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Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver

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SUMMARY

Although several abnormalities in gap junction (GJ) structure and/or function have been described in neoplasms, the molecular mechanisms responsible for many of the alterations remain unknown. The identification of a family of GJ proteins, termed connexins, prompted this study of connexin32 (Cx32), connexin26 (Cx26) and connexin43 (Cx43) expression during rat hepatocarcinogenesis. Using antibody, cDNA and cRNA probes, we investigated connexin mRNA and protein expression in preneoplastic and neoplastic rat livers. In normal liver, Cx32 is expressed in hepatocytes throughout the hepatic acinus, Cx26 is restricted to periportal hepatocytes, and Cx43 is expressed by mesothelial cells forming Glisson's capsule.

Most preneoplastic altered hepatic foci generated by diethylnitrosamine (DEN) initiation and either phenobarbital (PB) or 2,3,7,8-dichlorodibenzo-*p*-dioxin (TCDD) promotion exhibited decreased Cx32 or increased Cx26 staining. Foci from either protocol failed to display Cx43 immunoreactivity. In the majority of PB-promoted foci, Cx32 immunoreactivity decreased independently of changes in mRNA abundance. Continuous thymidine labeling, following cessation of PB promotion, showed that downregulation of Cx32 staining is reversible in foci that are promoter-dependent for growth, but irreversible in lesions that are promoter-independent for growth.

Hepatic neoplasms from rats initiated with DEN and promoted with PB or TCDD also displayed modified connexin expression. While all 24 neoplasms studied were deficient in normal punctate Cx32 and Cx26 staining, altered cellular localization of these proteins was apparent in some tumors. Immunoblotting of crude tissue extracts revealed that neoplasms with disordered Cx32 staining showed immunoreactive bands with altered electrophoretic mobility. These observations show that hepatomas may downregulate Cx32 expression through changes in the primary structure of Cx32 or by post-translational modifications. Northern blotting of total tumor mRNAs failed to demonstrate consistent changes in the abundance of Cx32, Cx26 or Cx43 transcripts. Some tumors expressed steady-state transcripts without observable immunoreactivity, indicating that some hepatomas downregulate connexin immunoreactivity independently of mRNA abundance. Increased levels of Cx43 mRNA and protein were found in several neoplasms, but immunostaining was always localized to nonparenchymal cells. Areas of bile duct proliferation and cholangiomas displayed Cx43 staining, whereas cholangiocarcinomas were deficient in immunoreactivity. These findings show that alterations in the expression of connexins, by either downregulation or differential induction, represent common modifications during hepatocarcinogenesis. Although our results imply that connexins represent useful markers for the boundary between tumor promotion and progression, preneoplastic and neoplastic rat hepatocytes fail to use a common mechanism to modify connexin expression.

Key words: hepatocarcinogenesis, connexin, gap junction

INTRODUCTION

Gap junctions (GJs) represent aggregates of transmembrane channels that provide cells with a mechanism to regulate the sharing of second messengers and other hydrophilic molecules less than about 1200 Da in mass. Recent studies have identified a family of GJ proteins, termed connexins, that form intercellular pores exhibiting different unitary conductance values and gating characteristics. Although the precise functional roles of the different connexins are unknown, normal biological processes including development, cell proliferation and modifications in organ physiology can be modulated by GJ-

mediated intercellular communication (GJIC) (Loewenstein, 1981; Larsen, 1989; Beyer et al., 1990; Musil and Goodenough, 1990; Bennett et al., 1991).

Since both positive and negative growth regulatory molecules may pass through GJs, a role of alterations in GJIC has been postulated for neoplastic development (Loewenstein, 1979). In accord with this hypothesis, many tumor promoters, oncogenic proteins and growth factors have been demonstrated to modulate GJIC (Loewenstein, 1990; Trosko et al., 1990; Musil and Goodenough, 1990; Yamasaki, 1991). Furthermore, subtractive hybridization of normal and tumor mRNAs has identified connexins as potential tumor suppressors (Lee et al., 1991). Tumorigenic cells transfected with either connexin32 (Cx32) or connexin43 (Cx43) exhibit growth retardation in vitro and in nude mice (Eghbali et al., 1991; Mehta et al., 1991; Naus et al., 1992).

Models of multistage hepatocarcinogenesis in the rat represent a useful system in which to study the biochemical and molecular mechanisms of neoplasia. Discrete populations of cells can be identified to study the stages of initiation, promotion and progression (Saeter and Seglen, 1990; Pitot et al., 1991). Approximately 90% of the total rat liver GJ protein has a predicted mass of 32 kDa (connexin32; Cx32), while a minor form has been termed connexin 26 (Cx26) (Paul, 1986; Zhang and Nicholson, 1989). Whereas Cx32 is expressed by hepatocytes throughout the rat acinus, Cx26 is restricted to periportal hepatocytes (Traub et al., 1989; Berthoud et al., 1992a). Connexin 43 (Cx43), first identified in cardiac myocytes, is expressed by several liver-derived cell lines (Asamoto et al., 1991; Spray et al., 1991) and in Glisson's capsule and perisinusoidal cells in fetal rat liver (Berthoud et al., 1992a). Furthermore, immortalized embryonic mouse hepatocytes express Cx32 and Cx26, but shift to Cx43 when serum is added to the medium (Stutenkemper et al., 1992).

During rat hepatocarcinogenesis Cx32 immunoreactivity and GJIC are reversibly downregulated in preneoplastic foci (Neveu et al., 1990). Unlike other markers of hepatic foci, these alterations were observed with several chemical carcinogenesis regimens (Neveu et al., 1990; Klaunig et al., 1990; Klaunig, 1991; Krutovskikh et al., 1991). In addition to alterations in Cx32, some hyperplastic nodules display increased Cx26 immunostaining (Sakamoto et al., 1992). While all hepatic neoplasms examined to date (from rats, mice and humans) display decreased abundance of morphologically identifiable GJs (Weinstein et al., 1976; Swift et al., 1983), the molecular mechanisms regulating these changes remain unclear. Janssen-Timmen and colleagues (1986) showed that rat hepatic neoplasms display reduced expression of Cx32 by immunofluorescence and western blotting. However, subsequent studies by our laboratory and others showed near normal levels of Cx32 mRNA in hepatic neoplasms from rats, mice and humans (Beer et al., 1988; Fitzgerald et al., 1989; Beer and Neveu, 1990; Oyamada et al., 1990; Sakamoto et al., 1992).

To better understand the regulation of connexins during rat hepatocarcinogenesis, we examined Cx32, Cx26 and Cx43 expression in preneoplastic and neoplastic lesions using cDNA, cRNA and antibody probes. The questions addressed were: (1) do modifications in Cx32 mRNA and protein expression commonly occur in rat hepatic neoplasms? (2) are changes in Cx32 expression reversible in promoter-independent lesions? and (3) do modifications in Cx26 and Cx43 mRNA or protein expression occur in preneoplastic or neoplastic rat hepatocytes?

MATERIALS AND METHODS

Animal experiments

Inbred Fisher F344 and outbred Sprague-Dawley rats (Harlan Sprague Dawley Co., Madison, WI) were maintained at 27°C on a 14 hour light/10 hour dark cycle and fed defined AIN-76 or crude NIH-07 diet (Teklad Test Diets, Madison, WI) and water ad libitum. Preneoplastic hepatic foci were examined in newborn female F344 rats initiated with a single non-necrogenic dose of diethylnitrosamine (DEN) (10 mg/kg, p.o.) and promoted with either 0.05% phenobarbital (PB) for 8 months or 0.1 μg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 14 months (Dragan et al., 1991). Promoter-independent lesions (Pitot et al., 1991), defined as those that continue to proliferate following withdrawal of PB promotion, as well as promoter-dependent foci, were induced in neonatal female F344 rats initiated with DEN (10 mg/kg, p.o.) followed by 8 months of 0.1% PB. At this time, osmotic minipumps (model 2001 Alzet, Alza Corp., Palo Alto, CA) containing 600 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL; specific activity, 84.0 Ci/mmol) were implanted i.p. (Neveu et al., 1990). Eight days following the implant, the animals were withdrawn from the PB-containing diet and placed on purified AIN-76 diet for 20 days before being killed. Hepatic neoplasms were generated by a single non-necrogenic dose of DEN (10-30 mg/kg, p.o.) followed by chronic promotion with either 0.05% PB or 0.1 μg/kg per day TCDD for 14 months (Dragan et al., 1991; Beer et al., 1988). Following their respective regimens, rats were killed, and representative sections of the liver were fixed for histopathology by rapid freezing on solid CO_2 for staining studies or in liquid nitrogen for mRNA and protein analyses.

Histopathology of neoplasms

The neoplastic nature of 22 lesions was evaluated by histopathological examination of H&E-stained sections as previously described (Beer et al., 1988). Tumors were dissected to remove surrounding liver tissue prior to isolation of protein and RNA samples. The histology of DEN/PB-treated neoplasms is listed in Fig. 4, below. Several hepatomas were also examined from rats treated with DEN/TCDD: TCDD1-hepatocellular carcinoma with mixed cell types with focal areas of lymphoid infiltration; TCDD2-hepatocellular carcinoma; TCDD3-hepatocellular carcinoma with fibrosis; TCDD4-hepatocellular carcinoma with bile duct proliferation, fatty metamorphosis and inflammation; TCDD5-neoplastic nodule with focal fibrosis, bile duct proliferation and inflammation; TCDD6-cholangiocellular carcinoma.

Antibodies

Because of the similarity of the amino acid sequences between the members of the connexin family (reviewed by Bennett et al., 1991), as well as the possibility of epitope masking, several antibodies generated against different cytoplasmic epitopes were employed to verify immunoreactivity (Table 1). A rabbit polyclonal antibody generated against the placental form of rat glutathione S-transferase (GST) identified altered hepatic foci (AHF; Hendrich et al., 1987). Immunocytochemistry and immunoblotting procedures were optimized so that no reactivity was observed when preimmune serum or nonspecific ascites fluid was substituted for the antibody preparations.

Immunoblot analyses

Crude homogenates were prepared by disrupting tissue for 30 seconds with a Brinkmann homogenizer (Brinkmann, Westbury, NY); 50 mg (wet weight) of tissue samples was disrupted in 1 ml of isolation

Table 1. Antibodies to connexins used in this study

Specificity	Name	Hapten	Species	Reference
Cx32	M12.13	Rat liver GJ*	Mouse†	Paul (1986)
	Lola	aa‡ 98-124	Rabbit§	Paul (1986
	594	Rat liver GJ	Sheep§·¶	Yamamoto et al. (1990)
	92b	Rat liver GJ	Rabbit§·¶	Yamamoto et al. (1990)
Cx26	LuLu	aa 101-119	Rabbit§·¶	Zhang & Nicholson (1989)
	a19	aa 105-119	Rabbit§·¶	(from E. Hertzberg)
Cx43	18A	aa 346-360	Rabbit§·¶	Yamamoto et al. (1990)
	Petunia	aa 252-271	Rabbit§	Beyer et al. (1989)
GST (*p*)		Rat GST (p)	Rabbit§	Hendrich et al. (1987)

*GJ, gap junctions; †monoclonal antibody; ‡amino acids; §polyclonal antibody; ¶affinity purified.

buffer A containing 4 mM $NaHCO_3$, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 mM EDTA, 5 mM diisopropylfluorophosphate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate. In addition, 50 mg of tissue samples was homogenized in 20 mM NaOH followed by 30 seconds of sonication at 50% power with a Branson sonifier 250 microtip (VWR, Chicago, IL). By centrifugation at 12,000 ***g*** for 15 minutes, NaOH-insoluble protein pellets were recovered, resuspended in 2 ml 20 mM NaOH, sonicated for 30 seconds at 50% power, repelleted at 12,000 ***g*** for 15 minutes, and then suspended in 1 ml of isolation buffer A (Hertzberg and Skibbens, 1984).

Samples were sonicated for 30 seconds at 50% power with a Branson sonifier 250 microtip, and protein concentrations were determined (Peterson, 1977). Proteins were solubilized in 2% SDS (Gallard-Schlesinger, Carle Place, NY), 62.5 mM Tris-HCl, 10% glycerol, and 50 mM dithiothreitol (Sigma), pH 6.8, for 30 minutes at room temperature (RT) and resolved by SDS-PAGE (Laemmli, 1970) with 3% to 5% stacking and 12.5% separating gels cast in a minigel apparatus (BioRad, Richmond, CA). Transfer of the protein to positively charged Immobilon-P membranes (Millipore, Bedford, MA) was carried out in modified Towbin transfer buffer (10% methanol) at 300 mA for 90 minutes at 4°C. The SDS-polyacrylamide gels were then stained with Coomassie Blue R-250 (Bio-Rad) to evaluate equal loading and transfer of proteins.

Nonspecific protein binding of the membranes was blocked with filtered Blotto (5% Carnation non-fat dry milk powder in 40 mM Tris-HCl, pH 7.4, 0.1% Tween-20, 0.05% sodium azide) for 1 hour at RT (Johnson et al., 1984). Primary antibodies were incubated with the blots for 2 hours at RT, washed several times in TBS (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.05% sodium azide), followed by incubation with the appropriate affinity-purified rabbit secondary antibody (Chappel, Malvern, PA) for 1 hour at RT. Antibody-connexin complexes were identified with 40 nCi/ml ^{125}I-Protein A (ICN, specific activity > 70 μCi/μg). Rainbow ^{14}C-methylated protein markers were used for molecular mass determinations (Amersham). Autoradiograms with XAR-5 film (Kodak, Rochester, NY) were exposed at −70°C with an intensifying screen.

Immunocytochemistry and immunofluorescence

Immunocytochemical staining of cryosections (6-8 μm) was performed as previously described (Neveu et al., 1990). Briefly, acetone-fixed sections were blocked for endogenous biotin and peroxidase activity, and incubated with primary antibody overnight at 4°C. Protein-antibody complexes were visualized by the biotin/streptavidin/peroxidase method with aminoethyl carbazole as the chromogen (Zymed Laboratories Inc., San Francisco, CA). Double staining of the placental form of glutathione S-transferase (GST) and connexins was performed with biotin-conjugated goat anti-rabbit antibody, followed by avidin conjugated to β-galactosidase and X-gal as the chromogen (Neveu et al., 1990). For quantitative stereological analysis, sections were also stained for gamma-glutamyltranspeptidase (GGT) activity with enzyme histochemistry (Hendrich et al., 1987). Nuclei were counterstained with Mayer's hematoxylin, and coverslips were applied with Crystal/Mount (Biomedia Corp., Foster City, CA). All slides were viewed with a Zeiss Axiophot microscope (Carl Zeiss, Germany) with either light- or dark-field microscopy.

Double immunofluorescence of Cx32 and Cx26 was performed with acetone-fixed cryosections. Sections were blocked for endogenous biotin (Neveu et al., 1990), incubated with an affinity-purified rabbit polyclonal antibody to Cx26 (α19, see Table 1), followed by incubation with goat anti-rabbit IgG conjugated with biotin (Sigma) for 1 hour at RT. A mouse monoclonal antibody to Cx32 (M12.13) was then applied for 2 hours at RT, incubated with Texas Red-conjugated to streptavidin (Vector), and then with goat anti-mouse IgG conjugated with FITC (Sigma). The sections were washed three times in phosphate-buffered saline between steps. To identify AHF in these sections, we incubated the slides with a third primary antibody to GST for 1 hour at RT, with goat anti-rabbit IgG conjugated to biotin for 1 hour at RT, and then with 7-amino-4-methyl-coumarin-3-acetic acid conjugated to streptavidin for 30 minutes at RT. Coverslips were placed on the sections with Fluoromount-G (Fisher, Chicago, IL) supplemented with 2.5% 1,4-diazabicyclo[2.2.2]octane to reduce quenching. Yellow (Cx32), red (Cx26) and blue (GST) fluorescences were visualized with epifluorescence illumination.

Northern blot analysis

Total RNA was isolated from pulverized, quick-frozen tissues by the acid-guanidinium method (Chomczynski and Sacchi, 1987), and northern blots were performed with 10 μg total RNA per lane (Beer et al., 1988). Ethidium bromide at a concentration of 0.2 μg/ml was added to the gels before electrophoresis to verify the integrity of the RNA as well as to monitor the equivalence of loading before and after transfer to GeneScreen Plus (Dupont, Boston, MA). *Eco*RI fragments of the cDNAs corresponding to nearly full-length coding mRNA for Cx32 (1.5 kb) (Paul, 1986), Cx26 (2.1 kb) (Zhang and Nicholson, 1989), and Cx43 (3.1 kb) (Beyer et al., 1989) were isolated from plasmid sequences by agarose electrophoresis and purified with GeneClean (Bio101 Inc., La Jolla, CA). Radiolabeled probes ([^{32}P]dCTP, 3000 Ci/mmol, Amersham, Arlington Heights, IL) were generated with the multiprime DNA labeling system (Amersham) followed by G-50 Quickspin purification (Boehringer Mannheim, Indianapolis, IN). Prehybridization and hybridization were performed as previously described (Beer et al., 1988). Blots were washed in 1× SSC with 1% SDS at 65°C for 60 minutes, 0.1× SSC with 1% SDS at 65°C for 60 minutes, and 0.1× SSC for 30 minutes at RT followed by exposure to XAR-5 X-ray film (Kodak) at −70°C with an intensifying screen (Dupont).

mRNA in situ hybridization

Sense and anti-sense radiolabeled ([^{35}S]thiophosphate-UTP, 1282 Ci/mmol, Amersham) riboprobes were transcribed in vitro from the Cx32 cDNA as previously described (Beer et al., 1988). The probes were hydrolyzed at pH 10.2 to lengths of about 200 nucleotides. The specificity of the probes was examined by northern blotting of normal liver and heart total RNA. In situ hybridization was performed as previously reported, with slight modifications (Beer et al., 1988; Nakatsukasa et al., 1990). Briefly, cryosections were thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides (Rentrop et al., 1986) and fixed in fresh 4% paraformaldehyde in PBS for 20 minutes at RT. Subsequently, the sections were incubated in PBS supplemented with 5 mM $MgCl_2$ for 10 minutes at RT, and then in 0.1 M glycine/0.2 M Tris-HCl, pH 7.4, for 10 minutes at RT. After being washed in phosphate-buffered saline, the sections were post-fixed in 4% paraformaldehyde for 20 minutes at RT. Washed sections were then dehydrated in 50, 75, 90 and 100% ethanol, air dried, and prehybridized at 45°C for 2 hours in 50% deionized formamide (Clonetech, Palo Alto, CA) containing 0.3 M NaCl, 100 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 20 mM vanadyl ribonucleosides (Bethesda Research Labs, Bethesda, MD), 1× Denhardt's solution, 0.05% yeast

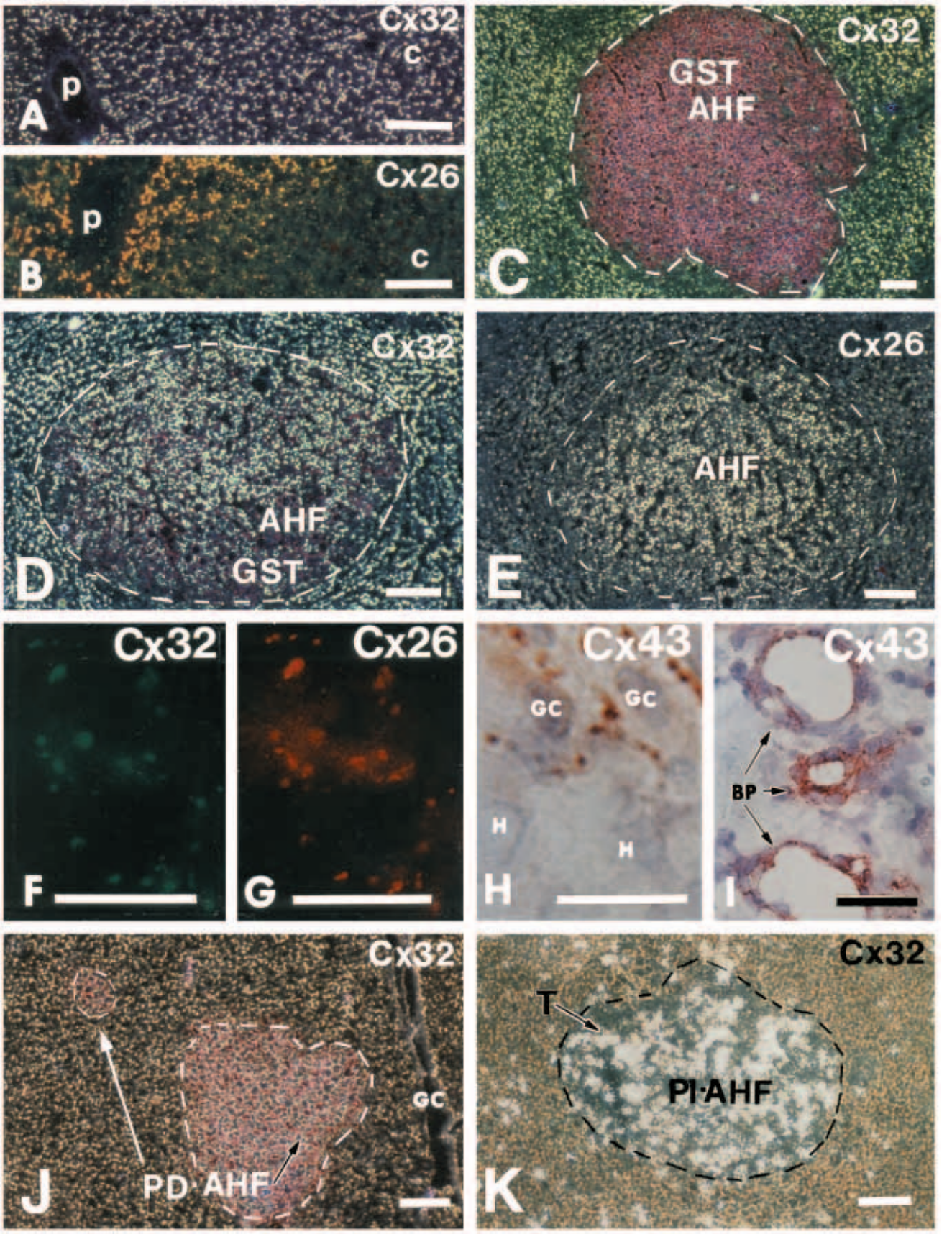

Cx32
C
P
A
Cx26
P
B
C
Cx32
GST
AHF
C
Cx32
AHF
GST
D
Cx26
AHF
E
Cx32
F
Cx26
G
Cx43
GC
GC
H
H
H
Cx43
BP
I
Cx32
GC
PD·AHF
J
Cx32
T
PI·AHF
K

Fig. 1

total RNA, 0.005% yeast tRNA, 0.05% sonicated salmon sperm DNA, 1 mM EDTA, and 10 mM DL-methionine. The sections were washed briefly in 2× SSC, air dried, and hybridized in the prehybridization buffer supplemented with 20% dextran sulfate (M_r 100,000; Sigma) and 1.5×10^8 cpm/ml of heat-denatured ^{35}S-labeled cRNA probe for 5 hours at 48°C. Washing of the sections, RNase treatment, dehydration, dipping in photoemulsion and development were performed as previously described (Beer et al., 1988; Nakatsukasa et al., 1990). After development, sections were coverslipped with Crystal/Mount (Biomedia Corp.) and viewed by both light- and dark-field microscopy.

RESULTS

Altered connexin immunostaining in altered hepatic foci

As previously described (Traub et al., 1989), the antibodies used in this study (Table 1) show that Cx32 is homogeneously distributed as discrete spots on hepatic membranes throughout the acinus in untreated rat liver (Fig. 1A), whereas punctate Cx26 staining is restricted to periportal hepatocytes (Fig. 1B). Modifications in Cx32 and Cx26 immunostaining were observed in foci generated by either PB or TCDD promotion. In both protocols, many foci that were positive for the placental form of glutathione S-transferase (GST) displayed diminished Cx32 immunoreactivity (Fig. 1C). A distinct population of GST-positive foci, exhibiting near-normal punctate Cx32 staining (Fig. 1D), was identified by increased Cx26 staining (Fig. 1E). Immunofluorescence microscopy of sections doubly stained for Cx32 (FITC) and Cx26 (Texas Red) showed that both proteins co-localize in Cx26-positive foci (Fig. 1F,G). Although Cx43 immunoreactivity was never observed in foci, staining was apparent between cells of the Glisson's capsule (Fig. 1H) and areas of biliary hyperplasia (Fig. 1I). Bile ducts present in portal triads from normal untreated rats did not express Cx43 immunoreactivity (data not shown).

The number of foci and the percentage of the liver occupied by foci exhibiting diminished Cx32 or enhanced Cx26 staining were examined by quantitative stereology as previously described (Hendrich et al., 1987; Neveu et al., 1990). In addition, we compared these parameters with those obtained using GST and γ-glutamyltranspeptidase (GGT) to mark foci in serial sections. Table 2 compares the number and volume percentage of the liver of foci scored by any one marker, with the cumulative number and volume of foci scored by any of the four markers (i.e. Cx32, Cx26, GST, GGT). Differential upregulation of Cx26 scored 43% of the total volume of foci promoted by TCDD, but only 6% of the total volume of lesions promoted by PB. In both protocols, approximately 75% of the volume of foci was identified by changes in either Cx32 or Cx26 staining.

Irreversible alterations in Cx32 staining in promoter-independent foci

Previously, we observed that about 94% of GST-positive foci exhibit a normal distribution of Cx32 staining if PB is withdrawn from the diet 20 days before killing the animal (Neveu et al., 1990). Utilizing osmotic minipumps containing [^{3}H]thymidine, we simultaneously examined the relationship of Cx32 staining and cell proliferation. Promoter-independent lesions were generated in rats initiated with DEN and promoted with PB for 8 months. PB was then withdrawn from the diet

Fig. 1. Immunostaining of Cx32 (A,C,D,F,J,K), Cx26 (B,E,) and Cx43 (H,I) protein in normal and preneoplastic rat livers. Cryosections were immunolabeled with specific antisera: M12.13 for Cx32, α19 for Cx26, and 18A for Cx43 (see Table 1). Antigen-antibody complexes were visualized with dark-field (A-E,J,K), fluorescence (F,G), or bright-field (H,I) microscopy (see Materials and Methods). (A) Cx32 in normal liver. Homogeneous acinar distribution between the portal tract (p) and central vein (v). (B) Cx26 in normal liver showing periportal (p) localization of staining. (C) Cx32 staining in DEN/PB-treated liver. Note decreased Cx32 staining in a GST-positive focus (purple). (D) Cx32 staining in DEN/TCDD-treated liver showing that most cells within the GST-positive focus exhibit normal Cx32 staining. (E) Cx26 expression in a serial section of DEN/TCDD-treated liver showing increased Cx26 expression in a focus. (F,G) Cx32 and Cx26 double immunofluorescence in DEN/TCDD-treated liver. High magnification shows that Cx32 (FITC) and Cx26 (Texas red) punctata colocalize in a DEN/TCDD focus. (H) Cx43 expression in normal liver viewed by bright-field microscopy. Glisson's capsule (GC) cells exhibit punctate Cx43 staining while hepatocytes (H) are negative. (I) Cx43 staining in DEN/PB-treated liver showing punctate staining in areas of biliary proliferation (BP). (J) Cx32 expression in DEN/PB-treated liver that was withdrawn from dietary PB 20 days prior to sacrifice. Unlike C, the GST-positive (purple), promoter-dependent foci (PD-AHF) exhibit punctate Cx32 staining (yellow dots). (K) Dark-field microscopy showing Cx32 staining (yellow dots) and [^{3}H]thymidine incorporation (T) (white dots) in a focus promoter independent (PI-AHF) for growth (see Materials and Methods). Note that Cx32 staining is not detected in PI-AHF. Key: AHF, altered hepatic foci; BP, bile duct proliferation; GC, Glisson's capsule cell; H, hepatocyte; T, [^{3}H]thymidine; PD-AHF, promoter-dependent altered hepatic foci; PI-AHF, promoter-independent altered hepatic foci. Bars: 50 μm (A-E, H-K), 10 μm (F-H).

Table 2. Quantitation of diminished connexin32 (Cx32) and enhanced connexin26 (Cx26) immunostaining in altered hepatic foci (AHF)

Protocol	% of AHF/liver	Volume % of AHF/liver
A. PB		
1. Cx32-deficient	81	76
2. Cx26-enhanced	4	7
3. GST	83	79
4. GGT	38	55
Any phenotype	(17,452±4771)	(3.91±0.67)
B. TCDD		
1. Cx32-deficient	32	34
2. Cx26-enhanced	30	43
3. GST	88	75
4. GGT	51	53
Any phenotype	(8052±672)	(14.4±3.7)

Percentage of total AHF(*Any* phenotype) identified by alterations in either GST, GGT, Cx32 or Cx26 expression. Serial cryosections were stained for the placental form of glutathione S-transferase (GST) and gamma-glutamyltranspeptidase (GGT) as previously reported (Hendrich et al., 1987), and for Cx32 and Cx26 as described above. Quantitation was performed on serial liver sections from female F344 rats given a single non-necrogenic dose of DEN (10 mg/kg, p.o.) and promoted with either (A) 0.05% PB for 8 months or (B) 0.1 μg/kg TCDD for 14 months (Dragan et al., 1991). The '*Any*' phenotype was obtained from the quantification of the altered expression of four markers in AHF (Hendrich et al., 1987). Sections from four rats were examined per group.

Table 3. Association of decreased Cx32 expression and enhanced proliferation in promoter-independent (PI) and promoter-dependent (PD) altered hepatic foci (AHF)

	AHF		'Normal' hepatocytes	
	PI	PD	CL	PP
No. of Cx32 spots*/ hepatocyte	1.6±1.1†	7.2±2.6	7.9±2.0	8.6±2.3
No. of labeled hepatocytes‡	79.2±13.1	27.5±12.6	13.5±5.2	15.4±5.8

'Normal' hepatocytes were grouped as periportal (PP) or centrolobular (CL). At least 1000 cells were assessed for each condition in sections from three animals.

*The number of Cx32 punctate areas per hepatocyte seen on cryosections double stained for GST and Cx32, followed by autoradiography (see Materials and Methods).

†Values ± the standard error.

‡Cx32, GST and [^{3}H]thymidine were simultaneously examined by dark-field microscopy (see Fig. 4.1J, K). Based on GST staining, hepatocytes were classified as either AHF or 'Normal' hepatocytes. AHF were classified as promoter independent if they continued to proliferate (more than 30% cells labeled) after PB was removed from the diet 20 days prior to killing.

for 20 days prior to killing. [^{3}H]thymidine incorporation was detected with photoemulsion and dark-field microscopy as previously described (Neveu et al., 1990). Fig. 1J shows normal Cx32 immunostaining observed in most GST-positive foci after cessation of PB promotion. Although no significant difference in the lobular distribution of hepatocyte [^{3}H]thymidine incorporation was observed after removal of PB, two populations of GST-positive foci were identified by differences in [^{3}H]thymidine incorporation (Table 3). Only promoter-independent foci, identified by their ability to proliferate in the absence of PB promotion, continued to exhibit decreased Cx32 staining (Fig. 1K). These findings indicate that irreversible downregulation of Cx32 staining correlates with foci that are promoter-independent for growth.

Analysis of connexin mRNA in preneoplastic livers

Fig. 2A demonstrates that Cx32 transcripts are homogeneously expressed in hepatocytes throughout the liver lobule with no detectable cRNA-mRNA hybrids in nonparenchymal cells. The specificity of the hybridization was apparent, since only background hybridization was observed when a sense probe was employed (Fig. 2B) or when sections were treated with RNase prior to hybridization. In contrast to decreased Cx32 immunoreactivity in foci, mRNA in situ hybridization of liver sections from rats initiated with DEN and promoted with PB showed that, of the 50 Cx32-deficient AHF (Fig. 2C), only four displayed reduced Cx32 message throughout the lesion (Fig. 2D). The remaining foci displayed levels of cRNA-mRNA hybrids comparable to surrounding non-neoplastic hepatocytes (Fig. 2E). A fraction of cells within these foci usually exhibited reduced Cx32 staining. Fig. 2F shows that Cx32 is absent in Glisson's capsule; Fig. 2G demonstrates Cx43 transcripts in these cells. These observations indicate that punctate Cx32 staining is downregulated in most foci in a manner independent of mRNA abundance.

Altered Cx32, Cx26 and Cx43 immunostaining in hepatic neoplasms

All 24 hepatic neoplasms, generated by DEN initiation and promotion with either PB or TCDD and examined for Cx32, Cx26 and Cx43 immunoreactivity, exhibited alterations in Cx32 and/or Cx26 staining. Although 15 of the tumors did not exhibit detectable Cx32 staining (Fig. 3A), most neoplastic hepatocytes within the other nine lesions exhibited either diffuse immunoreactivity (PB11; PB12; PB15) (Fig. 3B) or random punctate staining (PB5; PB9; PB10; PB13; TCDD2; TCDD4) (Fig. 3C). The disorganized Cx32 staining did not appear to be localized as discrete punctate areas on the plasma membrane between juxtaposed cells, but was randomly distributed (Fig. 3D) or localized in sinusoidal regions of the cell that did not contact neighboring tumor cells (Fig. 3E). Most neoplasms with altered Cx32 staining also displayed altered Cx26 staining. In contrast to the co-localization of Cx32 and Cx26 observed in foci, double immunofluorescence staining demonstrated that Cx32 (FITC) and Cx26 (Texas Red) immunoreactivities do not always co-localize in tumors (Fig. 3F, G).

All areas of bile duct proliferation, cholangiomas and cholangiocarcinomas were completely unreactive towards several antibodies to Cx32 and Cx26. Although Cx43 staining was present in 18 of 24 neoplasms, it was always restricted to non-parenchymal cell types. Both punctate and intracellular Cx43 staining was observed in areas of fibrosis (Fig. 3H) and bile duct proliferation (Fig. 3I). Whereas benign proliferations of bile ducts and cholangiomas always displayed Cx43 staining, all five cholangiocarcinomas were deficient in Cx43 immunoreactivity (Fig. 3J).

Alterations in connexin mRNA transcripts in hepatic neoplasms

Northern blotting determined that some of the observed alterations in Cx32, Cx26 and Cx43 immunoreactivities (see above) could be attributed to changes in steady-state mRNA abundance (Fig. 4). Ethidium bromide staining of the gel demonstrated that near-equivalent amounts of total RNA were loaded in each lane. Detectable qualitative alterations in the mobility of connexin mRNAs were not apparent in neoplasms relative to normal rat liver controls for Cx32 (1.6 kb) and Cx26 (2.1 kb) (Paul, 1986; Zhang and Nicholson, 1989), and relative to rat heart for Cx43 (3.1 kb) (Beyer et al., 1989). Cx32 mRNA abundance was greater than or equivalent to that of untreated adult liver in 11 of 16 neoplasms; the remaining samples displayed reduced or undetectable levels. Fig. 4 shows that the abundances of Cx26 and Cx43 mRNAs were low in normal adult rat liver, but abundant steady-state transcripts are apparent in many neoplasms (7/16-Cx26; 8/16-Cx43). Whereas increased Cx43 mRNA expression was identified in the neoplasms containing bile duct proliferation or fibrosis, specific alterations in Cx26 and Cx32 mRNA expression were not correlated with routine histopathology. Similar variations in the patterns of Cx32, Cx26 and Cx43 mRNA expression were seen in neoplasms from rats initiated with DEN and promoted with TCDD, mestranol or chlorendic acid (data not shown).

Western blot analysis of connexins in hepatic neoplasms

A representative immunoblot showing the spectrum of observed alterations in the abundance and/or electrophoretic mobility of Cx32, Cx26 and Cx43 in hepatic neoplasms from

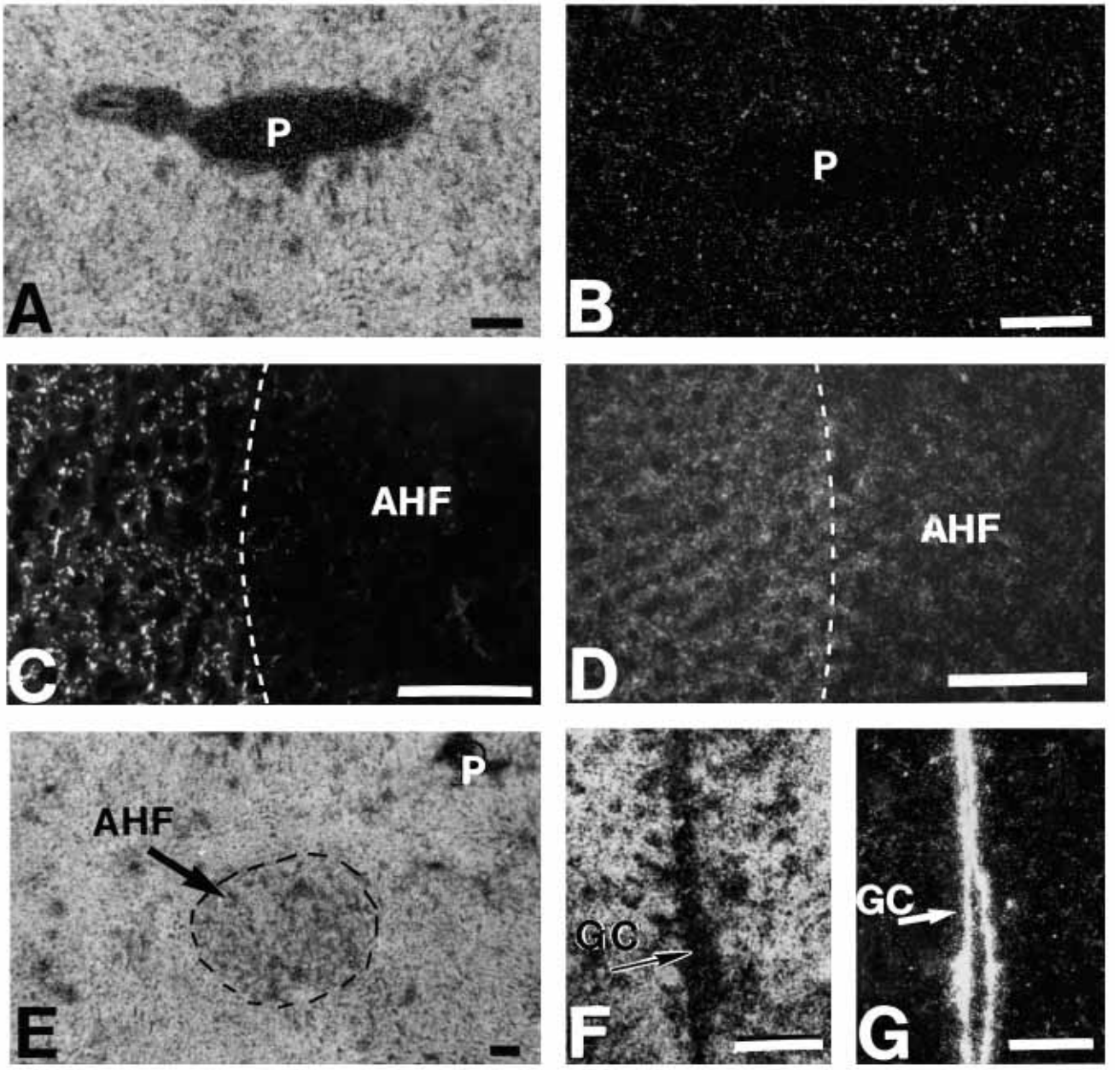
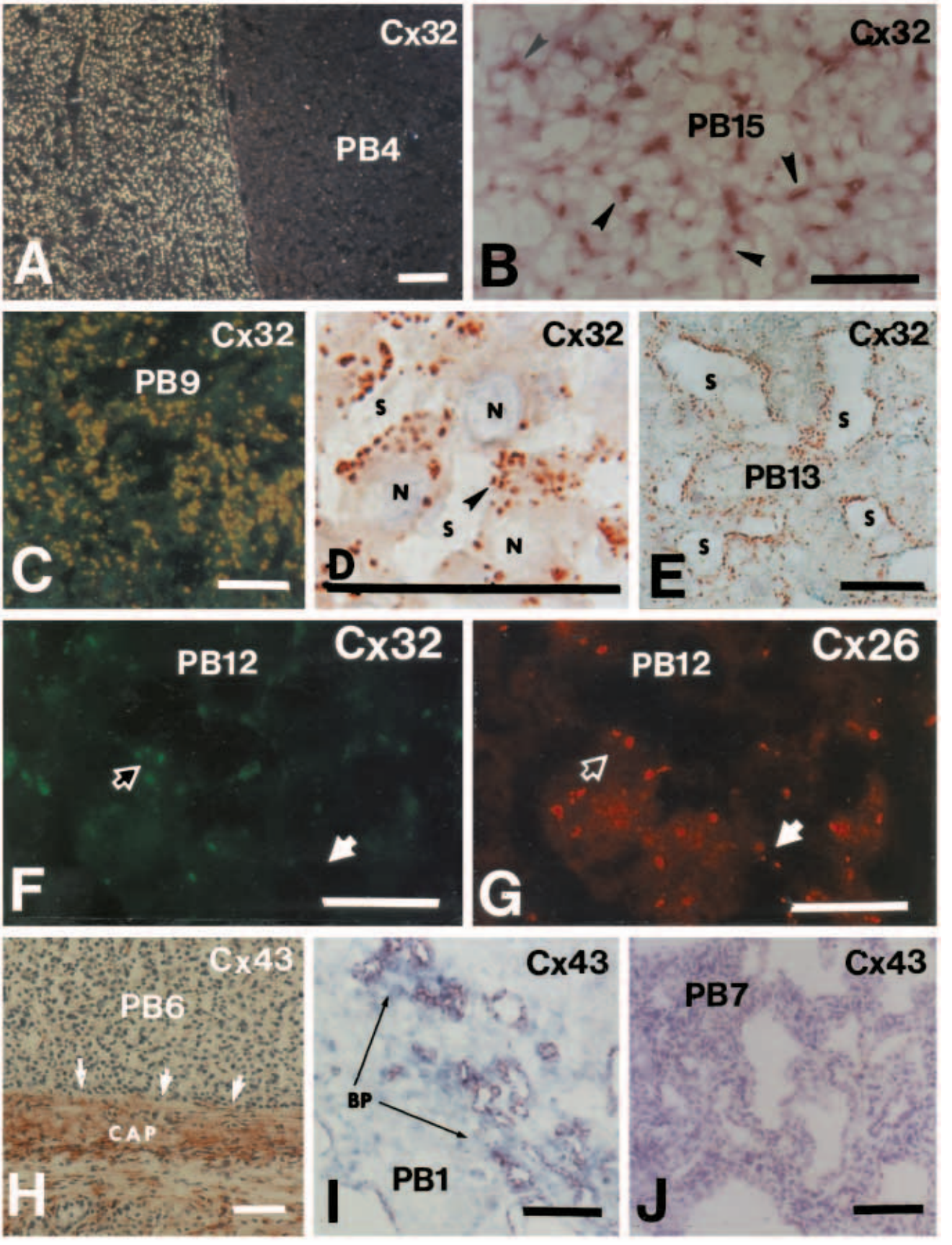


Fig. 2. In situ localization of Cx32 mRNA in livers from rats initiated with DEN and promoted with PB for 8 months. Cryosections were incubated with ^{35}S-labeled anti-sense (A,D,E,F), sense (B) Cx32 (A,B,D,E,F) cRNA probes. Autoradiograms of mRNA-cRNA hybrids appear as white dots when viewed by dark-field microscopy. (A) Cx32 mRNA expression in normal liver. While Cx32 mRNA was not observed in portal tracts (P), hybridization was observed in hepatocytes throughout the acinus. (B) No specific hybridization after incubation of a serial section with Cx32 sense cRNA probe. (C) Immunostaining of DEN/PB-treated liver with Cx32 viewed by dark-field microscopy showing reduced Cx32 punctata in an altered hepatic focus. (D) Hybridization of serial section of DEN/PB-treated liver with Cx32 anti-sense cRNA probe. Note a similar decrease in Cx32 protein (C) and mRNA expression (D). (E) Localization of Cx32 mRNA in liver from rats treated with DEN/PB. Note that most cells within the AHF express Cx32 mRNA. (F) Cx32 mRNA is not expressed by Glisson's capsule. (G) Cx43 mRNA expression in Glisson's capsule (GC) observed with an anti-sense Cx43 cRNA probe. AHF, altered hepatic foci; GC, Glisson's capsule; P, periportal. Bar, 50 μm.

rats promoted with PB is depicted in Fig. 5. As previously described (Hertzberg and Skibbens, 1984), monomer and dimer forms of Cx32 (27 and 47 kDa) were present in normal liver tissue homogenates. Although Cx32 immunoreactivity was observed in several neoplasms, the electrophoretic mobility of Cx32 was altered compared with that of normal liver. Tumors PB13, PB12 and PB15 displayed only the dimer form of Cx32; PB5 exhibited altered mobility at 31, 47 and 88 kDa; PB1 exhibited altered mobility at 29 kDa and PB9 exhibited only monomeric Cx32. The abnormal Cx32 immunoreactive bands in tumors were found to be localized to post-nuclear crude membranes (data not shown). Although anti-Cx26 antibodies failed to detect specific immunoreactivity in crude liver homogenates, a single immunoreactive band


Cx32
PB4
A
Cx32
PB15
B
Cx32
PB9
C
Cx32
S
N
N
S
N
D
Cx32
S
S
PB13
S
S
E
PB12
Cx32
F
PB12
Cx26
G
Cx43
PB6
CAP
H
Cx43
BP
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I
Cx43
PB7
J

Fig. 3

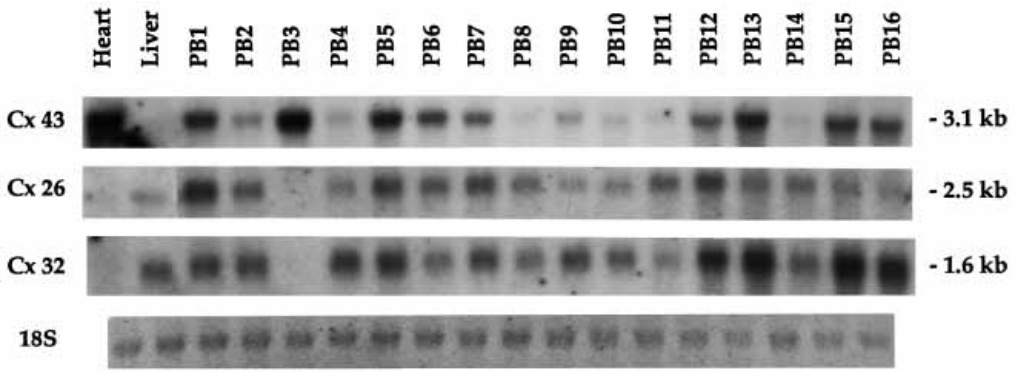

Fig. 4. Northern blot analysis of steady-state levels of Cx32, Cx26 and Cx43 mRNAs in untreated rat liver and rat heart as well as rat hepatic neoplasms (lanes PB1-16). Ethidium bromide staining shows equal loading of RNA. Mammalian and *E. coli* ribosomal RNAs were used as size markers. The histological diagnoses of the tumors are: PB1, neoplastic nodule with leukemic infiltration and bile duct proliferation; PB2, neoplastic nodule with focal areas highly suggestive of carcinomatous change; PB3, cholangiocellular and anaplastic hepatocellular carcinoma; PB4, neoplastic nodule; PB5, neoplastic nodule with areas of fibrosis and bile duct proliferation; PB6, hepatocellular carcinoma with fibrosis and inflammation; PB7, mixed hepatocellular-cholangiocellular carcinoma; PB8, hepatocellular carcinoma; PB9, hepatocellular carcinoma; PB10, neoplastic nodule with fatty metamorphosis and occasional bile duct proliferation; PB11, hepatocellular carcinoma; PB12, hepatocellular carcinoma containing multiple cholangiocarcinomas; PB13, neoplastic nodule with focal areas of cholangiomas and fibrosis; PB14, mixed hepatocellular-cholangiocellular carcinoma; PB15, neoplastic nodule with focal areas of chronic inflammation and fibrosis; PB16, hepatocellular carcinoma. Note that each tumor sample exhibits a unique pattern of connexin mRNA expression.

of 24 kDa was detected in NaOH-insoluble pellets of total tissue homogenates. Whereas the level of Cx26 was reduced in most neoplasms, immunoreactivity was apparent in PB1, PB5 and PB12. Increased Cx43 immunoreactivity at 43 kDa was observed in PB5, PB13 and PB15 (Fig. 5), which did not co-migrate with Cx43 (46 kDa) from rat heart (data not shown).

Comparison of northern and western blot experiments indicated that alterations in Cx32 mRNA in neoplasms were not always paralleled by changes in protein expression. For example, mRNA isolated from tumor PB4 exhibited normal levels of Cx32 mRNA, but failed to show immunoreactivity by staining (Fig. 3A) or western blotting (Fig. 5). A similar disparity between Cx32 mRNA and protein expression was observed in preneoplastic foci (Fig. 2C,D). Despite use of several different antibodies to Cx32 (Table 1), antigenic sites may have been masked in some tumors and AHF. Since Cx26 immunoreactivity was apparent in NaOH-insoluble pellets from several neoplasms that did not display staining, antigenic sites of this protein may also have been masked in tissue sections. Furthermore, some neoplasms were also observed to display increased Cx26 mRNA, but Cx26 protein was undetectable in NaOH-insoluble pellets. In either case, the masking of epitopes or the loss of protein expression demonstrates alterations in Cx26 protein expression.

Fig. 3. Distribution of Cx32, Cx26 and Cx43 immunoreactivity in hepatic neoplasms. Cryosections were incubated with antibodies as outlined in the legend to Fig. 1. Sections were viewed by either dark-field (A,C), bright-field (B,D,E,H,I,J), or fluorescence microscopy (F,G) (see Materials and Methods). (A) Cx32 staining in neoplastic nodule (PB4) from DEN/PB-treated rat. Note specific reduction in Cx32 staining in the neoplasm. (B) Cx32 staining in a GST-positive (purple) neoplastic nodule (PB15) showing diffuse intracellular localization. (C) Cx32 staining in hepatocellular carcinoma (PB9) showing disorganized punctate staining. (D) Higher-power visualization of Fig. 3C viewed by bright-field microscopy. (E) Sinusoidal localization of Cx32 in neoplastic nodule (PB13). (F,G) Double fluorescence localization of Cx32 (FITC) and Cx26 (Texas Red) in hepatocellular carcinoma PB13. Note that Cx32 and Cx26 staining did not always co-localize in this tumor. (H) Cx43 staining in the fibrotic capsule surrounding a hepatocellular carcinoma (PB6). (I) Cx43 staining of a neoplastic nodule (PB1) showing staining in regions of biliary hyperplasia. (J) Lack of Cx43 staining in hepatocellular cholangiocellular carcinoma (PB7). S, sinusoidal space; N, nucleus; CAP, fibroblastic capsule; BP, bile duct proliferation. Bar, 50 μm.

DISCUSSION

Alterations in connexin32 expression in hepatic foci and neoplasms

Our results confirm and extend previous observations that Cx32 staining is diminished in preneoplastic rat hepatocytes (Beer et al., 1988; Neveu et al., 1990; Krutovskikh et al., 1991). Although decreased Cx32 staining is reversible in rat hepatic foci (Neveu et al., 1990), we found that promoter-independent foci and neoplastic rat hepatocytes lack normal punctate distribution of Cx32 whether PB is present or absent (see Table 3). In agreement with these observations, mouse foci and adenomas exhibit a similar pattern of Cx32 staining after withdrawal of PB promotion (Klaunig, 1991). Krutovskikh and colleagues (1991) found that most GST-positive foci that cease to proliferate (i.e. remodel) following the Solt-Farber model of hepatocarcinogenesis display normal Cx32 expression and transfer the GJ-permeable dye Lucifer Yellow. Since the expression of several markers of rat hepatocarcinogenesis have been shown to be promoter dependent (Pitot et al., 1991), irreversible downregulation of Cx32 may represent a necessary change for promoter-independent growth or tumor progression.

Northern blotting (Fig. 4) and mRNA in situ hybridization (Fig. 2) showed that Cx32 mRNA is present in most preneoplastic and neoplastic hepatocytes and is comparable in abundance to untreated adult liver. While these observations concur with previous studies of rat, mouse and human hepatomas (Beer et al., 1988; Beer and Neveu, 1990; Oyamada et al., 1990), decreased Cx32 mRNA was seen in rat hepatic neoplasms induced by chronic administration of *N*-ethyl-*N*-hydroxyl ethylnitrosourea, or after a necrogenic dose of DEN

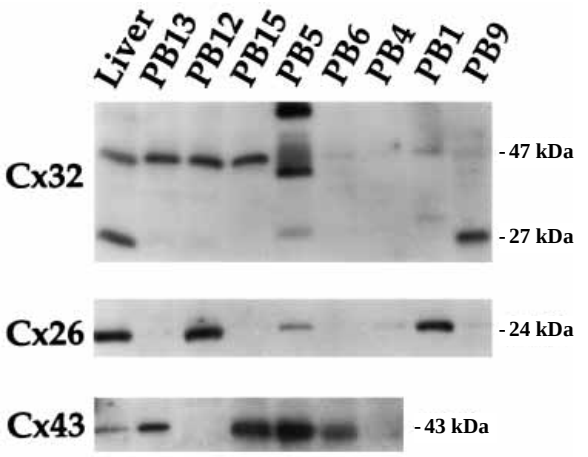

Fig. 5. Immunoblot detection of Cx32, Cx26 and Cx43 in normal and neoplastic rat liver. NaOH-insoluble protein fractions were examined for Cx32 and Cx26 (15 μg per lane), whereas crude tissue homogenates were used for Cx43 (75 μg/lane). Proteins were solubilized in SDS for 30 minutes at room temperature in order to reduce Cx32 oligomerization (see Materials and Methods). Samples were then subjected to SDS-PAGE, electroblotted to Immobilon membranes, and incubated with antibodies 594 (Cx32), α19 (Cx26), or 18A (Cx43) (see Table 1). Antigen-antibody complexes were examined with ^{125}I-Protein A followed by autoradiography. As with northern blot analyses (Fig. 4), each hepatic neoplasm exhibited a unique pattern of connexin immunoreactivity. Both monomer (27 kDa) and dimer (47 kDa) forms of Cx32 were observed in normal liver. Note that liver neoplasms exhibited either dimer (PB13, PB12, PB15) or monomer (PB9) forms of Cx32, but not both. Tumors PB5 and PB1 displayed Cx32 immunoreactive bands with altered mobility on SDS-PAGE. An identical pattern of immunoreactivities was observed with the anti-Cx32 antibody 92b. Furthermore, no bands were visualized in any of the samples when normal sheep serum or nonspecific ascites fluid was used in place of either Cx32 primary antibody. Cx43 immunoreactivity migrated at 43 kDa in normal rat liver and in tumors PB13, PB15, PB5 and PB6.

followed by several cycles of acetylaminofluorene and carbon tetrachloride (Fitzgerald et al., 1989; Sakamoto et al., 1992). Since we found no obvious correlation between the level of Cx32 mRNA and histopathology, the reported differences in the abundance of Cx32 mRNA may result from the different agents utilized to induce neoplasia. For example, a single administration of hepatotoxic levels of DEN or carbon tetrachloride transiently resulted in a greater than 90% reduction of Cx32 immunoreactivity in rat liver (Miyashita et al., 1992).

Several changes in the pattern of Cx32 immunostaining were evident in rat hepatic neoplasms (Fig. 3A-D). The diversity of both punctate and diffuse staining suggests that Cx32 can be sequestered in several different subcellular compartments. As described in nonhepatic neoplasms, intracellular staining may represent internalized GJs (Larsen, 1983). Alternatively, immunoreactivity may be sequestered in the smooth endoplasmic reticulum, lysosomes and other membrane components that are altered in neoplasms from rats initiated with DEN and promoted with PB (Feldman et al., 1981). Although immunoelectron microscopy is needed to define the intracellular localization of Cx32 in neoplasms, our results indicate that several post-translational mechanisms may alter the transport, assembly and/or turnover of Cx32 into GJs during hepatocarcinogenesis.

Alterations in connexin staining in some hepatomas may represent a secondary change because transfection of communication-deficient cell lines with various cell adhesion molecules can restore GJIC (Musil et al., 1990; Jongen et al., 1991). Interestingly, studies by Faris and colleagues (1991) show that the expression of the major liver cell adhesion molecule (cell CAM 105) is modified in many hepatic neoplasms.

Immunoblot analysis of crude extracts from neoplasms with modified Cx32 staining revealed several alterations in the migration of immunoreactive bands on SDS-PAGE (Fig. 5). The observed changes in mobility may result from alterations in the amino acid sequence of Cx32, folding of Cx32, or post-translational processing (Jaenicke, 1987; Amara et al., 1992). Several reports have described shifts in the electrophoretic mobility of Cx43 after protein phosphorylation, although these have not been observed for phosphorylated forms of Cx32 (Bennett et al., 1991). Additional studies will be necessary to determine what mechanisms post-translationally downregulate Cx32 expression during hepatocarcinogenesis.

Immunofluorescence studies of human hepatic neoplasms also found Cx32 immunoreactivity, but in a disorganized pattern (Oyamada et al., 1990; Wilgenbus et al., 1992). Since morphological studies demonstrate that GJs are diminished in cholestatic and neoplastic human livers (Robenek et al., 1981; Swift et al., 1983), the abnormal Cx32 staining could represent alterations similar to those we identified in rat hepatic neoplasms.

Differential expression of connexin26 during rat hepatocarcinogenesis

Although morphological studies show that some neoplasms display normal GJs (Larsen, 1983), differential expression of connexins could potentially alter GJIC without changing the morphological appearance or abundance of GJs. Consistent with this hypothesis, a subset of foci (44%) and neoplastic nodules (16%) generated by the Solt-Farber protocol exhibit increased Cx26 staining (Sakamoto et al., 1992). In this report, we show enhanced Cx26 protein in preneoplastic foci (Fig. 1D). Upregulation of Cx26 staining identified different numbers and volumes of foci in livers from rats promoted with either PB or TCDD. This observation suggests that modifications in Cx26 staining may be dependent on the nature of the promoting agent (Table 2). Furthermore, increased Cx26 staining was observed in foci generated by a choline/methionine-deficient diet, but not by the peroxisome proliferator WY-14,643 (data not shown).

As previously described in normal mouse and rat liver (Zhang and Nicholson, 1989; Traub et al., 1989), both Cx32 and Cx26 immunoreactivity co-localized in periportal hepatocytes and in Cx26-positive foci. In contrast, Cx32 and Cx26 immunoreactivity did not always co-localize in neoplasms (Fig. 3F, G). These results suggest that Cx32 and Cx26 have unique requirements for assembly/turnover or, less likely, that one connexin may be mutated, resulting in differential cellular localization. Unlike Cx32, diffuse Cx26 immunoreactivity was not observed in hepatomas. The disparity between increased Cx26 mRNA (Fig. 4) and decreased Cx26 immunostaining could be owing to antigen masking or to downregulation inde-

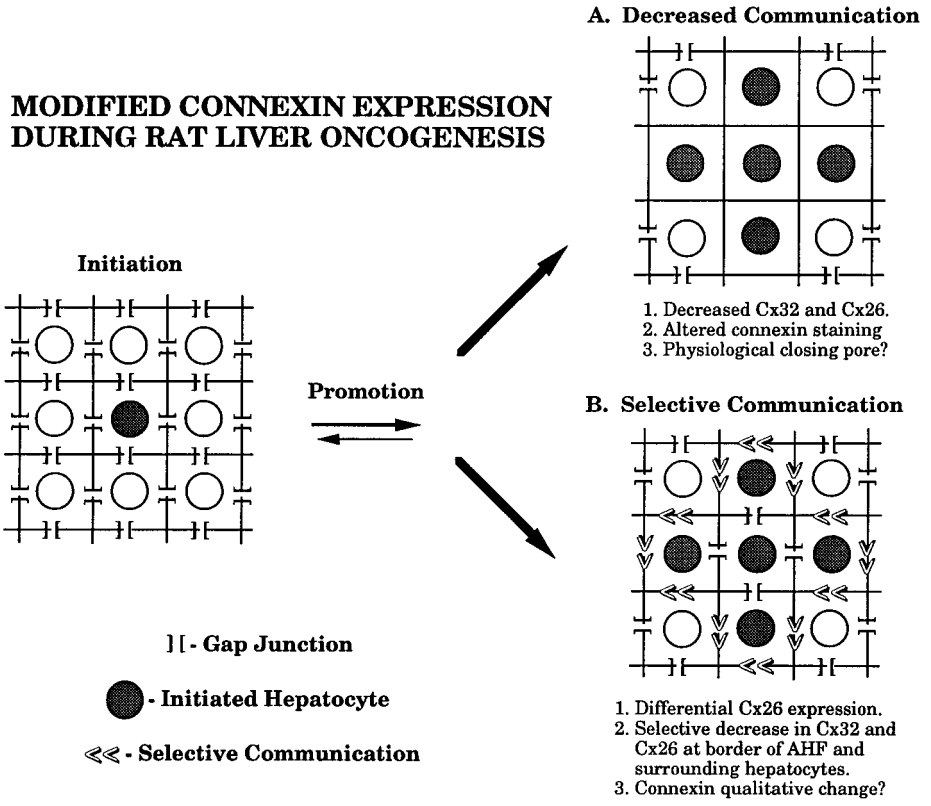

Fig. 6. Summary of alterations in connexin expression observed during rat liver oncogenesis. The schematic diagram depicts how the observed changes in connexin expression may effect homologous (A) and heterologous (B) communication between initiated cells (filled circles) and surrounding hepatocytes (open circles).

pendent of mRNA abundance. Similar results were seen in hepatic neoplasms generated by the Solt-Farber protocol (Sakamoto et al., 1992). Immunofluorescence analysis of two human hepatocellular carcinomas also failed to identify Cx26 immunoreactivity (Wilgenbus et al., 1992). Furthermore, post-transcriptional regulation has been partly implicated in the acinar heterogeneity of Cx26 in normal rat liver (Rosenberg et al., 1992).

The physiological consequence(s) of increased Cx26 staining in AHF may be important for the formation of selective GJIC and/or changing the gating properties of the intercellular channel. Heterotypic junctions formed with Cx32 and Cx26 in *Xenopus* oocytes exhibit gating properties different from corresponding homotypic junctions (Barrio et al., 1991). Therefore, GJIC may be restricted at the interface of Cx26-positive AHF and surrounding hepatocytes such that the passage of molecules of certain size, shape and/or charge may be limited.

Expression of connexin43 during rat hepatocarcinogenesis

In this study, we show that Cx43 mRNA (Fig. 4) and protein levels (Fig. 5) are enhanced in rat hepatic neoplasms. However, histological examination identified that the Cx43 staining was restricted to bile ducts, Glisson's capsule and cholangiomas (Figs 2, 3). Cholangiocarcinomas, thought to be derived from non-parenchymal liver cells (Saeter and Selgen, 1990), failed to exhibit Cx32, Cx26 or Cx43 immunoreactivity (Fig. 3). Tumor PB3 exhibited abundant Cx43 mRNA levels, but failed to display Cx43 staining. Increased Cx43 transcripts in human hepatic neoplasms (Oyamada et al., 1990) and Cx43 staining were restricted to non-parenchymal cell types (Wilgenbus et al., 1992). Although rat liver epithelial cells have the ability to switch between Cx43 and Cx32/Cx26 expression depending on culture conditions (Stutenkemper et al., 1992), we did not observe Cx43 expression in preneoplastic or neoplastic hepatocytes with several polyclonal antibodies generated against different epitopes of rat Cx43. Furthermore, foci from rats initiated with DEN and promoted with PB, TCDD, CI Solvent Yellow, WY-14,643, chlorendic acid, tamoxifen or ciprofibrate failed to exhibit Cx43 staining.

CONCLUSIONS

Evidence for a role of altered GJIC during oncogenesis has been strengthened by recent studies showing restoration of growth control following transfection of connexin cDNAs (Eghbali et al., 1991; Mehta et al., 1991; Naus et al., 1992). While transfection studies show that the mechanism(s) responsible for downregulation of GJIC in some neoplasms may be defeated by overexpressing connexin mRNAs, similar gene replacement strategies were not successful in several rat hepatoma cell lines (Mehta et al., 1991; M. J. Neveu, unpublished observation). In this study, we found that rat hepatic neoplasms utilize several different modifications to alter the expression of Cx32, Cx26 and Cx43. Fig. 6 summarizes how the observed changes may effect homologous (A) and heterologous (B) communication between initiated cells and surrounding hepatocytes. The wide spectrum of alterations in connexin mRNA and protein expression observed in this study is consistent with cell fusion analysis of GJIC-deficient tumorigenic cell lines that identified at least two genes that modulate GJIC (Loewenstein, 1979; MacDonald, 1982). Taken together,

the diverse array of mechanisms to downregulate connexin expression indicates that restoration of GJIC in some tumors could require the correction of several genetic loci/pathways. Since connexin expression was reversibly downregulated early during oncogenesis, upregulation of GJIC during tumor promotion may represent a more amenable target for cancer chemoprevention.

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