Journal of Biochemistry and Molecular Biology, Vol. 33, No. 5, September 2000, pp. 396-401

JOURNAL OF
Biochemistry and Molecular Biology

Screening of Differentially Expressed Genes by Desferrioxamine or Ferric Ammonium Citrate Treatment in HepG2 Cells

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Received 31 July 2000, Accepted 23 August 2000

A differential display method is used to identify novel genes whose expression is affected by treatment with ferric ammonium citrate (FAC) or desferrioxamine (DFO), an iron chelating agent in the human hepatoblastoma cell line (HepG2). These chemicals are known to deplete or increase the intracellular concentration of iron, respectively. Initially, we isolated seventeen genes whose expressions are down- or up regulated by the treatment of the chemicals, as well as their four differentially expressed genes that are designated as clone-1, -2, -3, and -4. These are further characterized by cDNA sequencing and Northern blot analysis. Through the cDNA sequencing, as well as comparing them to genes published using the NCBI BLAST program, we identified the sequence of the clone-1 that is up-regulated by the treatment of DFO. It is identical to the human insulin-like growth factor binding protein-1 (IGFBP-1). This suggests that the IGFBP-1 gene in the HepG2 cell is up-regulated by an iron depletion condition. Also, the expression of the clone-3 and -4 is up-regulated by FAC treatment and their cDNA sequences are identical to the human ferritin-light chain and human NADH-dehydrogenase, respectively. However, the sequence of the clone-2 has no significant homology to any other known gene. Therefore, we suggest that changes of the cellular iron level in the HepG2 cell affects the transcription of cellular genes. This includes human IGFBP-1, ferritin-light chain, and NADH-dehydrogenase. Regulation of these gene expressions may have an important role in cellular functions that are related to cellular iron metabolism.

Keywords: DDRT-PCR, Desferrioxamine, Ferric ammonium citrate, Iron regulatory protein, Transferrin receptor.

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Introduction

Iron (Fe) is an essential element that is required for cell multiplication and several other important cellular functions. Iron uptakes lead the binding of transferrin (Tf) to the transferrin receptor (TfR) on the cell membrane (Richardson and Ponka, 1995). The Tf-TfR complex formed is internalized by the receptor-mediated endocytosis (RME), then iron is released from the Tf-TfR complex by a process involving endosomal acidification and reduction (Klausner *et al.*, 1983; Nunez *et al.*, 1990). Cellular iron homeostasis is maintained by the coordinate regulation of iron uptake via the TfR, as well as by iron accumulation through the intracellular iron-storage capacity of ferritin (Coccia *et al.*, 1997). In higher eukaryotes, ferritin and TfR are responsible for the uptake and detoxification of iron (Klausner and Harford, 1989). The expression of both the TfR and ferritin is highly regulated by the amount of available iron (Mullner and Kuhn, 1988). For example, iron depletion results in a quantitative increase of TfRs (Ward *et al.*, 1982; Bridges and Cudkowicz, 1984; Mattia *et al.*, 1984), whereas the number of TfRs is decreased when the iron supply is sufficient (Rao *et al.*, 1986). However, the ferritin level in the cell is reversibly regulated by iron, compared to the regulation of the TfR number (Yu *et al.*, 1992). Free iron, as its non-protein-bound and low molecular weight form, causes mainly cellular damage by participating in the generation of hydroxyl radicals (Kontoghiorghes, 1995), the principal reagent of oxidative DNA damage and mutagenesis (Cerutti, 1985). The coordinate regulation of TfR and ferritin expression by iron via post-transcriptional mechanisms provides a rapid way for the cell to minimize the toxic effects of free iron (Yu *et al.*, 1992). However, desferrioxamine (DFO), a biological product derived from ferrioxamine B, is currently used in an attempt to prevent possible damage from the long-term exposure of iron overloaded (Cragg *et al.*, 1998). It can also be used as a tool to examine iron deficiency in the cell culture (Ye and Connor, 1999). Therefore, changes of cellular iron concentration can

be challenged by treatment of cells with ferric ammonium citrate (FAC) or DFO.

In this study, we report that several cellular genes, including human IGFBP-1, ferritin-light chain, and NADH-dehydrogenase genes, are differentially up-regulated by the treatment of FAC or DFO in HepG2 cells using an mRNA differential display technique (Liang and Pardee, 1992). We also suggest that over-expression of these genes may play an important role in the cellular functions that are related to cellular iron metabolism.

Materials and Methods

Materials Ferric ammonium citrate (13.75% by mass) and desferrioxamine were purchased from Sigma Chemical Co. (St. Louis, USA). The Dig-labeling kit and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, Germany. The MessageClean™ kit and DNase I were purchased from GenHunter Corp. (Brookline, USA) and the DNA sequencing kit and primers that were used in the differential display reverse transcription (DDRT)-PCR were obtained from Bioneer (Taejon, Korea). All other chemicals used in the RNA work were prepared in diethyl pyrocarbonate (DEPC)-treated water.

Cell culture The HepG2 cell, a human hepatoblastoma cell line, was grown in a RPMI 1640 medium (GIBCO-BRL) that was supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 and maintained at a density of 1.5×10^5 cells per ml. Approximately 12 h after the cell plating for cell attachment, the medium was replaced by fresh medium. Then, the cells were exposed to FAC (100 μg/ml) or DFO mesylate (100 μM) for 20 h in a RPMI-1640 medium. Untreated cell cultures (FAC- or DFO-free cells) were used as a control.

Preparation of total RNA Total RNAs were prepared from cultured HepG2 cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Briefly, the cells were washed in a phosphate buffered saline (PBS) buffer and lysed quickly in an ice-cold lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% Sarkosyl). After a quick centrifugation, a 1.2 volume of the SDS solution (2%) was added to the resulting supernatant and the mixture was treated with phenol/chloroform and chloroform. The extracted total RNAs were precipitated by ethanol and washed in 70% ethanol. The resulting RNAs were redissolved in DEPC-treated water. For removal of the residual DNAs that was contaminated in the prepared total RNA solution, the re-dissolved RNAs were treated with MessageClean™ Kit and Dnase I according to the manufacturer's protocols.

mRNA differential display A differential display (DD) analysis was performed as described previously (Liang and Pardee, 1992), but with some modifications (Park *et al.*, 2000). Each 1 μg of DNA-free RNAs that was prepared from untreated FAC- and DFO-treated HepG2 cells were reverse transcribed using the one-base anchored primer, H-T_{11}N (5'-AAGCT_{11}N-3', N represents G, A, C) in a reaction volume of 20 μl. Subsequently, each of these reaction products was amplified by PCR using the corresponding H-T_{11}N primer and 14 different arbitrary primers. Thermal Cycling Parameters, using the GeneAmp PCR System 4600 (Perkin-Elmer Corporation, Norwalk, USA), were as follows: 94°C for 30 sec, 42°C for 2 min, and 72°C for 30 sec for 30 cycles, followed by 72°C for 5 min. The amplified cDNAs were separated on a 6% DNA sequencing gel and visualized by the silver staining method (Bassam *et al.*, 1991).

Elution and re-amplification of cDNA fragments The differentially displayed cDNAs were directly transferred from the gel into the PCR tube using a sterile pipette tip. The gels were diluted with 18 μl of water and re-amplification procedures were performed using the initial set of primers (30 cycles of PCR at 94°C for 30 sec, 42°C for 2 min, and 72°C for 30 sec, followed by 72°C for 5 min). The amplified cDNAs were purified with Qiaquick spin columns according to the manufacturers protocol (Qiagen, Chatsworth, USA).

Northern blot analysis Northern blot analysis was conducted in order to verify that the genes on the initial display gel were differentially expressed. The gel-purified cDNAs were labeled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Mannheim, Germany). Northern blot hybridization was performed as previously described (Engler-Blum *et al.*, 1993). Then 20 μg of total RNA containing 0.1 μg/ml of ethidium bromide was separated on denaturing formaldehyde gels. The RNA was transferred onto Nytran-Plus membranes (Schleicher & Schull, Dassel, Germany) overnight by capillary transfer using 20× SSC. The membrane was cross-linked in a UVC 5000 Crosslinker (Hoefer Pharmacia Biotech Inc., San Francisco, USA) and pre-hybridized in a solution containing a 0.5% Bodhringer blocking reagent (20% SDS, 0.25 M sodium phosphate pH 7.2, and 1 mM EDTA) for 2 h. The concentration of labeled probes in the hybridization solutions was 2.5 ng/ml for cDNA. Hybridization was carried out for 16 h at 68°C with cDNA probes. After hybridization, the membranes were washed three times for 20 min at 65°C with a washing buffer (20 mM Na_2HPO_4, 1 mM EDTA, and 1% SDS). Subsequently, immunological detection procedures using anti-digoxigenin-AP conjugate (Boehringer Mannheim Biochemicals) were performed according to the manufacturer's instructions. The RNA bands were visualized and quantified by using the Fluor-S™ MultiImager system (Bio-Rad, Hercules, USA).

Cloning and sequencing of cDNAs Re-amplified cDNAs were cloned (Klimkait, 2000) into a pBluescript vector (Stratagene, La Jolla, USA). The recombinant plasmids were sequenced by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using T7 and T3 promoter primer. The cDNA sequence was compared through the BLAST DNA search program using GenBank and EMBL databases.

Results and Discussion

Induction of TfR mRNA by DFO To investigate the regulation of iron levels in HepG2 cells, a Northern blot

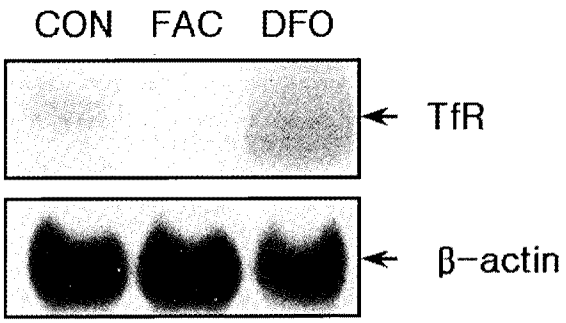

Fig. 1. Northern blot analysis of TfR mRNA expression in the HepG2 cell that was incubated for 24 h in the untreated control (CON), cells treated with ferric ammonium citrate (FAC), and cells treated with desferrioxamine (DFO). Hybridization with the β-actin probe is shown for normalization of RNA level.

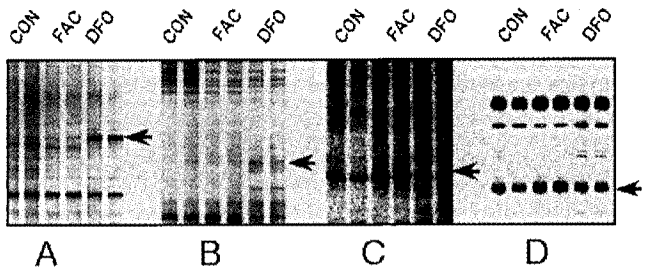

Fig. 2. Representative band patterns on DD analysis in the HepG2 cells using an arbitrary primer. Total RNA extracted from HepG2 cells; untreated cells (CON), cells treated with desferrioxamine (DFO), and cells treated with ferric ammonium citrate (FAC). Primer combinations used are as follows: (A) $AAGCT_{11}G$ and 5'-AAGCTTTGGTCAG-3', (B) $AAGCT_{11}A$ and 5'-AAGCTTATCGCTC-3', (C) $AAGCT_{11}G$ and 5'-AAGCTTGCCATGG-3', (D) $AAGCT_{11}G$ and 5'-AAGCTTCGA CTGT-3. Arrows (A)-(D) indicate signal demonstrating altered expression.

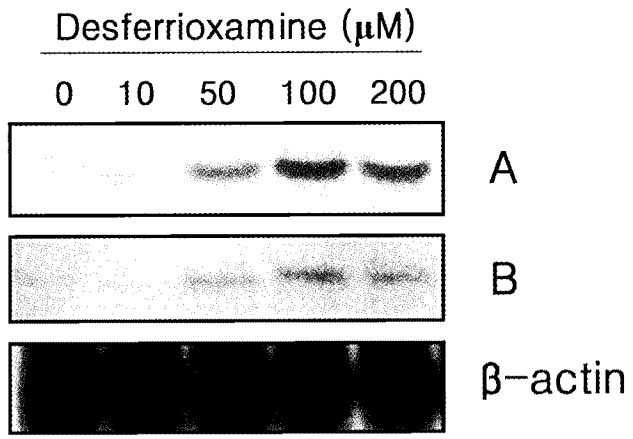

Fig. 3. Northern blot hybridization analysis of genes in the desferrioxamine (DFO)-treated HepG2 cell. HepG2 cells were left untreated (control) and in the presence of 10, 50, 100, 200 μM DFO. (A) IGFBP-1, (B) Unknown gene. Hybridization with the β-actin probe is shown for normalization of the RNA level.

analysis was performed with total RNAs obtained from untreated (CON), FAC-, and DFO-treated HepG2 cells (Fig. 1). β-actin was used as an endogenous RNA loading control and a transferrin receptor was used as an indicator of the iron level. The result showed that the transcription of TfR is up- or down-regulated in DFO- or FAC-treated HepG2 cells, respectively, as previously described (Ward *et al.*, 1982; Bridges and Cudkowicz, 1984; Mattia *et al.*, 1984; Rao *et al.*, 1986). From the results of the experiment shown in Fig. 1, the levels of FAC or DFO can modulate cellular iron levels in HepG2 cells.

Differentially amplified mRNAs Differentially displayed cDNAs were amplified using four RT templates from RNA derived from HepG2 cells that were subjected to FAC or DFO treatment compared to the untreated control. Two differentially displayed cDNAs (indicated as an arrow in panel A and B [Fig. 2]) showed density differences only in the DFO-treated cells, compared to the control. They are referred to as clone-1 and -2, respectively. Also, two other differentially displayed cDNAs in panel C and D (indicated as an arrow [Fig. 2]) were over-amplified in the FAC-treated HepG2 cells compared to the control and designated as clone -3 and -4, respectively. These four clones showed differences in intensity when compared to the control were directly recovered from the dried silver nitrate-polyacrylamide gel and re-amplified using the corresponding primer sets. The re-amplified cDNAs were cloned into the pBluescript vector for further characterization.

Northern blot analysis with differentially displayed cDNA fragments False-positives present a serious problem for differential display analysis because of artifacts of the PCR reaction. Therefore, in order to confirm the differential pattern of the cDNA displayed on a DNA sequencing gel, cDNA bands were recovered from the gels and re-amplified by PCR using the corresponding pair of primers. The resulting products were used as probes for the Northern blot analysis. Northern hybridization analysis showed that clone-1 and 2 are dose-dependently up-regulated by DFO treatment. The amount of the transcript hybridized to the clone-1 or clone-2 probe showed approximately a 8- or 4-fold increase in DFO-treated cells (over 100 μM concentration) for 20 h in comparison to the untreated control cells (Fig. 3, A or B), respectively. On the other hand, Northern blot analysis revealed that clone-3 and -4 are up-regulated only by FAC treatment, but they are not dose-dependent (Fig. 4). The amount of the transcript recognized by the clone-3 probe showed a 1.9-fold increase compared to the control at a 100 μg/ml concentration of FAC treatment, but there was no significant increase over a 100 μg/ml concentration of FAC treatment (Fig. 4, A). The amount of the transcript detected by

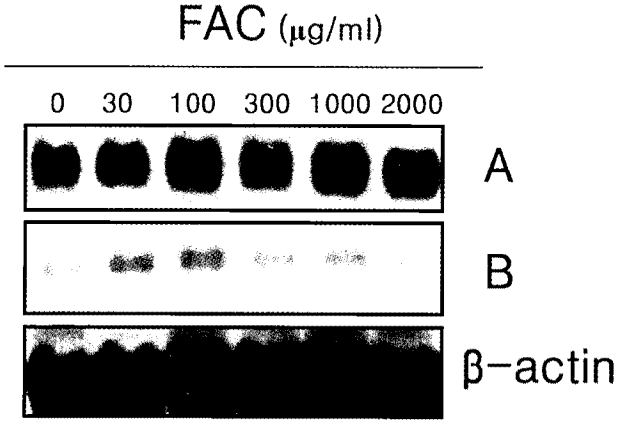

Fig. 4. Northern blot hybridization analysis of genes in ferric ammonium citrate (FAC)-treated HepG2 cell. HepG2 cells were left untreated (control) and in the presence of 30, 100, 300, 1000, 2000 μg/ml FAC. (A) Ferritin-light chain, (B) NADH-dehydrogenase. Hybridization with the β-actin probe is shown for normalization of RNA level.

the clone-4 probe showed a 2.3-fold increase compared to the control at a 100 μg/ml concentration of FAC treatment. However, over a 300 μg/ml concentration of FAC treatment it decreased to the control level (Fig. 4, B).

Nucleotide sequencing and homology searches in the databases The four differentially expressed cDNA fragments previously mentioned were completely sequenced and their nucleotide sequences were examined for sequence homologies to any other known genes from the GenBank and EMBL databases. Interestingly, the clone-1 sequences have a 100% identity to the human insulin-like growth factor binding protein-1 (IGFBP-1) gene (GenBank Accession No. M59316). On the other hand, the clone-3 and -4 sequences were 100% identical to the 3' end of the human ferritin light chain (GenBank Accession No. M11147) and human NADH-dehydrogenase (GenBank Accession No. NM 004549). However, the clone-2 sequences have no significant homology to any other known sequence (Table 1).

Interestingly, our results showed that IGFBP-1 is up-regulated by DFO treatment in the HepG2 cell. Furthermore, DFO, rather than being an iron chelator, was later found to be a potent stimulator of HIF-1 activation (Wang and Semenza, 1993). Previous results showed that treatment with DFO in Hep3B cells mimics hypoxic induction of the hypoxia inducible factor (HIF-1), which activates transcription by binding to the hypoxia responsive elements (HRE) (Bianchi *et al.*, 1999). Also, the hypoxia-induced IGFBP-1 mRNA expression in HepG2 cells was mediated by HRE containing a putative HIF-1 site, providing a precedent for direct regulation (Tazuke *et al.*, 1998). The expression of the ferritin light chain was not significantly regulated in HepG2 cells by FAC treatment. This suggests that ferritin regulation may occur at the translational level. However, intracellular and extracellular ferritin concentrations were strikingly increased by iron in dose- and time-dependent manners (Hubert *et al.*, 1993). Thus, an increased L-rich ferritin content is easily predictable in lens cells of individuals with a hereditary hyperferritinemia-cataract syndrome (Cazzola *et al.*, 1997). The dysregulated L-subunit synthesis was found to result from different point mutations in the noncoding sequence of genomic L-subunit

Table 1. Nucleotide sequences differentially expressed cDNA fragments. Reamplified cDNAs of clone-1, 2, 3, and 4 were cloned into the pBluescript-vector and sequenced with T3 and T7 primers. The sequences of the 3' and 5' flanking primers used in the PCR were shown in bold.

Clone	Effect of iron level	Homologous gene	Nucleotide sequences
Clone-1	DFO-Up	IGFBP-1	**aagctttttttttttt**ctacacagtaaaaacttgtactatgttaataacttgtcctatgtcaatttgtatatcatgaaacacttctcatcatattgtatgtaa gtaattgcatttctgctcttccaaagctcctgcgtctgtttttaaagagcatggaaaaatactgcctagaaaattcaaaatgaaataagagagagta gtttttcagctagtttgaaggaggacggttaacttgtatattccaccattcacatttgatgtacatgtgtagggaaagttaaaagtgttgattacataat caaagctacctgtggtgatgttgccacctgttaaaatgtacactggatatgttgttaaacacgtgtcgataatggaaacatttacaataaatattctgc atgg**ctgaccaaagctt**
Clone-2	DFO-Up	Unknown	**aagcttatcgatc**ataaataagtattataactttattaaaatgaagacaatattcaaaataatgcaacaaaatgaataaaatccttttgtccaatactg tacacataatgcagaaatcagtgcatttttcttaagcatgttttaaccttcatttagttcatactaaaatataataagctttaaatagctcaaataatattc agcagtttaaactgtaaacagcttgtttaactgttaagagaacattgcagtaatgtacctctgttagtgagcaccttctcttctgtgcttatctcttcaag ataaatacatggaaggatgtgaaaatcggaacaccaactatgtgtctcactgcatctaagtgaagcagccacagctgtgagagttttcaaagcag aaagatgctgatgtgacctctggaattcagacatactgagctatgggtcagaagtgttttacttaaaaagcaaacaatccccaggaaatactgaata ggaaccagcaacacaaggccagcttgtgttgtatgtttattaatacagtct**aaaaaaaaaaaagctt**
Clone-3	FAC-Up	Ferritin-light chain	**aagcttgccatgg**ccctggagaaaaagctgaaccaggccctttggatcttcatgccctgggttctgcccgcacggacccccatctctgtgactt cctggagactcacttcctagatgaggaagtgaagcttatcaagaagatgggtgaccacctgaccaacctccacaggctgggtggcccggagg ctgggctgggcgagtatctcttcgaaaggctcactctcaagcacgactaagagccttctgagcccagcgacttctgaagggccccttgcaaagt aatagggcttctgcctaagcctctccctccagccaataggcagctttcttaactatcctaacaagccttgga**caaaaaaaaaaagctt**
Clone-4	FAC-Up	NADH-dehydrogenase	**aagctttttttttttg**ctggatattatcttntaaaacgtgaagactacctgtatgctgtgagggccgtgaaatggtttggatatatgactagttnatcca gaggtttcctgaagaagataagaaaacatatggtgaaatttttgaaaaattccatccaatacgttgaagtcttcaaaatgcttgctccagtttcactga acctgctgtttctgaatttgatggaacatgtttcttatg**acagttgaagctt**

DNA, which behaves as a mRNA cis-acting element known as an iron regulatory element (IRE) (Girelli *et al.*, 1997).

NADH-dehydrogenase, which is also known as Complex I (Weiss *et al.*, 1991) or NADH-CoQ oxidoreductase, is responsible for transferring electrons from NADH to CoQ. NADH-dehydrogenase that is regulated by FAC may be due to Complex I, which contains a prosthetic group that has Fe-S (Ohnishi, 1998). It is hypothesized that Fe-S is affected by the reaction with iron.

The nature of the clone-2 differentially expressed by DFO in this study is unknown. This gene may be more important than the others because of its originality in the area of hypoxia research.

In this study, we identified four RNA transcripts whose level of expression reflected cellular iron concentrations by using the newly developed mRNA differential display method. It can be hypothesized that genes, apart from the above four genes, may be affected by iron (directly or indirectly). Therefore, further intensive investigations will be required to broaden our knowledge of the mechanism responsible for the differential gene expression induced by cellular iron levels.

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