Insulin's Regulation of c-fos Gene Transcription in Hepatoma Cells*

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In H4IIE rat hepatoma cells insulin interacts with its receptors to induce DNA synthesis and promote cell division. However, the postreceptor events that lead to DNA synthesis and cell division have not been well characterized. Previous studies indicate that insulin can regulate the expression of several genes in H4 cells. One of these genes is the proto-oncogene c-fos, a cellular gene whose deregulation has been implicated in the process of cellular differentiation and division. In the present work insulin is shown to regulate cellular c-fos mRNA accumulation and the transcription rate of the c-fos gene. Insulin caused a rapid, dose-dependent increase in the cytoplasmic concentration of c-fos mRNA which was maximal by 30 min. Preceding this, a more rapid 6–8-fold increase in transcription of the c-fos gene was observed. Induction of transcription was apparent following only 5 min of insulin addition. This is the most rapid effect of insulin yet demonstrated on the induction of gene expression. Protein synthesis inhibitors (cycloheximide, anisomycin) also induced the transcription of the c-fos gene. However, they stimulated a much greater increase in transcription than did insulin, and followed a different time course of action. The addition of insulin in combination with a protein synthesis inhibitor resulted in no greater increase in c-fos transcription than did the addition of a protein synthesis inhibitor alone. The nonadditivity of H4 cell c-fos gene expression may indicate a similar mode of action by insulin and protein synthesis inhibitors.

The proto-oncogene c-fos is one of the early response genes which is expressed in response to a variety of growth factors (1–9). The controlled expression of the normal cellular fos (c-fos) gene may be involved in the signaling mechanisms within the cell which lead to an increase in DNA synthesis and cell division. A rapid induction in the transcription of the fos gene was observed following growth factor or mitogen addition to quiescent fibroblasts, macrophages, myoblasts, and pheochromocytoma cells (for reviews see Refs. 10 and 11; also see Refs. 2, 3, 7, and 8). The enhanced expression of c-fos and human hepatoma cells (20–25). Insulin was also found to increase accumulation of c-fos mRNA in 3T3 cells (1, 2, 6). Recently, insulin was shown to increase accumulation of c-fos mRNA in a rat hepatoma cell line (26).

The c-fos gene is one of a group of genes thought to be involved in events leading to cell division. Since rat H4IIE hepatoma cells divide in response to insulin, we hypothesized that one of the initial actions of insulin would be to induce transcription of the c-fos gene. In the experiments presented here, insulin was found to induce c-fos gene transcription as well as protein synthesis inhibitors.

EXPERIMENTAL PROCEDURES

Materials—Sodium insulin was a gift from Dr. Ronald Chance, Eli Lilly Co. Swim's 77 media, fetal bovine serum, and horse serum were purchased from Gibco. EGF, fibroblast growth factor (FGF), vanadyl-ribonucleoside complex, and proteinase K were purchased from Bethesda Research Laboratories. RNasin was purchased from Promega. DNase I and RNase T1 were purchased from Worthington/Cappel Biochemicals (Freehold, NJ). [α-32P]UTP (3000 Ci/mM), [α-32P]UTP (3000 Ci/mM), and [3H]leucine (110 Ci/mM) were purchased from ICN Biochemicals. All other reagents were purchased from Sigma.

Cell Culture—H4IIE hepatoma cells (H4; American Type Culture Collection (ATCC)) were grown in 10-cm tissue culture plates in Swim's 77 medium with 5% fetal bovine serum and 5% horse serum in a 5% CO2 incubator at 85% humidity. Serum was withdrawn for 20–24 h prior to experiments when the cells were approximately 60% confluent (27).

Isolation and Quantification of Cytoplasmic mRNA—RNA was isolated by a modification of the method of Favaloro et al. (28). Cells were washed in Dulbecco's saline solution, scraped, and resuspended in a lysis buffer containing 10 mM Tris-HCl pH 8, 150 mM sodium chloride, 1.5 mM magnesium chloride, 0.5% Nonidet P-40, and 10 mM vanadyl-ribonucleoside complex. The nuclei were pelleted, and the supernatant was diluted with 1 volume of 200 mM Tris-HCl pH 9, 300 mM sodium chloride, 2% sodium dodecyl sulfate, 25 mM EDTA and then extracted with phenol/chloroform (1:1) and ethanol-precipitated. Northern gels and transfers were performed using 10 g of cytoplasmic RNA as described previously (29).

Hybridization to an excess of nick-translated PBR322 containing the entire mouse c-fos cDNA (pc-fos-3; ATCC) was performed as detailed previously (27). Nitrocellulose filters were washed extensively and exposed to Kodak X-ray film.

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1 The abbreviations used are: EGF, epidermal growth factor; FGF, fibroblast growth factor; Cyc, cycloheximide; IGF-I, insulin-like growth factor I; Anis, anisomycin; PDGF, platelet-derived growth factor; CHO, Chinese hamster ovary.
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XAR X-ray film. For quantification, densitometric scans were made of resultant autoradiograms. The integrated densitometric scan of the control for each experiment was arbitrarily set to unity and the densities of experimental samples were compared to those of controls.

Isolation of Nuclei and the Measurement of Transcription—To measure the elongation of transcripts in control or treated cells, nuclear mRNA run-off assays were performed as described previously (30). Nuclei were prepared from approximately 1 x 10^6 cells which were washed in Dulbecco’s salt solution and were scraped and resuspended in cell homogenization buffer (0.3 M sucrose, 2 mM magnesium acetate, 3 mM calcium chloride, 10 mM Tris-HCl, pH 8.0, 2.78 mM dithiotreitol, and 1 unit/ml RNasin). The cells were homogenized 15 strokes in a Dounce homogenizer. Two volumes of cell dilution buffer (2 M sucrose, 5 mM magnesium acetate, 10 mM Tris-HCl, pH 8.0, 2.75 mM dithiotreitol, 1 unit/ml RNasin) were added to the homogenized cells and this mixture was layered over cell dilution buffer. Nuclei were isolated by centrifugation at 30,000 x g for 45 min.

Nuclei were resuspended and incubated in a buffer containing 25 mM Tris-HCl, pH 8.0, 5 mM magnesium acetate, 12.5% glycerol, 100 mM ammonium sulfate, 1 mM ATP, 0.4 mM GTP and CTP, 5 mM dithiotreitol, 100 units/ml RNasin, 0.05 mM magnesium chloride, and 100 ng of [α-32P]UTP. Incubations were for 30 min at 26 °C and the reactions were stopped by the addition of 200 μg/ml Proteinase K. The reaction mixtures were then brought to 10 mM calcium chloride and incubated with DNase I (340 μg/ml). RNA was isolated by sequential sodium perchlorate/ethanol precipitation (31), phenol extraction, trichloroacetic acid precipitation, and sodium acetate/ethanol precipitation (32).

An aliquot of radioactively labeled RNA was counted in a β-scintillation counter. Labeled RNA was incubated with nitrocellulose on which 2 pg of plasmid DNA had been dotted. The plasmids used were pBR322 (for background controls), pBR322 containing a p-β-tubulin cDNA insert (33), or pBR322 containing the c-fos cDNA insert described above. Transcription of several other oncogenes was studied and the probes used were v-sis (pv-sis; ATCC), p53 (34), v-ros (35), erb-b (pAE Bam 0.5; ATCC), and myb (pCM 1.3 (36)). The number of counts/min of radioactively labeled RNA added to the nitrocellulose filters varied between 4 x 10^6 and 23 x 10^6 in the separate experiments. However, within each experiment the number of counts in the control and experimental groups were identical. The nitrocellulose was incubated with the radioactively labeled RNA for 4-6 h at 65 °C. The filters were then washed in 0.3 M sodium chloride, 0.03 M sodium citrate, treated with RNase A and RNase T1, and exposed to X-ray film. Following autoradiography, the extent of hybridization of labeled mRNA to the cDNA probes was quantitated by densitometric scanning of the autoradiograms. The densitometric signal of the experimental groups were expressed as a percentage of the control (untreated group) of each experiment.

5H1 Leucine Incorporation into Protein—Cells were deprived of serum for 24 h and then exposed to the protein synthesis inhibitors cycloheximide (Cyc) and anisomycin for 30 min. [5H]Leucine (2 μCi/ml) was added to each plate for a 120-min incubation. At the end of this incubation, cells were trypsinized, collected, and the protein was precipitated with 10% trichloroacetic acid at 4 °C for 30 min. The precipitated pellets were dissolved in ACS (Amerham Corp.) and then counted in a Beckman LS 3801 scintillation counter. The results are expressed as the percentage of leucine incorporated per 30-mm plate compared with the untreated control cells.

RESULTS

Regulation of c-fos mRNA by Insulin—Insulin increased the concentration of c-fos mRNA in a time- and dose-dependent manner. As shown in Fig. 1, insulin (5 x 10^-9 M) stimulated the cytoplasmic accumulation of c-fos mRNA in H4 cells (lanes b, c, and h) compared to cells not treated with insulin (lanes a, c, and f). Insulin increased c-fos mRNA levels approximately 10-fold and 8-fold by 30 and 60 min, respectively, followed by a decrease to 3-fold by 120 min (Table I). Concentrations of insulin greater than 5 x 10^-8 M did not result in a further rise in c-fos mRNA levels and lower concentrations gave proportionally less induction (data not shown).

Transcriptional Effects of Insulin on c-fos and Other Oncogenes—Several control experiments were performed to confirm the linearity of our nuclear run-off assays and have been presented previously (30). In the present studies, there was a small, but consistent 2-3-fold increase in c-fos transcription within 5 min of insulin addition (5 x 10^-5 M), reaching a maximum of 6-7-fold induction within 15 min (Fig. 2, A and B). This increase was short-lived since the induction declined to 4-fold in 30 min and returned to basal levels within 60-120 min. The effect of insulin on transcription of the c-fos gene was also determined at 24 h following insulin addition and was found to be comparable to transcription rates in basal, untreated cells (data not shown). Insulin had no effect on the transcription of the β-tubulin gene.

The ability of increasing concentrations of insulin to induce the transcription of c-fos mRNA is shown in Fig. 3. A minimal concentration of 5 x 10^-11 M insulin was necessary to obtain a 75% increase in transcription of c-fos compared to control values, with 5 x 10^-8 M insulin resulting in a maximum 7-fold stimulation. Higher concentrations of insulin resulted in no

a b c d e f g h

FIG. 1. Effect of insulin and cycloheximide on the cytoplasmic concentration of c-fos mRNA. H4 cells were deprived of serum for 24 h and treated with insulin or cycloheximide. RNA was prepared and blots were performed as described under “Experimental Procedures.” Shown are Northern transfers of three separate experiments. Lanes a, c, and f, control cells; lanes b, e, and h, insulin (5 x 10^-6 M) for 60 min; lanes d and g, cycloheximide (50 μg/ml) 60 min.

| Time (min) | n | Increase (range) |
|-----------|---|-----------------|
| 0         | 0  | 1.0             |
| 30        | 8  | 9.8 (3.1-19.4)  |
| 60        | 10 | 8.1 (2.8-21.3)  |
| 120       | 6  | 3.4 (1.2-6.7)   |

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Portions of this work (including part of “Results,” Figs. 5-8, and part of “Discussion”) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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The effects of insulin on the transcription of several other oncoproteins were studied and insulin was found to have no effect on transcription of the sis, p53, ras, erb-b, and myb oncoproteins (data not shown).

Transcriptional Effects of Serum and Other Growth Factors on the c-fos Gene—Fetal bovine serum has been shown to stimulate cell division of H4 cells (21). Since the c-fos gene is a growth-related gene, the effect of serum on the regulation of transcription of this gene was determined. The addition of 5 or 10% fetal bovine serum to 24-h serum-starved H4 cells resulted in a 6- or 8-fold increase in c-fos transcription, respectively (Fig. 4, serum lanes b and c). The induction of c-fos returned to basal transcription rates by 120 min following serum administration (Fig. 4, serum lanes d and e). This time course was similar to that observed with insulin treatment.

Two other growth factors, EGF and FGF, were ineffective in altering the transcription rate of the c-fos gene when added individually to serum-deprived H4 cells (Fig. 4, EGF- FGF lanes b and c). Likewise, transcription of the sis, p53, ras, erb-b, and myb oncogenes did not respond to the addition of serum, EGF, or FGF under the conditions tested (data not shown).

**DISCUSSION**

Growth factors such as EGF, platelet-derived growth factor, and FGF play important roles in the stimulation of DNA synthesis and cell division (11). The effects of insulin as a growth factor, however, have been poorly studied. Since insulin can interact with receptors for other growth factors, it has been difficult to determine if insulin's growth stimulatory effects are mediated through its own receptor or through other growth factor receptors. In the rat H4IIE hepatoma cell line used in the current experiments the growth-promoting effects of insulin were due solely to insulin's interaction with its own receptor (37-39).

Insulin has been shown to regulate the expression of several genes in rat hepatoma cells. For example, insulin stimulates the transcription of the p33 and glyceraldehyde-3-phosphate dehydrogenase genes (40, 41) while it inhibits the transcription of the phosphoenolpyruvate carboxylase gene (42, 43). In 3T3-L1 adipocytes insulin induced the accumulation of c-fos mRNA (5). However, the effects of insulin were not exclusive, since other growth factors, including platelet-derived growth factor, FGF, and bombesin all increased accumulation of c-fos mRNA in these cells (1, 2, 6). Recently, insulin was shown to increase the accumulation of c-fos mRNA in rat H35 hepatoma cells (26).

Evidence is presented in this report suggesting that insulin increases accumulation of c-fos mRNA by stimulating c-fos transcription. The induction of c-fos gene transcription occurred at physiological concentrations of insulin. This is the most rapid stimulatory effect of insulin on gene transcription yet shown, with significant induction of c-fos gene transcription evident within 5 min of insulin addition. The effects of insulin on c-fos gene transcription could be mimicked by serum but not by EGF or FGF. However, in a recently published report, higher concentrations of EGF than were

**FIG. 2.** Time course of insulin on the transcription of c-fos (fos) and β-tubulin (Tub) mRNAs. H4 cells were deprived of serum for 24 h and treated with insulin (5 × 10^{-11} M). Nuclei were then isolated by sucrose density ultracentrifugation. Transcription was measured using a nuclear run-off assay in which the isolated nuclei were incubated in the presence of [β-32P]UTP and the labeled RNA was hybridized to c-fos or β-tubulin cDNAs that were immobilized on nitrocellulose as described under “Experimental Procedures.” The filters were washed extensively and autoradiographed (A). For A, lane a, control cells (0 min); lane b, 5 min; lane c, 15 min; lane d, 30 min; lane e, 60 min; and lane f, 120 min. The rate of c-fos transcription was quantitated by densitometric scanning of the resultant autoradiograms (B). The autoradiographic signals of the experimental groups were expressed as a percentage of the untreated control cells. The results in B are the means of 4-10 separate experiments for each time point. The vertical lines indicate 1 S.E.

**FIG. 3.** Dose response of insulin on the transcription of c-fos mRNA. H4 cells were deprived of serum for 24 h and treated with insulin for 15 min at the indicated concentrations. Nuclei were isolated and transcription was measured as described in the legend for Fig. 2. The resultant autoradiograms were scanned and the results are the means of 3-10 separate experiments. The vertical lines indicate 1 S.E.

**FIG. 4.** Effects of addition of serum, EGF, or FGF on the transcription of c-fos mRNA. H4 cells were deprived of serum for 24 h and treated with serum, EGF, or FGF. Nuclei were isolated and transcription was measured as described in the legend for Fig. 2. For the serum treatments: lane a, control cells; lane b, 5%, 30 min; lane c, 10%, 30 min; lane d, 10%, 60 min; and lane e, 10%, 120 min. For the EGF-FGF treatments: lane a, control cells; lane b, EGF, 5 ng/ml, 30 min; lane c, FGF, 100 ng/ml, 30 min.
utilized in the present work increased c-fos mRNA levels in hepatoma cells (44).

An increase in c-fos mRNA accumulation in response to high concentrations of insulin was found in a Chinese hamster ovary cell line (CHO.T). This cell line was transfected with the human insulin receptor gene and expressed 1 million human insulin receptors/cell (45). A sequence in the c-fos 5'-flanking region, referred to as the serum-responsive element, was found to be necessary for an induction of the c-fos mRNA by insulin. However, there was no induction of c-fos mRNA by insulin in wild-type CHO cells that did not express this abnormally large number of human insulin receptors. Wild-type CHO cells did contain the normal complement of hamster insulin receptors and had normal insulin responses. The need for an abnormally high membrane concentrations of human insulin receptors to obtain insulin-induced increases of c-fos mRNA in the CHO.T cells questions the physiological significance of this insulin effect. In the present study, a cell line expressing endogenous rat insulin receptors (20,000-30,000/cell (46)) responded to insulin with a rapid increase in the transcription of the c-fos gene.

The rapidity of insulin’s effects suggest that few steps intervene between the binding of insulin to H4 cells and the stimulation of c-fos gene transcription. Additionally, the mechanism(s) by which insulin regulates transcription of this gene must be extremely quick since gene expression is induced as rapidly as can be measured. The regulation of this gene by physiological concentrations of insulin in cells containing normal levels of endogenous insulin receptors and the presumed involvement of transcriptional regulatory proteins make the regulation of this gene an interesting model in which to study the role of insulin in the control of gene expression and cell growth.

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Supplemental Material: Insulin's Regulation of the c-fos Gene Transcription in Neuroblastoma Cells

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RESULTS

Regulation of c-fos mRNA by Protein Synthesis Inhibitors: Either of two different protein synthesis inhibitors, cycloheximide or actinomycin D, induced the cellular accumulation of c-fos mRNA. The effects of cycloheximide were greater than those observed with actinomycin D treatment at 90 min. The levels of c-fos mRNA induced by cycloheximide were approximately 10-fold higher than control levels (Fig. 1, panel A), while actinomycin D effects were much smaller.

Transcriptional Effects of Protein Synthesis Inhibitors: The cycloheximide-induced increase in c-fos mRNA levels was greater than the growth factor induced levels (12, 3, 7, 4). The two protein synthesis inhibitors, cycloheximide and actinomycin D, acted through different mechanisms. Cycloheximide inhibited the transcription of c-fos mRNA when added in the absence of any agents. Following 15 min of cycloheximide (50 μg/ml) or actinomycin D (10 μg/ml) addition, c-fos mRNA transcription was stimulated by EGF and 4.3-kcalm (respectively, 5 A, 3A). These effects were similar to that of insulin (Fig. 2). However, unlike insulin's effects, cycloheximide inhibited c-fos mRNA transcription continued to increase after 15 min of treatment with cycloheximide (Fig. 3), indicating a posttranscriptional event. EGF and 4.3-kcalm stimulated c-fos mRNA transcription at 45 and 120 min following the addition of cycloheximide (Fig. 3).

The effects of cycloheximide on c-fos mRNA accumulation correlated with reductions in the expression of Fos proteins in the cell population. Cycloheximide concentrations of 50 and 500 μg/ml resulted in reductions of c-fos mRNA transcription by 85, 85 and 85%, respectively (Fig. 2). A stimulation of c-fos mRNA transcription by 30, 60, and 85%, respectively (Fig. 3). In a similar manner, anisomycin, a protein synthesis inhibitor, also prevented c-fos mRNA transcription at 20 min of treatment with cycloheximide (Fig. 3). Similar results were observed with several other inhibitors, including 200-250 μg/ml cycloheximide and 50 μg/ml anisomycin which were added 20 min after cycloheximide.

Effect of Addition of Insulin and Protein Synthesis Inhibitors: The effects of these inhibitors on the transcription of the c-fos gene were also examined. Insulin and protein synthesis inhibitors together reduced the accumulation of c-fos mRNA. As shown in Fig. 2, when insulin was added to the cultures 15 min after cycloheximide, the inhibition of c-fos mRNA accumulation was prevented. These results suggest that the proteins encoded by the c-fos gene are synthesized during the 15 min incubation period with cycloheximide and can be blocked by insulin.

Figure 1. TIME COURSE OF PROTEIN SYNTHESIS INHIBITION ON THE TRANSCRIPTION OF c-fos mRNA. The results were obtained from 24 h cultures of Neuro-2a cells treated with 50 μg/ml cycloheximide or actinomycin D (50 μg/ml). (A) The c-fos mRNA was measured at indicated time points. The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments. (B) The effect of cycloheximide on the transcription of c-fos mRNA. The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments. (C) The effect of actinomycin D on the transcription of c-fos mRNA. The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments.

Figure 2. EFFECTS OF CYCLOHEXIMIDE ON THE TRANSCRIPTION OF c-fos mRNA. (A) The results were obtained from 24 h cultures of Neuro-2a cells treated with 50 μg/ml cycloheximide or actinomycin D (50 μg/ml). (B) The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments. (C) The effect of cycloheximide on the transcription of c-fos mRNA. The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments.

Figure 3. EFFECTS OF CYCLOHEXIMIDE ON THE TRANSCRIPTION OF c-fos mRNA. (A) The results were obtained from 24 h cultures of Neuro-2a cells treated with 50 μg/ml cycloheximide or actinomycin D (50 μg/ml). (B) The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments. (C) The effect of cycloheximide on the transcription of c-fos mRNA. The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments.

Figure 4. EFFECT OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON PROTEIN SYNTHESIS CLONING INHIBITION IN 48 h CELLS. The results were obtained from 24 h cultures of Neuro-2a cells treated with 50 μg/ml cycloheximide or actinomycin D (50 μg/ml). (A) The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments. (B) The effect of cycloheximide on the transcription of c-fos mRNA. The ordinate shows the percentage of the control level at 0 time. The values are the means of 1-2 experiments.
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The two protein synthesis inhibitors stimulated c-myc gene transcription in the absence of insulin. This implies that the c-myc gene was under the control of a rapidly turning-on, inhibitory protein in 3T3 cells. In this model, decreased synthesis of this inhibitory regulator by substitution of Cyc or Anis resulted in an increase of c-myc gene transcription. The inhibitory factor was stabilized by the addition of protein synthesis inhibitors. In other words, protein synthesis inhibitors often augment growth factor induction of gene expression, EL, 81, 371.

In conclusion, insulin and protein synthesis inhibitors do not have an additive effect on cyclic transamination since the effects of protein synthesis inhibitors alone were minimal. Moreover, since the effects of protein synthesis inhibitors were as much larger than those of insulin, additional effects of insulin may not have been discernible. In addition, transcriptional activity may have been at maximal levels, with no further increases possible. An additive effect may have been masked.

If the effects of insulin and protein synthesis inhibitors are not additive, it suggests that insulin acts to increase the amount of a rapidly turning-on transcriptional inhibitory protein(s) by decreasing its synthesis. Alternatively, insulin may act by destabilizing the protein or by decreasing its activity.

Whether insulin acts through a mechanism similar to protein synthesis inhibitors or through a different mechanism, it is apparent that the effects of insulin and protein synthesis inhibitors are additive on c-myc expression. The results of protein synthesis inhibitors at least 180 min. Moreover, the effects of insulin-induced c-myc expression are not apparent in the absence of insulin, as for the entire duration of insulin treatment, the amount of c-myc mRNA was not increased. In contrast, following insulin treatment, insulin induced c-myc production a signal leading to the stimulation of gene transcription, as well as a termination signal to return transcription to basal levels.

Figure 1. EFFECTS OF ADDITION OF BOTH INSULIN AND EITHER CYCLOHEXIMIDE OR ANISOMYCIN ON THE TRANSCRIPTION OF c-MYC mRNA.

- 3T3 cells were deprived of serum for 24h and treated with insulin (100 ng/mL) for 30 min. Cyc or Anis (10 ng/mL) for 30 min. Insulin for 60 min. A combination of a protein synthesis inhibitor and anisomycin was added after the indicated times. The amount of c-myc mRNA was measured as described in the legend for Fig. 7. The results are the means of 4 separate experiments for Cyc and 2 separate experiments for Anis. The vertical lines indicate ± SE.
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