Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice

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Understanding the gene-expression patterns during liver regeneration may help to reveal how regenerative processes are initiated and controlled as well as shed new light onto processes that lead to liver disease. Using high-density oligonucleotide arrays, we have examined the gene-expression program in the livers of mice after partial hepatectomy. A time course was constructed for gene expression between 0 and 4 h after partial hepatectomy, corresponding to the priming phase of liver regeneration. The genomic program for liver regeneration involves transcription-factor generation, stress and inflammatory responses, cytoskeletal and extracellular matrix modification, and regulation of cell-cycle entry. The genome-wide changes that are observed provide a detailed and comprehensive map of the initial priming stage of liver regeneration.

The liver's ability to regenerate in mammals is relatively unique. Only a few species, including certain worms, insects, reptiles, and amphibians, can readily undergo various types of reparative regeneration including epimorphic reconstruction of entire body parts. In contrast, humans and larger mammals have little regenerative capacity (1, 2). Examples of organs and body parts that show reasonably good regenerative capacities are few and include the liver, fingertips, and peripheral nerves, and stem cells may be a source of the regenerative capacity (2). Among these types of reparative processes, liver regeneration stands out. The liver is capable of modulating its mass according to functional requirements, proliferating under conditions of functional deficiency, and undergoing apoptosis under functional excess. In both of these processes, the liver undergoes remodeling of the entire organ physiology to preserve normal histological organization (3, 4). Liver regeneration does not rely on stem cells, although liver stem cells may contribute to the process, and each cell type has the capacity to enter into a proliferative state and also alter their differentiation so that liver cells have innate progenitor cell characteristics (5, 6). Hepatocytes are the first cell types to enter into the cell cycle after functional deficiency in the liver (4), and they show an almost limitless capacity to proliferate (7). Also, during liver regeneration the liver cells continue to perform crucial metabolic functions such as glucose regulation, synthesis of many blood proteins, the secretion of bile, and biodegradation of toxic compounds required for homeostasis (3). Understanding the molecular mechanisms and genomic program of liver regeneration is of fundamental importance and is the first step toward controlling these events and other regenerative processes.

Liver regeneration can be initiated in several ways. Classical methods for initiating liver regeneration in animal models involve either partial hepatectomy (PHx) or injection of hepatotoxic compounds such as CCl_4. Pioneering studies by Taub and coworkers (8) as well as Fausto (3) have defined the roles of many immediate-early genes and cytokines in liver regeneration. On a molecular level, the entry of hepatocytes into cell cycle is stimulated by various cytokines and growth factors. Examples include IL-6, hepatocyte growth factor (HGF), epidermal growth factor, tumor necrosis factor (TNF)-α, transforming growth factor-α, insulin, and other receptor ligands that have been implicated in various stages of hepatocyte proliferation (3, 4). These ligands, through complex molecular mechanisms, activate transcription factors including NF-κB, signal transducer and activator of transcription 3 (STAT3), activator protein 1 (AP-1), and CCAAT/enhancer-binding protein (C/EBP)β that initiate a cascade of gene expression that ultimately is responsible for proliferation (9).

Before cell-cycle entry, quiescent hepatocytes (G_0) undergo a priming phase ($G_0 \rightarrow G_1$) during which the cells reenter the cell cycle and prepare for proliferation. The concept of priming phase in hepatocytes, originally proposed by Fausto *et al.* (3, 10–12), is the first stage of liver regeneration, the duration of which is species-dependent (13). For mice this stage lasts for ≈4 h after PHx. During this time, immediate-early genes such as c-*fos* and c-*jun* are induced (3, 8, 14, 15). In fact, many genes have been identified as being differentially expressed during hepatocyte priming and the following stages of the cell cycle leading up to DNA replication and mitosis. Known exogenous priming stimuli include sham surgery, protein deprivation, and collagenase treatment as well as infusion of TNFα, epidermal growth factor, or HGF (3, 4).

Given that the cells in a regenerating liver have progenitor cell character, we used functional genomic technologies to study cellular priming in mice. We have characterized the genome-wide expression changes in mice after ≈70% PHx over the course of 4 h, which corresponds to the priming stage of hepatocyte proliferation. Sham surgeries were conducted to eliminate responses that are caused by the surgery alone, and resected liver specimens functioned as a baseline for gene-expression changes in each mouse. We found that genes associated with transcription-factor production, stress and inflammatory responses, cytoskeletal and extracellular matrix modification, and regulation of cell-cycle entry all are involved in the early stages of liver regeneration. It is noteworthy that the liver is composed of many different types of cells, and most of its function is confined to hepatocytes (which represent ≈70% of the liver), Kupffer cells (macrophages), and bile ductule epithelium. Thus, it is possible that some of the changes reported may have occurred in nonparenchymal cells of the liver rather than hepatocytes.

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Materials and Methods

Mice, Tissues, and RNA Preparation. Groups of three 8–10-week-old male C57BL/6 mice were anesthetized and subjected to either sham operation or 70% PHx as described (16). At 1, 5, 10, 30, 90, and 240 min posthepatectomy, mice were killed, and liver tissue samples were harvested. Total RNA was isolated from these samples and the respective surgically resected liver sections as described (16) and analyzed by high-density oligonucleotide microarray, Northern blot, and RNase protection assay (RPA).

High-Density Oligonucleotide Microarray Analysis. Double-stranded DNA was synthesized from ≈5 μg of total RNA by using the SuperScript Choice system (Life Technologies, Grand Island, NY) and a primer containing poly(dT) and a T7 RNA polymerase

Abbreviations: PHx, partial hepatectomy; HGF, hepatocyte growth factor; TNF, tumor necrosis factor; RPA, RNase protection assay; MAPK, mitogen-activated protein kinase; C/EBP, CCAAT/enhancer-binding protein; PPAR, proliferator-activated receptor.

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Table 1. GeneChip analysis of changes in liver gene expression after partial hepatectomy

Acc.#	Gene name (synonyms)	Known in LR	10 min Δ	10 min E/S	30 min Δ	30 min E/S	90 min Δ	90 min E/S	240 min Δ	240 min E/S
Transcription factors										
V00727	c-fos	[Y]	22	13.5	46	18.6	58	22.7	25.8	20
J04115	c-jun	[Y]	19	2.2	49.6	2	13.9	15.3	33	2.8
M22326	zif/268 (EGR-1)	[Y]	8.5	6.4	18.6	2.5	3.2	7.8	4.7	12.1
M59821	pip92 (IER2)	[Y]	3.3	3	7	1.8	2.7	4.4	5.2	9.1
U19118	Irg-21 (ATF3)	[Y]			11	4.2	46	46	133	49.8
J03236	JunB	[Y]			5.3	2.4	3.5	3.3	--	--
M58691	TIS11	[Y]			3.8	2.3	--	--	--	--
X57638	PPARα				-2.4	-7	-11	-15	-30	-7
X16995	N10	[Y]					12.5	13.4	3.4	3
Y07836	stra 14						2.8	3	7.2	3.7
Y07688	NFI-X (CTF)						-2.3	-2.3	--	--
X63747	Zfp-38 / RU49						-2.2	-1.9	--	--
X74937	hepatocyte nuclear factor-3β						-2.2	-2	--	--
X01023	c-myc	[Y]							12.4	7.1
M60285	CREM (cAMP-REM)	[Y]							3.4	3.6
X95580	hypoxia-inducible factor 1α								2.7	1.8
J04103	ets-2								34	25
X78709	NRF1								-2	-1.9
X74938	hepatocyte nuclear factor-3γ								-3.1	-4.2
J04103	ets-2								34	25
AA008095	GF2I								-2.2	-1.9
U51992	ISGF3γ								-4	-5.1
J03168	interferon regulatory factor-2								-1.9	-3.4
M21065	interferon regulatory factor-1								-2.6	-2.6
Cell cycle control and apoptosis genes										
M64292	tis21(BTG2, PC3)	[Y]		2.8	2.9	7.8	2.5	27.3	33.2	30.5	8.9
AA138777	GADD45γ					16.7	4.3	64.7	5.6	19.7	0.7
X54149	MyD118	[Y]						8	10	66	45
L28177	GADD45α							5	8	27	23
Z72000	BTG3, tob5							1.9	2.1	6.1	2.8
Z14986	s-adenosylmethionine decarboxylase							-2.9	-4.2	-3.8	-3.5
D28492	caspase2 (casp-2)							-5.7	-4.6	--	--
M60523	ID-3 (IDB3; HLH462)							1.6	2.1	--	--
L20450	DNA binding protein							-4	-3	--	--
U10102	BCL-X (long)	[Y]								2.2	2
U09507	p21 (cip1, waf1)	[Y]								3.4	3.4
X58876	mdm2 (p53-associated protein)									1.9	1.8
L16462	BCL2-related prot. A1									4.1	3.3
X75926	abc1									3	3.1
X67644	gly96 (IEX1, IER3)	[Y]								3.6	2.5
U36799	Retinoblastoma-like protein 2									-3.4	-2.4
M90397	bcl-3									44	-2.8
U09416	retinoid X receptor interacting protein									-3.3	-5.4
Extra-cellular matrix/cell structure genes										
M32490	cyr61	[Y]	1.8	6	23.6	17	9.2	9.8	29	11.9
M36120	keratin 19 (CK19, KRT19)		4	-6	--	--	--	--	--	--
W63835	EST ~ α-actin, β-centractin		4	5	1	2	5	5	-0.6	-1.7
M33960	PAI-1	[Y]					104	103	102	89
X99963	rhoB						8	6	11	10
M73696	Glvr-1	[Y]					2.7	2	9.5	3.6
AA059763	tubulin, beta 2						3.5	3.4	2.7	5.1
M70641	FISP-12						11.7	4.1	4.8	2.6
AA088011	Hepsin (HPN)						3.6	3.4	--	--
L00919	protein 4.1						2.4	2.7	--	--
AA161979	kinesin heavy chain 5B (kif5b)						-4	-6	--	--
X81579	IGFBP-1 (IBP-1)	[Y]					6.9	2.3	4.5	2.1
AA002458	EST ~ actin polymerization inhibitor						2.8	2.9	--	--
X12789	cytokeratin 8 (endo A)						2	2.1	4.1	4
X16133	PRG1								2.9	2.2
X14194	entactin, nidogen (NID, ENT)								2.5	2.9
L41154	Transgelin (SM22-a, p27, TAGLN)								2	1.9
Z22532	syndecan-1								9	10
D10024	annexin II (lipocortin II)								2.4	2.1
AA107807	EST ~ β-actin, γ-actin								29.1	50.3
M21495	γ-actin								10	8.8

Acc.#	Gene name (synonyms)	Known in LR	10 min Δ	10 min E/S	30 min Δ	30 min E/S	90 min Δ	90 min E/S	240 min Δ	240 min E/S
Extra-cellular matrix/cell structure genes continued										
J04694	collagen (α-1 type IV)								14	3.2
M11686	cytokeratin 18 (cytokeratin endoB)								3.8	4.2
U16162	prolyl 4-hydroxylase alpha subunit								-6	-4
M68859	dystrophin								-1.7	-1.9
M31131	neural-cadherin								-2.5	-1.9
W18778	EST ~ β-tubulin								6.4	3.3
D87117	SAP102								-6	-4
Membrane associated genes										
M14757	MDR (ABCB1 MDR1)		3	3	--	--	--	--	--	--
M81445	Cx26 (GJB2)						2.1	2.5	--	--
X78874	clcn3 (CLC3)						-2.6	-2.5	--	--
U42467	leptin receptor precursor								13.9	2.8
U05265	gp49B								4.8	4.4
X76654	EAR2								-25	-3
Inflammation related genes										
J04596	GRO	[Y]			2.3	2.3	--	--	2.4	-1.9
M19681	MCP-1	[Y]					3.4	2.5	2.6	2
M34510	CD14	[Y]					14	17	151	106
J00424	IFNb						9.4	8.2	21	8.5
W29669	IFN-related developmental regulator 1 (PC4)						29	9.3	18.7	6.7
X56824	haem oxygenase 1 (HMOX1)								6.7	3.4
Glucose regulation genes										
M23384	glucose transporter 1								4.1	3.9
U00445	glucose-6-phosphatase (G6Pase)	[Y]							2.7	3.1
L41631	hexokinase D, hepatic isozyme								-3.2	-4.3
Signal transduction genes										
U36220	FK506 binding protein 51		1.8	3.9	--	--	--	--	2.1	-2.6
U12142	RVR (NR1D2)		6	5	--	--	--	--	--	--
L36435	MAF1 (KRML)		2	2.1	2	1.8	5	1.5	-2.7	-5.4
AA109491	liver fructose biphosphatase		-32	-19	--	--	--	--	--	--
W85446	PKC inhibitor 1 (HINT, PKCI)		-4	-5	--	--	--	--	--	--
W29562	adipocyte lipid BP				4.1	3.5	--	--	--	--
Z29532	Follistatin	[Y]					5	5	6.7	13.3
X76850	MAPK-activated prot. kinase 2						2.6	2.4	2.6	2
AA153484	SERCA2						12.7	23	5.2	4.9
D42124	MafK, NF-E2 p18 subunit						3.3	7.5	14	5.3
L31783	uridine kinase						-2.6	-2.2	-3.7	-2.7
AA030658	integrin linked kinase						3.8	6	--	--
W16171	Phosphatidylinositol-glycan phospholipase D1						2	2.9	--	--
J02980	alkaline phospatase: tissue non-specific, liver (AKP2)								80	89
X69620	inhibin/activin beta-B								28	16
U50413	phosphoinositide 3-kinase regulatory subunit p85α								12.3	11
M81477	PTP-2								5.9	2.8
U28138	Dvl-1								-2.7	-2.2
U34973	protein tyrosine phosphatase-like mRNA								-8	-4
Angiogenisis										
W13632	EST ~ angiotensinogen precursor						2.2	1.9	3	2.2
U26188	B61 (ephrin A)						4.2	33	--	--
D30782	Epiregulin								8	8
W17473	EST ~ angiotensinogen precursor								2.1	2
AA106347	angiotensinogen precursor								2.6	2
Metabolic and other gene types										
AA117646	sodium/bile acid cotransporter (NTCP)		-6	-7	--	--	--	--	--	--
U37438	CRP-ductin alpha				-21	-7	--	--	--	--
D49744	farnesyltransferase alpha subunit						-2.2	-2.5	-4.2	-4.8
U35886	arylamine n-acetyltransferase 2 (NAT-2)						-5	-4	-4	-2.9
AA162538	monocyte-activating polypeptide						3.7	8.4	--	--
AA166139	XPE UV-damaged DNA binding factor						3.9	3.7	--	--
X71642	GEG-154						-6	-4	--	--
U60987	glycerol-3-phosphate dehydrogenase, mitochondrial						-3.3	-5.8	--	--
X98096	G-rich box-binding protein						-5	-5	--	--
U70210	TR2L						-3	-4	--	--
M29325	L1Md-9						-7.1	-3.8	--	--
M13522	SAA-2								9.8	-0.1
M61737	FSP27								20	12.5

Fold change vs. resected liver specimens (Δ) for differentially expressed genes as well as the fold change vs. sham surgery (E/S) are listed in rows for time points 10, 30, 90, and 240 min. Genes are listed by GenBank accession number (Acc.#) and name and are grouped according to their function. The fold change for gene expression with values greater than 5 are highlighted; yellow corresponds to values >5; green corresponds to values >10; red corresponds to values >50. Genes that previously had been identified in liver regeneration, particularly in the priming stage prior to entry into cell cycle, are indicated with a [Y].

Table 1. (continued)

Acc.#	Gene name (synonyms)	Known in LR [Y]	10 min Δ	10 min E/S	30 min Δ	30 min E/S	90 min Δ	90 min E/S	240 min Δ	240 min E/S
Metabolic and other gene types continued										
M55154	Tissue transglutaminase (TGase, TGC)								3.8	2.8
AA003458	SERCA2								2.1	1.8
U36340	BKLF								3.3	4.4
X61940	dual-specificity protein phosphatase (MAPK phosphatase)								2.3	2.4
X13605	histone H3.3								6.3	2.9
Z46757	high mobility group 2								4	5.8
X64414	low density lipoprotein receptor								2.8	3.1
X14432	thrombomodulin								11.2	5.2
AA028547	Tx01								6.1	7
U44088	TDAG51								3.8	2.5
U27455	serine palmitoyltransferase2								2.6	2.3
M58004	C10 (SCYA6)								22	3.3
L07924	G nucleotide dissociation stimulator-ras-related GTPase								35	30
M59378	TNFR								4	2.6
J05663	Aldose reductase 1								11.7	18
M13685	mPRP								-3	-2.4
D45210	OZF (ZNF146)								-7.7	-3.8
D16333	coproporphyrinogen III oxidase (COX)								-3.1	-3.1
W65178	BMP1 precursor								-7	-4
AA097366	Gilz								-33	-12
U27838	P137								-4.4	-2.5
U29762	D-Box binding protein								3	-31
AA064330	hsp40								-2.2	-2
D49956	8-oxo-dGTPase								-2.3	-1.7
D49429	NCBP-29								-2	-2.8
U33557	folylpolyglutamate synthase								-2.3	-2.5
U41805	T1/ST2 precursor								-19	-25
U37351	PCEE								-1.9	-1.9
U02554	SAA-5 / SAA-4								-2.1	-4.3
X51971	carbonic anhydrase V								-14.5	-8.4
Z21524	Hex								-5.8	-2.4
L12030	SDF-1-β, PBSF								-2.5	-2.1
Metabolic and other gene types continued										
D78641	DGCR2/IDD								-2.5	-2.6
D67015	importin β-1								-3.9	-3.1
D86344	topoisomerase-inhibitor gene / MA-3								-2.5	-1.7
U70674	m-Numb								-7	-6
X76653	apolipoprotein AI regulatory protein-1 (ARP-1)								-2.7	-2.2
U42190	G/T mismatch binding protein								-3	-3
AA106163	NR1I3 orphan nuclear receptor								-2.6	-2.9
U73029	mirf6								-3.5	-2.2
L07037	Acetylglucosaminyl transferase								-52	-32
M94450	GRB7								-35	-23
M32010	Histidine-rich protein K4								-2.2	-1.8
L13593	pseudo-prolactin receptor (PRLR3)								-14	-7
J03482	histone H1.2								-2.3	-2.4
EST genes										
Z54179	EST		-2	-2	--	--	--	--	-4.1	-2.8
AA098418	EST ~ PIM-1						6.7	5.4	4	1.9
W13875	EST ~ myocin RLC-A						2.7	1.9	2.7	2.3
W15802	EST ~ PIM-1						5.7	4.1	3.2	1.9
W10081	EST ~ YPT1						7.9	7.9	8	5.6
AA107455	EST ~ EF-2						2.5	2.5	--	--
AA061461	EST ~ SREBP-1						5.25	3.4	--	--
AA067092	EST ~ SREBP-1						5.1	4.2	--	--
AA170375	EST ~ EIF-5A						2.8	2	--	--
AA098588	EST ~ ZF protein HRX						-4	-2	--	--
AA145127	EST ~ leukocyte elastase inhibitor						-2.7	-2.4	--	--
W16377	EST ~ myosin heavy chain								3.5	4.6
W48402	EST ~ SIR2								-17	-18
AA162205	EST ~ phosphatidylserine decarboxylase proenzyme								-10	-5
AA013581	EST ~ ZNF70 (zinc finger protein)								-10	-15
AA105081	EST ~ IF2								-3.2	-1.7
W08822	EST ~ ZF GLO3								-4.3	-4.4
W97102	EST ~ threonyl tRNA synthetase								-3.1	-2.2

promoter sequence (Genset, La Jolla, CA). *In vitro* transcription using double-stranded cDNA as a template in the presence of biotinylated UTP and CTP was carried out by using an Ambion (Austin, TX) *in vitro* transcription kit. Biotin-labeled cRNA was purified, fragmented, and hybridized to Mu6.5K arrays (Affymetrix, Santa Clara, CA). The arrays were washed and stained with streptavidin-phycoerythrin and then scanned with an Affymetrix GeneChip scanner. Primary image analysis was performed by using the GENECHIP 3.1 software, and images were scaled to an average difference value of 200 as described previously. Hybridizations were performed in duplicate, and only differential expression observed in both replicates was analyzed further. Comparison analyses for data at each time point were calculated by using the t_0 and sham as baselines. Gene-expression profiles were established from RNA samples isolated from four different mice per time point (two PHx and two sham).

Reverse Transcription–PCR, Northern Blots, and RPA. Probes for Northern blotting were amplified from RNA in the 240-min PHx time point by using the following primer pairs: Pip92Fwd (5′-GAGTCTGCAGCTATCCCTCG-3′), Pip92Rev (5′-CACGTTGAGCATATTGTCGG-3′); Tis21Fwd (5′-CCTAGCCAAGGTAAAAGGGG-3′), Tis21Rev (5′-GGTCCTCTCCATCTTAGCC-3′); and glvr-1Fwd (5′-GGTGGGATGTGCAGTTTTCT-3′), glvr-1Rev (5′-CCTTGTGCACGGTGTGATAC-3′). RNA samples were analyzed by electrophoresis and transferred to nitrocellulose membranes. The ^{32}P-labeled cDNA probes along with a ^{32}P-labeled probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) then were used to probe message levels by using established protocols (16). Quantitation of chemokines MCP-1 and GRO as well as *L32* was performed also by RPA exactly as described previously (17, 18).

Results and Discussion

We have examined the gene-expression changes in the liver of mice after PHx over a time course spanning 4 h by using high-density oligonucleotide arrays with probes for ≈6,500 genes. Liver RNA samples were removed at 1, 5, 10, 30, 90, and 240 min after PHx. No reproducible effects were observed at 1 and 5 min after PHx (data not shown). The experiments showed that 185 genes had altered gene expression for at least one of the remaining time points (Table 1). All of these genes showed higher induction or repression in regenerating livers than in sham controls, indicating the importance of including these controls in our analysis. We have excluded genes that were induced or repressed at similar levels in sham and regenerating livers, because these gene-expression changes are likely indicative of the effects of the surgery. It is important also to note that a wide variety of the genes observed to be differentially regulated according to microarray analysis have not been described previously in more limited studies of hepatocyte priming during liver regeneration after PHx.

Transcription Factors. During liver regeneration, liver cells are exposed to stresses associated with functional deficiency, and these stresses ultimately lead to cell proliferation (9, 19, 20). We have identified 19 immediate-early transcription-factor genes that are differentially regulated during the priming phase (Table 1), many of which overlap with previously established immediate-early genes implicated by Taub and coworkers (8, 15) and Fausto and coworkers (3, 10–12) that have established the importance of inflammation and protooncogenes in the early stages of liver regeneration. It is

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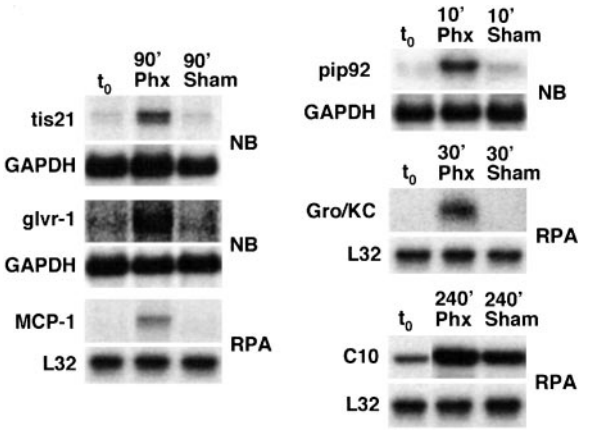

Fig. 1. Induction of *pip92*, *gro/KC*, *tis21*, *glvr-1*, *MCP-1*, and *C10* during the priming phase of liver regeneration. Age-matched C57BL/6 male mice (three mice per group) were subjected to 70% PHx and killed at different points afterward. Total RNA (20 or 10 mg) extracted from their livers were analyzed by Northern blot (NB) and RPA analyses, respectively, for the expression of *pip92*, *gro/KC*, *tis21*, *glvr-1*, *MCP-1*, and *C10*. Results from a representative mouse per group were compared with those obtained either in its own resected liver section (t_0) or in mice that were subjected to sham operation and killed at the same time point. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein light 32 (L32) were used to normalize either the amount of RNA bound to the membrane (NB) or the amount of RNA loaded in each lane (RPA).

well established that liver regeneration involves the posttranslational activation of the transcription factors NF-κB, STAT3, AP-1, and C/EBPβ by mechanisms connected to increased levels of cytokines and reactive oxygen species within the liver (9, 15, 19). Immediate-early genes such as the archetypal immediate-early genes c-*fos*, c-*jun*, and c-*myc* are up-regulated as a consequence (9). Immediate-early transcription factors generated in response to PHx represent a critical step in controlling the proliferation of hepatocytes within a regenerating liver. Cooperatively, they activate the proliferative program within quiescent hepatocytes.

Four immediate-early transcription factors were activated as early as 10 min after PHx: c-*fos*, c-*jun*, *Zif/268*, and *pip92*. Of these, c-*fos* and c-*jun* are primary immediate-early genes expressed by many cell types in response to cellular stress. Zif/268 (EGR-1) contains three zinc fingers recognizing a GC-rich sequence, which has been identified in the promoter regions of a number of genes including *PRL-1*, a mitogenic phosphatase associated with regenerating livers that plays an important role in cell growth in a number of different tissue types (21). The other immediate-early transcription factor in the 10-min time point is PIP92, the induction of which has been confirmed by Northern blot (Fig. 1). PIP92 encodes a short-lived proline-rich protein with no sequence homology to other known proteins. PIP92 is known to be stimulated by cytokines such as fibroblast growth factor and by mitogen-activated protein kinase (MAPK) signaling pathways and has been implicated in processes such as differentiation and stimulation of fibroblasts (22).

We observed high induction of ATF3, also known as liver-regenerating factor (LRF-1) because it is highly expressed in regenerating livers (23). Expression of ATF3 is known to modulate glucose homeostasis and other primary functions of the liver and likely plays a role in altering cell function before cell-cycle entry (24). Ets-2 is another immediate-early transcription factor that was highly induced after PHx. Ets-2, in conjunction with C/EBPα, and C/EBPβ, rapidly increases transcription from the p21 promoter via multiple binding elements within the enhancer region (25). Expression of Ets-2 has direct downstream effects on both cell-cycle progression and MAPK signaling.

We also observed that the gene encoding the peroxisome proliferator-activated receptor α (PPARα) was down-regulated during liver regeneration. PPAR genes mediate the proliferation of peroxisomes, which are organelles that participate in many primary functions of the liver including lipid metabolism, catabolism of purines, polyamines, and amino acids, H_2O_2-based respiration, cholesterol synthesis, and the production of bile acids (26). Down-regulation of PPAR linked to an immediate-early response to functional deficiency may be a natural response to the increase in H_2O_2 concentration in the remaining hepatocytes after PHx. Retinoic acid, a natural ligand of the retinoid X receptor (RXR), has been implicated as an important cytokine in regenerating livers (27). Consistent with a functional role of retinoic acid in liver regeneration, we observe the induction of Stra14 (stimulated by retinoic acid 14), the role of which in liver regeneration is unknown.

We also detected transient expression of other transcription factors such as hepatocyte nuclear factor 3(HNF-3)β and -γ, which are known to activate liver-specific genes such as albumin, and influence expression of genes involved in bile acid and glucose homeostasis (28, 29). HNF-3 isoforms mediate the hepatocyte-specific transcription of numerous genes important for liver function, and homozygous knockout mice do not survive embryonic development (28, 29). Gene expression of HNF-3 isoforms are reduced in the liver after injury by CCl_4 (30), a model system for studying liver regeneration, suggesting an important role for these transcription factors in response to damage and to controlling the differentiation state and proliferation of hepatocytes.

By 240 min, we observed transcription factors associated with the beginnings of tissue remodeling. One example is the hypoxia-induced factor (*HIF1α*), which is an angiogenic gene expressed during oxygen starvation (hypoxia) that promotes vascular growth (31). At this time point, three IFN-inducible transcription factors also were induced: IFN-stimulated gene factor-3 (ISGF-3) and IFN regulatory factors-1 and -2 (IRF-1 and IRF-2). ISGF-3 and IRF-1 have been identified as transcriptional activators of IFN-β signaling, whereas IRF-2 is thought to act as a repressor of such activity (32). Given that IFN-β was induced by 90 min after PHx and this cytokine has been involved with cell-growth suppression (33, 34), these results suggest that ISGF-3, IRF-1, and IRF-2 may differentially modulate regeneration. Although many of the immediate-early transcription factors induced after PHx have proliferative functions, several genes involved in regulation and arrest of the cell cycle were also induced. This apparent dichotomy must be linked with the precise control of cell-cycle entry and progression.

Cell-Cycle Genes. During hepatocyte priming in mice, we observed genes related to the cell cycle up-regulated as early as 10 min after PHx (Table 1). Consistent with the expression of both pro- and antiproliferative transcription factors, we observe the differential regulation of genes that stimulate and inhibit cell-cycle entry. Overall, we detected the differential regulation of 19 cell-cycle control genes during the time course of hepatocyte priming, and many are detected far earlier than reported previously. The majority of the genes are checkpoint genes at major cell-cycle transitions that can act to inhibit the cell cycle.

Three cell-cycle checkpoint genes induced during liver regeneration were *GADD45*, *TIS21*, and *p21*, which act at different cell-cycle transitions (Fig. 2). The levels of TIS21 mRNA, a p53-dependent growth arrest gene that inhibits the G_1/S transition (35), increased by 10 min and reached maximum levels by 90–240 min after PHx (Table 1) as also confirmed by Northern blot (Fig. 1). Immediate-early transcription factors regulate the expression of the cell-cycle checkpoints (25), thus implying that autonomous control of cell-cycle entry by the hepatocytes begins almost immediately. In addition, we observe the up-regulation of cell-cycle genes that are apoptosis inhibitors such as Bcl-2, Bcl-X, and GADD45, previously implicated in hepatocyte priming (3).

Signal Transduction. We observed differential regulation of several genes related to signal transduction. For example, we observe the

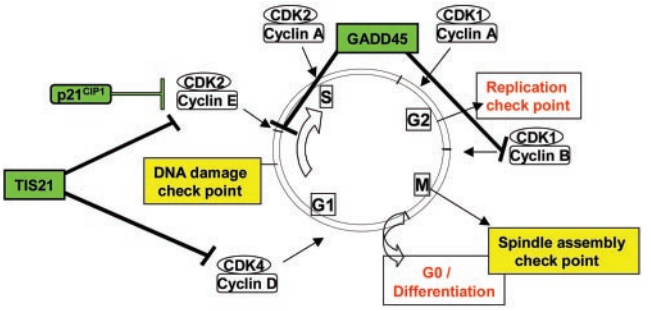

Fig. 2. The influence of observed genes on cell cycle.

differential regulation of genes related to the activation of MAPK and related pathways. MAPK signaling is mediated by increased expression of the cyclin-dependent kinase inhibitor protein p21, which is induced by Ets-2 and C/EBP (25), all of which are observed in our gene-expression profile. MAPK activation has been linked to interleukin-6 (IL-6) pathways (8) and is an essential component of the genetic program that leads quiescent hepatocytes into a proliferative state. Our results with regards to MAPK-related pathways are consistent with the findings of Taub and coworkers, which are discussed in detail elsewhere (8).

It is noteworthy also that follistatin and inhibin, inhibitors of activin A, have proliferative properties and were up-regulated during hepatocyte priming. Activin A is a member of the transforming growth factor-β superfamily and is known to be involved in kidney organogenesis and development (36). Activin A can block the activity of the key inflammatory cytokine IL-6, which is known to be critical in liver regeneration (37). Up-regulation of inhibitors of activin A therefore are likely to play a role in hepatocyte priming by enhancing the effects of IL-6, which leads to proliferation.

Extracellular Matrix/Cell Structure- and Membrane-Associated Genes. It is well established that modification of the extracellular matrix is integrally linked with liver regeneration (3). We have identified 28 differentially regulated genes that are associated with cell structure and extracellular matrix modification. Cyr61, an angiogenic extracellular matrix modifier (38, 39), appeared as early as 10 min after PHx and was overexpressed consistently during hepatocyte priming. Fisp12 (CTGF), another angiogenic extracellular matrix modifier that usually is observed in conjunction with Cyr61 (40), appeared only at the 90-min time point onward. The role of these two genes in liver regeneration has not been established previously, although they seem to be critical to the formation of new blood vessels and critical to proliferation in certain cancers (41). Both Cyr61 and Fisp12 are involved in wound healing and can stimulate chemotaxis and promote proliferation in endothelial cells and fibroblasts in culture and induce neovascularization *in vivo* (42). They also promote adhesive signaling responses that lead to sustained activation of p42/p44 MAPKs and prolonged gene-expression changes including up-regulation of MMP-1 (collagenase-l) and MMP-3 (stromelysin-l) mRNAs (42). The remainder of the genes began to be differentially expressed 90 min after PHx, suggesting that there is a lag between the initial response to PHx and extracellular modification. This was confirmed also by the fact that little or no induction of matrix metalloproteinases (MMP, proteinases known to play a role in tissue remodeling; ref. 43) was detected by microarray (Table 1) up to the 240-min time point.

It also is worth mentioning that plasminogen activator inhibitor 1 (PAI-1), a serine protease inhibitor that specifically inhibits plasmin activation (44) and liver regeneration, was very highly expressed from 90 min after PHx onward. PAI-1 specifically inhibits liver regeneration (3, 4) by forming a complex with the urokinase-type plasminogen activator (uPA) and contributes to the inactivation of HGF. HGF is a known stimulus of liver regeneration after priming (3, 4).

We also observed induction of RhoB, which regulates signal transduction from plasma membrane receptors (45, 46). RhoB is known also to regulate DNA synthesis and is expressed as a result of genotoxic stress (47). A number of other genes associated with cell adhesion and migration appear later in the priming phase (Table 1). Related to genes that are involved with cell adhesion and migration are those that are involved with cell–cell communication. Connexins comprise a class of cell membrane proteins that allow passive transport of small molecules between networked cells in tissues (48). Connexin 26, which shows antiproliferative behavior when overexpressed in human hepatoma cells (49), appeared up-regulated during liver regeneration. We also observed *clcn3* down-regulated during the priming phase of hepatocyte proliferation. This gene is a voltage-gated chloride channel for regulation of cell volume (50). Expression of both pro- and antiproliferative extracellular matrix-modifying genes, exemplified by Cyr61 and Fisp12 compared with plasminogen activator inhibitor 1 and RhoB, is consistent with observations of expression patterns of transcription factors and cell-cycle genes and again suggestive of tight control of cell-cycle entry during hepatocyte priming.

Inflammatory Responses. The pioneering work by Taub and coworkers regarding the role of IL-6 in liver regeneration in mice establishes the importance of inflammatory cytokines during hepatocyte priming (8). Inflammatory responses have been implicated in the priming of liver and other types of regeneration (51). For example, prevention of macrophage invasion impairs peripheral axonal regeneration, whereas implantation of macrophages into central nervous system nerves allows them to regenerate after axonal crush (51). Inflammation also is implicated in secondary degeneration after spinal cord injury (51). Inflammatory responses can be triggered by cytokines such as those of the TNF family, which activate immediate-early genes such as AP-1 (52). With the exception of IFN-β that, as mentioned before, was detected 90 min after PHx, other cytokines such as IL-6, IL-2, IL-3, IL-4, IL-5, TNF-α, TNF-β, and IFN-γ were not found in our samples, indicating that if induction of these genes occurred, it was below our detection limit. However, the messages for the chemoattractants MCP-1 and GRO were increased in the regenerating liver by 30 and 90 min after PHx, respectively. These chemokines can recruit monocytes/macrophages, which have the potential to exert both stimulatory and inhibitory influences on hepatocyte proliferation (53), in the liver after PHx (54). MCP-1 and GRO also may play a role in angiogenesis and tissue remodeling (55).

Other inflammatory genes included *CD14*, the lipopolysaccharide receptor, being highly induced after PHx, consistent with previous observations by Taub and coworkers (8), and the gibbon ape leukemia virus cell-surface receptor (*glvr-1*) that is involved in sodium/phosphate cotransport and induced during inflammatory responses (56). Up-regulation of the latter gene indicates an increase in the intracellular import of inorganic phosphate, which is required for activation of signaling pathways involving phosphorylation and nucleotide triphosphate synthesis. Northern blot analysis confirmed the increase in mRNA for the *glvr-1* gene in the regenerating liver (Fig. 1).

Glucose- and Metabolism-Related Genes. The liver plays an important role in maintaining metabolic and biosynthetic homeostasis. Glucose homeostasis is an important liver function and involves glycogenolysis, which breaks down glycogen into glucose, and gluconeogenesis, which involves synthesis of glucose from noncarbohydrate precursors (57). In a regenerating liver the majority of homeostatic responses involving metabolic functions occur after the priming phase of hepatocytes (15). In addition to previously mentioned genes *ATF3* and *PPAR*α, we observed the up-regulation of phosphoenol-pyruvate carboxykinase (*PEPCK*) and glucose-6-

phosphatase (*G6Pase*), which are known to be involved in maintaining glucose levels after the acute loss of liver mass posthepatectomy (15, 57). *G6Pase* is involved in glycogenolysis and associated with the endoplasmic reticulum (ER), where it hydrolyses the G6P into glucose and phosphate. Misregulation of this gene has been implicated in type 1 glycogen storage diseases (58). *PEPCK* is a gene involved in the synthesis of glucose from noncarbohydrate precursors. In addition, we observed up-regulation of the insulin-like growth factor-binding protein (IGFBP-1), which is induced by IL-6 and HGF, and is known to be up-regulated during the course of liver regeneration (59). *IGFBP-1* shares common promoter elements with other hepatic genes associated with the maintenance of metabolic homeostasis following large functional deficiency after PHx such as *G6Pase* (20).

Heme oxygenase 1 (HMOX1) is a protein that catalyzes the oxygen-dependent degradation of heme to biliverdin, free iron, and carbon monoxide. Of the two isoforms, the inducible HMOX1 primarily functions in the liver and the spleen. The increased expression of HMOX1 during liver regeneration is likely a reflection of the increased metabolic workload of the remaining hepatocytes after PHx and also may mediate oxidative stress by modulating iron levels and indirectly participate in antiproliferative proapoptotic pathways that control cell-cycle entry (60).

Uncharacterized Genes. We have identified a number of genes corresponding to expressed sequence tags that were differentially expressed after PHx in mice. The molecular function can be inferred for these genes based on sequence homology. For example, the genes AA107455 and *AA105081* appear to have high homology to elongation factor-binding proteins. The gene *AA145127* appears homologous to a serine protease inhibitor, and W08822 is homologous to a putative GTPase-activating protein for Arf. However, sequence homology does not reveal the cellular or physiological role of these proteins, and their specific roles in liver regeneration are unclear.

Conclusions. We have shown that the genome-wide expression profile of hepatocyte priming after PHx in mice is complex and covers different classes of proteins including transcription factors, metabolic enzymes, proteins associated with stress and inflammatory responses, and those involved in cytoskeletal and extracellular matrix modification. We have adapted a diagram originally produced by Fausto (3) to summarize these results (Fig. 3, which is published as supporting information on the PNAS web site, www.pnas.org). These genes are likely to have a broad effect on the liver. Along with the concept of hepatocyte priming (3, 10–12), it is worth mentioning that all these changes occurred well before DNA synthesis, suggesting that the transcriptional control of liver regeneration involves early and diverse cellular responses. It also was somewhat surprising to find that the expression levels of genes associated with the cell cycle over the time course of 4 h indicate that antiproliferative genes are favored during hepatocyte priming. These potential antiproliferative responses are in keeping with the concept of an autonomous control of cell-cycle entry by the hepatocytes and suggest that tight regulation of liver cell proliferation originates very early after a regenerative stimulus. Future experiments to determine which of the changes during hepatocyte priming are primary-cause factors in tissue regeneration may provide new insights into regenerative processes in mammals and potentially may lead to new approaches to the development of therapeutic agents for the treatment of liver diseases.

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