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Review

Complex analysis of genes involved in the inflammatory response: interleukin-1-induced differential transcriptome of cultured human hepatoma HepG2 cells★✪

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The systemic inflammatory reaction (acute phase response) is induced by many noxious stimuli but in all cases the inflammatory cytokines, such as interleukin-1-beta (IL-1β) and interleukin-6 (IL-6), are involved. Liver cell response to inflammation manifested by a characteristic change in the profile of synthesized plasma proteins (acute phase proteins) has been extensively studied. Here we describe a model system of cultured human hepatoma HepG2 cells stimulated with IL-1β to evaluate the transcriptome induced by this cytokine during 24 h of treatment. By using differential display analysis we found IL-1β-induced upregulation of several genes coding for cellular trafficking/motor proteins, proteins participating in the translation machinery or involved in posttranscription/posttranslation modifications, proteases, proteins involved in cellular metabolism, activity modulators, proteins of the cell cycle machinery and also some new proteins so far functionally not classified.

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Abbreviations: AP-1, activator protein-1; C/EBP, CAAT enhancer-binding protein; ERK, extracellularly regulated kinases; IFNγ, interferon-gamma; IL-1β, interleukin-1-beta; IL-6, interleukin-6; IL-8, interleukin-8; MAP kinases, mitogen activated protein kinases; MnSOD, Mn-dependent superoxide dismutase; NFκB, nuclear factor kappa B; PAI-2, plasminogen activator inhibitor type 2; LPS, endotoxin; SAGE, serial analysis of gene expression; STAT, signal transducer and activator of transcription; TNFα,

A LONG WAY FROM A GENOTYPE TO A PHENOTYPE

Recent successes in human genome sequencing might have created an illusion that we already understand the complex relation between the DNA sequence and gene expression and function in the organism. In fact, it is a long way from any genotype to specific phenotype: the most striking example is seen in the case of butterfly and caterpillar which contain identical genomes, but the pattern of gene expression is totally different in the two individuals. The crucial steps responsible for creating specific phenotypes involve gene activation and transcription, transcript processing, mRNA stability and translation into polypeptides and finally formation of mature, modified proteins, each of these steps being precisely regulated. Whereas regulation of individual genes has been in many cases elucidated, our knowledge of the overall picture is still patchy. As pointed out by Lockhart & Winzeler (2000) the biological and biomedical research is now in the state of transition being driven by the massive increase in the amount of available DNA sequence and the development of technologies to exploit it.

It is generally accepted that transient changes in the expression of particular sets of genes in certain types of cells are responsible for pathological phenomena, such as inflammation. Sequencing of the human genome opened new perspectives and challenges for understanding complex gene functioning. The global approach involves characterization of the transcriptome and proteome in cells participating in the inflammatory or „acute-phase" response. Up to now changes in gene expression during the acute phase reaction are only partly understood (for reviews see Kushner, 1982; Baumann & Gauldie, 1994; Koj, 1996; Moshage, 1997).

MOLECULAR BIOLOGY OF THE ACUTE PHASE RESPONSE

The acute phase response represents an early and unspecific but highly complex reaction of the animal organism to various noxious stimuli such as bacterial, viral or parasitic infection, mechanical or thermal trauma, ischaemic necrosis or malignant growth (for references see Koj, 1985; Kushner, 1982; Heinrich *et al.,* 1990; Sehgal, 1990; Koj *et al.,*1993). The systemic reaction to injury includes endocrine and metabolic alterations: fever, leukocytosis, release of several hormones and cytokines, activation of the clotting, complement and kinin-forming pathways, and drastic rearrangement of plasma protein synthesis in the liver (acute phase proteins). The cellular acute phase reaction clearly depends on cell phenotype but macrophages, fibroblasts, keratinocytes and endothelial cells play a dominant role in the initiation of inflammatory response. It appears that activation of the stress-responsive ERK and MAP kinases is a common triggering factor. These enzymes initiate signalling cascades leading finally to activation of transcription factors, such as NFκB, AP-1 or C/EBP that penetrate the cell nucleus and induce the expression of several genes, including cytokines (for references see Koj, 1996; Hatada *et al.,* 2001) (Fig. 1). The principal pro-inflammatory cytokines include IL-1β, TNFα and IFNγ, whereas the IL-6 family is held responsible for various symptoms of liver acute phase response (Koj, 1996). Although it is obvious that the inflammatory reaction at the cellular level is regulated by several factors, a simplified model adequate for molecular analysis of gene expression may be based on exposure of cell culture to individual cytokines or their combinations.

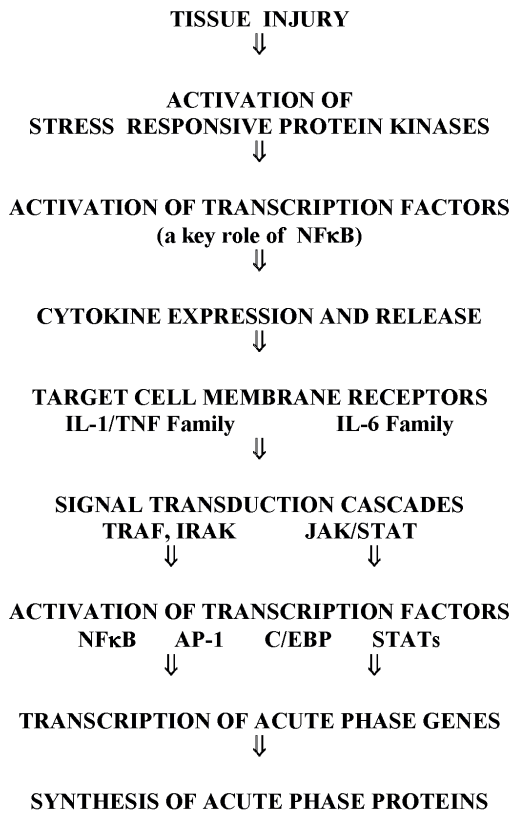

Figure 1. Two consecutive cascades during the acute phase response — from tissue injury to cytokine induction (the primary response) and from cytokines to acute phase proteins (the secondary response) (for further detail see Koj, 1996, and Moshage, 1997).

REGULATORY MECHANISMS OPERATE AT THE LEVEL OF TRANSCRIPTION AND TRANSLATION

Extensive studies have convincingly demonstrated that in animal cells gene expression is mainly regulated at the level of transcription. There are many reports of considerable agreement between the abundance of specific mRNAs in the liver cells and secretion rates of acute phase proteins, both *in vivo* and in hepatocyte culture (for early references see Koj, 1985). However, Kushner and co-workers showed that induction of human serum amyloid A in Hep3B cells by IL-6 and IL-1β involves both transcriptional and post-transcriptional mechanisms, such as regulation of nuclear processing of transcript, export of mRNA to the cytoplasm or regulation of mRNA stability (Jiang *et al.,* 1995). Considerable discrepancies between mRNA accumulation and the rate of translation have been reported by Dinarello and co-workers who found that adherence of macrophages to glass (Schindler *et al.,* 1990a) or exposure of macrophages to recombinant C5a protein (component of the complement system) (Schindler *et al.,* 1990b) stimulate transcription of IL-1 and TNF genes but not the synthesis and release of these cytokines. For these events to occur an additional signal must be provided (Dinarello, 1996), such as stimulation of cells with trace amounts of LPS (Haynes *et al.,* 2000). The importance of mRNA stability for efficient production of coded proteins was demonstrated by Beutler and co-workers in the case of TNFα (Han *et al.,* 1990), by Villarete & Remick (1996) for IL-8 and by Maurer & Metcalf (1996) for PAI-2. On the other hand, Kaspar & Gehrke (1994) emphasize the importance of the translation rate of IL-1β-mRNA in mononuclear cells stimulated either with the C5a component or with LPS. As pointed out by Calkhoven & Ab (1996) translational regulation is particularly important for transcription factors, but is often underestimated, and may include the initiation complex (Calkhoven *et al.,* 2002). All these data suggest that the relative abundance of mRNA for a given protein may not necessarily correspond to the proteome profile in a given cell.

A parallel analysis of proteome and transcriptome may provide yet another advantage in the identification of gene transcripts: during our studies on the intracellular inhibitor of elastase from horse leukocytes it appeared initially that β-thymosin amino-acid sequence constitutes an integral part of the inhibitor molecule until the gene sequence and polypeptide sequences were compared (Kordula *et al.,* 1993). For all the above mentioned reasons simultaneous analysis of transcriptome and proteome is recommended in order to understand the fine tuning of gene expression.

THE INFLAMMATORY TRANSCRIPTOME AND PROTEOME

The main purpose of our current studies is to identify characteristic sets of genes stimulated by proinflammatory factors such as bacterial endotoxin or interleukin-1 and other cytokines acting on cultured human cells. The model cells include macrophages deriving from blood monocytes, monocytic cell lines, and liver cells represented by differentiated human hepatoma HepG2. Secretory and cellular proteins are analysed with 2-dimensional gel electrophoresis followed by mass spectrometry (A. Dubin, in preparation). In order to obtain a complete picture of gene expression, RNA fractions isolated from control and cytokine-stimulated cells are studied by differential display and microarray techniques enabling direct comparison of the inflammatory transcriptome and proteome of these two types of cells. It should be recalled here that one of the first attempts to relate the mRNA and protein profiles in human, rat and mouse hepatocytes cultured with crude cytokine preparations was reported by us in 1984 (Baumann *et al.,* 1984). The proteins secreted by cultured liver cells were biologically labelled with [^{35}S]-methionine and separated by 2D-electrophoresis while mRNAs abundance was determined either directly by Northern blot or indirectly by translation in a cell-free system (Fig. 2). For obvious technical reasons the mRNA and protein analysis was limited to a dozen or so genes.

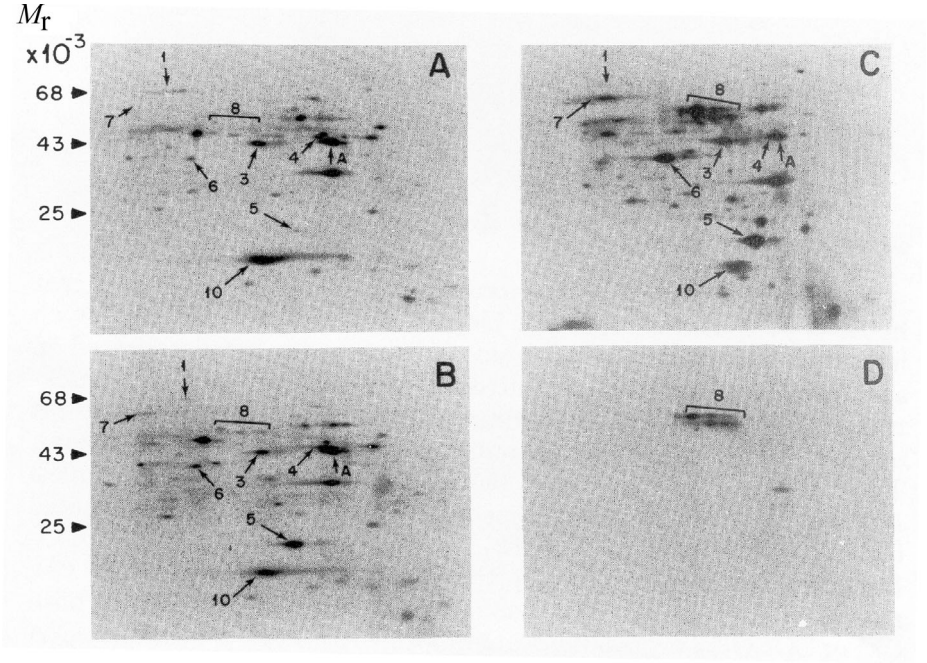

Figure 2. The effect of crude human cytokines on translatable mRNA in rat hepatocytes (from Baumann *et al.,* 1984, with permission).

Cultured rat hepatocytes were treated for 48 h with control culture media (A) or with media enriched in dexamethasone and crude cytokines (B). RNA was extracted from the cells and translated in a cell-free system; the products were separated by two-dimensional gel electrophoresis. For comparison, the *in vitro* translation products from RNA of a liver 48 h after *in vivo* inflammation were similarly separated (C). Identification of alpha-1-acute phase globulin (APG) was achieved by immunoprecipitation from the translation mixture of the liver RNA (D). Spots indicated by numbers represent the following proteins: albumin (1), alpha-1-antitrypsin (3), alpha-1-antichymotrypsin (4), alpha-1-acid glycoprotein (5), haptoglobin (6), hemopexin (7), APG (8), alpha-2-urinary globulin (10) and actin (A).

The currently available techniques used to evaluate mRNAs in comparative studies include subtractive hybridization (Duguid & Dinauer, 1990; Olivier *et al.,* 1999; Adams *et al.,* 2001; Kingsley *et al.,* 2001; Nishizuka *et*

al., 2001), differential display (Diachenko *et al.,* 1996; Fujimoto *et al.,* 2001; Grimshaw & Balkwill, 2001; Jheon *et al.,* 2001; Robert *et al.,* 2001), serial analysis of gene expression (SAGE) (Velculescu *et al.,* 1995; Argani *et al.,* 2001; Kuramasu *et al.,* 2001; Polyak & Riggins, 2001; Sun *et al.,* 2001) and microarray techniques (Schena *et al.,* 1995; Feng *et al.,* 2001; Narravula & Colgan, 2001; Vincenti & Brinckerhoff, 2001; Zhang *et al.,* 2001). Unfortunately, all these methods are expensive, laborious and might yield non-reproducible results. Critical evaluation of the above mentioned techniques in respect to advantages and possible pitfalls can be found in numerous publications (Knight, 2001; Aitman, 2001; Gabig & Węgrzyn, 2001; Dąbrowska, 2001; Bustin & Dorudi, 2001).

Despite the plethora of available methods recent reports on global analysis of genes involved in the acute phase response, especially those induced by proinflammatory cytokines, are rather scanty. The response of a cultured chondrocytic cell line to IL-1 was evaluated by Vincenti & Brinckerhoff (2001) with microarray analysis (Clontech, 1176 genes identified). The genes were further classified whether decreased or increased by at least twofold and sorted using Microsoft Excel. A subset of genes whose expression was increased by IL-1 was then confirmed using a radioactive RT-PCR assay with [^{32}P]ATP. Single-band products were resolved on native acrylamide gels and visualized by autoradiography. The affected genes (increased or decreased expression) were divided into four groups: transcription factors, cytokines and receptors, proteases and adhesion molecules, and signalling intermediates. The authors focused on transcription factors, important immediate early response genes such as NFκB and AP-1. Detailed analysis showed that *c-jun*, *JunB* and *Egr-1* were maximally expressed at 1 h and returned to almost control values after 2 h.

Olivier *et al.* (1999) investigated the liver acute phase response *in vivo* after injecting Freund's adjuvant into rats. After 4–8 h hepatic mRNA from control and stimulated animals was isolated and compared by subtractive hybridization. The authors identified genes coding for 23 known acute phase proteins, 31 genes coding for known proteins whose change in hepatic synthesis during the acute phase response had been unsuspected until then and 36 novel proteins containing domains related to other protein superfamilies. It is interesting that this search for inflammation-associated hepatic proteins of the rat identified mostly proteins of intracellular or membrane location.

The adult human liver transcriptome was analyzed by cDNA array hybridization by Yano and co-workers (2001). A total of 2418 unique gene transcripts were detected in five liver specimens obtained by surgical biopsy. Further analysis of these genes unexpectedly revealed signs of acute phase response in two of the liver specimens. This finding may be explained as the result of accompanying diseases in these patients. Clearly, the results obtained by Yano and co-workers should be verified in a model system of acute phase response.

INTERLEUKIN-1-INDUCED DIFFERENTIAL TRANSCRIPTOME OF CULTURED HUMAN HEPATOMA HEPG2 CELLS

In our experiments a comprehensive analysis was performed to measure the changes in transcriptome profile under IL-1 stimulation. Differential display analysis was used with cDNA from IL-1β treated (10 ng/ml) and untreated HepG2 cells. The efficiency of IL-1 stimulation was tested before each experiment through RT-PCR with primers specific for genes important in the acute phase response: α_1-antichymotrypsin and Mn-dependent superoxide dismutase (MnSOD). Total RNA was isolated from control and cytokine-stimulated cells exposed to IL-1 for differ-

ent time periods (0.5, 1, 2, 4, 6, 8, 12, 24 h). The synthesis of the first cDNA strand from 5 μg of total RNA was carried out using SuperScript RNaseH$^-$ reverse transcriptase (Promega). For differential display analysis the cDNA reaction mixtures from all samples were diluted 5 times and then 5 μl were taken for differential display PCR. The set of primers used in the DD-PCR designed by Clontech consists of ten arbitrary primers and nine oligo(dT) primers allowing for 90 combinations. The first three low-stringency cycles were performed at a low annealing temperature (40°C) to make possible imprecise binding of the arbitrary primers to various cDNAs. The next 25–30 cycles were done at a higher annealing temperature (55°C) to produce more specific products, decrease background and obtain reproducible results. The PCR products were separated on native polyacrylamide gels and detected by silver staining (Fig. 3). Different pairs of primers generated

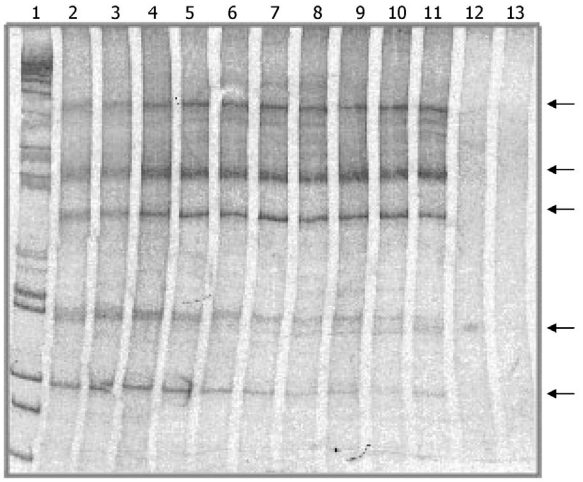

Figure 3. Differential display transcript pattern of HepG2 cells in response to IL-1β. PCR reaction was done with primers P3 and T4.

Lane 1, Size marker: λ-*Hin*dIII; lane 2, control, cDNA from unstimulated cells; lane 3–11, cDNA from cells stimulated with IL-1β for different time periods: 0.25, 0.5, 1, 2, 4, 6, 12, 18 and 24 h; lane 12, water control; lane 13, control RNA-PCR. Three upper arrows indicate up-regulated genes, two lower arrows, down-regulated genes.

various transcript profiles. Many of the primer combinations yielded no differentially expressed bands, but overall we observed 90 IL-1-modulated cDNA species from which 47 were reamplified, cloned to the pTZ57R vector (Fermentas) and sequenced. All the obtained sequences were found to have corresponding GenBank entries (National Center of Biotechnology Information). Homology searches revealed that 37 clones (79%) were significantly homologous to genes of known function. By contrast, no homology with known genes was found for 10 clones (21%). The sequences of the studied transcripts were long enough (200–600 bp) to match exactly some of the human genome sequences. Interestingly, they also matched rat and mouse sequences, confirming the high level of similarity of coding sequences among these organisms.

Based on the homology data all the identified transcripts modulated by IL-1β were classified into eight groups according to the function of the 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 the cell cycle machinery and those functionally unclassified (Table 1; for further details see Jura, Wegrzyn, Zarebski, Wladyka and Koj, in preparation).

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. The group of genes encoding proteins important for cellular energy metabolism was represented by 12 clones. Among them eight matched mitochondrial genes of respiratory Complex I, the first step in the electron transport chain of mitochondrial oxidative phosphorylation. This finding is probably of physiological relevance since IL-1β is known to stimulate energy metabolism of the cell (Dinarello, 1996). The enhanced expres-

sion of genes involved in the translation machinery (such as ribosomal proteins) and posttranscritpional/posttranslational modifications may be related to increased formation and secretion of acute phase proteins which occurs in liver cells under the effect of other cytokines, especially IL-6. As expected, stimulation of HepG2 cells with IL-1β alone caused no change in activation of genes coding for acute phase proteins but affected several other genes. This is in agreement with the report of Olivier *et al.* (1999) who found that acute phase response induced in rats *in vivo* affects not only secretory proteins but also many intracellular and membrane proteins. Our experiments now in progress indicate that much more specific changes related to acute phase response can be induced in HepG2 cells by a concerted action of IL-1 and IL-6 (Jura *et al.,* in preparation).

There is no precise data as to how many genes are expressed in HepG2 cells and how many of them are affected by IL-1. However, according to Zhang *et al.* (1997) and Jansen *et al.* (2001) who used SAGE analysis to compare the expression profiles of normal colon and cancer cells and of normally differentiated keratinocytes with those stimulated with TNFα, only 1% of genes responded to cytokine stimulation. Hastie & Bishop (1976) using cDNA-driven mRNA hybridization method estimated that in three different mouse tissues: kidney, brain and liver, around 11500–12500 genes were expressed from which 9500–10500 belong to the so-called "housekeeping" genes expressed in all cell types. The study done by Jongeneel *et al.* (2003) revealed that in normal breast epithelium and colon adenocarcinoma there is around 8500 transcripts expressed in both cell lines and additional 6000 showing cellular specificity. According to those data we could expect that under IL-1β stimulation around 100–150 genes would be modulated in HepG2 cells. This is in the range of values found in our experiments. However, the method used by us is not sensi-

Table 1. Differentially expressed and identified genes modulated by IL-1 in HepG2 cells

Clone number	Primer	GenBank Accession	Gen/Protein	Classification(s)/Function
1 (1F)	P1/T7	XM170518	Protein disulfide isomerase related protein (ERp72)	Posttranscription/posttranslation
50	P1/T6			modification
48.1	P2/T6	XM085471.2	Nuclear RNA binding protein (p54nrb)	Posttranscription/posttranslation modification
3 (4F), 7	P4/T7	NM_008448	Kinesin family member 5B (KIF5B)	Trafficking/motor proteins
29	P3/T5	NM079423.1	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle (MYL6)	Trafficking/motor proteins
		AY050643	Dynein heavy chain	Trafficking/motor proteins
19	P8/T7	NM001909.3	Cathepsin D (CTSD)	Proteases (lysosomal aspartic proteases)
21	P8/T7	AF244132.1	Short-chain alcohol dehydrogenase (HEP27)	Metabolism
24,44, 28/1	P2/T3, P2/T6	AAL54553.1	NADH dehydrogenase subunit 1	Metabolism
45/1,46,				
48, 63/4	P2/T6	BC014376	NADH dehydrogenase 4	Metabolism
27'	P10/T3	NM_173714	NADH dehydrogenase 6	Metabolism
49	P3/T6	NM002778	Prosaposin (PSAP)	Metabolism (lipids metabolism)
87.1, 91/1	P1/T9	NM_ 003900	Apolipoprotein B	Metabolism
90/1	P1/T9	BC029618	Glyceraldehyde-3-phosphate dehydrogenase	Metabolism
78	P3/T8	NM_000970	Ribosomal protein L6 (RPL6)	Ribosomal protein
53	P5/T6	NM000981.2	Ribosomal protein L19 (RPL19)	Ribosomal protein
17.1	P9/T4	AB061829	Ribosomal protein L26 (RPL26)	Ribosomal protein
2M (2F), 5/2	P1/T7		Ribosomal protein L41(RPL41)	Ribosomal protein
94/3	P3/T9	NM_001349	Aspartyl-tRNA synthetase (DARS)	Protein synthesis
28, 44.1,	P2/T6	BC039250.1	Ral guanine nucleotide dissociation stimulator-like 1 (RalGDS-like 1)	Signal transduction
55,	P5/T6			
88.3	P1/T9	X04506	Sequestosome 1 (SQSTM1)	Signal transduction
61.1	P6/T6	AB019987.1	*Homo sapiens* mRNA for chromosome-associated polypeptide-C	Cell cycle machinery
52	P1/T6	NM139323.1	Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein, (YWHAB)	Cell cycle machinery
54	P6/T6	L78671	CoxII/D-loop DNA fusion protein.	Functionally unclassified
31, 32B	P5/T5	NM153232.1	Hypothetical protein MGC20452, chrom.19	Functionally unclassified
32A	P5/T5	NM016121.1	NY-REN-45 antigen (NY-REN-45)	Functionally unclassified
59	P6/T6	AF432221	*Homo sapiens* CLL-associated antigen KW-8 mRNA, complete cds, (1280 bp mRNA)	Functionally unclassified

tive enough to identify changes in the level of transcripts represented by a few copies of a given mRNA per cell. Therefore, the changes elicited by IL-1β in HepG2 cells are likely to affect a higher number of genes than those described here but elucidation of this problem requires further studies.

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