J. Biochem. 131, 39–44 (2002)

Identification of Differentially Expressed Genes in Rat Hepatoma Cell Lines Using Subtraction and Microarray[1]

Fumihiko Tanaka, Naohiro Hori, and Kenzo Sato[2]

Department of Molecular Biology, School of Life Sciences, Faculty of Medicine, Tottori University, 86 Nishimachi, Yonago 683-8503

Received September 21, 2001; accepted October 17, 2001

Rat hepatoma Fao is a well-differentiated cell line that expresses numerous liver-specific enzymes and liver-enriched transcription factors, whereas the C2 cell line derived from Fao exhibits dedifferentiated phenotypes and fails to express these differentiated traits. Hence, to identify the gene related to the differentiation status in these cells, we analyzed differentially expressed genes between Fao and C2 cells using a microarray of 1,000 identified cDNA clones, and isolated a cDNA clone from subtractive library constructed by suppression subtractive hybridization. D6.1A, a member of the tetraspanin protein superfamily, was identified as a gene enhanced in C2 cells compared with Fao cells. Interestingly, expression of this gene was also induced following liver injury by CCl_4 and in liver regeneration following partial hepatectomy. These results suggest that the D6.1A gene is related to stimulation of cell proliferation and differentiation status in C2 hepatoma cells.

Key words: cDNA subtraction, dedifferentiation, hepatoma, microarray, tetraspanin.

Hepatocytes are highly specialized epithelial cells involved in the synthesis of numerous liver-specific proteins and enzymes, which operate in general metabolism, detoxification of drugs, generation of blood protein and homeostasis. However, most of these functions are down-regulated in carcinogenesis of the hepatocytes (*1, 2*).

Rat hepatoma cell line Fao, which is derived from the H4IIEC3 cell line adapted to growth *in vitro* from the rat hepatoma cell line Reuber H35, is well differentiated and expresses numerous liver-specific enzymes and transcription factors, whereas the C2 cell line, a derivative from Fao, exhibits dedifferentiated phenotypes (*3*). These phenotypes of C2 cell are sustained by culture at high temperature (41°C), and reversion to the hepatic phenotype occurs at low frequency upon culture in glucose-free medium (*4*). These cell lines provide an invaluable experimental system for investigating the differentiation mechanism of hepatocytes *in vitro*.

In the present study, to determine the gene expression profiles and identify the gene related to differentiation status of Fao and C2 cells, we analyzed the differentially expressed genes in these cells using a DNA chip microarray containing 1,000 identified cDNA clones and isolated cDNA from a subtractive library constructed by suppression subtractive hybridization. Among the differentially expressed genes, we focused on the D6.1A gene, expression of which is induced in C2 cells and liver injured by carbon tetrachloride.

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-859-34-8039, Fax: +81-859-34-8274, E-mail: kensato@grape.med.tottori-u.ac.jp

Abbreviation: SSH, suppression subtractive hybridization.

MATERIALS AND METHODS

Cell Culture—Reuber cells, a rat minimal deviation hepatoma cell line, were given by Dr. M. Nanba, Okayama University. Fao and C2 were obtained from Dr. Mary C. Weiss, Institute Pasteur, France (*3, 4*). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 units/ml of penicillin, and 100 μg/ml of streptomycin.

RNA Preparation and Northern Blot Analysis—Total RNAs were isolated from rat tissues and hepatoma cell lines using acid phenol–guanidinium thiocyanate–chloroform extraction as described previously (*5*). Poly(A)-enriched RNA was then purified using Oligo(dT) Latex (Rosche, Tokyo) (*6*).

Northern blot analysis was performed as described by Goldberg (*7*). Briefly, 10 μg of denatured total RNA was electrophoresed in 1% agarose gel, then transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, USA). The membrane was hybridized with cDNA probes labeled by the random primer method (*8*). The hybridized signals were detected by autoradiography on X-ray film or by BAS 1000, BioImage Analyzer (Fuji Film, Tokyo).

Microarray—Expression profile in hepatoma cell lines was determined using a high throughput microarray approach (*9*). A DNA chip spotted with 1,000 cDNAs from identified human genes (IntelliGene™ Human CHIP 1K Set I, version 1.0) was purchased from Takara Shuzo, Kyoto. Poly$(A)^+$ RNAs from Fao and C2 cells were labeled respectively with Cy3- and Cy5-dUTP by reverse transcription. The arrayed clones were hybridized with a 1:1 mixture of Cy3- or Cy5-labeled first-strand cDNAs in hybridization buffer containing 6× SSC, 0.2% SDS, 5× Denhardt's solution, and carrier DNA at 65°C for 14 h. The DNA chip was washed twice with 1.2× SSC with 0.2% SDS

at 55°C for 30 min, with the same buffer at 65°C for 5 min, then with 0.05× SSC at room temperature for 5 min. The fluorescence intensity was scanned with an Affimetrix 428 Array Scanner (Affimetrix, USA), and data were analyzed using ImaGene software (BioDiscovery, USA). Results were also analyzed by normalizing fluorescence intensities between experiments using a subset of cDNA clones.

Construction of the Subtractive cDNA Library by Suppression Subtractive Hybridization (SSH)—A subtractive cDNA library was prepared with poly(A)$^+$ RNAs purified from Fao and C2 cells by SSH (*10, 11*). SSH was performed with a PCR-Select cDNA Subtraction Kit (Clontech, USA) according to the manufacturer's protocol. Briefly, tester cDNA was synthesized from poly(A)$^+$ RNAs of objective cells, and driver cDNA was synthesized from the cells for withdrawal. The tester cDNAs were digested with *Rsa*I, and linked with adaptor oligonucleotides. The tester cDNA was hybridized with excess of driver cDNA digested with *Rsa*I (the first hybridization), and the remainder or subtracted single-stranded cDNA was converted double-stranded DNA by the second hybridization with another adaptor-linked first hybridization. The subtracted double-stranded cDNA thus carried distinct adapters at both ends of the cDNA fragment. The subtracted cDNA was amplified by PCR with primer set located in adapters, and inserted into pBluescript II phagemid.

Slot-Blot Differential Hybridization—Cloned cDNAs were alkaline-denatured with 0.4 M NaOH for 10 min at room temperature and slot-blotted on nylon membranes, followed by hybridization with probe RNAs from Fao and C2 cells by the same method as Northern blot hybridization. The RNA probes were prepared as follows. Poly(A)$^+$ RNAs were randomly digested by alkaline treatment (50 mM Tris-HCl, pH 9.5, 10 nM EDTA at 90°C 30 min). By this treatment, RNA was cleaved into fragments of approximately 100 nucleotides in length with 5′-OH ends (*12*). The RNA fragments were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

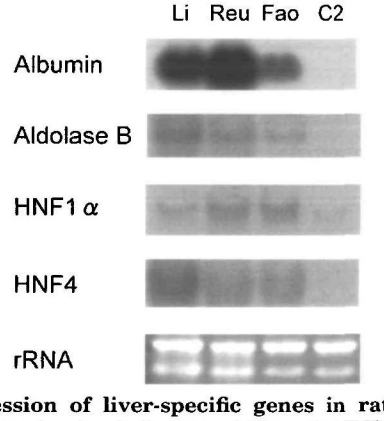

Fig. 1. **Expression of liver-specific genes in rat hepatoma cells.** Expression levels of albumin, aldolase B, HNF1α, HNF4 in rat liver (Li) and rat hepatoma cell lines, Reuber (Reu), Fao (Fao), C2 (C2) were determined by Northern blot analysis. Ten micrograms of total RNA was applied to a formaldehyde denaturing gel, transferred, and hybridized with corresponding ^{32}P-labeled cDNA. Amount of applied samples was normalized by ethidium bromide staining of the gel.

RESULTS

(1) Differentiation Levels of Reuber Cell Lineage, Fao and C2—Rat hepatoma Reuber H35 is a series of widely used cell lines that maintain well-differentiated characters. Similarly, Fao cells, which were isolated from H4IIEC3, a cell line derived from the Reuber cells, express liver-specific functions. On the other hand, C2 cells, a reversible variant cell line from the Fao cells, show decreased levels of certain liver enzymes and transcription factors. As shown in Fig. 1, cell lines Fao and C2, which were given by Dr. Mary C. Weiss and have been cultured in our laboratory, both

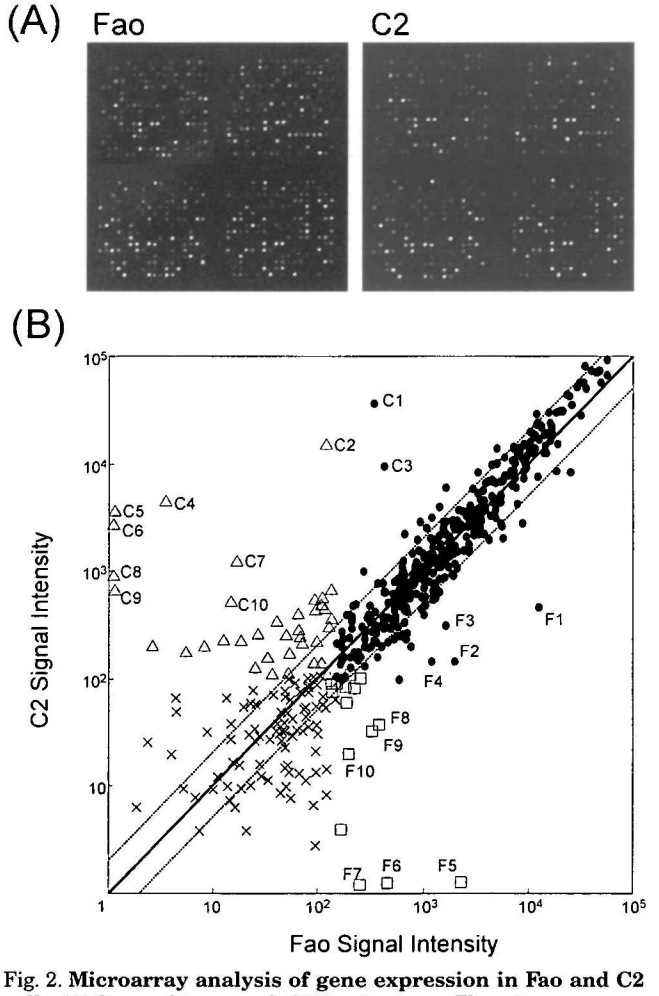

Fig. 2. **Microarray analysis of gene expression in Fao and C2 cells.** (A) Scanned images of cDNA microarray. The microarray was hybridized with a 1:1 mixture of Cy3- or Cy5-labeled first-strand cDNAs from Fao and C2 cells, respectively. The fluorescence intensity was scanned with Affimetrix 428 Array Scanner. (B) Scatter plot for expression profile comparison between Fao and C2 cell. Fluorescence intensities were analyzed using ImaGene software, and the values are corrected intensities representing levels of expression for the DNA element of the microarray. Closed circles show positive fluorescence signals to both probes, Xs indicate signal intensities below the lower limit of significance of detection of both probes, and open squares and triangles mean Fao-positive and C2-positive signals, respectively. C1 to C10 and F1 to F10 in the panel show differentially expressed genes in Fao and C2 cells, listed in Table II. Solid line shows $y = x$, and dotted lines indicate $y = 2x$ or $y = x/2$, where x is intensity of the Fao signal and y is that of the C2 signal.

retained their original characters. Fao cells abundantly expressed liver enzymes such as albumin and aldolase B and showed high levels of expression of HNF1α and HNF4, which are liver-enriched transcription factors targeting many liver-specific genes. On the contrary, C2 cells lost these gene expressions. Thus, the differentiated statuses of these cell lines were confirmed.

(2) Expression Profile of Genes Altered in Fao and C2 Cells—The profiles of gene expression in Fao and C2 are expected to afford a better understanding of the expression of hepatic function. Hence, we performed microarray analysis of genes differentially expressed between Fao and C2 cells. We used a cDNA microarray containing 1,000 nonredundant clones of defined human genes (IntelliGene Human Chip 1K; Takara), hybridized with Cy3-labeled Fao cDNA and Cy5-labeled C2 cDNA. The DNA chip was scanned with an Affimetrix 428 Array Scanner, and data were analyzed by computer software, BioDiscovery ImaGene Ver 4. As shown in Fig. 2A, scanned images of the cDNA array exhibit the expression pattern of genes in both cells. A scatter plot of the expression profiles with global normalization shows that many genes are commonly expressed by both cell types (Fig. 2B and Table I), and that the expression of a limited number of genes is enhanced or decreased in C2 cells (Fig. 2B and Table II). Expression of some genes (C1–C10), including vimentin and tissue inhibitor of metalloproteinase 2 genes, were greatly enhanced in C2 cells (Table II), while that of others, such as epoxide hydrolase 1 and translational inhibitor protein p14.5 genes (F1–F10), were greatly decreased in C2 cells during dedifferentiation from Fao cells. Several hundred genes were expressed at levels below the lower limits of detection, details of which are posted on our internet website (http://wwwlife.med.tottori-u.ac.jp/~molbio/data/jb.html).

TABLE I. **Representative genes expressed at a high level in Fao and C2 cells.**

Gene name	Accession No.	Intensity		C2/Fao ratio
		C2	Fao	
nuclease sensitive element binding protein 1	J03827	57977	45064	1.12
tubulin, alpha 1 (testis specific)	X06956	57679	63498	1.59
tyrosine 3-monooxygenase	U54778	50006	38261	1.10
tubulin, alpha 2	AF005392	48528	50844	1.51
glyceraldebyde-3-phosphate dehydrogenase	M33197	47182	50901	1.56
poly(rC)-binding protein 2	X78136	45725	48829	1.54
profilin 1	J03191	39436	50801	1.86
basic transcription factor 3	X53280	36006	34602	1.39
nucleolin	M60858	34380	54548	2.29
hnP similar to rat helix destabilizing protein	S63912	31830	18877	0.86
ribosomal protein L6	X69391	30743	38281	1.80
ribosomal protein S6	BE513192	26043	23775	1.32
ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2	X69908	23349	29729	1.84
solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	NM005888	21371	21399	1.44
antiquitin 1	S74728	19320	10305	0.77
Uba80 mRNA for ubiquitin	S79522	17477	12595	1.04
transcription elongation factor B (SIII)	Z47087	16808	14381	1.23
membrane nucleoside transporter	D14696	16576	15313	1.33
ubiquitin-activating enzyme E1	M58028	15782	20602	1.88
ribosomal rotein L5	U66589	15131	8500	0.81

TABLE II. **Expression profile of genes altered in Fao and C2 cells.**

No.[a]	Gene name	GenBank Accession No.	Intensity		Ratio	
			Fao	C2	C2/Fao	Fao/C2
C1	vimentin	AL133415	349	24865	71.2	—
C2	tissue inhibitor of metalloproteinase 2	AL110197	123	10212	82.8	—
C3	Cbp/p300-interacting transactivator	U65092	440	6372	14.7	—
C4	thrombospondin 1	X14787	4	2949	806.3	—
C5	transgelin 2	D21261	<1	1710	>1710	—
C6	annexin A1	X05908	<1	1449	>1449	—
C7	extracellular matrix protein 1	U68186	17	580	34.1	—
C8	EphB3	X75208	<1	543	>543	—
C9	transmembrane 4 superfamily member 1	M90657	<1	437	>437	—
C10	keratin 13	X14640	15	353	23.5	—
F1	epoxide hydrolase 1, microsomal (xenobiotic)	NM_000120	12878	309	—	41.6
F2	translational inhibitor protein p14.5	X95384	2018	99	—	20.2
F3	ketohexokinase (fructokinase)	X78678	1645	214	—	7.6
F4	p21/Cdc42/Rac1-activated kinase 1	U24152	1234	98	—	12.5
F5	betaine-homocysteine methyltransferase	U50929	2705	<1	—	>2705
F6	lectin, galactoside-binding, soluble, 2 (galectin 2)	AL022315	739	<1	—	>739
F7	group-specific component (vitamin D binding protein)	X03178	691	<1	—	>691
F8	nuclear RNA export factor 1 (Mex67, yeast, homolog)	AJ132712	365	24	—	15.0
F9	centromere protein F (350/400 kDa, mitosin)	U30872	341	22	—	15.5
F10	catalase	AL035079	213	13	—	16.1

[a]No's are matched with the number shown in Fig. 2B.

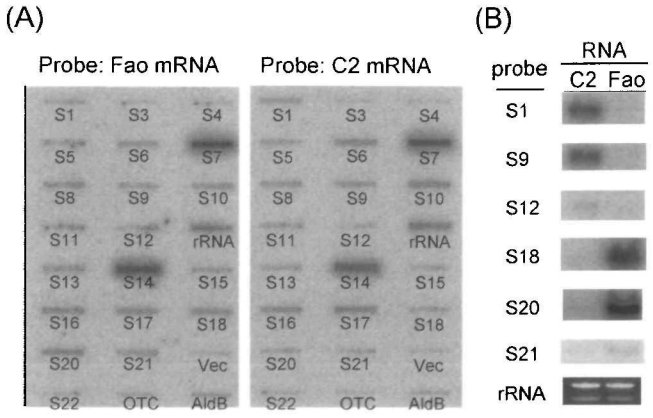

Fig. 3. **Screening of differentially expressed cDNA by subtraction PCR.** (A) Slot-blot hybridization with labeled RNAs from Fao and C2 cells. Clones isolated from SSH first screening were slot-blotted on nylon membrane, and hybridized with ^{32}P-labeled poly(A)$^+$ RNA. Clones S1 to S12 are isolated from C2 cDNA library subtracted with Fao mRNA, and clones S13 to S22 are from Fao cDNA library subtracted with C2 mRNA. Vector, rRNA, OTC, and AldB are cloning vector, cloned rRNA cDNA, ornitin transcarbamylase, and aldolase B cDNA spotted as controls for hybridization. (B) Northern blot hybridization of isolated clones as third screening. Total RNAs from Fao and C2 were electrophoresed, transferred, and hybridized with ^{32}P-labeled clone DNAs.

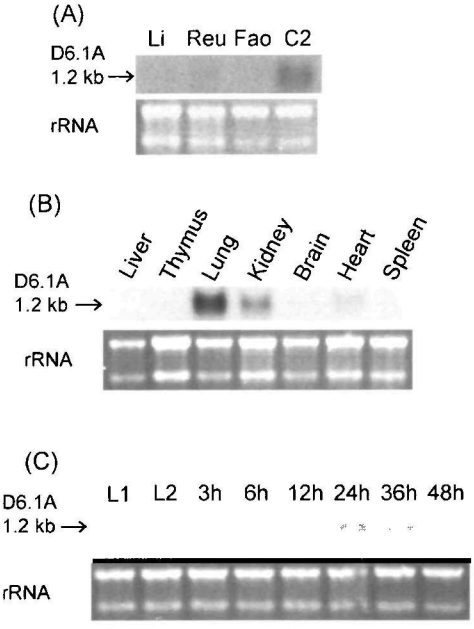

Fig. 4. **Northern blot analysis of D6.1A gene expression.** (A) D6.1A gene expression was determined by Northern blot hybridization of total RNA from rat liver (Li), Reuber (Reu), Fao (Fao), and C2 (C2) cells. (B) D6.1 gene expression was determined in various tissues. (C) Enhancement of D6.1A gene expression was examined with total RNA from CCl_4-treated rat liver. L1 and L2 are controls of non-treated rat liver. Rats were intraperitoneally injected with 2 ml of CCl_4:olive oil (1:1 mixture) per kg of body weight, and total RNA was extracted from the liver at the time indicated in the panel (3 h–48 h). All rats were fed *ad libitum* and received human care in compliance with the institution's guidelines for the care and use of laboratory animals in research.

(3) Screening of Fao and C2 Specific Genes by SSH—Profiles of gene expression revealed by the DNA chip used in this study are restricted to the 1,000 clones of the identified genes spotted on the chip. To identify other genes involved in the state of differentiation in the hepatoma cell lines, we screened genes dominantly expressed in Fao or C2 from the cDNA libraries constructed by subtracting Fao with C2 mRNA and by the inverted subtraction using the SSH protocol. To confirm the expression of the isolated genes in Fao and C2 cells in the second screening, individual clones randomly isolated from the two subtracted libraries were further analyzed by slot-blot hybridization probed with labeled Fao or C2 mRNA (Fig. 3A). Representative C2-dominant clones (S1 to S12) were isolated from C2 cDNA library subtracted with Fao mRNA, and Fao-dominant clones (S13 to S22) were isolated by the reverse subtraction. The clones S1 (rat cystatine C; Accession No. in GeneBank, X16957), S9 (the rat D6.1A; Y13275) and S12 (rat STOP protein; X93495) were abundantly expressed in C2 cells, whereas the clones S18 (the rat prothrombin; X52835), S20 (rat nucleus-encoded mitochondrial carbamyl phosphate synthetase I; M11710), and S21 (GTPI protein; AJ007972) were dominant in Fao cells. These observations were confirmed by Northern blot analysis of Fao and C2 mRNA using the respective cDNA clones as probes (Fig. 3B). The clones S7 (unknown) and S14 (rat cytochrome B; AF295545) were expressed at high level in both cell types in spite of subtracted clones. By this screening, we obtained cDNA clones that were not listed in the DNA array.

(4) D6.1A Gene Expression in Various Tissues and in Liver Injury—Among the C2-dominant clones, S9 was proved to be a cDNA for the rat D6.1A gene by homology search in the GeneBank database. The product of D6.1A gene is known to be a transmembrane protein belonging to the tetraspanin superfamily and to be induced in metastasis *in vivo*, but regulation of its expression has not been studied in detail. We are particularly interested in the gene induction in the dedifferentiated hepatoma C2 cells.

As shown in Fig. 4A, the D6.1A gene was expressed in C2 cells, but scarcely in the rat liver, Fao or Reuber cells, the parental cell line of Fao and C2. On the other hand, in normal rat tissues, the gene expression was clearly observed in lung and kidney, and slightly in heart, but not in liver, thymus, brain or spleen (Fig. 4B). The tissue-specific expression of the D6.1A gene cannot be well elucidated now. However, we found that D6.1A gene expression was enhanced in acute liver injury induced by treatment with carbon tetrachloride (CCl_4) in Wistar rat. As shown in Fig. 4C, when the liver injury was induced by intraperitoneal injection with CCl_4, the D6.1A gene expression was strongly stimulated at 24 h post-injection and maintained in significant level for up to 48 h. These observations suggested that the D6.1A gene expression might be induced in proliferative action in the cells stimulated by liver injury, as well as in active cell growth in the C2 cells.

DISCUSSION

Rat hepatoma cell line Fao is a well-differentiated line derived from Reuber minimum deviation hepatoma cells. C2 cells, which were obtained from the Fao cell population, fail to express these differentiated traits (*3*). However, the hepatic phenotype in C2 cells is recovered by culture in glu-

cose-free medium at a low frequency (*4*). Therefore, this cell line system is invaluable in *in vitro* experiments to investigate the differentiation mechanism of hepatocytes. Recently, hepatocyte nuclear factor 4 (HNF4) was reported to integrate the genetic programs of liver-specific gene expression and to act as a morphogen in dedifferentiated hepatoma cells (*13*). The present study showed that the expression of HNF4 gene was decreased in C2 cells, while the HNF4 was maintained at high level in Fao cells.

In this study, we examined the gene expression profiles of Fao and C2 cells which share a common origin but are in distinct differentiation states. Techniques currently available for analysis of gene expression are highly developed, involving DNA chips or microarray systems (*9*), expressed sequence tags (EST) (*14*) and serial analysis of gene expression systems (SAGE) (*15*). We chose microarray analysis using a DNA chip containing 1,000 identified human cDNAs. In Fao cells, the genes showing a high level of expression seem to be those involved in hepatic function, such as metabolism of nutrients (epoxide hydrolase 1, ketohexokinase), whereas in C2 cells the genes showing enhanced expression are those related to extracellular matrix components (tissue inhibitor of metalloproteinase 2, extracellular matrix protein 1, thrombospondin 1, keratin 13, and so on), membrane proteins and their regulators (annexin A1, EphB3, transmembrane 4 superfamily member 1), and cytoskelton (vimentin, transgelin). While some hepatic proteins such as albumin failed to show positive signals because of the low homology between human and rat genes, the difference in the gene expression profiles cannot be explained only by the disappearance of HNF1 and HNF4 gene expressions from the Fao cells.

To identify more genes that are differentially expressed in the Fao and C2 cells, we performed further screening of a cDNA subtraction library constructed by the SSH method (*10, 11*). Among the isolated clones, S9 clone was the rat D6.1A gene, which encodes a 235–amino acids transmembrane protein of the tetraspanin superfamily (*16, 17*), and has been isolated from metastatic cells. However, Cbp/p300-interacting transactivator (msg1), which is a nuclear protein enhanced in the low metastatic clone B16-F1 cell (*18*), was abundantly expressed in C2 cells. These observations suggested that the D6.1A gene product in C2 cells might be involved in malignancy of hepatocytes, rather than in metastasis. Most tetraspanin superfamily proteins serve as molecular facilitators for multifunctional integrin β1 family (*19*) and are involved in cell proliferation, cell adhesion, tumor metastasis, and signal transduction (*16, 19–22*). Interestingly, paxillin and α-actinin, mediator proteins of the integrin β1 signal, were slightly induced in C2 cells, and the cytoskeltal proteins vimentin and transgelin were highly enhanced, indicating the modulation of cell functions through the cytoskelton.

The D6.1A gene was expressed abundantly in lung and kidney in normal rat but hardly expressed in liver and other tissues. Interestingly, induction of the gene expression was also observed following liver injury in CCl_4-treated rat. Likewise, the stimulation of TM4SF4, a distinct member of tetraspanin family protein, was reported in regenerating liver following partial hepatectomy (*23*). These observations suggest that the function of D6.1A in normal lung and kidney tissues may be different from that in regenerating liver or liver injury, and also different from its function in the dedifferentiated hepatoma cells, depending on the variety of target molecules of the D6.1A protein. However, the question remains whether the D6.1 gene expression is directly concerned with dedifferentiation in C2 cells. To address this question, induction of the D6.1A gene in Fao cells and withdrawal of the expression from C2 cells are necessary in future study.

We thank Dr. Masatsugu Moriyama for his helpful discussion and critical reading of the manuscript, and Koichi Adachi for his excellent technical assistance.

REFERENCES

1. Clayton, D.F., Weiss, M., and Darnell, J.E. Jr. (1985) Liver-specific RNA metabolism in hepatoma cells: variations in transcription rates and mRNA levels. *Mol. Cell. Biol.* **5**, 2633–2641
2. Herbst, R.S., Neilsch, U., Sladek, F., Lai, E., Babiss, L.E., and Darnell, J.E. Jr. (1991) Differential regulation of hepatocyte-enriched transcription factors explains changes in albumin and transthyretin gene expression among hepatoma cells. *New Biol.* **3**, 289–296
3. Deschatrette, J. and Weiss, M.C. (1974) Characterization of differentiated and dedifferentiated clones from a rat hepatoma. *Biochimie* **56**, 1603–1611
4. Deschatrette, J., Moore, E.E., Dubois, M., and Weiss, M.C. (1980) Dedifferentiated variants of a rat hepatoma: reversion analysis. *Cell* **19**, 1043–1051
5. Chomoczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
6. Kuribayashi, K., Hirata, M., Hiraoka, O., Miyamoto, C., and Furuichi, Y. (1988) A rapid and efficient purification of poly(A)$^+$-mRNA by oligo(dT)-Latex. *Nucleic Acids Res. Symp.* **19**, 61–64
7. Goldberg, D.A. (1980) Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. *Proc. Natl. Acad. Sci. USA* **77**, 5794–5798
8. Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13
9. Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467–470
10. Gurskaya, N.G., Diatchenko, L., Chenchik, A., Siebert, P.D., Khaspekov, G.L., Lukyanov, K.A., Vagner, L.L., Ermolaeva, O.D., Lukyanov, S.A., and Sverdlov, E.D. (1996) Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: cloning of Jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate. *Anal. Biochem.* **240**, 90–97
11. Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., and Siebert, P.D. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**, 6025–6030
12. Favaloro, J., Treisman, R., and Kamen, R. (1980) Transcription maps of polyma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. *Methods Enzymol.* **65**, 718–749
13. Späth, G.F. and Weiss, M.C. (1998) Hepatocyte nuclear factor 4 provokes expression of epithelial marker genes, acting as a morphogen in dedifferentiated hepatoma cells. *J. Cell Biol.* **140**, 935–946
14. Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B., and Moreno, R.F. (1991) Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252**, 1651–1656
15. Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995) Serial analysis of gene expression. *Science* **270**, 484–487

16. Claas, C., Seiter, S., Claas, A., Savelyeva, L., Schwab, M., and Zoller, M. (1998) Association between the rat homologue of CO-029, a metastasis-associated tetraspanin molecule and consumption coagulopathy. *J. Cell Biol.* **141**, 267–280
17. Szala, S., Kasai, Y., Steplewski, Z., Rodeck, U., Koprowski, H., and Linnebach, A.J. (1990) Molecular cloning of cDNA for the human tumor-associated antigen CO-029 and identification of related transmembrane antigens. *Proc. Natl. Acad. Sci. USA* **87**, 6833–6837
18. Shioda, T., Fenner, M.H., and Isselbacher, K.J. (1996) *msg*1, a novel melanocyte-specific gene, encodes a nuclear protein and is associated with pigmentation. *Proc. Natl. Acad. Sci. USA* **93**, 12298–12303
19. Maecker, H.T., Todd, S.C., and Levy, S. (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J.* **11**, 428–442
20. Marken, J.S., Schieven, G.L., Hellstrom, I., Hellstrom, K.E., and Aruffo, A. (1992) Cloning and expression of the tumor-associated antigen L6. *Proc. Natl. Acad. Sci. USA* **89**, 3503–3507
21. Beinert, T., Munzing, S., Possinger, K., and Krombach, F. (2000) Increased expression of the tetraspanins CD53 and CD63 on apoptotic human neutrophils. *J. Leukoc. Biol.* **67**, 369–373
22. Zhang, X.A., Bontrager, A.L., and Hemler, M.E. (2001) Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific β1 integrins. *J. Biol. Chem.* **27**, 25005–25013
23. Liu, Z., Zhao, M., Yokoyama, K.K., and Li, T. (2001) Molecular cloning of a cDNA for rat TM4SF4, a homolog of human il-TMP (TM4SF4), and enhanced expression of the corresponding gene in regenerating rat liver. *Biochim. Biophys. Acta* **1518**, 183–189