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Identification of Expressed Sequence Tags of Genes Expressed Highly in the Activated Hepatic Stellate Cell

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Expressed sequence tags (ESTs) were generated from two 3'-directed cDNA libraries constructed from quiescent and activated rat hepatic stellate cell (HSC) to analyze the expression profiles of active genes in both cells. From quiescent and activated HSC, 694 ESTs and 779 ESTs, respectively, were obtained after excluding those having shorter than 30 bp. Among ESTs obtained from quiescent and activated HSC, 68 and 73 kinds of ESTs (186 clones and 236 clones), respectively, appeared more than once, implying that their genes are expressed highly in each cell type. 52 among 73 ESTs appeared only in the activated HSC, 47 among 68 ESTs only in the normal HSC, and 21 in both cells. The genes of these 52 ESTs were assumed to be expressed more highly in the activated HSC. To confirm the high expression of genes of which the ESTs appeared more than twice in the activated HSC, northern hybridization was carried out with RNAs derived from rat normal and fibrotic liver using each of 18 EST DNAs as probe. 13 ESTs showed more intense bands with RNA isolated from the fibrotic liver than normal liver. From these results, we confirm the positive correlation between abundance of transcript in activated HSCs and the expression level in fibrotic liver. The expression profile of the transcripts serves as an important tool in understanding the biological properties of HSC.

Key words: Expressed sequence tag (EST), Expression profile, Rat hepatic stellate cell, Liver fibrosis

INTRODUCTION

The molecular mechanism underlying the activation of the hepatic stellate cell (HSC) has been extensively studied during the last decade because activated stellate cells are thought to be a key player in the development of liver fibrosis (Eng and Friedman, 2000; Friedman, 2000; Pinzani and Gentilini, 1999). This activation is characterized by a transdifferentiation from a vitamin A-storing quiescent phenotype to a myofibroblast-like cell and accompanied by the expression of various genes of extracellular matrix matrices, cell growth factors, inflammatory cytokines, and receptors for growth factors (Kawada, 1997; Gressner, 1998). Analysis of the difference of gene expression between quiescent and activated HSC has provided profound insights into the cell activation mechanism (Ratziu *et al.*, 1998).

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We have undertaken a systematic survey of quiescent and activated HSC to identify new genes associated with HSC activation, using an expression-profiling method that is based on quantitative analysis of mRNA populations. Since *in vivo* mRNA populations are very difficult to directly analyze, indirect approaches are used. Sequencing of randomly selected clones in a 3'-directed cDNA library is one of the indirect methods for analyzing mRNA populations in a cell type or in a tissue (Matsubara and Okubo, 1993; Okubo *et al.*, 1998). It can be used for the identification of cell type-specific or tissue-specific genes upon comparison of sequencing results of two libraries (Lee *et al.*, 2000). Also, it can be assumed that the higher the frequency of appearance of an expressed sequence tag (EST) in a library, the higher is the expression level of an EST-containing gene. Although several methods have been developed recently to analyze the gene expression profiles in a cell-type or a tissue including the microarray of genes and serial analysis of gene expression (SAGE) (Velcuescu *et al.*, 1995), the analysis of a 3'-directed cDNA library is another method to analyze the gene

expression profile. In this approach we construct a 3'-directed cDNA library that faithfully represents the composition of mRNA in the same tissue, and sequence the cDNA moiety of qualitatively and quantitatively describing the population of the gene transcripts.

In this study, we describe active genes in the quiescent and activated HSC of rat according to their order of activity, and then describe some novel genes that are likely to be uniquely active in the activated HSC.

MATERIALS AND METHODS

Isolation and cultivation of HSCs

Rat HSCs were isolated from the livers of Sprague-Dawley rats weighing 450 to 500 g as described previously (Lee *et al.*, 2003), with additional modifications. Buoyants of HSCs were prepared by centrifugation using a double-layered (17%, 11.5%) metrizamide solution (Sigma, St. Louis, USA). After centrifugation at 1700 g for 15 min, HSCs were collected from the top layer. They were identified as HSCs by typical star-like configuration and vitamin A fluorescence, and by immunostaining with horseradish peroxidase coupled anti-desmin (Sigma). The purity was always high than 95%, and the viability was more than 90% as evaluated by a Trypan blue exclusion test. For culture, HSCs were plated in William's Medium E (WME, Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco BRL) and antibiotics (0.035 g/L penicillin and 100 mg/L streptomycin) in the cell density of 5×10^5 cells/mL for three hours. The cultures were subsequently washed with phosphate-buffered saline (PBS) to remove dead cells and cell debris. In the present study, the attached cells were designated quiescent HSCs and the attached cells that were cultured for 14 days were designated *in vitro* activated HSCs.

Construction of a 3'-directed cDNA library

Total mRNA was purified by affinity chromotography of isolated total RNA using an oligo (dT)-cellulose column. Construction of the 3'-directed cDNA libraries and transformation into *E. coli* has been described (Lee *et al.*, 2000). Briefly, cDNA was synthesized using a pUC19-based vector primer, digested by *dam* seneitive four base cutter *Mbo*I, circularized and transformed into *E. coli*. The resulting transformants had only the 3' cDNA portion between the poly A additional site and the nearest *Mbo*I or *Bam*HI site.

Analysis of nucleotide sequences

Sequencing templates of the randomly selected 3'-directed cDNA clones were prepared according to the method described previously (Okubo *et al.*, 1992). A Dye-Terminator DNA sequencing kit (Perkin-Elmer, palo Alto, CA) and a -20M13 universal primer (NEB 1211, Beverly, MA) were used for cycle sequencing. Which determines the sequence of the 3'-directed region in the 5' to 3' direction of the mRNA, on an ABI 377 automatic DNA sequencer (Perkin-Elmer).

EST sequences were compared with one another using a DNASIS program (Hitachi Software Engineering Co., Ltd., South San Francisco, CA). Similarity searches in GenBank databases of the sequences obtained were completed using the BLAST program (NCBI, http://www.ncbi.nlm.nih.gov). When an EST sequence had a similarity of more than 50% to a GenBank sequence over half of the size of the EST or longer than 100 nt, the EST sequence was regarded as being identified in GenBank.

Experimental fibrotic models

Liver fibrosis was induced in pathogen-free male Sprague-Dawley rats (200-250 g). The animals received humane care according to National Institute of Health (NIH) guidelines. Biliary cirrhosis was induced by ligation and excision of the bile duct (BDL/S) (Nan *et al.*, 2000). The animals were sacrificed after 28 days of obstruction and control animals were sham operated.

Measurement of expression levels of EST-containing Genes

To measure the expression levels of EST-containing genes in normal and BDL/S liver, about 20 mg of total RNA was blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Madrid, Spain). The probe was an EST DNA obtained by double digestion of a plasmid having each EST DNA with *Kpn*I and *Hind*III. The probe was labeled with a random priming kit (Amersham) and [α-^{32}P]dCTP (NEN Life Science Products). Northern hybridization was performed as described previously (Lee *et al.*, 2000). After northern hybridization, the band intensities on an X-ray film were measured by densitometric analysis (JX-330P, Pharmacia, USA), and the ratios of the intensities were determined.

RESULTS AND DISCUSSION

Gene expression profiles in quiescent HSC and in activated HSC

To identify genes expressed highly in the quiescent HSC or in the activated HSC, nucleotide sequences of randomly selected cDNA clones were determined. This was achieved through sequencing randomly selected clones from a non-biased cDNA library. This library represents the abundance of gene transcripts in the original mRNA population. Okubo *et al.* (1992) reported that about 1,000 randomly selected clones are enough to identify genes expressed highly in a specific cell-type

when the nucleotide sequences of those clones with each other and with those of cDNA clones in other cDNA libraries made from other cell-type or tissues. In this work, about seven hundred to eight hundred clones were selected randomly from two 3'-directed cDNA libraries made from the quiescent HSC and the activated HSC, and their nucleotide sequences were determined. In this 3'-directed cDNA library, the inserted cDNA is the region from the poly (A) site to the most proximal *Mbo*I site, and the sequencing determines the nucleotide sequence from the *Mbo*I site to the poly (A) site. Okubo *et al.* (1992) reported that every vector molecule in the library constructed by this method has only one cDNA insert, and thus the cDNA population in the cDNA library should be proportional to the mRNA population. We randomly chose 724 transformant colonies from the cDNA library of a quiescent HSC. Among the 724 clones analyzed, 30 were excluded from further analysis, since they had inserts shorter than 30 bp. Sequences of the remaining 694 clones were compared with each other (Table I). Out of 694 clones, 186 (26.8%) appeared more than once (redundant group) grouped in 68 different species, and 508 clones appeared only once (solitary group). Within the redundant group, one species appeared 15 times, one species 11 times, one species 5 times, eight species 4 times,11species3timesand45speciestwice.

We also analyzed the cDNA library prepared from activated HSC. Among the 815 clones analyzed, 36 were excluded from further analysis, since they had inserts shorter than 30 bp. Sequences of the remaining 779 clones were compared with each other (Table I). Out of 779 clones, 236 (30.3%) appeared more than once (redundant group) grouped in 73 different species, and 543 clones appeared only once (solitary group). Within the redundant group, one species appeared 16 times, two species 11 times, one species 9 times, two species 8 times, one species 7 times, seven species 5 times, one species 4 times, 11 species 3 times, and 47 species twice.

Table I. Summary of ESTs obtained from HSC

		Number of cDNA clones	EST species	Percentage (%)
	Total	724		
	< 30 nt	30		
	> 30 nt	694	576	100
Quiescent	**Redundant group**	186	68	26.8
HSC	15 times	15	1	
	11 times	11	1	
	5 times	5	1	
	4 times	32	8	
	3 times	33	11	
	twice	90	45	
	Solitary group	508	508	73.2
	Total	815		
	< 30 nt	36		
	> 30 nt	779	616	100
Activated	**Redundant group**	236	73	30.3
HSC	16 times	16	1	
	11 times	22	2	
	9 times	9	1	
	8 times	16	2	
	7 times	7	1	
	5 times	35	7	
	4 times	4	1	
	3 times	133	11	
	twice	94	47	
	Solitary group	543	543	69.7

Identification of genes expressed highly in quiescent HSC and in activated HSC

The expression profile of active genes in quiescent HSC is shown in Table II. We listed here 22 ESTs that appeared 3 times or more in descending order of abundance. The most abundant mRNA is that for immunoglobulin gamma chain which comprises about 8.1% of the redundant group. It is followed by those for anti-nerve growth factor 30 antibody, microsomal signal peptidase, thymosin beta-4, and so on.

The expression profile of active genes in activated HSC is shown in Table III. We listed here 26 ESTs that appeared 3 times or more in descending order of abundance. The most abundant mRNA is that for Glu-Pro dipeptide repeat protein which comprises about 6.8% of the redundant group. It is followed by those for α1 collagen type III, FISP-12, pF2 genomic DNA, and so on. The profile reflects unique features of the activated HSC physiology. Therefore, it includes a lot of genes that have been well known in extracellular matrixs, such as genes for collagen, fibronectin, biglycan, and so on (Friedman, 1999; Benyon *et al.*, 1998).

Therefore, these results show that the typical HSC functions well known from physiological and biochemical studies are maintained through very high rate of transcription of specific genes.

Confirmation of specific genes expressed in activated HSC

In order to identify specific genes expressed in activated HSC, we used a computerized data processing. Among ESTs found twice or more in activated HSC, the activated HSC specific genes were selected, except for extraxcelluar matrix such as collagen, fibronectin, and biglycan (Table IV).

In order to confirm if these genes expression could be induced following a fibrogenic stimulus *in vivo*, northern blot analysis, using EST cDNAs as probe was performed with RNAs derived from rat normal and BDL/S-operated

Table II. The expression profiles of genes in quiescent HSC

EST	GenBank Acc. No.[a]	Gene name	Frequency [b]	Size (bp)
nh29D09	M13802	Ig gamma-2b H-chain C	15	344
nh28E12	U39609	Anti-NGF 30 antibody	11	469
nh60C12	J04067	Microsomal signal peptidase	5	66
nh13B04	M34043	Thymosin beta-4	4	303
nh03E01	AF115770	BHE/Cdb tRNA-Lys gene	4	296
nh32H09	U40628	Glu-Pro dipeptide repeat protein	4	360
nh11C06	M55532	Carbohydrate binding receptor gene	4	296
nh35F05	X63561	Elongation factor1-α	4	537
nh35C01	L26087	Munc 18-1	4	274
nh32H11	X77168	pF2 genomic DNA	4	412
nh59G09		Unknown	4	67
nh09D05	X16956	beta-2 microglobulin	3	267
nh18D03		Unknown	3	456
nh20E03	X59051	Ribosomal protein S29	3	191
nh04C05		Unknown	3	190
nh06E06	L26268	Anti-proliferative factor	3	445
nh18E11		Unknown	3	180
nh34E08	X78327	Ribosomal protein L13	3	693
nh08D05		Unknown	3	330
nh33F02	X06407	21kD polypeptide under translational	3	192
nh08G02	M19533	Cyclophilin	3	234
nh11B08		Unknown	3	227

The 3'-directed cDNA clones identified in GenBank were determined by the BLAST program of the NCBI in 'nr' database.
[a]GenBank Acc. No. indicates GenBank entries already deposited.
[b]The frequency is the number of cDNA clones appearing among 694 clones.

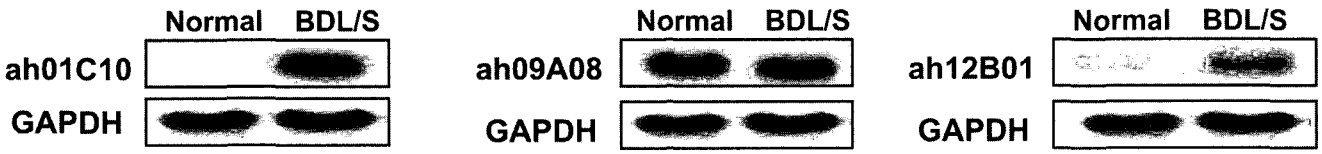

Fig. 1. Typical results of northern hybridization using GAPDH for quantity control. EST ah01C10 is hybridized only with fibrotic liver RNA. EST ah09A08 is hybridized with both normal and fibrotic liver RNA. EST ah12B01 is hybridized more intensively with fibrotic liver RNA than normal liver RNA.

liver. Typical results of northern blot analysis are shown in Fig. 1.

Among 18 ESTs, 7 ESTs hybridized only with fibrotic liver RNA. Indeed, several genes of the 7 ESTs have been described in HSCs; Thrombospondin-1 is play a role in the pathogenesis of liver fibrosis in patients with congenital hepatic fibrosis and the source is the HSC (El-Youssef *et al.*, 1999). Dystroglycan is expressed on the membrane of HSC and is up-regulated during liver fibrosis (Bedossa *et al.*, 2002). aB-crystallin is rapidly upregulated following HSC activation and represents an early marker for HSC activation. (Cassiman *et al.* 2001; Lang *et al.*, 2000). Additionally, we previously demonstrated that the expression of *O*-acetyl disialoganglioside synthase is induced after activation of the HSC, both *in vitro* and *in vivo* (Lee *et al.*, 2003). Through measuring the specific gene activities in pathophysiological condition, we confirm the positive correlation between abundance of transcript of activated HSCs and the expression level in fibrotic liver.

The rest 11 ESTs showed hybridization signals with both normal and fibrotic liver RNA However, 6 of the 11 ESTs hybridized more intensively with fibrotic liver RNA than normal liver RNA. These genesí expression in activated HSC has not been studied; SM22 appears secondary to smooth muscle actin during development and is also a marker for smooth muscle cell. The SM22 gene has widely been used to study the regulatory mechanisms of smooth muscle cell gene expression during cardiovascular development (Xu *et al.*, 2003). Profilin mRAN increased after acute and chronic treat-

Table III. The expression profiles of genes in activated HSC

EST	GenBank Acc. No.[a]	Gene name	Frequency [b]	Size (bp)
ah 80C03	U40628	Glu-Pro dipeptide repeat protein	16	266
ah05F09	X70369	Pro α1 collagen type III	11	208
ah01G01	M70642	FISP-12	11	211
ah13G04	X77168	pF2 genomic DNA	9	68
ah04H01	M62470	Thrombospondin1	8	114
ah05B04	X05834	Fibronectin	8	299
ah01E08	X06801	Vascular α-actin	7	165
ah14B01	U58829	Ferritin-H subunit	5	331
ah04A11	M73706	Ferritin large subunit	5	361
ah12B01	Z68618	SM22	5	429
ah26B09	J03464	Pre-procollagen α2(I)	5	83
ah10A12	J01436	Mitochondrial cytochrome B	5	425
ah13E04	J04067	Microsomal signal peptidase	5	195
ah26B09	J03464	Collagen α-2 type I	5	280
ah14G08	U27340	Sulfate glycoprotein	4	96
ah04E09	X52815	Actin γ isoform	3	348
ah08G07		Unknown	3	412
ah08F11	M27315	Cytochrome C oxidase	3	215
ah01C10	D84068	O-acetyl GD3 synthase	3	227
ah04F06		Unknown	3	287
ah09A08	X02918	Disulphide isomerase	3	162
ah10A04	U17834	Biglycan	3	126
ah13B01	L37206	Smooth muscle caldesmon	3	69
ah06A11		Unknown	3	121
ah08F12	Z78279	Collagen α1(I)	3	407
ah06E06	AJ011971	Wolframin	3	633

The 3'-directed cDNA clones identified in GenBank were determined by the BLAST program of the NCBI in 'nr' database.
[a]GenBank Acc. No. indicates GenBank entries already deposited.
[b]The frequency is the number of cDNA clones appearing among 779 clones.

Table IV. The list of genes of which ESTs did not appear in quiescent HSC

EST	Frequency	GenBank Acc. No.	Similarity (%)	Gene name	Northern blot analysis[a]	
					Normal	BDL/S
ah01G01	11	M70642	92	FISP-12	UD[b]	+
ah04H01	8	M62470	98	Thrombospondin 1	UD	+
ah12B01	5	Z68618	93	SM22	+	+++
ah01C10	3	D84068	95	O-acetyl disialoganglioside synthase	UD	+
ah09A08	3	X02918	96	Disulphide isomerase	+	+
ah04F09	2	D26154	96	Brain specific protein RB109	+	++
ah05B12	2	X96967	92	Profilin	+	++
ah05G01	2	M37041	97	Nuclear protein B23.1 and B23.2	+	++
ah06B01	2	S63521	99	Glucose-related protein GRP78	+	+
ah08H06	2	S77858	98	Non-muscle myosin alkali light chain	+	++
ah11B07	2	Y09257	86	NOV protein	UD	+
ah24A09	2	U19894	98	Target of the antiproliferative antibody	+	+
ah26C12	2	U43512	93	Dystroglycan	UD	+
ah28H08	2	AF134119	94	SKD1	+	+
ah14B04	2	AF107844	95	Neuroendocrine-specific golgi protein	+	+
ah14C04	2	J03026	95	Matrix Gla protein	UD	+
ah14D04	2	AB011081	92	Huntingtin interacting protein-2	+	++
ah14H09	2	X60351	93	αB-crystallin	UD	+

[a]The relative ratio of the band intensities on the lane of RNA from the normal and that from the BDL/S are shown. The intensity i ndicated by the symbol "++" is twofold of that indicated by "+", and so on.
[b]UD. Undetectable.

ments of carbon tetrachloride, which is associated with the increase of fibronectin, type-III and ñIV collagen mRNA (Nakamura *et al.*, 1994). B23 plays a role as an accessory factor in the nuclear import of the nuclear localization signals-containing proteins and that phosphorylation at sites in the highly acidic segments of the protein enhances the stimulatory effect (Szebeni *et al.*, 1997). For B23.1, the level of mRNA and protein expression was highest in Novikoff hepatoma cells, followed by testis, liver, and kidney. This suggests that B23.1 expression is correlated with the rate of proliferation of the tissue and/or the rate of ribosome biogenesis. Similarly, the B23.2 protein was barely detectable in the normal rat tissues, but significant quantities were found in the Novikoff cells (Wang *et al.*, 1993). Myosin light chain kinase expressed in rabbit liver may catalyze the phosphorylation of myosin light chain, which may play important roles in the regulation of hepatic cell functions (Ren *et al.*, 2001) and myosin light chain phosphorylation has been demonstrated as having potential role in cell migration (Suzuki *et al.*, 2004). Although our results provide evidence for the involvement of these genes in BDL/S liver, its role in the HSC activation remains to be clarified.

In conclusion, we performed a molecular biological quantification of transcripts in quiescent and activated HSCs. We discovered some candidate genes specific for activated HSCs. The expression profiles of the transcripts serve an important tool in understanding the biological properties of HSCs. Furthermore, marked upregulation of novel genes in injured liver suggest that these genes may play some roles in the pathogenesis of liver fibrosis. Studies of novel genes might thus open up the way for future therapeutic approaches to treat various liver disorders.

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