Reciprocal Regulation of Gene Transcription by Insulin

INHIBITION OF THE PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE AND STIMULATION OF GENE 33 IN A SINGLE CELL TYPE*

David T. W. Chu, Charles M. Davis†, Nancy B. Chrapkiewicz, and Daryl K. Granner§
From the Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tennessee 37232

Two H4IIE hepatoma cell genes, phosphoenolpyruvate carboxykinase (PEPCK) and gene 33 (g33), are reciprocally regulated by insulin. Quantitation of mRNA*PEPCK and mRNA^g33 in total RNA isolated from cells treated with insulin showed a 7-fold increase in mRNA^PEPCK amount and a 3-fold decrease of mRNA^g33. The cAMP analog 8-(4-chlorophenylthio)-cAMP-induced mRNA^PEPCK but had no effect on mRNA^g33. The responses to various insulins and related molecules showed that the insulin receptor mediates the effects of physiologic concentrations of insulin on each of these genes. This inverse pattern of regulation by insulin was further characterized by determining the transcription rates of both genes in nuclei isolated at various times after the addition of insulin and 8-(4-chlorophenylthio)-cAMP to H4IIE cells. Insulin increased the rate of synthesis of mRNA^g33 from 35 to 354 ppm and decreased the synthesis of mRNA^PEPCK from 1175 to 109 ppm. These effects of insulin occurred rapidly and reached their maxima by 60 min. In both cases, greater effects were observed as insulin concentrations were increased from 10^-12 to 10^-8 m. Although the effects of insulin were concentration-dependent for both genes, the PEPCK gene was significantly more sensitive to low concentrations of insulin than was gene 33. The reciprocal effects of insulin on the synthesis of mRNA^PEPCK and mRNA^g33 in H4IIE cells provide a means of investigating how a hormone can exert opposing effects on two genes in the same cell.

Many of the effects of hormones and their intracellular mediators result from alterations of the rate of transcription of specific genes. Studies to date have concentrated on how individual hormones either stimulate or, less commonly, inhibit transcription. The interesting question “How does a hormone (insulin, for example) stimulate transcription of some genes while inhibiting that of others?” becomes even more intriguing if one assumes that these opposing processes must be occurring simultaneously in some cells. Because the H4IIE clonal cell line exhibits both positive and negative effects on the amounts of specific mRNAs in response to insulin, it may be ideally suited for an analysis of this question.

We have previously shown that insulin’s inhibition of the phosphoenolpyruvate carboxykinase (PEPCK)1 gene in H4IIE cells is receptor-mediated, rapid in onset, quickly reversible, and independent of ongoing protein synthesis (1–3). Decreases in the rate of transcript initiation and elongation account for this effect (4). A region of DNA within 600 nucleotides of the cap site mediates the insulin response (5), which is dominant over the transcription-enhancing effects of cAMP and glucocorticoids (3), both of which act through discrete DNA elements in the PEPCK gene (6, 7).

The identification of hormone-responsive DNA elements and isolation of the related DNA-binding proteins are prerequisites for studies aimed at determining the mechanism of hormonal regulation of gene transcription. DNA elements involved in the action of glucocorticoid hormones (8–11), cAMP (6, 12–15), and phorbol esters (13, 16, 17) have been identified, and consensus sequences for each have been described (6, 11, 13, 14, 17–19). Considerable progress is being made toward the identification of proteins that interact with these sequences (19–22). Less is known about how insulin regulates transcription. A consensus insulin response element has not been described, and little is known about the proteins that interact with DNA to regulate transcription in response to this hormone, although a protein that binds to a fragment of DNA associated with the insulin (and serum) induction of the c-fos gene has recently been described (23).

A gene, whose transcription is increased by insulin in H4IIE cells, is required in order to analyze the question of how insulin can have opposing effects on different genes. Recently, Lee et al. (24) reported the isolation of a cDNA that is induced by insulin in rat liver. This cDNA, designated p33 because of the plasmid screening employed, detects an mRNA that encodes a protein of ~53 kDa whose function is unknown. The amount of this mRNA, a product of gene 33, is increased in liver (24) and H4IIE cells (25) in response to insulin. This induction in rat liver is known to be due to an increased rate of transcription of gene 33 (24), but detailed studies have not been reported using H4IIE cells.

In the present study we show that insulin stimulates transcription of gene 33 and inhibits transcription of the PEPCK gene in H4IIE cells. Both effects are mediated by the insulin receptor at physiologic concentrations of insulin, and both occur within the same time span. Analysis of the reciprocal regulation of these two genes in the H4IIE cell line should

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1 The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); mRNA^PEPCK, mRNA encoding PEPCK; mRNA^g33, mRNA transcribed from gene 33; 8-CPT-cAMP, 8-(4-chlorophenylthio)cAMP; IGF-1, insulin-like growth factor 1.

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† Trainee in the Vanderbilt Medical Scientist Training Program (T32 GM 07347).

§ To whom correspondence should be addressed.
allow us to address the question of how a hormone can exert opposing effects on different genes in the same cell.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The H4IIE cell line (26), a cloned derivative of the rat H4 hepatoma (27), was obtained from Dr. C. Hoffman, Veterans Administration Hospital, Hines, Ill. Attached cultures of H4IIE cells were propagated in α-minimal essential medium (GIBCO) supplemented with 2.2 g/liter NaHCO₃ and final concentrations (v/v) of 5% calf (HyClone), 3% newborn calf, and 2% fetal bovine serum (GIBCO). Cells were kept in serum-free α-minimal essential medium for 20–24 h prior to experiments.

**mRNA Quantitation**—The amount of specific mRNA was quantitated in either total cellular RNA prepared by guanidine isothiocyanate extraction and centrifugation through cesium chloride (28, 29) or by the cytodot method (30, 31). Probes used were a 1300-base pair EcoRI-PstI cDNA fragment isolated from pUC112.R3 for PEPCK (32) or pXS7.BS10+ (linearized plasmid pSP65.R3 which contains the 5.8-kilobase EcoRI genomic insert of pXPC112.R3 for PEPCK) (33). Nonspecific hybridization was detected by hybridization of total RNA synthesized after correction for nonspecific hybridization to nitrocellulose discs containing equal amounts of the respective cloning vectors, pSP65 or Rluescript M13+. All filters were hybridized simultaneously with the labeled transcripts. Hybridization efficiency was determined by the addition of [α-32P]UTP-labeled PEPCK gene and gene 33 cRNAs to the hybridization reaction. These were synthesized from the individual genomic DNA templates using SP6 and T7 RNA polymerases, respectively. Results are expressed as parts of PEPCK RNA and gene 33 RNA transcribed per million parts of total RNA synthesized after correction for nonspecific hybridization, the efficiency of hybridization, and for α-amanitin-resistant transcription. The latter (25 ± 5 ppb in 10 different experiments) was determined by incubating nuclei with 1.5 μg/ml α-amanitin.

**RESULTS AND DISCUSSION**

**mRNA Regulation by Insulin and cAMP**

H4IIE cells were treated with 0.1 mM 8-CPT-cAMP to test whether this nucleotide had any effect on mRNA and to enhance the production of mRNA and thereby accentuate the inhibitory effect of insulin on mRNA (2). Treatment of the cells with 8-CPT-cAMP for 3 h had virtually no effect on the level of mRNA (Fig. 1, lanes A and B), whereas treatment with insulin for 2 h in the presence or absence of 8-CPT-cAMP resulted in a 7-fold induction. Insulin is known to induce mRNA in liver (24) and H4IIE cells (25), but the lack of a response to 8-CPT-cAMP was somewhat surprising because Lee et al. (24) had shown that a cAMP derivative induced rat hepatocellular RNA (25). Our data suggest that the cAMP effect in liver is an indirect one or that this cAMP response has been lost in the H4IIE cells. We consider the former more likely, primarily because these cells retain many hepatic cAMP responses and of the interaction of mRNA with the cDNA. Lane A shows the autoradiograph formed as the result of the interaction of mRNA with the cDNA. Lane B shows the relative intensities of each pair of slots as determined by densitometric scanning. The control value is set at 1.0. Lanes C and D show the results of a similar analysis of mRNA determined on the same isolates of total RNA.

![FIG. 1. Effects of insulin and 8-CPT-cAMP on the levels of mRNA and mRNA on the levels of mRNA and mRNA](image)

Effects of using a clonal cell line to analyze gene regulation. In intact animals and complex tissues such as liver one cannot be sure which effector or cell is providing a given response. The effects of insulin and cAMP on mRNA and mRNA are illustrated in Fig. 1 (lanes C and D). The 8-CPT-cAMP treatment resulted in a 2.9-fold increase in mRNA levels while insulin treatment caused a slight decrease when given alone, and it reduced the cAMP-induced mRNA level to that of the control. These changes are not as dramatic as those published previously (3, 29), an effect that may be due, in part, to the use of a different line of H4IIE cells, which might have a different metabolism of mRNA.

Insulin and cAMP generally have opposing metabolic effects. This is certainly true in the case of PEPCK gene transcription where the balance between these two effectors determines the rate of synthesis of mRNA. Since this mRNA directs the synthesis of the enzyme PEPCK, changes of the transcription of this gene presumably alter the rate of gluconeogenesis. Recent studies show the linkage of these events in an H4IIE cell line. A different situation applies to gene 33, as cAMP is apparently not directly involved in regulating this gene, at least in H4IIE cells which are obviously capable of responding to this agent.

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1 A manuscript describing the isolation and structures of the cDNA for mRNA and of gene 33 is in preparation.

2 C. R. Kahn and D. K. Granner, manuscript in preparation.
Reciprocal Action of Insulin on Gene Transcription

FIG. 2. Effect of insulin and 8-CPT-cAMP on gene transcription: time course. Confluent cultures of H4IIE cells were placed in serum-free medium 24 h before harvest. At time -30 min the basal rate of transcription of the PEPCK gene (○) and of gene 33 (△) was determined. The remaining cells were treated with 0.1 mM 8-CPT-cAMP. The effect of 8-CPT-cAMP alone was assessed at times 0 and 120 (30 and 150 min of exposure to 8-CPT-cAMP, respectively; △ = PEPCK; ▲ = gene 33). Insulin, at 5 × 10⁻⁵ M, was added to all other cells at zero time, and the effect of this hormone on cells treated with 8-CPT-cAMP was assessed at the times shown in the abscissa. Each point in panel A represents the average ± S.D. of duplicate assays using aliquots from the same nuclear isolates. The open and closed circles represent the rate of transcription of the PEPCK gene and of gene 33, respectively. A direct comparison of the effect of insulin on the time course of induction of the two genes (○ = PEPCK, ● = gene 33) is illustrated in panel B. The data taken from the results plotted in panel A are represented as the percent of the maximal response at each time after the addition of insulin.

Effect of Insulin on Gene Transcription

Time Course—The reciprocal regulation of these two genes by insulin was further characterized using transcription run-on assays. The transcription rate of both genes was determined in common aliquots of nuclei that were isolated at various times after exposure of the cells to insulin. The cells were pretreated with 8-CPT-cAMP for 30 min in order to make the insulin effect on the transcription rate of the PEPCK gene and of gene 33, respectively. A direct comparison of the effect of insulin on the time course of induction of the two genes (○ = PEPCK, ● = gene 33) is illustrated in panel B. The data taken from the results plotted in panel A are represented as the percent of the maximal response at each time after the addition of insulin.

Later times, it remained severalfold higher than the control rate for at least 120 min. The addition of 5 × 10⁻⁵ M insulin at a time when the PEPCK gene was being transcribed at the maximal rate (30 min after the cyclic nucleotide analog was added) resulted in a rapid decrease in mRNAPEPCK synthesis, as previously described (3). Inhibition of PEPCK gene transcription was apparent after 15 min. Within 60 min the rate of PEPCK gene transcription was reduced to the basal level (from a maximum of 1175 to 10⁹ ppm), and by 90–120 min it was actually lower than the basal rate of transcription. In contrast, insulin caused a marked stimulation of gene 33 transcription. An increase in the transcription of this gene was apparent at 15 min (from 35 to 132 ppm), the maximum rate was reached 60 min after addition of insulin (a 10-fold increase to 354 ppm), and, although it declined somewhat, transcription continued to be elevated several fold over the basal rate at 2 h. There was no induction of gene 33 transcription by cAMP during the initial 30-min incubation nor during...
the additional 2-h period (Fig. 2A).

The inhibition of mRNA<sup>PEPCK</sup> synthesis and the stimulation of mRNA<sup>gene 33</sup> synthesis occurred over a very similar time span (Fig. 2A). When the data are expressed as the percent of maximal response (Fig. 2B) it is apparent that the kinetics of repression and induction are very similar; both responses occur rapidly (within 15 min) and both peak at about 60 min.

Since the repression of mRNA<sup>PEPCK</sup> synthesis occurs quickly and does not require ongoing protein synthesis (3), it has been suggested that a pre-existing transcription factor is altered, perhaps through covalent modification (35). If the effect on gene 33 transcription is independent of ongoing protein synthesis, it is conceivable that the same type of covalent modification of a transcription factor results in enhanced synthesis of gene 33 mRNA.

Concentration Response—The responses of the PEPCK gene and gene 33 to various concentrations of insulin are similar in that the regulation of both is concentration-dependent over a broad range (Fig. 3A). H4IE cells, pretreated with a maximally effective concentration of 8-CPT-cAMP for 30 min, showed a concentration-dependent decrease in transcription of the PEPCK gene after insulin addition. Substantial inhibition was achieved with as little as 10<sup>-12</sup> M insulin (from 1413 ± 50 to 1057 ± 45 ppm), half-maximal inhibition appeared to occur between 10<sup>-10</sup> and 10<sup>-9</sup> M, and maximal suppression, from the initial rate of 1413 ± 50 to 206 ± 18 ppm, was observed at 10<sup>-8</sup> M. In contrast, low concentrations of insulin were relatively less effective at stimulating the transcription of gene 33. Insulin at 10<sup>-12</sup> M increased transcription of gene 33 from the basal rate of 39 ± 5 ppm to 52 ± 9 ppm. Although a modest increase continued as the hormone concentration was increased to 10<sup>-10</sup> M, the largest change occurred at concentrations greater than this. At 10<sup>-8</sup> M insulin the response of gene 33 was still increasing whereas PEPCK transcription had reached a plateau.

These apparently different sensitivities to insulin are clarified by the representation in Fig. 3B, which presents the data as percent of the maximal response. The response of both genes appears to have two components. The first component occurs through the physiologic range of insulin concentration (10<sup>-11</sup>–10<sup>-8</sup> M), and a second occurs at concentrations greater than this. Both genes exhibit a curvilinear response at low insulin concentrations, although a given response of gene 33 requires 100 times as much insulin. A possible explanation of this difference in sensitivity to the hormone is that, although the effect of insulin on PEPCK transcription is known to be mediated by the insulin receptor (1,3), regulation of mRNA<sup>gene 33</sup> synthesis is accomplished through another receptor. Biologic responses mediated by the insulin receptor to various insulins, insulin analogs, and related molecules follows the hierarchy: insulin ≫ proinsulin > IGF-1 ~ desoctapeptide insulin. This relationship is well established and is used to define the role of the insulin receptor in a given process. The response of gene 33 transcription to various insulin-like molecules obeys this relationship, at least at low concentrations. Insulin is 50–100 times more potent than proinsulin which in turn is 5–10 times more effective than IGF-1 or desoctapeptide insulin (Fig. 4). Thus, the gene 33 response, although much less sensitive to insulin than is the response of the PEPCK gene, also appears to be mediated by the insulin receptor.

An alternative explanation of the differing sensitivities of gene 33 and PEPCK transcription to insulin is that varying concentrations of insulin result in different responses in H4IE cells. Such an occurrence is not unprecedented as successively greater degrees of occupancy of the adipose cell insulin receptor increase (in sequence) glucose oxidation, amino acid transport, and protein synthesis (36).

The break in the response curves at insulin concentrations greater than 10<sup>-10</sup> M, particularly in the case of gene 33, suggests that a second regulatory component may be operative. We noticed this phenomenon previously for PEPCK gene transcription (3), but it is more apparent in the case of gene 33. The dual component response could be mediated through two different classes of receptors. Very high concentrations of insulin activate the IGF-1 receptor, but H4IE cells have no detectable IGF-1 receptors (37), so this appears unlikely. Alternatively, it is conceivable that two postreceptor mechanisms are involved in regulating PEPCK gene and gene 33 transcription. Thus, low concentrations of insulin could act through distinct insulin response elements whereas high concentrations could activate a second response element. It is also possible that two intracellular messengers of insulin action, formed at a different insulin concentration, could account for this effect.

Conclusion

These results demonstrate that insulin reciprocally regulates the transcription of the PEPCK gene and of gene 33 in H4IE cells. The effects of insulin are exerted rapidly, in a concentration-dependent manner, and are mediated by the insulin receptor. We have developed a system which can be used to study the simultaneous stimulatory and inhibitory actions that insulin exerts on two specific genes in the same cell. We are currently trying to identify the cis-acting insulin-responsive elements and the corresponding trans-acting factors in an effort to understand this interesting and important regulatory mechanism.

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