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Comparative expressed-sequence-tag analysis of differential gene expression profiles in PC-12 cells before and after nerve growth factor treatment

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ABSTRACT **Nerve growth factor-induced differentiation of adrenal chromaffin PC-12 cells to a neuronal phenotype involves alterations in gene expression and represents a model system to study neuronal differentiation. We have used the expressed-sequence-tag approach to identify ≈600 differentially expressed mRNAs in untreated and nerve growth factor-treated PC-12 cells that encode proteins with diverse structural and biochemical functions. Many of these mRNAs encode proteins belonging to cellular pathways not previously known to be regulated by nerve growth factor. Comparative expressed-sequence-tag analysis provides a basis for surveying global changes in gene-expression patterns in response to biological signals at an unprecedented scale, is a powerful tool for identifying potential interactions between different cellular pathways, and allows the gene-expression profiles of individual genes belonging to a particular pathway to be followed.**

Comparisons of gene-expression profiles in a single cell line exposed to various external cues can provide information on the molecular basis of cellular differentiation. The rat pheochromocytoma cell line PC-12 differentiates into sympathetic-like neurons in response to nerve growth factor (NGF) and represents a model cell line for uncovering candidate mRNAs associated with the acquisition of a neuronal phenotype (1, 2). PC-12 cells possess many characteristics of adrenal chromaffin cells, including round cell body and the ability to synthesize, store, and release catecholamine neurotransmitters. Differentiation of adrenal chromaffin-like PC-12 cells to a neuronal-like phenotype is characterized by mitotic arrest, the elaboration of neuronal processes, and electrical excitability.

Current methods of comparing gene-expression profiles in different cell types, such as two-dimensional gel electrophoresis of cellular proteins (3) and differential screening of cDNA libraries (4–6), have been invaluable in providing the groundwork for assessing changes in gene-expression profiles during differentiation, but these techniques are ultimately limited in the amount of information they provide. The expressed-sequence-tag (EST) approach offers a rapid and efficient means of obtaining steady-state mRNA profiles (7–10) and thus can potentially be used in identifying differentially regulated mRNAs. Therefore, we have used the EST approach of high-throughput sequencing of cDNA clones to comprehensively assess the mRNAs found in untreated and NGF-treated PC-12 cells. Detailed expression profiles are presented here for a single cell type, in the absence of induction and during induction of differentiation by NGF treatment, which allowed for the identification of many differentially expressed transcripts by comparative EST analysis. Random sampling of the clones identified as differentially regulated by this approach was confirmed by RNA blot analysis, thereby demonstrating the potential of this technique in gene discovery and differential gene expression.*

EXPERIMENTAL PROCEDURES

cDNA Libraries. PC-12 cells (American Type Culture Collection) were cultured and treated with 2.5S NGF (50 ng/ml) as described (11). PC-12 cells undergo mitotic arrest and neuron-like phenotypic expression by day 7 of NGF treatment (12); hence, cDNA libraries were constructed from untreated PC-12 cells and PC-12 cells treated with NGF for 9 days. Total RNA was isolated from PC-12 cells using a single-step acid–phenol–thiocyanate protocol, and poly(A)$^+$ RNA was subsequently purified using oligotex-dT (Qiagen, Chatsworth, CA) as described (13). For comparative EST analysis of cDNA libraries, a cDNA library should be representative of the initial poly(A)$^+$ RNA population. The construction of unidirectionally cloned cDNA libraries from 10 μg of poly(A)$^+$ RNA is detailed elsewhere (43). Preparation of plasmid templates, automation of sequencing reactions, and comparison of ESTs to nucleotide and peptide data bases were done as described (7–9, 14). For estimating distinct mRNA species and aiding in the assignment of putative identifications, ESTs were treated as shotgun fragments and assembled into clusters of overlapping nucleotide sequences based on stringent overlap criteria (15).

Northern Blot Analysis. PC-12 cells were treated with NGF for 0, 3, 6, 9, and 12 days, and purified poly(A)$^+$ RNA was obtained. Northern blots were done as described (13). Because glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA levels did not change with NGF treatment according to comparative EST and Northern blot analysis, GAPDH mRNA levels served as an internal control for normalization. Levels of differentially regulated mRNAs were quantitated by scanning blots with a Molecular Dynamics PhosphoImager as described (13).

RESULTS AND DISCUSSION

In all, 3187 and 3251 cDNA clones were randomly selected from cDNA libraries of untreated and 9-day NGF-treated PC-12 cells, respectively, and partially sequenced from the 5′ and 3′ ends to generate a total of 7521 EST sequences. The average cDNA clone length was between 1.5 and 2 kb. Comparisons of the two EST data sets to each other, to sequences in the Human and Non-Human cDNA Database at The Institute of Genomic Research, and to public data-base sequences from GenBank, GenPept, Protein Identification

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Abbreviations: EST, expressed sequence tag; NGF, nerve growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. H31040-H35861).

Table 1. EST profiles in untreated and NGF-treated PC-12 cDNA libraries

Match category	− NGF all	+ NGF all	− NGF specific	+ NGF specific
Exact match	850 (288)	810 (287)	154 (110)	139 (109)
Nonexact match	843 (519)	751 (455)	411 (335)	347 (271)
No data-base match	2081 (1137)	2186 (1282)	1860 (1002)	1956 (1147)
Total	3774 (1944)	3747 (2024)	2425 (1447)	2442 (1527)

The 6438 ESTs were generated by sequencing cDNA clones from the 5′ ends; an additional 1083 ESTs were obtained by sequencing the 3′ ends of cDNA clones that had no data-base matches. "Exact match" category refers to ESTs with data-base matches of >95% nucleotide identity to rat sequences in GenBank or European Molecular Biology Laboratory. "Nonexact match" includes protein matches to other organisms and matches with <95% nucleotide identity to rat sequences. Number of ESTs in a match category is indicated. Numbers in parentheses are estimates of distinct mRNA species represented by ESTs with exact and nonexact matches. For ESTs with no data-base match, numbers in parentheses are estimates of distinct EST clusters (15). "− NGF all" and "+ NGF all" refer to all ESTs found in the untreated and NGF-treated PC-12 libraries, respectively. "− NGF specific" and "+ NGF specific" refer to all ESTs unique to the untreated and NGF-treated PC-12 libraries, respectively.

Resource, and Swiss-Prot were done as described (7–9, 14, 15). The combined EST data set consists of 2.6 Mb of cDNA sequence with an average EST length of ≈350 nt. Cumulative data-base match statistics and estimations of distinct mRNA species found in untreated and 9-day NGF-treated PC-12 cDNA libraries are shown in Table 1. ESTs with exact matches comprised 397 distinct mRNA species, ESTs with nonexact matches comprised ≈790 distinct mRNA species based on

Table 2. Redundant EST matches found in either untreated or 9-day NGF-treated PC-12 cDNA libraries

EST no.*	Putative identification	ESTs, no.†	Accession no.	Length, nt	ID, %	SIM, %
Untreated PC-12 library						
106019	ATPase inhibitor protein	3	GB:D13122	247	100.0	
105249	Activator 1 subunit 37 kDa	3	PIR:A45253	327	81.7	89.0
108300	α-Prothymosin	3	GB:X55326	399	98.3	99.3
105748	Cysteine-rich protein	3	SP:P21291	273	95.6	95.6
108400	Cytochrome *c* oxidase, VIII	3	GB:X06146	188	97.3	100.0
105353	DNA-binding protein TAXREB107	3	GB:X81987	273	93.0	95.6
107654	Dihydrolipoamide succinyltransferase	3	GB:D90401	353	96.3	97.8
108445	GTP-binding protein, β subunit	4	SP:P11016	360	99.2	99.2
108102	Glutaminyl-tRNA synthetase	4	SP:P07814	339	90.3	94.7
104756	*Homo sapiens* hypothetical protein	6	GP:D14696_1	240	96.3	97.5
107601	Inorganic pyrophosphatase	3	SP:P00817	330	60.9	73.6
106432	MEK kinase	3	GP:L13103_1	288	93.8	93.8
106586	NADH-ubiquinone oxidoreductase, 13-kDa subunit	3	SP:P23934	111	91.9	97.3
105326	NADH-ubiquinone oxidoreductase, 23-kDa subunit	3	PIR:A38409	330	91.8	94.5
106717	Protein disulfide isomerase, P5 protein	3	PIR:S19656	264	90.9	95.5
106817	Ribosomal protein L26	6	GB:X14671	360	97.2	98.1
108091	Ribosomal protein S21	6	SP:P05765	249	98.8	98.8
106968	SC2 synaptic glycoprotein	6	GB:S45663	322	99.1	99.4
107664	Sulfated glycoprotein 1 (SGP-1)	3	GB:M19936	363	98.3	98.3
107989	Transketolase	6	SP:P29401	348	93.1	97.4
108403	Ubiquinol-cytochrome-*c* reductase 7.2-kDa protein	3	SP:P00130	180	83.3	93.3
Nine-day NGF-treated PC-12 library						
109555	14-3-3 protein ε isoform	3	GB:M84416	314	95.2	96.8
109631	Infertility-related sperm protein 75 kDa	5	GP:S58544_1	246	37.8	52.4
109590	Adenylosuccinate synthetase	3	SP:P30520	366	78.7	82.8
110537	*Caenorhabditis elegans* hypothetical protein F55H2.2	3	GP:Z27080_2	210	75.7	87.1
111382	Calcium-dependent protease, small regulatory subunit	4	SP:P04632	297	88.9	91.9
109028	Clusterin	4	GB:M16975	338	97.9	98.5
111898	*Homo sapiens* hypothetical protein	3	GP:D14663_1	303	87.1	93.1
110299	Lysosomal membrane glycoprotein, 120 kDa	4	GB:M34959	300	98.3	99.7
110237	*Mus musculus* hypothetical protein Surfeit 4	4	GB:M63114	358	93.6	95.0
109929	Myelin basic protein-related	3	GB:X67319	316	87.2	87.8
112006	Nuclear transport protein NIP1	3	SP:P32497	234	42.3	64.1
110582	Prolylcarboxypeptidase	3	GP:S64262_1	219	36.0	61.3
109741	Proton pump, 116 kDa	3	GB:M58758	336	97.6	98.8
111939	Ras-related protein RAB-6	3	SP:P20340	288	96.9	96.9
111737	*Saccharomyces cerevisiae* ORF 5′ to RAD51	4	SP:P25451	303	59.4	77.2
111989	Superoxide dismutase (Cu/Zn)	5	GB:Y00404	372	99.5	99.5
109231	Translation initiation factor, SUI1	4	PIR:S31245	261	52.9	71.3

MEK, mitogen-activated/extracellular signal-regulated kinase; ID, identification; SIM, amino acid similarity; ORF, open reading frame.
*Representative EST.
†Data-base matches consisting of two redundant ESTs found in either untreated or NGF-treated PC-12 libraries can be found in GenBank and in The Institute for Genomic Research data base, which can be assessed through the World-Wide Web (URL :http://www.tigr.org/).

similarity searches and clustering of EST sequences, whereas ESTs with no data-base match were assembled into 2284 distinct EST clusters. The combination of these values represents the upper limit for estimating the number of distinct mRNA species from the two cDNA libraries. The actual number of individual species is likely to be less; a more precise value cannot be obtained with confidence at this time due to the low EST sampling size.

The mRNA population in mammalian cells is categorized into highly abundant, moderately abundant, and rare classes based on reassociation kinetics (16). It has been estimated that highly abundant (10 unique mRNAs at 10,000 copies each per cell), moderately abundant (500–1000 unique mRNAs at ≈100–400 copies each per cell), and rare mRNAs (8000–10,000 unique mRNAs at <5–10 copies each per cell) are proposed to represent 25%, 50%, and 25% of the total mRNA population, respectively (17). On the basis of these assumptions, >99% of the highly abundant, >85% of the moderately abundant, and <5% of the rare transcripts should be represented at least once in 3000 ESTs per library, based on a Poisson distribution for random sampling. Accordingly, two or more redundant ESTs (ESTs belonging to the same mRNA) in a cDNA library would represent a highly-to-moderately abundant mRNA. Our results are consistent with these estimates. In both cDNA libraries, ≈1500–1700 redundant ESTs with putative data-base matches were identified that comprise ≈700–800 distinct mRNAs (Table 2). For ESTs with no data-base match, we estimate that there are at least 400–500 redundant ESTs corresponding to ≈150–200 unique mRNAs in each library.

Many of the mRNAs found equally in both libraries likely represent "housekeeping" enzymes (43), such as elongation factor 1-α, GAPDH, and cyclophilin. The finding that these transcripts were unaltered by NGF treatment was confirmed by Northern blot analysis (data not shown). Other mRNAs, specific to cells of neuroendocrine origin, were also found equally in both libraries; these included the highly abundant mRNAs for the secretory proteins, secretogranin I (0.6–1% of ESTs) and secretogranin II (0.3–0.4%), and the catecholamine-synthesizing enzyme tyrosine hydroxylase (0.4–0.5%).

Of particular interest was the identification of ≈600 differentially regulated mRNA species, 227 of these mRNAs did not have data-base matches. Each of the ≈600 species was detected in (*i*) only one cDNA library, which contained two or more redundant ESTs or (*ii*) both libraries, with an EST ratio (untreated/NGF-treated or NGF-treated/untreated) >2. The differentially regulated mRNAs were evenly distributed among the two cDNA libraries with an equal number of up- and down-regulated genes found in each library. The ability to detect such a large number of putatively regulated mRNAs with the EST approach represents a substantial advantage over what has been possible with differential cDNA screening techniques (4–6). Some of the differentially expressed mRNAs identified with EST sequencing have previously been reported to be regulated by NGF, such as neurofilament L and neurofilament M (18) and SCG10 (superior cervical ganglion clone 10) (5) (Fig. 1). However, the vast majority of the clones (>95%) in our study were not previously identified as differentially regulated mRNAs in PC-12 cells (4–6). To confirm results obtained with the EST approach, Northern blots were probed by using ^{32}P-labeled cDNA clones of 15 mRNAs predicted by EST analysis to be differentially up- or down-regulated (Fig. 2). These ESTs appeared in low frequencies in either libraries. The ratio of ESTs (untreated/NGF-treated) ranged from 0/2 to 1/6 and 1/0 to 7/3 in the up- and down-regulated groups, respectively. The direction of change in mRNA levels predicted by EST analysis correlated with the Northern blot analysis in 12 of 15 cases. These findings are comparable, if not better, than previous methods used to

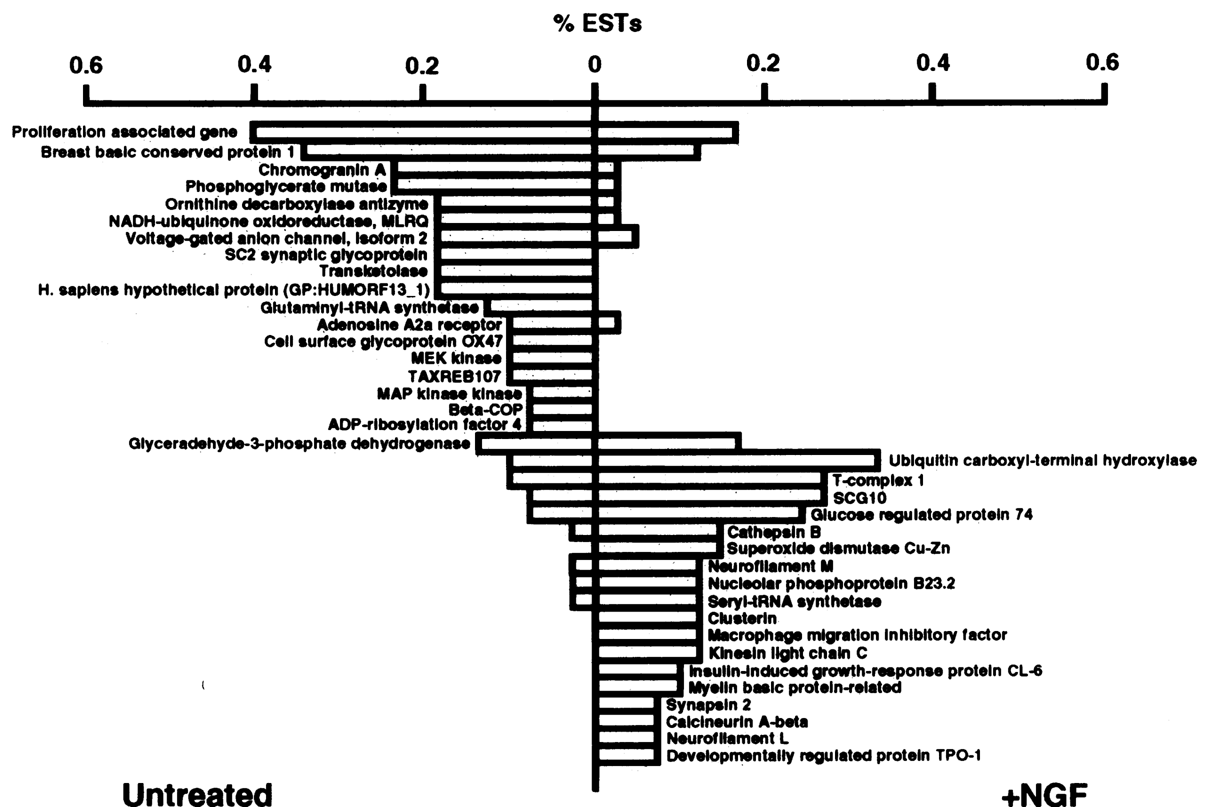

FIG. 1. Differential distribution of ESTs. Frequency of redundant ESTs as a percentage of the cDNA clones selected from untreated (− NGF) and 9-day NGF-treated (+ NGF) PC-12 cDNA libraries. *H. sapiens, Homo sapiens*; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MAP, mitogen-activated protein; SCG10, superior cervical ganglion clone 10.

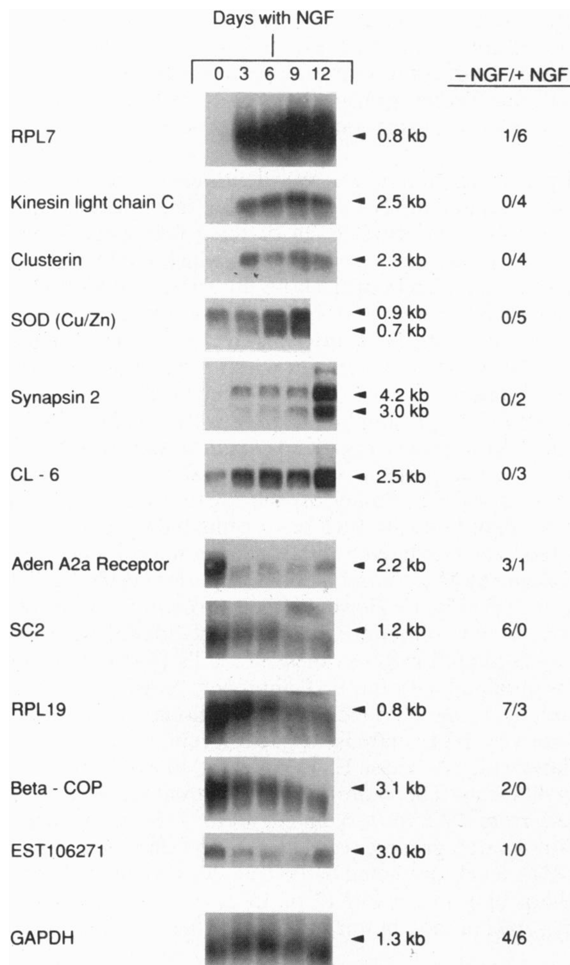

FIG. 2. Northern blots of putatively regulated mRNAs identified by EST analysis. Representative Northern blots showing time course of expression for putatively regulated mRNAs. Sizes of mRNAs species are indicated in kb. Ratio of ESTs in untreated (– NGF) and 9-day NGF-treated (+ NGF) PC-12 cDNA libraries is indicated. The 12 cDNA clones shown to be differentially regulated by EST and Northern analysis are as follows: ribosomal protein L7 (RPL7), kinesin light-chain C, clusterin, copper/zinc-containing superoxide dismutase (SOD), synapsin 2, insulin-induced growth-response protein CL-6 (CL-6), adenosine A_{2a} receptor (Aden A2a), synaptic glycoprotein SC2, ribosomal protein L19 (RPL19), β-COP, EST106271, and secretin receptor (blot not shown; EST ratio, 2/0). For another three cDNA clones that were represented by ESTs with exact matches, Northern and EST analysis did not correlate. These proteins were ribosomal protein S16, elongation factor 1-γ, and Na^+/K^+-ATPase α subunit; EST ratios (– NGF/+ NGF) were 6/0, 2/6, and 3/1, respectively. Data from Northern analysis are from two to four independent experiments.

identify differentially regulated genes (4–6, 19). The magnitude of change in mRNA levels observed by EST and RNA blot analysis did not correlate as well as the direction of change. Hence, the EST approach, at the sampling depths used here, is suitable for qualitative rather than quantitative comparisons.

The 12 differentially regulated mRNAs presented here represent a broad spectrum of proteins ranging from ribosomal proteins L19 (20) and L7 (21), the G protein-coupled adenosine A_{2a} (22) and secretin receptors (23), cell trafficking proteins kinesin light-chain C (24) and β-COP (named for coat protein) (25), a synaptic glycoprotein SC2 (26), a synaptic vesicle protein synapsin 2 (27), an insulin-induced growth response protein CL-6 (28), an enzyme involved in oxidative stress, superoxide dismutase (29), a protein of unknown function, clusterin (30), and a transcript without a data-base match, EST106271. NGF treatment of PC-12 cells produced a complex, time-dependent pattern of mRNA regulation, instead of a monotonic, coordinated pattern of regulation (Fig. 2). Some mRNAs were maximally induced by NGF within 3–6 days, such as the mRNAs encoding clusterin and CL-6 (2- to 7-fold increase), whereas others required at least 9–12 days, such as the transcripts for kinesin light-chain, superoxide dismutase, synapsin 2, and ribosomal protein L7 (2- to 12-fold increase). For down-regulated mRNAs, maximal decreases were achieved within 3 days for the adenosine A2a receptor, secretin receptor, SC2 and β-COP mRNAs (2- to 20-fold decrease), but at least 9 to 12 days were required for the ribosomal protein L19 mRNA (4-fold decrease). The novel mRNA represented by a nonredundant EST (EST106271) exhibited biphasic regulation, transiently decreasing 2.5-fold within 3 days before returning to untreated PC-12 levels by 12 days.

NGF had opposite effects on the mRNAs of ribosomal proteins L19 and L7. At present how changes in ribosomes may affect cellular differentiation is unclear; however, our data suggest that ribosomal protein content may be important in the differentiation of PC-12 cells. Differential expression of ribosomal proteins occurs during embryonic development of certain eukaryotes (31, 32). Our finding that synapsin 2 mRNA levels are up-regulated during differentiation of PC-12 cells is consistent with recent reports on the role of synapsins in neurite extension and synaptogenesis (33, 34). The progressive outgrowth of neuronal processes is also affected by the steady-state levels of microtubule-associated proteins, such as MAP-2, tau, and kinesin (2, 35–37). Cultured neurons depleted of kinesin by antisense oligonucleotides exhibit shortened neuronal processes (37). A role for this heterotetrameric protein in the elaboration of neuronal processes is supported by our observation that one of the subunits, kinesin light-chain C, was up-regulated in differentiated PC-12 cells. NGF deprivation is known to trigger apoptosis in sympathetic neurons *in vitro* (38). Our finding that superoxide dismutase is up-regulated by NGF treatment and the recent report that overexpression of superoxide dismutase delays apoptosis in transfected cultured neurons support the notion that superoxide generation is an important mediator of apoptosis (39).

The enormous differences in the number of copies per cell of each mRNA species currently limit quantitation of changes in mRNA levels by DNA sequencing to relatively moderate-to-abundant transcripts. Our data indicate that significant changes in the levels of many moderately abundant and abundant mRNAs occur during differentiation. This finding is significant because it suggests that changes in the levels of many moderate-to-abundant mRNA species, and not just rare transcripts, might be crucial in the process of cellular differentiation. Although nonabundant transcripts were identified with the EST approach, some rare mRNAs known to be regulated by NGF, such as the nicotinic (40) and the m4 muscarinic receptor mRNAs (41), were either represented by a single EST in the untreated PC-12 library or not at all in >6000 clones, respectively. Thus, the data set of "single-hit" (i.e., nonredundant) ESTs is less likely to be a dependable indicator of differentially regulated mRNAs as the data set of redundant ESTs, which appear preferentially or "exclusively" in a cDNA library. Nonetheless, the list of single-hit ESTs, by itself or in conjunction with a subtractive cDNA library approach, may still be a useful index of differentially regulated genes, as demonstrated by EST106271 (Fig. 2). It is not yet known whether the regulated mRNAs described in this study are directly responsible for differentiation or secondary to it. Further investigation is clearly warranted. Regardless, it should be emphasized that the comparative EST approach provides an unprecedented inventory of candidate genes, thereby generating additional and, in many instances, unfore-

seen leads into the molecular/cellular mechanisms of differentiation.

The comparative EST approach offers many advantages over other techniques (e.g., two-dimensional gel electrophoresis, differential display) (3, 19), such as the immediate availability of differentially regulated cDNA clones that can be used for overexpression and antisense knockout studies in undifferentiated or NGF-differentiated PC-12 cells. Comparative EST analysis also provides information on the mRNA diversity of a given cell type. The EST approach generates actual sequence data and not the less informative autoradiographic data provided by other methodologies (3, 19); hence, closely related members within a gene family can be distinguished. Our approach has identified cellular pathways (i.e., G protein-coupled receptors, ribosomal proteins) regulated by NGF through the trkA/Ras/mitogen-activated protein kinase signal transduction cascade (42), findings that, to our knowledge, were not previously known. We have also identified ≈200 other mRNAs encoding proteins without data base matches that may correlate with differentiation and warrant further investigation. Use of comparative EST analysis allows individual genes to be followed whether their expression is increased, decreased, or, just as importantly, unchanged. This analysis is not easily accomplished with other methods—for example, differential screening of cDNA libraries (4–6).

Application of the comparative EST approach can be readily extended to other cellular processes, such as development, homeostasis, cell-cycle regulation, apoptosis, cancer progression, and toxicological effects of drugs on gene expression. By establishing a large computer data base of EST sequences from different tissues and cells (43), a "computerized Northern blot" is generated that can serve as a powerful tool to determine gene-expression profiles and understand cellular biology and physiology.

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