

# Genes Regulated in Neurons Undergoing Transcription-dependent Apoptosis Belong to Signaling Pathways Rather than the Apoptotic Machinery\*<sup>§</sup>

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Neuronal apoptosis has been shown to require *de novo* RNA/protein synthesis. However, very few genes whose expression is necessary for inducing apoptosis have been identified so far. To systematically identify such genes, we have used genome-scale, long oligonucleotide microarrays and characterized the gene expression profile of cerebellar granule neurons in the early phase of apoptosis elicited by KCl deprivation. We identified 368 significantly differentially expressed genes, including most of the genes previously reported to be transcriptionally regulated in this paradigm. In addition, we identified several hundreds of genes whose transcriptional regulation has never been associated with neuronal apoptosis. We used automated Gene Ontology annotation, analysis of promoter sequences, and statistical tools to characterize these regulations. Although differentially expressed genes included some components of the apoptotic machinery, this functional category was not significantly over-represented among regulated genes. On the other hand, categories related to signal transduction were the most significantly over-represented group. This indicates that the apoptotic machinery is mainly constitutive, whereas molecular pathways that lead to the activation of apoptotic components are transcriptionally regulated. In particular, we show for the first time that signaling pathways known to be involved in the control of neuronal survival are regulated at the transcriptional level and not only by post-translational mechanisms. Moreover, our approach provides insights into novel transcription factors and novel mechanisms, such as the unfolded protein response and cell adhesion, that may contribute to the induction of neuronal apoptosis.

From the beginning of its description, apoptosis has been considered as an active process requiring RNA and protein synthesis, especially in neurons. Blockade by macromolecular synthesis inhibitors has long been used as a criterion to estab-

lish the apoptotic nature of a particular cell death (discussed in Ref. 1). Although identification of death receptor-induced pathways that do not require *de novo* protein synthesis has slightly restrained this notion (2), the question of the transcriptional control of apoptosis, the role it plays, and the nature of newly expressed genes still remains. The first studies on the transcriptional regulation of apoptosis were orientated by the concept of a preset rheostat based on the observation that the anti-apoptotic effect of Bcl-2 could be counteracted by pro-apoptotic Bax (3). Many studies were undertaken to examine possible expression variations of these two proteins. In fact, transcription of selective members of the Bcl-2 family was repeatedly reported during different forms of apoptosis, in particular in neurons. These data suggested that differential expression of certain genes may be essential to the initiation of apoptotic neuronal death. However, the number of genes whose differential expression could be essential to the initiation of apoptosis has become difficult to estimate. At least 20 Bcl-2 family members (4) and an increasing number of other components of the core apoptotic machinery (5) have been identified in mammals to date. Moreover, much less is known about early mechanisms leading to the activation of the apoptotic machinery, and they could also be regulated at the transcriptional level. The expression of all these components cannot be simultaneously detected by classical methods used to evaluate mRNA (reverse transcription (RT)<sup>1</sup>-PCR, Northern blotting, and differential display), which can give only a biased view of transcriptional regulations associated with apoptosis. The recent development of genome-scale methods of gene expression analysis now offers new perspectives (6).

Primary cultures of cerebellar granule neurons (CGN) are particularly suited to conduct this kind of study. CGN are one of the best characterized *in vitro* models of neuronal apoptosis (7), which depends on the synthesis of new RNA and proteins (8). Moreover, CGN constitute the most abundant neuronal population in the central nervous system of mammals and can be cultured *in vitro* up to 98% homogeneity. When dissociated from early postnatal mice, CGN can survive and differentiate in culture in the presence of serum and depolarizing levels of extracellular KCl ( $[KCl]_o = 25$  mM) (7). Depolarization is presumed to mimic the endogenous excitatory activity that is required for survival during cerebellar development *in vivo* (9). Lowering  $[KCl]_o$  to 5 mM in the absence of serum triggers apoptosis (8). This presumably mimics the naturally occurring

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains Supplemental Tables 1 and 2.

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<sup>1</sup> The abbreviations used are: RT, reverse transcription; CGN, cerebellar granule neuron(s); FDR, false discovery rate; GO, Gene Ontology; ER, endoplasmic reticulum; UPR, unfolded protein response; PI, phosphatidylinositol.

death that takes place in the external granular layer of newborn rat cerebellum (10).

In this study, we used genome-scale, long oligonucleotide microarrays to discover sets of genes associated with CGN apoptosis induction. We focused data analysis on components of the core apoptotic machinery. We identified several of these genes whose up-regulation has not been previously reported during neuronal apoptosis. However, genes involved in the execution of apoptosis were not significantly over-represented among differentially expressed genes.

#### EXPERIMENTAL PROCEDURES

**Primary Cultures of CGN**—CGN cultures were prepared from 7-day-old murine pups (C57Bl/6J mice, Charles River Laboratories) as described by Miller and Johnson (7) with slight modifications. Briefly, freshly dissected cerebella were incubated for 10 min at 37 °C with 0.25 mg/ml trypsin, and cells were dissociated in Hanks' balanced salt solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the presence of 0.5 mg/ml trypsin inhibitor and 0.1 mg/ml DNase I by several steps of mechanical disruption. The resulting cell suspension was centrifuged and resuspended in basal Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 20 mM KCl to achieve a final concentration of 25 mM. The cell suspension was filtered through a Falcon 40- $\mu\text{m}$  cell strainer and plated in a coated dish for 25 min to allow attachment of non-neuronal cells. Neurons were then resuspended, counted, and seeded at a density of  $25 \times 10^4$  cells/cm<sup>2</sup> in culture dishes previously coated with poly-D-lysine (BD Biosciences). The granule neurons were cultured at 37 °C in a humidified incubator with 6% CO<sub>2</sub> and 94% air for 6 days. To prevent proliferation of remaining non-neuronal cells, 10  $\mu\text{M}$  cytosine  $\beta$ -D-arabino furanoside was added to the culture medium 24 h after plating. At 6 days *in vitro*, granule neurons represented >98% of the cultured cells (data not shown).

**KCl Deprivation, Survival, and Apoptotic Assays**—CGN were washed and incubated for the indicated times in serum-free basal Eagle's medium supplemented with L-Gln, HEPES, antibiotics, and the *N*-methyl-D-aspartate antagonist (+)-MK-801 (1  $\mu\text{M}$ ) and containing either 25 mM KCl (K25 medium) or 5 mM KCl (K5 medium). We chose to use K25 medium as a control instead of the initial culture medium to exclude gene expression differences resulting from serum deprivation. Serum deprivation has been shown to induce the death of a small proportion of cultured CGN (7). However, this neuronal death proceeds mainly through necrosis (11). Moreover, we added 1  $\mu\text{M}$  MK-801 to both the K25 and K5 media to avoid any change in gene expression due to uncontrolled *N*-methyl-D-aspartate receptor stimulation by endogenously released glutamate. Neuronal survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the culture medium was replaced with K25 medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and neurons were returned to the incubator for 2 h. The medium was removed, and blue formazan produced by living cells was solubilized in Me<sub>2</sub>SO. The absorbance of blue formazan was estimated at 570 nm. To assess apoptosis, cytochrome *c* subcellular localization was detected by immunocytochemistry using an antibody from Pharmingen; caspase-3 activation was estimated by immunostaining with an antibody that specifically recognizes cleaved caspase-3 (Cell Signaling Technology); and nuclear condensation was visualized by Hoechst 33258 staining. Immunocytochemistry and Western blotting were performed as described previously (12).

**RNA Preparation and Real-time Quantitative RT-PCR**—Total RNA was extracted using the RNeasy kit and treated with the DNase I from the DNA-free™ kit (Ambion, Inc.) according to the manufacturer's instructions. Poly(A)<sup>+</sup> RNA was isolated from total RNA with Oligotex™ (Qiagen Inc.). Two rounds of hybridization with oligo(dT) beads were carried out successively to improve mRNA purity. RNAs were used to perform a two-step RT-PCR. Briefly, either 1  $\mu\text{g}$  of total RNA or 50 ng of mRNA were reverse-transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of 2.5  $\mu\text{M}$  N6 random primers and 0.5 mM dNTP. Either 10 or 0.5 ng of the resulting cDNA were used as template for real-time PCR using ABI Prism 7000 with the SYBR® Green PCR Master Mix (Applied Biosystems). Primers were designed with Primer Express™ software (Applied Biosystems). The sequences of all the primers used are provided in Supplemental Table 1. PCR was performed in a volume of 10  $\mu\text{l}$  in the presence of 300 nM specific primers. Thermal cycling parameters were 2 min at 50 °C and 10 min at 95 °C, followed by 40

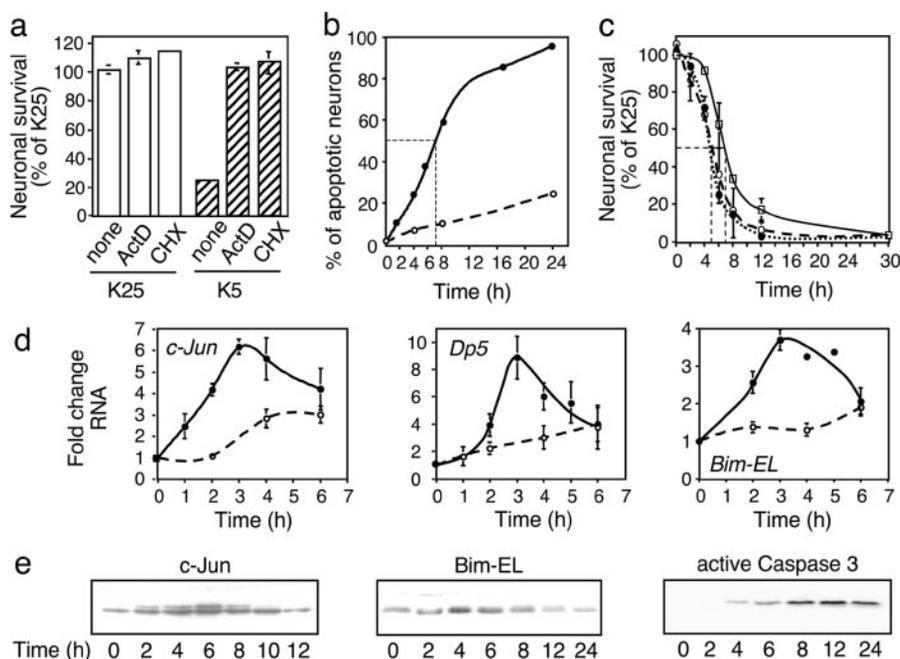
cycles at 95 °C for 15 s and 60 °C for 1 min. Data were analyzed with ABI Prism 7000 SDS software. The relative amounts of specifically amplified cDNAs were calculated with the comparative threshold cycle ( $\Delta\Delta\text{C}_t$ ) method (13). The  $\beta_2$ -microglobulin amplicon was used as a reference.

**Long Oligonucleotide Microarray Fabrication**—The long oligonucleotides of the Mouse Genome Oligo Set Version 2 (Operon) were used. Operon provided the following information about the oligonucleotides. The 16,463 70-mers were designed from representative sequences of the UniGene Database Build Mm.102 (February 2002) and the Mouse Reference Sequence (RefSeq) Database, each oligonucleotide representing a unique gene. An amino linker was attached to the 5'-end of each oligonucleotide. Oligonucleotides were designed to have melting temperatures of  $78 \pm 5$  °C. More than 98% of the oligonucleotides were within 1000 bases from the 3'-end of the available gene sequence. Oligonucleotides were selected to limit secondary structure. BLAST alignments were performed to exclude oligonucleotides that could cross-hybridize with other sequences of the Mouse UniGene Database. Each oligonucleotide had  $\leq 70\%$  of overall identity to any other gene and could not have >20 contiguous bases common to any other gene. Oligonucleotides were dissolved in Tris/EDTA and diluted twice in 2 $\times$  spotting solution (ArrayIt, TeleChem International, Inc.) to a final concentration of 10  $\mu\text{M}$ . Oligonucleotides were spotted using Amersham Generation III Array spotter on aminosilane-coated mirror glass slides (7-Star, Amersham Biosciences). Prior to hybridization, DNA was cross-linked to the slides by UV irradiation and washed twice with 0.2% SDS solution and twice with distilled water.

**cDNA Labeling**—Poly(A)<sup>+</sup> RNA was reverse-transcribed, and cDNA was labeled using the CyScribe cDNA post-labeling kit (Amersham Biosciences) in a two-step procedure according to the manufacturer's protocol. In the first step, aminoallyl-dUTP was incorporated during first-strand cDNA synthesis. A mixture of random nonamers and anchored oligo(dT) was used for priming the synthesis catalyzed by Cy-Script™ reverse transcriptase. For each reaction, 500 ng of mRNA were used as template, and incubation was carried out for 2 h at 42 °C. Following alkaline hydrolysis of the RNA template, the cDNA was purified on QIAquick PCR purification columns (Qiagen Inc.) according to the manufacturer's instructions, except that QIAquick wash buffer was replaced with 5 mM potassium phosphate buffer (pH 8.5) containing 80% ethanol, and cDNA was eluted with 4 mM potassium phosphate buffer (pH 8.5). In the second step, the aminoallyl-modified cDNA was chemically coupled to Cy3 and Cy5 *N*-hydroxysuccinimidyl esters. The coupling reactions were performed separately with Cy3 and Cy5 so that the cDNA derived from neurons incubated in K25 or K5 medium was labeled with a different dye. The coupling reaction was terminated by the addition of hydroxylamine. The fluorescent cDNAs were subsequently purified using the CyScribe GFX purification kit (Amersham Biosciences).

**Dual Color Microarray Hybridization**—Of the cDNA derived from each condition (K5 or K25 medium), one-half was labeled with Cy3 and the other half with Cy5 to perform dye swap hybridizations: K25-Cy3 + K5-Cy5 and K25-Cy5 + K5-Cy3. This enabled us to account for any bias in dye coupling or emission efficiency of Cy dyes. The two labeled cDNA preparations (0.9–1.2  $\mu\text{g}/\text{condition}$ ) were combined together with 10  $\mu\text{g}$  of oligonucleotide A80 and denatured at 95 °C for 5 min. They were subsequently added to microarray hybridization buffer (Amersham Biosciences) and applied to the microarrays in individual chambers of an automated slide processor (Amersham Biosciences). Hybridization was carried out at 37 °C for 12 h. Hybridized slides were washed at 37 °C successively with 1 $\times$  SSC and 0.2% SDS for 10 min, twice with 0.1 $\times$  SSC and 0.2% SDS for 10 min, with 0.1 $\times$  SSC for 1 min, and with isopropyl alcohol before air drying. Microarrays were immediately scanned in both Cy3 and Cy5 channels with an Amersham Generation III Array scanner with 10- $\mu\text{m}$  resolution. ArrayVision™ software (Imaging Research Inc.) was used for image analysis. A local background was calculated for each spot as the median value of the fluorescence intensities of four squares surrounding the spot. This background was subtracted from the foreground fluorescence intensity. Detection limit was calculated as (average + (3  $\times$  S.D.)) of the fluorescence intensities of three different negative controls spotted 12 times along each slide. Intensity values under this detection limit were replaced with the threshold value before any normalization and statistical analysis.

**Microarray Data Analyses**—Fluorescence intensity ratios from each microarray were normalized using the R package SMA (statistical microarray analysis) by the local weight regression (lowess) algorithm called print-tip (14). In this procedure, spotting variation is taken into account, and the normalizing quotient varies with the intensity of the spot. Once the two channels had been normalized for each microarray,



**FIG. 1. Determination of the optimal time for gene expression study.** *a*, CGN were washed and maintained in K25 or K5 medium for 30 h in the presence or absence of 1  $\mu$ g/ml actinomycin D (*ActD*) or 10  $\mu$ g/ml cycloheximide (*CHX*). Neuronal survival was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *b*, the time course of cell death was determined by switching CGN cultures to K5 (solid line) or K25 (dashed line) medium for increasing times and then assaying for the number of apoptotic neurons. Apoptosis was assessed by detection of cytochrome *c* release from mitochondria, activation of caspase-3, and chromatin condensation as described under "Experimental Procedures." The three curves were similar and are represented as one. *c*, to determine commitment points, cultures were switched to K5 medium at time 0. At increasing times after KCl deprivation, K5 medium was replaced with K25 medium (solid line), with K5 medium plus 1  $\mu$ g/ml actinomycin D (dotted line), or with K5 medium supplemented with 10  $\mu$ g/ml cycloheximide (dashed line). Neuronal viability was estimated after 30 h for these three conditions by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results are expressed as a percentage of control cultures maintained in K25 medium from time 0. Data are means  $\pm$  S.D. of triplicate determinations and are representative of three independent experiments. *d*, CGN cultures were switched to either K5 (solid lines) or K25 (dashed lines) medium for increasing times, and total RNA was extracted. mRNA contents for *c-jun*, *DP5*, and *bim-EL* were estimated under each condition by quantitative RT-PCR. -Fold change was calculated by comparison with neurons maintained in initial culture medium (time 0). Data are means  $\pm$  S.D. of three different reverse transcriptions and are representative of three independent experiments. Serum deprivation-induced death (*cf.* *b*) may explain the slight RNA increase observed in K25 medium. *e*, the culture medium was either unchanged (time 0) or replaced with K5 medium for the indicated times. Then, CGN were lysed, and proteins were analyzed by Western blotting with antibodies specific to *c-Jun* (Cell Signaling Technology), Bim (Stressgen Biotechnologies Corp.), and active caspase-3 (Cell Signaling Technology). Although anti-Bim antibody can detect the three Bim isoforms, only Bim-EL is expressed in CGN at a detectable level, as shown previously (19).

normalization was performed between array data by creating a matrix of normalized log intensity ratios across different slides. We then scaled the matrix such that each column had the same median absolute deviation as described by Yang *et al.* (14). Normalized data were analyzed using a variance estimate and a permutation technique known as SAM (significance analysis of microarrays; available at [www-stat.stanford.edu/~tibs/SAM/index.html](http://www-stat.stanford.edu/~tibs/SAM/index.html)) (15). SAM was used with the one-class response and 100 permutation parameters using the *k*-nearest neighbor approach for lost values. The delta value was obtained by fixing the false discovery rate (FDR) for both the median and 95th percentile to 1 or 5%. An FDR of 1% indicates that, on average, 1% of the genes called significantly regulated are not actually regulated (15).

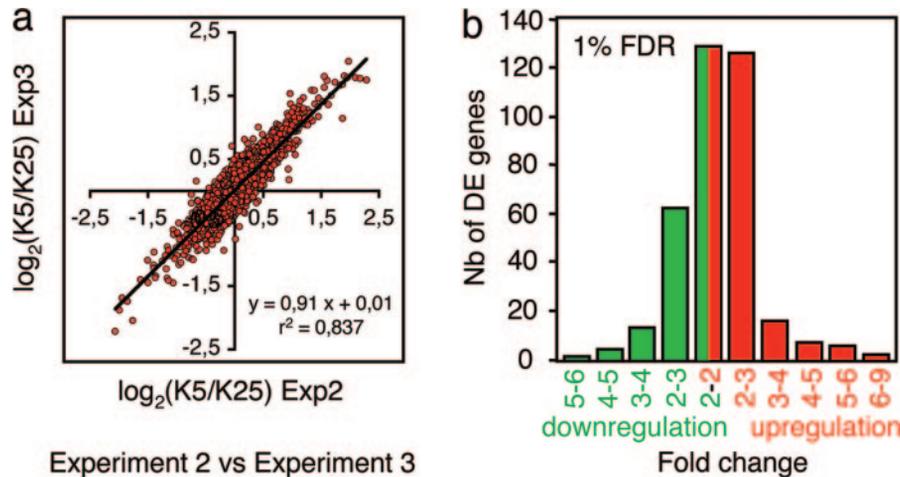
The eGOn Version 1.0 software of the Norwegian Microarray Consortium (available at [www.mikromatrise.no](http://www.mikromatrise.no)) was used to assign Gene Ontology (GO) terms to individual genes from the Oligo Set and the differentially expressed gene list. Annotated genes were then clustered in functional categories defined by the Gene Ontology Consortium (16). To simplify representation, only the third row of GO nodes have been considered, and redundancy was avoided when possible. Despite this, in many cases, single genes were associated with multiple GO identifiers. This reflects the biological reality that a particular protein may function in several processes, may contain domains that carry out diverse molecular functions, and may be active in multiple locations in the cell. This explains why the sum of gene numbers contained in the different categories is often greater than the total number of annotated genes. Statistical tests available on the eGOn Version 1.0 web site were performed to identify categories of genes in which differentially expressed genes were over-represented. A two-sided, one-sample binomial test was implemented, and a *p* value was calculated for each GO term. This test identified terms where the number of differentially expressed genes divided by the number of genes in the Oligo Set was greater than the overall proportion (the total number of differentially expressed

genes divided by the total number of genes in the Oligo Set).

Genomic sequences 1 kb upstream and 200 bp downstream from transcriptional start sites of genes from the Oligo Set and the differentially expressed gene list were retrieved from DBTSS (available at [dbtss.hgc.jp/index.html](http://dbtss.hgc.jp/index.html)). They were analyzed for the presence of consensus DNA-binding sites with Match™ software and the TRANSFAC Database (available at [www.gene-regulation.com/](http://www.gene-regulation.com/)) using high quality matrices to minimize the occurrence of false positive sites. A binomial test, implemented as described above, identified consensus binding sites over-represented in the promoters of differentially expressed genes compared with those of the Oligo Set.

## RESULTS

**Temporal Analysis of CGN Apoptosis**—CGN were completely protected from KCl deprivation-induced apoptosis by the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide (Fig. 1*a*). This supports the notion that *de novo* RNA and protein synthesis is required for neuronal apoptosis. Expression of the genes necessary for triggering apoptosis is likely to precede the point after which neurons are irreversibly committed to death. To determine this point, we performed a series of temporal analyses based on different apoptotic criteria such as cytochrome *c* release from mitochondria, caspase-3 activation, and chromatin condensation. The appearance of these hallmarks was approximately concomitant (Fig. 1*b*). We also determined the commitment points after which half of the neurons could no longer be rescued by actinomycin D, cycloheximide, or re-addition of 25 mM KCl to the medium (Fig. 1*c*) because, at that time, genes necessary to



**FIG. 2. Microarray analysis of CGN undergoing KCl deprivation-induced apoptosis.** *a*, long oligonucleotide microarrays were hybridized with labeled cDNAs derived from CGN incubated in K25 or K5 medium for 4 h. K5/K25 expression ratios were calculated using Cy3 and Cy5 normalized fluorescence intensities and are expressed as  $\log_2$  values. Intensities under the detection threshold were excluded from the analysis. The K5/K25 ratios obtained in one experiment (Experiment 2) are plotted against the K5/K25 ratios from another independent experiment (Experiment 3). Data are representative of three independent experiments. *b*, shown is the distribution of -fold change values of significantly differentially expressed (DE) genes identified by SAM with 1% FDR. -Fold change values ranged from 1.68 to 8.72 for up-regulated genes (red) and from 1.88 to 5.75 for down-regulated genes (green). The bicolor column represents genes that were significantly regulated with a -fold change below 2. It comprises 121 up-regulated genes and nine down-regulated genes. Only genes with a fluorescence intensity above the detection threshold were taken into account. *Nb*, number.

apoptosis induction have already been expressed. Our data were consistent with results reported by others in mouse CGN (17): transcriptional and translational commitment points were  $\sim 5$  h after deprivation. The temporal curves of apoptosis (Fig. 1*b*) and KCl rescue (Fig. 1*c*) indicated that 50% of the neurons were still alive after 7 h. These results suggested that expression of determinant genes preceded first manifestations of apoptosis by  $\sim 2$  h. This was confirmed by the expression time course of genes known to be implicated in the triggering of CGN apoptosis such as *bim-EL*, *DP5*, and *c-jun* (18–20): a peak of mRNA was determined at  $\sim 3$  h after KCl deprivation for the three genes (Fig. 1*d*). Moreover, Western blotting showed a peak of protein levels and phosphorylation of c-Jun after 6 h and a peak of Bim-EL after 4 h, both preceding massive cleavage and activation of caspase-3 (8 h) (Fig. 1*e*). These data prompted us to analyze the gene expression profile of CGN 4 h after KCl withdrawal, this time appearing as optimal to identify genes involved in the induction of neuronal apoptosis.

**Gene Expression Profiling of Apoptotic CGN**—Gene expression profiles of CGN maintained in K25 medium (control) and in K5 medium (apoptosis) for 4 h were compared using high density long oligonucleotide microarrays. Three independent experiments were performed using distinct neuronal cultures. For  $>50\%$  of the oligonucleotides, fluorescence intensities were above the detection threshold, indicating a good sensitivity. Data analysis also revealed good normalization (data not shown) as well as good reproducibility (Fig. 2*a*). SAM statistical analysis (15) was performed using an FDR of 1% to identify genes that were significantly differentially expressed. The analysis showed that 278 genes were significantly up-regulated and that 90 genes were significantly down-regulated in apoptotic neurons compared with controls. -Fold change values distributed as described in the legend to Fig. 2*b*. The regulations detected with microarrays were confirmed for 45 of 48 genes (94%) by real-time RT-PCR (Table I) (data not shown).

**Transcriptional Regulation of Apoptotic Genes**—To determine whether components of the core apoptotic machinery are regulated at the transcriptional level during CGN apoptosis, we examined individual microarray data for a repertoire of apoptotic genes. We used the lists of gene families defined by Reed *et al.* (5), based on evolutionarily conserved domains

shared by proteins involved in apoptosis and NF- $\kappa$ B induction. The expression measurements of the 229 genes contained in these different categories are presented in Supplemental Table 2. Only six genes were found to be significantly up-regulated after SAM performed with 1% FDR (Supplemental Table 2): *DP5*, *bim*, caspase-3, caspase-6, *Unc5h2*, and *Cide-A*. A less stringent SAM performed with 5% FDR identified four additional regulated genes: *Raidd/Cradd*, *mal/Tirap*, *Traf3*, and cytochrome *c* (Supplemental Table 2). All of these gene expression changes were confirmed by quantitative RT-PCR (Table I). However, K5/K25 medium ratios determined by quantitative PCR were too low for *mal/Tirap* ( $1.33 \pm 0.14$ ) and *Traf3* ( $1.72 \pm 0.26$ ) to warrant further analysis. Two additional genes that were not identified as significantly regulated by SAM but with a K5/K25 medium ratio on microarrays above 1.6 were found to be actually up-regulated during CGN apoptosis by real-time PCR: caspase-7 and p73 (Table I). In addition, *fasL* up-regulation that was not detected on microarrays but that had been previously reported during CGN apoptosis (21, 22) was found by quantitative PCR (Table I). Overall, only 11 genes of the 229 gene-containing list of the apoptotic machinery were found to be significantly regulated during CGN apoptosis.

In addition, most of the gene expression changes previously reported during CGN apoptosis and the regulations of some genes involved in signaling pathways controlling CGN survival were also detected both by our microarray measurements and by quantitative RT-PCR (Table I). For instance, we detected an increased level for the following genes: caspase-3; the Bcl-2 family members *bim* and *DP5*; the transcription factors *c-jun*, *c-fos*, and *egr-1*; the secreted glycoprotein neuronal pentraxin-1; the stress-responsive inhibitor of thioredoxin, *Vdup-1*; the potassium channel *Task-1*; and the insulin-like growth factor-binding protein *Igfbp5*, as observed by other investigators (7, 20–27). Additional transcriptional regulations that had been reported previously in KCl-deprived CGN were also detected by our microarray measurements (data not shown).

**Biological Characterization of the Global Gene Expression Profile of Apoptotic CGN**—To understand the biological meaning of our data, we annotated the 368 differentially expressed genes identified by our microarray measurements with the controlled vocabulary provided by the Gene Ontology Consor-

TABLE I

Transcriptional regulations of some genes potentially involved in the control of CGN survival and apoptosis

The expression ratios of some differentially expressed genes that are known to participate in survival signaling pathways, which had been reported previously to be up-regulated during CGN apoptosis (underlined), or from the apoptotic machinery (shaded) were measured. The K5/K25 expression ratio determined using microarrays and quantitative PCR, the *q* value (%) from SAM if available (the *q* value is an individual measure of significance in terms of FD Rate), the status of the oligonucleotide on the chip (OK, signal intensity above detection limit; <DL, signal intensity below detection limit) are shown for each gene. Data are means  $\pm$  S.D. of three independent experiments.

| Gene Name                                       | GenBank   | UniGene   | chips K5/K25 ratios |      |             |        | QPCR K5/K25 ratios |      |
|---|-----------|-----------|---------------------|------|-------------|--------|--------------------|------|
|   |           |           | average             | SD   | q value (%) | status | average            | SD   |
| <b>Genes upregulated during CGN apoptosis</b>   |           |           |                     |      |             |        |                    |      |
| <i>Igfbp1</i>                                   | NM_008341 | Mm.21300  | 5.10                | 2.19 | 0.470       | OK     | 21.25              | 1.09 |
| <i>Neuronal pentraxin 1</i>                     | NM_008730 | Mm.5142   | 4.60                | 2.05 | 0.470       | OK     | 15.99              | 3.04 |
| <i>DP5/Hrk</i>                                  | NM_007545 | Mm.31808  | 3.05                | 0.59 | 0.468       | OK     | 4.70               | 0.14 |
| <i>Egr-1</i>                                    | M22326    | Mm.181959 | 3.03                | 0.60 | 1.089       | OK     | 6.53               | 0.90 |
| <i>Unc5h2</i>                                   | AK018177  | Mm.290433 | 2.87                | 0.37 | 0.359       | OK     | 4.56               | 0.93 |
| <i>Vdup1</i>                                    | NM_023719 | Mm.271877 | 2.75                | 0.52 | 0.534       | OK     | 12.83              | 1.46 |
| <i>PI 3-kinase r (p85<math>\alpha</math>)</i>   | U50413    | Mm.3058   | 2.49                | 0.43 | 0.470       | OK     | 3.42               | 0.39 |
| <i>Caspase 3</i>                                | NM_009810 | Mm.34405  | 2.48                | 0.41 | 0.359       | OK     | 3.05               | 0.43 |
| <i>bim/Bod (S.L.EL)</i>                         | NM_009754 | Mm.141083 | 2.08                | 0.21 | 0.534       | OK     | 2.55               | 0.22 |
| <i>c-jun</i>                                    | NM_010591 | Mm.275071 | 2.03                | 0.45 | 0.468       | OK     | 3.68               | 0.49 |
| <i>Pten</i>                                     | NM_008960 | Mm.245395 | 1.84                | 0.13 | 0.470       | OK     | 2.36               | 0.24 |
| <i>Task1</i>                                    | AF065162  | Mm.10080  | 1.95                | 0.70 | 0.802       | OK     | 4.47               | 0.49 |
| <i>Cide-A (Dffa-likea)</i>                      | NM_007702 | Mm.449    | 1.76                | 0.23 | 0.805       | OK     | 2.17               | 0.23 |
| <i>Caspase 6</i>                                | NM_009811 | Mm.281379 | 1.80                | 0.52 | 0.635       | OK     | 2.52               | 0.52 |
| <i>Caspase 7</i>                                | NM_007611 | Mm.35687  | 1.69                | 1.23 | *           | OK     | 1.97               | 0.02 |
| <i>p73</i>                                      | AK014503  | Mm.103715 | 1.62                | 0.29 | *           | OK     | 3.02               | 0.47 |
| <i>Raidd/Cradd</i>                              | NM_009950 | Mm.17493  | 1.59                | 0.26 | 3.745       | OK     | 3.30               | 0.56 |
| <i>E2F-1</i>                                    | NM_007891 | Mm.18036  |                     |      |             | <DL    | 3.30               | 0.39 |
| <i>Fas ligand</i>                               | NM_010177 | Mm.3355   | 1.00                | 0.16 | *           | OK     | 2.60               | 0.49 |
| <b>Genes downregulated during CGN apoptosis</b> |           |           |                     |      |             |        |                    |      |
| <i>c-Fos</i>                                    | NM_010234 | Mm.246513 | 0.24                | 0.08 | 0.470       | OK     | 0.17               | 0.06 |
| <i>Mef2c</i>                                    | NM_025282 | Mm.24001  | 0.28                | 0.04 | 0.359       | OK     | 0.20               | 0.01 |
| <i>Igfbp5</i>                                   | L12447    | Mm.578    | 0.37                | 0.08 | 0.470       | OK     | 0.38               | 0.06 |
| <i>Cytochrome c</i>                             | NM_007808 | Mm.298102 | 0.64                | 0.06 | 4.940       | OK     | 0.48               | 0.09 |

tium (16) using eGOn Version 1.0 software. The GO vocabulary describes attributes of gene products according to three independent ontologies: molecular function (the biochemical activity of the gene product), biological process (the biological objective to which the gene product contributes), and cellular component (the place in the cell where a gene product is active) (16). Multifunctional proteins are assigned several annotations corresponding to their different functions. Meaningful biological categories were selected from the GO hierarchy by browsing these three ontologies, and annotated genes were clustered in these different categories (Fig. 3). Using a statistical test available on the eGOn Version 1.0 web site, we identified several GO annotations significantly over-represented in the sets of differentially expressed genes compared with the full Oligo Set printed on the arrays (Table II).

It is important to note that functional categories related to signal transduction, proteolysis, and transcriptional regulation represented an important part of both up- and down-regulated

genes in molecular function as well as in biological process ontologies (Fig. 3, *a* and *b*). In accordance with this, some annotations related to transcriptional and translational regulation were significantly over-represented in up-regulated genes (Table II). Likewise, categories related to signaling pathways and, to a lesser extent, to protease activity were among the most represented functional categories for both up- and down-regulated genes (Table II). "Transporters" (Fig. 3*a*) and "Transport" (Fig. 3*b*) categories also comprised an important proportion of genes transcriptionally regulated in KCl-deprived CGN. Consistently, genes involved in ion channel activity (in particular, potassium channels) represented the most significant categories of up-regulated genes in the three ontologies (Table II).

Distribution of up-regulated versus down-regulated genes was strikingly different in some GO categories. For example, up-regulated genes comprised "Apoptosis regulators," whereas this molecular function category was empty for down-regulated

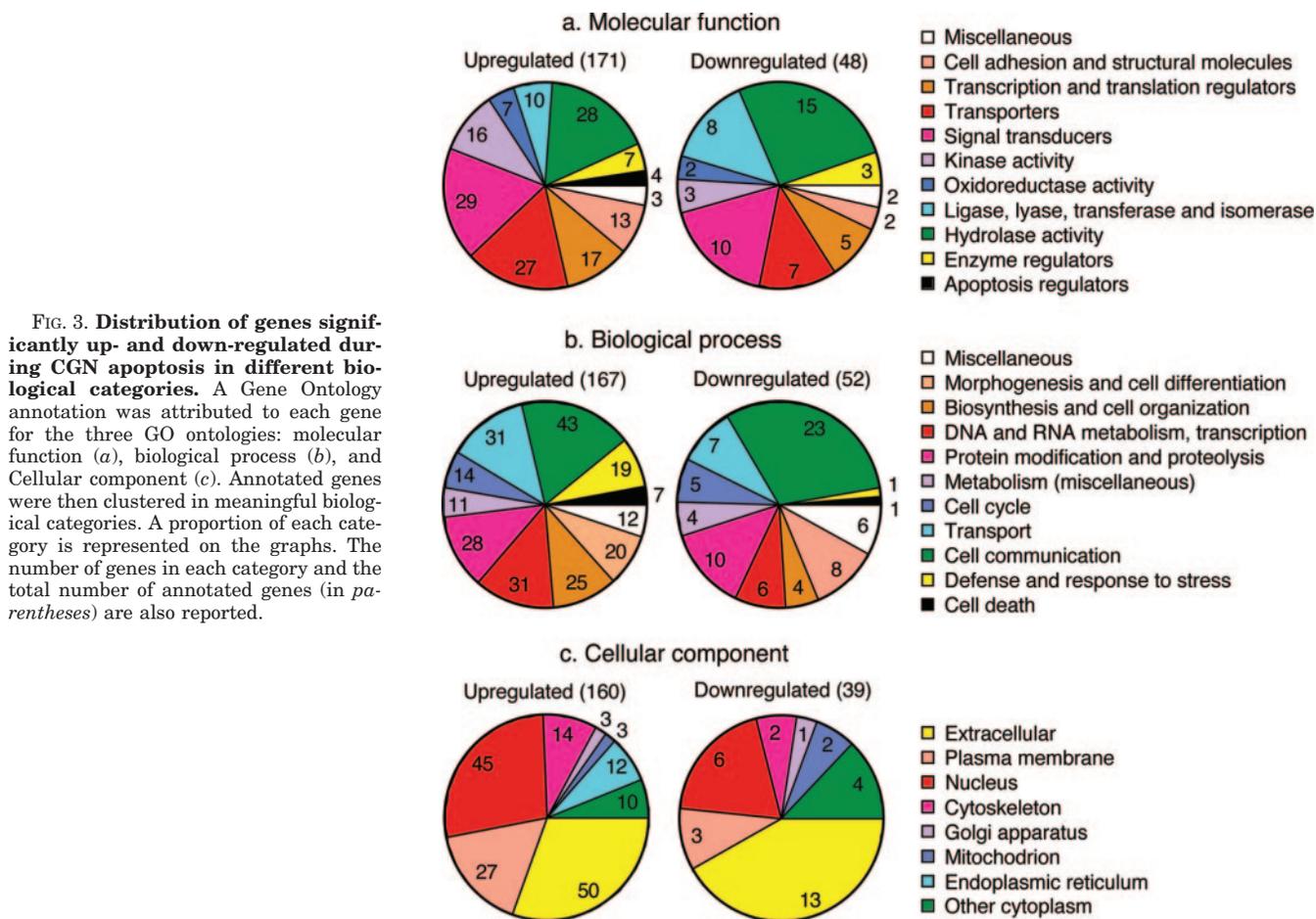


FIG. 3. Distribution of genes significantly up- and down-regulated during CGN apoptosis in different biological categories. A Gene Ontology annotation was attributed to each gene for the three GO ontologies: molecular function (a), biological process (b), and Cellular component (c). Annotated genes were then clustered in meaningful biological categories. A proportion of each category is represented on the graphs. The number of genes in each category and the total number of annotated genes (in parentheses) are also reported.

genes (Fig. 3a). Likewise, the biological processes “Defense and response to stress” and “Cell death” contained many more up-regulated than down-regulated genes (Fig. 3b). However, annotations related to apoptosis were not significantly over-represented among up-regulated genes (Table II). Surprisingly, an important proportion of up-regulated genes corresponded to proteins expressed in the endoplasmic reticulum (ER), whereas this category was empty for down-regulated genes (Fig. 3c).

Unexpected patterns also emerged from our analysis. Several functional categories related to lymphocyte activation and differentiation were significantly over-represented in up-regulated genes (Table II). The proportion of up-regulated genes related to cell adhesion such as “Cell matrix adhesion,” “Integrin-mediated signaling pathway,” “Cell motility,” “Integrin complex,” and “Focal adhesion” was also significantly higher than expected by chance (Table II). Notably, four integrin subunits ( $\alpha_v$ ,  $\beta_5$ ,  $\alpha_9$ , and  $\beta_2$ -like) were significantly induced under our conditions. Finally, functional categories annotated “Response to unfolded protein” and “Endoplasmic reticulum” were over-represented in up-regulated genes (Table II).

**Detection of the Unfolded Protein Response in KCl-deprived CGN**—To test whether processes pointed out by the GO analysis actually occur, we examined the possible activation of the unfolded protein response (UPR) in KCl-deprived CGN. The UPR is an adaptive response to ER stress that is orchestrated by transcriptional activation of multiple genes mediated by transcription factors such as XBP1 (28). The UPR also alters cellular patterns of translation. UPR induction activates the double-stranded RNA-activated protein kinase-like ER kinase PERK, which phosphorylates the  $\alpha$ -subunit of eukaryotic translation initiation factor-2, resulting in a general decrease in translational initiation and selective translation of specific

mRNAs such as *Atf4*. This transcription factor subsequently induces expression of *Chop/Gadd153* (29).

UPR activation in apoptotic CGN was first indicated by the up-regulation of a number of UPR-inducible genes, including the well known UPR markers *Grp78/BiP*, *Grp94*, and calreticulin (Fig. 4a) (28). We further confirmed that the UPR was activated in CGN by monitoring, at different time points after KCl withdrawal, the alternative splicing of *Xbp1* (Fig. 4b), which generates a potent transcriptional activator for many UPR target genes (30), and the induction of *Grp78/BiP*, *Perk*, and *Chop/Gadd153* (Fig. 4c).

**Identification of Transcription Factors Putatively Involved in Gene Regulation**—To gain further insights into the network of genes underlying the observed transcriptional regulations, we searched for consensus DNA-binding sites of transcription factors in the proximal promoters of differentially expressed genes. We used the data base generated by Suzuki *et al.* (31), who identified transcriptional start sites of 6875 mouse transcripts by mapping the most 5'-sequence of full-length cDNA clones on the MGSCv3 mouse genome assembly. Using the Match™ software (32), we identified putative DNA-binding sites significantly over-represented in the promoter sequences of differentially expressed genes compared with those of the Oligo Set: AP-4, NF-E2, NF-Y, and serum response factor in up-regulated genes and GATA-2, MyoD, Pax-6, and upstream stimulatory factor in down-regulated genes (Table III).

## DISCUSSION

RNA and protein synthesis has been clearly implicated in many models of neuronal death. However, limited progress has been made in identifying the genes whose expression is required for apoptosis induction. Whether these genes belong to

TABLE II  
GO annotations of differentially expressed genes  
with significant over-representation

Functional categories with a strong association with CGN apoptosis (low  $p$  values) were identified. Only GO nodes with  $p < 0.05$  and containing more than one differentially expressed (DE) gene are shown. For each GO term are indicated the number (Nb) of genes in the Oligo Set and in the differentially expressed gene list and, in parentheses, the number of differentially expressed genes expected if the proportion of genes annotated with this GO term was the same in the Oligo Set and the differentially expressed gene list ( $p = 1$ ). Annotations related to transcriptional and translational regulation, signaling, and proteolysis are underlined. Indicated are also the total numbers of annotated genes in the different gene lists. MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase kinase.

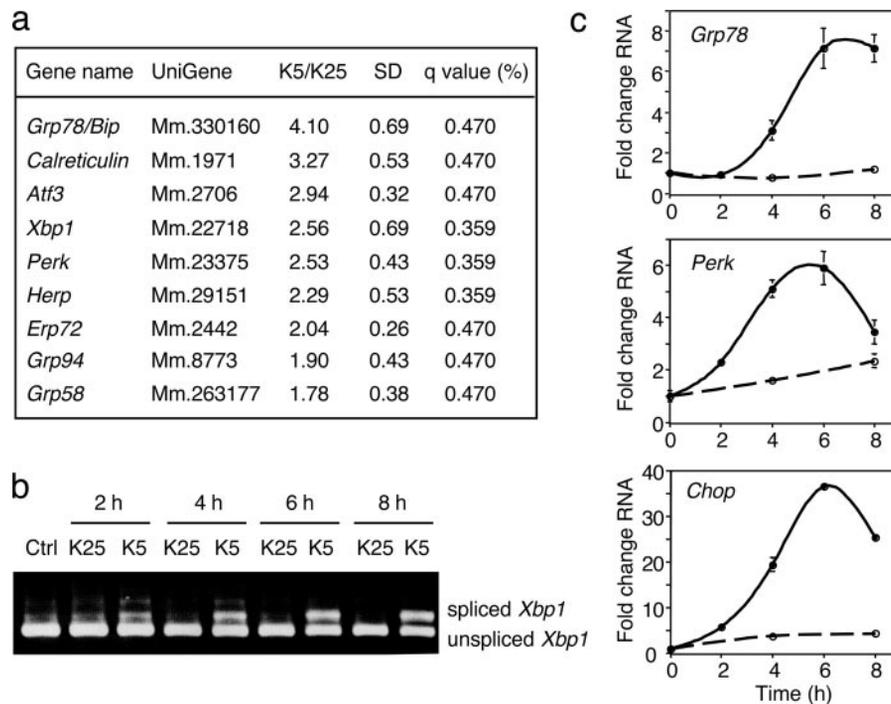
| GO identifier   | GO term   | Nb of genes |          |       |
|---|---|-------------|----------|-------|
|   |   | Oligoset    | DE genes | p     |
| <b>molecular function: upregulated genes (171) vs full oligoset (7945)</b>  |   |             |          |       |
| GO:0005085  | <u>Guanyl-nucleotide exchange factor activity</u>             | 47          | 4 (1)    | 0.037 |
| GO:0003714  | <u>Transcription co-repressor activity</u>                    | 29          | 3 (1)    | 0.048 |
| GO:0042578  | <u>Phosphoric ester hydrolase activity</u>                    | 176         | 9 (4)    | 0.029 |
| GO:0030693  | <u>Caspase activity</u>                                       | 10          | 2 (0)    | 0.037 |
| GO:0005216  | <u>Ion channel activity</u>                                   | 227         | 14 (5)   | 0.001 |
| GO:0005247  | <u>___ Voltage-gated chloride channel activity</u>            | 11          | 2 (0)    | 0.045 |
| GO:0005261  | <u>_ Cation channel activity</u>                              | 155         | 9 (3)    | 0.013 |
| GO:0005267  | <u>_ Potassium channel activity</u>                           | 75          | 7 (2)    | 0.002 |
| GO:0005249  | <u>___ Voltage-gated potassium channel activity</u>           | 47          | 6 (1)    | 0.001 |
| GO:0005244  | <u>_ Voltage-gated ion channel activity</u>                   | 98          | 11 (2)   | 0.000 |
| GO:0016329  | <u>Apoptosis regulator activity</u>                           | 73          | 4 (2)    | 0.146 |
| <b>molecular function: downregulated genes (48) vs full oligoset (7945)</b> |   |             |          |       |
| GO:0003925  | <u>Small monomeric GTPase activity</u>                        | 93          | 3 (1)    | 0.038 |
| GO:0005520  | <u>Insulin-like growth factor binding</u>                     | 17          | 2 (0)    | 0.009 |
| GO:0042578  | <u>Phosphoric ester hydrolase activity</u>                    | 176         | 5 (1)    | 0.009 |
| GO:0008081  | <u>_ Phosphoric diester hydrolase activity</u>                | 38          | 3 (0)    | 0.003 |
| GO:0004114  | <u>3',5'-cyclic-nucleotide phosphodiesterase activity</u>     | 16          | 2 (0)    | 0.008 |
| GO:0008237  | <u>Metalloproteinase activity</u>                             | 113         | 4 (1)    | 0.010 |
| <b>biological process: upregulated genes (167) vs full oligoset (7090)</b>  |   |             |          |       |
| GO:0007160  | <u>Cell matrix adhesion</u>                                   | 42          | 5 (1)    | 0.006 |
| GO:0007229  | <u>Integrin-mediated signaling pathway</u>                    | 41          | 4 (1)    | 0.031 |
| GO:0007243  | <u>Protein kinase cascade</u>                                 | 57          | 5 (1)    | 0.022 |
| GO:0000165  | <u>_ MAPKKK cascade</u>                                       | 18          | 3 (0)    | 0.016 |
| GO:0000187  | <u>___ Activation of MAPK</u>                                 | 4           | 2 (0)    | 0.006 |
| GO:0000075  | <u>Cell cycle checkpoint</u>                                  | 9           | 2 (0)    | 0.036 |
| GO:0006813  | <u>Potassium ion transport</u>                                | 92          | 7 (2)    | 0.012 |
| GO:0006928  | <u>Cell motility</u>  | 116         | 7 (3)    | 0.041 |
| GO:0006936  | <u>_ Muscle contraction</u>                                   | 40          | 4 (1)    | 0.029 |
| GO:0006633  | <u>Fatty acid metabolism</u>                                  | 34          | 4 (1)    | 0.016 |
| GO:0046456  | <u>_ Eicosanoids biosynthesis</u>                             | 16          | 3 (0)    | 0.012 |
| GO:0019376  | <u>___ Leukotriene biosynthesis</u>                           | 10          | 2 (0)    | 0.044 |
| GO:0006417  | <u>Regulation of protein biosynthesis</u>                     | 7           | 2 (0)    | 0.022 |
| GO:0046394  | <u>Carboxylic acid biosynthesis</u>                           | 41          | 5 (1)    | 0.005 |
| GO:0006366  | <u>Transcription from Pol-II promoter</u>                     | 128         | 8 (3)    | 0.022 |
| GO:0019886  | <u>Antigen processing, exogenous antigen via MHC class II</u> | 8           | 2 (0)    | 0.028 |
| GO:0046649  | <u>Lymphocyte activation</u>                                  | 17          | 4 (0)    | 0.001 |
| GO:0042113  | <u>_ B cell activation</u>                                    | 8           | 2 (0)    | 0.028 |
| GO:0046650  | <u>Lymphocyte differentiation</u>                             | 8           | 3 (0)    | 0.001 |
| GO:0006986  | <u>Response to unfolded protein</u>                           | 6           | 2 (0)    | 0.016 |
| GO:0006915  | <u>Apoptosis</u>  | 190         | 7 (4)    | 0.376 |
| <b>biological process: downregulated genes (52) vs full oligoset (7090)</b> |   |             |          |       |
| GO:0007242  | <u>Intracellular signaling cascade</u>                        | 511         | 10 (4)   | 0.010 |
| GO:0000188  | <u>_ Inactivation of MAPK</u>                                 | 3           | 2 (0)    | 0.000 |
| GO:0007264  | <u>_ Small GTPase-mediated signal transduction</u>            | 148         | 4 (1)    | 0.048 |
| GO:0006470  | <u>_ Protein amino acid dephosphorylation</u>                 | 84          | 3 (1)    | 0.048 |
| <b>cellular component: upregulated genes (160) vs full oligoset (7390)</b>  |   |             |          |       |
| GO:0005783  | <u>Endoplasmic reticulum</u>                                  | 279         | 12 (6)   | 0.040 |
| GO:0008305  | <u>Integrin complex</u>                                       | 25          | 4 (1)    | 0.004 |
| GO:0008076  | <u>Voltage-gated potassium channel complex</u>                | 27          | 5 (1)    | 0.001 |
| GO:0005925  | <u>Focal adhesion</u>   | 11          | 3 (0)    | 0.003 |

the core apoptotic machinery or whether apoptotic components are constitutively expressed has not been solved. In this study, these questions were addressed using a genome-scale, long oligonucleotide microarray method coupled with an automated GO annotation analysis. The global pattern of gene expression was elucidated in CGN 4 h after KCl deprivation. We identified 368 significantly differentially expressed genes, most of which had never been shown to be transcriptionally regulated during neuronal apoptosis. Although a few of these genes were part of the apoptotic machinery, the most significant number of regulated genes was related more to signal transduction than to apoptosis. Our data suggest that signaling pathways controlling apoptosis induction can be regulated at the transcriptional level, whereas the apoptotic machinery is mainly constitutive.

**Relevance of Microarray Analysis to Study Mechanisms Controlling Apoptosis**—Our microarray data appear to be reliable. Indeed, real-time RT-PCR detected significant changes in RNA levels for 94% of the 48 genes identified as differentially expressed by our microarray analysis. Moreover, our data confirmed most of the transcriptional regulations previously reported in CGN apoptosis. On the other hand, our microarray measurements did not detect the previously described up-regulation of *fasL* (22) and the transcription factor E2F-1 (33). Transcription of both genes was indeed induced under our conditions, as detected by quantitative PCR. This missing of expression regulations by microarray measurements may be attributable to different factors. For E2F-1, the corresponding oligonucleotide on the microarray produced signals below the detection threshold, possibly due to nucleic acid structure or low amounts of oligonucleotide available for hybridization. In the case of *fasL*, where the signal intensity was high, cross-hybridization with an abundant non-regulated transcript may have masked variations in expression. A similar cross-hybridization with a regulated abundant transcript could account for the 6% of gene expression regulations detected by the microarrays that could not be confirmed by quantitative PCR. Nevertheless, the false positive and missed regulation rates of our experiments remain low enough to enable reliable interpretation of the data.

A microarray study has previously been performed on cultured rat CGN undergoing apoptosis following KCl withdrawal, serum/KCl withdrawal, or kainic acid treatment (6). Besides the difference of species, the technology used was quite distinct from ours. Notably, these microarrays consisted of 7295 cDNA clones. Our microarrays provided greater coverage of the genome, with 16,463 genes represented. Moreover, the long oligonucleotides that we chose have been found to offer much higher specificity than cDNA probes while allowing a comparable sensitivity (34). Importantly, the culture conditions used in the study of Chiang *et al.* (6) were also quite different from ours. Only a small number of genes were found to be significantly regulated under their conditions after “KCl withdrawal alone.” We could identify mouse orthologs for only some of these genes, and the results were thus difficult to compare with ours. However, a few genes, including caspase-3 and *c-fos*, were found to be differentially expressed in both studies. At the time when the study of Chiang *et al.* (6) was published, specific methods for the statistical analysis of microarray data had not been developed; the Gene Ontology was in its early stages; and annotation tools were not available. We took advantage of these new tools to give a more comprehensive and informative view of the gene expression profile of CGN apoptosis.

Mapping of genes to the controlled vocabulary of the GO Database is a powerful tool to automatically extract potentially useful biological information from expression data. This approach still suffers from some limitations that are due mainly



**FIG. 4. KCl deprivation activates the UPR in CGN.** *a*, KCl withdrawal induced the transcription of several key players of the UPR. The UniGene cluster number, the K5/K25 expression ratio (mean  $\pm$  S.D. of three independent experiments) determined using microarrays, and the *q* value from SAM are indicated for each gene. *b*, CGN cultures were switched to either K25 or K5 medium for increasing times, and total RNA was extracted. Splicing of *Xbp1* transcripts was qualitatively estimated by RT-PCR, followed by PstI/BamHI digestion to produce the characteristic 298-bp (unspliced) and 375-bp (spliced) fragments. The band of spliced *Xbp1* was bigger than the band of unspliced *Xbp1* because splicing of *Xbp1* removed the PstI restriction site, rendering spliced *Xbp1* resistant to digestion. The smallest fragment of unspliced *Xbp1* digested by PstI was too small to be visible on the gel. The sequences of primers used to amplify both forms of *Xbp1* are provided in Supplemental Table I. *Ctrl*, control. *c*, CGN cultures were treated as described for *b*. mRNA contents for *Grp78*, *Perk*, and *Chop* were estimated by quantitative RT-PCR. -Fold change was calculated by comparison with neurons maintained in initial culture medium (time 0). Data are means  $\pm$  S.D. of triplicate determinations and are representative of three independent experiments.

TABLE III

*Consensus DNA-binding sites significantly over-represented in the promoter sequences of differentially expressed genes*

DNA-binding sites with a strong association with CGN apoptosis ( $p < 0.05$ ) were identified. For each DNA-binding sites are indicated the number (Nb) of sites in the promoters of genes from the Oligo Set and from the differentially expressed (DE) gene list and, in parentheses, the number of DNA-binding sites expected if the proportion of sites was the same in the Oligo Set and the differentially expressed gene list ( $p = 1$ ). The total numbers of DNA-binding sites found in the promoters of genes from the different lists are also indicated. Srf, serum response factor; Usf, upstream stimulatory factor.

| DNA binding sites (DBS)                             | Nb of DBS |          | <i>p</i> |
|---|-----------|----------|----------|
|   | Oligo Set | DE genes |          |
| Up-regulated genes (140) vs. full Oligo Set (6691)  |           |          |          |
| AP-4  | 39        | 3 (1)    | 0.040    |
| NF-E2   | 55        | 5 (1)    | 0.005    |
| NF-Y  | 261       | 12 (5)   | 0.006    |
| Srf   | 100       | 10 (2)   | 0.000    |
| Down-regulated genes (39) vs. full Oligo Set (6691) |           |          |          |
| GATA-2  | 30        | 2 (0)    | 0.013    |
| MyoD  | 187       | 5 (1)    | 0.004    |
| Pax-6   | 912       | 9 (5)    | 0.042    |
| Usf   | 1018      | 10 (6)   | 0.035    |

to the paucity of functional annotations for mouse genes and the subjectivity of the category selection process. Nevertheless, the ontology-based system of gene clustering can provide a pertinent analysis of results. For instance, it is consistent that genes encoding potassium channels represented the most significantly over-represented functional categories among genes up-regulated during  $K^+$ -dependent CGN apoptosis. The statistical analysis cannot indicate whether this phenomenon reflects an adaptive response of neurons to the change in medium

or whether it actively contributes to the triggering of neuronal apoptosis. However, it does suggest that GO annotation-based clustering can extract relevant information from microarray data despite its limitations. This notion is also supported by the important proportion of differentially expressed genes involved in signal transduction, transcriptional regulation, and proteolysis that is highlighted by the GO analysis. Indeed, it is generally assumed that neuronal apoptosis implicates regulation of signaling pathways, *de novo* RNA synthesis, and activation of different proteases. Keeping in mind that trends delineated by GO annotations can reflect real mechanisms, this kind of analysis can provide new information. For example, the proportion of genes identified as related to apoptosis is much more important in up-regulated than in down-regulated genes, suggesting that the induction of apoptosis requires the expression of pro-apoptotic proteins rather than the down-regulation of anti-apoptotic proteins. Analysis of the promoter sequences of co-regulated genes identified by microarray data can also give insights into novel functional gene networks. These co-regulations potentially result from the binding of the same transcription factor to the promoters of co-regulated genes. Therefore, identifying consensus DNA-binding sites and the transcription factors that bind to these sites may reveal unforeseen upstream signaling pathways. We took advantage of these different tools to draw new conclusions and to generate new hypotheses.

*Respective Role of Signaling Pathways Versus Apoptotic Machinery in Transcription-dependent Neuronal Apoptosis*—The major trend highlighted by our GO analysis is that genes encoding components of the core apoptotic machinery are not significantly over-represented among genes up-regulated during CGN apoptosis. This surprising observation can be explained by two different hypotheses. First, transcriptional reg-

ulation of a few key components may be sufficient to trigger neuronal apoptosis. The increased expression of the 11 genes that we identified may account for a limited number of critical events resulting in apoptosis induction. In this case, the hundreds of other differentially expressed genes would reflect either a side effect of the change in medium independent of the death process or adaptive responses to cell death, including expression of neuroprotective factors. The second possibility is that genes whose expression is required for inducing neuronal apoptosis are related to signaling pathways or enzymatic activities leading to the activation of key apoptotic components. This hypothesis is more consistent with the conclusions previously drawn by Weil *et al.* (1) from the effects of staurosporine and cycloheximide observed on a large panel of cell types. According to these results, all mammalian nucleated cells are capable of undergoing apoptosis and constitutively express all the protein components required to execute the death program. In those cases where inhibitors of RNA and protein synthesis inhibit apoptosis, it is presumably because macromolecular synthesis is required to activate the death program rather than to execute it (1).

Nevertheless, these two hypotheses are not mutually exclusive. It is likely that transcriptional regulations affecting both the apoptotic machinery and the signaling pathways leading to its activation contribute to the triggering of neuronal apoptosis. For instance, the BH3-only members of the Bcl-2 family, Bim and DP5, are known to repress anti-apoptotic proteins such as Bcl-2 and Bcl-x<sub>L</sub> (4). Up-regulation of these proteins may thus contribute to induction of neuronal apoptosis. Accordingly, *bim* deficiency and DP5 deletion have been separately shown to confer transient and partial protection against nerve growth factor deprivation-induced apoptosis of sympathetic neurons (35, 36). Moreover, our results reveal for the first time a modest but significant increase in downstream caspase-6 and caspase-7 expression in addition to the well known induction of caspase-3. Up-regulation of these caspases, which are in charge of the execution of the cell (37), is likely to participate in neuronal death by sustaining the degradation phase of apoptosis. Both caspase-3 and caspase-6 have been implicated in CGN apoptosis (38, 39). The induction of the death receptor UNC5H2, the adaptor protein RAIDD/CRADD, and the DNase regulator CIDE-A could also participate in CGN apoptosis, as these factors have all been shown to trigger apoptosis when overexpressed in different mammalian cells, including neurons (40–42). However, further studies are required to determine whether induction of these genes is necessary to CGN apoptosis.

Overall, a very limited number of genes belonging to the core apoptotic machinery are up-regulated during CGN apoptosis. As the protection afforded by actinomycin D and cycloheximide is very robust, it is unlikely that transcriptional control of CGN apoptosis relies only on a few apoptotic genes. It is plausible that different mechanisms leading to the activation of the apoptotic machinery are also regulated at the transcriptional level. This idea is supported by our GO analysis highlighting the important proportion of differentially expressed genes involved in signal transduction. Describing these latter genes in detail is outside the scope of this study. However, it is interesting to point out transcriptional regulations of some genes participating in transduction pathways that are known to mediate CGN survival. For example, it is well established that the phosphorylation cascade of the phosphatidylinositol (PI) 3-hydroxykinase/Akt pathway plays a crucial role in CGN survival (43, 44). It is therefore noteworthy that the tumor suppressor PTEN phosphatase and the p85 $\alpha$  regulatory subunit of PI 3-hydroxykinase are up-regulated early after KCl deprivation in CGN. PTEN negatively regulates PI 3-hydroxykinase/Akt

signaling (45). Therefore, increased expression of *Pten* should play an active role in inducing CGN apoptosis by switching off survival pathways. This is supported by the decreased sensitivity of *Pten*-deficient cells to various apoptotic stimuli (45) and by the accumulation of granule neurons, associated with an elevated activity of Akt, in the cerebella of mice with a brain-targeted disruption of *Pten* (46). The pro-apoptotic effect of PTEN should be reinforced by induction of p85 $\alpha$ . Recent studies have shown that, when p85 $\alpha$  is expressed at higher stoichiometric levels than the p110 catalytic subunit, p85 $\alpha$  monomers inhibit PI 3-hydroxykinase activation (47). In KCl-deprived CGN, the p85 $\alpha$  gene is up-regulated, whereas p110 gene expression is unchanged (data not shown). This should thus result in PI 3-hydroxykinase inhibition and should favor apoptosis induction, as suggested by increased PI 3-hydroxykinase activity in p85 $\alpha$ -deficient cells (47). Finally, it is interesting to note that the calcium-regulated transcription factor MEF2C is strongly down-regulated in our model. MEF2C has been implicated in activity-dependent neuronal survival (48). As transfection of CGN with dominant-interfering forms of MEF2 has been shown to induce apoptosis (48), it is likely that the strong repression of MEF2C participates in CGN death after KCl deprivation.

In view of this brief analysis, it appears that differential expression of genes involved in signal transduction is likely to take a more active part than transcriptional regulation of apoptotic machinery components in the control of CGN death. The few examples detailed above show for the first time that some crucial survival pathways can be modulated at the transcriptional level and not only by post-translational mechanisms. Moreover, our study provides insights into novel mechanisms that may contribute to induction of neuronal apoptosis.

*New Hypotheses Generated from Global Analyses of Microarray Data*—Mapping of differentially expressed genes to GO annotations identified a number of functional categories in which transcriptional regulations had never been detected during CGN apoptosis. Notably, genes related to the UPR and genes expressed in the ER were over-represented among up-regulated genes, suggesting that this process might play a role in neuronal apoptosis. The UPR is an adaptive response that has been evolved by eukaryotic organisms to ensure that the protein-folding capacity of the ER is not overwhelmed. Upon accumulation of unfolded proteins, UPR activation leads to reduced general protein translation and increased transcription of a characteristic set of genes by defined transcription factors, including XBP1, ATF6, and ATF4 (28). Consistent with a possible implication of the UPR in CGN apoptosis, our microarray data analyses identified the consensus DNA-binding sites of NF-E2 and NF-Y as over-represented in the promoter sequences of up-regulated genes. Indeed, transcription of UPR-inducible genes mediated by ATF6 requires the presence of NF-Y (28), and the NF-E2 consensus binding site can bind the transcriptional activator Nrf2 (NF-E2-related factor-2), which is activated by the ER-resident kinase PERK in response to ER stress (49). Using various biochemical criteria, we confirmed that the UPR was actually activated during KCl deprivation-induced CGN apoptosis. However, the positive or negative role of the UPR in CGN apoptosis remains to be determined. The UPR can promote protective stress adaptation by increasing the folding capacity of the ER and by increasing resistance to oxidative stress (28, 49). Alternatively, prolonged activation of the UPR can lead to apoptosis through different pathways, including expression of pro-apoptotic genes by the b-ZIP transcription factor CHOP (28, 29). The sustained expression and activation of PERK, followed by the prolonged and very high induction of *Chop*, may contribute to CGN apoptosis, further

substantiating the role of the UPR in neurodegenerative diseases (50). However, this hypothesis requires further experiments.

GO analysis of our microarray data also highlighted an important proportion of genes involved in cell adhesion among up-regulated genes. Notably, several integrins were induced during CGN apoptosis. Integrins are known to promote cell survival (51), and their induction could thus reflect a compensatory mechanism triggered by the detachment of neurons from substrate subsequent to death. However, expression of these genes preceded the first hallmarks of apoptosis by several hours. This suggests that integrin induction could contribute to CGN death by actively promoting a form of apoptosis called integrin-mediated death (52). This process is distinct from anoikis, which is caused by the loss of adhesion *per se*. However, functional studies are required to determine the positive or negative role of increased integrin expression in neuronal apoptosis.

In conclusion, our gene expression study indicates that transcriptional regulation of neuronal death can act at multiple levels, from plasma membrane receptors and phosphorylation cascades to transcription factors, and not only at the level of the apoptotic machinery. Moreover, our observations shed new light on neuronal apoptosis and show for the first time that the UPR is activated during CGN death. Apoptosis involves both transcription-dependent and -independent mechanisms. Gene expression profiling cannot prefigure subcellular localization and post-translational modifications that finally determine the activity of gene products. Only functional studies of individual genes can establish their actual role and mechanisms of action within apoptotic pathways. Nevertheless, our microarray data, highlighted by GO annotations and statistical analysis, have generated new hypotheses that could not be generated by any other approach. These hypotheses open new fields of investigation to elucidate molecular mechanisms leading to neuronal apoptosis. They will be used to guide future mechanistic studies. The potential function of some genes identified by our microarray analysis is being investigated by overexpression and silencing experiments. These studies may reveal unsuspected molecular pathways controlling the survival/death fate of neurons and ultimately contribute to the identification of new target genes for the treatment of neurodegenerative diseases.

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