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mRNA Levels of Catalytic Subunits of Protein Phosphatases 1, 2A, and 2C in Hepatocarcinogenesis

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The mRNA levels of three phosphoseryl/phosphothreonyl protein phosphatases, PP1, PP2A and PP2C, in rat liver have been determined by Northern blot analysis in various stages of rat chemical hepatocarcinogenesis using a Solt-Farber model. Five weeks after administration of diethylnitrosamine, the mRNA levels of $PP1_{\alpha}$, PP2A and PP2C were elevated 8, 29 and 11 times, respectively, as compared to those of the control livers. However, in primary hepatoma induced according to the Solt-Farber model, the mRNA levels of all three protein phosphatases were dramatically decreased to normal levels or even to much lower levels, whereas the mRNA level of glutathione *S*-transferase placental form, a tumor marker protein, was greatly elevated as compared with that of the control livers. In a poorly differentiated hepatoma AH13, a line of rat ascites hepatoma, the mRNA level of $PP1_{\alpha}$ was 5.6 times higher than that of the control livers, whereas the mRNA level of PP2C was almost the same as that of the control livers and the level of PP2A mRNA was distinctly lower than that of the control livers. These data appear to suggest some involvement of protein phosphatases in hepatocarcinogenesis.

Key words: Protein phosphatase — Gene expression — Northern blot analysis — Rat liver — Hepatoma cell

Reversible phosphorylation of protein has now been recognized as a major regulatory mechanism of various cellular functions.[1-3] The fact that most oncogene products have protein kinase activity strongly suggests that cell growth is also controlled by phosphorylation/dephosphorylation of target proteins.[4,5] In the past several years, our knowledge of protein phosphatases has enormously increased. At present, four principal phosphoseryl/phosphothreonyl protein phosphatases, termed PP1, PP2A, PP2B and PP2C, are known.[1] The nucleotide sequences of the cDNAs of these molecular species have been reported.[6-11] Recently, evidence suggesting important roles of protein phosphatases in growth control of cells has been accumulating, although the mechanisms involved are not known in any detail. Suganuma *et al.* reported that okadaic acid, a potent inhibitor of PP1 and PP2A, has strong promoter action in skin carcinogenesis.[12] In contrast, Sakai *et al.* reported flat reversion by okadaic acid of *raf* and *ret*-II transformants.[13] Mutations of PP1 gene induce abnormal cell division.[14-16] PP2A was reported to exist as complexes with viral oncogene proteins.[17]

We previously reported the separation, purification and characterization of protein phosphatases from rat livers, and neoplastic alterations of the phosphatase activities in rat hepatoma cells.[18-21] Also, we showed that PP2C was replaced by the novel phosphatase H and that the enzyme activity of a divalent cation-inhibited protein phosphatase termed phosphatase N was tremendously increased in AH13.[22,23] On the basis of their nature and properties, phosphatases H and N are supposed to belong to the PP2C and PP1 types, respectively. Recently, we reported the nucleotide sequence of the $PP1_{\alpha}$ cDNA from rat kidney, and a remarkable elevation in the $PP1_{\alpha}$ mRNA level in AH13.[10] To elucidate the physiological significance of the neoplastic alterations of protein phosphatase, we have analyzed by Northern blot analysis the expression of the mRNAs of protein phosphatases PP1, PP2A and PP2C during hepatocarcinogenesis and in AH13. In the present paper, the significance of these results is discussed in relation to hepatocarcinogenesis.

MATERIALS AND METHODS

Animals, tissues, and hepatomas Male Sprague-Dawley rats (150–200 g) were subjected to a carcinogenic treat-

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ment using a Solt-Farber model.[24, 25] Animals were initially injected with DEN[7] (200 mg/kg ip), followed by a diet containing 0.02% (w/v) AAF for 4 weeks, starting 2 weeks after administration of DEN, then subjected to partial hepatectomy 1 week after initiation of AAF. Primary hepatoma was also induced in male Sprague-Dawley rats (150–200 g) according to the Solt-Farber model. AH13, a line of rat hepatoma, was inoculated intraperitoneally into Donryu rats.[26] The hepatoma cells were harvested 5 days after inoculation, washed with physiological saline and stored at −80°C until use.

Poly(A)$^+$ RNA preparations One gram of liver tissue was homogenized with a Polytron homogenizer (Kinematica, Switzerland) in a solution of 5 *M* guanidinium thiocyanate, 50 m*M* Tris-HCl, pH 7.5, 25 m*M* EDTA and 0.1 *M* 2-mercaptoethanol (soln. A). Cold ethanol (0.3 volume) was added to the homogenate, and the mixture was centrifuged for 5 min at 10,000 rpm at −10°C. The pellet was suspended in soln. A, homogenized and centrifuged for 3 min at 10,000 rpm at 4°C. After ethanol precipitation, the RNA preparations were suspended in 6 *M* guanidine hydrochloride, 25 m*M* EDTA and 0.1 *M* 2-mercaptoethanol, and precipitated with ethanol. The precipitates were dissolved in water, applied to an oligo(dT) cellulose column, washed with washing buffer (10 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 0.1% SDS, 0.1 *M* NaCl), and then eluted with elution buffer (10 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 0.02% SDS). The eluates were used as poly(A)$^+$ RNA fraction for Northern blot analysis.

cDNA probes The *Pst*I-*Sma*I fragment of 600 bp, the *Bst*EII-*Bgl*II fragment of 410 bp, and the *Pst*I-*Sal*I fragment of 550 bp were prepared from cDNAs of $PP1_\alpha$, $PP2A_\alpha$ and $PP2C_1$, respectively.[8-10] The full-length GST-P cDNA was a generous gift from Dr. Masaharu Sakai (Hokkaido University) and Dr. Masami Muramatsu (University of Tokyo).[25, 27, 28] The DNA fragments were labeled with [α-^{32}P]dCTP by using the Multiprime DNA Labeling Kit (Amersham International plc, Buckinghamshire, England) and used for Northern blot analysis as probes.

Northern blot analysis of RNA Northern blot analysis was performed as described elsewhere.[10] Five μg of poly(A)$^+$ RNA was electrophoresed on 1.2% agarose gel containing 6% formaldehyde. RNA was transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After being baked at 80°C for 2 h under vacuum, the filters were prehybridized in 5×SSPE (1×SSPE; 1.8 *M* NaCl, 0.01 *M* NaH_2PO_4, 0.1 m*M* EDTA), 5×Denhardt's solution, 100 μg/ml denatured DNA and 50% formamide overnight at 42°C. Hybridization was performed in the same solution with the multiprime radioactive cDNA probes described above overnight at 42°C. Filters were then washed twice in 5×SSPE at room temperature for 10 min, followed by washing at 50°C for 30 min in 5×SSPE containing 0.1% SDS, followed by 0.1×SSPE containing 0.1% SDS at 65°C for 2 h. Filters were exposed to XRP-5 X-ray film (Eastman Kodak Company, Rochester, NY) at −80°C with intensifying screens. The density was scanned with a densitometer (Shimadzu dual-wavelength TLC scanner). The filters were reused several times after the probes had been removed by washing the filters in 0.1×Denhardt's solution containing 2 m*M* EDTA and 5 m*M* Tris-HCl, pH 8.0, at 70°C for 2 h.

Chemicals Guanidinium thiocyanate and guanidine hydrochloride were purchased from Fluka Chemie AG (Buchs, Switzerland) and Wako Pure Chemical In-

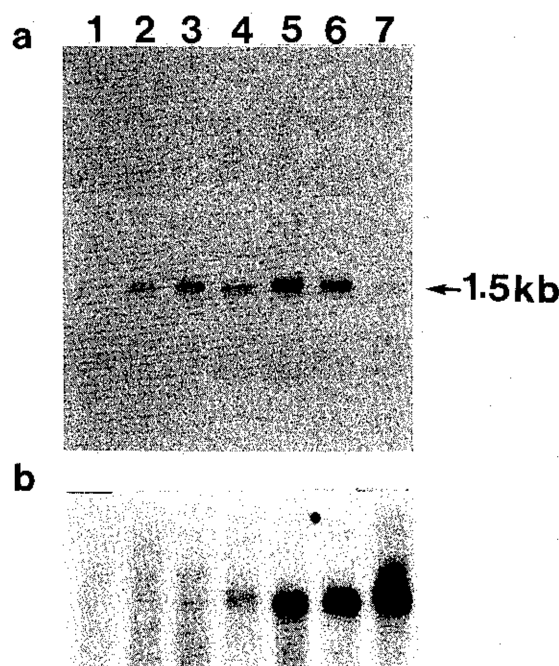

Fig. 1. Expression of PP1 mRNA in hepatocarcinogenesis. Five μg samples of poly (A)$^+$ RNA in livers at various stages of chemical carcinogenesis according to the protocol of the Solt-Farber model and in a primary hepatoma induced according to the same protocol were subjected to Northern blot analysis using a) *Pst*I-*Sma*I fragment of $PP1_\alpha$ cDNA and b) GST-P cDNA as probes, respectively. Other conditions are described in the text. Lane 1, control; lanes 2–6, 2, 3, 4, 5, and 6 weeks after administration of DEN, respectively; lane 7, primary hepatoma induced according to the Solt-Farber model.

[7] Abbreviations used in this paper: DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; MeDAB, 3′-methyl-4-dimethylaminoazobenzene; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; GST-P, glutathione *S*-transferase placental form.

dustries. Ltd. (Osaka), respectively. [α-^{32}P]dCTP and oligo(dT) cellulose were purchased from DuPont/NEN Research Products (Boston, MA) and Pharmacia LKB Biotechnology AB (Uppsala, Sweden), respectively.

RESULTS

Expression levels of PP1 mRNA Expression levels of PP1 mRNA were analyzed by Northern blot analysis in livers at various stages of chemical hepatocarcinogenesis according to the Solt-Farber protocol and in a primary hepatoma induced according to a Solt-Farber model. As shown in Fig. 1, a single band of 1.5 kb was detected in all samples employed, corresponding to mRNA of $PP1_{\alpha}$.[10] After administration of DEN, the mRNA level gradually increased, reached the maximum 5 weeks after administration (8 times higher than that of control livers) and then began to decline. In primary hepatoma induced according to a Solt-Farber model, the mRNA level was similar to the control level. Under identical conditions, the expression of GST-P mRNA was remarkably elevated as previously reported.[25]

Expression levels of PP2A mRNA The mRNA levels of PP2A were determined under identical conditions of Northern blot analysis except that the probe for PP2A was used. As already reported, two mRNA bands (a major one of 2.0 kb and a minor one of 2.7 kb) were detected.[8] As shown in Fig. 2, the major band increased during hepatocarcinogenesis, being 29 times higher at 5 weeks after administration of DEN than that of the control livers. In the primary hepatoma, the mRNA level of PP2A was similar to the control level.

Expression levels of PP2C mRNA Figure 3 shows the results of Northern blot analysis of PP2C. With PP2C, a similar gene expression pattern to those of PP1 and PP2A was observed during hepatocarcinogenesis. Five weeks after administration of DEN, the mRNA level of PP2C was 11 times higher than that of the control rats. In the primary hepatoma, the PP2C mRNA level was lower than that of the control livers.

mRNA levels of protein phosphatases in AH13 In accordance with our previous results,[10] the mRNA level of $PP1_{\alpha}$ in AH13 was 5.6 times higher than that of the control livers (Fig. 4). As the primary hepatoma induced by MeDAB showed a slight increase in the gene expression as compared to the control livers (not shown), these results strongly suggest an enhancement of the gene expression of $PP1_{\alpha}$ during progression of hepatocarcino-

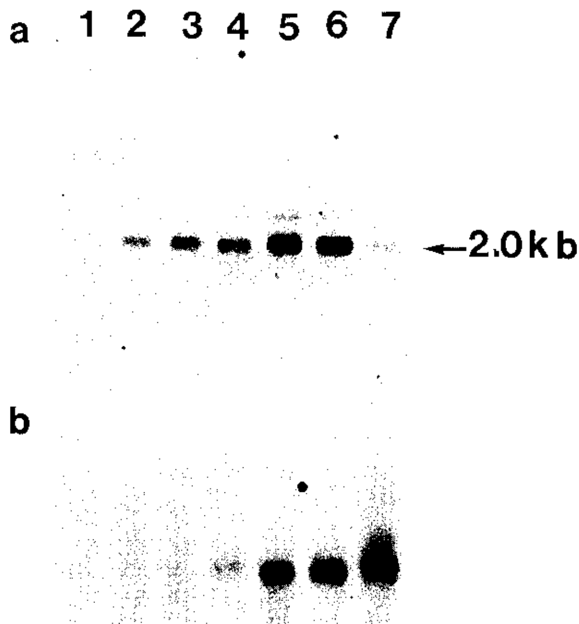

Fig. 2. Expression of PP2A mRNA in hepatocarcinogenesis. Experimental conditions in a) and b) were essentially identical to those in Fig. 1 except that *Pst*I-*Sma*I fragment of $PP1_{\alpha}$ cDNA was replaced by *Bst*EII-*Bgl*II fragment of $PP2A_{\alpha}$ cDNA. Lane 1, control; lanes 2–6, 2, 3, 4, 5, and 6 weeks after administration of DEN, respectively; lane 7, primary hepatoma induced according to the Solt-Farber model.

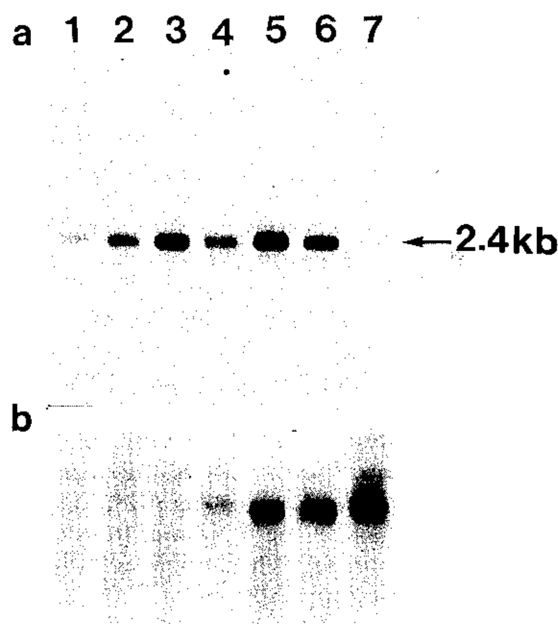

Fig. 3. Expression of PP2C mRNA in hepatocarcinogenesis. Experimental conditions in a) and b) were essentially identical to those in Fig. 1 except that the *Pst*I-*Sma*I fragment of $PP1_{\alpha}$ cDNA was replaced by the *Pst*I-*Sal*I fragment of PP2C cDNA. Lane 1, control; lanes 2–6, 2, 3, 4, 5, and 6 weeks after administration of DEN, respectively; lane 7, primary hepatoma induced according to the Solt-Farber model.

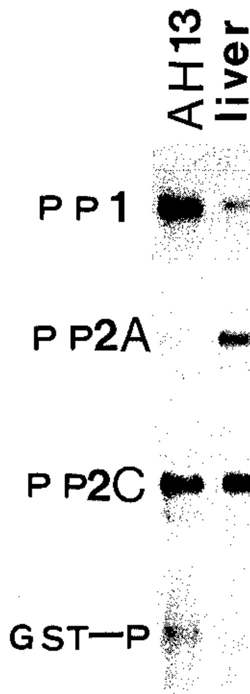

Fig. 4. Expression of PP1, PP2A and PP2C mRNAs in AH13 and in liver. Five μg samples of poly $(A)^+$ RNAs from AH13 hepatoma cells and control rat livers were subjected to Northern blot analysis. Probes used for PP1 and GST-P, PP2A, and PP2C were identical to those described in the legends to Fig. 1, Fig. 2, and Fig. 3, respectively. Other conditions are described in the text.

genesis. In striking contrast to $PP1_\alpha$, the gene expression of PP2A was much lower in AH13 than the control livers, whereas there was no alteration in the mRNA level of PP2C.

DISCUSSION

The present study demonstrates that expression levels of mRNAs of protein phosphatases $PP1_\alpha$, PP2A and PP2C were remarkably elevated in livers at preneoplastic stages of hepatocarcinogenesis, but was decreased in primary hepatoma to below the control level. In AH13, a poorly differentiated hepatoma line, the mRNA level of $PP1_\alpha$ was greatly and selectively elevated as compared with that of the control livers, whereas the mRNA levels of PP2A and PP2C in AH13 were decreased and unchanged from the control values, respectively.

Two weeks after administration of DEN, altered foci appeared in the livers. But, upon administration of AAF at this stage, the foci mostly disappeared because of the toxicity of AAF. Thereafter the area of hyperplastic nodules gradually increased. The ratios of area of hyperplastic nodule cells to area of normal liver cells at 4, 5, and 6 weeks after administration of DEN were 0.05–0.10, 0.25–0.30, and 0.42–0.54, respectively.[25] The mRNA levels were significantly increased 3 weeks after administration of DEN. At this stage, hyperplastic nodules were very small, and the mRNA levels of GST-P were also very low. Then the phosphatase mRNA levels further increased, but the patterns of this increase varied from experiment to experiment. In most cases, the mRNA levels at 4 weeks after administration of DEN were slightly lower than those at 3 weeks and 5 weeks after the administration as shown in Figs. 1–3. However, in some cases, the mRNA levels at 4 weeks after the administration were intermediate between those at 3 weeks and 5 weeks after the administration. So we have presented what we regard as typical data. We speculate that the increase at 3 weeks after the administration of DEN might be ascribed to the response of normal liver cells to DEN, the levels being transiently decreased 4 weeks after the administration probably because of the toxicity of AAF to liver cells, and then increased because of the formation of hyperplastic nodules. So the levels at 4 weeks after the administration seem to reflect the influences of at least two factors, i.e., response to DEN and formation of hyperplastic nodules.

The decreases of the mRNA levels in primary hepatoma were unexpected, because previous studies revealed elevation of the mRNA levels of PP1 and PP2A in hepatoma cells.[8, 10] In the primary hepatoma presented here, in which the mRNA levels of protein phosphatases were all decreased, the mRNA level of GST-P, a tumor marker protein, was dramatically elevated, as already reported.[25, 27, 28] These results exclude the possibility that the decrease in the mRNA levels in the primary hepatoma was due to artifacts during preparation of the RNAs used. Increase in protein kinase activities during hepatocarcinogenesis and in hepatomas has been reported.[29, 30] We have also reported an increase in casein kinase 1 activity in AH13.[26] Moreover, Beer *et al.* reported the elevation in rat hepatoma cells of mRNA for c-*raf*, which encodes a protein kinase catelyzing the phosphorylation of serine and threonine.[31] Also, Maridor *et al.* reported that expressions of mRNA for all three subunits of casein kinase II were high in early embryos and decreased substantially during embryogenesis, suggesting involvement of this protein kinase in control of cell proliferation.[32] Persons *et al.* reported that transfection of NIH 3T3 fibroblasts with protein kinase C enhanced tumorigenicity via enhancement of the mRNA levels of the PKC-I.[33] These results, taken together, suggest that the mRNA levels of protein kinases might be predominant, at least in primary hepatomas, over those of protein phosphatases.

Recently, evidence demonstrating involvement of both phosphorylation and dephosphorylation in the cell cycle has been accumulating.[34-36] Taking account of these facts, our present results appear to show an important role of protein phosphatases in the regulation of cell growth at preneoplastic stages of hepatocarcinogenesis. It is of geat interest that there was a striking difference in gene expressions of protein phosphatases among livers at preneoplastic stages, primary hepatoma, and transplanted, poorly differentiated hepatoma AH13. Since not only PP2A but also PP1 can form heterooligomers and their activities are subject to multiple control mechanisms, these alterations in mRNA levels might not necessarily reflect the protein phosphatase activities. But these features of the gene expression may represent a new control mechanism of protein phosphatase. It is tempting to suggest a relationship of the elevation of $PP1_\alpha$ mRNA levels to malignancy during the progression of hepatocarcinogenesis.

We previously reported a decrease in the enzyme activity of PP2C and emergence of the novel protein phosphatase H.[22] We also found in AH13 a marked increase in protein phosphatase N belonging to the PP1 category.[23] These previous results may be reflections of the increase in gene expression of PP1 presented in this paper. Attempts to elucidate the relationship between those enzymatic alterations previously reported and the present data on the mRNA levels are in progress.

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