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# Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: Coordinate regulation with c-*fos* or c-*myc*

(growth factors/gene expression/mRNA stability/protooncogenes)

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ABSTRACT **We have previously identified by cDNA cloning 5 mRNAs that appear in resting BALB/c 3T3 cells soon after growth stimulation by serum or platelet-derived growth factor. Five additional mRNAs of this class are described in this report. The mRNAs reached peak levels between 40 and 120 min after serum addition and rapidly decayed thereafter. All 10 RNAs were superinduced in the presence of cycloheximide. Nuclear run-on experiments indicated that the increase in the mRNAs is the result of rapid transcriptional activation of their genes on stimulation by serum or platelet-derived growth factor. Superinducibility by cycloheximide is due to two effects: prolonged transcription and stabilization of mRNAs. This overall pattern of regulation is similar to that of the c-*fos* or c-*myc* protooncogenes reported previously. We hypothesize that these newly identified "immediate early" genes play a role in the proliferative response induced by growth factors.**

The growth of mammalian cells is regulated by polypeptide growth factors (1). How such factors induce cell growth is a key question, relevant not only to the mechanisms of cell proliferation and neoplasia but more generally to the response of cells to a variety of extracellular ligands, such as developmental or differentiation factors, classical hormones, or neurotransmitters. The interaction of a growth factor with its specific surface receptors on resting cells rapidly induces a cascade of biochemical events including the formation of phosphoinositide metabolites, phosphorylation of proteins, and a transient increase in pH and free intracellular $Ca^{2+}$ (2). One or more of these biochemical events is thought to generate a signal within the nucleus that activates a set of specific growth-related genes, followed by the ordered expression of other genes and the onset of DNA replication (3). This sequential expression of specific genes during the transition of cells from a resting to a growing state suggests that there is a genetic program for growth analogous to the developmental program of a complex virus (4–6).

We and others have been interested in identifying genes that may be part of this putative growth program, understanding how they are regulated, and characterizing their encoded proteins. One way to identify growth factor-responsive genes is to search for changes in the expression of known genes encoding suspected regulatory proteins after stimulation of resting cells with serum or a purified growth factor. In this way the protooncogenes c-*fos* and c-*myc* have been found to be activated in a variety of cells (7–10), as have a number of other genes for which probes are available. Altered expression of other specific genes has been inferred by the appearance of new proteins (11–14) or translatable mRNAs (15) after growth stimulation. A more general way to identify genes activated by mitogenic stimuli is by differential screening of cDNA libraries prepared from RNA of resting cells exposed to serum or growth factors (16–22). This approach has led to the identification of a number of mRNAs that appear at specific times between stimulation and DNA synthesis.

In this communication we concentrate on the expression of 10 genes recently identified by cDNA cloning that are activated almost immediately after resting mouse 3T3 cells are stimulated with serum or a purified growth factor (22). Since these genes do not require protein synthesis for expression, we refer to them as "immediate early" genes by analogy to the immediate early genes (4–6) of viruses. Regulation of the newly described genes occurs at the transcriptional and post-transcriptional levels and appears to be coordinate with the regulation of c-*fos* or c-*myc*. Our results thus point to a class of genes, of which c-*fos* and c-*myc* are members, that make up the primary genomic response of 3T3 cells to growth factors.

## MATERIALS AND METHODS

**Cell Culture.** BALB/c 3T3 clone A31 cells were obtained from the American Type Culture Collection, recloned, and maintained in Eagle's minimal essential medium with Earle's salts (GIBCO), glutamine (2 mM), and 10% fetal calf serum (MEM-10). Quiescent cells were prepared by growth in MEM-10 to confluence followed by incubation in MEM-0.5 for 3 days. Serum stimulation of quiescent cells was accomplished by changing the medium to MEM-20. Where indicated, cycloheximide was added to 10 μg/ml; anisomycin (Sigma), to 26 μg/ml; and pactamycin (a gift from Upjohn), to 20 μg/ml. Fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) were from Collaborative Research and were used at 100 and 50 ng/ml, respectively.

**cDNA Libraries.** For differential hybridization screening, we used the cDNA library described previously (22). Another cDNA library, from which long cDNA clones were isolated, was prepared from the same RNA source using the procedure of Gubler and Hoffman (23).

**RNA Analysis and Transcription Assay.** Total cellular RNA was isolated using the procedure of Chirgwin *et al.* (24); poly(A)$^+$ RNA was purified using an oligo(dT)-cellulose column (25). RNA blots were prepared after electrophoretic separation in a denaturing formaldehyde/agarose gel (26) and RNA was immobilized on nitrocellulose filters. RNA was visualized by autoradiography following hybridization to various labeled cDNAs. The chicken β-actin (27), mouse c-*fos* (28), and mouse c-*myc* (29) clones were provided by D. Cleveland (Johns Hopkins University) or the American Type

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Abbreviations: FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; kb, kilobase(s); MEM-*X*, minimal essential medium/*X*% fetal bovine serum.

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Culture Collection. Nuclear run-on transcription assays were carried out according to the procedure of Groudine *et al.* (30) as modified by Greenberg and Ziff (9).

## RESULTS

**Identification of Five Additional Immediate Early mRNAs.** We previously described the identification via cDNA cloning of 5 mRNAs that appear rapidly in BALB/c mouse 3T3 cells following stimulation of resting cells with serum, PDGF, FGF, or phorbol ester (22). cDNA clones derived from this class of mRNA were detected by differentially screening a phage λgt10 library prepared from the mRNA of cells stimulated with serum for 3 hr in the presence of cycloheximide, thereby limiting the stimulated mRNAs to those derived from immediate early genes. Approximately 150,000 plaques were surveyed, and more than 800 were found to hybridize preferentially to the cDNA probe prepared from stimulated cell RNA. From this pool we obtained 5 sequence-specific classes of cDNA clones in addition to the 5 previously reported (22). Thus we have isolated a total of 10 different cDNAs derived from immediate early RNAs. These clones did not hybridize to probes for actin, *fos*, and *myc*. Partial nucleotide sequences of each of the 10 cDNAs did not reveal sequence identity with any cDNA or gene segment in the GenBank† data bank. After the initial isolation of these cDNAs, another library was constructed from which we isolated longer cDNA clones corresponding to each RNA. These cDNAs contain at least two-thirds of their corresponding RNA sequences and were used as probes in this study.

To characterize the mRNAs corresponding to the new cDNA clones, RNAs from quiescent or serum-stimulated cells were fractionated by electrophoresis in formaldehyde/agarose gels and hybridized to labeled cDNA probes (Fig. 1). Clones 3CH268, -462, -465, -475, and -482 hybridized to poly(A)$^+$ mRNAs of about 3.5, 1.2, 2.1, 2.2, and 2.4 kilobases (kb), respectively. The time course of appearance of these RNAs following serum stimulation is shown in Fig. 2. Each RNA is undetectable or is at a low level in resting cells and appears within 20 min of growth stimulation (except for 3CH482), reaching a maximal level by about 90 min and rapidly declining thereafter. This time course is similar to that for the immediate early mRNAs reported previously (22) and for the protooncogene c-*fos* (9). The increase in 3CH482 mRNA is somewhat delayed, resembling that of the c-*myc* RNA (7–9). Also, like the other immediate early RNAs, 3CH268, -462, -465, -475, and -482 RNAs are superinducible by serum in the presence of the protein synthesis inhibitor cycloheximide (Fig. 2).

We determined whether the newly detected serum-responsive RNAs also respond to purified growth factors by stimulating quiescent cells with purified PDGF or FGF in place of serum (Fig. 3). Each growth factor was used at a concentration that has maximal mitogenic effect. Like the immediate early RNAs reported earlier (22), all five of the new RNAs respond to PDGF and to FGF with kinetics similar to that found after serum stimulation. We take this as evidence that the rapid and transient increases in these RNAs after exposure to serum or growth factor is part of the early cellular responses to mitogenic stimulation.

**Transcriptional Activation of Immediate Early Genes.** Since increases in mRNA levels can be due either to enhanced transcription or to post-transcriptional changes, including increased mRNA stability, we determined whether the rapid rise in the 10 immediate early RNAs identified by cDNA cloning is due to transcriptional activation of their genes. For this purpose, nuclei were isolated from resting cells and from cells stimulated for various times with either serum or PDGF, and nuclear run-on assays were carried out (9, 30). As shown in Fig. 4 (see also Fig. 6), transcripts were not detected or were barely so for any of these 10 genes in nuclei from resting cells. However, within minutes after serum stimulation, transcripts were evident and they increased in amount for 20–60 min for most of the genes and thereafter rapidly declined. By 3–4 hr the entire set of genes was nearly inactive again. This response is similar to that of the c-*fos* and actin genes (9, 31). The response of gene 3CH482 (like the kinetics of RNA accumulation) resembles that of the c-*myc* gene (see Fig. 6). Some of the nuclear run-on experiments were repeated with PDGF in place of serum. As shown in Fig. 5, PDGF rapidly stimulates transcription of each of the immediate early genes; for most of them, maximal activity is found before 30 min. Thus, like c-*fos* or c-*myc*, all 10 immediate early genes are rapidly and transiently activated by purified growth factor or serum, resulting in stimulation of transcription that is shut-off within 3–4 hr. This burst of transcription appears to account for the appearance of the immediate early RNAs.

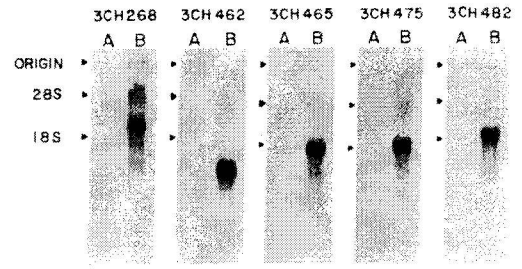


FIG. 1. RNA blot hybridization. Two micrograms of poly(A)$^+$ RNA from either quiescent cells (lanes A) or cells stimulated with MEM-20 and cycloheximide for 3 hr (lanes B) was resolved by formaldehyde/agarose gel electrophoresis. The RNA samples were then transferred to nitrocellulose filters and hybridized to various labeled cDNAs. Arrows indicate the origin and the positions of the 18S and 28S ribosomal RNAs.

**Effect of Inhibition of Protein Synthesis on the Shut-Off of Transcription.** Greenberg *et al.* (32) have shown that transcription of the c-*fos* and β-actin genes in BALB/c 3T3 cells stimulated by serum is prolonged in the presence of inhibitors of protein synthesis. The same is true of the 10 growth-related genes we have identified (Fig. 6). Nuclei from cells stimulated

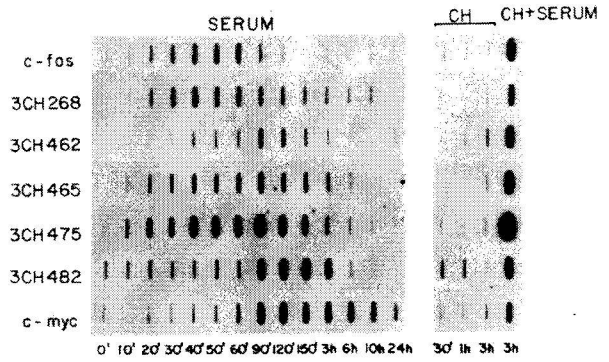


FIG. 2. Accumulation and decay of RNAs stimulated by serum. Total RNA (3 μg per sample) isolated from quiescent BALB/c 3T3 cells and cells stimulated with 20% serum (MEM-20) for the indicated times (10 min to 24 hr) was immobilized on nitrocellulose filters and hybridized to labeled DNA of various clones. Accumulation of RNAs in response to stimulation by serum, cycloheximide (CH), or CH plus serum was detected by autoradiography; exposure times were different for the various probes.

†National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Cambridge, MA), Tape Release 44.0.

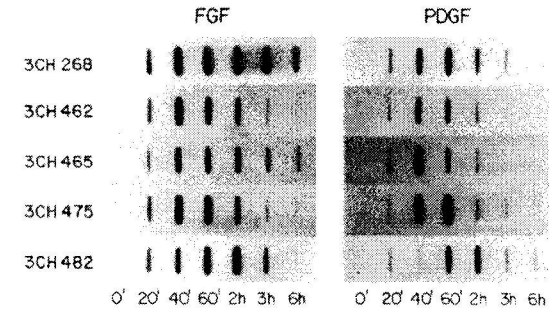


FIG. 3. Response of immediate early RNAs to purified growth factors. Quiescent 3T3 cells were stimulated by the addition of either purified FGF to 100 ng/ml or PDGF to 50 ng/ml and incubated for the indicated times (20 min to 6 hr). Total RNA (6 μg per sample from FGF-treated cells and 2 μg from PDGF-treated cells) from cells so treated was immobilized on nitrocellulose filters, hybridized to labeled DNA of various clones, and then autoradiographed. Exposure times were different for the various probes and for the PDGF vs. FGF experiment.

with serum for various times in the presence or absence of cycloheximide were used in run-on transcription assays. The levels of transcription of these genes after 30 min of stimulation were nearly identical in cells treated with or without cycloheximide. By 2–4 hr of stimulation, however, when transcription in serum-stimulated cells was substantially reduced, transcription in cells treated with both serum and cycloheximide remained at high levels. Cycloheximide thus inhibits the transcriptional shut-down, suggesting that protein synthesis is required to repress the entire class of immediate early genes.

We show below that cycloheximide greatly prolongs the half-lives of immediate early mRNAs. To exclude the possibility that a similar effect of cycloheximide on nuclear transcripts could account for the nuclear run-on results, we reduced the transcription reaction from 30 min to 5 min—less than the apparent half-lives of the RNAs. The transcripts should thus be much less affected by any degradative mechanism. The results were essentially the same as those obtained with 30-min incubations, indicating that protein synthesis is required for transcriptional repression of these genes.

**Effect of Inhibition of Protein Synthesis on the Stability of Immediate Early RNAs.** Immediate early RNAs characteristically have short half-lives (Fig. 2; refs. 7–9, 17, and 33–36). In view of the superinduction of these RNAs by cycloheximide and the previous demonstration that the half-lives of

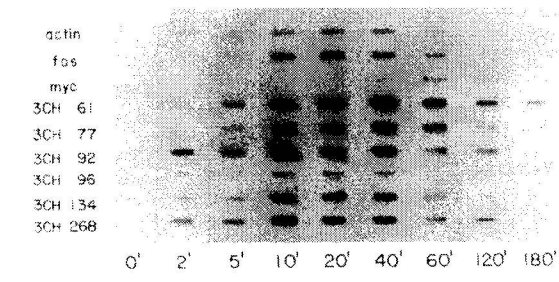


FIG. 4. Kinetics of transcriptional activation of immediate early genes after serum stimulation. Cell nuclei were isolated from either quiescent cells or cells stimulated with 20% serum (MEM-20) for the indicated times (2–180 min) and used in nuclear run-on transcription reactions in the presence of labeled nucleoside triphosphates. The labeled transcripts [equal counts ($10^7$ dpm/ml) from each reaction were used] were hybridized to denatured DNA of various clones immobilized on nitrocellulose filters. Clones 3CH61, -77, -92, -96, and -134 are new isolates of those described in ref. 22.

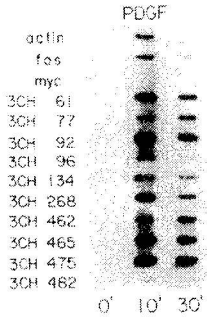


FIG. 5. Transcriptional activation of immediate early genes by purified PDGF. Isolated cell nuclei from either quiescent cells or cells stimulated with PDGF (50 ng/ml) for 10 or 30 min were used in nuclear run-on transcription reactions. Labeled transcripts (equal counts from each reaction) were hybridized to denatured DNA of various clones immobilized on nitrocellulose filters.

c-*fos* and c-*myc* mRNAs are prolonged in the presence of an inhibitor of protein synthesis (7–9, 34, 37), we determined the effect of inhibition of protein synthesis on the stability of the immediate early RNAs detected by our probes. Quiescent cells were stimulated with serum for 50 min and then treated with actinomycin D, after which the decay of the RNAs was followed in the absence of transcription. As shown in Fig. 7 *A* and *B*, the RNAs decayed rapidly under these conditions, with apparent half-lives of 15–30 min in most cases. (The observed stability of 3CH482 RNA may be due to the low level of stimulation of this RNA at 50 min.) However, when the cells were stimulated with serum in the presence of cycloheximide and then treated with actinomycin D, the levels of all the RNAs remained rather constant over a 2-hr period (Fig. 7*C*). We conclude that protein synthesis is required for the rapid decay of this class of immediate early RNAs.

Cycloheximide-dependent inhibition of RNA degradation is consistent with at least two hypotheses. First, a newly synthesized protein may be required for degradation of these RNAs; and second, degradation may be coupled to the process of translation. We attempted to distinguish between these possibilities by stimulating the cells with serum for 50 min, then adding cycloheximide and actinomycin D simultaneously. If a newly synthesized protein is involved in degradation, it should be made during the first 50 min and continue to be active for at least a short time thereafter. If, however, degradation is coupled to translation, it should be inhibited within a few minutes by cycloheximide. As shown

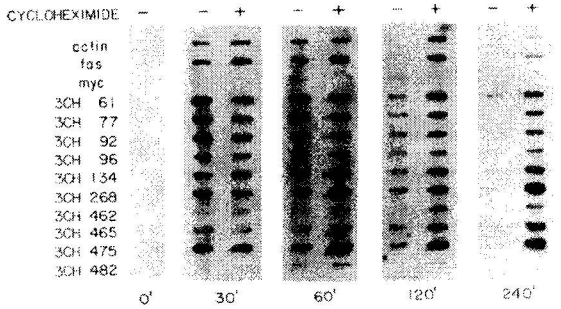


FIG. 6. Effect of cycloheximide on transcription of immediate early genes. Cell nuclei were isolated from quiescent cells and cells stimulated with 20% serum for the indicated times (30–240 min) in the presence (lanes +) or absence (lanes –) of cycloheximide (10 μg/ml). Labeled transcripts (equal counts from each reaction) made in nuclear run-on transcription reactions using these nuclei were hybridized to denatured DNA of various clones immobilized on nitrocellulose filters.

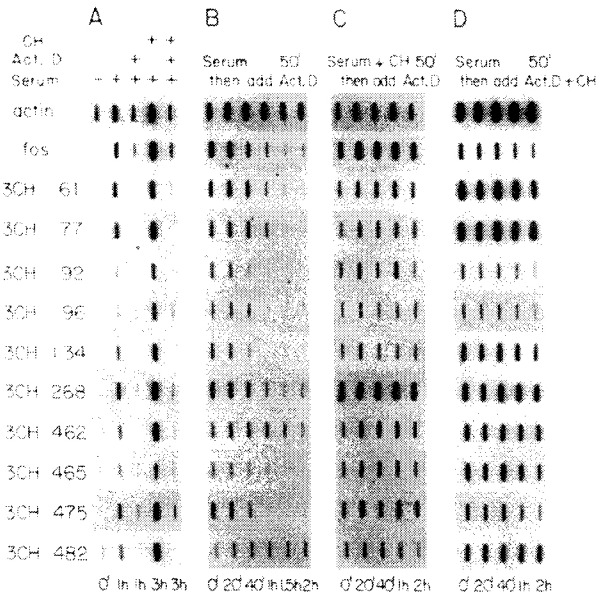


FIG. 7. Effect of cycloheximide on the decay of immediate early RNAs. Total RNA (5 μg per sample) from cells treated in various ways was immobilized to nitrocellulose filters and hybridized to labeled DNA of various clones. (*A*) Quiescent cell RNA (0 min) or RNA from cells treated (+) with serum (MEM-20), cycloheximide (CH; 10 μg/ml), and/or actinomycin D (Act. D; 1 μg/ml) for 1 or 3 hr. (*B*) RNA from cells stimulated with MEM-20 for 50 min, then treated with Act. D for the indicated times (20 min to 2 hr). (*C*) RNA from cells stimulated with MEM-20 and cycloheximide for 50 min, then treated with Act. D; the cells were harvested at the indicated times (20 min to 2 hr). (*D*) RNA from cells stimulated with serum for 50 min, then treated with Act. D and cycloheximide; the cells were harvested at the indicated times (20 min to 2 hr).

in Fig. 7*D*, no degradation of the immediate early RNAs is evident after simultaneous addition of actinomycin D and cycloheximide. (Similar results were obtained with two other inhibitors of protein synthesis, anisomycin and pactamycin.) These results thus favor the hypothesis that inhibitors of protein synthesis block mRNA degradation by their effect on the process of translation, although it remains possible that a protein required for RNA degradation must be continuously synthesized.

## DISCUSSION

A number of genes that are induced by mitogenic signals have been identified in mammalian cells. The earliest responsive group, transcriptionally activated within a few minutes of exposure to serum or growth factors, includes the protooncogene c-*fos*, the actin gene (9), and 9 of the genes described in this report: 3CH61, -77, -92, -96, -134, -268, -462, -465, and -475. A second group of genes, whose RNAs accumulate somewhat later and for a more extended period, includes c-*myc* (7–9), KC, JE (17), and 3CH482. We refer to genes that respond rapidly to growth factors in the absence of protein synthesis as "immediate early genes" by analogy to the immediate early genes of DNA viruses (4–6). Genes with this type of response have also been termed "competence genes" (17, 38), since in BALB/c 3T3 cells PDGF induces a state in which the cells can progress to DNA synthesis in response to other growth factors, termed "progression factors" (39). Whether products of any of the genes so far identified contribute to competence is not yet known. In fact, except for c-*fos* (40) and c-*myc* (41, 42), there is currently no evidence that any of the immediate early genes is required for cell growth.

The rapidity of the transcriptional response of the immediate early genes suggests that their promoters may be poised for activation in quiescent cells and that transcription begins as soon as an appropriate second messenger is generated through the interaction of growth factor with receptor. The precise signal that activates transcription is not known. Recent studies on the activation of c-*fos* by serum growth factors reveal that a 22-nucleotide palindromic sequence upstream of the promoter is required for transcriptional activation (43, 44); this sequence element is recognized by a cellular factor present in resting as well as stimulated cells. The actin gene, which is transcriptionally activated with c-*fos*, competes with the c-*fos* promoter for binding of the cellular factor, whereas the c-*myc* gene, transcriptionally activated later, does not (44). It is possible that those genes activated coordinately with c-*fos* may be controlled by a common mechanism.

Expression of immediate early genes is attenuated in at least two ways after the onset of transcription. First, the RNAs are degraded rapidly; second, transcription ceases within a few hours. Their accumulation is therefore transient. Inhibition of protein synthesis by cycloheximide counteracts both measures of control, leading to superinduction. Similar patterns of regulation have been described previously for certain interferon-responsive genes (45) and for c-*fos* and c-*myc* (32).

How inhibition of protein synthesis leads to stabilization of RNA or prolongs transcription is not clear. One interpretation of the transcriptional effect of cycloheximide is that after the initial burst of transcription, a newly synthesized protein acts as a specific transcriptional repressor, which could be the product of one or more immediate early genes. Regarding stabilization of RNA, the fact that three different protein synthesis inhibitors were effective indicates that it is inhibition of translation *per se* and not another unrelated effect of these drugs that is responsible. The observation that cycloheximide is fully effective even when added 50 min after serum suggests that degradation of the RNAs may be coupled to the process of translation rather than due to *de novo* synthesis of a regulatory protein or nuclease. Similar conclusions have been reached for the c-*myc* (34) and histone H4 mRNAs (46). Since only particular mRNAs decay rapidly, there would have to be a sequence-specific step in such a coupled degradative pathway. In the case of c-*fos* mRNA, it has been shown that a segment of the 3′-untranslated region is responsible for its instability (37); removal of this region converts c-*fos* to a transforming gene, presumably due to overproduction of the c-fos protein (47). Another unstable mRNA, encoding granulocyte-macrophage colony-stimulating factor, has an A+U-rich sequence in the 3′-untranslated region that has recently been found to be responsible for the instability of this RNA (48). This sequence is found in the 3′-untranslated region of a number of labile mRNAs, including those for certain lymphokines and cytokines and for the c-fos and c-myc proteins (48). Similar sequences are also present in the 3′ regions of several of the immediate early RNAs we have identified (unpublished results).

Aside from their possible importance in cell proliferation, these tightly regulated immediate early genes could serve as a paradigm for studying coordinate gene regulation by extracellular ligands more generally. Rapid activation of specific genes, including c-*fos*, can be induced by nicotine or other agents that induce an influx of extracellular $Ca^{2+}$ in neuronally differentiated PC12 cells (49, 50), suggesting a role for gene activation in response to neurotransmitters. An analogy has been drawn between the mechanisms that control cell growth and the acquisition of long-term memory (51, 52); both processes may involve the activation of a specific set of genes.

As noted above, the activation of immediate early genes in response to serum or growth factor stimulation of resting cells is followed by the ordered expression of other genes preceding DNA synthesis (3, 16–22, 38, 53), a pattern that resembles the developmental program of DNA viruses. Based on this analogy one can anticipate the possibility that some of the products of the genes described in this report may be regulators of genes expressed later and that many of the latter encode proteins more directly concerned with the duplication of cellular components. Genetic and biochemical experiments will be needed to evaluate this model and to determine whether the newly identified immediate early genes do play a role in regulating cell growth.

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