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 has been observed in a variety of differentiating cells (12–14) and after stimulation of the central nervous system (15–17).

Insulin regulates normal growth and development of mammals. When this hormone was deficient, or when cells were resistant to the actions of this hormone, normal growth was impaired (18, 19). The ability of insulin to induce DNA synthesis was shown in a variety of cell types in culture including human fibroblasts, mouse 3T3 fibroblasts, mouse pre-adipocytes, hamster kidney cells, rat liver cells, and rat and human hepatoma cells (20–25). Insulin was also found to regulate the c-fos gene in 3T3-L1 adipocytes (5). However, this effect of insulin on c-fos induction was not exclusive. Other growth factors, including platelet-derived growth factor, epidermal growth factor (EGF),1 and bombesin, also increased 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 was 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). [*32P]UTP (3000 Ci/mM), [*3P]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 salt solution, scraped, and resuspended in a lysis buffer containing 10 mm Tris-HCl pH 8, 140 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 7.5, 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-OMAT film.

1The 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-run-off* assays were performed as described previously (30). Nuclei were prepared from approximately 1 × 10^8 cells which were washed in Dulbecco's salt solution and were scraped and resuspended in cell homogenization buffer (0.3 mM sucrose, 2 mM magnesium acetate, 5 mM calcium chloride, 10 mM Tris-HCl, pH 8.0, 2.78 mM dithiothreitol, and 1 unit/ml RNasin). The cells were homogenized 15 strokes in a Dounce homogenizer. Two volumes of cell dilution buffer (2.0 mM sucrose, 5 mM magnesium acetate, 10 mM Tris-HCl, pH 8.0, 2.75 mM dithiothreitol, 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 × 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, 5 mM dithiothreitol, 100 units/ml RNasin, 0.05 mM EDTA, and 100 μg of [α-32P]UTP. Incubations were for 45 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 μg of plasmid DNA had been dotted. The plasmids used were pBR322 (for background controls), pBR322 containing a 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 Barn 0.5; ATCC), and myb (pCM 1.3 (36)). The nitrocellulose filters varied between 4-70% at 65°C. The filters were then washed in 100 mM 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 counts in the control and experimental groups were identical. The densitometric signal of the experimental groups were expressed as a percentage of leucine incorporated per 35-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 × 10^{-9} M) stimulated the cytoplasmic accumulation of c-fos mRNA in H4 cells (lanes b, c, and f) compared to cells not treated with insulin (lanes a, e, and h). 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 × 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 × 10^{-9} 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 × 10^{-11} M insulin was necessary to obtain a 75% increase in transcription of c-fos compared to control values, with 5 × 10^{-8} M insulin resulting in a maximum 7-fold stimulation. Higher concentrations of insulin resulted in no

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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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**Table I**

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**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 × 10^{-8} M) for 60 min; lanes d and g, cycloheximide (50 μg/ml) 60 min.
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**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^{-9} 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 [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.

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 carboxykinase 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 further increase in c-fos transcription. The transcription of the β-tubulin gene was not affected by any concentration of insulin.

The effects of insulin on the transcription of several other oncogenes were studied and insulin was found to have no effect on transcription of the sis, p53, ras, erb-b, and myb oncogenes (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).

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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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REFERENCES


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

**Joseph L. Mendes**

**RESULTS**

Regulation of c-fos cDNA by Protein Synthesis Inhibitors — Either of two different protein synthesis inhibitors, cycloheximide (20 μg/ml) or actinomycin D (10 μg/ml) induced the cellular accumulation of c-fos mRNA. The effects of cAMP or cAMP and forskolin on c-fos mRNA were even more marked than those observed with insulin treatment at 30 min. The levels of c-fos mRNA induced by cAMP or forskolin were approximately 10-fold higher than control levels (Fig. 1). RNA prints analyses, lanes d and g.

Transcriptional Effects of Protein Synthesis Inhibitors on c-fos and Other Genes — Protein synthesis inhibitors usually have a small or no effect in stimulating c-fos gene transcription when added alone. However, when added in combination with a growth factor, these inhibitors can result in a much greater induction of transcription than the growth factor alone (2). The two protein synthesis inhibitors, cycloheximide and actinomycin D, induced the transcription of c-fos mRNA when added in the absence of any other agents. Following 13 min of CAMP (10 μg/ml) or forskolin (10 μg/ml) addition, c-fos transcription was stimulated by 1.5- and 1.6-fold, respectively (Fig. 5, A, K). These effects were similar to those of insulin (Fig. 2). However, unlike insulin's effects, cycloheximide and actinomycin D continued to increase after 15 min of incubation (Figs. 6, A, B). In contrast, forskolin stimulation of c-fos transcription at 10 and 15 min following addition of CAMP or forskolin (Fig. 5).

The effects of cAMP and forskolin on transcription of the c-fos gene occurred at concentrations that exceeded the incorporation of [3H]thymidine into DNA cell proteins. CAMP or forskolin, in concentrations of 10 and 50 μM, resulted in reductions of insulin incorporation by 85% and 80%, respectively (Fig. 5, A, B). Actinomycin D, added at concentrations of 10 and 50 μM, resulted in reductions of base incorporation of 85% and 80% (Fig. 5, A, B). A combination of CAMP at 10 μM and forskolin at 50 μM, resulting in stimulation of c-fos transcription 2.5-fold, resulted in a stimulation of base incorporation of 25% (Fig. 5, A, B). The results indicated that the c-fos gene is regulated at both transcriptional and posttranscriptional levels.

**Figure 1. Time Course of Protein Synthesis Inhibition on the Transcription of c-fos mRNA** — Each inhibitor and protein synthesis inhibitors induced the transcription of the c-fos gene. However, it was noted whether cAMP and protein synthesis inhibitors together would increase c-fos transcription more than either agent alone. As shown in Fig. 2, when Actinomycin D was added for the last 15 min of a 60 min incubation with either CAMP or forskolin, there was no additional increase in c-fos transcription. The results indicated that the c-fos gene is regulated at both transcriptional and posttranscriptional levels.

**Figure 2. Dose Response of Cycloheximide and Actinomycin D on Transcription of c-fos mRNA** — No cells were deprived of serum for 24 h and treated with CAMP or forskolin at the indicated concentrations for 60 min. [3H]thymidine was added to the culture media for 2 hr after the stimulus was observed and measured in a [3H]thymidine counter. The data was expressed as a percentage of the insulin incorporation in untreated cells. The results are the means of 3 separate experiments.
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MATERIALS AND METHODS

Figure 1. EFFECTS OF ADDITION OF BOTH INSULIN AND EITHER CYCLOSPORINE OR AMINOREXIN ON THE TRANSCRIPTION OF GADD45 gene.

In cells deprived of insulin for 24h and treated with insulin (50 nM) for 30 min, Cyc Rs, 30 pg/ml for 60 min, Amin Rs. 25 pg/ml for 60 min of a combination of a protein synthesis inhibitor and an eicosanoid synthase inhibitor, resulted in an increase in GADD45 gene transcription. Treatment with Cyc or Amin resulted in a decrease in GADD45 gene transcription. The results are the means of 3 separate experiments for Cyc and Amin. The vertical lines indicate 1 SE.

DISCUSSION

The two protein synthesis inhibitors stimulated GADD45 gene transcripion in the absence of insulin. This implies that the gadd45 gene was under the control of a rapidly turning-on, inhibitory protein in the cells. In this model, decreased synthesis of this inhibitory regulator by inhibition of Cyc or Amin resulted in an increase of gadd45 gene transcription. In contrast, increased transcription of the transcription factor was inhibited by the addition of protein synthesis inhibitors. In culture, protein synthesis inhibitors often augment growth factor induction of gene expression.12,21,47. Moreover, it is essential that insulin and protein synthesis inhibitors did not have an additive effect on cyclosporine transcription at times when the effets of protein synthesis inhibitors alone were minimal. However, 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 maximum capacity, with no further increase possible. An additive effect may have been underestimated. If the effects of Insulin and protein synthesis inhibitors are not additive, it suggests that Insulin acts on expression by its ability to increase the transcription of the gene. Alternatively, Insulin may act by decreasing the activity of the transcriptional factors.

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 cyclosporine were additive, with the effects of protein synthesis inhibitors prevented for at least 18h by Insulin. Insulin's effects were short-lived even when present in the incubation medium for the entire period. Employment of a growth factor which possesses a short life span is ideal for the efficient examination of growth factor activities. In the case of cyclosporine transcription, Insulin added alone produces a signal leading to the stimulation of gene transcription, as well as a termination signal to return transcription to basal levels.
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J L Messina


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