Neurosecretion Competence
A COMPREHENSIVE GENE EXPRESSION PROGRAM IDENTIFIED IN PC12 CELLS*§

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The phenotype of neurosecretory cells is characterized by clear vesicles and dense granules, both discharged by regulated exocytosis. However, these organelles are lacking completely in a few neurosecretion-incompetent clones of the pheochromocytoma PC12 line, in which other specific features are maintained (incompetent clones). In view of the heterogeneity of PC12 cells, a differential characterization of the incompetent phenotype based on the comparison of a single incompetent and a single wild-type clone would have been inconclusive. Therefore, we have compared two pairs of PC12 clones, studying in parallel the transcript levels of 4,200 genes and 19,000 express sequence tags (ESTs) by high density oligonucleotide arrays. After accurate data processing for quality control and filtration, a total of 755 transcripts, corresponding to 448 genes and 307 ESTs, was found consistently changed, with 46% up-regulated and 54% down-regulated in incompetent versus wild-type clones. Many but not all neurosecretion genes were profoundly down-regulated in incompetent cells. Expression of endocytosis genes was normal, whereas that of many nuclear and transcription factors, including some previously shown to play key roles in neurogenesis, was profoundly changed. Additional differences appeared in genes involved in signaling and metabolism. Taken together these results demonstrate for the first time that expression of neurosecretory vesicles and granules is part of a complex gene expression program that includes many other features that so far have not been recognized.

Expression of two classes of secretory organelles, small translucent vesicles (clear vesicles) and dense content granules of larger size (DGs), is the typical trait of neurosecretory cells.

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§ The abbreviations used are: DG, dense content granule; NI, neurosecretion incompetent; WT, wild-type; EST, expressed sequence tag; AD, average difference; CV, coefficient of variation; MFC, minimum detectable -fold change; CG, chromogranin; SG, secretogranin; IP3, inositol 1,4,5-trisphosphate; CGA and CGB, chromatogranins A and B; H+-pump, vacuolar H+-pump, vATPase; ICA, inositol cell antigen 512; Rab3A and C, small G protein; STX1, syntaxin; SNAP25 and SNAP23, soluble NSF attachment proteins of 25 and 23 kDa; SV2 A–C, synaptic vesicle protein 2 A–C; VAMP 1 and 2, vesicle-associated membrane proteins 1 and 2 (synaptobrevin).
In the present study, neurosecretion competence has been analyzed in a broad perspective. Using a high density oligonucleotide array approach, we provide for the first time a global view of gene expression profiles related to neurosecretion competence. To ensure the highest confidence to our analyses, we removed the bias due to single events by analyzing two independent NI PC12 clones (which most likely have the same genetic defect, because no complementation is observed after fusion between them, see Ref. 13), compared with two independent WT PC12 clones. Our analysis is focused on genes expressed at similar levels in both clone pairs (WT/WT and NI/NI) and at significantly different levels between pairs (WT/NI).

EXPERIMENTAL PROCEDURES

WT and NI clones were generated and isolated at different times and with slightly different procedures from PC12 populations (ATCC) that had been transfected with vectors coding for neomycin resistance. WT-15 and NI-27 were obtained following transfection with a pMV7 vector; NI-Trk with an episomal vector carrying the TrkA cDNA, WT-CG with an empty pcDNA vector (Invitrogen). All clones were grown in Dulbecco's modified Eagle's medium 10% HS (Euroclone) and 5% FCIII (HyClone) in 10% CO2.

Sample Preparation and Chip Hybridization—Aliquots of clones at three different passages were lysed in 10 ml of RNAzolB (AMS Biotechnology), and total RNA, extracted according to the manufacturer's protocol, was used for target preparation (14). Briefly, 20 μg of total RNA were reverse-transcribed using an oligo(dT)-T7 primer; the target was biotin-labeled by in vitro transcription and fragmentation. The fragmented cRNAs (45 μg) from different clones were mixed with four spiked controls (Bioll, BioC, BioD, and Cre at 1.5, 5, 25, and 100 pm, respectively) and applied to Affymetrix GeneChip rat genome U34 arrays. A total of 26,202 rat sequences were tested on three arrays (corresponding to 4,204 independent genes in GenBankTM and 19,408 ESTs in UniGene, build 34) and hybridized overnight at 45 °C. After washing (at 50 °C) and antibody amplification, the arrays were stained and scanned in a Hewlett-Packard scanner. The raw data, exported from the Affymetrix GeneChip software program, were subsequently analyzed with ad hoc programs. For comparisons between arrays, the median signal intensity of each chip was normalized to the median signal intensity of all chips. For each gene the mean AD, the standard deviation, the NI/WT -fold change and the t test p value of the change were calculated. ADs from undetectable genes (≤ 0) were all set to 20 AD to make calculations of -fold changes possible.

Analysis of the Chip Data Quality—Different parameters were used to assess the quality of experimental data. The coefficient of variation (CV) was computed for each gene exhibiting a mean average difference (AD) of significance. CV is directly related to the reproducibility of the data: 1) the smaller the CV the more precise the measurement; 2) the coefficient of variation (CV), derived from standard deviation of ADs divided by the mean AD of each transcript. CV is a detailed analysis was performed to assess the quality of the data is a major concern of the GeneChip hybridization approach. To solve the problem in our experimental conditions where the vertical lines indicate absolute value) was calculated using the standard curve method (15).

RESULTS

Comparison of Gene Expression between WT and NI Clones—To obtain fully reliable data characterized by high sensitivity and reproducibility, we optimized sample collection and data analysis. Details about the procedures are given under “Experimental Procedures.” For each clone, triplicate RNA samples were collected from different passages on different days and hybridized to a set of arrays testing rat mRNA and EST sequences. For each comparison the following parameters were calculated: 1) the normalized mean average difference (AD), representing the hybridization intensity of each transcript; 2) the coefficient of variation (CV), derived from standard deviation of ADs divided by the mean AD of each transcript. CV is directly related to the reproducibility of the data: the smaller the CV the more precise the measurement; 3) a t test p value calculated for each transcript in each comparison, based on six AD measurements.

The global AD values, obtained by pair-wise comparisons of the NI and WT clones, are shown in the scatter plots of Fig. 1 (A and B). Between the two WT clones the fraction of the analyzed transcripts showing invariant expression (difference ≤2-fold, where the vertical lines indicate absolute value) was 79%. The remaining 21% transcripts, most of which are of the low expressor category, were up- or down-regulated in one clone with respect to the other (Fig. 1A). A slightly larger difference in expression levels was observed between the two NI clones (Fig. 1B), with 70% of the transcripts invariant and 30% variant. The comparison between NI and WT clones showed 33% (NI-Trk/WT-15) and 29% (NI-27/WT-15) variant transcripts (Fig. 1, C and D, respectively). Similar observations were made when comparing either one of the NI clones with the second WT clone, WT-CG (not shown).

Northern Blot Analysis—Total RNA (20 μg) or poly(A)RNA (5 μg) from WT and NI clones were separated on 1.2% agarose-2.2 mM formaldehyde denaturing gels and transferred (Turboblotting system, Schleicher & Schuell) onto nylon membranes (Byodyne A, Pall). Hybridization probes were reverse transcription-PCR products obtained from WT-15 cDNA, eluted from agarose gel (Qiaex II extraction kit, Qiagen) and labeled with [32P]dCTP (3000 Ci/mmol, Redivue, Amer sham Biosciences) by the random priming reaction (Readyprime kit, Amersham Biosciences). Amplification primers were designed on the full-length cDNA sequences reported in GenBankTM accession numbers given in Table I (see Supplemental Material). The cDNAs for Phox2a, Phox2b, and MASH1 were a kind gift of Dr. J. F. Brunet. All probes were confirmed by sequencing. Hybridizations, carried out over night at 65 °C, were followed by washes at 65 °C in 0.1x SSC-0.1% SDS.

Quantitative Reverse Transcription-PCR—For each sample, cDNA was synthesized from 1 μg of DNase-treated total RNA, previously used for microarray target preparation, using random primers. Quantitative PCR was performed on a Lightcycler using SYBR green detection. Primer sequences were the following: glyceraldehyde-3-phosphate dehydrogenase (GenBankTM accession number M17701): 5' forward, TGGCAGATTGTGACATCAAGAA; 3' reverse, TGCTGTGTAAGTCAGAGA; ribosomal phosphoprotein (GenBankTM accession number X15096): 5' forward, CTAGTGCTCTACTCCTCA; 3' reverse, GGGGCTTAGTCGAAGAGACC; rat Munc18-1 (GenBankTM accession number L26087): 5' forward, TGAATGCTAGCAGCTGTTGCT, 3' reverse, TCACTCTGACCTTTGACCTCT.

Samples contained Fast-start DNA master SYBR green I mix (Roche Molecular Biochemicals), 3 mM MgCl2, and 500 nM of each primer in a 20-μl volume. The PCR reaction conditions were as follows: 10 min at 95 °C, 30 cycles of 10 s at 95 °C, 5 s at 60 °C, and 15 s at 72 °C, followed by a melting curve analysis. The expression levels were normalized to the levels of either glyceraldehyde-3-phosphate dehydrogenase or rat acidic ribosomal phosphoprotein. Relative mRNA abundance was calculated using the melting curve method (15).
3200 and 6400. Because the CV is directly correlated to the minimal detectable -fold change (Fig. 2B), CV values < 30% allowed the detection of significant -fold changes ≥2, whereas with larger CVs the MFC became much larger. A CV of 45% still allows the detection of a 7-fold change. This analysis indicated that AD values below 100, corresponding in WT-15 to 16.8% of the whole analyzed transcript population (Fig. 2C), were largely unreliable. Therefore, only transcripts with AD values of ≥100 were considered for further analysis. Fig. 2D shows the distribution of the CVs for the 16,860 transcripts with AD ≥ 100. Of these, 76% exhibited CV values < 30% and 12% values in the 30–45% range. Only 12% (mostly with ADs in the low 100–200 range) had CV values > 45%, incompatible with precise MFC measurements.

Based on this quality evaluation, we proceeded to the comparative analysis of transcript expression in NI and WT clones. To be called “differentially expressed,” a transcript had to meet the following requirements: 1) in at least one pair of clones, its AD value had to be ≥100; 2) its average NI/WT clone pair -fold change had to be ≥2; 3) with a lower change the transcript was considered invariant; 3) within both the NI and the WT pairs the -fold change had to be <2 or >-2 (see Fig. 3, A and B); and 4) in at least one NI/WT comparison the t test p value of the change had to be ≤0.05.

NI Cells are Bona Fide Neuroendocrine Cells—Despite the lack of neurosecretion competence, NI cells have an overall gene expression profile very similar to WT neurosecretory PC12, as shown in Fig. 1 (C and D). In fact about 70% of their tested genes are similarly expressed. In previous studies, NI-27 cells had already been shown to express markers typical of the WT neurosecretory PC12 cells, such as tyrosine hydroxylase, neuronal-kinesin, synapsin I, neurofilament H, and voltage-operated Ca2+ channels (8, 11). This list can now be expanded based on the present analysis. Among the ~4200 known genes, 74 transcripts of recognized neuronal specificity were found to be expressed at similar levels in both NI- and WT-cells (Table III, Supplemental Material). Among these genes are neuronal voltage-operated Ca2+ and Na+ channels subunits; cytoskeletal proteins, such as MAP1 and MAP2; markers of the dopaminergic phenotype, such as the transcription factor Nurr1 and the sodium-dependent dopamine transporter; and others. Furthermore a few neuronal-specific transcripts, such as N-methyl-D-aspartic acid receptor 1, neurexin II, and presynaptic protein sap102, are up-regulated in NI clones (see Table I, Supplemental Material). The neurosecretory nature of NI cells was further

Fig. 1. Scatter plot analysis of gene expression levels. In each panel the mean transcript AD values (ranging from 0 to 88,000) of the two clones indicated in the abscissa and ordinate were plotted one against the other in a logarithmic scale. The diagonal line crossing the intersection shows 100% identity in expression. The parallel lines above and below the intersecting line indicate cut-offs at 2- and 5-fold difference. The color scale from blue to red shown in A indicates t test p values from 0 to 1 and is valid for all panels. The histogram inset in each panel shows the distribution of the analyzed genes, grouped according to -fold changes: 0–2; 2–6; 6–12; 12–25; 25–50; 50–200; >200, negative or positive depending on whether up- or down-regulated in the clone of the abscissa. In the insets the histograms of the 0–2 groups, representing invariant genes, are shown in white; the percentages of up-regulated, invariant, and down-regulated genes in the clone of the abscissa are indicated above the histograms.
confirmed by the inspection of gene expression in a fibroblast line, 3Y1, analyzed in an analogous microarray study (16).

Identification of Up- and Down-regulated Transcripts—When the raw data were filtered according to the above specified criteria, the number of variant transcripts dropped from a total of 6,686 and 5,875 (observed by separate comparison of NI-Trk and NI-27 to WT 15, respectively) to 755. Of the latter, 46%, i.e. 346 (176 encoding proteins with known function; 52 with unknown function, and 118 ESTs) were up-regulated, whereas 54%, i.e. 409 (169 known, 51 unknown, and 189 ESTs) were down-regulated in the NI clones (Figs. 3 C,4 A, and 4 B).

Within these populations, high differential expression (-fold change >10) was observed in 83 (43 known, 11 unknown, and 29 ESTs) up-regulated and 70 (34 known, 12 unknown, and 24 ESTs) down-regulated transcripts. In addition, 82 transcripts (38 known, 5 unknown, and 39 ESTs), which were clearly expressed in WT clones (median AD = 656, range 112–40,000), remained undetectable (Fig. 5 C) in both NI clones (AD < 20). Up- and down-regulated transcripts with known function, grouped in the pie chart slices of Fig. 4, are listed individually in the Supplemental Material (Table I). The information provided includes, for each gene, the accession number, the function, and the average WT/NI -fold expression change. Gene transcripts undetectable in the NI clones are also given in the Supplemental Material (Table II).

The genes with known function, either down-regulated, up-regulated, or undetectable in the NI clones, were assigned to functional classes as shown in the pie charts of Figs. 4 (C and D) and 5 (D–F). The assignment was made based on Swiss-Prot and GenBank annotations and on recent publications. Whenever possible, transcripts were annotated based on their degree of homology with known genes (BLAST probability value scores < 10E-20). Genes of general significance, involved in metabolism, signaling, and coding for receptors, appeared in both the up- and down-regulated groups. The same occurred with smaller groups of genes (cytoskeleton, adhesion, and membrane trafficking). Nuclear factors were more numerous among the down-regulated (36, i.e. 21%) than among up-regulated (23, i.e. 13%) transcripts. The numerous genes involved in neurosecretion were found exclusively among down-regulated genes (see Fig. 4, compare C to D). Among the genes up-regulated ≥10-fold, the contribution of the various groups was only slightly changed with respect to the genes up-regulated ≥2-fold (compare Fig. 4 C to Fig. 5 D). In contrast, among down-regulated genes the contribution of neurosecretion was markedly higher (26 to 13%, compare Fig. 4 D to Fig. 5 E). In the groups of genes undetectable in the NI clones (Fig. 5 F) the contribution of neurosecretion and nuclear factors was around 17% each, the rest being contributed by metabolism, receptors, signaling, and others.

Genes Involved in Specific Cell Functions—We then focused on genes whose products are either directly involved or indirectly linked to neurosecretion, including those dealing with transcription, membrane trafficking, receptors, and signaling.
Genes involved in other cellular processes were not studied in detail.

In NI clones, the extensive down-regulation of the genes coding for proteins directly involved in neurosecretion, already shown in Figs. 4D and 5E, is illustrated in the schematic picture of Fig. 6, where CVs and DGs are presented as a single organelle docked to the plasmalemma. Of the genes coding for three cytosolic proteins necessary for neurotransmitter biogenesis and DG/CV circulation, two (tyrosine hydroxylase, the limiting enzyme in catecholamine biosynthesis (17), and synapsin 1, a protein binding CVs of the reserve pool (18)) were unchanged, whereas the third, the acetylcholine-synthesizing enzyme choline acetylase (19) was down-regulated. Also down-regulated were fatty amide hydrolase, which degrades neuro-modulatory fatty acid amides (20), and GTP cyclohydrolase I (21), which is involved in the biogenesis of amine neurotransmitters (22). The genes coding for the proteins of the DG lumen destined to be discharged by exocytosis, chromogranins and secretogranins (CGs and SGs, see Refs. 23 and 24; PC12 cells do not express the convertases, PC1 and PC2 (25)), were all considerably down-regulated. Also down-regulated were the SH2 domain of Grb2 (26), a SH2 domain-containing protein-tyrosine kinase (27), and the SH2 domain of Grb2 (28), a SH2 domain-containing protein-tyrosine kinase (29). The possibility that the low level observed in WT-CG was due to a unique defect of the clone remains open.

Invariant expression predominated in the case of cytosolic proteins that work as members of multimeric complexes involved in exocytosis and/or by binding to critical domains of the DG/SV and plasma membranes (see Ref. 1). In this group, down-regulation was evident only for the genes of the GTP-binding proteins, Rab3A (30), Rab3B (31), and Rab5 (32), and for one of their targets, Rabphilin 3A (33, 34). The gene for dopamine-beta-hydroxylase (35), the noradrenaline-synthesizing enzyme localized both in the membrane and within the lumen of DGs, was also down-regulated in the two NI clones, however, only with respect to WT-15. The possibility that the low level observed in WT-CG was due to a unique defect of the clone remains open.

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group, inclusion of the Munc18-1 gene, a syntaxin1-binding protein (40), was confirmed by quantitative PCR (see Supplemental Material, Fig. S1). On the other hand, the genes of proteins known to operate in exocytotic processes distinct from neurosecretion, including the SNAREs SNAP23 and cellubrevin (41), were not down-regulated but rather invariant or up-regulated in the NI clones (Fig. 6 and Supplemental Material, Table I).

Genes related to the other investigated functions will be presented only briefly. For details refer to the Supplemental Material (Table I and information therein). In NI clones, a clear dissociation emerged between two processes often considered coupled to each other in neuroendocrine cells, i.e. regulated exocytosis and endocytosis (42). Despite the absence of the first, the genes of the second process were in fact invariant. Likewise, genes of other forms of trafficking, including the endosome-to-lysosome shuttle, were invariant or up-regulated. In contrast, profound changes were observed in the expression of genes involved in receptors and signaling proteins. Here the most impressive result was the opposite regulation of the channels inducing Ca\textsuperscript{2+} release from intracellular stores: ryanodine 2 (−15-fold) and IP\textsubscript{3} (+4-fold) receptors (43, 44). The latter change was accompanied by the parallel up-regulation of various IP\textsubscript{3}-generating receptors (P2u; prostaglandin E\textsubscript{2} receptors).

Changes in nuclear factors were analyzed searching for their possible involvement in neurosecretion competence expression. Many homeobox genes (45) were expressed normally in NI clones, whereas others, such as HOX3D, were down-regulated. For a few more, including Phox2a (46) and Isl-1 (47), down-regulation was highly variable in the two NI clones (−19 and −5fold, −52 and <−2-fold in NI-27 and NI-Trk, respectively), thus they did not reach the threshold to be included in Table I of the Supplemental Material. Among the other transcription factors, many (e.g. Myt1, Maf1, Pet-1, and MITF-2B) were strongly down-regulated, independently of their protein structure: helix-loop-helix, Zn\textsuperscript{2+} fingers, BRLZ domain, and multidomain. Other nuclear factors (e.g. Kruppel-like factor4, Hox2.2, and Skn-1a) were up-regulated, even to a considerable extent (>10-fold).

To verify results for a few transcription factors and to expand the investigation to two more that were not included in the microarrays, the WT and NI clones were analyzed also by Northern blotting. Fig. 7 (A–C) confirms the profound down-regulation of three genes: Maf1 (48), eHAND (49), and CREM/ICERII isoforms (50, 51). In the case of Isl-1 and Phox2a the down-regulation result was reinforced by Northern blotting, because the discrepancy between the two NI clones appeared less pronounced (Fig. 7, D and E). Of the two transcription factors not arrayed on GeneChips and investigated in only one NI clone, NI-27, Phox2b (52) appeared strongly down-regulated, whereas Mash1 (53, 54) was normally expressed (Fig. 7, F and G).

**DISCUSSION**

So far, the investigation of neurosecretion has been largely carried out in specialized cell lines, including PC12. The latter is known to be heterogeneous, composed by a variety of clones. In most studies reported in the literature this property was given little attention. Single, homogeneous clones were em-
employed only in a low fraction of cases mostly to investigate issues that do not require inter-clone comparisons, such as the effects of treatments, intracellular trafficking, and exocytotic membrane fusion (see, among others, Refs. 55 and 56).

Our aim, a molecular definition of neurosecretion competence in PC12 cells, required in contrast the direct comparison of NI and WT clones. In previous studies carried out by Northern and Western blotting, the degree of heterogeneity of the various PC12 clones had been underestimated. Indeed, of 29 gene products investigated in a single NI and a single WT clone, all the 11 mRNAs/proteins directly related to neurosecretion were found down-regulated, whereas the others (related to membranes, cytoskeleton, signaling, and endocytosis) appeared in contrast invariant (8, 11, 13). Neurosecretion competence had been therefore envisaged as a highly specific process that does not require inter-clone comparisons, such as the effects of treatments, intracellular trafficking, and exocytotic membrane fusion (see, among others, Refs. 55 and 56).

Rather, a considerable number of neuronal-specific transcripts are expressed in NI and WT clones, with the expected neurotransmitter release response; NI clones are both competent for neurosecretion, because fusion of their cells fails to induce complementation, i.e. reappearance of neurosecretion (13); 2) In addition to the neuronal proteins previously shown to be normally expressed in NI-27 (8, 11), the present analysis has revealed that many more gene expression programs distinct from neurosecretion competence. This conclusion was unexpected, because of the well-known mechanistic coordination existing between exocytosis, endocytosis, and membrane recycling.

A question that might be asked is whether up- or down-regulation of non-neurosecretory genes has a role in the competence program. For many such genes the problem remains open. However, the reversed expression of ryanodine2 and IP31 receptors is expected to induce major changes in the control of cytosolic [Ca2+]i, a parameter of great importance in neurosecretion (57). In incompetent cells, the homeostasis of the cation depends primarily on G protein-coupled receptors and on their effectors (58). On the other hand, incompetence appears to affect neither endocytosis nor other membrane trafficking processes. These and other membrane-based functions appear therefore to be part of one or more gene expression programs distinct from neurosecretion competence.

What are the mechanisms of competence expression? Recently Kim et al. (59) reported that neurosecretory cells loose their DGs following down-regulation of the luminal protein,
CGA, and that fibroblasts acquire DGs when transfected with its cDNA. These events were attributed not to changes in gene expression but to an on/off process based on the inactivation and activation, respectively, of DG degradation. Based on the data from Kim et al., therefore, it may be concluded that neurosecretion competence depends on CGA protein levels. However, previous data of the literature (60, 11) and experiments carried out in various cell types, including the PC12–27 NI clone2 appear incompatible with this conclusion. Moreover, the results of the present report demonstrate that neurosecretion competence is largely regulated at the transcript level, because in incompetent cells the expression of many genes coding for proteins of DG/CV and of their exocytotic machinery is downregulated, often to levels 10-fold. Whether the control is transcriptional or post-transcriptional cannot be argued from the present results. Although the latter results were expected based on previous Northern and Western blots, the invariant expression of other genes coding for proteins of DG/CV membranes and their exocytotic fusion was a surprise. Some of these proteins might be functional also in NI cells. The H+ pump is active also at organelles other than DG/CV, and the regulatory proteins might function in other types of membrane fusions. This however is hard to imagine for synaptogyrin I and munc18-1. The first is a membrane protein of DG/CV active in the regulation of their exocytotic fusion (34); the second is a syntaxin1 binding protein that plays a key role in the assembly of the fusogenic complex (1). Moreover, previous Western blots had shown that the Munc-18 protein is strongly down-regulated in the NI-27 clone (11, 61). Taken together these results suggest that loss of competence is sustained not only by down-regulation of specific gene expression but also by additional processes possibly occurring at the post-transcriptional, translational, and/or the protein turnover level, which appear to play the predominant role in the expression of individual proteins (see also Refs. 62 and 63).

Finally, how is neurosecretion competence controlled? Previous NI cell fusion and cDNA library transfection experiments had suggested the involvement of one or more master genes.

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2 Giordano, T., Malosio, M. L., and Meldolesi, J., unpublished results.
coding for specific suppressor(s) or enhancer(s) that in the NI clones could be up- or down-regulated, respectively (13, 11). One or more master genes could control the coordinate expression of operative genes directly involved in the competence program. The mechanisms of this hypothesized control remain, however, unclear also because neurosecretion genes for which information is available: 1) are not clustered together but are widely distributed in the genome and 2) show no common sequences in their regulatory elements. Expression of a considerable number of transcription and other nuclear factor genes appears profoundly changed in NI versus WT clones. In previous studies a few general regulatory genes, i.e. Mash1, Phox2a, Phox2b, and Isl-1, were shown to play important roles in neurogenesis (6, 53). Except for Mash1, which in the NI-27 clone is normally expressed, the others were found to be all down-regulated (although with some discrepancy between the two NI clones), suggesting their possible involvement also in our cellular model. However, preliminary experiments in which the cDNAs of Phox2a, Phox2b, and Isl-1 were individually transfected into NI cells failed to induce any recovery of the neurosecretory phenotype. At the moment it may be too early to exclude these factors from the control of competence, because their work could take place not independently, but coordinately in multifactorial complexes (see Ref. 64).

In conclusion, the neurosecretion competence program is beginning to disclose its secrets. The extensive microarray comparison of NI and WT PC12 clones, carried out by a strictly controlled and filtered experimental approach, was aimed at distinguishing specific differences from unspecific inter-clone heterogeneities. The results demonstrated (a) the down-regulation of many genes coding for proteins directly involved in neurosecretion, whereas expression of other genes in the same group appeared unaffected, but also (b) the changed expression of numerous other genes and processes, many of which had not
been even envisaged yet. The events induced in the cell by the acquisition of neurosecretion appear therefore to be multiple and complex. Further deciphering of these events could ultimately open the way not only to a better understanding of the competence program but also to possible applications at both the biotechnological and pathological levels.

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