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Activated *ras* oncogene collaborates with HBx gene of hepatitis B virus to transform cells by suppressing HBx-mediated apoptosis

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The hepatitis B virus HBx protein is a promiscuous transactivator implicated in the development of hepatocellular carcinoma. The ectopic expression of HBx fails to transform both primary and immortalized rodent cells, but rather induces apoptosis. Furthermore, most transgenic mice harboring HBx do not develop liver tumors. Thus, it remains unclear whether and how HBx contributes to oncogenesis. Here, we show that HBx collaborates with activated H-*ras* to transform immortalized rodent cells. Indeed, REF52 cells transfected by both HBx and activated H-*ras* were morphologically transformed and were able to grow in soft agar. Remarkably, nude mice injected with REF52 cells transfected by both HBx and activated H-ras developed tumors, whereas the mice injected with REF52 cells transfected by either gene alone did not. Thus, we concluded that HBx could contribute to neoplastic transformation of cells in collaboration with other oncogenes, such as H-*ras*, that renders cells to overcome the HBx-mediated apoptosis. Further, we found that HBx mediated apoptosis was suppressed by activated H-*ras* through activation of the phosphatidylinositol-3 kinase and Akt pathway. Data presented here firmly established the oncogenic potential of HBx during multistage carcinogenesis. *Oncogene* (2001) **20,** 16–23.

Keywords: hepatitis B virus; X gene; apoptosis; H-*ras* oncogene

Introduction

Hepadnaviruses is a family of small enveloped DNA viruses characterized by their pronounced liver tropism (Ganem and Varmus, 1987). Human hepatitis B virus (HBV), a prototype member of the family, is a leading cause of acute and chronic viral hepatitis in humans worldwide. Earlier epidemiological evidence has indicated that chronic HBV infection is the major etiological factor of the hepatocellular carcinoma (HCC) (Beasley *et al*., 1981). Despite the compelling epidemiological evidence, to what extent the viral gene products contribute to hepatocarcinogenesis is still in question. Animal models of hepadnaviruses including woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) have been invaluable to further substantiate the link between HBV infection and HCC (Buendia, 1992). It was noted that three mammalian hepadnaviruses share a regulatory gene termed X, whereas no counterpart of this gene is found in the distantly related nononcogenic duck hepatitis B virus (DHBV) (Ganem and Varmus, 1987). The HBx, a 154 amino acid gene product of the X gene, has been known as a promiscuous transcriptional transactivator since it is capable of transactivating gene expression by acting on a wide range of viral and cellular regulatory elements (Yen, 1996). This led to suggest that HBx might play a role in liver carcinogenesis. Until recently, its role during the infection cycle remained elusive, since HBx has been shown to be dispensable for viral genome replication in tissue culture (Blum *et al*., 1992). Subsequently, the requirement of HBx gene product for productive viral infection has been firmly established in the woodchuck model (Chen *et al*., 1993; Zoulim *et al*., 1994). Thus, HBx is an essential viral protein during the viral infection cycle *in vivo*, although its specific function pertaining to the infection cycle still remains unknown.

On the other hand, transcriptional transactivator properties of HBx have been demonstrated on a variety of viral and cellular regulatory elements (Yen, 1996). HBx does not directly bind to DNA and may stimulate transcription by interacting with transcription factors or with the basal transcription machinery of host RNA polymerase II and III (Cheong *et al*., 1995; Haviv *et al*., 1998; Maguire *et al*., 1991; Qadri *et al*., 1996; Williams and Andrisani, 1995). Besides its nuclear function as a transcriptional transactivator, several studies indicated that HBx influences cellular signaling pathways in the cytoplasm as well, which is a function consistent with the predominant cytoplasmic localization of HBx *in vivo* and in most experimental systems (Benn and Schneider, 1994; Natoli *et al*., 1994). It has been shown that HBx activates *ras in vivo*, inducing a cytoplasmic signaling cascade linking *ras*, *raf*, and MAP kinases, which leads to transcriptional transactivation and the stimulation of proliferation in quiescent cells (Benn and Schneider, 1994; Natoli *et al*., 1994). On the other hand, it was reported that HBx either

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induces apoptosis or sensitizes cells to apoptotic stimuli (Chirillo *et al*., 1997; Kim *et al*., 1998; Su and Schneider, 1997). In addition, HBx has been shown to activate transcriptional factors NF-kB (Chirillo *et al*., 1996; Lucito and Schneider, 1992; Su and Schneider, 1996), and *src* oncogene (Klein and Schneider, 1997). More recently, HBx was shown to activate Jak/stat signaling as well (Lee and Yun, 1998). Regarding its cellular location, dual role of HBx has been demonstrated, that is, a nuclear function as a transcriptional transactivator as well as a cytoplasmic function as an activator of mitogenic signaling (Doria *et al*., 1995). Thus, HBx has pleiotropic activities that might involve in viral pathogenesis and carcinogenesis.

Numerous attempts have been made to examine the oncogenic potential of HBx in cell culture (Hohne *et al*., 1990; Oguey *et al*., 1996; Shirakata *et al*., 1989; Tarn *et al*., 1999). However, its transforming ability was barely measurable only when cells were immortalized by other oncogenes, such as SV40 T-antigen (Hohne *et al*., 1990) or TGF-α (Oguey *et al*., 1996; Tarn *et al*., 1999). Further, most transgenic mouse harboring the HBx gene did not result in serious liver diseases or tumors (Billet *et al*., 1995; Guidotti *et al*., 1995; Koike *et al*., 1994a; Lee *et al*., 1990). Only in certain transgenic lineage of CD-1 strain, HBx weakly promoted tumorigenesis, where HBx was expressed at high levels (Kim *et al*., 1991). Nonetheless, a second mouse lineage with lower HBx expression developed liver tumors at the same rate as the normal CD-1 mouse (Koike *et al*., 1994a). In addition, HBx was shown to potentiate c-*myc*-induced liver oncogenesis in transgenic mice (Terradillos *et al*., 1997). Taken together, data obtained from these transgenic studies suggested that HBx had no acute transforming activity, but its overexpression in a certain genetic background might induce tumor formation, possibly in collaboration with activated cellular oncogene(s) in multistage transformation. Although it is generally believed that HBx is a cofactor in hepatocarcinogenesis (Dandri *et al*., 1996; Paterlini *et al*., 1995; Su *et al*., 1998), direct evidence for oncogenic contribution of HBx is still lacking.

To examine the oncogenic potential of the HBx protein, we tested whether HBx collaborates with other oncogenes to transform cells using several assay methods including colony formation assay, morphological change, anchorage independent growth in soft agar, and tumor formation in nude mouse. In contrast to a previous report (Kim *et al*., 1998; Schuster *et al*., 2000), significant collaboration between HBx and activated H-*ras* oncogene was observed. Our observation that HBx mediated apoptosis was suppressed by activated H-*ras* underlies the molecular basis of the collaboration between HBx and H-*ras*. Importantly, we demonstrated that REF52 cells transfected by both HBx and H-*ras* were morphologically transformed and had the ability to grow on soft agar. Remarkably, nude mice injected with HBx and H-*ras* cotransfected REF52 cells developed tumors, whereas the mice injected with REF52 cells transfected by either gene alone did not. These data demonstrating the oncogenic activity of HBx define its role during the multistage carcinogenesis.

Results

*HBx gene collaborates with H-*ras *to transform cells*

To investigate the role of HBx in oncogenesis, we examined the transforming ability of HBx by colony formation assay. Rat2 cells were transfected by various combinations of HBx and other oncogenes and selected for G418 resistance (Table 1). As expected, cotransfection of the *ras* and *myc* gene in Rat2 cells resulted in a higher number of colonies than when H-*ras* was transfected alone. On the other hand, consistent with other reports (Chirillo *et al*., 1997; Kim *et al*., 1998), only a few colonies were observed when cells were transfected with the HBx expression plasmid alone. This number is even smaller than that resulting from the pSV2neo control, suggesting that HBx might have induced apoptosis. Interestingly, cotransfection of HBx and H-*ras* expression plasmids yielded significantly higher number of colonies than either HBx or H-*ras*

Table 1 Colony formation assay of Rat2 and NIH3T3 cells

	Number of colonies[a]			
	Exp. 1		*Exp. 2*	
Transfected genes (1 μg)	*Rat2*	*NIH3T3*	*Rat2*	*NIH3T3*
Neo only	nd[b]	nd	27	12
HBx	10	1	2	2
H-*ras*	28	25	25	47
H-*ras* + HBx	49	47	74	39
H-*ras* + myc	48	53	43	45

[a]The number of colonies formed (>0.2 mm in diameter) was counted 2–3 weeks after transfection. [b]nd, not determined

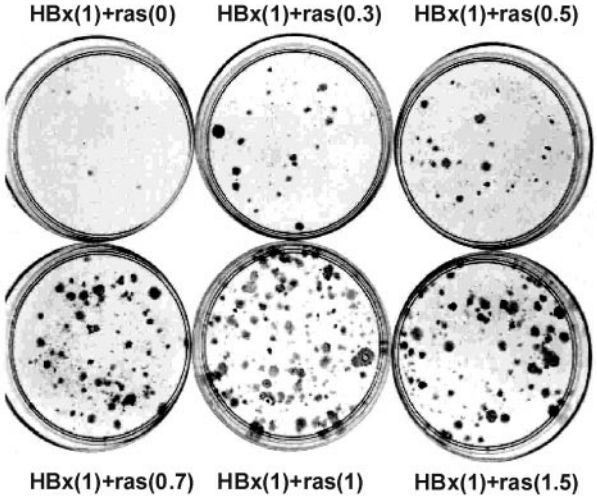
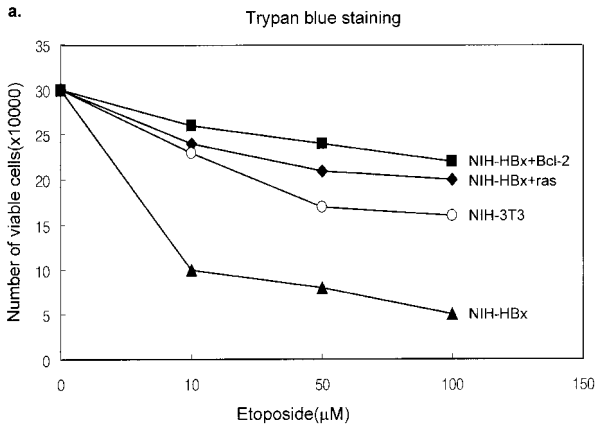
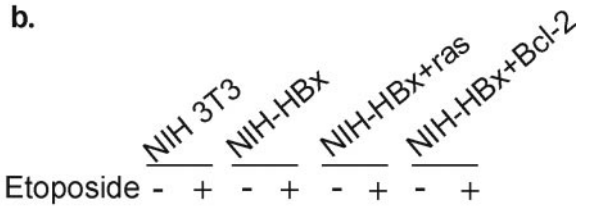
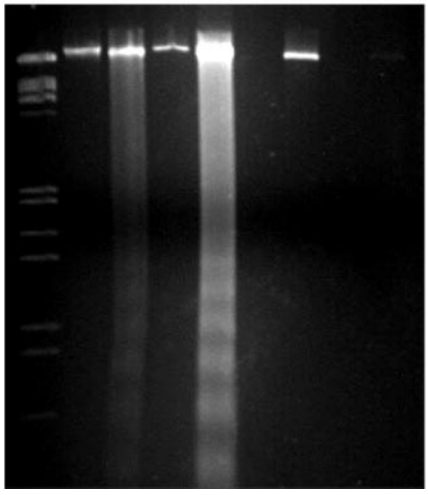
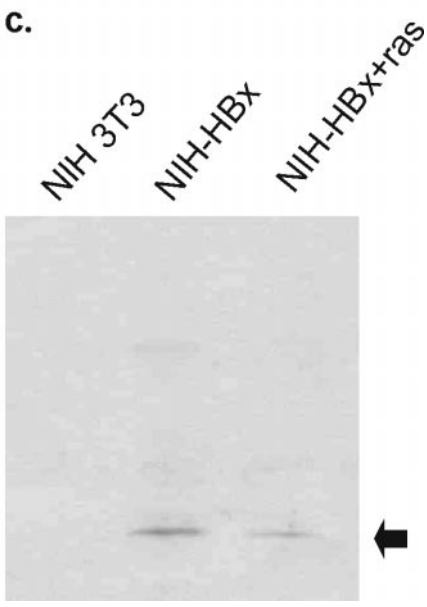


Figure 1 Colony formation assay in NIH3T3 cells. The result from a typical experiment is shown. The amount of plasmid DNA transfected is indicted in parentheses. HBx, pSV-X(SV40 early promoter); H-ras, pEJ 6.6 (H-*ras*)









alone did, comparable to that with H-*ras* plus *myc* (Table 1). Thus, it appeared that HBx significantly collaborated with the activated H-*ras* to transform Rat2 cells. Similar results were obtained with NIH3T3 cells (Figure 1 and Table 1). Our result is not consistent with a previous observation that the ectopic expression of HBx using the CMV promoter suppressed colony formation by other oncogenes including H-*ras* (Kim *et al*., 1998). Our own experiment using CMV promoter also agreed with the previous report (data not shown). On the other hand, in our experiment using the moderate SV40 promoter to express HBx, we observed a measurable collaboration between HBx and H-*ras* (Table 1). Given that HBx is present in hepatocyte of infected animal at a very low copy number (less than 40 000–80 000 molecules per cell) (Dandri *et al*., 1996), an experiment using a moderate SV40 promoter is more likely to reflect the physiological concentration of HBx in hepatocytes. Thus, subsequent experiments were carried out using the SV40 promoter to express the HBx protein.

H-ras *oncogene suppresses HBx mediated apoptosis*

Since HBx can induce apoptosis upon various stimuli (Chirillo *et al*., 1997; Kim *et al*., 1998; Su and Schneider, 1997), it is possible that the HBx-induced apoptosis was suppressed by the cotransfected H-*ras*. To substantiate this notion, apoptosis assays were performed with cells derived from the above foci. Cell death was measured either by trypan blue staining for viable cells or by agarose gel electrophoresis analysis for DNA fragmentation (Figure 2). When cells were treated with a lower concentration of etoposide (10 μM), cell viability of parental NIH3T3 cells were not affected. In contrast, cell death was evident in the NIH-HBx cells (Figure 2a). On the other hand, cell viability was reduced even in parental NIH3T3 cells if cells were treated with a higher concentration of etoposide (100 μM). The result indicated that HBx sensitized cells to apoptosis upon DNA damage. Interestingly, we found that cell viability were not significantly reduced in NIH3T3 cells transfected with HBx plus H-*ras*, even when cells were treated with a

Figure 2 Evidence that the ectopic expression of H-*ras* inhibits HBx-mediated apoptosis. (**a**) Viable cell counts after trypan blue staining. Cells were plated in DMEM containing 5% FBS at a density of 3×10^5 cells/60 mm plate: NIH3T3(○), NIH-HBx(▲), NIH-HBx + ras(◆), NIH-HBx + Bcl-2(■). At 24 h, the cells were treated with the indicated concentration of etoposide. After another 24 h of incubation, the cells were stained in PBS containing 0.04% trypan blue solution and counted with a hemacytometer. (**b**) Analysis of DNA fragmentation after etoposide treatment. Cells were plated in DMEM containing 5% FBS at a density of 1×10^6 cells per 100 mm plate. Next day, the cells were treated with 10 μM of etoposide for an additional 1 day. Both adherent and floating cells were collected and DNAs were prepared and subjected to electrophoresis in 1.4% agarose gels. (**c**) Western blot analysis of HBx protein. HBx protein was detected by immunoblotting with rabbit polyclonal anti-HBx peptide (residue 144–154). HBx protein, 17 kDa, is indicated by an arrow

higher concentration of etoposide (100 μM). It is noted that the extent of protection against the etoposide-induced apoptosis by H-*ras* was comparable to that by Bcl-2 (Figure 2a). In addition, DNA fragmentation analysis results were consistent with those of viable cell counts (Figure 2b). Likewise, upon serum starvation, apoptosis was observed in the NIH-HBx cells, but not in the NIH-HBx plus H-*ras* cells (data not shown). These results are consistent with the notion that the HBx-mediated apoptosis was suppressed by H-*ras*. In addition, Western blot analysis indicated that similar levels of HBx protein were expressed in both HBx transfected cells (Figure 2c). Thus, the possibility that HBx expression in NIH-HBx plus H-*ras* cells was suppressed by the cotransfected H-*ras* was excluded.

H-ras *oncogene suppresses HBx mediated apoptosis through PI(3)-Akt pathway*

Next, we sought to examine the signal transduction pathway involved in the *ras*-mediated suppression of the apoptosis. Recently, it has been documented that the activated Akt oncogene suppresses apoptosis by phosphorylating Bad (Datta *et al*., 1997), a pro-apoptotic member of the Bcl-2 family. In addition, it was reported that the activation of Akt, induced by *ras*, blocks apoptosis by phosphorylating caspase-9 (Cardone *et al*., 1998). Since the activated *ras* can activate Akt via PI(3) kinase (Datta *et al*., 1996), we examined whether the *ras* might suppress the HBx-mediated apoptosis via Akt activation. To this end, NIH3T3 cells were transiently transfected by various expression plasmids as indicated, along with pcDNA3.1-lacZ plasmid (Figure 3a). Two days after transfection, apoptotic cells with dense nuclear staining were counted as described (Miura *et al*., 1993). As shown in in Figure 3a, it was found that approximately 12% of transfected cells were subjected to apoptosis by HBx transfection. On the other hand, the ectopic expression of H-*ras* suppressed the HBx-mediated apoptosis significantly. When a dominant negative mutant of Akt (Akt-dn, which is a kinase dead mutant) was cotransfected into cells along with HBx and the *ras*, the number of apoptotic cells was increased to a level similar to that in HBx only transfected cells. This result suggested that the suppression of the HBx-mediated apoptosis by H-*ras* was inhibited by Akt inactivation. In contrast, cotransfection of the Akt-dn with HBx did not increase apoptosis relative to that of HBx alone. In addition, H-*ras* alone did not induce significant level of apoptosis, whereas cotransfection of the Akt-dn with H-*ras* did increase apoptosis. Thus, it seems that anti-apoptotic signal triggered by H-*ras* was suppressed by the Akt-dn.

Similarly, we next examined the involvement of the PI(3)-Akt pathway in the suppression of the apoptosis by treatment of cells with Wortmannin, a potent inhibitor of PI(3) kinase. Consistent with the above result, Wortmannin treatment suppressed the protective effect of activated H-*ras*, as Wortmannin treatment

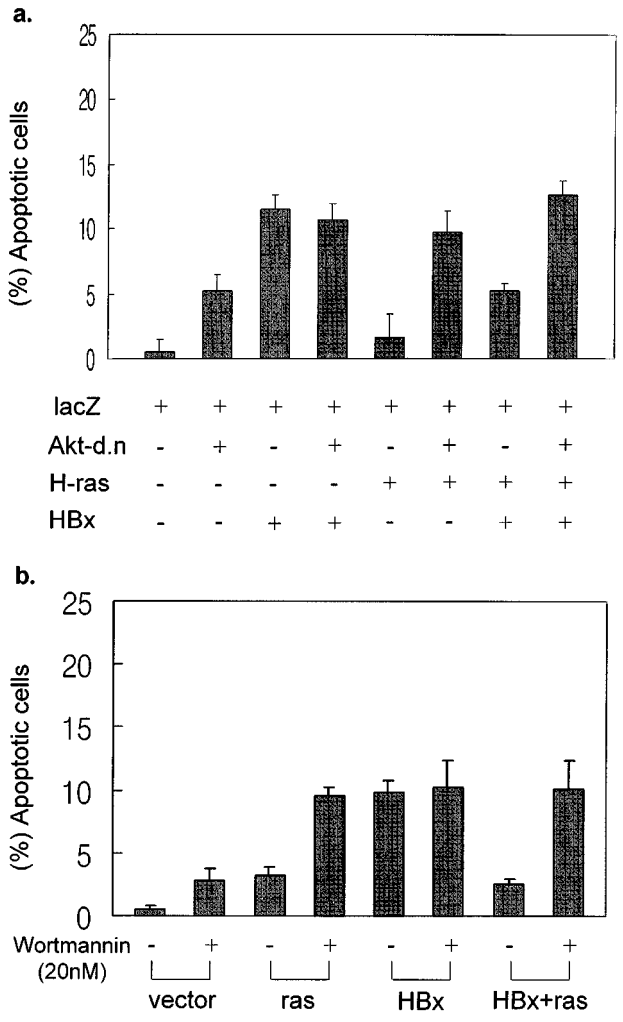


Figure 3 Evidence that the activation of Akt is involved in the inhibition of HBx induced apoptosis by H-*ras*. (**a**) Transient transfection assay to examine the effect of Akt-dn on the apoptosis. NIH3T3 cells were transiently transfected with 0.3 μg of pcDNA3.1-lacZ (Invitrogen) plus various combinations of the indicated plasmids: 0.7 μg each of pSV-X, pEJ 6.6 (H-*ras*), or pcDNA-Akt-dn. Akt-dn is a dominant negative mutant of Akt in which its kinase domain is mutated. Two days after transfection, apoptotic cells with dense nuclear staining were counted. (**b**) Transient transfection assay to examine the effect of Wortmannin on the apoptosis. Transfection was done as in panel (**a**), but some cells were treated with Wortmannin (20 nM), a potent inhibitor of PI(3)-kinase, as indicated. Four hours after transfection, cells were treated with Wortmannin (20 nM) and incubated for another 48 h. Percentage of apoptotic cells with standard deviations from triplicate cultures is indicated. Basal level of apoptosis observed in the vector only control ($\sim$ 10%) was subtracted

in HBx plus H-*ras* transfected cells enhanced apoptosis to a level similar to that in HBx transfected cells (Figure 3b). However, Wortmannin treatment did not affect the apoptosis induced by HBx. In addition, Wortmannin treatment did increase the apoptosis of cells transfected by H-*ras*. Similar to those of ectopic expression of Akt-dn, it is possible that Wortmannin treatment inhibited the survival signal triggered by H-*ras*. Taken together, data obtained are consistent with

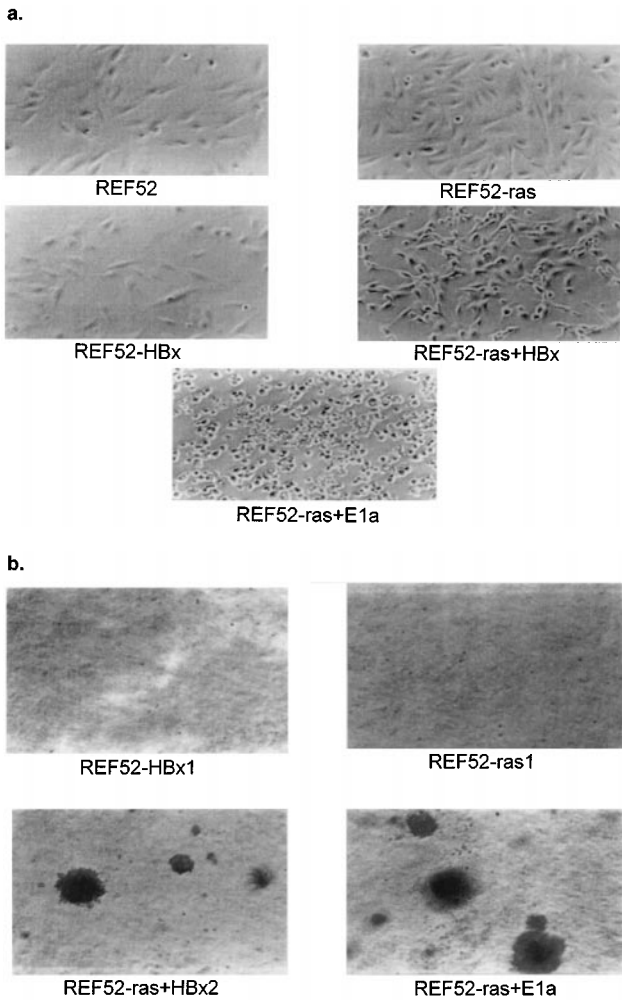


Figure 4 Tumorigenicity of REF52 cells stably transfected by HBx and H-*ras*. (**a**) Morphology of REF52 cells transformed by the indicated oncogenes. Phase contrast photomicrographs of transformed REF52 cells as well as parental REF52 cells. (**b**) Anchorage-independence of HBx and H-*ras* transformed cells. G418-resistant colonies recovered after transfection were plated by layering single-cell suspensions (10^5 cells in 5 ml) in 0.37% Noble agarose (Difco) on 60 mm plates containing 4 ml of 0.9% Seakem agarose (FMC). Colonies (>0.2 mm in diameter) were counted after 21 days

a notion that the activated H-*ras* suppressed HBx-mediated apoptosis via the PI(3) kinase-Akt pathway.

*REF52 cells transfected by H-*ras *and HBx are fully transformed*

The observation that the HBx-mediated apoptosis was suppressed by activated *ras* led us to examine the transforming ability of HBx transfected rodent cells in soft agar and in nude mice. It has been reported that REF52 cells transfected either by *ras* or *myc* alone do not form tumors in nude mice, whereas REF52 cells transfected by both *ras* and *myc* can form tumors (Franza *et al*., 1986). Therefore, we employed REF52 cells, which is an appropriate cell line for transformation assay. Thus, REF52 cells were transfected with various test plasmids along with pSV2neo for selection. Several colonies from each set of transfection were picked and examined for morphological transformation (Figure 4a). The G418-resistant colonies isolated from HBx or H-*ras* transfected REF52 cells were morphologically indistinguishable from parental REF52 cells. In contrast, the morphology of HBx plus H-*ras* transformants resembled cells transformed by H-*ras* plus E1a, consisting of round and loosely adherent cells (Figure 4a). Next, we tested four cell lines derived from H-*ras* plus HBx transfection for anchorage-independent growth in soft-agar (Table 2 and Figure 4b). Three out of four REF52 cells transfected by both HBx and H-*ras* formed colonies in soft agar, whereas the cells transfected by either H-*ras* or HBx alone did not (Table 2). As expected, the cells transfected by both H-*ras* and E1a also grew in soft agar. Subsequently, the tumor forming ability was tested by subcutaneously injecting these REF52 cells into nude mice. Remarkably, nude mice injected with REF52 cells transfected by both HBx and H-*ras* formed tumors. Three out of four REF52-H-*ras* plus HBx cell lines injected into nude mice gave rise to tumors (Table 2). On the other hand, REF52 cells transfected with either H-*ras* or HBx did not form any visible tumors. Thus, we concluded that REF52 cells transfected by both HBx and H-*ras* were fully transformed, as evidenced by morphological transformation, anchorage-independent-growth, and tumor formation in nude mouse.

Table 2 Transforming phenotypes of HBx and H-*ras* transfected REF52 cells

Cell lines	*Colony formation in soft-agar (10^5 cells)*[a] *Exp. 1*	*Exp. 2*	*Exp. 3*	*Tumorigenicity in nude mice*[b]
REF52-*ras*1	0	nd[c]	0	0/4
REF52-*ras* + E1a	2104	nd	1064	4/4
REF52-HBx	0	nd	0	0/4
REF52-*ras* + HBx1	nd	0	0	0/4
REF52-*ras* + HBx2	1488	3720	1896	4/4
REF52-*ras* + HBx3	nd	552	512	3/4
REF52-*ras* + HBx4	nd	2360	928	4/4

[a]The number of cells forming colonies (>0.2 mm) within 3 weeks was counted. [b]Tumorigenicity is expressed as the number of tumor forming animals/number of injected animals. [c]nd, not determined

Discussion

In this report, we provide direct evidence for the contribution of HBx in tumorigenesis, in that activated H-*ras* oncogene collaborates with HBx by suppressing the HBx-mediated apoptosis. We examined the oncogenic potential of HBx by colony formation assay using NIH3T3 cells that is considered as a standard cell line for transformation assay. Since HBx is present at a very low copy number in hepatocytes of infected animals (Dandri *et al*., 1996), we speculated that overexpression of HBx might lead to a misinterpretation of outcomes. Thus, we employed the SV40 promoter to express HBx, instead of the strong CMV

promoter (Kim *et al*., 1998). Indeed, we found that H-*ras* collaborated with HBx to transform cells by suppressing HBx-mediated apoptosis. We demonstrated that REF52 cells transfected by both H-*ras* and HBx have transformed morphology and gained the ability to grow on soft agar. Further, we demonstrated the ability of HBx and H-*ras* transfected REF52 cells to form tumors in nude mouse. To our knowledge, this is the first experimental evidence that the contribution of HBx to tumorigenesis is clearly demonstrated both in cell culture and *in vivo*.

It was noted that HBx expression level is critical in the decision as to whether cells are subjected to apoptotic death (Terradillos *et al*., 1998). When HBx was expressed from the strong CMV promoter, collaboration of HBx with H-*ras* was not detected in colony formation assay (Kim *et al*., 1998; Schuster *et al*., 2000). Consistently, it has been reported that a low level expression of HBx sensitizes cells to apoptotic stimuli while overexpression of HBx triggers apoptotic death in transfected cells (Terradillos *et al*., 1998). In fact, HBx protein is hardly detectable in hepatocytes of infected patients (Su *et al*., 1998). In woodchuck liver, it has been convincingly demonstrated that HBx protein level was extremely low (less than 40 000–80 000 molecules per cell) (Dandri *et al*., 1996). Thus, the modest level of HBx expression from the SV40 promoter is more likely to reflect the physiological effect of HBx in cells.

Several studies have indicated that HBx influences cellular signaling pathways. It was reported that HBx activates *src* (Klein and Schneider, 1997) and the *ras*/*raf*/ERK (extracellular signal-regulated kinase) pathway, which leads to transcriptional transactivation and the stimulation of proliferation in quiescent cells (Benn *et al*., 1996; Natoli *et al*., 1994). In addition, it was reported that HBx activates c-Jun N-terminal kinases (JNK) via MEKK-1 (MEK kinase) (Benn *et al*., 1996). Subsequently, HBx has been implicated in both deregulation of cell cycle control or cell proliferation (Benn and Schneider, 1995; Koike *et al*., 1994b) and apoptosis (Chirillo *et al*., 1997; Kim *et al*., 1998). Thus, it appears that HBx has pleiotropic activities that may or may not be related to oncogenesis. Signals emanating from HBx expression trigger pleiotropic biological outcomes, some of which, as in the case of proliferation (*ras*/*raf*/ERK, *src*) and apoptosis (JNK), may be contradictory. Related to this notion, we interpreted our findings as to the suppression of the HBx-mediated apoptosis by the activated H-*ras* leads to stimulation of cell proliferation without subjecting to apoptotic death, although our experiment did not rule out a possibility that the activating Akt signaling may contribute to cell proliferation via alternative pathway, such as progression through G1/S arrest. The net outcome of HBx expression is presumably dictated by downstream interactions that independently potentiate or mitigate each effector pathway in response to other signals. Apoptosis normally eliminates cells with damaged DNA, that is, those mostly likely to engender a neoplastic clone. The proapoptotic activity of HBx may contribute to viral oncogenesis by exerting a selective pressure that favors the emergence of mutated cells. Further studies on the mechanism of the HBx-mediated apoptosis may provide new insights into the mechanisms of viral persistence and pathogenesis as well as the contribution of HBx in hepatocarcinogenesis.

Regarding collaboration with cellular oncogenes, HBx has been shown to collaborate with c-*myc* for tumor formation in transgenic mouse (Terradillos *et al*., 1997). In this mouse model, HBx alone has no direct pathological effect, but has accelerated tumor formation induced by c-*myc*. Likewise, HBx expression has been shown to lead to anchorage-independent growth of AML12 cell line, mouse hepatocyte cell line derived from TGF-α transgenic mouse (Tarn *et al*., 1999). In these cells, it is presumed that either c-*myc* or TGF-α collaborated with HBx to exhibit the transforming phenotype. However, the molecular mechanisms by which c-*myc* or TGF-α collaborated with HBx are not known. Taken together, it becomes apparent that HBx is not acutely oncogenic, but contributes to oncogenesis by stimulating proliferation of cells that have already obtained additional mutation, such as the activation of H-*ras* or c-*myc*.

Tumorigenesis is a multistage process, which involves activation of cellular oncogenes and inactivation of tumor suppressor genes. Cooperation between oncogenes to transform cells is well-documented in transformation of primary rodent cells. The initial paradigm of this cooperation is the one between *ras* and *myc* oncogene (Land *et al*., 1983). Subsequently, it has been shown that Ela- or *myc*-induced apoptosis is suppressed by *ras* (Kauffmann-Zeh *et al*., 1997; Lin *et al*., 1995), whereas the ras-induced senescence is blocked by Ela or *myc* (Serrano *et al*., 1997). This interaction is now understood as a molecular basis of the collaboration between *ras* and *myc*. We propose that along with adenovirus Ela and *myc*, HBx belongs to a class of oncogenes, which alone induces apoptosis, but can collaborate with other oncogenes such as H-*ras* to transform cells.

Materials and methods

Plasmids

HBx expression plasmid, pSV-X, was a gift from CH Lee (Spandau and Lee, 1988). The H-*ras* expression plasmid, pEJ 6.6(H-*ras*), was a gift from RA Weinberg (Land *et al*., 1983). pRSV-*myc*, pE1a(13S), pcDNA-Akt-dn, and pCMV-Bcl-2 were obtained from Drs BS Yang, DS Im, J-K Jung and Y-J Oh, respectively.

Cells and colony formation assay

NIH3T3 cells or Rat2 cells were transfected with 1 μg of pSV-X, pEJ 6.6(H-*ras*), or pRSV-*myc* plasmid DNAs along with 0.2 μg of pSV2neo plasmid DNA per 3×10^5 cells/60 mm plate as described (Franza *et al*., 1986). The total amount of transfected DNA was kept constant using salmon

sperm DNA as a carrier. At 20 h post-transfection, the cells were split at a 1:3 or 1:4 ratio and cultured in the presence of 200 μg per ml of G418. After 2–3 weeks of selection, the cells were fixed in methanol: acetone (1:1) solution for 10 min, stained with 0.1% Giemsa in 6% methanol for 10 min, and the number of colonies was counted. REF52 cells were transfected by calcium phosphate precipitation with 5 μg of pSV-X, pEJ 6.6(H-*ras*), or pE1a(13S) DNAs along with 1.0 μg of pSV2neo DNAs per 1×10^6 cells/100 mm plate. At 20 h post-transfection, the cells were washed in PBS and cultured in the presence of 50 μg/ml of G418. After 3 weeks of selection, G418-resistant colonies were isolated.

Viable cell counts

Cells were plated in DMEM containing 5% FBS at a density of 3×10^5 cells/60 mm plate. Next day, the cells were washed and fed with DMEM containing 5% or 1% FBS and the indicated amount of etoposide. After another 24 h of incubation, the cells were washed and stained in PBS containing 0.04% trypan blue solution. The viable cells (trypan blue excluded) were counted with a hemacytometer.

DNA fragmentation analysis

DNA fragmentation assay was done as described (Hockenbery *et al*., 1990). Briefly, cells were plated in DMEM containing 5% FBS at a density of 1×10^6 cells per 100 mm plate. Next day, the cells were treated with 10 μM of etoposide for an additional 1 day. Both adherent and floating cells were collected and incubated in ice-cold lysis buffer (0.5% Triton X-100, 5 mM Tris, pH 7.4, 20 mM EDTA) for 30 min. The lysates were centrifuged at 12 000 r.p.m. for 15 min, and the supernatant was transferred to a new tube. DNA was extracted by phenol extraction and ethanol precipitation and subjected to electrophoresis in 1.4% agarose gels at 60 V for 6 h.

Transient apoptosis assay

NIH3T3 cells (1×10^5) were seeded in each well of 12-well plates. Next day, cells were transiently transfected with 0.3 μg of pcDNA3.1-lacZ (Invitrogen) plus various expression plasmids. Salmon sperm DNA was used to adjust total DNA to equal amount. Two days after transfection, apoptotic cells with dense nuclear staining were counted as described (Miura *et al*., 1993).

Soft agar assay

G418-resistant colonies recovered after transfection were tested for anchorage-independent growth as described (Kohl and Ruley, 1987). Cells were plated by layering single-cell suspension (10^5 cells in 5 ml) in 0.37% Noble agarose (Difco) on 60 mm plates containing 4 ml of 0.9% Seakem agarose (FMC). Agarose layers contained DMEM supplemented with 10% FBS. Cells were fed weekly by addition of 5 ml of media containing 0.37% agarose. To facilitate colony counting, the 60 mm plate was divided into four parts and only one part was counted.

Tumor formation in nude mice

Tumorigenicity of transfected cell lines was measured as follows. Cells were harvested by trypsinization, washed twice with sterile phosphate-buffered saline, and resuspended at 2×10^7 cells per ml. Aliquots (0.1 ml) were injected subcutaneously into 6- to 8-week-old Balb/c athymic nude mice. Mice were observed at periodic intervals. The photograph was taken at 3 weeks after injection.

Western blot analysis

Equal amounts of whole cell protein extracts were resolved by 15% SDS–PAGE, transferred to nitrocellulose, immunoblotted with rabbit polyclonal anti-HBx peptide (carboxy-terminal of HBx peptide, residue 144–154), visualized with the ECL system (Amersham).

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