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Research

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Abstract

Background: Rat Nb2-11C lymphoma cells are dependent on prolactin for proliferation and are widely used to study prolactin signaling pathways. To investigate the role of this hormone in the transcriptional mechanisms that underlie prolactin-stimulated mitogenesis, five different techniques were used to isolate differentially expressed transcripts: mRNA differential display, representational difference analysis (RDA), subtractive suppressive hybridization (SSH), analysis of weakly expressed candidate genes, and differential screening of an organized library.

Results: About 70 transcripts were found to be modulated in Nb2 cells following prolactin treatment. Of these, approximately 20 represent unknown genes. All cDNAs were characterized by northern blot analysis and categorized on the basis of their expression profiles and the functions of the known genes. We compared our data with other cell-cycle-regulated transcripts and found several new potential signaling molecules that may be involved in Nb2 cell growth. In addition, abnormalities in the expression patterns of several transcripts were detected in Nb2 cells, including the constitutive expression of the immediate-early gene *EGR-1*. Finally, we compared the differential screening techniques in terms of sensitivity, efficiency and occurrence of false positives.

Conclusions: Using these techniques to determine which genes are differentially expressed in Nb2 lymphoma cells, we have obtained valuable insight into the potential functions of some of these genes in the cell cycle. Although this information is preliminary, comparison with other eukaryotic models of cell-cycle progression enables identification of expression abnormalities and proteins potentially involved in signal transduction, which could indicate new directions for research.

Introduction

Prolactin is a pleiotropic hormone whose numerous actions are associated with reproduction, growth and development, water and electrolyte balance, metabolism, behavior and immunoregulation [1]. The prolactin-dependent rat Nb2 lymphoma (Nb2-11C) is widely used as a model in which to study signal transduction and transcriptional mechanisms that underlie prolactin-stimulated mitogenesis (reviewed in [1-3]). Deprivation of lactogen induces a block in the early G1 phase of the Nb2 cell cycle [4]. The addition of physiological concentrations of prolactin to synchronized G0/G1-arrested cultures reinitiates cell-cycle progression [5], which is characterized by the induction of growth-related genes such as those for c-Myc, β-actin and ornithine decarboxylase (ODC) and

hsp70-like genes [6]. This list has recently been extended to include the genes for interferon regulatory factor-1 (IRF-1) [7], cyclins D2 and D3 [8], proto-oncogene Pim-1 [9], growth factor independence-1 (Gfi-1) [10], and rat nuclear distribution c (Rnudc) [11]. The identification of other prolactin-regulated genes in proliferating Nb2 cells would help to elucidate the relationships between prolactin-activated proteins and genes induced or repressed by prolactin, and lead to a better understanding of the role of prolactin in proliferation regulatory mechanisms. In this study, five differential screening techniques were applied at different stages of the Nb2 cell cycle. Differential display [12], representational difference analysis (RDA [13]) and suppressive subtractive hybridization (SSH [14]) consist of selective and/or suppressive cycles of PCR using cDNA prepared from the cell populations or tissues to be compared. The two other techniques used were the screening of an organized library [15] and the analysis of weakly expressed candidate genes. These two methods are based on the hybridization of DNA macro- or microarrays on nylon filters using complex probes generated from radiolabeled transcribed cDNA isolated from the cell populations to be compared.

We have characterized known and unknown transcripts identified by these five techniques, adding information relative to their expression peak or expression variations during Nb2 cell proliferation. Whenever possible, prolactin-induced transcripts were compared with those in other eukaryotic models of cell-cycle progression such as *Saccharomyces cerevisiae* and normal human fibroblasts. This comparison allowed us to establish non-exhaustive lists of cell-cycle-regulated transcripts. Regulated mRNAs were classified with respect to their functional characteristics and to their conservation from yeast to vertebrates. On the basis of this analysis, new signaling molecules presumably involved in Nb2 proliferation are proposed. Furthermore, we have detected expression profile abnormalities in Nb2 lymphoma cells, and we discuss the consequence of one, the constitutive expression of the immediate-early gene *EGR-1*.

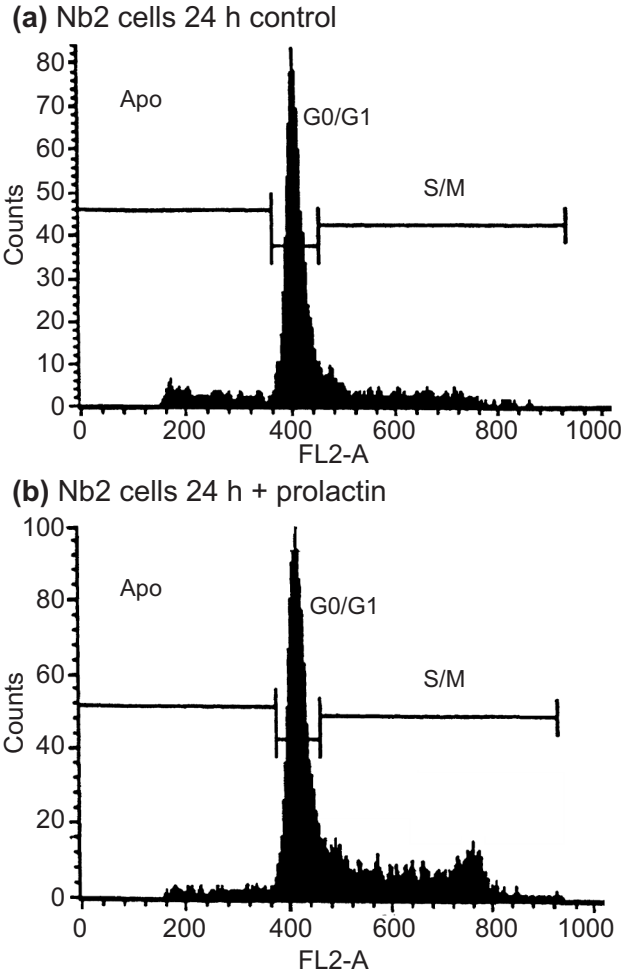

Figure 1
Cell-cycle analysis of synchronized Nb2 cells stimulated by prolactin. Nb2 cells were serum deprived for 24 hours, then incubated with no PRL or PRL at 20ng/ml before cells were collected for analysis. DNA content (FL2-A) *versus* cell number is presented in each panel. **(a)** Profiles obtained with control cells; **(b)** profiles obtained with cells incubated with PRL. From left to right, profiles correspond to cells in apoptosis (Apo, area below 400 on the x axis), in G0/G1 (peak centered on 400 on the x axis), or in S/M phase (area above 400 on the x axis).

Results

Application of the different screening techniques to Nb2 cells

When deprived of lactogen, 80-85% of an Nb2 cell culture is synchronized in growth arrest [5] (Figure 1). Addition of prolactin to the culture reinitiates cell-cycle progression and cell proliferation. Using differential display, we first compared RNAs from synchronized Nb2 cells stimulated for various times with prolactin. In addition, three different RDA and SSH subtractive libraries were prepared. One RDA library allowed the identification of transcripts expressed at a higher level during proliferation (12 hours prolactin-stimulated) compared with growth arrest, and two SSH subtractive libraries were used to compare expression profiles in growth arrest and G1 (mix of cells stimulated with prolactin for 2, 4, 6 and 8 hours) and *vice versa*. Messenger RNAs from Nb2 cells were used to differentially screen an organized library of rat brain cDNA. Finally, the expression of 91 weakly expressed candidate genes was also compared in growth-arrested, early (mix of 2, 4, 6 and 8 hours prolactin-stimulated Nb2 cells), intermediate (10, 12 and 14 hours) or late (20, 22 and 24 hours) proliferative phase and unsynchronized Nb2 cells.

Most of the potential positive clones isolated by differential display, RDA, SSH and screening of rat brain organized

library were analyzed by northern blot to eliminate false positives and to evaluate variations in the expression of each clone during the Nb2 proliferative response. The remaining cDNA clones were tested using reverse northern blot to rapidly eliminate false-positive cDNAs. Briefly, PCR products corresponding to potential positive clones were screened by hybridization with complex probes generated from the populations tested. This step was necessary because of the high rate of false-positive clones generated by the earlier protocols used for differential display. The method has since been improved and may now have a better readout. Sequencing of these clones enabled us to identify known transcripts and to determine which ones were of unknown genes.

Together, the different techniques enabled the isolation of about 70 known or unknown differentially expressed transcripts potentially involved in the resumption of cell proliferation by quiescent cells. A summary of the data is presented in Table 1. Examples of expression profiles obtained by northern blot using the isolated cDNAs as probes are shown in Figure 2. We did not isolate transcripts for known molecules such as histones or cyclins; it is, however, of interest to note that the expression of the rat homolog of Cdc21, the adenosine nucleotide translocator Ant-2, the nuclear export factor CRM-1 (exportin), and unknown transcripts DD3, 4-16 and 4-15 (Figure 2) are induced during Nb2 cell proliferation. In contrast, expression of the unknown transcript 6-4 is decreased during G1 phase, but this transcript is much more abundant in unsynchronized Nb2 cells. Northern blots indicate that some of these cDNA probes identify several distinct transcripts, probably generated by alternative splicing (ANT-2, CRM-1, CD45 (leukocyte common antigen), DD3, 4-15), which are not necessarily all induced in the same manner. Interestingly, two opposite expression profiles are observed for the two transcripts recognized by the cDNA probe identified as CD45 (clone from the SSH library G1 phase growth arrest). Indeed, the longer transcript is progressively repressed during Nb2 cell-cycle progression, whereas the shorter form is induced. These examples emphasize that northern blot analysis provided new information that could not be obtained using other methods (such as reverse northern blot, arrays or DNA chips). As shown in Table 1, about 20 of the differentially expressed cDNAs that were isolated correspond to unknown transcripts whose expression is modulated during Nb2 proliferation. Most of these unknown cDNAs share significant homology with several mouse and human expressed sequence tags (ESTs) isolated from various libraries, suggesting that the corresponding transcripts are ubiquitously expressed and have a role in cell proliferation in one of the different functional categories described below.

Putative signaling molecules potentially involved in Nb2 cell survival and/or cell-cycle progression

The list of differentially expressed transcripts has been completed with Nb2 cell transcripts described in previous reports (Table 2). These differentially expressed genes can be found in almost all the subclasses listed in Table 2, including, for example, those for receptors (such as the prolactin receptor [16], the T-cell receptor γ chain [17], the vitamin D3 receptor, the thromboxane A2 and the prostaglandin F2 receptors (our present results)), transcription factors (IRF-1 [7], c-Myc [18], Zfx (our present results)), and T-cell survival and apoptosis molecules (Bag-1 [19], Bcl-2, Bax [20], Ant-2 (our present results)). Although all the signaling molecules are not necessarily regulated at the transcriptional level, it can be hypothesized that those translated from cell-cycle-modulated transcripts are involved in Nb2 cell-cycle progression. This hypothesis seems to be confirmed, as we have found that several molecules previously described as transducers in Nb2 cells are encoded by cell-cycle-modulated transcripts (for example, phosphatidylinositide 3-kinase (PI 3-kinase), phospholipase Cγ1 (PLCγ1), and focal adhesion kinase (FAK) p125). Consequently, it is tempting to speculate that at least some of the cell-cycle-modulated transcripts may encode transducers of Nb2 cell proliferation. For example, the stress kinase p38 mitogen-activated protein kinase (p38 MAP kinase), whose transcript is induced in Nb2 cells upon prolactin stimulation [21], may be involved in prolactin-induced signaling pathways. This hypothesis is reinforced by the fact that p38 MAP kinase seems to be required for the optimal activation of T cells by interleukin (IL)-12 and IL-2 and for the regulation of serine phosphorylation of STAT transcription factors [22]. The GD3 ganglioside synthase, which mediates the propagation of CD95-generated apoptotic signals in hematopoietic cells [23], may also be involved in the regulation of survival and apoptosis in Nb2 cells. It is also possible that receptors, such as the prostaglandin F2, thromboxane A2 and vitamin D3 receptors, galectin-8 and CD45 and their ligands, may be involved in the signaling pathways required for Nb2 cell survival and proliferation.

Functional classification of cell-cycle-regulated transcripts

We identified 70 differentially expressed genes in proliferating Nb2 cells. Although this number is not negligible, it is of course not exhaustive, as the number of genes involved in cell-cycle modifications could be as high as several hundred. However, estimations of the number of genes modulated using other proliferation and cell-cycle models, such as yeast [24-26] or human fibroblasts [27,28], are equally limited. We compared these different models, adding new information generated from large-scale differential screening techniques (microarrays and DNA chips). On the basis of these analyses, approximately 7% of transcripts from yeast (416 out of 6,220) and 6% from normal human fibroblasts (517 out of 8,600) display cell-cycle-dependent fluctuations. All the yeast cell-cycle-regulated transcripts are not, however, regulated in vertebrates, and *vice versa*.

These transcripts were classified into 10 different functional categories as shown in Table 2: cell cycle (cyclins and cell-cycle

Table 1

Differentially expressed transcripts found in Nb2 cells during cell-cycle progression using five different screening techniques

Identity	Accession number	Expression variations
Differential display		
Unknown DD3	No EST	Three transcripts (2, 2.8, 3.7 kb) expressed in G1 and G1/S, (>4-fold induction)
Representational difference analysis: G1/S–GA		
ATP synthase β subunit	M19044	Induced in G1/S, G2 (2-fold induction)
Aldehyde dehydrogenase	U79118	Induced in late G1 (2-fold induction)
Enolase α1	NM012554	Induced in late G1 (2-fold induction)
Dynein heavy chain	D13896	Induced in G1, G1/S (3-fold induction)
TCP-1 ε	D43950(h)	Induced in G1/S, G2 (3-fold induction)
αCOP (COPA)	U24105	Induced in late G1 (2-fold induction)
Cdc21 homolog	D26089 (m)	Peaks in G1/S (>8-fold induction)
Ribosomal protein L26	AI716351	Induced in late G1 (2-fold induction)
Itm1	AI956728 (h)	Induced in G1/S (2-fold induction)
Unknown T22	AI053031 (h)	6 kb, 2-fold induction in G1/S
Unknown T34	AI235326	2 kb, 2-fold induction in G1/S
Subtractive suppressive hybridization: G1–GA		
Galectin-8	U09824	Induced in G1 (2-fold induction)
Hsp86	X16857	Induced in late G1 (>3-fold induction)
TCP-1 η	AA900460	Induced in late G1 (2-fold induction)
Ribosomal protein L13A	X68282	Induced in late G1 (2-fold induction)
Ribosomal protein L12	AA900142	Induced in late G1 (2-fold induction)
Ribosomal protein L3	AI687295	Induced in G1 (2-fold induction)
	Y00441	Induced in G1 (2-fold induction)
β_2-microglobulin	D12771	Induced from late G1 to G2 (>5 fold induction)
Adenine nucleotide translocator ANT-2	NM011342 (m)	Induced in G1 (2-fold induction)
Sec-22	NM012562	Induced in G1 (2-fold induction)
L-fucosidase	U91538	Induced in G1/S (>3-fold induction)
CRM-1 homolog/exportin 1	X81839	Induced in G1/S (>3-fold induction)
Ubiquitin/ribosomal protein S27a	A47416	Induced in G1/S (>3-fold induction)
Ubiquitin/ribosomal protein S30 (FAU)	AF195142 (m)	3-fold induction in G1 (1.5 kb)
Unknown 4-2 (mouse selenoprotein R mRNA)	H35219	2-fold induction in G1/S (2 and 4 kb)
Unknown 4-4 (human KIAA0081)	No EST	2-fold induction in G1 (1 kb)
Unknown 4-11	AU035826	4-fold induction in G1 (1 and 1.5 kb)
Unknown 4-15	AF046001	4-fold induction in G1 (2.5 kb)
Unknown 4-16 (human ZNF207 or mouse Zep)	No EST	3-fold induction in G1 (1.5 kb)
Unknown 4-20	AW435432	2-fold induction in G1
Unknown 4-27 (new ribosomal protein L15 type)	AI121996 (m)	2-fold induction in G1
Unknown 4-49	AW246248 (h)	2-fold induction in G1
Unknown 4-58 (SH3, Rab GAP, TBC domain)	No EST	2-fold induction in G1
Unknown 4-59		
Subtractive suppressive hybridization: GA–G1		
Spermidine/spermine N-acetyl transferase (SSAT)	AA955996	Repressed transiently in G1
Leukocyte common antigen (alternative splicing) CD45	Y00065	Switch between two transcripts (one repressed, the other induced in G1)
ZFX	X75171	Repressed transiently in G1
Ribosomal protein S8	AA874997	Repressed transiently in G1
Ribosomal protein S13	L01123	Repressed transiently in G1

Table 1 (continued)

Identity	Accession number	Expression variations
Unknown 6-2	No EST	Repressed transiently in G1
Unknown 6-3 (hypothetical protein expressed in thymocytes)	AJ237585 (m)	Repressed transiently in G1
Unknown 6-4	No EST	Repressed in G1
Unknown 6-9	No EST	Repressed transiently in G1
Unknown 6-10	No EST	Repressed transiently in G1
Unknown 6-12 (human protein KIAA0710)	AB014610 (h)	Repressed transiently in G1
Unknown 6-45 (homolog to mouse PARP-2)	NM009632 (m)	Repressed transiently in G1
Differential screening of a rat organized library: G2–GA		
Prothymosin α	M86564	Induced in G2
Cyclophilin	M19533	Induced in G2
ATP synthase β subunit	M19044	Induced in G1/S and G2
Tubulin α2	AA686718	Induced in G1/S and G2
GaPDH	X02231	Induced in G2
Phosphoglycerate kinase	M31788	Induced in G1/S and G2
MRG1 related protein	U65093	Induced in G1/S and G2
Unknown BO1	No EST	Induced in G2

Analysis of weakly expressed candidate genes: UN, GA, G1, G1/S, G2

Name	Accession number	Fold induction	Kinetics
Ganglioside synthase (GD3)	D84068	> 4	UN GA G1 G1/S G2
PI3 kinase	D64045	> 3	
Phospholipase Cγ1	M34667	> 3	
Bax	S76511	> 2	UN GA G1 G1/S G2
P53	X13058	> 2	
FAKp125	AF020777	> 3	
14-3-3 ε	M84416	> 2	
14-3-3 η	D17445	> 2	UN GA G1 G1/S G2
Vitamin D3 receptor	J09838	3	
Glycine transporter	M88595	3	UN GA G1 G1/S G2
Thromboxane A2 receptor	D32080	2	UN GA G1 G1/S G2
Phosphatidylinositol transfer protein	D17445	2	UN GA G1 G1/S G2
RexB/NSP	U17604	> 2	UN GA G1 G1/S G2
Glucocorticoid receptor	M14053	2	UN GA G1G1/S G2
Ga3PDH	J04147	2	UN GA G1G1/S G2
Zif268 = EGR1	U75398	2	UN GA G1 G1/S G2

Nb2 cells: UN, unsynchronized; GA, growth arrested; G1, G1 phase; G1/S, G1/S transition; G2, G2 phase. PC12, rat pheochromocytoma PC12 cells. 18S and 28 S, ribosomal RNA. Whenever possible, rat accession numbers (GenBank) are written in the second column. When this sequence is not in known for the rat, (h) or (m) indicates that the accession number corresponds, respectively, to a human and a mouse cDNA homologous to those of the rat. 'No EST' means that our rat sequence does not correspond to any previously described EST in mammals. For some of the unknown cDNAs, an estimation of the size of the corresponding transcript(s) as well as the fold induction is given in the right column 'Expression variations', and for weakly expressed candidate genes, the expression kinetics are shown.

Table 2

Functional classification of cell-cycle-regulated transcripts found in Nb2 cells

Category	Transcript
Cell cycle: cyclins and cell-cycle regulators	
Transcripts that have a cell-cycle-modulated homolog in yeast	Cyclin E1 [78], peaks in G1 EGR-1, immediate-early gene (constitutive expression in Nb2 cells) Cdc5-like protein [79], peaks in M Cdk2, Cdk5 [78], peak in G1
Cell-cycle-modulated transcripts with a yeast homolog (not modulated)	Cyclin B1, peaks in G2/M
Cell-cycle-modulated transcripts with no yeast homolog	Cyclin B2, peaks in G2 Cyclin D2 [8], peaks in G1 Cyclin D3 [8], peaks in G1
Nucleotide metabolism, DNA replication and repair	Cdc21 homolog Spermidine/spermine N-acetyl transferase (SSAT) Ornithine decarboxylase (ODC) [80] *S*-adenosylmethionine decarboxylase [80] Prothymosin α PARP-2 (Unknown 6-45)
Chromatin structure	Histones H2A, H2B, peak in S in mammals and in yeast
Cytoskeleton	Myosin heavy chain Tubulin α, β β-actin [6] Clone 15 = rNUDC [81] FAK p125
Cell-surface antigens, adhesion molecules and signaling molecules (involved in apoptosis, survival and/or proliferation)	
Growth factor	FGF-2 [82]
Surface molecules	Prolactin receptor Nb2 form [16] T-cell receptor γ chain [17] T-cell receptor α chain [17] GnRH receptor [83] Glucocorticoid receptor Galectin-8 Leukocyte membrane glycoprotein, CD45 Vitamin D3 receptor Thromboxane A2 receptor β_2-microglobulin
Cytoplasmic and/or nuclear signaling molecules	p38 Map kinase [21] Pim-1 [9] Gfi-1 [10] Stathmin [84] PI3 kinase p110 α Phospholipase Cγ1 14-3-3 η and ε Bax p53 RexB/NSP Phosphatidylinositol transfer protein α 4 phosphoprotein [79]
Transcription factors	IRF-1 [7] c-Myc [18] c-Fos (not in Nb2 cells [18]) MRG1-related protein E2F-1 [78] Zfx

Table 2 (continued)

Category	Transcript
Heat shock, stress response and chaperones	Cyclophilin (B) TCP-1 ε and η Hsp70 (not expressed in Nb2 cells) Hsp70-like = Nb29 [41] Hsp27 Hsp86 β-actin α_2-tubulin Rdnuc (Golgi-associated protein) [11] Myosin heavy chain Focal adhesion kinase (FAK)
Metabolism (energy)	Phosphoglycerate kinase Enolase α Aldehyde dehydrogenase ATP synthase β subunit
Protein and RNA synthesis, modifications and degradation	
Ribosomal proteins	L3, L12, L13A, new ribosomal protein L15 type (unknown 4-27) S8, S13
Glycosylation factor	Itm1
Elongation factor	EF-2 [77]
Inter-compartment transport and trafficking	CRM-1 = exportin 1 Sec-22 Glycine transporter Unknown 4-58 (SH3, Rab GAP, TBC domain: putative nuclear pore protein)
Unknown function(s)	FGF-responsive Non/p54nrb Unknowns T22, T34, 4-2, 4-4, 4-11, 4-15, 4-20, 4-49, 4-59, BO1 Unknowns 6-2, 6-3, 6-4, 6-9, 6-10, 6-12

regulators); nucleotide metabolism, and DNA replication and repair; chromatin structure; cytoskeleton, cell surface antigens, adhesion molecules and signaling molecules (involved in apoptosis, survival and/or proliferation); heat shock, stress response and chaperones; metabolism (energy); protein and RNA synthesis, modifications and degradation; inter-compartment transport and trafficking; and unknown function(s).

These functional categories agree with previous observations concerning cell-cycle-regulated transcripts in various eukaryotic models. Indeed, cell-cycle-dependent mRNA fluctuations have been observed for genes involved in many cellular processes, including control of mRNA transcription [29], responsiveness to external stimuli [30] and subcellular localization of proteins [31]. Genetic studies have revealed that the activity of cell-cycle regulatory proteins is required for normal DNA repair [32], meiosis [33] and multicellular development [34,35]. These observations suggest that, in eukaryotic cells, diverse biological events depend on maintenance of this periodicity.

Expression abnormalities

The loss of appropriate cell-cycle regulation leads to genomic instability [36] and is believed to have a role in the etiology of both hereditary and spontaneous cancers [37-40]. In Nb2-11C cells, several growth-related genes that display abnormalities in their expression patterns were observed. These abnormalities may be the cause or the consequence of the tumor phenotype of Nb2 cells.

Using the candidate gene approach (Figure 3a), striking expression abnormalities were observed. For example, Nb2 cells display an abnormal response to heat shock. Indeed, whereas the *hsp70*-like mRNA is upregulated following lactogen stimulation [41], no expression of the inducible *hsp70* (GenBank X74271) gene was detected. As components of the heat-shock response are involved in normal cell-cycle-progression, the abnormalities observed in Nb2 cells may have important consequences for their growth. Furthermore, in comparison with mammalian models of cell-cycle progression, expression abnormalities of immediate-early genes are observed in Nb2 cells. Indeed, the expression of c-*fos* remains undetectable in our model (Table 2) as well as in starved Nb2 cells, which resume proliferation after prolactin stimulation [18]. In contrast, the expression of *EGR-1* (also termed *Zif268* / *KROX24* / *ETR103* / *NGFIA* / *TIS8* / *GOS30*) is constitutive in Nb2 cells. This peculiarity, observed using the candidate gene approach (Figures 3b,4), was confirmed by northern blot (Figure 3c). The gene has been shown in

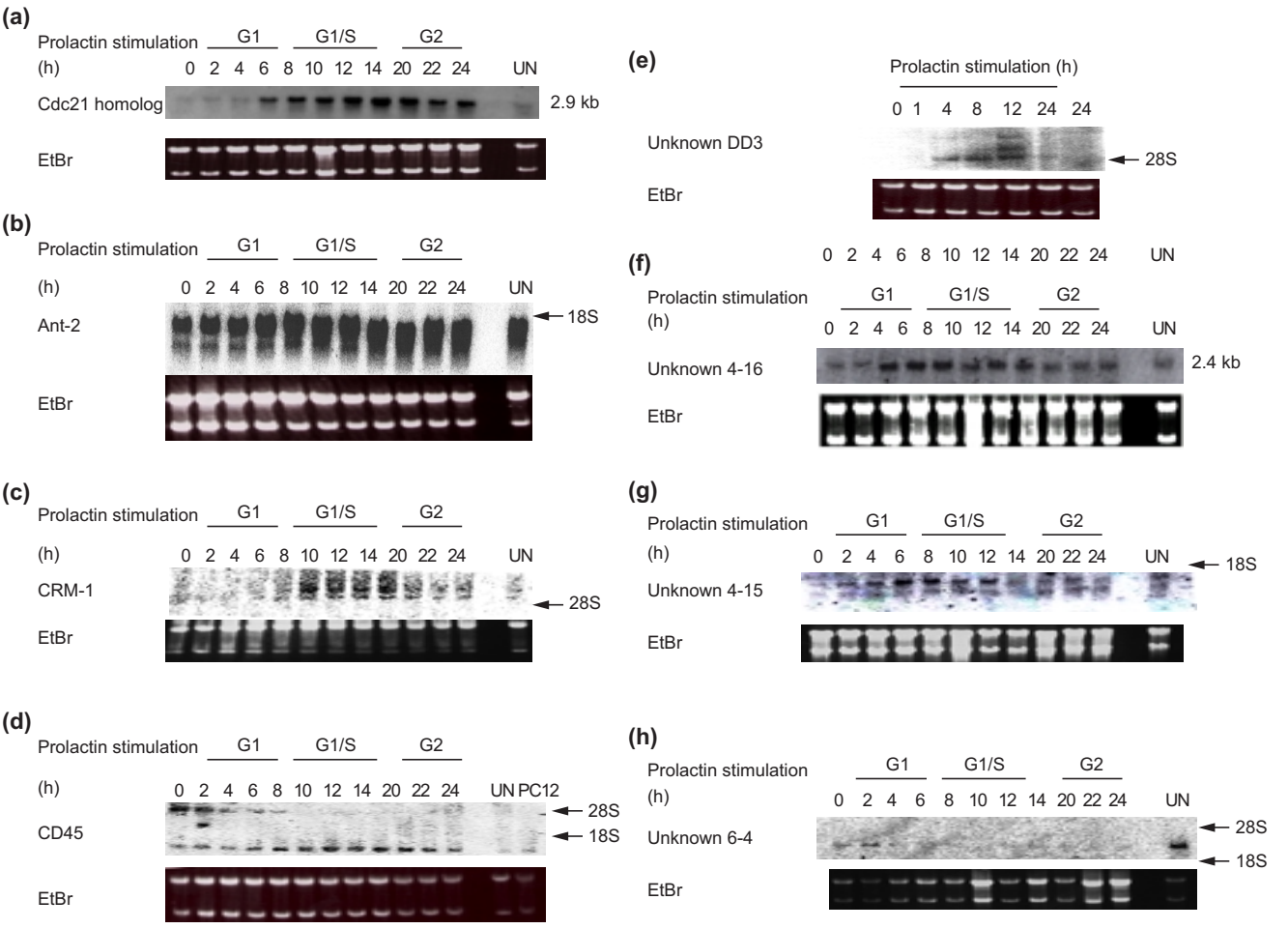

Figure 2
Expression profiles of various known and unknown transcripts during Nb2 cell cycle progression. Samples of total RNA (10 μg) were loaded per lane and blots were hybridized with the indicated cDNA probes. Ethidium bromide staining (EtBr) of the gels is shown as a control (18S and 28S rRNA). **(a)** Cdc21 homolog; **(b)** Ant-2; **(c)** CRM-1; **(d)** CD45; **(e)** unknown DD3; **(f)** unknown 4-16; **(g)** unknown 4-15; **(h)** unknown 6-4.

numerous model systems to have induction kinetics similar to c-*fos*, characterized by a rapid transient expression requiring *de novo* transcription between 15 and 30 minutes after the mitogenic stimulus [42-47].

Discussion

New putative signaling molecules in Nb2 cells

In this study we have identified new regulated genes encoding potential signaling molecules. Genes encoding proteins involved in cell survival, apoptosis, proliferation and/or cell-cycle progression may or may not have cell-cycle-dependent expression. Most of these proteins (listed in Tables 1,2) are known to be activated by post-translational mechanisms; little is known, however, about their regulation at the transcriptional level.

In our experiments, for example, PI 3-kinase, PLCγ1 and ganglioside synthase GD3 share identical expression profiles, characterized by lower expression in unsynchronized than in synchronized Nb2 cells. This indicates that they may share similar regulation mechanisms. In high-density Nb2 cell cultures [48], secreted growth factors may be involved in this negative regulation. PI 3-kinase and/or PLCγ1 have been implicated in cell-cycle progression, proliferation, survival, transformation and apoptosis in different cellular models [49-52]. Thus, ganglioside synthase may also take part in similar processes in immune cells. Indeed, ganglioside synthase GD3 is highly expressed in various human cancer cell lines, is upregulated in activated T lymphocytes [53], and has been implicated in Fas-mediated apoptosis [23]. It may therefore be of interest to determine whether prolactin is able to activate, directly or indirectly, the activity of GD3 and the Fas signaling pathways.

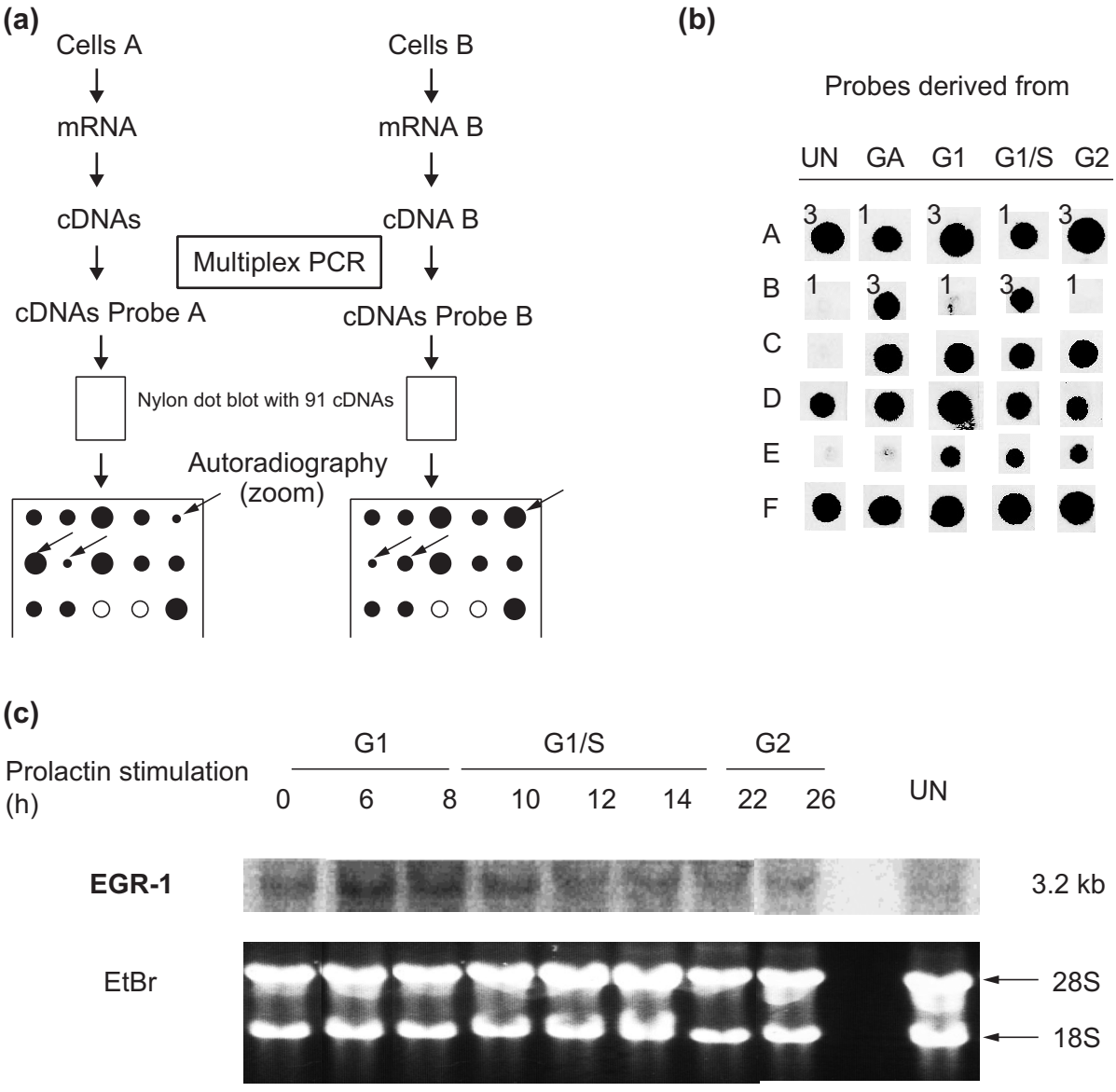

Figure 3
Analysis of candidate gene expression. **(a)** General principles. Messenger RNAs from the different cell populations (cells A and B) are reverse transcribed. Multiplex PCR is then performed using specific primer pairs to amplify the cDNAs of interest. The resulting mixture of PCR products is radiolabeled and these complex probes are used to hybridize identical membranes spotted with the candidate gene cDNA targets. After autoradiography, the intensities of the hybridization signals are compared and quantified. Arrows indicate the positions of differentially expressed genes. The absence of hybridization (open circles) indicates that the candidate gene is not expressed. **(b)** Efficiency of the technique and examples of differentially expressed genes. The expression of different candidate genes was compared in either unsynchronized (UN), growth-arrested (GA), G1 phase (G1), G1/S transition (G1/S) or G2 phase (G2) cultures of Nb2 cells. The efficiency of the technique was controlled using equivalent amounts of rabbit α and β globin cDNAs, which were included on the nylon membranes along with the candidate gene targets. The two globin cDNAs were added in different amounts (50 or 150 ng) to each cDNA population before co-amplification. For each population tested, filters were hybridized with both globin probes, but only representative hybridization signals are shown, for either α (Panel A) or β (Panel B) globin. Numbers 1 and 3 represent the relative amount of the control rabbit globin cDNAs added, and are reflected in the differences in the intensity of the hybridization signals. Thus, a threefold difference in the quantity of a particular transcript in the initial population generates a clear difference in the intensity of the corresponding hybridization signals. Rows C, D, E and F are examples of the results obtained with ganglioside synthase GD3, EGR-1, FAK p125 and Stat3, respectively. Except for Stat3, which is not differentially expressed in probes UN, GA, G1, G1/S and G2, the three other genes showed a clear differential expression. **(c)** Northern blot analysis showing the constitutive expression of EGR-1 during Nb2 cell-cycle progression. Growth-arrested Nb2 cells were stimulated with ovine prolactin and collected after various periods of stimulation corresponding to different stages of the cell cycle (G1, G1/S and G2). The expression of EGR-1 was evaluated by northern blot using 10 μg of total RNA from the various times following prolactin stimulation. Ethidium bromide (EtBr) staining of the gel is shown as a control (18S and 28S rRNA).

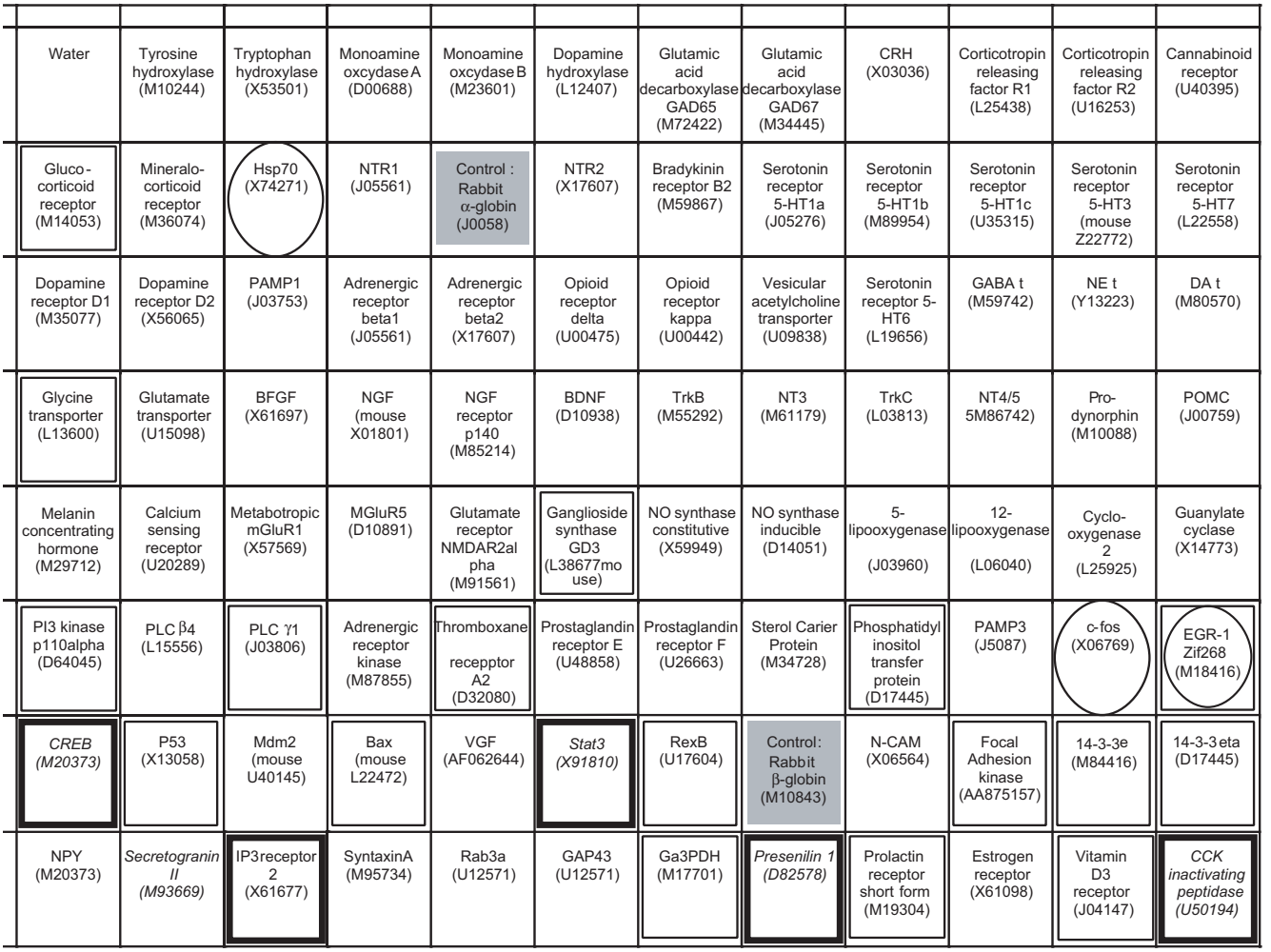

Figure 4
Schematic representation of rat candidate genes on a nylon filter. Squares with names and accession numbers represent the places where the cDNAs were spotted. The solid gray boxes correspond to the controls (rabbit α and β globin). The boxes enclosed in a thick black square represent differentially expressed genes in Nb2 cells; the boxes enclosed in a thin black square represent genes that are repressed, but not differentially in Nb2 cells; and those enclosed in an oval correspond to expression abnormalities in Nb2 cells.

Expression profiles of genes for Bax, p53, 14-3-3 ε and FAK are characterized by increased mRNA expression during the G1, G1/S and G2 phases in comparison to the growth-arrested or unsynchronized Nb2 cells (Table 1). These kinetics suggest that prolactin may have a direct effect on the transcription of these genes. Indeed, in myeloid cells [54] and in proliferating prostate cells [55], FAK expression is induced by various cytokines. This molecule is located at the signaling crossroads of cell growth and attachment, and is involved in dynamic cytoskeletal rearrangements [56]. In Nb2 cells, prolactin has been shown to increase *bax* mRNA expression in 8 hours [20]. The thromboxane A2 receptor, which is highly expressed in immature thymocytes, has also been shown to mediate DNA fragmentation and apoptosis [57]. It is possible that, in Nb2 cells, prolactin could also counteract thromboxane-induced apoptosis, as is the case for glucocorticoids.

It is not known at present whether these expression profiles are common to all dividing mammalian cells or only to a particular subclass of immune-system cells. Alternatively, these profiles could be the consequence of the genetic abnormalities displayed by Nb2 cells. It is of interest that the expression of the p38 MAP kinase gene is modulated in Nb2 cells [21] but not in normal human fibroblasts [27,28], suggesting that this regulation is specific to the T-cell lineage. Different arguments exist in favor of the existence of cross-talk between the JAK/STAT and p38 MAP kinase

pathways, at both the translational and transcriptional levels. As the expression of ganglioside synthase GD3 is restricted to the brain and the hematopoietic lineage, the regulation of the transcript and the involvement of the protein in the regulation of survival and apoptosis may also be shared by these tissues. In contrast, the cell-cycle-dependent expression of Ant-2, observed in both Nb2 cells (our results) and human fibroblasts [27,28], suggests that this feature is common to all dividing mammalian cells. These speculations require further experimentation at the protein level for confirmation.

Expression abnormalities

We report for the first time, to our knowledge, the constitutive expression of the transcription factor EGR-1 in synchronized proliferating cells. EGR-1 has a role in differentiation and development, in normal growth and in virus-induced growth and immortalization (reviewed in [58]). Many of these effects may be related to complex cooperative and competitive mechanisms between the three transcription factors Sp1, EGR-1 and Wt1, which often have overlapping binding sites in target promoters.

Several arguments suggest that EGR-1 may act as a tumor suppressor [59-61], and that its anti-oncogenic function could be due to the transcriptional induction of the gene for transforming growth factor β1 (TGFβ1), which suppresses growth by an autocrine mechanism in the late G1 phase of the cell cycle [62,63]. Exogenous TGFβ inhibits Nb2 cell growth [64], suggesting that these cells are still sensitive to the anti-proliferative action of TGFβ, but are unable to synthesize or activate TGFβ on their own, despite their constitutive EGR-1 expression. It is possible that, in Nb2 cells, the anti-proliferative effect induced by the constitutive expression of EGR-1 is suppressed by other abnormalities such as a deficiency in the production of active TGFβ.

Other studies, however, argue in favor of the existence of anti-apoptotic and/or pro-proliferative properties of EGR-1 [65-68]. In this context, the constitutive expression of EGR-1 in Nb2 cells suggests that this transcription factor may have both anti- and pro-proliferative effects, as previously described for other proteins such as p53 [69]. These dual and antagonistic functions may constitute a protective mechanism against tumor formation. In this model, three oncogenic abnormalities would have to occur in order to generate continuous tumor growth: immortalization; activation of all the transduction pathways required for proliferation; and suppression of all the anti-proliferative and apoptotic properties resulting from proto/anti-oncogene modifications.

Further studies are required to confirm the integrity of the EGR-1 protein and its constitutive expression in Nb2 cells and to understand the relationships between the EGR-1 target genes and their signaling pathways.

Comparison of various differential screening techniques

We have compared the advantages and drawbacks of four differential screening techniques. Of the four approaches, differential display analysis presents several advantages: it is easy, rapid, does not require large amounts of biological material, and it allows the comparison of multiple transcriptomes in a single experiment. The occurrence of false-positive clones is, however, quite high. In our experimental conditions, we isolated 20 potential positive cDNAs; only two, however, presented a relatively distinct cell-cycle-modulated expression profile. The problem with this technique lies in the low reproducibility of the PCR reaction and the occurrence of non-differential PCR products, which are recovered together with differentially expressed transcripts from the acrylamide gel. Several methods (such as reverse northern blots) have been proposed to circumvent this problem, albeit with rather limited success. Another disadvantage is that the identification of cDNA clones may be difficult if the model system studied has not previously been used in an extended EST sequencing program.

RDA and SSH are based on the same principle. These techniques are easy to use and enable the rapid generation of RDA or SSH subtractive libraries. The proportion of false-positive cDNAs can be less than 10% (our data), but if the differences between the two libraries are discrete, this number is increased. RDA or SSH cDNA clones correspond to sequences positioned in the middle portions of transcripts (generally coding regions), and the sequencing of each cDNA clone enables their identification independently of the model used. These techniques have two principal disadvantages: only two different transcriptomes can be compared in one experiment, and these approaches are far from being exhaustive. Although they should facilitate the detection of low-level transcripts, this is often not the case, as non-optimal conditions appear preferentially to select cDNAs corresponding to highly expressed transcripts.

The screening of an organized library can be compared with the use of DNA arrays, and the detection of a wide range of differential transcripts. This approach theoretically allows the screening of transcripts corresponding to unknown genes. False-positive cDNAs are relatively rare (10-20%) if the amount of DNA fixed on the high-density nylon filters is strictly controlled. The detection threshold for these techniques, however, does not allow the detection of weakly expressed differential transcripts and remains a major limitation.

The principle of the analysis of weakly expressed candidate genes is derived from that of macroarrays. In that case, the detection threshold is increased by a step of moderate PCR amplification for each candidate gene in the complex probes, but allows only a semi-quantitative detection of differentially expressed candidate genes. Internal controls are included to monitor the efficiency of the technique.

Cell-cycle-regulated transcripts in mammals

To facilitate the comparative analysis of cell-cycle-regulated transcripts, we classified them into ten different functional categories (Table 2). This classification is in agreement with the target genes of growth-related transcription factors. Indeed, putative c-Myc target genes are involved in the cell cycle, apoptosis, DNA metabolism and dynamics, energy metabolism and macromolecular synthesis [70]. Nevertheless, as transcriptional gene activation or inhibition result from a complex multifactorial *cis* and *trans* regulation, it is necessary to integrate the various components of this regulation and of post-transcriptional modifications to explain the origin of differential expression.

The current functional classification is too restrictive and does not take into account the interactions between these functional categories. For example, the regulation of proto-oncogene and cytokine mRNAs and proteins is particularly complex. The regulatory processes involved include transcriptional control, nuclear export and import of transcripts and proteins, translation, heat-shock pathways (Hsc70-Hsp70), and the ubiquitin- and proteasome-mediated degradation of mRNA and proteins [71]. Interestingly, transcripts of many proteins involved in these processes (such as Hsp proteins, translation factors, and CRM-1) were found to be induced by prolactin in Nb2 cells in this study, as well as in other models. Moreover, the abnormal heat-shock response described in Nb2 cells may perturb this regulation and have important consequences for tumor progression.

Comparison of cell-cycle regulated transcripts in yeast and higher eukaryotes

Data are still lacking for an extensive comparison of cell-cycle-modulated transcripts in unicellular (yeast) and multicellular organisms (essentially mammals) and for deduction of evolutionarily conserved features. Several conclusions can be drawn, however. In almost all the functional subclasses detailed in Table 2, homologous proteins are regulated at the transcriptional level in both yeast and mammals. For example, the yeast cyclins, Cdc proteins, histones and their mammalian homologs are usually regulated in the same fashion during the cell cycle. These observations have been well documented in numerous studies [72] and suggest that the functions of the corresponding proteins and the regulation of their transcripts are both conserved. A list of yeast cell-cycle-regulated transcripts that have known mammalian homologs has been established [73]. In this list, 26 out of 99 of the yeast cell-cycle-regulated transcripts correspond to proteins involved in nucleotide synthesis, DNA replication and repair. Within the cytoskeleton subclass, the transcripts for yeast MYO3 and its mammalian homolog, myosin I heavy chain, both peak at the G2/M phase of the cell cycle, whereas TUB3 and mammalian tubulin γ peak in G1. Proteins in several classes and subclasses do not have yeast homologs. For example, 'adhesion molecules, extracellular matrix and cellular surface antigens' are specific to multicellular organisms.

There are numerous cellular and physiological differences between yeast and multicellular organisms, and some of the molecular divergences observed may reflect these differences. In unicellular organisms the growth priorities are to proliferate as long as enough nutrients are available. In contrast, in multicellular organisms the integrity of the organism is paramount, and individual cell behavior is highly controlled. Therefore proliferation is basically prohibited, and occurs in a cell lineage only if the environment sends the appropriate combination of signals to unlock all the growth-inhibition mechanisms. Despite these differences, cell-cycle checkpoints located just before and at the end of mitosis are essential and similar in all organisms. These two controls ensure that all the DNA has been correctly replicated before the cell enters mitosis and that the condensed chromosomes are properly aligned on the division spindle before anaphase. Thus, all proteins potentially involved in such controls may be regulated similarly from yeasts to animals and plants.

The present study illustrates how data from various large-scale differential screening analyses can be integrated into specific and/or global biological studies. Such comparisons of gene expression profiles are of value in understanding general expression profiles of all dividing cells and in analyzing differences between unicellular and multicellular organisms. They can also identify new signaling molecules and explain how different signals and transduction pathways could regulate the proliferation of different cell types. They can help elucidate the function of proteins, and finally they can identify abnormal patterns of gene expression in transformed and tumor cells. With the increasing amount of information being generated from microarrays and DNA chips, the potential value of comparative analyses will be all the greater.

Materials and methods

Cell culture

Suspension cultures of lactogen-dependent Nb2-11C lymphoma cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 10% lactogen-free horse serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 5 mM HEPES pH 7.3, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively) at 37°C with 5% CO_2. Cultures were rendered quiescent by transferring cells into starvation medium (cell density about 1.5×10^5 cells/ml), deficient in FCS and β-mercaptoethanol, for 24 hours. Under these conditions, about 80-85% of the cells were arrested in the G0/G1 phase (Figure 1a). Ovine prolactin (Sigma) was added at a concentration of 20 ng/ml to starvation medium to reinitiate growth (Figure 1b). Cells were collected after various periods of prolactin stimulation and total RNA was extracted. Unsynchronized Nb2 cells were collected from high-density cultures (10^6 cells/ml).

RNA extraction, poly(A)+ RNA preparation, cDNA synthesis, northern blot and reverse northern blot analyses

For each cell population to be compared, RNA was prepared by acid guanidinium-thiocyanate-phenol-chloroform extraction [74] and poly(A)+ RNA was isolated using magnetic oligodT (Dynabeads, Dynal). Double-stranded cDNA was transcribed using a commercial kit (Boehringer). Northern blots were performed using the formaldehyde/formamide procedure and reverse northern blots as described in [75]. The Vacugene transfer system (Pharmacia), nylon filters (Hybond N+, Amersham) and the hybridization solution (ExpressHyb, Clontech) were used following the manufacturer's instructions. The cDNAs were radiolabeled using the Readyprime kit (Amersham).

mRNA differential display, representational difference analysis (RDA), subtractive suppressive hybridization (SSH) and organized library screening

Total RNA treated with DNase I was prepared from Nb2 cells treated for 0, 2, 4, 6, 8, 12 or 24 h with prolactin, and used in mRNA differential display using the GenHunter kit (GeneHunter Corporation, TN, USA). Poly(A)+ mRNA extracted from Nb2 cells treated with prolactin for 12 h was used for RDA according to the protocol described in [13]. SSH [14] was performed using poly(A)+ RNA obtained by mixing mRNA from Nb2 cells stimulated with ovine prolactin for 2, 4, 6 and 8 h. An organized rat brain library was screened as described in [15].

Preparation of complex probes, multiplex PCR and differential screening of candidate genes

In this technique, nylon filters dotted with candidate genes are hybridized with complex cDNA probes from different cell populations, to compare expression levels (see, for example [76]). Originally developed and validated by SANOFI-Recherche (unpublished data), the nylon filters contain 91 rat candidate genes (PCR products), including signaling molecules and transcription factors (Figure 4). These cDNAs were selected with the idea of defining a panel of genes whose expression is likely to be modulated in response to a proliferative signal. To increase the sensitivity of the approach, the mRNAs of interest are co-amplified by reverse transcription polymerase chain reaction (RT-PCR), using primers specific for the 91 candidate genes (the multiplex PCR step), before their use as hybridization probes (Figure 3a). Under these conditions, moderate PCR amplification allows the detection of weakly expressed genes and the evaluation of their differential expression in a semi-quantitative manner. mRNAs are amplified by RT-PCR, and the number of PCR cycles is adapted to their relative abundance in the population tested (16, 21, 24 or 26 cycles are performed for the detection of relatively abundant, moderately expressed, weakly expressed and very weakly expressed transcripts, respectively). For each cell population analyzed, the multiplex PCR products are then mixed, radiolabeled by random priming with α-^{32}P-labeled dCTP, and used as hybridization probes against the 91 candidate genes. Hybridization signals are quantified by densitometry (Visiomic) for each of the different populations. During the development of this technique, control studies involving repeated hybridizations with replicate filters showed minimum variation of signal response (data not shown).

The efficiency of the technique was controlled by including rabbit α and β globin cDNAs on the nylon membranes along with the candidate genes. Defined amounts (50 or 150 ng) of the globin cDNAs were also added to the cDNAs mixtures before the multiplex PCR step. As expected, when these different amounts of rabbit globin cDNAs were added to the complex cDNAs, spots of different intensities were obtained (Figure 3b), indicating that this approach could detect at least a threefold difference in mRNA expression between two cell populations.

Figure 4 shows the list of the candidate genes analyzed and indicates their expression pattern in Nb2 cells.

Sequencing

The potential positive clones isolated by differential display, RDA, SSH or screening of the organized library were sequenced with a dye terminator kit using the ABI Prism system (Perkin-Elmer).

Bioinformatics

To identify the sequenced cDNAs, BLAST and UniGene from NCBI were used [77]. For comparison, we have also consulted databases of transcripts differentially expressed during cell-cycle progression in human fibroblasts [27,28] and in *S. cerevisiae* [24,25].

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