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Identification of Genes Differentially Expressed by Hypoxia in Hepatocellular Carcinoma Cells

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In order to identify genes differentially expressed under hypoxia (1% O_2, 5% CO_2, balance N_2), we performed mRNA differential display analysis using total RNA extracted from hypoxic and normoxic HepG2, human hepatocellular carcinoma (HCC) cells. Of the differentially expressed genes by hypoxia, some of cDNA fragments were cloned and sequenced. The expression patterns of these clones by hypoxia were confirmed by Northern blot analysis and the quantitative RT-PCR. Down-regulated genes by hypoxia have homology to cDNA sequences encoding cytochrome oxidase subunit II and ADP/ATP translocase, respectively. Up-regulated gene by hypoxia was identified as *Homo sapiens* oscillin. Moreover, novel genes induced by hypoxia represent partial sequences of cDNAs that have not been reported or functionally identified. Up- or down-regulated expression of these genes in response to hypoxia may contribute to human hepatocarcinogenesis.

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Hypoxia, the condition of low cellular oxygen tension, is a feature of both physiological (1) and pathological conditions including ischemia, fibrosis (2), and neoplasia (3). Mammalian cells respond to hypoxia by increasing the expression of several genes that are related in erythropoiesis (4), angiogenesis (5), apoptosis (6), glycolysis (7), xenobiotic detoxification, and/or by decreasing the expression of genes that are involved in gluconeogenesis and Krebs' cycle (8). Thus, hypoxia may work as an essential trigger for significant genetic reprogramming with selective gene induction and down-regulation.

Human solid tumors including HCC, even those less than 1 cm in a diameter (i.e., at the limits of clinical detection), may have substantial hypoxic fractions. Many reports strongly indicated that hypoxia in various cancer cell lines is a practical signal to induce angiogenic factors (9), some transcriptional factors (10, 11), and so on. Therefore, it is highly probable that the expression of many other genes that have not been reported yet is regulated in the hypoxic microenvironment of the tumors.

In this study, we have used the technique of mRNA differential display (12) to detect genes differentially expressed under hypoxia in HepG2 human HCC cells. The HepG2 cells are the permanent cell culture models available to study oxygen-regulated expression (13, 14). This differential display strategy enabled us to identify genes that were up- or down-regulated in hypoxic (1% O_2) HepG2 cells as compared to normoxic (21% O_2) control cells. Present study demonstrated that hypoxia decreased mRNA levels of cytochrome oxidase subunit II and ADP/ATP translocase and also increased those of oscillin and novel genes. Our results are the first one describing the use of differential display to identify differentially expressed genes under hypoxic conditions in HCC cells.

MATERIALS AND METHODS

Cell culture and Hypoxic treatment. HepG2 cells, human HCC cells (2.0×10^6 cells) were plated in 75 cm^2 flask and cultured in minimum essential medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL), 100 units/ml of penicillin and 100 μg/ml of streptomycin. For hypoxic condition, cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2. Oxygen tension (pO_2) in the incubator (Forma Scientific) was either 140mm Hg (21% v/v, normoxia) or 7mm Hg (1% v/v, hypoxia).

RNA isolation. Total RNA to be used for Northern blot analysis was isolated according to the single step guanidium thiocyanate-phenol-chloroform extraction procedure using Trizol (Gibco/BRL) according to the manufacturer's instructions. For differential display analysis, possible DNA contamination was removed by treating the obtained RNA with RNase-free DNase I for 30 min at 37°C. After phenol/chloroform (3/1) extraction and ethanol precipitation, RNA was resuspended in DEPC-treated H_2O.

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Differential display analysis. Differential display was carried out using RNAmap kit (Genhunter Corp., Brookline, MA). DNA-free total RNA (0.4 μg) was reverse-transcribed using $T_{12}MA$, $T_{12}MT$, $T_{12}MG$, and $T_{12}MC$ primer where M represent a degenerated mixture of dA, dG, dC. 1/10 of reverse transcribed mixture was used directly for PCR amplification in the presence of α-S^{35} ATP (1200 Ci/mmole, Amersham, Arlington, IL) using Taq polymerase (Amplitaq, Perkin Elmer, CA, U.S.A). The 20 different primer sets used for PCR amplification were the combination of five 5′-AP (arbitrary primer) with the four 3′-$T_{12}MN$ primer that were used for initial cDNA synthesis. The cycle parameters were as follows: 94°C for 30s, 40°C for 2min, 72°C for 30s for 40 cycles, followed by 72°C for 5min. The amplified cDNAs were separated on a 6% DNA sequencing gel and autoradiographed with X-ray film. To determine cDNA size, partial sequencing of the DNA from bacteriophage M13mp18 by the chain-termination method was performed on the same gel as used in the differential display.

Recovery and reamplification of cDNAs. Differentially displayed cDNAs were cut from the dried sequencing gel, and the DNA was recovered and reamplified, using the same primer set and PCR conditions as used in the differential display except for dNTP concentrations of 20 μM and no radioisotope. The amplified cDNA was purified with Quiaquick spin columns according to the manufacturer's conditions (Quiagen, Hilden, Germany).

Cloning and sequencing of cDNAs. The reamplified cDNA bands were cloned into plasmid pGEM-T vector using pGEM-T vector system (Promega, Madison, WI) according to the manufacture's instructions. Plasmid DNA was purified using a Wizard Plus Miniprep Kit (Promega Corporation). Plasmid DNA sequencing of cloned cDNAs with either T7 or SP6 primer was carried out using the Sequenase version 2.0 DNA sequencing kit (United States. Biochemicals, Cleveland, OH) following the manufacture's instructions or using the ABI PRISMTM Dye Terminator cycle sequencing ready reaction kit protocol with the Automated DNA sequencer (Perkin Elmer ABI PRISMTM model). Comparison of DNA homology and Protein homology with GenBank and the EMBL data base was performed using BLAST and FASTA.

Northern blot analysis. Total RNAs (20 μg) were electrophoresed on 1.2% agarose / formaldehyde agarose gels, transferred to Zeta-probe membranes (Bio-Rad, Hercules, CA). cDNAs obtained from the differential display reaction used as probe for Northern blot analysis. Probes were [α-^{32}P] dCTP labeled using the Rediprime DNA labelling system (Amersham) and hybridized to filters overnight at 42°C. Filters were washed at 42°C in 2× SSC, 0.1% SDS for 15min and 0.5× SSC, 0.1% SDS for 15 min. The filters were then exposed to X-ray film for 1 to 4 days.

Reverse Northern blot analysis. Reamplified bands were resolved on 1.5% agarose gel, applied to nylon membranes (Zeta-Probe membrane; Bio-Rad). 5 μg of total RNA from normoxic or hypoxic HepG2 cells were reverse transcribed as descibed above, except oligo$(dT)_{12\text{-}18}$ was used as a primer. Radioactive DNA probe was produced from the cDNA reaction by the Rediprime DNA labelling system (Amersham). Probes were added to each filter and hybridized in (0.5M sodium phosphate/ 7% SDS) overnight at 65°C. Filters were washed in 20mM sodium phosphate/ 0.1% SDS at 65°C for 15min and exposed for 1 day.

Reverse transcription-polymerase chain reaction (RT-PCR). 5 μg of each DNA -free total RNA sample, prepared as above, was converted into cDNA with Superscript II RNase H- reverse transcriptase and oligo$(dT)_{12\text{-}18}$(GIBCO BRL) according to the manufacturer's instructions. Equal amounts of cDNA were subsequently amplified by PCR in a 50 μg reaction volume containing 1× PCR buffer, 200 μM dNTPs, 1.5mM $MgCl_2$, 0.2 μM of each specific primer and 2.5U Taq DNA polymerase. Control amplification were performed with β-actin to confirm the use of equal amounts of RNA and to allow PCR products to be quantitated comparatively. The sequences of the primers for candidate clones were designed from the sequence of the cloned differential display products. Amplification products were electorophosed on 1.8% (w/v) agarose gels and visualized with ethidium bromide. DNA was then transferred to nitrocellulose membrane, hybridized with [α-^{32}P] dCTP-labeled probe, an oligonucleotide sequence included in the amplified fragments, and subjected it to autoradiography on Kodak XAR film. Quantitation of bands was performed with densitometer (Molecular Dynamics).

RESULTS

To identify genes whose expression was altered in response to hypoxia in HepG2 cells, we performed mRNA differential display using total RNAs extracted from hypoxic and normoxic HepG2 cells incubated for 2, 8, and 16 h. A total of 5 arbitrary 10-mer primers were used in combination with all 4 $T_{12}MN$ primers. Utilizing the application of 20 different primer sets, our results

TABLE 1

Summary of Hypoxic-Regulated Differential Display Clones in HepG2 Cells

Clone	Effect of hypoxia	Northern blot/RT-PCR conformation	Identity/Homology
A-21	down	Confirmed	Human cytochrome oxidase subunit II (385/390:98%)
G-3	down	Confirmed	Human ADP/ATP translocase (150/154:97%)
G-4	up	Confirmed	Human DNA sequence from PAC 528L19 chromosome 6q23 (221/221: 100%)
A-8	up → down	Confirmed	*Rattus norvegicus* rsec 8 (101/108: 93%)
T-7	up	Confirmed	*Homo sapiens* oscillin (hLn) (298/307:97%)
G-21	up → down	Confirmed	Unknown
A-27	up → down	Confirmed	Unknown
G-5	down	Not confirmed	Unknown
G-11	up	Not confirmed	Unknown
G-12	up	Not detectable	Unknown

The letter 'up' indicated that, by visual inspection, the mRNA differential display band was more intense in the hypoxia than in the normoxia, while the letter 'down' indicated the reverse. Homology or identity for each clone, excluding the PCR primer sequences, was identified as the highest scoring homologue from BLAST searches of GenBank.

yielded 29 up-regulated and 22 down-regulated cDNA fragments under hypoxic conditions. All of these were successfully recovered from the dried gel, reamplified, and used as probes for reverse Northern blotting (which included a positive control, band C-4). Among them, 10 bands were observed to be differentially expressed by the reverse Northern blotting (data not shown). These 10 cDNAs were cloned into the vector pGEM-T and then sequenced in both directions. The results were summarized in Table 1. Next, each of 10 subcloned cDNA inserts excised from the vectors was used as probe for Northern blot analysis to confirm its expression. A time course of hypoxic treatment on HepG2 cells was performed, including a normoxic control for every time point. Two of the clones, identified as encoding human cytochrome oxidase subunit II (COXII) (Genbank accession no. X15759) and human ADP/ATP translocase (ANT) (Genbank accession no. J03592), showed time-dependent down-regulation in their expression under hypoxia. In contrast, the level of clone G-4 mRNA was increased by hypoxia within 4h and reached a maximum levels at 24h. Surprisingly, we found that the nucleotide sequence of clone G-4 completely matched to based 73261-73481 of human DNA sequence from PAC 528L19 on human chromosome 6q23 (Genbank accession no. Z84486). In case of clone G-21, mRNA level was increased up to 8h exposure of hypoxia and then declined (Fig. 1). To investigate the expression of candidate clones that had no hybridization signal in Northern blot analysis, perhaps due to their low copy messages, RT-PCR analysis was performed. Clone T-7, showing a significant homology to *Homo sapiens* oscillin (Genbank accession no. AF029914), revealed that its expression elicited a 2-fold increase by hypoxia compared to normoxia at 8h. The expression of clone A-8 was induced at early time points, 2h and 4h exposure of hypoxia, and then declined (Fig. 2). In addition, a search of protein data base using BLASTAX indicated that the amino acid sequence of A-8 clone was completely matched to amino acid 55-92 of *Rattus norvegicus* of rSec8 (Genbank accession no. 2143962) and *Mus musculus* mSec8, both of which have region to interact with GTP-binding protein (Fig. 3). Finally, the remaining clones including clone G-21 are novel genes as they have no significant homology in Genbank.

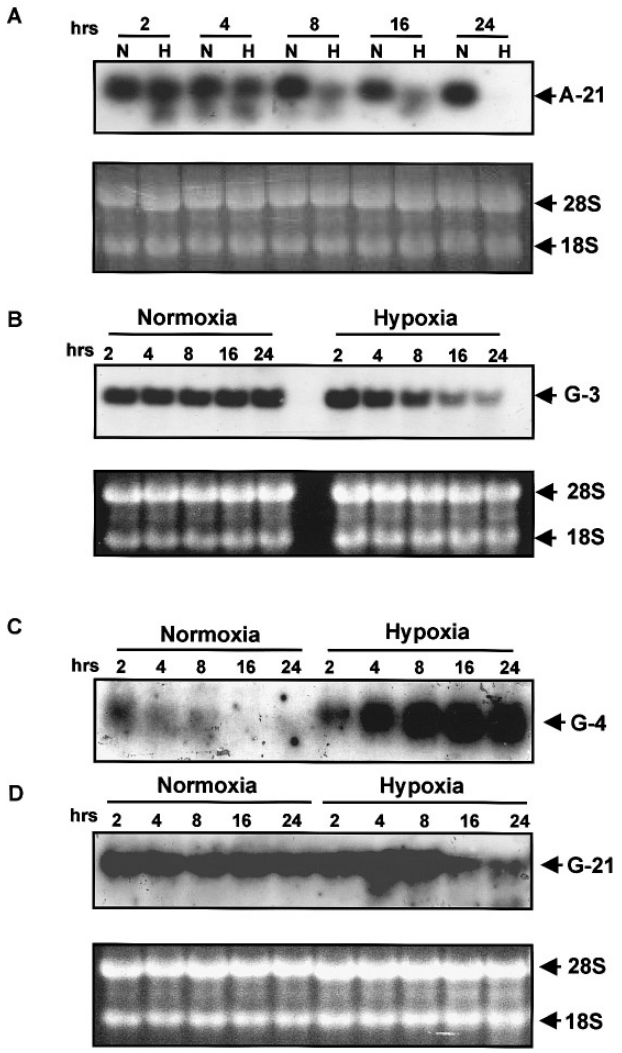

FIG. 1. Time-dependent Northern blot analysis of differential display clones regulated by hypoxia in HepG2 cells. Northern blot analysis of total RNA from normoxia or hypoxia-treated HepG2 cells for indicated time point. The blot was probed with ^{32}P-labeled differentially displayed cDNAs; A, clone A-21, B, clone G-3, C, clone G-4, and D, clone G-21. Lower panels; Ethidium bromide-stained RNA samples before transfer; Ribosomal RNA markers (18S and 28S) are indicated. N, normoxia; H, hypoxia.

DISCUSSION

In this study, the mRNA differential display method was applied to identify genes whose expression is differentially regulated under hypoxia in HepG2 cells. We identified that under the hypoxic condition, human cytochrome oxidase subunit II (COXII) and human ADP/ATP translocase (ANT) were down-regulated, whereas *Homo sapiens* oscillin and clone G-4 were up-regulated. In addition, the expression of clone G-21 and A-8 was up-regulated up to 8h of hypoxia and then down-regulated.

As one of the electron-driven proton pumps of oxidative phosphorylation, cytochrome oxidase plays an important role in determining the rate of energy production in yeast and other eukaryotes (15, 16) and as an oxygen sensor in hepatocytes (17). The functions of ADP/ATP translocase, encoded by a nuclear gene (18), is well known to maintain cytosolic ATP concentration by translocation newly synthesized ATP from inner side of the mitochondria to the cytosol, and ADP from cytosol to the inner side of the mitochondria (19). These reported data indicate that the down-regulation of both genes by hypoxia is responsible, at least in part, for the depletion of ATP under hypoxic condition. The deple-

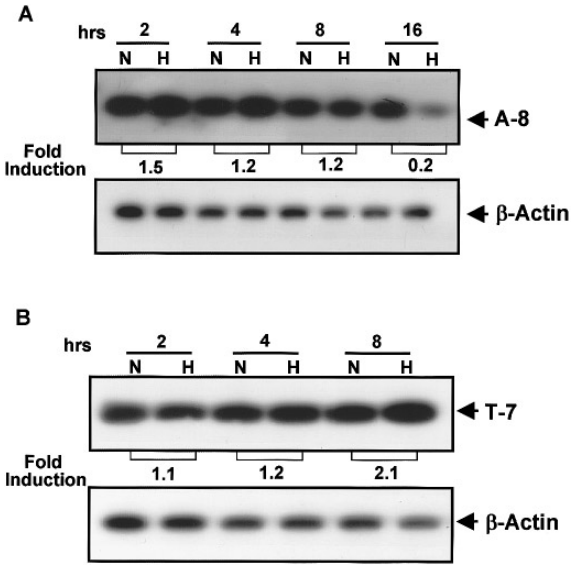

FIG. 2. Expression of hypoxic-regulated genes detected by RT-PCR. RT-PCR analysis was performed as described under "Materials and Methods" using total RNA (5 μg) isolated from cells incubated under hypoxia (H) and normoxia (N) for each time point. The quantity of PCR products were confirmed by Southern blot analysis using the probes an oligonucleotide sequence included in the amplified fragments; A, clone A-8 and B, clone T-7. Control amplifications were performed with β-actin to confirm the use of equal amounts of RNA.

tion of ATP may subsequently disrupt many energy dependent mechanisms, *e.g.*, the functioning of ATP-dependent membrane bound pumps, thereby resulting in a rise of intracellular calcium level and a fall of pH. The changes in $[Ca^{2+}]$ or pH induce the activation of intracellular endonuclease, resulting in cleavage of DNA in specific patterns of nucleosome length fragments and then leading to the apoptotic cell death (20). Others and we have reported recently that hypoxia induces apoptosis in various tumor cells including HepG2 cells (21, 22). Furthermore, recent studies indicated that hypoxia provides a physiological selective pressure in some tumors for the expansion of variants that have lost their apoptotic potential (23). Therefore, our results suggest that ATP depletion caused by reduced level of COXII and ANT mRNA under hypoxia may be a significant factor in the induction of apoptotic cell death by hypoxia. During this process, apoptosis resistant cells might be selected and these might finally contribute to the malignancy of HCC.

Homo sapiens oscillin is a specific protein that was correlated with the ability of sperm extracts to trigger Ca^{2+} oscillations in fertilization (24, 25) and to mediate Ca^{2+} release from intracellular stores (26). Recent reports indicated that the change of intracellular $[Ca^{2+}]$ by hypoxia drives the induction of many genes (27, 28) including VEGF gene (29). The function of VEGF as a potent angiogenic factor (30) contributes to the progress of neovascularization in many tumors such as glioma, breast cancer, and HCC *etc.*, suggesting that $[Ca^{2+}]$ plays an important role in tumor neovascularization via the induction of VEGF expression under hypoxia. Thus, it is likely that $[Ca^{2+}]$ regulated by many factors including oscillin is an important signal transducer in hypoxia-mediated neovascularization in HCC.

The deduced amino acid sequence of clone A-8 is completely identical to a part of N-terminal of rSec8, mammalian homologs of yeast proteins essential for secretion (31). N-terminal of rSec8 contains region, which interacts with the GTP-binding protein such as Rab3a. In addition, the deduced amino acid sequence of this clone has partly ~40% sequence identity with COOH-terminal domain of β-adrenergic receptor kinase (β ARK) (32, 33). This domain is also involved in the interacting with $\beta\ \gamma$ subunit of heterotrimeric G proteins (34) known to activate β ARK. Therefore, it is likely that the putative motif of clone A-8 is essential for the interaction with GTP-binding protein. Taken together, we suggest that clone A-8 may play a role in acting as a relay integrating cellular signal transduction pathways from hypoxia. However, for the verification of this, it is still required the full-length sequence for clone A-8 and functional characterization of the encoded protein.

Most interestingly, clone G-4 sequence is completely matched to the part of chromosome 6q23 that submitted by large-scale sequencing of human genome project, although its product and role have not yet been determined. The human chromosome 6 is reported to encode genes involved in genetic diseases including arthritis, diabetes, haemochromatosis, and *etc.*. Haemochromatosis is an autosomal recessive disorder of iron metabolism. The end effects of this disease include liver cirrhosis and HCC. Moreover, the chromosomal aberrations of chromosome 6q are significantly elevated in HCC (35). Therefore, clone G-4 showing hypoxia-induced expression in HepG2 human HCC cells may play a role during hepatocarcinogenesis. Further studies are now going to elucidate the detailed role of this clone.

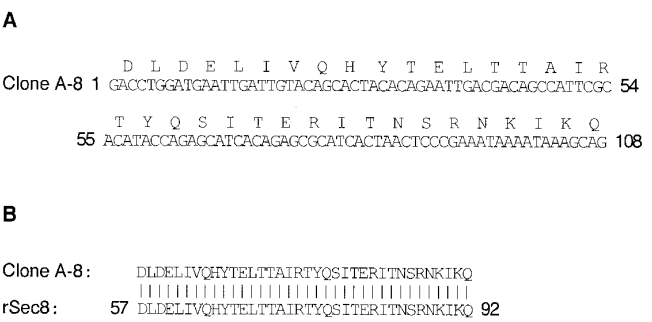

FIG. 3. Nucleotide and deduced amino acid sequence of clone A-8. A, The deduced amino acid sequence is shown by the single letter code above the nucleotide sequence of clone A-8. B, Comparison of deduced nucleotide amino acid sequence clone A-8 with partial protein sequence of *Rattus norvegicus* rsec8 (aa 57-92).

In summary, we have demonstrated the utility of mRNA differential display as a molecular screen for differentially regulated genes in the human HCC cells under hypoxia; genes of known and unknown function have been identified. Further investigations about these genes differentially expressed under hypoxia will helpful to make the gene profile which provide much information of gene regulation during tumorigenesis. Furthermore, elucidating the molecular base about tumorigenesis will be important for understanding the process of tumor progression and also for application the basic knowledge of tumors to clinical and therapeutic fields.

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