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c-*myc* gene amplification during hepatocarcinogenesis by a choline-devoid diet

(hepatocellular carcinoma/liver/oncogene/dietary carcinogenesis)

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ABSTRACT **Liver tumors arise in rats fed a choline-devoid diet without added carcinogens. We found amplification of the c-*myc* gene in 13/13 of these tumors. The amplification ranged from 2- to 70-fold and was accompanied by an increase in c-*myc* gene expression. Amplification of c-*myc* was larger in tumors of rats fed a choline-devoid diet followed by a choline-supplemented diet than in tumors from animals fed a choline-devoid diet exclusively. In the former animals, low levels of c-*myc* gene amplification were also detected in nontumorous regions of tumor-bearing livers. The choline-devoid diet provides an *in vivo* experimental model for the induction of gene amplification in the rat liver. In this setting, amplification of the c-*myc* gene may be an early and critical event in carcinogenesis.**

There is evidence that the c-*myc* gene is involved in the control of normal cell division and in the process of cell transformation (1). In the liver, increased c-*myc* expression is seen during liver regeneration (2) and in hepatocellular carcinomas (3–8).

Rats fed a choline-devoid (CD) diet, without exposure to chemical carcinogens, develop hepatocellular carcinomas after 14–16 months (9–11). The mechanism of this carcinogenesis is unknown, but major consequences of the diet include cell damage and a marked increase in hepatocyte turnover (12). DNA adducts were not detected in the livers of these animals, ruling out the possibility that contamination of the diet or the rats' environment with chemical carcinogens was responsible for the liver pathology and tumor induction (13). The CD diet also causes a general reduction in hepatocyte DNA methylation (14, 15).

During a study of *ras* genes, we noticed changes in endogenous viral sequences in tumor DNA and surmised that the changes may be characteristic of CD diet hepatocarcinogenesis (16). As liver cell death and regeneration persist during CD diet feeding, we undertook studies of c-*myc* expression and gene structure.

In an earlier study, groups of rats were fed a continuous diet, either CD or choline-supplemented (CS), for 14–16 months, with tumor incidences of 26% and 0%, respectively (11, 17). In later studies, groups were fed sequential diets—CD diet for 3, 6, 9, and 12 months followed by CS diet for a combined total of 16 months; respective tumor incidences were 13%, 27%, 33%, and 73% (17). All tumors were well to moderately well differentiated hepatocellular carcinomas of trabecular, adenomatous, or mixed type. In this paper we report our analysis of c-*myc* gene structure and expression in these livers and tumors.

MATERIALS AND METHODS

Experimental Animals, Diets, and Tumors. All studies were performed on male Fisher 344 rats weighing 90–100 g when fed CD or CS diets, as described (11, 16, 17). In a first series of experiments, the animals were fed continuous CD or CS diets. Larger tumors became palpable at 13–14 months; smaller tumors were found on killing animals at 14 months. In a second series of experiments, the diets were fed sequentially, first the CD diet for 3–12 months and then the CS diet for a combined total of 16 months.

Recombinant DNA Clones. We derived subclones from a genomic clone of the mouse c-myc clone provided by P. Leder (Harvard University, Cambridge, MA). Probes were based on the map of Stanton *et al.* (18). A 5.1-kilobase (kb) *Xba* I–*Bam*HI fragment, subcloned into pBS (Stratagene), contained the entire second and third exons and flanking sequences. A 0.5-kb *Pst* I probe was from within the second exon. A v-sis clone, pR12 (19), was provided by S. Tronick (National Institutes of Health, Bethesda, MD). It consists of a 1.2-kb *Pst* I fragment of simian sarcoma virus in pBR322. Our hybridization probe was a 0.9-kb *Pst* I–*Xba* I fragment. We used a segment of the Kirsten sarcoma virus to detect rat endogenous virus sequences (16). This was a 0.4-kb *Eco*RI–*Sst* II fragment of clone HiHi-3, provided by E. M. Scolnick (Merck Sharpe & Dohme Research Laboratories, West Point, PA).

Nucleic Acid Purification and Hybridization Methods. DNA and total RNA were purified from tumors and livers as described (16). Southern blotting and hybridization were also carried out as described (20). DNA was labeled by the random primer method (21) to a specific activity of 4×10^8 dpm/μg and hybridized to DNA blots at 1×10^6 dpm/ml using reduced stringency hybridization conditions [40% (vol/vol) formamide, room temperature, ≈44°C below the melting temperature] (16). RNA hybridization used the same specific activities and probe concentrations, but normal stringency conditions (50% formamide, 37°C, ≈20°C below the melting temperature) were used.

Hybridization Quantification. DNA concentration and hybridization intensity were both quantified by scanning densitometry. Gels contained at least two lanes of normal control DNA (designated CS in the figures). The DNA concentration was determined by optical density at 260 nm, but the DNA concentration still varied in the gels. This was caused by impurities in the DNA and by the high viscosity of the solutions. Accordingly, to more accurately quantify the amount of DNA loaded, we photographed gels used for blotting and scanned the photographic negative. After blotting, the membrane was examined under UV light to make sure that transfer was uniform. Densitometric analysis of standard hybridizations (data not shown) showed that hybridization intensity was linear with DNA concentration, while fluorescence intensity of the ethidium-stained gel conformed to a second order relationship. These data were computer-fitted to give the empirical relationship $y = -0.39 + 3.89x + 1.66x^2$, where x is the scanned fluorescence

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Abbreviations: CD, choline devoid; CS, choline supplemented.

intensity and y is the DNA concentration. Corrected DNA concentration was determined from this equation, and the hybridization levels for each membrane were normalized to the average value for control lanes.

RESULTS

Tumor DNA from rats fed the continuous CD diet showed increased hybridization to a c-*myc* gene probe (Fig. 1). Although the increased hybridization to tumor DNA is clear in the figure, the individual gel lanes showed additional variation of hybridization intensity because DNA concentration was not equivalent. For actual quantification (presented below), we measured both the hybridization intensity and the amount of DNA in each lane. We defined gene amplification as representing increased c-*myc* hybridization in proportion to the amount of DNA in the lane, in the absence of a similar increase in a rat chromosome 7 (c-*sis*; refs. 23 and 24) control or in other controls. We did not study c-*sis* gene expression. The same liver and tumor nucleic acid preparations had been previously studied with *ras* gene and rat endogenous virus probes (16). No Ki-*ras*, Ha-*ras*, or N-*ras* gene abnormalities were found, which provided additional controls for our c-*myc* gene analysis. However, upon hybridization with a probe that detects endogenous virus segments in the rat genome, several of the tumors showed a marked increase in the intensity of a band at 2.6 kb. This appears to be another gene amplification (16).

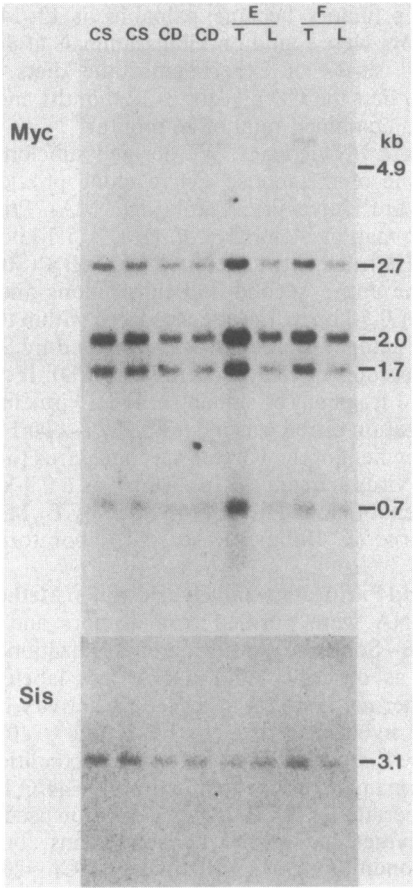

FIG. 1. Southern blot analysis of c-*myc* and c-*sis* DNA in tumors and livers of animals fed a continuous CD diet. Each lane contains 5 μg of *Pvu* II-digested DNA, resolved on a 1% agarose gel. The lanes designated CS contain DNA from normal livers of animals fed the control CS diet for 14 months. CD designates two DNA preparations from animals fed a CD diet for 14 months; these animals did not have tumors. The CS lanes appear darker because they contain somewhat more DNA. Tumors (lanes T) and adjacent liver tissue (lanes L) were obtained from animals E and F after they had been fed the CD diet alone for 14–16 months. The sizes of the fragments are given in kb. The DNA preparations are the same as those analyzed for *ras* genes and endogenous viral segments in Chandar *et al.* (16). This is the rehybridization of the blot from the left side of figure 6 in that paper. (*Upper*) Hybridization with the 5.1-kb c-*myc* probe containing the entire second and third exons and flanking sequences. This probe hybridizes to *Pvu* II digest fragments of the rat c-*myc* gene at 2.7, 2.0, 1.7, and 0.7 kb (ref. 22; J.L. and N. Crawford, unpublished results). The band at 4.9 kb represents cross hybridization to another gene, possibly N-*myc*. An extra band above 4.9 kb in lane T from animal F represents contaminating plasmid DNA, verified by hybridization studies (data not shown). (*Lower*) Subsequent hybridization of the same filter with a c-*sis* gene probe.

We then studied tumors from animals fed the sequential diets (Fig. 2). These tumors also had c-*myc* gene amplifications; several were larger than those observed with the continuous CD diet. In addition, gene amplifications were also apparent in nontumorous regions of tumor-bearing livers. The endogenous virus probe (16) showed increased intensity of the 3.2-kb band in about half of the tumors but none of the livers. The intensity of this band did not correlate with c-*myc* hybridization intensity, indicating an independent gene amplification (data not shown).

Histologic analysis was used to evaluate the finding of gene amplification in nontumorous portions of the livers. The tumors were tan to white and easily distinguished from the dark brown hepatic parenchyma. However, our observation could be explained by the presence of tiny tumor nodules or individual tumor cells throughout the livers. All livers were examined microscopically with at least three full cross sections. The specific segments taken for DNA extraction were split, and a facing portion of each was examined histologically. Among all the nontumorous liver segments that we analyzed for this paper, we observed only one microscopic tumor nodule. This nodule represented a minute fraction of the total tissue in that liver. It is thus unlikely that tumor cells account for the gene amplification in the liver segments. In animal 58, for example, the tumor had a 9-fold gene amplification and the nontumorous liver had a 3.5-fold gene amplification. The liver segment would have to have been contaminated with 30% tumor cells to account for this level of gene amplification. Another example, tumor 51, actually had lower gene amplification than the nontumorous liver. This suggests that c-*myc* gene amplification can continue to evolve in the liver as well as the tumor. Since none of the nontumorous liver portions contained a significant tumor cell population, the most likely explanation for our observations is that c-*myc* gene amplification is present outside of the carcinomas. However, we recognize that this observation needs to be verified by more rigorous analysis. After 16 months of sequential CD and CS diets, the nontumorous liver regions and non-tumor-bearing livers showed generally normal hepatic trabeculae (but with moderate fibrosis), marked nuclear pleomorphism, and occasional hepatocyte mitoses.

Relative c-*myc* transcript levels were measured by dot-blot hybridization of total RNA (Fig. 3). RNA blot hybridization of the c-*myc* probe (data not shown) demonstrated the usual rat transcript of 2.3 kb (2). Nontumorous livers of animals fed the CD diet showed a small increase in c-*myc* transcript levels. Nontumorous regions of some tumor-bearing livers had higher levels, while most tumors had even higher levels.

Fig. 4 shows comparative quantifications of gene and transcript levels. All of the tumors had c-*myc* gene amplification. Tumor frequency (17) and extent of gene amplification were both greater when the CD and CS diets were fed sequentially. A possible source of bias in our study relates to the selection of tumors for analysis. Usually, only large tumors could be used for DNA and RNA extraction, whereas smaller tumors were documented mainly by histopathologic

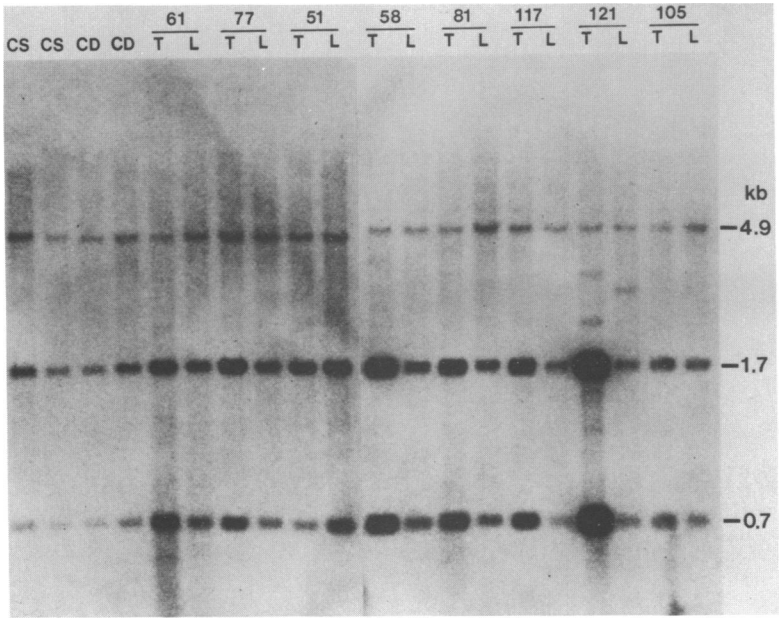

FIG. 2. Southern blot analysis of tumors obtained on consecutive feeding of CD and CS diets. *Pvu* II digests (5 μg) were electrophoresed in 1% agarose, blotted to nitrocellulose, and hybridized to a c-*myc* probe. CS and CD represent animals fed either diet exclusively for 14 months. These four preparations are different from the CD and CS preparations of Fig. 1, but they represent the same experimental conditions. Individual experimental animals are designated by numbers. Rats 61 and 77 were fed the CD diet alone for 16 months. All other tumors were obtained after feeding the CD diet for periods ranging from 3 to 12 months followed by feeding the CS diet for a combined total of 16 months. CD diet intervals were 3 months for animal 51; 6 months for rats 58 and 81, 9 months for rats 117 and 121, and 12 months for rat 105. Other experimental details are as in Fig. 1 except that the hybridization probe was the 0.5-kb *Pst* I fragment from the second exon of the mouse c-*myc* gene (18). This probe hybridizes to the 1.7- and 0.7-kb c-*myc* bands and also hybridizes strongly to the 4.9-kb non-c-*myc* band, which can be considered an internal hybridization control. This 4.9-kb band is useful for comparing the relative DNA loading of each lane. The extra bands in lanes T and L from animal 121 have not been further characterized. T, tumor; L, adjacent liver tissue.

examination. However, a few of the latter tumors were included in the analyses and did show c-*myc* gene amplification.

c-*myc* transcript levels (Fig. 4*B*) were slightly elevated in the livers of non-tumor-bearing animals fed the CD diet for 14 months and significantly elevated in livers (and tumors) of animals that had tumors. Most of these latter animals were fed the CD diet for longer than 14 months. The livers and tumors with the largest gene amplifications had the highest transcript levels, but even livers without gene amplification showed elevation. To distinguish mechanisms for elevated c-*myc* transcript levels, we calculated the relative expression per gene (Fig. 4*C*). Animals fed the continuous CD diet had the highest transcript levels per gene; their livers had the most cell damage and hence the greatest stimulation for liver regeneration. Some tumors with large gene amplifications had relatively low levels of transcripts. The CD diet had been stopped in these animals. They consequently had less liver damage and hence less stimulation to regenerate. Alternatively, these tumors might have contained some c-*myc* genes that could not be normally stimulated or a mixture of functional and nonfunctional genes within the amplified population.

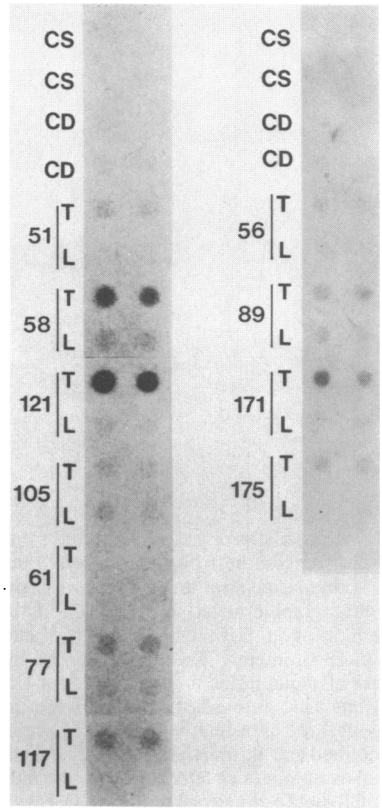

FIG. 3. Dot-blot hybridization analysis of c-*myc* transcripts. Total RNA samples (0.4 and 2.0 μg) were applied to nylon membranes in a filtration manifold. Hybridization with the 0.5-kb *Pst* I fragment of c-*myc* exon 2 was in 50% formamide at 37°C. The two illustrated strips represent separate hybridizations with different CS and CD controls. Animal numbers and other labels are as in Fig. 2.

DISCUSSION

Our data show two striking phenomena: (*i*) the uniform presence of c-*myc* gene amplification in CD diet-induced tumors, and (*ii*) the presence of gene amplification in nontumorous portions of some of the tumor-bearing livers. We have observed another apparent gene amplification in these tumors (16), of a genomic region containing an endogenous retroviral segment. However, this amplification was present in only about half of the tumors analyzed in this paper, and its levels did not correlate with c-*myc* gene amplification levels. We thus conclude that the CD diet causes gene amplification of several loci, but that cells with c-*myc* gene amplification are selected during this carcinogenesis process. Feeding the CD and CS diets sequentially gave larger gene amplifications, and c-*myc* gene amplification was detected even in the tumors that arose in rats fed the CD diet for only 3 months. It is apparent, therefore, not only that further c-*myc* gene amplification occurs after the CD diet is discontinued, but also that this genomic alteration occurs early during CD diet feeding. The CD diet induces liver damage, which stimulates most hepatocytes to proliferate, but proliferating cells persist months after the damaging diet has been stopped (17). We hypothesize that cell proliferation in this setting enlarges gene amplification and that cells with c-*myc* gene amplification eventually acquire autonomous proliferation in these livers. However, the observation of c-*myc* gene amplification in nontumorous liver regions indicates that this is one precancerous event and that other(s) must occur to result in hepatocellular carcinoma induction. These events

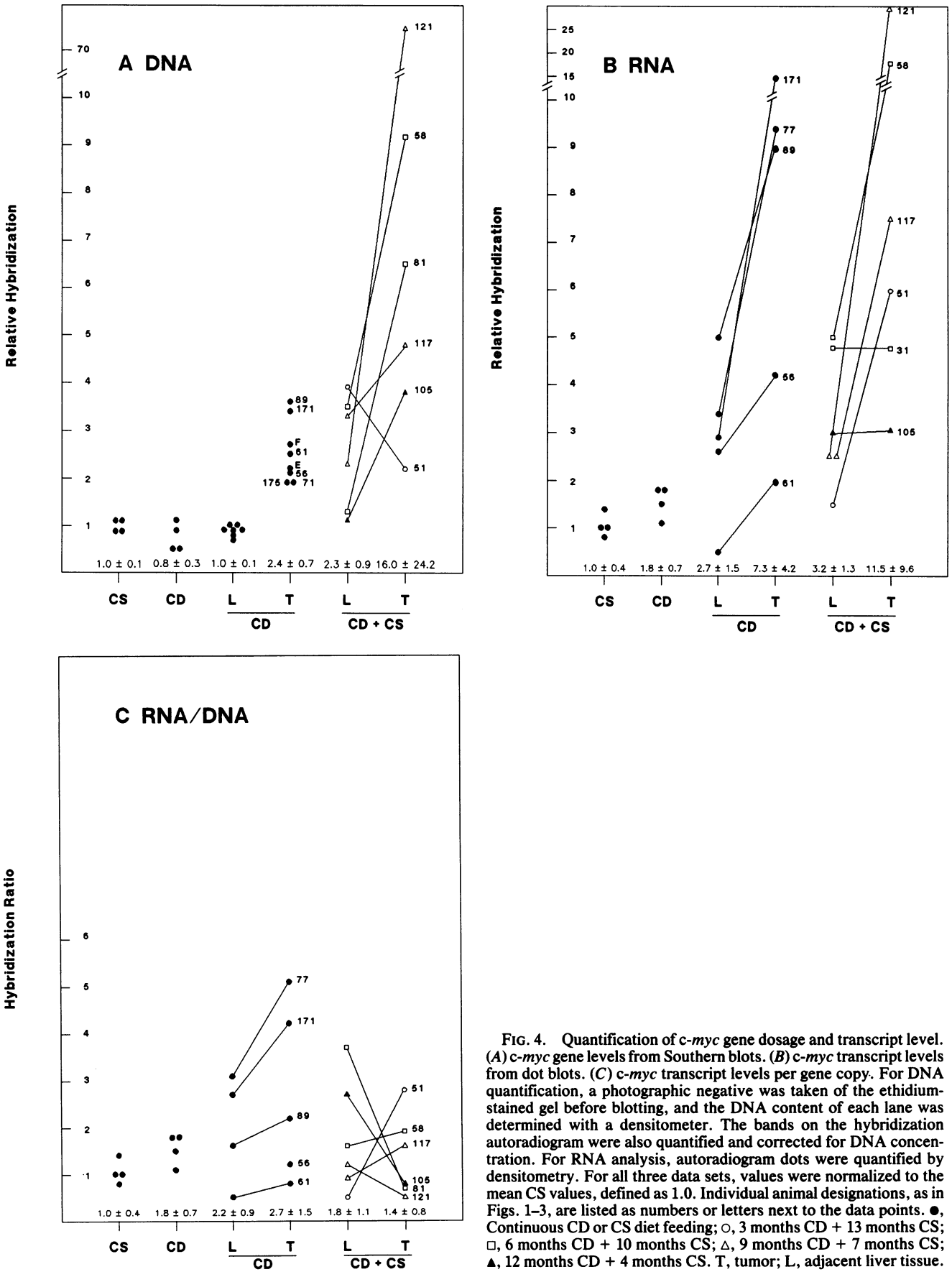

FIG. 4. Quantification of c-*myc* gene dosage and transcript level. (*A*) c-*myc* gene levels from Southern blots. (*B*) c-*myc* transcript levels from dot blots. (*C*) c-*myc* transcript levels per gene copy. For DNA quantification, a photographic negative was taken of the ethidium-stained gel before blotting, and the DNA content of each lane was determined with a densitometer. The bands on the hybridization autoradiogram were also quantified and corrected for DNA concentration. For RNA analysis, autoradiogram dots were quantified by densitometry. For all three data sets, values were normalized to the mean CS values, defined as 1.0. Individual animal designations, as in Figs. 1–3, are listed as numbers or letters next to the data points. ●, Continuous CD or CS diet feeding; ○, 3 months CD + 13 months CS; □, 6 months CD + 10 months CS; △, 9 months CD + 7 months CS; ▲, 12 months CD + 4 months CS. T, tumor; L, adjacent liver tissue.

could include amplification of other genes or other kinds of mutations.

The increases in c-*myc* transcripts observed in the present studies probably resulted from two processes: (*i*) stimulation of c-*myc* expression by the intrinsic mechanisms of cell division and (*ii*) c-*myc* gene amplification. However, other

c-*myc* gene mutations that alter expression or epigenetic changes, such as altered DNA methylation, should also be considered. During liver regeneration, stimulated expression leads to a 2- to 5-fold increase in c-*myc* transcript levels (2), the same range we observed in both livers and tumors. This range of increase appears to be sufficient for, and a marker of, hepatocyte proliferation. The stimulated c-*myc* expression observed with rats fed the CD diet could therefore have resulted from the extracellular stimuli that induce regeneration, as a consequence of the liver damage caused by the diet (9, 17). Alternatively, the stimulation could represent an intrinsic alteration in the cellular pathway that regulates c-*myc*.

c-*myc* gene amplification has been found in a small proportion of rat liver tumors induced with chemical carcinogens (refs. 3 and 7; J.L. and N. Crawford, unpublished results). Other oncogenes, notably *ras* and *raf*, have been detected in some rat and mouse hepatomas induced with chemical carcinogens (25–27), but Reynolds *et al.* (28) detected virtually no transforming genes in spontaneous hepatomas of F344 rats. One consistent finding, though, has been an elevation of c-*myc* transcripts in hepatocellular carcinomas (3–8).

Gene amplification may lead to overexpression of the c-*myc* gene in the absence of a primary stimulation of gene expression. Since stimulated hepatocyte c-*myc* gene levels are only a few times the unstimulated levels (2), even a small gene amplification could provide enough additional c-*myc* expression to deregulate cell proliferation.

Since c-*myc* amplification has been previously reported in a few hepatocellular carcinomas, we are not reporting a novel association. However, our study demonstrates several unusual aspects of carcinogenesis. (*i*) The CD diet provides an experimental system for reproducibly inducing tumors with gene amplification. (*ii*) There is an invariant association of c-*myc* gene amplification with CD diet carcinogenesis. (*iii*) The size of c-*myc* gene amplifications is manipulable by the experimental conditions of carcinogenesis. We are not aware of any experimental carcinogenesis system in which the reproducible induction of gene amplification has been described, although several describe the reproducible induction of point mutations, for example, in the Ha-*ras* gene (29). Our observations may not be surprising, but they integrate a second kind of genetic damage into the pathway of experimental tumor induction, since the CD diet acts without mutagenizing carcinogens. Thus, our results provide an additional perspective on the process of carcinogenesis and an important experimental system.

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