in 20 ml of 50 mM Tris–HCl, (pH 8), 8 M urea and the solution was cleared by centrifugation at 14,000 × *g* for 20 min at 4 °C. The resulting solution was filtered and applied to a Ni-NTA column (QIAgen). The subsequent washing and elution steps were all carried out with buffer A (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl) but at different pHs. The column was washed first with buffer A, pH 8 and than with buffer A, pH 6.3. Bound protein was eluted with a linear pH gradient between 6.3 and 4.5 in buffer A. The column eluate was collected in 2 ml fractions and 10 μl aliquots were analyzed by SDS–PAGE. Peak fractions were pooled and dialyzed against renaturing buffer (10 mM Pipes, pH 7) at 4 °C with several changes of the buffer. Dialyzed protein was cleared by centrifugation at 14,000 × *g* and used for the UV cross-linking and in vitro p53 binding assays.

2.5. Circular dichroism analysis

The espectroscopic CD analysis of the HBx proteins was performed on a JASCO J-810 spectropolarimeter, coupled to a Peltier PFD 425S system for stable temperature at 20 °C. The far UV-CD spectra (260–190 nm) were collected using a quartz cuvet with an optical path of 1 mm. The final spectra were obtained from at least four independent scans and by subtraction of the solvent spectrum. The obtained data were converted into values of residual molar ellipticity θ, and processed by the program *CDNN deconvolution* (http://bioinformatik.biochemtech.uni-halle.de/cdnn/) to obtain the predicted relative percentage of secondary structure elements for each of the analyzed HBx proteins at the different buffer conditions.

2.6. UV cross-linking assay of HBx with RNA oligonucleotides

The purified 6xHis-HBx and 6xHis-mini-HBx proteins were incubated with an AU-rich [γ-^{32}P]-RNA 38-mer oligonucleotide (GUGAUUAUUUAUUAUUUAUU-UAU UAUUUAUUUAUUUAG) (Rui et al., 2001) in a final volume of 25 μl containing 20 mM Hepes, 10 mM $MgCl_2$, 60 mM KCl and 10% glycerol. After incubation on ice for 30 min, the opened reaction tubes were exposed to the ultraviolet light (254 nm) for 5 min in a distance of 100 mm (UV Stratalinker 2400). The complexes were resolved by SDS–PAGE under denaturing conditions and visualized after a 2–6 h exposition on a BAS-IP MS 2325 (Fujifilm) by phosphorimager scan (Bio-Imaging Analyzer BAS-1800II; Fujifilm).

2.7. Co-precipitation assay of GST-p53 with HBx

We used the expressed and purified proteins GST, GST-p53 and HBx in its various mutant forms to perform pull down assays in vitro. First, we immobilized GST-p53 protein or GST control protein on glutathione beads. The coupled beads were than incubated with wt-HBx(1–154), mini-HBx(18–142) or mini-HBx(-Cys) (Fig. 1). After incubation and extensive washings with PGDE buffer (25 mM Pipes, pH 6, 50 mM KCl, 80 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1% Triton X-100, 5% glycerol) the beads were collected and bound protein was analyzed by SDS–PAGE.

2.8. Yeast one-hybrid system based p53 transcriptional activation assay

The vector p53blue (Clontech) was generated by inserting three tandem copies of the consensus p53 binding site

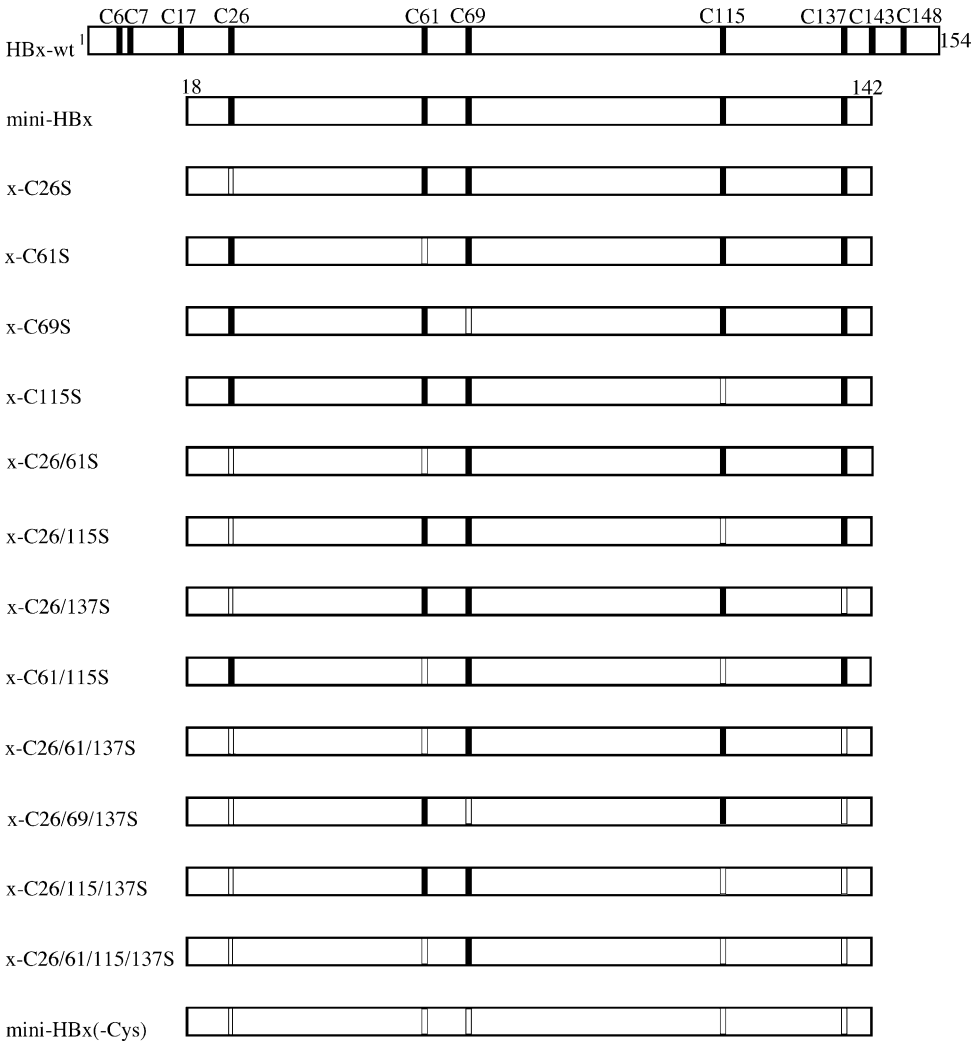

Fig. 1. Schematic representation of the different HBx protein variants indicating the introduced cysteine/serine mutations. The mini-HBx cysteine/serine point mutants were generated through site directed mutagenesis methodology and confirmed by sequencing analysis. The DNA fragments were cloned into the bacterial expression vector pET28a+ and the HBx constructs were expressed as 6xHis fusion proteins. The numbers indicate the amino acid residues present in the truncated HBx proteins. The cysteine residues are indicated in the HBx constructs and marked as full boxes while the serine residues are marked as empty boxes.

via *Eco*RV and *Sal*I sites into the vector pLacZi (Clontech). This vector was linearized with ApaI and transformed into yeast strain W303 (important features of its genotype: *ade2-1/ade2-1; ura3-1/ura3-1; his3-11,15/his311,15; trp1-1/trp1-1; leu2-3,112/leu2-3,112*) for integration into its genome and generation of the yeast reporter strain W303-p53blue which was used for the one-hybrid transcriptional activation assay described further. Next, W303-p53blue cells were or were not transformed with pGAD424 or pGAD424-p53(1–393) plasmids. In a second step these transformands were co-transfected with empty pBTM116, or pBTM116 encoding for lexA in fusion with: HBx(1–154), mini-HBx(18–142) or mini-HBx(-Cys).

The degree of activation of the p53 promotor lacZ reporter construct was then assessed by an ONPG (*o*-nitrophenyl -β-D-galactopyranoside) liquid assay (Clontech). Briefly, equal quantities of yeast cells were centrifuged and disrupted in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) by freeze–thawing. Twenty-five millimolar 2-mercaptoethanol and 10 mM ONPG (each dissolved in Z-buffer) were added to each reaction. After 1 h at 30 °C the reaction was stopped by adding 0.3 volumes of 1 M Na_2CO_3. After mixing and centrifugation the OD of the supernatants was measured at 420 nm using a spectrophotometer. All experiments were done in triplicate and the average value was plotted as arbitrary units.

3. Results

3.1. Cloning and expression of HBx fusion proteins

The full-length 6xHis-HBx protein (wt) and the mini-HBx cysteine/serine point mutants (Fig. 1) were expressed in *E. coli* cells transformed with the plasmid pET28a+ that had the *HBx* gene of the *ayw* subtype inserted in frame into its cloning

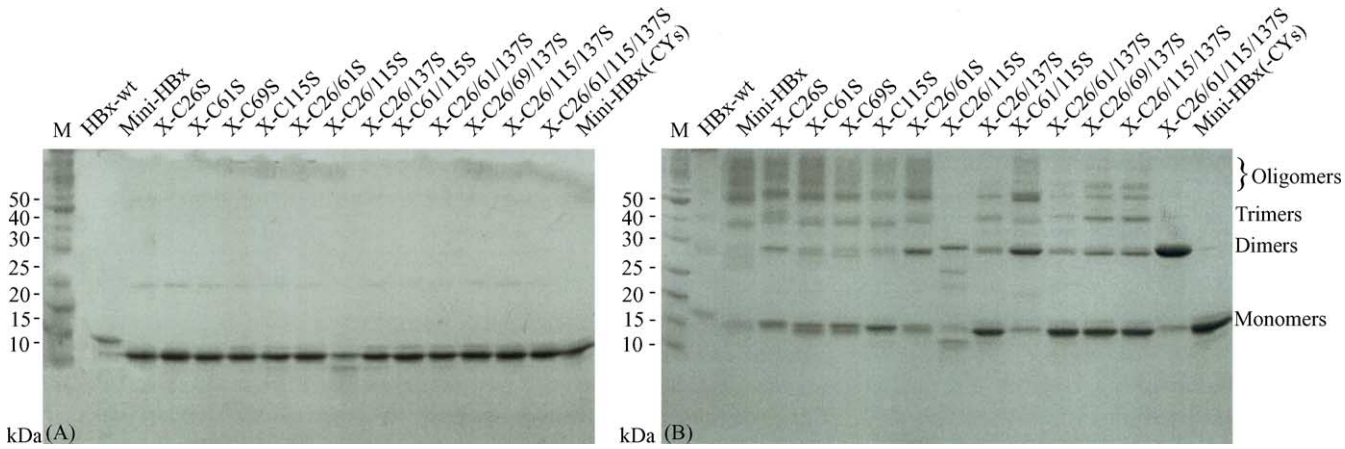

Fig. 2. SDS–PAGE analysis of mini-HBx cysteine/serine point mutants under reducing (A) and non-reducing (B) conditions. One liter of bacterial culture was induced with 1 mM IPTG for 3 h at 37 °C. Harvested cells were lysed and HBx-inclusion bodies were solubilized in 8 M urea solution after previous purification. The solubilisate was applied to a Ni-NTA superflow column wich had been equilibrated with buffer A (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8). The column was subsequently washed with buffer B (like buffer A, but pH 6.3) and proteins were eluted in one step with buffer C (like buffer A but pH 4.5). Aliquots of purified proteins were dissolved in SDS loading buffer containing 5% of 2-mercaptoethanol (A) or not (B). The 15% SDS–PAGE was stained with Coomassie blue. Approximately 1 μg of proteins were loaded in each lane. The molecular weights of the marker proteins (M) are indicated on the left side. The HBx protein point mutants are indicated on the top.

site. The expression of 6xHis-HBx and the 6xHis-mini-HBx fusion proteins resulted in proteins of 18 and ∼15 kDa that were exclusively found in the insoluble inclusion body fraction of the bacterial cell lysate.

3.2. Site-directed mutagenesis

Our aim was to address the importance of Cysteines for the biological activity of HBx protein and we decided to produce several point mutants of mini-HBx applying a site-directed mutagenesis methodology. Another group had shown before that a truncated HBx protein, spanning amino acids 58–140 maintained its full trans-activation function (Kumar et al., 1996). In order to remove some Cysteines and hydrophobic amino acids from the N- and C-terminal regions of HBx we produced a 6xHis-mini-HBx(18–142) protein in *E. coli*. Next, we produced mutant 6xHis-mini-HBx(-Cys) proteins, where the remaining five Cysteines were substituted by serine residues individually or in different combinations, applying a site-directed mutagenesis methodology (Fig. 1). It has been shown previously that a Na, K-ATPase had its 23 Cysteines changed to serine residues and this mutant protein had kept its full functional and structural integrity (Hu et al., 2000). All generated mini-HBx cysteine/serine point mutants have 124 amino acids (residues 18–142) and lack 6–10 Cysteines in comparison to the full-length HBx-wt protein that contains 10 cysteine residues (Fig. 1). All mutations were confirmed by DNA sequencing.

3.3. Purification of the 6xHis-HBx fusion proteins by Ni-NTA chromatography

Since the full-length 6xHis-HBx and the 6xHis-mini-HBx fusion proteins were found in the inclusion bodies we solubilized them with 8 M urea to purify the protein under denaturing conditions on a Ni-NTA column (Ljungquist et al., 1989; Porath et al., 1975; Rui et al., 2001). The column was washed and eluted in buffers containing 8 M urea and the collected fractions were analyzed by SDS gel electrophoresis. The recombinant 6xHis-HBx fusion proteins were dialyzed against 10 mM Pipes (pH 7) and used in the UV cross-linking and in vitro p53 binding assays.

All 6xHis-mini-HBx cysteine/serine point mutants were analyzed by SDS–PAGE under reducing (Fig. 2A) and non-reducing (Fig. 2B) conditions, in order to addresss if di- or multimerization of the mini-HBx proteins would occur. Under non-reducing conditions, it can be observed that the x-C26/61/115/137S point mutant is mostly found as a dimer while the mutant mini-HBx(-Cys) (x-C26/61/69/115/137S) is completely monomeric due to the lack of all five cysteine residues. The vast majority of point mutants was found in a di- and oligomeric forms due to the formation of inter-molecular disulphide bonds. Under reducing conditions, all inter-molecular disulphide bonds were reduced so that all the 6xHis-mini-HBx point mutants have the same electrophoretic mobilities and migrate as monomers (Fig. 2).

3.4. Circular dichroism spectroscopic analysis of HBx-wt, mini-HBx and mini-HBx(-Cys) proteins

Next, we wanted to address if the presence or absence of cysteine residues in the protein HBx influences its secondary structure content measured by far UV circular dichroism spectroscopy. We tested the different 6xHis-HBx protein variants using two different buffer conditions against which the samples had been repeatedly dialyzed: pure water (Fig. 3) and PGDE buffer (25 mM Pipes, pH 6, 50 mM KCl, 80 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1% Triton X-100, 5% glycerol) (Table 1).

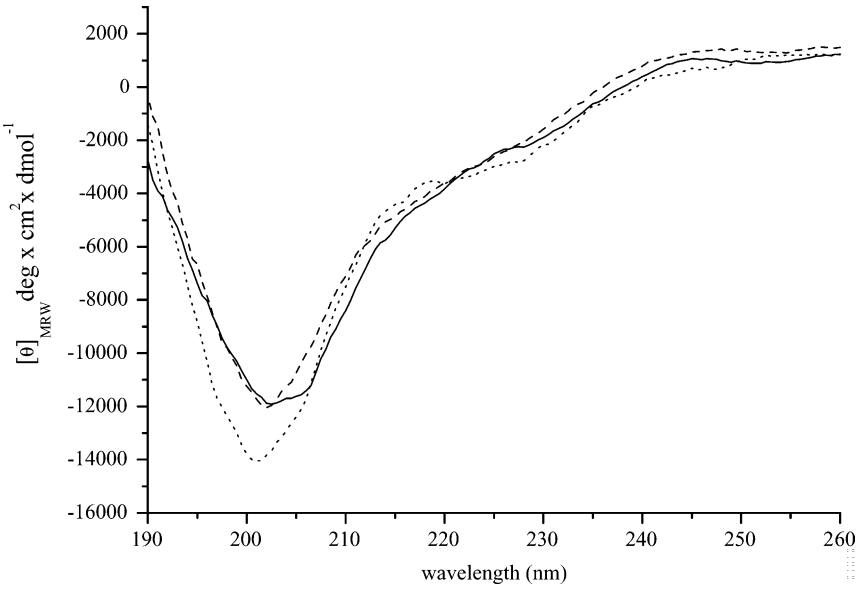

Fig. 3. Circular dichroism analysis of three HBx proteins. Far-UV CD spectra at 20 °C of the purified 6xHis-HBx (solid line), 6xHis-mini-HBx (dashed line), and 6xHis-mini-HBx(-Cys) (dotted line) in pure water at pH 6. The protein concentration was 5 μM for each sample. Spectra were background corrected and the observed optical activity was expressed as the mean residue molar ellipticity $[\theta]_{MRW}$ (deg × cm^2 × $dmol^{-1}$).

It is remarkable that the values of the secondary structure contents of the three different tested HBx protein variants in the same buffer condition are very similar (Table 1, Fig. 3). The values are very similar between 6xHis-HBx compared to 6xHis-mini-HBx, suggesting that their secondary structure contents are almost identical. 6xHis-mini-HBx(-Cys), which lacks all Cysteines, also shows comparable values for the different secondary structure elements. It is noteworthy that there was relatively little variation in the content of secondary structure comparing the three different protein versions of the HBx when tested in the same buffer (Table 1). 6xHis-HBx, 6xHis-mini-HBx and 6xHis-mini-HBx(-Cys) had for example very close values of β-sheet content in buffer PGDE (42, 42.6 and 43.7%, respectively). Comparing water and PGDE buffer, 6xHis-HBx for example showed a rather large decrease of its β sheet content from 45.9 to 42% respectively. In consequence of this, 6xHis-HBx random coil content increases from 23.3% in water to 29.5% in PGDE. In summary, these data suggest that the buffer conditions have a larger influence on the random coil content of the different HBx protein variants than the presence or absence of cysteine residues in the different protein variants, since HBx has ten Cys residues, 6xHis-mini-HBx fivc and 6xHis-mini-HBx(-Cys) no cysteine residue.

3.5. HBx and mini-HBx lacking one to all Cysteines interact with AU-RNA oligonucleotides

We decided to employ the HBx ability to bind to singlestranded DNA in vitro as described by Qadri and coworkers (Qadri et al., 1996) as an assay for one potential biological activity of HBx. The binding of HBx to singlestranded nucleic acids can be tested by an electrophoretic mobility shift assay (EMSA) in which a radioactively labeled RNA oligonucleotide 38-mer was used (Rui et al., 2001) instead of the ssDNA probes described by Qadri et al. (1996). Here, we used an UV cross-linking assay instead of a conventional EMSA assay, since it is more sensitive. We found (Fig. 4), that not only the full-length 6xHis-HBx fusion protein but also all 6xHis-mini-HBx cysteine/serine point mutants proteins were able to bind to the RNA oligonucleotide (AU-38) and caused its retardation in the denaturing gel. Along with the 38-mer AU-rich oligonucleotide we also tested an AU-rich 21-mer oligonucleotide (data not shown) and found that

Table 1
Assessment of the relative secondary structure content of the complete and mutant 6xHis-HBx protein variants in different buffers as measured by CD spectroscopy analysis (see Section 2 for details and Fig. 3)

Secondary structure element	6xHis-HBx		6xHis-mini-HBx		6xHis-mini-HBx(-Cys)	
	Water	PGDE[a]	Water	PGDE[a]	Water	PGDE[a]
α-Helix	14.2	12.9	13.9	13.5	14.4	14.1
β-Sheet	45.9	42	45.6	42.6	42.3	43.7
β-Turn	16.6	15.6	16.4	15.9	16.4	16.4
Random coil	23.3	29.5	24.1	28	26.9	25.8

[a] PGDE buffer: 25 mM Pipes, pH 6, 50 mM KCl, 80 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1% Triton X-100, 5% glycerol.

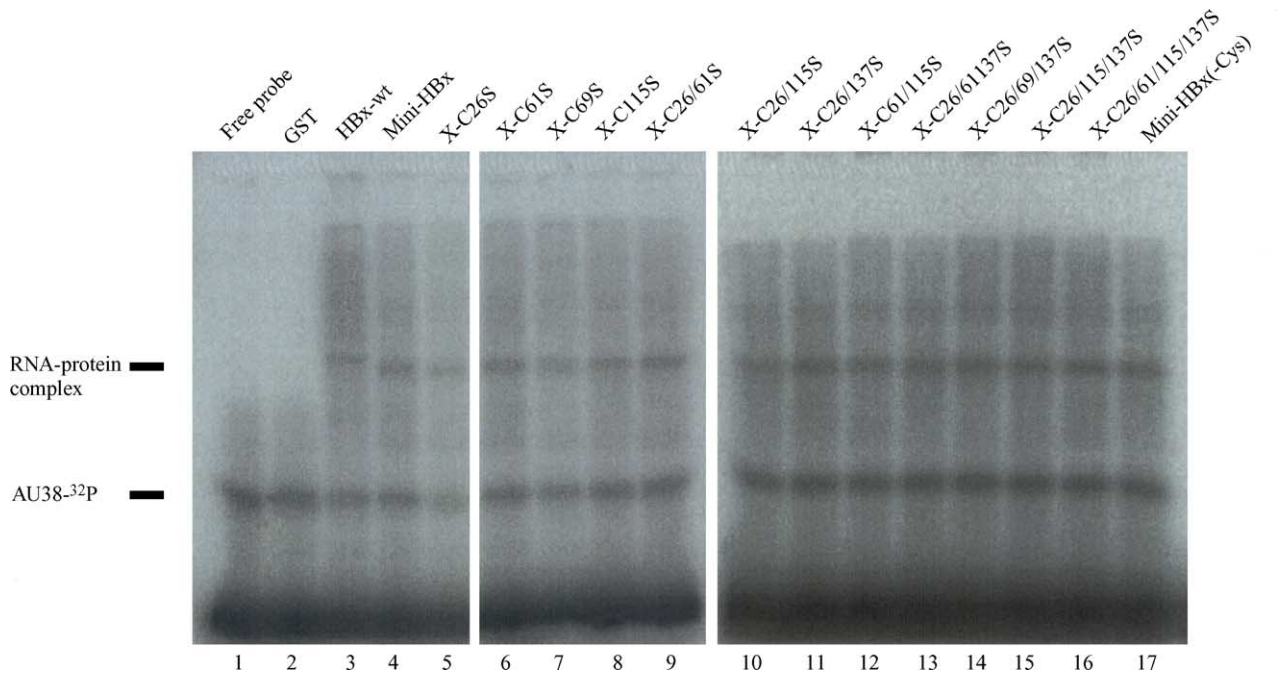

Fig. 4. The cysteine residues of the HBx protein are not important for its interaction with an AU-rich RNA oligonucleotide (AU-38). UV cross-linking assay with the HBx proteins and AU-38 mer oligonucleotide. After incubation on ice for 30 min, the protein–oligonucleotide complexes were exposed to the ultraviolet light (254 nm) and resolved by SDS–PAGE under denaturing conditions. The 12.5% SDS gel was visualized by phosphorimager scan. Lanes 1–17, radioactively labeled AU-rich RNA oligonucleotide AU-38 (120 fmol per lane). Proteins: lane 1, nil; lane 2, GST (0.76 nmol); lane 3, 6xHis-HBx-wt (0.35 nmol); lanes 4–17, 6xHis-mini-HBx with the indicated point mutations (0.36 nmol per lane). The complexes of RNA and protein and the free probe are indicated on the left side.

mini-HBx(-Cys) binds to these oligonucleotides just like mini-HBx and HBx-wt. In summary, these results indicate that the lack of the Cysteine in the 6xHis-mini-HBx point mutants do not alter its interaction with RNA.

3.6. Mini-HBx(-Cys) interacts with p53 protein as HBx or mini-HBx

HBx has not only been shown to interact with singlestranded DNA and RNA but also with many different cellular proteins. The large group of HBx interacting proteins contains members of diverse functional groups of proteins (Murakami, 1999), especially transcription factors, including p53. Based on the functional importance of p53 and its well documented interaction with HBx (Feitelson et al., 1993; Elmore et al., 1997), we decided to test our mutant HBx proteins ability to interact with human p53 protein in vitro. We found that 6xHis-mini-HBx(-Cys) is as capable of interacting with GST-p53 as is full-length 6xHis-HBx-wt and the 6xHis-mini-HBx (Fig. 5). This interaction is specific for p53 since the control glutathione sepharose beads containing only unfused GST control protein did not bind any of the HBx-wt or mutant proteins.

3.7. HBx, mini-HBx and mini-HBx(-Cys) increase the p53 dependent β-galactosidase reporter gene activity in a yeast one-hybrid transcription activation assay

It has been previously described that HBx binds to p53 (Feitelson et al., 1993) and is capable to increase the transcription activity of p53 regulated promoters (Ahn et al., 2002), probably by stabilizing the p53-multimerization upon binding. Such a stabilization had been described for HBx upon binding to other transcription factors (Schneider and Schepartz, 2001). We wanted to test if HBx in its mutated forms is still capable of transactivating a p53 promotor in the presence of human p53. Therefore, we established a yeast one-hybrid system based transcription activation system. The strain W303 was transfected with a plasmid containing the *LacZ* gene (encoding β-galactosidase) under control of a p53 promotor region. Next, additional transfections of the yeast expression plasmids (pGAD424) encoding human p53 in fusion with the Gal4 activation domain on the one hand and of a second yeast expression plasmid (pBTM116) encoding full-length or the mutant mini-HBx proteins fused to the lexA DNA binding domain were performed. The co-transfected yeast clones were than tested for the activation of the *p53* reporter gene encoding β-galactosidase (ONPG enzymatic assay of the β-galactosidase activity). As expected, the presence of only the p53 encoding plasmid caused a relatively strong activation of the reporter gene (Fig. 6, column 2) but remained on the same level when an empty pBTM116 had been co-transfected into the cells (column 3). The co-transfection of a pBTM116 plasmid containing the cDNA coding for full-length HBx-wt or the two mutant HBx proteins (mini-HBx or mini-HBx-Cys), all caused an equally high increase in the level of β-galactosidase expression in these cells. Full-length HBx caused an increase of the transcriptional activation by factor 1.69, mini-HBx by factor 1.68 and mini-HBx(-Cys) by

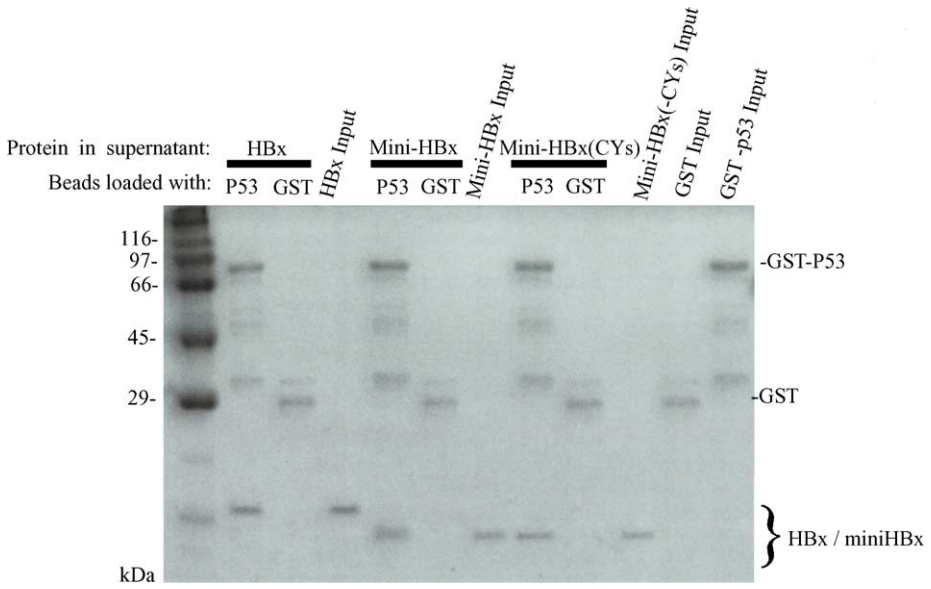

Fig. 5. HBx, mini-HBx and mini-HBx(-Cys) interact with human protein GST-p53. In vitro pull down assay with recombinant purified proteins. Briefly, glutathione sepharose beads were coupled with GST control protein or GST-p53 (denominated p53 above the figure) fusion protein. Next the loaded beads were incubated with wild type 6xHis-HBx, 6xHis-mini-HBx or 6xHis-mini-HBx(-Cys) proteins as indicated above the figure. After washing the bound proteins were analyzed by SDS–PAGE. Input control lanes indicate the positions of the corresponding protein bands, which are pointed out on the left side of the figure.

factor 1.87. This strongly suggest that the mini-HBx as well as mini-HBx(-Cys), where all five Cys had been replaced by Ser, are fully capable to cause the increase of the p53 transcriptional activity. This increase of the transcriptional activation by p53 is however not mediated by the lexA protein fusion part in the expressed constructs, since the expression of lexA alone did not cause a significant increase in the transactivation of the reporter gene (1.1-fold increase only) (Fig. 6).

4. Discussion

Here, we wanted to test if the HBx cysteine residues are relevant to the structural and functional aspects of the HBx protein. It has been previously shown for other proteins, that replacement of Cys residues to Ser residues in intracellular proteins might still preserve the proteins functions. In the extreme and remarkable case of the Na, K-ATPase all

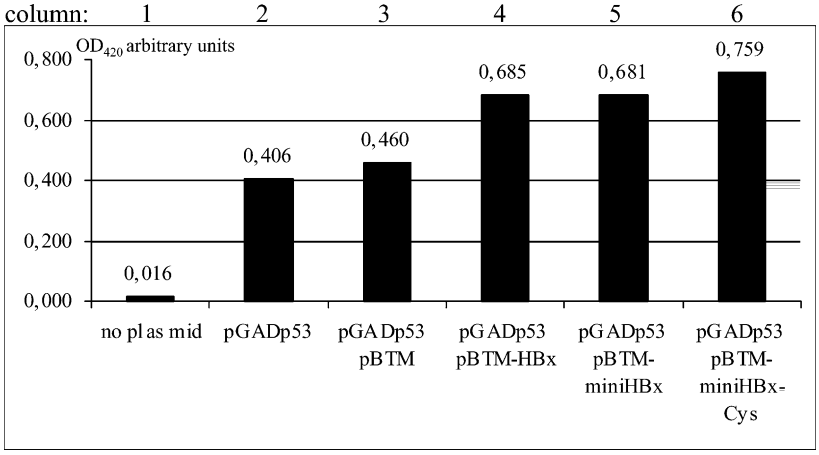

Fig. 6. HBx, mini-HBx and mini-HBx(-Cys) increase the p53 dependent β-galactosidase reporter gene activity in an yeast one-hybrid based transcription activation assay. W303-p53 blue yeast cells were transformed with no vector (column 1) or with pGAD424-p53 vector encoding full-length human p53 in fusion with the Gal4-activation domain (columns 2–6). These same strains were or were not (columns 1 and 2) co-transformed with: pBTM116 vector containing no insert DNA (column 3), pBTM116-HBx(1–154) (column 4), pBTM116-mini-HBx(18–142) (column 5), and pBTM116-mini-HBx(-Cys) (column 6). Freshly grown co-transformed colonies were then grown in liquid culture. After adjusting equal cell numbers the cells were pelleted, lysed and their cell lysates were submitted to a quantitative β-galactosidase assay based on the OPNG reaction (see Section 2 for details). Each point represents the average value of three independent experiments in arbitrary units.

23 Cysteines of the protein sequence have been changed to serine residues and the resulting mutant protein had kept its functional and structural integrity (Hu et al., 2000). We set out to construct by site-directed mutagenesis 14 6xHis-mini-HBx proteins containing one to five cysteine-to-serine point mutations. We found that all of our mini-HBx point mutant proteins can bind to the AU-rich oligonucleotide (AU-38). Furthermore, we tested the capacity of selected mutants to interact in vitro with the human p53 tumor suppressor protein (Feitelson et al., 1993) and to influence its transcription activation function (Takada et al., 1995; Ahn et al., 2002). We observed that the three proteins HBx-wt, mini-HBx with and mini-HBx without Cys residues all bound in a specific way in vitro to p53 and that all three proteins showed also a significant stimulating effect on p53 transcriptional activation. In summary, these data suggests that the Cys residues of HBx are of minor importance for its activity RNA and p53 binding and for its enhancement of p53 transcription activation.

In the literature the outcome of the interference of HBx with p53 is still a matter of debate since both inhibitory effects of HBx on p53 function (Wang et al., 1994) as well as stimulatory effects (Ahn et al., 2002) have been described. This could be due to variations based on the different cell types and/or promotor systems studied. Ahn and co-workers (Ahn et al., 2002) found that the activity of endogenous cellular p53 on a p53 responsive, p21-promotor controlling a luciferase reporter gene was activated by the presence of the HBx protein. These authors speculated that this increase in p53 activity by HBx could cause an increase in hepatocyte cell death rate, which is indeed frequently observed in severe chronic hepatitis (Papakyriakou et al., 2002). In case of the HBx protein seemingly contradictive functional consequences are not a novelty, since its main predicted functions range from the induction of apoptosis (Pollicino et al., 1998; Elmore et al., 1997) to participation in cellular transformation (Koike et al., 1994), oncogenesis and tumor survival (Henkler and Koshy, 1996; Shirakata et al., 1989). However, in the liver, which is chronically infected with HBV, probably both an increased tumor growth and a compensating increase in hepatocelular apoptosis or necrosis, leading to liver cirrhosis, may keep a balanced state for prolonged periods of time. Therefore it is tempting to speculate that HBx with its pleiotropic functions, which are mediated by many protein–protein interactions with diverse cellular proteins, could contribute to both cell growth and apoptosis at the same time, depending on the cellular state and on the phase of infection of the cell.

In conclusion, our finding that the cysteine residues of the HBx protein can be either deleted or substituted with serines without influencing HBx functional interactions with either RNA nor p53 protein might have far reaching consequences to our understanding of the functional mechanisms underling HBx. In fact it could be assumed, that HBx is able to interact with such a multitude of different molecules, including singlestranded nucleic acids and a vast variety of cellular proteins that include both transcriptional activators (Murakami, 1999) as well as many signaling proteins (Yen, 1996), because it is able to adapt different conformations upon binding to its target molecule. Further functional interaction studies of the mutated cysteine-free HBx with other important cellular protein partners are required in order to confirm whether our findings hold true for other interacting proteins, too. In the moment we are trying to express and purify mini-HBx(-Cys) in large scale for possible structural studies including crystallization trials and nuclear magnetic resonance spectroscopy analysis. Such studies had been impossible in the past due to the fact that bacterially derived HBx protein has the strong tendency to form inter-molecular disulfide bridges and aggregate, therefore preventing any detailed structural characterization. However, structural data on the HBx protein would be of great value in order to understand how this intriguing viral protein achieves to adapt to so many different cellular protein targets and thereby mediate its manifold cellular functions.

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Artigo III

Expression and spectroscopic analysis of a mutant hepatitis B virus onco-protein HBx without cysteine residue.

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* Contribuíram igualmente para o trabalho

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Expression and spectroscopic analysis of a mutant hepatitis B virus onco-protein HBx without cysteine residues

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Abstract

Chronic infection of the hepatitis B virus (HBV) is one of the causes leading to liver cancer. The 3.2 kb genome of HBV encodes four proteins: core antigen, surface antigen, a DNA polymerase and the X protein (HBx). The biological functions of HBx are not fully understood. It has been shown that HBx is a potent trans-activator, which activates transcription of many cellular and viral promoters indirectly via protein-protein interactions. These transactivating activities of HBx may contribute to the development of hepatocellular carcinoma. In this paper a truncated mini-HBx(-Cys) (18–142) protein, where the cysteines had been either deleted or substituted by serines, was constructed by site-directed mutagenesis and overexpressed as a 6xHis fusion protein in *Escherichia coli*. The 6xHis-mini-HBx(-Cys) protein was isolated from inclusion bodies, purified by Ni-affinity chromatography under denaturing conditions and refolded by sequential dialysis. The structure of the 6xHis-mini-HBx(-Cys) protein was analyzed by circular dichroism, fluorescence and one-dimensional NMR spectroscopic assays. The data presented here suggest that HBx is unstructured but has a propensity to gain secondary structure under specific experimental conditions. Its conformational flexibility might partially explain its functional complexity, namely its capacity to interact with a wide array of signaling proteins, transcriptional regulators and nucleic acids.

Keywords: Viral hepatitis; Hepatitis B virus X protein; Viral transactivator; Onco-protein; Circular dichroism; Fluorescence

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major malignant diseases in the world, ranking fifth as the most common cancer, and it is believed that chronic hepatitis B virus (HBV) infection is a major global cause of HCC (Arbuthnot and Kew, 2001; Waris and Siddiqui, 2003). The 3.2 kb HBV partially double-stranded DNA genome is the smallest among mammalian viruses and predominantly infects human hepatocytes in the liver (Seeger and Mason, 2000). The compact HBV genome includes four ORFs that encode at least seven translation products through the use of varying in frame initiation codons. These translation products include three surface antigens (HBsAg): the envelope glycoproteins preS1, preS2, and S; core (C) and e antigens (HBcAg and HBeAg); viral polymerase (P); and the X protein (HBx) (Lo et al., 1988; Caselmann, 1996).

Both the X gene and its product are well conserved among mammalian hepadnaviruses (Kidd-Ljunggren et al., 1995; Hildt et al., 1996; Murakami, 1999). HBx is a small, 17 kDa protein and produces antibodies in sera of infected humans and naturally infected animals. Although the specific function of HBx in natural infection remains elusive, its presence is necessary during viral replication and for the establishment of viral infection in vivo in animals (Chen et al., 1993; Zoulim et al., 1994). HBx has been shown to activate the transcrip-

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tion of a variety of viral and host cellular genes (Caselmann, 1996; Murakami, 2001). This indiscriminate or 'promiscuous' trans-activation activity appears to be via an indirect process, since HBx does not have any classical double-stranded DNA-binding domain. It appears rather to act indirectly on transcription and signaling, through protein–protein interactions (Maguire et al., 1991).

Due to its both nuclear and cytoplasmatic localization, HBx can interact with transcription factors, stimulating the transcriptional machinery in the nucleus as well as interfering with signal transduction pathways in the cytoplasma of the cell (Murakami, 1999; Henkler et al., 2001). It has been reported that HBx activates host genes such as the proto-oncogenes c-*myc* (Balsano et al., 1991), c-*fos* (Avantaggiati et al., 1993) and c-*jun* (Twu et al., 1993), and interacts with cellular proteins like TBP (TATA-binding protein) (Qadri et al., 1995; Wang et al., 1997) and the cAMP-responsive element binding protein (CREB) (Williams and Andrisani, 1995). HBx also activates transcription factors such as ATF-2 and NF-κB (Maguire et al., 1991; Twu et al., 1989), as well as the Ras-Raf-Mitogen-activated protein (MAP) kinase signaling cascade (Benn and Schneider, 1994), and the JAK/STAT signal transduction pathways (Lee and Yun, 1998; Arbuthnot et al., 2000). Besides, HBx has also been reported to interact both in vivo and in vitro with the tumour suppressor protein p53 (Feitelson et al., 1993; Wang et al., 1994; Truant et al., 1995; Elmore et al., 1997), what implicates an important role of the HBx protein in the regulation of cell cycle control (Benn and Schneider, 1995) and programmed cell death (Wang et al., 1995; Chirillo et al., 1997; Arbuthnot et al., 2000).

Lastly, HBx may be responsible for hepatocarcinogenesis by modulating DNA repair in the host cell (Arbuthnot et al., 2000), due to its interaction with tumour suppressor protein p53, thus affecting the p53-mediated repair pathway, and UVDDB (UV-damaged DNA-binding protein) (Becker et al., 1998; Groisman et al., 1999). These results are consistent with a model in which HBx acts as a cofactor in hepatocarcinogenesis by preventing the cell from repairing damaged DNA, thus leading to an accumulation of DNA mutations and, eventually, cancer (Butel et al., 1996; Capovilla et al., 1997; Anthony, 2001).

Previous studies had also shown that the HBx protein is able to bind to single stranded DNA (Qadri et al., 1996) and an AU-rich RNA oligonucleotide in a gel shift assay (Rui et al., 2001). This nucleic acid binding activity might be relevant for HBx oncogenic character. It has been previously shown that HBx is able to displace the AUF1 protein from its binding site on the RNA oligonucleotide, which could cause the stabilization of AU-rich proto-oncogenes mRNAs and thereby contribute to the transforming phenotype (Rui et al., 2001).

A truncated protein mini-HBx(18-142) (-Cys), where the remaining five cysteines have been replaced by serine residues, was generated by applying a site-directed mutagenesis methodology. Expression in *Escherichia coli* BL-21 cells and protein purification by Ni-affinity chromatography from inclusion bodies were optimized, yielding 6xHis-mini-HBx(-Cys) protein in a high degree of purity. Far-UV CD and fluorescence spectroscopy were used to determine the overall secondary and tertiary structure of the refolded protein, and the percentage of secondary structure was calculated using the *CDNN deconvolution* program. A one-dimensional ^{1}H NMR spectrum was taken and collected data are in agreement with previous CD and fluorescence spectroscopy assays.

2. Materials and methods

2.1. Plasmid construction by site-directed mutagenesis

The truncated mini-HBx gene (372 bp), coding for amino acids 18–142 of the HBx protein, was amplified by PCR using the full-lenght HBx DNA (subtype *ayw*) inserted into pCMV-Ad (kindly provided by Dr. R. Schneider, New York) as a template DNA. The mini-HBx gene was directionally cloned into the bacterial expression vector pET28a+ (Novagen) via *Nde*I and *Bam*HI restriction sites. The recombinant plasmid pET28a+/mini-HBx(18–142) was used as a template DNA in the following PCR reactions in order to generate a complete cysteine/serine point mutant using the "*QuickChange*^{TM} *Site-Directed Mutagenesis*" kit (Stratagene). The cysteine/serine point mutations were confirmed by DNA sequencing. The recombinant plasmid pET28a+/mini-HBx(-Cys) contains a 6-histidine tag (6xHis) encoding sequence that allows the purification of the recombinant fusion protein via Ni-affinity chromatography.

2.2. Expression and purification of 6xHis-mini-HBx(-Cys) fusion protein from inclusion bodies

E. coli strain BL-21, transformed with the mini-HBx (-Cys) DNA containing pET28a + vector, was grown in LB medium containing kanamycin (50 μg/ml) to an $OD_{600} = 0.7$. The culture was subsequently induced at 37 °C for 4 h with 1 mM IPTG (isopropylthio-β-D-galactoside). Cells were harvested by centrifugation and the bacterial pellet was resuspended in lysis buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 5 mM DTT, 1 mM PMSF) containing lysozyme (1 mg/ml). After incubation on ice for 30 min, cells were lysed by three subsequent freeze-thaw cycles. Genomic DNA was digested by addition of DNAse (1 μg/ml), and after incubation for 20 min at 37 °C, the samples were sonicated and the pellet was separated by centrifugation (18.600 × g for 15 min at 4 °C). The pellet was ressuspended in the washing buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 5 mM DTT, 3 M Urea, 1% Triton X-100) and then centrifuged at 9.500 × g for 15 min at 4 °C for three times or until the supernatant was clear.

The pellet containing the 6xHis-mini-HBx(-Cys) inclusion bodies was solubilized in 50 mM Tris–HCl (pH 8), 8 M urea and the solution was cleared by centrifugation at 13.700 × g for 20 min at 4 °C. The resulting solution was filtered and applied to a Ni-NTA column (QIAgen). The sub-

sequent washing and elution steps were all carried out with buffer A (8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl) but at different pHs. The column was washed first with buffer A, pH 8 and than with buffer A, pH 6.3. Bound protein was eluted with a linear pH gradient between 6.3 and 4.5 in buffer A. The column eluate was collected in 2 ml fractions and 10 μl aliquots were analyzed by SDS-PAGE. The proteins 6xHis-HBx(1–154) and 6xHis-mini-HBx(18–142) were expressed and purified according to the same protocol.

2.3. Refolding of purified protein

The denatured purified protein in eluted fractions was refolded by dialysis at 4 °C in 10 mM Pipes (pH7) with several changes of buffer. After this, the protein was again dialyzed against pure water. Dialyzed protein was cleared by centrifugation at 2000 × g and used in all spectroscopic assays. Protein concentration was measured by determining the absorption in a 1 cm path length cell using a JASCO UV/Vis spectrophotometer. For 6xHis-mini-HBx(-Cys) protein, a molar extinction coefficient at 280 nm of 6970 $M^{-1} \times cm^{-1}$ was used.

2.4. Western blotting

Proteins were separated by SDS-PAGE using a 12.5% acrylamide gel and then transferred to the PVDF membrane (*Immobilon*™*-P*, Millipore) using a *Semi-Dry Blotting System* (W.E.P. Company). The membrane blocked in 5% BSA in TBS (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.2) was probed with primary antibody (anti-4xHis at 1:3000) or antiserum anti-HBx (at 1:100). The produced antiserum is a polyclonal rabbit anti-6xHis-HBx(1-154) antibody that has been generated by six immunizations of rabbits with 1 mg of recombinant 6xHis-HBx(1–154) protein. Secondary antibodies (anti-mouse antibody and anti-rabbit antibody) conjugated with HRP were used at a dilution of 1:5000, and immunoreactive proteins were detected using the *Western Blotting Luminol Reagent* (Santa Cruz Biotechnology).

2.5. UV cross-linking assay of HBx with an AU-rich RNA oligonucleotide

The purified 6xHis-HBx(1–154), 6xHis-mini-HBx(18–142) and 6xHis-mini-HBx(-Cys) proteins were incubated with a radiolabeled AU-rich [γ-^{32}P]-RNA 38-mer oligonucleotide (GUGAUUAUUUAUUAUUUAUU-UAUUAUUUAUUUAUUUAG) (Rui et al., 2001) in a final volume of 25 μl containing 20 mM Hepes, 10 mM $MgCl_2$, 60 mM KCl and 10% glycerol. After incubation on ice for 30 min, the opened reaction tubes were exposed to the ultraviolet light (254 nm) for 5 min in a distance of 100 mm (UV Stratalinker 2400). The complexes were resolved by SDS-PAGE under denaturing conditions and visualized after a 2–6 h exposition on a BAS-IP MS 2325 (Fujifilm) by phosphorimager scan (Bio-Imaging Analyzer BAS-1800II; Fujifilm).

2.6. CD spectroscopy

Circular dichroism (CD) experiments were performed at the indicated temperatures on a JASCO J-810 spectropolarimeter coupled to a *Peltier* PFD-425S system for temperature control. Measurements in the far-ultraviolet region (190–260 nm) were carried out with a 1 mm path length quartz cell (Hellma). The protein concentration was 5–15 μM in aqueous solution at pH 6. Four scans were averaged for each sample and the contribution of the buffer was always subtracted. The observed optical activity was expressed as the mean residue molar ellipticity $[\theta]_{MRW}$ (degree $cm^2\ dmol^{-1}$). The secondary structure of the recombinant 6xHis-mini-HBx(-Cys) protein was predicted by *CDNN deconvolution* software (http://bioinformatik.biochemtech.uni-halle.de/cdnn/).

2.7. Fluorescence spectroscopy

The intrinsic fluorescence emission spectra were measured by AMINCO-BOWMAN (Series 2) luminescence spectrometer, using a 10 mm path length quartz cell (Hellma). The excitation wavelength was set at 295 nm, and the emission spectra were collected over a range of 310–430 nm, and recorded at 25 °C or at 37 °C. The protein concentration was 2 μM in aqueous solution at pH6. NaCl was added to a final concentration of 100 mM, and Gdn-HCl to 6 M. Spectra were background corrected and values of λ_{max} were obtained from the emission spectra.

2.8. NMR spectroscopy

The one dimensional ^{1}H NMR experiment was carried out on a Varian INOVA 500AS spectrometer operating at 499.730 MHz for ^{1}H frequency, at a temperature of 25 °C. The proton chemical shifts were referenced to the water signal that was set at 4.8 ppm. Water suppression was achieved by low power continuous wave irradiation during the relaxation delay or using the WET pulse technique. An NMR sample of ∼400 μM of 6xHis-mini-HBx(-Cys) in aqueous solution containing 5% D_2O at pH 6 was used in the one-dimensional ^{1}H NMR assay.

3. Results

3.1. Cloning and expression of 6xHis-mini-HBx(-Cys) fusion protein

The 17 kDa protein HBx consists of 154 amino acids, contains 10 cysteine residues and is translated during the viral infection. The HBx protein has been involved in several important steps on the HCC development due to its ability to induce liver cancer in transgenic mice (Kim et al., 1991;

Koike, 2002) and to transactivate a variety of viral and cellular promoters (Twu et al., 1990; Yen, 1996; Caselmann, 1996). HBx has also been reported to stimulate the transcription machinery in the nucleus by indirect activation of transcription factors and cell signaling pathways, deregulating the cell cycle checkpoints that might lead to an uncontrolled cellular proliferation (Benn and Schneider, 1995; Kim et al., 1998). Nevertheless, the exact function of HBx is still unknown and its structural determination would be of great value in the study of HCC development mediated by HBx.

However, the detailed study of HBx functions has been complicated due to the difficulty in HBx isolation from HBV-infected liver tissues and from transfected cell lines because of its low expression level and its short half-life (Hildt et al., 1996; Urban et al., 1997). Besides, it has been shown previously that HBx protein is predominantly expressed in the insoluble inclusion body fraction of the bacterial cell lysate (Jameel et al., 1990; Marczinovits et al., 1997; Rui et al., 2001). It also tends to form intracellular aggregates when expressed in insect cells using a baculovirus expression system (Urban et al., 1997). Of the 14 liters of HBx transformed *E. coli* induction, only 0.05% of the recombinant HBx protein produced was isolated from the soluble fraction (Truant et al., 1995). In order to improve HBx poor solubility several investigators have tested a series of induction and expression conditions in *E. coli*, including the co-expression of thioredoxin or the chaperones GroEL and GroES (Rui et al., 2001), but none of this has shown any improvement on HBx solubility (Hildt et al., 1996). The reducing environment inside the *E. coli* and the lacking of specific chaperones, present in eukaryotic cells, that could be involved in the correct folding and formation of disulphide bonds, might contribute to the accumulation of the HBx protein in the inclusion body fraction of the bacterial cells.

Another group had shown before that a truncated HBx protein, spanning amino acids 58–140 maintained its full transactivation function (Kumar et al., 1996). In order to remove some cysteines and hydrophobic amino acids from the N- and C-terminal regions of HBx a 6xHis-mini-HBx(18–142) protein was produced in *E. coli*. Moura and co-workers demonstrated in a recent study, that the mini-HBx(18–142) protein retains its functional activity to bind to both the interacting protein p53 and also to RNA (Moura et al., 2005). Furthermore, they showed that the circular dichroism spectra of the wt HBx and mini-HBx(18-142) are practically identical, suggesting that both proteins have similar conformations (Moura et al., 2005). Finally, when the mini-HBx(18–142) protein was used as a bait protein in a yeast two-hybrid screen, it was like wt HBx capable to interact with both new as well as previously identified proteins (Rui et al., unpublished observation), including RXR (Retinoid X receptor) and UVDDB (UV-damaged DNA-binding protein) (Kong et al., 2000; Sitterlin et al., 1997). However, it was observed that the remaining five cysteine residues in this protein tend to result in the formation of incorrect intermolecular disulfide bridges, leading to the aggregation of the mini-HBx(18–142) protein in vitro. Therefore, it was decided to produce the mutant 6xHis-mini-HBx(-Cys) protein, where the remaining five cysteines were substituted by serine residues, by applying a site-directed mutagenesis methodology. It has been shown previously that a Na, K-ATPase had its 23 cysteines changed to serine residues and this mutant protein had kept its full functional and structural integrity (Hu et al., 2000).

The full length HBx gene (*ayw* subtype) was used as a DNA template in PCR reactions in order to generate a truncated mini-HBx(-Cys) gene. The mini-HBx(-Cys) gene has 124 amino acids (residues 18–142) and lacks all 10 cysteine residues (Fig. 1). The cysteine/serine point mutations (Cys26Ser, Cys61Ser, Cys69Ser, Cys115Ser and Cys137Ser) were confirmed by DNA sequencing. Both amino acid sequences of the full length HBx (1–154) and the truncated mini-HBx(-Cys) contain a tryptophan residue that can be used as an intrinsic probe in fluorescence emission experiments.

3.2. Purification of the 6xHis-mini-HBx(-Cys) fusion protein by Ni-NTA chromatography

An induced protein band of ~15 kDa was observed in SDS-PAGE (Fig. 2A, lane 2) when pET28a/mini-HBx(-Cys) transformed BL-21 cells were induced with 1 mM IPTG. After lysing the cells, the 6xHis-mini-HBx(-Cys) protein remained in the insoluble fraction. The pellet containing inclusion bodies was purified first in order to eliminate cell debris

Fig. 1. Amino acid sequence alignment of full-length HBx(1–154) and mini-HBx(-Cys) proteins. All five remaining cysteine residues were mutated to serine residues and are indicated by boxes. An arrow indicates the tryptophan residue present in both amino acid sequences.

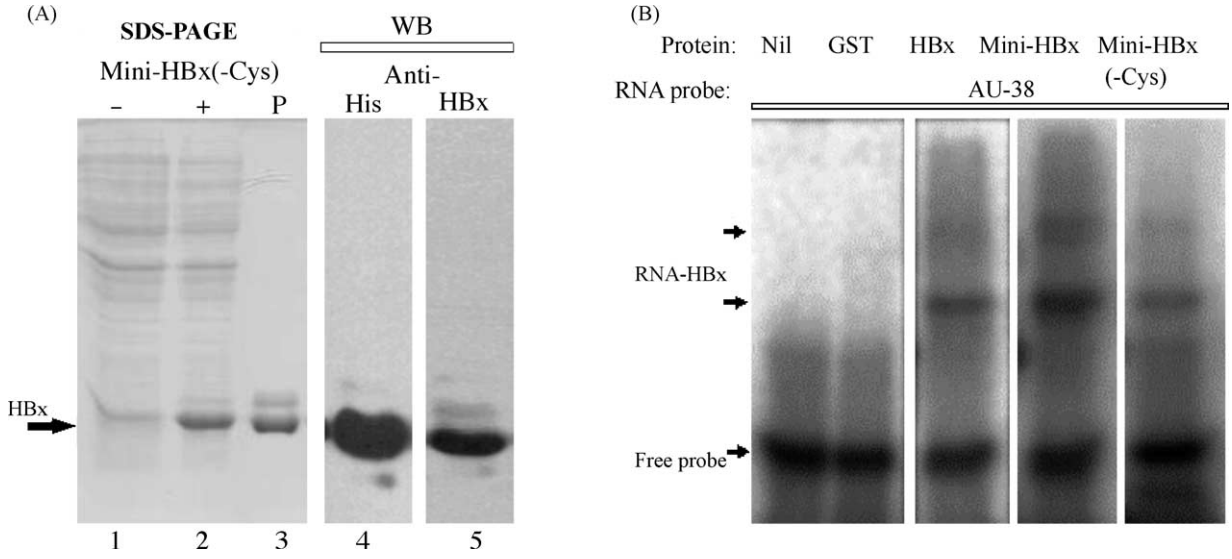

Fig. 2. SDS-PAGE, Western blot and test of biological activity of the expression and purification of 6xHis-mini-HBx(-Cys) from inclusion bodies. (A) Lane 1, cell lysate of transformed BL21 cells before induction; lane 2, lysate of transformed cells after 4 h IPTG induction at 37 °C; lane 3, purified protein after preparation of inclusion bodies and affinity purification using a Ni-NTA column under denaturing conditions (8 M urea); lane 4, immunoblot of purified protein developed with an anti-His monoclonal antibody; lane 5, immunoblot of purified protein using a rabbit antiserum against 6xHis-HBx(1–154) protein. The recombinant protein has a molecular weight of ∼15.6 kDa and it is exclusively found in the insoluble inclusion body fraction of the bacterial cell lysate. (B) UV cross-linking assay with the three different HBx proteins and an AU-rich 38-mer RNA oligonucleotide. After incubation on ice for 30 min, the protein-oligonucleotide complexes were exposed to the ultraviolet light (254 nm) and resolved by SDS-PAGE under denaturing conditions. The 12.5% SDS gel was visualized by phosphorimager scan. All lanes: radioactively labeled AU-rich RNA oligonucleotide AU-38 (120 fmol per lane). Lane Nil: no protein; lane GST: GST control protein; lane HBx: 6xHis-HBx(1–154); lane Mini-HBx: 6xHis-mini-HBx(18–142); lane Mini-HBx(-Cys): 6xHis-mini-HBx(-Cys) (0.36 nmol of protein per lane). The complex of HBx and RNA and the free probe are indicated on the left side of the panel.

and other impurities. After extensive washing, the inclusion bodies were disrupted with 8 M urea for protein solubilization. The protein was purified via Ni-affinity chromatography under denaturing conditions (Ljungquist et al., 1989; Rui et al., 2001), yielding protein at high concentration (Fig. 2A, lane 3). The identity of the protein band was confirmed by immunoblotting with anti-His (Fig. 2A, lane 4) and anti-HBx rabbit antiserum (Fig. 2A, lane 5). To remove the urea, the protein was dialyzed against 10 mM Pipes at pH 7 and followed by another dialysis against pure water at pH 6. The concentration of the recombinant purified 6xHis-mini-HBx (-Cys) protein in aqueous solution was determined and the purified protein was used in the spectroscopic assays (CD, fluorescence, NMR) and in the UV cross-linking assay.

3.3. The mutated protein 6xHis-mini-HBx(-Cys) interacts with RNA

The HBx ability to bind to single-stranded DNA in vitro was employed as described by Qadri and co-workers (Qadri et al., 1996) in an assay for one potential biological activity of HBx. The binding of HBx to single-stranded nucleic acids can be tested by an EMSA (Electrophoretic Mobility Shift Assay) in which a radioactively labeled RNA oligonucleotide 38-mer was used (Rui et al., 2001) instead of the ssDNA probes described by Qadri et al. (1996). Here, a UV cross-linking assay instead of a conventional EMSA assay was used, since it is more sensitive. It was found, that not only the full length 6xHis-HBx(1–154) fusion protein but also the 6xHis-mini-HBx(18–142) and 6xHis-mini-HBx(-Cys) mutant proteins were able to bind to the RNA oligonucleotide (AU-38) and caused its retardation in the denaturing gel (Fig. 2B). These results indicate that the lack of the cysteines in the 6xHis-mini-HBx(-Cys) mutant does not alter its activity to interact with RNA. This result is further corroborated by recent studies from our laboratory, which show that the mutant protein mini-HBx(-Cys) like wild type HBx, retains its full capacity to interact with the human tumor suppressor protein p53 in vitro and in vivo (Moura et al., 2005).

3.4. CD spectra and secondary structure analysis of 6xHis-mini-HBx(-Cys) protein

The overall conformation of the 6xHis-mini-HBx(-Cys) protein was measured by far-UV CD analysis (Fig. 3). The CD spectra of the protein at different temperatures are depicted in the Fig. 3A. At 4 °C the far-UV CD spectrum shows a minimum at ∼200 nm and a shoulder at 225–230 nm, indicative of a high content of non-ordered secondary structure. Raising the temperature, the minimum shifts to ∼202 nm and the shoulder acquires a different shape. The assignment of the secondary structure elements according to the CDNN software indicates that there is an increase in the β-sheet (3.3%) and alpha helical (1.3%) contents upon raising the temperature, while the non-ordered secondary structure reduces from 39.4% to 37%. After cooling down the protein to 4 °C, the per-

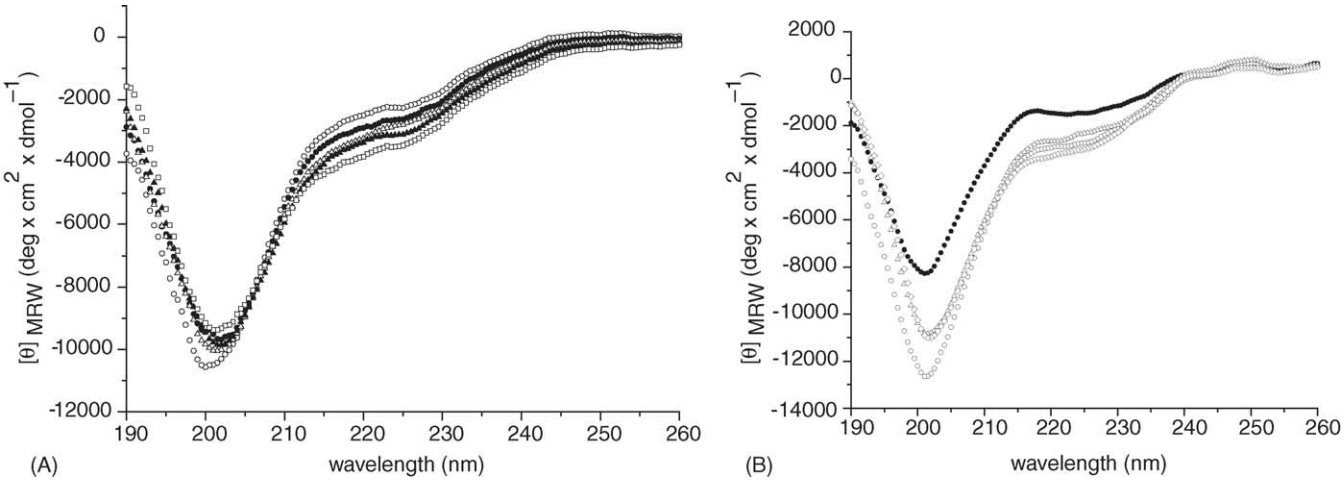

Fig. 3. Circular dichroism analysis of the 6xHis-mini-HBx(-Cys) protein. (A) Far-UV CD spectra of the purified 6xHis-mini-HBx(-Cys) protein in pure water at pH 6. The protein concentration was 15 μM (0.24 mg/ml) and the temperature was raised from 4 °C to 60 °C. Spectra were collected at five different temperatures: 4 °C (open circles), 20 °C (closed circles), 30 °C (open triangles), 40 °C (closed triangles), and 60 °C (open squares). (B) Far-UV CD spectra at 25 °C of the purified of 6xHis-mini-HBx(-Cys) protein in pure water at pH 6 with or without TFE. The protein concentration was 5 μM (0.08 mg/ml) in the different experimental conditions: pure water (closed circles), 0.5% TFE/water (open circles), 1% TFE/water (open triangles), and 2% TFE/water (open diamonds). Spectra were background corrected and the observed optical activity was expressed as the mean residue molar ellipticity $[\theta]_{MRW}$ (° cm^2 dmol^{-1}).

centage of secondary structure elements of the 6xHis-mini-HBx(-Cys) protein at 4 °C according to the CDNN software returned to be identical to that of the protein, which had not been heated to 60 °C. This shows that the conformational changes observed are fully reversible and could indicate that the protein 6xHis-mini-HBx(-Cys) is relatively stable to heat denaturation.

The far-UV CD spectra of the protein 6xHis-mini-HBx (-Cys) upon NaCl addition show that the shape of six spectra is very similar (data not shown). However, the assignment of the secondary structure elements (Table 1) shows that the extended structure (β-sheet) contents have a higher contribution in the sample containing 100 mM NaCl (40.6%) than in the sample without NaCl (36.4%). Moreover, the alpha-helical content increases 1.2% upon 100 mM NaCl addition, changing from 7.8% to 9% of its contribution to the secondary structure. However, there is still a great contribution of the non-ordered structure, accounting for 32.9% of the secondary structure in the protein sample containing 100 mM NaCl.

On the other hand, the shape of CD spectra changed slightly upon TFE addition (Fig. 3B). The ellipticity values at 200 nm, −8.000° cm^2 dmol^{-1} for 6xHis-mini-HBx (-Cys) in pure water and −13.000° cm^2 dmol^{-1} in 0.5% TFE/water, are indicative of a gain in alpha-helical content. The assignment of the secondary structure elements by CDNN software shows that the alpha-helical content increases from 6.7% to 8.5% upon TFE addition. Nevertheless, the extended structure (β-sheet) reduces by 4%, and the non-ordered structure changes from 35.6% to 36.3% of its contribution to the secondary structure of the 6xHis-mini-HBx(-Cys) protein.

3.5. Fluorescence spectra of refolded protein

Fluorescence emission spectra provide useful information about the protein tertiary structure organization. The recombinant 6xHis-mini-HBx(-Cys) protein has one tryptophan residue and one tyrosine residue (Fig. 1). Upon excitation at 295 nm, only the tryptophan residue is excitated and the fluorescence emission spectra are depicted in Fig. 4. The fluorescence data were collected at two different temperatures.

At 25 °C (Fig. 4A), the protein exhibits a maximum centered at 342 nm in aqueous solution, but after the addition of 100 mM NaCl the maximum is shifted to 336 nm, indicating that there is less exposition of the tryptophan residue to the solvent. Raising the temperature to 37 °C (Fig. 4B), the maximum of the intrinsic fluorescence emission does not

Table 1
Assessment of the relative secondary structural elements of the 6xHis-mini-HBx(-Cys) protein upon increasing NaCl addition as calculated from CD spectra (see Section 2 for details)

Addition to water at pH 6	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
None	7.8	36.4	23.6	35.5
10 mM NaCl	8	37.7	22.8	35.2
25 mM NaCl	8.1	37.8	22.3	35
50 mM NaCl	8.4	35.6	23.3	35.5
75 mM NaCl	8.2	37.1	22.6	35.4
100 mM NaCl	9	40.6	21.5	32.9

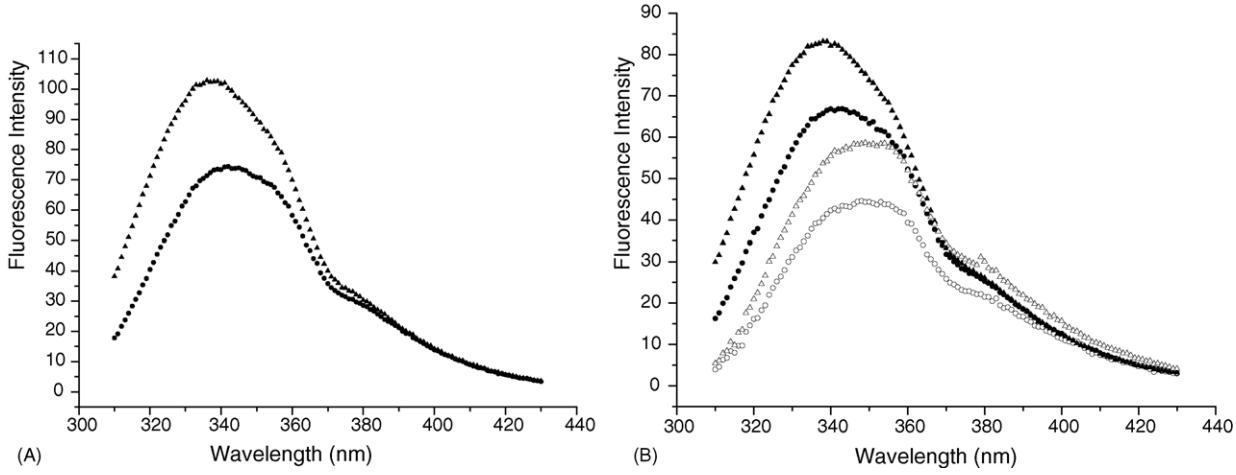

Fig. 4. Fluorescence emission spectra of purified 6xHis-mini-HBx(-Cys) protein at 25 °C (A) and 37 °C (B). The protein concentration was 2 μM (0.03 mg/ml) in the following different experimental conditions: pure water (closed circles), 100 mM NaCl (closed triangles), 6 M Gdn-HCl (open circles), and 100 mM NaCl + 6 M Gdn-HCl (open triangles). The excitation wavelength was 295 nm and spectra were background corrected.

change (343 nm in pure water), but in the 100 mM NaCl solution there is again a 'blue shift' to 338 nm of the tryptophan fluorescence emission due to its localization within the protein 'hydrophobic core'. Besides, under denaturing conditions (6 M Gdn-HCl), the maximum of fluorescence emission is shifted to 348 nm (pure water) or 349 nm (100 mM NaCl), indicating that the tryptophan residue is now more exposed to the solvent as a free tryptophan in aqueous solution. These data are in agreement with the CD spectra that showed an increasing structural gain upon NaCl addition (Table 1).

3.6. NMR analysis

The one-dimensional ^{1}H NMR spectrum (Fig. 5) indicates that the recombinant 6xHis-mini-HBx(-Cys) protein is only partially structured in aqueous solution at pH 6. The absence of proton signals under 0 ppm, as well as signal dispersion be-

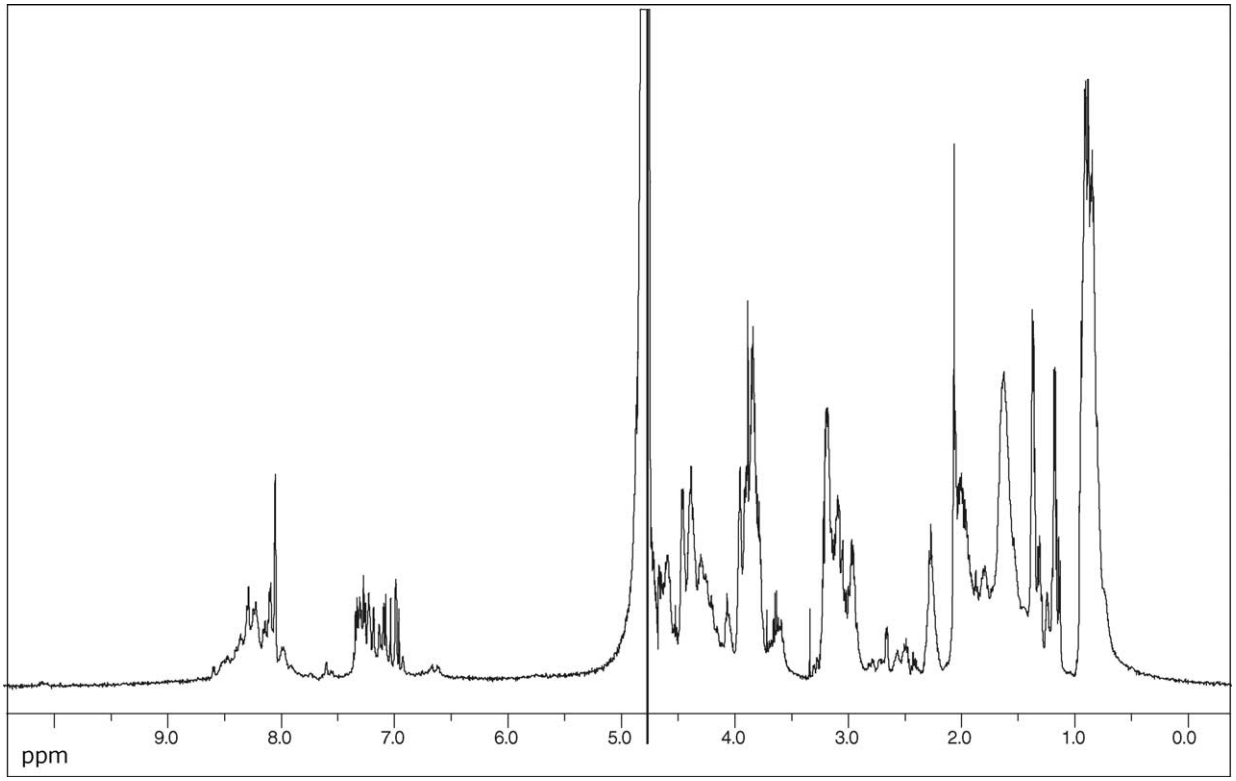

Fig. 5. One-dimensional ^{1}H NMR spectrum of 6xHis-mini-HBx(-Cys) protein. The NMR sample contained ~400 μM protein in 95% H_2O/5% D_2O at pH 6.

tween 7.0 ppm and 8.5 ppm are indicative of an unstructured polypeptide. This result is in agreement with previous spectroscopic assays, including far-UV CD (Fig. 3; Table 1) and fluorescence emission spectra (Fig. 4). Interestingly, there is a proton signal duplicated around 10 ppm that is assigned to the indolic NH which belongs to the tryptophan residue, leading us to speculate that there may be two different kinds of protein structure populations. However, it was not possible to assign the chemical shifts for the 6xHis-mini-HBx(-Cys) protein in a two-dimensional ^{1}H-TOCSY experiment (data not shown) due to a too crowded fingerprint region (NH-Hα). In short, the NMR data confirmed that the 6xHis-mini-HBx (-Cys) protein is rather unstructured in aqueous solution at pH 6.

4. Discussion

Hepatitis B virus infection is one of the major causes of hepatocellular carcinoma and the X protein has been suspected to be one of the oncogenic viral proteins of HBV (Anthony, 2001; Murakami, 2001). Several studies have demonstrated that HBx is a 'promiscuous' multifunctional viral regulator that affects viral replication and viral proliferation, directly or indirectly (Hildt et al., 1996). HBx protein is further known to modulate transcription and interferes with diverse signaling pathways, thereby contributing either to carcinogenesis or causing apoptosis (Yen, 1996; Chirillo et al., 1997; Madden and Slagle, 2001). This modulation seems to occur mainly through protein-protein interactions because HBx does not possess any classical nucleic acid binding domains. Previous studies however demonstrated, that HBx binds to single stranded DNA and RNA in a specific fashion, although it cannot bind to double stranded DNA (Qadri et al., 1996; Rui et al., 2001). The exact function and structural features of HBx remain unclear.

A truncated mini-HBx(18–142) protein that had its cysteines mutated to serine residues was generated and expressed, utilizing a site directed mutagenesis methodology. The 6xHis-mini-HBx(-Cys) protein was highly expressed in the *E. coli* insoluble fraction, and after its purification under denaturing conditions, was refolded by sequential dialysis and used in spectroscopic assays. It was shown that the 6xHis-mini-HBx(-Cys) protein was able to interact with an AU-rich RNA oligonucleotide (Fig. 2B) and with the human tumor suppressor protein p53 (Moura et al., 2005), confirming that the 6xHis-mini-HBx(-Cys) protein like the wild-type protein possesses biological activity after its renaturation upon refolding.

CD spectroscopic experiments showed that the 6xHis-mini-HBx(-Cys) protein is rather unstructured in aqueous solution but has a propensity to gain secondary structure upon NaCl or TFE addition. 6xHis-mini-HBx(-Cys) protein maintained its secondary structure up to 60 °C, but upon NaCl or TFE addition there were structural changes in the CD spectra that could be followed using the CDNN deconvolution program. In both cases, there was an alpha-helical gain in the secondary structure elements of the 6xHis-mini-HBx (-Cys) protein, with a reduction of the non-ordered structure. In addition to that, the intrinsic emission fluorescence spectra showed that upon NaCl addition there was less exposition of the tryptophan residue to the solvent, suggesting that the 6xHis-mini-HBx(-Cys) protein can change its overall conformation under specific experimental conditions.

In agreement with our finding that HBx changes its structural features under specific experimental conditions, it has been shown previously that several proteins involved in regulatory functions are completely or partly unstructured in solution, and they become structured only upon binding to their target molecule (Dyson and Wright, 2002). For instance, the binding of the zinc finger domain of the nuclear receptor RXR to DNA results in an induced folding of the dimerization region, which is dynamically disordered in the free protein (Holmbeck et al., 1998). Moreover, upon binding to the cellular oncoprotein MDM2, the transactivation domain of p53 tumor suppressor protein undergoes a coil-to-helix folding transition (Kussie et al., 1996). In short, the intrinsic lack of structure may confer to HBx functional complexity, including the ability to bind to several different targets in different conformations, and the fine control over binding affinity.

Not surprisingly, the HBx protein does not contain any significant structural motifs nor sequence homologies with other known transactivating factors, which could indicate a possible mechanism of transcriptional activation mediated by HBx. Besides, it has been shown that HBx stimulates the transcriptional machinery in the nucleus through protein-protein interactions with several transcription factors, and interacts with cellular components of signal transduction pathways in the cytoplasm (Murakami, 1999). Given this highly "promiscuous" behavior, it is tempting to speculate that the intrinsically unstructured HBx protein may become folded upon binding to its target molecules.

It is known that HBx protein is essential in the early stages of the HBV infection, as well as for the establishment of the viral infection in vivo, and its continuous expression might be responsible for the cellular transformation leading to cirrhosis and HCC development (Arbuthnot et al., 2000; Murakami, 2001). Besides, it is very difficult to detect HBx protein in the HBV infected liver due to very low amounts of the protein in the hepatocytes (Urban et al., 1997). As in the case for many signaling and transcriptional processes, the low cellular concentration of a regulatory protein and its target might affect their efficient binding if the protein has a restricted conformational flexibility. On the other hand, despite its low concentration in the cell, the unfolded polypeptide can bind weakly to its target molecule, and could in a second step fold as it "reels" onto its target, according to the "fly casting" mechanism proposed by Shoemaker and co-workers (Shoemaker et al., 2000). In this context, the inherent flexibility of the HBx protein could allow changes in the local and global structure in response to different molecular targets, and might explain the interaction of HBx with multiple

cellular partners. The coupled folding and binding processes might play an important role in the function of many proteins, as well as in the transactivating activity mediated by HBx onco-protein.

In summary, our circular dichroism and fluorescence experiments showed that the 6xHis-mini-HBx(-Cys) protein is rather unstructured in aqueous solution. Its thermal stability might be related to its conformational flexibility, which could be an important key to understand the HBx protein. Moreover, its adaptability might allow HBx to interact with such an enormously wide array of cellular proteins and thereby interfere simultaneously in diverse cellular processes ranging from transcriptional regulation, apoptosis and DNA repair to signaling mechanisms in general.

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Artigo IV

Interaction of the hepatitis B virus protein HBx with the human transcription regulatory protein p120E4F in vitro

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Interaction of the hepatitis B virus protein HBx with the human transcription regulatory protein p120E4F in vitro

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Abstract

Infection with the hepatitis B virus has been identified as one of the major causes of liver cancer. A large body of experimental work points to a central role for the virally encoded protein HBx in this form of carcinogensis. HBx is expressed in HBV-infected liver cells and interacts with a wide range of cellular proteins, thereby interfering in cellular processes including cell signaling, cycle regulation and apoptosis. In order to identify possible new targets of the HBx protein, we performed a yeast two-hybrid screen using a truncated protein mini-HBx(18–142) as the bait. In addition to known interacting partners, such as RXR and UVDDB1, we identified several new candidates including the human transcriptional regulatory protein p120E4F, which has been implicated in the regulation of mitosis and the cell cycle. In vitro pull down experiments confirmed the interaction and transcription activation assays in the yeast demonstrated that HBx protein was able to repress GAL4AD-p120E4F-dependent activation of a reporter gene under the control of E4F binding sites found in the adenovirus E4 promoter and the HBV enhancer II region. We also showed that the cysteine residues in HBx are necessary for its interaction with UVDDB1 but not for the interaction with RXR or p120E4F. The possible functional relevance of the interaction between HBx and E4F proteins is discussed in the contexts of cellular transformation and host-virus co-evolution.

1. Introduction

Viral hepatitis is an important global public health problem, particularly in developing countries. According to the World Health Organization (WHO), approximately two billion people worldwide have had contact with the hepatitis B virus (HBV) and of those approximately 325 million are chronically infected. About 50% of chronically infected HBV patients develop liver cirrhosis or hepatocellular carcinoma, and some experimental evidence points to the viral protein HBx as a causative agent for the cellular transformation, since some strains of HBx transgenic mice have a strong tendency to develop liver cancer (Kim et al., 1991; Yu et al., 1999). Other studies with HBx transgenic mice on the other hand have not confirmed an elevated risk for liver cancer (Lee et al., 1990; Reifenberg et al., 1997), suggesting that the manifestation of the disease could depend on the locus of genomic insertion, the configuration of the HBx gene construction used in the different studies or other yet uncharacterized factors. Not withstanding the unresolved question of the role of HBx in oncogenesis, the majority of studies on HBx function suggest that its pleiotropic functions and capacity to contribute to tumor growth are due to its ability to interact with a diverse array of biological macromolecules including single stranded DNA (Qadri et al., 1996a), RNA (Rui et al., 2001) and a broad assortment of cellular proteins. The latter include the transcription factors RXR (Kong et al., 2000), TBP (Wang

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et al., 1998), RPB5 (Cheong et al., 1995), TFIIB (Haviv et al., 1998), C/EBPβ (Choi et al., 1999), ATF-2 (Schneider and Schepartz, 2001) and CREB (Barnabas and Andrisani, 2000), the DNA repair proteins UVDDB1 (Bontron et al., 2002), ERCC2 (Qadri et al., 1996b) and ERCC3 (Wang et al., 1994), the tumor suppressor protein p53 (Chung et al., 2003) and several other proteins of diverse functions including HSP60 (Tanaka et al., 2004) and HIF-1α (Moon et al., 2004). Other studies showed that HBx interacts strongly with the bZIP elements present in some of the transcription factors mentioned above (C/EBPβ, ATF-2 and CREB), all of which have important functions in hepatocyte physiology.

In utilizing yeast two-hybrid experiments to discover new interacting partners of HBX, we identified the p120E4F transcription factor. p120E4F and its mouse orthologue phiAP3 are members of the GLI-Kruppel family of proteins, which are characterized by conserved zinc finger and linker (HTGEKPY/FXC) motifs (Fognani, 1993; Fernandes and Rooney, 1997), and the genes encoding these proteins (E4F1) are expressed at low levels in a wide variety of human and murine tissues (Rooney et al., 1998a). Previous analyses of the E4F protein showed that it exists in two cellular forms, the predominant full length 120 kDa protein (p120E4F) and a less abundant 50 kDa N-terminal proteolytic fragment (p50E4F) (Fernandes and Rooney, 1997; Raychaudhuri et al., 1987, 1989). Both forms contain a zinc finger region that is responsible for binding to the major regulatory element [ATGACGTAAC] in the adenovirus E4 promoter (Fernandes and Rooney, 1997; Rooney et al., 1998b) and both are regulated by the adenovirus E1A oncoprotein. However, the two forms are functionally dissimilar: p120E4F is a repressor of the E4 promoter and is down-regulated by E1A whereas p50E4F stimulates the E4 promotor in an E1A-dependent manner (Fernandes and Rooney, 1997; Raychaudhuri et al., 1987, 1989; Fernandes and Rooney, 1999). Regulation of the DNA binding activity of both proteins may involve E1A-stimulated phosphorylation (Fernandes and Rooney, 1997; Rooney et al., 1998b).

These and other recent studies have implicated p120E4F in the control of the cell cycle (Fajas et al., 2001; Rooney, 2001; Rizos et al., 2003) and p50E4F in the regulation of apoptosis (Fernandes et al., 1999). Despite of this, the exact function of the E4F proteins remains to be elucidated. One impediment has been that the E4F1 gene appears to be absolutely essential for embryonic development and cell division, as E4F1-deficient mouse embryos die at pre-implantation stages and E4F1-null blastocysts show an increased incidence of apoptosis and problems with the progression of mitosis and chromosomal segregation (Le Cam et al., 2004). Interesting, a number of GLI-Kruppel family members have been functionally implicated in embryonic development and neoplasia (Ruppert et al., 1988).

Here, we show that HBx can physically interact with the E4F proteins in vivo and in vitro. In a yeast-based transcriptional activation assay, we found that a fusion protein containing E4F linked to the GAL4 activation domain can activate the expression of a β-galactosidase gene under the control of the E4 adenovirus promoter and that HBx inhibits this activation to a significant degree. Furthermore, using the same assay system, we also found that E4F can bind to a DNA sequence found in the enhancer region II of the genome of HBV. Finally, we mapped the protein regions responsible for the interaction between E4F and HBx. The possible functional implications of this new HBx protein interaction with E4F in the context of viral infection and hepatocellular carcinogenesis are discussed.

2. Results

2.1. Yeast two-hybrid screen to identify HBx interacting proteins

We screened a human fetal brain cDNA library to identify new HBx-interacting proteins and thereby get new insights in its putative cellular functions. We used a truncated HBx protein as a "bait", which spans the amino acid residues 18–142 and appears to be fully functional in all biological assays thus far, as shown by us and others (Rui et al., 2005; Moura et al., 2005; Kumar et al., 1996). This truncated form eliminates the first 17 and last 12 amino acids of HBx, which are rather hydrophobic and contain non-conserved Cys residues, to improve its solubility and to reduce artifactual effects in the interaction screening. The mini-HBx bait construct did not show auto-activation in the yeast two-hybrid system (not shown) and was therefore deemed suitable for the library screen. Our screen identified seven different proteins that interacted with mini-HBx(18–142) (Table 1). Two of these were RXR and DDBP1, which had been previously described as cellular HBx interacting protein partners (Kong et al., 2000; Bontron et al., 2002), and thus confirmed that mini-HBx(18–142) functions appropriately as a bait in this assay. Among the other mini-HBx interacting proteins were several proteins involved in tumorigenesis or transcription regulation (Table 1) including the human transcription factor p120E4F, which had been implicated in cell cycle control. Although, we used a fetal human brain cDNA library in the screen, the observed interaction of HBx with p120E4F could be biologically significant, since p120E4F is ubiquitously expressed and found in human and murine liver (Rooney et al., 1998a).

2.2. RXR interacts with a cysteine free mini-HBx protein whereas UVDDB1 does not

Prior to studying the interaction of HBx with p120E4F, we wanted to determine if HBx interaction is distinguishable among its partners. In previously studies, we had generated a cysteine free mini-HBx(18–142) protein, which we named mini-HBx(-Cys) (Rui et al., 2005; Moura et al., 2005), where all cysteines had been either deleted or substituted to serines. We had demonstrated that the CD spectrum of the mini-HBx(-

Table 1
Human proteins that were identified to interact with mini-HBx(18–142) in the yeast two-hybrid system

Interacting protein	Number of clones	Interaction previous known	Coded protein residues (retrieved/complete sequence)	Complete name—possible function/s	Citations
E4F1	3	No	176–784/784	Transcription factor E4F1. Involved in the control of the cell cycle.	Rooney et al. (2001)
RXR	3	Yes	137–533/533	Retinoid X Receptor. Nuclear receptor. Participates in signaling pathways.	Ahuja et al. (2003)
DDBP1	2	Yes	1–1158/1158	DNA Damage-Binding Protein 1. DNA repair and nucleotide excision.	Stohr et al. (1998)
MKRN1	1	No	64–482/482	Makorin ring finger protein-1. Development of the central nervous system.	Gray et al. (2000)
SHFM3	1	No	465–1244/1244	Split Hand/Foot Malformation-3. Associated to the formation of feet and hands.	Mollerat et al. (2003)
mtb1	1	No	446–534/534	Similar to "lethal malignant brain tumor l" of *Drosophila melanogaster*. Suppressor of brain tumors.	Koga et al. (1999)
pva1	1	No	96–200/200	Similar to Pv-Alu of *Plasmodium vivax*. Expressed in the erythrocytic of the parasite	Dhar et al. (1998)

Cys) protein did not differ from that of mini-HBx or wt-HBx and that it interacts with both RNA and human p53 in vitro similarly to wt-HBx or mini-HBx (Moura et al., 2005). These results suggested that the cysteine residues of HBx are not required for the described functions. Here, we wanted to test whether the proteins RXR or UVDDB1 can interact not only with mini-HBx but also with wt-HBx and mini-HBx(-Cys) (Fig. 1A).

We used the yeast two-hybrid assay to test direct one-on-one interactions of the above mentioned protein constructs. RXR interacted with wt-HBx, even stronger with mini-HBx(18–142) and still showed a significant interaction with mini-HBx(-Cys) (Fig. 1B). This suggests that the cysteine residues in HBx are again not important for this interaction. UVDDB1 on the other hand showed a different interaction pattern. Although it strongly interacted with wt-HBx, it showed a slight decrease in the interaction with mini-HBx and a complete abrogation of the interaction with mini-HBx(-Cys). This is the first demonstration that the cysteine residues in HBx are essential for an interaction (Fig. 1C) and indicates that HBx interacts with various proteins through different amino acid residues.

2.3. Mapping the wt-HBx binding site on the E4F transcription factor using the yeast two-hybrid system

Full length p120E4F (784 amino acids) contains a number of different functional domains including three zinc finger domains, containing one, three and six putative zinc finger motifs, respectively, a proline-rich region and a helix-turn-helix domain (Fig. 2A). The intervening regions between these domains are of unknown function and structure. Therefore, we generated a series of truncation mutants of p120E4F that are fused to the Gal4 activation domain in the yeast two-hybrid expression vector pGAD424 to determine which domains are required for the interaction with HBx (Fig. 2B). All constructs containing either the second or third cluster of zinc-finger motifs showed strong activity, suggesting that the regions located between amino acids 173–262 and 428–784 are important for the interaction with HBx. For example, a small fragment containing the N-terminal cluster of three zinc-finger motifs E4F(173–262) interacts almost as strongly with HBx as does wt p120E4F, as was also true for the fragment E4F(428–784) containing all six of the zinc-finger motifs in the C-terminal end. No activity was observed with either the empty vector pGAD424 (encoding only the Gal4 activation domain) or with an unrelated protein (Gal4-AD-Fez1 construct) (Surpili et al., 2003), indicating that the interaction of E4F sequences with HBx was specific.

2.4. Confirmation and mapping of the interaction between HBx and E4F in vitro

We confirmed the interaction of HBx and p120E4F by using purified recombinant proteins in an in vitro "pull down" assay. Various fragments of p120E4F (Fig. 3A) were expressed as GST fusion proteins, coupled to glutathione sepharose beads and incubated with 6xHis-HBx. After the beads were pelleted and washed, Western blot analysis showed that HBx was pulled down with the following E4F protein fragments: GST-E4F(1–262), GST-E4F(173–262), GST-E4F(263–784) and GST-E4F(428–784) (Fig. 3B). For the most part, these results confirmed that amino acids in either the second or third zinc finger clusters participated in the HBx interaction. Further localizing the site of interaction in the third zinc finger cluster, the E4F fragment GST-E4F(263–486) that contained only the first two zinc finger motifs did not interact with HBx, indicating that amino acids in the latter four zinc finger motifs were important. The observed interaction of HBx with the aforementioned GST-E4F fusion proteins appeared specific since we did not observe any interaction of HBx with the control protein GST. The one anomaly of these results is that a fusion protein containing the entire second cluster and the first two zinc finger motifs in the third cluster [GST-E4F(173–486)] did not interact with HBx. However, because the smaller intrinsic

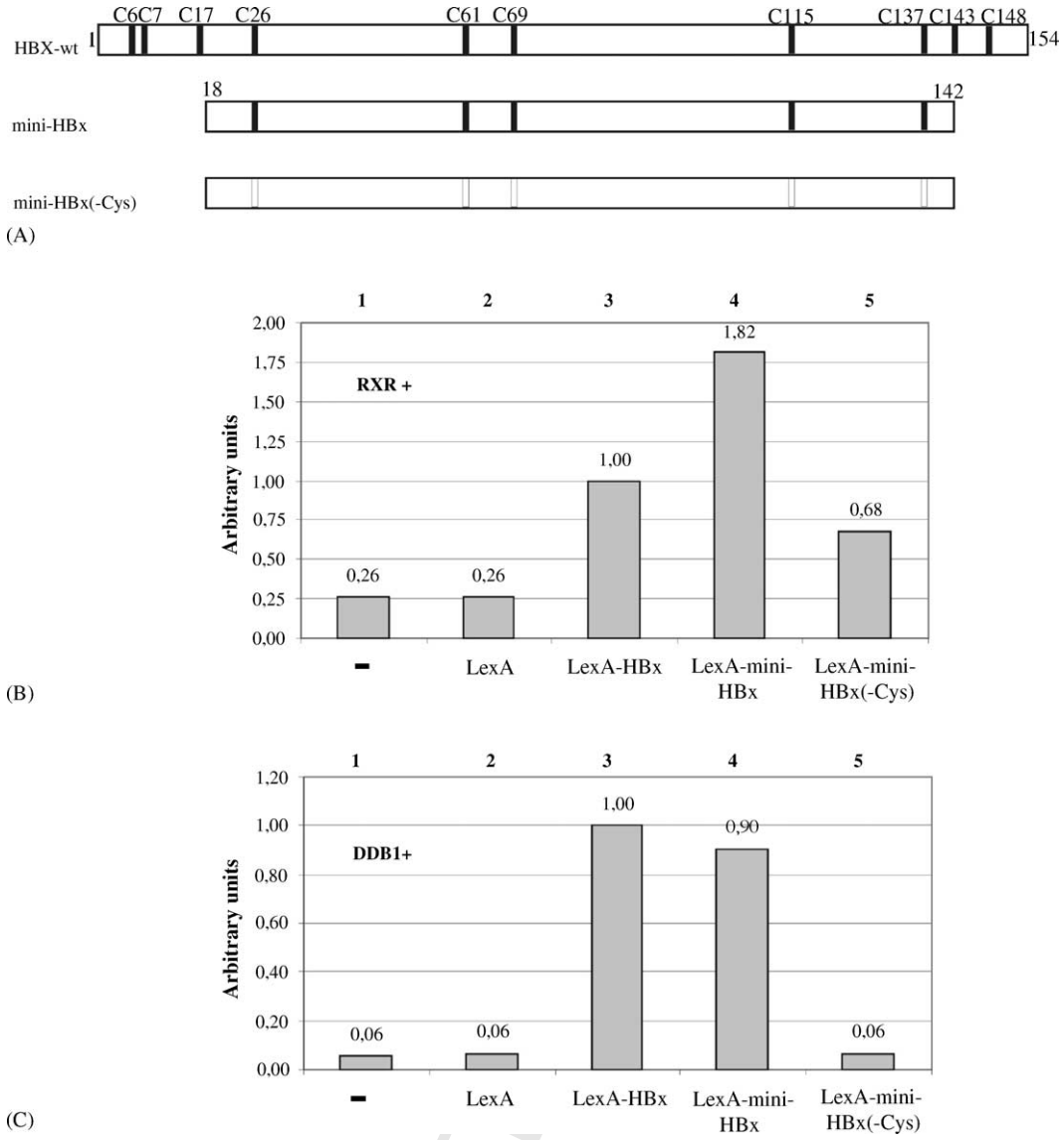


Fig. 1. HBx with and without cysteines interacts with RXR, but HBx without cysteines does not interact with UV-DDB1 protein. (A) Schematic presentation of the three tested protein forms of HBx used in this study: wt-HBx containing 10 cysteine residues, mini-HBx lacking residues 1–17 and 143–154, and thereby also lacking five cysteine residues, and finally, mini-HBx(-Cys), which lacks all 10 cysteine residues. (B) Interaction of the three HBx proteins with the RXR protein in the yeast twohybrid system. The transcriptional activation function, which is indicative of the interaction between the two proteins, was expressed as the β-galactosidase activity in arbitrary units. (C) Same as (B), but this time the interacting protein UV-DDB1 was tested instead of the RXR protein.

fragment GST-E4F(173–262) was able to interact, it could be speculated that the interaction site is somehow buried or interfered with by the C-terminal extension in the larger protein.

2.5. Gal4-AD-E4F fusion proteins activate a promoter containing the adenovirus E4F binding site in yeast

As a prelude to test the influence of the HBx protein on the function of p120E4F, we established a yeast one-hybrid transcriptional activation test system. As a first step, we constructed a reporter plasmid for p120E4F by inserting three tandem copies of the E4F binding site from the adenovirus E4 promoter adjacent to the promoter in pLACZi that controls expression of β-galactosidase and used this to generate a yeast reporter strain. The reporter strain was then transfected with either a control plasmid (pGAD424), a construct expressing wt p120E4F fused to the Gal4 activation domain (GAL4AD-wt-E4F), or the clone we originally isolated with mini-HBx in the two-hybrid screen (GAL4AD-E4F-173–784) (Fig. 4B, 1–3). We observed no activation of the reporter gene lacZ in cells transfected with control plasmid, strong activation in cells transfected with GalAD-wt-E4F and an intermediate level of activation in cells transfected with GalAD-E4F(173–784), corresponding to 28% of that observed with the wt-E4F protein. This suggests that the

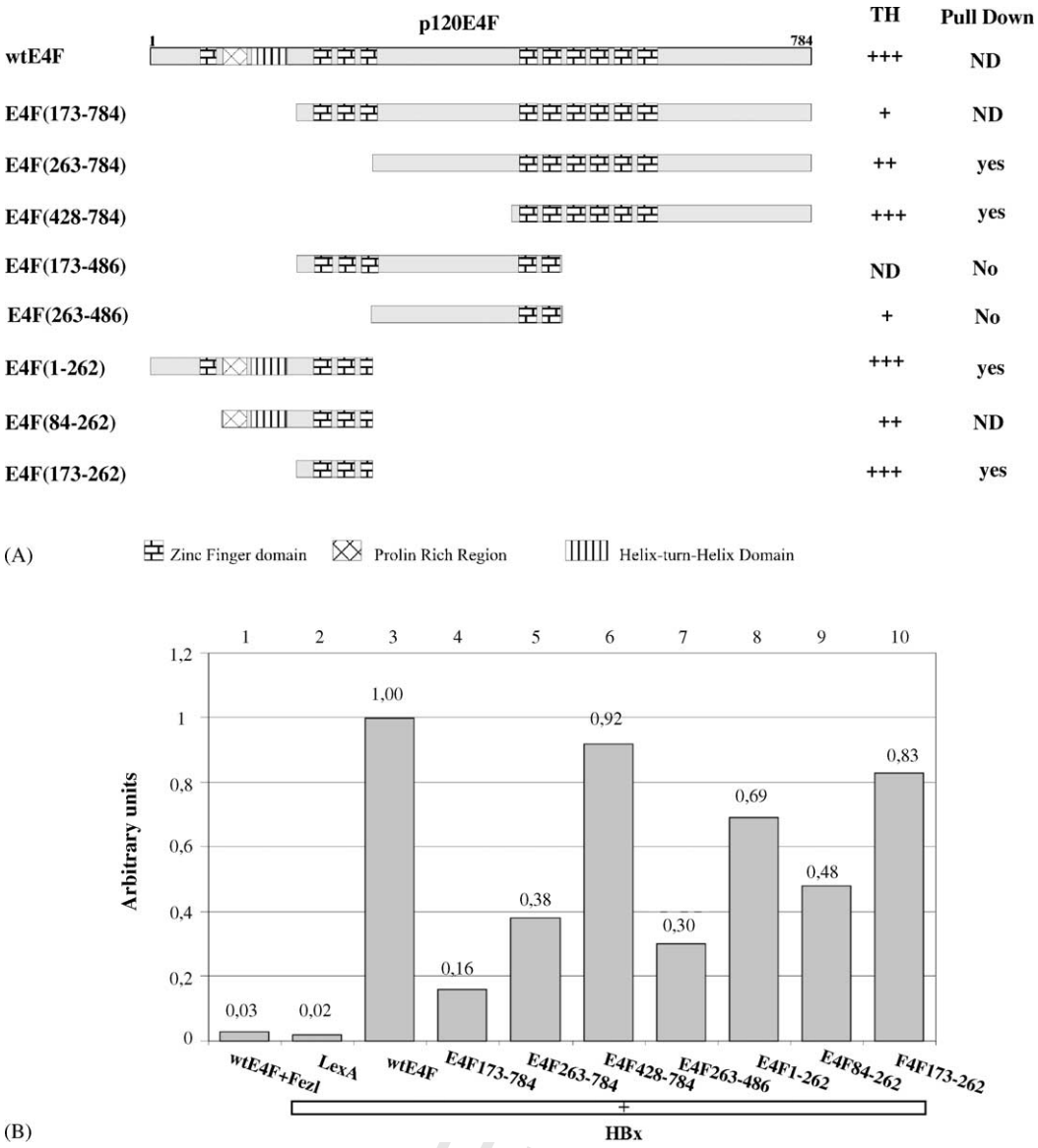


Fig. 2. HBx interacts with E4F in the yeast two-hybrid system. (A) Schematic figure of the generated deletion mutants of the E4F protein. The interaction in the TH (yeast two-hybrid assay) and the pull down experiment (Fig. 3) is indicated in form of a table at the right side of this figure panel. (B) Quantification of the interaction of the different deletion constructs of the E4F protein with HBx in the yeast two-hybrid assay. β-Galactosidase activity was expressed as arbitrary units.

GAL4-E4F fusion protein binds to the E4F binding site and functions as a transcriptional activator in the yeast. The reduced activation seen with GAL4AD-E4F(173–784) may reflect the fact that deletion of the first 84 amino acids in the E4F sequence reduces E4F DNA binding stability (Rooney et al., 1998b).

2.6. E4F is an activator of a reporter gene under the control of the enhancer II promoter region from the HBV genome

Examination of the HBV genomic sequence shows that a close copy of the E4F binding site is present in the enhancer II region (Fig. 4A). Therefore, we modified the above reporter plasmid by replacing each canonical E4F binding site with the putative site found in the HBV enhancer II region and tested the ability of GAL4AD-E4F proteins to activate β-galactosidase expression. The enhancer II construct is not activated by empty pGAD vector (Fig. 4B, column 4) but is significantly activated by Gal4AD-wt-E4F (Fig. 4B, column 5). Interestingly, the construct isolated from the two-hybrid assay [Gal4AD-E4F(173–784)] had 5.4 times higher activity than wt-E4F (Fig. 4B, column 6), perhaps suggesting that the truncated protein binds to the site in the enhancer II region better than to the canonical site. Regardless, these results show that E4F pro-

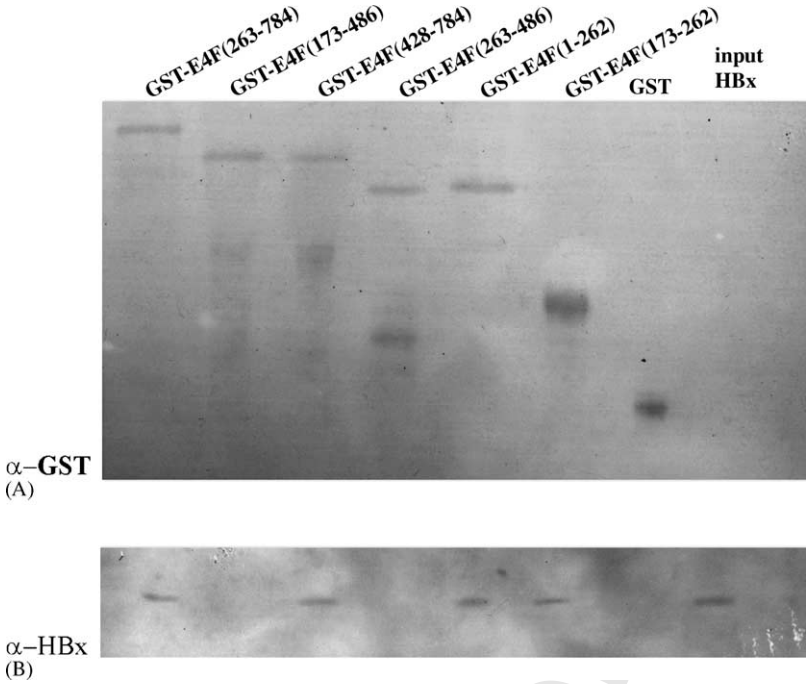


Fig. 3. Purified recombinant proteins 6xHis-HBx and GST-E4F interact in vitro. (A and B) Result of the in vitro pull down assay. (A) GST-E4F fusion protein was coupled to glutathione sepharose beads. An aliquot of the coupled beads as well as GST control protein were run out on SDS-PAGE and analyzed by anti-GST Western blot. (B) The coupled beads were washed and incubated with purified 6xHis-HBx protein. After three additional washes, the beads denatured and analyzed for bound proteins in an anti-HBx Western blot. Input HBx control protein had also been run out on the SDS-PAGE previous to Western blot.

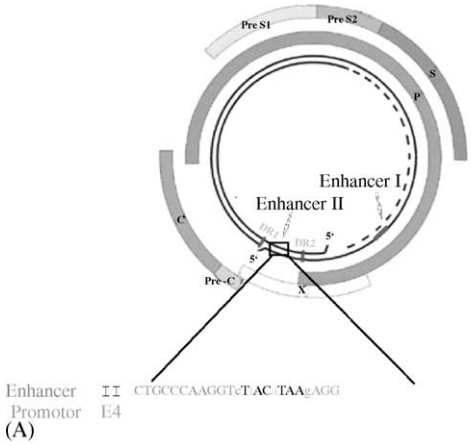
teins can bind to the HBV enhancer II region and trans-activate a reporter gene under its control, when fused to the Gal4AD.

2.7. HBx interferes with promoter activation by GAL4AD-E4F proteins

Finally, we tested the ability of HBx to influence trans-activation by Gal4AD-E4F proteins and the requirement for HBx cysteine residues (Fig. 5). Upon co-transfection of lexA-HBx, lexA-mini-HBx and lexA-mini-HBx(-Cys) constructs into the yeast reporter strain containing the E4 reporter plasmid and a Gal4AD-wt-E4F expression vector, we found that all three HBx proteins had an inhibitory effect on trans-activation of the reporter gene by GAL4AD-wt-E4F, reducing β-galactosidase expression by 35–45%. By contrast, co-transfection of the lexA parent construct had no effect. The inhibition appears to be a specific effect, since we observed a quite different effect in previous comparable studies with HBx and the transcription factor p53 (Moura et al., 2005). In that case, all three types of HBx protein had a stimulatory effect on the transactivation activity of p53 and not an inhibitory one as observed for E4F here. Given that the interaction of p120E4F with HBx in the two hybrid assay (Fig. 2) did not prohibit the promoter activation by GAL4AD-wt-E4F, it is possible that the HBx inhibitory effect seen here reflects an interference with DNA binding, either due to direct blocking of the p120E4F DNA binding domain by HBx or an HBx-induced conformational change in p120E4F.

3. Discussion

The action of the HBV-encoded HBx oncoprotein is thought to be the primary cause of hepatocellular carcinoma arising during chronic HBV infection. Therefore, we performed a yeast two-hybrid screen, using the mini-HBx(18–142) onco-protein as bait and a human fetal brain cDNA library as a prey, to identify potential HBx-interacting proteins. A number of new HBx partners were identified in addition to several previously known partners, including the human transcription factor p120E4F. p120E4F is ubiquitously expressed in both humans and mice (Rooney et al., 1998a) and thus its expression in liver suggests that its interaction with HBx could be biologically significant. We performed several additional analyses that confirmed the interaction between HBx and p120E4F. In addition, we also present evidence that the cysteine residues in HBx may be differentially required for the interaction of HBx with various protein partners. Using two previously identified HBx-interacting proteins: RXR (Kong et al., 2000) and UVDDB1 (Bontron et al., 2002) (Fig. 1), we found that full length HBx, mini-HBx(18–142) and the mini-HBx(-Cys) mutant were all able to interact with RXR in the yeast two-hybrid system, whereas the mini-HBx(-Cys) mutant was unable to interact with the UVDDB1 protein. In previous reports, we showed that the mini-HBx(-Cys) protein is able to interact with p53 and RNA and that it has the same random coil conformational structure as full length wt-HBx, as shown by CD spectroscopy (Moura et al., 2005; Rui et al., 2005). These data



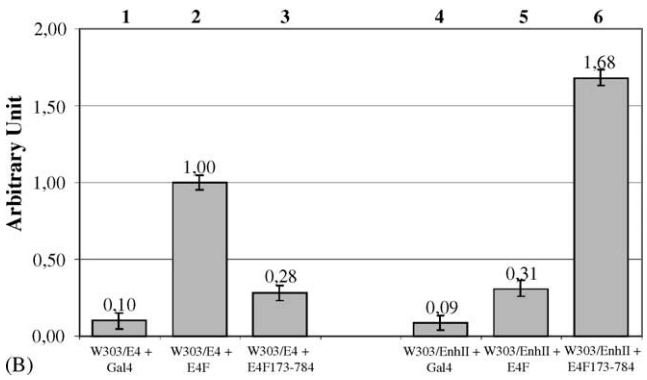
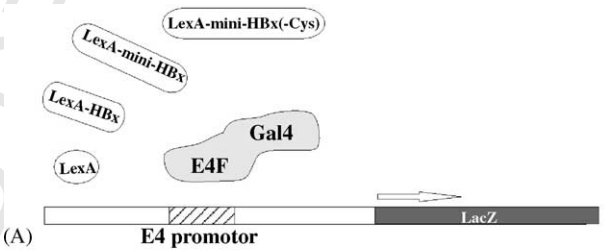


Fig. 4. GalAD-E4F ativates the transcription of a E4 promotor- and an HBV enhancer II-controlled β-galactosidase reporter gene in the yeast one-hybrid system. (A) Schematic figure of the organization of the genome of HBV and indication of the localization of the two enhancer regions I and II. (B) Amplification below the panel: enhancer region II shows a 60% sequence identity with the minimal promotor element controlled by E4F. (C) Quantiative activation of the β-galactosidase gene controlled by the E4 promotor (left) or by the HBV enhancer II region (right). Shown is the dependence of the expression of the β-galactosidase on the expression, absence, presence of the full lenght Gal4AD-E4F fusion protein or the GalAD-E4F(173–784) fusion protein. β-Galactosidade activity was expressed as arbitrary units.

collectively suggested that the cysteine residues in the HBx protein are not required for the majority of HBx interactions or functions tested to date. In contrast, the inability of mini-HBx(-Cys) to interact with UVDDB1 clearly shows that the cysteine residues may indeed be important for some HBx functions, dependent on the particular interacting partner, and thus warrants further study with a greater spectrum of HBx-interacting proteins. The fact that mini-HBx(-Cys) inhibited GAL4ADwtE4F to the same extent as wt-HBx (Fig. 5) points out, however, that the cysteine residues may play a role in only a limited number of HBx interactions.

p120E4F has been described previously as a transcriptional repressor involved in the control of cell cycle progression, mitosis and cytokinesis (Fernandes et al., 1998; Fajas, 2001; Rooney, 2001; Rizos et al., 2003, LeCam et al., 2004). A truncated form of p120E4F, termed p50E4F, has also been implicated in the regulation of apoptosis (Raychaudhhuri et al., 1987; Fernandes et al., 1997; Fernandes and Rooney, 1999). Therefore, any potential involvement of E4F proteins in HBx-induced transformation warrants a more detailed understanding of their physical interaction with HBx. We utilized an in vitro pull-down assay in addition to yeast one- and two-hybrid systems as experimental models to evaluate the interaction of HBx with E4F proteins and its possible influence on their function. Our results showed that HBx can independently interact with two zinc finger domains, one of which functions as the DNA binding domain for both p120E4F and p50E4F while the other resides solely within p120E4F (Rooney et al., 1998b). We also found that co-expression of HBx with a fusion protein consisting of p120E4F and the Gal4 activation domain inhibited promoter activation by the E4F fusion protein in the yeast one-hybrid assay by almost 50% (Fig. 5). This suggests that HBx either binds to the DNA binding domain in E4F proteins or interferes with its function in an indirect way. While it is also possible that this effect could be due to HBx interfering with the attraction of components of the general transcriptional machinery, the lack of a prohibitive effect in the yeast two-hybrid assay is consistent with an effect on promoter binding. This assay also showed that the cysteine residues in HBx are dispensable for this inhibitory activity.

The adenovirus E1A onco-protein functions analogously to HBx in that it also influences the transcription of both viral and cellular genes. E1A has been shown to block the repressor function of p120E4F in vivo (Fernandes and Rooney,



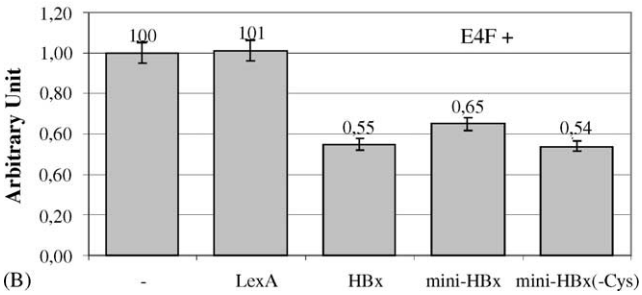


Fig. 5. HBx with and without cysteine residues is able to inhibit the Gal4AD-E4F fusion protein dependent expression of β-galactosidase by 50% in the yeast one-hybrid system. (A) Schematic figure panel showing the possible interfereance of the HBx proteins on the Gal4AD-E4F driven E4 promotor activity controlling the lac-Z gene expression. (B) Quantitation of the β-galactosidase activity (in arbitrary units). The dependance of the β-galactosidase expression on the persence of Gal4AD-E4F and the three different HBx protein constructs were analyzed [lexA-wt-HBx, lexA-mini-HBx and lexA-mini-HBx(-Cys)].

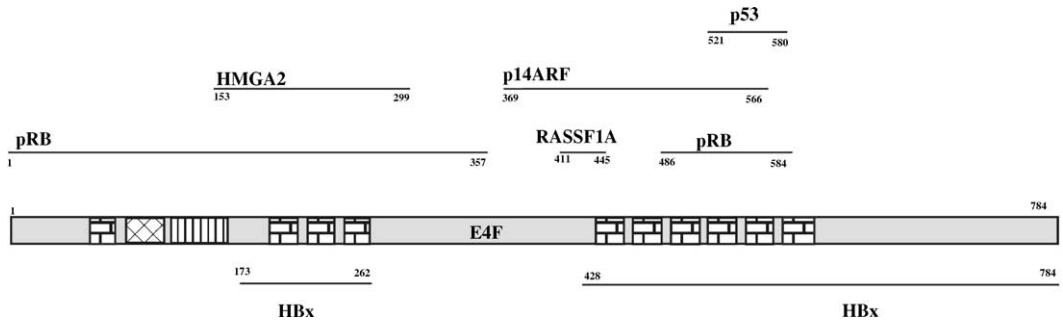


Fig. 6. Schematic representation. The protein domains of E4F with indication of the mapped protein regions shown to be involved in the interactions with the shown proteins, including HBx and the HBx interacting proteins p53 and pRB.

1999) and thereby promote the transcription of the early viral genes during adenovirus infection (Flint and Shenk, 1989). Aside from its activation of early viral genes, E1A has also been found to have an influence on the promoters controlling a number of cellular genes, including those encoding ATF, Oct-1 and Sp1 (Jones and Lee, 1991). HBx has been found to regulate the transcription of many of these same genes (Choi et al., 2001; Antunovic et al., 1993; Gomez-Gonzalo et al., 2001), supporting the hypothesis that HBx and E1A target a common set of cellular components to achieve host cell transformation. Our results suggest that the association with E4F proteins is one of those common events. This hypothesis is further supported by experimental results showing that HBx can partially substitute for the transcription regulatory function of E1A during adenovirus infection (Schaack et al., 1996). In addition, it is noteworthy that E4F and HBx share several interacting protein partners, including pRB and p53; the binding regions in p120E4F for HBx and other E4F interacting proteins are summarized in Fig. 6. Interestingly, another protein called E4-binding protein 4 (E4BP4 or NF-IL3), a human bZIP transcription factor that can bind to the adenovirus E4 promoter, has been shown to also bind to the HBV enhancer II region (Ishida et al., 2000) (Fig. 5A) through the same element that shares 60% sequence identity with the E4F binding site. E4BP4 has been implicated in the regulation of apoptosis, cell survival, inflammatory responses and the circadian rhythm and thus would represent another ideal candidate for achieving cellular transformation (Cowell et al., 1992; Cowell, 2002).

The discovery of a DNA motif in the HBV genome enhancer II region that shares 60% sequence identity with the minimal E4F DNA recognition site suggests additional similarities between HBV and adenovirus. The enhancer region of the HBV genome stimulates the transcription of the genes encoding surface proteins I and II and the HBx protein itself (Ishida et al., 2000). It is possible that p120E4F, the most abundant form of E4F in the human cell, could bind to enhancer region II and repress HBV viral transcription. In this context, it is tempting to speculate that the viral protein HBx, possibly by interacting with p120E4F, could have a counteracting function. HBx would decrease p120E4F repressor activity, thereby activating HBV transcription. Alternatively, or perhaps in addition, HBx could inhibit the negative role of p120E4F in the regulation of the cell cycle.

4. Materials and methods

4.1. Construction of plasmids

HBx pET28a: full length HBx DNA (subtype *ayw*) (Galibert et al., 1997; Lo et al., 1988) inserted into pCMV-Ad (kindly provided by Dr. R. Schneider, NYU, New York) was used as a template DNA in the PCR reactions, in order to amplify the full length HBx gene (510 bp). The full length HBx gene was directionally cloned into the bacterial expression vector pET28a+ (Novagen) via *Nde*I and *Xho*I restriction sites. The truncated "mini-HBx" gene (372 bp) was amplified by PCR using the full length HBx DNA as a template DNA. The "mini-HBx" gene was directionally cloned into the bacterial expression vector pET28a+ (Novagen) via *Nde*I and *Bam*HI restriction sites. The recombinant plasmid pET28a+/mini-HBx(18–142) was used as a template DNA in the PCR reactions in order to generate a complete cysteine/serine point mutant [mini-HBx(18–142)-Cys] using the "*QuickChangeTM Site-Directed Mutagenesis*" kit (Stratagene) (Moura et al., 2005; Rui et al., 2005). The cysteine/serine point mutations were confirmed by DNA sequencing. The recombinant plasmids pET28a+/HBx contain a six-histidine tag (6xHis) encoding sequence that allows the purification of the recombinants fusion proteins via Ni-affinity chromatography. pBTM116-HBx: the HBx(1–154), mini-HBx(18–142) and mini-HBx(18–142)-Cys (not coding any Cysteines) were amplified by PCR and subcloned in frame with the lexA DNA binding domain in the yeast expression vector pBTM116 (Bartel and Fields, 1995) via *Eco*RI and *Bam*HI restriction sites (Moura et al., 2005).

pGEX5x2-E4F and pGAD424-E4F: a pCITE plasmid containing the full length human E4F had been generated previously as described (Rooney et al., 1998b). The gene coding human E4F(1–784) protein was amplified and used to create full length and truncated "E4F" genes from this vector with specific primers which were inserted in frame with the Gal4 activation domain gene, via *Eco*RI and *Xho*I

sites, into the cloning site of the yeast expression vector pGAD424 (Clontech). A similar approach was used to clone E4F fragments into the bacterial expression vector pGEX5x2 (Amersham Pharmacia Biotech) in frame with GST. The oligonucleotides for the full length construct were: sense: GGC*GGAATTC*CCATGGAGGGCGAGATGGCAGTGC and anti-sense: GGC*GAGCTC*CTAGACGATGACCGTCTGCACC. In a similar approach oligonucleotides were generated to amplify fragments that encode the E4F constructs spanning the following indicated amino acid sequences: E4F(173–784); E4F(263–784); E4F(428–784); E4F(173–486); E4F(263–486); E4F(1–262); E4F(84–262); E4F(173–262). These fragments were inserted in the pGAD424 and pGEX5x2 vectors. E4 and enhancer II promoters constructs in vector pLacZi: the vectors pLacZi-E4 and pLacZi-enhancer II were generated by inserting three tandem copies of E4F recognition sites found in the adenovirus E4 promoter $(CATGACGTAA)_3$ and HBV enhancer II $(TGCCCAAGGTCTTACATAAGAGG)_3$ into the yeast vector pLacZi (Clontech) via *Eco*RI and *Xho*I sites. The oligonucleotides used to generate these constructs were: EnhII-S: AATTCTTGCCCAAGGTCTTACATAAGAGGTTGCCCAAGGTCT TACATAAGAGGTTGCCCAAGGTCTTACATAAGAGGC; EnhII-AS: TCGAGC CTCTTATGTAAGACCTTGGGCAACCTCTTATGTAAGACCTTGGGCAACCTCTTATGTAAGACCTTGGGCAAG; E4-S: AATTCATGACGTAACATGACG TAACATG ACGTAAC; E4-AS: GAGGTTACGTCATGTTACGTCATGTTACGTCAT. All vector constructs were confirmed by DNA sequencing.

4.2. Expression of proteins and purification of the GST-E4F fusion protein

Escherichia coli strain BL-21 was transformed with human E4F containing pGEX-5x2 or empty pGEX-5x2 vector. Cells were grown in LB medium containing ampicillin to an $OD_{600} = 0.7$, followed by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and induced for 3 h at 37 °C. Cells were harvested by centrifugation and E4F proteins were purified from the soluble protein fraction of the bacterial lysate using glutathione-sepharose 4B (Amersham) as described previously for other GST fusion proteins (Moraes et al., 2003).

4.3. Purification of 6xHis-HBx fusion proteins from inclusion bodies

E. coli strain BL-21 was transformed with the either HBx, or mutant mini-HBx DNAs containing pET28a + vector. Cells were grown in LB medium containing kanamycin (pET) to an $OD_{600} = 0.7$, followed by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and induced for 3 h at 37 °C. Cells were harvested by centrifugation and HBx proteins were purified from inclusion bodies as described previously (Moura et al., 2005; Rui et al., 2005).

Briefly, a bacterial pellet derived from a 1 l culture was re-suspended in lysis buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 5 mM DTT, 1 mM PMSF) containing lysozyme (1 mg/ml). After incubation on ice for 30 min, cells were lysed by three subsequent freezethaw cycles. Genomic DNA was digested by addition of DNAse (1 μg/ml) and incubation for 20 min at 37 °C. After incubation, the samples were sonicated and the pellet was separated by centrifugation at 18,600 × *g* for 15 min at 4 °C. The pellet was re-suspended in washing buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 5 mM DTT, 3 M Urea, 1% Triton X-100) and then centrifuged at 9500 × *g* for 15 min at 4 °C for three times or until the supernatant was clear. The pellet containing the HBx inclusion bodies was solubilized in 20 ml of 50 mM Tris–HCl, (pH 8), 8 M Urea and the solution was again centrifuged at 14,000 × *g* for 20 min at 4 °C. The resulting solution was filtered and applied to a Ni-NTA column (QIAgen). The subsequent washing and elution steps were all carried out with buffer A (8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl) but at different pHs. The column was washed first with buffer A, pH 8 and than with buffer A, pH 6.3. Bound protein was eluted with a linear pH gradient between 6.3 and 4.5 in buffer A. The column eluate was collected in 2 ml fractions and 10 μl aliquots were analyzed by SDS-PAGE. Peak fractions were pooled and dialyzed against renaturing PGDE buffer (25 mM pipes, pH 6, 50 mM KCl, 80 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1% Triton X-100, 5% glycerol) at 4 °C with several changes of the buffer. Dialyzed protein was cleared by centrifugation at 14,000 × *g* and used for the in vitro E4F binding assays.

4.4. Co-precipitation assay of GST-E4F with HBx

We used the expressed and purified proteins GST, GST-E4F in its various deletion mutant forms and 6xHis-HBx protein to perform pull down assays in vitro. First, we immobilized GST-E4F protein or GST control protein on 10 μl glutathione beads using PBS (pH 7.4) as the coupling and washing buffer. The coupled beads were than equilibrated with buffer PDGE (see above) and incubated with 6xHis-HBx(1–154) for 20 min at room temperature on an end-over-end mixer. After three washes with 0.5 ml of PGDE buffer the beads were collected and bound protein was analyzed by SDS-PAGE.

4.5. Yeast one-hybrid system based transcriptional activation assay

pLacZi-E4 and pLacZi-enhancer II vectors were linearized with ApaI and stably transformed into yeast strain W303 to generate reporter strains W303-E4 and W303-EnhII that were used in one-hybrid transcriptional activation assays described below (the W303 genotype: *ade2-1/ade2-1*; *ura31/ura3-1*; *his3-11,15/his311,15*; *trp1-1/trp1-1*; *leu2-3,112/leu2-3,112*). W303E4 and W303-enhancer II yeast cells were transformed with pGAD424 or the indicated pGAD424-E4F plasmids and in a second step were cotrans-

fected with empty vector pBTM116 or pBTM116 encoding lexA-fusions with HBx(1–154), mini-HBx(18–142) or mini-HBx(18–142)-Cys. The degree of activation of the promoter-lacZ reporter construct was then assessed by an ONPG (*o*-nitrophenyl β-D-galactopyranoside) liquid assay (Clontech). Briefly, equal quantities of yeast cells were centrifuged and disrupted in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) by freeze-thawing. 25 mM 2-mercaptoethanol and 10 mM ONPG (each dissolved in Z-buffer) were added to each reaction. After 1 h at 30 °C the reaction was stopped by adding 0.3 volumes of 1 M Na_2CO_3. After mixing and centrifugation the OD of the supernatants was measured at 420 nm using a spectrophotometer. All experiments were done in triplicate and the average values were plotted as arbitrary units.

4.6. Western blotting

Proteins were separated by SDS-PAGE using a 12.5% acrylamide gel and then transferred to the PVDF membrane (*ImmobilonTM-P*, Millipore) using a *Semi-Dry Blotting System* (W.E.P. Company). The membrane was blocked in 5% BSA in TBS (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.2) was probed with primary antibody (anti-GST) or anti-HBx serum (from rabbit). Secondary anti-mouse and anti-rabbit antibodies conjugated with HRP (1:5000) were used for detection using the *Western Blotting Luminol Reagent* (Santa Cruz Biotechnology).

4.7. Yeast two-hybrid assay

The yeast two-hybrid assay was performed using the L40 yeast strain that was maintained on yeast extract/peptone/dextrose (YPD) plates. Cells were transformed with the indicated plasmids using the lithium acetate procedure and grown on selective minimal medium plates with synthetic dextrose (SD) in the absence of Trp and Leu. Protein interaction studies were performed on SD plates without Leu, Trp and His (SD-Leu-Trp-His-). Two-hybrid transformants were streaked out, grown at 30 °C for 48 h, and analyzed for their ability to grow in SD-Leu-Trp-His- and regulate β-galactosidase expression.

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V - Discussão

HBx e sua interação com RNA

Em concordância com dados da literatura, a proteína HBx foi encontrada na fração insolúvel do lisado bacteriano, na forma de corpos de inclusão (Gupta, A. et al, 1995; Marczinovits, I. et al, 1997; Qadri, I. et al, 1995; Qadri, I. et al, 1996a). A existência de um meio citoplasmático reduzido na *E.coli* poderia ser responsável pela formação incorreta das pontes dissulfeto intermoleculares das 10 cisteínas presentes na proteína HBx, assim como a ausência de chaperones específicas que poderiam auxiliar no enovelamento correto da proteína. Por outro lado, a presença de um grande número de resíduos hidrofóbicos na seqüência de aminoácidos da proteína HBx, ou uma possível toxidez da proteína para a *E.coli*, podem também favorecer sua insolubilidade e acúmulo na forma de corpos de inclusão.

Em nossos estudos, a purificação prévia dos corpos de inclusão permitiu um alto grau de pureza da proteína HBx. A obtenção da proteína HBx na forma de corpos de inclusão impede a sua degradação proteolítica mas, por outro lado, a necessidade de sua extração com solução desnaturante de uréia 8 M (ou de guanidina-HCl 6 M) traz uma grande desvantagem: a difícil obtenção de proteína renaturada, corretamente enovelada e biologicamente ativa, através de diálises seqüenciais.

Para testar a atividade biológica da proteína HBx renaturada, utilizamos a capacidade da proteína HBx em se ligar a uma fita simples de ácidos nucléicos, como descrito por Qadri e colaboradores (Qadri, I. et al, 1996b). Como a molécula de DNA de fita simples é semelhante à molécula de RNA, resolvemos estudar a interação da proteína HBx com oligonucleotídeos de RNA ricos em adenina e uracila (AU). Estes motivos ricos em AU estão presentes em vários mRNAs que apresentam alta taxa de síntese e degradação (*turnover*) como, por exemplo, os mRNAs de proto-oncogenes *c-myc*, *c-fos* e de citocinas GM-CSF, IL-2 e TNF.

Nós mostramos em estudos *in vitro* que a proteína HBx pode se ligar com alta especificidade a um RNA rico em adenina e uracila (Rui, E. et al, 2001). Nossos dados permitem especular que a ligação da proteína HBx a estes mRNAs poderia causar uma estabilização *in vivo* destes mRNAs de proto-oncogenes, contribuindo para a transformação celular que levaria ao desenvolvimento do carcinoma hepatocelular (HCC).

A relevâncias das cisteínas para HBx

Os resíduos de aminoácidos de cisteínas têm a capacidade de formar ligações covalentes por meio das pontes dissulfetos. As formações de pontes dissulfetos em muitas proteínas desempenham um papel importante na estabilidade da conformação espacial da molécula e, conseqüentemente em sua atividade biológica. É conhecido que em alguns casos a substituição das cisteínas por aminoácidos com características similares não afeta significantemente a estrutura e a função de certas proteínas (Hu, Y.K. et al, 2000), porém em outras situações esta substituição pode inibir completamente sua atividade biológica (Karnik, S.S. et al, 1988; Petrov, V.V. et al, 1997; van Iwaarden, P.R. et al, 1991).

Deve-se ater especial atenção à presença de resíduos de cisteína em uma proteína pois isto tem importantes implicações para pesquisas que abordam temas estruturais. Muitas vezes a obtenção de grandes quantidades de proteínas, com alto grau de pureza, provêm de um sistema procariótico de expressão; no entanto, organismos como *E. coli* possuem um citoplasma redutor que dificulta a correta formação de pontes dissulfeto. Como conseqüência, as proteínas produzidas por este organismo podem não ser funcionais, seja pelas formações inespecíficas de pontes dissulfeto, seja pela agregação quando em solução, ou ainda na formação de corpos de inclusão (proteínas insolúveis). Uma proteína onde suas cisteínas foram permutadas por serinas e com suas principais funções biológicas pouco alteradas é de grande interesse para estudos estruturais e evolutivos.

Um estudo anterior já tinha investigado a ocorrência de pontes dissulfeto na proteína HBx (Gupta, A. et al, 1995), outros trabalhos sugeriram que esta proteína poderia sofrer dimerização e que as pontes dissulfetos poderiam participar deste evento. Para investigar a participação dos resíduos de cisteína na funcionalidade da proteína HBx, construímos uma série de proteínas mutantes, onde as cisteínas foram substituídas por serinas, para testar cada um das proteínas quanto à sua funcionalidade. Para testar a integridade funcional das HBx mutantes, decidimos adotar a capacidade da proteína viral em se ligar aos ácidos nucléicos de simples fita, como descrito por Qadri e colaboradores (Qadri, I. et al, 1996b) e posteriormente em nosso trabalho (Rui, E. et al, 2001). Tal decisão se fundamenta no fato de que este ensaio pode ser facilmente reproduzível em laboratório, e a interação entre ácido nucléico e proteína pode ser revelada por experimentos como EMSA e/ou UV-*cross-linking*. Surpreendentemente, todas as proteínas testadas tiveram a capacidade de interagir com a sonda de RNA AU38 (tabela 01) (de Moura, P.R.

et al, 2005). Com esse resultado podemos sugerir que a atividade biológica que nós observamos em ensaios de UV-*cross-linking* deve-se possivelmente à proteína HBx na forma monomérica e que a presença das cisteínas não seriam relevantes para a ligação ao RNA AU38, uma vez que o mutante mini-HBx(-Cys) não há qualquer cisteína e mesmo assim exibiu atividade biológica com similar intensidade quando comparada com a proteína HBx selvagem.

Motivados pelos resultados obtidos com os ensaios de interação de RNA e HBx, fomos impulsionados a fazer novas perguntas:

Qual a importância das cisteínas para a interação proteína-proteína?

A proteína HBx sem cisteína ainda preserva a capacidade de se ligar a outras proteínas importantes como p53, UV-DDB1, RXR ou às proteínas membro da família bZip como CREB, ATF3 e ICERIIγ (Barnabas, S. e Andrisani, O.M., 2000; Reddi, H. et al, 2003)?

Como já conhecido, HBx é uma proteína multifuncional que desencadeia efeitos pleotrópicos nos hepatócitos. Esta capacidade deve-se em parte à característica da proteína HBx em interagir com outras proteínas da célula hospedeira. Importantes interações de HBx com UV-DDB1 (Lee, T.H. et al, 1995), XAPC7 (Huang, J. et al, 1996), XAP2 (Meyer, B.K. et al, 1998), XIP (Melegari, M. et al, 1998) e p55sen (Sun, B.S. et al, 1998) foram descobertas pelo emprego da técnica de duplo-híbrido. Estas proteínas foram isoladas, identificadas e as suas ligações com a proteína HBx selvagem caracterizadas. Para responder as perguntas acima submetemos a proteína mini-HBx ao ensaio de duplo-híbrido a fim de isolar proteínas já descritas e identificar possíveis novas proteínas que interagem com HBx e, em um segundo momento, testar se a interação também ocorre com a proteína mini-HBx(-Cys).

Como resultado da seleção da biblioteca por proteínas que interagissem com mini-HBx, identificamos 2 proteínas já descritas em interagir com a proteína viral selvagem: *Retiniod X receptor* (RXR); *UV-damage DNA-binding*1 (UV-DDBP1), e uma potencial nova proteína, *E4 transcription factor* (E4F) (Rui, E. et al, 2005a). Para estudar a relevância das cisteínas em um contexto de interação proteína-proteína, realizamos experimentos com RXR, UV-DDB1, E4F e, como controle positivo, a proteína p53.

Ao realizar os ensaios quantitativos com p53 e E4F percebemos que as 3 formas de HBx pouco diferenciaram em interagir com as duas proteínas humanas, concluímos que neste contexto as cisteínas foram pouco relevantes na interação HBx-p53 (de Moura, P.R. et al, 2005) e HBx-E4F (Rui, E. et al, 2005a). Porém, ao verificar se o mesmo aconteceria com a proteína RXR,

observamos que as substituições dos aminoácidos fez com que HBx perdesse aproximadamente 30% da afinidade para RXR, mas preservando ainda a capacidade em interagir com este fator (Rui, E. et al, 2005a). Entretanto, os resultados com UV-DDB1 revelaram que a substituição das cisteínas desfavoreceu completamente a formação do complexo mini-HBx(-Cys)-UV-DDB1. Porem, de acordo com a literatura, as deleções N- e C-terminal tiveram pouca influência nos teste quando comparado a proteína HBx selvagem.

Acreditamos que estas descobertas tenham importantes conseqüências para o entendimento do mecanismo de ação de HBx. Sabendo que a proteína viral é capaz de interagir com dezenas de proteínas humanas é coerente indagar se esta capacidade seria em decorrência de uma grande flexibilidade estrutural, onde os resíduos de cisteínas teriam um papel secundário na estabilidade conformacional de HBx. Entretanto é muito importante ressaltar que testamos HBx sem cisteínas com apenas 4 proteínas humanas, dentre dezenas outras conhecidas. Antes de quaisquer especulações mais profundas a respeito do papel das cisteínas na funcionalidade de HBx é necessário que outros grupos venham confirmar nossos resultados com uma gama maior de técnicas e proteínas diferentes.

Apesar de uma primeira tentativa de cristalização da proteína HBx ter falhado, impedindo dessa forma a utilização de métodos cristalográficos e difração de raio-X para a elucidação da estrutura tridimensional da proteína viral, nossos estudos estruturais por técnicas de dicroísmo circular e ressonância nuclear magnética nos mostraram que a proteína HBx apresenta uma estrutura não-periódica (Rui, E. et al, 2005b). Em nossos ensaios observamos ainda que, em determinada condições experimentais, a proteína HBx pode alterar as porcentagens das estruturas secundárias. Tais informações suportam um modelo de proteína bem flexível, que tendesse a assumir estruturas tridimensionais específicas com determinados substratos. Tal característica traria a vantagem de um ajuste fino de interação proteína-proteína que visasse um estado mínimo de energia.

E4F em um contexto celular

Em nossos experimentos de duplo-híbrido achamos a proteína E4F interagindo com HBx.

O cDNA de E4F (*E4 transcription factor*) codifica uma proteína de 784 aminoácidos com 86% de identidade com o fator nuclear φ-AP3 de camundongo (Fernandes, E.R. e Rooney, R.J., 1997). Ambas as proteínas possuem seqüências conservadas nos domínios *zinc fingers* que as

remetem como membros da família GLI-Kruppel (Fognani, C. et al, 1993). Os genes da família GLI-Kruppel são caracterizados por possuírem um domínio conservado presentes em muitos *fingers* (HTGEKP(Y/F)XC), e membros desta família estão implicados nos processos de desenvolvimento embrionário e em certas neoplasias (Ruppert, J.M. et al, 1988). Análises na seqüência de aminoácidos da proteína E4F mostraram que a proteína apresenta uma região rica em prolina, um domínio *helix-turn-helix* e 4 dois domínios *zinc fingers* na região N-terminal qual abrange os primeiros 262 aminoácidos (Fernandes, E.R. e Rooney, R.J., 1999); este fragmento, conhecido por p50E4F, foi isolado de células HeLa por afinidade a uma seqüência específica de DNA do promotor E4 do adenovírus como um polipeptídeo de 50kDa (Raychaudhuri, P. et al, 1987; Raychaudhuri, P. et al, 1989), o que sugere que a proteína sofra proteólise endógena.

Na região C-terminal (262-784) encontram-se outros 6 domínios *zinc finger*, porém não há estudos referentes ao fragmento C-terminal da proteína E4F. A proteína E4F humana é expressa em baixa concentração em uma grande variedade de tecidos e existe sob duas formas até agora detectada, uma predominante de 120kDa (p120E4F) e outra menos abundante de p50E4F. Ambas as formas reconhecem o mesmo sítio no promotor E4 do adenovírus, mas com efeitos funcionais opostos: enquanto p120E4F reprime o promotor, a forma p50E4F estimula sua atividade (Fernandes, E.R. et al, 1998). A expressão do gene E1A(13S) exerce forte influência sobre a atividade de E4F por meios de eventos que envolvem a fosforilação da proteína humana. Ensaios de transfecção transiente mostraram que na presença de E1A(13S) o fragmento de p50E4F estimulou o promotor E4, porém, a proteína completa p120E4F agiu como repressor do promotor somente na ausência da proteína viral, o efeito repressor foi anulado na presença de E1A(13S) (Fernandes, E.R. et al, 1998).

Acumulam-se evidências de que E4F esteja envolvida no controle do ciclo celular. Um efeito fisiológico da expressão ectópica de p120E4F em fibroblastos de rato é a parada do ciclo celular próximo a transição da fase G1 para S, mas este resultado foi observado somente com a proteína completa e não com o fragmento amino-terminal p50E4F (Fernandes, E.R. e Rooney, R.J., 1999), indicando um específico envolvimento da metade carboxi-terminal da proteína nesta função. Neste contexto, estudos mostraram que a interrupção do ciclo celular, intermediada por E4F, é dependente da presença de p53 funcional.

O produto do gene supressor de tumor p53 está envolvido na manutenção da integridade do genoma, além de múltiplas outras funções como: ativador e repressor da transcrição; modulação de

fatores relacionados no reparo ao DNA; regulação da apoptose e controle do ciclo celular (Harms, K. et al, 2004). A regulação do crescimento e divisão celular por p53 requer a capacidade de reprimir e ativar genes celulares específicos, como também associar-se em complexos com outras proteínas. Entretanto, a formação do complexo E4F-p53 não influencia a capacidade de p53 de se ligar ao DNA (Sandy, P. et al, 2000), indicando que a supressão do ciclo celular por p120E4F pode envolver outros genes que não são diretamente regulados pelas seqüências consenso de ligação ao DNA seja de p53 ou de E4F, pois mesmo com a *deleção* do domínio de ligação ao DNA de E4F observou-se a supressão do ciclo celular. Isto sugere que p120E4F é um importante elemento dentro de uma complexa rede de interações proteína-proteína na qual p53 está envolvida. No entanto, p53 é uma das primeiras proteínas a ser descrita interagindo com E4F. Uma segunda proteína, descoberta interagindo com E4F foi a pRB (*retinoblastoma protein*).

As proteínas membros da família pRB participam na transdução de sinal do ciclo celular para o controle transcricional de um conjunto de genes que regulam processos de crescimento e diferenciação celular, e esta função está na capacidade de ligarem e modularem a atividade de diferentes fatores de transcrição (Fajas, L. et al, 2000). E4F interagiu com pRB somente quando as células encontravam-se em estado quiescentes, e o complexo não foi detectado com as células em estado proliferativo, mostrando que a interação E4F-pRB é dependente do ciclo celular. Ensaios utilizando células deficientes em pRB (pRB-/-) apresentaram evidências que o efeito antiproliferativo de E4F é fortemente influenciado pela presença de pRB. Descobriu-se ainda que pRB estimula a ligação de E4F ao seu sítio no DNA, porém a pRB não foi detectada no complexo p120E4F-DNA em ensaios de *gel shift* (Fajas, L. et al, 2000).

Outro estudo encontrou E4F associada a ARF. O p14ARF é um gene supressor de tumor que pode induzir uma forte parada no crescimento ou morte celular em resposta a estímulos hiperproliferativos oncogênicos, tais como os desencadeados pela expressão do gene E1A do adenovírus, que neste caso interrompe o ciclo em G1 e G2 ou induz a célula para apoptose (de Stanchina, E. et al, 1998). p14ARF também ativa uma via de resposta de p53 por interagir e inibir Mdm2, um antagonista de p53 (Weber, J.D. et al, 1999). Animais *knock-out* para ARF têm alta propensão para a formação de tumor e, em células deficientes em ARF há progressão do ciclo celular mesmo após a ocorrência de lesões ao DNA (Khan, S.H. et al, 2000). Foi demonstrado que p14ARF liga-se nos resíduos 551 a 566 de p120E4F para formar um complexo envolvido na modulação do ciclo celular. Em ensaios de transfecção transiente verificou-se que ambas as

proteínas sozinhas foram hábeis em parar o ciclo celular, porém, a co-expressão de p120E4F e p14ARF inibiu fortemente a ocorrência da fase S, indicando um efeito sinergístico entre as duas proteínas. Entretanto, tal fenômeno só é observado na presença de p53 funcional, o que corrobora com os experimentos de imunoprecipitação que demonstram a formação do complexo p14ARF-p120E4F-p53 *in vivo* (Rizos, H. et al, 2003).

Uma outra investigação determinou que os aminoácidos 411 a 445 de E4F formam um importante ponto de interação com a proteína RASSF1A (Fenton, S.L. et al, 2004). Em vários tipos de linhagens de células tumorais verificou-se que não ocorria a expressão do gene RASSF1A; por outro lado, a expressão do gene RASSF1A teve a capacidade de diminuir a formação de tumor em camundongos, suprimir a formação de colônias e impedir o crescimento celular (Burbee, D.G. et al, 2001; Dammann, R. et al, 2000). Assim aparecem fortes evidências de que a inativação de RASSF1A é importante na patogênese de cânceres humanos, o que conduz este gene para a lista de genes supressores de tumor. Ensaios de expressão e co-expressão de E4F e RAASF1A em células de mamíferos demonstraram que RASSF1A, quando sozinha, influencia muito pouco na distribuição das células pelas fases do ciclo celular, mas possui um efeito potencializador quando expressa junto com E4F na parada do ciclo celular na fase G1 (Fenton, S.L. et al, 2004). Apesar dos esforços para compreender o papel de E4F nas atividades celulares, a função desta proteína em células normais ainda não está completamente elucidada, mas já se sabe que a geração de ratos *knock-out* viáveis não é possível, pois os embriões deficientes em E4F morrem no estágio pré-implantação, enquanto que cultura de blastócitos E4F-/- exibem problemas no progresso mitótico, má segregação cromossômica e aumento da apoptose, sugerindo um papel crucial de E4F para a progressão mitótica e durante a fase inicial do embrião (Le Cam, L. et al, 2004).

Até onde os estudos sobre E4F avançaram verificou-se uma forte relação desta proteína com a regulação do ciclo celular e, curiosamente, todas as 5 proteínas conhecidas hoje em interagir com E4F são proteínas supressoras de tumor (pRB, HMGA2, p14ARF, p53 e RASSF1A). As pesquisas com E4F estão apenas no início, visto que existem poucos artigos sobre o tema.

E4F, uma proteína alvo de dois vírus

Os adenovírus podem causar uma variedade de doenças humanas associadas geralmente com infecções do trato gastrointestinal, respiratória e conjuntivites (Horwitz, M.S., 1996). Há estudos

que mostram que os produtos dos genes E1A, E1B e E4, de alguns tipos de adenovírus, têm potencial oncogênico (Thomas, D.L. et al, 2001). O ciclo de infecção do adenovírus pode ser divido em duas fases: a primeira fase, ou fase inicial, compreende a entrada do vírus dentro da célula hospedeira e a passagem do genoma viral para o núcleo da célula; posteriormente, o vírus promove uma seletiva transcrição e tradução dos primeiros genes alvos, e tais eventos modulam as funções celulares que facilitam a ativação de outros genes (genes tardios) os quais promovem a replicação do DNA viral; a segunda fase, ou fase tardia, abrange a biosíntese de proteínas estruturais e a maturação do vírus infectante (Russell, W.C., 2000). O primeiro gene viral a ser transcrito é o E1A (Nevins, J.R. et al, 1979). Este transcrito primário sofre diferentes *splicings* que resultam em 5 distintos mRNAs (13S, 12S, 11S, 10S e 9S) (Stephens, C. e Harlow, E., 1987). Uma das principais funções dos produtos do gene E1A do adenovírus é ativar a transcrição dos primeiros genes virais (*early viral genes*) durante a primeira fase do ciclo lítico (Flint, J. e Shenk, T., 1989). Para este fim, E1A tem como alvo um número de distintos fatores de transcrição celulares. Muitos destes fatores celulares identificados até o momento são proteínas que interagem diretamente com seqüências específicas do DNA viral (Jones, N.C. et al, 1988).

Estudos do promotor E4 do adenovírus identificaram vários fatores de transcrição que são alvos de E1A, dentre eles destacam-se: os membros da família ATF (*Activating Transcription Factor*), os quais compartilham similar ou idênticos elementos de ligação ao DNA (TGACGT) e que fazem parte de uma classe de fatores de transcrição denominada bZip; Oct-1, um fator de transcrição eucarioto ubiqüamente expresso que reconhece o octâmero ATGCAAAT; Sp1 (*Specificity protein 1*), um fator de transcrição que possui especificidade para a seqüência GGGCGG e E4F, proteína humana envolvida na regulação do ciclo celular e que se liga ao motivo TGACGTTAAC do promotor E4 (Jones, C. e Lee, K.A., 1991). Para nossa admiração foi intrigante saber que HBx pode também regular a função de ATF (Choi, C.Y. et al, 1997), Oct-1 (Antunovic, J. et al, 1993), Sp1 (Gomez-Gonzalo, M. et al, 2001) e muito provavelmente, como sugere nosso estudo, a E4F. E mais, que a proteína HBx pode parcialmente substituir a função do gene E1A durante o processo de infecção do adenovírus (Schaack, J. et al, 1996).

Muito curiosamente, ATF, E4F e E4PB4 (*E4 Binding Protein 4*) (Cowell, I.G. et al, 1992), um fator de transcrição humano caracterizado como bZip e relacionado com a sobrevivência e apoptose celular, resposta inflamatória e mecanismos cicardianos (Cowell, I.G., 2002) reconhecem a mesma seqüência de DNA. Para nossa surpresa, um grupo que pesquisou fatores de hepatócitos,

com capacidade de se ligar a seqüências de DNA do *enhancer* II do HBV, descobriu E4BP4 interagindo com a região compreendida entre os nucleotídeos 1640 a 1663 do genoma do HBV (Ishida, H. et al, 2000). Ao alinharmos as seqüências reconhecidas por E4F e E4B4 com a esta região do genoma viral, observamos uma homologia entre as seqüências. A descoberta nos conduziu obrigatoriamente a questionar se E4F poderia ligar-se ao DNA do HBV via seqüência do *enhancer* II e se HBx estaria de alguma forma exercendo um efeito regulador em E4F.

E4F interage com *enhancer* II do HBV

Para esclarecer se E4F também seria capaz de reconhecer o DNA do HBV, optamos por empregar ensaios de ativação da transcrição baseado no sistema de mono-híbrido. Com este intuito construímos leveduras recombinantes que tinham em seu genoma o gene repórter LacZ sob o controle do promotor E4, usado como controle positivo ou a seqüência do *enhancer* II, para teste. Os testes mostraram que E4F selvagem também é capaz de reconhecer o *enhancer* II do HBV, embora com menor afinidade quando comparado ao seu sítio de ligação ao DNA do promotor E4. Aqui cabe reafirmar que outros pesquisadores evidenciaram que E4F, quando completa, exerce um efeito repressor da transcrição (Fajas, L. et al, 2001), possivelmente por ligar-se ao DNA e impedir a montagem da maquinaria de transcrição, fato que não foi observado com o fragmento p50E4F. Para verificar se HBx apresenta alguma regulação na interação de E4F ao DNA, empregamos novamente o experimento de ativação da transcrição baseado no sistema de mono-híbrido. Observamos que a presença de HBx no sistema diminui a ativação do gene repórter em aproximadamente 50%. Poderíamos concluir que HBx agiria de forma similar ao produto do gene E1A, que inibe o efeito repressor da proteína E4F no promotor E4 e dessa forma possibilita a transcrição dos primeiros genes do adenovírus.

O *enhancer* II, presente no genoma do HBV, foi caracterizado por estimular a transcrição de SP-I, SP-II e X, há porém fortes evidências que a transcrição do RNA pré-genomico também seja dependente da presença deste elemento (Lee, J.H. e Rho, H.M., 2001). Sabe-se ainda que E4F completa (p120E4F) é a forma mais abundante dentro da célula. Essa proteína, supressora da transcrição, poderia associar-se ao *enhancer* II e inibir o processo transcricional do HBV. Neste contexto especulamos que HBx, ao interagir com E4F, diminuiria o efeito repressor, proporcionando desta maneira o prosseguimento da replicação viral no hospedeiro. Paralelamente, a inibição desta proteína por HBx poderia acarretar alterações no processo de regulação do ciclo

celular, função sobre a qual E4F parece exercer forte influência, resultando em uma acelerada proliferação das células infectadas com HBV.

VI - Conclusões

- A proteína HBx apresenta alta insolubilidade em sistema procarioto de expressão, entretanto processos de purificação e renaturação permitem obter a proteína com alto grau de pureza e biologicamente ativa.

- Mostramos neste trabalho que HBx pode interagir com RNA rico em adenina e uracila. Ensaios de EMSA mostram-se reprodutíveis, rápidos e confiáveis como método para testar a funcionalidade da proteína produzida em *E. coli*.

- A geração de proteínas truncadas possibilitou mapear sítios de ligação de HBx ao RNA e mutações pontuais nos resíduos de cisteínas permitiram avaliar a funcionalidade da proteína viral em condições experimentais.

- As substituições das cisteínas por serinas reduziram a interação de HBx com RXR e inibiram completamente a interação com UV-DDB1, mas não alteraram a capacidade de interagir com E4F, p53 e RNA AU38.

- Os dados de CD e NMR nos mostraram que a proteína HBx apresenta uma estrutura não uniforme e com variação nas estruturas secundárias em condições experimentais, sugerindo uma proteína flexível que possivelmente ajusta sua estrutura conforme o substrato.

- O ensaio de duplo-híbrido selecionou E4F como uma nova proteína capaz de interagir com HBx. A proteína E4F foi capaz de reconhecer e ligar-se com o *enhancer* II do HBV e ainda, a presença de HBx no sistema inibiu a ligação de E4F ao DNA viral.

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VIII - Anexos

Tabela 1 - Oligonucleotídeos desenhados para a manipulação de genes, clonagens, mutações, geração de proteínas truncadas e sequenciamento.

Nome	Enzima de restrição	Seqüência
Oligonucleotídeos para a manipulação do gene de HBx		
cBHX-AS-pBTM	SalI	GCGGTCGACTTAGGCAGAGGTAGGAAAG
cHBX-S-pBTM	EcoRI	CGC***GAATTC***ATGGCTGCTAGGTTGAGCTGC
PET-X-AS	XhoI	GTT***CTCGAG***TTAGGCAGAGGTGAAAAAG
PET-X-S	EcoRI	AC***GAATTC***CATATGGCTGCTAGGTTGTGCTGCC
X-MUT-AS	BamHI	CG***GGATCC***TTAGACCAATTTATGCCTACAGCC
X-MUT-S	EcoRI/NdeI	G***GAATTC CATATG***CGTCCCGTCGGCGCTGAATC
X-GST-S	BamHI	GC***GGATCC***TTGAGCTGCCAACTCGATCCTGCGCGG
X-GST-S2	BamHI	GC***GGATCC***GAGACCACCGTGAACGCCCAC
X-GST-AS	EcoRI.	GC***GAATTC***TTAGGCAGAGGTGAAAAAGTTGC
XWV-AS3	EcoRI/Xba I	TG***GAATTC TCTAGA***TTATTAGCGACGTGCAGAGGTGAAGCG
XWV-AS2	EcoRI/Xba I	TG***GAATTC TCTAGA***TTATTAGACCAATTTATGCCTACAGCCTC
XPWW-S	HindIII	GCACTG***AAGCTT***GGTTCTGGTTCTGGTGACGACGATGACAAGATGGCTGC TAGGTTGTGC
XWST-AS	XBaI	AGTC***TCTAGA***TTATTACTTCTCGAACTGTGGGTGAGACCAGGCACAGGTG AAAAAGTTG
HBx-1-S	HindIII	GCACTG***AAGCTT***GGTTCTGGTTCTGGTGACGACGATGACAAGATGGCTGC TAGGTTGTGCTGC
HBx-48-S	HindIII	GCACTG***AAGCTT***GGTTCTGGTTCTGGTGACGACGATGACAAGGACCACGG GGCGCACCTCTCT
HBx-80-S	HindIII	GCACTG***AAGCTT***GGTTCTGGTTCTGGTGACGACGATGACAAGGAGACCAC CGTGAACGCCCAC
HBx-154-AS	XBaI	AGTC***TCTAGA***TTATTAGGCAGAGGTGAAAAAGTTGCATGG
HBx-78-AS	XBaI	TGGAATTC***TCTAGA***TTATTAGCGACGTGCAGAGGTGAAGCG
HBx-142-AS	XBaI	TGGAATTC***TCTAGA***TTATTAGACCAATTTATGCCTACAGCC
Oligonucleotídeos para mutações pontuais no gene de HBx		
C26	Nde I	CGG***CATATG***CGTCCCGTCGGCGCTGAATCC**a**GCGGACGACCC
C61		CGGACTCCCCGTC**a**G**c**GCCTTCTCATCTGC
C61 AS		GCAGATGAGAAGGC**g**C**t**GACGGGGAGTCCG
C69		CTCATCTGCCGGACCC**a**GTGCACTTCGCTTC
C69 AS		GAAGCGAAGTGCAC**t**GGGTCCGGCAGATGAG
C115		GGCATACTTCAAAGACT**c**TCTCTTCAAAGACTGGGAGGAG
C115 AS		CTCCTCCCAGTCTTTGAAGAGA**g**AGTCTTTGAAGTATGCC
C137	BamHI	CG***GGATCC***TTAGACCAATTTATGCCTA**g**AGCCTCC
Oligoribonucleotídeos utilizados como sonda para ensaios de EMSA		
AU-38		GAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUAG
AU-21		UUAUUAUUUAUUUAUUAUUUA
AU-21 Mut		UUAUUAUAUAUAUAUUAUAUA
AU-11		UUUAUUUAUUA
poli U (25)		UUUUUUUUUUUUUUUUUUUUUUUUU
poli A (25)		AAAAAAAAAAAAAAAAAAAAAAAAA
poli C (25)		CCCCCCCCCCCCCCCCCCCCCCCCC
poli G (25)		GGGGGGGGGGGGGGGGGGGGGGGGG
Oligonucleotídeos para clonagem e geração de proteínas truncadas E4F		
E4F-S	EcoRI	GGCG***GAATTC***CCATGGAGGGCGAGATGGCAGTGC
E4F84-S	EcoRI	GGCG***GAATTC***CCCGGGCCCCTCCGGAGGCCCTG

E4F173-S	EcoRI	GGCG***GAATTC***CCCTCGCAGGGGAGGGTGAGCAGG
E4F263-S	EcoRI	GGCG***GAATTC***CCCTGACCCGGCACCTCAAGTCTC
E4F428-S	EcoRI	GGCG***GAATTC***CCGCCTCAGCGGTGCCCAGGACC
E4F-AS	XhoI	GGC***GAGCTC***CTAGACGATGACCGTCTGCACC
E4F262-AS	XhoI	GGC***GAGCTC***TTATGCACCCGACTCCCGGAAGCT
E4F486-AS	XhoI	GGC***GAGCTC***TTATGAGCCCTTCTCTCGGAAGCC
Oligonucleotídeos para geração de leveduras transgenicas		
EnhII-S	EcoRI	***AATTC***TTGCCCAAGGTCTTACATAAGAGGTTGCCCAAGGTCTTACATAAG AGGTTGCCCAAGGTCTTACATAAGAGGC
EnhII-AS	XhoI	***TCGAG***CCTCTTATGTAAGACCTTGGGCAACCTCTTATGTAAGACCTTGGG CAACCTCTTATGTAAGACCTTGGGCAAG
E4-S	EcoRI	***AATTC***ATGACGTAACATGACGTAACATGACGTAAC
E4-AS	XhoI	***TCGAG***GTTACGTCATGTTACGTCATGTTACGTCAT
Oligonucleotídeos para sequeciamento		
PBTM-AS		CAACCTGCAGCCTCAGTAAATATCTGTTAAGGA
PBTM-S		AACAACGTCGACCTGCGGAGCTCAGTTACAGCTTG
ScrAmp-S		CTATTCGATGATGAAGATACCCCACCAAACC
ScrpAmp-AS		GTGAACTTGCGGGGTTTTTCAGTATCTACG
T7		TAATACGACTCACTATAGGG
T3		AATTAACCCTCACTAAAGGG
pET-S		TAATACGACTCACTATAGGG
pET-AS		GCTAGTTATTGCTCGGCGG
PLacZi-S		CTGTTCGGAGATTACCGAATC
Gal4		TACCACTACAATGGATG

Tabela 2 - Soluções, tampões e meios de cultura

Nome	**Componentes**
solução Ânodo I	300 mM Tris-HCl pH 8,0, 20% metanol
solução Ânodo II	25 mM Tris-HCl pH 8, 20% metanol
solução Cátodo	25 mM Tris-HCl pH 8,0, 20% metanol, 40 mM ácido -capróico
solução coomassie blue staining	50% (v/v) metanol, 10% (v/v) ácido acético e 0,25% (v/v) Commassie Blue
solução de descoloração	12,5% (v/v) metanol e 5% (v/v) ácido acético
solução desnaturante 6M guanidina	50 mM Tris-HCl (pH8.0)e 6 M guanidina-HCl
solução desnaturante 8M uréia	50 mM Tris-HCl (pH8.0)e 8 M uréia
solução EDTA	0.5M EDTA (pH8.0)
tampão OPA (One-Phor-All Buffer Plus)	100 mM Tris-acetato (pH 7.5), 100 mM acetato de magnésio e 500 mM acetato de potássio
tampão 10mM Pipes (pH 6.0)	10mM Pipes (pH 6.0)
tampão 10mM Tris-HCl (pH7.4)	10mM Tris-HCl (pH7.4)
tampão AI	10 mM Tris-HCl, 100 mM fosfato de sódio, 8 M uréia, (pH8.0)
tampão BI	10 mM Tris-HCl, 100 mM fosfato de sódio, 8 M uréia, (pH 6.3)
tampão BS	PBS e 20mm imidazol
tampão CI	10 mM Tris-HCl, 100 mM fosfato de sódio, 8 M uréia, (pH 4.5)
tampão CS	PBS e 200mm imidazol
tampão citrato-fosfato	Ácido cítrico e 50mM Na_2HPO_4
tampão de amostra	50 mM Tris-HCl (pH 6,8); 2 mM EDTA; 1% (w/v) SDS; 1% (w/v) β-mercaptoetanol; 8% (w/v) glicerol; 0,025% (w/v) azul de bromofenol
tampão de corrida TBE	1 M Tris base, 500 mM ácido bórico, 10 mM EDTA
tampão de corrida Tris-Glicina	25 mM Tris base; 250 mM glicina; 0,1% (w/v) SDS
tampão de diálise	10 mM Pipes (pH 6.0)
tampão de eluição BG	50 mM Tris-HCl (pH 8.0) e 10 mM glutationa reduzida

tampão de extração	50 mM Tris-HCl (pH 8.0), 8 M uréia ou 6 M guanidina-HCl
tampão de lavagem 1	50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 3 M uréia, 1% Triton X-100
tampão de lavagem 2	50 mM Tris-HCl (pH 8.0), 5 mM EDTA,
tampão de lise	50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 5 mM DTT, 1 mM PMSF
tampão PBS	137 mM NaCl, 2,7 mM KCl, 10,1 mM Na_2HPO_4 e 1,76 mM KH_2PO_4, (pH 7.4)
tampão PGDE	25 mM Pipes, pH 6, 50 mM KCl, 80 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0,1% Triton X-100, 5% glycerol
tampão TAE	40 mM tris-HCl, 1 mM EDTA, pH 8,0
tampão Taq DNA Polimerase	50 mM KCl, 1,5 mM $MgCl_2$, 10 mM Tris-HCl, (pH 9.0)
tampão TBS 1X	20 mM Tris-HCl, 150 mM NaCl, pH 7,2
tampão Tris pH 8,0	Solução estoque 0.5M Tris-HCl (pH 8.0)
meio SOC	0,5% extrato de levedura, 2% peptona, 10 mM NaCl, 2,5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glicose
meio LB (Luria-Bertani)	1% de peptona, 0,5% de extrato de levedura e 0,5% de NaCl
meioYPD	1% extrato de levedura, 2% peptona, 2% glicose
tampão de ligação EMSA	50 mM de acetato de potássio, 5 mM de Tris-HCl pH 7,5, 5 mM de acetato de magnésio, 10 μg/ml de BSA e 4% de glicerol
tampão de Cross-Linking	20 mM Hepes, 10 mM $MgCl_2$, 60 mM KCl e 10% glicerol.
solução de bloqueio	5% de BSA e 0,1% de Tween-20 em tampão TBS 1X

Lista de Abreviações

A	Adenina
Aa	aminoácido
AD	domínio de ativação (*Activation domain*)
APS	persulfato de amônio
ATP	adenosina 5'-trifosfato
BD	domínio de ligação (*Binding domain*)
BSA	albumina bovina sérica
CD	Dicroísmo Circular
D_2O	água deuterada
dATP	2'-deoxiadenosina 5'-trifosfato
dCTP	2'-deoxicitidina 5'-trifosfato
DEPC	dietil-pirocarbonato
dGTP	2'-deoxiguanosina 5'-trifosfato
DISC	Complexo sinalizador indutor de morte celular
DNA	ácido desoxirribonucléico
DOTAP	*N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate*
DTT	ditiotreitol
dTTP	2'-deoxitimidina 5'-trifosfato
EDTA	ácido tetra-acético dissódico (etilenodinitrilo)
EMSA	Ensaio de retardamento de migração em gel
FPLC	*Fast Purification Liquid Chromatography*
GST	glutathione-S-transferase
H	Histidina
HBSS	*Hank`s Balanced Salt Solution*
HBV	Vírus da Hepatite B
HCC	Carcinoma Hepatocelular
HBC	Vírus da Hepatite C
IPTG	isopropil-β-D-tiogalactopiranosídeo
kb	Quilobases
kDa	Quilodaltons
L	Leucina
LB	Luria Bertani

mA	mili-Ampére
MCS	sítio de clonagem múltipla (*Multiple cloning site*)
MOPS	ácido 4-morfolino propano sulfônico
mRNA	RNA mensageiro
Ni-NTA	ácido níquel-nitrilotriacético
nm	nanômetro
NOESY	*Nuclear Overhauser and Exchange Spectroscopy*
nt	nucleotídeo
OD_{600}	densidade ótica a 600 nm
ONPG	o-nitro fenil -(-D- galactopiranosídico (ONPG)
ORF	*Open reading frames*
pb	pares de base
PCR	reação de polimerase em cadeia (*Polimerase Chain Reaction*)
PDB	*Protein Data Bank*
PEG	polietileno-glicol
pH	Potencial hidrogeniônico
PM	Peso molecular
PMSF	Fluoreto de fenil metil sulfonila
ppm	Partes por milhão
PTP	Complexo da transição do poro
PVDF	Fluoreto de polivinila
RMN	Ressonância Magnética Nuclear
RNA	Ácido ribonucléico
rpm	Rotações por minuto
SDS	Dodecil-sulfato de sódio
SDS-PAGE	*SDS polyacrylamide gel electrophoresis*
TOCSY	*Total Correlated Spectroscopy*
Tween 20	Monolaurato de polioxietileno (20) sorbitano
U	Uracila
UTR	Região não traduzida
UV	Ultravioleta
W	Triptofano
YNB	*Yeast nitrogen base*