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Differentially expressed genes in C6.9 glioma cells during vitamin D-induced cell death program

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Abstract

C6.9 rat glioma cells undergo a cell death program when exposed to 1,25-dihydroxyvitamin D3 (1,25-D3). As a global analytical approach, we have investigated gene expression in C6.9 engaged in this cell death program using differential screening of a rat brain cDNA library with probes derived from control and 1,25-D3-treated cells. Using this methodology we report the isolation of 61 differentially expressed cDNAs. Forty-seven cDNAs correspond to genes already characterized in rat cells or tissues. Seven cDNAs are homologous to yeast, mouse or human genes and seven are not related to known genes. Some of the characterized genes have been reported to be differentially expressed following induction of programmed cell death. These include PMP22/gas3, MGP and β-tubulin. For the first time, we also show a cell death program induced up-regulation of the c-*myc* associated primary response gene CRP, and of the proteasome RN3 subunit and TCTP/mortalin genes. Another interesting feature of this 1,25-D3 induced-cell death program is the down-regulated expression of transcripts for the microtubule motor dynein heavy chain/MAP 1C and of the calcium-binding S100β protein. Finally 15 upregulated cDNAs encode ribosomal proteins suggesting a possible involvement of the translational apparatus in this cell program. Alternatively, these ribosomal protein genes could be up-regulated in response to altered rates of cellular metabolism, as has been demonstrated for most of the other isolated genes which encode proteins involved in metabolic pathways. Thus, this study presents to our knowledge the first characterization of genes which are differentially expressed during a cell death program induced by 1,25-D3. Therefore, this data provides new information on the fundamental mechanisms which participate in the antineoplastic effects of 1,25-D3 and on the machinery of a cell death program in a glioma cell line.

Keywords: Vitamin D; brain; gene expression; apoptosis; differential screening

Abbreviations: 1,25-D3, 1,25-dihydroxyvitamin D3; PMP22, peripheral myelin protein 22; MGP, matrix gamma carboxyglutamic acid protein; CRP, cysteine-rich protein; TCTP, translationally controlled tumor protein; VDR, vitamin D receptor; ORF, open reading frame; UGG, unknown glioma gene; SPARC, secreted protein acidic and rich in cysteine; ON, osteonectin

Introduction

It is presently established that cell death is most often an active process, triggered by precise signals which induce crucial biochemical changes in target cells, resulting in many instances from changes in the pattern of gene expression. However, the precise molecular mechanisms which promote these cell death programs are poorly understood. They may be expected to vary from one cell type to another, while different stimuli have the potential to induce a cell death program by activating independent or overlapping metabolic pathways in the same cell. Previous studies have demonstrated that some genes play a critical role in the triggering of/or progression to cell death. These include nuclear regulatory factors encoded by the *p53* gene (Lowe *et al*, 1993; Hermeking and Eick 1994) and the proto-oncogene c-*myc* (Evan *et al*, 1992), or genes such as *gadd45*, whose expression is controlled by the *p53* protein (Kastan *et al*, 1992; Carrier *et al*, 1994). However, numerous studies evidenced the role of non-nuclear components, such as members of the *bcl2–bax* gene family (Larsen 1994), proteases belonging to the family of interleukin 1-β converting enzyme (Gargliardini *et al*, 1994), or receptors mediating a death signal, such as the TNFα receptor (Yonehara *et al*, 1989), the low affinity NGF receptor (p75) (Barrett and Bartlett 1994) or the Fas/apo-1 receptor (Trauth *et al*, 1989). Many other gene products were reported to be also involved in this process. These include enzymes or structural proteins participating in second messenger transduction cascades, cell cycle progression, cell shape and housekeeping cytoplasmic mitochondrial functions. It has become clear, therefore, that a cell death program cannot be only described by monitoring the expression of some specific markers, but represent profound changes in cell homeostasis, leading to new metabolic situations that eventually promote cell death.

In order to get an overview of the genetic changes occurring during the accomplishment of a cell death program, we have undertaken an approach based on the identification of genes that are differentially expressed in cells that were either in growth-phase, or committed to die. Such genes are potential candidates that are implicated, directly or not, in the cell death mechanism. This methodology was applied in the case of rat C6 glioma cells, whose death was induced by a 24 h-treatment with

1,25-dihydroxyvitamin D_3 (1,25-D3) (Naveilhan *et al*, 1994; Baudet *et al*, 1996a,b).

1,25-D3 is the most active metabolite of vitamin D. This hormone exerts genomic effects by interacting with a nuclear receptor, referred to as Vitamin D Receptor (VDR), which is structurally related to the receptors of thyroid hormones and retinoic acids (Haussler 1986). 1,25-D3 exerts a tight control on calcemia and bone metabolism. However, the hormone also acts on the immune system. It controls the production of several lymphokines, inhibits T cell proliferation, promotes macrophage differentiation, and has *in vivo* an overall immunosuppressive effect (for review see Thomasset 1994). A growing body of evidence indicates that 1,25-D3 is also active in the central nervous system (CNS). Binding sites of 1,25-D3 were detected in certain neurons and non-neuronal cells (Stumpf and O'Brien 1987, Stumpf *et al*, 1992; Musiol *et al*, 1992), while the mRNA of its receptor, VDR, was identified in the human hippocampus (Sutherland *et al*, 1992) and *in vitro*, in rat primary astrocytes (Neveu *et al*, 1994a). Astrocytes constitute a target for the hormone. They respond to 1,25-D3 by an enhanced production of neurotrophic factors such as NGF (Neveu *et al*, 1994a) or neurotrophin 3 (Neveu *et al*, 1994b). Furthermore, 1,25-D3 enhances the responsiveness of these cells by increasing the expression of the VDR gene. The hormone also promotes its own catabolism by inducing a gene encoding vitamin D-24-hydroxylase (Naveilhan *et al*, 1993). Astrocytes tolerate high concentrations of 1,25-D3, even when cultured in a serum-free medium, that is in the absence of serum vitamin D-binding protein which should limit the concentration of free hormone. This is not the case of malignant C6 glioma cells. Treatment of these cells during 24 h with 10^{-8}–10^{-7} M, 1,25-D3 results 1 week later in the death of a part of the population, provided that cells are maintained in a chemically defined medium (Naveilhan *et al*, 1994). This cytotoxic effect of 1,25-D3 appeared more or less pronounced, depending on the origin of the C6 cells, a fact which is likely to reflect strain variations, observed from laboratory to laboratory on the basis of other criteria (Röser *et al*, 1991; Gubits *et al*, 1992). Therefore, C6 cells were subcloned and a subclone, referred to as C6.9, was selected on the basis of its high susceptibility to the cytotoxic action of 1,25-D3 (Baudet *et al*, 1996a). In C6.9 cells, 1,25-D3 treatment induces accumulation of mRNAs of c-*myc*, *p53* and *gadd45* genes (Baudet *et al*, 1996b). Furthermore, cells are largely protected against the toxic action of 1,25-D3 if they are treated with cycloheximide (Baudet *et al*, 1996b). These data indicate that 1,25-D3 cytotoxicity corresponds to a cell death program, that requires protein synthesis. The interest of this experimental system is strengthened by the fact that glioma form evolutive tumors which are generally fatal in human (for review see Janus *et al*, 1992). Therefore, C6.9 cells appeared adequate to undertake a characterization of genes that are differentially expressed in treated and untreated populations. For this purpose, we have screened an organized library of rat brain cDNA, with complex probes prepared from mRNAs extracted from growing C6.9 cells, and from cells collected 3 days after a 24 h treatment with 1,25-D3. An advantage of organized libraries is that they can be reused, and this permits an overview of a panel of genes whose expression may be compared under different experimental conditions. The present work describes data obtained following the screening of 7680 clones. About 90 of these were found to correspond to mRNAs whose levels do either increase, or decrease, following C6.9 cell treatment with 1,25-D3.

Results and Discussion

Most of the substractive hybridizations or differential screenings performed to characterize genes involved in cell death have been designed to isolate genes specifically expressed during this process. The reasonable assumption that a cell death program is directly controlled by the induction of several genes which are not expressed or expressed at very low levels in exponentially growing cells should not, however, exclude the possibility that cell death might ultimately result from a dysregulation in the expression of some housekeeping genes. The possibility that subtle variations in the levels of expression of this kind of genes could be an important aspect of a cell death program has retained until now little attention. As a first attempt to characterize genes expressed in normal non-apoptotic tissues whose expression is altered during the course of a cell death program, we used a cDNA library derived from Poly(A)$^+$ RNA extracted from adult rat brain. The screening of this library was performed with two complex ^{32}P-labeled cDNAs probes prepared from Poly(A)$^+$ RNA isolated from C6.9 cells 3 days after a treatment of 24 h with either 1,25-D3 or its vehicle. This time point was chosen to allow the detection of changes in RNA expression occurring well before the death of cells, which takes place around day 6 (Baudet *et al*, 1996a), and because a peak in the expression of c-*myc*, *p53*, *gadd45*, IL-6 and VEGF in 1,25-D3-treated C6.9 cells has been recently reported to occur at day 3 (Baudet *et al*, 1996b).

Characterization of the differentially expressed genes

7680 organized clones were screened. In most instances, spot intensity associated with a same clone remained constant. However, signal intensity turned out to diverge after normalization of the spot intensity, by a factor of at least 1.5-fold in 88 clones. These clones were subjected to a confirmatory step based on a Northern blot analysis using total RNA extracted at day 3 from C6.9 cells treated for 24 h with 1,25-D3 or vehicle alone. Thus, Northern blots have been serially hybridized with the 88 plasmids purified from the selected bacterial clones. An example illustrating these different steps of analysis is presented in Figure 1. Figure 1A focuses on a spot (01.11-06) obtained following the screening of the library with complex probes prepared from control (a) or treated (b) cells. Hybridizations with the cloning vector to control bacterial growth are in (c) and (d). The computer analysis of these autoradiograms is presented in Figure 1B. The underexpression of the gene corresponding to clone 01.11-06 (Figure 1A), identified according to its DNA sequence as being osteonectin, was further confirmed by

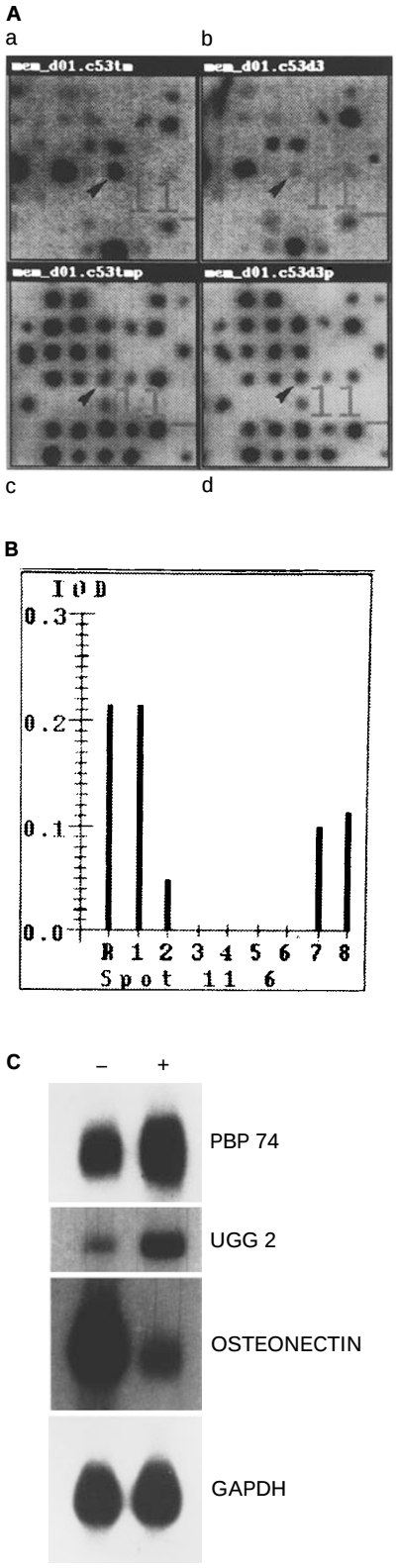

Figure 1 Overview of the different steps of analysis: selection of the spot 01.11-06 which represents the Osteonectin gene. (**A**) Autoradiogram of one of the nitrocellulose membrane containing a part of the cDNA organized library obtained after the labelling with complex probes prepared from control C6.9 cells (a) or 1,25-D3 treated cells (b). Arrowhead indicates the spot 01.11-06 which corresponds to a gene differentially expressed. (c) and (d) represent respectively the same membranes labelled with the plasmid alone as control of the bacterial clone growth. (**B**) Computer densitometric analysis of the spot 01.11-06 presented in **A**. R and 1 represent the relative density of the spot 01.11-06 pointed out in **A**(a). 2 represents the relative density of the spot 01.11-06 pointed out in **A**(b). 7 and 8 represent the relative density of the spots pointed out respectively in **A**(c) and **A**(d). (**C**) Northern blot analysis of the mRNA transcript modulations 3 days after a 24 h treatment with 1,25-D3 (+) or with vehicle alone (−) and serially labelled with three of the cDNA isolated in the course of this differential screening. These correspond to Osteonectin (01.11-06), UGG2 (01.15-17), PBP74 (02.48-14) and GAPDH probes

Northern blot analysis (Figure 1C). Northern blot analyses concerning two other genes, which are this time up-regulated in the course of this cell death program, are also presented in Figure 1C. These results were obtained with clones 02.48-18 and 01.15-17, which correspond respectively to a protein involved in cellular senescence called PBP74 or mortalin, and to a novel sequence called UGG2 for Unknown Glioma Gene 2. Using this experimental approach, we were able to select 61 clones encoding sequences corresponding to mRNAs which are effectively regulated during the 1,25-D3-induced cell death program of C6.9 cells. The 17 remaining clones, for which the subsequent Northern blot analyses revealed a different regulation than that expected on the basis of the initial differential screening, were discarded.

Overview of the differentially expressed genes

According to the computer-assisted sequence comparison, these 61 clones can be subdivided into three classes (see also Table 1).

1 Forty-seven clones (approximately 77%) carry inserts with known sequences already detected in rat cells or tissues. It is noteworthy that the length of these inserts is often equal or even longer in 5′ than that already described.
2 Seven clones (approximately 11.5%) carry inserts whose sequences are homologous to mouse, human or yeast genes.
3 Seven clones (approximately 11.5%) carry inserts with novel sequences, including 3 cDNAs containing a potential Open Reading Frame (ORF).

Inserts carried by the 61 clones corresponding to RNA differentially expressed were partially sequenced, and sequences were compared to the GENBANK/EMBL sequence databases. Because of the redundancy of several sequences, the number of different cDNAs isolated after this differential screening was reduced to 45. An overview of these 45 cDNAs is presented in Tables 2 and 3 where they are classified as 'genes induced' (Table 2a), 'genes repressed' (Table 2b), 'homologous sequences induced' (Table 3a), 'homologous sequence repressed' (Table 3b) and 'unknown sequences induced' (Table 4). In addition, data presented in this table give the name of the corresponding genes or proteins, the lengths of each insert (bp), the factor of induction/repression of the corresponding mRNA assessed by Northern blot analysis (d°), and the ACCESS NUMBER in the GENBANK/EMBL sequence or the SWISSPROT/PIR databases.

Table 1 Summary of the results obtained after the differential screening of the adult rat brain cDNA library and the Northern blot analysis

Selected clones after differential screening	88
• Selected clones after Northern Blot analysis	61
• Known sequences	47
• Homologous sequences	7
• Unknown sequences	7
• unknown, with potential ORF	3
• unknown, without ORF	4

Cellular roles of the differentially expressed genes

Another way to present the results of this differential screening is to classify the identified genes with respect to their cellular role. A first attempt of classification is presented in Table 5. In this table genes are grouped into the following six broad categories of biological roles: (1) Cell signalling/Cell communication, (2) Cell structure/Mobility, (3) Cell/Organism defense, (4) Gene/protein expression, (5) Metabolism, and (6) Unclassified. This table does not address to the specific involvements of the different cloned genes in the C6.9 cell death program, but rather gives an overview of the genetic changes, affecting all the general cell functions, which occur in the course of this process. Therefore the discussion has been focused here on the regulation of a dozen of these genes and on the increased expression of ribosomal protein genes.

Osteonectin/SPARC and dynein heavy chain/MAP 1C genes

One of the aims of this work was to try to characterize essential house-keeping gene(s), such as those encoding for vital enzymatic reactions, whose expression could be dramatically decreased in the onset of a cell death program. Among the genes characterized here, three genes are down regulated by more than a factor of five. They encode for Peripheral Myelin Protein (PMP22/gas3) (Spreyer *et al*, 1991), osteonectin/SPARC (Mason *et al*, 1986) and Dynein Heavy Chain/MAP 1C (Mikami *et al*, 1993). The observed down-regulation of osteonectin/SPARC and dynein heavy chain/MAP 1C genes was rather unexpected.

Osteonectin (ON) also known as basement membrane protein (BM40) or Secreted Protein Acidic and Rich in Cysteine (SPARC) displays a high degree of interspecies sequence conservation (Termine *et al*, 1981; Mann *et al*, 1987; Sage *et al*, 1984). It was initially discovered in mineralized tissues (Young *et al*, 1986) and its expression was recently demonstrated in adult rat brain (Mendis *et al*, 1995). The cellular functions of ON/SPARC include effects on matrix deposition and on cell proliferation. An important feature of ON/SPARC is its ability to abrogate the interactions of cells with cytokines such as PDGF or bFGF (reviewed by Lane and Sage 1994). Therefore, the consequence of the down-regulation of ON/SPARC observed here would be to increase cell responsiveness to cytokines. This suggests that the decreased expression of ON/SPARC could be, together with the induction of VEGF and IL-6 expressions (Baudet *et al*, 1996b), a part of the mechanisms observed under stressful conditions to enhance cell viability.

On the other hand, the down-regulation of the Dynein Heavy CHain/MAP 1C (Mikami *et al*, 1993) gene could be directly connected to the mechanisms involved in the course of apoptosis. The Dynein Heavy Chain/MAP 1C encodes the heavy chain of a molecular complex called dynein that has been found to be responsible of intracelular movements associated with microtubules. These include transport of endosomes, lysosomes and the elements of the Golgi apparatus (for review see Vallee 1993; Barton and Goldstein 1996). Its localization to kinetochores is also suggestive of a role in the movement of the chromosomes to the pole. In addition, evidences have been presented demonstrating that dynein plays a role in mitotic spindle formation (Vaisberg *et al*, 1993). Therefore, in view of these functions, this gene fulfills the criteria of a house-keeping gene whose down-regulated expression could induce lethality. This assumption was further reinforced by the fact that loss of function of the dynein light chain has been reported to cause widespread apoptotic cell death in *Drosophila melanogaster* (Dick *et al*, 1996). Experiments with antisense oligonucleotides could help to clarify the effect of a decrease in dynein activity on cell viability. An intriguing point will be to determine whether a reduction of this microtubule motor could be involved in the mechanisms responsible of the margination of clumped nuclear chromatin observed in apoptotic cell.

Matrix Gla protein, β-tubulin, TCP-1-delta, PMP22/gas 3 and PBP74/mortalin genes

Matrix Gla protein (MGP), β-tubulin and PMP22/gas3 correspond to genes already described in other experimental systems to be over-expressed during a cell death program. However, it should be pointed out that PMP22/gas3, which has been associated with the induction of apoptosis in Schwann cells (Fabbretti *et al*, 1995), is down-regulated here. Interestingly, the underexpression of the PMP22/gas3 gene is known to increase the proportion of cells that enter the S+G2/M phases (Zoidl *et al*, 1995), a situation recently found to occur during the course of the cell death program studied here (Baudet *et al*, 1996b).

Matrix Gla (gamma carboxyglutamic acid) Protein (MGP) (Price *et al*, 1987) is a Vitamin K dependent protein and serves as a substrate for the enzyme γ-carboxylase which converts glutamic acid to a γ-carboxyglutamic acid (Price *et al*, 1987). MGP mRNA has been detected in many tissues including brain (Fraser and Price 1988; Hale *et al*, 1988; Price *et al*, 1987; Rice and Price 1994) and the MGP protein is secreted by a large variety of cells in culture (Rannels *et al*, 1993; Fraser and Price 1988; Hale *et al*, 1988). This gene is induced in rat prostate following castration, which causes apoptosis of androgen-dependent prostate cells (Briehl and Miesfeld 1991).

One of the selected cDNA clones encodes the rat β-tubulin (Ginzburg *et al*, 1980). This up-regulation of β-tubulin can be correlated to the enhanced expression of TCP-1-delta (clone 03.25-13) since this latter protein is a

Tables 2 and 3 Characterization of the analyzed cDNA clones selected after the differential screening of 7680 of the adult rat brain library. The data represent the name of the genes induced (2a), repressed (2b), and the homologous sequences induced (3a), repressed (3b) corresponding to the differentially expressed cDNA clones

TABLE 2:
a. *Genes induced*

Clone	Frequency	Size of insert (bp)	Putative sequence	d°	Access Number
04.40-05	1	1000	Ribosomal protein S2	+1.5	RO:RRRPS2
03.31-27	1	500	Ribosomal protein S6	+2	RO:RATRPS
02.03-22	2	800	Ribosomal protein S8	+2	RO:RNRPS8
01.34-08	1	700	Ribosomal protein S9	+4.7	RO:RNRPS9
02.10-17	1	600	Ribosomal protein S12	+2.4	RO:RATRPS12
01.40-08	1	600	Ribosomal protein S17	+3.6	RO:RATRPS17
03.35-15	1	600	Ribosomal protein S18	+2.7	RO:RRRPS18A
01.22-21	3	600	Ribosomal protein S25	+2	RO:RRRPS25
03.35-27	4	800	Ribosomal protein L9	+1.9	RO:RRRPL9
03.08-06	1	1000	Ribosomal protein L13a	+1.7	RO:RNRPL13A
01.43-19	1	650	Ribosomal protein L23	+1.5	RO:RRRPL23A
04.36-07	1	600	Ribosomal protein L26	+2.3	RO:RRRPL26
01.05-26	1	560	Ribosomal protein L44	+2	SW:RL44_RAT
03.29-05	1	500	Ribosomal phosphoprotein P1	+1.5	RO:RRRP1
02.06-05	1	500	Ribosomal phosphoprotein P2	+1.6	RO:RRRPP2
02.16-11	1	2500	28S RNA	+2	RNRRNA04
02.29-12	4	1300	Mitochondrial gene 16S RNA	+2.5	MIRNXX
03.37-13	1	850	Proteasome RN3 subunit	+3.3	RO:RATRN3
01.46-28	2	1700	Cysteine-rich protein	+7.1	RO:RNU09567
03.10-29	2	600	Transthyretin	+2.5	RO:RNTTHY
01.22-09	1	1300	Rap 1B	+2.7	RO:RNU07795
03.39-27	2	600	Matrix Gla Protein	+3.8	RO:RATMGP
02.33-10	1	1200	β-Tubulin	+4.3	RO:X03369
02.07-05	1	1100	Phosphoglycerate mutase type B	+2.5	RO:S63233
02.34-24	1	1800	Cytosolic Aspartate Aminotransferase	+2.3	RATCASPAT
02.40-24	1	1100	Cytochrome C Oxydase subunit I	+1.5	RO:S79304
b. *Genes repressed*					
01.10-17	1	1200	Peripheral Myelin Protein 22	−6.8	SW:PM22_RAT
01.11-06	4	1300	Osteonectin	−7.7	SW:SPRC_RAT
01.11-01	1	1400	Dynein Heavy Chain MAP 1C	−5.6	SW:DYHC_RAT
03.30-07	1	1400	Aldolase C	−1.5	SW:ALFC_RAT
02.18-27	2	1800	β subunit s-100 Protein	−3.5	RO:RATs100b

TABLE 3:
a. *Homologous sequences induced*

Clone	Frequency	Size of insert (bp)	Putative sequence	Identity	d°	Access Number
03.25-13	1	1300	TCP-1-Delta	100% mouse	+1.6	RO:MUSCHAP
02.42-23	1	1500	KOX30	90% human	+2	PR:HSKOX30
02.48-14	1	1000	PBP74	100% mouse	+2.4	RO:MUSPBP74
02.30-19	1	950	Proteolipid Protein A1	52% yeast	+1.8	SP:PPA1_YEAST
03.22-18	1	900	Translationally Controlled Tumor Protein	100% mouse	+2.3	RO:MML21KD1
b. *Homologous sequences repressed*						
02.13-21	2	1000	Stearoyl-CoA desaturase	90% mouse	−4.4	RO:MUSSCD2
01.26-20	2	2000	Probable E1-E2 ATPase YIL048W	52% yeast	−1.7	SW:YIE8 YEAST

Frequency indicates the number of cDNA clones which encodes the corresponding sequence. d° is the fold of increase (+) or decrease (−) in comparison with the control after densitometric analysis of the Northern blot autoradiogramms. Northern blot analyses have been verified in two different experiments and correlate with the results of the differential screening. ACCESS NUMBER: Number of access in the GENBANK/EMBL sequence or SWISSPROT/PIR databases

member of cytoplasmic chaperonins, which supports the correct folding of β-actin and tubulin (Gao *et al*, 1992; Yaffe *et al*, 1992). It is known that the relative β-tubulin immunofluorescence, as measured by FCM in human leukemic T-cell line CCRF-CEM treated by a cytotoxic drug, increased in apoptotic populations compared to untreated cells (Pittman *et al*, 1994). It has also been recently found that in *S. cerevisae*, genetic configurations resulting in an increase in the ratio of β-tubulin to α-tubulin, causes microtubule disassembly and cell death (Archer *et al*, 1995). It remains now to determine whether the increased expression of β-tubulin observed here affects the ratio of the microtubule constituents. Finally the classification of PBP74, also called mortalin, with this set of genes is motivated by its involvement in the determination of cellular senescence (Wadhwa *et al*, 1993).

Table 4 Sequencing analysis and Northern blot analysis of inserts with unknown nucleotide sequences. d° is the fold of increase (+) in comparison with the control after densitometric analysis of the Northern blot autoradiogramms. Northern blot analyses have been verified in two different experiments and correlate with the results of the differential screening

Clone	Particularity	Insert (bp)	d°	mRNA (kb)
Unknown sequences				
UGG1 (01.14-23)	EST	1200	+2.4	3.5
UGG2 (01.15-17)	None	700	+5.5	10
UGG3 (02.03-12)	None	2400	+6.3	1.2
UGG4 (02.22-12)	ORF 52 AA	1000	+2.6	1.2
UGG5 (02.33-08)	ORF 97 AA and signal peptide	2000	+2.2	>10
UGG6 (02.38-23)	ORF 63 AA	1100	+2.1	1.5
UGG7 (03.45-21)	EST	1100	+2.1	1.4/1.8

Table 5 List of the identified genes classified with respect to their cellular role

Genes related to	Identification
Cell signalling/ Cell communication	Transthyretin RAP1B
Cell structure/ Motility	PMP22 β-Tubulin Dynein Heavy Chain MAP1C Matrix Gla Protein Osteonectin Proteolipid protein A1
Cell/Organism defense	PBP74
Gene/Protein expression	Cysteine-rich protein TCP-1-delta Proteasome RN3 subunit Ribosomal proteins (S2, S6, S8, S9, S12, S17, S18, S25, L9, L13a, L23, L26, L44) Ribosomal phosphoproteins (P1, P2) 28S RNA Mitochondrial gene 16S RNA
Metabolism	Cytosolic Aspartate Aminotransferase Cytochrome C Oxydase subunit I Stearoyl CoA Desaturase Aldolase C Phosphoglycerate mutase B Probable E1-E2 ATPase YIL048W
Unclassified	β-subunit S-100 protein TCTP

Cysteine-rich protein and proteasome RN3 subunit genes

These two genes, which have not been associated until now with cell death, are up-regulated by more than a factor of three in C6.9 cells committed to die.

Cysteine-rich protein (CRP) gene encodes a member of a protein superfamily containing a double zinc finger-like motif. The expression of this gene is developmentally regulated in rat brain (McLaughlin *et al*, 1994), but its biological function is not yet understood. In human quiescent fibroblasts, CRP has been shown to be a primary response gene displaying coordinate serum induction with c-*myc* (Wang *et al*, 1992). This finding is interesting in light of recent work showing that c-*myc* gene is overexpressed at day 3 in C6.9 treated with 1,25-D3 (Baudet *et al*, 1996a,b). It is noteworthy that c-*myc* expression may induce apoptosis when combined with a block of cell proliferation (Evan *et al*, 1992). Therefore, data presented here raise the possibility that the coordinated expression of CRP and c-*myc* genes in C6.9 cells cultured in serum-free medium could be directly involved in the cell death program.

cDNA clone 03.37-13 specifies proteasome RN3 subunit (Thomson *et al*, 1993). This protein is one of the numerous components of rat proteasomes, which degrades proteins conjugated to ubiquitine in an ATP-dependent mode (Waxmann *et al*, 1987; Hough *et al*, 1987). This proteasome plays a major role in non lysosomal pathways of protein turnover (Goldberg and Rock 1992). Experimental evidences implicate this proteolytic pathway in the degradation of mitotic cyclins (Deshaies *et al*, 1995), oncoproteins as c-Myc (Ciechanover *et al*, 1991), or the tumor suppressor protein p53 (Scheffner *et al*, 1990). It is noteworthy, in this respect, that an overall increase in the amounts of the 26S proteasome complex takes place during programmed cell death (Dawson *et al*, 1995). This suggests that an increased expression of this gene could be detected in the course of other cell death programs.

Ribosomal protein and translationally controlled tumor protein genes (TCTP)

An important feature of this differential screening is the high number of clones encoding ribosomal proteins, which account for about one third of all selected cDNAs. The various ribosomal protein genes are widely dispersed in the genome. They are transcribed at very similar rates owing to the equivalent strenghts of their promoters (Hariharan *et al*, 1989), and a coordinate regulation, at various levels of gene expression, operates to maintain the proper stoichiometry of the ribosomal components (Mager, 1988). In yeast, most of the ribosomal protein gene promoters contain one or two sites for a global regulator, RAP1p (Klein and Struhl 1994; Kraakman *et al*, 1993), and the transcription of ribosomal proteins is coordinated with rRNA synthesis (Warner, 1989). Conserved motifs where also found in chicken (Maeda *et al*, 1993) or mouse (Genuario *et al*, 1993) ribosomal protein genes, and a coregulation of a set of ribosomal proteins and rRNA was already described in rat liver after a glucocorticoid stimulation (Flusser *et al*, 1989). As we know that these genes are very conserved throughout species, the existence of such a global regulator or common motif of regulation probably exists in the rat genome. The overexpression of several ribosomal protein genes can be compared to the up-regulation of TCTP, another gene isolated in the course of this differential screening, since this gene shares a feature observed on the 5′ terminus of the transcripts of some mouse ribosomal protein genes, a run of Ts followed by a GC-rich segment (Chitpatima *et al*, 1988; Wagner and Perry 1985). This finding raises the possibility that this conserved sequence could be a target for a transcriptional factor induced during this cell death program. Electrophoretic mobility shifts assays experiments will help to clarify this

point. Another intriguing feature of TCTP mRNA is that it occurs as untranslated mRNP particles unable to interact with the translation machinery in mouse tumor cell line, in spite of an open reading frame encoding a protein of 172 amino acids (Chitpatima *et al*, 1988).

It is presently not clear whether the differential expression of several ribosomal protein genes plays an active role in this cell death program, or if it only reflects the metabolic disturbance occuring during this cell death process. As outlined up-there, the assembly of functional ribosomes requires precise stochiometries and coordinate expression of more than 80 genes. Therefore, any dysfunction of ribosome assembly could theorectically lead to formation of aberrant and toxic particles. Interestingly, this overexpression of several ribosomal protein genes is not observed in astrocytes treated with 1,25-D3, which have been previously reported to respond to 1,25-D3 by the induction of several genes including VDR, but fail to induce a cell death program (data not shown). The involvement of the translational machinery in cell death has been limited until now to the synthesis of novel proteins at the onset of apoptosis. Nevertheless, our results suggest that a possible link between ribosomes and cell death could exist. Consistent with this hypothesis is the existence of ribonucleoprotein complexes composed of 5S RNA and ribosomal L5, mdm2 and p53 proteins, whose function is however still unknown (Marechal *et al*, 1994).

Unknown sequences (Table 5)

Sequence analysis of seven clones did not reveal known or homologous sequences. They were then called Unknown Glioma Gene (UGG). Three of them present a putative Open Reading Frame (ORF) which could suggest the translation of polypeptides composed of at least 52 amino-acids for the clone UGG4, 63 amino-acids for the clone UGG6, and 97 amino-acids for the clone UGG5. The finding of a putative signal peptide suggests that the potential protein corresponding to the clone UGG5 could be secreted.

Two other unknown sequences have already been indexed in the Gene Databank as human EST after a randomly and automatized sequencing. The clone UGG1 was reported to be a non-codant sequence and the clone UGG7 has not been further analyzed.

The extensive study of these genes will be of great interest to make up the synthetic scheme proposed in Figure 2.

In spite of active research, little is known on the biochemistry and on the genetics which control cell death. In a preliminary approach to solve the puzzle of a cell death program in a rat glioma cell line, we have characterized 61 genes whose expression is differently regulated in the course of this process. In a previous work, we reported that this process was also associated with increased expression of genes such as c-*myc*, *p53*, *gadd45*, VEGF and IL6 (Baudet *et al*, 1996b). A synthetic picture of the possible interaction of these corresponding proteins in the course of the 1,25-D3-induced-C6.9 cell death program is presented in Figure 2. However, it is important to mention that all the data of this differential screening concern mRNA levels and do not account for possible translational or post-translational events. Several differentially expressed genes which have not been extensively discussed here have been reported there. These include Rap1B, which belongs to the Ras family, and S100β and aldolase C genes. An interesting feature concerning these two later genes, which are down-regulated here, is that their corresponding proteins interact together (Zimmer and Van Eldick 1986). In addition, disruption of the microtubular cytoskeleton is known to cause a specific reduction in the level of S100 protein mRNA in C6 cells (Dunn *et al*, 1987). S100 protein is a low molecular weight, Ca^{2+}-binding protein (Pritchard and Marston 1991), then a reduction of S100β mRNA could result in a decreased calcium-buffering potential of the cells, and increased susceptibility to the excessive calcium loading, often reported to occur during programmed cell death. A major task will be now to determine how all these variations in gene expression are integrated in a common cell death mechanism. In this regard, the possibility exists that, in view of the biological importance of cell death, several distinct and independent programs, each of them sufficient to induce cell death, could be active simultaneously in the same cell during a given event of cell death program. This could further complicate our ability to elucidate the individual components of a specific cell death program event.

Materials and Methods

Cell culture

Clone C6.9 used in this study was isolated from the rat glioma C6 cell line (Benda *et al*, 1969; Baudet *et al*, 1996a) by the method of limiting dilution in microtiter plates. The microtiter plates were screened for wells containing single cells 24 h after seeding. Cells were maintained in F12 medium supplemented with 10% fetal calf serum and were used between passages 5–20. For the experiments, cells were rinsed once with PBS and incubated for 24 h in a serum-free medium consisting of F12 medium supplemented with insulin (2.5 μg/ml), transferrin (2.5 μg/ml) and selenium (2.5 ng/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Then, 1,25-D_3 was added to the cultures at 5×10^{-8} M and left during 24 h. At the end of this incubation period, media were replaced by a serum-free medium devoided of 1,25-D_3. Control cultures were treated with vehicle alone (ethanol).

Preparation of complex cDNA probes for differential screening

Total RNA was extracted following the LiCl/urea method (Auffray and Rougeon 1980) from C6.9 cells harvested 3 days after a 24 h treatment with 5×10^{-8} M of 1,25-D3 or ethanol, and submitted to chromatography on oligo(dT)-cellulose (Aviv and Leder 1972). Two cDNA complex probes were simultaneously prepared from 1 μg of poly(A)-rich mRNAs using oligo(dT)12-Apa1 primer adapter, in presence of [^{32}P]dCTP (with a specific activity of 3000 d.p.m./ng cDNA) and SuperScript II reverse transcriptase (B.R.L., Charbonnière, France). cDNAs obtained were precisely quantified and cDNA patterns verified on denaturing gels. Then, the two cDNA probes corresponding to C6.9 cells treated or not with 1,25-D3 were labeled with high specific

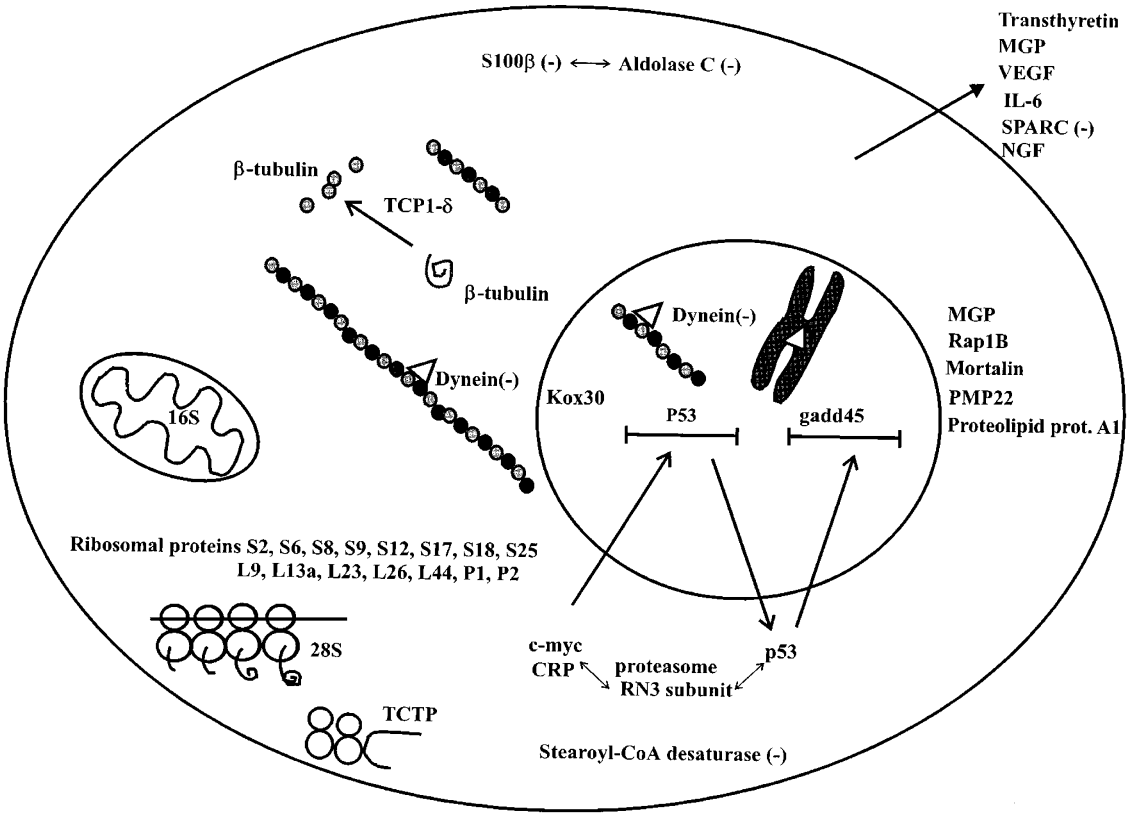

Figure 2 Schematic representation of the localization and of the possible interactions between the proteins corresponding to genes differentially expressed during C6.9 cell death program. ↔ illustrates physical interactions

radioactivity using the random primer DNA Labelling System for BRL (2×10^9 d.p.m./μg). Non-incorporated nucleotides were separated by two successive chromatographic steps on a Biogel P10 (BioRad, Ivry sur Seine, France) column.

cDNA library and high density filters

The precise procedure is described in Perret *et al* (submitted for publication). Briefly, the cDNA library was prepared from rat brain mRNA followig the primer adapter method described by Caput *et al* (1986). The cDNA library was constructed using the cloning vector pT7T3 18 (Pharmacia) and transformed by electroporation in highly competent DH5α bacteria (10^{10} c.f.u./mg DNA). The cDNA library was organised in plates of 1536 well-dishes using a modified fluorescence activated cell sorter (FACS IV Becton Dickinson). After bacterial growth, each plate was replicated onto several identical nylon membranes (Filter Pall) with a Biomek 1000 workstation. After overnight incubation at 37°C, bacteria on membranes were lysed and treated as described by Nizetic *et al* (1991).

Hybridization and differential screening analysis

Two replicas of five 1536 well-dishes were hybridized for 48 h at 42°C with complex probes prepared from C6.9 cells treated or not with 1,25-D3. For each filter 50 ng of the probe labeled as described above was added to 5 ml of hybridization mix (formamide 50%, Denhardts 5×, SDS 0.1%, 6×SSC, DNAss 100 μg/ml). After hybridization, filters were washed once in 2×SCC, 0.1% SDS at room temperature and then successively in 0.1×SSC, 0.1 SDS at 42°C for 30 min and 65°C for 60 min.

The hybridized filters were revealed in a phosphorimager screen system (Molecular dynamics), the files were transferred to SUN Sparc 10 workstation and comparisons were performed using BioImage program (Millipore, USA).

Then, each filter was re-hybridized by a radiolabelled oligonucleotide recognizing pT7T3 18 vector to determine the quantity of plasmid on filters. The normalisation of the spot intensity by assaying plasmid quantity permitted to eliminate artefactual variations.

DNA sequencing and computer analysis

For each of the selected clones, around 300–500 bp were sequenced using a dyed terminator sequencing kit from Applied Biosystem (ref: 402122). The sequences thus obtained, corresponding to the 5′ ends of the coding strands of the inserted cDNAs, were compared with the whole set of known nucleic sequences (GENBANK/EMBL sequence databases) in order to ascertain whether they are already known or if they are homologous or related to known sequences.

For each unknown sequence, the presence of an Open Reading Frame (ORF) was investigated and the longest ORF was compared with the whole set of protein sequences stored in SWISSPROT/PIR databases.

RNA isolation for Northern blot analysis and cDNA probe preparation

Total RNA was extracted following the LiCl/Urea method (Auffray and Rougeon 1980) from C6.9 cells treated or not with 1,25-D3, as described above. Identical amounts of glyoxal-treated RNA were then subjected to electrophoresis, transferred to a Hybond N membrane (Amersham, Les Ulis, France) and hybridized as previously described (Wion *et al*, 1991) with cDNA inserts carried by the plasmids of each bacterial clone selected in the library. Plasmids were purified according to the plasmid mini-procedure from Promega (Charbonnière, France), and labelled with [^{32}P]dCTP by random priming as described above. Standardization of RNA loading was routinely controlled by hybridization of the blots with a GAPDH cDNA (Fort *et al*, 1985). Blots were subjected to autoradiography, and autoradiograms were analyzed by densitometric tracing. Probes giving a signal differing by a factor of at least 1.5-fold, when hybridized with RNAs extracted from control or 1,25-D3 treated cells, were selected for further analyses.

Evaluation of the length of inserts

Each selected bacterial clone was cultured overnight. Bacteria were lysed under alkaline conditions, and plasmidic DNA was purified with the Qiagen KIT (COGER, Paris, France). Electrophoresis on a 1% analytical gel permitted the estimation of the size of inserts, after a double digestion with two different restriction enzymes: *Hind*III and *Bam*HI, this in comparison with a lane containing a size ladder made of Marker 2 (λ/*Hind*III. *Eco*RI double digestion) from EUROGENTEC (Angers, France).

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