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Biochimica et Biophysica Acta 1689 (2004) 120–133

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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Identification of changes in the transcriptome profile of human hepatoma HepG2 cells stimulated with interleukin-1 beta

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Received 29 December 2003; received in revised form 27 February 2004; accepted 2 March 2004
Available online 19 March 2004

Abstract

Interleukin-1 (IL-1) is the principal pro-inflammatory cytokine participating in the initiation of acute phase response. Human hepatoma HepG2 cells were exposed to 15 ng/ml of IL-1beta for times ranging from 1 to 24 h and the total RNA was isolated. Then cDNA was obtained and used for differential display with 10 arbitrary primers and 9 oligo(dT) primers designed by Clontech. Validation of observed changes of differentially expressed known genes was carried out by RT-PCR or Northern blot analysis. Out of 90 cDNA strands modulated by IL-1, 46 have been successfully reamplified and their sequencing indicates that they represent 36 different cDNA templates. By GenBank search, 26 cDNA clones were identified as already known genes while 10 showed no homology to any known gene. The identified transcripts modulated by IL-1 in HepG2 cells code for intracellular proteins of various function: trafficking/motor proteins (3 genes), proteins participating in the translation machinery or posttranscriptional/posttranslational modifications (7 genes), proteases (1 gene), proteins involved in metabolism (6 genes), activity modulators (3 genes), proteins of the cell cycle machinery (2 genes) and those functionally unclassified (4 genes). Majority of genes responded to IL-1 within 1 to 6 h (early genes), while two were late response genes (12–24 h) and four showed prolonged response over the whole 24-h period. Most of the observed changes of expression were in the range of two- to threefold increase in comparison to control untreated cells. Among identified genes, no typical secretory acute phase protein was found. The obtained results suggest that IL-1 affects the expression of several genes in HepG2 cells, especially those engaged in the synthesis and modifications of proteins.
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Keywords: Inflammatory transcriptome; Acute phase protein; Inducible gene expression; Differential display; RT-PCR; Northern blot analysis

1. Introduction

The principal cytokines initiating the inflammatory reaction include interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), whereas IL-6 and related cytokines are held responsible for enhanced production of acute phase proteins in the liver cells [1,2]. Although the inflammatory and acute phase responses are highly complex phenomena, they can be analysed in a model system of cell culture stimulated by cytokines. Rat and human liver cell cultures provided a plethora of data on the mechanism of induced synthesis of plasma proteins (see Ref. [3]), but relatively little is known about cytokine-induced changes in the expression of intracellular proteins of hepatocytes. Moreover, the role of IL-1 in hepatocyte metabolism and liver-specific gene expression has only been partially elucidated and is limited to some acute phase proteins [4].

So far, very few comprehensive studies have been conducted with the aim at finding all genes involved in the acute phase reaction in the liver cells. Olivier et al. [5] investigated the liver acute phase response by subtractive hybridization after injecting rats with Freunds adjuvant. The authors identified genes coding for 23 known acute phase proteins, 31 genes coding for other proteins up to now not related to the inflammatory response, and 36 novel proteins of unknown function. The adult human liver transcriptome was analysed by cDNA array hybridization by Yano et al. [6]. A total of 2418 gene transcripts were assigned to the liver tissue obtained by surgical biopsy from five subjects.

Abbreviations: IL-1β, interleukin-1 beta; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; IL-6, interleukin 6

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doi:10.1016/j.bbadis.2004.03.002

In two of these patients, gene expression pattern resembled a model acute phase response. Clearly, the results obtained by Olivier et al. [5] and Yano et al. [6] derive from a complex system in which the involvement of individual cytokines cannot be properly evaluated. For this reason, we decided to use highly differentiated human hepatoma HepG2 cells that are known to respond to several cytokines in a manner similar to liver cells (see Ref. [7]). HepG2 cells were cultured in vitro and treated with relatively low doses of IL-1β in order to simulate the initial stages of the inflammatory response. The culture was maintained for time periods ranging from 1 to 24 h after exposure to IL-1 and differential display method was used to characterize changes in the transcript profile. A preliminary report of these studies was presented during 30th Annual Conference "Progress in Molecular Biology" in Zakopane [8].

2. Materials and methods

2.1. Cell culture

HepG2 cells (from ATCC, passages 11-16) were cultured at 37 °C and 5% CO_2 in Dulbecco Modified Eagle Medium (DMEM) with 1000mg/l D-glucose (Gibco/BRL) supplemented with 10% foetal bovine serum (FBS), 2.5 μg/ml plasmocin (Invivogen). Cells were grown in plastic Petri dishes (10 cm in diameter, TPP, Switzerland) to approximately 70% confluence. At 15 h before IL-1β treatment, they were washed twice in PBS and placed in DMEM containing 0.5% FBS without plasmocin. Then cells were stimulated with human IL-1β (kindly provided by Dr. C. Dinarello, Denver, CO, USA) at a final concentration of 15 ng/ml for different time periods (1, 2, 4, 6, 12, 18, 24 h). The responsiveness of cells to IL-1β was demonstrated by increased levels of mRNA coding for manganese superoxide dismutase and by modulation of IL-6 signalling as reported elsewhere [9].

2.2. Differential display analysis

Total RNA was isolated from unstimulated (control) and IL-1-stimulated cells according to the Chomczynski and Sacchi [10] method. Briefly, cells were treated with GTC solution (4 M guanidinum thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1), RNA precipitated with isopropyl alcohol, washed with 70% ethanol and the pellet was dissolved in DEPC-treated water. The first strand of cDNA was synthesized from 5 μg of total RNA in 20 μl using SuperScript RNaseH$^-$reverse transcriptase (Promega). Reaction mixtures were diluted five times and then 5 μl was taken to the differential display analysis. Primers used in the PCR reaction (10 arbitrary primers and 9 oligo(dT) primers) were designed by Clontech.

Sequences of arbitrary primers: p1: attaaccctcactaaatgctgggga; p2: attaaccctcactaaatcggtcatag; p3: attaaccctcactaaatgctggtgg; p4: attaaccctcactaaatgctggtag; p5: attaaccctcactaaagatctgactg; p6: attaaccctcactaaatgctgggtg; p7: attaaccctcactaaatgctgtatg; p8: attaaccctcactaaatggagctgg; p9: attaaccctcactaaatgtggcagg; p10: attaaccctcactaaagcaccgtcc.

Sequences of oligo(dT) primers: t1: cattatgctgagtgatatctttttttttaa; t2: attatgctgagtgatatctttttttttac; t3: cattatgctgagtgatatctttttttttag; t4: cattatgctgagtgatatctttttttttca; t5: cattatgctgagtgatatctttttttttcc; t6: cattatgctgagtgatatctttttttttcg; t7: cattatgctgagtgatatctttttttttga; t8: cattatgctgagtgatatctttttttttgc; t9: cattatgctgagtgatatctttttttttgg.

After three low-stringency cycles (94 °C–1 min; 40 °C–1 min;72 °C–5 min) 25–30 high-stringency cycles were done (94 °C–1 min; 55 °C–1 min;72 °C–1 min). The amplified cDNA fragments were separated by electrophoresis on polyacrylamide gels (200 × 200 × 1 mm) with 4% concentration of upper half and 6% of lower half of the gel to separate fragments of 200–2500 bp. The PCR products were visualised by the silver staining method.

2.3. Band recovery and sequence analysis

Differentially expressed amplified cDNA fragments were excised from the dried gel, soaked in water and reamplified using the same set of primers as in original PCR. After agarose gel purification (Qiagen) PCR products were cloned to pTZ57R vector from InsT/A clone™ PCR product cloning kit (Fermentas). After transformation, three clones from each bacterial plate were checked by restriction analysis using *Eco*RI and *Hin*dIII restriction enzymes. One of the analysed clones was sequenced using PCR sequencing method and a primer labelled with [γ^{32}P]dATP according to the manufacturer (Perkin Elmer).

2.4. Computational analysis

Sequences of differentially expressed genes were determined by searching the GenBank data at the National Center for Biotechnology Information using BLAST program.

2.5. RT-PCR

Validation of observed changes of differentially expressed known genes was carried out by semiquantitative RT-PCR or Northern blot analysis. PCR primers were designed on the basis of data obtained in cycle sequencing and GenBank data. The sequence of reverse transcriptase-PCR (RT-PCR) primers is available from the authors on request. cDNA was prepared from 1-μg RNA isolated from the control sample or after IL-1β stimulation for different time periods (1, 2, 4, 6, 12, 18, 24 h). Amplification consisted of 25 cycles with fivefold diluted cDNA. RT-PCR samples (8 μl) were electrophoresed through a 1.5% agarose gel. All RT-PCR products were verified by measuring the amounts of elongation factor 2 (EF2) transcript in

each sample obtained by RT-PCR with the use of EF2-specific primers. PCR products were visualised by ethidium bromide staining and the changes in gene expression were measured using Multiimage Scanner and software Quantity One (BioRad).

2.6. Northern blot analysis

Total RNA was isolated from control and IL-1-stimulated cells (after 1, 2, 4, 6, 12, 18 and 24 h of stimulation) using the guanidinum/phenol extraction procedure [10]. Ten micrograms of total RNA were separated in 1% formaldehyde agarose gel and blotted to a nitrocellulose membrane. Prehybridization and hybridization were carried out at 65 °C in 1% SDS, 1 M NaCl, 10% dextran sulfate solution. The cDNA probes for selected genes were amplified using standard primers for M13 vector which are present in pTZ57R vector. PCR products were purified (Qiagen) and labelled with [α-^{32}P]dCTP using the random primer labelling kit (Promega). After the washing procedure, RNA blots were exposed to phosphoimage screen and then read out using Molecular Imager FX and software Quantity One (BioRad).

2.7. Calculation of changes in the level of expression

To analyse the results from Multiimage Scanner (RT-PCR) and Molecular Imager FX (Northern blot analysis), the densitometric readings of Quantity One software were calculated using Microsoft Exel. The value of intensity of each analysed band was divided by the value of intensity EF2 band for RT-PCR, or 28S for Northern blot analysis, and the final results were calculated as percentage values of the control. The reported values represent the mean of three independent experiments. Statistical analysis was carried out with the use of *t*-test.

3. Results and discussion

In order to evaluate the transcriptional profile of hepatoma cells stimulated with IL-1β, a comprehensive analysis of cDNA from control and cytokine-treated cells was carried out using differential display (Fig. 1). To exclude contamination of RNA preparation with DNA, the amplification procedure was also performed directly on RNA (100 ng) samples. Using a combination of 10 arbitrary and 9 oligo(dT) primers, 90 PCR reactions were completed. Different pairs of primers generated various transcript profiles. Many of these combinations did not yield any differentially expressed band but, overall, we found 90 IL-1-modulated cDNA species. All differentially expressed bands, excised from the gel, were subjected to reamplification but only 46 were successfully reamplified and cloned to pTZ57R vector. The problems with reamplification of the remaining 44 bands are the consequence of a low expression level resulting in poor recovery of cDNA from the gel and possible contamination with acetic acid leading to the inhibition of *Taq* polymerase.

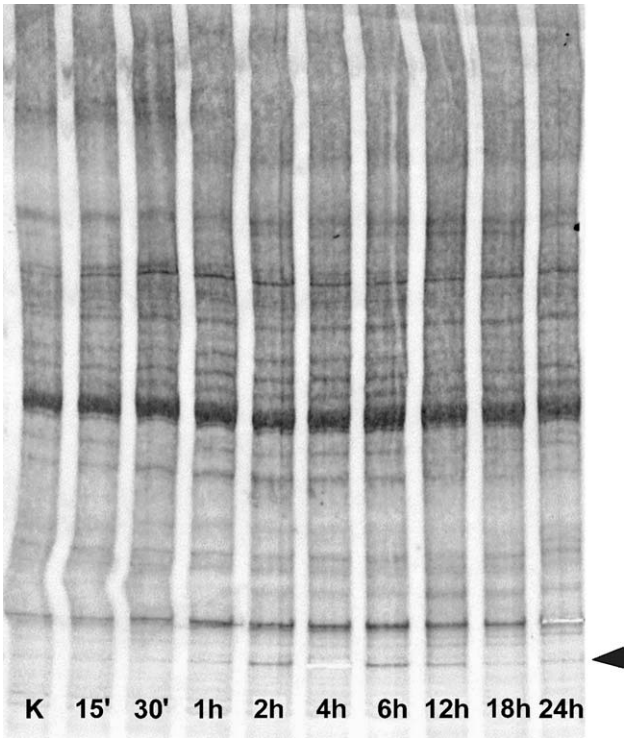


Fig. 1. Differential display analysis. A representative picture of DD-PCR products separated on polyacrylamide gels and detected by the silver staining procedure. In this particular case, PCR reaction was done with primers P1/T7. One of the differentiated bands (marked with an arrow) represents the cDNA fragment encoding RPL19.

All identified transcripts with known full cDNA and aminoacid sequence could be classified into several groups according to the function of proteins encoded by these transcripts: trafficking/motor proteins, proteins participating in the translation machinery or posttranscriptional/posttranslational modifications, proteases, proteins involved in cellular metabolism, activity modulators, proteins of cell cycle machinery and those functionally unclassified (Table 1). Many of the identified genes were represented by more than one clone, e.g. NADH-ubiquinone oxidoreductase subunit 1 was represented by five clones and subunit 4 by two clones. Altogether, eight clones matched mitochondrial genes. Another group represented by several clones matched genes involved in the translation machinery: four ribosomal proteins (RPL6, RPL19, RPL26, RPL41), aspartyl-tRNA synthetase and two proteins involved in the posttranscriptional/posttranslational modifications (protein disulfide isomerase ERp72 and nuclear binding protein p54nrb).

Changes in the expression of identified genes with a known function were in all cases confirmed by semiquantitative RT-PCR or Northern blot analysis with RNA from control sample and after IL-1β stimulation for different time periods (1, 2, 4, 6, 12, 18, 24 h; Fig. 2A-I). Comprehensive

Table 1
Classes of genes responding to IL-1 stimulation in HepG2 cells

	GenBank accession	Classification
Early response genes (1–6 h)		
Kinesin family member 5B	NM_008448	Trafficking/motor proteins
Myosin, light polypeptide 6 (MYL6)	NM_079423.1	Trafficking/motor proteins
Ral guanine nucleotide dissociation stimulator-like 1	BC039250.1	Activity modulators
Nuclear RNA binding protein (p54nrb)	XM085471.2	Posttranscription/ posttranslation modification
Protein disulfide isomerase related protein (ERp72)	XM170518	Posttranscription/ posttranslation modification
NADH dehydrogenase subunit 1	AAL54553.1	Metabolism
NADH dehydrogenase subunit 4	BC014376	Metabolism
NADH dehydrogenase subunit 6	NM_173714	Metabolism
Ribosomal protein L41 (RPL41)	NM_021104	Protein synthesis
Ribosomal protein L26 (RPL26)	AB061829	Protein synthesis
Ribosomal protein L19 (RPL19)	NM_000981.2	Protein synthesis
Ribosomal protein L6 (RPL6)	NM_000970	Protein synthesis
Aspartyl-tRNA synthetase	NM_001349	Protein synthesis
CLL-associated antigen KW-8	AF432221	Functionally unclassified
NY-REN-45 antigen	NM_016121.1	Functionally unclassified
Chromosome-associated polypeptide C (hCAP-C)	AB019987.1	Cell cycle machinery
Apolipoprotein B	NM_003900	Metabolism
Short chain alcohol dehydrogenase (HEP27)	AF244132.1	Metabolism
Glyceraldehyde-3-phosphate dehydrogenase	BC029618	Metabolism
Late response genes (8–24 h)		
Tyrosine 3 monooxygenase/ tryptophan 5-monooxygenase activation protein	NM_139323.1	Cell cycle machinery
Sequestosome 1 (SQSTM1/p62)	X04506	Activity modulators
Prolonged response (1–24 h)		
Cathepsin D (CTSD)	NM_001909.3	Proteases (lysosomal aspartic proteases)
Dynein heavy chain (DNN)	AY050643	Trafficking/motor proteins
MGC20452/EID-2	NM_153232.1	Functionally unclassified
Prosaposin	NM_002778	Metabolism

Expression analysis is not presented for cDNA encoding CoxII/D-loop DNA fusion protein (L78671). For details see text.

analysis of transcript profile under different time of exposure to IL-1β revealed three types of gene response: early response (1–6 h), late response (12–24 h) and prolonged response (1–24 h) (Table 1, Figs. 2 and 3).

The trafficking/motor proteins are represented by three genes encoding one component of the hexameric protein-alkali light chain-myosin [11], kinesin-family member 5B [12] and the heavy chain of dynein [13]. Confirmation of differential display analysis by RT-PCR revealed up-regulation of these genes (Figs. 2A and 3A). Myosin and dynein transcripts show a prolonged increase following IL-1 treatment whereas kinesin represents the early response gene. Myosin is an important component of muscle cells but is also essential in the maintenance of cellular structures and movements of other cells. This protein exists in phosphorylatable and nonphosphorylatable forms which arise from two different transcription start sites and alternative splicing of the same gene [14]. So far, the up-regulation of transcription of myosin alkali chain was described in T7D human breast cancer cells under ethanol exposure [15], so the response to IL-1 is a novel observation. It is known that cytoskeletal proteins are important in the progression of apoptosis [16]; thus, it is possible that the increase in transcript level of myosin light chain elicited by prolonged exposure to IL-1 may preceed cell death. Kinesins and dyneins are involved in movements of organelles within cells. Using RT-PCR, we observed a prolonged increase of dynein transcript with 2.5-fold up-regulation after 24 h of IL-1 exposure. The level of transcript encoding kinesin increased twofold after 6 h of IL-1 exposure and then declined to control values after 24 h. Zhu et al. [17] observed IL-1-induced alteration in cell shape and in the cytoskeletal organisation of fibroblasts and related them to the effect of cytoskeleton organisation on the IL-1 signal transduction. Vinall et al. [18] showed that morphogens (e.g. bone morphogenetic protein-7) and cytokines (IL-1) change in chondrocytes the expression level of cytoskeletal proteins, such as tensin, talin, paxillin and focal adhesion kinase. Such data suggest that probably changes in the transcript level of other cytoskeletal proteins, so far not determined, are important in regulation of cell structure during adaptation to the stress. The genes identified by us enlarge the group of cytoskeletal proteins directly involved in IL-1-elicited response.

A prolonged and late increase in the expression level was observed for cathepsin D, a lysosomal aspartic protease (Figs. 2B and 3B), important in degradation and processing of many proteins. Despite extensive research on cathepsin D, rather little is known about the role of cytokines in its regulation. Whitaker et al. [19] studied the expression level of cathepsin D induced by cytokines and leupeptin in astrocytes. Under serum-free conditions, leupeptin increased by 1.4–2-fold the protein level and Northern blot analysis showed concomitant changes in mRNA. By contrast, no changes were observed in the level of cathepsin D after exposure of astrocytes to INFγ, TNF-α, IL-1β, LPS and calcium ionophore. Kudo et al. [20] studied in vitro metabolic degradation of human interleukin-1beta (IL-1β) using lysates of rat kidney lysosomes, and identified proteases involved in this process. The results suggest that cathepsin B, cathepsin L, and cathepsin D in kidney lysosomes are involved in the metabolic degradation of human IL-1β. Using Northern blot analysis, we found that in HepG2 cells treated with IL-1, the level of mRNA coding for cathepsin D

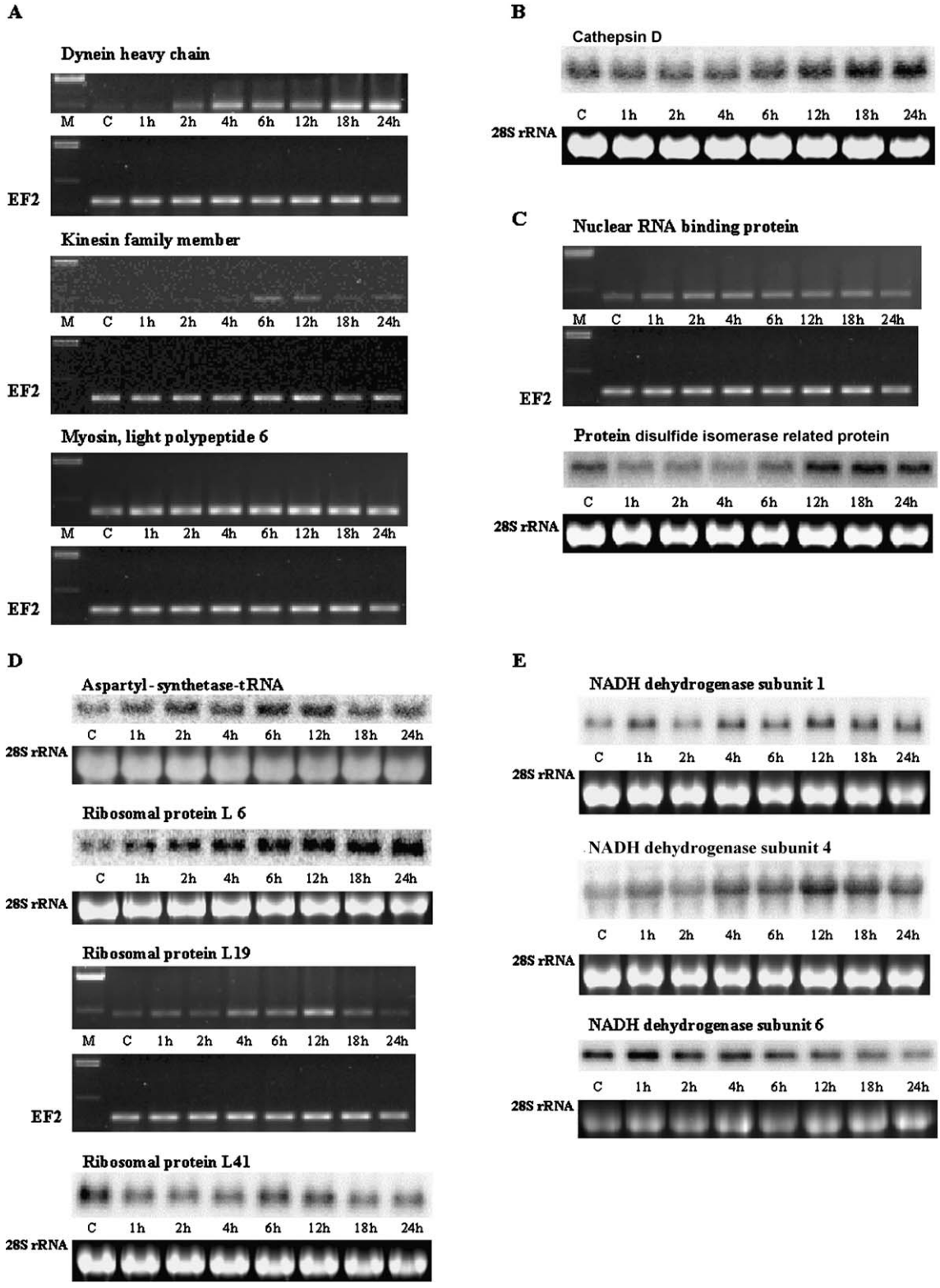


Fig. 2. Representative pictures of Northern blots or RT-PCR analysis obtained for the examined genes in the order presented here (A-I). The reference for Northern blots was 28S rRNA, and for RT-PCR the product of the EF2 gene.

increases twofold (Figs. 2B and 3B). It may be speculated that this represents a regulatory mechanism to terminate the stimulatory action of IL-1β.

The group of genes coding for proteins participating in posttranscriptional/posttranlational modifications includes nuclear binding protein-p54nrb, a product associated with

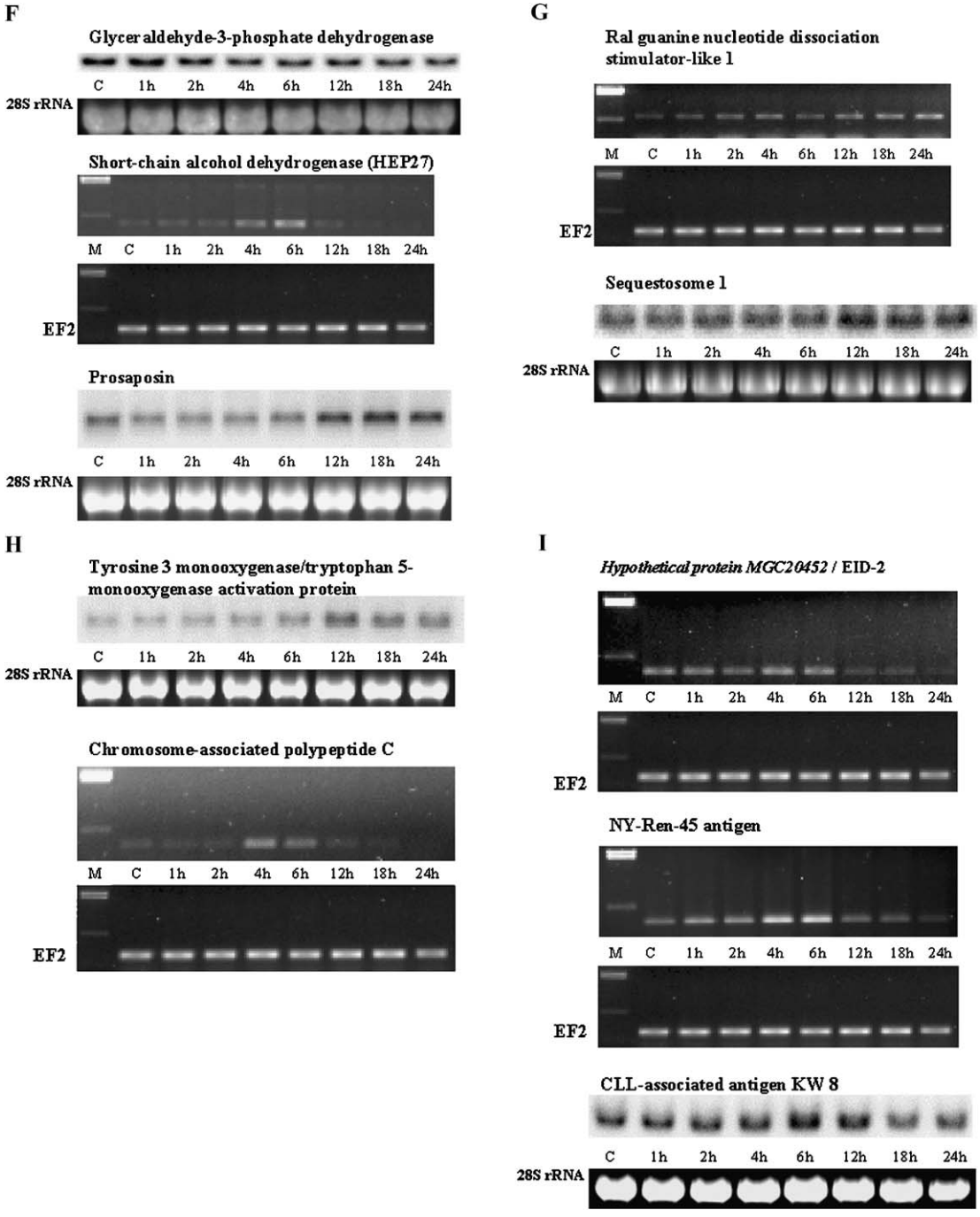
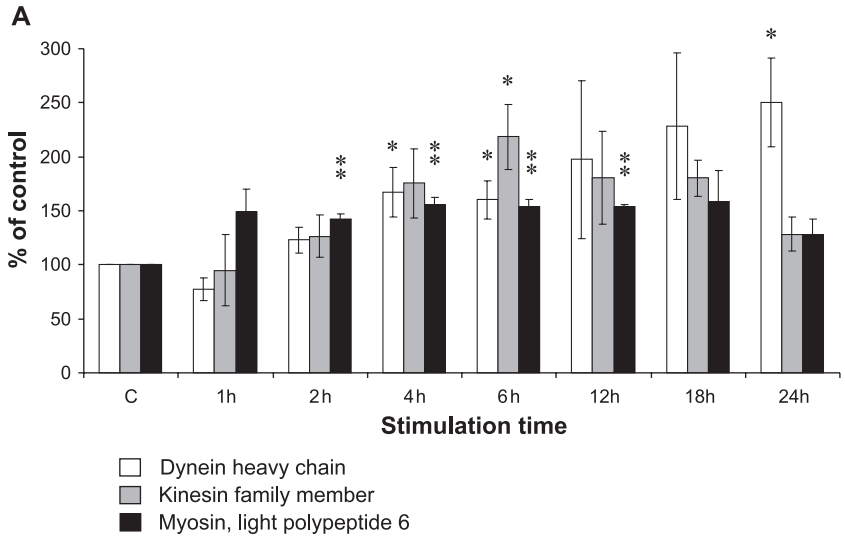


Fig. 2 (*continued*).

pre-mRNA processing [21,22], and another protein important for folding and/or assembly of proteins: disulfide isomerase ERp72. Our results indicate that nuclear binding protein-p54nrb belongs to the group of early response genes and is up-regulated in HepG2 cells by 1.7-fold after 2 h and returns to the baseline level after 24 h of IL-1β treatment (Figs. 2C and 3C). It has been shown that p54nrb interacts with PSF (polypyrimidine tract binding protein-PTP associated splicing factor); these two proteins bind U5snRNA and are involved in splicing [23]. Ample evidence indicates that p54nrb participates not only in RNA splicing but also in other steps of pre-mRNA processing such as 5′-end cap and 3′-end formation. By binding to the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II, the nuclear binding protein p54nrb may fold this domain into a form active in pre-mRNA processing [22].

Another representative of posttranscriptional/posttranlational modification class is ERp72, belonging to the family of protein disulfide isomerases (PDIs), present in the endoplasmic reticulum (ER), involved in the formation of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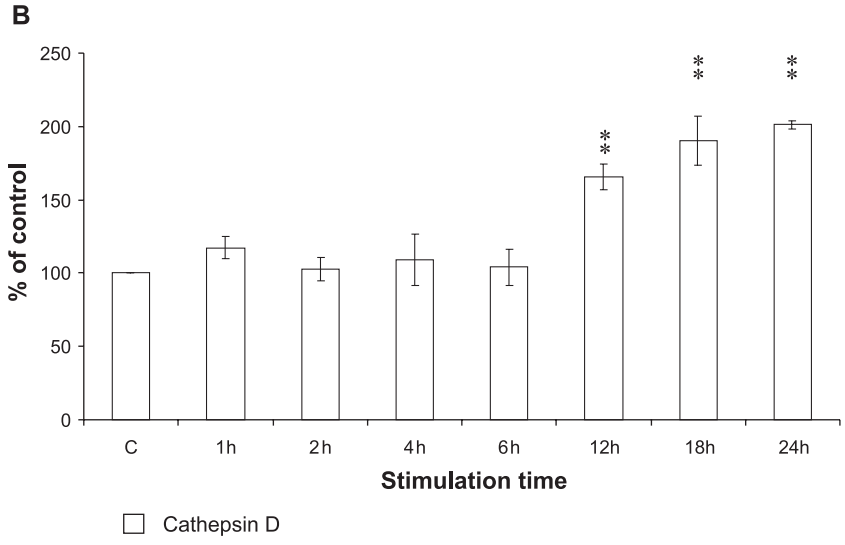
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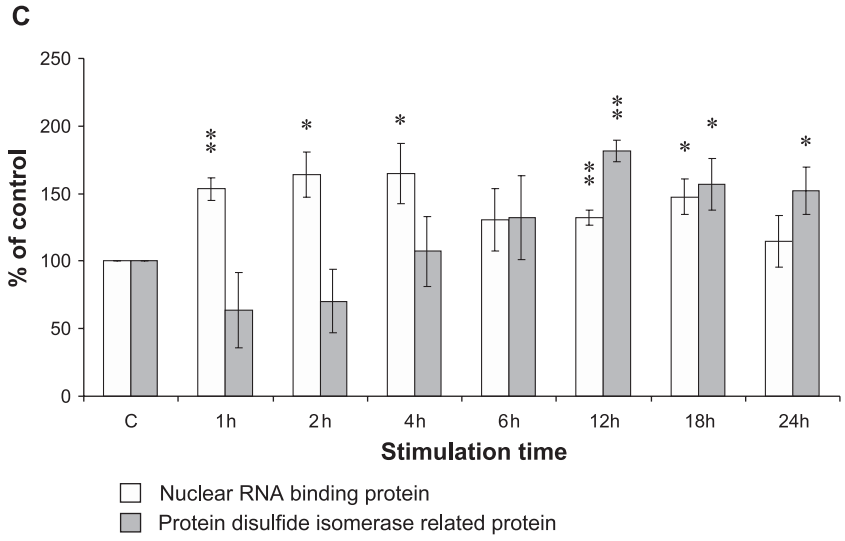
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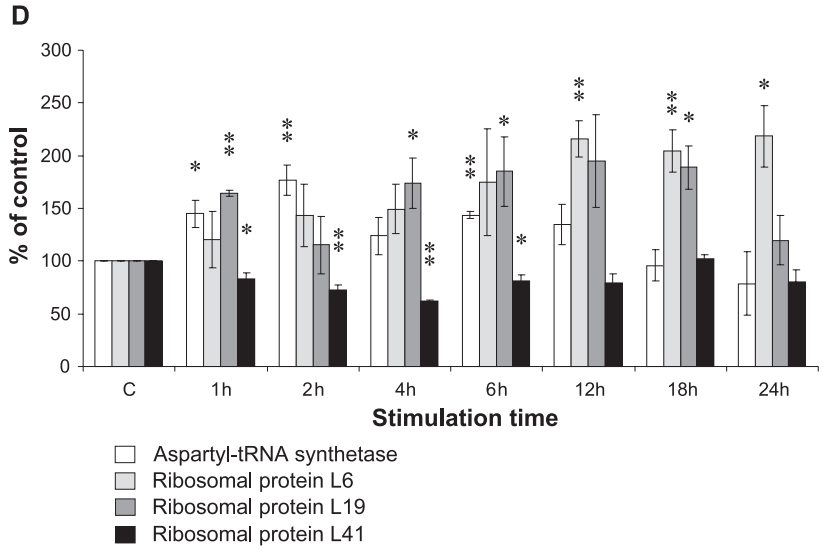
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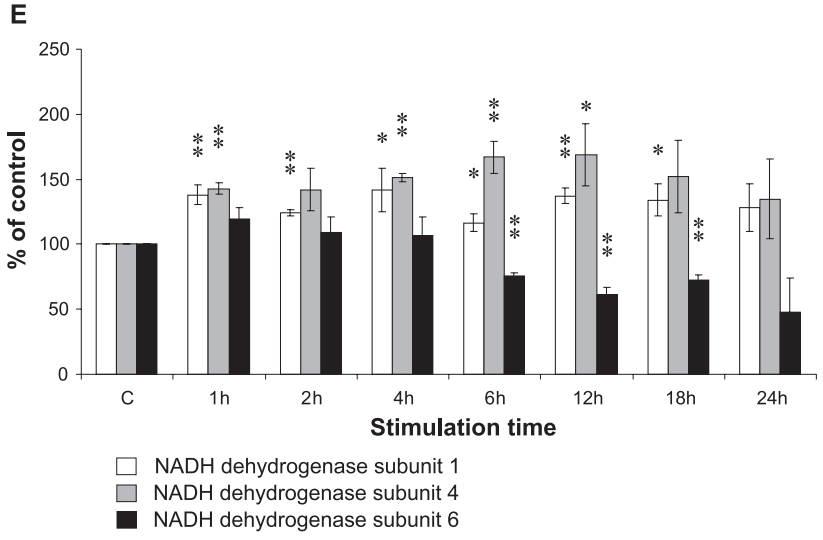
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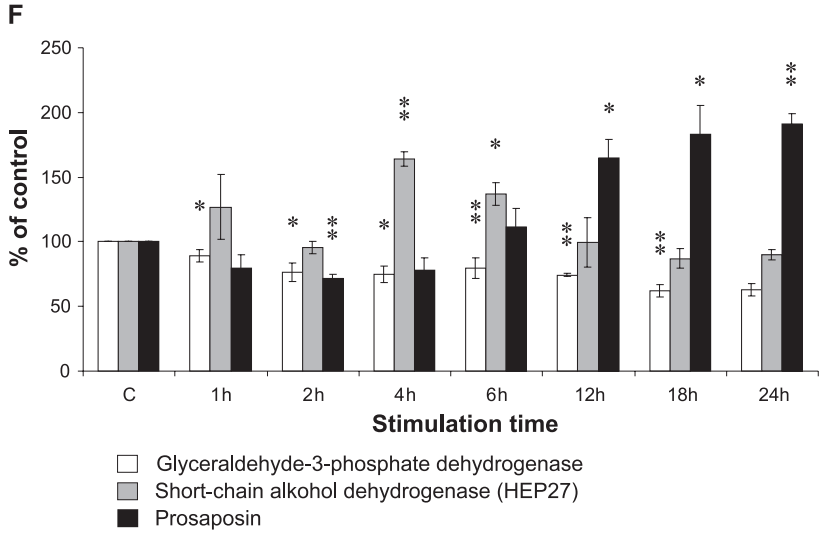
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Prosaposin

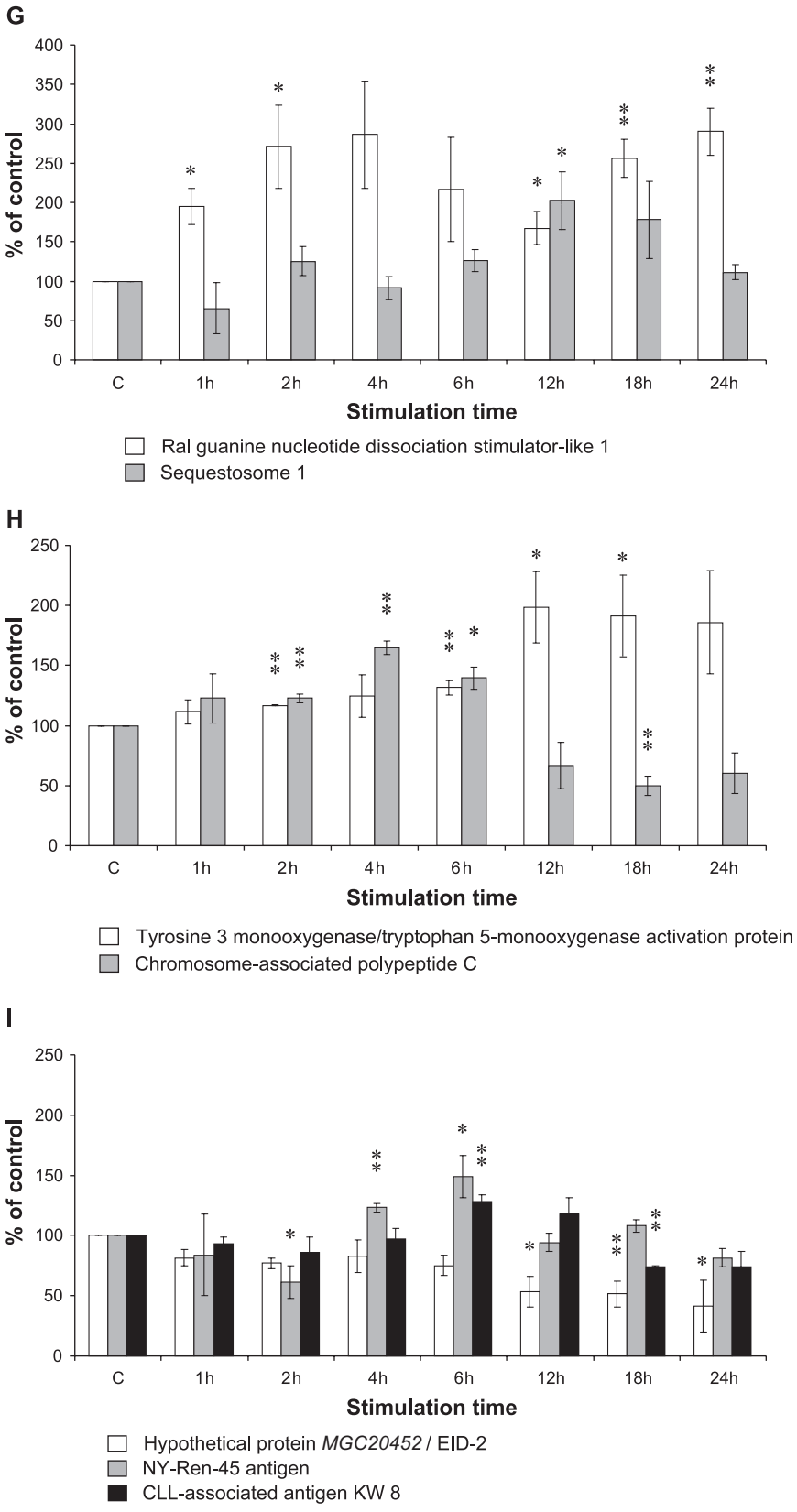

Fig. 3. Results of densitometric analysis of relative changes in mRNAs abundance as determined by Northern blot or RT-PCR (mean ± S.E. of three independent experiments). Statistical analysis was carried out with the use of *t*-test; $*P<0.05$; $**P<0.025$. (A) Trafficking/motor proteins; (B) lysosomal proteases; (C) proteins involved in posttranscriptional/posttranslational modifications; (D) proteins involved in translation machinery; (E and F) proteins involved in metabolism; (G) activity modulators; (H) proteins involved in cell cycle machinery; (I) proteins functionally unclassified.

disulfide bonds and functioning as a molecular chaperone in the folding and/or assembly of membrane and secretory proteins [24,25]. The Erp72 mRNA and protein levels were found to increase in neuroblastoma cells exposed to hypoxia and this corresponded to a neuroprotective effect in the rat brain ischemia [26]. We observed that after 1 h of exposure of HepG2 cells to IL-1β, the mRNA level for ERp72 decreases by 30% in comparison to the control but then it rises to a maximum at 12–18 h (Figs. 2C and 3C). It is interesting that the gene important for mRNA processing is maximally active during the first 2 h of IL-1 exposure, whereas that one coding for the product involved in protein modifications reaches the maximal expression later. This would indicate that regulation of p54nrb and ERp72 genes controlling the quality of transcripts and final protein products is important in cell adaptation to stress response elicited by IL-1β. Although we cannot provide data on the level of proteins coded by these two genes, it is very likely that HepG2 cells respond to IL-1β by activating the machinery responsible for synthesis and processing of transcripts important in the acute phase response. This is further supported by the observed changes in the expression of transcripts encoding ribosomal proteins (RPL6, RPL19, RPL41) and aspartyl-tRNA synthetase (Figs. 2D and 3D). It looks that during cytokine-elicited stress, cells increase the levels of proteins involved in the translation machinery in order to respond appropriately to the environmental changes.

From 36 known genes identified as differentially expressed in HepG2 cells exposed to IL-1β, seven represent those involved in cellular energy metabolism. NADH dehydrogenase subunits 1, 4 and 6 encoded by mitochondrial DNA are among 41 polypeptides of respiratory Complex I. Another transcript encoding HEP27 protein has been already identified by Heinz et al. [27] as up-regulated during monocyte to dendritic cell differentiation. The gene contains two alternative promoter regions: a hepatocyte-specific promoter induced by the histone deacetylase inhibitor sodium butyrate in several cell types, and a second upstream promoter which was specifically active in monocyte-derived dendritic cells. A possible role of the HEP27 protein has yet to be explained. Gabrielli et al. [28] predicted from cDNA the amino acid sequence indicating that functional domains of HEP27 are similar to the SCAD family (short-chain alcohol dehydrogenase enzymes family). In our study, we observed up-regulation of HEP27 transcript in HepG2 cells with a maximum increase after 6 h of IL-1 treatment (2.5–2.7-fold increase; Figs. 2F and 3F).

Confirmation of the results of differential display by Northern blot analysis indicates that another transcript encoding a protein involved in metabolism-prosaposin is up-regulated, with a 1.8-fold increase after 24 h of IL-1 treatment. Our results indicate that this gene belongs to the prolonged response genes (Table 1). Prosaposin is present in the brain and muscle, but processed saposins are found in the spleen, liver and kidney [29]. Proteolytic cleavage of prosaposin generates four sphingolipid activator proteins (saposin A, B, C, D) [30] responsible for the metabolism of lipids. There is evidence that prosaposin is involved in signal transduction. Earlier studies showed an up-regulation of prosaposin in differential display screening of mRNA extracted from breast cancer cell lines (T47D, MCF7) after treatment with HPR (*N*-4-hydroxyphenylretinamide), a synthetic retinoid which has antiproliferative and apoptotic effects in vitro against several malignant cell lines. Panigone et al. [31] suggested that chemopreventive activity of HPR arises from its effect via prosaposin on integrin receptors, which are down-regulated resulting in reduced metastatic activity of tumor cells. It was also shown that prosapeptides, synthetic peptides (14–22 residues) from the C domain, induce differentiation and prevent cell death in a variety of neuronal cells [32,33]. Their action leads to enhanced phosphorylation of mitogen-activated kinases in Schwann cells, ERK1 and ERK2, which are involved in the MAPK pathway [34]. In many cellular systems, MAPK activation leads to changes in gene expression, especially the genes associated with cellular proliferation and differentiation [35]. Our data showing up-regulation of prosaposin mRNA may provide additional evidence that IL-1β activates molecular mechanisms essential for cell survival rather than apoptosis.

Other proteins involved in cellular metabolism and affected by IL-1β in HepG2 cells include apolipoprotein B and glyceraldehyde-3-phosphate dehydrogenase (Table 1). Yokoyama et al. [36] observed a significant increase in apoB mRNA after 1 h of stimulation of HepG2 cells with IL-1 while the maximum mRNA level was reached 3 h after stimulation and persisted for up to 18 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme involved in cytosolic energy production. Aldred and Schreiber [37] showed that the level of mRNA for GAPDH is decreased after induction of acute inflammation during the first 80 h, suggesting that proinflammatory cytokines affect cellular glucose degradation. Using Northern blot analysis, we observed a down-regulation of GAPDH (Figs. 2F and 3F). Regulation of genes involved in glucose metabolism is probably of physiological relevance since IL-1 is known to affect the energy balance [38].

The results of both differential display analysis and Northern blot indicate the up-regulation of mRNA coding for sequestosome 1 (SQSTM1/p62), a protein which noncovalenly binds ubiquitin [39]. Ubiquitination of cellular proteins is an important step initiating degradation of many proteins such as cell surface receptors, mitotic cyclins, oncoproteins, transcription regulators such as IκB, NFκB, c-Jun and c-Fos and tumor suppressor p53 [39]. Sanz et al. [40] showed that p62 interacts with atypical kinase C (aPKC) which is important in cell proliferation and survival. p62 mediates intracellular signalling through the IL-1/TNF pathways towards NFκB known to have a protective role by inducing antiapoptotic factors [41]. Down-regulation of p62 severely reduces NFκB activation by IL-1 and TRAF6 [40].

According to our observations, the gene encoding sequestosome 1 is from the category of late response genes under IL-1 stimulation. It is possible that activation of p62 is promoted by aggregation and sequestration of abnormal proteins. Increased amounts of unfolded or misfolded proteins are observed in pathogenesis of many diseases [42]. It may be expected that such phenomena occur frequently during a stress response and up-regulation of p62 after IL-1 exposure helps to protect cells against accumulation of inappropriately folded proteins. The formation of Mallory bodies (MB), considered as a pathological form of sequestosome, has been observed in alcoholic hepatitis and other liver disorders. The MBs consist of abnormal proteins (keratins), heat shock proteins (HSP25, HSP70 and HSP70) and also of p62. The role of p62 in MBs formation is unclear but, undoubtedly, it takes part in ubiquitination of proteins assigned for degradation.

To the group of signal transducers belongs also Ral guanine nucleotide dissociation stimulator-like 1 (RGL), involved in Ras and Ral signalling pathways as a downstream effector protein [43]. RGL transcript is highly up-regulated under IL-1β exposure: we observed a biphasic response with the maximum after 4 and 24 h (Figs. 2G and 3G). Nothing is known about the function of this protein in acute phase response but, according to reports of Ljungdahl et al. [44], NFκB is a downstream mediator of Ras signaling pathway. Thus, we suggest that RGL activation may promote cell survival and acute phase protein synthesis.

The last group of identified transcripts with a known function includes proteins involved in cell cycle machinery (Figs. 2H and 3H). Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAB) belongs to the 14-3-3 family, highly conserved from bacteria to humans and plants. Proteins that interact with 14-3-3 isoforms are involved in signal transduction by binding to phosphoserine/threonine-containing proteins, and thus affect cell cycle regulation, cell survival, apoptosis, proliferation and stress response [45,46]. We observed over twofold up-regulation of YWHAB mRNA after 12 h of IL-1 exposure. Recently, it was shown that 14-3-3ε protein encoded by *YWHAE* gene is important in brain development and neuronal migration [46]. It would be interesting to analyse function of YWHAB protein in macrophages since probably it is important for macrophage migration during inflammation.

Another transcript belonging to the cell cycle machinery codes for chromosome-associated polypeptide C, belonging to the multiprotein complex: 13S condensin. The complex plays a key role in chromosome condensation and segregation in mitosis. We observed 1.7-fold up-regulation of this transcript after 4 h of IL-1 treatment followed by a decrease after 12 h in comparison with control (Figs. 2H and 3H). Hagstrom and Meyer [47] reported recently that condensin interacts also with different sets of proteins performing various roles, such as regulation of gene expression, participation in the DNA-damage checkpoint-signalling pathway and organization and orientation of the centromere.

In the group of transcripts encoding proteins functionally not characterized, we observed modulated expression for a hypothetical protein MGC20452, NY-REN-45 antigen, CLL-associated antigen KW8 and a transcript encoding CoxII/D-loop fusion protein. Recently, Ji et al. [48] reported that MGC20452 protein (renamed EID-2) is involved in skeletal muscle differentiation. This nuclear protein has the ability to associate with p300 and inhibit its acetyltransferase activity. EID-2 is a homologous protein to EID-1 which is also responsible for the inhibition of skeletal muscle-specific transcription through association with p300 [49]. We observed that IL-1 stimulation leads to a prolonged decrease in EID-2 transcript level (Figs. 2I and 3I). This suggests that EID-2 is in fact not a skeletal muscle specific protein but is responsible for transcription inhibition in HepG2 cells under IL-1 exposure. It would be interesting to define what kind of transcriptional coactivators with histone acetyltransferase activity interact with EID-2. Another transcript, NY-REN-45 antigen, was earlier cloned by screening of cDNA libraries derived from renal cell carcinoma with autologous antibody (SEREX) [50]. The antigens detected by SEREX method represented proteins with different functions: transcription factors, DNA and RNA binding proteins, metabolic enzymes, molecular chaperones, cytoskeletal proteins, membrane-associated proteins. The novel antigen REN-45 cDNA consists of a 4.42-kb transcript with 2445 nucleotides of the open reading frame. The function of this protein has to be studied, but amino acid composition shows certain similarity to potassium channel proteins with several nuclear signals and single transmembrane domain [50], so it is possible that under specific stimulation, the protein can be translocated to the nucleus. We observed 1.6–1.7-fold increase of mRNA NY-REN-45 after a 6-h exposure to IL-1.

Using BLAST search, we found that one of the detected transcripts matched CLL-associated antigen KW8 [51] and carboxyl terminus of Hsc70-interacting proteins (CHIP) cDNA. Both transcripts have the same sequence, which was identified in different laboratories with the use of various methods. CHIP was identified by the yeast two-hybrid screen as a protein interacting with Hsp70 and Hsc70. A characteristic feature of this protein depends on the presence of the tetratricopeptide repeat domain, typical for proteins with many cellular functions, such as mitosis, protein transport and development [52]. For heat shock proteins, several such chaperone cofactors were identified with a tetratricopeptide repeat domain. These proteins participate in regulation of protein folding and transport. The suggested role for CHIP is mediation of interactions between chaperoning and ubiquitin-proteasome systems [52]. We observed around 1.5-fold increase of the transcript coding for CHIP protein after 6 h of IL-1β stimulation (Figs. 2I and 3I). More detailed data are needed to elucidate whether both transcripts, NY-REN-45 antigen and CHIP

(CLL-associated antigen KW8), are indeed engaged in the IL-1 signal transduction pathway in HepG2 cells.

We are not presenting the expression analysis data for the transcript encoding CoxII/D-loop fusion protein. This is a novel fusion cDNA generated by an 8037-bp deletion in mitochondrial DNA. The deletion encompasses the cytochrome oxidase II gene (nt 8030–8034) and the D-loop region (nt 16071–16075) and is observed in various diseases and during aging [53]. From several Northern blot experiments done for this transcript, only one was readable while the rest presented unspecific hybridization with three bands visible. They may have appeared because not all cells, and not all mitochondria, contain the big deletion (heteroplasmy) and therefore the molecular probe used in our experiments could hybridize to different mitochondrial or genomic transcripts.

Changes in the transcript level of RPL26 in the IL-1-activated HepG2 cells were also not analysed in our experiments since modulated synthesis of this ribosomal component in response to different activators in various cell lines has been already described by many authors [54,55]. For this reason, our expression studies were limited to transcripts encoding RPL6, RPL19 and RPL41. The third protein presented in Table 1 and not analysed by RT-PCR or Northern blot is apolipoprotein B. As mentioned above, the detailed expression studies for ApoB were done by Yokoyama et al. [36].

Ten of forty-six templates identified by us in differential display as modulated by IL-1β had no homology with known and functionally classified genes deposited in GenBank entries. We are planning to study these sequences in the near future since these yet uncharacterized transcripts can be involved in specific acute phase response of HepG2 cells stimulated by IL-1β. Using Northern blot analysis, we have already confirmed that four of these transcripts are indeed modulated by exposure of HepG2 cells to IL-1 for different time periods (data not shown).

In the described experiments, we have not detected changes in the mRNAs coding for any of the main transcription factors, probably because of their very low abundance in a cell. According to Jongeneel et al. [56], many transcription factors are coded for by less than 10 mRNA copies per cell. The great majority of highly expressed genes are housekeeping genes responsible for cytoskeletal structures or enzymes involved in protein synthesis and energy metabolism [57].

One should remember that differential display analysis belongs to indirect screening methods. Primers used in PCR reaction are not specific to any cDNA and low stringency conditions enable annealing them to various accidental templates. Also for this reason, not all transcripts known to be modulated by IL-1 treatment have been amplified and visible on polyacrylamide gel. We were not able to detect transcripts encoding metallothionein 1 and 2 [58], phospholipase A2 [59], ferritin [60] and many other genes described earlier as regulated by IL-1 in HepG2 cells. This is clearly an inherent weakness of the differential display analysis.

We are aware that the use of microarray technique might yield a different transcriptome profile of gene expression in HepG2 cells stimulated with IL-1β. The chip technology is a powerful method which permits to monitor the activity of several thousand of genes but the results are difficult to standardize and interpret. In our case, the pattern of expression of 25 genes of known function that are modulated by IL-1 exposure was confirmed by Northern blot and/or RT-PCR in three independent experiments involving three separate cultures used for isolation and analysis of RNA (cf. Figs. 2 and 3). Thus, our results clearly indicate that differential display is a useful method of screening for changes of expression of several genes. However, the final proof of real differences in gene expression will be only possible after evaluating the corresponding proteome profile. This requires measuring changes in the level of individual proteins coded by specific mRNAs and we are currently engaged in such studies.

In conclusion, differential display analysis permits to detect subtle changes in the IL-1-induced gene expression in cultured human hepatoma cells, provided the changes in the level of individual transcripts are verified by Northern blot or RT-PCR. The technique is also useful in detecting IL-1-responsive genes of as yet unknown function but with sufficiently abundant transcripts. We have identified 10 of such gene transcripts in HepG2 cells stimulated with IL-1. On the other hand, differential display, being based on accidental annealing of arbitrarily designed primers, may miss altogether some important components of the transcriptome. The transcripts identified by us in differential display analysis of HepG2 cells were stimulated with IL-1 encode cytoplasmic and nuclear proteins but not secreted proteins from the class of acute phase reactants. The latter are affected mainly by IL-6 or by a synergistic action of IL-1 and IL-6 (see Refs. [3,61]). It appears that the cellular response to IL-1 promotes the process of cell survival and adaptation by up-regulating the early response genes playing an important role in cytoskeletal organisation, cellular metabolism, protein synthesis, posttranscriptional and posttranlational modifications and cell cycle machinery. As the late response genes are activated those responsible for the control of protein quality. A few genes—those coding for cathepsin D, dynein and prosaposin—represent the prolonged response under IL-1 stimulation. Clearly, further studies employing different methods of transcriptome and proteome analysis are needed to verify and expand the results reported here.

Acknowledgements

This work was partially supported by a grant (PBZ/039/P04/2001) from the State Committee for Scientific Research (Warsaw, Poland).

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