Differential Regulation of Multiple Gap Junction Transcripts and Proteins during Rat Liver Regeneration

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Abstract. The mRNA and protein expression of α_1 (connexin 43), β_1 (connexin 32), and β_2 (connexin 26) gap junction genes were examined in the regenerating rat liver after 70% partial hepatectomy (PH). Expression of β_1 and β_2 steady-state mRNA levels changed minimally until 12 h after PH when both transcripts decreased to approximately 15% of baseline values. A similar decrease in assembled connexin levels was detected by immunoblot and indirect immunofluorescence at 18 h after PH. Both transcripts simultaneously increased between 24 and 42 h and again rapidly decreased by 48 h post-PH. β_1 and β_2 assembled gap junction protein expression increased at 48 h post-PH and rapidly decreased by 56 h. By 72 to 84 h post-PH, β_1 and β_2 mRNA and assembled protein expression returned to near baseline levels and were maintained. Interestingly, inhibition of protein synthesis with cycloheximide completely inhibited disappearance of the β_2 transcript, in contrast to β_1 mRNA which was unaffected. Nuclear run-on assays showed no change in transcriptional rates for either gene during the regenerative period. However, both β_1 and β_2 transcripts exhibited significantly decreased mRNA half-lives at 12 h post-PH (3.8 and 3.7 h, respectively) relative to those at 0 h (10.9 and 6.1 h, respectively). Surprisingly, although the transcriptional rate for α_1 was similar to that observed for β_2, no α_1 transcripts were detectable by northern or RNase protection analysis. The results suggest that in the regenerating rat liver, β_1 and β_2 gap junction genes are not regulated at the transcriptional level. Rather, the cyclical modulation of their steady-state transcripts is regulated primarily by posttranscriptional events of which mRNA stability is at least one critical factor in the control process.

Gap junctions are specialized regions of intercellular contact allowing cell-to-cell communication, via ions and small molecules up to 1,500 kD, through channels formed by the connexin family of proteins (Kumar and Gilula, 1992). The patency of these channels is controlled by a variety of factors including intracellular pH, Ca^{2+}, and cAMP concentrations, of which the latter is known to influence the phosphorylation state of the connexin proteins (for review see Kumar, 1991). Since these channels have no apparent chemical selectivity, they allow molecular equilibrium to be established between neighboring cells, and the propagation of chemical and electrical signals (Loewenstein, 1985).

A gap junction contains two hemi-channels or connexons, one from each of the adjacent cell plasma membranes (Kumar and Gilula, 1992). Each connexon is an oligomeric assembly of protein subunits, the connexins, which are closely related members of a multigene family whose integral membrane protein products exhibit topological similarities. Members of the gap junction family share many characteristics, including the presence of a set of cysteine residues in the extracellular loops which join four transmembrane domains, of which one is capable of forming part of the channel. β_1 (connexin 32) and β_2 (connexin 26) are the two major gap junction proteins in the liver, and they have been colocalized to the same gap junctional plaques (Nicholson et al., 1987). A high level of expression of the β_1 and β_2 gap junction proteins and undetectable levels of α_1 (connexin 43) are correlated with the hepatocyte-specific phenotype (Stutenkemper et al., 1992). In fact, loss of both β_1 and β_2 and detectable expression of α_1 are typically associated with dedifferentiation of hepatocytes. For example, in hepatocellular carcinomas, both β_1 and β_2 gap junction expression are significantly reduced (Sakamoto et al., 1992).

Gap junctions can transmit growth controlling signals, and consequently, they have been proposed to be instrumental in the regulation of cell growth (Loewenstein and Rose, 1992). For example, transformed cells exhibit decreased gap junctional communication (Beer et al., 1988), and the growth rate of tumor cells in situ is inversely correlated with the level of intercellular communication (Loewenstein and Rose, 1992). To further evaluate the regulation of gap junctions during controlled cell growth in vivo, a model of liver regeneration was examined following 70% partial hepatectomy (PH)[1]. The adult hepatocyte is normally a quiescent,

1. *Abbreviations used in this paper*: $ASGP_R$, asialoglycoprotein receptor; LR, liver regeneration; PH, partial hepatectomy; SSPE, sodium chloride sodium phosphate EDTA.

highly differentiated cell. However, a decrease in liver mass or traumatic cell loss induces hepatocytes to replicate. The regenerative response after 70% PH represents a fairly synchronous process in which a wave of DNA replication peaks around 24 h post-PH, and this is followed 6 to 8 h later by mitosis. A second, smaller peak of DNA synthesis occurs at 48 h, and this represents a less synchronous wave of cell division. Hepatocyte as well as nonparenchymal cell replication continues until liver mass and cell number are restored (Fausto, 1990). Previous studies have examined the morphologic changes in gap junctions in regenerating liver (Yee and Revel, 1978; Yancey et al., 1979; Meyer et al., 1981; Hendrix et al., 1992*a*). In each of the studies, dramatic changes in gap junction abundance were observed during the first 24 to 48 h during which time they exhibited a cyclical phase of disappearance and reappearance. It has been postulated that the disappearance of gap junctions in the regenerating liver coincides with DNA synthesis to functionally segregate the replicating cell from its neighbor (Dermietzel et al., 1987). Furthermore, the changes in gap junction expression cannot be explained only by dispersal and reuse of the subunits, but rather involves degradation and resynthesis of the individual proteins (Traub et al., 1983; Iwai et al., 1991; Hendrix et al., 1992*a*).

The present study extends the analysis of modulation of gap junctions in the regenerating liver, and investigates potential mechanisms involved in their regulation. Immunohistochemical localization was used to determine the distribution and expression patterns of α_1, β_1, and β_2 gap junction plaques in regenerating livers until 96 h after 70% PH. Northern blots and immunoblots from the same tissues were used to correlate the expression of β_1 and β_2 mRNA and assembled gap junction proteins. The results suggest that changes in gap junction abundance coincide with the cell cycle pattern of the regenerating liver. Furthermore, modulation of the β_1 and β_2 gap junction steady-state transcript levels appears to be regulated at the posttranscriptional level where changes in mRNA stability represent at least one critical factor in the control process.

Materials and Methods

Partial Hepatectomies and Half-life Determinations

Male Sprague-Dawley rats (250–275 g) (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were maintained on a standard 12-h light-dark cycle and fed commercial laboratory chow ad libitum. They underwent 70% PH by removal of the median and left lateral lobes under ether anesthesia between 8 a.m. and noon as previously described (Higgins and Anderson, 1931). At various times post-PH, the animals were sacrificed, the liver tissue removed, rinsed in normal saline, and a 0.5-cm cube from the right lower lobe excised and embedded in OCT (Baxter Scientific, Minneapolis, MN) for immunohistology. The remaining tissue was flash-frozen in liquid nitrogen. Heart tissue was obtained from control animals and processed similarly. Animals used for the mRNA half-life determinations received an intravenous bolus of 50 μg per 100 g body weight (b.w.) α-amanitin (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 150 μg per 100 g b.w. actinomycin D (Boehringer Mannheim Biochemicals) i.p. at either 0 or 12 h post-PH. The animals were sacrificed at various times postinjection, the remnant liver removed and processed. To inhibit protein synthesis, an i.p. injection of 5 mg per 100 g b.w. cycloheximide (Sigma Immunochemicals, St. Louis, MO) (Morello et al., 1990) was administered at either 0 or 6 h post-PH.

Preparation of Gap Junctions

Gap junctions were isolated from rat liver as previously described (Zimmer et al., 1987). In brief, 2 g of liver was chopped, placed in 1 ml buffer (1 mM $NaHCO_3$, 1 mM PMSF, 2.5 mM EDTA, pH 7.0) at 4°C and dounce homogenized using 50 strokes. An equal volume of 40 mM NaOH was added, and the suspension sonicated for 10 s using a micro-ultrasonic cell disrupter (Branson, Inc., Danbury, CT). After the addition of 5 ml of 20 mM NaOH, the samples were incubated on ice for 40 min and then centrifuged at 35,000 *g* for 30 min at 4°C. The resulting pellets were resuspended in 1 ml of the same buffer, and the centrifugation step repeated. The pellets containing gap junctions were resuspended in 100 μl of 1 mM $NaHCO_3$ for analysis. The protein concentration of the samples was determined using a protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Immunoblot of Gap Junction Proteins

Affinity-purified antibodies prepared against synthetic peptides corresponding to rat β_1 and β_2 gap junction proteins were used for immunoblot analysis as previously described (Risek et al., 1990). SDS-PAGE and immunoblots were performed (Milks et al., 1988), using 15% polyacrylamide gels for separation of liver membrane proteins. Prestained molecular weight standards from Bio-Rad Laboratories were used as markers. After electrophoretic transfer to nitrocellulose, the blots were incubated with 3% BSA for 3 h at room temperature, followed by overnight incubation with β_1 or β_2 specific antibodies (1:100 dilution) at 4°C. The blots were washed with several changes of TBS, and incubated with ^{125}I-protein A for 1.5 h. After washing with TBS, the blots were exposed to Kodak X-Omat film (Rochester, NY).

Indirect Immunofluorescence Microscopy of Gap Junction Proteins

Immunolabeling of the gap junction proteins in regenerating liver was carried out on 6–8-μm-thick cryosections placed on gelatinized slides. The sections were fixed in methanol for 5 min at −20°C. After rinsing in PBS, the specimens were incubated in PBS containing 3% BSA and 3% normal goat antisera for 1 h to reduce nonspecific labeling. Sections were incubated at 37°C for 2 h with the anti-β_1S peptide, anti-β_2J peptide, or anti-α_1S peptide antibodies (Risek et al., 1990). After removal of the unbound antibodies by washing with PBS, sections were incubated with the secondary antibodies (FITC-conjugated anti-rabbit IgG) at 37°C for 1 h. Slides were washed with PBS and mounted in 90% glycerol containing 0.1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Immunolabeling was analyzed using a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) with epifluorescence. Photographs were taken using Kodak T-Max 400 black and white film (Rochester, NY).

Thin-section Electron Microscopy

Rat liver was dissected and cut into 1 × 2-mm pieces. Tissues were fixed in modified Karnovsky's fixative (1.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2) for 1 h at room temperature and then postfixed in 1% OsO_4 in the same buffer for 1 h. After rinsing several times, tissues were incubated in 0.05 M cacodylate, pH 7.4, containing 0.25% tannic acid for 1–2 h and stained en bloc in uranyl acetate for 1–2 h. After dehydrating through graded ethanol, the tissues were embedded in Poly/Bed 812 (Polysciences, Inc. Warrington, PA). Thin sections were cut with a diamond knife, mounted on copper grids, poststained in uranyl acetate and lead citrate, and then examined with a Philips CM-12 electron microscope.

Northern Analysis and Densitometery

Total RNA was isolated from liver tissue as previously described (Chomczynski and Sacchi, 1987). Poly(A)$^+$-enriched RNA, obtained by oligo-dT (New England Biolabs, Beverly, MA) chromatography was electrophoresed on 1% agarose, 2.2 M formaldehyde, 1× MOPS (pH 7.0) denaturing gels, and transferred by passive capillary diffusion to MagnaGraph nylon membrane (Micron Separations, Inc., Westboro, MA). The large molecular weight RNA ladder from (GIBCO-BRL, Gaithersburg, MD) was used for nucleic acid standards. cDNAs used were an EcoRI 1.4-kb fragment of rat β_1 (Kumar and Gilula, 1986), an EcoRI 2.2-kb fragment of rat β_2 (Risek

et al., 1990), an EcoRI 2.4-kb fragment of rat α_1 (Beyer et al., 1987), and 0.9-kb PstI cDNA clone of the rat $ASGP_R$ (Holland et al., 1984). The probes were ^{32}P-labeled with α-^{32}P-dCTP (3,000 Ci/mmole) (Amersham Corp., Arlington Heights, IL) by random priming (Feinberg and Vogelstein, 1983) using a commercial kit (United States Biochemical Corp., Cleveland, OH). Hybridizations were done under stringent conditions in 50% formamide, 5× SSPE, 5× Denhardt's, 0.2% SDS, and 200 μg/ml yeast RNA for 18 to 24 h at 42°C. Membranes were washed twice for 15 min at room temperature in 1× SSPE, 0.1% SDS, twice at 37°C in 0.1× SSPE, 0.5% SDS, and twice at 65°C in 0.1× SSPE, 0.5% SDS. Autoradiography was done with Kodak XAR film (Rochester, NY) at −70°C using an intensifying screen. Several exposures were done to ensure linearity of the films for densitometric analysis.

Video-densitometry was performed using a Macintosh II (Apple Computer, Cupertino, CA) coupled to a Data Translation DT2255 video digitizer (Data Translation, Marlboro, MA) and a JVC GX-N8 video camera (JVC Corporation of America, Elmwood Park, NJ) as previously described (Correa-Rotter et al., 1992). Quantitation of autoradiograms utilized the NIH Image 1.4 Densitometric Analysis Program normalized to the $ASGP_R$ mRNA transcript (Diehl et al., 1990*a*).

Transcriptional Rate Analysis

Isolation of nuclei and transcriptional rate assays were performed using modifications of a previously described method (Diehl et al., 1990*b*). Approximately 1 g of frozen liver was pulverized in liquid nitrogen and dounce-homogenized in ice cold buffer containing 0.32 M sucrose, 3 mM $MgCl_2$, 1 mM Hepes, pH 6.8, and 5 mM DTT. The homogenate was centrifuged at 1,000 *g* for 10 min in an SS-34 Sorvall rotor at 4°C. The crude nuclear pellet was resuspended in 7 ml of 1.65 M sucrose, 5 mM $MgCl_2$, 1 mM Hepes, pH 6.8, 5 mM DTT, layered over a 4 ml cushion of 2.1 M sucrose, 3 mM $MgCl_2$, 1 mM Hepes, pH 6.8, 5 mM DTT, and centrifuged at 70,000 *g* for 1 h in a SW-40 Ti rotor (Beckman Instruments, Mountainview, CA) at 4°C. The nuclei were resuspended in 100 μl of 20% glycerol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 140 mM KCl, 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 100 U placental RNase inhibitor (United States Biochemical Corp.) and 50 nmoles each of ATP, CTP, and GTP (Boehringer Mannheim Biochemicals). 100 μCi of α-^{32}P-UTP (3,000 Ci/mmole) (Amersham Corp.) was added and the suspension incubated for 30 min at 30°C with periodic mixing. The nuclei were lysed by adjusting the solution to 0.3 M NaCl. DNA was digested by adding 12 μl of a 1 mg/ml solution of RQI DNase (Promega Biotec, Madison, WI) followed by a 15-min incubation at 30°C. The reactions were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). The solution was adjusted to 0.3 M sodium acetate and the RNA precipitated twice in 3 vol of ethyl alcohol for 30 min at −70°C. MagnaGraph nylon membranes to which 3 μg of the indicated cDNA fragment was bound by slot blot were hybridized with labeled RNA at 3.0×10^6 cpm/ml for 4 d under stringent conditions at 42°C. In addition to the α_1, β_1, and β_2 gap junction cDNAs, a 1.2-kb rat β-fibrinogen cDNA, cloned in this laboratory, and pGEM-3Z were included in the nuclear run-on assays. The pGEM RNA synthesis system (Promega Biotec) was used to generate α_1 sense, α_1 antisense and vector control RNA. After hybridization, the membranes were washed for 15 min twice at room temperature in 1× SSPE, 0.1% SDS, twice at 42°C in 1× SSPE, 0.1% SDS, and twice at 42°C in 0.1× SSPE, 0.5% SDS. Autoradiography and densitometry were performed as described above.

RNase Protection Assay

RNase protection assays were done according to the manufacturer's instructions in the Ambion RPA kit (Austin, TX). The probe for the α_1 gap junction mRNA was a 338-bp HindIII/HindII fragment corresponding to nucleotides 210 to 548 (Risek et al., 1990) which was subcloned into pGEM-3Z. A ^{32}P-labeled cDNA probe was generated from the T7 RNA polymerase promoter in pGEM-3Z according to the manufacturer's protocol (Promega Biotec) and was labeled with α-^{32}P-CTP (800 Ci/mmole) (Amersham Corp.). The reaction yielded a 378-bp fragment containing 40 nucleotides of the polyclonal region 5′ to the HindII cloning site. Poly(A)$^+$-enriched RNA from heart and liver were hybridized overnight at 45°C with 1.0×10^5 cpm of probe per 0.625 μg of RNA according to the manufacturer's specifications. After hybridization, 200 μl of a 1:200 dilution of the RNase A/T1 mixture was added and the solutions incubated for 30 min at 37°C. Samples were electrophoresed on a 7.5% acrylamide, 8 M urea, 1.5 mm gel, dried, and exposed to Kodak XAR film at −70°C for 4 h using an intensifying screen.

Results

Gap Junction mRNA Expression in Regenerating Rat Liver

The mechanisms responsible for the regulation of β_1 and β_2 gap junction proteins in the regenerating liver are largely unknown. The mRNA steady-state transcript levels for both β_1 and β_2 gap junction proteins were examined to determine whether they are modulated during the first 96 h of liver regeneration (LR). As shown in Fig. 1, there was little change in transcript expression for both β_1 and β_2 during the first 12 h of rat LR after 70% PH. However, at 12 h post-PH, β_1 and β_2 transcripts rapidly declined to approximately 15 to 20% of their 0-h values. Almost in parallel, both transcripts began to increase at 18 to 24 h and returned to near normal levels by 42 h post-PH. This cycle of expression for both transcripts repeated itself between 48 and 72 h post-PH. By 96 h post-PH, both mRNAs returned to near baseline levels. Thus, the steady-state transcripts encoding β_1 and β_2 gap junction proteins were modulated during LR and exhibited cyclical periods of expression which were closely associated with the major waves of DNA synthesis observed in the regenerating rat liver (Fausto, 1990).

The expression of α_1 gap junction mRNA was also examined in the regenerating liver. Interestingly, no detectable signal was observed by northern blot analysis when poly(A)$^+$-enriched RNAs from varying time points through 96 h were analyzed for α_1 transcript expression (data not shown). RNase protection assays were performed to increase the sensitivity of detection. Poly(A)$^+$-enriched RNA was isolated from rat liver and heart, which has been shown to express abundant α_1 gap junction mRNA (Beyer et al., 1987). As expected, RNA isolated from rat heart showed the protected 338-bp band at two concentrations (Fig. 2). Conversely, no protected band was detected in liver poly(A)$^+$-enriched RNA samples even under conditions where four times as much material was assayed and much longer autoradiographic exposure times were used. The upper band represents undigested probe and does not indicate the existence of α_1 protected transcript.

Gap Junction Protein Expression in Regenerating Rat Liver

To determine the relationship between the steady-state transcript and protein levels for β_1 and β_2 assembled gap junctions in the regenerating liver, immunoblots were performed on samples derived from the same tissue used for northern analysis (Fig. 3). It was observed that at certain time points such as 18 h post-PH, it was not possible to detect a signal in total homogenates. Therefore, to increase the sensitivity of the assay, sodium hydroxide resistant fractions were prepared for each liver sample as described in Materials and Methods. No signal was observed with either of the preimmune antibodies (data not shown). β_1 and β_2 gap junction proteins, migrating at 32 and 26 kD, respectively, showed a reproducibly small increase in intensity from 6 to 12 h post-

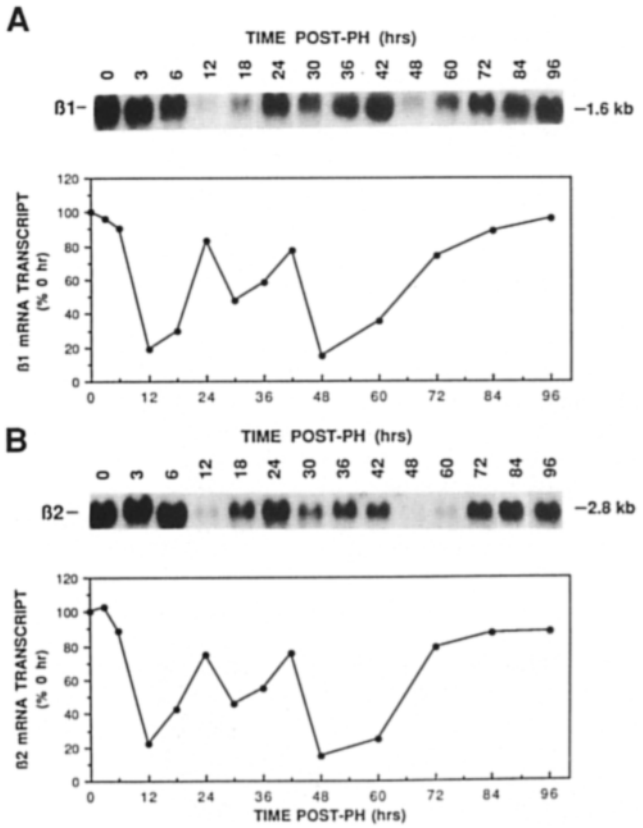

Figure 1. Northern analysis of steady-state transcript levels encoding β_1 and β_2 gap junction proteins in regenerating rat liver. Membranes were hybridized with ^{32}P-labeled cDNA probes for (*A*) β_1 and (*B*) β_2 mRNAs at varying times post-PH. Transcript sizes are indicated at the right. Blots were then stripped and rehybridized with the ^{32}P-labeled $ASGP_R$ cDNA to verify lane loading. Hybridizations for the β_1 and β_2 transcripts were carried out with 5 and 15 μg/lanes poly(A)$^+$-enriched RNA, respectively. Videodensitometric analysis of the autoradiograms was performed according to Materials and Methods and normalized to the $ASGP_R$ signal. Expression of the transcripts as the percent of 0 h values is shown in the accompanying graphs through 96 h of regeneration. The results are representative of four separate experiments.

PH which then decreased dramatically from 18 to 42 h. β_1 gap junction protein peaked at 48 h post-PH, in contrast to β_2 protein which showed only a small increase in signal. Both proteins then decreased between 56 and 60 h post-PH,

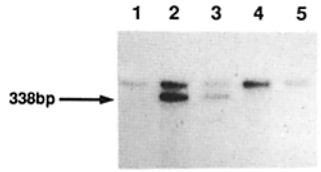

Figure 2. RNase protection analysis to determine α_1 gap junction mRNA expression in the regenerating rat liver. An α_1 gap junction riboprobe corresponding to nucleotides 210 to 548 of the NH_2-terminal coding region of the cDNA was synthesized as described in Materials and Methods. The riboprobe was incubated with varying concentrations of poly(A)$^+$-enriched RNA from heart and liver. (Lane *1*) Riboprobe, yeast RNA and RNase; (lane *2*) 1.25 μg and (lane *3*) 0.625 μg from heart; and (lane *4*) 5.0 μg and (lane *5*) 2.5 μg from liver. The upper band represents undigested probe. The lower band, indicated by the arrow, represents the 338-bp protected fragment of α_1 transcript from heart.

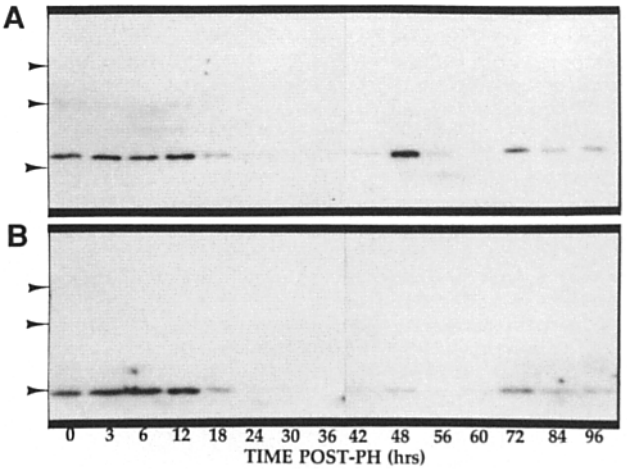

Figure 3. Immunoblot analysis of β_1 and β_2 gap junction proteins from regenerating rat liver. Alkali-insoluble fractions of total homogenate were electrophoresed on SDS–polyacrylamide gels as described in Materials and Methods. After transfer, the blots were probed with antibodies to β_1 gap junctions (*A*) and β_2 gap junctions (*B*). Molecular weight markers are indicated by arrowheads that correspond to 27.5, 49.5, and 80 kD in order of decreasing migration. Equal amounts of protein sample were loaded per lane.

and returned to near baseline levels by 72 h post-PH. After some loss of intensity at 84 h, the β_1 and β_2 gap junction proteins remained invariant through 96 h post-PH. The pattern of expression of the assembled gap junction proteins was offset approximately 6 h from those of their respective transcripts. However, at certain time points such as 24 h, there was a lack of precise temporal synchrony between changes in steady-state protein and mRNA transcript levels implying that the biosynthesis of the β_1 and β_2 gap junction proteins was regulated at multiple levels, and not simply by the steady-state mRNA levels.

It was important to corroborate results from the immunoblot analyses for the β_1 and β_2 assembled gap junction proteins. Thus, control (0 h) and regenerating liver from the same tissue used for northern and immunoblot analyses were analyzed by indirect immunofluorescence using anti-β_1S and anti-β_2J peptide antibodies. In non-regenerating liver, β_1 and β_2 gap junction proteins were both present. Anti-β_1S immunolabeling (Fig. 4, *A* and *B*) was detected in essentially all cells of the acinus. However, anti-β_2J immunolabeling (Fig. 5, *A* and *B*) was detectable only in peripheral zones of acini. While no immunoreactivity for the β_2 gap junction protein in the area of the central vein was observed, a strong signal was present near the interlobular vessels (data not shown). The signal was usually punctate or short linear in appearance and confined to the periphery of cells. No signal was observed using preimmune antibodies (data not shown).

In partially hepatectomized animals, the distribution of β_1 (Fig. 4 *C*) and β_2 (Fig. 5 *C*) gap junction antigens did not change significantly during the first 12 h post-PH as determined by indirect immunofluorescence. However, as previously shown by immunoblot analysis both β_1 (Fig. 4 *D*) and β_2 (Fig. 5 *D*) expression began to decrease by 18 h post-PH. The levels of β_1 and β_2 were at their minimum 36 h post-PH (Figs. 4, *F* and 5 *F*), and then increased by 42 h, peaking at 48 h post-PH (Figs. 4 *H* and 5 *H*). Similar to 0 h liver, β_1 antigen was detected in all cells of the aci-

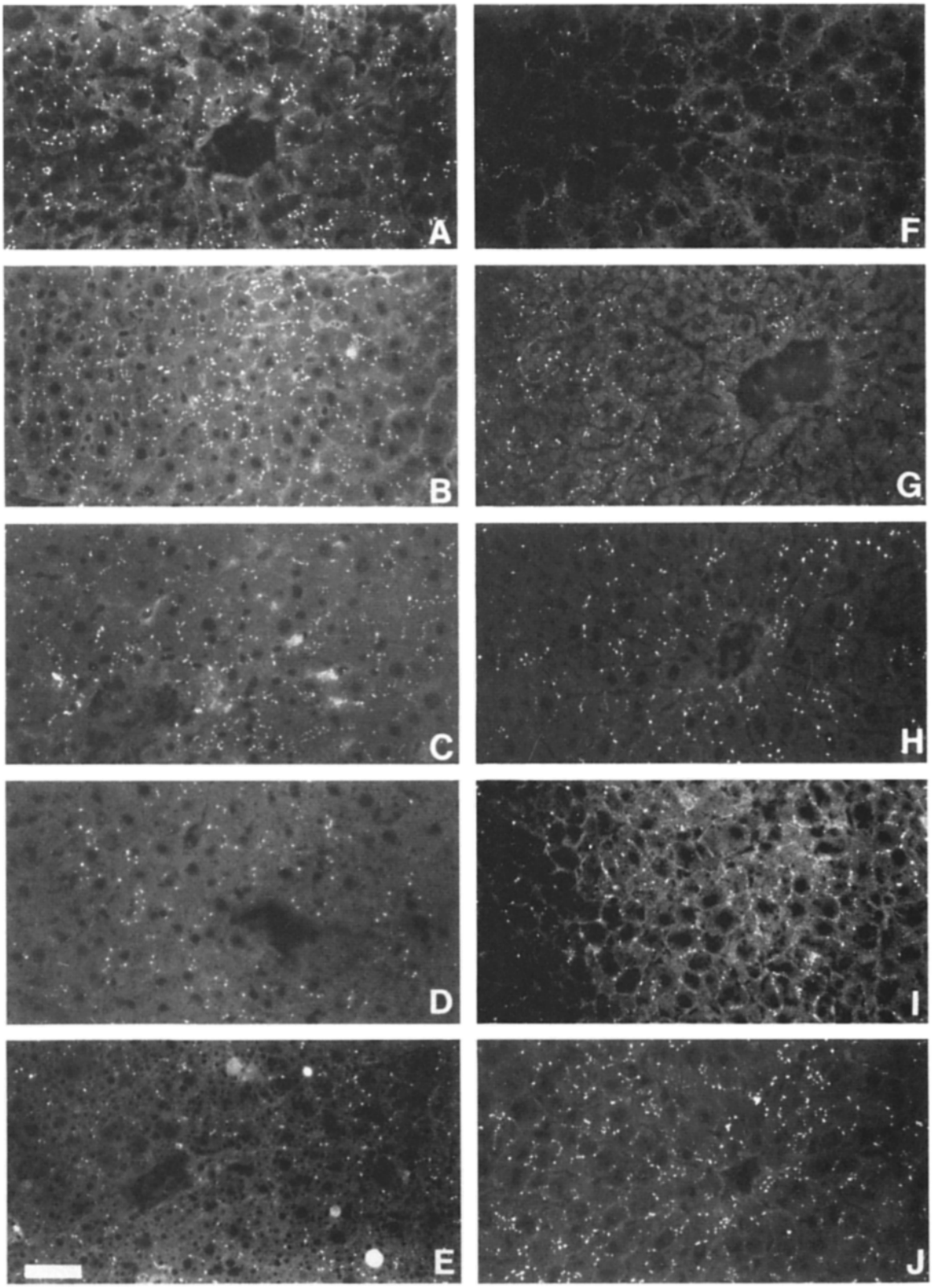

Figure 4. Indirect immunofluorescence of β_1 gap junction antigen in regenerating rat liver. (*A*) Central vein of control (0 h) liver. (*B*) Peripheral zone of 0 h liver showing a uniform distribution of immunofluorescence in the rat liver acinus. Distribution of β_1 gap junction antigen in regenerating liver (*C*) 12, (*D*) 18, (*E*) 24, (*F*) 36, (*G*) 42, (*H*) 48, and (*I*) 60 h post-PH. (*J*) 84 h regenerating liver demonstrating the recovery of immunofluorescence to near control levels. Bar, 40 μm.

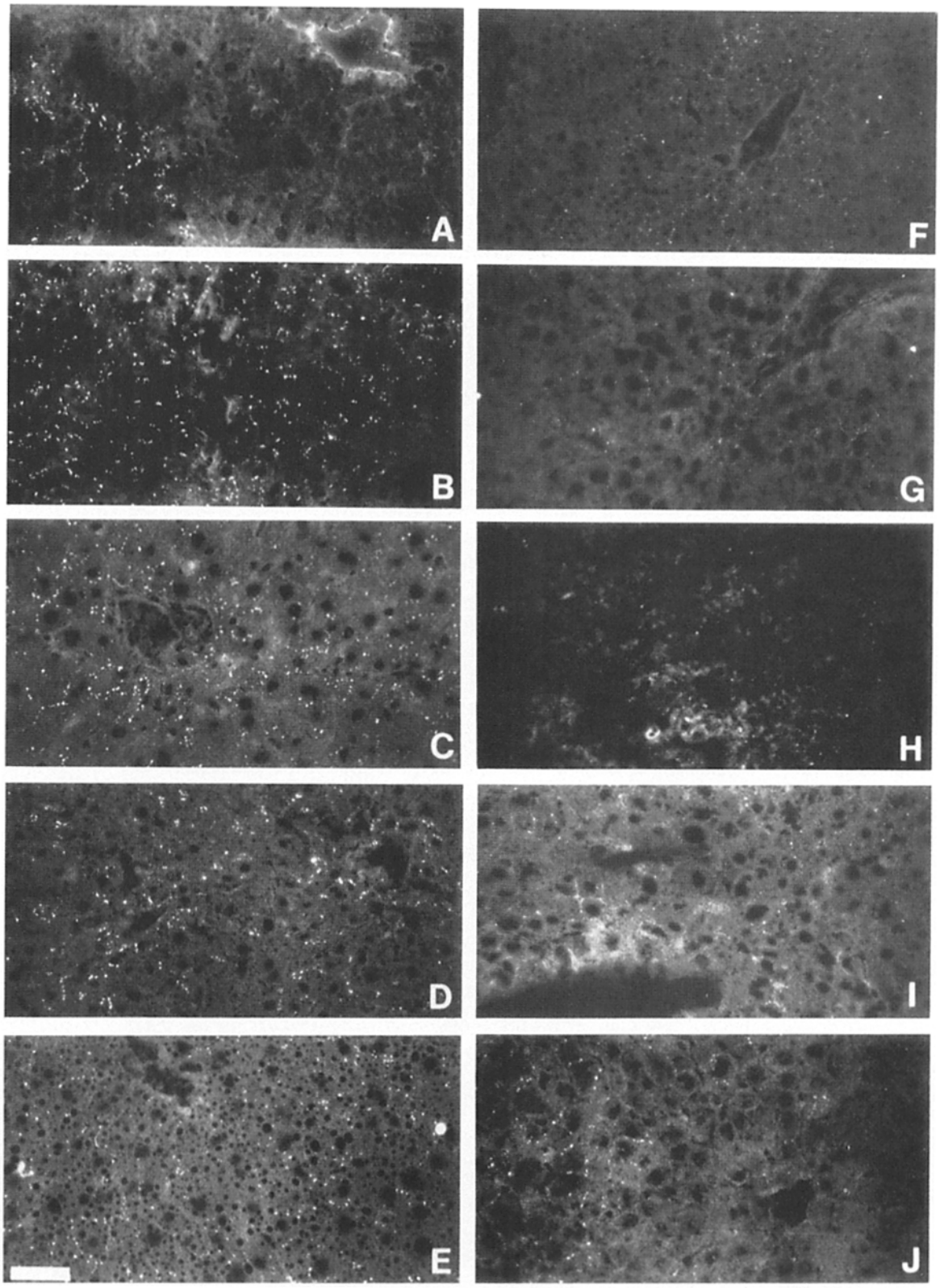

Figure 5. Indirect immunofluorescence of β_2 gap junction antigen in regenerating rat liver. (*A*) Central vein of control (0 h) liver. (*B*) Peripheral zone of 0 h liver acinus showing that β_2 gap junction plaques are localized predominantly in this region. Distribution of β_2 gap junction antigen in regenerating liver (*C*) 12, (*D*) 18, (*E*) 24, (*F*) 36, (*G*) 42, (*H*) 48, (*I*) 60, and (*J*) 84 h post-PH. Bar, 40 μm.

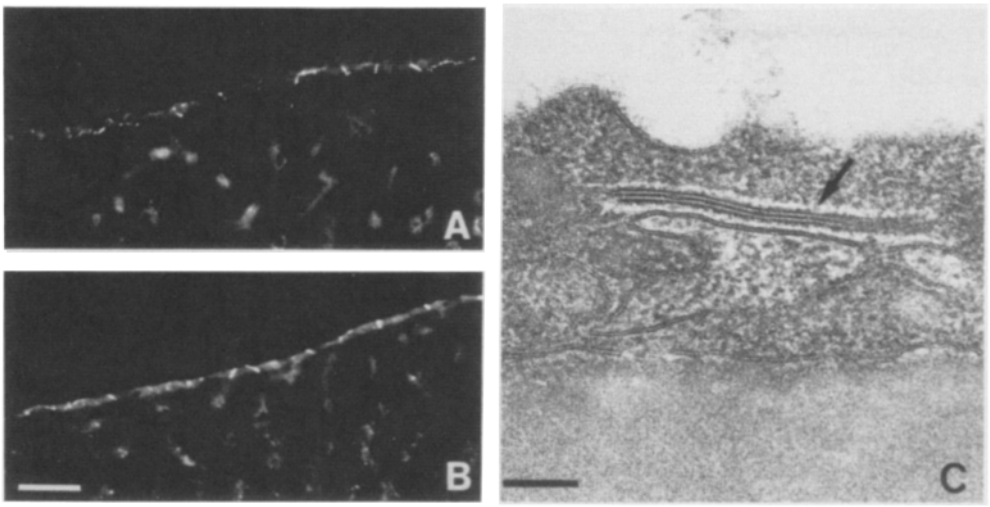

Figure 6. Indirect immunofluorescence localization of α_1 gap junction antigen in peritoneal mesothelial cells of Glisson's capsule. (*A*) Control (0 h) liver. (*B*) 36 h regenerating liver. Scattered labeling of parenchymal cells was nonspecific and occurred when sections were incubated with preimmune antibodies. (*C*) Thin section electron micrograph of a gap junction, indicated by the arrowhead, between peritoneal mesothelial cells. Bars: (*B*) 40 μm; (*C*) 100 nm.

nus, whereas β_2 immunolabeling was less intense and located primarily in the interlobular zones. Both β_1 and β_2 antigen levels decreased again at 60 h (Figs. 4 *I* and 5 *I*), and then increased by 72 h post-PH. By 84 h post-PH, the patterns of immunofluorescence for both gap junction antigens were similar to control levels (Figs. 4 *J* and 5 *J*). There was no detectable labeling with the preimmune antibodies (data not shown).

The expression of α_1 gap junction protein was also investigated in control and regenerating rat liver. By indirect immunofluorescence using an anti-α_1S peptide antibody, α_1 gap junction antigen in control liver was located only in a single layer of peritoneal mesothelial cells in Glisson's capsule, which covers most of the liver's free surface (Fig. 6 *A*). A background staining of parenchymal cells was present when sections were also incubated with preimmune antibodies (data not shown). Thin-section EM of 0 h liver confirmed the presence of gap junctions between cells in Glisson's capsule (Fig. 6 *C*). In contrast to the observed modulation in the levels of both β_1 and β_2 gap junction proteins during LR, there was no detectable change in expression of α_1 antigen by indirect immunofluorescence during the regenerative period (Fig. 6 *B*).

Effect of Cycloheximide on mRNA Expression

The existence of cycloheximide-superinducible genes in eukaryotic systems is well established (Hofbauer and Denhardt, 1991). The increase in steady-state mRNA after inhibition of protein synthesis appears to be regulated at the transcriptional and/or posttranscriptional level (Sobczak et al., 1989; Noguchi et al., 1992). In some cases labile *trans*-acting protein factors have been implicated in the regulation of certain steady-state transcripts (Morello et al., 1990; Bernstein et al., 1992; Noguchi et al., 1992; Klausner et al., 1993). Therefore, it was of interest to examine the effect of protein synthesis inhibition on β_1 and β_2 mRNA expression in control and regenerating livers. Rats were injected with either vehicle or cycloheximide at 0 or 6 h post-PH, and livers collected at various times up to 6 h postinjection. Northern analysis of poly(A)$^+$-enriched RNA isolated from the liver specimens indicated that the 1.6-kb β_1 steady-state mRNA levels were unaffected relative to 0 h values both pre- and post-PH (Fig. 7 *A*, *a* and *b*). In contrast, steady-state expression of the 2.8-kb β_2 transcripts increased sixfold by 4 h postinjection in the non-hepatectomized group (Fig. 7 *B*, *a*). The injection of cycloheximide at 6 h post-PH not only eliminated the disappearance of the β_2 transcripts but also led to an approximately 18-fold increase in mRNA expression 10 h post-PH (Fig. 7 *B*, *b*).

Transcription Rate Analysis

Nuclear run-on assays were performed to examine the potential role of transcriptional rate changes in the regulation of steady-state mRNA expression observed for β_1 and β_2 in the regenerating liver. The rate of β_1 transcription was significantly greater than that of β_2, consistent with their observed levels of steady-state transcript expression (Fig. 8). However, there were no significant changes in transcriptional rates for β_1 and β_2 genes during the regenerative period. Conversely, there was a marked increase in transcription rate following PH for the acute-phase reactant β-fibrinogen as previously reported (Milland et al., 1990). Based on these results, it appeared that the regulation of β_1 and β_2 mRNA levels in the regenerating liver was controlled by posttranscriptional processes.

Interestingly, the α_1 gap junction gene (Fig. 8) was transcribed at a rate equal to that observed for β_2 in nuclear run-on assays. To ensure that the apparent transcription of α_1 was specific, nuclear run-on assays were repeated using hybridizations to both sense and antisense cDNAs. In fact, there was complete absence of transcriptional product using sense cDNA, in contrast to the intense signal generated by that of the antisense (Fig. 9), indicating that the observed results were specific.

It was also important to investigate whether transcriptional rate changes were responsible for the increased steady-state levels of β_2 transcripts observed after cycloheximide. Nuclei were isolated from vehicle- and cycloheximide-injected animals, and nuclear run-on assays performed. No detectable changes in transcription rate for the β_2 gap junction gene between the two groups were observed (data not shown). In contrast, transcriptional activity for the proto-oncogene *c-myc* increased threefold in the cycloheximide-treated animals as previously reported (Sobczak et al., 1989) (data not shown).

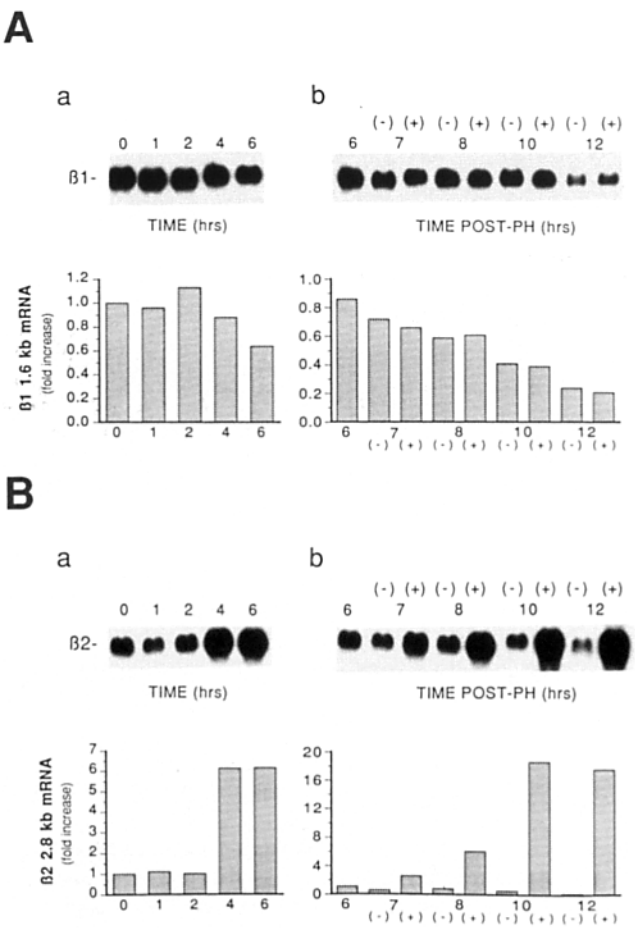

Figure 7. Effect of cycloheximide on steady-state (*A*) β_1 and (*B*) β_2 gap junction transcript levels. (*a*) Cycloheximide was administered at 0 h as described in Materials and Methods and livers collected at the indicated times. (*b*) Cycloheximide (+) or vehicle (−) was administered 6 h post-PH, and regenerating livers collected until 12 h post-PH. The northern blots were hybridized with the ^{32}P-labeled cDNA probes, stripped and rehybridized with ^{32}P-labeled $ASGP_R$ cDNA to verify lane loading. Video-densitometric analysis of the autoradiograms was done as described in Materials and Methods. Each of the signals was normalized to that of the $ASGP_R$ transcript and expressed in comparison to the 0 h steady-state transcript levels. The results are shown in the accompanying histograms, and they are representative of three separate experiments.

In Vivo mRNA Transcript Half-life Determinations

The invariant transcription rates for the β_1 and β_2 gap junction genes and the effect of cycloheximide on β_2 mRNA expression suggested that posttranscriptional events were responsible for the changes in steady-state transcript levels observed in the regenerating liver. Therefore, to establish that altered mRNA stability during LR could account for observed changes in steady-state transcript values of the two gap junction genes, mRNA half-lives were determined in vivo at times corresponding to maximal and minimal transcript expression. The transcriptional inhibitors α-amanitin and actinomycin D were administered at 0 and 12 h post-PH, and samples collected at 0, 2, 4, 6, 8, and 12 h postinjection. The combination of drugs irreversibly inhibited essentially all transcriptional activity within 30 min of injection (data not shown). Poly(A)$^+$-enriched mRNA obtained from the treated and untreated animals was subjected to northern analyses, and the signals from the decaying β_1 and β_2 transcripts quantitated densitometrically. Linear regression analysis of the data (Fig. 10) indicated that the steady-state transcript levels of the gap junction genes were, in fact, associated with significant changes in message half-life ($t_{1/2}$). There was a 65% decrease in the half-life of β_1 mRNA between 0 and 12 h post-PH. Similarly, there was a 40% decrease in the half-life of β_2 mRNA between the two time points.

Discussion

This is the first detailed report that examines the expression of three (α_1, β_1, and β_2) gap junction products during LR by transcriptional, steady-state mRNA, and protein analysis. Furthermore, unlike previous studies, this analysis is extended to periods well beyond immediate- and delayed-early gene expression (Haber et al., 1993). Although modulations of the β_1 and β_2 gap junction transcripts and assembled proteins were observed, no changes in transcriptional rates for either gene were detectable during the regenerative period. Furthermore, it was found that the transcriptional rate for the α_1 gap junction gene was equal to that observed for β_2, even though α_1 transcripts were undetectable by northern analysis or RNase protection. However, immunofluorescence studies did indicate that the α_1 antigen was present in Glisson's capsule. The half-lives of the β_1 and β_2 transcripts were determined in vivo at varying time points post-PH, and appeared to account for the changes observed in the steady-state mRNA levels. Also, results from the cycloheximide studies indicated that protein factors that are involved in the regulation of β_1 transcript expression are different from those that regulate the β_2 transcript.

The mammalian liver represents a remarkable example of position-specific gene expression. In the present study, identification of assembled gap junction proteins by indirect immunofluorescence indicated that β_1 gap junction protein was distributed uniformly throughout the entire acinus, while β_2 gap junction protein was primarily located in the more peripheral zones. The results are consistent with previous observations that β_2 gap junction mRNA was almost three times more abundant in the periportal than in the pericentral cells, while β_1 mRNA was equally distributed (Rosenberg et al., 1992). Numerous studies have shown that hepatocytes in various locations within the acinus may perform very different functions (Kuo and Darnell, 1991). For example, several enzymes in the urea cycle are synthesized by most hepatocytes, but not by those which surround central veins (Gaasbek-Janzen et al., 1984). Such a difference in the pattern of distribution suggests that β_2 gap junctions may play an important role in regulating nutrient exchange. In fact, the composition of gap junctions may affect their functional capacity for intercellular communication. The unaltered distribution of the two gap junction proteins during LR strongly suggests position-dependent gene expression.

The functional relationship between gap junction expression and the cell cycle remains speculative. Our results suggest that in the regenerating liver a strong temporal association exists. The initial disappearance of gap junctions during LR coincides with the first wave of DNA synthesis. Decreas-

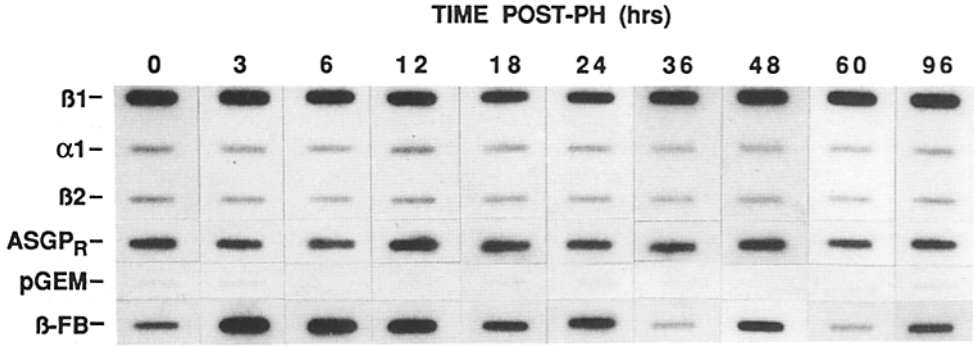

Figure 8. Transcriptional rate analysis of genes encoding α_1, β_1, and β_2 gap junction proteins in regenerating rat liver. Nuclei were isolated from remnant livers following PH and used to perform nuclear run-on assays as described in Materials and Methods. Slot blots were hybridized with ^{32}P-labeled RNA nuclear run-on products from the indicated timed samples. Autoradiograms were quantified using video-densitometry. The figure is representative of results from four separate experiments. (β-FB, β-fibrinogen.)

ing expression of the assembled gap junctions continues until 36 h post-PH which is associated with termination of the first mitotic phase (Fausto, 1990; Miyashita, et al., 1991). Furthermore, the loss of gap junctions at 56 to 60 h post-PH coincides relatively closely to the second major wave of DNA synthesis and mitosis observed in the regenerating rat liver (Fausto, 1990). The results are consistent with the previous observation that expression of the 28-kD gap junction protein in hepatocytes of regenerating rat liver is diminished during periods of cellular replication (Dermietzel et al., 1987). Also, it has been shown in normal human mammary epithelial cells that β_2 is a cell cycle-regulated gene expressed at moderate levels during G_1 and S phases and significantly increased in late S and G_2 phases. In contrast, α_1 (connexin 43) is constitutively expressed at a uniformly low level throughout the cell cycle (Lee et al., 1992).

The observed decrease in gap junctions during LR may not simply reflect a change to a more proliferative state. For example, their synthesis has been shown to be affected by the composition of extracellular matrix (Spray et al., 1987) which is also altered during LR (Martinez-Hernandez et al., 1991; Glück et al., 1992). Also, certain hepatotoxins reduce β_1 gap junction protein expression without inducing DNA synthesis (Miyashita et al., 1991). The reappearance of gap junctions at 72 h post-PH may represent a critical event in the regulation of LR. It corresponds to the peak expression of transforming growth factor β1 (Fausto, 1990), which has been previously shown to suppress LR (Russell et al., 1988)

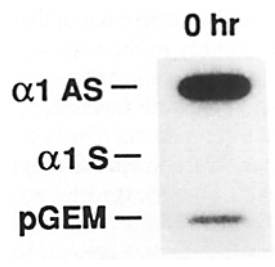

Figure 9. Transcriptional activity of α_1 gap junction gene using antisense probe in control liver. Nuclei were isolated from nonregenerating liver and processed for nuclear run-on assays as described in Materials and Methods. A slot blot to which either 3 μg of sense or antisense α_1 gap junction cDNA was bound was hybridized with ^{32}P-labeled RNA nuclear run-on products as described. (α_1 *AS*, α_1 antisense RNA; α_1 *S*, α_1 sense RNA; and *pGEM*, pGEM RNA.)

and enhance the extent of gap junction communication in vitro (van Zoelen and Tertoolen, 1991).

The mechanisms which control gap junction expression in the regenerating liver after PH are poorly understood. While the zonation of β_2 mRNA synthesis in nonreplicating liver appears to be controlled transcriptionally, posttranscriptional mechanisms seem to regulate the abundance of the connexin mRNAs (Rosenberg et al., 1992). During the first 12 h, a slight increase in the abundance of gap junction proteins in the regenerating liver was observed. Although many factors may be responsible for this change, cAMP, which increases biosynthesis of β_1 gap junctions in hepatocytes (Traub et al., 1987), is markedly enhanced by 6 h post-PH (Diehl et al., 1992). Whatever the putative mechanisms, the observed changes of the β_1 and β_2 gap junction products cannot be explained simply by dispersal and reassembly of gap junction subunits.

Expression of the β_1 and β_2 steady-state transcripts was investigated to correlate their changes with those of the assembled proteins. The significant decreases in transcript expression for both genes by 12 h and then again at 48 h post-PH preceded the decreases in protein levels by 6 h. However, at various times post-PH, especially 24 h, the β_1 and β_2 gap junction protein levels did not accurately reflect the expression of the respective mRNAs. This could be due to: (*a*) underestimation of the amount of connexin protein due to its intracellular localization and the method of isolation; (*b*) changes in translational efficiency; and (*c*) changes in the half-life of the proteins. The half-life of the β_2 gap junction protein in the rodent liver is approximately 2 h in cultured hepatocytes (Traub et al., 1989). Similarly, the half-life of the β_1 gap junction protein varies from approximately 1 to 3 h in vitro (Traub et al., 1987) to approximately 10 h in vivo (Yancey et al., 1981). These varying rates of decay and the rapid loss of gap junctions in cultured primary hepatocytes (Spray et al., 1987) imply that the rate of degradation of the gap junction proteins is accelerated during LR. In contrast, certain integral membrane proteins exhibit decreases during LR resulting from inhibition of synthesis rather than enhanced degradation (Bartles et al., 1991). Regulation of these events at the translational level would not be unique to the regenerating liver. It appears to be a critical factor in modulating gene expression in several other systems (Gross

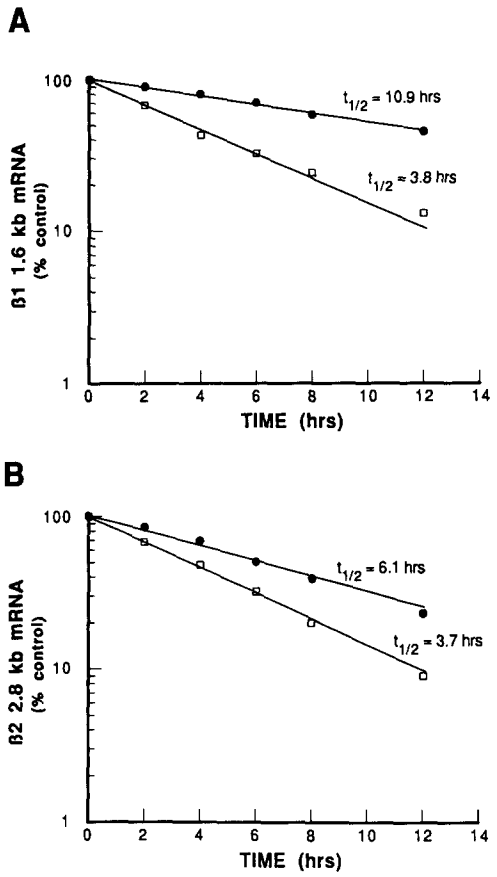

Figure 10. In vivo half-lives ($t_{1/2}$) of β_1 and β_2 gap junction transcripts in control and regenerating liver. At 0 and 12 h post-PH, rats were injected with actinomycin D and α-amanitin as described in Materials and Methods. Poly(A)$^+$-enriched RNA was prepared from remnant tissue harvested at 2-, 4-, 6-, 8-, and 12 h postinjection, and analyzed by Northern blot. Video-densitometric analyses of the autoradiograms were done and were standardized at each time point to the $ASGP_R$ mRNA. The apparent half-lives, determined by linear regression analysis, are indicated. (*A*) 1.6-kb β_1 transcript at 0 h (●) and 12 h post-PH (□). (*B*) 2.8-kb β_2 transcript at 0 h (●) and 12 h post-PH (□). Each data point is the mean of five experiments done on separate days using different animals. At each time point, the degree of variance within the group was less than 10 to 15%. To determine the half-life of the transcripts at 12 h, twice the concentration of poly(A)$^+$-enriched RNA per lane was used compared to 0 h.

and Merrill, 1989; Walden et al., 1989; Aloni et al., 1992; Klausner et al., 1993). It is also possible that a pool of connexin proteins exists which is undetectable by immunofluorescence. In this regard, α_1 gap junction protein has been shown to accumulate in Golgi vesicles of the rat myometrium before parturition (Hendrix et al., 1992*b*) and is present in intracellular compartments in preimplantation embryos (Sousa et al., 1993). Also, it has recently been shown that connexin 32 is present in a number of hepatocyte subcellular compartments including Golgi, lysosomes, and the ER (Rahman et al., 1993). Yet, in each case the protein was undetectable using morphological or immunocytochemical approaches.

Cycloheximide-induced stabilization of mRNA can result from either transcriptional and/or posttranscriptional processes (Sobczak et al., 1989; Morello et al., 1990; Noguchi et al., 1992). In the present study, inhibition of protein synthesis by cycloheximide markedly affected the steady-state transcript levels of the β_2 gap junction gene in control and regenerating liver. In contrast, no significant changes were seen in β_1 mRNA expression suggesting that different protein factors are involved in the regulation of the two transcripts. Such factors may also regulate the differential expression of gap junction proteins during hepatocarcinogenesis (Sakamoto et al., 1992). In the absence of transcriptional rate changes, the effect of cycloheximide on β_2 gene expression is apparently controlled at the posttranscriptional level. Furthermore, differences in accumulation of β_2 mRNA postcycloheximide at 0 and 6 h post-PH suggest that in the regenerating liver, its expression is controlled by additional factors. For example, changes in the steady-state levels of mRNA by cycloheximide could conceivably result from inhibition of certain *trans*-acting labile proteins involved in mRNA degradation (Brewer, 1991; Noguchi et al., 1992; Klausner et al., 1993). Similar factors may be associated with posttranscriptional control of other genes such as c-*fos*, c-*myc*, the insulin receptor, and HMG-CoA reductase (Shyu et al., 1989; Bernstein et al., 1992; Levy and Hug, 1992; Roitelman and Simoni, 1992).

Alterations in mRNA stability have been shown to be important in regulating the steady-state transcript levels in hepatocytes, both pre- (Simonet and Ness, 1989; Johnson et al., 1991; Levy and Hug, 1992) and post-PH (Noguchi et al., 1992). The substantially shortened half-lives for the β_1 and β_2 transcripts at 12 h post-PH would certainly implicate this posttranscriptional process. The decreased half-lives observed in this study for the two transcripts at 12 h post-PH accounts for approximately 80% of the difference in the steady-state levels at 6 and 12 h post-PH. Since the levels of expression for both mRNAs increased by 18 h post-PH, it is possible that the half-lives determined at 12 h post-PH may not represent the period of most rapid mRNA decay. Thus, the decreased stability of the β_1 and β_2 transcripts could be entirely responsible for the observed changes in their steady-state levels and, with the delay in disappearance of the proteins, could conceivably be regulated by translation-dependent mRNA destabilizing complexes (Savant-Bhonsale and Cleveland, 1992). However, other posttranscriptional processes may also be involved such as capping and splicing of the gap junction hnRNAs (Gudas et al., 1988; Cohen et al., 1989) and nucleocytoplasmic transport of their mature mRNAs (Leppard and Shenk, 1989; Piñol-Roma and Dreyfuss, 1992).

Gap junctions containing α_1 antigen were identified by indirect immunofluorescence in Glisson's capsule. However, transcripts for α_1 could not be detected by northern analysis or RNase protection assays, due most likely to the very low abundance of the RNA. The results are consistent with previous studies which report virtually undetectable levels of α_1 gap junction protein in well-differentiated hepatocytes (Stutenkemper et al., 1992). Therefore, it was surprising that the transcriptional rate of the α_1 gene as determined by nuclear run-on analysis was as high as that of β_2. This suggests that posttranscriptional mechanisms also appear to regulate α_1 gap junction mRNA levels. However, one cannot rule out the possibility that it represents a unique example of "illegitimate" transcription (Chelly et al., 1989). Post-

transcriptional processes, rather than changes in gene transcription, may in fact be responsible for the observed up-regulation of α_1 gap junctions in extended cultures of primary hepatocytes (Stutenkemper et al., 1992) and hepatocellular carcinomas (Oyamada et al., 1990).

In conclusion, the regenerating liver represents a dynamic system for studying regulation of gap junctions. It has been observed that posttranscriptional mechanisms are mainly responsible for changes in the levels of three gap junction proteins. Although nucleocytoplasmic processing appears to regulate α_1 gene expression, that of the β_1 and β_2 genes is regulated primarily by changes in mRNA stability. Furthermore, the factors involved in the regulation of the two mRNA half-lives are different. The results suggest a diverse group of control processes responsible for gap junction expression during LR and provide a basis to determine the functional relationship between gap junction formation and the cell cycle.

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