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Differential Changes in Expression of Gap Junction Proteins Connexin 26 and 32 during Hepatocarcinogenesis in Rats

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We examined expressions of the gap junction proteins, connexin 26 (Cx26) and 32 (Cx32), in preneoplastic and neoplastic lesions during rat hepatocarcinogenesis. A marked reduction in the number of Cx32-positive gap junctions was observed in 17% of the glutathione *S*-transferase placental form-positive foci, whereas 44% of the foci showed increased expression of Cx26. Most hyperplastic nodules exhibited decreased expression of Cx32, whereas 16% of the nodules showed increased expression of Cx26. In hepatocellular carcinomas, expressions of both Cx32 and Cx26 were significantly reduced. These results suggest that the expressions of Cx32 and 26 are differentially regulated during hepatocarcinogenesis, and that the decrease in Cx32 expression occurs earlier, whereas reduction in Cx26 expression occurs later in association with promotion and progression of carcinogenesis.

Key words: Gap junction — Connexin — Hepatocarcinogenesis

Gap junctions are composed of a family of structural proteins called connexins,[1)] which oligomerize into intercellular channels, and mediate transfer of low-molecular-weight metabolites and ions between the cells in contact. Gap-junctional intercellular communication (GJIC[2]) is suggested to be involved in metabolic cooperation, cell differentiation and growth control.[2)] So far, approximately ten different homologous connexin sequences have been cloned and characterized in the mouse or rat genome.[3)] Among them, connexins 32 (Cx32)[4)] and 26 (Cx26)[5,6)] are expressed by hepatocytes. It has been reported that the ratio of Cx32 to Cx26 is ~10:1 in isolated rat liver gap junction plaques and ~2:1 in isolated mouse liver gap junction plaques.[5,7)]

There is increasing evidence that GJIC plays an important role in carcinogenesis.[8–10)] In rat chemical hepatocarcinogenesis, several laboratories have reported a significant decrease in the amount of Cx32 in persistent nodules and hepatocellular carcinomas (HCC).[11–14)] Krutovskikh *et al.*[15)] recently described a significant decrease in Cx32 expression in glutathione *S*-transferase placental form (GST-P)-positive foci at early stages of hepatocarcinogenesis using the Solt-Farber protocol. Although the expression of Cx26 has been shown to decrease after partial hepatectomy,[7)] little information is currently available on the changes of Cx26 expression during hepatocarcinogenesis in rats.

We report here evidence that the expression of Cx26 and Cx32 is differentially regulated during chemical hepatocarcinogenesis in rats.

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[2] Abbreviations: GJIC, gap-junctional intercellular communication; Cx connexin; HCC, hepatocellular carcinoma; GST-P, glutathione *S*-transferase placental form; AAF; acetylaminofluorene; PH, partial hepatectomy.

MATERIALS AND METHODS

Animals and treatment The Solt-Farber protocol[16)] was used to induce liver lesions in male Fischer 344 rats (body weight, 150 g). This protocol was initiated by an i.p. injection of diethylnitrosamine (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl at the dose of 200 mg/kg. Two weeks later, the rats were fed a diet (Oriental Yeast Co., Ltd., Tokyo) containing 0.02% 2-acetylaminofluorene (AAF) for a 2-week period; in the middle of the AAF selection period, a 70% partial hepatectomy (PH) was performed. The animals were killed at 4 weeks, 5 months and 1 year after the beginning of the experiment and the livers were frozen in liquid nitrogen.

Immunofluorescence Frozen serial sections (6 μm) were fixed with acetone for 5 min at −20°C and dried. The sections were incubated with one of the following antibodies for 2 h at room temperature: a rabbit polyclonal antibody against a Cx26-specific peptide (amino acid residues 101–119), a rabbit anti-J-peptide antiserum against connexin 32[17)] (1/80 dilution), or anti-rat GST-P antibody[18)] (1/2500 dilution). After washing in PBS, the sections were reacted with swine fluorescein-conjugated anti-rabbit immunoglobulins (1/100; DAKO, Copenhagen, Denmark) for 1 h and examined under a fluorescence microscope. Some sections were double-stained for Cx26 and Cx32, by utilizing mouse monoclonal antibody against Cx32[19)] and swine rhodamine-conjugated anti-mouse immunoglobulins (1/100; DAKO). Some sections were stained with hematoxylin and eosin (H&E) for histopathological examination. After immunostaining, randomly selected areas of the liver lesions including GST-P-positive foci, nodules and HCCs were photographed at the magnification of ×40. The number of fluorescent spots on hepatocyte plasma membranes was

counted using an image analysis system (LA-555, Pias, Inc., Osaka).

Northern blot analysis Total RNA was isolated from normal rat livers, hyperplastic nodules and HCCs using a single-step thiocyanate-phenol-chloroform extraction method.[20] The concentration of RNA was determined by measuring absorption at 260 nm and by the intensity of RNA-ethidium bromide staining on the gel. Northern analysis was conducted using γ-^{32}P-labeled cDNA probes for Cx26[6] and Cx32.[4] After exposure to film, northern blots were reprobed with a cDNA β-actin probe to confirm that we had loaded similar amounts of RNA from each sample.

RESULTS

The rabbit polyclonal antibody against Cx26-specific peptide and anti-J-peptide antisera against Cx32 revealed regular macular staining on membranes between adjacent hepatocytes of a non-treated rat (Fig. 1). While Cx32 was uniformly distributed in hepatocytes within the liver lobules, Cx26 was prominent in the peripheral zones and almost absent in hepatocytes located near the central vein, in accordance with the findings reported by Traub *et al.*[7]

Figure 2 shows sequential changes in the number of Cx26- and Cx32-immunoreactive spots observed in hepatocytes located in the areas free from preneoplastic or neoplastic lesions. A significant decrease in the number of Cx32-positive spots was observed in the hepatocytes which composed the area free from preneoplastic lesions at the 4th week of the experiment, in agreement with the report of Krutovskikh *et al.*[15] On the other hand, the average number of Cx26-positive spots in the areas free from liver lesions did not show any significant change, though the uneven distribution of Cx26 in the liver lobules became less prominent, presumably due to the reorganization of liver lobules after AAF-treatment and PH.

Figure 3 demonstrates the relative distributions of GST-P-positive foci at 4 weeks, hyperplastic nodules at 5 months and HCCs at 1 year. The numbers of Cx26- or Cx32-positive gap junctional spots are shown as percentages of those in surrounding hepatocytes. The numbers of Cx32- and Cx26-positive spots varied substantially among foci at the 4th week. No foci had fewer Cx26-positive spots than the surrounding hepatocytes. Conversely, 44% of the foci had more (>50%) Cx26-positive spots than the surrounding hepatocytes (Fig. 4B). In contrast, 12% of the foci exhibited a marked decrease in the number of Cx32-positive spots to less than half of

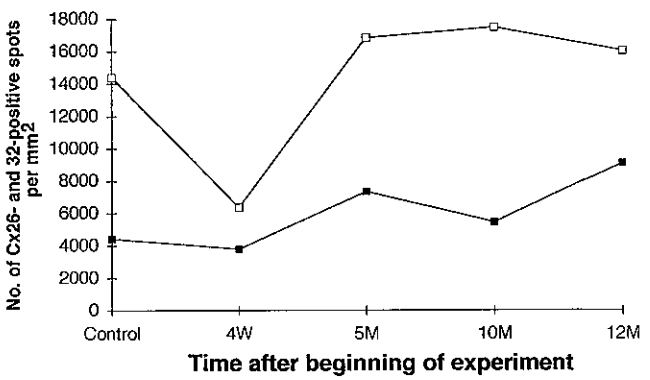

Fig. 2. Sequential changes in the numbers of Cx26- (■) and Cx32-immunoreactive spots (□) in rat liver parenchyma free from focal lesions (GST-P-negative area). W, weeks; M, months.

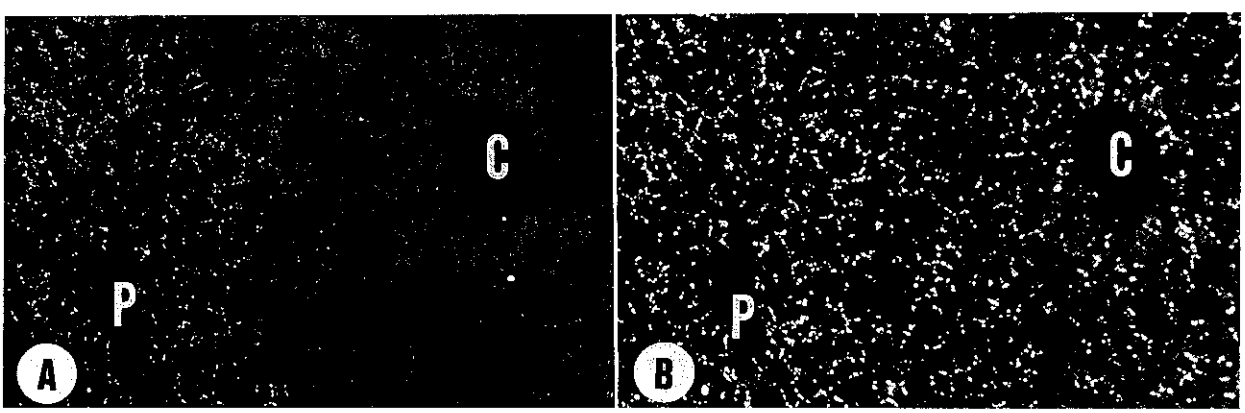

Fig. 1. Double immunofluorescent staining of Cx26 (A) and Cx32 (B) in the same field of a normal rat liver. Cx26-positive spots are most prominent around the portal area (P) and decrease in number towards the central vein (C), whereas Cx32-positive spots are localized uniformly within the lobule.

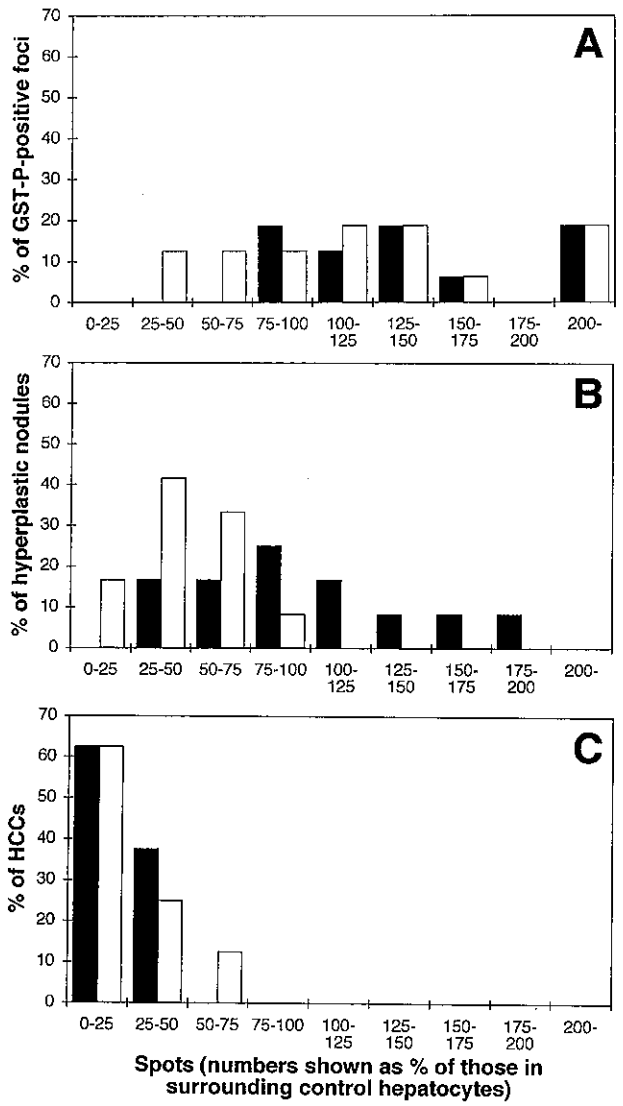

Fig. 3. Relative distributions of GST-P-positive foci at 4 weeks (A), hyperplastic nodules at 5 months (B) and HCCs at 1 year (C) in terms of the number of Cx26- (solid bars) or Cx32-positive spots (open bars).

that seen in the surrounding hepatocytes (Fig. 4D), while only 25% of the foci showed a significant increase (>50%) in the number of Cx32-positive spots.

Hyperplastic nodules at 5 months demonstrated substantial variations in the number of Cx26-positive spots. Unlike the foci at 4 weeks, 25% of the nodules showed a significant decrease (>50%) in the number of Cx26-positive spots (Fig. 5B), although 16% of the nodules still had an increased number (>50%). No nodules exhibited a marked increase in the number of Cx32-positive spots. In fact, a large decrease was observed in 58% of the nodules (Fig. 5C).

Most HCCs at 1 year (8/8 for Cx26 and 7/8 for Cx32) demonstrated a striking reduction (>50%) in the numbers of both Cx26- and Cx32-positive spots (Fig. 6).

Northern blot analysis revealed that there was no clear reduction in Cx26 mRNA in HCCs, whereas a noticeable decrease in the Cx32 mRNA was found in HCCs (Fig. 7).

DISCUSSION

A limited amount of information exists on the changes in expression of various kinds of connexins in hepatocytes under physiological and pathological conditions. Traub *et al.*[7] observed that the amounts of Cx26 and Cx32 decreased and increased similarly with time in mouse embryonic hepatocytes in culture and suggested that the appearance of both proteins in hepatic plasma membranes is similarly regulated. They indicated that the expression of both proteins is also similarly regulated in mouse hepatocytes after partial hepatectomy. In contrast, the present study has demonstrated that expression of Cx26 and Cx32 was differentially regulated during rat chemical hepatocarcinogenesis. A decrease in the number of Cx32-positive spots occurred in preneoplastic foci at an early stage, at which time no decrease in the number of Cx26-positive spots was observed. At later stages the number of Cx26-positive spots was reduced in some hyperplastic nodules. Finally, all HCCs examined had a significant reduction in both Cx26 and Cx32 protein expression. Northern blot analysis revealed that there was a significant decrease in the steady-state level of Cx32 mRNA in HCCs, as earlier studies had reported,[12, 13] but no clear reduction in that of Cx26 mRNA. Therefore, it appears that a post-transcriptional mechanism may be important for the reduction of the number of Cx26-positive spots in HCC while a transcriptional decrease in the Cx32 gene may be responsible for the observed decrease in the number of Cx32-positive gap junctions.

Transformed cells *in vitro* and *in vivo* have a decreased GJIC capacity among themselves[8] or with surrounding normal cells.[21] It has been discovered that some tumor-promoting agents can block GJIC[22, 23] and it was proposed that inhibition of GJIC may be involved in the clonal expansion of initiated cells by releasing them from suppressive control exerted by surrounding normal cells via GJIC.[8, 22] Our present results suggested that, in multistage carcinogenesis of rat livers, a decrease in Cx32 expression is an early event related to initiation or early promotion and that reduction in Cx26 expression may be a later phenomenon associated with promotion and progression of preneoplastic lesions to malignant tumors. Our findings described above are in good agreement with the previous report by Krutovskikh *et al.*[15] showing that

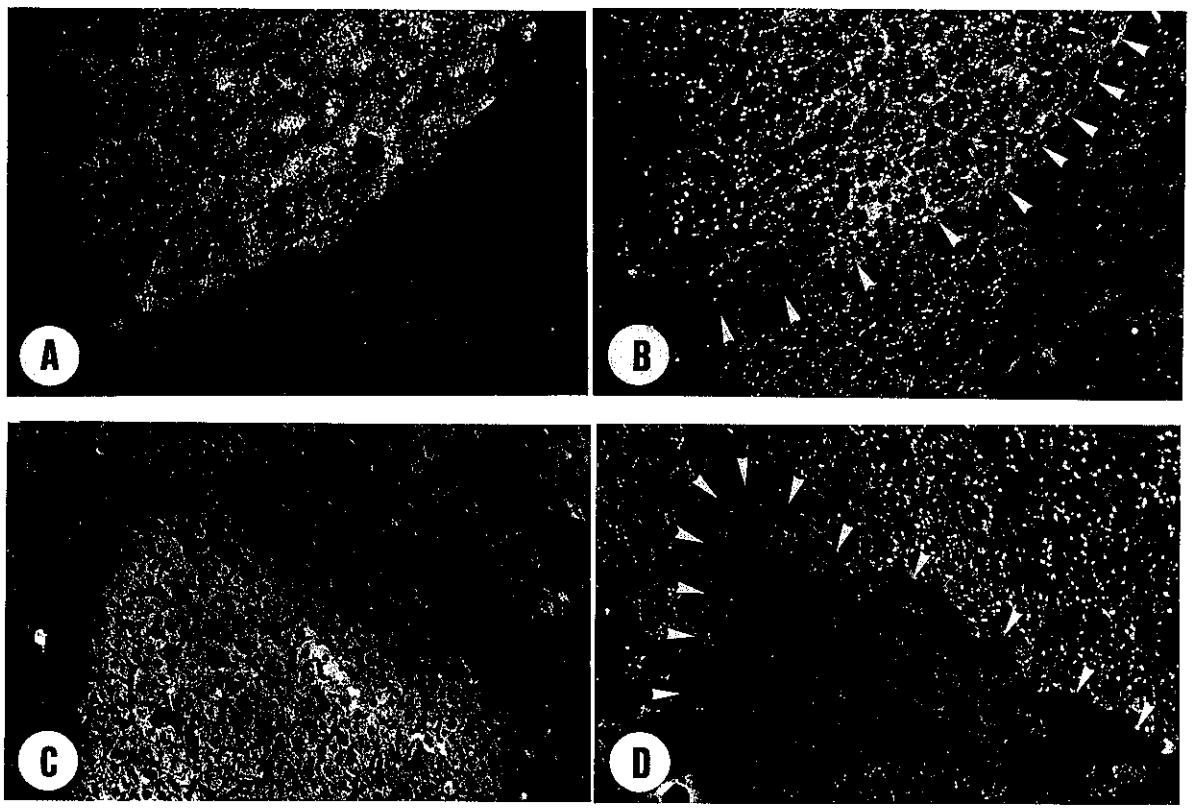

Fig. 4. Double immunofluorescent staining of GST-P (A, C) and, Cx26 (B) or Cx32 (D) in the livers at 4 weeks. A and B are taken from the same field as are C and D. The number of Cx26-positive spots (B) is increased in the GST-P-positive focus (A), whereas that of Cx32 (D) is decreased in the other focus (C).

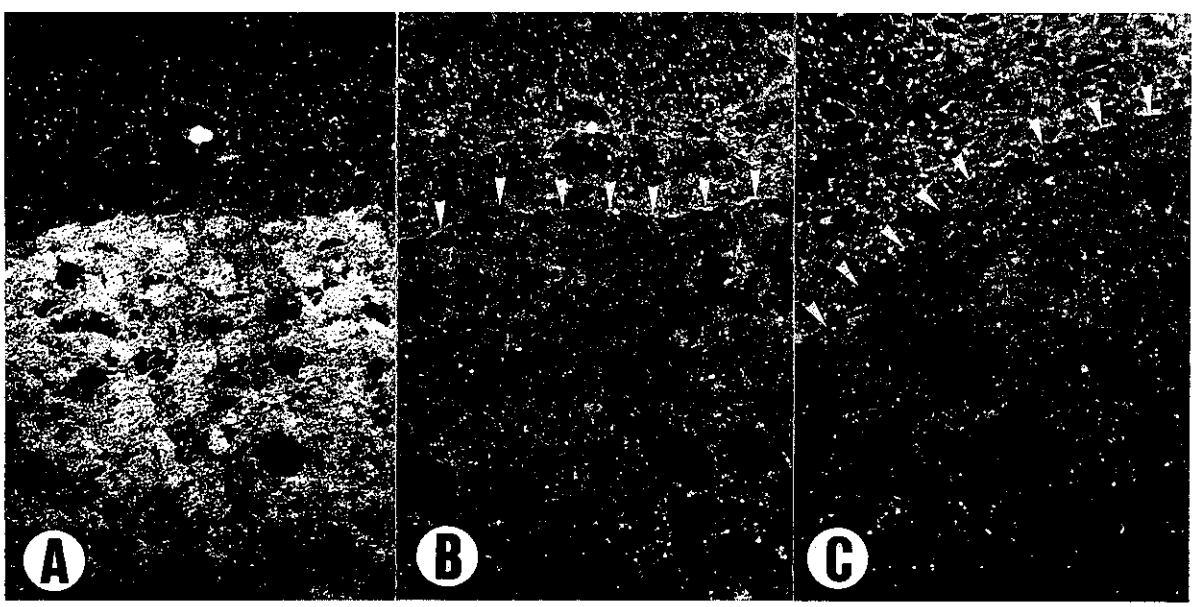

Fig. 5. Essential reductions of Cx26- (B) and Cx32- (C) positive spots in the GST-P-positive nodule (A) at 5 months. Serial sections.

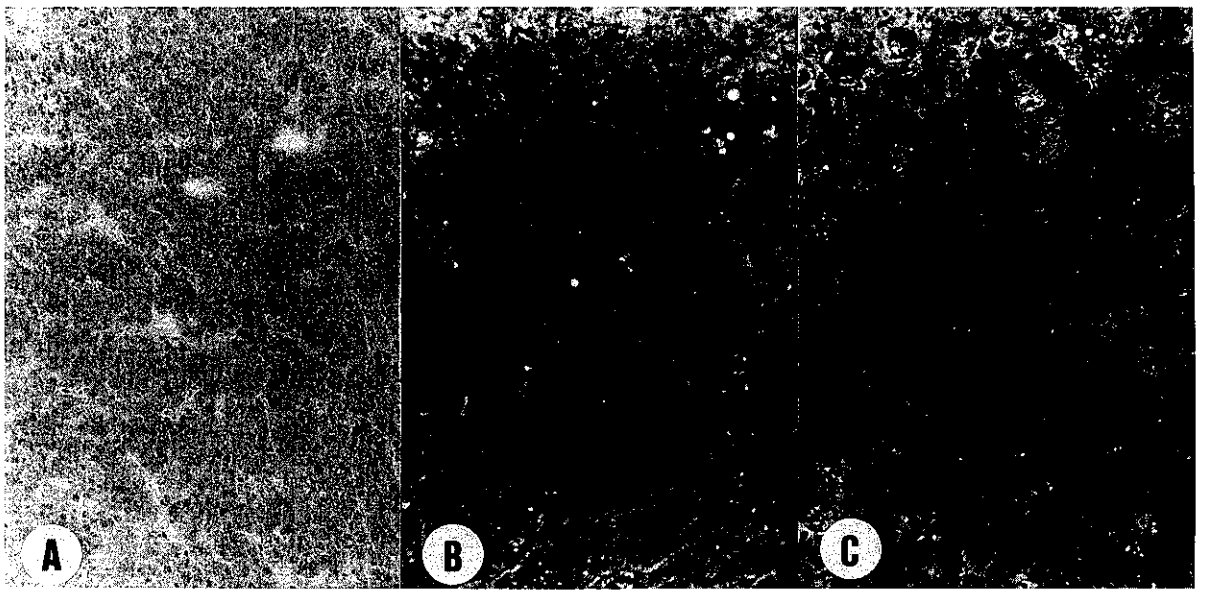

Fig. 6. Total absence of Cx26- (B) and Cx32-positive spots (C) in hepatocellular carcinoma at 1 year. (A) H&E.

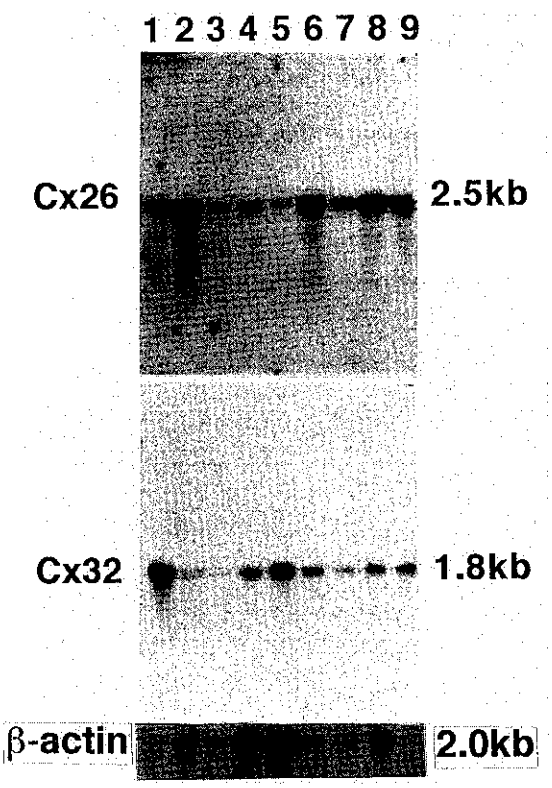

Fig. 7. Northern blot analysis of transcripts of Cx26, Cx32 and β-actin in a rat liver control, in hepatocellular carcinomas and in a hyperplastic nodule using the same filter. Lane 1: liver of untreated rat. Lanes 2–9 except for lane 5: hepatocellular carcinomas. Lane 5: hyperplastic nodule.

GST-P-positive foci had lower GJIC than surrounding hepatocytes and that HCCs showed a further reduction of GJIC as demonstrated by microinjection/dye transfer assay. We suppose that, among hyperplastic nodules, those with lower expression of Cx26 may preferentially advance to HCCs.

Recent studies on cloning and characterization of new connexin genes suggested that coexpression of more than one connexin occurs in most, if not all, cell types.[1,3] For example, hepatocytes express both Cx26 and Cx32, which co-localize to the same junctional plaques.[5–7] However, it remains unclear whether different connexin genes encode gap junction proteins with different functions. Barrio *et al.*[24] recently studied properties of coupling from expression of Cx complementary RNAs in *Xenopus* oocyte pairs. They indicated that Cx26 and Cx32 can form homotypic and heterotypic junctions and that properties of the hemichannels contributed by the two connexins in the heterotypic case were different from those in homotypic junctions. They further demonstrated that when one oocyte was injected with Cx32 cRNA and the other with a varying ratio of Cx32 and Cx26 cRNAs, the properties of channels were modulated. In the present study, some preneoplastic lesions showed an increase in the number of Cx26-positive spots and a decrease in that of Cx32-positive spots, but other lesions exhibited decreases in both proteins. Therefore, it seems plausible that during hepatocarcinogenesis, GJIC may be modified not only by the loss of both Cxs, but also by differential expression of Cx26 and Cx32.

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REFERENCES

1) Beyer, E. C., Paul, D. L. and Goodenough, D. A. Connexin family of gap junction proteins. *J. Membr. Biol.*, **116,** 187–194 (1990).
2) Hertzberg, E. and Johnson, R. "Gap Junctions. Modern Cell Biology Vol. 7" (1988). Alan R. Liss, Inc., New York.
3) Willecke, K., Hennemann, H., Dahl, E., Jungbluth, S. and Heynkes, R. The diversity of connexin genes encoding gap junctional proteins. *Eur. J. Cell Biol.*, **56,** 1–7 (1991).
4) Paul, D. L. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.*, **103,** 123–134 (1986).
5) Nicholson, B. J., Dermietzel, R., Teplow, D., Traub, O., Willecke, K. and Revel, J. P. Two homologous protein components of hepatic gap junctions. *Nature*, **329,** 732–734 (1987).
6) Zhang, J. T. and Nicholson, B. J. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J. Cell Biol.*, **109,** 3391–3401 (1989).
7) Traub, O., Look, J., Dermietzel, R., Brümmer, F., Hülser, D. and Willecke, K. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.*, **108,** 1039–1051 (1989).
8) Loewenstein, W. R. and Kanno, Y. Intercellular communication and the control of tissue growth. Lack of communication between cancer cells. *Nature*, **209,** 1248–1249 (1966).
9) Yamasaki, H. Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis*, **11,** 1051–1058 (1990).
10) Klaunig, J. E. and Ruch, R. J. Role of inhibition of intercellular communication in carcinogenesis. *Lab. Invest.*, **62,** 135–146 (1990).
11) Janssen-Timmen, U., Traub, O., Dermietzel, R., Rabes, H. M. and Willecke, K. Reduced number of gap junctions in rat hepatocarcinomas detected by monoclonal antibody. *Carcinogenesis*, **7,** 1475–1482 (1986).
12) Beer, D. G., Neveu, M. J., Paul, D. L., Rapp, U. R. and Pitot, H. C. Expression of the c-*raf* protooncogene, gamma-glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. *Cancer Res.*, **48,** 1610–1617 (1988).
13) Fitzgerald, D. J., Mesnil, M., Oyamada, M., Tsuda, H., Ito, N. and Yamasaki, H. Changes in gap junction protein (connexin 32) gene expression during rat liver carcinogenesis. *J. Cell Biochem.*, **41,** 97–102 (1989).
14) Neveu, M. J., Hully, J. R., Paul, D. L. and Pitot, H. C. Reversible alteration in the expression of the gap junctional protein connexin 32 during tumor promotion in rat liver and its role during cell proliferation. *Cancer Commun.*, **2,** 21–31 (1990).
15) Krutovskikh, V. A., Oyamada, M. and Yamasaki, H. Sequential changes of gap-junctional intercellular communications during multistage rat liver carcinogenesis: direct measurement of communication *in vivo*. *Carcinogenesis*, **12,** 1701–1706 (1991).
16) Solt, D. B. and Farber, E. New principle for the analysis of chemical carcinogenesis. *Nature*, **263,** 702–703 (1976).
17) Milks, L. C., Kumar, N. M., Houghten, R., Unwin, N. and Gilula, N. B. Topology of the 32-kd liver gap junction protein determined by site-directed antibody localizations. *EMBO J.*, **7,** 2967–2975 (1988).
18) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. Purification, induction and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **82,** 3964–3968 (1985).
19) Takeda, A., Kanoh, M., Shimazu, T. and Takeuchi, N. Monoclonal antibodies recognizing different epitopes of the 27-kDa gap junction protein from rat liver. *J. Biochem.*, **104,** 901–907 (1988).
20) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162,** 156–159 (1987).
21) Enomoto, T. and Yamasaki, H. Lack of intercellular communication between chemically transformed and surrounding nontransformed BALB/c 3T3 cells. *Cancer Res.*, **44,** 5200–5203 (1984).
22) Yotti, L. P., Chang, C. C. and Trosko, J. E. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science*, **206,** 1089–1091 (1979).
23) Murray, A. W. and Fitzgerald, D. J. Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. *Biochem. Biophys. Res. Commun.*, **91,** 395–401 (1979).
24) Barrio, L. C., Suchyna, T., Bargiello, T., Xu, L. X., Roginski, R. S., Bennett, M. V. L. and Nicholson, B. J. Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by applied voltage. *Proc. Natl. Acad. Sci. USA*, **88,** 8410–8414 (1991).