Expression of the c-raf Protooncogene, γ-Glutamyltranspeptidase, and Gap Junction Protein in Rat Liver Neoplasms


ABSTRACT

Female Harian Sprague-Dawley and F-344 rats were subjected to a 70% hepatectomy and 18 h later given one dose of 30 mg diethylnitrosamine/kg body weight. Beginning 1 week later, the animals were fed a diet containing 0.05% phenobarbital. Groups of rats were sacrificed 6 and 15 months later, and the livers were either frozen for cryostat sectioning or used to isolate RNA. Primary liver tumors present in these animals were used for RNA isolation, and a portion was taken for histopathological analysis. Eleven of 13 primary lesions, consisting of either neoplastic nodules or hepatocellular carcinomas, showed elevated levels of mRNA for the c-raf protooncogene. Increased c-raf mRNA in these tumors appeared to be unrelated to their cellular proliferative status inasmuch as the levels of c-raf mRNA did not correlate with levels of H4 histone mRNA. Decreased expression of the major rat liver gap junction protein mRNA was observed in all of the primary tumors. Immunocytochemical analysis using an anti-gap junction antibody revealed a decrease in gap junction immunoreactivity in some but not all preneoplastic focal lesions. All preneoplastic foci having positive γ-glutamyltranspeptidase enzyme staining also exhibited a marked increase in γ-glutamyl transpeptidase mRNA as determined by in situ hybridization. The possible relation of alterations of the mRNA levels of c-raf and the gap junction protein to the further development of preneoplastic foci is discussed.

INTRODUCTION

The appearance of preneoplastic focal lesions (AHF) in rat liver following the administration of a hepatocarcinogen is believed to reflect one or more of the early stages in multistage hepatocarcinogenesis (1–3). Since usually many foci are induced, yet few tumors arise (3), only a certain subset of foci may have neoplastic and malignant potential. It is therefore important to distinguish, if possible, the AHF of the latter population if we are to increase our understanding of the early stages of the neoplastic process. Characterization of the phenotypic expression of certain marker enzymes (4, 5) has been one approach utilized; however, the heterogeneity of AHF phenotypes (5) has been a major obstacle in relating any specific phenotype(s) to cancer formation. An important finding potentially related to the basis of neoplasia is the altered expression of specific protooncogenes in different liver tumors induced with various protocols of hepatocarcinogenesis (6–10). Since oncogenes are more tightly coupled to cellular regulation versus specific gene expression (23), and their reduction may play a role in tumor promotion (24, 25) as well as in the early stages of cell proliferation (22) and their reduction may play a role in the regulation of cell proliferation (22) and their reduction may play a role in tumor promotion (24, 25) as well as in the early stages of cell proliferation (22), 26, 27). A reduction in the expression observed in preneoplastic foci compared with normal hepatocytes. In addition, we examined the expression of the major gap junction protein in normal liver and primary liver tumors using immunocytochemistry. Gap junctions have been shown to play an important role in cell-cell communication (21), to affect the regulation of cell proliferation (22) and specific gene expression (23), and their reduction may play a role in tumor promotion (24, 25) as well as in the early stages of the neoplastic process (26, 27). A reduction in the expression of this gene may also be related to the progression of some AHF to malignancy.

MATERIALS AND METHODS

Female Sprague-Dawley and F-344 rats (Harian Sprague-Dawley, Madison, WI), weighing 150–175 g, were subjected to a 70% hepatectomy according to the method of Higgins and Anderson (28). Eighteen h later they were given a single intragastric instillation of 30 mg of DEN/kg body weight (Eastman Organic Chemicals, Rochester, NY) in distilled water and then fed a diet containing 0.05% phenobarbital. Animals were sacrificed after 6–15 months on the above regimen, and portions of their livers were frozen for cryostat sectioning (11), with the remainder used for RNA isolation according to the method of preneoplastic foci should be discovered by investigating their regulation. However, oncogenic alterations are not always observed in preneoplastic foci (11), suggesting that the elevated expression of protooncogenes in some tumors may be a nonessential or secondary alteration, occurring during multistage hepatocarcinogenesis.

In this report we attempt to characterize further the role of altered protooncogene expression during the early stages of hepatocarcinogenesis on the basis of two assumptions: (a) protooncogenes that show consistent alteration in primary liver tumors are those most likely to play a key role in the progression of AHF to malignancy; and (b) because only relatively few such lesions progress to neoplasia, AHF must be examined as individual entities. This supposition necessitates the examination of specific gene expression within individual AHF by in situ types of analysis (12–14). The homogenization of a liver containing foci or the mass isolation of cells of AHF encompassing different phenotypes (11) are not methods which allow such studies.

Rapp et al. (15) have demonstrated that the v-raf/oncogene is the transforming gene of the murine retrovirus, 3611-MSV, and the chromosomal localization of c-raf is known in the human (16) and the mouse (17). The cytoplasmic gene product of c-raf is distantly related to the src family of oncogenes (18, 19). Unlike other protein kinases in this family, the one coded by c-raf catalyzes the phosphorylation of serine and threonine rather than tyrosine (20).

For determination of the feasibility of examining the expression of the c-raf and other protooncogenes in individual early focal lesions, the optimal conditions for in situ hybridization of cellular mRNA for the gene GGT were examined. GGT was used to optimize the in situ procedure because of the increased expression observed in preneoplastic foci compared with normal hepatocytes. In addition, we examined the expression of the major gap junction protein in normal liver and primary liver tumors using immunocytochemistry. Gap junctions have been shown to play an important role in cell-cell communication (21), to affect the regulation of cell proliferation (22) and specific gene expression (23), and their reduction may play a role in tumor promotion (24, 25) as well as in the early stages of the neoplastic process (26, 27). A reduction in the expression of this gene may also be related to the progression of some AHF to malignancy.
Chirgwin et al. (29). Primary tumors that were present in livers were dissected free of normal liver, a portion taken for histological diagnosis, and the remainder also used for RNA isolation. For histopathological analysis, portions of the normal liver and tumors were fixed in 10% buffered formalin, and 5-μm sections were stained with hematoxylin and eosin.

The isolation and purification of GGT-positive hepatocytes from rat livers containing GGT-positive foci were performed as previously described (11), according to the "panning" method of Hanigan and Fitot (30). GGT-positive hepatocytes were isolated from animals 6 months after the initiation of the treatment protocol described above and used for RNA isolation and analysis of the mRNA transcribed from the GGT gene.

3P-labeled DNA probes were prepared by nick translation of the following clones: the 2.1-kb human c-raf-1 fragment (16); the human H4 histone genomic DNA subcloned into pBR322 (31); the 780-bp GGT fragment (32); and the 1.5-kb cDNA for the rat liver gap junction protein (33). The Northern blot filters were prehybridized in 50% deionized formamide, 1% SDS, 1 M NaCl, and 10% dextran sulfate overnight at 42°C. Two of 5 × 10^6 dpm of nick-translated, heattreated DNA/ml and 200 μg of salmon sperm DNA/ml were then added and the filters hybridized for an additional 18 h at 42°C. After hybridization, the filters were washed twice in 2x SSC at room temperature for 15 min, followed by two 30-min washes at 55°C with 2x SSC containing 1% SDS. The filters were then washed twice in 0.1x SSC at room temperature for 30 min and sealed in plastic wrap. Autoradiograms were prepared with Kodak XAR 5 X-ray film and exposed at −70°C for 1-5 days with intensifying screens. Autoradiograms were quantified by scanning with 1KB soft laser scanning densitometer (LKB Instruments, Inc.).

In Situ Hybridization. The expression of mRNA for the genes GGT and c-raf was examined by a modification of in situ hybridization procedures previously described (12-14). The procedure is given in detail in the text.

Thaw-mounted cryostat sections were immediately frozen (dry ice) after placement onto poly-L-lysine-treated slides. Poly-L-lysine-treated slides were prepared by a wash in 100% ethanol, double-distilled H2O, air dried, treated with 50 μg/ml poly-L-lysine in 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA, and dried at 80°C. The frozen sections were then fixed for 20 min in freshly prepared 4% paraformaldehyde in PBS at room temperature. All solutions prior to prehybridization should be treated with or made up with water treated with 0.05% diethylpyrocarbonate to reduce exogenous RNase activity. We have found that the fixed sections could be dehydrated in an ethanol series and stored for at least 1 month at −70°C. These sections should be rehydrated in an ethanol series before the next step.

The sections were treated with 0.05 N HCl for 10 min in double-distilled H2O, 0.2% Triton X-100 in PBS at room temperature for 10 min, and 100 mM triethanolamine, pH 8.0, with 25 mM acetic anhydride for 10 min at room temperature with two 5-min washes in PBS between each step. The slides are immersed in 2x SSC 0.3 M NaCl-0.03 M sodium citrate for 30 min at 37°C and then prehybridized for 2 h in a moist chamber at 45°C by overlaying of a solution containing 50% deionized formamide, 10% dextran sulfate, 2x SSC, 20 mM dithiotreitol, 5 mM EDTA, pH 7.6, 1x Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate, 200 μg/ml SS DNA, 100 μg/ml polyadenylate, and 200 μg/ml yeast tRNA. The sections were covered with a siliconized, baked coverglass. The SS, polyadenylate, and tRNA were boiled for 3 min each step. The slides are immersed in 2x SSC for 1 h at 37°C (change SSC several times), the slides were treated with 25 μg/ml RNase A in 0.5 M NaCl-10 mM Tris (pH 8.0)-1 mM EDTA for 30 min, washed in 2x SSC for 2 h at 37°C (change SSC as above), 0.3x SSC for 3 h at room temperature (change SSC as above), and finally dehydrated for 5 min in 70 and then 90% ethanol containing 0.3 M NH4 acetate.

The air-dried slides are then dipped in NTB-2 photo emulsion diluted 1:1 with double-distilled H2O and exposed at 4°C for 4-8 days in the presence of desiccant. The autoradiographs are then developed in Kodak D-19 for 5 min at 16°C, rinsed with H2O, and fixed for 5 min (in Kodak Fixer Rapid Fix). The slides can also be developed less accurately with XAR X-ray film placed over the slides to examine the mRNA distribution in preneoplastic foci (12). The sections are lightly counterstained with hematoxylin and viewed and photographed with dark- or bright-field illumination. Serial sections were stained for GGT enzyme activity according to the method of Rutenberg et al. (34).

The 35S-labeled cRNA probe was synthesized by using a 780-bp fragment of the GGT cdna (32) subcloned into the Gemini transcription vector (Promega Corp., Madison, WI). This 780-bp EcoRI fragment, which is appropriate size for in situ hybridization (14), was shown to hybridize to the 2.2-kb GGT mRNA in isolated liver GGT-positive hepatocytes and kidney mRNA. The mRNAs were transcribed with [35S]UTP to form high specific activity probes that could be detected in situ using either NTB-2 photo emulsion or XAR X-ray film. The sense probe provided an excellent control for nonspecific binding.

Immunocytochemistry. The distribution of gap junctional complexes in normal rat liver and preneoplastic foci was examined with an antibody against the major M, 27,000 rat liver gap junction protein (33). Preimmune serum from the same rabbit was used as a control. Ten-μm serial frozen sections were fixed in acetone for 10 min at room temperature, hydrated in PBS, and incubated with the serum diluted 1:200 in PBS for 45 min at room temperature. Visualization of the antigen-antibody complexes was obtained by the peroxidase-antiperoxidase method of Sternberger et al. (35).

RESULTS

Northern Blot Analysis of mRNAs Transcripts in Primary Liver Tumors and During Liver Regeneration. RNA from 13 primary rat liver tumors, obtained as described in "Materials and Methods," and livers from rats at 0, 2, 10, and 20 h following partial hepatectomy were separated in agarose-formaldehyde gels; the blot was examined for the abundance of transcripts of the c-raf-1 protooncogene, H4 histone, the M, 27,000 rat liver gap junction protein, and GGT genes (Fig. 1). Compared with the relatively low abundance of the c-raf mRNA in normal rat liver, 11 of 13 primary tumors examined contained elevated levels of the 3.0-kb mRNA for this protooncogene (Fig. 1A). The tumor shown in lane 5 contained approximately a 20-fold increase of c-raf mRNA compared with normal liver. We have previously described variable expression of the H-ras and c-myc protooncogenes in similar primary liver tumors induced with DEN and promoted with dietary administration of phenobarbital (11). Additionally, we found undetectable levels of mRNA for the c-fos protooncogene in these same 13 primary liver tumors (data not shown). The consistent increased expression of the c-raf gene in hepatic nodules and cancers may, therefore, suggest its relationship to the progression of neoplastic growth.

At 20 h after partial hepatectomy, an approximately 4-fold increase in the levels of c-raf mRNA was observed, which corresponds to the peak of DNA synthesis (36-38). To determine whether the increased expression of c-raf in the tumors may be correlated with increased DNA synthetic activity, we examined the relative abundance of transcripts for H4 histone...
gene (Fig. 1B). The tight coupling of histone and DNA syntheses (31) provides a qualitative estimate of the extent of DNA synthesis by examination of the expression of the histone gene mRNA. Consistent with the reported increase in DNA synthesis at 20 h following partial hepatectomy (36–38), an increase in the 430-bp H4 histone mRNA was also observed. The extent of H4 histone gene expression among the primary liver tumors, however, was observed to vary substantially. Furthermore, there appeared to be no correlation between the extent of tumor c-raf and H4 histone expression. This finding suggests that the increased c-raf expression observed in the primary tumors is not a direct result of increased DNA synthetic and/or proliferative activity.

The histological analysis of these primary lesions revealed that increased c-raf expression is present in neoplastic nodules as well as in hepatocellular carcinomas. There does not appear to be an increase in c-raf expression with increasing malignancy, however. Interestingly, the age-matched control liver (Fig. 1B, lane 8) shows slight elevation of c-raf mRNA over the control and 2- and 10-h animals. Histological analysis of this tissue revealed occasional focal lesions and bile duct proliferation, which might have contributed to the increase in c-raf. The possible induction of c-raf expression by phenobarbital needs to be investigated. Thus, determination of changes in c-raf expression in individual AHF may be important for our understanding of a role of this protooncogene in the early stages of neoplastic development.

The expression of the rat liver M, 27,000 gap junction protein gene was examined in normal, regenerating, and tumor tissues by use of a cDNA coding for this protein (33). This cDNA recognized a 1.6-kb mRNA in all the tissues examined (Fig. 1C). Normal liver also possesses a much larger mRNA, which is recognized by this probe and which may represent an mRNA precursor to the smaller molecule (33). The 1.6-kb mRNA was fairly abundant in normal liver and was observed to decrease to 50% of normal levels at 2 h and to 10% by 10 h following partial hepatectomy; however, an increase to 25% of normal levels occurred by 20 h. The reason for this dramatic decrease is not certain, but the numbers of gap junctional complexes have been observed to decrease during liver regeneration (39). A significant reduction in the expression of the 1.6-kb gap junction mRNA was also found in most of the primary tumors. The reason for the lack of detection of gap junction mRNA in the tumors of lanes 6 and 7 (Fig. 1C) and the age-matched liver (lane 8) is not clear. It does not appear to have resulted from incomplete mRNA transfer in those regions. The reduction of gap junction mRNA in most of the liver lesions is consistent with a recent report of reduced gap junction protein in hepatocellular carcinomas induced with N-methyl-N-nitrosourea as detected by a monoclonal antibody (40). Because of the significant reduction in the gap junction mRNA in the animals 2 and 10 h following a partial hepatectomy, the reduced mRNA in the tumors may represent an increased percentage of the cell population stimulated to undergo DNA synthesis. It appears from examination of the H4 histone mRNA, however, that most tumors (i.e., Fig. 1C, lanes 1, 2, 9, and 12–14), while not actively undergoing DNA synthesis, show reduced gap junction mRNA.

We also examined the mRNA for the enzyme, GGT (Fig. 1D). Consistent with the lack of expression of this enzyme in normal adult liver, we did not observe any expression of GGT and mRNA in either normal liver, that from an age-matched control fed a diet containing 0.05% phenobarbital, or in the livers of rats following partial hepatectomy. We did observe
variable expression of the 2.2-kb mRNA in the primary tumors. Some tumors (Fig. 1D, lanes 2 and 14) contain very little if any GGT mRNA, while in two tumors (Fig. 1D, lanes 6 and 7) an mRNA doublet was observed. Whether these doublet mRNAs represent two isoforms of the enzyme, variable polyadenylate content, or differential processing of the primary transcripts is not readily apparent. A recent report, which shows a difference in the molecular weights of the GGT mRNAs found in liver and kidney (41), suggests that the doublet could consist of both kidney and liver transcripts. More work will have to be done to explore this possibility. Slight nonspecific binding of the probe to the 28 and 18S ribosomal mRNA was observed in this Northern blot, appearing as the light bands above and below the GGT mRNA.

Examination of GGT mRNA in Preneoplastic Foci. To determine the contribution of AHF to the total extent of expression of a specific gene when examining whole liver is difficult. The mRNA from the liver in lane 8 (Fig. 1D) which contained foci failed to show any 2.2-kb GGT mRNA. This result suggested either that foci do not express increased GGT mRNA, as do many of the primary tumors, or that the GGT mRNA of foci present in the liver is diluted by the mRNA from the vast majority of the normal hepatocytes that do not express GGT. To examine these possibilities, we probed a Northern blot of RNA from GGT-positive hepatocytes that had been enriched from a rat liver containing many GGT-positive foci. This animal had received DEN 20 h after a 70% hepatectomy and was fed a diet containing 0.05% phenobarbital for 6 months. The GGT-positive hepatocytes were isolated by use of anti-GGT antibody with a panning procedure, and the RNA was purified as described earlier (11). Fig. 2 illustrates a Northern blot containing RNA from the initial starting cell population, which contained both the GGT-positive and the normal GGT-negative cells, the GGT-negative hepatocytes, and the GGT-positive foci hepatocytes. With the GGT cDNA (32) as a probe, the GGT-positive hepatocytes were found to contain the 2.2-kb GGT mRNA, whereas the normal GGT-negative hepatocytes did not. The starting cell population showed some GGT mRNA, since it contained both GGT-positive and -negative hepatocytes. These results demonstrate that increased GGT enzyme activity is also accompanied by increased GGT mRNA in cells of AHF.

In Situ Hybridization. The results described above were obtained by isolating hepatocytes from many different AHF and do not provide information regarding gene expression within individual foci. Since the study of the role of protooncogenes in the early stages of AHF development requires knowledge of the frequency and potential heterogeneity of expression among these lesions, we chose to examine gene expression by in situ hybridization. The following results demonstrate the feasibility of using this technique by examining the expression of GGT mRNA in individual foci.

To demonstrate the detection of GGT mRNA in individual liver foci, serial frozen sections of rat liver containing focal lesions were either stained for GGT enzyme activity or hybridized with the antisense and/or sense GGT RNAs and processed for autoradiography as described in “Materials and Methods.” As shown in Fig. 3, increased GGT enzyme activity in foci is accompanied by an increase in GGT mRNA. This confirms the increased GGT mRNA observed in isolated GGT-positive hepatocytes (Fig. 2). Consistent with the low levels of GGT mRNA observed in some primary tumors (Fig. 1D, lanes 2 and 14), focal lesions that do not express GGT enzyme activity were also observed not to contain increased hybridization GGT mRNA (Fig. 3, bottom). Hybridization with the sense-strand GGT RNA demonstrates only background labeling (Fig. 4), thus showing the specificity of hybridization with the antisense GGT RNA.

Similar experiments are presently underway to determine c-raf-1 mRNA expression in these preneoplastic foci. However, preliminary results suggest that the change between the basal levels of expression in normal hepatocytes and in some preneoplastic foci is minimal.

Immunocytochemical Localization of the Gap Junction Protein in Normal and Preneoplastic Liver. As shown in Fig. 1, many primary tumors showed a reduction in the mRNA for the M, 27,000 gap junction protein. To determine whether a reduction of the gap junction protein is observed in preneoplastic foci, we examined frozen sections of normal liver containing foci using an antibody directed against a rat liver gap junction fusion protein which had been expressed in bacteria by the cloned cDNA (33). This antibody effectively detects the gap junctional complexes between hepatocytes in intact liver (Fig. 5A). No labeling was observed with the preimmune control serum (Fig. 5B). Examination of liver containing focal lesions revealed a diminution of immunoreactivity of the gap junctions in some AHF (Fig. 5C). At higher magnification (Fig. 5D) it is apparent that although gap junction complexes are present within these foci, their relative abundance is decreased. These results are consistent with the reported decreased gap junction immunoreactivity in liver lesions induced with N-methyl-N-nitrosourea (40).

DISCUSSION

We have previously reported that primary liver tumors induced with diethylnitrosamine and promoted with dietary ad-
Fig. 3. Serial frozen sections of rat liver containing AHF, which were either stained for GGT enzyme activity (left) by the histochemical method of Rutenberg et al. (34) or used for in situ hybridization with antisense GGT 35S-labeled RNA (right). In the dark-field images of the autoradiograms after in situ hybridization, the silver grains of the exposed emulsion appear white. These results demonstrate that increased GGT enzyme activity in AHF was accompanied by increased GGT mRNA. In the focal lesion (bottom) which contained areas GGT negative for enzyme activity (GN), the corresponding in situ autoradiogram demonstrates that no hybridization with GGT mRNA occurs in these areas. All x 150.
ministration of phenobarbital vary considerably in their expression of the H-ras and c-myc protooncogenes (11). In contrast, we have observed that many primary liver tumors present in rat livers following the same treatment protocol contain elevated expression of the mRNA for the c-raf-1 protooncogene. Histological analysis revealed that tumors composed of both hepatocellular carcinomas and nodular lesions showed increased c-raf mRNA.

The actual role of c-raf in these tumor cells is not certain, but mutational activation of the c-raf gene has been found in NIH 3T3 cells transfected with DNA from a hepatocellular carcinoma induced by 2-amino-3-methylimidazo[4,5-f]quinoline (42), in a human gastric cancer (43), and glioblastoma cell lines (44). More recently it was shown that c-raf activation in these assays may occur as an artifact of the transfection process (45, 46). Our present studies suggest that increased levels of
normal c-raf mRNA, as judged by the similar size of the transcripts in normal and neoplastic liver, occurs in many primary liver tumors in vivo. Recently, analysis of normal c-raf transcript shows an atypical sequence before its initiating ATG (47). According to Kozak’s rules the atypical sequence CATCA is not an efficient sequence for initiation of translation (48). Therefore, it is possible that the mRNA increase in these tumors may not directly correlate with c-raf protein concentrations. We have begun to examine the level of c-raf at the protein level in preneoplastic foci and tumors using c-raf antibodies.

ACKNOWLEDGMENTS

We would like to thank Susan Carlson for her histological expertise and Susan Hanika, Mary Jo Markham, and Kristen Adler for excellent secretarial assistance.

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