Expression of c-Ki-ras, c-Ha-ras, and c-myc in Specific Cell Types During Hepatocarcinogenesis

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We examined the expression of six proto-oncogenes in (i) whole rat liver and isolated liver cell populations during the course of hepatocarcinogenesis induced by a choline-deficient diet containing 0.1% ethionine and (ii) fetal rat liver at different stages of development. The abundance of c-Ki-ras, c-Ha-ras, and c-myc transcripts in polysomal polyadenylated RNA from liver cells increased by 2 weeks after the start of the carcinogenic diet. c-Ki-ras and c-myc expression remained elevated during the 35 weeks of the diet, whereas c-Ha-ras transcripts increased transiently. A primary tumor sampled at 35 weeks after the carcinogenic diet was started contained high levels of both c-Ki-ras and c-myc RNA. The abundance of c-src transcripts was unchanged throughout carcinogenesis; c-abl and c-mos transcripts were not detected in either preneoplastic or neoplastic livers. To determine which cell types within the liver contained proto-oncogene transcripts, we isolated hepatocytes, oval cells, and bile duct cells from normal and preneoplastic livers. The results indicate that proto-oncogenes are expressed differentially in these cell types during hepatocarcinogenesis and that the expression of c-Ki-ras and c-myc is high in oval cells throughout carcinogenesis. In developing livers, c-Ki-ras, c-Ha-ras, and c-myc transcript levels were high at 17 days of gestation but reached the low values characteristic of adult rat livers between 20 days of gestation and 3 days after birth.

The activation of proto-oncogenes has been implicated in transformation in vitro and tumorigenesis in vivo, but the role of these genes in the sequential events leading to tumor formation is as yet unclear. Temporal changes in proto-oncogene transcript abundance have been noted during development (27, 28), regeneration (16, 17), and preneoplasia (1) in vivo as well as during differentiation and stages of the cell cycle in vitro (3, 6, 15, 22). Although the functions of proto-oncogenes in normal tissue have not been fully characterized, it is generally assumed that they are involved in control of growth.

Liver carcinogenesis is an established experimental system for the study of cancer induction in animals. Profound changes in the cellular and biochemical composition of the liver accompany hepatocarcinogenesis. Traditional theories have argued that during carcinogenesis, mature hepatocytes (parenchymal cells) "dedifferentiate," losing some properties of the differentiated state and reacquiring characteristics of fetal liver cells (40). Alternative theories suggest that "stem cells," which are induced to proliferate by carcinogens and can be prevented from completing differentiation, might exist in mature livers (26, 32). Evidence from this laboratory, as well as from others, suggests that "oval cells," which often proliferate during early preneoplastic stages of chemical hepatocarcinogenesis (10), fulfill some of the criteria expected for liver stem cells (21, 24, 35, 42). Oval cells contain oncodevelopmental gene products such as alpha-fetoprotein and fetal liver isozyme forms of aldolase and pyruvate kinase (8, 19-21, 23, 42). Moreover, oval cells contain hybrids of fetal and adult forms of aldolase and pyruvate kinase as well as functional markers associated specifically with mature hepatocytes (such as albumin mRNA), indicating the transitional nature of some of the oval cells (21, 29, 42).

We have developed methods for the purification of oval cells and hepatocytes from preneoplastic livers of rats fed a carcinogenic diet which induces widespread oval cell proliferation before the development of well-defined hepatocellular carcinomas. This system has enabled us to explore the following questions about proto-oncogene expression during hepatocarcinogenesis. (i) Do changes in proto-oncogene transcript abundance accompany early preneoplastic stages of hepatocarcinogenesis? (ii) Are proto-oncogene transcripts differentially expressed in various cell populations in preneoplastic livers? (iii) Can the proliferation of a particular cell type (oval cells or hepatocytes) be correlated with increased levels of proto-oncogene transcripts? (iv) Are the transcripts of proto-oncogenes in liver cell populations during hepatoma induction similar (quantitatively and qualitatively) to the transcripts of the genes in the actual tumors? (v) Are the patterns of proto-oncogene expression which characterize hepatocarcinogenesis similar to patterns which occur at different stages of development?

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 130 to 150 g were fed a standard laboratory chow, a choline-deficient diet containing 0.1% ethionine (CDE diet), or a choline-deficient diet alone (CD diet) (36); the last two diets were prepared by Teklad Test Diets, Madison, Wis. Rats receiving the carcinogenic diet were killed 2 to 35 weeks after the diet was started. Neoplastic nodules which developed in the liver of a rat given the CDE diet for 35 weeks were excised from surrounding tissue and processed without further cell separation. Fetal rats were obtained from Sprague-Dawley rats at 17 and 20 days of gestation; neonatal rats were killed 3 and 14 days after birth. Livers removed from fetal or neonatal rats were immediately frozen in liquid nitrogen and stored at -70°C. To induce the proliferation of bile ducts in the liver, the common bile ducts of two adult Sprague-Dawley rats

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were ligated. These animals were kept on standard laboratory chow until they were killed 3 to 4 weeks after the operation.

Preparation of polysomal poly(A)⁺ RNA from whole livers. Livers were homogenized, and polysomes were isolated by magnesium precipitation as previously described (11, 31, 34). Polysomal RNA was extracted with phenol-chloroform and fractionated by polyuridylylate-Sepharose affinity chromatography to obtain polyadenylated [poly(A)⁺] RNA.

Cell separation and purification. The isolation of oval cells, bile duct cells, and hepatocytes from rat livers was performed as described previously (42), with minor modifications: (i) elutriated fractions 3, 4, and 5 were pooled to maximize the yield of oval cells and bile duct cells from preneoplastic and bile duct ligated livers; (ii) the elutriation step was omitted entirely in the isolation of bile duct cells from normal livers because of the low abundance of these cells; (iii) to increase the purity of hepatocytes obtained from preneoplastic livers, the elutriation rotor speed was kept at 1,000 rpm, and cell fractions were collected at pump speeds of 15, 20, 25, 30, and 40 ml/min; fractions collected at 20 to 40 ml/min were either pooled or prepared separately for RNA extraction.

Isolation of total RNA from purified cells. Total cellular RNA was isolated from purified cell preparations as well as from fetal and neonatal livers by using the guanidine thiocyanate method of Chirgwin et al. (4). Cell pellets or whole tissues were homogenized in 0.5 ml of guanidine thiocyanate homogenization buffer, passed three times through a 21-gauge needle, diluted to 8.5 ml with homogenization buffer, and layered onto a 3.5-ml cushion of 5.7 M CsCl in 25 mM sodium acetate (pH 5). RNA was pelleted by centrifugation for 20 h at 28,000 rpm in a Beckman SW40 Ti rotor. RNA pellets were rinsed with ethanol and redissolved in 0.1 ml of water, to which was added 0.65 ml of guanidine hydrochloride buffer and 0.02 ml of 1 M acetic acid. RNAs were further purified by two successive precipitations with 0.5 and 2 volumes of ethanol.

Hybridization and DNA probes. ³²P-labeled probes were prepared by nick translation (33) of the following oncogene clones: the BS-9 fragment for *Ha-ras* (9); the HiHi380 fragment for *Ki-ras* (9); the 2.2-kilobase (kb) *Pst*I fragment of *v-myc* (41); the 4.5-kb *Bam*HI-*Xho*I fragment of λ *Momyc1* containing all three exons (39) or p*Momyc1.8*, a subclone containing sequences in the 5' *c-myc* exon (D. Steffen, personal communication) for *myc*; the pHT10 insert of *v-mos* for *mos* (30); the 3.0-kb *Eco*RI fragment of *v-src* for *src* (7); and the pABLsub9 insert for *abl* (J. Y. W. Wang and D. Baltimore, personal communication). RNAs were either spotted directly onto 20× SSC-treated nitrocellulose filters (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) or separated in 1.1% agarose-formaldehyde gels and transferred to nitrocellulose filters (2). For size markers on Northern blots, either ³²P-end-labeled *Hind*III fragments of phage lambda DNA were included in gels or ribosomal RNA bands in gels were stained with ethidium bromide and photographed under UV illumination. Standards ranging from 1 to 100 pg of each oncogene DNA were included on dot blot filters to determine the sensitivity of the procedure and the specificity of the probes (17). The filters were incubated with the labeled probes in hybridization buffer for 72 h at 42°C as described previously (16), except for the filters hybridized with the HiHi380 probe, which were incubated at 50°C (17). Ribosomal RNA contamination of poly(A)⁺ RNA was estimated by hybridization of appropriate filters with a cloned *Xenopus* 18S rDNA fragment

provided by S. Gerbi. After hybridization, the filters were washed and autoradiographs were prepared. Appropriate autoradiographs were quantified by scanning at 550 nm in a recording spectrophotometer. As previously shown (17), there is a linear relationship between the amounts of RNA blotted on the filter and the absorbance of the bands on Northern blots. Filters were hybridized with different probes after removal of the previous probe by incubation in 0.1× SSC–0.1% sodium dodecyl sulfate at 100°C and preparation of an autoradiogram to ensure adequate removal of the label.

RESULTS

Proto-oncogene transcripts in whole livers and tumors. We determined the size and abundance of *c-Ki-ras*, *c-Ha-ras* and *c-myc* transcripts in polysomal poly(A)⁺ RNA obtained from whole livers of rats maintained on the CDE diet, standard rat chow, or the CD diet for 2 to 35 weeks (Fig. 1; Table 1). The CDE diet, which contains the carcinogen ethionine, leads to the development of hepatocellular carcinomas in rats fed the diet for 30 to 35 weeks. The CD diet, which does not contain ethionine, does not lead to oval cell proliferation or tumor formation within the period studied and so serves as a control diet. In both the CD and CDE diets, a small degree of hepatocyte replication takes place, probably in response to cell death (13, 14).

The polysomal poly(A)⁺ RNA preparations used had similar poly(A) contents. Hybridization of the same filter with various probes (oncogenes, actin, alpha-fetoprotein, albumin) showed a lack of change (*src*), no detectable expression (*mos*, *abl*), or increased expression characteristic for each gene (*Ha-ras*, *Ki-ras*, *myc*, actin, alpha-fetoprotein, albumin) in preneoplastic livers and tumors. Only the oncogene data are presented here. The abundance of *c-Ki-ras*, *c-Ha-ras*, and *c-myc* transcripts changed during carcinogenesis, with each transcript displaying a specific pattern. As previously reported (17), the *Ki-ras* probe hybridizes with liver RNAs of approximately 5.0 and 2.1 kb in length (Fig. 1a). The size of the transcripts was invariant throughout the course of carcinogenesis and in the primary tumor. The abundance of the two *Ki-ras* transcripts in polysomal poly(A)⁺ RNA increased significantly by 2 weeks after the start of the CDE diet but changed little in livers of animals that were fed the CD diet. In the experimental series shown in Fig. 1, the 5.0- and 2.1-kb transcript levels were 15-fold and 8-fold higher than normal at 2 weeks after the start of the carcinogenic diet (Table 1). The abundance of these transcripts in polysomal poly(A)⁺ RNA from liver cells, although variable, remained elevated up to the time of development of tumors. In liver RNA after 17 weeks of the diet (the latest time surveyed before the emergence of the primary tumor), as well as in the sampled tumor, the 2.1-kb transcript was 10-fold higher in abundance than normal, and the 5.0-kb transcript was at least 20-fold higher in abundance than in normal liver.

In normal, preneoplastic, or neoplastic livers, the *Ha-ras* probe hybridized with a 1.2-kb transcript. In contrast to the sustained elevation of *Ki-ras* transcript abundance, *Ha-ras* transcript abundance changed only slightly during hepatocarcinogenesis. The *Ha-ras* transcript level was approximately threefold higher than normal in rats on the CDE diet for 2 weeks but was at or slightly below normal levels throughout the rest of the feeding period (Fig. 1b; Table 1). A small increase in the *Ha-ras* transcript abundance in the primary tumor and in rats that were fed the CD diet for 9 weeks was also detected. Although small and difficult to

FIG. 1. Analysis of *c-Ki-ras* (a), *c-Ha-ras* (b), and *c-myc* (c) mRNAs during liver carcinogenesis. Polysomal poly(A)⁺ RNAs (5 µg) from normal adult rat livers (N), livers of animals maintained on the CDE diet for 2, 4, 8, or 17 weeks, tumor nodules excised from the liver of a rat maintained on the CDE diet for 35 weeks (35T), or livers of rats maintained on the CD diet alone for 2, 4, or 9 weeks were fractionated in 1.1% agarose-formaldehyde gels, blotted to nitrocellulose, and hybridized to ³²P-labeled probes as described in the text. The probes used were HiHi380 (*Ki-ras*), BS-9 (*Ha-ras*), and the 4.5-kb *Bam*HI-*Xho*I fragment of λ *Momyc*1 (*myc*). The marker lane (M) contained a *Hind*III digest of phage λ DNA end-labeled with ³²P.

TABLE 1. Relative amounts of *c-Ki-ras* and *c-Ha-ras* mRNA in polysomal poly(A)⁺ RNA^a

RNA source ^b	Amt of <i>c-Ki-ras</i> mRNA:		Amt of 1.2-kb <i>c-Ha-ras</i> mRNA
	5.0 kb	2.1 kb	
Norm	1	1	1
Norm	<1	<1	1
2CDE	15	8	3
4CDE	6	6	1
8CDE	15	6	<1
17CDE	>20	10	<1
35T	>20	9	2
2CD	1	2	<1
4CD	1	<1	<1
9CD	2	2	2

^a Appropriate autoradiographs (in linear range of spectrophotometer) were quantified spectrophotometrically by scanning at 550 nm in a recording spectrophotometer. Absorbance values for normal liver (Norm) in the first row were arbitrarily assigned a value of 1.

^b Abbreviations are the same as in Fig. 1.

quantify accurately, the transient changes in *Ha-ras* transcript levels were consistently detected in several different groups of animals that were fed different batches of the CDE diet.

The determination of the sizes of *c-myc* transcripts during liver carcinogenesis was complicated by the lack of resolution of discrete RNA bands (Fig. 1c). This pattern is not due to nonspecific RNA degradation, since the same filters hybridized with *ras* probes showed well-resolved transcripts. An increase in the abundance of *c-myc* transcripts was consistently detected after 2 weeks of the CDE diet (the earliest time point examined) and was maintained throughout carcinogenesis and in the primary tumor, although the extent of the increase varied from experiment to experiment.

The expression of three additional proto-oncogenes (*mos*, *abl*, and *src*) was examined and found to be either undetectable (*mos* and *abl*) or unchanged (*src*) in whole liver and tumor polysomal poly(A)⁺ RNA from animals on the CDE and control diets (data not shown).

Proto-oncogene transcripts in isolated cell populations. Hepatocytes and oval cells can both be purified from preneoplastic livers up to 4 weeks after the start of the CDE regimen. Although oval cells can be isolated with the same degree of purity at later times, this is not possible for hepatocytes because hepatocyte fractions isolated from the livers of rats on the CDE diet for 6 weeks or more are heavily contaminated with other cell types. In the livers of rats maintained on the CDE diet, oval cells were first detected at 2 to 4 weeks of feeding. The mean recovery of oval cells after cell dissociation and elutriation of nonparenchymal cells from the livers of single animals on the diet was 5.2×10^6 , 1.7×10^8 , 4.7×10^8 , and 5.1×10^8 cells at 2, 4, 9, and 16 weeks of feeding, respectively.

Total RNA was prepared from purified oval cells and hepatocytes from preneoplastic livers and from hepatocytes and bile duct cells from normal livers. The RNA was separated by electrophoresis in 1.1% agarose-formaldehyde gels, blotted onto nitrocellulose filters, and hybridized with *Ki-ras*, *Ha-ras*, and *myc* probes. In comparison with normal hepatocytes, *c-Ki-ras* transcript levels were elevated in hepatocytes and oval cells isolated from carcinogen-fed animals (Fig. 2a and 3a; Table 2). Analysis of six different samples of hepatocytes and five samples of oval cells suggested that *Ki-ras* expression increases progressively in oval cells, whereas in hepatocytes it reaches a maximum at 2

FIG. 2. Analysis of *c-Ki-ras* (a), *c-Ha-ras* (b), and *c-myc* (c) mRNAs in total RNA prepared from whole tissues and purified cells. Total RNAs (20 μ g) from 17-day fetal liver (FL-17d), 20-day fetal liver (FL-20d), 3-day neonatal liver (NN-3d), 14-day neonatal liver (NN-14d), normal parenchymal cells (hepatocytes) and parenchymal cells from animals that were fed the CDE diet for 2 to 4 weeks (PC-N, PC-2, and PC-4, respectively), oval cells from animals that were fed the CDE diet for 4, 9 or 16 weeks (OC-4, OC-9, OC-16), and normal adult kidney cells (KID) were electrophoresed in 1.1% agarose-formaldehyde gels, blotted to nitrocellulose, and hybridized to 32 P-labeled probes as described in the text. The probes used in (a) and (b) were HiHi380 (*Ki-ras*) and BS-9 (*Ha-ras*). In (c), FL-17d, FL-20d, NN-3d, and NN-14d were hybridized with pMyc1.8, and in the remaining lanes, extracts were hybridized with the entire 4.5-kb *Bam*HI-*Xho*I fragment of λ Momycl (*myc*). The marker lane (M) contained a *Hind*III digest of phage λ DNA end-labeled with 32 P.

weeks and decreases thereafter (Fig. 3a). Hybridization of whole-cell RNA from hepatocytes and bile-duct-enriched cell preparations isolated from normal rats indicated that normal bile duct cells contain higher levels of *Ki-ras* transcripts than hepatocytes (Fig. 3a, BDC-N) and that their abundance does not change when biliary cells proliferate 3 to 4 weeks after ligation of the common bile duct (Fig. 3a, BDL-3 and BDL-4).

c-Ha-ras transcript levels showed more conspicuous changes in hepatocytes than in oval cells. Maximum levels in hepatocytes were found at 2 weeks (Fig. 2b and 3b), whereas different preparations obtained from individual rats that were fed the CDE diet for 4 weeks showed generally lower levels with a high degree of variation. Elevations of *c-Ha-ras* transcript levels in oval cells were relatively small (Fig. 2b and 3b; Table 2) and did not progress further with longer feeding times.

The relative levels of *c-myc* transcripts in isolated cell RNA more closely matched those of *c-Ki-ras* (Fig. 3c). The *c-myc* levels were highest in later-stage oval cells (16 weeks of the CDE diet), when they were approximately sixfold greater than in normal hepatocytes. Both preneoplastic liver hepatocytes and normal bile duct cells contained more *c-myc* transcripts than did normal hepatocytes.

Proto-oncogene expression during liver development. We determined whether there were changes in the sizes and abundances of *c-Ki-ras* and *c-Ha-ras* transcripts in whole livers during development and compared the fetal and neonatal liver transcripts with those found in oval and parenchymal cells during carcinogenesis. Samples (20 μ g) of the various total RNAs were separated by electrophoresis in 1.1% agarose-formaldehyde gels and transferred to nitrocellulose filters. The relative concentration of *c-Ki-ras* transcripts was highest at 17 days of gestation and dropped dramatically by 20 days of gestation and 3 days after birth (Fig. 2a; Table 2). The 2.1-kb *c-Ki-ras* band was the major transcript detected in whole liver RNA during all stages of development sampled (Fig. 2a), but at 17 days of gestation, there appeared to be a second transcript of 1.6 kb. The larger, 5.0-kb, transcript detected in polysomal poly(A)⁺ preparations was barely detectable in total RNA preparations. The 1.2-kb *c-Ha-ras* band observed in polysomal poly(A)⁺ RNA from whole liver was also observed in total RNA from developing livers (Fig. 2b). The *c-Ha-ras* transcript level was highest at 17 days of gestation and decreased later in liver development, although this decline was less precipitous (a threefold difference between 17 days of gestation and 3 days postpartum) than that observed for *c-Ki-ras*. *c-myc* levels were high in 17-day fetal liver and appeared to decrease with increasing age (Fig. 2c). However, the lack of complete resolution of discrete RNA bands, alluded to above, made exact quantification impossible.

DISCUSSION

On the basis of the results presented in this paper and previous work from our laboratories, it is now possible to describe the patterns of gene expression of three proto-oncogenes (*c-myc*, *c-Ha-ras*, and *c-Ki-ras*) in rat liver from early fetal development through the adult stage and during nonneoplastic (regenerative) and neoplastic growth (12, 16, 17).

Once developmental growth of the liver ceases at about 28 days of age in rats, DNA synthesis and cell division in individual hepatocytes may occur only once or twice during the lifespan of the animal. However, hepatocytes do not lose the capacity for replication, as illustrated by the massive growth response which takes place during liver regeneration after partial hepatectomy. In contrast to compensatory growth, liver carcinogenesis generally involves massive changes in cell populations and a long interval between the initial stimulus and the development of tumors. One of the major questions in liver carcinogenesis is to identify the cells which eventually give rise to tumors. In the system we have described, there is extensive proliferation of oval cells which

FIG. 3. Analysis of *c-Ki-ras* (a), *c-Ha-ras* (b), and *c-myc* (c) RNAs in total RNA prepared from purified cells. Total RNA (10, 5, or 1 μ g) from parenchymal cells (hepatocytes) (PC), normal bile duct cells (BDC-N), bile duct cells from bile duct ligated livers (BDL-3 and BDL-4), normal adult whole kidney cells (KID), and oval cells (OC) was dotted onto a nitrocellulose filter, and the same filter was hybridized sequentially with 32 P-labeled probes as described in the text. The probes used were HiHi380 (*Ki-ras*), BS-9 (*Ha-ras*), and the 2.2-kb *Pst*I fragment of *v-myc* (*myc*). *n*CDE refers to the number of weeks (*n*) for which each donor animal was maintained on the CDE feeding regimen.

have many of the properties expected of a liver stem cell and which contain oncodevelopmental markers associated with liver carcinogenesis (21, 24, 42).

Transcripts from the *c-Ha-ras*, *c-Ki-ras*, and *c-myc* genes are relatively abundant in fetal liver at 17 days of gestation. For *c-Ki-ras*, there is a rather abrupt decrease in abundance between 17 and 20 days of gestation. At 20 days of gestation, the abundance of *c-Ki-ras* transcripts is similar to that of normal adult hepatocytes. The significance of the appear-

ance of a 1.6-kb *c-Ki-ras* transcript at 17 days is not clear at this time. In contrast to *c-Ki-ras*, the level of *c-Ha-ras* transcripts decreases much more gradually during intrauterine development. The measurements of *c-Ki-ras*, *c-Ha-ras*, and *c-myc* transcripts in fetal and neonatal livers suggest that the expression of these three genes decreases as liver development progresses, but the interpretation of these findings is complicated by the presence of hemopoietic cells in fetal livers. In developing rat livers, the number of hemopoietic cells decreases drastically at 21 days of gestation, and the proportion of hepatocytes relative to other cells increases progressively from 15 days until the time of birth (18). The shift in cell populations is accompanied by a corresponding decrease in the frequency of hepatocyte replication, although cessation of hepatocyte replication does not occur until approximately the fourth week after birth. Therefore, the patterns of expression of the three proto-oncogenes may reflect the progressive conversion of hepatocytes to a nonreplicating state or the decrease in the level of hemopoietic cells in fetal and neonatal liver.

During carcinogenesis in rats on the CDE diet, *c-Ha-ras* transcripts show a modest increase at 2 to 4 weeks, with the changes confined primarily to hepatocytes. In contrast, the abundance of *c-Ki-ras* transcripts increases throughout carcinogenesis and is progressively higher in oval cells. When tumors ultimately emerge, they contain very high levels of *c-Ki-ras* transcripts, whereas the level of *c-Ha-ras* transcripts is only slightly higher than normal. The change in the abundance of *c-myc* transcripts during carcinogenesis follows a pattern similar to that of *c-Ki-ras*. *c-myc* transcript levels are very low in normal parenchymal cells, increase

TABLE 2. Relative amounts of *c-Ki-ras* and *c-Ha-ras* mRNA in total RNA^a

RNA source	Amt of 2.1-kb <i>c-Ki-ras</i> ^b mRNA	Amt of 1.2-kb <i>c-Ha-ras</i> mRNA
17-day fetal liver	28	4
20-day fetal liver	5	2
3-day neonatal liver	<1	<1
14-day neonatal liver	<1	<1
Normal hepatocytes	1	1
2CDE hepatocytes	14	5
4CDE hepatocytes	10	3
4CDE oval cells	11	<1
9CDE oval cells	13	1
16CDE oval cells	24	<1

^a Appropriate autoradiographs (in linear range of spectrophotometer) were quantified spectrophotometrically by scanning at 550 nm in a recording spectrophotometer. Absorbance values for normal adult hepatocytes were arbitrarily set as 1.

^b The 5.0-kb *c-Ki-ras* band in polysomal poly(A)⁺ RNA was barely detectable in total RNA preparations and was therefore not quantified.

progressively in oval cells during carcinogenesis, and are elevated in the tumors which develop by 35 weeks of feeding.

When adult livers regenerate after partial hepatectomy, the level of *c-myc* transcripts increases before DNA synthesis, whereas the levels of both *c-Ha-ras* and *c-Ki-ras* transcripts increase at the time or slightly after DNA synthesis begins (16, 17). The changes in these transcript levels are much more marked for *c-Ki-ras*, but the levels of the transcripts from all three genes return to normal at the end of the first wave of DNA synthesis. Therefore, during regenerative growth *c-myc* and *c-ras* gene expression correlates, respectively, with reentry into the cell cycle and DNA replication in hepatocytes. In rats on either the CDE or the CD diet, a low level of hepatocyte replication may take place (13, 14). The transient elevation of *c-Ha-ras* and *c-Ki-ras* transcripts in hepatocytes after 2 weeks of the CDE diet and after 9 weeks of the CD diet may reflect the division of these cells.

During CDE carcinogenesis, proliferation and probable differentiation of oval cells occur. It is conceivable that the high expression of *c-Ki-ras* and *c-myc* transcripts in oval cell populations during carcinogenesis reflects the proliferation of oval cells. However, it is interesting to note that Sirica and Cihla (37) reported that the rate of DNA synthesis in isolated oval cells decreases with increasing time on the CDE regimen. Since *c-Ki-ras* and *c-myc* transcript abundance in oval cells increases progressively throughout the feeding period, it is possible that the levels of oncogene transcripts may become independent from the rate of oval cell replication at the later stages of carcinogenesis. If oval cells may, under special conditions, function as facultative liver stem cells, the concept of defective terminal differentiation as a cause of neoplastic transformation in hepatocarcinogenesis becomes more tenable.

The work reported here on hepatocarcinogenesis induced by feeding rats the CDE diet is in substantial agreement with the conclusions reached by Makino et al. (25), who used the carcinogen 3-methyl-4-dimethylaminoazobenzene in studies of *c-Ha-ras* and *c-myc* expression in rat liver tumors. Both studies suggest that the elevated expression of *c-myc* is associated with hepatocarcinogenesis, whereas *c-Ha-ras* expression might be related to hepatocyte proliferation. Corcos et al. (5) have reported that elevated expression of *c-Ki-ras* and *c-Ha-ras* was found in "perineoplastic" hepatocytes surrounding tumor nodules, whereas elevated expression of *c-myc* transcripts was found only in tumor tissue. Neither our studies nor those of Makino et al. (25) or Corcos et al. (5) have examined the transforming capacity of proto-oncogenes during hepatocarcinogenesis. It is possible that mutations in some proto-oncogenes, which may be associated with increases in transcript levels, occur in cells which are the direct precursors of the tumors. This possibility might be explored by assaying DNA from purified cell populations for its transforming capacity in suitable *in vitro* systems. Until these studies are completed, it is difficult to dissociate the contributions of enhanced cell division and cell transformation to the increase in the expression of some proto-oncogenes during carcinogenesis and in tumors (38).

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