MOLECULAR AND CELLULAR BIOLOGY, Apr. 1986, p. 1050–1057
0270-7306/86/041050-08$02.00/0

Vol. 6, No. 4

Effect of Protein Synthesis Inhibitors on Growth Factor Activation of c-*fos*, c-*myc*, and Actin Gene Transcription

MICHAEL E. GREENBERG, ANNE L. HERMANOWSKI, AND EDWARD B. ZIFF*

Department of Biochemistry and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016

Received 28 October 1985/Accepted 16 December 1985

Stimulation of quiescent 3T3 cells with purified growth factors or of the pheochromacytoma cell line PC12 with nerve growth factor results in the rapid transient induction of c-*fos*, c-*myc*, and actin gene transcription (M. E. Greenberg and E. B. Ziff, Nature [London] 312:711–716; M. E. Greenberg, L. A. Greene, and E. B. Ziff, J. Biol. Chem. 26:14101–14110). We used protein synthesis inhibitors to investigate whether synthesis of new proteins plays a role in the rapid induction and subsequent repression of the transcription of these genes. Pretreatment of quiescent 3T3 cells with the inhibitor anisomycin before growth factor stimulation caused a superinduction of c-*fos* and c-*myc* mRNA levels upon growth factor addition. Nuclear runoff transcription analyses of 3T3 cells indicated that anisomycin potentiated c-*fos*, c-*myc*, and also actin expression at the transcriptional level, possibly by inhibiting transcriptional repression. Somewhat different results were obtained when PC12 cells were incubated with either anisomycin or cycloheximide. In PC12 cells protein synthesis inhibitors superinduced nerve growth factor activation of c-*fos* mRNA production but completely abolished the activation of c-*myc*. The results suggest that in PC12 cells c-*fos* transcription is activated by a protein-synthesis-independent mechanism, whereas c-*myc* stimulation requires new protein synthesis. The difference in the effect of anisomycin on growth factor activation of c-*myc* expression in 3T3 versus PC12 cells may be due to differential stringency of protein synthesis inhibition in the two cells or could reflect cell type differences in c-*myc* regulation.

Proto-oncogenes are normal cellular genes whose alteration by mutation or deregulation has been implicated in the process of tumorigenesis (reviewed in reference 4). Recent experiments have identified a role for several proto-oncogenes in the control of normal cell growth and differentiation. For example, the expression of two of these genes, c-*fos* and c-*myc*, is transiently activated within minutes after quiescent fibroblasts or lymphoid cells are stimulated to enter the cell cycle (6, 11, 19, 25, 27, 29) and when the pheochromocytoma cell line PC12 is induced to differentiate with nerve growth factor (NGF) (13, 18). Both c-*fos* and c-*myc* encode nuclear proteins (1, 12, 32) whose function is unknown, although c-*myc* has recently been shown to interact with DNA (32) and to play a direct role in the progression of quiescent 3T3 cells into the cell cycle (2, 23).

The induction of c-*fos* and c-*myc* expression appears to be part of a general transcriptional response to growth factors. Other cellular genes such as those for β-actin (15, 19) and ornithine decarboxylase (ODC) (24) and a group of genes identified by the differential screening of cDNA libraries (10) are also transiently activated early after growth factor addition to quiescent cells. The kinetics of activation and repression of transcription is distinct for different genes. β-Actin gene and c-*fos* transcription peak within 10 min after growth stimulation, whereas ODC gene and c-*myc* expression is maximal approximately 60 to 120 min after treatment (18, 19). Little is known about the mechanism by which growth factors induce these transcriptional changes, although the activation of c-*fos* and actin genes occurs so rapidly that it most likely does not require new protein synthesis. The observation that c-*fos* and actin gene expression is stimulated before the increased transcription of c-*myc*, ODC, and other genes raises the question of whether activation of the synthesis of c-*fos*, actin, or other rapidly induced proteins might be required for the subsequent activation of genes such as c-*myc*.

To analyze the mechanisms by which genes are induced and subsequently repressed after growth factor stimulation, we examined the role of protein synthesis in the transient activation of the early response genes c-*fos*, β-actin, c-*myc*, and ODC. We found that the transcription of these four genes is differentially affected by the protein synthesis inhibitor anisomycin. In 3T3 cells all four transcripts are superinduced in the presence of protein synthesis inhibitors; however, in PC12 cells anisomycin blocks c-*myc* transcriptional induction. Our results suggest that there are multiple nuclear and cytoplasmic mechanisms by which growth factors affect gene expression.

MATERIALS AND METHODS

Cell culture. Quiescent cultures of BALB/c-3T3 A31 cells were prepared as previously described (19). Before stimulation, 3T3 cells were incubated in 20 ml of serum-free Dulbecco modified Eagle medium (DMEM) with or without 100 μM anisomycin (Pfizer, Inc., Groton, Conn.) or 10 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml for 30 min. Cultures were stimulated to progress from G0 into the G1 phase of the cell cycle by the addition of 15% calf serum or 1 unit of purified porcine platelet-derived growth factor (PDGF, kindly provided by Paul Stroobant and Michael Waterfield) per ml. The maintenance and stimulation of PC12 cultures with NGF is described elsewhere (18). When cultures were pretreated with anisomycin (100 μM) or cycloheximide (10 μg/ml), the protein synthesis inhibitors were added as concentrated solutions directly to the culture medium, and the cells were then stimulated 30 min later by the addition of NGF to 50 ng/ml.

Nuclear runoff transcription assay and Northern analysis.

* Corresponding author.

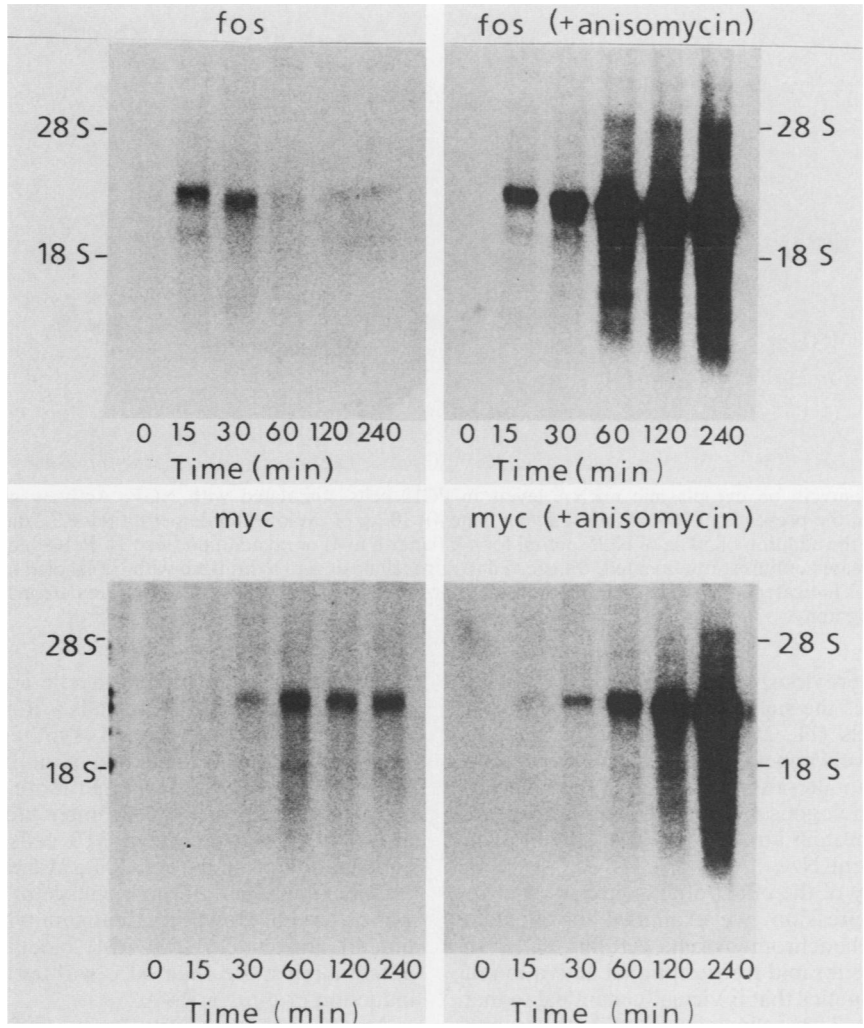

FIG. 1. Kinetics of cytoplasmic mRNA induction after serum stimulation in the presence or absence of anisomycin. Quiescent BALB/c-3T3 cells were incubated with 100 μM anisomycin–DMEM (right) or DMEM alone (left) for 30 min and then stimulated by the addition of 15% calf serum. Polyadenylated cytoplasmic RNA was isolated from cells at various times after serum addition (0 to 240 min), fractionated, transferred to nitrocellulose, and hybridized with ^{32}P-labeled fragments of c-*fos* or c-*myc* as indicated in Materials and Methods. The positions of 28S and 18S rRNA markers are indicated. The radioactivity is visualized by autoradiography.

Nuclei and cytoplasmic RNA were isolated from BALB/c-3T3 or PC12 cells at various times after stimulation as described by Greenberg and Ziff (19). The details of the nuclear runoff transcription assay and sources of cloned plasmid DNAs have been described elsewhere (19). For Northern analysis polyadenylated cytoplasmic RNA was fractionated on a formaldehyde-agarose gel and transferred to nitrocellulose as described by Chen-Kiang et al. (8). The RNA was hybridized to a nick-translated 1-kilobase internal *Pst*I fragment from pv-*fos*-1 (14), a ^{32}P-labeled 1-kilobase *Pst*I third exon fragment from pM-c-*myc*54 (34), or a ^{32}P-labeled 600-base-pair internal *Pst*I fragment from pODC934 (3).

RESULTS

Anisomycin potentiates mRNA production. Stimulation of quiescent BALB/c-3T3 cells with 15% calf serum or purified PDGF caused a rapid transient increase in the 2.2-kilobase cytoplasmic c-*fos* mRNA as previously reported (19, 27, 29). The level of cytoplasmic c-*fos* mRNA was maximal approximately 15 min after serum stimulation, and thereafter its level rapidly decreased (Fig. 1). c-*myc* cytoplasmic mRNA synthesis was also induced; however, the activation occurred subsequent to c-*fos* activation (6, 19, 25) (Fig. 1). To determine whether the activation of c-*fos* and c-*myc* mRNA synthesis in BALB/c-3T3 cells is dependent on protein synthesis, we stimulated quiescent cells with growth factors in the presence of anisomycin, a stringent inhibitor of protein synthesis. In these cells, anisomycin inhibits protein synthesis more than 97% as measured by the decrease in radioactively labeled amino acid incorporation into protein (data not shown). When quiescent BALB/c-3T3 cells were treated with 100 μM anisomycin for 30 min and then stimulated with serum, both c-*fos* and c-*myc* cytoplasmic mRNAs were rapidly induced, and the kinetics of c-*fos* mRNA induction relative to that of c-*myc* was unaffected (Fig. 1). However, pretreatment with anisomycin caused a dramatic increase in the amount of c-*fos* or c-*myc* mRNA that accumulated after growth factor stimulation (Fig. 1), and under these conditions the mRNAs remained in the cytoplasm for a longer period of time. We obtained similar results with cycloheximide (data not shown), a less stringent inhibitor of protein

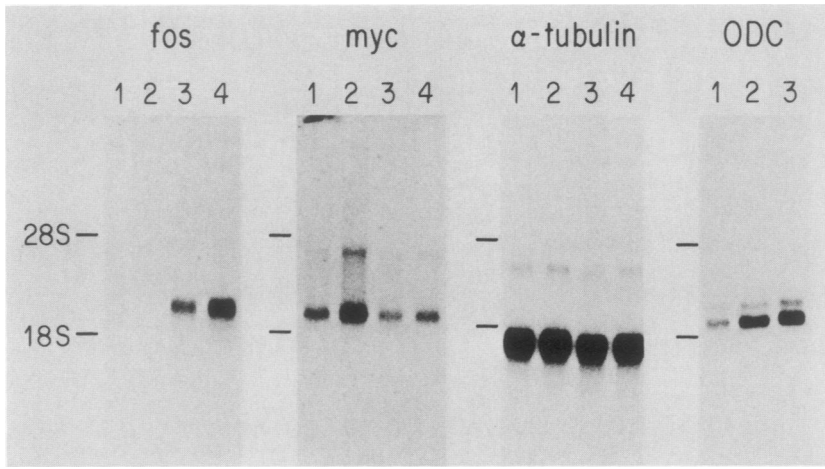

FIG. 2. Effect of anisomycin on cytoplasmic mRNA levels in PC12 cells stimulated with NGF. Actively growing PC12 cells were preincubated for 30 min in the presence of 100 μM anisomycin (lane 4); 10 μg of cycloheximide per ml (lane 3), or no addition (lanes 1 and 2) and then stimulated by the addition of 50 ng of NGF per ml for 4 h (lanes 2 to 4) or no addition (lane 1). Polyadenylated cytoplasmic RNA was isolated from NGF-treated cultures, fractionated, transferred to nitrocellulose, and hybridized with ^{32}P-labeled fragments of c-*fos*, c-*myc*, ODC, or α-tubulin genes as indicated in Materials and Methods. The positions of 28S and 18S rRNA markers are indicated. The radioactivity is visualized by autoradiography.

synthesis, confirming previously reported results showing that this inhibitor causes the superinduction of c-*fos*, c-*myc*, and actin mRNA levels (11, 25, 27, 29). The increase in cytoplasmic mRNA levels appears to be specific for certain mRNAs inasmuch as no alteration was seen in the levels of mRNA sequences homologous to another proto-oncogene, c-*raf*, that encodes a protein kinase (data not shown) or in the levels of α-tubulin mRNA.

To test the generality of the effect of anisomycin on c-*fos* and c-*myc* mRNA expression, we examined its effect in another cell line, the pheochromocytoma cell line PC12. In these cells NGF causes a rapid transcriptional activation of c-*fos* and c-*myc* with kinetics that is virtually identical to that observed in quiescent 3T3 cells stimulated with growth factors (18) (see Fig. 4). The level of c-*fos* cytoplasmic mRNA was very low in unstimulated PC12 cells (Fig. 2, lane 1). It peaked within 15 min after NGF addition (18), and by 4 h after NGF treatment, the time point shown in lane 2 of Fig. 2, the c-*fos* mRNA returned to the level present in unstimulated cells. c-*fos* mRNA was superinduced when PC12 cells were treated with 100 μM anisomycin or 10 μg of cycloheximide per ml for 30 min before NGF addition for 4 h (Fig. 2, compare lanes 1 through 4). However, in contrast to the results obtained in 3T3 cells, when PC12 cells were preincubated with anisomycin and then treated with NGF for periods varying between 0 and 4 h, the induction of c-*myc* mRNA levels was completely abolished (Fig. 2; M. E. Greenberg and E. B. Ziff, unpublished observations). The expression of another NGF-inducible mRNA, that for ODC, was potentiated slightly if cells were pretreated with anisomycin for 30 min and then stimulated with NGF (Fig. 2). In a control experiment, tubulin mRNA levels were found to be unaltered in NGF-stimulated PC12 cells incubated in either the presence or absence of anisomycin (Fig. 2).

We considered several alternative explanations for the difference between the effect of anisomycin on c-*myc* expression in PC12 cells and 3T3 cells. One possibility is that anisomycin is a more stringent inhibitor of protein synthesis in PC12 cells than in 3T3 cells. Analysis of the level of protein synthesis, by measuring the incorporation of [^{35}S]methionine into trichloroacetic acid-preciptable protein (21), indicated that in PC12 cells a 30-min pretreatment with anisomycin inhibits protein synthesis more than 99%, whereas in 3T3 cells the extent of inhibition is somewhat less (95 to 97%). We tested whether more stringent inhibition of protein synthesis in 3T3 cells might block the superinduction of c-*myc*. When quiescent 3T3 cells were treated with a combination of inhibitors, 100 μM anisomycin and 10 μg of cycloheximide per ml, no blockage of c-*myc* superinduction was observed. However, treatment with a higher concentration of anisomycin (500 μM) blocked the c-*myc* mRNA potentiation more than 50% without affecting the level of induction of c-*fos* mRNA.

An alternate possibility for the difference in the two cell lines is that an activator or repressor of c-*myc* expression is more labile in one cell type than the other. In this case a longer or more stringent inhibition of protein synthesis with anisomycin might yield different results. Longer pretreatment of 3T3 cells with protein synthesis inhibitors before serum stimulation was not effective in preventing c-*myc* stimulation in 3T3 cells, suggesting that the block in c-*myc* expression is not due to differential lability of a c-*myc* activator.

In summary, the observation that growth factor stimulation of c-*fos* mRNA expression is not inhibited but rather is potentiated, even under the most stringent conditions of inhibition of protein synthesis, indicates that activation of this gene is independent of new protein synthesis. In PC12 cells c-*myc* activation is protein synthesis dependent; however, in 3T3 cells this question is not yet resolved.

Protein synthesis inhibitors affect nuclear transcription. The experiments described thus far analyzed the effects of protein synthesis inhibitors on growth factor regulation of mRNA levels. We next examined the effects of inhibitors upon growth factor induction of c-*fos*, c-*myc*, actin, and ODC gene transcription and upon nuclear RNA stability. We tested the possibility that anisomycin directly affects c-*fos* and c-*myc* transcription by measuring the level of transcription in vitro in nuclei isolated from fibroblasts that had been pretreated for 30 min in the presence or absence of anisomycin and then stimulated for various periods with calf

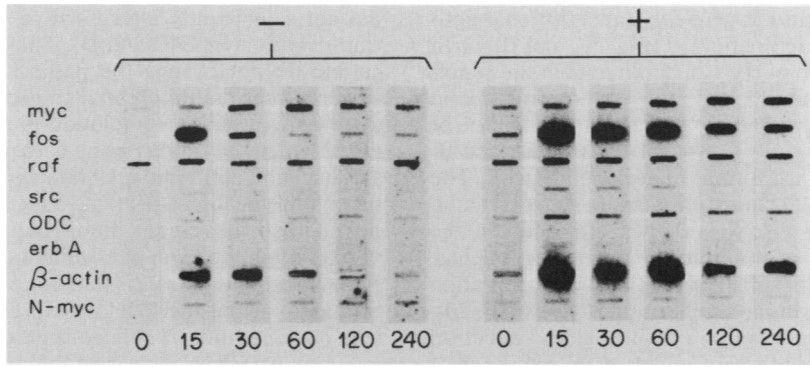

FIG. 3. Analysis of gene transcription after serum stimulation in the presence or absence of anisomycin. Quiescent BALB/c-3T3 cells were incubated with 100 μM anisomycin–DMEM (+) or DMEM alone (−) for 30 min and then stimulated by the addition of 15% calf serum. Nuclei were isolated at various times after serum addition (0 to 240 min), runoff assays were performed, and ^{32}P-labeled RNA was hybridized to plasmid DNAs that had been spotted onto nitrocellulose. The radioactivity is visualized by autoradiography.

serum. We have previously shown using a runoff transcription assay that growth factor stimulation of BALB/c-3T3 cells results within minutes in the transient induction of c-*fos* and β-actin gene transcription and somewhat later in the stimulation of c-*myc* transcription (19). Pretreatment of 3T3 cells with 100 μM anisomycin for 30 min caused a small increase in the level of c-*fos*, c-*myc*, and actin gene transcription in quiescent cells even without growth factor stimulation (Fig. 3). This increase in transcription after 30 min of treatment of quiescent 3T3 cells with anisomycin was somewhat variable (see Fig. 6); however, longer incubations with anisomycin clearly resulted in a substantial increase in c-*fos* transcripts (data not shown). Moreover, preincubation of quiescent 3T3 cells with anisomycin resulted in a substantial increase in the magnitude and duration of the c-*fos*, actin, and ODC gene transcriptional activation that occurred upon serum stimulation (Fig. 3). There appeared to be some variability in the response of c-*myc* transcription to anisomycin (compare Fig. 3 and 6). Nevertheless, in several experiments anisomycin was observed to superinduce c-*myc* transcription two- to threefold (Fig. 3). The potentiation of c-*myc* mRNA by protein synthesis inhibitors was much greater than the increase seen at the transcriptional level, suggesting that in 3T3 cells the effect of anisomycin is primarily to stabilize c-*myc* mRNA. The effect of protein synthesis inhibitors on c-*fos* transcription was more substantial and may account in large part for the superinduction of the c-*fos* mRNA (Fig. 1).

Similar effects of anisomycin or cycloheximide on c-*fos* transcription were obtained when growth-arrested 3T3 cells were treated with purified PDGF (data not shown). In all cases, the potentiation of transcription in the presence of the inhibitor was selective for genes such as c-*fos*, c-*myc*, ODC, and actin and was not seen for several other genes including c-*raf* and N-*myc* (Fig. 3). Treatment with anisomycin also did not reproducibly affect the number of counts of [^{32}P]UTP incorporated into total RNA.

When the pheochromocytoma cell line PC12 was incubated with anisomycin for 30 min and then stimulated with NGF, c-*fos* transcription was superinduced (Fig. 4) in a manner virtually identical to that seen for this gene in 3T3 cells. However, the NGF-stimulated induction of c-*myc* transcription was completely inhibited by anisomycin (Fig. 4). The effect of these drugs on c-*myc* mRNA levels in NGF-treated PC12 cells shown in Fig. 2 is due at least in part to a protein-synthesis-inhibitor-dependent transcriptional block.

Role of nuclear transcript stability. We considered several alternative explanations for the mechanism by which anisomycin enhances the levels of c-*fos*, c-*myc*, and actin nuclear transcripts in 3T3 cells. One possibility, corresponding to transcriptional control, is that there exists a repressor of transcription whose synthesis is activated or which is stabilized by growth factor stimulation but that this repressor is not synthesized or decays rapidly in the presence of inhibitors of protein synthesis. Another explanation, corresponding to nuclear transcript stability, is that within minutes after their synthesis, c-*fos*, c-*myc*, and actin gene transcripts are degraded by a newly synthesized or activated, highly specific RNase. To rationalize the nuclear runoff transcription data shown in Fig. 3 with the analysis of cytoplasmic mRNA shown in Fig. 1, one or more sequence-specific nucleases would have to act both in the nucleus and in the cytoplasm and would have to be labile, not synthesized, or otherwise inactivated in the presence of protein synthesis inhibitors. Several experiments were carried out to test for the presence of a transcript-specific RNase. If such a nuclease was active in isolated nuclei during the labeling of newly transcribed RNA in the runoff transcription assay, less degradation of c-*fos*, c-*myc*, or actin gene transcripts might be expected if the assay incubation time were shortened. This would give rise to an increased hybridization of ^{32}P-labeled c-*fos*, c-*myc*, or actin RNA during the period of repression.

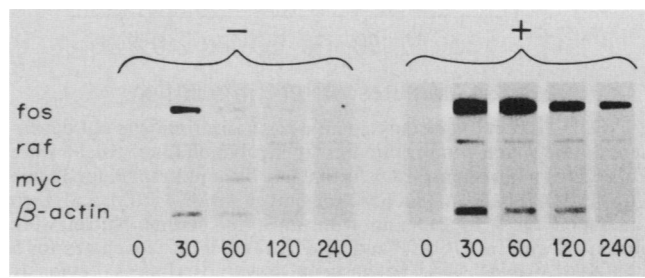

FIG. 4. Analysis of gene transcription after NGF stimulation of PC12 cells in the presence or absence of anisomycin. Actively growing PC12 cells were incubated with (+) or without (−) 100 μM anisomycin for 30 min and then stimulated by the addition of 50 ng of NGF per ml. Nuclei were isolated at various times after NGF addition (0 to 240 min), runoff assays were performed, and ^{32}P-labeled RNA was hybridized to plasmid DNAs that had been spotted onto nitrocellulose. The radioactivity is visualized by autoradiography.

The kinetics and magnitude of c-*fos* transcriptional activation and repression were unaffected (Fig. 5A and B) when the duration of incubation of the isolated nuclei in the runoff assay was shortened from 30 to 5 min. Decreasing the in vitro incubation time also had no effect on the ability of anisomycin to potentiate c-*fos* transcription (Fig. 5A and B) or c-*myc* and actin transcription (data not shown). The results of this 5-min runoff transcription assay suggest that in 3T3 cells the repression of c-*fos*, c-*myc*, and actin in the absence of anisomycin is not due to a specific nuclear RNase. This conclusion is substantiated by the observation that the addition of heparin, an inhibitor of RNases (16, 20), to the runoff transcription reaction mixture had no effect on c-*fos* transcription either in the presence or in the absence of anisomycin (Fig. 6, compare panels A and B). Furthermore, the inclusion in the runoff assay of sarcosyl or ammonium sulfate, reagents which release proteins from RNA and decrease the specificity of sequence-specific RNases (20), did not significantly alter c-*fos*, c-*myc*, actin gene, or c-*raf* transcription (Fig. 6C and D). That sarcosyl and ammonium sulfate do not change the pattern of gene expression also suggests that the differential transcription of c-*fos* observed after growth factor stimulation is not the result of a differential block in transcriptional elongation. Agents such as sarcosyl and ammonium sulfate should remove histones and other chromosomal proteins that might be blocking RNA polymerase movement along the DNA (16, 17, 20). The variability in the level of c-*raf* and c-*erbB* expression seen in Fig. 6 most likely reflects fluctuations in the runoff transcription assay since the level of expression of these two genes was observed not to change reproducibly in many different experiments. The nuclear runoff analysis of c-*fos* and actin transcription under the various conditions described supports the conclusion that subsequent to their activation the expression of these genes is repressed at the level of transcription by a protein-synthesis-dependent mechanism.

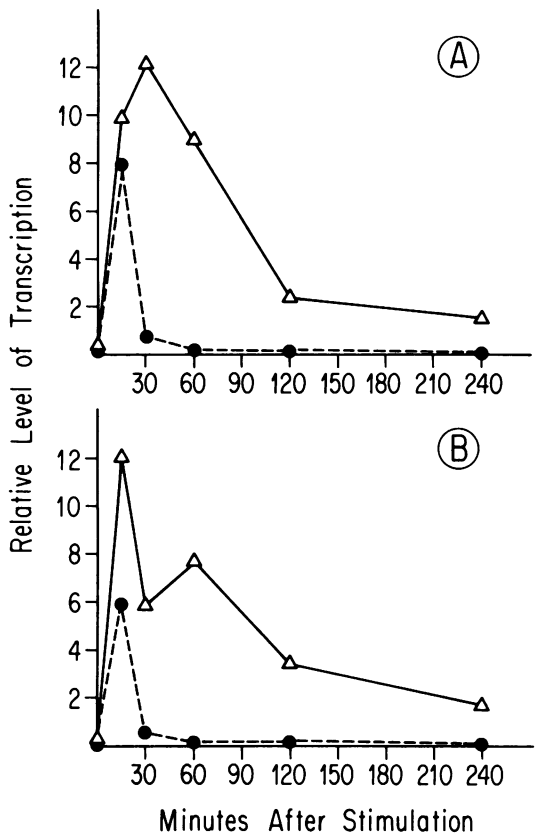

FIG. 5. Effect of anisomycin on c-*fos* transcription is not dependent on the runoff transcription assay incubation time. Nuclei were isolated from quiescent 3T3 cells that had been pretreated for 30 min with DMEM (●) or 100 μM anisomycin–DMEM (△) and then stimulated for 0 to 240 min with 15% calf serum. Nuclei were incubated with [α-^{32}P]UTP and cold nucleotide triphosphates for 5 min (A) or 30 min (B) to label nascent RNA transcripts as previously described (18). ^{32}P-labeled RNA was hybridized to the pv-*fos*-1 plasmid spotted onto nitrocellulose. The radioactivity was detected by autoradiography. Relative signal intensities were calculated by densitometric scanning of the autoradiograms, and the results were plotted. The experiments shown in panels A and B were performed on separate days. The relative levels of transcription calculated by densitometric scanning of the autoradiogram from the experiment shown in panel B were normalized to the values obtained for the experiment shown in panel A.

DISCUSSION

We used inhibitors of protein synthesis to examine which of the steps in growth factor activation and repression of c-*fos*, c-*myc*, and actin gene expression are dependent on the appearance of new proteins or the continued synthesis of labile proteins. We were particularly interested in examining the possibility that growth factor stimulation of a rapidly induced nuclear protein such as c-*fos* might be necessary for the subsequent activation of c-*myc* transcription. As will be discussed, the results of experiments with PC12 cells are consistent with this possibility. However, different results were obtained with 3T3 cells, and the question of the involvement of c-*fos* or another induced protein in c-*myc* activation remains open.

Previous studies have shown that cytoplasmic c-*fos* and c-*myc* mRNAs are superinduced when 3T3 cells are stimulated with growth factors in the presence of cycloheximide (11, 25, 27, 29). Elder et al. (15) have reported results of nuclear runoff transcription experiments indicating that pretreatment with cycloheximide causes an increase in the magnitude and duration of epidermal growth factor-stimulated activation of actin transcription. Our experiments extend these previous studies in several ways. We found that the c-*fos* and c-*myc* cytoplasmic mRNA expression is potentiated not only when growth factor-stimulated 3T3 cells are pretreated with cycloheximide, but also upon pretreatment with another protein synthesis inhibitor, anisomycin. Employing a stringent inhibitor of protein synthesis may be particularly important if one considers that in addition to blocking protein synthesis these inhibitors cause a dramatic increase (up to 100-fold) in the level of specific cytoplasmic mRNAs. If, for example, the average level of protein synthesis is blocked 90 to 95% (as is the case with cycloheximide) but the absolute number of c-*fos* or c-*myc* mRNAs is increased ~100-fold, then there could result a severalfold increase in the amount of c-*fos* or c-*myc* protein synthesized. For this reason the use of anisomycin is advantageous since it is a very stringent inhibitor, blocking protein synthesis 99%. Under these stringent conditions it will be important to use specific antibodies to determine whether anisomycin does effectively block the synthesis of the c-*fos* and c-*myc* proteins.

An advantage of using several different inhibitors of protein synthesis in these experiments is that compounds such as anisomycin and cycloheximide act by different mechanisms: cycloheximide blocks peptide bond formation while anisomycin inhibits association of the ribosomal subunits

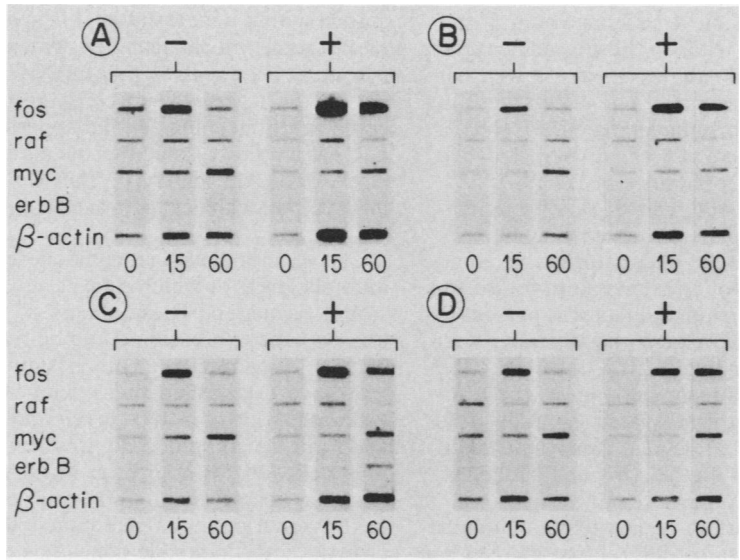

FIG. 6. Effect of sarcosyl, heparin, and ammonium sulfate on the kinetics of proto-oncogene transcriptional activation. Quiescent BALB/c-3T3 cells were incubated with DMEM (−) or 100 μM anisomycin–DMEM (+) for 30 min and then stimulated by the addition of 15 percent calf serum. Nuclei were isolated from untreated 3T3 cells (0) or 3T3 cells treated with calf serum for 15 or 60 min. Runoff assays were performed as described in Materials and Methods with the following modifications: reaction mixture contained no change (A), 1 mg of heparin per ml (B), 0.5 percent sarcosyl (C), or 0.4 M ammonium sulfate (D). ^{32}P-labeled RNA was hybridized to plasmid DNAs pv-*fos*-1, pv-*raf*, pM-c-*myc*-54, pv-*erbB*, and pH-β-actin bound to nitrocellulose. The radioactivity is visualized by autoradiography.

(28). The fact that these two inhibitors of protein synthesis affect transcription similarly makes it unlikely that the effect of the drugs is a nonspecific one that is unrelated to protein synthesis inhibition. In particular, the finding that anisomycin (an inhibitor of polysome formation) potentiates c-*fos* and c-*myc* mRNA rules out polysome protection as a possible mechanism for the increase in mRNA half-lives.

c-*myc* mRNA levels are not always superinduced by treatment with protein synthesis inhibitors. As shown above, in PC12 cells both cycloheximide and anisomycin inhibit c-*myc* mRNA expression. The difference in the effect of these reagents on c-*myc* mRNA levels in 3T3 and PC12 cells is paradoxical. Both 10 μg of cycloheximide per ml and 100 μM anisomycin block induction of c-*myc* mRNA completely in PC12 cells while significantly potentiating mRNA expression in 3T3 cells. Experiments with high concentrations of anisomycin in 3T3 cells suggest that the difference in response to protein synthesis inhibitors could reflect the fact that the inhibition is more stringent in PC12 cells. The activation of c-*myc* may be mediated by the same factor in these two cell lines; however, the activating factor might be present in limiting amounts in PC12 cells. The difference in c-*myc* regulation in PC12 and 3T3 cells could also result from the fact that the growth states of the two cell lines used in our experiments were different. The 3T3 cells were quiescent before the addition of calf serum, while the PC12 cells were actively dividing before the addition of NGF. It remains possible, however, that the mechanisms regulating c-*myc* transcription are different in the two cell lines. Perhaps growth factors such as PDGF and NGF activate c-*myc* transcription by distinct pathways. It is equally plausible that the variation in the response of c-*myc* to anisomycin treatment in the two cell lines arises from an unidentified difference in the structures of the c-*myc* genes resulting from mutation. Distinguishing between these various alternative models for c-*myc* regulation must await further investigation. It is clear, however, that in PC12 cells, NGF activation of c-*myc* expression requires new protein synthesis. As shown by the nuclear runoff transcription analyses, the failure to superinduce c-*myc* mRNA in PC12 cells in the presence of protein synthesis inhibitors results at least in part from a failure to induce transcription of the c-*myc* gene.

In the absence of protein synthesis inhibitors, growth factor treatment of both BALB/c-3T3 and PC12 cells causes an increase in c-*myc* expression. Using the nuclear runoff transcription assay, we reproducibly observed that the induction of c-*myc* mRNA by growth factors is regulated at least in part at the level of transcription. However, in both 3T3 and PC12 cells the extent of c-*myc* transcriptional activation is somewhat variable (compare Fig. 3, 4, and 6; see references 18 and 19) from one experiment to another. Since this variability is not seen with other growth factor-regulated genes such as c-*fos*, it could reflect a variable loss or inactivation of specific c-*myc* transcriptional factors during the isolation and incubation of nuclei in the different experiments. A recent publication by Blanchard et al. (5) reports that in Chinese hamster lung fibroblasts growth factor activation of c-*myc* is due to alterations in mRNA stability and is not the result of changes in c-*myc* transcription. The apparent discrepancy between our results and those of Blanchard et al. may be the result of cell type differences or differences in the method for isolating nuclei and performing the runoff transcriptional assay.

The finding that the activation of c-*fos* transcription precedes that of c-*myc* temporally (18, 29), coupled with the present observation that in PC12 cells c-*myc* stimulation requires protein synthesis while c-*fos* transcriptional activation does not, suggests that growth factors activate these two genes by distinct mechanisms. The c-*fos* protein could play a role in c-*myc* activation. Inhibition of c-*fos* protein synthesis by pretreatment of cells with anisomycin might be the cause of the block in c-*myc* transcription. It is also possible that another unidentified labile protein mediates growth factor induction of c-*myc* transcription.

Since c-*fos* and actin gene transcriptional activation do not require new protein synthesis, the binding of PDGF and

NGF to their receptors must elicit a cellular event(s) that utilizes preexisting proteins and leads to these specific rapid transcriptional changes. Recent studies have shown that c-*fos* expression can be stimulated in a range of cell types in response to many different factors, including PDGF, NGF, epidermal growth factor, phorbol esters, dibutyryl cyclic AMP, forskolin, and calcium ionophores (13, 18, 19, 25, 27, 29; M. E. Greenberg, L. A. Greene, and E. B. Ziff, unpublished observations), all of which act at least in part by activating protein kinases. Protein phosphorylation could play a key role in the activation of c-*fos* and actin transcription. In one such model, a transcriptional factor is present in an inactive form either in the cytoplasm or associated with the plasma membrane. Upon binding of growth factor to the plasma membrane and protein kinase activation, the transcriptional factor becomes phosphorylated so that it is activated and translocated to the nucleus. In the nucleus it would bind to regulatory regions on the c-*fos* or actin genes, thereby activating their transcription.

Interestingly, the action of various agents that can induce c-*fos* transcription initially activates different types of protein kinases, for example, a tyrosine kinase (for PDGF and epidermal growth factor [31, 35]), the c-kinase (for phorbol esters [7]), the cyclic AMP-dependent kinase (for dibutyryl cyclic AMP and forskolin [26]), or a Ca^{2+}-calmodulin-dependent kinase (for calcium ionophores [9]). If a phosphorylation event does mediate the effects of growth factors on c-*fos* transcription, the activator must serve as a substrate for many different kinases, or else the signaling mechanism of these different agents must converge before the phosphorylation of the c-*fos* activator.

Inhibitors such as anisomycin could either stabilize such a transcriptional activator or inhibit the production (or activity) of a repressor, thereby potentiating the level of c-*fos* and actin transcripts detected by the runoff assay with isolated 3T3 cell nuclei. The rapid repression of c-*fos* and actin transcription that normally follows activation in growth factor-stimulated cells is significantly delayed in the presence of anisomycin. This is consistent with an autoregulatory role for c-*fos*, c-*myc*, actin, or another early response gene. For example, a newly synthesized growth factor-induced protein might be required to repress c-*fos* and actin transcription. If protein synthesis is blocked, the transcription of these genes would remain at a high level. An alternative to this mechanism is that gene expression is turned off by a labile repressor whose synthesis is not regulated by growth factors and that decays in the presence of anisomycin. The observation that anisomycin treatment can induce somewhat the level of c-*fos* and actin gene transcription in quiescent 3T3 cells that have not been stimulated with growth factors (Fig. 2) is consistent with this last possibility.

The runoff transcription experiments presented in Fig. 5 and 6 suggest that the failure to repress c-*fos* and actin in 3T3 cells in the presence of anisomycin is not a consequence of the decay of a sequence-specific RNase that normally degrades these RNA transcripts immediately after synthesis in the nucleus. However, we cannot completely rule out the alternative that there exists a potent, fast-acting RNase that functions in both the nucleus and the cytoplasm. Regardless of the mechanism by which RNA expression is reduced in the nucleus subsequent to its activation, and what effect anisomycin has on this process, it is clear that growth factor-activated c-*fos* and c-*myc* cytoplasmic mRNAs normally have a very short half-life. In the absence of inhibitors of protein synthesis the c-*fos* mRNA is preferentially degraded within minutes after it is synthesized. There appears to be some mechanism by which a cytoplasmic RNase specifically recognizes this mRNA. The inactivation or decay of the RNase when cells are treated with anisomycin could partly account for the superinduction of cytoplasmic mRNA synthesis seen after the addition of protein synthesis inhibitors. Alternatively, the RNase might only attack mRNA molecules which are actively being translated on polysomes.

The experiments presented here suggest that there are multiple levels at which c-*fos*, c-*myc*, and actin gene expression is regulated. These effects of protein synthesis inhibitors on gene expression could be relevant to a large class of early-response genes. This is supported by earlier studies that have established that cycloheximide treatment causes superinduction of mRNA levels and nuclear transcription for actin mRNA in epidermal growth factor-stimulated embryonic cells (15), β-interferon mRNA in transfected Chinese hamster ovary cells (33), and early-region adenovirus mRNAs during virus infection (30). A different group of growth factor-inducible genes has a requirement of protein synthesis for activation (21). In eucaryotic cells stimulated with growth factors and other agents, a variety of transcriptional activators and repressors may mediate the activation of gene expression in the nucleus while sequence-specific RNases may dictate the mRNA half-life in the cytoplasm.

ACKNOWLEDGMENTS

We thank Lloyd Greene for providing PC12 cells and purified NGF, Adriana Rukenstein for assistance with cell culture and Willy Welch for his help in the preparation of the manuscript.

This work was supported by American Cancer Society grant MV-75 and Public Health Service grants GM 30760 and P30 CA 16087 from the National Institutes of Health. E.B.Z. is the recipient of American Cancer Society Faculty Research Award FRA 207 and is an associated member of the Kaplan Cancer Center. M.E.G. was supported by a postdoctoral fellowship from the National Institutes of Health (5 F32-CA07515-02).

LITERATURE CITED

1. **Abrams, H. D., L. R. Rohrschneider, and R. N. Eisenman.** 1982. Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell **29**:427–439.
2. **Armelin, H. A., M. C. S. Armelin, K. Kelly, T. Stewart, P. Leder, B. H. Cochran, and C. D. Stiles.** 1984. Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. Nature (London) **310**:655–660.
3. **Berger, F. G., P. Szymanski, E. Read, and G. Watson.** 1984. Androgen-regulated ornithine decarboxylase mRNAs of mouse kidney. J. Biol. Chem. **259**:7941–7946.
4. **Bishop, J. M.** 1983. Cellular oncogenes and retroviruses. Annu. Rev. Biochem. **52**:301–354.
5. **Blanchard, J.-M., M. Piechaczyk, C. Dani, J.-C. Chambard, A. Franchi, J. Pouyssegur, and P. Jeanteur.** 1985. c-*myc* is transcribed at a high rate in G0 arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. Nature (London) **317**:443–451.
6. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonenshein.** 1984. Cell-cycle control of c-*myc* but not c-*ras* expression is lost following chemical transformation. Cell **36**:241–247.
7. **Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka.** 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. **257**:7847–7851.
8. **Chen-Kiang, S., D. J. Wolgemuth, M.-T. Hsu, and J. E. Darnell.** 1982. Transcription and accurate polyadenylation *in vitro* of RNA from the major late adenovirus 2 transcription unit. Cell **28**:575–584.

9. **Cheung, W. Y.** 1980. Calmodulin plays a pivotal role in cellular regulation. Science **207**:19–27.
10. **Cochran, B. H., A. C. Reffel, and C. D. Stiles.** 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33**:939–947.
11. **Cochran, B. H., J. Zullo, I. M. Verma, and C. D. Stiles.** 1984. Expression of the c-*fos* gene and of an *fos*-related gene is stimulated by platelet-derived growth factor. Science **226**: 1080–1082.
12. **Curran, T., A. D. Miller, L. Zokas, and I. M. Verma.** 1984. Viral and cellular *fos* proteins: a comparative analysis. Cell **36**:259–268.
13. **Curran, T., and J. T. Morgan.** 1985. Superinduction of the c-*fos* by nerve growth factor in the presence of peripherally active benzodiazepines. Science **229**:1265–1268.
14. **Curran, T., G. Peters, C. Van Beveren, N. M. Teich, and I. M. Verma.** 1982. FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. J. Virol. **44**:674–682.
15. **Elder, P. K., L. J. Schmidt, T. Ono, and M. J. Getz.** 1984. Specific stimulation of actin gene transcription by epidermal growth factor and cycloheximide. Proc. Natl. Acad. Sci. USA **81**:7476–7480.
16. **Ferencz, A., and K. H. Seifart.** 1975. Comparative effect of heparin on RNA synthesis of isolated rat-liver nucleoli and purified RNA polymerase A. Eur. J. Biochem. **53**:605–613.
17. **Gariglio, P., J. Buss, and M. Green.** 1974. Sarkosyl activation of RNA polymerase activity in mitotic mouse cells. FEBS Lett. **44**:330–333.
18. **Greenberg, M. E., L. A. Greene, and E. B. Ziff.** 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. **260**:14101–14110.
19. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311**:433–438.
20. **Groudine, M., M. Peretz, and H. Weintraub.** 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. Mol. Cell. Biol. **1**:281–288.
21. **Hendrickson, S. L., and C. D. Scher.** 1983. Platelet-derived growth factor-modulated translatable mRNAs. Mol. Cell. Biol. **3**:1478–1487.
22. **Horwitz, M. S., C. Brayton, and S. G. Baum.** 1973. Synthesis of type 2 adenovirus DNA in the presence of cycloheximide. J. Virol. **11**:544–551.
23. **Kaczmarek, L., J. K. Hyland, R. Watt, M. Rosenberg, and R. Baserga.** 1985. Microinjected c-*myc* as a competence factor. Science **228**:1313–1315.
24. **Kahana, C., and D. Nathans.** 1984. Isolation of cloned cDNA encoding mammalian ornithine decarboxylase. Proc. Natl. Acad. Sci. USA **81**:3645–3649.
25. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
26. **Krebs, E. G., and J. A. Beavo.** 1979. Phosphorylation-dephosphorylation of enzymes. Annu. Rev. Biochem. **48**: 923–959.
27. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. Nature (London) **312**:711–716.
28. **Lewis, J. B., and M. B. Mathews.** 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell **21**:303–313.
29. **Muller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature (London) **312**:716–720.
30. **Nevins, J. R.** 1981. Mechanisms of activation of early viral transcription by the adenovirus E1a gene product. Cell **26**: 213–220.
31. **Nishimura, J., J. S. Huang, and T. F. Deuel.** 1982. Platelet-derived growth factor stimulates tyrosine-specific protein kinase activity in Swiss mouse 3T3 cell membranes. Proc. Natl. Acad. Sci. USA **79**:4303–4307.
32. **Persson, H., and P. Leder.** 1984. Nuclear localization and DNA binding properties of a protein expressed by human c-*myc* oncogene. Science **225**:718–721.
33. **Ringold, G. M., B. Dieckmann, J. L. Vannice, M. Trahey, and F. McCormick.** 1984. Inhibition of protein synthesis stimulates the transcription of human β-interferon genes in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA **81**:3964–3968.
34. **Stanton, L. W., R. Watt, and K. B. Marcu.** 1983. Translocation, breakage and truncated transcripts of c-*myc* oncogene in murine plasmacytomas. Nature (London) **303**:401–406.
35. **Ushiro, H., and S. Cohen.** 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J. Biol. Chem. **255**:8363–8365.