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A search for genes modulated by interleukin-6 alone or with interleukin-1β in HepG2 cells using differential display analysis

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Abstract

Interleukin-1 and interleukin-6 are principal cytokines involved in regulation of expression of acute-phase proteins. In the joint action of both cytokines IL-1 can suppress or enhance the IL-6-dependent induction of gene expression. Here, we report changes in the transcriptome profile of HepG2 cells exposed to IL-6 alone, or IL-1 and IL-6. Cytokine-responsive genes were identified by differential display analysis. Validation of observed changes in the transcript level was carried out using the slot blot method. Out of 88 cDNA species modulated by IL-6, only 38 represent different known genes whereas 18 clones match genomic clones in NCBI data with hypothetical cDNA sequences (the remaining 32 clones showed no homology with the database or represented several clones of the same gene). In the experiments with HepG2 cells prestimulated for 3 h with IL-1 and then stimulated with IL-6, 43 cDNA fragments were amplified. Twenty-three of them represent known genes while 10 clones have inserts matching hypothetical cDNA sequences in NCBI data. The identified transcripts modulated by IL-6 or both cytokines in HepG2 cells code for intracellular proteins of various function. The largest groups represent genes engaged in metabolism, protein synthesis and signaling pathways. Among all genes identified as differentially regulated under stimulation by IL-6, or IL-1/IL-6, six were detected in both types of stimulation. None of the typical genes coding for plasma acute phase proteins was identified in our experiments. This indicates that differential display cannot be used to characterize the profile of a given transcriptome. On the other hand, it is a useful technique for detection of new genes responding to IL-6 alone or IL-6 in combination with IL-1.

Keywords: Acute phase response; Human hepatoma HepG2; Inflammatory cytokine; Differential display

1. Introduction

The acute phase reaction has been defined as a characteristic response of the animal organism to various forms of injuries (for description of molecular mechanism and earlier references see [1–3]). The systemic acute phase reaction is initiated by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor, as well as by acute phase cytokines from the interleukin-6 family. These cytokines are released from activated macrophages, fibroblasts, keratinocytes or endothelial cells and stimulate liver and other tissues to synthesis of acute phase proteins. The cytokine-modulated expression of genes coding for liver-derived plasma proteins is either upregulated (positive acute phase proteins) or downregulated (negative acute phase reactants) [1]. Moreover, the action of IL-1 and IL-6 can be synergistic or antagonistic: synthesis of α_1-acid glycoprotein or serum amyloid A induced by IL-1 is further enhanced by IL-6, whereas expression of fibrinogen is IL-6 dependent and IL-1 behaves as a negative regulator [1,4]. Although abundant information is available on the expression of genes coding for individual plasma and cellular proteins modulated by various cytokines very few attempts have been made so far to compare the profile of the whole transcriptome analysed by currently available techniques such as subtractive hybridization, differential display or microarrays (for review, see [3]).

Abbreviations: IL-1β, interleukin-1 beta; IL-6, interleukin-6; DD-PCR, differential display-polymerase chain reaction; AGP, α_1-acid glycoprotein; HPT, haptoglobin; MnSOD, manganese-dependent superoxide dismutase

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By using cultured human hepatoma cells (HepG2 cells) and differential display analysis, we have already shown that the cellular response to IL-1 is highly complex and encompasses activation of genes encoding intracellular proteins of various function: trafficking/motor proteins, proteins participating in the translation machinery or post-transcriptional/posttranslational modifications, proteases, proteins involved in metabolism, activity modulators and proteins of the cell cycle machinery [5]. In order to expand these studies, we evaluated changes in gene expression of HepG2 cells stimulated by IL-6, or by the joint action of IL-1 and IL-6. According to our experimental protocol cells were pretreated with IL-1 for 3 h and then stimulated with IL-6. This was based on the observation of Uhlar and Whitehead [6] who showed that stimulation with IL-1 prior to IL-6 was essential for maximal synergistic transcriptional induction of human serum amyloid A promoter inserted upstream of a luciferase reporter gene. Similar results were obtained by Thorn et al. [7] in hepatic and epithelial cell lines stimulated with TNFα and IL-6. Such order of cytokine delivery is also close to the sequence of events in vivo [8]. On the other hand, several authors [9,10] reported that pretreatment of HepG2 cells with IL-1 inhibits formation of the IL-6-induced STAT complexes that are involved in the expression of many acute phase protein genes [11].

In the experiments described here carried out with the employment of differential display analysis, we found that the number of transcripts modulated by IL-6 alone is much higher than after pretreatment of cells with IL-1 before stimulation with IL-6. This indicates that inhibitory effects of IL-1 on the acute phase response prevail over possible synergistic action of the two tested cytokines. Although we identified several new genes not known to be controlled by IL-1 and/or IL-6, we did not detect mRNAs coding for acute phase plasma proteins. This may be due to accidental annealing of the used primers with the most abundant mRNA species while certain transcripts remain undetected. However, such an outcome represents a serious limitation of the differential display technique.

2. Materials and methods

2.1. Cell culture and cytokine treatment

HepG2 cells (from ATCC, passages 11–16) were grown to 70% confluence in plastic Petri dishes (10 cm in diameter, TPP, Switzerland), at 37 °C and 5% CO_2 in Dulbecco Modified Eagle Medium (DMEM) with 1000 mg/L D-glucose (Gibco/BRL) supplemented with 10% foetal bovine serum (FBS). At 24 h before the experiment cells were placed in DMEM containing 0.5% FBS. Finally, HepG2 cells were stimulated with IL-6, or prestimulated for 3 h with IL-1 and then stimulated with IL-6, for the time indicated. Human IL-1β was a gift from Dr. C. Dinarello (Denver, CO, U.S.A.) and human recombinant IL-6 was purchased from ICN (USA). The responsiveness of HepG2 cells to these cytokines was evaluated in each series of experiments by comparing changes in the levels of mRNA coding for known acute phase proteins (Fig. 1). Changes in the level of appropriate mRNAs were determined by Northern blot as recently described by us [5]. In the experiments with differential display analysis, final concentrations of IL-1β in the tissue culture media were 15 ng/ml, and IL-6–25 ng/ml.

2.2. Differential display analysis

Expression studies were carried out with the use of total RNA isolated from unstimulated (control) and IL-6-stimulated cells, or from control and cells prestimulated by IL-1β (3 h) and then stimulated with IL-6 for different time periods. Differential display analysis was carried out with cDNA synthesized on the whole RNA template, with oligo(dT) primers. Each PCR reaction, done with 10 oligo(dT) and 9 arbitrary primers (Clontech, USA) (shown in Table 1), consisted of two stages: in stage one, three cycles were performed at low

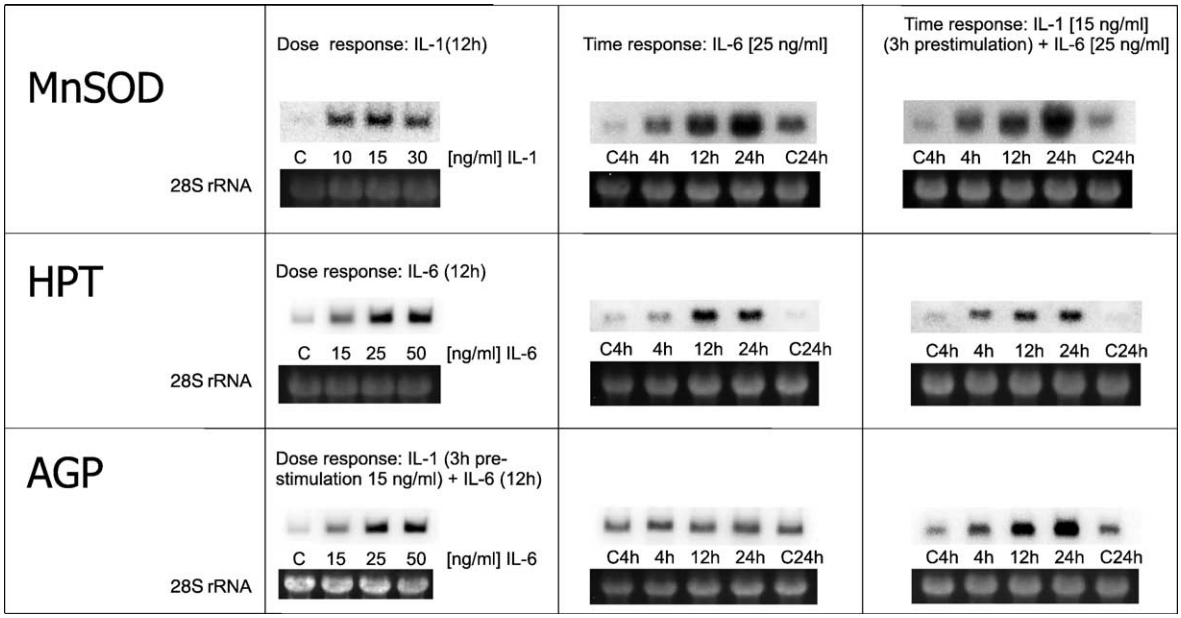

Fig. 1. Time and dose dependent changes in the level of transcripts encoding acute phase proteins: mitochondrial manganese superoxide dismutase (MnSOD), haptoglobin (HPT) and α_1-acid glycoprotein (AGP) in HepG2 cells stimulated with IL-1, IL-6 or mixture of IL-1/IL-6. To establish the optimal times and doses for both cytokines cells were treated with 10, 15, 30 ng/ml of IL-1; 15, 25, 50 ng/ml of IL-6 during 4, 12 and 24 h. Unstimulated cells served as a control (C). In case of MnSOD only the smaller transcript is presented. For other details see Results.

Table 1
The sequence of primers designed by Clontech and used in DD-PCR

Arbitrary primers	Oligo(dT) primers
P1: 5′-ATTAACCCTCACTAAATGCTGGGGA	T1: 5′-CATTATGCTGAGTGATATCTTTTTTTTTAA
P2: 5′-ATTAACCCTCACTAAATCGGTCATAG	T2: 5′-CATTATGCTGAGTGATATCTTTTTTTTTAC
P3: 5′-ATTAACCCTCACTAAATGCTGGTGG	T3: 5′-CATTATGCTGAGTGATATCTTTTTTTTTAG
P4: 5′-ATTAACCCTCACTAAATGCTGGTAG	T4: 5′-CATTATGCTGAGTGATATCTTTTTTTTTCA
P5: 5′-ATTAACCCTCACTAAAGATCTGACTG	T5: 5′-CATTATGCTGAGTGATATCTTTTTTTTTCC
P6: 5′-ATTAACCCTCACTAAATGCTGGGTG	T6: 5′-CATTATGCTGAGTGATATCTTTTTTTTTCG
P7: 5′-ATTAACCCTCACTAAATGCTGTATG	T7: 5′-CATTATGCTGAGTGATATCTTTTTTTTTGA
P8: 5′-ATTAACCCTCACTAAATGGAGCTGG	T8: 5′-CATTATGCTGAGTGATATCTTTTTTTTTGC
P9: 5′-ATTAACCCTCACTAAATGTGGCAGG	T9: 5′-CATTATGCTGAGTGATATCTTTTTTTTTGG
P10: 5′-ATTAACCCTCACTAAAGCACCGTCC	

primer binding temperature (40 °C), which enables non-specific binding of an arbitrary primer to different cDNA templates. In the second stage, 25 cycles were performed at higher primer binding temperature, which led to the decrease of background resulting from amplification of non-specific products, and the repeatability of obtained results. We developed a method for fractionating PCR products in polyacrylamide gels of $1.5 \times 200 \times 200$ mm, having two different acrylamide concentrations: 8% in the lower, and 6% in the upper part of the gel. The use of two different concentrations enabled us to retain in the lower part of the gel products of 200–600 bp, while 6% acrylamide of the upper part of the gel enabled correct separation of fragments ranging from 600 to 2200 bp. The silver staining method was used to visualize individual bands (Fig. 2). Differentiating bands were excised with a scalpel from the gel immediately after the end of electrophoresis, or after the gel had been dried. The excised gel fragments were incubated in sterile water for 1 h in order to release cDNA used subsequently for reamplification. The products were separated on agarose gel, purified with a PCR product purification kit (Qiagen, USA) and cloned into type T/A pTZ57R vector (Fermentas, Lithuania). The obtained colonies (3 colonies from each plate) were subjected to alkaline lysis, and then sequenced.

2.3. Sequencing

Clones with a ligated insert were sequenced in one direction using the reverse primer M13 lacZ in a Perkin Elmer 9700 Thermal Cycler at 96 °C for 10 s, 50 °C for 5 s, 60 °C for 2 min for 25 cycles. Cycle sequencing reaction was performed in 20 μl of total reaction mixture consisting of: 4 μl ABI Prism BigDay Terminator v. 1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, UK), 2 μl 5× Sequencing Buffer (Applied Biosystems, UK), 3.2 pmol of sequencing primer and approximately 100–200 ng of DNA. Sequencing reaction products were precipitated with the mixture of 50 μl absolute ethanol and 2 μl 3 M sodium acetate pH 4.6 followed by washing in 70% ethanol. Electrophoretic separation of sequencing products was carried out using an ABD3100 DNA Sequencer with 50 cm capillaries. Data analysis was performed with ABI Prism Sequencing Analysis Software v.2.1. Sequences of differentially expressed genes were determined by searching the GenBank data at the National Center for Biotechnology Information using BLAST program.

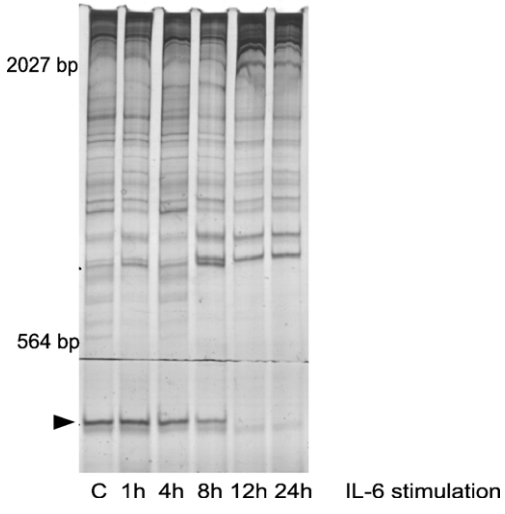

Fig. 2. Representative picture of differential display analysis. PCR products were separated on native polyacrylamide gel and visualized by silver staining. Differentially expressed transcript encoding EID-1-like inhibitor of differentiation-2 (EID2) was marked on a gel. Confirmation of modulated expression of EID2 under IL-6 stimulation presents Fig. 3A.

2.4. Slot blot

Cytokine-dependent modulated expression of all known genes identified in differential display analysis was confirmed by the use of slot blot method. The slot blot apparatus (Schleicher and Schuell, Germany) was assembled according to the manufacturer. A positively charged nylon filter (Hybond NX; Amersham Biosciences, UK) was wetted in 10× SSC. Gene fragments corresponding to identified cDNAs, encoding known transcripts, were amplified with the use as a template pTZ57R constructs containing respective cDNA fragments and primers complementary to the vector's sequence. PCR products were purified (Qiagen, USA), denatured in 95 °C, 4 min, cooled on ice and loaded into the slots. Each slot was loaded with 200 ng of the PCR product corresponding to an individual gene. In the experiment with IL-6 38 probes on one nylon filter represented cDNA of known genes. In the experiment with IL-1/IL-6, 23 different probes were loaded. In both experiments genomic DNA (50 ng) served as control.

Validation of observed changes in gene expression was performed for 3 independent experiments: unstimulated cells (control) and cells stimulated for different time periods: 4, 12, 24 h. Thus, all together 12 membranes were used for one experiment with IL-6 stimulation and additional 12 membranes in the study of IL-1/IL-6 action.

Prehybridization and hybridization were carried out at 65 °C in 1% SDS, 1 M NaCl, 10% dextran sulfate solution. As a molecular probe, the whole cDNA reaction mixture was used. The reaction was carried out from 5 μg of total RNA in 20 μl using SuperScript RNaseH$^-$ reverse transcriptase (Promega, USA), in the presence of [α-^{32}P]dCTP. All slot blot signals were verified by measuring the background of the signal obtained from the control sample (50 ng of genomic DNA). After washing procedure slot blots were exposed to a phosphoimager screen and then red out using Molecular Imager FX and software Quantity One (Biorad, USA).

2.5. Calculation of changes in the level of expression

Slot blot densitometric readings were calculated using Microsoft Excel. The value of intensity of each analysed band corresponding to individual gene and appropriate time of stimulation was divided by the value of intensity of genomic DNA and the final results were calculated as fold change in respect to the control (see Fig. 3, Tables 2 and 3). The reported values represent the mean of three independent experiments; statistical analysis was carried out with the use of *t*-test.

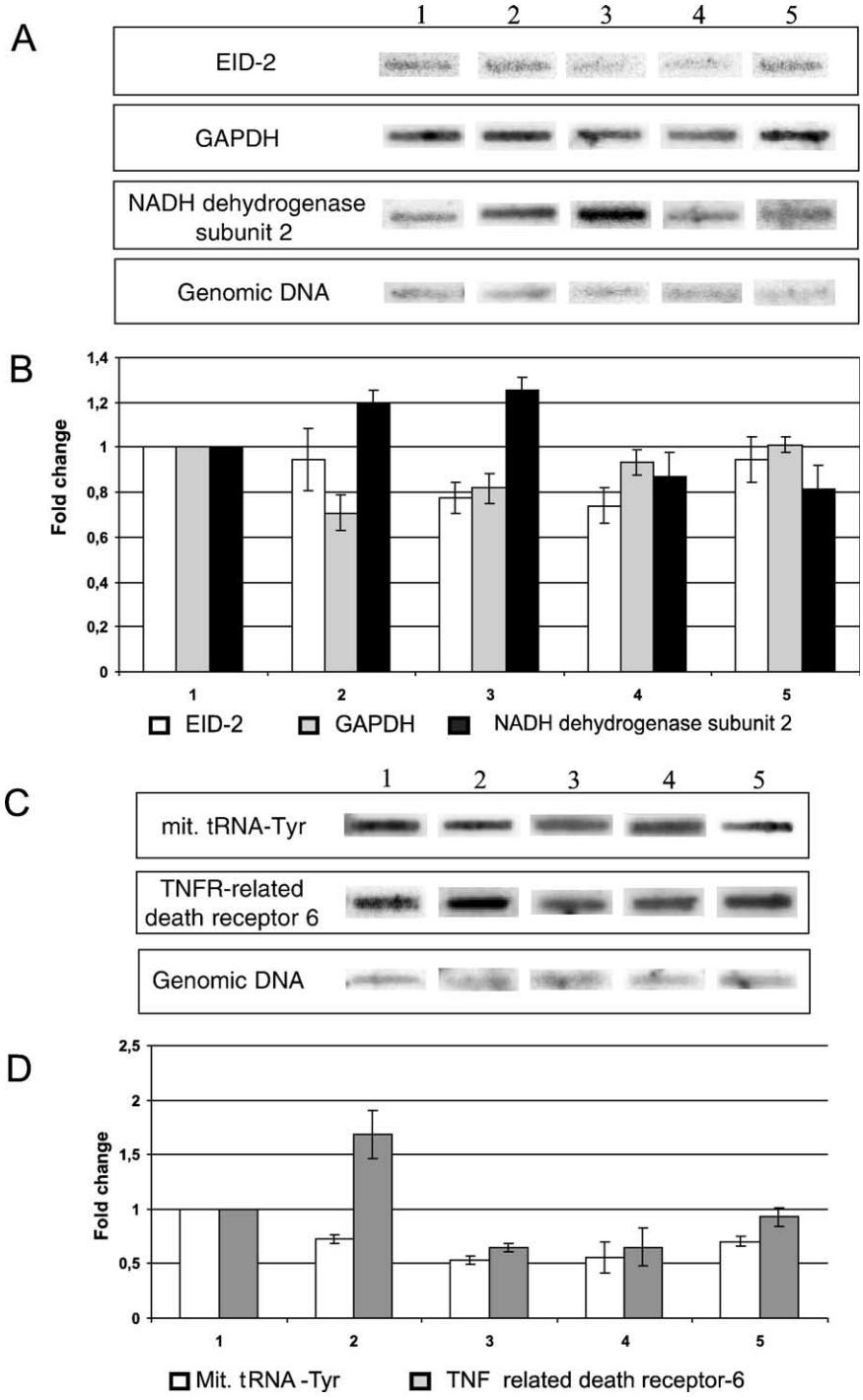

Fig. 3. A representative picture of slot blot analysis showing changes in the expression of some of the identified genes after stimulation of HepG2 cells with IL-6 (A, B) or the mixture of IL-1/IL-6 (C, D). Fold change corresponds to the ratio of intensity of each analysed band and intensity of genomic DNA band divided by the corresponding value of 1 h control. For each experiment two controls are shown: unstimulated cells collected after 1 h and 24 h incubation. (A) Individual bands show the transcript level of genes coding for EID-1-like inhibitor of differentiation-2 (EID2), GAPDH, and NADPH dehydrogenase subunit 2. (B) Results of densitometric analysis of relative changes in mRNAs abundance as determined by slot blot (mean ± SE of 3 independent experiments). Statistical analysis was carried out with the use of t-test. Lane 1—control, unstimulated HepG2 cells harvested after 1 h of incubation; lane 2—HepG2 cells stimulated with IL-6 for 4 h; lane 3—HepG2 cells stimulated with IL-6 for 12 h; lane 4—HepG2 cells stimulated with IL-6 for 24 h; lane 5—control, unstimulated HepG2 cells harvested after 24 h of incubation. (C) Individual bands show the transcript level of genes coding for mitochondrial tRNA-Tyr and TNFR-related death receptor 6 after 3 h prestimulation with IL-1 followed by stimulation with IL-6 for different time periods (4, 12, 24 h). (D) Results of densitometric analysis of relative changes in mRNAs abundance as determined by slot blot (mean ± SE of 3 independent experiments). Statistical analysis was carried out with the use of t-test. Lane 1—control, unstimulated HepG2 cells harvested after 1 h of incubation; lane 2—HepG2 cells stimulated with IL-1 for 7 h and IL-6 for 4 h; lane 3—HepG2 cells stimulated with IL-1 for 15 h and IL-6 for 12 h; lane 4—HepG2 cells stimulated with IL-1 for 27 h and IL-6 for 24 h; lane 5—control, unstimulated HepG2 cells harvested after 24 h of incubation.

Table 2
Classes of 38 genes responding to stimulation of HepG2 cells by IL-6 for the time indicated

Clones	GenBank Accession No.	Genes	Fold change					Classification
			C 1 h	4 h	12 h	24 h	C 24 h	
8	AY495240.1	NADH dehydrogenase subunit 1	1	2.22±0.45	3.39±0.19*	3.70±0.32▲	2.44±0.34♦	Metabolism
1	AY495242.1	NADH dehydrogenase subunit 2	1	1.19±0.06♦	1.25±0.06♦	0.87±0.11	0.81±0.11	Metabolism
1	AY495242.1	NADH dehydrogenase subunit 3	1	1.61± 0.16♦	1.78±0.16▲	2.62±0.10*	1.13±0.07	Metabolism
2	AY495242.1	NADH dehydrogenase subunit 4	1	1.47±0.20	2.30±0.28♦	1.98±0.06*	1.36±0.22	Metabolism
2	AF275320	Glyceraldehyde-3-phosphate dehydrogenase	1	0.71±0.08♦	0.82±0.07	0.93±0.06	1.01±0.04	Metabolism
1	NM_001643.1	Apolipoprotein A-II (APOA2)	1	0.44±0.05*	0.66±0.03*	0.69±0.12	0.78±0.14	Metabolism
1	NM_002225.2	Isovaleryl Coenzyme A dehydrogenase	1	0.47±0.07▲	0.77±0.10	0.79±0.08	1.11±0.12	Metabolism
3	BC000629.2	Aspartyl-tRNA synthetase	1	0.63±0.09♦	1.00±0.15	1.23±0.08♦	0.91±0.09	Protein synthesis
1	AY495242.1	tRNA-Tyr	1	1.23±0.02*	0.49±0.01*	0.60±0.14♦	0.74±0.16	Protein synthesis
1	BC067738.1	Eukaryotic translation elongation factor 1 gamma	1	0.72±0.12	1.10±0.08	1.51±0.13♦	1.07±0.03	Protein synthesis
2	NM_002212.2	Integrin beta 4 binding protein (ITGB4BP)	1	0.74±0.10▲	0.72±0.11▲	0.86±0.08	1.08±0.04	Protein synthesis
1	NM_001013.2	Ribosomal protein S9	1	0.47±0.09▲	0.47±0.12♦	0.34±0.06*	0.55±0.10♦	Protein synthesis
3	BC006781.1	Myosin, light polypeptide 6	1	0.88±0.05	0.83±0.07♦	0.83±0.09	0.82±0.07	Trafficking
1	X65873.1	Kinesin (heavy chain)	1	0.59±0.08▲	1.33±0.10♦	0.51±0.04*	0.84±0.10	Trafficking
2	NM_001153.2	Annexin A4 (ANXA4)	1	0.48±0.12♦	0.71±0.19	1.77±0.27♦	0.80±0.23	Trafficking
1	NM_002778.1	Prosaposin	1	0.88±0.05	1.42±0.26	1.46±0.07▲	0.82±0.11	Signaling intermediates
1	AF029838	Alpha 7 neuronal nicotinic receptor	1	0.80±0.02*	1.19±0.06♦	0.79±0.09	0.88±0.03♦	Signaling intermediates
1	BC005852.1	SPRY domain-containing SOCS box protein SSB-1	1	0.57±0.02*	1.28±0.09♦	1.47±0.06▲	0.67±0.12♦	Signaling intermediates
1	BC058925.1	Intersectin 1 (SH3 domain protein),	1	0.68±0.05▲	1.14±0.06	1.31±0.17	0.83±0.02▲	Signaling intermediates
1	BC022850.2	Transmembrane 9 superfamily protein member 4	1	0.83±0.18	1.77±0.04*	1.19±0.03▲	0.87±0.39	Signaling intermediates
3	AF068868.1	TNFR-related death receptor-6 (DR6)	1	0.63±0.05▲	0.94±0.05	0.98±0.02	0.81±0.07	Signaling intermediates
1	NM_000801.2	FK506 binding protein 1A, 12 kDa (FKBP1A)	1	0.91±0.06	1.18±0.14	1.70±0.22♦	0.82±0.19	Signaling intermediates
1	BC001522.2	GRIP1 associated protein 1	1	0.98±0.29	0.75±0.07♦	1.15±0.10	0.97±0.16	Signaling intermediates
1	NM_0031035	SON DNA binding protein (SON)	1	0.52±0.02*	0.77±0.07♦	1.07±0.10	1.19±0.03▲	DNA-binding proteins
1	BC006175.1	v-jun sarcoma virus 17 oncogene homolog	1	1.74±0.18♦	3.27±0.67♦	1.52±0.31	0.47±0.19	DNA-binding proteins
1	NM_003675.2	PRP18 pre-mRNA processing factor 18 homolog	1	0.71±0.15	0.60±0.06▲	0.86±0.14	0.77±0.13	Posttranslation modification
1	BC011754.2	Disulfide isomerase related protein	1	0.72±0.22	2.22±0.26▲	1.11±0.22	0.85±0.42	Posttranslation modification
1	BC012141.1	Nuclear RNA binding protein (p54nrb)	1	1.33±0.05▲	0.72±0.15	0.72±0.00*	0.83±0.15	Posttranscription modification
1	NM_003330.2	Thioredoxin reductase 1 (TXNRD1), transcript variant 1	1	0.66±0.11♦	0.69±0.00*	0.86±0.10	1.12±0.05	Stress protection
1	AF003529.2	Glypican 3 (GPC3)	1	0.67±0.16	0.44±0.05*	0.56±0.13♦	0.98±0.13	Cell proliferation
1	AY422170.1	p53-inducible protein (PINH)	1	1.32±0.12	0.77±0.11	0.41±0.16♦	0.54±0.04*	Cell proliferation
2	BC000379.2	Ubiquitin B	1	0.71±0.14	0.75±0.06♦	0.72±0.12	0.97±0.11	Proteolysis
1	AF221130	Chromatin remodeling factor WCRF180	1	0.84±0.06♦	0.61±0.07▲	0.46±0.06▲	0.72±0.13	Transcription
1	AY251272.1	CREBBP/EP300 inhibitor 2 (EID2)	1	0.95±0.14	0.77±0.07♦	0.74±0.08♦	0.95±0.10	Transcription
1	NM_006698.1	Bladder cancer associated protein (BLCAP)	1	0.66±0.07▲	0.61±0.02▲	0.84±0.04♦	0.82±0.03▲	Functionally unclassified
1	BC032833.2	Ankyrin repeat domain 13 (NY-REN25)	1	0.69±0.17	1.01±0.11	1.28±0.07♦	1.07±0,31	Functionally unclassified
1	BC011625.2	Zinc finger protein 503	1	1.64±0.04*	1.26±0.16	1.16±0.14	0.76±0.14	Functionally unclassified
1	BC000687.2	Translocation associated membrane protein 1	1	0.42±0.34	0.63±0.06▲	0.78±0.04▲	0.81±0.06♦	Functionally unclassified

For legend see Table 3.

3. Results

3.1. Effectiveness of stimulation of HepG2 cells by IL-6 and IL-1/IL-6

In order to establish the experimental protocol, HepG2 cells were treated with various concentrations of IL-1β or/and IL-6. The dose and time-responses to these cytokines were obtained for mRNAs coding for well-known acute phase proteins: mitochondrial manganese superoxide dismutase (MnSOD) [12] and two plasma proteins: haptoglobin (HPT) and α_1-acid glycoprotein (AGP) [13] (Fig. 1). On the ground of these results, we selected concentrations of cytokines used in the differential display analysis: 15 ng/ml of IL-1β and 25 ng/ml of IL-6.

As shown in Fig. 1, maximal abundance of the tested mRNAs occurred either after 12 h of exposure to IL-6 alone (HPT), or after 24 h exposure to IL-6 when the cells were pretreated with IL-1β (MnSOD, AGP). The additive effects of IL-1 and IL-6 are clearly visible for the two latter proteins belonging to class I, whereas human HPT represents class II being regulated by IL-6 alone [1].

3.2. Differential display analysis of IL-6-induced transcriptome changes in HepG2 cells

After stimulation of HepG2 cells with IL-6 we found 100 differentiating PCR products from which 88 were successfully reamplified and cloned to pTZ57R vector as described in Materials and methods. After searching the NCBI-BLAST database we found homology sequence for 77 different cDNAs. Many of the identified genes were represented by more than one clone, thus from 77 identified genes 56 cDNA sequences corresponded to 38 different known genes (Table 2). The remaining 18 clones contained new transcripts: their sequences matched BAC and PAC clones in NCBI database with the identity ranging from 85 to 100%. These sequences represent new full size cDNA identified already by the complex transcriptome sequencing analysis [14], or recognized as open reading frames in big genomic clones; therefore, they have to be cloned and characterized at 5′ and 3′ ends. The cloning procedure was not successful for 12 clones, in which vector sequences were inserted.

Table 3
Classes of 23 genes responding to stimulation of HepG2 cells by IL-1/IL-6 for the time indicated

Clones	GenBank Accession No.	Genes	Fold change					Classification
			C 1 h	7/4 h	15/12 h	27/24 h	C 24 h	
3	AY495241.1	NADH dehydrogenase subunit 1	1	0.47±0.01*	0.76±0.07♦	0.82±0.09	0.80±0.03▲	Metabolism
1	NM_004925.3	Aquaporin 3 (AQP3),	1	2.23±0.32♦	1.02±0.15	1.29±0.22	1.45±0.16♦	Metabolism
2	NM_001013.2	Ribosomal protein S9 (RPS9)	1	0.54±0.11♦	0.53±0.10▲	0.41±0.08▲	0.50±0.07▲	Protein synthesis
1	BC020515.1	Ribosomal protein S14	1	0.90±0.12	0.46±0.07▲	0.62±0.10♦	1.14±0.06	Protein synthesis
1	AK495323.1	Mit. tRNA-Ile	1	0.45±0.11▲	0.44±0.06*	0.62±0.02*	0.82±0.10	Protein synthesis
1	AY570526.1	Mit. tRNA-Tyr	1	0.73±0.04▲	0.53±0.04*	0.55±0.14♦	0.70±0.04▲	Protein synthesis
1	AY570526.1	Mit. 16S ribosomal RNA	1	0.54±0.03*	0.72±0.05▲	0.63±0.10♦	0.73±0.00*	Protein synthesis
1	BC012141.1	Nuclear RNA binding protein (p54nrb)	1	0.85±0.09	0.56±0.08▲	0.49±0.06▲	0.97±0.18	Posttranscription modification
1	AF239156	Deformylase-like protein mRNA	1	1.70±0.22♦	1.17±0.11	1.50±0.10▲	1.05±0.13	Posttranslation modification
2	BC037428	RAVER1	1	0.82±0.08	0.79±0.04♦	0.71±0.08♦	0.69±0.11♦	RNA processing
2	BC006781.1	Myosin, light polypeptide 6	1	0.91±0.06	0.87±0.04♦	0.77±0.06♦	0.78±0.05▲	Trafficking
1	AF151978	Amino acid transporter B0+ (ATB0+) mRNA	1	0.90±0.01▲	1.33±0.01*	1.25±0.18	1.43±0.01*	Transport
1	BC012137.1	Syntaxin 5A	1	1.27±0.01*	1.14±0.04♦	1.27±0.04▲	1.06±0.05	Transport
1	BC013017	CD63 antigen (melanoma 1 antigen)	1	0.39±0.13▲	0.64±0.15	0.65±0.13♦	0.73±0.12	Signaling intermediates
2	AF068868.1	TNFR-related death receptor-6 (DR6)	1	1.68±0.23♦	0.65±0.04*	0.65±0.17	0.93±0.08	Signaling intermediates
1	AF035158	Serine/threonine protein phosphatase catalytic subunit	1	1.47±0.13♦	0.83±0.07	0.87±0.05	1.03±0.05	Signaling intermediates
1	NM_001238.1	Cyclin E1 (CCNE1)	1	0.55±0.09▲	0.39±0.04*	0.92±0.14	1.14±0.01*	Cell proliferation
1	AF003529.21	Glypican 3 (GPC3)	1	0.86±0.10	0.70±0.08♦	0.62±0.01*	0.90±0.02▲	Cell proliferation
1	HSU75285	Survivin	1	1.66±0.08▲	1.18±0.07	1.03±0.02	1.24±0.14	Cell proliferation
1	BC007897.2	Proteasome 26S subunit,	1	0.77±0.03▲	0.60±0.06▲	0.60±0.07▲	0.78±0.15	Proteolysis
1	BC010902.1	CGI-150 protein	1	0.79±0.01*	0.76±0.05▲	0.65±0.03*	0.94±0.25	Functionally unknown
2	AF277316.1	PWP1-interacting protein 8 percursor (PWP1)	1	1.42±0.07▲	1.04±0.03	1.04±0.07	1.08±0.08	Functionally unknown
1	NM_006698	Bladder cancer associated protein (BLCAP)	1	0.74±0.05▲	0.59±0.11♦	0.55±0.02*	0.91±0.00*	Functionally unknown

Fold change is a ratio of densitometric value of each analysed band corresponding to individual gene at appropriate time of stimulation to the densitometric value of intensity of genomic DNA divided by the corresponding value of 1h control (see Fig. 3). Therefore, the ratio value below 1 indicates down-regulation of gene expression, and above 1 indicates up-regulation. Time of cells collection for the analysis shows the exposure to the tested cytokines, e.g., 7/4 indicates 7 h exposure to IL-1 and 4 h exposure to IL-6. The reported values represent the mean of three independent experiments. Statistical significance: ♦$P<0{,}05$; ▲$P<0{,}01$; *$P<0{,}001$.

3.3. Differential display analysis of transcriptome changes in HepG2 cells stimulated with IL-1β followed by IL-6

In case of HepG2 cells prestimulated for 3 h with IL-1 and then stimulated with IL-6, we excised 81 bands from the DD gels, and 43 of them were successfully reamplified. All in all, 30 clones corresponded to known genes. Several genes, such as NADH dehydrogenase subunit 1, ribosomal protein S9, RAVER1, myosin light polypeptide 6, TNFR-related death receptor 6 and PWP1-interacting protein 8 precursor were represented by more than one clone, so finally 23 different known genes were identified (Table 3). Ten clones had inserts matching in NCBI data new cDNA clones or big genomic clones with hypothetical cDNA sequences. Three of all identified clones instead of cDNA insert contained vector sequences. In this experiment the number of differentially expressed bands seen on a polyacrylamide gel was considerably smaller in comparison with experiments where cells were treated with IL-6 only. Majority of these differentially expressed bands were detected in a reproducible fashion in DD analysis, but for some of them, we were not able to optimize reaction conditions required for successful reamplification. Thus, almost half of the excised bands were not reamplified, especially when the bands were recovered from the dried gels.

4. Discussion

4.1. Response of HepG2 cells to IL-6 and IL-1

The results shown in Fig. 1 indicate that the employed treatment of HepG2 cells with IL-6 alone or after prestimulation with IL-1 leads to the development of typical acute phase response. When comparing the presented data with previous results [5] where HepG2 cells were stimulated with IL-1 alone, we found that among transcripts identified as differentially regulated by cytokines eight are controlled by both IL-1 alone and IL-6 alone. These transcripts encode proteins representing different functional groups: (a) engaged in metabolism (NADH dehydrogenase subunit 1, glyceraldehyde-3-phosphate dehydrogenase); (b) synthesis and processing of transcripts (nuclear RNA binding protein [p54nrb], aspartyl-tRNA synthetase, CREBBP/EP300 inhibitor 2 [EID2]); (c) cellular structure (kinesin 5 B, myosin-light polypeptide 6); and (d) signal transmission (prosaposin). Moreover, NADH dehydrogenase subunit 1 and myosin-light polypeptide 6 are the only two genes responding to IL-1, IL-6 and the mixture of two cytokines.

Eighteen clones in the experiment when cells were stimulated with IL-6, and ten clones after stimulation with IL-1/IL-6, did not show homology with known and functionally classified genes deposited in GenBank entries. We are currently engaged in the studies of these yet uncharacterized transcripts since they appear to be involved in specific acute phase response of HepG2 cells stimulated by both cytokines. Using slot blot analysis, we have already confirmed that most of them are indeed modulated by exposure of HepG2 cells to IL-6 or IL-1/IL-6 (data not shown).

When analysing all DD gels in experiments with IL-6 as the only cytokine used for HepG2 cell stimulation, we observed that the number of differentially expressed bands was much higher in comparison with the experiments where two cytokines (IL-1/IL-6) were used. This inhibitory effect of IL-1 on the expression of some IL-6-inducible genes is a well known phenomenon and can be explained by synthesis of specific proteins (SOCS) or activation of MAP kinase pathway [10,15], but these suggestions must be experimentally verified.

4.2. Possible biological functions of detected by us genes coding cellular proteins regulated by IL-6 and IL-1

Besides two main groups of genes engaged in metabolism and protein synthesis, there is a large group of genes coding for proteins that play an important role as signalling molecules. In the latter group significant changes in the level of expression were observed for FK506 transcript: almost 2-fold up-regulation was found after 24 h stimulation with IL-6. The protein encoded by FK506 is a potent immunosuppressive agent, highly effective in preventing organ transplant rejection in humans. It was earlier reported that FK506 has a profound anti-inflammatory effect [16]. Unfortunately, we did not detect a cDNA fragment of this gene in the experiment when cells were treated with IL-1/IL-6.

One of the genes modulated by IL-1/IL-6 codes for a subunit of proteasome 26 S (Table 3). This protein complex is an important player in NF-κB signaling pathway, responsible for degradation of IκB-inhibitor. The loss of IκB permits translocation of NF-κB to the nucleus [17] and subsequent binding to the target genes. But Shen et al. [9] proposed a model for a cross-talk between IL-1 and IL-6 transduction pathways with an additional important role for proteasome 26 S. According to this model IL-1 inhibits IL-6-induced STAT-1 tyrosine phosphorylation by a proteasome-dependent mechanism, followed by a down-regulation of IL-6-induced STAT activation. Our slot blot experiment data show quite significant decrease in the transcript level for a proteasome subunit. Therefore, regarding the model of Shen [9] the decrease in the transcript level may be associated not only with IκB degradation but also with inhibition of STAT1 activation. It has been generally accepted now that the ubiquitin-proteasome system is also involved in the transcriptional regulation (for references, see Muratani and Tansey [18]).

Among all genes identified as differentially regulated under stimulation by IL-6 or IL-1/IL-6 six were the same, detected in both experiments. These include: NADH dehydrogenase subunit 1, ribosomal protein S9, myosin-light polypeptide 6, TNFR-related death receptor-6 (DR6), glypican 3 and bladder cancer-associated protein. Only TNFR-related death receptor-6 protein product has been known so far to be engaged in NF-κB signaling pathway. This protein belongs to the family of TNF receptors and interacts with TRADD. Ectopic expression of DR6 gene in mammalian cells induces apoptosis and activation of both NF-κB and JNK [19]. It is interesting that the transcript level of TNFR-related death receptor-6 is decreased to one half after 4 h of IL-6 stimulation, and only after 24 h returns to

control values. But a totally different effect is observed after exposure of cells to IL-1/IL-6: almost 2-fold up-regulation occurs during the first 4 h following addition of cytokines and later the transcript level decreases. A synergistic effect of IL-1/IL-6 indicates that TNFR-related death receptor 6 belongs to type 1 acute phase reactants [1].

We presume that glypican-3 (GPC3) is also directly engaged in NF-κB signaling pathway, even if this property has not been described so far. Literature data show that GPC3 plays a negative role in cell proliferation, and blocking of endogenous GPC3 expression with an antisense transcript promotes growth of Hep3B cells [20].

Cytokine-modulated expression of several clones containing inserts coding for NADH dehydrogenase subunit 1 and ribosomal protein S9 (Table 2) is also understandable. As we have shown in the earlier study, the exposure of HepG2 cells to IL-1 leads to activation of the machinery responsible for cell metabolism and protein synthesis [5].

The last gene belonging to the group of genes modulated by IL-1/IL-6, bladder cancer associated protein, has unknown functions and its involvement in the acute phase reaction should be examined in detail.

The most frequently identified transcript (8 clones after IL-6 stimulation and 3 clones after IL-1/IL-6 stimulation), with quite significant changes in its level, encodes NADH dehydrogenase subunit 1. After times ranging from 12 to 24 h of HepG2 cells stimulation with IL-6, we observed more than 3-fold up-regulation of the transcript level (Table 2). It should be added that all 4 subunits of NADH dehydrogenase are up-regulated by the cytokines. In distinction to cells stimulated with IL-6 alone, pretreatment with IL-1 leads to a significant down-regulation of the transcript level. The direct role of NADH dehydrogenase subunit 1 in the inflammatory processes has not been evaluated so far. Arai et al. [21] studied the importance of reactive oxygen metabolites (ROMs) in up-regulation of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells after stimulation with TNFα. They showed that NADH dehydrogenase together with cytochrome bc_1 and complex NADPH oxidase are important enzymic sources of ROMs involved in this signaling. Specific inhibitors of these enzymes significantly decrease TNF-induced ICAM-1 expression. The significance of ROMs in mediation of signal transduction from various stimuli, including cytokines, endotoxins and PMA was postulated earlier but the source of ROMs was not clear [22,23]. Our studies provide additional evidence that mitochondrial enzymes, including NADH dehydrogenase subunit 1, may play an important role in the inflammatory processes. Both cytokines modulate the expression of NADH dehydrogenase subunit 1 but it looks that the joint action of IL-1/IL-6 inhibits its expression and in consequence ROMs production.

4.3. Technical problems encountered in the employed differential display analysis

In both types of cell stimulation some of the excised bands could not be amplified (12 in the IL-6 transcriptome profile analysis and 38 in the IL-1/IL-6 experiment). As we had noticed previously one of the reasons explaining difficulties with reamplification is low expression level of some genes resulting in poor recovery of cDNA from the gel [5], especially when the reaction was carried out after drying of the gel.

In two types of experiments (IL-6 alone or with IL-1), the unstimulated cells served as control. RNA was isolated from unstimulated cells after 1 h and 24 h of incubation. Comparing the densitometric values of both controls, we observed some differences in the transcript level of several genes, such as NADH dehydrogenase subunit 1, ribosomal protein S9, v-jun sarcoma virus 17, p53-inducible protein, zinc finger protein 503 (Tables 2 and 3). Many of identified genes belong to "housekeeping genes" and their protein products fulfil general functions of cells. Therefore, it can be expected that modulation of expression of these genes may not be controlled solely by cytokines but is probably influenced by other factors. It is possible that some tissue culture supplements, or confluency of cultured cells, may also affect gene transcription or stability of mRNAs. For this reason these housekeeping genes may not always be reliable reference check-points, although, some of them are still frequently used in the expression studies. Genes encoding proteins responsible for cellular metabolism (such as GAPDH) [24], for protein synthesis (such as elongation factor-2) [25] or cytoskeletal proteins (such as beta-actin) [26] are most often employed as control in Northern blot analysis, semiquantitative PCR and real-time PCR. Using Northern blot analysis, we observed significant changes in the transcript level of genes mentioned above in HepG2 cells stimulated with proinflammatory cytokines (data shown only for GAPDH; Table 2). This is in agreement with earlier studies, showing that activation of murine microvascular endothelial cells (MME) with TNF-α and IFN-γ resulted in a strong elevation of GAPDH mRNA level [27].

For these reasons, we decided to use genomic DNA as a standard during slot blot analysis. Since all PCR products (200 ng) on a slot blot membrane, representing different cDNA fragments, are complementary to a defined region of genomic DNA we used 50 ng of genomic DNA to establish the background of the slot blot experiment, to control pipetting errors and verify slot blot data. The value of intensity of each band corresponding to individual gene and appropriate time of stimulation was divided by the value of intensity of the spot from genomic DNA. All statistical data were validated when 3 independent experiments were performed. This procedure is depicted in detail in Fig. 3 for three genes modulated by IL-6 alone (A, B) and two genes modulated by IL-1/IL-6 (C, D).

4.4. Evaluation of differential display analysis as a method of studying changes in the profile of transcriptome induced by cytokines in HepG2 cells

The data presented in Tables 2 and 3 show that the identified genes encode cellular proteins engaged in metabolism, protein synthesis, trafficking, signal transduction, transcription, DNA interaction, posttranscription/posttranslation modifications, cell proliferation and proteolysis but none of these belong to typical plasma acute phase proteins. There are several possible reasons

why cDNA sequences encoding plasma acute phase proteins were not found in our DD-PCR analysis. The differential display is a simple screening method based on the PCR reaction. The major feature of this method is the use of arbitrary primers, which consist of different combinations of nucleotides. These primers bind to random templates and even at optimal PCR conditions are not sufficient to amplify all cDNAs which are expressed in a cell at any given time. Thus, this method enables to detect changes in the expression level of only some of all expressed genes. Another explanation for the lack of cDNA sequences encoding plasma acute phase proteins is related to the mechanism of PCR reaction. If more than one pair of primers is used in a PCR mix, for example as it is in multiplex PCR, the smaller fragments are amplified more efficiently than the larger fragments. Using specific primers, as is done for multiplex PCR in deletion screening of the dystrophin gene or profiling of STR (short tandem repeats) loci, it is possible to optimize reaction conditions to obtain several bands of different size, amplified with the same efficiency [28,29]. Using arbitrary primers in DD-PCR we were obtaining PCR fragments ranging from 600 to 2200 bp. Primers annealing to random cDNA templates make impossible the choice of optimal conditions to generate all products with equal efficiency. In many cases, we observed strong signals from small fragments and weak signals from large fragments (Fig. 2). Then, if PCR products are amplified with a very low or a very high efficiency, the differences in the real amplicon increase or decrease are not visible. Thus, part of these PCR products are false-negatives because the real changes in the expression level were not detectable. Therefore, by analysing DD-PCR products on polyacrylamide gels we are able to observe changes in the transcript level only for those cDNA templates for which the reaction conditions appear to be optimal.

One more reason for the lack on our list of genes encoding plasma acute phase proteins may be the colony screening procedure. In our experiments, PCR products with a visible difference in the expression level under different time check points were excised from gel, reamplified and cloned to a plasmid vector. Then only 3 colonies from each plate were subjected to sequencing. In several cases, we have observed that the collected colonies do not contain the same insert. We assume that the appearance of more than one cDNA in one excised PCR product is quite a frequent phenomenon and screening done only for three colonies out of all colonies grown on the plate might decrease the real number of differentially expressed genes.

The last, and probably the most important reason explaining that in our study, we have not found a single plasma acute phase protein gene is the ratio of intracellular proteins to plasma proteins. It has been assumed that there are not more that one hundred of acute phase proteins in plasma [13], whereas a differentiated human cell expresses around 10,000 genes [30]. Since we detected cytokine-induced differences for 56 cDNA (38 known genes and 18 hypothetical cDNA) in IL-6 stimulation study and for 33 cDNA (23 known genes and 10 hypothetical cDNA) in IL-1/IL-6 stimulation study, we might have missed all mRNAs coding for plasma acute phase protein genes.

4.5. Concluding remarks

The changes reported here in the transcriptome of HepG2 cells stimulated with IL-6 or IL-1/IL-6 appear to be more complex than anticipated so far. After stimulation with 2 cytokines we identified 61 genes encoding cytoplasmatic and nuclear proteins (Tables 2 and 3). Moreover, we found 28 templates representing hypothetical proteins or cDNA fragments complementary to BAC/PAC clones, therefore, belonging to genes not identified so far. For all known genes the detailed analysis concerning changes in the transcript level was carried out and is presented in Tables 2 and 3. Our experiments suggest also that the inflammatory response of HepG2 cells is a high energy-consuming process for the cells involved because many of the identified genes participate in cellular metabolism, such as mitochondrial genes of respiratory complex I.

Up to now, there is no precise data as to how many genes are expressed in HepG2 cells under proinflammatory cytokine stimulation and all available methods used in the transcriptome analysis offer certain advantages or disadvantages [3]. The results presented here provide additional new information on changes in gene expression of HepG2 cells stimulated with IL-6 or IL-1/IL-6 for times ranging from 1 to 24 h. However, our experiments indicate also that the differential display analysis–being a valuable tool in discovering unknown cytokine-responsive genes–cannot be used for evaluation of the transcriptome profile because it may miss altogether products of transcription of several genes for the reasons discussed in detail above.

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