Oncogene (1999) 18, 1677–1687

http://www.stockton-press.co.uk/onc

The translation initiation factor, hu-Sui1 may be a target of hepatitis B X antigen in hepatocarcinogenesis

Zhaorui Lian[1], Jingbo Pan[1], Jie Liu[1], ShuMin Zhang[1], Minghua Zhu[4], Patrick Arbuthnot[2], Michael Kew[2] and Mark A Feitelson*[,1,3]

[1]*Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA;* [2]*Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa;* [3]*Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, USA;* [4]*Department of Pathology, Fourth Military Medical College, Xi'an, Shanxi, PRC*

The role of hepatitis B virus X antigen in the development of hepatocellular carcinoma was explored by stably transfecting HepG2 cells with an X antigen expression vector, and identifying the differences in gene expression that distinguish X positive from X negative cells by subtractive PCR. One differentially expressed gene, the human homolog of *sui1* (hu-*sui1*), encodes a translation initiation factor whose expression was suppressed by X antigen in HepG2 cells. Hu-Sui1 was also expressed in nontumor liver but not in tumor cells from patients with hepatocellular carcinoma. Introduction of hu-*sui1* into HepG2 cells inhibited cell growth in culture, in soft agar, and partially inhibited tumor formation in nude mice. Hence, the suppression of hu-*sui1* by X antigen may result in the abrogation of negative growth regulation and contribute to the development of hepatocellular carcinoma.

Keywords: translation initiation; hepatitis B X antigen; Sui1; hepatocellular carcinoma

Introduction

The hepatitis B virus (HBV) carrier state, when acquired early in life, is associated with a very high risk for the development of hepatocellular carcinoma (HCC) (Szmuness, 1978; Beasley and Hwang, 1984). The finding that HBV and related viruses (hepadnaviruses) (Robinson *et al*., 1982) make a genetic contribution to HCC (Seeger *et al*., 1991), suggests that one or more virus encoded proteins play a central role in hepatocarcinogenesis. The findings that hepatitis B X antigen (HBxAg) transforms a mouse hepatocyte cell line (Hohne *et al*., 1990; Seifer *et al*., 1991), and that HBxAg binds to and inactivates the tumor suppressor, p53 (Feitelson *et al*., 1993; Wang *et al*., 1994; Truant *et al*., 1995; Takada *et al*., 1995), implies that HBxAg contributes to HCC. This is further supported by observations that HBxAg/p53 costaining and complex formation correlate with the development of liver tumors in X transgenic mice with sustained high levels of HBxAg expression (Kim *et al*., 1991; Ueda *et al*., 1995; Koike *et al*., 1994a). The findings that HBxAg is a *trans*-activating protein (Twu and Schloemer, 1987; Rossner, 1992; Henkler and Koshy, 1996), and that virus DNA fragments integrated into HCC cells often encode HBxAg with *trans*-activating (Zhou *et al*., 1987; Chen *et al*., 1988; Wollersheim *et al*., 1988; Zahm *et al*., 1988; Matsubara and Tokino, 1990; Takada and Koike, 1990; Unsal *et al*., 1994) and transforming (Luber *et al*., 1996) activities, further suggest that the targets of HBxAg transcriptional regulation mediate hepatocellular transformation. Since HBxAg binds to and alters the activity of several transcription factors (Maguire *et al*., 1991; Autunovic *et al*., 1993; Cheong *et al*., 1995; Qadri, *et al*., 1995; Haviv *et al*., 1996; Henkler and Koshy, 1996), and may also alter the activity of signal transduction pathways (Kekule *et al*., 1993; Benn and Schneider, 1994; Natoli *et al*., 1994; Su and Schneider, 1996), experiments were designed to test the hypothesis that the introduction of HBxAg into HepG2 cells is associated with altered patterns of host cell gene expression that regulate cell growth. This report describes the isolation, identification and preliminary functional characterization of a natural HBxAg effector molecule which appears to negatively regulate cell growth. The results also imply that HBxAg possesses a novel *trans*-repressive transcriptional activity that may be relevant to the mechanism whereby HBV contributes to hepatocarcinogenesis.

Results

Establishment of HepG2X and HepG2CAT cells

Recombinant retroviruses encoding the full length HBxAg polypeptide or bacterial CAT protein were used to infect HepG2 cells. Following 14 days of selection in G418, which eliminated most of the uninfected cells, cell lysates were prepared and checked for expression of the recombinant proteins. For the detection of CAT activity, equivalent amounts of cell lysates were incubated with ^{14}C-chloramphenicol, and the extent of acetylation analysed by thin layer chromatography and autoradiography. CAT activity was present in HepG2CAT, but not in HepG2X cells (Figure 1a). For the detection of HBxAg, lysates prepared from 5×10^6 cells were immunoprecipitated with monoclonal anti-x (Feitelson and Clayton, 1990; Feitelson *et al*., 1990b, 1993). The immunoprecipitates

*Correspondence: MA Feitelson, Room 222 Alumni Hall, Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107-6799, USA
Received 1 June 1998; revised 21 September 1998; accepted 6 October 1998

were then analysed by SDS–PAGE and Western blotting using a mixture of X peptide antisera, as described (Feitelson and Clayton, 1990; Feitelson *et al*., 1990a, 1993). The results show that HBxAg was present in lysates from HepG2X, but not from HepG2CAT cells (Figure 1b). When immunoprecipitations were performed with anti-FLAG in the place of anti-x, identical results were obtained (data not shown). Together, these findings show that both of the recombinant retroviruses are expressing the expected products in HepG2 cells.

PCR select cDNA subtraction, verification, and full length cloning of the C2 fragment

Equivalent amounts of poly(A)$^+$ RNA were isolated from confluent cultures of HepG2X and HepG2CAT cells and subjected to PCR select cDNA subtraction according to instructions provided by the manufacturer. Among the ten cDNA fragments generated by subtractive hybridization, which potentially distinguish HepG2X from HepG2CAT cells, two cDNA fragments were present in HepG2CAT cells but absent in HepG2X cells. Given that HBxAg appears to inactivate the tumor suppressor, p53 (Feitelson *et al*., 1993; Wang *et al*., 1994; Truant *et al*., 1995), it was of considerable interest whether the action of HBxAg also resulted in the down-regulated expression of other negative growth regulatory molecules. Hence, the fragment designated as C2, which appeared to be turned off in HepG2X cells, was cloned in pT7Blue(R) and both DNA strands individually sequenced. The cloned PCR fragment was 447 bp long (see underlined sequence in Figure 2). When this sequence was compared to entries in GenBank (Benson *et al*., 1997), the C2 insert showed 91% homology over a 425 bp region with a clone identified as coming from a molecule whose cDNA is highly enriched in human senescent fibroblasts (N95547) (Figure 2). These findings are consistent with the idea that C2 is part of a cDNA that encodes a protein involved in negative growth regulation.

In order to verify that the mRNA containing C2 sequences is differentially expressed in HepG2X compared to HepG2CAT cells, these cells were analysed by *in situ* hybridization (ISH) with a C2 probe. By this approach, the C2 mRNA was easily detected in HepG2CAT cells but not in HepG2X cells

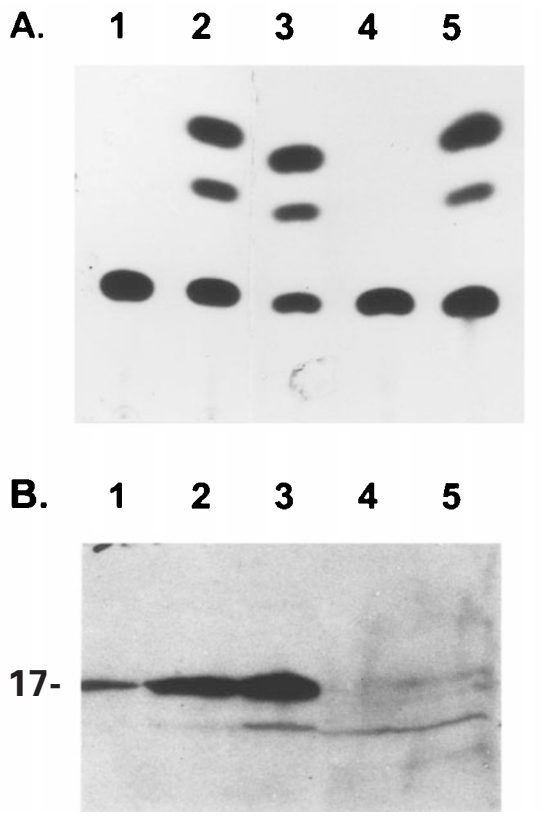

Figure 1 Documentation of HBxAg and CAT expression in recombinant retrovirus infected HepG2 cells. To maximize production of HBxAg and CAT, infected cultures were selected for 14 days in G418, lysed, and scored for CAT activity with ^{14}C-chloramphenicol (**a**) or for p17 HBxAg by Western blot using a well characterized anti-x (**b**). (**a**) shows a CAT assay of HBxAg (lanes 1 and 4) or CAT expressing (lanes 2 and 3) HepG2 cells from two independent experiments, and of CAT expressing PA317 cells (positive control; lane 5). (**b**) shows a Western blot of HBxAg from a bacterial lysate (lane 1) prepared as described (Horiike *et al*., 1991), from HepG2X cells from two independent experiments (lanes 2 and 3), and from HepG2CAT cells from two different experiments (lanes 4 and 5)

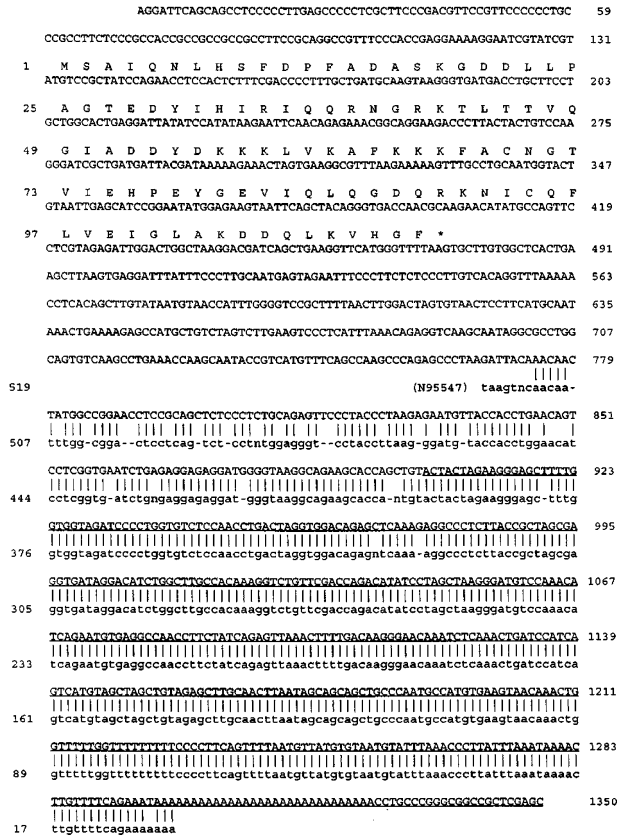

Figure 2 Sequence of the full length cDNA encoding the human homolog of the yeast *sui1* gene product. The hu-Sui1 polypeptide is encoded near the 5′ end of the full length cDNA, and is denoted by single letter amino acid residue abbreviations directly above the corresponding nucleic acid sequence. The translation stop codon is indicated by an asterisk (*) following amino acid residue 113. The hu-*sui1* cDNA clone (denoted by uppercase base abbreviations) is highly homologous to a fragment of a clone from GenBank (N95547; denoted by lowercase base abbreviations) which is overexpressed in sensecent human fibroblasts. The C2 fragment, obtained from PCR select cDNA subtraction, spans bases 903–1350 of full length hu-*sui1* cDNA, and is indicated by an underline in the corresponding portion of the full length clone

(Figure 3a and b, respectively). Parallel experiments using a G3PDH probe yielded positive signals in both cell types, while the use of no probe or an irrelevant probe yielded negative results (data not shown). The differential expression of the mRNA containing the C2 sequences was also assessed by standard Northern blot analysis using 10 μg of whole cell RNA from each cell type. The results show a major band of 1.3 kb in HepG2CAT cells, and a relatively faint faster migrating band, with an apparent mobility of about 1.2 kb, in HepG2X cells (Figure 3c). The reason for the slight differences in the apparent size of the C2 RNA in HepG2X compared to HepG2CAT cells is not clear. However, RNA quantitation by gel scanning revealed that the amount of C2 mRNA in HepG2CAT cells was 9–10-fold higher than in HepG2X cells. Hence, the C2 mRNA is downregulated in the presence of HBxAg.

The ISH and Northern blot analyses verified that the C2 mRNA was differentially expressed in HepG2X and HepG2CAT cells. In order to further study the structure and function of the protein encoded by this mRNA, the full length cDNA (from human placenta) was obtained by RACE PCR, the product cloned into pT7Blue(R) vector, and the insert sequenced. The results (Figure 2) show a full length clone exactly 1.35 kb in length, which encodes a small protein of 113 amino acids near its 5′ end that has 100% homology with the human homolog of the yeast translation initiation factor, hu-Sui1 (Fields and Adams, 1994; Kyrpides and Woese, 1998). Other than its regulatory role in translation initiation, hu-Sui1, does not appear to have any recognizable motifs that would suggest additional functions. The region of the cDNA clone originally found to be highly homologous to the C2 probe turns out to be in the 3′ untranslated half of the cDNA (Figure 2). These results suggest that the introduction of HBxAg results in the altered expression of a protein whose function is associated with the regulation of translation, implying that HBxAg may contribute to hepatocarcinogenesis, in part, by altering gene expression at the level of translation initiation.

*Detection of hu-*sui1 *transcripts in tumor and nontumor cells from HBV infected patients*

The above data suggests that hu-Sui1 may play a role in hepatocarcinogenesis. If this is true, then this protein may be differentially expressed in tumor and adjacent nontumor cells from HBV infected patients with HCC. To test this hypothesis, a panel of tumor/nontumor tissue pairs from HCC patients were analysed by ISH using the C2 probe. Among this group, 14 patients were from South Africa, while the remaining 23 were from mainland China (Table 1). The results show that hu-*sui1* mRNA is easily detectable in nontumor tissue from both groups, but that it is rarely present in tumor tissues from the same patients (Table 2). For example, 13 of 14 South African patients (93%) and 22 of 23 Chinese patients (96%) had detectable hu-*sui1* mRNA by ISH in nontumor cells. In contrast, only one South African (7%) and five Chinese (22%) had detectable hu-*sui1* mRNA by ISH in tumor tissue. Among the Chinese patients with detectable hu-*sui1* mRNA in

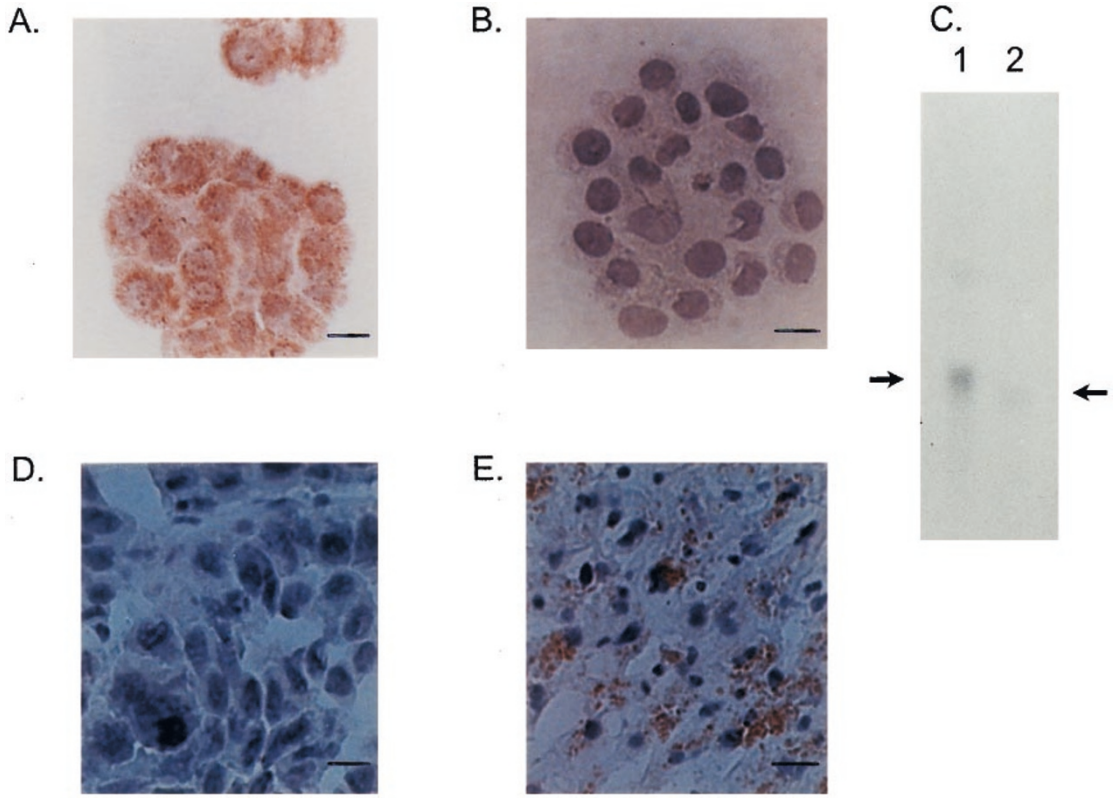

Figure 3 Differential expression of hu-*sui1* sequences in cultured cells and tissues. (**a**) HepG2CAT cells evaluated by ISH. (**b**) HepG2X cells evaluated by ISH. (**c**) Northern blot analysis of HepG2CAT (lane 1) and HepG2X (lane 2) RNA showing bands at 1.3 kb and 1.2 kb, respectively. Fresh frozen sections from tumor (**d**) and surrounding nontumor (**e**) tissues from a representative patient analysed for hu-*sui1* mRNA by ISH. Bars represent 35 microns

Table 1 Characteristics of HCC patients used in this study

Patient group	*African*	*Chinese*
Number tested	14	23
Race	13 black, 1 caucasian	23 Chinese
Gender	14 male, 0 female	17 male, 6 female
Age range:	14–72 years	31–68 years
mean:	39 years	48 years
No. $HBsAg^+$ of total tested	8 of 14 tested (57%)	9 of 23 tested (39%)
No. $HBeAg^+$ of total tested	2 of 13 tested (15%)	14 of 23 tested (61%)
No. anti-HBc^+ of total tested	13 of 14 tested (93%)	19 of 23 tested (83%)
No. anti-HBe^+ of total tested	2 of 13 tested (15%)	10 of 23 tested (43%)
No. anti-HBs^+ of HBs^- tested	5 of 6 HBsAg [−] cases	6 of 14 HbsAg [−] cases

Table 2 Summary of ISH results for the C2 probe in tumor/nontumor pairs for HCC patients from South Africa and China.

South Africa patients																								
case no:	1	2	3	4	5	6	7	8	9	10	11	12	13	14										
tumor	0	0	0	0	2	0	0	0	0	0	0	0	0	0										
nontumor	3	3	3	3	4	3	3	3	2	4	3	2	0	3										
Chinese patients																								
case no:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
tumor	2	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	
nontumor	3	2	4	3	3	4	3	3	4	0	4	4	3	3	3	4	3	3	3	3	4	4	4	

ISH staining is estimated as follows. 0: no signal; 1: ISH signal in $<10\%$ of cells; 2: ISH signal in 10–25% of cells; 3: ISH signal in 25–50% of cells; 4: ISH signal in $>50\%$ of cells

HCC, three of the five had only trace amounts of signal in less than 10% of the tumor cells. Nontumor tissue signals were often more intense and widespread compared to tumor tissue (Figure 3d and e). These patterns were observed in both HBsAg positive and negative patients with HCC from both ethnic groups (Tables 1 and 2). These results are consistent with previous findings of integrated HBV DNA in HCCs from anti-HBs positive patients (Shafritz *et al*., 1981), and of HBxAg in the livers and tumors of HBsAg negative HCC patients (Zhu *et al*., 1993). As outlined above for HepG2 cells, both positive and negative controls gave the expected results in tissue sections (data not shown). The distribution of hu-*sui1* transcripts by ISH was confirmed by immunohistochemical staining using the antibodies prepared herein, which showed a predominant localization of hu-Sui1 in the cytoplasm of nontumor cells (data not shown). This suggests that the ISH positive cells were actually expressing protein. HBxAg had a very similar staining pattern (data not shown), as reported previously (Wang *et al*., 1991a,b). These results support the hypothesis that hu-*sui1* is differentially expressed in tumor compared to nontumor tissue, which is also consistent with its having a role in hepatocarcinogenesis.

Preliminary functional characterization of hu-Sui1

The findings that hu-*sui1* mRNA is overexpressed in senescent human fibroblasts, and that it is downregulated in tumors, suggest that the hu-*sui1* gene may encode a negative regulator of cell growth. To test this hypothesis, full length hu-*sui1* cDNA was subcloned into pcDNA3, and the recombinant plasmid used for transient transfection experiments in HepG2X and HepG2CAT cells. Transfected cells were then prepared for FACS analysis 24 and 48 h later. Preliminary work showed that the transfection frequencies ranged from roughly 30–35% of plated cells (data not shown). The results show that an average of 32.2% of the HepG2CAT cells transfected with pcDNA3-hu-*sui1* were in S phase 48 h posttransfection (Figure 4a), compared to an average of 44.8% of the cells transfected with the pcDNA3 vector ($P<0.01$) (Figure 4b), suggesting that hu-Sui1 significantly inhibits DNA synthesis in HepG2CAT cells. pcDNA3-hu-*sui1* overexpression also depressed DNA synthesis in HepG2X cells. In this experiment, an average of 32.7% of the HepG2X cells transfected with pcDNA3-hu-*sui1* were in S phase 48 h posttransfection (Figure 4c), while an average of 50.8% of the cells transfected with the pcDNA3 vector were in S phase ($P<0.001$) (Figure 4d). These observations suggest that hu-Sui1 is a negative regulator of cell growth.

If hu-Sui1 is a negative growth regulator whose downregulation by HBxAg contributes to the development of HCC, then stable overexpression of hu-Sui1 should alter the clonability of HepG2 cells in soft agar and the development of tumors in nude mice. Accordingly, HepG2 cells were transfected with pcDNA3-hu-*sui1*, pcDNA3-HBx or pcDNA3 and selected in G418 for 4 weeks. Cultures were then lysed and equal amounts of protein analysed for hu-Sui1 by Western blotting using a mixture of peptide antisera raised against hu-Sui1 synthetic peptides (Figure 5a). Cultures overexpressing hu-Sui1 had a single prominent band at 13 kDa (lane 1), which is close to the 12.3 kDa expected based upon the size of the corresponding open reading frame (Figure 2). Cells transfected with pcDNA-HBx showed little or no detectable hu-Sui1 by Western blotting (lane 2), which is consistent with the suppression of this protein in the presence of HBxAg (Figure 3). Endogenous hu-Sui1 was observed in pcDNA3 transfected cells (lane 3). Comparison of the hu-Sui1 levels in lanes 1 and 3 by

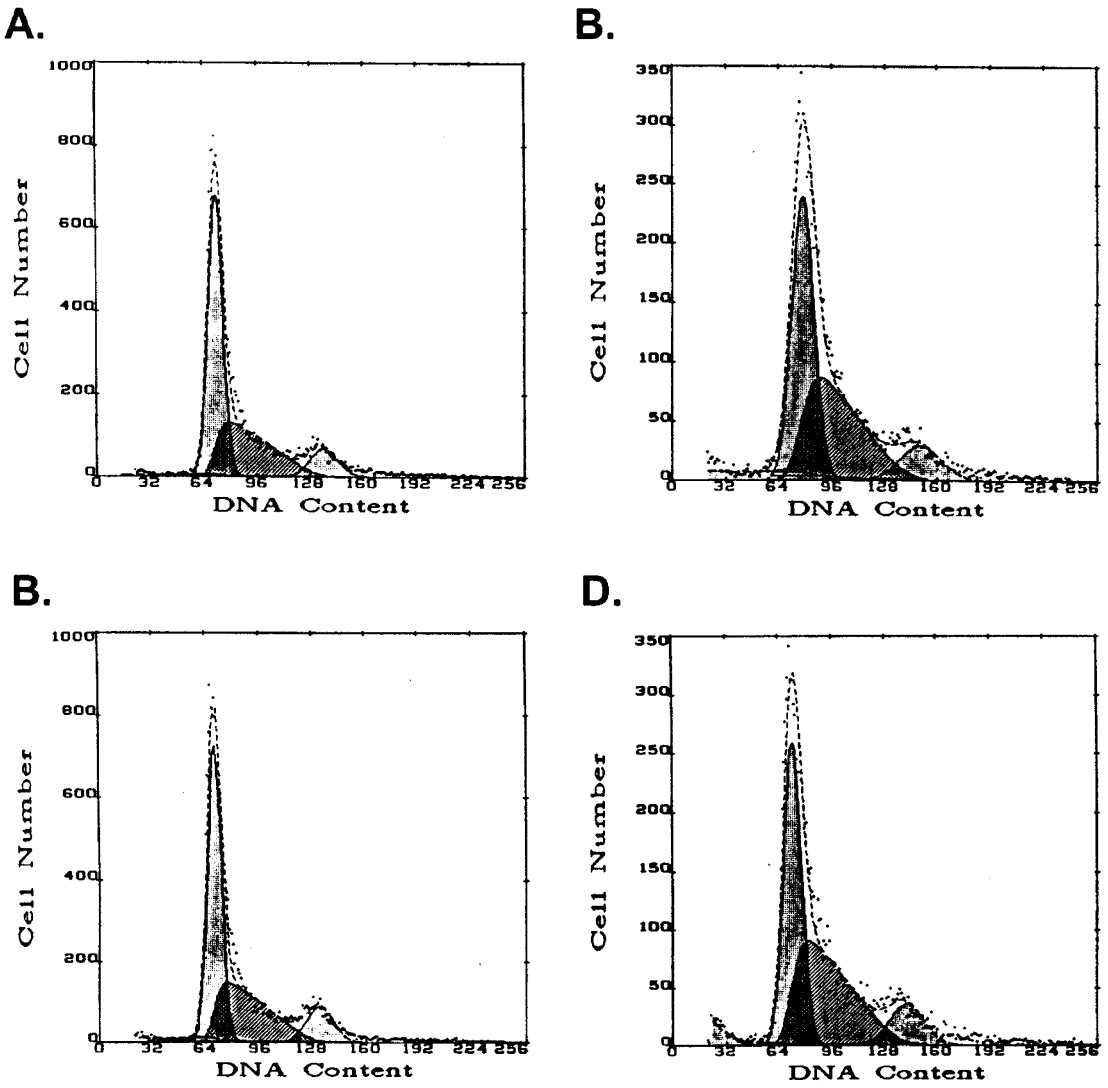

Figure 4 Cell cycle (FACS) analysis of HepG2CAT and HepG2X cells transiently transfected with pcDNA3-hu-*sui1*. Transfection was carried out in triplicate in each of three independent experiments, and the cells evaluated by FACS analysis 48 h posttransfection. HepG2CAT cells transfected with pcDNA3-hu-*sui1* (**a**) or with pcDNA3 (**b**). HepG2X cells transfected with pcDNA3-hu-*sui1* (**c**) or with pcDNA3 (**d**)

scanning showed an increase of about 3–4-fold in cultures overexpressing this antigen.

To test whether hu-Sui1 suppresses anchorage independent growth in soft agar, HepG2 cells were transfected with pcDNA3, pcDNA3-hu-*sui1*, or pcDNA3-HBx and the cultures grown in G418 for 4 weeks. Twenty thousand G418 resistant cells were then seeded into soft agar, and the number of colonies counted after 21 days. Hu-Sui1 overexpressing HepG2 cells yielded an average of 90 ± 28 colonies (Figure 5b) while control HepG2 cells yielded 291 ± 36 colonies ($P < 0.001$) (Figure 5c), suggesting that hu-Sui1 overexpression significantly depressed the growth of HepG2 cells in soft agar. In parallel experiments, HepG2 cells transfected with pcDNA3-HBx also yielded fewer colonies (163 ± 22) compared to HepG2 cells transfected with vector alone ($P < 0.001$) (Figure 5d). These results are consistent with previous observations of HBxAg mediated cell loss (Kim *et al*., 1998; Chirillo *et al*., 1997). pZeoSV-hu-*sui1* was then cotransfected into HepG2 cells with pcDNA3-HBx or a control vector, the cells selected in zeocin plus G418 for 4 weeks, and 2×10^4 of the resulting cells from each culture seeded into soft agar. After another 3 weeks, the hu-Sui1 overexpressing cells making HBxAg yielded an average of 116 ± 11 colonies in soft agar compared to control cells transfected with pZeoSV-hu-*sui1* or pcDNA3-HBx alone ($P > 0.5$) (Figure 5e). These results indicate that hu-Sui1 suppresses the growth of HepG2 cells in soft agar whether or not HBxAg is present, at least under these experimental conditions.

The ability of hu-Sui1 to suppress tumor formation was determined by injecting stably transfected HepG2-pcDNA3-hu-*sui1* cells into nude mice and measuring the number of mice that developed tumors over a 2 month period. The results, summarized in Table 3, show that hu-Sui1 overexpressing HepG2 cells formed tumors in a significantly smaller percentage of injected mice compared to HepG2 cells stably transfected with pcDNA3 ($P = 0.0012$). Although a larger percentage of mice injected with HBxAg positive cells developed tumors compared to animals injected with hu-Sui1 overproducing cells, these differences were not statistically significant ($P > 0.3$). The time of tumor appear-

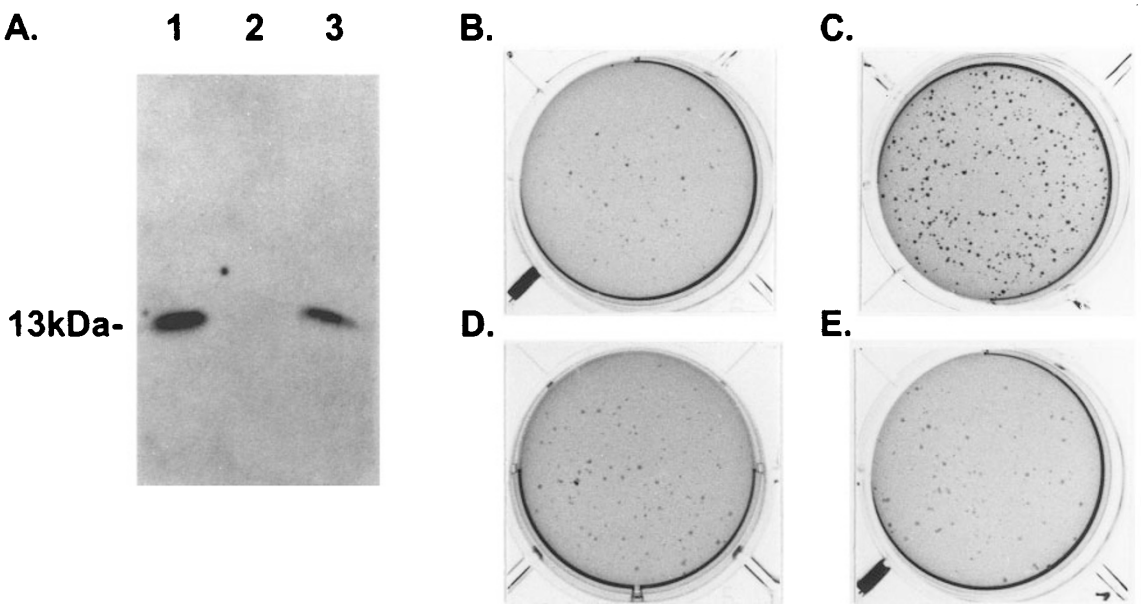

Figure 5 Presence of hu-Sui1 in HepG2 cells and growth of such cells in soft agar. (**a**) Western blot analysis of hu-Sui1 from HepG2 cells stably transfected with pcDNA3-hu-*sui1* (lane 1), with HepG2 cells stably transfected with pcDNA3-HBx (lane 2), and with HepG2 cells stably transfected with pcDNA3 (lane 3). (**b–e**) show the soft agar assay results from hu-Sui1 overproducing HepG2 cells (**b**), from pcDNA3 transfected HepG2 cells (**c**), from pcDNA3-HBx transfected HepG2 cells (**d**), and from HepG2 cells cotransfected with pCDNA3-HBx plus pZeoSV-hu-*sui1* (**e**). The results shown are typical of triplicate experiments

Table 3 Growth of tumors in nude mice injected with HepG2 cells stably transfected with pcDNA3, pcDNA3-hu-*sui1* or pcDNA3-HBx

	No. of mice	*No. (%) of mice with tumors*	*Average diameter of tumors* (cm)	*Size range of tumor* (cm)
HepG2-				
pcDNA3	21	16 (76)	0.94	0.5–1.25
pcDNA-hu-*sui1*	10	2 (20)	0.50	0.50
pcDNA3-HBx	10	5 (50)	0.95	0.5–1.50

ance did not differ among the various groups of animals. Tumors were only discernable at the site of injection in all cases. The histological appearance of the tumors, which ranged from differentiated and trabecular to undifferentiated, was the same among the mice in the various categories analysed (data not shown). When the two tumors from pcDNA3-hu-*sui1* transfected cells were analysed for the presence of pcDNA3-hu-*sui1* sequences by PCR using primers which spanned the vector-insert junction, no signal was observed, suggesting that the tumors no longer expressed exogenous hu-Sui1 (data not shown).

*Relationship between HBxAg associated trans-regulatory function and hu-*sui1 *suppression*

In order to assess whether the suppression of hu-*sui1* mRNA by HBxAg is related to HBxAg *trans*-activation, mutants of HBxAg which were positive or negative for *trans*-activation were made and tested for polypeptide production and stability by *in vitro* translation (data not shown). Recombinant plasmids encoding the mutant HBxAg polypeptides were then stably transfected into HepG2 cells. Total cell RNA from G418 resistant cultures was then isolated, and Northern blot analysis was performed using the probes for hu-*sui1*, HBx, or G3PDH. The results show an inverse correlation between HBxAg *trans*-activation activity and the levels of hu-*sui1* mRNA in HepG2 cells (Figure 6). Hence, the downregulation of hu-*sui1* mRNA in HepG2X compared to HepG2CAT cells appears to be associated with the ability of HBxAg to regulate gene expression at the transcriptional level. These results not only confirm the PCR select cDNA subtraction, where hu-*sui1* mRNA is expressed in predominantly HBxAg negative cells (Figure 3), but also suggest that HBxAg has a *trans*-suppression function which maps to a portion of the *trans*-regulatory domain, and that hu-*sui1* is a natural target of that suppression.

Discussion

HBxAg appears to suppress a gene whose mRNA, or a related mRNA, is overexpressed in senescent human fibroblasts. This result suggests that HBxAg may inhibit the expression of a negative growth regulatory molecule encoded by this transcript. This finding also suggests that HBxAg may possess a *trans*-suppression function which is likely to be relevant to the mechanism whereby X antigen contributes to the development of HCC. The finding that this cellular mRNA encodes the human homolog of the translation initiation factor, Sui1, whose function is to work with eIF-2 to enable the initiator $tRNA^{MET}$ to establish ribosomal recognition of an AUG codon (Yoon and Donahue, 1992), implies that the expression of hu-*sui1* contributes to the regulation of protein translation. In this context, it is proposed that the intrahepatic accumulation of HBxAg during chronic infection (Wang *et al*., 1991a,b) contributes to the development of HCC, in part, by transcriptionally repressing the expression of hu-*sui1*, resulting in altered patterns of host gene expression at the level of translation

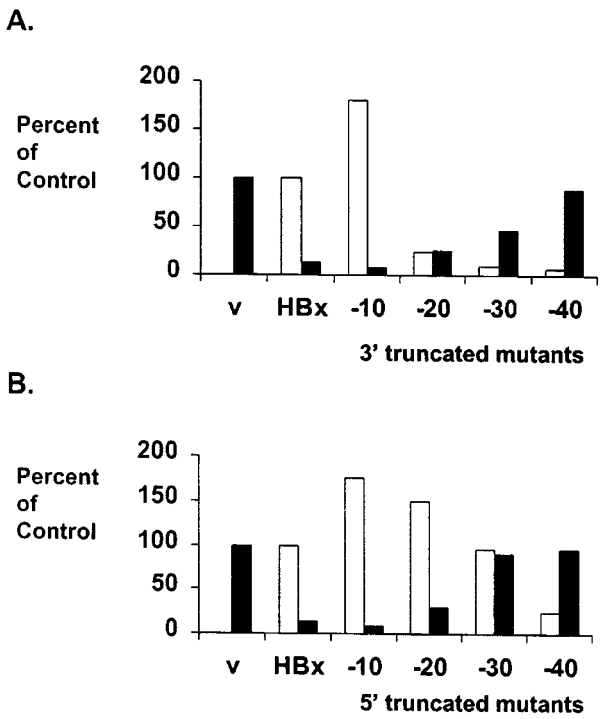

Figure 6 Relationship between HBxAg *trans*-activation function and the levels of hu-*sui1* mRNA. HepG2 cells were transiently transfected with pGL2-HIV-LTR (the reporter plasmid) together with pcDNA3 (vector or v), pcDNA3-HBx (or HBx), or a pcDNA3 construct encoding an HBxAg mutant truncated at its 3′ end by 10, 20, 30 or 40 amino acids (**a**). In (**b**) 5′ truncated mutants were used. Luciferase activity (white bars) was measured 48 h later. All luciferase values were calculated relative to the percent of the control value, which in this case was the luciferase activity stimulated by full length HBxAg (HBx lane). The endogenous hu-*sui1* mRNA value in each transfection (black bars) was scored by Northern blotting and normalized to endogenous G3PDH. Calculations of the RNA levels were then based upon those observed in the control, which in this case was the hu-*sui1* mRNA levels in the vector (v) transfected cultures. All experiments were conducted in triplicate. The extent of experimental variation was within 6%

initiation. The finding that Sui1 also maintains the integrity of translation by inhibiting frame-shifting (Cui *et al*., 1998) implies that the inhibition of hu-*sui1* expression by HBxAg may promote frameshifting within the HBV genome, as suggested earlier (Feitelson, 1986), and in selected host genes whose altered expression contributes to carcinogenesis.

The finding that hu-*sui1* transcription is lower in HepG2X compared to HepG2CAT cells (Figure 3), and that HBxAg stimulates cellular growth (Hohne *et al*., 1990; Koike *et al*., 1994b; Benn and Schneider, 1995), implies that the downregulation of hu-*sui1* promotes the translation of growth stimulatory molecules and/or prevents the translation of proteins that mediate growth suppression. The fact that hu-*sui1* mRNA is easily detected in nontumor tissue but absent from HCC cells (Figure 3, Table 2), implies that its expression is incompatible with the continued cellular growth characteristic of tumors, and that its differential expression is relevant to the pathogenesis of HCC. This implication is strengthened by the findings that hu-Sui1 overexpression inhibits growth in culture (Figure 4), decreases growth in soft agar (Figure 5), and reduces the tumorigenicity of HepG2 cells in nude mice (Table 3). Although these findings support the existence of a potentially novel step in the development of HCC, the regulation of translation initiation has been documented to be important to the pathogenesis of other tumor types. For example, overexpression of eIF-2 and/or eIF-4E have been shown to be associated with transformation in several tissue types (Rosenwald *et al*., 1993; Kerekatte *et al*., 1995; Shantz *et al*., 1996). In this light, the finding that overexpression of hu-*sui1* negatively regulates cell growth, and that the overexpression of eIF-2 and/or eIF-4E are associated with the stimulation of cell growth, implies that the integrity of translation initiation is carefully regulated by the ratio of these factors in the cell. Hence, it is proposed that the sustained intrahepatic expression of HBxAg, combined with its strong nuclear localization in the livers of patients with cirrhosis and dysplasia (Wang *et al*., 1991a), brings about a downregulation in the expression of hu-*sui1*, thereby altering the number and types of mRNAs which get translated. If continued work supports this model, then it would be the first demonstration that the mechanism of DNA virus associated carcinogenesis involves altered patterns of gene expression regulated at the level of translation initiation.

The downregulation of the negative growth regulator hu-Sui1 by HBxAg (Figure 6) is consistent with the previous findings that HBxAg inactivates several other functionally similar molecules in other pathways. For example, HBxAg binds to and functionally inactivates the tumor suppressor and negative growth regulator, p53 (Feitelson *et al*., 1993; Wang *et al*., 1994; Truant *et al*., 1995). p53 regulates the entry of cells into the S phase of the cell cycle at the G1/S restriction point (Kasten *et al*., 1991; Lane, 1992), and also participates in senescence related pathways in the cell (Wright and Shay, 1992; Bond *et al*., 1996; Smith and Pereira-Smith, 1996). The p53 effector, $p21^{WAF1/CIP1/SDI1}$, which negatively regulates the cell cycle by inhibiting G1/S cyclin dependent kinases (Xiong *et al*., 1993; Harper *et al*., 1995), and which is a known senescence factor (Hensler and Pereira-Smith, 1995), is also inactivated by HBxAg (Wang *et al*., 1995). HBxAg also binds to and inactivates another factor overexpressed in human senescent fibroblasts, $p55^{sen}$ (Sun *et al*., 1998). The latter is developmentally regulated, and is closely related to a ligand of the protein Notch, which is involved in the regulation of cell growth and fate during embryogenesis (Artavanis-Tsakonas *et al*., 1995). These combined results suggest that HBxAg contributes to multistep hepatocarcinogenesis by inactivating a series of negative growth regulatory molecules in distinct pathways. They also suggest that the *trans*-suppression of hu-*sui1*, and perhaps other genes by HBxAg, play important roles in the pathogenesis of HCC. These findings are consistent with the lengthy incubation between virus infection and the appearance of tumors (30 – 50 years), since it takes many years for intrahepatic levels of HBxAg to accumulate to inactivate these pathways. These combined results are also consistent with an emerging common denominator whereby HBxAg mediates transformation on the molecular level.

The likelihood that the documented growth stimulatory properties of HBxAg (Benn and Schneider, 1994, 1995) trigger the induction of negative growth regulatory pathways within the cell, provides a mechanism whereby transformation is prevented, even in the presence of HBxAg, for many years or decades after

infection. In the context of multistep carcinogenesis, the HBxAg mediated suppression of hu-Sui1 would be only one step in this process. If this step is not rate limiting, which appears to be the case in HepG2 cells, the triggering of other negative growth regulatory pathways as part of a cellular response to HBxAg would result in the suppression of cell growth, reflected herein as fewer colonies in soft agar (Figure 5d) and fewer tumors in mice (Table 3). This may be due to an HBxAg mediated increase in the rate of apoptosis compared to growth, as suggested earlier (Kim *et al*., 1998; Chirillo *et al*., 1997). It is proposed that during the course of chronic liver disease, increased viral DNA integration results in increased intracellular HBxAg. The latter inactivates a number of key negative growth regulatory pathways, as indicated above, but as long as some are intact, the hepatocytes respond by growth arrest or apoptosis. The former condition supports HBV replication, which is favored in quiescent cells (Ozer *et al*., 1996), while the latter condition prevents untimely cell growth. The increased intracellular concentration of HBxAg may effect DNA repair pathways (Becker *et al*., 1998; Lee *et al*., 1995), resulting in the accumulation of mutations. When the key negative growth regulatory pathways are finally inactivated, the growth stimulatory property of HBxAg becomes the dominant phenotype and autonomously growing foci finally develop. Hence, the inactivation of hu-Sui1 may be a necessary but not sufficient step in the mechanism whereby HBxAg contributes to hepatocellular transformation.

Materials and methods

Cell lines and culture

HepG2 cells, a differentiated cell line derived from a human hepatoblastoma (Aden, *et al*., 1979; Knowles *et al*., 1980), were cultured on type-1 rat tail collagen (Becton Dickinson, Franklin Lakes, NJ, USA) coated tissue culture dishes or plates. Cells were grown in Earle's MEM supplemented with 10% heat inactivated fetal calf serum (FCS), 100 μM MEM non-essential amino acids, 1 mM sodium pyruvate, as well as standard concentrations of penicillin plus streptomycin. The retrovirus packaging cell line PA317 (Danos, 1991) was also grown on plastic dishes in the same medium.

Plasmids

The retroviral vector plasmid, pSLXCMVneo, was used to clone the HBV X gene (Valenzeula *et al*., 1980) or the bacterial chloramphenicol acetyltransferase (CAT) gene sequences for these studies, as described (Vile, 1991; Duan *et al*., 1995). Briefly, pSLXCMV-CAT was constructed by inserting a 726 bp *Hind*III-*Bam*HI fragment containing the CAT gene into the *Hpa*I-*Bgl*I site of the pSLXCMV polylinker. PSLXCMV-FlagX was constructed by inserting a 920 bp *Mlu*I-*Bgl*I fragment of Flag-HBx DNA into the *Mlu*I-*Bgl*II site of the pSLX-CMV polylinker. In both cases, the recombinant proteins were expressed under control of the CMV immediate-early promoter. The rationale for using the FLAG epitope in the HBx containing construct is that it provided an alternative way to detect HBxAg polypeptides in HepG2 cells using anti-FLAG. Additional details of the procedures used for the preparation and use of recombinant retroviruses have been published (Danos, 1991; Vile, 1991; Cepko, 1992). Recombinants were used to transform HB101. Minipreps were prepared and the DNA used for sequence analysis at the DNA sequencing facility of Thomas Jefferson University.

pcDNA3 (Invitrogen, San Diego, CA, USA) was used to clone full length hu-*sui1* cDNA under the control of the immediate early CMV promoter. pcDNA3 was also used for the cloning and characterization of full length and truncated HBxAg polypeptides. Subcloning of full-length and truncated HBxAg polypeptides into p-CITE-3c was conducted in order to verify their production and stability by *in vitro* translation prior to further characterization.

The 525 bp *Kpn*I-*Hind*III fragment from the human immunodeficiency virus-type 1 long terminal repeat (HIV-LTR) was cloned into the pGL2-Basic vector (Promega, Madison, WI, USA) following digestion of the vector with these enzymes, yielding a recombinant plasmid in which the firefly luciferase reporter gene is under control of these HIV-LTR sequences. This recombinant (pGL2-HIV-LTR) was used to test the *trans*-activation properties of HBxAg mutants, and is based upon previous work in which the HIV-LTR is responsive to *trans*-activation by wild type HBxAg (Twu *et al*., 1989).

The hu-*sui1* open reading frame was subcloned into the pZeoSV(+) expression plasmid (Invitrogen, San Diego, CA, USA). Briefly, pcDNA3-hu-*sui1* was digested with *Kpn*1 plus *Xho*1, and the fragment directly inserted into the corresponding sites within the pZeoSV(+) polylinker. pZeoSV carries the Zeocin™ resistance gene that allows selection with zeocin in both mammalian and bacterial cells.

Preparation of recombinant retroviruses and infection of HepG2 cells

Approximately 1×10^6 PA317 cells/100 mm dish were transfected using standard calcium phosphate precipitation using 15 μg of pSLXCMV-FLAGX or 15 μg of pSLXCMV-CAT. At 24, 48, and 72 h after transfection, the medium was removed and processed through a 0.45 μm filter to remove PA317 cells. Filtered supernatants were used immediately for infection of HepG2 cells, as described (Duan *et al*., 1995). Virus was also quantitated by infection of NIH3T3 cells with different dilutions of PA317 cell culture supernatant, followed by G418 selection for 7 days, and then visual inspection of the number of colonies. Five ml of recombinant retrovirus-enriched supernatant was used to infect 1×10^6 target HepG2 cells/100 mm dish in the presence of polybrene (8 μg/ml) for 24 h. Fresh virus supernatant was added after 24 and again after 48 h so that the cells were exposed to HBxAg or CAT producing virus for a total of 72 h. All of these infections were carried out in log phase cultures. HepG2 cultures which received at least 5×10^5 CFU/ml of virus were then passaged at 1 : 2 and selected by incubation in G418 (800 μg/ml; GIBCO/BRL, Grand Island, NY, USA) for 14 days in order to maximize the fraction of cells producing HBxAg or CAT. G418 colonies were then expanded in normal growth medium and used for analysis.

Detection of CAT activity and HBxAg polypeptide in HepG2 cells

CAT assays were performed essentially as described (Wang *et al*., 1994). Briefly, 5×10^6 HepG2CAT cells in a 100 mm dish were lysed by addition of 0.9 ml of 1 × report lysis buffer (Promega, Madison, WI, USA) for 15 min and harvested by scraping. Cells were pelleted and 180 μl of cell lysate was used for a standard CAT assay. After incubation with ^{14}C-chloramphenicol, acetylated forms were separated by thin-layer chromatography. Alternatively, lysates prepared from 5×10^6 HepG2X cells were assayed for the 17 kDa HBxAg by Western blotting using a mixture of rabbit anti-x peptide antibodies, as described (Feitelson and Clayton 1990; Feitelson *et al*., 1990a). Horseradish peroxidase conjugated

goat anti-rabbit Ig (Accurate, Westbury, NY, USA) and ECL substrate (Amersham, Arlington Heights, IL, USA) were used for detection.

PCR select cDNA subtraction, cloning, sequencing and identification of a cDNA from a putative HBxAg effector

The differences in gene expression which distinguish HepG2X from HepG2CAT cells were determined by using a commercially available subtraction hybridization approach (the PCR-select cDNA subtraction kit from Clontech (Palo Alto, CA, USA) according to the instructions provided by the manufacturer. Briefly, whole cell RNA was extracted separately from HepG2X and HepG2CAT cells using the Qiagen RNeasy RNA kit (Qiagen, Inc., Valencia, CA, USA) and the quality of the extraction was determined by assaying for 18S and 28S rRNAs by agarose gel electrophoresis and ethidium bromide staining. PCR-select cDNA subtraction was carried out by reverse transcriptase (RT)–PCR starting with 2 μg of poly(A)$^+$ RNA isolated from the HepG2X and HepG2CAT cell lines. Adaptors were then ligated to a fraction of *Rsa*I digested cDNAs generated by RT–PCR. The cDNAs were subjected to two rounds of subtractive hybridization against the PCR products from the cells in which the comparison was being made. The resulting products, now enriched for differentially expressed genes, were PCR amplified using primers that matched the sequence of the adaptors (in the CLONTECH Advantage cDNA PCR kit). Following agarose gel electrophoresis, the unique fragments were then eluted from the gels (using the QIAGEN gel extraction kit) and cloned into pT7Blue(R) T vector (Novagen, Madison, WI, USA). Positive clones were selected by blue-white phenotype. Recombinant DNAs were isolated from minipreps of individual clones, digested by *Rsa*I to check insert size, and then both strands individually analysed by sequence analysis in the DNA sequencing facility at the Kimmel Cancer Institute of Thomas Jefferson University. The sequences obtained were then compared to those in GenBank using the FASTA command in the GCG software package for homology to known genes (Benson *et al*., 1997).

Patient samples

The HCC and surrounding nontumor liver tissues used for analysis were obtained from two different sets of patients. Twenty three paired tumor/nontumor samples came from as many Chinese patients who had undergone surgery for the removal of their tumors. Most patients lived in and around Xi'an and were treated at the Fourth Military Medical University. Fourteen additional paired tumor/nontumor samples were obtained from South African patients. Half of these were HBV carriers (serum HBsAg positive) while the remaining patients, except for one, had evidence of past HBV infection (detectable anti-HBs and/or anti-HBc). Formalin fixed, paraffin embedded tissues, fresh frozen blocks, and -80°C snap frozen paired liver and tumor samples from individual patients were collected from most patients, used for diagnostic purposes, and were then made available for these studies. Analogous pieces of uninfected human liver from two individuals were available to serve as controls. Additional characteristics of these patients are presented in Table 1.

In situ *hybridization*

The gene fragment obtained from PCR select cDNA subtraction was used as a probe in *in situ* hybridization (ISH) to validate the subtraction hybridization procedure in HepG2X compared to HepG2CAT cells. The probe was then used to screen tumor/nontumor tissue pairs by the same approach to see whether expression was confined to either tumor or untransformed liver cells. In both applications, ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according to the instructions provided by the manufacturer (Oncor, Gaithersburg, MD, USA).

Northern blot analysis

Total cellular RNA was extracted from confluent HepG2CAT and HepG2X cells. Approximately 10 μg of total RNA from each cell line was then analysed by denaturing (formaldehyde) agarose gel electrophoresis. RNA integrity was assessed by visualization of 18S and 28S ribosomal RNA bands after ethidium bromide staining. Northern blot analysis was carried out under stringent hybridization and washing conditions using a ^{32}P-radiolabeled C2 probe made by random priming using the Prime-A-Gene labelling kit (Promega, Madison, WI, USA). Results were detected by autoradiography and quantitated by gel scanning. A probe for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used for normalization of the results.

Cloning of full length C2 cDNA and identification of hu-Sui1 as the encoded product

The full length cDNA clone containing the C2 sequences was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Briefly, one 3′ and one 5′ gene specific primers were synthesized. PCR was performed with human placental cDNA as template using these primers together with an adaptor primer to get the 3′ or 5′ cDNA specific products in separate amplification reactions. The products were cloned into pT7Blue(R) T vector (Novagen, Inc., Madison, WI, USA) and sequenced. The appropriate 3′ and 5′ gene specific fragments were then digested with suitable restriction enzymes and cloned into pcDNA3 (Invitrogen, San Diego, CA, USA) at the chosen site(s), and the integrity of the full length clone verified by DNA sequencing.

Preparation and use of hu-Sui1 antisera

The full length cDNA from hu-*sui1* was used to deduce the corresponding amino acid sequence using the TRANSLATE program. The amino acid sequence was then subjected to analysis in the PEPTIDESTRUCTURE and PLOTSTRUCTURE programs in order to identify hydrophilic peptides likely to be on the surface of hu-Sui1 that would be suitable candidates for solid phase peptide synthesis (Benson *et al*., 1997). Accordingly, peptides spanning inclusive residues 52–69 and 75–94 were made in the Peptide Synthesis Facility at the Kimmel Cancer Center of Thomas Jefferson University. The peptides were coupled to keyhole limpet hemocyanin carrier and used to raise antisera in New Zealand rabbits (2 rabbits/peptide), as described (Feitelson *et al*., 1988). Antisera were initially characterized in specific ELISAs prior to use in Western blotting. Each antiserum was used at a dilution of 1 : 2000, and blotting was conducted by using a mixture of antisera from both specificities. A mixture of these antibodies was also used for immunohistochemistry on slides prepared from formalin fixed paraffin embedded tissues, as described (Wang *et al*., 1991a,b). Preimmune serum and preincubation of primary antibodies with an excess of the corresponding synthetic peptide(s) served as controls (Feitelson and Clayton, 1990; Feitelson *et al*., 1990a).

Construction and characterization of HBxAg mutants

In order to determine if there was a relationship between HBxAg *trans*-activation and hu-*sui1* mRNA levels, a series of HBxAg polypeptide mutants were made. Accordingly, a series of amino- and carboxy-terminal truncation mutants were made, since the HBxAg *trans*-activating function has been mapped to residues within these regions (Kwee *et al*., 1992; Runkel *et al*., 1993; Marakami *et al*., 1994; Takada and Koike, 1994). Using pcDNA3-HBx (or p-CITE-3c-HBx), deletions of 10, 20, 30 or 40 amino acids from either end were generated by PCR. For amino-terminal deletions, primers within the 5′ region of the gene were synthesized to incorporate a new methionine codon at the beginning of each amino-terminal truncated product. For carboxy-terminal deletions, primers within the 3′ end of the X gene were synthesized to incorporate a new translation stop codon at the end of each truncated product. Each primer also had an *Eco*RI recognition site (present only at the very ends of the amplicons) so that the PCR products could be digested and cloned directly back into pcDNA3 (or p-CITE-3c). Verification of each mutant was carried out by DNA sequence analysis. *In vitro* translation was carried out to ascertain whether the mutant polypeptide product from each construct was produced. Unprogrammed reticulocyte lysate was included as a negative control, while wild type HBxAg, which is known to be stable following *in vitro* translation (Feitelson *et al*., 1993; Wang *et al*., 1994; Truant *et al*., 1995), was used as a positive control. Although the pcDNA3 recombinants produced readily detectable proteins upon transfection of HepG2 cells, the pCITE-3c T vector yielded higher levels of *in vitro* translated products, so that the template used for mutagenesis was dependent upon the intended application.

Transfections and FACS analysis

To assess the effect of hu-Sui1 on cell growth, approximately 1×10^5 HepG2X or HepG2CAT cells were seeded into 60 mm diameter plates, allowed to grow overnight, and each plate transiently transfected with 5 μg of pcDNA3-hu-*sui1* using SuperFect (QIAGEN, Valencia, CA , USA). At 24 and 48 h after transfection, cells were recovered, fixed, stained with propidium iodide, and subjected to fluorescence activated cell sorting (FACS) analysis for DNA content in the FACS facility at Thomas Jefferson University. All experiments were conducted in triplicate, and the results evaluated blindly.

In order to stably transfect clones expressing wild type and mutant HBV polypeptides, 1×10^6 HepG2 cells were seeded per 100 mm collagen coated dishes in 10 ml of complete medium (see above), and incubated overnight at 37°C in 5%CO_2. Cultures which were 50–60% confluent were then transfected with 10 μg of the appropriate plasmid using SuperFect transfection reagent (QIAGEN, Valencia, CA, USA) according to the instructions provided by the manufacturer. After a 3 h incubation, dishes were each washed once with 5 ml of PBS. Transfected cells were selected in complete medium containing 800 μg G418 per ml for 4 weeks with several changes of medium until the cultures were confluent. Northern blot analysis of hu-*sui1*, HBx, and G3PDH mRNAs were then conducted as described above. Hu-*sui1* mRNA was detected using the full length (hu-*sui1*) probe. HBx mRNA was detected using the full length X gene (462 bp) excised from pcDNA3-HBx by *Hind*III plus *Kpn*1. G3PDH was obtained by PCR amplification of cDNA prepared from human placenta using specific primers. The PCR product was then gel purified and isolated using the QIAEX II gel extraction kit (QIAGEN, Valencia, CA, USA). All probes were radiolabeled by random priming using [^{32}P]dCTP and then used for hybridization as described above.

Growth of cells in soft agar and in nude mice

HepG2 cells stably transfected with plasmids encoding hu-Sui1 or HBx were tested for anchorage independent growth in soft agar and for tumorigenicity in nude mice. For growth in soft agar, 1×10^4 cells/well were seeded in triplicate into six well plates, and allowed to grow for 21 days. The colonies were then counted under code. For tumorigenicity assays, 1×10^7 cells were injected subcutaneously at a single site in nude mice. The mice were observed over 6 weeks for tumor formation. The mice were then sacrificed and the tumors recovered for further analysis.

Statistics

The relationship of hu-*sui1* gene expression to cell growth in the presence or absence of HBxAg was determined by calculating the significance level, *P*, using the Student's *t*-test.

Acknowledgments
This work was supported by NIH grants CA48656 and CA66971 to MF. EMBL Data Base accession number L26247.

References

Aden DP, Fogel A, Plotkin S, Damjanov I and Knowles BB. (1979). *Nature,* **282,** 615–617.
Artavanis-Tsakonas S, Matsuno K and Fortini ME. (1995). *Science,* **268,** 225–232.
Autunovic J, Lemieux N and Cromlish JA. (1993). *Cell. Mol. Biol. Res.,* **39,** 463–482.
Beasley RP and Hwang LY. (1984). In: Vyas GN, Dienstag JL and Hoofnagle JH (eds). *Viral Hepatitis and Liver Disease*. Grune and Stratton, Inc.: New York, pp. 209–224.
Becker SA, Lee TH, Butel JS and Slagle BL. (1998). *J. Virol.,* **72,** 266–272.
Benn J and Schneider RJ. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 10350–10354.
Benn J and Schneider RJ. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 11215–11219.
Benson DA, Boguski MS, Lopman DJ and Ostell J. (1997). *Nuc. Acids Res.,* **25,** 1–6.
Bond J, Haughton M, Blaydes J, Gire V, Wynford-Thomas D and Wyllie F. (1996). *Oncogene,* **13,** 2097–2104.
Cepko C. (1992). Current protocols in molecular microbiology. Section III: In: Ausubel FM (ed.) *Transduction of genes using retrovirus vectors*. Current Protocols, 9.10.1–9.10.7.
Chen JY, Harrison TJ, Lee CS, Chen DS and Zuckerman AJ. (1988). *Hepatology,* **8,** 518–523.
Cheong JH, Yi MK, Lin Y and Murakami S. (1995). *EMBO J.,* **14,** 143–150.
Chirillo P, Pagano S, Natoli G, Puri PL, Burgio VL, Balsano C and Levrero M. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 8162–8167.
Cui Y, Dinman JD, Kinzy TG and Peltz SW. (1998). *Mol. Cell. Biol.,* **18,** 1506–1516.
Danos O. (1991). Methods in molecular biology, Practical molecular virology: In: Collins, M (ed.). *Viral vectors for gene expression*. Human Press, Inc.: Clifton, NJ, 8, pp. 17–27.
Duan LX, Zhu M, Bagasra O and Pomerantz RJ. (1995). *Human Gene Ther.,* **6,** 561–1573.

Feitelson MA. (1986). *Hepatology,* **6,** 191 – 198.
Feitelson MA, Millman I, Duncan GD and Blumberg BS. (1988). *J. Med. Virol.,* **24,** 121 – 136.
Feitelson MA and Clayton MM. (1990). *Virology,* **177,** 367 – 371.
Feitelson MA, Clayton MM and Blumberg BS. (1990a). *Gastroenterology,* **98,** 1071 – 1078.
Feitelson MA, Clayton MM and Phimister B. (1990b). *Virology,* **177,** 357 – 366.
Feitelson MA, Zhu M, Duan LX and London WT. (1993). *Oncogene,* **8,** 1109 – 1117, 1993.
Fields C and Adams MD. (1994). *Biochem. Biophys. Res. Commun.,* **198,** 288 – 291.
Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, Fox MP and Wei N. (1995). *Mol. Biol. Cell.,* **6,** 387 – 400.
Haviv I, Vaizel D and Shaul Y. (1996). *EMBO J.,* **15,** 3413 – 3420.
Henkler F and Koshy R. (1996). *J. Viral. Hepatitis,* **3,** 109 – 121.
Hensler PJ and Pereira-Smith OM. (1995). *Am. J. Pathol.,* **147,** 1 – 8.
Hohne M, Schaefer S, Seifer M, Feitelson MA, Paul D and Gerlich WH. (1990). *EMBO J.,* **9,** 1137 – 1145.
Horiike N, Blumberg BS and Feitelson MA. (1991). *J. Infect. Dis.,* **164,** 1104 – 1112.
Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW. (1991). *Cancer Res.,* **51,** 6304 – 6311.
Kekule AS, Lauer U, Weiss L, Luber B and Hofschneider PH. (1993). *Nature,* **361,** 742 – 745.
Kerekatte V, Smiley K, Hu B, Smith A, Gelder F and De Benedetti A. (1995). *Int. J. Cancer,* **64,** 27 – 31.
Kim CM, Koike K, Saito I, Miyamura T and Jay G. (1991). *Nature,* **351,** 317 – 320.
Kim H, Lee H and Yun Y. (1998). *J. Biol. Chem.,* **273,** 381 – 385.
Knowles BB, Howe C and Aden DP. (1980). *Science,* **209,** 497 – 499.
Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T and Kurokawa K. (1994a). *Hepatology,* **19,** 810 – 819.
Koike K, Moriya K, Yotsuyanagi H, Iino S and Kurokawa K. (1994b). *J. Clin. Invest.,* **94,** 44 – 49.
Kwee L, Lucito R, Aufiero B and Schneider RJ. (1992). *J. Virol.,* **66,** 4382 – 4389.
Kyrpides NC and Woese CR. (1998). *Proc. Natl. Acad. Sci. USA.,* **95,** 224 – 228.
Lane DP. (1992). *Nature,* **358,** 15 – 16.
Lee TH, Elledge SJ and Butel JS. (1995). *J. Virol.,* **69,** 1107 – 1114.
Luber BL, Arnold N, Sturzl M, Hohne M, Schirmacher P, Lauer U, Wienberg J, Hofschneider PH and Kekule AS. (1996). *Oncogene,* **12,** 1597 – 1608.
Maguire HF, Hoeffler JP and Siddiqui A. (1991). *Science,* **252,** 842 – 844.
Marakami S, Cheong JH and Kaneko S. (1994). *J. Biol. Chem.,* **269,** 15118 – 15123.
Matsubara K and Tokino T. (1990). *Mol. Biol. Med.,* **7,** 243 – 260.
Natoli G, Avantaggiati ML, Chirillo P, Costanzo A, Artini M, Balsano C and Levrero M. (1994). *Mol. Cell. Biol.,* **14,** 989 – 998.
Ozer A, Khaoustov VI, Mearns M, Lewis DE, Genta RM, Darlington GJ and Yoffe B. (1996). *Gastroenterology,* **110,** 1519 – 1528.
Qadri I, Maguire HF and Siddiqui A. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 1003 – 1007.
Robinson WS, Marion P, Feitelson MA and Siddiqui A. (1982). In: Szmuness W, Alter HJ and Maynard JE (eds). *Viral Hepatitis, 1981 International Symposium*. Franklin Institute Press: Philadelphia, pp. 57 – 68.
Rosenwald IB, Rhoads DB, Callanan LD, Isselbacher KJ and Schmidt EV. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 6175 – 6178.
Rossner MT. (1992). *J. Med. Virol.,* **36,** 101 – 117.
Runkel L, Fischer M and Schaller H. (1993). *Virology,* **197,** 529 – 536.
Seeger C, Baldwin B, Hornbuckle WE, Yeager AE, Tennant BC, Cote P, Ferrell L, Ganem D and Varmus HE. (1991). *J. Virol.,* **65,** 1673 – 1679.
Seifer M, Hohne M, Schaefer and Gerlich WH. (1991). *J. Hepatol.,* **13**(Suppl 4), S61 – S65.
Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ and Kew MC. (1981). *N. Engl. J. Med.,* **305,** 1067 – 1073.
Shantz LM, Coleman CS and Pegg AE. (1996). *Cancer Res.,* **56,** 5136 – 5140.
Smith JR and Pereira-Smith OM. (1996). *Science,* **273,** 63 – 66.
Su F and Schneider RJ. (1996). *J. Virol.,* **70,** 4558 – 4566.
Sun BS, Zhu X, Clayton MM and Feitelson MA. (1998). *Hepatology,* **27,** 228 – 239.
Szmuness W. (1978). *Prog. Med. Virol.,* **24,** 40 – 69.
Takada S and Koike K. (1990). *Proc. Natl. Acad. Sci. USA,* **87,** 5628 – 5632.
Takada S and Koike K. (1994). *Virology,* **205,** 503 – 510.
Takada S, Tsuchida N, Kobayashi M and Koike K. (1995). *J. Cancer Res. Clin. Oncol.,* **121,** 593 – 601.
Truant R, Antunovic J, Greenblatt J, Prives C and Cromlish JA. (1995). *J. Virol.,* **69,** 1851 – 1859.
Twu JS and Schloemer RH. (1987). *J. Virol.,* **61,** 3448 – 3453.
Twu JS, Rosen CA, Haseltine WA and Robinson WS. (1989). *J. Virol.,* **63,** 2857 – 2860.
Ueda H, Ullrich SJ, Gangemi JD, Kappel CA, Ngo L, Feitelson MA and Jay G. (1995). *Nature Genetics,* **9,** 41 – 47.
Unsal H, Yakicier C, Marcais C, Kew M, Volkmann M, Zentgraf H, Isselbacher KJ and Ozturk M. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 822 – 826.
Valenzuela P, Quironga M, Zaldivar J, Gray P and Rutter WJ. (1980). In: *Animal Virus Genetics*. Academic Press, Inc.: NY, pp. 57 – 70.
Vile R. (1991). Methods in molecular biology, Practical molecular virology: In: Collins, M (ed.). *Viral vectors for gene expression*. Human Press, Inc.: Clifton, NJ, 8, pp. 1 – 15.
Wang W, London WT, Lega L and Feitelson MA. (1991a). *Hepatology,* **14,** 29 – 37.
Wang W, London WT and Feitelson MA. (1991b). *Cancer Res.,* **51,** 4971 – 4977.
Wang XW, Forrester K, Yeh H, Feitelson MA, Gu J-R and Harris CC. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 2230 – 2234.
Wang XW, Gibson MK, Vermeulen W, Yeh H, Forrester K, Sturzbecher HW, Hoeijmakers JHJ and Harris CC. (1995). *Cancer Res.,* **55,** 6012 – 6016.
Wollersheim M, Debelka U and Hofschneider PH. (1988). *Oncogene,* **3,** 545 – 552.
Wright WE and Shay JW. (1992). *Exp. Gerontol.,* **27,** 383 – 389.
Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and Beach D. (1993). *Nature,* **366,** 701 – 704.
Yoon H and Donahue TF. (1992). *Mol. Cell. Biol.,* **12,** 248 – 260.
Zahm P, Hofschneider PH and Koshy R. (1988). *Oncogene,* **3,** 169 – 177.
Zhou YZ, Butel JS, Li PJ, Finegold MJ and Melnick JL. (1987). *J. Natl. Cancer Inst.,* **79,** 223 – 231.
Zhu M, London WT, Duan LX and Feitelson MA. (1993). *Int. J. Cancer,* **55,** 571 – 576.