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The Immediate-Early Growth Response in Regenerating Liver and Insulin-Stimulated H-35 Cells: Comparison with Serum-Stimulated 3T3 Cells and Identification of 41 Novel Immediate-Early Genes

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Liver regeneration provides a unique system for analysis of mitogenesis in intact, fully developed animals. Cellular immediate-early genes likely play an important role in cell cycle regulation and have been extensively studied in mitogen-stimulated fibroblasts and lymphocytes but not in liver. We have begun to characterize the immediate-early growth response genes of mitogen-stimulated liver cells, specifically, regenerating liver and insulin-stimulated Reuber H-35 hepatoma cells, and to address differences in growth response between different cell types. Through subtraction and differential screening of cDNA libraries from regenerating liver and insulin-treated H-35 cells, we have extensively characterized 341 differentially expressed clones and identified 52 immediate-early genes. These genes have been partially sequenced and subjected to Northern (RNA) blot analysis, and 41 appear to be novel. Surprisingly, two-thirds of these genes are also expressed in BALB/c 3T3 cells, but only 10 were identified in previous studies of 3T3 cells, and of these, 6 include well-known genes like *jun* and *fos*, and only 4 are novel. Approximately one-third of the immediate-early genes identified in mitogen-stimulated liver cells or serum-stimulated NIH 3T3 cells are expressed in a tissue-specific fashion, indicating that cell type-specific regulation of the proliferative response occurs during the immediate-early period. Our findings indicate that the immediate-early response is unusually complex for the first step in a regulatory cascade, suggesting that multiple pathways must be activated. The abundance of immediate-early genes and the highly varied pattern of their expression in different cell types suggest that the tissue specificity of the proliferative response arises from the particular set of these genes expressed in a given tissue.

The liver constitutes one of the few normally quiescent tissues in an adult body that have the capacity to regenerate (16, 23, 42). As a result, it provides a unique, multicellular, physiologically normal system in which to study the mitogenic response of epithelial cells. In rats, following 70% hepatectomy, the cells in the remaining, intact, liver lobes rapidly resume proliferation (23). They initiate the first round of DNA synthesis within 12 to 16 h postsurgery and continue to traverse the cell cycle until the liver regains its initial mass in about 10 days, whereupon the cells again become quiescent (23, 42). Multiple factors, including circulating hormones, growth factors, and nervous input, participate in the regulation of this response (13, 16, 42, 46, 59), but the actual mechanism remains incompletely understood.

While the liver constitutes an excellent system in which to study mitogenesis, the use of an intact animal model limits experimental manipulation. The minimal-deviation Reuber H-35 hepatoma cell line (52) provides an alternative system in which to examine aspects of liver cell proliferation not readily addressed with intact animals. In a response mediated by the insulin receptor, physiologic concentrations of insulin alone can stimulate serum-starved, quiescent H-35 cells to resume proliferation (29). While this characteristic differs from normal hepatocyte physiology, it permits examination of the response to a single mitogen.

Immediate-early growth response genes, which participate in the transition from quiescence into the G1 phase of the cell cycle (31, 32), have been identified primarily in mitogen-treated fibroblasts (2, 31, 32) and lymphocytes (65). These genes are characterized by their transcriptional activation and, often, superinduction in the presence of a cycloheximide-induced protein synthesis blockade (2, 31). Immediate-early genes fall into three known categories (2, 27): transcription factors like the Jun (4, 24, 45, 54, 55), Fos (33, 40, 44, 45, 50), Krox-Egr-Zif-nerve growth factor I (10, 35, 36, 43, 60), and Nur77-steroid hormone receptor (22, 57) families; secreted proteins that may have autocrine or paracrine functions, like JE (28, 53) and KC (48, 56); and structural proteins, like actin (2, 32, 58). Although the mechanisms are not understood, these proteins are important in regulating the subsequent phases of G1 and driving the cells through the cell cycle. In regenerating liver, immediate-early gene expression begins as early as 15 min posthepatectomy with c-*fos* induction (30) and includes other genes, such as c-*myc* (19), *ets-2*, and c-*myb* (3). Similar gene expression can be observed after insulin stimulation of H-35 cells (61).

Increased expression of immediate-early genes appears to be due to signals mediated by mitogens that cause modifications of preexisting cellular proteins and transcription factors (2, 32). Therefore, the intracellular milieu before mitogenic stimulation plays an important role in shaping the immediate-early response. It seems likely that this milieu and the genes expressed vary with the cell type. Unlike fibroblasts (2) and lymphocytes (65), epithelial cells have not been used in extensive studies of immediate-early growth response genes. These cells differ morphologically and functionally from the previously studied cells and are more

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commonly involved in tumorigenesis than are other cell types (17).

To understand the proliferative response in epithelial cells in general and liver cells in particular, we have begun identifying and characterizing immediate-early genes which are unique to proliferating epithelial cells through differential-screening and subtraction analyses of cDNA libraries from the two systems described above. We report here the identification of 41 nonoverlapping sequences believed to represent novel immediate-early genes. We also report that the expression characteristics of these and other known immediate-early growth response genes differ significantly among regenerating liver, insulin-stimulated H-35 cells, and serum-stimulated 3T3 cells.

MATERIALS AND METHODS

Cell culture. H-35 cells were grown in Dulbecco modified Eagle medium (low glucose; GIBCO) supplemented with 5% fetal bovine serum (FBS; GIBCO), 5% calf serum (GIBCO), 2 mM L-glutamine (Flow), and 100 U of penicillin per ml–50 U of streptomycin per ml (P-S; Flow) as previously reported (29). To produce quiescence, the medium was changed to serum-free Dulbecco modified Eagle medium for 72 h, at which time the cells were between 50 and 80% confluent. Following serum deprivation, cells were treated for the indicated periods with insulin (10^{-8} M; Sigma), serum (20% FBS), and cycloheximide (10 μg/ml; Sigma). BALB/c 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% FBS, L-glutamine, and P-S. Medium was changed to Dulbecco modified Eagle medium plus 0.5% FBS, L-glutamine, and P-S for 48 h to produce quiescence, and thereafter, cells were treated with various agents as described above and in the figure legends. Cells were harvested by scraping in 4 M guanidine thiocyanate buffer and stored at −70°C (14). Samples were homogenized (Polytron; Brinkmann) before RNA preparation as described below.

Rat tissue preparation. For regenerating liver, female Fischer rats (160 to 200 g; Bantin-Kingman) were ether anesthetized and subjected to midventral laparotomy with approximately 70% liver resection (left lateral and median lobes) (23). For cycloheximide-treated samples, rats were treated with 50 mg of cycloheximide per kg of body weight (5% solution in phosphate-buffered saline) intraperitoneally. When cycloheximide treatment was coupled with partial hepatectomy, the rats were injected with cycloheximide 15 min before surgery. Sham-operated rats were subjected to midventral laparotomy with minimal trauma to the liver. Animals were allowed to recover for the times indicated in the figure legends before decapitation and isolation of the remaining liver lobes. Tissue was immediately homogenized in 4 M guanidine thiocyanate buffer (14) and stored at −70°C.

RNA and blot preparation. Frozen homogenates were thawed, layered over a 5.6 M CsCl–25 mM sodium acetate cushion, and centrifuged at 175,000 × *g* for 18 h (14). RNA was suspended in H_2O, phenol-$CHCl_3$ extracted, ethanol-sodium acetate precipitated, suspended in H_2O, and quantified by optical density at 260 nm. For Northern (RNA) blots, 10 μg of heat-denatured total RNA per lane was separated by electrophoresis in a 1% agarose–0.6% formaldehyde–morpholinepropanesulfonic acid (MOPS) denaturing gel and transferred to Optibind (Schleicher & Schuell) supported nitrocellulose. DNA dot blots were prepared with 2 μg of recombinant plasmid DNA per dot prepared as recommended by Schleicher & Schuell, filtered through a Minifold apparatus, and immobilized on Optibind nitrocellulose. The DNA dot blots in Fig. 3 were prepared as described previously (2).

Probes and hybridization. Recombinant plasmids or isolated cDNA inserts were labeled by incorporation of [α-^{32}P]dCTP (New England Nuclear) by nick translation (Bethesda Research Laboratories Nick Translation Reagent Kit). [α-^{32}P]dCTP-labeled first-strand cDNA probes were synthesized by using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and the Bethesda Research Laboratories cDNA Synthesis Kit. Hybridization buffer consisted of 10% dextran sulfate, 40% formamide, 0.6 M NaCl–0.06 M sodium citrate, 7 mM Tris [pH 7.6], 0.8× Denhardt solution, and 0.002% heat-denatured, sonicated salmon sperm DNA for nick-translated probes and 50% formamide, 0.75 M NaCl–0.075 M sodium citrate, 0.5% sodium dodecyl sulfate (SDS), and 5× Denhardt solution (2) for cDNA probes. Blots were hybridized at 42°C for 16 h (nick translated) or 40 h (cDNA) and washed twice for 10 min each time at 60°C in 0.015 M NaCl–0.0015 M sodium citrate–0.1% SDS (nick translated) or 0.015 M NaCl–0.0015 M sodium citrate (cDNA) before exposure to film (Kodak X-OMAT AR5).

cDNA library construction and screening. The insulin-treated H-35 cell cDNA library was prepared for us by Invitrogen from RNA isolated as described above from serum-starved H-35 cells stimulated for 3 h with insulin (10^{-8} M) plus cycloheximide (10 μg/ml) and poly(A)$^+$ selected by passage over an oligo(dT) column (Collaborative Research). The cDNA inserts were cloned into the *Eco*RI site of lambda gt10 by using *Not-Eco*RI linkers. Another library was made by using the Invitrogen Lambda Librarian Kit and poly(A)$^+$ RNA isolated from regenerating livers 3 h after pretreatment with cycloheximide (50 mg/kg) and partial (70%) hepatectomy. cDNA inserts from the regenerating liver library were ligated into the multicloning site in Lambda Zap II (Stratagene) by using *Not-Eco*RI linkers (Invitrogen).

The libraries were plated on lawns of susceptible bacterial hosts at a density of about 3,000 plaques per 150-mm-diameter petri dish, and duplicate lifts (Biotrans; 1.2-μm pore size; ICN) were made. Lifts were hybridized with cDNA probes from either quiescent or mitogen-cycloheximide-treated RNAs from the appropriate cell type or with a cDNA probe which was subtraction enriched for differentially expressed inserts by using the Invitrogen Subtractor Kit and a quiescent rat liver cDNA library purchased from Stratagene. Following hybridization, these lifts were washed, autoradiographed, and compared to identify plaques expressed more abundantly in mitogen-treated cells. Differentially expressed plaques were secondarily screened and plaque purified by low-density plating, duplicate lifts, and differential hybridization with cDNA probes as before. cDNA inserts from recombinant bacteriophage of interest were subcloned (H-35 library) into pGEM-4Z (Promega) or excised in vivo (regenerating liver library) to produce pBluescript clones and nick translated for initial analysis (hybridization to a panel of all recombinant phage clones of interest and to Northern blots containing mitogen-stimulated and quiescent RNAs from H-35 cells, regenerating liver, and BALB/c 3T3 cells). Unique differentially expressed clones were preliminarily sequenced (approximately 300 bp at each end) by the Sanger method and T-3, T-7, and SP6 primers (Promega), and the sequences were compared with other reported sequences by using IntelliGenetics software and data bases (release 5.37).

TABLE 1. Identification of at least 52 immediate-early genes on the basis of differential and subtraction-enriched probe screening of 210,000 primary plaques from two liver cell cDNA libraries[a]

Library	No. of recombinant plaques screened[b]	No. of differentially expressed clones[c]	No. of clones examined[d]	No. of nonoverlapping sequences[e]	No. of novel clones[f]
Insulin-stimulated H-35 cells	150,000	908	169	31	25
Regenerating liver	20,000	106	106	14	11
Subtraction-probed regenerating liver	40,000	107	66	7	5

[a] The number of clones identified in each of the different libraries is presented.
[b] Approximate number of insert-bearing phage plaques differentially screened.
[c] Number of clones which hybridized more strongly to the mitogen-cycloheximide probe than to the unstimulated probe.
[d] Number of clones examined through cross-hybridization, Northern blot analysis, and partial sequence analysis.
[e] Number of clones or groups of clones which did not cross-hybridize with any of the other clones examined.
[f] Number of clones with no homology to sequences in the IntelliGenetics data bases (Release 5.37) or not previously described as immediate-early (IE) genes.

RESULTS

Identification and characterization of 52 immediate-early genes in liver cells. To identify liver-specific immediate-early genes, we constructed two different cDNA libraries: one from Reuber H-35 cells treated for 3 h with insulin-cycloheximide and one from regenerating liver 3 h after partial hepatectomy in the presence of cycloheximide. These libraries were differentially screened by using cDNA probes from quiescent and mitogen-cycloheximide-stimulated cells. In addition, 40,000 clones from the regenerating liver library were screened with a subtraction-enriched cDNA probe. We screened a total of approximately 210,000 primary plaques and identified 1,121 differentially expressed clones (Table 1). Of these clones, 341 were compared through cross-hybridization and this resulted in identification of 52 nonoverlapping clone families. Northern blot analysis confirmed that these were, in fact, immediate-early genes because their mRNA abundance increased following mitogen stimulation either with or without a cycloheximide-induced protein synthesis blockade. We sequenced approximately 600 bp from most of these genes and compared them with the sequences in the IntelliGenetics data bases. Forty-one of these genes do not share significant homology with any of the sequences on record or have not previously been associated with the growth response (gene 33 and insulinlike growth factor-binding protein 1) and are therefore considered novel.

To characterize the tissue specificity of expression of these genes, we hybridized radiolabeled recombinant plasmids representing the 52 differentially expressed genes to Northern blots from regenerating liver, insulin-stimulated H-35 cells, and serum-stimulated BALB/c 3T3 fibroblasts. In light of the morphologic and functional differences between fibroblasts and hepatocytes, immediate-early genes shared by these two cell types are likely to participate in the mitogenic response of many different cell types. Therefore, clones which were expressed in fibroblasts and at least one of the liver cell lines were assumed to be expressed in multiple cell types while those which lacked expression in BALB/c 3T3 cells were considered to be, to some extent, liver specific in their expression.

The clones were grouped by patterns of expression in the various cell types studied. Figure 1 presents a classification flowchart of the various groups and should be used as a key for interpretation of the subsequent figures. While this subdivision is an oversimplification due to the spectrum of variation within a group, it creates groups of genes which presumably share some regulatory and, perhaps, functional features. Tables 2 and 3 present comprehensive lists of the genes within each group and summaries of the results of the initial analysis of these genes, including clone and mRNA sizes; relative intensities of expression in mitogen-cycloheximide-stimulated H-35 cells, regenerating liver, and BALB/c 3T3 cells; and homology to previously identified genes. Figure 2 illustrates the results of Northern blot analyses for typical genes in many of the different groups.

Expression of many immediate-early genes in multiple cell types. During proliferation, cellular components must be duplicated and segregated into daughter cells. Therefore, it seems reasonable that most of the genes involved in proliferation would be expressed in many or all proliferating tissues. Consistent with this hypothesis, most (37 [72%] of

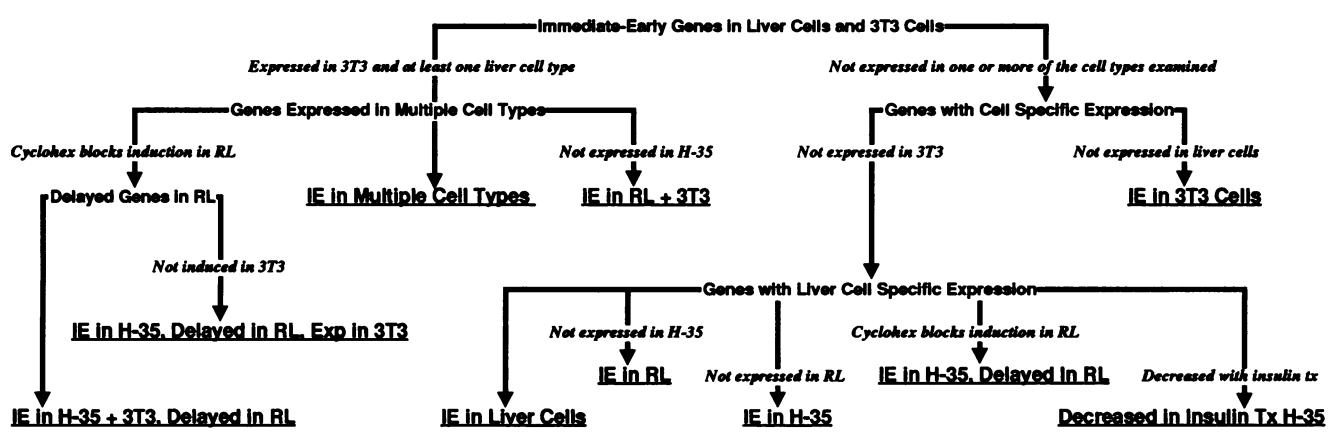

FIG. 1. Grouping of immediate-early genes on the basis of expression characteristics. This flow chart indicates the categories and subcategories into which the immediate-early genes were divided in this study. The italicized comments indicate the distinguishing characteristics of each group. Abbreviations: IE, immediate-early gene; Tx, treated; Exp, expressed; RL, regenerating liver.

TABLE 2. Genes expressed in multiple cell types[a]

IE gene pattern and clone[b]	Gene product[c]	Size of cL/RNA (kbp)[d]	Summary of Northern blot and dot blot analyses[e]							
			H-35		RL				3T3	
			SF	IC	Q	HC	C	E	SD	SC
In multiple cell types										
CL-2	Histone H-3	1.8/1.8 + 1.0	++	+++	±	+++		I	−	+
CL-19*	Actin	2.0/2.0	+	++	±	+		I	++	++++
RL-33	Ubiquitin	1.5/2.5 + 2.7	++++	++++	++	++++	=	I	++++	++++
CL-154*	KR24/Egr-1	2.2/3.3	−	+	−	+	=	I	−	+++
CL-Fos1*	c-Fos	1.7/2.2	−	±	−	+		I	−	++
RL-25*	c-Jun	0.6/2.7 + 3.0	−	+++	−	++	<	I	−	+
SL-290*	Jun-B	0.7/2.1	−	++	−	+		I	−	±
RL-37*	Gene 33	2.0/3.2	−	+	±	>+	<	I	−	++
RL-87*	LRF	2.1/2.1	−	>+	−	±	=	I	−	±
CL-4	?	0.9/2.0	±	++	−	+	=	I	−	+
CL-6	?	1.8/3.0 + 5.5	+	+++	±	++	<	I	−	++
CL-14	?	1.7/1.7	±	>++	±	++	=	I	−	<++
CL-20	?	1.0/2.3 + 4.0	+	>++	±	+	<	I	+	>+
CL-36	?	1.4/4.0	±	>+	−	+	<	I	±	>±
CL-38	?	0.35/3.4 + 5.0	−	±	−	±		I		
CL-48	?	1.4/1.4	++	+++	±	+		I	−	±
CL-73	?	1.6/3.2	−	+	−	±		I		
CL-87	?	1.2/1.7	+	++	±	>±	=	I	±	>±
CL-97	?	1.5/2.7	−	++	−	±	<	I	−	+
CL-183	?	1.7/2.2 +3.5	+	>++	+	++	<	I	±	++
CL-211	?	1.7/4.4	±	+	±	+		I		
RL-14	?	1.0/1.6	±	++	±	+++	<	I	−	++
RL-27		2.0/3.0	++	>++	±	++	=	I		
RL-30*	?	1.7/2.1	−	±	−	+	=	I	−	++
RL-98*	?	1.5/4.7	±	+	−	+	=	I	±	++
SL-314	?	1.0/3.0 +3.3	++++	++++	±	++++		I	−	+++
SL-339	?	1.0/1.4	+	++	±	+		I	−	+
SL-371		1.2/2.1	±	++	−	++		I	±	++++
In RL and 3T3, absent in H-35										
RL-53	?	1.6/3.9	−	−	−	+++	<	I	−	+
SL-353	?	2.7/3.0	−	−	−	+++		I	+	+++
In H-35 and 3T3, delayed in RL										
CL-68	MHC Class 1	1.4/1.8	−	±	±	+	=	D	−	+
CL-184*	Tropomyosin	1.6/1.6	±	+	±	±	<	D	−	++
CL-61	?	1.3/1.7 + 4.7	±	++	±	±	<	D	−	±
CL-141	?	1.2/1.3	±	++	±	±	<	D	−	+
CL-182	?	1.9/2.4, 3 + 3.8	++	++++	±	±	<	D	−	+
In H-35 cells, delayed in RL, expressed in 3T3										
CL-22	?	1.0/2.3 + 4.5	++	+++	−	−	<	D	±	±
CL-31	?	1.2/1.6	+	>+	−	−	<	D	+	+

[a] The 52 immediate-early genes identified in regenerating liver and insulin-stimulated H-35 cells were not expressed equally in all cell types. The clones are grouped on the basis of predominant patterns of expression, as in Fig. 1.

[b] The prefix designates the origin as follows: CL, H-35 cell library; RL, regenerating liver library; SL, subtraction-enriched probing of the regenerating liver library. *, clones also identified by Almendral et al. (2) as immediate-early genes in serum-stimulated NIH 3T3 cells.

[c] Clones were partially sequenced (approximately 600 bp) and checked for homology against the available data banks from IntelliGenetics, Inc. (release 5.37). ?, no significant homology to reported sequences. KR24, Krox-24; LRF, liver regeneration factor; MHC class 1, novel major histocompatibility complex class 1 allele or gene; P, protein. Size cl/RNA: Size of cDNA insert-size of mRNA detected on Northern blot analysis in kilobase pairs.

[e] Relative strength of hybridized signal is expressed as follows: −, no signal; ±, barely detectable signal; + to ++++, increasing relative signal strength. For Reuber H-35 hepatoma cells (H-35): SF, serum-free medium, 3 days with no serum; IC, treated for 3 h with insulin (10^{-8} M) and cycloheximide (10 μg/ml). For regenerating liver (RL): Q, quiescent, untreated liver; HC, 3 h after 70% hepatectomy with intraperitoneal cycloheximide (50 mg/kg of body weight) pretreatment; C, 3 h after intraperitoneal treatment with cycloheximide (50 mg/kg of body weight) (<, signal strength less than HC; =, signal strength equal to HC); E, expression pattern in regenerating liver; I, immediate-early gene; D, delayed gene. For BALB/c 3T3 cells (3T3): SD, serum deprived, 48 h in 0.5% FBS; SC, treated for 3 h with 20% FBS plus cycloheximide (10 μg/ml).

TABLE 3. Genes with liver-specific expression[a]

IE gene pattern and clone	Gene product	Size of clone/RNA (kbp)	Summary of Northern blot and dot blot analyses							
			H-35		RL				3T3	
			SF	IC	Q	HC	C	E	SD	SC
In liver cells										
CL-79	Albumin	2.5	±	>+	+	>+		I	−	−
RL-9	Viral envelope protein	0.5/7.0	±	+	±	+	<	I	−	−
CL-34	?	1.7/1.7 +1.3	±	+	±	+		I	−	−
CL-58	?	0.8/2.5 ± 4.7	±	+	±	+	=	I	−	−
CL-142	?	1.0/2.4	±	+	±	±		I	−	−
CL-180	?	2.0/2.0 + 5.0	±	++	−	++		I	−	−
RL-104	?	1.0/1.7	±	+	−	+	<	I	−	−
In H-35, delayed in RL										
CL-39	?	2.5/2.0 + 3.2	−	+	−	−			−	−
CL-131	?	0.7/2.0	±	+	−	−			−	−
In RL, decreased in insulin-treated H-35										
RL-15	IGF-binding protein[b]	1.5/1.8	+	±	±	+++	<	I	−	−
SL-351	PEPCK[c]	1.0/2.5	+	±	±	++		I	−	−
In RL										
RL-5	IP-10	0.8/1.3	−	−	−	+++	<	I	−	−
SL-332	?	1.5/3.4	±	±	−	++		I	−	−
RL-1	?	1.0/2.6	−	−	±	+++	<	I	−	−
In H-35										
CL-35	?	1.5/4.1	−	±	−	−			−	−

[a] For an explanation, see the footnotes to Table 2.
[b] IGF, insulinlike growth factor.
[c] PEPCK, phosphoenolpyruvate carboxykinase.

52) of the immediate-early genes identified in regenerating liver or insulin-stimulated H-35 cells are expressed in multiple cell types (Tables 2 and 3). Some genes within this group encode structural proteins, like actin and tropomyosin (1), but others encode known activators of transcription, like c-Fos, Krox-24–Egr-1–Zif268–nerve growth factor A, and the Jun family. In addition, we identified a gene that is highly expressed in liver regeneration and encodes a novel leucine zipper protein designated liver regeneration factor (24a). Apparently, the products of these genes activate transcription in a relatively tissue-nonspecific manner. Interestingly, within this group, we also identified gene 33, which has been described as a hormonally responsive gene (25, 34, 41, 62) but has not been previously reported to be an immediate-early gene (43a). These findings suggest that most of the immediate-early genes, including many of the transcription factors, are expressed in a wide variety of mitogen-treated cells.

Since 72% of the genes were expressed in BALB/c 3T3 fibroblasts, we were interested in knowing which of these had been previously identified by Almendral et al. in their extensive characterization of the immediate-early response in serum-stimulated NIH 3T3 cells (2). By comparing partial DNA sequences of the genes in Tables 2 and 3 (60a) with partial sequences from the 77 genes identified by Almendral et al. (4a), all of which contained at least the 3′ ends of the genes, we found that other than previously described immediate-early genes (those for JunB [54], c-Jun [55], Krox-24–Egr-1 [35, 60], Fos [44], actin [2, 32, 58], and tropomyosin [2, 58]), only four genes, those for 33-M4 (34), liver regeneration factor-U56, RL-30–X97, and RL-98–O25, were common to both groups. Thus, although the analysis by Almendral et al. was extensive, we identified an additional 27 immediate-early genes expressed in mitogen-treated fibroblasts. Combining these results showed that serum-stimulated 3T3 cells express more than 100 immediate-early genes.

Likewise, we were interested in determining how many of the immediate-early genes identified in serum-stimulated 3T3 cells were also expressed in insulin-treated H-35 cells and regenerating liver. We hybridized dot blots of recombinant plasmids containing the 77 immediate-early genes of Almendral et al. with quiescent and stimulated cDNA probes from our cell lines. Of the 77 genes, 13 were not expressed at sufficiently high levels in any cell type to be categorized (data not shown). However, we found that most of the remaining genes, 41 (64%) of 64 were expressed in at least one of the mitogen-stimulated liver cell types (Fig. 3). These results indicate that there are more than 70 immediate-early genes in hepatic cells and further support the idea that many immediate-early genes are common to a variety of mitogen-treated cells.

Cell type specificity of nearly one-third of immediate-early clones identified in liver cells and fibroblasts. While we expected to identify a few liver-specific immediate-early genes, we discovered that 15 (28%) of 52 genes are not expressed in fibroblasts and, thus, exhibit at least some degree of cell type specificity (Tables 2 and 3). Similarly, 23 (36%) of 64 clones from serum-stimulated fibroblasts are not

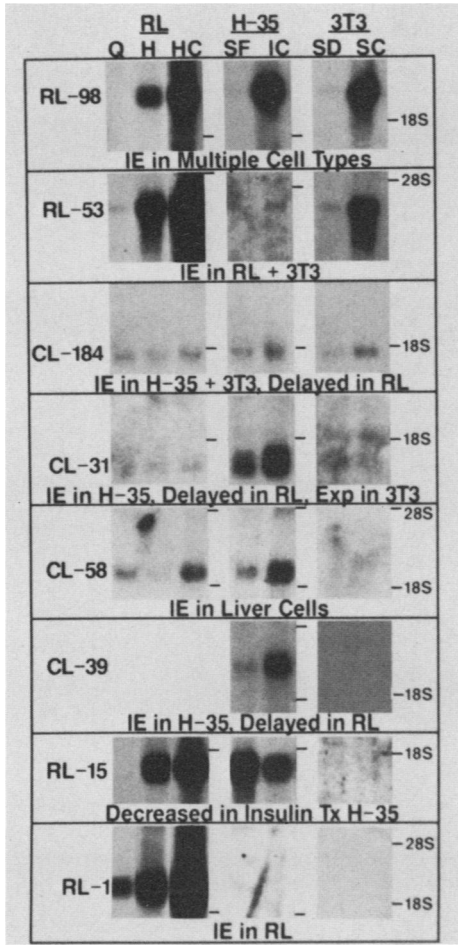

FIG. 2. Expression of immediate-early genes in a tissue-specific manner. Northern blots of representative genes from each of the categories contain 10 μg of total RNA per lane immobilized on nitrocellulose and hybridized with ^{32}P-labeled probes. 18S and 28S, positions of the respective rRNA bands. Labeling is the same as in Table 2, except for RL (regenerating liver): H, 1 h post 70% hepatectomy. Abbreviations: IE, immediate early; delayed, fails to increase in abundance in the presence of cycloheximide; Exp, expressed; Tx, treated.

expressed in either of the liver cell systems (Figure 3). Some of these genes encode proteins with cell type-specific functions, such as liver proteins involved in gluconeogenesis (i.e., phosphoenolpyruvate carboxykinase [SL-351]) or homeostasis (i.e., albumin [CL-79]) or fibroblast proteins involved in wound healing (i.e., tissue factor [21]). Presumably, genes in this group also encode proteins which tailor the proliferative response to the specific requirements of a particular cell type. Secreted proteins involved in paracrine or autocrine regulation, such as interleukins (27, 51), could fall into this category.

Among the interesting 3T3 cell-specific immediate-early genes are those for FosB (64), Fra-1 (11, 12), and Krox-20–Egr-2 (8, 9, 26) (Fig. 3). These genes are members of the same families as those for the relatively ubiquitous proteins c-Fos (11, 64) and Krox-24–egr-1 (35, 36, 60), but they are not induced in either regenerating liver or insulin-stimulated H-35 cells. Genes in both groups, ubiquitous and tissue specific, contain serum response elements within their promoters (8, 18, 63). This element has been implicated in the control of both induction and repression of immediate-early gene expression (18, 63). However, these results suggest that in addition to the serum response factor (49), other nuclear factors control the expression of these genes.

The liver cell-specific group contains 10 immediate-early genes for which partial DNA sequence analysis has been uninformative (Table 2). However, within this group, we have identified an abundantly expressed immediate-early gene, RL-15, which encodes the rat homolog of human insulinlike growth factor-binding protein 1 (5, 6). This protein potentiates the effects of insulinlike growth factor (15) and, thus, may act in a paracrine or autocrine fashion to coordinate the proliferative response of parenchymal and nonparenchymal cells in the liver. Like phosphoenolpyruvate carboxykinase (20, 61), its expression decreases in response to insulin treatment of H-35 cells (Fig. 2), indicating that insulin may not be a primary growth factor during liver regeneration (40a). In addition, we cloned a gene that encodes a protein with extensive homology to the envelope proteins of several retroviruses from different species (37, 47). Apparently, a virus is integrated fairly ubiquitously into the rat genome, since this gene is expressed in both H-35 cells and regenerating liver. Its transcription may be activated by the same machinery which activates expression of immediate-early genes. Alternately, it may have integrated downstream from an immediate-early gene promoter. Its absence in the BALB/c 3T3 cell line is probably related to species differences, since those cells are murine in origin.

Some of the liver cell-specific genes are expressed in only one of the two liver cell systems (Tables 2 and 3). This reflects the difference between normal liver cells and immortalized Reuber H-35 hepatoma cells. Expression of these genes is not necessarily restricted to one cell type, as illustrated by IP-10 (RL-5), a gene, first cloned in lymphocytes (39), that is induced in regenerating liver but not in serum-stimulated fibroblasts. Thus, expression of some of these genes, while restricted, is not limited to a single cell type.

Expression of some immediate-early genes in insulin-stimulated H-35 cells as delayed genes in regenerating liver. A time course analysis of expression of the immediate-early genes in regenerating liver and insulin-stimulated H-35 cells (Fig. 4B; Northern blots not shown) demonstrated that some of the genes identified in H-35 cells function as delayed genes during liver regeneration. Pretreatment with cycloheximide blocks the increased expression of these genes following partial hepatectomy, presumably because their expression depends upon a protein factor(s) not present in quiescent liver cells. However, in H-35 cells, nothing is required for increased expression beyond insulin-induced activation of preexisting factors, and thus, they function as immediate-early genes. Interestingly, most of these genes are also expressed during the immediate-early period in BALB/c 3T3 cells, which emphasizes that the intracellular milieu of factors in these immortalized tissue culture cell lines differs from the physiologically normal state.

Constitutive expression of some immediate-early genes in H-35 cells. The altered internal milieu of H-35 cells is further demonstrated by the fact that while normal liver cells require multiple growth factors to resume proliferation, H-35 cells can proliferate upon stimulation with insulin alone (29). Thus, it follows that some of the genes normally activated by other mitogens in hepatic cells are constitutively expressed in H-35 cells. Therefore, we are interested in genes AE36, S48, TT13 (Fig. 3), and SL-314 (Tables 2 and 3), which are expressed at similar levels in both serum-starved and insulin-

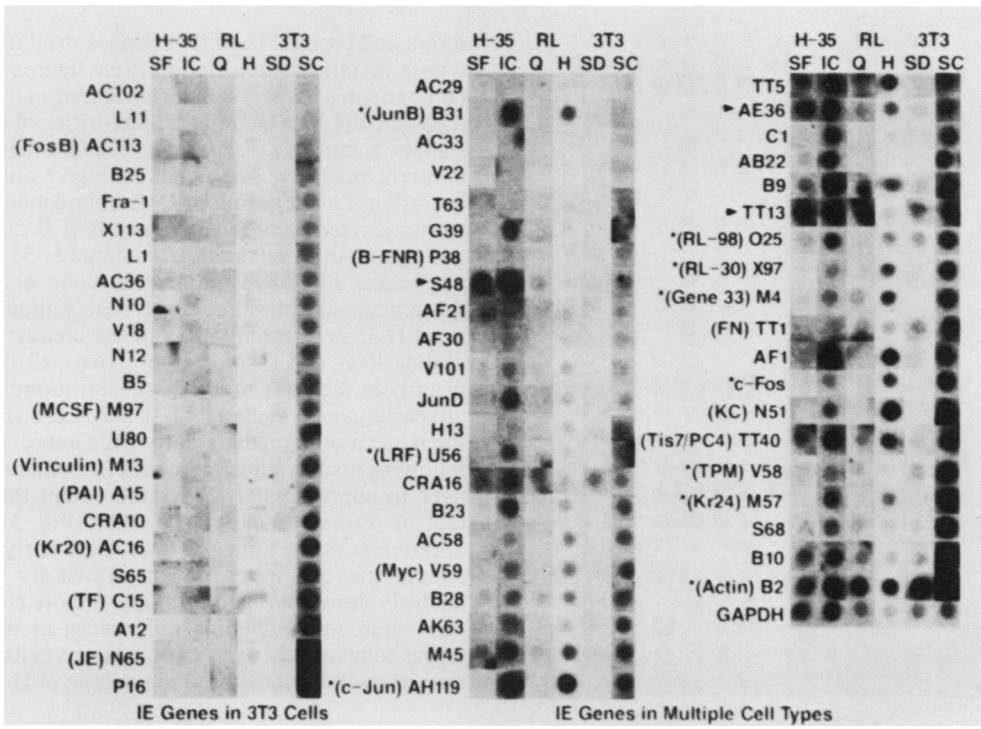

FIG. 3. Identification of many immediate-early genes in serum-stimulated NIH 3T3 cells that are also expressed in liver cells and dramatic variation in the relative abundances of the mRNAs among cell types. The genes are arranged according to strength of expression in serum-cycloheximide-treated BALB/c 3T3 fibroblasts and grouped on the basis of predominant patterns of expression, as in Fig. 1. The names of identified genes or clones from our screening which correspond to those of Almendral et al. (2) are enclosed in parentheses. Genes which are constitutively expressed in H-35 cells are marked with arrowheads. The dot blots contained 1 μg of insert-bearing plasmid per spot and were hybridized with ^{32}P-labeled cDNA probes synthesized from total RNA isolated at the various times. Labeling is as in Table 2. Abbreviations: MCSF, macrophage colony-stimulating factor; PAI, plasminogen activator inhibitor; Kr20, Krox-20–Egr-2; TF, tissue factor; B-FNR, β-fibronectin receptor; FN, fibronectin; TPM, tropomyosin; Kr24, Krox-24–Egr-1–Zif268–Ngfi-a. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) served as an unstimulated control. *, clones also identified by Almendral et al. as immediate-early genes in serum-stimulated NIH 3T3 cells.

cycloheximide-treated H-35 cells despite their clear induction following mitogen treatment in 3T3 cells and regenerating liver. Premature activation or constitutive expression of these genes may provide the basis for H-35 cell tumorigenesis and/or responsiveness to insulin.

Induction of expression of different subsets of immediate-early genes by sham surgery and cycloheximide treatment. As a control for immediate-early gene induction in response to surgery alone, we performed sham surgery in which rats were anesthetized and subjected to midventral laparotomy with minimal hepatic trauma. This resulted in increased expression of a subset of immediate-early genes which includes several genes that encode structural proteins, gene 33, and other genes of unknown function (Fig. 4A and B). Most of the immediate-early genes expressed following sham surgery are also induced in insulin-treated H-35 cells and serum-stimulated 3T3 cells, which suggests that they are involved in cellular proliferation and are not just produced in response to the stress of surgery. Time course studies of several of these genes indicate that the sham-induced expression is more transient than that induced by regeneration (60a).

As a further analysis, we examined the expression of members of this collection of immediate-early genes in the livers of rats treated with cycloheximide alone. Gene expression ranged from very little increase above quiescent levels to an increase equivalent to that observed following partial hepatectomy in the presence of cycloheximide (Tables 2 and 3; Northern blots not shown). Thus, cycloheximide alone alters the expression of a subset of genes, perhaps through mRNA stabilization or perturbation of the liver by the drug or its carrier. However, the time course data in Fig. 4 demonstrate that the 52 genes identified in liver cells are not merely artifacts of cycloheximide treatment, since they are induced during the immediate-early period by partial hepatectomy alone.

Different kinetics and abundance of immediate-early gene expression among insulin-stimulated H-35 cells, serum-stimulated BALB/c 3T3 fibroblasts, and regenerating liver. While some of these immediate-early genes are tissue specific and others are expressed in a less tissue-restricted manner, most are tissue nonspecific. However, these may also participate in tissue-specific regulation of cellular proliferation if their abundance or the timing of their expression differs between cell types. We found striking differences in the levels of expression of some genes among different cell types. For example, of the 169 differentially expressed clones analyzed from the H-35 cell cDNA library, 54 were homologous to CL-6 and 24 corresponded to CL-4. Although both CL-4 and CL-6 are expressed in all of the cell types examined, neither of these genes was identified by differentially screening the regenerating liver or 3T3 cell (2) cDNA library (Tables 1 and 2), presumably because their level of expression in these cells makes detection difficult.

Figures 3 and 4 further emphasize the differences in the levels of expression of immediate-early genes among dif-

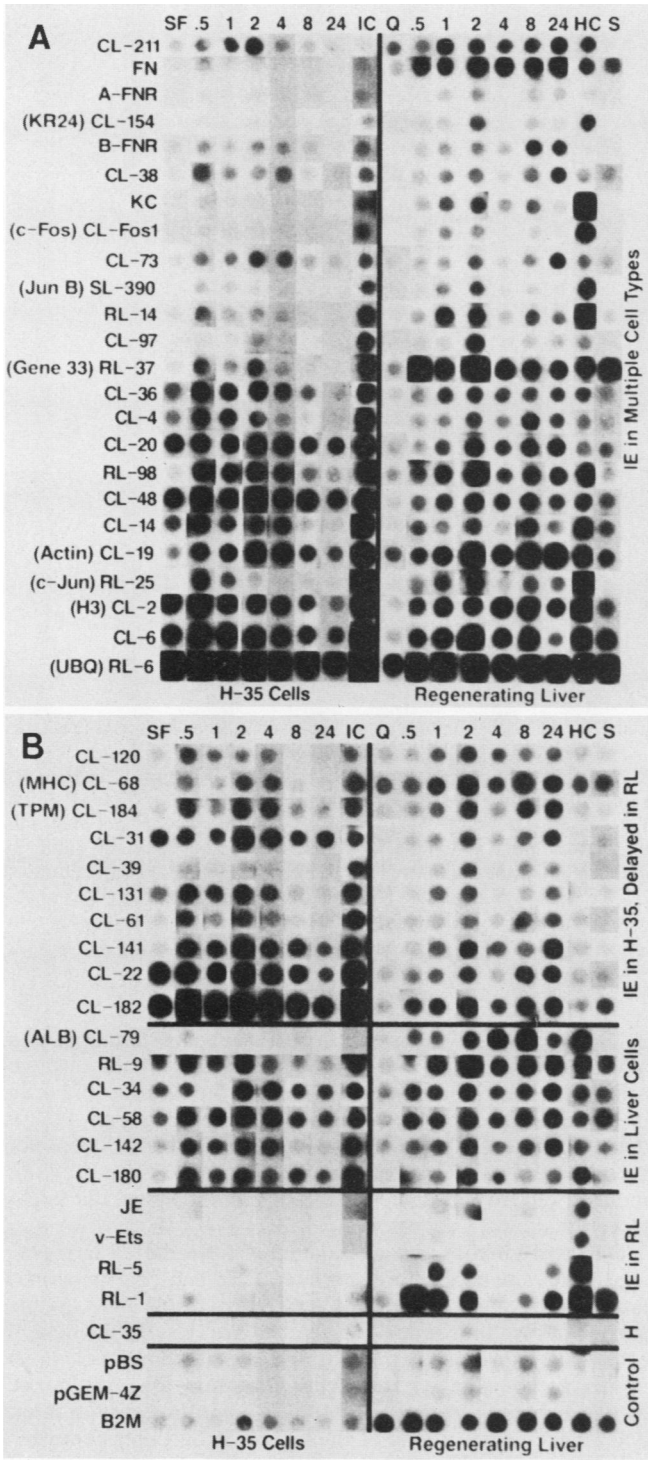

FIG. 4. Differences in the time courses of expression of many immediate-early genes between insulin-stimulated H-35 cells and regenerating liver and presence of some immediate-early genes in H-35 cells as delayed genes in regenerating liver. The genes are grouped in the categories described in Fig. 1. Within each group, the genes are arranged by strength of expression in insulin-cycloheximide-treated Reuber H-35 cells, except for the regenerating-liver-specific genes, which are arranged by strength of expression in that tissue. pBS (pBluescript) and pGEM-4Z were negative controls, and B2M (β-2-microglobulin) was a constitutively expressed positive control. Labeling is similar to that in Table 2, except that 0.5, 1, and 2, etc., indicate time after treatment of serum-starved cells with insulin (10^{-8} M) (H-35) or after partial hepatectomy (RL). S, 1 h post sham surgery (laparotomy without hepatectomy). Abbreviations: FN, fibronectin; A-FNR, α-fibronectin receptor; KR24, Krox-24 or Egr-1; B-FNR, β-fibronectin receptor; H3, histone H3; UBQ, ubiquitin; MHC, novel major histocompatibility class 1 gene or allele; TPM, tropomyosin; ALB, albumin, IGF-R, insulinlike growth factor 1 receptor; IGF, insulinlike growth factor 1; AFP, α-fetoprotein; AFGF, acidic fibroblast growth factor; EGF-R, epidermal growth factor receptor. The dot blots contained 2 μg of insert-bearing plasmid per spot and were hybridized with ^{32}P-labeled cDNA probes synthesized from total RNA isolated at the various times.

ferent cell types. The cDNA probes used in the experiments whose results are depicted in these figures prevent accurate comparison of expression levels among cell types, but within a given cell type, the relative intensities of the hybridization signals accurately reflect the relative abundances of the different mRNAs. The genes in Fig. 3 and 4 are arranged according to increasing mRNA abundance in one cell type (serum-cycloheximide-stimulated BALB/c 3T3 cells in Fig. 3 and insulin-cycloheximide-stimulated H-35 cells in Fig. 4) to emphasize differences in the relative levels of expression of these genes in the other cell types. Although insulin-stimulated H-35 cells retain many of the characteristics of regenerating liver (19, 52, 61), these two cell types differ significantly in the peak mRNA concentrations of some immediate-early genes, such as CL-4 and CL-6 (Fig. 4A). In fact, the pattern of immediate-early gene expression in H-35 cells following insulin stimulation differs from that of regenerating liver to approximately the same extent that it differs from that of serum-stimulated 3T3 cells (Fig. 3). There are also differences in the timing of immediate-early gene expression among the cell types, as illustrated by the time course analysis shown in Fig. 4. Multiple factors could contribute to this variability, including differences in the mitogens, differing cellular milieus in the quiescent cells, and differences caused by the transformed phenotype of H-35 and 3T3 cells.

DISCUSSION

We have identified approximately 100 immediate-early genes in serum-treated 3T3 cells and over 70 in regenerating liver and insulin-treated H-35 cells. The overlapping patterns of expression of these genes among different cell types is illustrated in Fig. 5. In light of the limited sensitivity of differential screening analysis, it is likely that more immediate-early genes remain to be identified. Thus, the immediate-early response involves an unusually large number of genes for the first step in a response cascade. Apparently, the rapidity with which cells must resume proliferation and traverse the cell cycle and the large number of new proteins which must be synthesized make it temporally impossible for cells to respond through a single pathway which initially activates a small number of genes.

The abundance of immediate-early genes and the highly varied pattern of their expression in different cell types suggest that the tissue specificity of the proliferative response is not due to expression of a few cell type-specific genes. Rather, the specificity of the response seems to arise from the particular set of immediate-early genes expressed in a given tissue or in response to a given growth factor. Although most of the immediate-early genes examined here, including many of the transcription factors, are expressed in multiple cell types, their levels of expression vary significantly. Since many transcription factors function as dimers

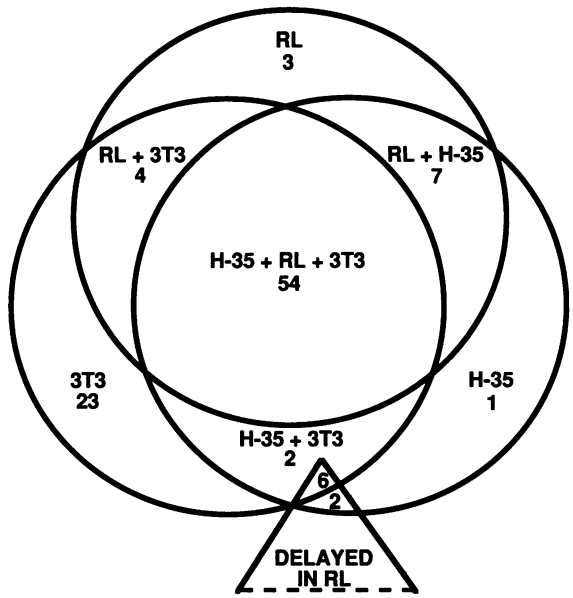

FIG. 5. Expression of large numbers of immediate-early genes in regenerating liver (RL), insulin-treated H35 cells, and serum-treated 3T3 cells. Numbers of immediate-early genes identified and the cell types in which they are expressed, i.e., regenerating liver (RL), 3T3 cells (3T3), and insulin-treated H-35 cells (H35) are indicated. Numbers of genes that showed delayed expression in regenerating liver are indicated (delayed in RL).

(7, 45), their relative concentrations may alter in a tissue-specific manner the transcription of genes involved in later aspects of the proliferative response. In addition, the particular combination of secreted immediate-early proteins, such as IP-10 (38), KC (48, 56), and insulinlike growth factor-binding protein 1 (15, 40a), produced by a cell type may shape the proliferative response through paracrine or autocrine interactions.

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