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Alterations in Specific Gene Expression and Focal Neoplastic Growth During Spontaneous Hepatocarcinogenesis in Albumin-SV40 T Antigen Transgenic Rats‡

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Transgenic rats containing the mouse albumin promoter and enhancer directing the expression of simian virus (SV40) T antigen (T Ag) exhibited a 100% incidence of hepatic neoplasms by 24–36 wk of age. These transgenic rats exhibited expression of large T Ag and c-*myc* protein within focal basophilic lesions and nodules, but not in surrounding hepatocytes. At 24 wk of age, female TG+ rats exhibited a significantly greater number of lesions and a much greater percentage of the liver occupied by TG+ focal hepatic lesions than did their male TG+ littermates. Previous studies on these animals [Sargent et al., Cancer Res 1997;57:3451–3456] demonstrate that at 12 wk of age approximately one-third of metaphases in hepatocytes exhibit a duplication of the 1q3.7-1q4.1 region of rat chromosome 1, with the smallest common region of duplication being that of 1q4.1. Duplication of the 1q3.7-1q4.3 region is also noted in many primary hepatic neoplasms resulting from the multistage model of Initiation-Promotion-Progression (IPP) [Sargent et al., Cancer Res 1996;56:2985–2991]. This region is syntenic with human 11p15.5 and mouse 7qter, which have been implicated in the development of specific neoplasms. Within the syntenic region was a cluster of imprinted genes whose expression we investigated in livers and neoplasms of TG+ rats. H19 was expressed in almost all of the neoplasms, but not in normal adult liver cells. Igf2 expression was detected in the majority of hepatic neoplasms of female TG+ rats, but in a relatively smaller number of neoplasms of TG+ males. The expression of $p57^{Kip2}$ (Kip2), a cyclin-dependent kinase inhibitor that was also in the imprinted region, exhibited some variable increased expression predominantly in hepatic neoplasms from livers of female TG+ rats. Other imprinted genes within the imprinted gene cluster—insulin II (*Ins2*), Mash2 (which codes for a basic helix-loop-helix transcription factor), and Kvlqt1 (coding for a component of a potassium transport channel)—showed no consistently different expression from that seen in normal hepatocytes. Another gene, also located on the long arm of chromosome 1, that showed changes was the ribonucleotide reductase M1 subunit (*Rrm1*), in which an increase in its expression was found. This was seen in hepatic neoplasms of TG+ rats of both sexes compared with surrounding normal-appearing liver. Because hepatic neoplasms developing in livers of rats treated with chemical carcinogens commonly exhibit an increased expression of c-*myc* mRNA, expression of this gene was investigated in focal lesions and livers of TG+ rats, although c-*myc* was not located on chromosome 1. c-*myc* mRNA was increased in focal lesions, nodules, and neoplasms in both male and female TG+ rats compared with adult and surrounding liver. Immunostaining for c-*myc* protein demonstrated detectable levels in isolated single cells as well as focal lesions and neoplasms. Thus, the enhanced c-*myc* expression, common to all hepatic neoplasms in this system, coupled with enhanced expression of Igf2 in female TG+ rats, may be responsible for the increase in growth rate in hepatic neoplasms of female TG+ rats compared with that in livers of male TG+ rats and may contribute to neoplastic progression in the liver of this transgenic model. Published 2004 Wiley-Liss, Inc.†

Key words: albumin-SV40 transgenic rat; insulin growth factor 2; H19 gene expression; hepatomas; c-*myc*

INTRODUCTION

The pathogenesis of hepatic neoplasia has been elucidated to a significant degree in the rodent [1–3], and the multistage nature of the development of hepatic neoplasia has been best delineated in the rat [4]. In our laboratory, we were interested in developing a model for the characterization, quantitation, and mechanism of each of the stages of initiation,

Abbreviations: IPP, initiation-promotion-progression; T Ag, T antigen; AHF, altered hepatic foci; LOI, loss of imprinting; SD, Sprague-Dawley.

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promotion, and progression [4–6]. Utilizing the Initiation-Promotion-Progression (IPP) protocol developed in our laboratory [7], we demonstrated that a significant number of early neoplastic hepatocytes exhibited specific chromosomal abnormalities: duplication of all or part (1q3.7-1q4.3) of chromosome 1, and the loss of all or part of the short and long arms of chromosomes 3 and 6, respectively [8]. No other significant chromosomal abnormalities were noted in these studies of early, chemically induced lesions with this protocol. Our laboratory, in conjunction with the Biotechnology Laboratory of the University of Wisconsin-Madison, developed a transgenic line of rats in which the transgenic construct was the mouse albumin promoter-enhancer 12-kb region linked 5′ to the SV40 T antigen (*TAg*) gene [9]. As part of the ongoing cytogenetic investigation of hepatic neoplasms in our laboratory, we analyzed the karyotype of hepatic neoplasms occurring in the transgenic strain as well as that of early focal lesions and the surrounding liver when the animals were 3 mo of age. Hepatic neoplasms of the transgenic animals exhibited karyotypic changes strikingly similar [10] to those seen in chromosomes of neoplastic hepatic lesions induced in the IPP protocol. In livers of 3-mo-old transgenic animals that contained focal basophilic lesions, the only significant karyotypic change identified was a trisomy of a region of the long arm of chromosome 1, the smallest common region of duplication being that of 1q4.1. This region was found within the trisomic region noted in chromosome 1 from the lesions induced by the IPP protocol [7]. Neoplasms developing in the livers of transgenic animals exhibited deletions of all or part of the short and long arms of chromosomes 3 and 6 as well as the trisomy of the 1q3.7-1q4. region in chromosome 1.

In view of this striking similarity in the cytogenetic changes seen in spontaneous hepatomas of the transgenic rats and of chemically [8] and virally [11] induced neoplasms of rat hepatocytes, we felt it was important to study the alteration of the expression of genes found in this chromosomal region. Because the most readily reproducible example of this karyotypic change was in hepatic neoplasms of the transgenic rats, we chose to use this model to study the expression of genes in the trisomic region of chromosome 1. This choice was further guided by the fact that the chromosomal regions syntenic to 1q3.7-1q4.3 in the rat are the distal region of mouse chromosome 7q (specifically 69–72 cM) and human 11p15.5 [12]. Several of the imprinted genes in the cluster exhibited aberrant expression and in human liver carcinomas [13,14] and hepatoblastomas [15]. Thus, we have examined the expression of several genes, both imprinted and nonimprinted, within this chromosomal region in the liver and in hepatic neoplasms that arise in the albumin-SV40 T Ag transgenic (TG+) rat.

MATERIALS AND METHODS

Transgenic rats carrying the albumin-SV40 T Ag transgene developed hepatic neoplasms exhibiting the pattern of hepatocellular carcinomas or adenocarcinomas, with 100% incidence by 4–9 mo of age [9]. Hepatoblastoma patterns were noted earlier in the development of these neoplasms (before 4 mo), but in many neoplastic lesions all of the histologic patterns could be identified, although usually one predominated. In general, no difference in the pattern of expression of the genes we studied was seen between neoplasms exhibiting the different histologic patterns. We did not attempt to investigate changes in gene expression during progression of the lesions, but as can be noted from Figure 1, the expression of Igf2, T Ag, and c-*myc* occurred even in small focal lesions as well as in later lesions (Figures 2–4). In the present study, a Sprague-Dawley transgene-negative female was crossed with a transgene-positive male, resulting in F_1 litters that were roughly half transgene-negative and half transgene-positive (genotypically heterozygous for the transgene). The transgene, T Ag, is expressed in a dominant manner in neoplastic hepatocytes in the transgene-positive rats. The time course for the development of T Ag-expressing areas was assessed as a function of the age of the animals.

Immunohistochemistry

At sacrifice, the liver was excised and a portion was fixed in 10% neutral buffered formalin for pathological diagnosis. In addition, sections of the three main lobes of the liver were placed adjacent to one another on filter paper and frozen as a block on solid carbon dioxide. Where appropriate, serial sections of these frozen samples were cut at 10-μm thickness by cryostat and placed on poly-L-lysine-coated slides. The sections were stained with hematoxylin and eosin or for c-*myc* expression. Immunohistochemical staining was performed by an adaptation of the avidin-biotin complex method of Hsu et al. [16]. Endogenous biotin was blocked by an addition of excess avidin, and residual avidin sites were blocked by addition of biotin. Excess biotin was removed by washing. Endogenous peroxidase activity was blocked by an addition of 0.3% hydrogen peroxide in methanol. Non-specific protein binding was blocked by soaking the tissue in a protein-rich solution consisting of 5% normal goat serum with 1% BSA in PBS. T Ag and c-*myc* antibodies were obtained from Oncogene Science (Cambridge, MA) and were used at a 1:200 dilution. The primary antibody for Igf2 was obtained from Upstate Biotech, Charlottesville, VA and was used at a 1:100 dilution. The tissue sections were incubated with one of these primary antibodies overnight at 4°C. The primary antibody was drained, and a biotinylated secondary antibody (goat anti-mouse or goat anti-rabbit IgG) was incubated with

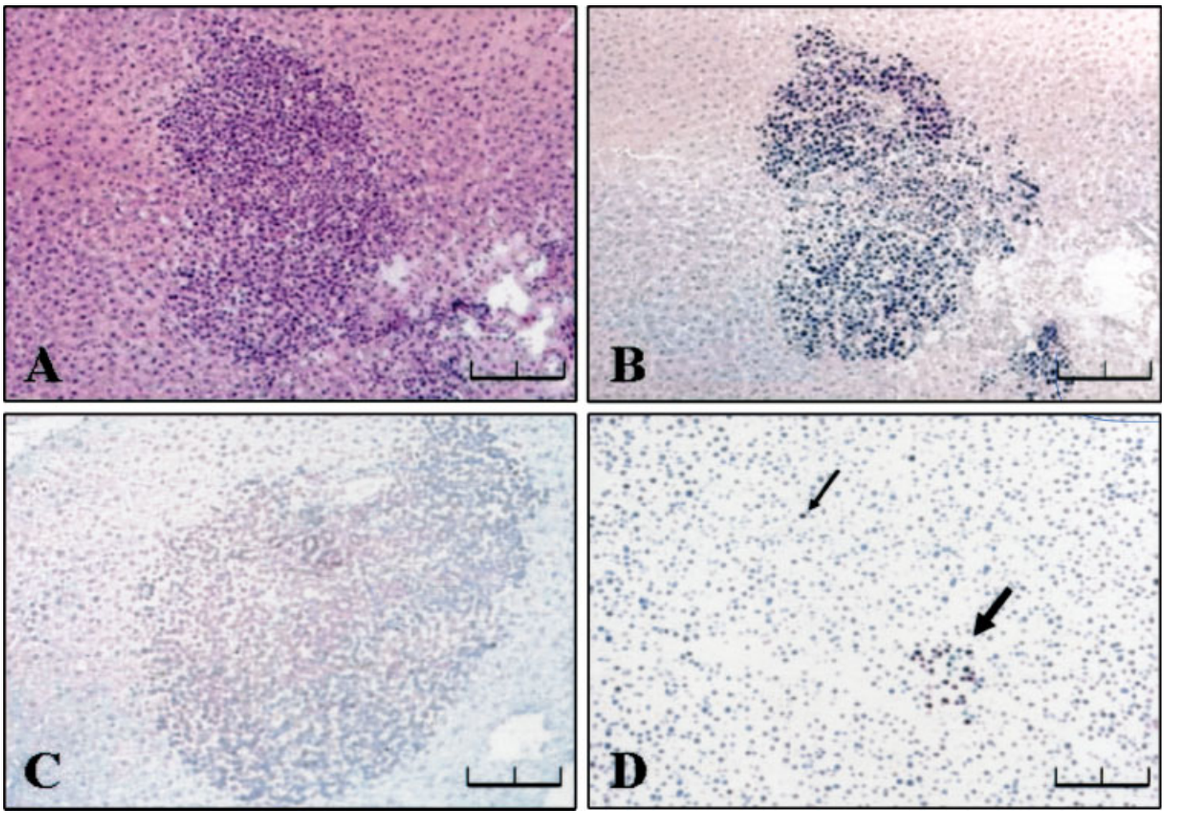

Figure 1. H&E-stained focus in liver of albumin-SV40 T antigen (T Ag) transgenic rat (A); serial section from (A) of the focus stained for T Ag (B); section of focus stained for expression of Igf2 protein by immunohistochemistry (C); and immunohistochemical staining of nuclei of small focus (large arrow) and single hepatocyte (small arrow) for expression of c-*myc* protein by immunohistochemistry (D). See Materials and Methods for techniques utilized. Scale in each photomicrograph represents 0.2 mm.

the tissue section for 30 min. Next, the streptavidin horseradish peroxidase link was added to the sections for 30 min, followed by chromagen development with amino ethylcarbazole (AEC). A tyramide amplification step was included to enhance visualization of this potent growth factor that was present at low concentration.

Stereology and Statistics

The number and size of the altered hepatic foci (AHF) were scored by the method of quantitative stereology as applied to focal hepatic growth by Campbell et al. [17]. Each serial tissue section was projected onto a Summagraphics digitizer, and the AHF were identified and quantitated by their expression of the large T Ag of SV40. The number of AHF per cm^3 of liver was calculated according to the method of Saltykov [18] as adapted to the liver by Campbell et al. [17]. On the basis of the individual liver weights, the number of AHF per liver was then calculated. The method of Delesse [19] was used to calculate the percentage of the liver occupied by AHF. Comparison of differences between groups was done with the nonparametric Mann-Whitney statistic, and $P \leq 0.05$ was considered significant. The criteria for the histological analysis of liver lesions were those of Squire and Levitt [20], Maronpot et al. [21], and those previously described from this laboratory [9].

RNA Expression Analysis

Additional pieces of liver and of visible neoplasms were snap frozen and stored at $-70^{\circ}C$ until analyzed for gene expression. RNA was prepared by the guanidinium-thiocyanate method [22].

For Northern blot analysis of gene expression in hepatic tumors, RNA was isolated from visible liver neoplasms, surrounding liver areas, normal adult Sprague-Dawley livers, and livers of 5-d-old Sprague-Dawley neonates. Thirty micrograms of each RNA sample was electrophoresed and blotted on Hybond-N^+, utilizing the NorthernMax TM kit (Ambion, Austin, TX) with downward capillary transfer, according to the manufacturer's specifications. These blots were hybridized overnight at $42^{\circ}C$ in ULTRAhyb (Ambion) hybridization buffer.

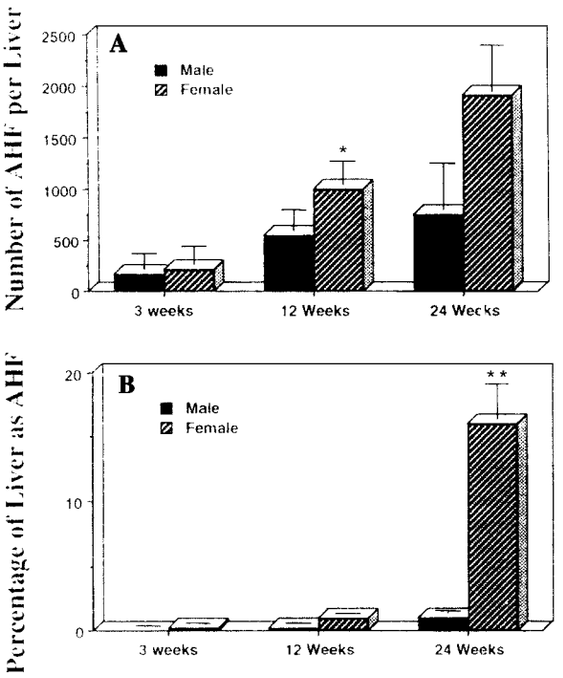

Figure 2. The focal hepatic expression of the large T Ag of SV40 as a function of age and sex in albumin-SV40 T Ag transgenic rats determined from the stereological parameters for the number of altered hepatic foci (AHF) per liver (A) and the percentage of the total liver occupied by the focal lesions (B). The columns represented the number and volume percentage of T Ag-positive foci, and the T at the top of the column, the standard error of the mean. Five to six rats were utilized for each point. $*P < 0.05$; $**P < 0.01$.

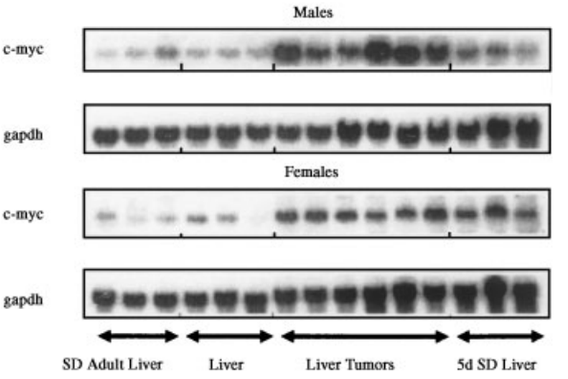

Figure 3. Northern blot analysis of c-*myc* gene expression in livers of male and female albumin-SV40 T Ag transgenic rats. Lanes 1–3 are of liver from three normal adult Sprague-Dawley rats; lanes 4–6 are of grossly normal-appearing liver from tumor-bearing rats; lanes 7–12 are individual liver neoplasms; and lanes 13–15 are from livers of 5-d-old nontransgenic rats. See Materials and Methods for experimental details.

Ambion's Strip-EZTM DNA probe synthesis and removal kit were used to generate and remove randomly primed probes labeled with α^{32}P-dATP (Amersham, Arlington Heights, IL). The blots were consecutively probed with Igf2, H19, Kip2 (p57), Rrm1, c-*myc*, and GAPDH. Blots were washed twice for 5–10 min at 42–48°C with 2× SSC, 0.1% SDS, and twice for 15–20 min at 42–48°C with 0.1× SSC, 0.1% SDS. After quantitation of the radioactivity present with a PhosphorImager (Molecular Dynamics, Amersham Biosciences Corp., Piscataway, NJ), blots were exposed to X-ray film for varying periods of time. Similar blots were carried out with RNA isolated from liver and neoplasms of the TG+ rats by the following probes: Mash2, Kvlqt1, cyclin D1, preproinsulin 2, Ha-*ras*.

The sources of the probes used to examine gene expression on these blots are as follows. The Igf2 cDNA was obtained from Dr. J. Eggenschwiler of Columbia (as the Efstratiadis clone 27), and the 540-bp *Eco*RI to *Bam*HI insert was used as a probe. The rat cDNA for H19 was obtained from Dr. Leibovitch of the Laboratory of Molecular Oncology of the Institute Gustave Roussy and was excised as a 2.3-kb insert with *Eco*RI. Mouse Mash2 cDNA was obtained from Dr. Andras Nagy of Mount Sinai Hospital in Toronto. The mouse Kip2 cDNA was obtained from Dr. Stephen Elledge of Baylor University. The rat Kvlqt1 probe was a gift from Dr. Koichi Takimoto (University of Pittsburgh) and was a 933-bp partial cDNA clone obtained by digesting the PGEM1 plasmid with *Apa*I and *Sac*I. The c-*myc* probe was a 0.5 kb *Pvu*II-*Hind*III fragment from a pMMTV-Sma *myc* clone obtained from ATCC. The Tssc3 probe was obtained from ATCC (#63409) consisting of a mouse clone. A 1.0-kb insert was cut out with *Eco*RI and *Not*I and used as a probe. The rat cyclin D1 clone pHsCYCD1-H1223 was a gift from Dr. David Beach, and the 610-bp fragment excised with *Nco*I was used as a probe. A rat preproinsulin 2 clone was obtained from ATCC (#67109), and a 170-bp *Bam*HI to *Hind*III insert was used as a probe. The 670-bp *Hind*III to *Pst*I insert from the ATCC HB-11 clone (#41013) was used as a probe for Ha-*ras*. A clone containing human Rrm1 cDNA was obtained from ATCC (#99484) and a 2.38-kb insert was excised with *Sac*I and *Pst*I digestion. The rat GAPDH probe was obtained from Dr. Norman Drinkwater (McArdle Laboratory), and the 1.3-kb *Pst*I digestion product was used as a probe. Probes were randomly labeled with ^{32}P-dCTP (Amersham) with either the Megaprime or Redi-prime kit (Amersham).

RESULTS

T Ag and c-*myc* Proteins in Foci, Nodules, and Neoplasms

Although large neoplasms and nodules were readily apparent grossly in livers of TG+ rats, microscopic focal lesions could be discerned only by histologic examination. Normal (non-transgene-bearing) littermates did not develop preneoplastic or neoplastic lesions during the course of this experiment, as would be expected for young Sprague-Dawley rats. Livers of TG+ littermates contained small numbers

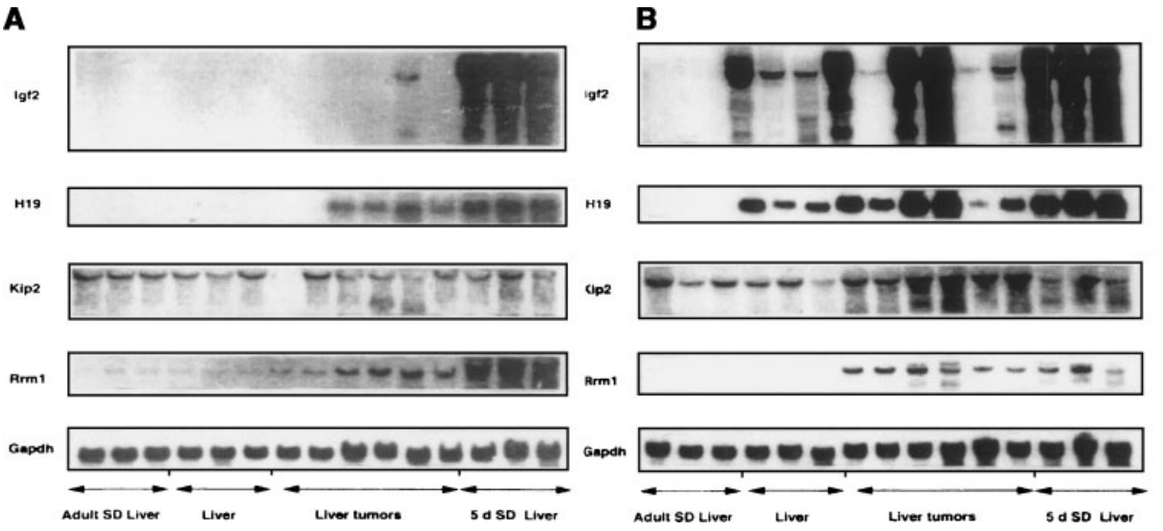

Figure 4. Northern blot analysis of gene expression in livers and neoplasms of male (A) and female (B) transgenic and non-transgenic (SD) rats. The description of the lanes is the same as in Figure 3. See Materials and Methods for further experimental details.

of T Ag-positive focal hepatic lesions at the earliest time point examined (3 wk of age). Malignant neoplasms were not detected in this series of animals at 3 wk of age, but by 12 wk, a 4% incidence (2 of 37 rats) of hepatic neoplasms was observed in the TG+ rats, both females. TG+ rats at 24 wk of age had a marked increase in incidence of malignant neoplasms, in that 50% of the rats (8 of 16), predominantly females, contained hepatic neoplasms. All of the TG+ rats contained focal basophilic hepatic lesions expressing T Ag. These lesions were largely basophilic, as previously reported [9]. One example may be seen in Figure 1A. These focal basophilic hepatic lesions exhibit large T Ag expression as well as expression of c-*myc* (Figure 1B and D). These lesions are histologically and immunochemically distinct from the clear eosinophilic lesions that arise spontaneously but infrequently in these rodents [23].

The focal expression of large T Ag (Figure 1B) can be quantitated to ascertain the number and growth of the transgene-induced hepatic lesions (Figure 2). At 24 wk, there was a significant elevation by stereologic analysis in the number of T Ag-positive foci and small nodules compared with the 3-wk time point in female, but not in livers of male TG+ rats (Figure 2, upper panel). A significant difference in the number of hepatic T Ag-positive foci between the male and female transgene-positive progeny was observed only at 24 wk.

The gender difference was even more striking when the percentage of the liver occupied by T Ag-positive foci was examined (Figure 2, lower panel). This parameter reflects changes in the cell number within the foci and, therefore, growth of the focal lesions. The total volume occupied by these microscopic lesions was not significantly different in livers of TG+ males and females at the 3-wk time point, but the percentage of the liver occupied by T Ag, positive lesions was significantly greater in female than in male TG+ siblings at both 12 and 24 wk of age. In males, the percentage of liver occupied by T Ag-positive lesions was significantly increased at 24 wk compared with either 3 or 12 wk. In the females, the volume fraction of T Ag-expressing focal areas was significantly elevated at each of the time points examined after 3 wk. Lesions expressing the placental form of glutathione transferase, as seen in the livers of rats treated with chemical carcinogens, were rarely found [24].

As noted in Figure 1D, expression of c-*myc* at the protein level was detectable by immunostaining in all focal hepatic lesions arising in the livers of these TG+ rats. Previous studies demonstrate that the T Ag of the SV40 virus sequesters and binds the p53 protein as well as other nuclear DNA-binding proteins, such as members of the retinoblastoma protein family and p300 [25]. c-*myc* protein is detectable in nuclei of very early focal lesions that express the T Ag as well as in isolated single cells (Figure 1D) in which we have not noted the expression of T Ag. At least in vitro, functional c-*myc* is necessary for the induction of DNA synthesis by the SV40 T Ag [26]. We thus examined hepatic neoplasms from these transgenic rats for the expression of c-*myc* mRNA (Figure 3). There was a substantial increase in the expression of c-*myc* in the neoplasms of transgenic animals, both male and female, compared with the surrounding liver and nontransgenic liver. In TG+ males the expression appeared to be somewhat higher in most instances and was significantly higher than in neonatal liver from nontransgenic rats.

Expression of Genes in the 1q3.7-1q4.3 Region

Previous studies on the cytogenetic changes that accompany rat hepatocarcinogenesis in this and other models [8,11] indicate that the earliest detectable change is a duplication of all or part of rat chromosome 1. The minimal region of non-overlapping duplication in the TG+ rats is 1q4.1 [10]. This region contains a cluster of imprinted genes [27] that are present in syntenic regions of human chromosome 11p15.5 [28] and the distal portion of mouse chromosome 7 [29]. Thus, the expression of a number of genes in the imprinted cluster were studied in liver neoplasms, in samples of liver showing no gross evidence of neoplasms, and in livers from control adult and 5-d-old Sprague-Dawley rats. Some of these data are seen in Figure 4. The expression of *H19*, *Igf2*, and *Kip2*, imprinted genes within the cluster, showed differential expression of mRNA in rats carrying the transgene from age- and sex-matched controls. Igf2 and H19 are not expressed in hepatic tissue of adult rats of either sex. The expression of Igf2 in livers of female TG+ (Figure 4B) may be due to the presence of focal lesions and small nodules in the tissue grossly identified as nonneoplastic liver (Figure 1B). However, in the case of Igf2, it may be argued that the expression was due to contaminating foci and small nodules that were present in the tissue, because immunohistochemistry indicated that only the focal lesions expressed Igf2 (Figure 1C). This argument could not be used for the expression of H19, which has no known protein counterpart, because its expression could only be determined by in situ hybridization on microscopic slides. However, an alternative explanation is that in females hepatocytes surrounding preneoplastic and neoplastic lesions do in fact express these two genes. Abnormal expression of these genes in nonneoplastic tissue adjacent to breast carcinomas has been reported in the human [30,31]. Also significant is the fact that no expression of *Rrm1*, a nonimprinted gene, occurs in adult nontransgenic or in surrounding liver of TG+ rats in either males or females.

As noted in Figure 4A, only one of six neoplasms in TG+ males expressed even a low level of *Igf2*, whereas all six neoplasms from females expressed this gene, albeit two of them at relatively low levels. The multiple bands seen in most of the samples showing Igf2 expression have been reported previously [32] and in part are due to the multiple promoters of this gene. H19 and Igf2 are both expressed in neonatal liver, but not in adult Sprague-Dawley liver in either sex (Figure 4). H19 expression occurred in all six hepatic neoplasms in the females and apparently in only four in the males. Longer exposure of chromatograms revealed that the two neoplasms showing no apparent expression of H19 exhibited expression of this gene. In a more extensive study (Table 1), 24 of 26 hepatic neoplasms from females and 21 of 25 from TG+ male rats expressed H19. Thus, the expression of H19 was found in most of the neoplasms examined in both sexes. The expression of the imprinted gene, *Kip2*, was seen in essentially all samples of both livers and neoplasms, but neoplasms from female TG+ rats consistently showed an increased expression of this gene. In another study (data not shown), six out of seven hepatic neoplasms from TG+ female rats exhibited a significant increase in the expression of Kip2. The expression of several additional genes present in the imprinted cluster [27], including Kvlqt1 (Kcnq1), coding for a protein involved in potassium ion transport; Ins2, coding for a preproinsulin; Tssc3, coding for a gene implicated in Fas-mediated apoptosis [33]; and Mash2, which encodes a basic helix-loop-helix transcription factor [34] was also examined. None of these genes were reproducibly differentially expressed in the livers or neoplasms of the rats examined (data not shown).

We also attempted to examine the expression of several nonimprinted genes whose positions were near the imprinted cluster. Of those genes studied—Ha-*ras*, the placental form of glutathione *S*-transferase, cyclin D1, and Rrm1—only Rrm1 showed significant differential expression in the hepatic neoplasms studied (Figure 4A and B), in the TG+ surrounding liver, and in adult nontransgenic liver. As with the imprinted genes *H19* and *Igf2*, significant expression of this gene occurred in neonatal nontransgenic liver. The product of Rrm1 is necessary for DNA synthesis and has been shown to be expressed in hepatic neoplasms in previous studies [35].

DISCUSSION

The principal and, until recently, the only way to induce hepatocarcinogenesis in the rat was by the administration of carcinogenic chemicals. Over the last two decades a number of model systems of chemically induced hepatocarcinogenesis, several of which delineate two or more of the stages of hepatocarcinogenesis [7,36,37], have been developed in an attempt to understand the pathogenesis of neoplasia after administration of chemicals. The development of transgenic models of carcinogenesis permits study of the mechanism of hepatocarcinogenesis in which the expression of a transgene in the TG+ rat is carcinogenic [9]. The SV40 viral oncogene has been utilized as a transgene under the regulation of a variety of promoters and enhancers in the mouse [38,39] and the rat [9] for the production of neoplasia. The TG+ rats of the strain utilized in this study all expressed T Ag in focal, nodular, and malignant lesions in the liver. These lesions appeared spontaneously in about equal numbers in males and females, but the growth of the focal lesions and early nodular lesions was much greater in the livers of female TG+ rats (Figure 2). The observed gender

Table 1. Expression of *IgF2* and *H19* mRNAs in a Series of Albumin-SV40 T Antigen (T Ag) Positive Hepatic Neoplasms in the Rat*

Females			Males		
Neoplasm no.	Igf2	H19	Neoplasm no.	Igf2	H19
1	H	H	1	M	M
2	M	M	2	M	T
3	H	H	3	T	T
4	—	—	4	—	—
5	L	M	5	—	—
6	T	L	6	T	M
7	L	L	7	M	H
8	L	L	8	—	H
9	H	H	9	—	H
10	M	H	10	—	L
11	M	H	11	—	—
12	—	M	12	—	—
13	M	H	13	—	H
14	M	H	14	—	T
15	M	M	15	—	T
16	—	M	16	—	M
17	M	M	17	—	M
18	H	H	18	M	M
19	—	L	19	—	L
20	L	M	20	L	H
21	L	L	21	—	T
22	L	L	22	T	M
23	M	H	23	—	L
24	—	—	24	—	L
25	M	M	25	—	L
26	L	H			

*Each neoplasm was examined by Northern analysis for expression levels of the mRNAs of each of the two individual genes, *Igf2* and *H19*.
Tumors tested for expression levels of both genes: H, high; M, medium; L, low; T, trace; —, not detected. See Materials and Methods for details of analysis.

differences may result from the different hormonal environment of the male and female. Estrogens and other steroid hormones may directly or indirectly affect T Ag expression [40]. Estrogen may also play a role in the relatively high levels of expression of the growth factor, Igf2, which is expressed in both the neoplasms and the surrounding liver in female TG+ rats, but not in most male TG+ rats. Promoter 3 of the rat *Igf2* gene is the major contributor to the development of the Igf2 transcript family [41], and this promoter is to a great extent highly conserved across species. In the mouse, binding of an AP1 complex contributes to the regulated expression of the Igf2 promoter [42], indicating a possible role for the estrogen receptor in the transactivation of this gene [43]. Thus, the estrogen receptor dependence on AP1-activation of Igf2 may contribute to the earlier onset and higher growth rate of T Ag-expressing hepatic lesions in the female compared with the male TG+ rats.

The carcinogenic potency of the SV40 T Ag is largely due to the many protein products and cellular processes perturbed by this oncogenic protein [44,45]. Besides its sequestration and hence inactivation of p53, T Ag can bind pocket proteins, the best known of which is the retinoblastoma protein and many of its family of proteins, as well as the ubiquitous p300 protein [45]. Undoubtedly, these interactions are important in the carcinogenic action of the oncogenic viral protein, but other properties also probably contribute to its carcinogenic action. In cultured cells, c-*myc* is required for the T Ag induction of DNA synthesis in these cells [26], while the maintenance of cell transformation in culture seems to require both a functional large T Ag and a metabolically stabilized p53 [46]. Enhanced c-*myc* expression has been described in many examples of rodent [47,48] as well as human hepatocellular carcinomas [50]. In rat hepatocarcinogenesis, increased expression of c-*myc* occurs primarily in the later stages of neoplastic development, although some increased expression may be seen in foci and nodules [49,51]. Interestingly, in chemically induced hepatic nodules and carcinomas, males exhibit a higher expression of c-*myc* than do females [52]. In many instances, both in the human [50] and in animal

models [49], the increase in c-*myc* is due to an amplification of the gene. The enhanced expression of this gene, seen in Figure 3, conforms to these findings in chemically induced hepatic neoplasms, but we do not know whether the increased expression is due to amplification. However, the finding of increased c-*myc* expression in nuclei of single hepatocytes in 3- and 12-wk-old TG+ rats (Figure 1D) suggests that this change occurs extremely early in this model.

While it is clear that T Ag expression, together with p53 sequestration and enhanced c-*myc* expression, probably played major roles in the development of neoplasia in these TG+ rats, the cytogenic abnormalities noted and their similarities to such abnormalities in chemically induced hepatocarcinogenesis have stimulated a closer investigation of the expression of genes in the duplicated region of the long arm of chromosome 1, 1q3.7-1q4.1 [8,10]. This is especially true because this is the duplication seen in at least a third of the metaphases occurring in the livers of TG+ rats that have essentially no neoplasms and relatively few small foci and nodules [10]. The potential number of genes in the 1q4.1-1q4.3 region of chromosome 1 is somewhat over 700, as now evidenced from data obtainable through the rat genome database [53,54]. We have made a study of the expression of several other genes and ESTs in this region (Nesterova and Pitot, unpublished observations) and found no alteration in the neoplasms. Thus, the altered expression of the genes described in this study represents the principal positive result of our efforts. The regions in the mouse and the human that were syntenic with this region in the rat, respectively 7q and 11p1.3-11p1.5, reported to be associated with the development of specific neoplasms in these species. Specifically, a trisomy of chromosome 7 occurs in the early development of mouse epidermal carcinomas [55], and a deletion of the 11p1.3-11p1.5 region is found in a significant number of Wilms' tumors [56] and lung carcinomas [57] in the human.

The paternally imprinted gene, *H19*, located in close proximity to the maternally imprinted gene, *Igf2*, is first isolated on the basis of its expression during embryogenesis and differentiation [58,59]. In embryonic muscle cells, H19 expression is reportedly associated with a tumor suppressor activity [60]. The role of H19 expression is unknown, because a protein product has not been ascribed to this gene, prompting the suggestion that it may function as an RNA [61]. H19 is expressed monoallelically in a variety of fetal tissues, but in the adult only muscle, thymus, and lung express the gene [62]. However, H19 has been shown to be expressed in a variety of neoplasms, especially in the human, where the expression is biallelic with loss of imprinting (LOI) [63,64]. Studies in our laboratory [64] have demonstrated biallelic expression of H19 in TG+ rats. Overexpression of H19 reportedly enhances tumor progression in human breast cancer cells [65]. With cDNA microarray technology to determine genes upregulated by the expression of H19, Ayesh et al. [66] came to similar conclusions for a role of H19 expression in promoting cancer progression, angiogenesis, and metastasis. Vernucci et al. [67] demonstrate that the H19 endodermal enhancer is required for Igf2 activation as well as the development of neoplasms in experimental liver carcinogenesis in transgenic mice.

The insulin-like growth factor 2 gene product is a potent rat liver mitogen [68] that can additionally inhibit apoptosis [69]. These two actions of this growth factor can lead to a selective growth advantage for cells expressing Igf2. The expression of the *Igf2* gene has been associated with the progression of neoplasms in several transgenic models of hepatocarcinogenesis [70,71]. Re-expression of Igf2 has additionally been implicated as a late event in human hepatocellular carcinoma development [72], as well as in several models of rat hepatocarcinogenesis [67,73]. Expression of Igf2 is generally low in the adult liver, and re-expression has been attributed to several possible factors, including a LOI at the Igf2 locus [74], a loss of heterozygosity of the H19 locus or of its regulatory region [75,76], or an altered methylation status of the *Igf2* gene [77,78]. Human hepatoblastomas differ from Wilms' tumor in that H19 expression may not be depressed in concert with an increase in Igf2 expression, indicating that LOI of this locus is not the only explanation for increased Igf2 expression [79]. Mouse transgenic models of hepatocarcinogenesis induced by T Ag expression also demonstrate co-expression of these reciprocally imprinted genes [80,81].

Although, it is clear that T Ag expression is a driving force in the transformation of hepatocytes to neoplasia in TG+ rats, the striking similarity of the trisomy of the 1q3.7-1q4.3 region of the karyotype in early and neoplastic lesions of TG+ rats, rats treated with the IPP protocol [8], and hepatocytes transfected with an oncogenic virus [11] suggests an additional component in hepatocarcinogenesis by all three of these mechanisms. In TG+ rats the LOI and overexpression of H19 in almost all neoplasms, as well as the overexpression of Igf2 predominantly in neoplasms of female rats, may contribute significantly to the growth rate of these lesions, especially in females (Figure 2). Because biallelic expression of both the *H19* and *Igf2* genes has been reported in primary hepatocellular carcinomas [74], and because the carcinogen diethylnitrosamine, which is used in the IPP protocol [7], appears to induce Igf2 expression during the early stages of hepatocarcinogenesis [82], it is possible that altered expression of these imprinted genes during chemically induced hepatocarcinogenesis may play a role similar to that proposed here for transgenic hepatocarcinogenesis.

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