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ORIGINAL CONTRIBUTION

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A two-dimensional map and database of soluble nuclear proteins from HepG2 cells as reference for identification of nutrient-regulated transcription factors

Summary *Background* Eukaryotic cells of higher organisms are able to regulate gene transcription in response to changes in the supply of nutrients. In hepatocytes, extracellular glucose levels affect transcription of genes that encode enzymes engaged in glycolysis, gluconeogenesis and lipogenesis. While glucose response elements have been located within a few model gene promoters, the identity of glucose-sensing transcription factors and the mechanisms of their activation remain to be elucidated. *Aim of the study* We intended to establish a two-dimensional map of nuclear proteins as a reference for identification of nutrient-regulated transcription factors. *Methods* Human hepatoma HepG2 cells were used for the preparation of nuclear extracts. 150–200 µg of the protein mixture were analyzed by 2-dimensional gel electrophoresis (2-DE) and silver-stained protein spots were identified by MALDI-TOF mass spectrometry. *Results* Nuclear extracts capable of transcriptional initiation and elongation and containing low amounts of cytoplasmic contaminations were prepared. 543 spots between 17 and 100 kDa and pI 3.7 and 8.8 have been resolved. From these, 65 spots were analyzed by MALDI-TOF mass spectrometry and 53 spots were identified as known proteins of which six represented transcription factors. Regulation by glucose was shown for the activator protein-1 component cJun. Since cJun was not visible on the silver stained 2-DE gel, western blotting of 1-DE gels and immunological detection had to be used in this case. The data were used to construct an online database. *Conclusions* A 2-DE map and database of soluble nuclear proteins is presented. The identification of several transcription factors was possible on the silver-stained gels. However, further fractionation of the nuclear extracts will facilitate the detection of larger numbers of transcriptional regulators. The database and 2-DE map shown here may provide a useful reference for the identification of transcription factors from liver nuclei that are activated by different stimuli, e. g., nutrients.

Key words HepG2 cells – Nuclear extract – Transcription – Mass spectrometry – Proteomics

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Introduction

Higher organisms adapt their cellular metabolism to the availability of nutrient substrates. This ability is conferred by extracellular signals including circulating hormones and nutrients themselves. Short-term regulation involves the change of catalytic activities, while long-term regulation involves changes in gene expression and synthesis of enzymes that are responsible for the cellular nutrient turnover [1]. For example, it has been shown *in vitro* and *in vivo* that high levels of glucose activate genes for enzymes that regulate glycolysis and lipogenesis, but decrease the mRNA levels for gluconeogenetic

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enzymes in hepatocytes [2–4]. The molecular mechanisms that underlie the glucose-regulated transcription remain to be elucidated [5]. DNA elements that are able to transfer the glucose-sensitivity to heterologous promoters have been identified in several genes. Although transcription factors that bind to these elements have been identified [5–7], their actions are not sufficient to mediate the effect of glucose on gene expression, indicating that additional factors must exist [5–7].

Inducible transcription factors are usually activated by post-translational modifications, including protein phosphorylation/dephosphorylation [8], glycosylation [9], translocation into the nucleus as a result of ligand binding [10] or loss/change of binding partners [11] and proteolytic cleavage [12]. In the past, analyses of nutrient-inducible regulatory nuclear proteins were usually either restricted to selected model proteins (for an example see [7]) or relied on molecular biological methods, such as differential display RT-PCR [13] or subtractive hybridization [14]. However, a systematic approach to identify inducible transcription factors at the protein level has not yet been described.

Two-dimensional gel electrophoresis (2-DE) is the method of choice to resolve complex mixtures of proteins and also for the separation of proteins that differ by post-translational modifications [15]. Thus, 2-DE offers the opportunity to identify inducible regulatory proteins by comparisons of nuclear extracts from cells that have been previously challenged by variations in nutrient supply. The establishment of a precise 2-DE map of proteins that serves as a reference is an essential prerequisite for this purpose.

The combination of 2-DE and highly sensitive analytical methods, such as immunoblotting and peptide-mass fingerprinting [16], improved the identification of protein spots from 2-DE gels. Consequently, proteome analyses of selected model organisms have been established. The IPG-DALT technique (2-DE with immobilized pH gradients in the first dimension; [17]) significantly increased the reproducibility of 2-DE gels from independent research groups and greatly facilitated the establishment of online 2-DE databases. To date, the databases contain only partial proteomes of a number of organisms displayed on 2-DE gel images derived from tissues or cell lines (for an index of these databases see *http://www.lmnb.ncifcrf.gov/EP/table2Ddatabases.html*).

A drawback of the current databases stems from the fact that protein extracts from whole cells or tissues are commonly being used as starting material for 2-DE. This protocol does not allow the identification of many proteins from cell organelles because they represent only a minor proportion of the total cellular protein. It is therefore important to establish 2-DE maps of proteins derived from cell organelles. To date only a limited number of 2-DE maps from nuclear proteins is available despite the great importance of this organelle [18–21] and only one on-line 2-DE database of nuclear proteins exists (http://www.expasy.ch/cgi-bin/ch2d-compute-map?NUCLEI_LIVER).

In this article the construction of an online 2-DE database is presented for proteins from transcriptionally active nuclear extracts of HepG2 cells, a human hepatoma cell line. HepG2 cells have been chosen because they serve as a model to investigate the regulation of hepatic gene expression by nutrients [22]. The database is a necessary prerequisite for the identification of transcription factors activated by nutrients.

Results

Preparation of nuclear extracts

A critical step in the construction of 2-DE databases from subcellular extracts is the purity of the compartment of interest. Therefore, we selected a method based on vigorous removal of cytoplasmic materials by shearing forces and subsequent isolation of the nuclei by density centrifugation. If isolated by this procedure and visualized by phase contrast microscopy, nuclei appeared to be largely free from cytoplasmic contaminants (Fig. 1). To prove the presence of transcription factors in our extract, we analyzed its transcriptional activity. A plasmid carrying a G-free cassette under the control of the adenovirus major late promoter [23] was incubated in the presence of nuclear extract and in the absence of

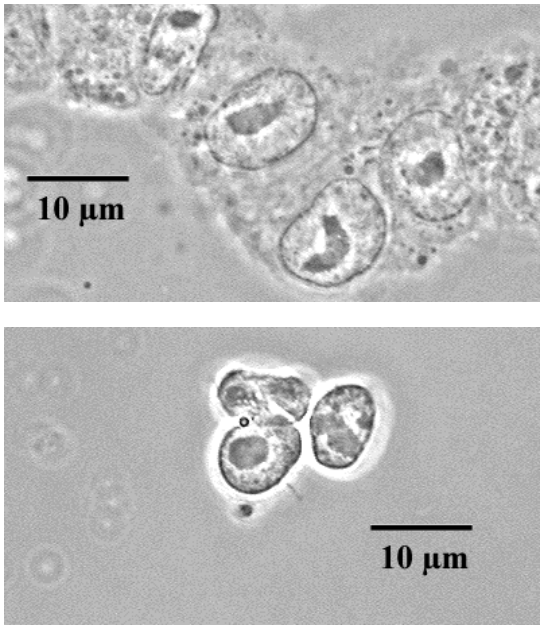

Fig. 1 Purification of HepG2 cell nuclei. The phase contrast micrographs show HepG2 cells following homogenization by a motor-driven potter (**A**) and nuclei after the first ultracentrifugation (**B**).

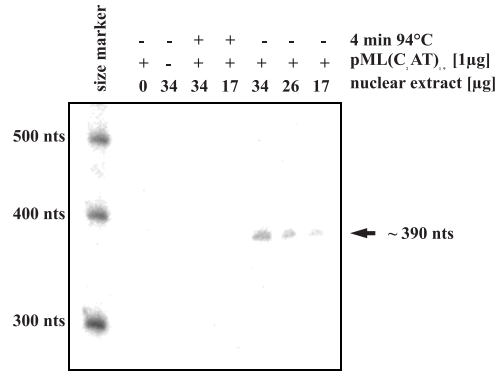

Fig. 2 In vitro transcription of pML(C_2AT)$_{19}$ using nuclear extracts of HepG2 cells. Transcriptional assays were conducted with various amounts of extracts, in the presence of plasmid DNA template. For control, incubations were also carried out using heat-inactivated extracts or in the absence of DNA template. A radiolabeled 100 bp DNA ladder was used as a size standard.

GTP. Under these conditions, transcription begins at the initiation site and terminates at the first cytidine nucleotide of the template DNA strand located 390 nucleotides further downstream. Fig. 2 shows a sharp band of the predicted size of 390 nucleotides on the gel image that indicates correct transcriptional initiation and elongation. The HepG2 nuclear extracts contain therefore all factors required for the highly complex processes of specific transcriptional initiation and elongation.

Two-dimensional gel electrophoresis and spot identification

The nuclear extracts gave rise to 543 protein spots following separation on the 2-DE gels and silver-staining (Fig. 3). The spots covered a molecular weight region from 17 to 100 kDa and a pI range from 3.7 to 8.8. Sixty-five spots of varying staining intensities and extent of separation from neighboring spots were chosen and, after tryptic digest in the gel matrix, submitted to MALDI-TOF mass spectrometry. The peptide masses obtained were used to search the non-redundant trEMBL and SwissProt databases resulting in the identification of 53 spots corresponding to 39 different proteins (Table 1). The difference is explained by the fact that some proteins gave rise to several spots on the 2-DE gels. The remaining 12 spots also resulted in high quality peptide fingerprints (Fig. 4). However, the Peptide Search algorithm failed to identify corresponding proteins in the databases.

Six of the 38 identified proteins represented transcriptional regulators. The first, ZFM1 (spot 56), is a zinc finger protein derived from a gene that is probably responsible for multiple endocrine neoplasia type 1 [24]. The second is α-CBP1 (spot 296), a protein that regulates transcription by binding to the canonical CCAAT-box

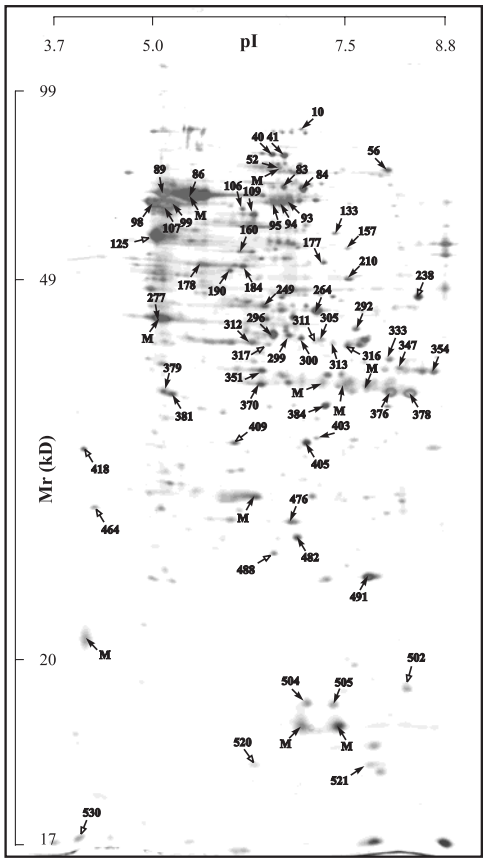

Fig. 3 Image of 2-DE gel. A transcriptionally active HepG2 nuclear extract was separated on a 2-DE gel and silver-stained. pH and Mr scales are displayed on top and on the left hand of the image. Identified spots (solid arrows) are indicated by numbers (see also Table 1). Open arrows refer to spots for which tryptic peptide mass spectra have been obtained but could not be identified in the databases. *M* location of 2-DE standard proteins. The 2-DE gel is representative for three gels made from three independent samples.

[25]. Finally, we identified FBP (far upstream element (FUSE)-binding protein, spots 83, 84). FBP regulates c-myc expression, is induced during apoptosis and has been implicated in cellular proliferation and differentiation [26, 27]. Three other proteins, CLP–36 (spot 370), ZF36 (spot 184) and DJ25J6.2 (spot 249) represent *bona fide* transcriptional regulators. CLP–36 belongs to the LIM domain containing proteins. Zinc fingers and the LIM domain are zinc-binding amino acids found in various proteins that function in protein-protein interactions and transcriptional regulation [28]. An extrapolation of the number of identified transcriptional regulators predicts that there will be more then 30 transcription factors present on our 2-DE gel.

In addition to the six transcription factors, we identified various other proteins that serve important functions in gene expression. Hsc71 (P11142, spot 89), Hsp70 (P08107, spots 98 and 99), and transformation-sensitive protein IEF SSP 3521 (P31948, spot 109) function as

Table 1 HepG2 proteins identified on 2-DE gels

No.	Accession no.[l]	Protein description	Experimental pI/Mr [kD]	% of sequence covered by peptides	Note
10	P11586	C-1-tetrahydrofolate synthase	6.9/86.1	21.8	
40	O00301	KSRP/Ribonuclease 6 precursor	6.5/78.0	40.0	
41			6.7/77.6	35.0	a)
52	O00571	Dead box protein 3, RNA helicase	6.7/73.8	23.7	b)
56	Q15637	Transcription factor ZFM1	8.0/73.6	15.2	c)
83	Q12828	FUSE binding protein	6.7/69.3	37.0	
84			7.0/68.7	32.8	
86	P02769	Serum albumin precursor.	5.6/67.2	62.6	k)
89	P11142	Heat shock cognate 71 kd protein	5.2/67.5	29.1	
93	P14866	Heterogeneous nuclear	6.8/65.8	42.8	
94		ribonucleoprotein l	6.7/65.5	6.8	
95			6.6/65.5	38.0	
98	P08107	Heat shock 70 kd protein 1	5.1/64.6	43.5	
99			5.3/65.0	27.1	
106	P33240	Cleavage stimulation factor; 64 kd subunit	6.2/64.0	22.5	
107	Q07244	Heterogeneous nuclear ribonucleoprotein k	5.2/63.4	39.1	
109	P31948	Transformation-sensitive protein IEF SSP 3521	6.3/62.4	28.9	
133	X56494	Gene for M1-type and M2-type pyruvate kinase	7.4/58.7	54.0	
157	Q01518	CAP1 human adenylyl cyclase-associated protein	7.6/55.7	26.9	
160	Q9 UMS4	Nuclear matrix protein NMP 200	6.2/55.1	17.5	m)
177	P00390	Glutathione reductase	7.2/53.1	22.4	
178	P31943	Heterogeneous nuclear ribonucleoprotein h	5.6/52.9	40.1	
184	P16415	ZF36 human zinc finger protein	6.2/52.5	25.4	m, n)
190	P78371	Chaperonin containing t-complex polypeptid 1, beta subunit	6.0/57.5	34.0	
210	P34897	Serine hydroxymethyltransferase	7.6/50.3	36.2	
249	Q9 UJL4	DJ25J6.2 zinc finger protein	6.5/46.2	22.5	

Table 1 *Continued*

No.	Accession no.[l]	Protein description	Experimental pI/Mr [kD]	% of sequence covered by peptides	Note
264	Q12771	p37 AUF1 RNA-binding protein, hnRNP d	7.2/45.4	39.3	d)
277	P02571	Actin, cytoplasmic 2	5.1/44.4	41.3	e)
292	Q12771	p37 AUF1 RNA-binding protein, hnRNP d	7.7/42.9	40.5	d)
296	Q13157	alpha-CP1	6.6/42.3	59.0	f)
299	Q12771	p37 AUF1 RNA-binding protein, hnRNP d	6.8/42.17	37.4	d)
300	Q92524	26s protease regulatory subunit p42	7.0/41.8	53.2	g)
305	Q12771	p37 AUF1 RNA-binding protein, hnRNP d	7.2/41.5	25.2	d)
312	Q99729	ABBP-1	6.3/41.3	24.8	
313	P78406	RAE1	7.4/41.1	23.6	h)
333	Q12771	p37 AUF1 RNA-binding protein, hnRNP d	8.1/39.3	21.0	d)
347	P51991	Heterogeneous nuclear ribonucleoprotein a3	8.2/38.3	29.0	
351	Q12771	p37 AUF1 RNA-binding protein, hnRNP d	6.4/38.0	46.8	d)
354	P22626	Heterogeneous nuclear ribonucleoproteins a2/b1	8.65/37.89	29.0	i)
370	O00151	Lim domain protein CLP-36	6.4/36.6	73.9	
376	P22626	Heterogeneous nuclear	8.1/35.8	22.9	j)
378		ribonucleoproteins a2/b1	8.3/35.7	42.5	j)
379	Q9NQG5	Hypothetical protein dJ1057B20.2	5.2/35.8	28.2	
381			5.3/35.6	35.2	
384	P13995	NAD dependent methylene-tetrahydrofolate hydrogenase/methenyltetrahydrofolate cyclohydrolase mitochondrial precursor	7.3/34.5	29.4	
403	P25388	Guanine nucleotid-binding protein beta subunit-like protein 12.3 (p205)	7.2/31.7	19.2	
405	P32322	Pyrroline-5-carboxylate reductase	7.0/31.4	34.2	
476	P17080	GTP-binding nuclear protein Ran	6.8/26.3	67.6	
482	Q99714	replace by: 3-hydroxyacyl-CoA dehydrogenase type II	6.9/25.5	42.0	
491	Q06830	Natural killer cell enhancing factor a	7.8/23.6	43.7	
504	P05092	Peptidyl-prolyl cis-trans isomerase a	7.0/19.6	43.3	
505	P05092	Peptidyl-prolyl cis-trans isomerase a	7.4/19.6	35.0	
521	P07 737	Profilin I	7.9/18.4	75.5	

a) O00301 displays 97.2 % identity with Q92945, but measured peptide mass of 1557 679 Da is a tryptic peptide from O00301
b) Spot 52 contains also standard protein (hen egg white conalbumin)
c) Q15637 displays 99.5 % identity with Q92745
d) It is not possible to discriminate between Q14101, Q14103, Q12771, and A54601with our peptides
e) Spot 277 also contains standard protein (bovine muscle actin)
f) Q13157 displays 99.7 % identity with Q15365
g) Q92524 displays 97 % identity with P49719, but measured peptide mass of 2226 122 Da is a tryptic peptide from Q92524
h) P78406 displays 98.9 % identity with O15306
i) The observed peptide mass of 1057.4 Da (AAs 4–12) is specific for hnRNP B1
j) It is not possible to discriminate between hnRNP A2 and hnRNP B1 with our peptides
k) Standard protein (bovine serum albumin), but also in culture medium of HepG2 cells
l) The accession numbers refer to the trEMBL or Swiss-Prot data bases that can be viewed by http://www.expasy.ch/cgi-bin/sprot-search-ful
m) Some peptides matched also with a second protein. Further sequencing is necessary for proof of identity
n) Peptides generated by tryptic digest were contaminated with peptides derived from keratin

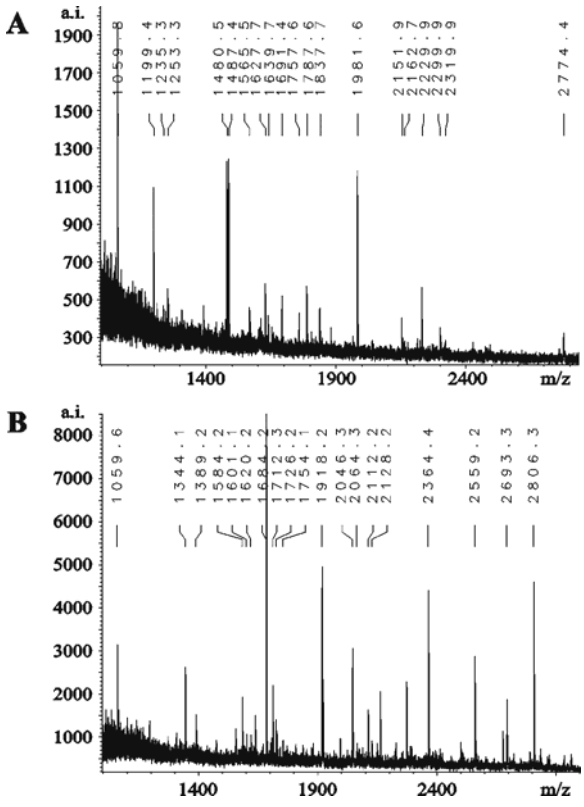

Fig. 4 MALDI-TOF peptide mass fingerprint spectra of proteins following 2-DE and tryptic digest. Spots 89 (**A**) and 125 (**B**) were cut out of the gel, digested with trypsin and submitted to MALDI-TOF mass spectrometry. Internal calibration was performed using one peak of the matrix ions (m/z = 379.4; not shown) and one peptide peak (m/z = 2163.06) resulting from autodigestion of trypsin.

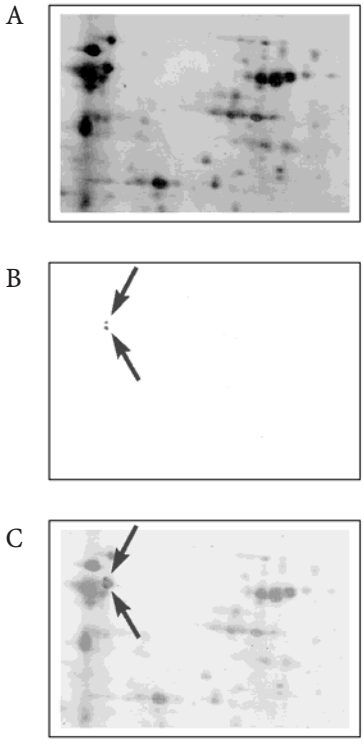

Fig. 5 Detection of p65 by western blotting of the 2-DE gel. The nuclear proteins from HepG2 cells were separated on a 2-DE gel and transferred onto a membrane which was silver-stained and documented (**A**). The same membrane was destained as described previously [25] and immunostained using an antiserum specific for the NFκB subunit p65 and a secondary antibody conjugated to alkaline phosphatase (**B**). **C** The spot patterns shown in **A** and **B** were superimposed. Arrows indicate the position of immunostained protein spots.

chaperones for nuclear steroid hormone receptors [29, 30]. RAE1 (P78406, spot 313) is involved in pre-mRNA transport [31], ABBP–1 (Q99729, spot 312) in pre-mRNA editing [32], AUF1 (Q12771, spots 264, 292, 299, 305, 333, 351) in pre-mRNA stability [33] and cleavage stimulation factor (P33240, spot 106) in pre-mRNA polyadenylation [34]. Remarkably, the yeast homologs of two of these, RAE1 and transformation-sensitive protein IEF SSP 3521, participate in the cellular response evoked by changes in food supply [35].

The small proportion of transcription factors that we detected (six out of 39 identified proteins) prompted us to examine whether the concentration of transcriptional regulators in our soluble nuclear extracts is too low to permit their detection by silver staining. We therefore analyzed the extracts for the presence of a well-known transcriptional activator, nuclear factor kB (NFκB), following western blotting of the 2-DE gel. To achieve high accuracy in the correlation of a silver-stained protein spot to its immunostained counterpart, we employed the method that we developed recently. It allows silver- and immunostaining of protein spots on the same membrane [36]. Fig. 5 shows that this method readily allowed the immunological detection of the NFκB subunit p65, which was not visible on the silver-stained membrane. Similar results were obtained for cJun, a subunit of the activator protein-1 (AP-1) [36].

Establishment of an online database

The pattern of the nuclear HepG2 proteins was used to construct an online database [37]. The protein spots are consecutively numbered and can be accessed via a clickable gel image. For each identified spot the accession and identification number, date, name, description, organism, pI and Mr is available. All data are presented in tabular form. The database also allows full text searches. Moreover, additional information is available via links to the SWISS-PROT database.

Corresponding spot and image files are being created following user query by common gateway interface (CGI)-scripts. Tryptic peptide masses of the unidentified proteins are available through the program MALDI-SEARCH that we have developed for this purpose. This program compares peptide masses of query proteins

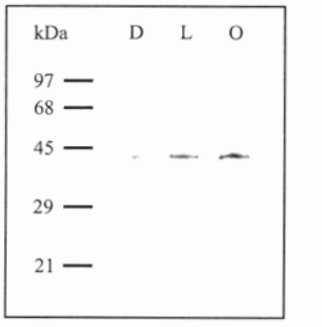

Fig. 6 Induction of the transcription factor cJun in absence of glucose or presence of L-glucose. Following incubation of HepG2 cells in presence of 4.5 g/l D-glucose (D), L-glucose (L) or in absence of glucose (0), 8 µg of the respective cell nuclear extract were separated on a 15 % SDS-PAA gel and transferred onto a membrane. The expression of cJun proteins was visualized by incubation of the membrane with anti-cJun antibodies and an alkaline phosphatase-conjugated secondary antibody. The size of protein standard proteins is indicated on the left.

specified by the users with those determined in our study. Because all data are stored in a structured query language (SQL) database, all updates are immediately available. This greatly facilitates the administration of the database. Importantly, this strategy bears the advantage that other research groups can coordinately update the database via a world wide web (WWW) interface.

Regulation of AP-1 by glucose

Nuclear extracts of HepG2 cells previously incubated in the absence or presence of D- or L-glucose were submitted to SDS polyacrylamide gel electrophoresis and analyzed on western blots with an antibody directed against cJun, a component of AP–1. In the presence of D-glucose, cJun protein was hardly detectable (Fig. 6). However, when D-glucose was omitted or substituted for by L-glucose, cJun was easily identified. This observation indicates that the glucose levels in the medium regulate AP–1 and therefore suggest that they also affect transcription in HepG2 cells.

Discussion

In this report we have analyzed soluble nuclear extracts from HepG2 cells on 2-DE gels. A hepatocyte cell line was selected because the liver is exposed to the greatest variations in glucose concentrations under physiological conditions. The quality of the nuclear extracts was proven by the demonstration of correct transcriptional initiation and elongation. It was also devoid of the abundant histone and nuclear matrix proteins [38], which facilitated the display of proteins of lower abundance. The absence of the latter is nicely demonstrated by the observation that none of the proteins identified in this report were found in the 2-DE database of nuclear matrix proteins from human liver [19]. In addition, our experimental protocol minimized the presence of cytoplasmic contaminants. This is supported by visual inspection of the nuclei and by the 2-DE analysis of the nuclear extract. Only nine protein spots out of 65 were of non-nuclear origin, but derived from mitochondria (spots 384 and 482, Table 1), the cytosceleton or the cytosol (spots 10, 157, 177, 210, 277, 405 and 491; Table 1). When the 2-DE pattern of nuclear and cytoplasmic protein extracts were compared, about 10 % of the cytoplasmic spots also appeared in the nuclear pattern (data not shown). These observations support a recent note that cytoplasmic contaminants can not completely be avoided in nuclear preparations [20]. On the other hand, a nuclear localization of at least some of these "cytoplasmic" proteins can also not be excluded. For peptidyl-prolyl cis-trans isomerase we observed four spots in the 2-DE pattern of total HepG2 cell extracts but only two of them (spots 504 and 505, Table 1) were identified in the nuclear extract (result not shown). It appears, therefore, that variants of this enzyme exist, some of which may occur in the nucleus. Moreover, two of the cytoplasmic proteins, natural killer enhancing-factor A (spot 491) and glutathione reductase (spot 177), protect the cells against oxidative stress. Several transcriptional regulators such as NFκB and AP–1 are sensitive to the redox potential [39]. This implies that enzymes involved in redox regulation are also present in the cell nucleus.

To evaluate the reproducibility of the 2-DE analysis, three gels of three independent samples of unstimulated HepG2 cells were compared and about 90 % of the detected protein spots proved to be reproducible in all three gels. This observation is well in agreement with findings of others (17). However, spot intensities varied considerably which made a quantitative comparison difficult. To ensure a correct identification, a given protein spot was cut out from 2–3 different gels and analyzed separately by mass spectrometry.

From the 543 spots of the 2-DE gel, 65 of different staining intensity, M_r and IP were analyzed by mass spectrometry and for all of them we obtained high quality peptide finger prints. We therefore conclude that also the weakly stained spots contain sufficient material, allowing their successful analysis. The data also suggest that the intensity and the protein content of a spot are not clearly correlated. Of the 65 spots 53 were identified, representing 39 different proteins. Only five of the identified proteins (spots 10, 89, 93/94/95, 491, 504, Table 1) were contained in the SWISS–2DPAGE databases including the HepG2 2-DE database (http://www.expasy.ch/ch2d/). Most likely the low abundance of these nuclear proteins in total cellular protein extracts prevented their previous detection on 2-DE gels. This observation strongly supports the demand for 2-DE databases obtained from subcellular compartments.

Six of 38 identified proteins represented transcriptional regulators. An extrapolation of the number of identified transcriptional regulators predicts that there will be more then 30 transcription factors present on our 2-DE gel. While we detected six transcription factors as silver-stained spots on our 2-DE gel, the well-known inducible regulators of transcription, NFκB and AP–1, were not seen. Only the more sensitive western blotting method with immunological detection allowed the identification of these proteins. We therefore conclude that the silver staining method was not sensitive enough to detect these proteins in HepG2 nuclear extracts. Further enrichment of such proteins seems to be required to allow the identification of a larger number of transcriptional regulators by silver-staining. Affinity chromatography on immobilized DNA or RNA to separate the abundant RNA binding proteins from DNA binding transcription factors would probably be useful. The 2-DE map presented here facilitates the monitoring of the success of such purification steps.

In a final experiment we used glucose as a stimulator for the following reasons: First, consumption of food with a high glycemic index increases the risk for insulin resistance syndrome and subsequently for cardial vascular diseases [40]. Second, glucose has been shown to regulate gene expression in liver [1, 2]. Semenkovich, for instance, has shown for the fatty acid synthase (FAS) gene as a model that glucose can regulate gene expression in HepG2 cells [41]. In agreement with this observation we observed a decrease of FAS activity in cells cultivated in the presence of L-glucose (data not shown). However, more importantly, glucose deprivation resulted in the induction of cJun protein, a component of the AP–1 complex. This observation indicated that glucose concentrations affected transcription in HepG2 cells and confirms recent studies which showed glucose sensitivity of cJun in squamous SiHa- and pancreatic INS–1 cells [42, 43].

Twelve spots escaped their identification although high quality tryptic peptide maps were obtained. At present we can not completely exclude that post-translational modifications changed the peptide masses in a way preventing their identification. However, this possibility appears unlikely because the masses of the analyzed peptides represented high proportions of the proteins. These spots likely represent novel proteins not yet contained in the public nucleic acid and polypeptide databases. Since the amount of sequence information is continuously increasing, it is important that the MALDI-TOF spectra of the unidentified proteins are available [44]. Therefore, we developed a program MALDI-SEARCH that allows researchers, via a web browser, to compare peptide spectra they determined or predicted from DNA sequence information with those of the unidentified proteins in our database. Thereby, it will be possible for the first time to determine the position of a novel protein on a 2-DE gel solely on the basis of its DNA sequence. Moreover, our database is of great value for the identification of protein coding regions in DNA sequences that accumulate in the course of the human genome project. In this respect, it is noteworthy that the two proteins DJ25J6.2 (Q9 UJL4) and dJ1057B20.2 (Q9NQG5) corresponding to spots 249, 379 and 381 are hypothetical proteins, i. e., the peptide sequence was predicted from chromosomal DNA sequence determined by the human genome project. The development of the genome project and our database will finally complete the HepG2 nuclear protein map.

Materials and methods

Chemicals

All chemicals and media for cell culture were purchased from GIBCO BRL (Eggenstein, Germany), chemicals for 2-DE from Pharmacia (Freiburg, Germany), the 2-DE standard from BIO-RAD (Munich, Germany), chemicals for silver staining from Merck (Darmstadt, Germany).

Cell culture

HepG2 cells were grown in 150 cm^2 flasks at 5 % CO_2 in RPMI1640 supplemented with 5 % fetal calf serum (FCS), 5 % new born calf serum, 2 mM L-glutamine. For analysis of the glucose sensitivity of AP–1, the medium was exchanged for RPMI1640 supplemented with 10 % FCS, 2 mM L-glutamine, 4.5 g/l D-glucose (25 mM) when cells were grown to 30–50 % confluence. After 24 h cells were washed in Puck's saline and incubation continued for 6 h in RPMI1640, 3 % bovine serum albumin, 2 mM L-glutamine, and 4.5 g/l D-glucose. Then the cells were washed again and incubated for another 12 h in the same culture medium containing 4.5 g/l D-glucose or L-glucose or lacking any glucose. Thereafter, cell number and viability were determined by staining with trypan blue and cells were scraped off the flasks, washed in Puck's saline, and shock frozen in liquid nitrogen.

Preparation of nuclear extracts

Nuclear extracts were prepared according to Gorski et al. [38]. Cells (2 x 10^8) were homogenized by a motor-driven 5 ml Teflon-glass homogenizer in 10 mM Hepes (pH 7.6), 25 mM KCl, 1 mM EDTA, 2 M sucrose, 10 % glycerol, 0.15 mM spermine, 0.5 mM spermidine, 0.5 % Triton-X100. The homogenate was layered on a 1 ml cushion of the same buffer not containing Triton-X100. Nuclei were sedimented by centrifugation (SW60, 28 200 rpm, 30 min, 4°C) and thereafter resuspended in 10 mM

Hepes (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 % glycerol, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethysulfonyfluoride, 0.2 µM Aprotinin, 2.3 µM Leupeptin and adjusted to 10 A_{260} units per ml and, at 4°C, 1/10 volume 4 M ammonium sulfate (pH 7.9) was added dropwise. The extract was gently shaken for 30 min. The viscous lysate was then centrifuged (90 Ti, 37 500 rpm, 60 min, 4°C) to pellet chromatin. Soluble nuclear proteins that are contained in the supernatant were precipitated by addition of 0.3 g/ml ammonium sulfate and recovered by centrifugation (90 Ti, 37 500 rpm, 25 min, 4°C). Proteins were dissolved in 25 mM Hepes (pH 7.6), 40 mM KCl, 0.1 M EDTA, 10 % glycerol, 1 mM EDTA and dialyzed 1 h against the same buffer which was changed once. Material that was precipitated during dialysis was removed by centrifugation, the protein concentration was determined using a spectrometric method described previously [45] and the nuclear extracts were frozen in small aliquots in liquid nitrogen.

In vitro transcription

In vitro transcription assays are performed essentially as described by Gorski et al. [38]. Various amounts of nuclear extracts were added to transcription buffer (10 mM Hepes (pH 7.6), 3 % glycerol, 25 mM KCl, 6 mM $MgCl_2$, 0.6 mM ATP, 0.6 mM CTP, 35 µM UTP, 200 µM O-methyl-GTP, 2 U RNasin, 7 µCi [α-^{32}P]UTP, 1 µg plasmid DNA template (pML(C_2AT)$_{19}$; [23]). Incubation was carried out for 30 min at 37 °C and stopped by addition of 15 volumes 25 mM NaCl, 1 % sodium dodecylsulfate (SDS), 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, 20 µg tRNA, 40 µg proteinase K, extracted with phenol/chloroform and nucleic acids precipitated with ethanol. In vitro synthesized transcripts were analyzed on denaturing 4 % polyacrylamide gels. The gels were analyzed using a PhosphoBioImager (BAS 2000, Raytest, Straubenhardt, Germany).

Two-dimensional gel electrophoresis and Western blotting

High resolution gel electrophoresis was carried out according to Görg et al. [17]. 42 µl 1.4 M DTT, 4 µl Pharmalyte 3–10, 4 mg CHAPS, 108 mg urea and 6 µl 2-DE-standard were added to 100 µl of nuclear extract (150–200 µg protein) and incubated 1 h at room temperature with shaking. Thereafter Immobiline-stripes (11 cm, pH 3–10) were rehydrated in this solution. Following isoelectric focusing (6000Vh, 20 °C) stripes were incubated for 15 min subsequently in 0.01 g/ml DTT, 6 M urea, 30 % glycerol, 2 % SDS, 0.05 M Tris/HCl (pH 8.8) and 0.045 g/ml iodoacetamide, 6 M urea, 30 % glycerol, 2 % SDS, 0.05 M Tris/HCl (pH 8.8). Stripes were then placed on top of a 15 % SDS-polyacrylamide gel and fixed with 1 % agarose. Following electrophoretic separation (13.5 cm x 20 cm x 0.75 mm; 1 h, 15 mA; 4 h, 20 mA) gels were silver stained and digitized using a ARCUSII-Scanner (Agfa, Mortsel, Belgium) with a resolution of 300 dpi and stored as 16 bit-TIFF-files [46]. Spot recognition and numbering were carried out automatically by the program Phoretix V.4.00 (Nonlinear Dynamics Ltd, New Castle upon Tyne, England) under visual control. To evaluate the reproducibility three independent samples of unstimulated HepG2 cells were analyzed by 2-DE. Qualitative comparison of the three 2-DE gels revealed about 90 % identical spots. Spot intensities varied considerably which made a quantitative comparison difficult. Western blotting of 1- and 2-DE gels and silver staining, destaining and immunostaining are described elsewhere [36]. Antisera directed against cJun and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Tryptic digest, extraction of peptides from gels and MALDI-analysis

Protein spots were cut from the gel, submitted to digestion with modified trypsin (Roche Diagnostics, Mannheim, Germany) and the peptides were extracted by washing alternatively with 5 % HCOOH and acetonitrile according to Shevchenko et al. [47]. The solvent was evaporated, peptides dissolved in 10 µl 5 % HCOOH and concentrated over a miniaturized reverse-phase column [48]. α-Cyano-4-hydroxy cinnamic acid (15 µg/µl in 70 % acetonitrile, 30 % (v/v) 0.1 %TFA was used as the matrix. Mass spectra were recorded using a Reflex MALDI-TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany) in the reflector mode. The same protein spot out of different gels was analyzed 2–3 times following identification by searching the nonredundant trEMBL and Swiss-Prot databases using the Peptide Search program (*http://www.mann.embl-heidelberg.de/peptidesearchpage.html*) based on peptide maps with a mass accuracy of less than 0.3 Dalton. The criteria for positive identification of proteins were set as follows: 1) at least five matching peptides, 2) about 20 % of the amino acid sequence of the identified protein should be covered by the matching peptides and 3) molecular weight and pI of the identified protein should match the estimated values obtained from image analysis. Similar criteria have been described earlier [49].

WWW-database

The individual spot parameter (coordinates in the gel, spot diameter, pI, MWG) were transferred into an SQL-

database. The WWW- and SQL server was set up on a 586-PC using Linux (http://www.suse.de) as an operating system. Using this method perl-scripts (http://www.perl.com) use the gd-library (*http://www.genome.wi.mit.edu/pub/software/WWW/GD.html*) to create appropriate pages dynamically upon user request. Data of individual spots are stored in an SQL database (Hughes Technologies Pty Ltd., http://Hughes.com.au) and accessed by corresponding perl-scripts via an SQL-Perl interface (T. Bunce, England).

Electronic supplementary material

The URL of the 2-DE database is http://pc57.dife.de

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