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Selective Transcription of An Insulin-Regulated Gene in Nuclear Extracts of Rat Hepatoma Cells[1]

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We have previously shown that when H_4 hepatoma cells are pretreated with insulin, plant lectins, phorbol esters, or insulin mediator, the steady state concentration of gene 33 mRNA is markedly increased. The increase in gene 33 mRNA concentration with insulin is due to an increase in the transcription rate of this gene. In the present report we demonstrate that nuclear extracts prepared from H_4 hepatoma cells pretreated with insulin exhibit enhanced transcription of gene 33 RNA from a DNA template containing the cap site and 1500 bp upstream of the 33 gene. This is a stable effect of insulin on the nuclear RNA polymerase II system since it is observed in frozen and thawed nuclear extracts as well as fresh nuclear extracts.

The binding of insulin to its plasma membrane receptor has been shown to have a variety of actions on cellular metabolism and growth. The mechanisms by which insulin exerts its pleotropic effects are not clear, although both

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the generation of mediators and the production of a set of decreased and increased polypeptide phosphorylations have been shown to occur (see 1 for a review). Insulin has also been shown to regulate the expression of several cellular RNAs, including phosphenol pyruvate carboxykinase, albumin, amylase, growth hormone (2-7), and an mRNA designated p33 that is found in both rat liver (8) and cultured hepatoma cells (9). Insulin induction of gene 33 mRNA is primarily due to an increase in the rate of transcription of the gene (8,9). A genomic clone for gene 33, including the start site for transcription and 1500 bp of 5' regulatory region, has been recently isolated by Tindal et al. (10). To begin to define factors involved in insulin-mediated increases in gene 33 transcriptional activity, we wanted to design an in vitro assay in which the transcriptional activity of the 33 gene would mimic those activities seen in vivo. We report here that in high salt nuclear extracts of the rat H_4 hepatoma cell line, gene 33 is correctly transcribed. Furthermore, the transcription rate of gene 33 is selectively increased when cells have been exposed to insulin prior to preparation of the nuclear extracts. The enhancing effect of insulin is seen on both fresh nuclear extracts and nuclear extracts that have been frozen and thawed. This suggests that in nuclear extracts of H_4 cells, the modification in the factor(s) that enhance transcriptional activity of the gene 33 template after insulin treatment is relatively stable. Tissue specific expression of a variety of genes has been seen in vitro using nuclear and whole cell extracts including immunoglobulins (11,12) α-globin (13), mouse albumin (14), growth hormone (15), and rat prolactin (16). As far as we are aware, this is the first system described in which the inductive action of insulin on the expression of a gene in vivo can be partly reproduced in vitro. This system should allow us to begin to define and purify those factors that mediate the actions of insulin on gene 33 transcription in the intact cell.

Materials and Methods

Materials. The sources of materials were as follows: Swim's 77 cell culture medium, fetal calf serum, and horse serum from Gibco Laboratories; porcine

insulin from Lilly; transfer RNA, cycloheximide, and phenylmethylsulfonyl fluoride (PMSF) from Sigma; restriction endonucleases (Pvu II, Hind III, Bam HI, and Sma I) and SP6 RNA polymerase from Promega Biotec; DNase I and RNase T2 from Bethesda Research Laboratories; [α-^{32}P] UTP from ICN Radiochemicals; protein assay reagents from Bio-Rad. Other reagents were of analytical grade from commercial sources.

Cell Culture. The H4IIEC3 (H4) cell line, a clonal derivative of the rat H35 Reubner hepatoma, was obtained from Dr. D. F. Steiner (University of Chicago). Cells were grown in 15 cm Falcon tissue culture plates in Swim's 77 medium supplemented with 5% fetal calf serum, 5% horse serum and 100 μg/ml gentamicin. Serum was withdrawn for 20-24 h prior to experiments.

Preparation of Nuclear Extract. Nuclear extracts were prepared essentially as described by Dignam et al. (17) with slight modifications. After incubation, confluent cells (about 5 x 10^8) were washed with cold phosphate-buffered saline and scraped from the culture plates and harvested by centrifugation (1000 x g, 5 min, 5°C). Pelleted cells were then suspended in 1 ml of cold hypotonic HEPES buffer (10 mM, pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.075 % NP-40 and were allowed to stand on ice for 10 min. The partially lysed cells were again pelleted and resuspended as before for complete lysis. Following centrifugation (1000 x g, 5°C, 8 min), the pelleted nuclei were suspended in 0.5 ml of extraction buffer consisting of 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF, and were homogenized by 10 strokes with a Dounce homogenizer. The suspension was stirred gently with a magnetic stirring bar for 30 min at 5°C and then was centrifuged for 30 min at 25,000 x g at 5°C. The resulting clear supernatant, designated the nuclear extract, was divided into small aliquots, and was either used fresh or was frozen in liquid nitrogen and stored at -70° until used.

Standard Conditions for in vitro Transcription Assay. Assays were carried out in a final volume of 10 μl essentially as described by Wildeman et al. (18). The components of the reaction mixture were as follows: 7 mM HEPES (pH 7.9), 7.6% (v/v) glycerol, 0.4 mM DTT, 0.03 mM EDTA, 0.1 mM PMSF, 0.08 mM

NaCl, 1.2 mM $MgCl_2$, 23 mM KCl, 500 μM each of three unlabeled nucleoside triphosphates (ATP, GTP and CTP), 3 μM [α-^{32}P]UTP (600 Ci/mmol), 2 mM spermidine, 1 μg nuclear extract protein diluted in the extraction buffer and linearized DNA (100 ng of Hind III cut gene 33 and 200 ng of Sma I cut PMLHI (19) or Hind III cut ISG-54K (20). After a 60 min preincubation of DNA template and extract in the absence of nucleotides, KCl and spermidine, transcription was carried out by addition of the remaining components and incubation for 20 or 30 min at 30°C. After extraction, [^{32}P] RNA was analyzed by electrophoresis on 4% polyacrylamide-7 M urea gels followed by autoradiography. Bands representing the specific transcripts were cut from the gels and counted; regions of equal area above and below the specific bands were excised, counted and the average of these values was subtracted as a blank.

Isolation of Total Cellular RNA. The method used to prepare total cellular RNA was that of Chirgwin et al. (21) with slight modification as previously described (9,22).

Hybridization of [^{32}P] RNA probe to cellular RNA. [^{32}P]-RNA complementary to the gene 33 mRNA was synthesized as previously described (22). Total cellular RNA (3 μg) was used for solution hybridization at 55°C and unhybridized probes were removed by RNase T2 (22). After extraction, [^{32}P] RNA-RNA hybrids were analyzed by electrophoresis on 4% polyacrylamide-urea gels followed by autoradiography.

Results

Nuclear extracts were prepared from H_4 rat hepatoma cells as described by Dingham et al. (17). In initial experiments, conditions were optimized for transcription of the gene 33 in extracts prepared from untreated cells. The effects of incubation time, temperature, KCl, and $MgCl_2$ concentrations were examined using three different DNA templates: the gene 33, the adenovirus major late promotor (PMLHI) and an interferon-regulated gene, ISG-54K. In addition, the effects of extract concentration and DNA template concentration were optimized for each extract preparation, as was the effect of

preincubation of the template with the extract prior to determining the rates of transcription. The optimal conditions for transcription of the three DNA templates were similar, to those described by Dingham et al. for transcription of the adenovirus major late promotor using nuclear extracts of Hela cells (17).

After conditions were optimized, we studied the effect of time of preincubation of H_4 hepatoma cells with insulin on the transcription rate of nuclear extracts prepared from these cells. As shown in Fig. 1, the transcription rate of gene 33 increased 2-fold after 15 and 30 min of insulin treatment and then decreased slightly at 60 min. This reproduces quite well the time-course of the insulin stimulation of gene 33 transcription in H_4 hepatoma cells, although the magnitude of the increase observed with nuclear extracts from hepatoma cells is smaller than that seen in nuclear run-off

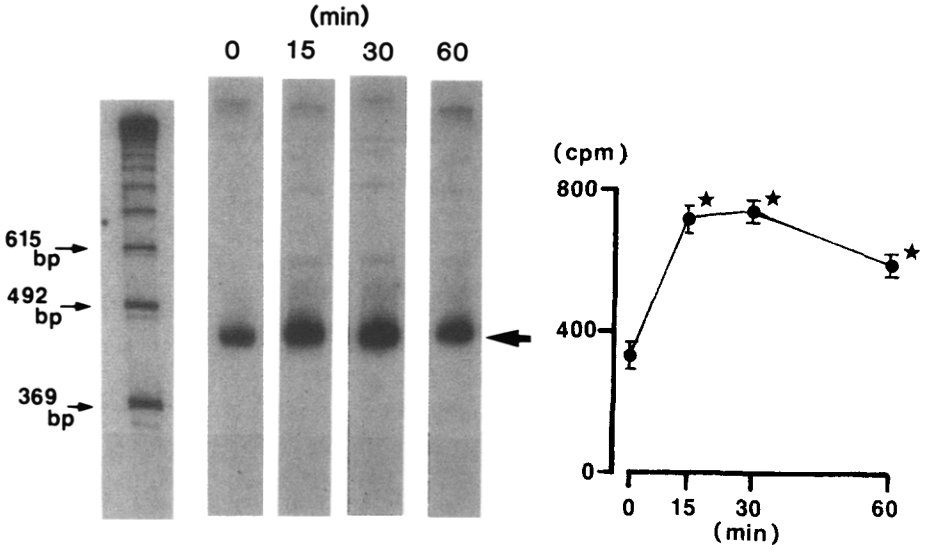

Fig. 1. Time course of in vivo treatment of H4 cells with insulin on gene 33 transcription rate in vitro . Nuclear extracts prepared from H4 cells which had been treated with insulin (1×10^{-7} M) for a designated period of time were assayed for p33 transcription under the standard conditions. After extraction, [^{32}P] RNA was analyzed by electrophoresis on 4% polyacrylamide-7 M urea gels followed by autoradiography. The bands were cut from the gels and radioactivity counted as described. The arrow indicates gene 33 transcript. Results plotted on the right are means $\pm$ S.E.M. from three cell plates. Asterisks indicate significant differences from control, 0 min-incubation ($p < 0.01$).

assays performed on nuclei from liver (2- to 3- fold in hepatoma nuclear extracts versus about 8-fold in liver nuclei) (8).

The specificity of the action of insulin was next studied. As shown in Fig. 2 and summarized in Fig. 3, insulin increased the transcription of all 3

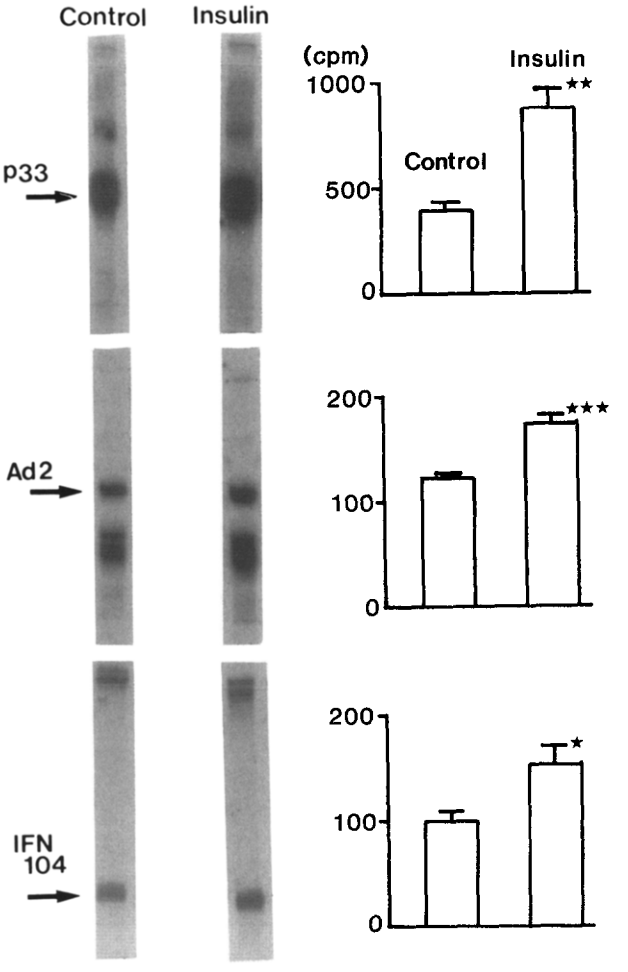

Fig. 2. Effect of in vivo treatment of H4 cells with insulin on gene 33, PMLHI or ISG-54K transcription rate in vitro. Extracts were prepared from control or insulin (1 x 10^{-7} M, 30 min)-treated cells as described and were assayed for gene 33, Ad2 or IFN104 in vitro transcription under the standard conditions. After extraction, [^{32}P] RNA was analyzed by electrophoresis on 4% polyacrylamide-7 M urea gels followed by autoradiography. The bands were cut from the gel and radioactivity counted as described. The arrow indicates each transcript. Results are means ± S.E.M. from three cell plates. Asterisks indicate significant differences between control and insulin (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

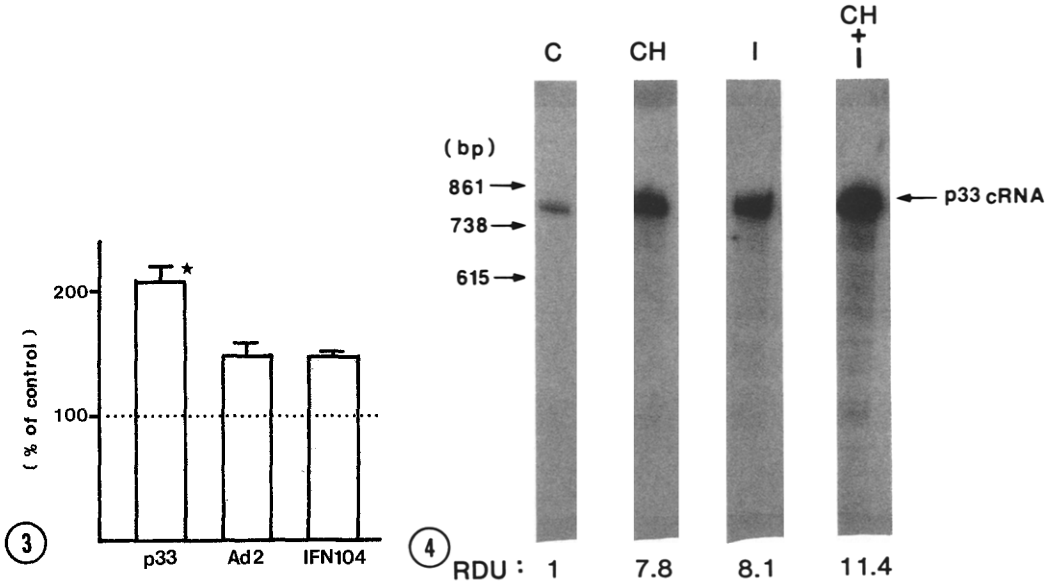

Fig. 3. Predominant stimulation of gene 33 in vitro transcription by in vivo treatment of H4 cells with insulin. Three separate experiments using the same procedure as shown in Fig. 2 were carried out. Results are means ± S.E.M. of insulin-control ratio. The asterisk indicates significant difference of transcription stimulation between gene 33 and the other two, templates PMLHI and ISG-54K ($p < 0.01$).

Fig. 4. Effect of cycloheximide and/or insulin on gene 33 mRNA accumulation in H4 cells. H4 cells were incubated for 15 min with or without cycloheximide (30 μg/ml), and then treated with or without insulin (1×10^{-7} M) for 30 min. Total cellular RNA was prepared, hybridized to [^{32}P] gene 33 cRNA and analyzed by using gel electrophoresis. The relative amount of gene 33 mRNA was determined by densitometric tracing of the resultant autoradiographs. The control was arbitrarily set to a RDU of 1. C, control; CH, with cycloheximide (30 μg/ml) for 45 min; I, with insulin (1×10^{-7} M) for 30 min; CH + I, with cycloheximide (30 μg/ml) for 15 min directly followed by insulin (1×10^{-7} M) for 30 min. This is a representative result of two separate experiments.

DNA templates (gene 33, adenovirus major late promoter and ISG-54K). However, the effect of insulin was 50% greater on the rate of transcription of the gene 33 template compared with the other 2 templates. The difference between the effect of insulin on gene 33 and on adenovirus major late

promoter and ISG-54K was statistically significant. Thus, the specificity of insulin action as measured in nuclear extracts in vitro, is not as strict as insulin's actions in hepatoma H_4 cells in vivo where only stimulation of gene 33 mRNA accumulation is observed, and neither the mRNA corresponding to ISG-54K or the PMLHI is induced by insulin (data not shown).

To determine whether protein synthesis was required for the stimulating effect of insulin on gene 33 mRNA accumulation in intact hepatoma cells, we studied the effect of cycloheximide. As shown in Fig. 4, cycloheximide alone stimulated gene 33 mRNA accumulation 7-fold under these conditions. Insulin alone caused a 8-fold increase, and the two together resulted in an 11-fold increase. Thus cycloheximide did not inhibit the action of insulin, but rather led to increased accumulation of gene 33 specific mRNA. The effects of cycloheximide are similar to its action on a number of other mRNA's that are regulated by growth factors (23-27).

Experiments were also performed with nuclear extracts prepared from cells treated with cycloheximide or insulin alone or in combination. As shown in Fig. 5, similar results were obtained for the rate of transcription of the gene 33 template as in intact cells. Cycloheximide alone, as well as insulin alone, led to a 2-fold increase in the rate of transcription of the gene 33 template, while the presence of the two together led to a slightly greater than a 2-fold increase over basal rates of transcription. Similar effects (although smaller in magnitude) were observed with the PMLHI and with the ISG-54K templates. Thus, the effect of cycloheximide in intact cells is reproduced in nuclear extracts, and reinforces the relative specificity of this system compared with the effects of insulin seen in vivo. It is interesting to note that both ISG-54K and the adenovirus major late promoter show increased transcriptional activity in vivo with cycloheximide treatment (23,28). The effects that are seen in nuclear extracts may partially reflect the in vivo responses of these genes to inhibitors of protein synthesis. However they also may be non-specific in that we have not examined a promotor that shows no change in in vitro transcriptional action after cycloheximide treatment of cells.

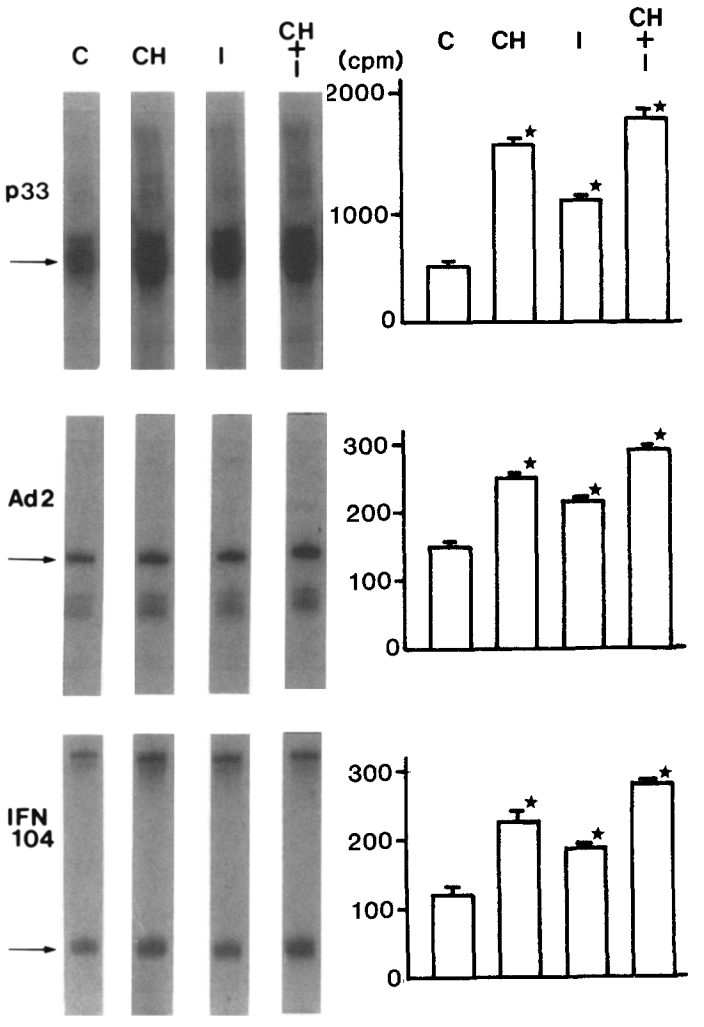

Fig. 5. Effect of in vivo treatment with cycloheximide and/or insulin on gene 33, PMLHI or ISG-54K transcription rate in vitro. H4 cells were incubated for 15 min with or without cycloheximide (30 μg/ml), and then directly treated with or without insulin (1 x 10^{-7} M) for 30 min. Nuclear extracts were prepared from the cells as described in "Materials and Methods", and assayed for gene 33, PMLHI or ISG-54K in vitro transcription under the standard conditions. After extraction, [^{32}P] RNA was analyzed on 4% polyacrylamide-7 M urea gels followed by autoradiography. The bands were cut from the gels and counted as described. The arrow indicates each transcript. Results are means $\pm$ S.E.M. from three cell plates. Asterisks indicate significant differences from control ($p < 0.01$). C, control; CH, with cycloheximide (30 μg/ml) for 45 min; I, with insulin (1 x 10^{-7} M) for 30 min; CH + I, with cycloheximide (30 μg/ml) for 15 min directly followed by insulin (1 x 10^{-7} M) for 30 min.

Discussion

Insulin has been shown to selectively increase the transcription of gene 33 mRNA in both cultured rat H_4 hepatoma cells and in the liver of the intact rat (8,9). Recently, the rat gene corresponding to the 33 cDNA has been isolated and sequenced by Kenney and his coworkers (10). A 2 kb fragment of this gene containing the transcriptional start site and 1500 bp 5' to the cap site was used in the present experiments to design an in vitro assay for transcription of this template in nuclear extracts. We have shown that nuclear extracts prepared from rat H_4 hepatoma cells accurately initiate transcription on this DNA template. Furthermore, nuclear extracts prepared from cells treated with insulin show a selective increase in gene 33 transcription compared with untreated extracts, and compared with other genes that are not insulin-responsive. The action of insulin on gene 33 transcription in vitro shows a time-dependence of increased transcription similar to that seen in vivo. The effects of cycloheximide and insulin on the extracts also mimic the effects seen in vivo. Although the effects of prior insulin treatment on transcription of gene 33 template in nuclear extracts are less than the changes in the rate of transcription seen in vivo, the actions of insulin are reproducible with different extract preparations. It is also well known that transcription of RNA polymerase II gene products in nuclear extracts is permissive, in the sense that the regulation of transcriptional activity often does not reflect the activity seen in vivo. A number of tissue-specific genes have been shown to be selectively transcribed using nuclear or whole cell extracts derived from the appropriate cell, and the differences in transcriptional activity seen in vitro are often smaller than those observed in vivo (11-16). These systems are comparable to the results we described for insulin induction of gene 33 in vitro. As far as we are aware the present report is the first to show that a gene responsive to a hormone which mediates its actions via a plasma membrane receptor can be selectively transcribed using nuclear extracts. Using this cell free system, an assay is now available to begin a functional analysis of those factors

involved in insulin regulation of gene 33 transcription, and will allow us to compare these results to the DNA protein binding sites that are presumably involved in hormonal regulation of this gene in vivo.

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