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Insulin Induction of *pip 92*, *CL-6*, and Novel mRNAs in Rat Hepatoma Cells

Katherine Dobson Bortoff,[1] Chang-Cheng Zhu,[1] Yaroslav Hrywna,[1] and Joseph L. Messina[1,2]

[1]*Department of Physiology and Cell and Molecular Biology Program, SUNY Health Science Center, Syracuse, NY;*
[2]*Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL*

Insulin directly affects many aspects of cellular metabolism. An additional, poorly studied effect of insulin is the regulation of multiple genes whose products are important in many cellular functions. Using differential screening techniques, we identified insulin-regulated genes induced in insulin-treated rat H4IIE (H4) hepatoma cells. Two of the mRNAs identified were homologous to the previously characterized mouse *pip* 92 and rat *CL-6* immediate-early genes. The *pip* 92 clone was initially isolated from mitogen-stimulated mouse Balb/c 3T3 fibroblasts, whereas the *CL-6* clone was first isolated from regenerating rat liver. In this article, we demonstrated that in rat H4 cells, the transcription rates of both *pip 92* and *CL-6* are induced by insulin alone. Additionally, we showed that the transcription rates of two other genes, whose sequences are not homologous to any other sequences in gene bank ("novel genes"), were rapidly and transiently induced by insulin. These results demonstrate that insulin regulates the expression of several novel genes with a time-course similar to members of the immediate-early response gene family.

Key Words: Insulin; anisomycin; hepatoma cells; gene expression; immediate early genes.

Introduction

Insulin is important for normal growth and development in mammals *(1,2)*. Insulin also influences cellular metabolism by affecting glucose transport, enzyme activity, and protein synthesis *(3–6)*. One action of insulin that has been less well studied to date is its ability to alter the concentrations of specific mRNAs. It has been demonstrated that insulin induces transcription of several genes in rat H4IIE (H4) hepatoma cells and inhibits the transcription of several others *(6–13)*. Several of the induced genes are often referred to as immediate-early genes (IEGs), include the *c-fos*, *c-myc*, gene-33, β-actin, γ-actin, and α-tubulin genes, which are induced by serum and other growth factors in different tissues *(7,8,10,14–18)*. Many protein products of the IEGs, such as *c-fos* and *c-myc*, are thought to be important in regulating cell division and differentiation *(14,19,20)*. Previous work suggests that regulation of *c-fos* and regulation of *c-myc* gene expression are also important actions of insulin *(10,17,18,21)*. We decided to investigate whether additional genes may be regulated by insulin treatment of cultured hepatoma cells.

It has been demonstrated that insulin, along with other growth factors, participates in inducing DNA synthesis and cell division in many cell types, and that H4 cells will divide in response to the addition of insulin alone *(22–25)*. However, the exact mechanism of this regulation by insulin remains unclear. We hypothesized that a better understanding of which genes are regulated by insulin and eventually determining their role may give insight into this action of insulin. In addition, insulin has numerous metabolic effects on the liver, many of which are also observed in insulin-treated cultured H4 cells. Thus, genes responsive to insulin in H4 cells were identified through differential screening of an insulin/anisomycin-treated cDNA library, isolated from H4 cells.

Two genes selected from our library were previously isolated as *pip* 92 and *CL-6* from Balb/c3T3 cells and regenerating liver, respectively *(26–28)*. Sequence analysis of *pip 92* identified the gene product as a proline-rich protein with an extremely short half-life that is localized in the cytoplasm or possibly secreted *(26,27)*. It was characterized as an IEG inducible by serum, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) in Balb/c 3T3 fibroblasts *(29)*. Sequence analysis of *CL-6* resulted in the identification of a highly hydrophobic hepatic protein. It was characterized as an abundant induced mRNA in regenerating liver and inducible by a combination of insulin and cycloheximide in H35 cells, but the function is not yet known *(28)*.

Once cDNA clones were selected for these two genes, we demonstrated that *pip 92* and *CL-6* mRNAs are induced by insulin alone in rat H4 cells and that this increase was

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Author to whom all correspondence and reprint requests should be addressed: Joseph L. Messina, PhD, Department of Pathology, Division of Molecular and Cellular Pathology, Volker Hall, G019, 1670 University Blvd., University of Alabama at Birmingham, Birmingham, AL, USA 35294-0019. E-mail: messina@vh.path.uab.edu

Table 1
Summary of Clones Isolated by Differential Hybridization

Clone	Size of insert, kb	Size of mRNA, kb	Reference
pip 92	1.4	1.7	*(27)*
CL-6	2.0	3.1	*(28)*
7-34	0.9	1.8	New[a]
7-46	1.1	3.0, 2.1	New[a]

[a]No similarity of partial sequence of these two genes to any other sequence deposited in Genbank.

owing solely to a stimulation of transcription. Furthermore, we identified several additional cDNA clones for previously unidentified genes (designated 7-34 and 7-46), whose transcription rates and mRNA levels were induced by insulin.

Results

Selection of pip 92, CL-6, *7-34 and 7-46 mRNAs by Differential Screening*

In the differential screening analysis of insulin/anisomycin-treated H4 cells, several insulin/anisomycin-regulated clones were selected. We initially sequenced 150–400 bp of each clone and compared them to the sequences contained in the Genbank and EMBL data bases. Several of the clones selected showed no significant homology with any of the sequences on record and therefore are being considered novel.

Two of the clones selected were homologous to *pip 92*, cloned by Lau and Nathans *(29)* and *CL-6*, cloned by Mohn et al. *(30)*. Other clones selected for this study, based on a high degree of induction by insulin, were designated 7-34 and 7-46. The sizes of the cDNA inserts selected are summarized in Table 1. The rat clone homologous to mouse *pip* 92 was 1.4 kb and identified an mRNA that was approx 1.7 kb in rat H4 hepatoma cells. The reported size of *pip* 92 mRNA in mouse Balb/c 3T3 cells is approx 1.5 kb, excluding the poly-A tail *(27)*. The mRNA recognized by our cDNA via Northern analysis could have included the poly-A tail, since it was larger than 1.5 kb. Alternatively, the rat homolog of mouse *pip 92* might be slightly larger than that in the mouse. The insert size of 7-34 was 0.9 and probed for an mRNA that was approx 1.8 kb, which ran slightly below the 18S rRNA band. The insert size of 7-46 was 1.1 kb and probed for two mRNAs, of approx 3.0 and 2.1 kb (Table 1). Northern analysis showed that these clones were responsive to insulin, since their mRNA levels were increased following insulin stimulation, with or without pretreatment with anisomycin. Additional DNA sequencing of these novel cDNAs (800 bp of 7-34 and 960 bp of 7-46) again resulted in no significant homology with any of the sequences on record and, therefore, are being considered novel.

Stimulation of Insulin-Regulated Clones

It has previously been reported that mRNAs coding for *pip 92* and *CL-6* are rapidly induced in mitogen-treated fibroblasts and regenerating liver, respectively *(27,28)*. To confirm that the clones we selected from our library followed the same levels of induction previously noted for *pip* 92 and *CL-6* in different cell lines, we performed Northern analysis. H4 cells were deprived of serum for 24 h and treated with 1×10^{-8} *M* insulin. This corresponds to a postprandial concentration of insulin in rats *(31)*. The mRNAs were isolated at different times following insulin addition, and an equal amount of the respective mRNAs was probed with the *pip 92* and *CL-6* clones selected from our library (Fig. 1A,B). The increase in *pip* 92 mRNA levels was eight- and sevenfold above basal levels after 1 and 2 h of insulin treatment, respectively.

The protein synthesis inhibitor, anisomycin, was included because IEGs are often superinduced or the mRNA is stabilized in the presence of a protein synthesis inhibitor *(29,32,33)*. Additionally, we have found from previous studies in H4 hepatoma cells that anisomycin's effects are generally greater and more rapid than those of cycloheximide *(34,35)*, two agents that inhibit protein synthesis through different mechanisms *(8,34,35)*. Following treatment with anisomycin (100 μ*M*), alone or together with insulin, *pip* 92 levels were superinduced to 135- and 150-fold above basal levels (Fig. 1B). There was no significant effect with any treatment on the β-tubulin mRNA levels.

Addition of insulin for 1 and 2 h led to a fivefold increase in *CL-6* mRNA levels. Anisomycin alone also stimulated the accumulation of *CL-6* mRNA. After a 2-h incubation with anisomycin, or insulin and anisomycin together, there was an 11- or 9-fold induction, respectively, of *CL-6* mRNA (Fig. 1B). Induction of both the *pip 92* and *CL-6* mRNAs by anisomycin was greater than that exhibited by incubation with insulin alone for 1 or 2 h. Anisomycin has a more pronounced effect on *pip* 92 than on *CL-6* mRNA expression, suggesting differing control mechanisms for these two mRNAs (*see* Discussion).

The novel clones 7-34 and 7-46 were also rapidly induced by insulin. Following insulin addition, 7-34 mRNA levels were increased 2.5-fold above basal levels by 0.5 h, peaking at 4.5-fold above basal by 1 h and returning toward baseline by 1.5–2 h, whereas there was no effect on β-tubulin mRNA (Fig. 2 A,B). The appearance of a larger band is coincident with insulin treatment that results in an increased level of 7-34 mRNA. This may be a differentially spliced or preprocessed form of 7-34, which runs slightly faster than 28S rRNA. In the presence of anisomycin, 7-34 mRNA levels were superinduced 25-fold above basal levels. Following the addition of insulin together with anisomycin, 7-34 mRNA accumulation increased 30-fold (Fig. 2C). Insulin induced the mRNA level of clone 7-46 fivefold above baseline by 1 h. Clone 7-46 mRNA levels returned to baseline after

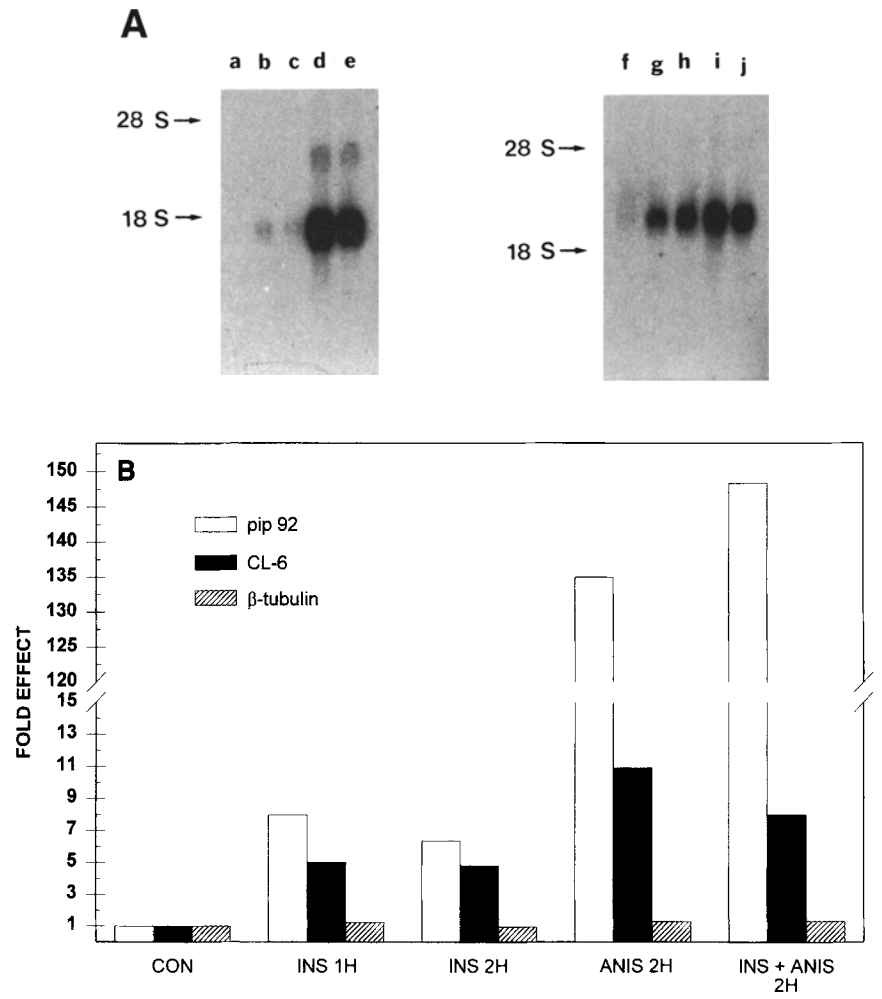

Fig. 1. Effects of insulin and anisomycin on the cytoplasmic concentrations of *pip 92* and *CL-6* mRNA. H4 cells were deprived of serum for 24 h and treated with the following: Lanes a and f, no treatment; lanes b and g, insulin (1×10^{-8} *M*) for 1 h; lanes c and h, insulin (1×10^{-8} *M*) for 2 h; lanes d and i, insulin (1×10^{-8} *M*) + anisomycin (100 μ*M*) for 2 h; lanes e and j, anisomycin (100 μ*M*) for 2 h. The respective mRNAs were prepared and blots were performed as described in the text. Panel (**A**) shows a representative experiment using *pip 92* as a probe (left panel) and *CL-6* as a probe (right panel). Densitometric scans of the results of *pip 92*, *CL-6*, and β-tubulin mRNA levels are graphed in panel (**B**).

2 h of insulin exposure (data not shown). The kinetics displayed by these insulin-regulated mRNAs is characteristic of IEGs as is the apparent "superinduction" of these genes in response to protein synthesis inhibitors *(29)*.

Transcription of the Insulin-Regulated Clones

To determine whether increases in the mRNAs isolated from the insulin/anisomycin library was due to an increased rate of transcription, nuclear run-on assays were performed. Transcription rates of the *pip* 92 and *CL-6* mRNAs were rapidly and transiently induced by insulin. In fibroblasts, *pip 92* transcription occurs as early as 2 min in response to mitogens *(29)*. In H4 cells *pip 92* transcription was already greatly induced at 14-fold above baseline by 0.25 h following insulin addition, declining to 9- and 8-fold by 0.5 and 1 h, respectively, and returning toward baseline by 2 h (Fig. 3A,B). The increase of *pip 92* transcription was sufficient to account for the increase in *pip 92* mRNA levels by insulin.

Insulin also rapidly increased the transcription of *CL-6* mRNA, but with slightly slower kinetics compared to the induction of *pip 92* transcription. Following 0.25 h of insulin addition, there was no change in the transcription rate of *CL-6* mRNA, but by 0.5 and 1 h, transcription of *CL-6* was elevated eight- and sixfold above basal levels, respectively. Transcription of *CL-6* returned to basal values by 2 h (Fig. 3A,B). As with *pip 92*, the increase in *CL-6* transcription was sufficient to account for the increase in *CL-6* mRNA following insulin addition. *CL-6* has been reported to be an abundant mRNA in regenerating liver or H35 cells stimulated with a combination of insulin plus cycloheximide *(27)*. However, this is the first direct evidence demonstrating that *CL-6 is* transcriptionally regulated by insulin. As has been previously published, insulin had no effect on transcription of β-tubulin, which was used as a control *(8,13,36,37)*.

Since the library was made from insulin plus anisomycin-treated cells, we next asked whether there was

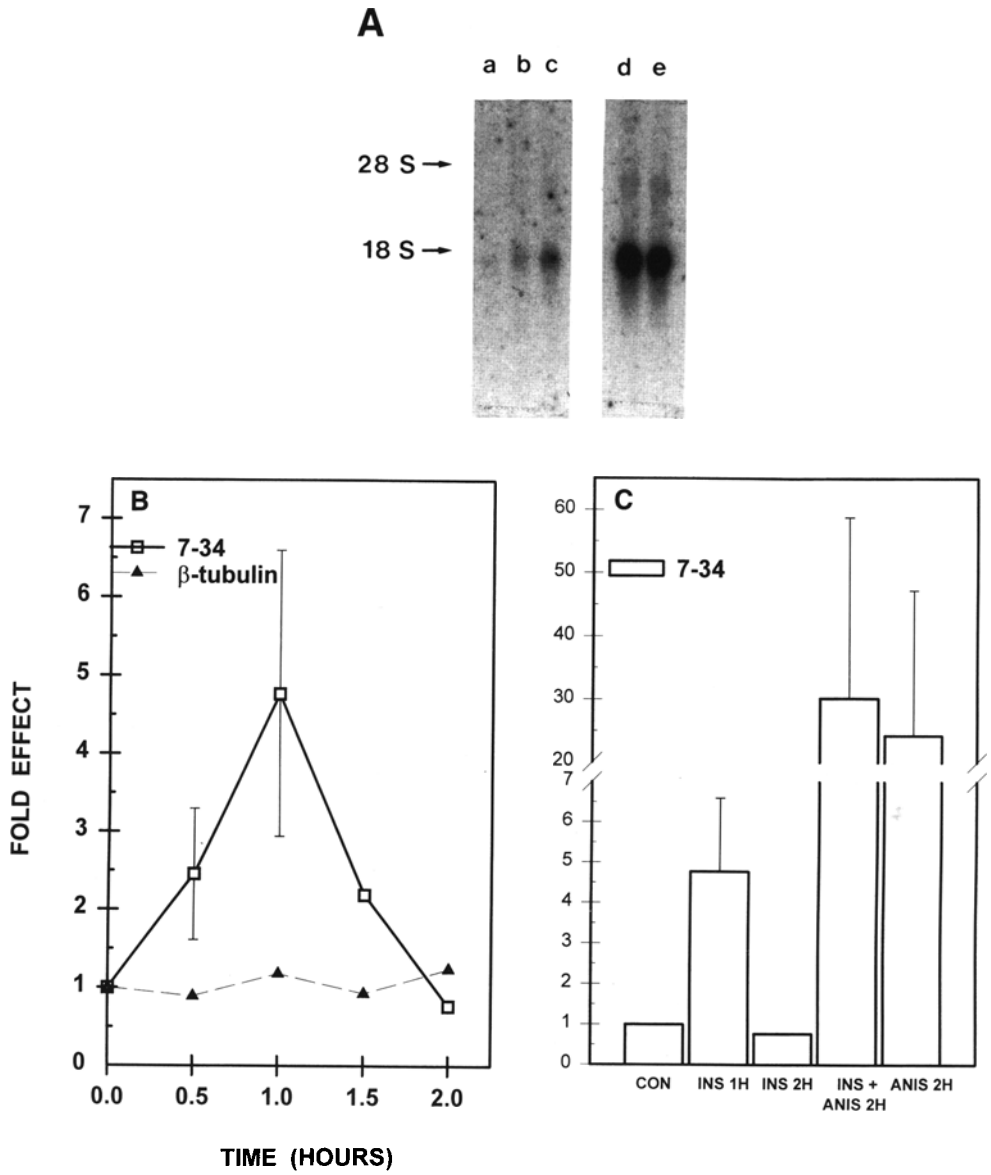

Fig. 2. Effects of insulin and anisomycin on the cytoplasmic concentration of 7-34 mRNA. H4 cells were deprived of serum for 24 h and treated with the following: Lane a, no treatment; lane b, insulin (1×10^{-8} *M*) for 0.5 h; lane c, insulin (1×10^{-8} *M*) for 1 h; lane d, insulin (1×10^{-8} *M*) + anisomycin (100 μ*M*) for 2 h; lane e, anisomycin (100 μ*M*) 2 h. The respective mRNAs were prepared and blots were performed as described in the text. Panel **(A)** shows the representative experiment using 7-34 as a probe. The results are graphed in panels (**B**; insulin time-course) and (**C**). The data expressed in (B) and (C) are the means of three experiments for insulin at 0.5 and 1 h, two experiments for anisomycin alone or together with insulin, and one experiment for insulin at 1.5 and 2 h. The vertical line, where present, represents 1 standard error of the mean (SEM).

an effect of anisomycin separate from the insulin effect on transcription of these genes *(10,34)*. Following incubation with anisomycin for 1 h, both *pip 92* and *CL-6* mRNA transcription rates were rapidly increased 35- and 25-fold, respectively. After 2 h, transcription of *pip 92* was still elevated by 22-fold, whereas transcription of *CL-6* decreased to sevenfold above basal values (Fig. 3C). Addition of insulin and anisomycin together for 1 h had similar effects on *pip 92* and *CL-6* transcription as did anisomycin alone. However, transcription of *pip 92* was significantly lower when exposed to insulin and anisomycin together for 2 h (10-fold), compared to anisomycin alone for 2 h (22-fold), a level of transcription intermediate between the still elevated levels with anisomycin alone and the complete return to basal levels of transcription following 2 h of insulin alone. This suggests an inhibition of the anisomycin effect with the two agents added together at this time-point. Also demonstrated in Fig. 3C is the induction of *pip 92* gene transcription at sevenfold, similar to the effect of insulin, but only twofold above baseline for *CL-6*, less than the insulin effect, following exposure to phorbol-12-myristate-13-acetate (PMA) for 1 h. This suggests a possible involvement of the protein kinase C (PKC) signaling pathway in the regulation of the *pip 92* gene.

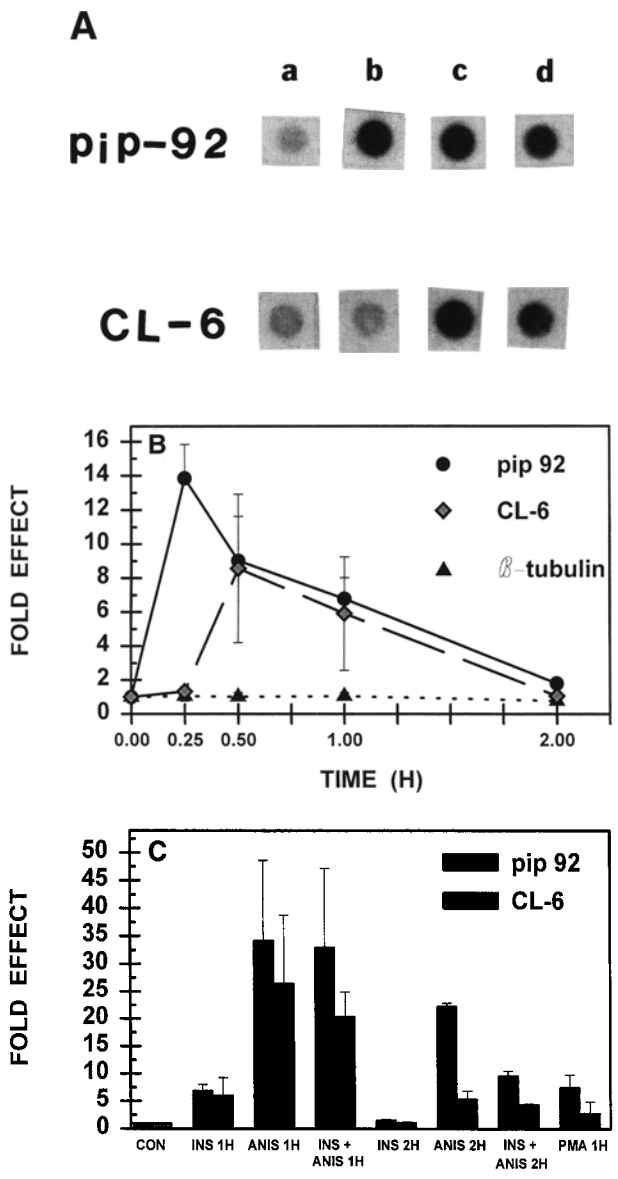

Fig. 3. Effects of insulin, anisomycin, or PMA on transcription of the *pip 92* and *CL-6* genes. H4 cells were deprived of serum for 24 h and treated with insulin (1×10^{-8} *M*), anisomycin (100 μ*M*), a combination of insulin and anisomycin, or PMA (1 μg/mL), for the indicated times. Nuclei were isolated and transcription was measured as described in the text. Panel **(A)** shows a representative run-on transcription assay with the following treatments: untreated H4 cells (a), insulin for 0.25 h (b), insulin for 0.5 h (c), insulin for 1 h (d). The respective cDNAs dotted on the nitrocellulose are: *pip 92* (top line), *CL-6* (bottom). The results, graphed in (**B**; insulin time-course) and (**C**) are the means of three separate experiments for *pip 92* and *CL-6* and six experiments for β-tubulin. The vertical line, where present, represents 1 SEM. The SEM of β-tubulin is smaller than the size of the triangles in (B).

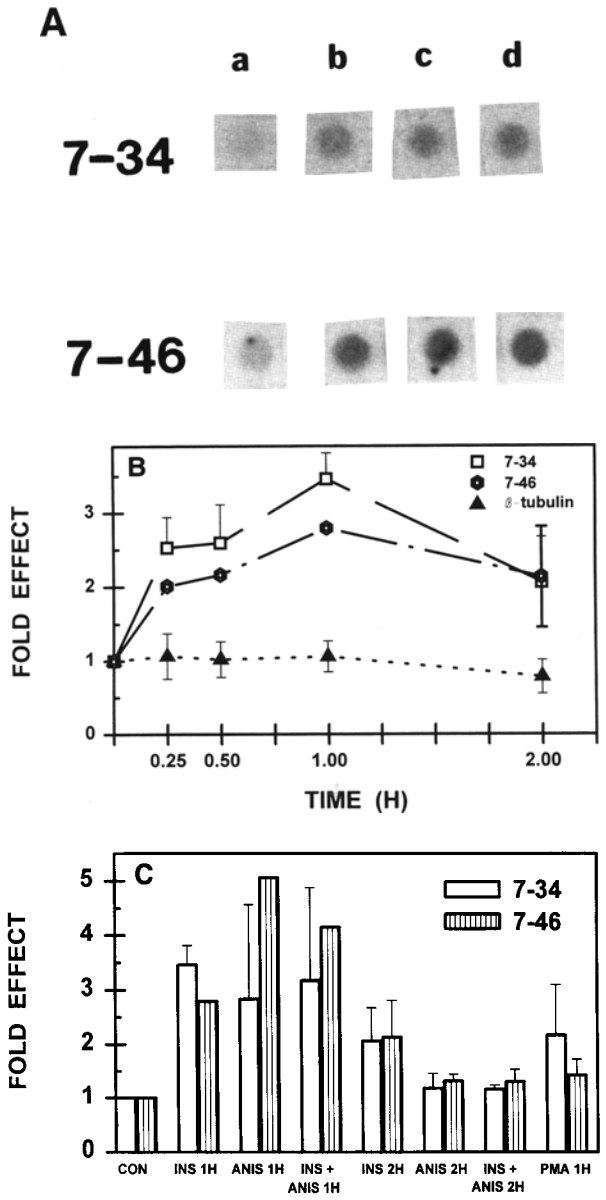

Fig. 4. Effects of insulin, anisomycin, and PMA on transcription of the 7-34 and 7-46 genes. H4 cells were deprived of serum for 24 h and treated with insulin 1×10^{-8} *M* for the specified times. Nuclei were isolated, and transcription was measured as described in the text. Panel **(A)** shows a representative run-on transcription assay with the following treatments: untreated H4 cells (a), insulin for 0.25 h (b), insulin for 0.5 h (c), insulin for 1 h (d). The respective cDNAs dotted on the nitrocellulose are: 7-34 (top line), 7-46 (bottom). The results graphed in (**B**; insulin time-course) for 7-34 are the mean of three experiments. The results for 7-46 at 0.25–1 h are for one experiment and the mean of three experiments at 2 h. The results graphed for β-tubulin are the means of six experiments. The results graphed in (**C**) are the mean of three experiments for 7-34. For 7-46, $n = 1$ at all of the 1-h time-points (except PMA for 1 h where $n = 3$), and $n = 3$ at the 2-h time-points. The vertical line, where present, represents 1 SEM.

The transcription rates of the 7-34 and 7-46 mRNAs were increased 2.0- to 2.5-fold above baseline following exposure to insulin for 0.25 and 0.5 h. Transcription rates of both mRNAs peaked at 3.2- and 2.5-fold, and remained elevated at twofold above baseline for up to 2 h, the longest time-point tested (Fig. 4A,B). Again, transcription of β-tubulin, a control mRNA, was unaffected by insulin *(8,13,36,37)*.

The presence of anisomycin, alone or together with insulin for 1 h, also increased 7-34 transcription. However, there was no significant difference between the level of induction demonstrated by insulin alone, anisomycin alone,

or insulin and anisomycin together. At 2 h, the presence of anisomycin may have attenuated the insulin induction of 7-34 and 7-46, bringing transcription rates back to baseline (Fig. 4C).

The novel clones also showed varying responses to PMA. Following exposure to PMA for 1 h, 7-34 transcription was induced slightly over twofold, whereas there was no significant effect on 7-46 transcription (Fig. 4C).

Discussion

Differential screening has been used to isolate several IEGs in many cell types in response to various mitogens. We report here the isolation of several clones from a rat H4 hepatoma cell cDNA library treated with insulin and anisomycin. The protein synthesis inhibitor, anisomycin, was included because IEGs are often superinduced or the mRNA is stabilized in the presence of the inhibitor *(29,32,33)*. Therefore, the effects of insulin alone were carefully studied when subsequently examining the regulation of these clones.

Our results confirmed that insulin increased mRNA accumulation of the previously characterized mouse *pip 92* gene and rat *CL-6* gene, and induced several novel clones isolated from the library. Additionally, we have demonstrated that insulin alone can rapidly increase the transcription rates of *pip 92* and *CL-6* in H4 cells. The increased *pip 92* and *CL-6* transcription rates, observed in the present study, are sufficient to account for the increased cytoplasmic concentrations of the mRNAs, suggesting a singular effect of insulin on these genes. This is the first evidence demonstrating that insulin alone induces transcription of the previously cloned mouse *pip 92* gene and rat *CL-6* gene in cultured rat hepatoma cells.

The amino acid sequences of *pip 92* and *CL-6* have been deduced, yet the functions of their gene products are unknown. The *pip-92* protein is hydrophilic and lacks potential N-linked glycosylation sites, and is localized to the cytoplasm or can possibly be secreted *(27)*. Conversely, the protein product of *CL-6 is* highly hydrophobic and contains an N-linked glycosylation site, suggesting that it is associated with the membrane *(28)*.

The presence of insulin together with anisomycin, or anisomycin alone, markedly increased *pip 92* transcription. However, the increase in *pip 92* transcription by anisomycin (35-fold) was not enough to account for the cellular increases in *pip 92* mRNA (135-fold). Thus, there may be a secondary effect of anisomycin on the regulation of *pip 92* mRNA. The striking increase in *pip 92* gene expression by anisomycin differs from previous reports in which cycloheximide was found to have no effect on *pip 92* expression in mouse fibroblasts *(29)*. The observed difference may be due to the different cell types used in previous experiments (fibroblasts; *29*), compared to the present work (hepatoma cells). Additionally, we have found from previous studies in H4 hepatoma cells that anisomycin's effects are generally greater and more rapid than those of cycloheximide *(34,35)*, two agents that inhibit protein synthesis through different mechanisms *(8,34,35)*.

Insulin together with anisomycin, or anisomycin alone, also increased *CL-6* transcription. This increase following 1 h of anisomycin (25-fold) was larger than the increase in *CL-6* mRNA levels measured at 2 h (11-fold). Since transcription rates fall rapidly between 1 and 2 h (from 25- to 7-fold), an mRNA with a short half-life could have decreased dramatically during this time, from some higher level (not measured in the present study) to the 11-fold increase above basal observed at 2 h. The cytoplasmic stability of *CL-6* mRNA has not been studied, but a short half-life of approx 30 min would be similar to many other IEGs *(15,27,38)*, resulting in the mRNA levels observed at 2 h (11 -fold). However, mRNA stability studies must be performed on *CL-6* mRNA to determine its half-life.

The increase in 7-34 and 7-46 transcription rates by insulin accounted for the increased level of cytoplasmic accumulation observed, whereas the effects of anisomycin on the expression of these novel genes were complex. The degree of induction of transcription rates by anisomycin did not always correlate with the magnitude of increase in mRNA concentrations. For example, anisomycin and insulin together had a moderate effect on 7-34 transcription. However, anisomycin alone or together with insulin had a significantly greater effect on mRNA accumulation (25- to 30-fold), which could not be accounted for by the level of transcription alone (threefold). This would suggest a posttranscriptional effect of anisomycin on 7-34 mRNA. Conversely, the levels of anisomycin-induced 7-46 transcription (four- to fivefold) were enough to account for the amount of 7-46 mRNA levels (data not shown). This varied response of *pip 92, CL-6*, and the novel clones to anisomycin raises many possibilities. Instances where transcription rates were increased significantly more than mRNA levels (*CL-6*) may signify that anisomycin is regulating synthesis of protein(s) involved in posttranscriptional processes (necessary for presentation of the mature mRNA in the cytoplasm) or may be inhibiting synthesis of proteins that stabilize this mRNA. Instances where increases in transcription rates were insufficient for the increased levels of mRNAs (*pip 92*, 7-34) suggest that anisomycin may act to stabilize the mRNAs by preventing synthesis of proteins that may degrade the mRNAs.

Anisomycin is known to affect intracellular signaling pathways involved in the regulation of these transcripts. Recent studies indicate that anisomycin is a strong inducer of the jun-kinase (JNK), which by phosphorylating transcription factors, such as *c-jun*, can regulate gene expression *(39–41)*. Thus, activating this pathway may lead to the changes in expression of *pip 92*, *CL-6*, 7-34, and 7-46 obtained in the present study. Since there is no additivity of the insulin and anisomycin effects, one might suggest that

insulin is working through activating JNK activity. However, for *pip 92* and *CL-6*, the effects of anisomycin are far greater than that of insulin, and it might be difficult to measure additivity, even if present. For 7-34 and 7-46, the effects of insulin and anisomycin are smaller and about equivalent, so additivity could be measured if insulin and anisomycin were activating different signaling systems. We have not directly measured JNK activation and published data are controversial concerning whether insulin can activate JNK activity both in vivo and in vitro *(42–47)*. Thus, additional data, including measuring JNK activation directly following insulin or anisomycin alone or in combination in this cell line, would be necessary to conclude whether insulin might be stimulating transcription via activation of JNK.

There also is evidence that protein synthesis inhibitors increased diacylglycerol and PKC activity in cultured myocytes *(48)*. Our results are consistent with earlier studies suggesting PKC activation in insulin-stimulated gene expression *(36,49,50)*. However, in the present studies, PMA addition resulted in only moderate increases in transcription of *pip 92, CL-6,* and 7-34, and no effect on 7-46. Thus, activation of PKC could not account for the larger increases in transcription by anisomycin, again suggesting the importance of the JNK signaling pathway.

Previous work has demonstrated that for many insulin-induced genes, activation of the PKC signaling pathway is necessary for insulin to exert its full effects in H4 cells. Pretreatment of H4 cells with PMA for 24 h, which reduces functional PKC activity, inhibited the ability of PMA to increase transcription of gene 33, *c-fos* and the β-actin genes *(36,49,50)*. Transcription of the genes examined in the present study demonstrated variability in response to PMA. Although it has been reported that the *CL-6* protein contains putative consensus PKC phosphorylation sites *(28)*, this is the first report demonstrating that *CL-6* transcription is induced by PMA. It was demonstrated that *pip 92* mRNA levels were increased by PMA in fibroblasts *(29)*, whereas this is the first evidence demonstrating that *pip 92* transcription is PMA-inducible in hepatoma cells, suggesting that the involvement of PKC in *pip 92* regulation may not be tissue-specific. In these experiments, at 1 h following addition, both insulin and PMA induced *pip 92* transcription to approximately the same extent (seven- to eightfold) leaving open the possibility that insulin could be working through a PKC-dependent mechanism in regulation of this gene. The possibility that insulin also may be using the PKC signaling pathway for the induction of 7-34 gene transcription cannot be ruled out, since both insulin and PMA induced transcription of this gene to approximately the same extent (2.5- to 3.5-fold). Conversely, PMA treatment had no significant effect on 7-46 transcription, suggesting that insulin-induced 7-46 transcription must be signaling by working through a PKC-independent pathway in the regulation of this gene. The induction of transcription by insulin for all of the genes in this study was not the result of nonspecific increases in transcriptional activity in the cells, since β-tubulin gene transcription was unchanged in the presence of insulin.

These results extend the observations that insulin plays a role in the regulation of several hepatic genes. The data presented demonstrate that individual IEGs may have independent responses to the same commonly used factors, such as insulin, anisomycin, and PMA, suggesting a specific regulation of these genes and implying the use of multiple signaling pathways in insulin's regulation of these genes. The identity and function of the protein products of these novel genes await further study.

Experimental Procedures

Materials

Porcine insulin was a gift from Ronald Chance (Eli Lily and Co., Indianapolis, IN). Swim's 77 medium, fetal bovine serum, calf serum, and horse serum were obtained from Gibco (Grand Island, NY). The Fast Track mRNA Isolation, cDNA library, and Microwave screening kits for nucleic acid probes were purchased from Invitrogen (Carlsbad, CA). Oligo $(dT)_{12-18}$ primer, reverse transcriptase (Superscript 11), dNTPs, RNase H, and proteinase K were purchased from Gibco BRL (Grand Island, NY). The random primer labeling kit was obtained from Stratagene, the *fmole* DNA Sequencing System kit was purchased from Promega (Madison, WI), the T7 and Sp6 promoter primers were purchased from New England Biolabs (Beverly, MA), and Sequagel rapid was purchased from J. T. Baker. RNAsin was obtained from Promega, DNase I and RNase T, from Worthington/Cappel Biochemicals (Freehold, NJ), Inc., and anisomycin and all remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO) International Chemical and Nuclear Corp. (ICN Radiochemicals, Inc., Costa Mesa, CA) supplied the [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [α-^{32}P]UTP, and Eastman Kodak, Co. (Rochester, NY) supplied the XAR-5 X-ray film and intensifying screens.

Cell Culture

Rat H4IIE hepatoma cells (H4; American Type Culture Collection, Rockville, MD) were maintained in monolayer cultures in Swim's 77 medium supplemented with 2% fetal bovine serum, 3% calf serum, and 5% horse serum in a 5% CO_2 incubator *(38)*. Experiments were initiated after serum was withdrawn for 20–24 h when cells were approx 70–80% confluent.

Differential Screening of cDNA Libraries

Libraries were constructed from H4 cells treated with insulin and anisomycin. The cDNA libraries were plated, transferred in duplicate to nylon membranes, and then denatured on Whatman paper saturated with 2X SSC + 5% SDS. The replica nylon membranes were heated in a

microwave oven and then probed with radiolabeled cDNAs synthesized from mRNA isolated from H4 cells treated with insulin and anisomycin for 2 h, or cDNAs synthesized from mRNA from untreated cells. Following hybridization and autoradiography, any colonies that were differentially expressed were selected for a second round of screening. Bacterial suspensions containing the clones of interest were dotted onto nylon filters in duplicate. Resultant replica filters were then probed again with insulin and anisomycin-treated and untreated cDNAs. The desired colonies were selected for partial DNA sequence analysis of the cDNA inserts following four rounds of differential screening.

DNA Sequencing

The template cDNAs were combined with the respective end-labeled primer, buffer, and sequencing grade *Taq* DNA Polymerase, and sequenced as described *(fmole* DNA sequencing kit, Promega) using an MJ Research thermocycler. The reactions were electrophoresed on a 6% polyacrylamide/bis-acrylamide gel, the gel was exposed to X-ray film, and sequences obtained were compared to the sequences in the EMBL and Genbank data bases using the GCG analysis program (University of Wisconsin).

Isolation of mRNA and Northern Transfer

The Fast Track mRNA isolation kit (Invitrogen) was used to isolate poly [A+] mRNA. The mRNA was electrophoresed on a 1.2% agarose, 2.2 *M* formaldehyde denaturing gel, and transferred to nitrocellulose membranes. Following prehybridization, the hybridization was initialed by the addition of randomly labeled cDNAs. The membranes were washed, and the concentration of mRNA was estimated by densitometric scanning of the resultant Northern blots using a Shimadzu densitometer. The degree of hybridization of the cDNA used to probe mRNAs in treated samples were compared to RNA from the untreated control in each experiment where the signal obtained from the untreated control was arbitrarily set to 1 *(38,51)*.

Transcription Assays

Transcription rates of the mRNAs of interest were measured in isolated nuclei by the nuclear run-on method exactly as described previously *(38)*. Resultant autoradiograms were densitometrically scanned, and each experimental value was compared to the control in each experiment.

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