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Global gene expression patterns during neural differentiation of P19 embryonic carcinoma cells

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Abstract The nervous system is composed of many different types of neurons and glia cells. Differentiation of these cell types is regulated by various intrinsic transcriptional programs as well as extrinsic signals. Studies of neural differentiation have been focused on the roles of individual factors. Here we profiled global gene expression patterns during neural differentiation of P19 embryonic carcinoma cells. Grouping of the genes induced during P19 neural differentiation into functional categories reveals a set of important transcription factors and extracellular signaling pathways, many of which are also involved in neural development *in vivo*. In addition, clustering of the induced genes according to their temporal expression pattern reveals 6 groups of genes, each with distinct kinetics, suggesting the existence of different phases in P19 neural differentiation. Our studies provide a temporal array of global pictures of the gene expression patterns used during neural differentiation. The results of this study provide the framework for subsequent analysis of the effects of various intrinsic and extrinsic factors on neural differentiation.

Key words cDNA microarray · neural differentiation · P19 cells

Introduction

The mammalian nervous system is composed of many different types of neurons and glia cells. Elucidation of the mechanisms that regulate the differentiation of these cell types is essential for understanding the development of the nervous system. Neurogenesis is accomplished by the successive development of neural progenitor cells, which generate neurons, astrocytes and oligodendrocytes, and precursor cells, which persist into adulthood, and are thought to have restricted developmental and proliferative potentials (Temple and Qian, 1996). A large number of transcription factors are sequentially expressed during neurogenesis, suggesting the existence of a complex interacting set of transcriptional events that control the developmental fate and the specific phenotypes of the resulting differentiated cells. Among these factors, proteins of the basic helix-loop-helix (bHLH) class play a central role not only in the acquisition of a neural fate and the determination of neuronal lineage but also the specification of phenotypes of the terminally differentiated neurons (Brunet and Ghysen, 1999). In *Drosophila*, these factors can be classified into two groups: the proneural genes and the neurogenic genes. The expression of proneural genes, such as achaete-scute genes and atonal, provides neural competence to groups of ectodermal cells. The neurogenic genes are in turn activated by the proneural genes and specify neuronal identity (Jan and Jan, 1993). In vertebrates, homologues of the *Drosophila* proneual and neurogenic gene have been found. However, the evidence for a similar transcriptional regulatory cascade has been lacking, as the time of onset and restricted patterns of expression of reported bHLH genes excludes them from a role in neural determination (Guillemot, 1999). Neural differentiation does not depend solely on intrinsic factors but also on extrinsic signals. Signals coming from soluble molecules, such as fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), Wnt proteins and retinoic acid (RA), extracellular matrix as well as cell-cell interactions regulate the proliferation, lineage commitment and differentiation of the different neural progenitor and precursor cells (Sasai and De Robertis, 1997; Frade and Rodriquez, 2000). How the intrinsic transcriptional programs

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and extrinsic signals are integrated in neural differentiation remains to be determined.

The P19 embryonic carcinoma cell line has proved to be a particularly tractable system for studying neuron and glia differentiation. P19 cells are pluripotent and can be induced to differentiate into derivatives of all three germ layers: endoderm, mesoderm and ectoderm, depending on the nature of chemical inducers and the culture conditions (Runnicki and McBruney, 1987). After treatment with high concentrations of retinoic acid (RA) and aggregation, P19 cells can differentiate into neurons, glia and fibroblast-like cells (Jones-Villeneuve et al., 1982). The fibroblast-like cells remain uncharacterized. But these cell types appears in a reproducible manner temporally: fibroblast-like cells emerge first, followed by neurons, and glia cells. This temporal pattern is similar to that seen in explants of brain from rat emryos. Furthermore, cells with similar morphology are also seen in cultures of central and peripheral nervous system (Jones-Villeneuve et al., 1982). All these suggest that RA-induced P19 neural differentiation in part mimics the development of nervous system.

RA has also been implicated in the development of vertebrate nervous system *in vivo*. RA is involved in the stimulation of axon outgrowth, the migration of neural crest cells and the specification of rostrocaudal position in the developing central nervous system (Maden and Holder, 1992). Studies of RA-induced P19 neural differentiation have led to the discovery of a number of genes that are important for neural development *in vivo* (for review see Bain et al., 1994). But these studies have focused on the roles of individual intrinsic or extrinsic factors. Little is known about transcriptional and signaling networks and how their interactions influence cell fate and the terminal differentiated phenotypes of the resulting cells. The advent of DNA microarray technology allows the examination of thousands of genes simultaneously (Schena et al., 1998). Here we report the initial dissection of the transcriptional networks that control the cell fate determination during neural differentiation under the regulation of RA signaling. In our experiments we determined the genome-wide expression pattern of terminally differentiated neuron populations derived from P19 cells using a microarray containing 9000 cDNA clones. To determine the sequence leading up to terminal neural differentation, we analyzed RA-induced P19 gene expression patterns at 12 time points, ranging from 1 hour to 8 days after induction. Out of the 9000 clones in our array, 555 are induced during neural differentiation. Homology searches of these 555 clones revealed 309 unknown expressed sequence tags (ESTs) and 246 known genes. Hierarchical clustering of the 555 genes showed 6 distinct patterns of temporal expression. This study provides a wealth of data during the dynamic process of cell commitment and neural differentiation and provides the basis for subsequent studies on specific pathways involved in neurogenesis.

Methods

Microarray preparation

A set of sequence verified mouse GEMarray EST inserts (Incyte/Genome Systems) plus 212 selected mouse genes from the IMAGE collection were PCR amplified and spotted onto polylysine coated microscope slides using a custom designed robot (Cheung et al., 1999). Prior to use, the arrays were UV crosslinked and chemically blocked using 0.7g of Succunic anhydride, 45 mls of N-methylpyrrilidinone (Aldrich) and 5 mls of 0.2M Boric acid pH 8.0 for 30 minutes. After blocking the slides were rinsed in 0.1% SDS and denatured in 94°C water f,or 5 minutes. The arrays were then washed in cold ethanol, dried and then prehybridized with 35% formamide, 4× SSPE, 2.5× Denhardts, 0.5% SDS and 100 µg/ml Salmon sperm DNA for 1 hour at 50°C in a submerged hybridization chamber (Telechem International).

Cell culture and induction

P19 cells were purchased from American Type Culture Collection. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, Without sodium pyruvate (life technologies GLUTAMAX^{TM} modification), supplemented with 2.5% fetal calf serum and 7.5% heat inactivated calf serum. Dishes were sub-cultured on alternate days. Retinoic acid-induced differentiation was performed by culturing cells in aggregates over a solid layer of 1% agar in the presence of 1 µgM RA for 4 days and then plating the cells onto tissue-culture surface without RA. Enriched neurons were obtained by growing cell at 5 µg/ml cytosine arabinoside 1 day after plating the cells onto tissue culture surface until day 12 (Runnicki and McBruney, 1987). The experimental RNA was harvested from cells treated with RA and control RNA was harvested from logarithmatically growing P19 cells.

Fluorescent probe preparation

RNA was harvested directly from the plates using the guanidine isothiocyanate method from the RNeasy Maxi kit (Qiagen) following manufacturer's protocol. 100 µg of total RNA was re-suspended in water to a volume of 19 µl with 1 µg of oligo dT primers (Life technologies), and reverse transcribed using 2.5 mM dATP, dGTP, dCTP, 1 mM dTTP, 10 mM DTT, 1 µl Rnasin (Promega), 4 µl of Cy5 or Cy3 labeled dUTP in a final volume of 40 µl. Two µl of Superscript II (life technologies) was added and the reaction incubated at 42°C for one hour, followed by the addition of another 2 µl of the reverse transcriptase for an additional hour of incubation. The reaction was stopped by heating at 94°C for 2 minutes and brought to a volume of 100 µl with water; the RNA was removed by the addition of 2 µls of RNAse ONE (Promega) and incubated at 37°C for 15 minutes. Both probe solutions were combined in a Microcon YM50 spin column and centrifuged at 12000×*g* for 10 minutes. After three washes with water, the combined probe was eluted from the column in a final volume of 6.5 µl and brought to a final volume of 19 µl containing 35% formamide, 4× SSPE, 2.5× Denhardts and 0.5% SDS. The probe was allowed to prehybridize with 1 µl of blocking reagent containing Cot-1 DNA, tRNA and poly dA for 1 hour at 50°C. Then it was added to the microarray and incubated at 50°C for 18–24 hours.

Data collection and analysis

After hybridization, the slide was washed in 500 ml of 0.2× SSC/0.1% SDS for 10 minutes followed by a 0.2× SSC wash and finally dried by centrifugation. After the slide was scanned with GenePix 4000 scanner (Axon), the signal intensities for each element was calculated using the software program GenePixPro from Axon. To reduce inter

array variability we performed repeat arrays as well as utilizing the wealth of *a priori* knowledge of this system to select expression level cutoffs. Throughout the experiments any elements present in areas containing excessive noise, printing artifacts or high regional areas of background were eliminated from further calculations, also any elements with a net total intensity less than 250 in either channel were likewise not considered due to the higher amount of variability in replicate arrays. The two channels were normalized by using the average spot signal intensity across the whole array. After the data had been preprocessed and entered into an Access database, queries were run to produce sets of genes meeting reproducibility, signal ratio and net intensity thresholds. For the comparison of expression patterns between neurons and P19 cells, two independent experiments were carried out. Only genes with relative ratio greater than or equal to 2 in both experiments were considered to be upregulated in neurons. For the time-course studies, only genes that had three consecutive time points with relative ratio greater than or equal to 2 during the time-course were considered to be induced genes. The selected genes were analyzed using the hierarchical algorithms in Cluster from Stanford. The ratio values were transformed into Log2 for analysis and Jackknife Correlation Coefficient (Heyer et al., 1999) was used with Average Linkage Clustering.

Results and Discussion

Consistency of our microarray results

In our microarray analysis, cDNA from total RNA of both RA-treated cells and control cells was labeled with Cy5 or Cy3, respectively, and hybridized to the arrays. The expression level of each gene was represented by a relative ratio of its intensity in samples to that in controls. The false color images of the hybridization results, as shown in Fig. 1A, D and E, were made by representing Cy3 signal as green and the Cy5 signal as red and merging the two images. The spot will appear yellow if Cy3 signal is equal to Cy 5 signal, green if Cy3 signal is stronger than Cy5 signal, and red if Cy5 signal is stronger than Cy3 signal.

To test the reliability of our microarray results, the same sample of RNA was labeled with Cy5 or Cy3 and hybridized to an array. A section of the merged color image, the plot of the Cy5 intensities against the Cy3 intensities and the distribution of the relative ratios are shown in Fig. 1A, B and C, respectively. Most of the spots appear yellow, Cy3 and Cy5 intensities are similar with a correlation of 0.97 and the distribution of the relative ratios is centered around 1. All these indicate that most genes have a relative expression ratio close to 1, just as expected, and suggest that the intrinsic variation introduced by labeling and hybridization within an array is small. To test array to array variation, two independent hybridizations to different microarrays were done to compare the same set of experimental and control RNAs. The lower panels of Fig. 1 show the same section of the two merged images and the plot of the relative ratios for all genes from these two independent hybridizations. The two experiments give very similar results: the two merged color images are almost identical and the correlation between the two ratios is 0.96.

Gene expression pattern of neurons derived from P19 cells

Previous studies have shown that when P19 cells are grown in suspension in the presence of RA and subsequently plated onto tissue culture dishes, round neuron-like cells with extensive processes develop within a few days (Jones-Villeneuve et al., 1982). Neurons can be enriched by growing the RA-treated cells in the presence of 5 μg/ml cytosine arabinoside (Runnicki and McBruney, 1987). While these are interesting observations, little is known about the genes involved. We took advantage of these observations to compare the gene expression pattern of the resulting neurons with that of logarithmatically growing P19 cells by two independent microarray hybridizations. Consistent with the dramatic change in morphology, there is an equally dramatic difference in gene expression between neurons and P19 cells: out of the 9000 clones on the array, 918 were shown to be preferentially expressed in neurons in both hybridizations. The identity of these clones can be obtained from our website. Shown in Table 1 is the subset of the genes known to be consistent with neuronal cell phenotype. These include genes involved in neurotransmitter metabolism, such as acetylcholinesterase, glutamic acid decarboxylase 1(Gad1) and somatostatin, genes encoding neurotransmitter receptors, such as nicotinic acetylcholine receptor beta subunit, N-methyl-D-aspartate (NMDA) receptor glutamate-binding subunit and gamma-aminobutyric acid (GABA) B1a receptor, and genes encoding ion channels, such as voltage-dependent calcium channel beta-3 subunit and alpha-1-G subunit. The expression of these genes suggests the induction of cholinergic, GABAergic, glutamaergic and somatostatinergic neurons. This result is in accordance with previous observations (Staines et al., 1994) and suggests that our microarrays can provide a "barcode" for neuronal cell types and therefore an assay for factors that promote an individual cell type at the expense of others.

In developing embryos, extracelluar cues, consisting of secreted molecules, cell-cell contact and cell-extracellular matrix interaction, are responsible for neurite outgrowth and axon guidance. Genes encoding members of a number of these signaling pathways are expressed during P19 neural differentiation. For example, pleiotrophin, a cytokine that induces neurite outgrowth (Li et al., 1990), is expressed in P19 derived neurons, as is plexin 2, a receptor for semaphorin/collapsin (Tamagnone et al., 1999). Consistent with plexin2 expression, Ulip, a member of TUC(TOAD/Ulip/CRMP) family of proteins, is also expressed in neuronally differentiated P19 cells. The TUC proteins mediate semaphorin/collapsin signal transduction (Quinn et al., 1999). The expression of both plexin 2 and Ulip suggests a role for semaphorin/collapsin signaling in P19 neural differentiation. Another guidance cue molecule, Ephrin B3, is also expressed in P19 derived neurons. Ephrin-Eph receptor

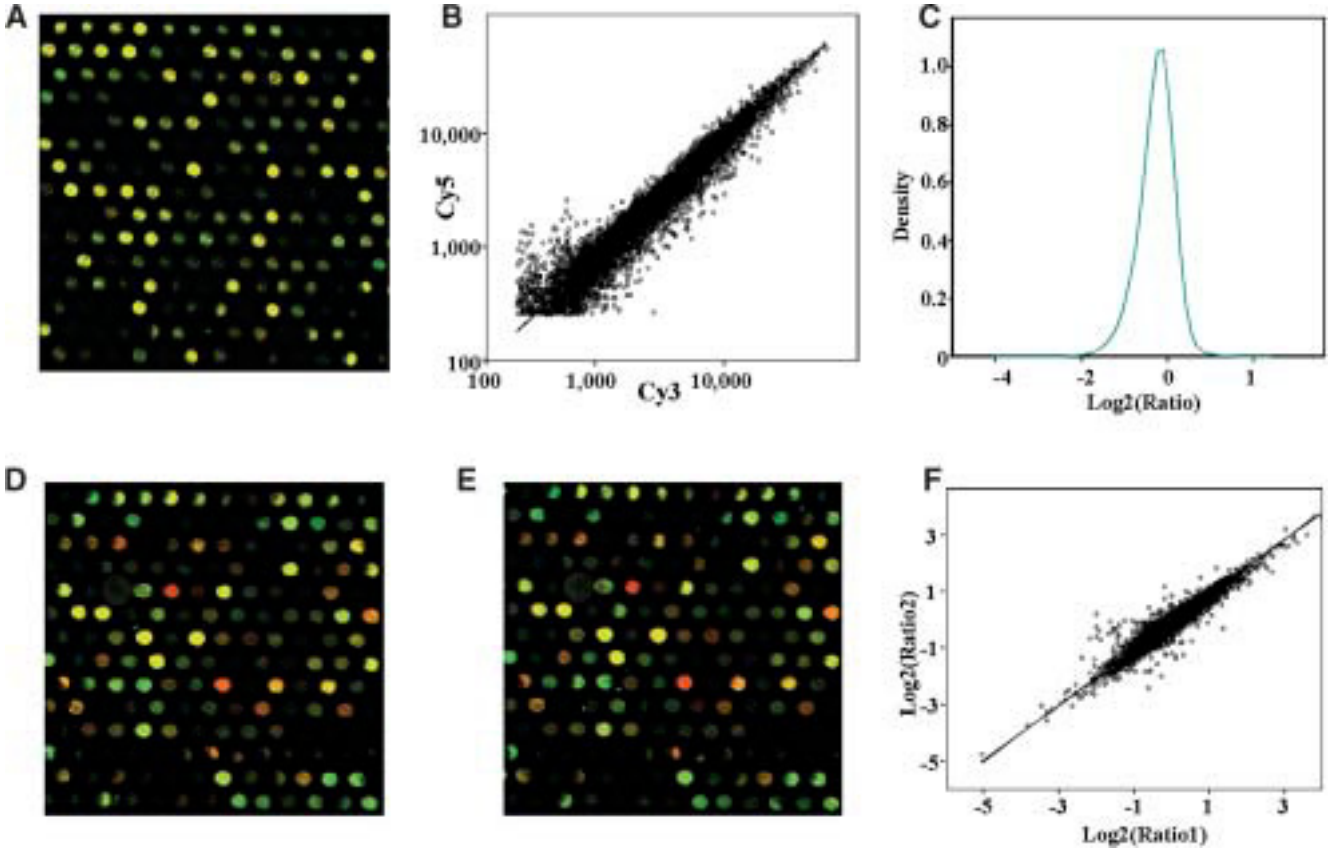

Fig. 1. Variability within an array and between arrays. *Upper panels*: the same sample of RNA was labeled with either Cy5 or Cy3 and hybridized to an array. A representative area of the merged color images, a plot of the Cy5 intensities versus the Cy3 intensities, and the distribution of the relative ratios are shown in Fig. 1A, B and C, respectively. *Lower panels*: two independent hybridization using the same set of RNA samples were carried out. Identical sections of the two merged images are shown in Fig. 1D and E and the plot of the relative ratios for all genes from these two independent hybridizations is shown in Fig. 1F.

system has been shown to mediate contact-dependent repulsion involved in axon guidance (Mellitzer et al., 2000). A role for the Ephrin-Eph receptor system in P19 neural differentiation is further supported by the expression of SHEP1, a SH2 domain-containing Eph receptor-binding protein that transmits Ephrin-Eph signals downstream. Finally, extracellular matrix proteins, entactin, s-laminin and tenascin-C, are also expressed in the P19-derived neurons. These proteins have been shown to play a role in neurite outgrowth or axon guidance (Kim and Wadsworth, 2000; Hunter et al., 1989; Faissner, 1997). mRNAs for three cell adhesion molecules, N-cadherin, neural cell adhesion molecule (NCAM-140) and neural cell adhesion molecule L1, are also expressed. These cell adhesion molecules may be involved in the transmission of signals from extracellular matrix or cell-cell contact.

Neurite formation requires extensive cytoskeleton remodeling. Microtubules are the prominent components in the neurite shaft and the central domain of the neural growth cone. Microtubules also provide structural support and act as substrates for the fast axonal transport of vesicles (Valtorta and Leoni, 1999). Consistent with these important roles of microtubules in neurons, a number of microtubule proteins, such as beta-tubulin, microtubule associated testis specific serine/threonine protein kinase (Mtssk), microtubule-associated protein 2 (MAP2), MAP4, MAP1A and light chain 3 subunit and syntrophin-associated serine-threonine protein kinase are preferentially expressed in P19 derived neurons. In addition to the microtubule network, there is a rapid rearrangement of the actin cytoskeleton during neurite outgrowth (Valtorta and Leoni, 1999). Correspondingly, proteins that have important roles in regulating the actin system, such as drebrin (Shirao, 1995), profiling 2 (Pfn2) (Schluter et al., 1997) and rhoB (Tapon and Hall, 1997), are upregulated in P19-derived neurons.

Induction of the neural phenotype correlates with two large increases in gene expression

The temporal pattern of gene transcription during P19 neural differentiation was followed at time points 1, 3, 6, 10 and 16 hours and 1, 2, 3, 4, 5, 6 and 8 days after RA treatment. The gene expression pattern of P19 cells

Table 1 Neuron-specific genes found in neurons derived from P19 differentiation

Classification	GeneBank number	Gene description
Cytoskeleton	AA061838	Beta-tubulin T beta15 [*R. norvegicus*]
	AA034730	Drebrin
	W54533	Drebrin
	AA016762	Microtubule associated testis specific serine/threonine protein kinase (Mtssk)
	AA386889	Microtubule-associated protein 2 (MAP2) [*R. norvegicus*]
	AA003769	Microtubule-associated protein 4 (MAP4)
	AA386890	Microtubule-associated protein tau
	AA498356	Microtubule-associated proteins 1A and 1B light chain 3 subunit [*R. norvegicus*]
	AA003408	*Mus musculus* neuronal-specific septin 3 (Sep3)
	AA386891	Neurofilament protein NF-66
	AA217908	Non-muscle alpha-actinin 1
	AA230924	Non-muscle myosin light chain 3 (MLC3nm)
	AA032658	Profilin 2 (Pfn2)
	W33989	rhoB gene [*R. rattus*]
	AA002708	Syntrophin-associated serine-threonine protein kinase
	AA032848	Syntrophin-associated serine-threonine protein kinase
Ion channel	W90827	Voltage-dependent calcium channel beta-3 subunit (cchb3)
	AA049807	Voltage-gated calcium channel, alpha-1-G subunit (cchna1)
Neurite outgrowth signaling	AA239404	Entactin
	W88036	Entactin
	AA178037	Ephrin-B3 (EFNB3) [*H. sapiens*]
	W62969	Fyn protooncogene Fyn(T) (L-fyn(T)
	AA034594	N-cadherin/cadherin 2 (Cdh2)
	AA242226	N-cadherin/cadherin 2 (Cdh2)
	AA288224	Neural cell adhesion molecule L1 [*R. norvegicus*]
	W54389	Neural cell adhesion molecule (NCAM-140)
	AA049699	OSF-1 Pleiotrophin
	AA511430	Plexin 2
	AA036176	s-laminin [*R. norvegicus*]
	AA270625	Tenascin C
	W18828	Ulip protein/dihydropyrimidinase-like 3
Neurotransmitter metabolism	AA030863	Acetylcholinesterase
	W59173	Glutamic acid decarboxylase 1 (Gad1)
	AA386894	Somatostatin (Smst)
Neurotransmitter receptor	AA036134	GABA-B1a receptor mRNA
	AA386892	Nicotinic acetylcholine receptor beta subunit
	AA386893	NMDA receptor glutamate-binding subunit mRNA [*R. norvegicus*]
Vesicular trafficking	W13111	Dynamin
	AA218406	RAB11B, member RAS oncogene family
	W82220	Rab3A gene
	AA270037	Rab3B protein [*R. norvegicus*]
	AA030574	Rab3B protein [*R. norvegicus*]
	AA435275	Syntaxin 13 [*R. norvegicus*]
	AA111166	Syntaxin 7 (Syn7)
	AA061343	Vesicle associated membrane protein (VAMP-2)

at these 12 time points was compared to that of the untreated, logarithmatically growing P19 cells by microarray hybridization. The number of induced genes at any given time points is shown in Fig. 2. There are two dramatic increases in the number of induced genes during P19 neural differentiation. Between hour 16 and day 2, the number of activated genes increases four fold from 103 to 424. We believe this increase reflect the induction of genes required for the commitment step in neural differentiation. This hypothesis is consistent with previous morphological observations showing that a 48 hour pulse of RA is sufficient for neural differentiation (Jones-Villeneuve et al., 1982). The larger increase in the number of expressed genes occurs between day 5 and 6: the number of activated genes increases from 494 to 814. This change reflects sets of genes involved in the terminal differentiation of neurons, neurite outgrowth and guidance molecules and correlates with morphological changes in the cell population.

Functional clustering of genes induced during P19 neural differentiation

Of the 9000 cDNA clones, a subset of 555 clones whose expression is substantially elevated by two fold or more in three consecutive time points were selected for analysis. These stringent selection criteria were chosen to

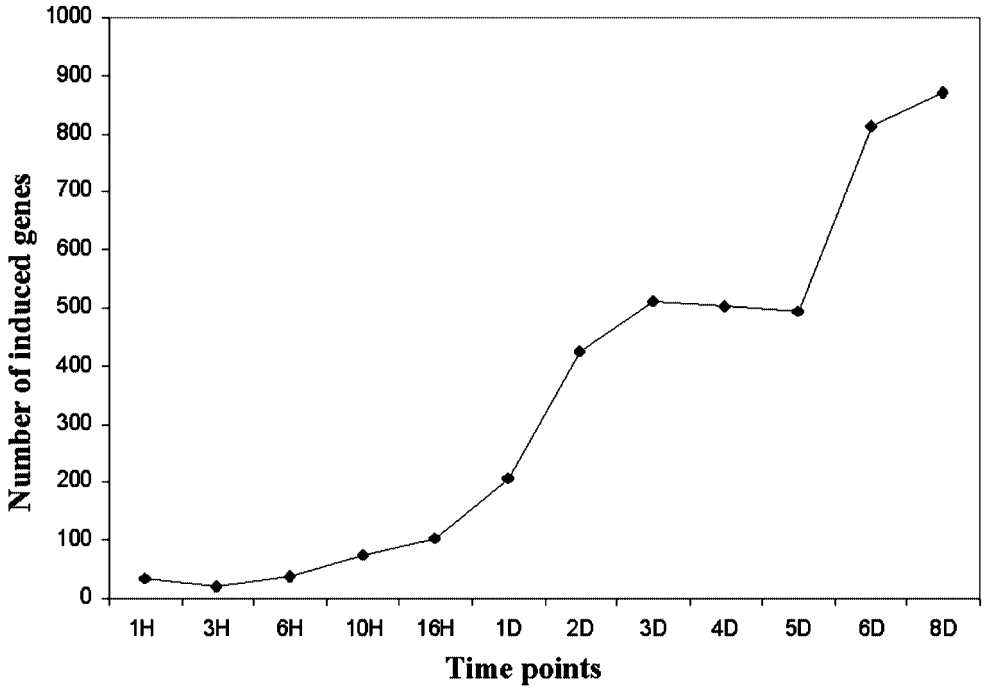

Fig. 2 The number of genes induced by RA at different time points during P19 neural differentiation. The graph shows the number of genes with more than two-fold induction at time points hour 1, 3, 6, 10 and16 and day 1, 2, 3, 4, 6 and 8 during P19 neural differentiation.

identify genes utilized over a reasonable time frame of the differentiation process. Genes whose expression is highly transient were missed in the presentation of our data, however, genes induced in only one time point are more likely to be in error. All of our raw data will be available on our website for those interested in seeing the expression level of all the genes on our chips. Among these 555 genes, 309 are ESTs, the GeneBank numbers of which are listed in Table 1 of the Appendix. The remaining 246 clones encode 224 known genes with 16 genes represented twice and 3 genes represented three times (Table 2). Among these known genes, at least 15 previously been shown to be RA inducible in P19 cells (denoted by * in Table 2). Among these 15 genes are Stra1 and Stra13, two genes that were discovered in a subtraction hybridization screen for RA inducible genes in P19 cells (Bouillet et al., 1995; Boudjelal et al., 1997). The presence of these genes in our stringent selection of genes induced during the process of differentiation also validates our microarray results.

To better understand the gene expression pattern during P19 neural differentiation, these known genes were classified into functional groups, based published reports (Table 2). The major functional groups identified include apoptosis, cell-cycle control, cell surface molecules, cytoskeleton, extracellular matrix, extracellular or intracellular signaling, metabolism, neurotransmission, transcription factor and vesicular trafficking. The temporal expression patterns for all these genes can be found in Fig. 1 of the Appendix. For the purpose of brevity, we will focus our discussion on the following three groups: transcription factors, extracellular signaling and cell-cycle control.

The first group of genes is involved in transcriptional regulation (Fig. 3A). Among this group of genes, 5 are from the murine Hox complex: Hox A5, Hox A7, Hox B6, Hox B9 and Hox D8. In our studies, Hox A5 is induced earlier than Hox A7 and Hox B6 is induced earlier than Hox B9. These observations are consistent with previous data showing that the Hox genes are activated in a sequential order from the 3′ most to the 5′ most gene within a collinear Hox gene cluster (Simeone et al., 1990; 1991). In addition, Pbx2, a Hox co-activator (Knoepfler and Kamps, 1997), is induced by RA, as described previously. Concomitant with activation of Hox genes by RA, we observed the repression of mPh1/Rae28, a member of the polyhomeotic gene family (Nomura et al., 1994). The polyhomeotic genes in turn belong to the Polycomb group of genes (Pc-G), which are involved in Hox gene regulation (Krumlauf, 1994). mPh1/Rae28 has been shown to negatively regulate Hox gene expression, as in mPh1/Rae28-deficient mouse embryos, the expression boundaries of Hox genes are expanded (Takihara et al., 1997). The coincidence of mPh1/Rae28 repression with activation of Hox genes implies that the repression of mPh1/Rae2 is responsible for the activation of Hox genes in response to RA. Interestingly, mPh2, another member of the polyhomeotic genes (Hemenway et al., 1998), isactivated, as mPh1 is repressed. The specific temporal expression patterns of mPh1/Rae28 and mPh2 suggests that they might play different roles in the regulation of Hox genes within the

Table 2 RA-induced genes during P19 neural differentiation

Function	GeneBank number	Gene description	Cluster
Apoptosis	AA177976	Apoptosis-related RNA binding protein (Napor-3) Mrna	4
	AA051441	Bcl2-X	5
	W82116	DAP-kinase related protein 1	6
	W34612	Gh/tissue transglutaminase (Tgm2) mRNA	2
	AA268162	*M. musculus* mRNA for calpain-like protease	2
Cell cycle	AA387446	Cdc25 phosphatase homolog (cdc25B)	5
	AA067318	Cyclin G	5
	W16205	TIS21/BTG2	6
	AA154848	TIS21/BTG2	6
Cell surface molecule	AA109951	Beta-2 microglobulin(B2M)	6
	W98974	CD24a antigen	5
	W10683	Lymphocyte antigen 6 complex, locus H (Ly6h)	6
	W83922	Membrane glycoprotein LIG-1	5
	AA242226	N-cadherin/cadherin 2 (Cdh2)	6
	AA034594	N-cadherin/cadherin 2 (Cdh2)	6
	W40832	NLRR-1 for leucine-rich-repeat protein	6
	AA050218	PETA-3 (CD151)	5
	AA511430	Plexin 2	2
	AA250069	Retinoic acid early transcript 1, alpha (rae-1)	3
	AA268592	Transforming growth factor beta induced 68 kDa (Tgfbi) mRNA	6
Chromatin	AA049416	Histone H1.1	6
	AA050562	Meiotic cohesin REC8 mRNA	1
Cytoskeleton	AA061838	Beta-tubulin T beta15 [*R. norvegicus*]	4
	W54533	Drebrin	5
	AA034730	Drebrin	5
	AA048116	Ena-vasodilator stimulated phosphoprotein (Evl)	6
	W08134	Lethal giant larvae homolog 2 (LLGL2) [*H. sapiens*]	1
	AA016762	Microtubule associated testis specific serine/threonine protein kinase (Mtssk)	3
	AA386889	Microtubule-associated protein 2 (MAP2) [*R. norvegicus*]*	4
	AA028410	Microtubule-associated protein tau*	4
	W14559	Nomyosin, light polypeptide 1, alkali; skeletal, fast (MYL1)	5
	AA030447	Peripherin(prph1)	6
	AA032658	Profilin 2 (Pfn2)	6
	AA139628	Profilin 2 (Pfn2)	6
	W33989	rhoB gene [*R. norvegicus*]	6
	W50677	Tubulin [*R. norvegicus*]	5
DNA repair	AA067927	Protein kinase, DNA activated, catalytic polypeptide (prkdc)	2
DNA replication	W91387	DNA polymerase delta 1, catalytic domain	2
	W18484	Mitochondria DNA polymerase gamma	6
	AA000318	Replication protein A3 (14kD) (RPA3) [*H. sapiens*]	5
ER protein	W82577	ER transmembrane protein	5
	AA000093	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1 (KDELR1)	6
	AA016913	Reticulocalbin	5
Extracellular matrix	AA276537	Agrin gene	6
	W54287	Biglycan	5
	AA016661	Chondroitin sulfate proteoglycan 5 (neuroglycan C) (Cspg5)	4
	AA509765	Endomucin-1 mRNA	6
	W88036	Entactin	6
	AA237378	Extracellular matrix protein 1 (ECM1)	N/A
	AA049136	Fibrillin 2 (fbn2) gene	6
	AA175306	Fibrillin 2 (fbn2) gene	5
	AA145458	Fibronectin	3
	AA268927	Glucuronosyltransferase I (GlcAT-I)	5
	W62484	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1 (Hs3st1)	N/A
	AA032581	Heparan sulfate 6-sulfotransferase 1	6
	W20986	Keratin complex 2, gene 6a (Krt2–6a)	4
	AA016810	Mesothelial keratin K7 (type II) [*H. sapiens*]	6
	AA034564	Procollagen type V alpha 2 (Col5a-2)	6
	W99856	Procollagen, type V, alpha 1 (Col5a1)	6
	W33786	Procollagen, type VI, alpha 1 (Col6a1)	4
	W16221	Procollagen, type VI, alpha 1 (Col6a1)	4
	W98013	Procollagen, type XI, alpha	6
	W89883	Procollagen, type III, alpha 1 [*H. sapiens*]	6
	W87014	*R. norvegicus* mRNA for gal beta 1,3 galNAc alpha 2,3-sialyltransferase	5
	W47914	Sialyltransferase 8 (alpha-2, 8-sialytransferase) B (Siat8b)	4
	W85526	s-laminin [*R. norvegicus*]	5
	AA036176	s-laminin [*R. norvegicus*]	5
	AA270625	Tenascin C	4

Table 2 Continued

Function	GeneBank number	Gene description	Cluster
Extracellular signaling	W82677	Bone morphogenic protein (BMP-1)	5
	AA033292	Bone morphogenic protein (BMP-1)	6
	W88094	CXCR-4/fusin	4
	AA059653	Delta-like 1 homolog	6
	AA015264	Delta-like 1 homolog	6
	AA253928	Endothelial monocyte-activating polypeptide I	4
	AA178037	Ephrin-B3 (EFNB3) [*H. sapiens*]	6
	AA467534	Frizzled-4	N/A
	AA030294	Frizzled-1	5
	AA250120	Glial cell line derived neurotrophic factor family receptor alpha(gfra1)	5
	AA049480	BMP-3/GDF-10	4
	AA013895	*Homo sapiens* host cell factor 2 (HCF-2) mRNA	6
	W10072	Insulin-like growth factor 1	2
	AA030603	Insulin-like growth factor II (Igf2)	6
	AA472764	MK gene for midkine*	3
	AA222497	MK gene for midkine*	5
	W98998	Notch-1 mRNA*	6
	AA049699	OSF-1 Pleiotrophin	6
	AA034782	Parathyroid hormone receptor*	5
	W53765	Pigment epithelium-derived factor SDF3	2
	AA209592	Retinoid X receptor alpha	5
	AA473070	Secreted frizzled-related sequence protein 3 (Sfrp3)	6
	AA003297	Stra1/LERK-2/Eplg2 protein/cek5 receptor protein-tyrosine kinase ligand*	5
	W53962	Transforming growth factor beta 2*	4
Intracellular signaling	AA014945	Abelson murine leukemia oncogen	3
	W10726	ADP-ribosylarginine hydrolase	6
	AA002439	Annexin V	5
	AA002514	Brain fatty acid-binding protein (B-FABP) gene	4
	AA176037	Calcium/calmodulin-dependent protein kinase-related peptide [*R. norvegicus*]	4
	W89518	Annexin A2 (anxa2)	4
	W83974	Carboxypeptidase E	6
	W98395	Cell adhesion kinase (Cak)	6
	W81912	Cellular retinoic acid binding protein II (CRABP-II)*	5
	W83609	Cellular retinol binding protein 1 (mCRBPI)*	5
	AA144377	Cyclic nucleotide phosphodiesterase (PDE3A)	6
	AA000227	Diacylglycerol kinase [*R. norvegicus*]	3
	W62969	Fyn protooncogene Fyn(T) (L-fyn(T)	6
	AA027478	G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein (GIT1) [*R. norvegicus*]	3
	AA050000	GTPase-activating protein GAPIII	6
	W57092	Guanine nucleotide exchange factor LFC	6
	AA543573	Hagoromo/WD40-containing F-box protein mRNA	6
	W82182	Heat shock factor binding protein 1 (HSBP1) (*H. sapiens*]	6
	AA002985	HIC-5	2
	W99117	HIC-5	2
	AA241784	Insulin-like growth factor binding protein 5	6
	AA170009	Myristoylated alanine rich protein kinase C substrate	6
	AA175094	Myristoylated alanine rich protein kinase C substrate	6
	AA051250	Neurotrophic tyrosine kinase receptor type 3 (Ntrk3)	6
	AA123762	Neutral sphingomyelinase	5
	AA277332	p38delta MAP kinase mRNA	N/A
	AA032457	Placental alkaline phospatase mRNA	1
	AA067153	Platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit/LIS-1	6
	W99102	Prion protein (PrP) mRNA	4
	AA087796	PDZ domain-containing protein PDZK1	5
	AA032966	protein tyrosine phosphatase TD14 (PTP-TD14) [*R. norvegicus*]	3
	AA200201	proto-oncogene (Frat1)	6
	AA268862	RA70*	5
	W48230	Ral guanine nucleotide dissociation stimulator (Rgds)	6
	AA237366	Serine-threonine protein kinase/AKT8 provirus v-akt oncogene	6
	AA154763	Sphingosine kinase (SPHK1b)	5
	AA032723	Spinophilin [*R. norvegicus*]	5
	AA033417	src homology 2 domain-containing transforming protein D (Shd)	6
	W42241	Stathmin-like-protein RB3 mRNA	4
	AA105224	Tetraspan TM4SF (Tspan-6)	6
	AA222201	TIS11B/BRF1	5
	AA060205	TIS11B/BRF1	5
	AA285969	Ubiquitin carboxy-terminal hydrolase L1 (Uchl1)	4

Table 2 Continued

Function	GeneBank number	Gene description	Cluster
	AA050167	Ubiquitin-conjugating enzyme E2–16 KD [*S. cerevisiae*]	6
	W78330	Ufo oncogene homolog	6
	AA144080	v-ski avian sarcoma viral oncogene homolog (SKI) [*H. sapiens*]	3
Metabolism	AA473227	CHOT1 choline transporter [*R. norvegicus*]	6
	W99217	Dimethylarginine dimethylaminohydrolase 2 (Ddah2)	6
	AA068366	Liver tricarboxylate carrier mRNA [*R. norvegicus*]	6
	AA475004	Low density lipoprotein receptor	6
	W80096	Low-density lipoprotein receptor-related protein (LRp105) [*R. norvegicus*]	6
	AA245413	Mitochonrial carnitine/acylcarnitine carrier protein	6
	AA289571	Phosphorylase kinase alpha I	3
	AA217366	Pyruvate dehydrogenase kinase, isoenzyme 3 (PDK3) [*H. sapiens*]	5
	W18057	Sarcomeric mitochondrial creatine kinase [*R. norvegicus*]	4
Neuroactivity	AA031207	Cocaine and amphetamine regulated transcript (Cart)	4
	AA273669	NMDA receptor glutamate-binding subunit mRNA [*R. norvegicus*]	5
	AA220458	SC2 synaptic glycoprotein [*R. norvegicus*]	6
	W20888	Somatostatin (Smst)	6
	W90827	Voltage-dependent calcium channel beta-3 subunit (cchb3)	4
	AA049807	Voltage-gated calcium channel, alpha-1-G subunit (cchna1)	5
Nuclear envelope	AA270364	Multispanning nuclear envelope membrane protein nurim (NRM29) mRNA [*H. sapiens*]	5
	AA065748	Nuclear pore complex glycoprotein p62	6
	AA271353	Nuclear transport factor 2 (placental protein 15) [*H. sapiens*]	3
RNA splicing	W09867	snRNA-associated Sm-like protein LSm6 mRNA [*H. sapiens*]	N/A
	AA467142	U7 snRNA gene	6
Transcription factor	AA049602	AF9/MLLT3	6
	AA023994	AP-2, alpha (Tcfap2a)*	4
	W89370	AP-2.2 gene (aka AP-2gamma) (Tcfap2c)*	1
	AA198553	Bach protein 2	6
	W62249	CIZ mRNA for Cas-associated zinc finger protein [*R. norvegicus*]	5
	W90859	COUP-TF1/Ear3	6
	AA036604	CtBP1 protein	6
	AA059930	DNA binding protein NFI-B (NfiB)	4
	W55827	DNA binding protein NFI-X (NfiX)	6
	W40683	Early B-cell factor	6
	AA472070	GATA-6	3
	AA000036	General control of amino acid synthesis-like 1 (yeast) (Gcn5l1)	6
	AA289093	Hey1/Hesr-1	6
	W88001	Hey1/Hesr-1	6
	AA033195	Homeobox A5*	5
	W15055	Homeobox A7	N/A
	AA008326	Homeobox B6	6
	W97853	Homeobox B9	6
	W99235	Homeobox D8	6
	W48116	Inhibitor of DNA binding dominant negative helix-loop-helix gene (Id4)	4
	AA237169	LZIP-1 or LZIP-2	6
	W89502	Mesenchyme homeobox 1	2
	W67089	mPh2	6
	W70909	MRFX2 mRNA/rfx2	6
	AA050726	Paired mesoderm homeobox/Phox1	4
	AA387552	Paired-like homeodomain transcription factor (DRG11)	4
	AA062140	Paired-like homeodomain transcription factor (Pitx3)	4
	AA163180	Pleiomorphic adenoma gene-like 2 (PLAGL2) [*H. sapiens*]	6
	AA272260	Smooth muscle LIM protein (Crp2/SmLim)	4
	W14398	Sox4	6
	AA220077	Sox9 [*H. sapiens*]	5
	AA064241	Stra13*	5
	AA003969	Transcription factor PBX2 (PBX2)*	3
	AA037955	Transcriptional repressor RP58 (rp58)	6
	AA124575	Transcriptional repressor RP58 (rp58)	6
	AA185217	Transcriptional repressor RP58 (rp58)	6
	W14113	Twist gene homolog	N/A
	AA028696	Zinc finger protein (Mtsh1) mRNA	5
Vesicular trafficking	AA063753	abc1 mRNA	5
	AA266938	Brain ARF2	6
	AA260594	Clathrin-associated protein 17 (AP17)	6
	AA061343	Vesicle associated membrane protein (VAMP-2)	6
	AA060361	X11/Mint2	6

Table 2 Continued

Function	GeneBank number	Gene description	Cluster
Miscellaneous	W61574	AF1q	4
	AA275865	AF1q	4
	W98956	Amyloid precursor-like protein(APLP2)	4
	AA168437	Brain acid-soluble protein 1 (BASP1)	4
	W08432	Brain protein 44-like protein (Brp44l) mRNA	4
	W08056	bup gene, 5′ of bmi-1 proviral insertion locus	6
	AA027699	C184M protein	5
	AA498788	CGI-38 protein [*H. sapiens*]	6
	AA050300	Cold-inducible RNA-binding protein (CIRP)	6
	W83904	Cyclophilin C (cyp C)	6
	AA242287	DXImx39e protein (DXImx39e)	6
	AA399831	DXS6673E for mental retardation Xq13.1 or ZNF261	1
	AA060697	DXS6673E for mental retardation Xq13.1 or ZNF261	1
	W37018	DXS6673E for mental retardation Xq13.1 or ZNF261	1
	AA051563	ETO/MTG8-related protein ETO-2	4
	W15931	Glioblastoma amplified sequence (Gbas)	6
	AA015221	Hematopoietic stem/progenitor cells protein MDS033 (MDS033)	6
	AA022130	Heme-binding protein	5
	AA011823	HU-antigen/Elav14	4
	W62925	Leptin receptor gene-related protein	6
	AA023656	Lipoma HMGIC fusion partner (LHFP) mRNA [*H. sapiens*]	4
	W98359	MAGE tumor antigen D1 (MAGE-D1) [*H. sapiens*]	5
	W08774	MMLP gene for muscle LIM protein	6
	AA003408	*Mus musculus* neuronal-specific septin 3 (Sep3)	6
	W66992	Nedd-4-like ubiquitin-protein ligase WWP2 mRNA [*H. sapiens*]	6
	W82737	Neural precursor cell expressed developmentally downregulated Nedd9 (Nedd9)	6
	W34802	Numblike (m-nbl)	6
	AA000945	P311/Pentylenetetrazol-related mRNA (PTZ-17)	6
	W62819	P311/Pentylenetetrazol-related mRNA (PTZ-17)	6
	AA259807	P311/Pentylenetetrazol-related mRNA (PTZ-17)	6
	AA413622	p53 responsive (EI24)	6
	AA030780	Peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase (Peci)	6
	AA473010	Peroxisomal membrane protein PMP 24	6
	W82416	PIPPin	4
	W82945	PIPPin	4
	W89268	Pseudo theta 2 globin gene	6
	AA015434	Retinoic acid-responsive protein (Stra6)*	5
	AA276440	Selenoprotein P	N/A
	AA007970	Stannin	6
	W97985	Tax interaction protein 1 mRNA [*H. sapiens*]	4
	AA111643	Tax interaction protein 1 mRNA [*H. sapiens*]	6
	AA117023	Thioredoxin-dependent peroxide reductase (tpx)	6
	W18828	Ulip protein/dihydropyrimidinase-like 3	6
	AA014335	von Hippel-Lindau disease (VHL) gene	6
	AA501052	Xin	4

mixed population of cells generated from undifferentiated P19 EC cells.

Genes encoding bHLH family of transcription factors have been implicated in the determination and differentiation of neurons. Included among these are the proneural gene Mash1 and the neurogenic gene NeuroD. Because our microarrays do not contain these genes, we can not assess their expression patterns directly. However, another bHLH gene family, the Ebf/Olf family, is also involved in neural determination and differentiation (Dubois et al., 1998; Prasad et al., 1998; Garel et al., 1999), and is represented in our arrays (Garel et al., 1999). We observed that a member of the Ebf/Olf family, Ebf, is activated by RA during P19 neural differentiation. Ebf induction is significantly earlier than the reported induction of proneural and neurogenic genes (We showed that Ebf is activated 10 hours after RA treatment while Mash1 and NeuroD have been reported by others to be activated on day 3 and 4, respectively Itoh et al., 1997). This result suggests the very testable hypothesis that Ebf function is potentially upstream of and regulates the expression of the proneural and neurogenic genes.

Activator protein-2, which include AP-2α, AP-2β and AP-2.2, plays an important role in activation of neural gene expression (Oulad-Abdelghani et al., 1996). Our data confirm the previous observation that AP-2α and AP-2.2 genes are induced by RA in P19 cells (Oulad-Abdelghani et al., 1996; Philipp et al., 1994). Furthermore, we extended these findings by demonstrating that

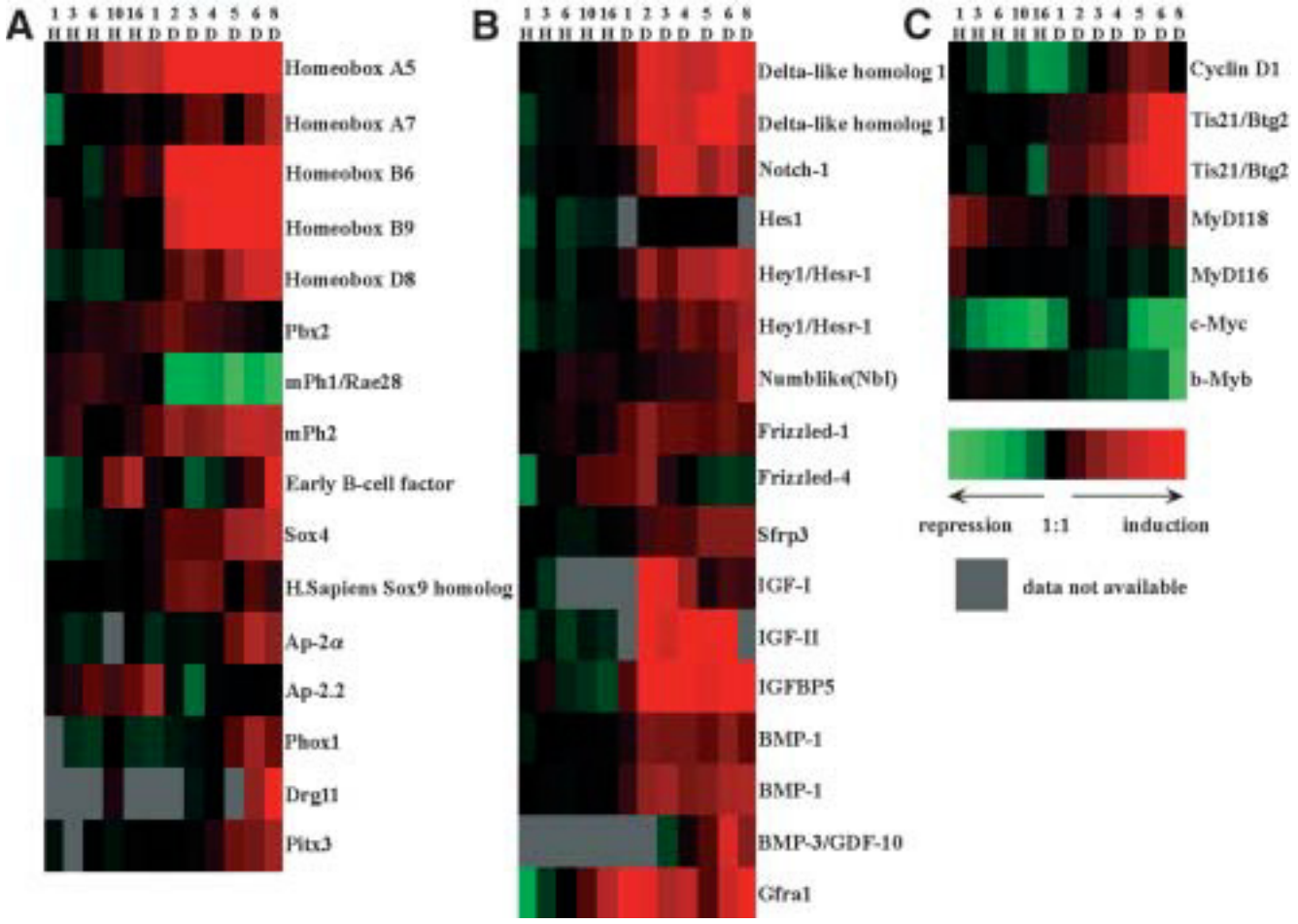

Fig. 3 Expression pattern of a selected set of genes induced by RA during P19 neural differentiation. The expression pattern of a selected set of genes belonging to transcription factor (**A**), extracellular signaling (**B**) and cell cycle control (**C**) is shown. The expression level of each gene is shown in a horizontal bar. The intensity of red and green color is proportional to the relative gene induction (*red*) or repression (*green*).

AP-2.2 responds early to RA: it is activated within 6 hours of RA treatment, and its expression is lost by day 2. In contrast, AP-2α does not respond to RA until day 5, after which it persists. Taken together this suggests that AP-2α and AP-2.2 function differently during P19 neural differentiation.

Three paired homeodomain-containing genes, Phox1, Pitx3 and Drg11, are expressed during the final stage of P19 neural differentiation. Pitx3 has been shown to be expressed by mesencephalic dopaminergic neurons (Smidt et al., 1997), whereas Drg11 expression is restricted to sensory neurons and a subset of their CNS targets (Saito et al., 1995). The presence of both of these transcripts in our cell populations makes it probable that multiple neuronal subtypes are generated in our system. It will be of considerable interest to determine the target genes of these factors and the resulting phenotypes generated by their ectopic expression in these cells.

Besides transcription activators, a number of transcription repressors, Hey1, Id4, mPh2, Stra13, Rp58 and Twist, are also activated during P19 neural differentiation. The activation of these genes during P19 neural differentiation clearly indicates that this program is a balance between transcription activation and repression. All together, there are 38 transcription factors found in 224 known genes (Table 2). This significant percentage (~18%) raises the possibility that a large number of novel transcription factors are likely to be among the 309 ESTs identified in our study.

The second group of genes is involved in signaling (Fig. 3B). Prominent among this group are Delta-like homolog 1 and Notch-1. The Delta-Notch pathway operates in neural differentiation. The neural determination genes induce the expression of Delta and activate Notch signaling in lateral cells, resulting in the inhibition of determination gene expression and inhibition of differentiation (Artavanis-Tsakonas et al., 1999). Notch signaling is mediated by transcription repressors belonging to the *Drosophila* hairy and enhancer of split genes, Hes1 and Hes5. Activation of Hes1 inhibits both neuronal and glial differen-

tiation (Sasai et al., 1992) while activation of Hes5 promotes gliogenesis in the expense of neurogenesis (Hojo et al., 2000). Our results showed that during P19 neural differentiation, Hes1 expression is not changed even when Notch-1 and Delta-1 are activated. This is consistent with previous observation that expression of a constitutively activated form of Notch in P19 cells inhibits neurogenesis but not gliogenesis in this cell culture model system (Nye et al., 1994). While our array cannot directly assess Hes5 expression, a novel member of the Hes family, Hey1, is activated during P19 neural differentiation, suggesting its involvement in the inhibition of neuronal differentiation but not glia differentiation. Finally, a Numb-like gene, Nbl, is expressed at day 6 and 8 of P19 neural differentiation. Both Numb and Nbl are inhibitors of Notch signaling pathway. In the developing neocortex, Numb is expressed in neural progenitor cells while Nbl is expressed in postmitotic neurons (Zhong et al., 1997). The activation of Nbl suggest that cell fate determination through Notch signaling in P19 neural differentiation involves both cell-extrinsic (mediated by Delta) and cell-intrinsic (mediated by Nbl and possibly Numb) mechanisms.

Another major signaling pathway involved in neurogenesis is Wnt signaling pathway. Wnt proteins interact with members of Frizzled family of receptors to regulate many of the early events in the developing nervous system (Patapoutian and Reichardt, 2000). We observed that two members of the frizzled receptor gene family, Frizzle-1 and Frizzled-4, are activated by RA each with distinct temporal expression patterns. Frizzled-4 is induced as early as 10 hours after RA treatment but its expression lasts only until day 2. In contrast, Frizzled-1 does not responds to RA until day 1 but remains overexpressed for the remainder of the time course. Secreted frizzled-related protein 3 (Sfrp3), which shares amino acid similarity with Frizzled receptors, and act as Wnt antagonists by competing for Wnt proteins, is also activated during late stage of P19 neural differentiation. This result emphasizes the importance of attenuation of Wnt signaling during late stages of P19 neural differentiation. In fact it has been shown that expression of Wnt-1 can induce some aspects of early neural differentiation but persistence of expression perturbs later differentiation process (Patapoutian and Reichardt, 2000).

The third signaling pathway we observed is the insulin-like growth factor (IGF) pathway. Both IGFs and IGF receptors are expressed in the nervous system during early embryonic development and IGF signaling has been found to be important for growth cone motility, neurite outgrowth and the inhibition of apoptosis during neural differentiation (Feldman et al., 1997). Consistent with these roles, both IGF-I and IGF-II are induced by RA. Concomitant with the expression of IGFs, IGF binding proteins (IGFBP), IGFPBP-5, is also induced. IGFBP-5 possibly plays a role in the regulation of IGF signaling during P19 neural differentiation.

The fourth signaling pathway is the BMP pathway. Activation of BMP1 starts at day 1 and lasts until day 8. BMP1 is a metalloprotease that degrades chordin, a BMP4 antagonist (Wardle et al., 1999) and BMP4 has been shown to promote gliogenesis during P19 neural differentiation (Bani-Yaghoub et al., 2000). Therefore the activation of BMP1 might be involved in gliogenesis during P19 neural differentiation. BMP3 is also induced by RA, suggesting it plays a role in neural differentiation.

In addition to these signaling pathways, our analysis also suggests the involvement of other pathways in P19 neural differentiation. For example, glial cell line derived neurotrophic factor(GDNF) family receptor alpha (gfra1) is induced by RA as early as 10 hours after RA treatment and the upregulation persists throughout the timecourse, indicating a role for GDNF in the early steps of P19 neural differentiation. The involvement of all these pathways emphasize the importance of extrinsic signals for neural differentiation and our data suggest numerous experimental modifications of these signaling events which might influence cell behavior and differentiation fate. Based on the global nature of gene expression we have documented, we believe that the microarray system described here would serve as a useful tool to monitor the consequences of altering these signaling pathways and the time course experiment reported here serves as the baseline for these subsequent experiments.

The third group of genes is involved in the control of cell cycle (Fig. 3C). The expression of cyclin D1 has three stages. Three hours after RA treatment expression of cyclin D1 begins to decrease and it reaches its lowest level at between hour 16 and day 1. Cyclin D1 expression then steadily increases until it reaches its highest level between day 5 and 6, and at day 8, the expression of cyclin D1 returns to the control level. This expression pattern suggests 3 stages in cell-cycle progression during P19 neural differentiation. First, RA causes an early arrest in cell cycle. This arrest in cell cycle might coincide with the commitment stage of P19 neural differentiation. MyD118 and MyD116, genes that cause cell growth arrest in myeloid differentiation (Liebermann and Hoffman, 1998), are induced 1 hour after RA treatment, just prior to the decrease in cyclin D1 expression, and might be involved in this early cell-cycle arrest. After P19 cells are committed into neural progenitor and precursor cells, the progenitor and precursor cells reenter the cell cycle, as suggested by the increase of cyclin D1 expression from day 2 to day 6. This coincides with the activation of Tis21/Btg2, a gene indicative of the switch from proliferative to neuron-generating cell divisions in neuroepithelial (NE) cells (Iacopetti et al., 1999). Finally, the post-mitotic neurons exit cell-cycle and the expression of cyclin D1 returns to normal. Interestingly, MyD118 but not MyD116 is activated at this stage. The expression of c-Myc and b-Myb genes, two genes whose expression is correlated with cell proliferation (Weston, 1998; Nasi et al., 2001) is also shown. There are two

drops in c-Myc expression during P19 neural differentiation. Consistent with early cell-cycle arrest, the expression of c-Myc drops immediately after RA induction until day 1. Expression of c-Myc returns to normal at day 2 and 3 and then decreases steadily until day 8. In contrast, expression of b-Myb does not change until day 2 when it starts to decrease. The decrease of c-Myc and b-Myb expression at day 5 and 6 seems to be incongruous with the increase of cyclin D1 expression, but it might represent novel properties of neuron-generating cell divisions compared with proliferation in the germinal zones.

Clustering of genes induced during P19 neural differentiation using hierarchical clustering

Genes can be classified not only by their functions but also by their temporal expression patterns. Insights into the function of uncharacterized genes can be gained by their association with known genes in the same clusters. We employed a modified version of the hierarchical clustering method in the Stanford Gene Cluster software (Eisen et al., 1998). Instead of using the correlation coefficient provided in the software, we implemented the jackknife correlation coefficient for the clustering. Compared to the correlation coefficient, the jackknife correlation coefficient is more robust to data with single outliers (Heyer et al., 1999). The 555 RA-induced cDNA clones fall into six clusters with distinct expression kinetics following RA induction (Fig. 4 and 5). The known genes found in each cluster are summarized in Table 2. The ESTs found in each cluster are listed in Table 1 of the Appendix.

RA modulates transcription of its target genes through binding to nuclear receptors RARs and RXRs. Based on the difference in binding affinities and kinetics for different promoters, RA-induced genes are likely to show different patterns of expression after RA induction, as was observed in our cluster analysis. The activation of some genes is obvious as early as 3 hours after RA treatment but their expression drops off after day 1. These genes, represented in cluster 1, include transcription factor AP-2.2 and placental alkaline phosphatase. Genes in cluster 3 are activated between 10 and 16 hours after RA treatment and their expression lasts until day 4. A number of genes involved in intracellular signaling, such as Abelson murine leukemia oncogene (Abl), diacylglycerol kinase, G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein, protein tyrosine phosphatase TD14 (PTP-TD14) and v-ski are included in this group. Genes in cluster 2 have a transient expression pattern: expression is induced after 1 day of RA treatment but decreases after day 4. The late responsive genes are represented in cluster 4, 5 and 6: the induction of these genes starts at day 5, 1 and 2, respectively and they remain active

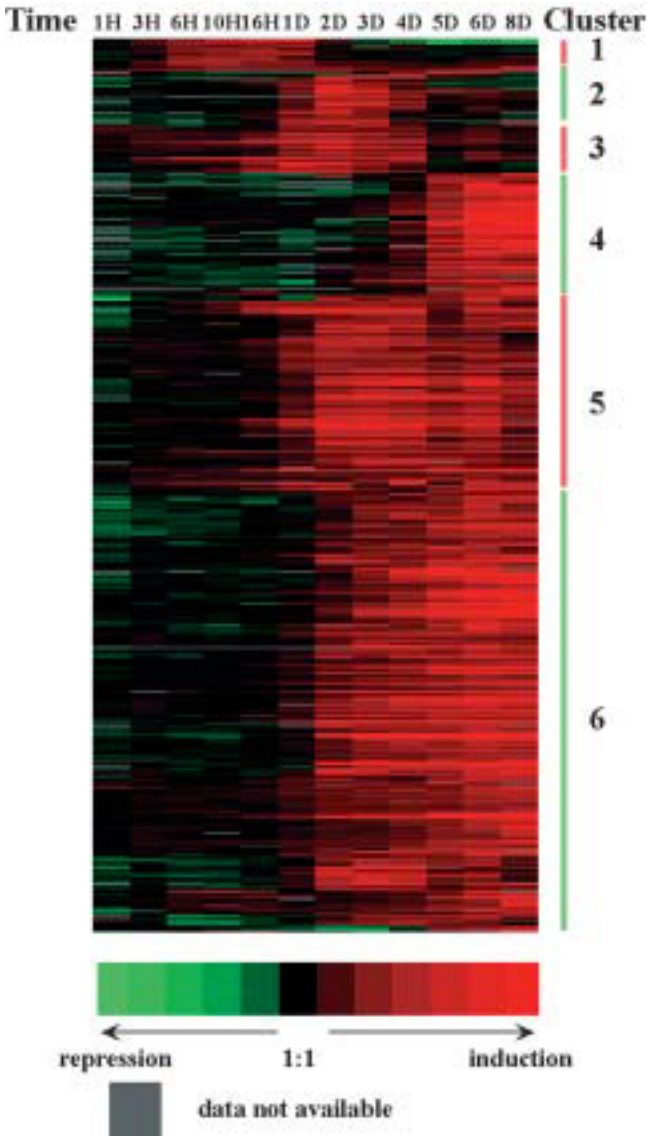

Fig. 4 Cluster analysis of temporal gene expression patterns during P19 neural differentiation. A total of 555 genes selected for this analysis were clustered into several groups on the basis of the similarity of their temporal expression pattern. The expression level of each gene is shown in a horizontal bar. The intensity of red and green color is proportional to the relative gene induction (*red*) or repression (*green*).

throughout the time course. The expression profiles of cluster 5 are somewhat heterogeneous. Some genes, such as cellular retinol binding protein 1 (mCRBPI), cellular retinoic acid binding protein II (CRABP-II), Stra1, Stra6, Stra13, Homeobox A5 and GDNF family receptor alpha(Gfra1) are activated before day 1 while the majority of the genes are activated at day 1. Finally, cluster 4 includes a number of neuron-specific genes, such as microtubule-associated protein 2 (MAP2), microtubule-associated protein tau, amyloid beta (A4) precursor-like protein 2 (Aplp2) and chondroitin sulfate proteoglycan 5/neuroglycan C, consistent with the fact that neurite

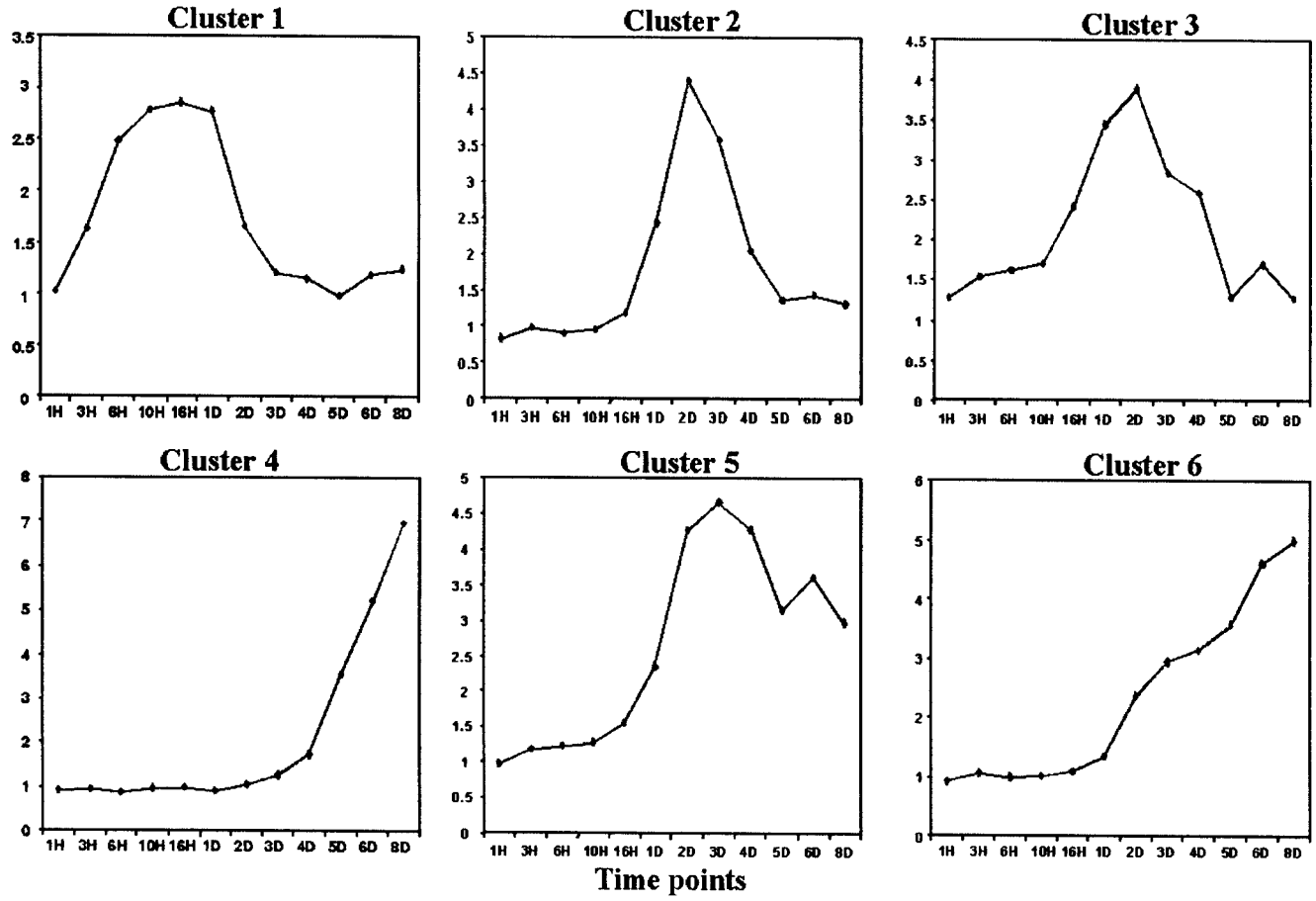

Fig. 5 Average expression profiles for each gene cluster. The expression profile of each gene in a cluster was averaged. Six clusters were derived from the cluster analysis, showing different induction kinetics.

outgrowth and other neural morphological changes occur during this period.

In conclusion, we profiled global gene expression patterns during P19 neural differentiation, using microarrays containing 9000 cDNA clones. Based on our criteria, 555 genes were induced by RA and selected for analysis. Grouping of the 222 known genes by function reveals that important transcription factors and extracellular signaling pathways that function in neural development *in vivo* are also involved in P19 neural differentiation. In addition, clustering of the induced genes according to their temporal expression pattern reveals 6 modes of expression with distinct kinetics, suggesting the existence of different phases in P19 neural differentiation. Taken *in toto*, this study integrates thirteen global snapshots of the gene expression patterns used during neural differentiation into an initial dynamic picture of this complex process. This study has generated many new and testable hypotheses for the role of specific genes in neural differentiation in cell culture and *in vivo*. Our results reported here form the framework for study these studies of the effect of various intrinsic and extrinsic factors on gene expression in neural differentiation.

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