Multihormonal Regulation of Phosphoenolpyruvate Carboxykinase Gene Transcription

THE DOMINANT ROLE OF INSULIN*

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Kazuyuki Sasaki, Timothy P. Cripe, Stephen R. Koch, Teresa L. Andreone, Daniel D. Petersen, Elmus G. Beale, and Daryl K. Granner‡

From the Veterans Administration Medical Center and the Departments of Internal Medicine and Biochemistry, Diabetes and Endocrinology Research Center, University of Iowa College of Medicine, Iowa City, Iowa 52240

We used a nuclear RNA transcription elongation assay to show that cAMP analogs and dexamethasone cause a selective increase of transcription of the P-enolpyruvate carboxykinase gene in H4IIE hepatoma cells. 8-(4-chlorophenylthio)-cAMP increased transcription within 5 min and the maximal rate, generally 10–15-fold above the basal rate, was attained by 30 min. This increase was of sufficient magnitude to account for the effect on mRNA_{PEPCK} (for example, where PEPCK is phosphoenolpyruvate carboxykinase) accumulation. After the initial increase, and with continued presence of cAMP, transcription of this gene declined to a new steady-state level which was 2–3 times the basal value. The effect of cAMP analogs on P-enolpyruvate carboxykinase gene transcription was obtained in the absence of protein synthesis. This, and the rapidity of the response, indicates that the effect of cAMP is exerted directly on the P-enolpyruvate carboxykinase gene. Dexamethasone results in a specific, 6-fold increase of transcription, sufficient to account for the increase of mRNA_{PEPCK} which follows treatment of H4IIE cells with this glucocorticoid.

When 1 nM insulin was added to either untreated H4IIE cells, or cells first treated with a cAMP analog or dexamethasone, there was a marked reduction of cytoplasmic mRNA_{PEPCK}. The inhibitory effect of insulin was readily reversible, as cells regained the basal level of mRNA_{PEPCK} and full responsiveness to cAMP within 1 h after removing insulin. The transcript elongation assay was used to show that insulin inhibits transcription of the gene coding for mRNA_{PEPCK}. The concentration of insulin required for 50% inhibition was 2–5 μM, whereas approximately 200 μM of proinsulin was required to achieve the same inhibition of transcription. This effect was specific, since insulin did not affect the synthesis of total RNA; it was rapid, as 5 nM insulin decreased the rate of P-enolpyruvate carboxykinase gene transcription by 50% within 15 min; and it also does not require ongoing protein synthesis. The magnitude and kinetics of the response suggest that the primary action of insulin in the regulation of P-enolpyruvate carboxykinase synthesis is exerted at the level of mRNA_{PEPCK} transcription. The insulin-mediated inhibition of mRNA_{PEPCK} transcription was noted in untreated cells and in cells first treated with 8-(4-chlorophenylthio)-cAMP, dexamethasone, or both of these agents. Hence, among these compounds, insulin is the dominant regulatory molecule.

A principal action of hormones is to regulate the production of proteins which govern metabolic processes. That steroid and thyroid hormones accomplish this purpose by controlling the expression of specific genes is now amply documented, and the first step in this action involves the binding of the ligand to intracellular receptors. The hormone-receptor complex then binds in a site-specific manner to DNA, and thereby regulates the transcription of specific genes (1, 2), including a small effect on the P-enolpyruvate carboxykinase1 gene in rat liver (3).

Evidence that hormones which bind to receptors on the plasma membrane also regulate the expression of specific genes is accumulating (3–9). In most instances the internalization of the hormone-receptor complex is not a prerequisite for action. This implies that another molecule or process mediates the intracellular action of this large class of hormones. In many cases the interaction of peptide hormones with their specific receptors results in the activation of adenylate cyclase which, in turn, increases cAMP within the cell. Although cAMP has well-defined effects on gene transcription in prokaryotic cells, until very recently the effect of this intracellular mediator on the synthesis of eukaryotic proteins was thought to be exerted at the level of mRNA translation (10).

Cyclic AMP has now been shown to regulate the mRNAs that code for several proteins in eukaryotic cells including tyrosine aminotransferase (11, 12), P-enolpyruvate carboxykinase (13, 14), lactate dehydrogenase (9), prolactin (5, 6), albumin (15), alkaline phosphatase (16), haptoglobin (17), and discoidin (18). In a number of these instances cAMP, or various analogs, regulate gene transcription, as quantitated by measuring RNA elongation in isolated nuclei (4–7, 9, 13, 18, 19).

For example, Maurer (5) found that cAMP increases pro-

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‡ VA Medical Investigator.

1 The abbreviations used are: P-enolpyruvate carboxykinase, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); mRNA_{PEPCK}, mRNA coding for P-enolpyruvate carboxykinase; Bz_{cAMP}, N^6-O^-diethyl-cAMP; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; dexamethasone, 3α-fluoro-16α-methylprednisolone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HEPES, 4(2-hydroxyethyl)l-piperazineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
The effect of cAMP on prolactin gene transcription in cultured GH3 pituitary tumor cells was subsequently demonstrated (6). Transcription of the gene coding for the A subunit of lactate dehydrogenase increases after the addition of either isoproterenol or Bt-cAMP to C6 glioma cells (9). The observation that Bt-cAMP enhances transcription of the P-enolpyruvate carboxykinase gene in rat liver and hepatoma cells provides a third example (3, 4, 19). It is likely that mammalian cells may commonly employ this mechanism of action of cAMP to regulate the synthesis of specific proteins.

Although insulin influences the rate of synthesis of many proteins, this action has not been extensively studied. An effect of insulin on ribosomes and/or translation factors could be mediated by this mechanism, especially since stimulatory and inhibitory responses have been noted (30; this paper). Insulin has also been reported to stimulate RNA synthesis and RNA polymerase activity (22, 23), an indication that the hormone may regulate protein synthesis at a pretranslational site. Observations that the messenger RNAs coding for albumin (24), amylose (25), pyruvate kinase (26, 27), a2-γ-globulin (28), fatty acid synthetase (29), tyrosine aminotransferase (30), casein (31), and P-enolpyruvate carboxykinase (4, 13, 32) change in response to insulin treatment support this hypothesis. These changes could result from effects of insulin on a number of processes including an alteration of the rate of cytoplasmic mRNA degradation, an effect on transport from the nucleus to cytoplasm, or on the synthesis, modification, processing, or degradation of the specific RNA in the nucleus.

We have studied the regulation of P-enolpyruvate carboxykinase in H4IIE cells, a clone of Reuber H4 tumor hepatoma cells (33, 34), in an effort to learn how insulin regulates the synthesis of a specific protein. Concentrations of insulin in the 10−12 to 10−2 M range decrease H4IIE cell cytoplasmic mRNAPEPCK and P-enolpyruvate carboxykinase synthesis rapidly (4, 32). This effect is mediated through the insulin receptor (32) and it appears to be due to decreased synthesis of mRNAPEPCK (4).

Our ultimate goal is to understand how a number of hormones, each of which presumably has a unique mechanism of action, provide an integrated biological response. Transcription of the gene coding for mRNAPEPCK is thus of particular interest since it is increased by both cAMP (3, 4) and glucocorticoid hormones (19) and it is decreased by insulin (4). Because several obstacles are encountered in studying cellular and subcellular regulation in intact animals, a cultured cell system in which first the separate, and then the concerted, actions of these hormones on gene transcription can be studied would be quite useful. In this report we present a detailed analysis of the individual and combined effects cyclic AMP analogs, dexamethasone and insulin have on transcription of the P-enolpyruvate carboxykinase gene in H4IIE hepatoma cells, a system which satisfies these initial requirements.

**EXPERIMENTAL PROCEDURES**

**Materials**—Swinn's S-77 medium was purchased from Grand Island Biological Corp. and calf serum was obtained from Flow Laboratories.

2 Portions of this paper (including part of "Experimental Procedures") were 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 available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-151, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Inc. Bt-cAMP, 8-bromo-CAMP, and RNase A were obtained from Sigma. S-77 supplemented with 2.4 mM CaCl2, 1.2 mg/ml of glucose, 10 μg/ml of phenol red, 6 mM NaHCO3, 60 μM Tricine, 60 μM cystine, 2 mM glutamine, 50 μg/ml of neomycin sulfate, and fetal and newborn calf sera at final concentrations of 2.5% (v/v) each. The final pH was adjusted to 7.6 with NaOH. The cells were changed to serum-free medium 24 h prior to harvest.

Quantitation of mRNAPEPCK—Total cell poly(A)+ RNA was isolated (35) and quantitated using the dot-blot hybridization technique described previously (14). The cDNA probe was a 1300 base pair Small-SphI fragment isolated from pPC165, a cloned recombinant DNA plasmid and labeled by nick translation (36). The results were analyzed by a densitometer scan of an autoradiogram (14).

Isolation of Nuclei—Cells were scraped into 5 ml of homogenization buffer containing 0.25 M sucrose, 10 mM HEPES at pH 8.0, 10 mM NaCl, 20 mM dithiothreitol, and 10% (w/v) Triton X-100, and homogenized in a Dounce homogenizer at 0−4°C. Nuclei were isolated by centrifugation at 600 × g for 5 min and were washed twice by homogenization in fresh buffer. After the final centrifugation through a cushion of a homogenization buffer containing 1 M sucrose, the nuclei were suspended in 50 mM HEPES at pH 8.0 containing 40% (v/v) glycerol, 5 mM MgCl2, 0.1 mM EDTA, and 2 mM dithiothreitol. The concentration of nuclei was measured by diluting an aliquot in 10% (v/v) sodium dodecyl sulfate and measuring the absorbance at 260 nm. The final total nuclear acid concentration was adjusted to 1.5 mg of DNA/ml (30 A260 units).

Transcription Elongation Assay—The elongation of nascent RNA transcripts on the P-enolpyruvate carboxykinase gene was measured by a modification of the procedure described by McKnight and Palmiter (37). Twenty μl of isolated nuclei was incubated at 26°C for 45 min in a final volume of 50 μl containing (as final concentrations): 20 mM HEPES at pH 8.0, 5 mM MgCl2, 30 mM NaCl, 0.5 mM MnCl2, 16% (v/v) glycerol, 0.04 mM EDTA, 2 mM dithiothreitol, 0.4 mM each of ATP, CTP, and GTP, and 50 μCi of [α-32P]UTP (400−600 Ci/mmol). The reaction was terminated by digestion with 100 μg/ml of DNase I and 100 μg/ml of proteinase K in the presence of 100 μM CaCl2 and 25 μg/ml of RNAse-free RNAse for 20 μg/ml of DNase I and 100 μg/ml of proteinase K in the presence of 100 μg/ml of 95% ethanol. The RNA was collected by centrifugation, dissolved in 200 μl of 20 mM Tris-HCl at pH 8.0 with 10 mM CaCl2. The process of enzyme digestion, phenol/chloroform extraction, ether extraction, and ethanol precipitation was repeated, except that the first digestion was for 60 min at 37°C and the second, in the presence of EDTA and sodium dodecyl sulfate, was for 30 min. The samples were then precipitated with 0.5 M NaCl, 20 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate, and 33% (v/v) formamide. Hybridization was conducted as described by McKnight and Palmiter (37) using nicktranslated DNA probes, respectively. pPCC112.R3 is a 5.8-kilobase EcoRI fragment of genomic DNA inserted into the EcoRI site of pBR322. All of the insert represents P-enolpyruvate carboxykinase DNA that is transcribed into precursor mRNAPEPCK.
The isolation and characterization of this plasmid will be described elsewhere.\(^4\) The filters for hybridization were prepared as follows: pAPC112.R3 or pBR322 were digested with EcoRI, boiled for 1 min in 2 M NaCl and 0.2 M \(\text{NH}_4\)OH, and cooled on ice. Ethanol was added to give a final concentration of 4% (v/v) and 0.5-\(\mu\)g aliquots of DNA were applied to 6-mm diameter circles of nitrocellulose. The filters were dried, baked for 2 h at 80 °C in vacuo, and washed for 30 min with a buffer containing 10 mM Tris at pH 7.5, 0.3 M NaCl, 2 mM EDTA, and 0.1% (w/v) sodium dodecyl sulfate. Hybridization was carried out for 2-3 days at 45 °C in the presence of 6000-7000 cpm of \(^{32}\)P-labeled RNA, prepared as previously described, to monitor hybridization efficiency.\(^4\) The filters were washed with washing buffer (37), incubated with RNases A and T1, washed again, then the RNA was eluted, and radioactivity was measured in a scintillation spectrometer. The results are expressed as parts of P-enolpyruvate carboxykinase RNA transcribed per one million parts of total RNA transcribed. The general features of this assay, including the calculation of parts per million, are described in detail in the protocol.

RESULTS

Time Course of Induction of Transcription and mRNA\(^{PEPCK}\) by 8-CPT-cAMP—Although Bt,cAMP was an effective inducer of P-enolpyruvate carboxykinase gene transcription, the results varied considerably between experiments. Since this could be due to metabolism of Bt,cAMP, which is rapidly deacetylated into the inactive N\(^6\)-, and the inactive O\(^2\r\)-methylbutyryl forms (39), the nonmetabolizable analog 8-CPT-cAMP, was used in most experiments. In the experiment illustrated in Fig. 1A, the maximal rate of transcription was obtained 30 min after the addition of 8-CPT-cAMP. This was maintained for about 3-4 h then there was a gradual decline to a new steady state which was maintained at approximately 2-3 times the basal rate for at least 72 h. The amount of total cellular mRNA\(^{PEPCK}\) changed commensurate with, but slightly slower than the rate of transcription, as would be expected if transcription is the principal site of mRNA\(^{PEPCK}\) regulation. Since the maximal rate of transcription occurred by 30 min, the first time examined in this experiment, it seemed possible that a larger increase might occur even earlier, and that transcription could increase soon after addition of the inducer. Fig. 1B represents a composite of the results of several different experiments designed to examine the early time course of induction. Transcription of the P-enolpyruvate carboxykinase gene doubled within 5 min after the addition of 8-CPT-cAMP and the peak rate of transcription occurred at 30 min. An experiment which compared the concentration-response curves for 8-CPT-cAMP and forskolin, a diterpene which results in a concentration-dependent increase of endogenous cAMP, was conducted. Not surprisingly, different concentrations of each were required for equivalent inductions, yet the response curves were superimposable (data not shown). Half-maximal induction was reached with 30 \(\mu\)M 8-CPT-cAMP or 3 \(\mu\)M forskolin, a concentration of the latter which results in about a 6-fold increase of cAMP in H4IIE cells.\(^5\) Forskolin also caused an equally rapid induction of transcription in H4IIE cells (data not included).

Transcription of the P-enolpyruvate Carboxykinase Gene Becomes Desensitized to CAMP—The observation that P-enolpyruvate carboxykinase gene transcription declines rapidly after the maximal rate is achieved, even though the intracellular concentration of the inducer is presumably unchanged, raises the question of whether the cell becomes desensitized to the nucleotide. The experiment described in Table 1 was performed to examine this possibility. Cells acutely shifted to serum-free medium were tested for responsiveness to 8-CPT-cAMP and, after a 30-min incubation, a

\(^{4}\) E. G. Beale, N. Chrapkiewicz, and D. K. Granner, manuscript in preparation.

**FIG. 1. Time course of 8-CPT-cAMP induction.** Cells were treated with 0.1 mM 8-CPT-cAMP for the times indicated. P-enolpyruvate carboxykinase gene transcription was measured by the run-off assay, and mRNA\(^{PEPCK}\) was measured by dot-blot hybridization. The results in panel A are shown as the mean ± S.D. of the per cent of maximal induction for duplicate samples, and each curve represents a separate experiment. The maximal mean values for P-enolpyruvate carboxykinase gene transcription was 1030 ppm, and for mRNA\(^{PEPCK}\), an amount it was 2.72 integrator units (see Beale et al. (14) for an explanation of this measurement). The results in panel B show the rate of gene transcription, expressed as the mean ± S.D. from six assays, at various times after the addition of 8-CPT-cAMP to H4IIE cells. Details of the assay and calculations were as described under "Experimental Procedures" and in the legend accompanying MiniPrint Fig. 1.

15-fold induction (107 to 1569 ppm) occurred. Twenty-four h later the basal rate of transcription was unchanged but the cells continuously exposed to 8-CPT-cAMP now had a rate only twice that of control cells (205 versus 96 ppm). If additional inducer was added at this time very little response was seen (an increase from 205 to 312 ppm). That cells are capable of responding to an inducer after being held in serum-free medium for 24 h is illustrated by the fact that forskolin increased the rate of transcription in such cells from the basal rate of 130 to 1842 ppm. The next part of the experiment was designed to test whether reinuduction failed because of some peculiar response to the 8-CPT-cAMP analog, whereas endogenous cAMP might induce transcription. This is not the case for forskolin, with the attendant increase of intracellular cAMP, also failed to stimulate P-enolpyruvate carboxykinase gene transcription in cells exposed to 8-CPT-cAMP for 24 h.
Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA&lt;sub&gt;P&lt;sub&gt;PEPPCK&lt;/sub&gt; synthesis</th>
<th>ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>107 ± 17</td>
<td>1569 ± 323</td>
</tr>
<tr>
<td>8-CPT-cAMP for 30 min</td>
<td>1569 ± 323</td>
<td>205 ± 38</td>
</tr>
<tr>
<td>8-CPT-cAMP for 24 h</td>
<td>8-CPT cAMP, 30 min</td>
<td>432 ± 22</td>
</tr>
<tr>
<td>8-CPT-cAMP for 24 h</td>
<td>8-CPT cAMP, 30 min</td>
<td>312 ± 22</td>
</tr>
<tr>
<td>None</td>
<td>30C50, 30 min</td>
<td>130 ± 118</td>
</tr>
<tr>
<td>None</td>
<td>Forskolin, 30 min</td>
<td>1842 ± 18</td>
</tr>
<tr>
<td>8-CPT cAMP for 24 h</td>
<td>Mes0, 30 min</td>
<td>142 ± 91</td>
</tr>
<tr>
<td>8-CPT cAMP for 24 h</td>
<td>Forskolin, 30 min</td>
<td>251 ± 21</td>
</tr>
</tbody>
</table>

Transcription Decreases Rapidly Following Insulin Treatment—Decreased cytoplasmic mRNA<sub>P<sub>PEPPCK</sub> could result from accelerated degradation in the nucleus or cytoplasm, an inhibition of transport from the nucleus to the cytoplasm, or an inhibition of some step of the production (including transcription, processing, and chemical modification) of this specific RNA. We recently presented data which suggests that the principal effect of insulin is to inhibit P-enolpyruvate carboxykinase gene transcription (4). The purpose of the experiments described next was to evaluate this action of insulin in more detail.

Nuclear and cytoplasmic P-enolpyruvate carboxykinase RNA decrease with a t<sub>1/2</sub> of 20–40 min after H41IE cells are exposed to insulin (4). One might expect insulin to inhibit transcription with a t<sub>1/2</sub> of less than 20 min if this is the primary effect of the hormone. This was tested in the experiment illustrated in Fig. 2A. H4IE cells were first exposed to 8-CPT-cAMP which caused a rapid, 9-fold increase in transcription within 30 min after 8-CPT-cAMP was added. Transcription continued at the maximal rate of 1500 ppm for an additional 60 min then decreased to 800 ppm 150 min after 8-CPT-cAMP addition. Addition of insulin 30 min after the cyclic nucleotide was added resulted in a rapid decrease in P-enolpyruvate carboxykinase gene transcription. Detectable inhibition was noted at 5 min and by 10 min after adding insulin the rate of P-enolpyruvate carboxykinase gene transcription was reduced to the basal level. The t<sub>1/2</sub> of this inhibition is thus approximately 12–15 min, notably faster than the decrease in cytoplasmic mRNA<sub>P<sub>PEPPCK</sub> (4, 14, 35, 40). Two h after insulin was added transcription had decreased to 100 ppm, a value less than the uninduced rate of 1500 ppm. Transcription was also reduced to the basal rate when 5 nM insulin was added to H4IE cells previously treated with 50 μM forskolin. Insulin thus inhibits P-enolpyruvate carboxykinase gene transcription when cAMP is added to the cells exogenously, in the form of analogs, or when endogenous cAMP levels are high.

Physiological Concentrations of Insulin Inhibit Transcription—H4IE cells are remarkably sensitive to insulin (4, 32), thus it is not surprising that 1 pm insulin caused a significant decrease in transcription (Fig. 2B). The half-maximal decrease occurred with 2–5 pm insulin and 10<sup>−8</sup> M insulin reduced transcription to the level measured in untreated cells. Prolinulin also decreased P-enolpyruvate carboxykinase gene transcription, but approximately 30–50-fold higher concentrations were needed to achieve an inhibition equivalent to that obtained with insulin. These insulin concentration effects are virtually identical to those obtained when either mRNA<sub>P<sub>PEPPCK</sub> or the rate of synthesis of P-enolpyruvate carboxykinase are measured in H4IE cells (4). This provides additional evidence in support of the hypothesis that transcription is the primary site at which insulin regulates mRNA<sub>P<sub>PEPPCK</sub> amount and subsequent enzyme synthesis. The 30–50-fold difference in sensitivity to insulin and proinsulin supports our previous contention that this effect is mediated by the insulin receptor (32).
Heretofore the inhibitory effects of insulin on P-enolpyruvate-carboxykinase gene transcription were noted in cells which had been exposed to agents which enhance transcription of this gene. It is possible to observe an inhibition of P-enolpyruvate-carboxykinase gene transcription by insulin in H4IIE cells that have not been treated with inducers, as is shown in Table 2. In the absence of cAMP, insulin concentrations of $10^{-11}$ and $10^{-9}$ M decreased P-enolpyruvate-carboxykinase gene transcription from the basal rate of 177 ppm to 71 and 25 ppm, respectively. In this experiment nuclei incubated in $\alpha$-amanitin at 1 $\mu$g/ml also showed an incorporation of 25 ppm, so $10^{-9}$ M insulin abolished transcription of this gene.

The inhibition of mRNA$^{PEPCK}$ is rapidly reversible—The repression of mRNA$^{PEPCK}$ by insulin is exerted quickly (4) and, given the rapid response of mRNA$^{PEPCK}$ during feeding and fasting cycles (41), should be readily reversible. In the experiment shown in Table 3, mRNA$^{PEPCK}$ decreased from 0.24 integrator units to undetectable levels during a 3-h exposure of H4IIE cells to insulin. When cells first incubated with insulin were washed at pH 6.0 to remove receptor-bound insulin (41), mRNA$^{PEPCK}$ returned to the level found in untreated cells within 3 h. The fact that such cells also respond to Bt2cAMP (mRNA$^{PEPCK}$ increased from 0.18 to 1.40 integrator units, a value similar to that in Bt2cAMP-treated cells not pretreated with insulin) indicates that the wash was effective in removing insulin. Cells washed before the additions exhibited this same response to insulin, and Bt2cAMP induced mRNA$^{PEPCK}$ from 0.22 to 1.76 integrator units. Thus, the pH 6.0 wash per se did not impair the subsequent responsiveness of these cells to either insulin or Bt2cAMP.

The effects of 8-CPT-cAMP and insulin do not require protein synthesis—The hormonal induction of several different mRNAs is prevented when protein synthesis is inhibited (43-45). This is generally interpreted to mean that the hormone acts by inducing a regulatory protein which has a rapid turnover time, rather than by acting directly on the gene in question (46). The injection of Bt2cAMP into rats increases the amount of mRNA$^{PEPCK}$ whether or not protein synthesis is allowed to proceed (14, 40). Comparable studies with insulin have heretofore not been described, nor has the action of inhibitors of protein synthesis on transcription of the P-enolpyruvate-carboxykinase gene, as mediated by these effectors, been described. Such studies in intact animals would be difficult to interpret given the demonstrated ability of cycloheximide to stabilize mRNA$^{PEPCK}$ against degradation (13, 14, 40), an effect which might obscure the repression by insulin. We therefore elected to address these questions using H4IIE cells. The 10-fold decrease in mRNA$^{PEPCK}$ caused by insulin in H4IIE cells (from 0.32 ± 0.08 integrator units to 0.03 ± 0.01) was not affected by cycloheximide, emetine, or puromycin at concentrations (10 $\mu$M, 10 $\mu$M, and 1 $\mu$M, respectively) that inhibited protein synthesis by greater than 95%, as assessed by incorporation of $[^3H]$leucine into trichloroacetic acid-precipitable material. Under these conditions the inhibitors had no effect on total RNA concentration. Parenthetically, the stabilization of mRNA$^{PEPCK}$ by cycloheximide appears to be an effect noted only in intact animals.

We then tested for similar effects in the RNA transcript elongation assay. In our first experiment H4IIE cells were incubated in cycloheximide and 8-CPT-cAMP for 60 min, after which transcription was assessed. In this instance P-enolpyruvate-carboxykinase gene transcription was markedly enhanced by the cyclic nucleotide, but the relative rate was 25% less than that observed in cells not exposed to cycloheximide (see Table 4). With the observation that induction occurred within minutes, and achieved the maximal rate by 30 min (Fig. 3), correspondingly shorter incubations with the inhibitor were employed. At early times (up to 30 min) transcription of the P-enolpyruvate-carboxykinase gene is unaffected by cycloheximide. From the data presented above, one might also expect that cycloheximide would not block the effect that insulin exerts on transcription of the P-enolpyruvate-carboxykinase gene. That this is so is illustrated in Table 4. Cycloheximide had no effect on transcription in either untreated or insulin plus 8-CPT-cAMP-treated cells, nor did it affect total RNA transcription under these conditions. These observations indicate that the regulation of P-enolpyruvate-carboxykinase gene transcription by cAMP and insulin does not require the synthesis of a protein with a rapid turnover time.

Summary of the Action of Insulin on mRNA$^{PEPCK}$ in H4IIE Cells—The data shown in Tables 2 and 4 and Fig. 2, coupled with previous observations of insulin repression of mRNA$^{PEPCK}$ (4, 13, 32), indicate that the primary site of insulin action is exerted at the level of transcription of this gene. As a test of this hypothesis we plotted changes of the transcription rate, the amount of the putative primary transcript, the amount of the mature mRNA$^{PEPCK}$ in the nucleus, and the amount of cytoplasmic mRNA$^{PEPCK}$, as a function of time after insulin addition (see Fig. 3). P-enolpyruvate-carboxykinase gene transcription appears to decrease first fol-

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**Table 2**

Effect of insulin on mRNA$^{PEPCK}$ transcription

<table>
<thead>
<tr>
<th>Insulin concentration</th>
<th>mRNA$^{PEPCK}$ synthesis</th>
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<tr>
<td>$10^{-11}$ M</td>
<td>177 ± 12</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>71 ± 3</td>
</tr>
<tr>
<td>0</td>
<td>25 ± 14</td>
</tr>
</tbody>
</table>

**Table 3**

Reversibility of the insulin repression of mRNA$^{PEPCK}$

An initial incubation of confluent cultures of H4IIE cells was done with or without insulin for 3 h. At the end of this preincubation the cells were washed at pH 6.0 to remove bound insulin (42). This wash was carried out as follows. The culture medium was aspirated and the cells were quickly rinsed four times with a solution containing 10 mM sodium phosphate at pH 6.0, 0.15 M NaCl, and 0.1% (w/v) bovine serum albumin. The cells were incubated for 10 min at 37 °C in the same solution and then washed four times with the same solution but at pH 7.5. Fresh medium was added following the washing procedure and the incubation was continued for 3 h in the presence of the effectors shown in the Table. All of the incubations were performed in the absence of serum. Insulin was at a final concentration of 0.1 nM; Bt2cAMP and theophylline were at 0.5 mM and 1 mM, respectively. Poly(A)$^+$ RNA was extracted and assayed by dot-blotted hybridization as described under "Experimental Procedures." Integration units are expressed as the mean ± S.D. of triplicate RNA samples.

**Table 4**

<table>
<thead>
<tr>
<th>Addition during 3 h preincubation</th>
<th>pH 6 wash</th>
<th>Additions during 3 h incubation</th>
<th>mRNA$^{PEPCK}$ hybridized</th>
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</thead>
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<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0.24 ± 0.04</td>
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<tr>
<td>None</td>
<td>Insulin</td>
<td>Not detectable</td>
<td></td>
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<tr>
<td>None</td>
<td>+</td>
<td>None</td>
<td>0.22 ± 0.06</td>
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<tr>
<td>None</td>
<td>+</td>
<td>Insulin</td>
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<tr>
<td>Insulin</td>
<td>+</td>
<td>Bt2cAMP</td>
<td>1.76 ± 0.10</td>
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<tr>
<td>Insulin</td>
<td>+</td>
<td>Bt2cAMP</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>Insulin</td>
<td>+</td>
<td>Bt2cAMP</td>
<td>1.40 ± 0.36</td>
</tr>
</tbody>
</table>
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Table 4

Cycloheximide does not influence the effects that 8-CPT-cAMP and insulin have on P-enolpyruvate carboxykinase gene transcription.

H4IIE cells, grown as described under "Experimental Procedures," were prestimulated with 0.1 mM 8-CPT-cAMP for 30 min and then were incubated for an additional 60 min in the presence or absence of 10 μM cycloheximide plus 8-CPT-cAMP. Insulin, where indicated, was added to a final concentration of 5 nM 30 min before the cells were harvested. Nuclei were isolated and transcription was assayed as described above. Results are expressed as the mean ± S.D. of triplicate assays. In the experiment in which mRNA amount was determined, cells were incubated with the inhibitors of protein synthesis for 3.5 h and insulin (1 nM) was present for the last 1 h prior to cell harvest. Poly(A)+ RNA was isolated and quantitated as described under "Experimental Procedures." Cycloheximide, emetine, and puromycin inhibited [3H]leucine incorporation into trichloroacetic acid-precipitable protein by 95, 100, and 100%, respectively, and did not affect total RNA levels. In this and each of three other experiments, cycloheximide and emetine had no effect on basal levels of mRNAPEPCK.

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>Total RNA synthesis (cpm × 10^-6)</th>
<th>mRNAPEPCK synthesis (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.44 ± 0.16</td>
<td>186 ± 30</td>
</tr>
<tr>
<td>+cycloheximide</td>
<td>1.55 ± 0.14</td>
<td>195 ± 22</td>
</tr>
<tr>
<td>8-CPT-cAMP</td>
<td>1.14 ± 0.15</td>
<td>1397 ± 88</td>
</tr>
<tr>
<td>+cycloheximide</td>
<td>1.63 ± 0.18</td>
<td>1196 ± 112</td>
</tr>
<tr>
<td>8-CPT-cAMP + insulin</td>
<td>1.39 ± 0.26</td>
<td>447 ± 28</td>
</tr>
<tr>
<td>+cycloheximide</td>
<td>1.34 ± 0.17</td>
<td>383 ± 36</td>
</tr>
</tbody>
</table>

Effect of Hormone Combinations on mRNAPEPCK and on P-enolpyruvate Carboxykinase Gene Transcription—It has been suggested that cAMP is the primary regulator of P-enolpyruvate carboxykinase synthesis in rat liver and that insulin decreases P-enolpyruvate carboxykinase synthesis by reducing the intracellular concentration of cAMP (12, 41, 47-52). This effect of insulin could be due to enhanced hydrolysis of cAMP through increased phosphodiesterase activity (53, 54). The induction of P-enolpyruvate carboxykinase by a cyclic nucleotide that cannot be metabolized, or by an agent other than cAMP, should be unaffected by insulin if insulin acts in this manner. We therefore investigated insulin action in H4IIE cells in which mRNAPEPCK was induced by either 8-bromo-CAMP, a fully active but nonmetabolizable derivative (55), or by dexamethasone, a glucocorticoid which induces enzymes in a CAMP-independent manner (56). Dexamethasone and 8-bromo-CAMP resulted in 4- and 15-fold increases of mRNAPEPCK, respectively, and insulin completely inhibited both of these induction processes (Table 5).

The observations of multihormonal regulation of mRNAPEPCK were extended using the nascent transcript elongation assay. The relative rate of transcription of mRNAPEPCK, which was 108 ppm in untreated H4IIE cells, increased to 606 ppm following dexamethasone, to 1355 ppm in cells treated with 8-CPT-cAMP, and to 1519 ppm in cells treated with the combination of 8-CPT-cAMP plus dexamethasone (see Table 6). Five μM insulin decreased mRNAPEPCK transcription in otherwise untreated H4IIE cells to 70 ppm, and in cells treated with dexamethasone, 8-CPT-cAMP, and 8-CPT-cAMP plus dexamethasone to 58, 365, and 520 ppm, respectively. The dominant role of insulin is apparent in all combinations, yet...
there is a fundamental difference in the responses. In this and other similar experiments in which a 30-min exposure to hormones was employed, insulin did not always completely reduce transcription to the basal level whenever a cAMP analog was present (see Fig. 2A for a detailed analysis of the kinetics of the insulin response in 8-CPT-cAMP-treated cells), but it always totally inhibited the effect of dexamethasone. In fact, the rate of isotope incorporation in the presence of 1 μg/ml of α-amanitin in this experiment was 71 ppm, so insulin completely abolished transcription in basal H4IIE cells, or in cells exposed to dexamethasone. None of the individual hormones, or the various combinations, had any demonstrable effect on the synthesis of total RNA, so again these effects were very specific.

DISCUSSION

Few examples of regulation of the transcription of mammalian genes by peptide hormones or their intracellular mediators have been reported and examples of multimodal regulation of transcription of a single gene involving peptides are even rarer (3–9). In this paper we show that cAMP and glucocorticoids exert a rapid stimulatory effect on transcription of the P-enolpyruvate carboxykinase gene in H4IIE cells. Insulin inhibits transcription of this gene, and overrides the actions of the stimulatory agents.

Glucagon, acting through cAMP, provides the major stimulus for P-enolpyruvate carboxykinase synthesis. In this paper we show that transcription of mRNAPEPK increases within 5 min after the addition of cyclic AMP analogs, or after intracellular cAMP is increased by treating H4IIE cells with forskolin, and achieves its maximal rate within 30 min (Fig. 1). The kinetics of the induction of P-enolpyruvate carboxykinase gene transcription in H4IIE cells and rat liver are similar (cf. this paper and Ref. 19) and compare favorably with the rapid increase of prolactin gene transcription which follows the addition of cAMP to pituitary cells (5, 6). This, coupled with the observation that protein synthesis is not required (Table 4) indicates that cAMP is directly affecting P-enolpyruvate carboxykinase gene transcription and that no rapidly synthesized protein intermediates are necessary (46).

The 10–15-fold increase of P-enolpyruvate carboxykinase gene transcription noted after 30 min is of sufficient magnitude to account for the changes in cytoplasmic mRNAPEPK seen after cAMP treatment. Regulation of the steps involved in processing, chemical modification, and degradation of the primary transcript must therefore play a negligible role in this induction, and transport from the nucleus to the cytoplasm likewise cannot be a major site of control. The effects of cAMP are highly selective since fewer than 1% of the peptides in the liver cell are in the domain regulated by cAMP and no effect of this nucleotide on total RNA synthesis was observed (Miniprint, Table 1). The desensitization of P-enolpyruvate carboxykinase gene transcription that develops after prolonged exposure of H4IIE cells to 8-CPT-cAMP (Fig. 1A and Table 1) may be a common feature of genes regulated by this nucleotide. Desensitization may occur in rat liver since the 7–10-fold increase in P-enolpyruvate carboxykinase gene transcription noted after Br2-cAMP injection is not maintained even if continued injection of the nucleotide are given (19). The transcription of the lactate dehydrogenase A gene in C6 glioma cells reaches its maximal value 4 h after addition of isoproterenol, which acts through cAMP, to the culture medium (9), and at 6 h the rate of transcription is significantly lower. This may also represent desensitization, although these cells were not challenged with cAMP, and the possibility that down-regulation of the isoproterenol receptor is involved must be considered (57). A similar situation is noted vis à vis with thyrotropin-releasing hormone regulation of prolactin gene transcription in GH4 cells. A 7–12-fold increase is seen within 30 min, and the process begins to decrease within 1 h, so that by 36 h only a 2-fold induction remains. The addition of thyrotropin-releasing hormone results in no additional response during this time (7). The “burst-attenuation” kinetics of transcription may be due to different mechanisms in all these instances, including receptor down-regulation, or may be due to a common molecular mechanism at the gene level. If the latter is the case, the definition of this process should materially aid in understanding how these molecules regulate gene expression.

Having demonstrated an action of cAMP on P-enolpyruvate carboxykinase gene transcription, we are faced with defining its mechanism of action. The rapidity of the process, and the fact that protein synthesis is not required, indicates that an allosteric or covalent modification of a regulatory factor may be involved. Based on well-defined cytoplasmic actions of cAMP, a protein phosphorylation-dephosphorylation mechanism is an obvious possibility. This would require either the presence of a cAMP-dependent protein kinase/protein phosphatase system in the nucleus, or the translocation of a protein kinase and/or protein phosphatase from the cytoplasm to the nucleus, or the translocation of a regulatory phosphoprotein to the nucleus. Jungmann and co-workers (9) have proposed a model in which isoproterenol induces a cAMP-dependent protein kinase to translocate into the nucleus of C6 glioma cells and they suggest that this then results in the phosphorylation of histones and/or RNA polymerase II. The substrate of phosphorylation is of paramount importance if such a mechanism is involved. Rosenfeld and colleagues (6, 7, 58) have found a chromatin-associated basic protein in GH4 cells which is rapidly phosphorylated after cAMP or thyrotropin-releasing hormone treatment, and this modification precedes the increase of prolactin gene transcription. There is no evidence, as yet, which couples this observation to an effect of cAMP on specific gene transcription. Full activation of protein kinase occurs in less than 1 min after adding 8-CPT-cAMP to H4IIE cells, so, on kinetic grounds, protein kinase activation may be involved. H4IIE cells also contain the basic nuclear protein described above so the possibility that this phosphoprotein is involved in P-enolpyruvate carboxykinase gene transcription also exists. Finally, it is also conceivable that the induction mechanism involves a CAMP-binding protein, independent from protein kinase. This would be analogous to the catabolite regulatory protein system in prokaryotic cells (59), but such a mechanism has not yet been demonstrated in eukaryotic cells.

Glucocorticoid hormones also stimulate P-enolpyruvate carboxykinase synthesis, but to a lesser extent than glucagon/cAMP (60). One would expect glucocorticoids to be proportionately less effective in stimulating transcription of the P-enolpyruvate carboxykinase gene, if this is the primary site of control. The data presented in Table 6 shows this to be the case. While transcription increased 10-fold after 8-CPT-cAMP, dexamethasone resulted in a 6-fold increase. In contrast to the effect noted in H4IIE cells, Lamers et al. (3) measured only a 25% increase in the rate of transcription of this gene in rat liver. This is probably due to the fact that glucocorticoid hormones stimulate insulin release (60), which

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6 D. K. Granner, unpublished observations.
7 M. Rosenfeld and D. Granner, unpublished observations.
in turn inhibits P-enolpyruvate carboxykinase gene transcription (4) and thereby obscures the action of the glucocorticoid hormone.

The data presented in this paper provide several clues as to how insulin regulates P-enolpyruvate carboxykinase gene transcription. First, whether transcription (Fig. 2B), cytoplasmic mRNA \( ^{PRK} \text{pepk} \) (32), or P-enolpyruvate carboxykinase synthesis were measured (32), the effect is achieved at physiological concentrations of insulin, as the half-maximal decrease is achieved with 2–5 pm insulin (Fig. 2B). Second, the observation that proinsulin (Fig. 2B), insulin-like growth factor-I, and multiplication stimulating activity (data not presented) are 20–50 times less effective supports our previous contention that this effect is mediated by the insulin receptor. Third, the effect of insulin is quite specific, as total RNA transcription is not affected (Table 4). Although the inhibition of P-enolpyruvate carboxykinase gene transcription is the first example of a transcriptional effect by insulin (4), two other demonstrations of control at this level have been reported and more will likely follow. Insulin, in conjunction with estrogen, stimulates transcription of the ovalbumin gene (61) and regulation of albumin gene transcription by insulin has recently been reported (62). Other obvious candidates include the genes that code for the mRNAs shown to be increased by insulin, namely tyrosine aminotransferase (30), amylase (25), pyruvate kinase (27, 28), \( \alpha_1 \)-globulin (28), casin (21), and fatty acid synthetase (29). Since each of these mRNAs is induced, any general model of transcriptional control by insulin will have to account for both positive and negative effects. Fourth, the action of insulin is readily reversible (Table 3); it is rapid, as the half-maximal decrease is achieved in less than 15 min (Fig. 2A); and protein synthesis is not required (Table 4).

Finally, the inhibitory action of insulin is dominant; it is exerted even when maximally effective concentrations of 8-CPT-cAMP or dexamethasone, or the combination of these two, are present (Tables 5 and 6). These two inducers presumably act by different mechanisms and, in fact, they have additive effects on P-enolpyruvate carboxykinase transcription (Table 5). It is interesting that the induction by dexamethasone appears to be more sensitive to repression by insulin than that caused by cAMP, at least it occurs more rapidly. Insulin must affect P-enolpyruvate carboxykinase transcription by a mechanism that is different from and not dependent on the mechanisms employed by cAMP and dexamethasone, since it affects transcription in the absence of either of these inducers (Table 6).

The regulation of P-enolpyruvate carboxykinase synthesis in H11E cells offers a unique opportunity to study multihormonal regulation of gene transcription. It is possible that all three effectors converge through a common regulatory factor. The rapidity of the effects of cAMP and insulin, and the observation that neither requires ongoing protein synthesis, is compatible with a transcription regulatory process that involves an allosteric or covalent modification of existing proteins; for example, a protein phosphorylation-dephosphorylation system requiring specific protein kinases and phosphoprotein phosphatases. Such a mechanism is well-established for cAMP. Insulin, whether it acts via activation of the tyrosine kinase on its receptor, and a subsequent phosphorylation cascade, or via a unique intracellular mediator, which is quantitated using assays which involve changes in catalytic activity due to phosphorylation or dephosphorylation, may act in an analogous manner. The final link is the action of glucocorticoid hormones. Evidence that the glucocorticoid receptor may also be a protein kinase (64), has been presented. The concerted action of these hormones could thus be explained if a protein exists which regulates transcription of the P-enolpyruvate carboxykinase gene in proportion to its extent of phosphorylation.

Acknowledgments—We thank Cathy Caldwell for technical assistance and Sara Paul and Cathy Kaufman for secretarial assistance.

REFERENCES

Multihormonal Regulation of Gene Transcription


Additional references are found on p. 15251.
Supplementary Material 1

Multihormonal Regulation of Phosphoenolpyruvate Carboxykinase Gene Transcription.


Characterisation of a Nuclear Transcription Assay - The nuclear RNA transcript accumulation assay was employed to study the effects of hormones on transcription from a non-functional nuclease-sensitive sequence. Initial exposure of intact cells to the hormone and subsequent isolation and incubation of the nuclei in a buffer containing 40 Gy/ml of the hormone resulted in most reported instances, since rates of incorporation into the specific RNA molecules have not generally been quite so slow. The ratio of turnover of nucleolar RNA did not vary significantly, but it is not likely to be slower than that of cytoplasmic RNA, which has a half-life of about 20 minutes. Therefore, it might not be possible to distinguish between hormone-induced changes in RNA synthesis. The hormone first introduced a steady state which the rate of hormone could not be affected by the labeling time, this indicates that transcription and RNA stability was being quantitated. In subsequent experiments an incubation time of 45 min was routinely employed to maximize the amount of RNA synthesized. As expected, in the presence of inhibitors of RNA synthesis, a decrease in the amount of RNA was observed. The effects of these inhibitors on transcription from the same cells were multiplicative: the mRNA could readily be demonstrated, or from cells which had been exposed to 40 Gy/ml for 40 min (see Miniprint Fig. 1B). The observation that survival was higher, even at the highest RNA concentrations, suggests that the labeling conditions in the presence of inhibitors of RNA synthesis was optimal for the measurement of transcription in intact cells which were within their limits in all of the experiments described above.

Unlabelled RNA should compete for binding to the filter if the binding of the labelled, nascent RNA is specific. A transcription reaction was performed using nuclei isolated from cells that had been exposed to 30 Gy with 0.1 M 40 Gy/ml. The 40 Gy/ml RNA was extracted and mixed with various concentrations of poly(dT)TNA which was isolated from HeLa cells that had been treated for 30 Gy with 0.1 M 40 Gy/ml and exposed to 40 Gy/ml. The mixture was then exposed to 40 Gy/ml for 40 min (see Miniprint Table 1). In Experiments 1, 30 Gy/ml increased transcription from a basal rate of 80% at 728 to 200% and 400% by 40 Gy/ml. Unlabelled transcription of the poly(N)-cytosine polynucleotide gave rise to a 10-fold increase in specific activity and the highest concentration employed resulted in a 100% inhibition (Miniprint Fig. 1C).

Effect of forskolin on polynucleotide binding. The general features of the transcription of the phosphoenolpyruvate carboxykinase gene and the effects of forskolin were assessed using the nuclear RNA transcript accumulation assay and the results are shown in Miniprint Table 1. In Experiment 1, forskolin increased transcription from a basal rate of 80% at 728 to 200% and 400% by 40 Gy/ml. Unlabelled transcription of the poly(N)-cytosine polynucleotide gave rise to a 10-fold increase in specific activity and the highest concentration employed resulted in a 100% inhibition (Miniprint Fig. 1C).

REFERENCES