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Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model

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ABSTRACT **Overexpression of the c-*myc* oncogene is associated with a variety of both human and experimental tumors, and cooperation of other oncogenes and growth factors with the *myc* family are critical in the evolution of the malignant phenotype. The interaction of hepatocyte growth factor (HGF) with c-*myc* during hepatocarcinogenesis in a transgenic mouse model has been analyzed. While sustained overexpression of c-*myc* in the liver leads to cancer, coexpression of HGF and c-*myc* in the liver delayed the appearance of preneoplastic lesions and prevented malignant conversion. Furthermore, tumor promotion by phenobarbital was completely inhibited in the c-*myc*/HGF double transgenic mice, whereas phenobarbital was an effective tumor promoter in the c-*myc* single transgenic mice. The results indicate that HGF may function as a tumor suppressor during early stages of liver carcinogenesis, and suggest the possibility of therapeutic application for this cytokine.**

Deregulation of c-*myc* expression has been implicated in the development of a wide variety of experimentally induced and naturally occurring tumors (1), including hepatocellular carcinoma (HCC) (2, 3). In addition, the interaction of several other oncogenes and growth factors with members of the *myc* family during neoplastic development may be critical in the evolution of the malignant phenotype. Expression of transforming growth factor type α (TGF-α), a potent hepatotrophic mitogen activating the epidermal growth factor tyrosine kinase receptor and synthesized in hepatocytes during liver regeneration, is also frequently detectable in human hepatic tumors (3).

To explore possible synergistic effects of nuclear oncogenes such as the c-*myc* and growth factors in tumorigenesis, we recently established a transgenic mouse model coexpressing c-*myc* and TGF-α in the liver and demonstrated a dramatic acceleration of neoplastic development in this organ (4, 5) as compared with tumor formation when either of these transgenes were expressed alone (6–8). Similar cooperation of epidermal growth factor with c-*myc* during hepatocarcinogenesis has recently been shown (9). Although TGF-α and epidermal growth factor seem to act as powerful liver tumor promoters, the role played by hepatocyte growth factor (HGF) in the development of HCC is less clear. HGF, initially identified in serum of partially hepatectomized rats as a potent mitogen for hepatocytes in culture (10), is expressed in mesenchymal cells of different tissues, including nonparenchymal liver cells (10–12). HGF induces motility (13–15), proliferative activity, and morphogenesis in hepatocytes and many other extrahepatic cell types (16, 17). Moreover, HGF is a potent angiogenic factor *in vitro* and *in vivo* (18, 19), and is involved in hematopoiesis (20) and local regulation of fibrinolysis and coagulation (21, 22). The pleiotropic effects of HGF imply a physiological function as an essential paracrine and endocrine modulator of mesenchymal–epithelial interactions during development and repair/regeneration of tissues (10, 17, 23–26). This modulatory activity is mediated by the HGF receptor encoded by the c-*met* protooncogene expressed in most of epithelial tissues including the liver (27, 28). Numerous reports have shown expression of HGF and/or its receptor in tumors of different tissues and in cell lines (27, 29), suggesting that the HGF/c-met dependent signaling may also be involved in neoplastic development, conceivably during tumor progression (27, 30–33). Nevertheless, the role of HGF during hepatocarcinogenesis is still controversial.

It has been shown *in vitro* that HGF can inhibit the growth of many transformed cell types, including HCC cell lines, by a cytostatic mechanism (34–38), but chemical carcinogenesis studies *in vivo* have not produced consistent results. In fact, Liu *et al.* (39) have reported that intraportal infusion of HGF in rats inhibits the proliferation of diethylnitrosamine-induced neoplastic liver nodules, whereas Yaono *et al.* (40) have described enhancement of preneoplastic hepatic foci development in rats treated with a similar protocol. In contrast to the TGF-α transgenic mice (7), no neoplastic transformation occurred in recently established transgenic mice overexpressing HGF in the liver (41). These observations suggest that the involvement of HGF/c-*met* signal transduction system in the neoplastic process of the liver might differ fundamentally from the TGF-α/epidermal growth factor receptor system. Guided by this notion, we wished to characterize the effects of HGF upon the neoplastic process in a transgenic mouse model in which we had earlier established the impact of TGF-α on carcinogenesis (4, 5).

MATERIALS AND METHODS

Transgenic Mice. The c-*myc* and c-*myc*/HGF transgenic mice were generated by using the mouse c-*myc* and the human HGF (h-HGF) recombinant cDNA constructs as reported (4, 37). Several lines of both c-*myc* and h-HGF transgenic mice were developed, displaying similar phenotype (4, 5, 37, 41). Animal housing and care were in accordance with National Institutes of Health guidelines. The transgenic offsprings were identified by DNA dot-blot and Southern analysis of tail DNA using nick-translated c-*myc* probe or by PCR amplification of a 581-bp h-HGF product.

Macroscopical and Histological Analyses. During the study 8 or 10 mice from each group were sacrificed monthly between the first and ninth month of age, while 12 mice from each group were used at 10, 12, 14, and 16 months of age. Body weights

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Abbreviations: HCC, hepatocellular carcinoma; TGF-α, transforming growth factor type α; HGF, hepatocyte growth factor; h-HGF, human HGF; HCA, hepatocellular adenoma; PB, phenobarbital; wt, wild type; TGF-β, transforming growth factor type β; TGFβR, TGF-β receptor; Ab, antibody.

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were recorded, and livers were removed, weighed, and examined for macroscopic lesions. For morphological, immunohistochemical, and molecular analysis, parts of the livers were fixed in 10% formalin or promptly frozen in liquid nitrogen and stored at −80°C. All fixed tissues were embedded in paraffin and sections stained with hematoxylin/eosin (H&E). Two sections from each lobe of nontumorous livers and all grossly visible tumors were examined and the latter diagnosed as hepatocellular adenomas (HCAs) or HCCs (42). The c-*myc*/HGF mice did not develop HCCs throughout the time course.

Mitotic and Apoptotic Indices. Mitotic and apoptotic indices were scored with a light microscope on hematoxylin/eosin-stained livers from each animal sacrificed. Random evaluation of 5000 hepatocytes/mouse from nontumorous areas was performed in blind experiments, and final values obtained by averaging data from three investigators. In tumorous areas, depending on lesion size, the indices were determined either by evaluation of all the cells or as described above for nontumorous areas. Recognition of apoptotic cells by morphological criteria was as reported (43).

Northern Blot Analysis. RNA isolation and Northern blot analysis of 10 μg of mRNA from each sample were performed as described (43). The ^{32}P-labeled probes utilized were (*i*) a fragment of the h-HGF gene corresponding to the coding region from bp 122 to 969 (kindly provided by R. Zarnegar, University of Pittsburgh School of Medicine), (*ii*) a 1.9-kb fragment coinciding to the pLEC1 construct used to generate the c-*myc* transgene (4), and (*iii*) a 985-bp fragment of rat transforming growth factor type β1 (TGF-β1) cDNA. The rat β-actin cDNA was used as an internal standard. After hybridization, the membranes were exposed to Kodak XAR film or analyzed by PhosphorImager (Molecular Dynamics) to quantify mRNA expression.

Immunoblot Analysis. Western blot analysis of mouse c-met and tyrosine autophosphorylation of this receptor were performed on nonneoplastic and neoplastic liver samples from two animals per time point as described (28, 44, 45). A rabbit polyclonal antibody (Ab) against the intracellular 21 C-terminal amino acids of mouse c-met (44) (Santa Cruz Biotechnology) or an antiphosphotyrosine monoclonal Ab (Upstate Biotechnology, Lake Placid, NY) were used. Reactions were revealed by enhanced chemiluminescence system (ECL; Amersham).

ELISA Assay. Serum h-HGF levels were determined in mouse blood collected from the retro-orbital cavity with an ELISA kit specific for the active form of h-HGF (Otsuka America Pharmaceutical, Rockville, MD) (46).

Immunohistochemistry. Immunohistochemistry for h-HGF and c-met was performed with a goat polyclonal anti-h-HGF Ab (R & D Systems) and the anti-mouse c-met Ab described above. After deparaffinization and blocking of endogenous peroxidase, liver sections from two animals per time point and from all the neoplastic lesions were microwaved for 10 min in 10 mM sodium citrate (pH 6.0) and then incubated overnight at 4°C in Tris-buffered saline (TBS) containing 0.05% Triton X-100, 1% BSA, 1% mouse serum, 1.5% blocking serum, and 1 μg of primary Ab per ml. The reaction was detected by the Vectastain ABC Elite kit (Vector Laboratories) with diaminobenzidine as a substrate. As negative controls the anti-h-HGF Ab was preincubated with recombinant h-HGF (1:20 w/w; R & D Systems), while the anti-c-met Ab was neutralized by a control peptide (Santa Cruz Biotechnology). Immunostainings for type I and II TGF-β receptors (TGFβR-I and TGFβR-II) were performed by using rabbit polyclonal Abs against the cytoplasmic kinase domain of either receptor. TGF-β1 was detected as reported (47).

Tumor Promotion by Phenobarbital (PB). Mice were given PB 0.05% in their food pellets starting at 3 weeks of age. Ten to 12 transgenic mice and 5 wild-type mice (wt) were sacrificed at each time point and further analyzed as described for the untreated animals.

RESULTS

Inhibition of Hepatocarcinogenesis by HGF in c-*myc* Transgenic Mice. Homozygous female C57BL/6J × CBA/J mice bearing the pLEC1 mouse albumin enhancer/promoter c-*myc* fusion gene were crossed with male heterozygous FVB transgenic mice expressing h-HGF driven by the albumin regulatory elements. As expected, ≈50% of the mice coexpressed c-*myc* and h-HGF (c-*myc*/HGF), and the other 50% expressed only c-*myc* in the same final background. (C57BL/6J × CBA/J) × FVB hybrid were used as control animals. Due to the higher incidence of tumors, only male mice were analyzed. In both transgenic lines during the first year of life the liver weight/body weight ratio, a measure of liver growth, was moderately higher than in control mice (Fig. 1*A*). However, the liver weight of c-*myc* mice increased significantly after 12 months of age reaching 10% of the body weight by 16 months, reflecting the formation of large tumor masses. In contrast, the liver weight of c-*myc*/HGF mice older than 12 months did not differ from that of younger animals.

The onset of histologically detectable hepatic abnormalities occurred in the c-*myc* line at 4 months of age and consisted of perivascular dysplastic cells (Fig. 1*B*). Subsequently a progression from mild to severe dysplasia was observed and all c-*myc* mice had large dysplastic hepatocytes by the age of 14 months (Fig. 1*B*). Dysplastic cells were detected in 15% of the c-*myc*/HGF mice at 12 months of age, and at 16 months 67% of the mice were affected by mild hepatic dysplasia (Fig. 1*B*). Preneoplastic lesions (foci of cellular alteration) (42) appeared as early as 7 months in 30% of the c-*myc* animals and foci were present in all the mice between 12–16 months (Fig. 1*C*). In contrast the c-*myc*/HGF mice showed preneoplastic foci only after 1 year and more than 50% of the mice were still not affected at 16 months of age (Fig. 1*C*).

The first benign hepatic neoplasms (HCAs) were detected at 8 months in the c-*myc* mice, while the first HCC appeared at 10 months (Fig. 1*D*). The incidence of both types of tumors remarkably increased in older c-*myc* animals, and at 16 months ≈60% of the mice had multiple HCCs (Table 1). In the c-*myc*/HGF mice the onset of lesions was seen at 13 months (Fig. 1*D*). Histological examination showed that, even at 16 months of age, these mice were affected only by small benign lesions (Table 1), composed of cells resembling hepatocytes. Furthermore, at each time point the number of c-*myc*/HGF mice carrying benign lesions was always lower than that

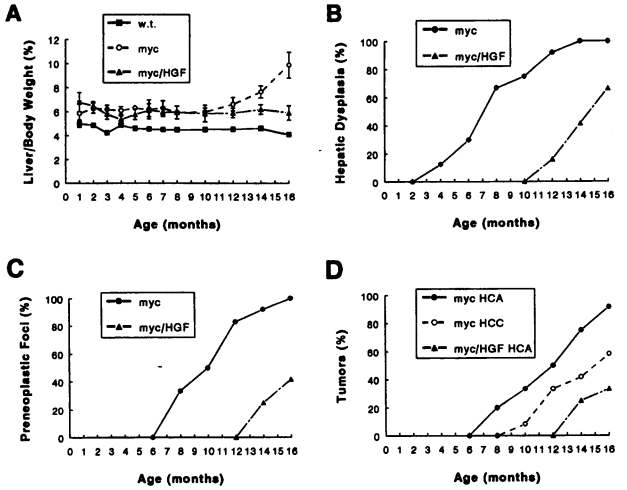

FIG. 1. Liver growth and neoplastic development in c-*myc* and c-*myc*/HGF transgenic mice. (*A*) Liver weight (% of body weight) during ontogenesis of wt and transgenic mice. Each time point represents mean ± SEM of 8–12 mice. Also shown is the time course and incidence of dysplasia (*B*), preneoplastic foci (*C*), and tumors (*D*). Values are percentages of animals affected at each time point.

observed in the c-*myc* mice affected by HCAs and/or HCCs (Fig. 1*D*). In addition to displaying earlier onset and higher incidence, the tumors arising in c-*myc* were larger and more numerous than HCAs appearing in c-*myc*/HGF mice, which were never more than two per liver (Table 1). The c-*myc* HCCs were either of the trabecular or the solid histological type, varying from well-differentiated to poorly differentiated tumors with cell polymorphism, atypia, and areas of hemorrhagic necrosis. These tumors ended up becoming confluent and replacing most of the liver parenchyma. None of the lesions described above were detected in the wt mice throughout the time course of this study. Taken together, these data suggest that hepatocytes overexpressing c-*myc* progressively develop HCC but the coexpression of HGF inhibits the appearance of preneoplastic lesions (Fig. 2) and prevents the progression toward malignant phenotypes.

Expression of Transgenes and c-met Receptor. The expression of transgenes was first assessed by Northern blot analysis in all mice used in the analysis (Fig. 3*A*). The probe used for the detection of h-HGF also recognized the transcript for the endogenous gene in both transgenic lines. The expression of endogenous mouse HGF mRNA did not show significant variations between the transgenic lines and the wt mice. The c-*myc* transgene was equally and strongly expressed in both transgenic lines.

The serum levels of active h-HGF in the c-*myc*/HGF mice, measured by ELISA, ranged from 1.5 to 5 ng/ml (Fig. 3*C*) and were detectable throughout the entire experimental time course, demonstrating a continuous production of h-HGF. These values corresponded to the range of HGF plasma levels detected in patients with chronic liver diseases and HCC (46, 48, 49). These values were also consistent with the h-HGF serum levels reported in the HGF monotransgenic mice (41), and similar to the concentrations of HGF used to inhibit the growth of various tumor cell lines including HepG2 cells (35). Interestingly, these concentrations of h-HGF are able to stimulate DNA synthesis in normal rat and human hepatocytes (16). As expected, no h-HGF was detected in the c-*myc* and wt mice. The expression of h-HGF in the liver of c-*myc*/HGF mice was also confirmed by immunohistochemistry (Fig. 3*D*).

Western blot analysis, performed with an Ab against the intracellular C-terminal domain of the c-met receptor β subunit, showed two bands, corresponding to the p170 uncleaved $\alpha\beta$ precursor and the mature p140 β subunit (27, 44). Both bands were expressed more strongly in the c-*myc*/HGF mice (Fig. 3*B*), consistent with HGF being capable of inducing the expression of its own receptor, as described (27). Further analysis revealed that the 140-kDa band was the one most intensely reactive with antiphosphotyrosine Ab, indicating a proper activation of the mature HGF receptor (data not shown).

However, in the livers of both transgenic lines, c-met expression, as assessed by immunohistochemistry and immunoblotting, was lower in preneoplastic and neoplastic lesions than in the surrounding parenchyma (Fig. 3 *E–G*). We analyzed 26 c-*myc* preneoplastic foci and 14 c-*myc*/HGF foci by immunohistochemistry and detected a clear downregulation of c-met in 23 and 12 foci, respectively. We also examined c-met expression in all of the HCAs detected in c-*myc*/HGF mice (10 tumors) and in all tumors of c-*myc* mice. In the former group eight HCAs displayed strong downregulation of c-met expression and only two HCAs had modest reduction as compared with surrounding nontumorous tissue. Similarly, all of the c-*myc* tumors showed much lower c-met immunoreactivity than the peritumorous parenchyma. We confirmed that c-met downregulation is a common feature of c-*myc*-induced hepatocarcinogenesis by immunostaining liver sections obtained from c-*myc* and c-*myc*/TGF-α transgenic mice generated in a different genetic background as reported (4, 5). In these mice 8 of 8 c-*myc* tumors and 7 of 8 c-*myc*/TGF-α tumors presented significantly lower c-met levels, while one c-*myc*/TGF-α tumor had heterogenous downregulation (data not shown). The immunohistochemical data were also supported by Western analyses showing decrease of both c-met protein and autophosphorylation in liver homogenates of transgenic mice affected by preneoplastic and neoplastic lesions (Fig. 3*B*). Taken together the findings indicate that in our transgenic models (pre)neoplastic cells may respond differently from normal hepatocytes to HGF. Decreased expression of c-met protein was revealed with an Ab against 21 C-terminal amino acids of the mouse c-met, which is a region of the receptor reported to be essential for important biological functions in epithelial cells (45). The reduced c-met autophosphorylation also supports this interpretation, whereas the possibility of alternate HGF-dependent pathways is unlikely since the other members of the MET family, Ron and Sea, do not bind HGF (50). Nevertheless, the decreased levels of c-met receptor seen in the (pre)neoplastic lesions of the c-*myc*/HGF mice appear sufficient to prevent and/or delay malignant conversion in the presence of h-HGF. Moreover, the inhibitory activity of h-HGF transgene on hepatocarcinogenesis was not mediated by induction of TGF-β1, a known inhibitor of hepatocyte growth and proliferation, since comparable expressions of TGF-β1 mRNA and protein were detected in the liver of both transgenic lines. Immunohistochemical analysis of TGFβRs performed on serial sections from the samples used for c-met

Table 1. Neoplastic lesions in c-*myc* and c-*myc*/HGF transgenic mice at 16 months

	c-*myc*	c-*myc*/HGF
No. of mice with HCA	11/12 (92%)	4/12 (33%)
Average size of HCAs, cm	0.8 × 0.9	0.4 × 0.4
± SD, cm	0.1 × 0.3	0.1 × 0.2
No. of HCAs/liver*	Multiple	1.5
No. of mice with HCC	7/12 (58%)	0/12 (0%)
Average size of HCCs (cm)	1.2 × 1.4	
± SD, cm	0.3 × 0.3	
No. of HCCs/liver	Multiple	0

No liver tumors were detected in wt mice at the same age.
*Only livers bearing tumors were considered.

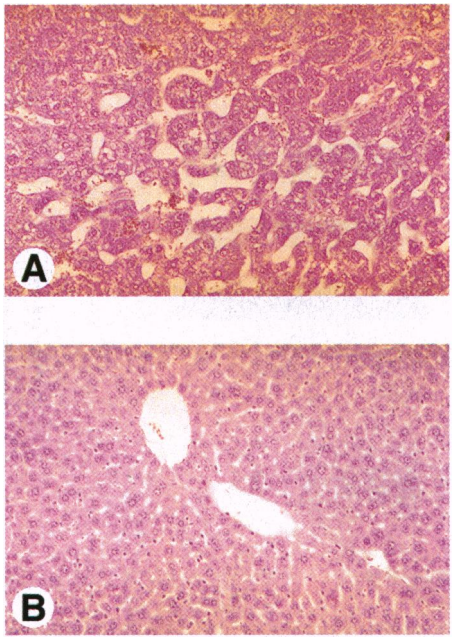

FIG. 2. Inhibition of c-*myc* induced hepatocarcinogenesis by h-HGF. (*A*) Trabecular HCC in a 12-month-old c-*myc* mouse. (*B*) c-*myc*/HGF liver at the same age is essentially normal. (Hematoxylin/eosin, ×200.)

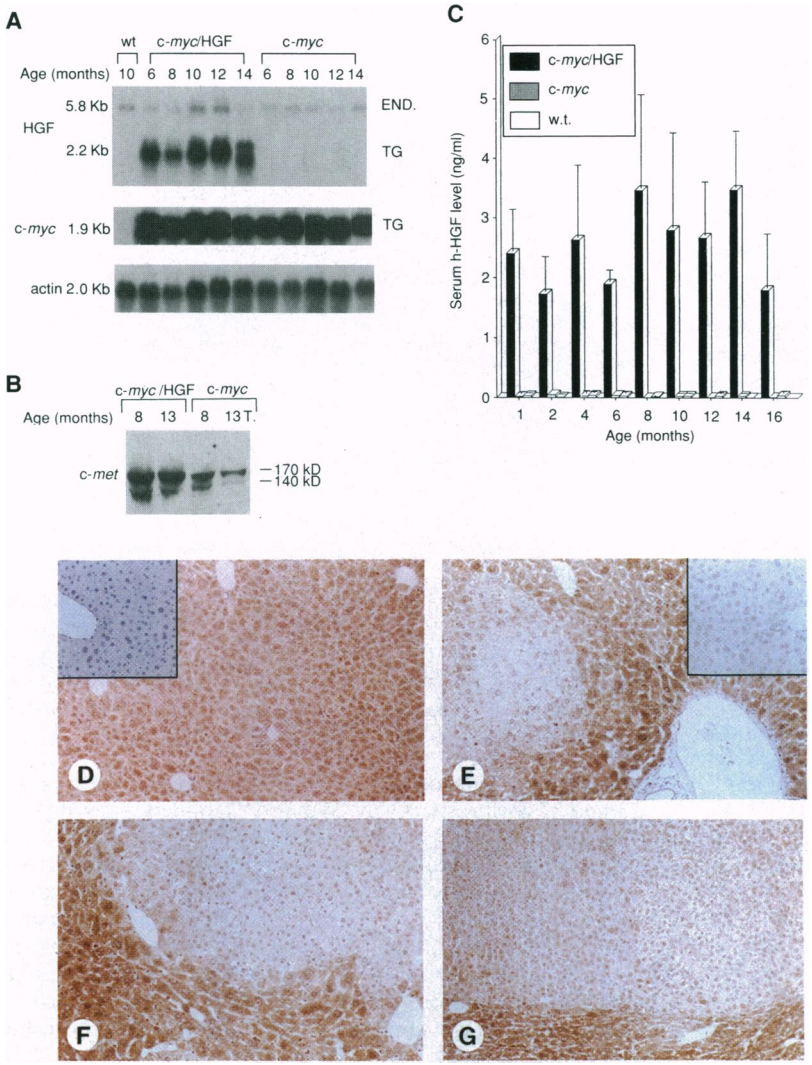

FIG. 3. Expression of transgenes and c-met receptor. (*A*) Northern blot analysis of mRNA expression in liver of wt (lane 1) and transgenic mice (c-*myc*/HGF, lanes 2–6; c-*myc*, lanes 7–11). The probe for the h-HGF transgene (TG) also recognized the endogenous mRNA (End). (*B*) Western blot analysis of c-met receptor expression in livers of transgenic mice. The letter T indicates a tumor sample. (*C*) Serum h-HGF levels in c-*myc*/HGF mice. The values for c-*myc* and wt mice represent the assay background. Each bar represents mean value ± SE of three animals per time point. (*D*) Expression of h-HGF in hepatocytes of a 12-month-old c-*myc*/HGF mouse assessed by immunohistochemistry. (*Inset*) Negative control as indicated in materials and methods. (×125.) (*E*) Immunohistochemistry for c-met receptor in a 16-month-old c-*myc*/HGF mouse. The preneoplastic focus shows lower expression of HGF receptor. (*Inset*) Negative control as indicated in materials and methods. (×200.) Downregulation of c-met receptor in preneoplastic focus (*F*) (×150) and hepatic tumor (*G*) (×125) of c-*myc* mice.

immunohistochemistry showed that all of the (pre)neoplastic lesions in c-*myc* and c-*myc*/HGF mice expressed TGFβR-I. However, while all of the c-*myc*/HGF (pre)neoplastic lesions expressed TGFβR-II at the same levels as the adjacent parenchyma, 40% of the c-*myc* foci and 60% of the c-*myc* tumors displayed downregulation of TGFβR-II. This suggests that HGF overexpression could, either directly by an unknown mechanism(s) or indirectly by affecting cellular differentiation, regulate the sensitivity of transformed cells to TGF-β. In this context, it is of interest that preliminary results on TGFβRs expression in c-*myc*/TGF-α double transgenic mice showed that the vast majority of preneoplastic foci and hepatic tumors in these mice downregulate the TGFβR-II (E.S.-R. and S.S.T., unpublished results). These results support the notion that TGF-α and HGF have opposite effects on c-*myc*-induced hepatocarcinogenesis.

Cell Proliferation and Apoptosis in Transgenic Mice. Since overexpression of c-*myc* can induce either cell proliferation, or cell death, by still unclear mechanisms (51), mitotic and apoptotic indices were scored in nontumorous as well as tumorous parts of the liver parenchyma. We reasoned that this approach would provide insight into the mechanism(s) by which overexpression of c-*myc* in the liver may influence growth and tumor development in this organ as well as how HGF may modulate these processes (Fig. 4). Very high and parallel mitotic and apoptotic activities were observed in nontumorous tissues of both transgenic lines, thereby preventing an excessive net liver growth prior to the appearance of large tumors. Two waves of mitosis and apoptosis were observed, peaking at 1 and 6 months of age after which both activities gradually declined. The first peak of mitosis was identical in the two transgenic lines, with levels about 5-fold above the wt mice, and coincided with the period of juvenile liver growth that normally occurs in mice during the first 6 weeks of life (Fig. 4 *A* and *B*). These data suggest that c-*myc* provided the major stimulus for enhancing the physiological growth of the liver in these young transgenic mice. In contrast, the second increase in mitotic activity occurred between 4 and 8 months, when the mitotic activity in normal mouse liver is close to zero, resulting in 100- and 50-fold increase over control in c-*myc* and c-*myc*/HGF mice, respectively. It is noteworthy that the mitotic index in the c-*myc* mice was significantly higher ($P < 0.001$, Student's *t* test) than in the c-*myc*/HGF animals. Considering that an abnormally high mitotic activity may increase the probability of acquiring and/or "fixing" mutations and other chromosomal abnormalities, this period of time might be critical for the selection of malignant clones in the c-*myc* transgenic livers. Although both transgenic mouse lines displayed similar levels of net cell proliferation (mitosis minus apoptosis), the tumor incidence (Fig. 1 and Table 1) is much higher in the c-*myc* mice. These data suggest that the higher overall rate of cell replication seen in the c-*myc* livers provides an environment that favors the escape of initiated cells from the homeostatic apoptotic elimination and permits their progression toward a more malignant phenotype. Alternatively, hepatocytes may acquire capacity for neoplastic development

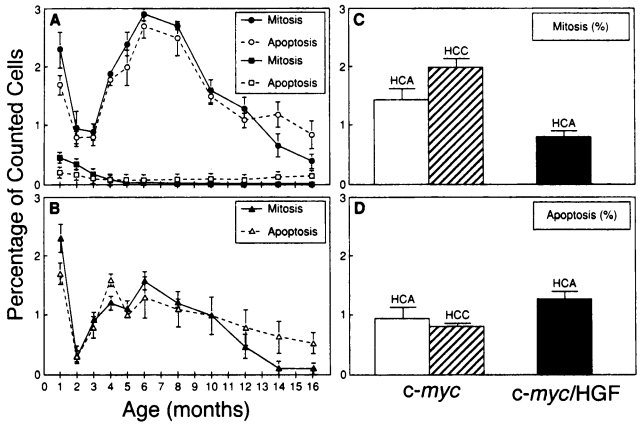

FIG. 4. Mitotic and apoptotic indices in the liver of transgenic mice. (*A*) Indices in wt mice (squares) and in nontumorous tissues of c-*myc* mice (circles). (*B*) Indices in nontumorous tissues of c-*myc*/HGF mice (triangles). Mitotic (*C*) and apoptotic (*D*) index in HCAs (white bar), and HCCs (hatched bar) of c-*myc* mice, and in HCAs (black bar) of c-*myc*/HGF mice.

at similar rates in the two transgenic lines due to c-*myc* overexpression, but HGF selectively inhibits the proliferation of these cells. The elevated mitotic index in c-*myc*/HGF line as compared with control animals may therefore to a large extent reflect the rapid but controlled proliferation of normal hepatocytes. This is consistent with the observation that HGF at concentrations of 1–15 ng/ml is capable of blocking growth of human HCC cell lines but has opposite effects on normal primary hepatocytes (35, 37, 38). The twofold increase in DNA synthesis in HGF transgenic mice, as well as increased rate of regeneration after partial hepatectomy, with no liver tumor formation after 18 months (41), also supports this explanation.

The selective growth inhibition of (pre)neoplastic cells by HGF is also reflected in the growth patterns of tumors and benign lesions in the c-*myc* and c-*myc*/HGF mice, respectively (Fig. 4 *C* and *D*). While the lesions and peri-tumorous tissues of both transgenic lines exhibited similar patterns of apoptosis (Fig. 4 *A*, *B*, and *D*), the mitotic index was higher in c-*myc* tumors than in the small c-*myc*/HGF HCAs (Fig. 4*C*). The rate of cell death in these lesions of c-*myc*/HGF mice was sufficient to offset to a large extent the increase in cell proliferation (the ratio of mitosis/apoptosis in the c-*myc*/HGF HCAs was 3- to 4-fold lower than in the c-*myc* tumors; data from Fig. 4 *C* and *D*), thus resulting in slower growth of the HCAs. Also, the downregulation of c-met receptors seen in (pre)neoplastic lesions of both transgenic lines (Fig. 3 *E–G*), suggests that this event may be required for initiated hepatocytes to escape the selective growth control by HGF during liver tumor progression.

Inhibition of PB Tumor Promotion by HGF. PB is considered a potent nongenotoxic liver tumor promoter in rodents treated with chemical carcinogens. Although several mechanisms by which PB promotes liver carcinogenesis have been proposed, the notion that during PB treatment initiated hepatocytes have a distinct growth advantage over mitoinhibited normal hepatocytes has recently attracted increased interest (52). PB has also been shown to promote growth of transplanted HCCs (53) and more recently to collaborate with TGF-α in accelerating hepatocarcinogenesis of TGF-α transgenic mice (54). Increased plasma levels of HGF have been found in rats treated with PB and this observation has generated the hypothesis that HGF could trigger proliferation in preneoplastic cells during PB promotion (55, 56). Since this hypothesis is in apparent conflict with our data, we wished to test if PB was capable of promoting to the same extent the formation of liver tumors in the c-*myc* and c-*myc*/HGF transgenic mice.

PB was administered in the diet to both transgenic lines and to wt mice from 3 weeks to 10 months of age. The PB treatment resulted in acceleration and significant increase of the HCA and HCC incidence in c-*myc* mice as compared with the untreated c-*myc* mice (Table 2). The HCAs and HCCs appeared 2 months earlier than in untreated mice, and by 10 months of age 100% and 40% of PB-treated c-*myc* mice had HCAs and HCCs, respectively. These results suggest that hepatocytes overexpressing c-*myc* respond to PB promotion in a manner similar to hepatocytes initiated by chemical carcinogens. However, in striking contrast with the c-*myc* mice, no tumor formation was detected in the c-*myc*/HGF mice after 10 months of PB treatment (Table 2). We therefore hypothesize that there is either no production of initiated hepatocytes in c-*myc*/HGF mice over the first 10 months of life or more likely that HGF can suppress the growth of these initiated cells.

DISCUSSION

The results of our study show that the sustained overexpression of c-*myc* in the liver indeed leads to cancer. More importantly, our data reveal that the interaction of the nuclear oncogene c-*myc* with different growth factors acting via tyrosine kinase receptors can result in profoundly different outcome of the neoplastic process. We have previously shown that coexpression of TGF-α in the c-*myc* transgenic mice is capable of dramatically accelerating the c-*myc*-induced neoplastic development in the liver resulting in severe hepatic dysplasia by the second month of age, appearance of HCC 2 months later, 100% HCC frequency at 8 months, and survival <1 year (4, 5). In contrast, the entire c-*myc*-driven oncogenic process in the liver is strikingly inhibited by coexpression of HGF. In addition to complete lack of HCC development in the c-*myc*/HGF mice, the c-*myc* and c-*myc*/HGF transgenic lines display remarkable differences in the rate at which preneoplastic and neoplastic lesions develop in the liver, as well as in the characteristics of these lesions. Liver cell dysplasia, preneoplastic foci, and HCAs appeared many months later in c-*myc*/HGF mice and were never as severe and frequent as in the c-*myc* mice (Figs. 1 and 2). It should be emphasized that these two transgenic lines are of the same genetic background, indicating that the observed differences are due to the expression of h-HGF.

The discovery that no malignant conversion of the neoplastic liver lesions was found in the c-*myc*/HGF mice is of particular importance. The observation that the expression and autophosphorylation of c-met receptor were decreased in both the preneoplastic lesions and tumors suggests that both the level and intactness of the HGF/c-met signal transduction system may be required for effective suppression of the neoplastic process in this transgenic mouse model. In this context it is interesting to note that in a recent study (57) on the relationship between c-met expression and malignant grade of human HCC the authors found an uneven distribution

Table 2. Tumor promotion by PB

Line	Age, months	Incidence of HCA		Incidence of HCC	
		No.	%	No.	%
c-*myc*	6	0/10	0		
	8	2/10	20	0/10	0
	10	4/12	33	1/12	8
c-*myc*-PB	6	5/10	50		
	8	8/10	80	2/10	20
	10	10/10	100	4/10	40
c-*myc*/HGF	6	0/10	0		
	8	0/10	0	0/10	0
	10	0/12	0	0/12	0
c-*myc*/HGF-PB	6	0/10	0		
	8	0/10	0	0/10	0
	10	0/10	0	0/10	0

of the c-met expression in HCCs in contrast to a homogeneous pattern in normal liver and benign lesions. Also, our recent study on a series of 95 human HCCs showed downregulation in 35% and no change in 47% for c-met expression compared with nontumorous tissues (A.K. & S.S.T., unpublished results). Based on the present data it is possible to interpret these results as indicating that at least a percentage of the cells in human HCCs are not subject to the HGF/c-met dependent suppression and could therefore proliferate regardless of HGF serum levels. However, the c-*met* protooncogene has been shown to be overexpressed in a certain percentage of different types of carcinoma (27, 29, 32) and sarcoma (33) as well as being an efficient transforming agent of NIH 3T3 cells (27, 44). It therefore seems likely that c-*met* could play an important role in malignant and metastatic phenotypes of tumors different from those generated in our c-*myc* transgenic mice.

The c-*myc*/HGF mice, in contrast to the c-*myc* mice, are completely resistant to PB promotion. These data suggest that the HGF/c-met system is effective in inhibiting, possibly via apoptosis and/or growth inhibition, the expansion of the initiated cell population. However, further work is needed to properly address questions regarding which stage(s) of liver carcinogenesis is most sensitive to the HGF/c-met dependent inhibition.

The present results are also supported by previous findings *in vitro* (34–38) showing that HGF selectively blocks the growth of transformed hepatocytes and stimulates the proliferation of the normal ones. HGF can, in light of its complex spectrum of activities, be considered a homeostatic liver modulator essential for the development of this organ (25, 26) as well as for hepatic regeneration and repair (10, 17). Our results indicate that in addition to all these important functions HGF may, at least in the liver, also act as a tumor suppressor.

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