Rapid Insulin-stimulated Accumulation of an mRNA Encoding a Proline-rich Protein*

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By differential hybridization screening of a cDNA library derived from insulin-stimulated cells, we selected a clone which hybridized to an mRNA species that rapidly accumulated in response to insulin. The insert from this clone encoded a putative polypeptide of M, 33,600, pl 11.2; because the protein was enriched in proline residues (14.4 mol %) and contained three Pro-Pro-Pro-Pro repeats, we have tentatively labeled it tris-tetraprolin 1 (TTP). The function of this protein is not known, but it contains two regions very rich in proline (30–40 mol %); similar proline-rich regions have been shown to be involved in transcriptional activation by other proteins.

The mRNA (2.0 kilobases) encoding the TTP protein was essentially undetectable in serum-deprived HIR 3.5 cells, but accumulated dramatically within 10 min of stimulation by insulin. This effect appeared to be due to insulin acting through the intrinsic protein-tyrosine kinase activity of its own receptor. Insulin induction of TTP mRNA accumulation was prevented by actinomycin D and superinduced by cycloheximide. Accumulation of TTP mRNA was also stimulated by a variety of growth factors and active phorbol esters; however, the insulin effect was virtually normal in cells depleted of protein kinase C. A single TTP gene appeared to be present in the mouse genome. This gene joins the group of genes whose members are rapidly transcribed in response to insulin and other mitogens.

Stimulation of gene transcription is an important cellular response to insulin (1–3). In a recent study of NIH 3T3 fibroblasts overexpressing normal human insulin receptors (HIR 3.5 cells) (4), we found that of ~25 proteins whose biosynthesis was rapidly (within 1 h) stimulated by insulin, ~25% were apparently stimulated mainly or exclusively as a result of increased transcription (5). An example of one of these early transcriptional responses to insulin is the stimulated expression of the c-fos proto-oncogene (6–8). Insulin-stimulated accumulation of c-fos mRNA can be detected as early as 10 min after exposure of insulin; since the c-fos protein is thought to be an important trans-acting regulator of the expression of other genes, it is possible that the c-fos protein plays a role in mediating the expression of other insulin-sensitive genes whose transcription is stimulated with a somewhat slower time course.

We have begun a series of studies aimed at identifying other genes whose transcription is rapidly stimulated by insulin. Initial studies have involved screening a cDNA library derived from insulin- and cycloheximide-stimulated HIR 3.5 cells with cDNAs from both insulin-treated and control cells. Of eight cDNA clones hybridizing preferentially to the cDNAs from the insulin-treated cells, one was identical to a gene (egr-1) previously identified by differential hybridization of a serum-stimulated fibroblast cDNA library (9). Six other clones remain uncharacterized. We describe here the characteristics of a final clone representing a cellular gene that is rapidly stimulated at the transcriptional level by insulin and several other mitogens.

MATERIALS AND METHODS

Cells and Culture—NIH 3T3 HIR 3.5 cells (4) (a generous gift from Dr. J. Whitaker, State University of New York, Stony Brook, NY) were grown and made quiescent as described in Refs. 5 and 6. 3T3-L1 cells were induced to differentiate into adipocytes as described (6).

Preparation of cDNA Library—Quiescent serum-deprived HIR 3.5 cells were treated with 0.1 mM cycloheximide for 1 h and then stimulated with 70 nM insulin for an additional 1 h. Poly(A)+ RNA was prepared by oligo(dT)-cellulose chromatography of total cellular RNA as described (10). 7.5 μg of poly(A)+ mRNA was used to make a cDNA library in the λZAPII bacteriophage vector by standard techniques (Strategene, La Jolla, CA). The resulting library contained ~1.4 × 106 recombinant phage; these were used for screening without amplification.

Differential Hybridization Screening of cDNA Library—Phage were incubated with Escherichia coli (XL1 Blue) for 15 min and then plated at low density in 55 150-mm dishes (1–4 plaque-forming units/cm²). A duplicate set of nitrocellulose filters was prepared from each dish. Prehybridization was performed for 3 h at 60 °C in 6 × SSC (SSC = 0.15 M sodium chloride and 15 mM sodium citrate), 5 × Denhardt’s solution, 0.5% SDS, 0.1% sodium pyrophosphate, and 0.1 mg/ml denatured salmon sperm DNA. The filters were then hybridized at 60 °C for 24 h with 32P-labeled cDNA probes synthesized using Moloney murine leukemia virus reverse transcriptase as described (11) from poly(A)+ RNA obtained from quiescent HIR 3.5 cells or cells stimulated with 70 nM insulin for 1 h in the presence of cycloheximide. In all cases, ~1.5 × 106 cpm/ml was present in the hybridization solution containing 8 × SSC, 1 × Denhardt’s solution, 0.1% sodium pyrophosphate, 10 mM EDTA, and 0.1 mg/ml denatured salmon sperm DNA. Filters were washed extensively in 1 × SSC, 0.1% SDS at 52 °C. After exposure to film at ~70 °C overnight, plaques preferentially hybridizing to probes derived from the insul

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J085618.
stimulated cells were rescreened by spotting on a grid (5–10 plaque-forming units/grid), and duplicate filters were obtained and hybridized as described above. Phage reacting positively on secondary screening were rescued by superinfection with R408 helper phage as described in the protocol supplied by Stratagene. Infection of E. coli (XLI-Blue) with the resulting filamentous phage stock produced transformed colonies with the desired inserts cloned into Bluescript SK(−) plasmid vectors.

**Northern Blot Analysis**—Total cellular RNA from cultured cells was prepared by lysis of cells with guanidinium thiocyanate and centrifugation of cell lysates through a cesium chloride cushion (12). RNA from mouse tissues was prepared by homogenization and used for Northern blotting as described (12). Probes used were 32P-labeled cDNA inserts purified from low-melting-point agarose gels as described (13).

**Genomic Southern Blot Hybridization**—Genomic DNA from mouse, rat, bovine, and human cells or tissues was digested overnight with restriction enzymes as described (12). Following gel electrophoresis and blot transfer to nylon filters, the filters were hybridized with cDNA probes as described above for Northern hybridization. In vitro transcription and translation—Plasmid Bluescript SK(−) was rescued by XZAPII according to the instructions supplied by Stratagene. The plasmid containing the complete proposed open reading frame (see below) was linearized with XbaI and transcribed using T7 polymerase to obtain sense mRNA. The antisense mRNA was obtained by linearizing the same plasmid with HindIII and transcribed using T3 polymerase. In vitro transcription was performed in a rabbit reticulocyte lysate system (Promega Biotec) with [35S]methionine (100 μCi/reaction). After a 1-h incubation at 30 °C, the reactions were stopped, and aliquots with equal counts were analyzed on 7 or 9% denaturing polyacrylamide gels.

**DNA Sequencing and Sequence Analysis**—Nucleotide sequencing of Bluescript SK(−) plasmids containing the inserts of interest was performed by the dyeode chain termination method (14) using a T7 DNA polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.). In some regions containing sequence compressions, 7-deaza-dGTP or 7-deaza-dITP (Pharmacia LKB Biotechnology Inc.) was substituted for dGTP in the sequencing reaction.

### RESULTS

**Differential Screening**—A cDNA phagemid library was prepared from HIR 3.5 cells stimulated with insulin in the presence of cycloheximide, and ~13,000 recombinants from this library were screened in duplicate by differential hybridization using cDNA probes prepared from poly(A)+ RNA from either quiescent HIR 3.5 cells or cells treated with insulin and cycloheximide. Plaques preferentially hybridizing to cDNAs prepared from stimulated cells were analyzed by DNA sequence analysis. Out of eight positive clones identified, one proved to be identical to egr-1 (9). Six other clones remain to be characterized. A final clone (designated DU1 32-1) was characterized for this paper.

**cDNA Sequence of DU1 32-1**—The insert from DU1 32-1 proved to be 1465 bp in length in addition to a putative poly(A) tail of 36 A residues. The insert from DU1 32-1 was used to rescreen the same library, and a clone containing a 1.7-kb insert (DU2) was selected. Fig. 1 shows the nucleotide sequence of this longer insert, which contained 1774 nucleotides in addition to the poly(A) tail. This poly(A) tail was used to rescreen the same library, and a clone containing a 1.7-kb insert (DU2) was selected. This poly(A) tail was used to rescreen the same library, and a clone containing a 1.7-kb insert (DU2) was selected. The putative 3' untranslated region also contained three repeats of the sequence ATTTA, which is a potential signal for rapid mRNA degradation (Fig. 1) (15).

Both strands of DU1 32-1 and DU2 were sequenced at least once, and many times in regions rich in G and C. The first base of DU1 32-1 was equivalent to base 282 of DU2 (Fig. 1); and from this position to the 3' end, the nucleotide sequences of these two clones were identical.

**Amino Acid Sequence of DU2 Open Reading Frame**—The sense strand of DU2 contained a single open reading frame extending from nucleotides 32 to 988 that encoded a putative polypeptide of 319 amino acids (Fig. 2), with a calculated M, of 35,553. This open reading frame begins with the only ATG in the entire sequence that is in the optimum context for translational initiation (GCCCAATGG) (17) (Fig. 1). Translation of the sense mRNA from DU2 resulted in a single major protein band (Fig. 3); several gel analyses using different percentages of acrylamide revealed an average approximate M, of 34,000. No such translational product was formed by translation of the antisense mRNA (Fig. 3).

The sequence of the putative protein encoded by this open reading frame (Fig. 2 and Table I) is interesting in several respects. It is very rich in Pro (14.4 mol %) and Ser (17.2 mol %) residues; in addition, its relative paucity of Asp and Glu and abundance of Arg residues (7.2 mol %) result in an overall percentage of 33.553. This open reading frame begins with the only ATG in the entire sequence that is in the optimum context for translational initiation (GCCCAATGG) (17) (Fig. 1). Translation of the sense mRNA from DU2 resulted in a single major protein band (Fig. 3); several gel analyses using different percentages of acrylamide revealed an average approximate M, of 34,000. No such translational product was formed by translation of the antisense mRNA (Fig. 3).

The search for potential functional motifs failed to reveal consensus sequences for ATP binding, protein kinase, GTP binding, or nuclear localization signals (see “Discussion”). Computer secondary structure analysis (Fig. 4) revealed no hydrophobic regions that might correspond to membrane-spanning segments, no potential glycosylation sites, and few regions of α-helix, in keeping with the proline richness of the protein. The amino terminus contains the only significant predicted region of α-helix in the protein and also contains Leu residues spaced 7 amino acids apart; this is reminiscent of a truncated leucine zipper domain (18).
Northern analysis using the DU1 32-1 cDNA insert as the probe. The level of mRNA was barely detectable in quiescent cells, but increased dramatically within 10 min of insulin stimulation and reached maximum expression between 30 and 45 min. Thereafter, the mRNA levels decreased rapidly to reach low but not base-line levels at 2 h (Fig. 5, upper). Beginning 30–45 min after stimulation of the cells by insulin, there was a gradual decrease in the size of TTP mRNA, which probably reflects its rapid degradation.

TTP mRNA expression was also dependent on insulin concentration (Fig. 5, lower): the level of mRNA started to increase in the presence of 0.07 nM insulin and reached a maximum at ~70 nM. The time course of insulin induction of TTP mRNA is remarkably similar to that seen for insulin induction of c-fos mRNA in these and other (6–8) cells; however, TTP mRNA induction begins to occur at substantially lower insulin concentrations than does induction of c-fos mRNA in these and other (6–8) cells.

It is likely that insulin-stimulated accumulation of TTP mRNA occurs at the level of transcription since actinomycin D (4 μM for 15 min) completely blocked insulin-stimulated accumulation of TTP mRNA at all time periods studied (data not shown). In the presence of cycloheximide alone (0.1 mM), a slight accumulation of TTP mRNA was observed after 1 h (Fig. 8). Insulin stimulation of the cells in the presence of cycloheximide, however, led to superinduction of TTP mRNA (Fig. 6). Under these conditions, mRNA levels were the highest at 2 h. This pattern is characteristic of genes whose mRNAs have very short half-lives.

To determine the half-life of TTP mRNA, quiescent HIR 3.5 cells were stimulated with 70 nM insulin for 30 min and subsequently treated with actinomycin D. This procedure permits measurement of degradation of accumulated transcripts in the absence of ongoing transcription. As shown in Fig. 7, TTP mRNA levels decreased rapidly, reaching low levels 30 min after the addition of actinomycin D and essentially undetectable levels at 60 min. The estimated half-life was 15–30 min.

Treatment of HIR 3.5 cells with phorbol 12-myristate 13-acetate (PMA) (1.6 μM) produced a response similar to that of insulin (Fig. 8). After overnight incubation of the cells with 16 μM PMA (a treatment known to down-regulate protein kinase C to levels ~5% of control in these cells), induction of TTP mRNA by PMA was abolished, whereas insulin-stimulated expression was not affected (Fig. 8). Similarly, serum (which can induce c-fos expression by both protein kinase C-dependent and -independent mechanisms) (6) induced TTP mRNA accumulation both in the normal and down-regulated cells (Fig. 8).

A variety of other growth factors were able to induce TTP mRNA accumulation (Fig. 9). Serum (20% fetal calf serum) had the greatest effect. Although expression of TTP mRNA was induced to a minor degree at higher concentrations of insulin-like growth factor I (IGF-I) (80 nM), 8 nM IGF-I had no effect compared to the significant accumulation induced by 8 nM insulin. These results suggest that the insulin-stimulated accumulation of TTP mRNA is a consequence of insulin binding to its own receptors in these cells. The expression observed in the presence of a high concentration of IGF-I could have resulted from binding to the insulin receptors by IGF-I or could be a consequence of IGF-I binding to its own receptors.

Further evidence that insulin stimulation of TTP mRNA


3 D. M. Haupt and P. J. Blackshear, unpublished data.

# Table I

<table>
<thead>
<tr>
<th>Amino acid composition of TTP</th>
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</table>

Total amino acids = 319
Calculated $M_r$ = 33,593
Estimated $p_I$ = 11.2

# FIG. 3. In vitro translation of DU2. The DU2 cDNA in both sense and antisense orientations was transcribed in vitro as described under "Materials and Methods." The resulting mRNA was translated in the presence of [35S]methionine in a rabbit reticulocyte lysate translation system, and the reaction products were subsequently analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Molecular weight standards are shown to the left. Analysis of several such gels using different percentages of acrylamide yielded an average $M_r$ of 34,000 for the principal translation product.

# TABLE II

<table>
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<th>Insulin Induction of TTP mRNA</th>
<th>Amino acid composition of TTP</th>
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sity of Wisconsin, Madison, WI) of the National Biomedical Research Foundation Data Base (release 23.0; 12/89) revealed no apparent biologically significant similarities between the amino acid sequence of TTP and known proteins. However, a search of the SwissProt Data Base (release 13.0; 1/90) revealed a region of sequence identity with a proposed open reading frame encoded by the "TIS11" phorbol ester-induced cDNA clone described by Varnum et al. (19) (see "Discussion").

Characteristics of TTP mRNA Expression—To study the regulation of TTP mRNA expression by insulin and other agonists, total cellular RNA was isolated from serum-deprived HIR 3.5 cells or cells stimulated with 70 nM insulin for varying periods of time. TTP mRNA expression was measured by


D. M. Haupt and P. J. Blackshear, unpublished data.
Insulin Induction of TTP mRNA

Fig. 4. Secondary structure analysis of TTP. The predicted sequence of TTP was analyzed using the program PeptideStructure (Genetics Computer Group, University of Wisconsin, Madison, WI), and the results of this analysis are displayed here. KD, Kyte-Doolittle; Surf., Surface Prob., surface probability; CF, Chou-Fasman; GOR, Garnier-Osguthorpe-Robson; Glycosyl. sites, glycosylation sites. See the Genetics Computer Group Program Manual for further documentation.

Fig. 5. Time course and concentration dependence of effect of insulin on TTP mRNA accumulation in HIR 3.5 cells. Upper, [32P]-labeled DU1 32-1 cDNA was hybridized to Northern blots of total cellular RNA (15 µg/lane) from serum-deprived HIR 3.5 cells exposed to 70 nM insulin for the indicated times. Lower, quiescent cells were stimulated with the indicated concentrations of insulin for 30 min; 15 µg of total cellular RNA from these cells was then used in each lane for Northern analysis. The major species of TTP mRNA migrates just above the 18S ribosomal RNA.

accumulation is mediated through activation of functional insulin receptors comes from experiments in rat-1 fibroblasts expressing high numbers of normal or mutant human insulin receptors. As shown in Fig. 10, stimulation of normal rat-1 cells with various concentrations of insulin for 45 min led to low levels of TTP mRNA accumulation; these cells express only ~1700 rat insulin receptors/cell (20). In contrast, HIRc-B cells, which express >10^6 human insulin receptors/cell (20), displayed marked increases in TTP mRNA accumulation after insulin treatment. In A/K 1018 cells (expressing ~2 x 10^6 mutated human insulin receptors in which lysine is substituted for alanine at residue 1018, destroying the intrinsic tyrosine kinase activity of the receptor) (20), insulin completely failed to stimulate an increase in TTP mRNA expression (Fig. 10). Interestingly, all three of the rat-1 cell derivatives expressed readily detectable levels of TTP mRNA in the

Fig. 6. Superinduction of TTP mRNA accumulation by insulin in presence of cycloheximide. Serum-free HIR 3.5 cells were incubated with either 0.1 mM cycloheximide (CHx) or 70 nM insulin (Ins) or were pretreated with 0.1 mM cycloheximide for 1 h and then exposed to 70 nM insulin for the indicated times (CHx + Ins). Each lane contained 15 µg of total cellular RNA. See the legend to Fig. 5 for further details.

Fig. 7. Degradation of TTP mRNA. Serum-deprived HIR 3.5 cells were exposed to control conditions (C) or 70 nM insulin (I) for 30 min, at which point 4 µM actinomycin D was added to the incubation medium. After further incubation with actinomycin D for the times indicated, the cells were washed and used for the preparation of total cellular RNA and Northern blotting. Each lane contained 15 µg of total cellular RNA.
Insulin Induction of TTP mRNA

**Fig. 8.** Insulin-stimulated TTP mRNA accumulation in HIR 3.5 cells depleted of protein kinase C. Serum-deprived HIR 3.5 cells were preincubated overnight with 0.1% (v/v) MeSO or 10 μM PMA and then exposed to 0.1% MeSO (C), 70 nM insulin (I), 1.6 μM PMA (P), or 20% fetal calf serum (S) for 30 min at 37 °C. Preparation of total cellular RNA (15 μg/lane) and Northern blotting analysis were performed as described in the legend to Fig. 5.

**Fig. 9.** Induction of TTP mRNA accumulation by growth factors and other mitogens. Serum-deprived HIR 3.5 cells were incubated for 30 min with various growth factors at the indicated concentrations. Total cellular RNA was then prepared, and 15 μg/lane was subjected to Northern blotting analysis as described in the legend to Fig. 5. EGF, epidermal growth factor (Collaborative Research, Inc., Lexington, MA); FGF, fibroblast growth factor (Collaborative Research, Inc.); FCS, fetal calf serum (GIBCO/Bethesda Research Laboratories); Ins, purified pork insulin (Squibb Novo, Princeton, NJ); PDGF, platelet-derived growth factor (Amgen Biologicals, Thousand Oaks, CA); DMSO, dimethyl sulfoxide (Sigma). IGF-I was from Amgen Biologicals, and PMA was from Sigma. PMA was dissolved in MeSO, and the final concentration of MeSO in the incubation medium was 0.1%. IGF-I was dissolved in acetic acid; the final concentration of acetic acid in the incubation medium (0.01 mM) did not induce TTP mRNA accumulation (data not shown).

**Fig. 10.** Effect of insulin on accumulation of TTP mRNA in rat-1, HIRC-B, and A/K 1018-B fibroblasts. Serum-deprived rat fibroblasts were incubated with the indicated concentrations of insulin for 45 min; other details of cell culture and preparation will be described elsewhere. Preparation of RNA (15 μg/lane) and Northern analysis were performed as described in the legend to Fig. 5. Rat-1 cells (a) are normal rat fibroblasts; HIRC-B cells (b) are rat-1 cells that stably express ~1.25 × 10^6 normal human insulin receptors/cell; and A/K 1018-B cells (c) express ~2.2 × 10^6 human receptors in which the lysine at position 1018 has been replaced with an alanine, thus destroying the ATP-binding site and intrinsic protein kinase activity. See Ref. 20 for further details.

**Fig. 11.** Effect of insulin on TTP mRNA accumulation in HIR 3.5 cells, 3T3-L1 fibroblasts, and 3T3-L1 adipocytes. Serum-deprived HIR 3.5 cells and 3T3-L1 fibroblasts and adipocytes were exposed to control conditions (C) or 70 nM insulin (I) for 30 min. RNA isolation (15 μg/lane) and Northern analysis were performed as described in the legend to Fig. 5.

Basal or serum-deprived state. The reason for this difference from the mouse cells used elsewhere in this paper is not known, but could reflect a species difference in regulated expression of the TTP gene.

In agreement with the observations cited above that cells expressing high numbers of normal insulin receptors exhibit a high degree of insulin-induced expression of TTP mRNA, differentiation of 3T3-L1 fibroblasts into adipocytes, which results in a marked increase in insulin receptor expression (21, 22), resulted in much greater insulin stimulation of TTP mRNA production (Fig. 11). Induction of TTP mRNA accumulation by insulin in these cells also occurred after down-regulation of protein kinase C (Fig. 12). It is interesting to note that the adipocytes appeared to express modestly higher levels of TTP mRNA than the fibroblasts, both in the basal state and after a maximal stimulus, e.g. PMA for 30 min. These data suggest that this gene is modestly induced by adipocyte differentiation per se. Insulin-stimulated expression of this gene was also seen in other insulin-responsive cell
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FIG. 12. Effect of protein kinase C depletion on induction of TTP mRNA by insulin, PMA, and serum. Serum-deprived 3T3-L1 fibroblasts (a) and adipocytes (b) were incubated overnight with 0.1% MeSO or 1 µM PMA. A further incubation was carried out for 30 min with 0.1% MeSO (C), 70 nM insulin (I), 1.6 µM PMA in 0.1% MeSO (P), or 20% fetal calf serum (S). RNA isolation (15 µg/lane) and Northern blotting analysis were performed as described in the legend to Fig. 5.

FIG. 13. Expression of TTP mRNA in various mouse tissues. Total cellular RNA from adult male BALB/c mouse tissues was prepared as described under "Materials and Methods," and 15 µg of each sample was subjected to Northern blotting as described in the legend to Fig. 5. The molecular sizes of the RNA standards are shown to the left.

types, such as H4IIEC3 hepatoma cells (Ref. 23 and data not shown).

Hybridization of the DU1 32-1 cDNA insert to total RNA isolated from various mouse tissues revealed low levels of expression in all tissues tested, requiring long exposure of the autoradiograph (Fig. 13). However, the highest levels of expression appeared to occur in kidney and lung, with intermediate levels in fat, heart, liver, and spleen; barely detectable levels in skeletal muscle and testes; and undetectable levels in brain.

Southern blot analysis of genomic DNA from mouse, rat, cow, and human demonstrated that a single major gene is apparently present in all four species studied (Fig. 14). In addition to the major bands seen in Fig. 14, longer exposure of the autoradiograph revealed lesser degrees of hybridization to several other bands, raising the possibility that related genes exist.

DISCUSSION

We have described in this study a cDNA clone whose corresponding cellular gene appears to be rapidly transcribed in response to insulin in several insulin-sensitive cell types. The response of this gene to insulin appears to be virtually identical to that of the c-fos gene in these and other (6-8) responsive cells. Specific similarities include the following. 1) The time courses of response to insulin are very similar, with the earliest significant accumulation of mRNA occurring at 10 min, peaking at 20 to 45 min, and falling off by 1-2 h. 2) Both responses appear to be primarily or exclusively transcriptional, as demonstrated by complete inhibition by actinomycin D pretreatment of the cells. 3) Both mRNAs are very unstable, with half-lives of <30 min. 4) As a consequence, both genes are superinduced in the presence of cycloheximide, which is presumably inhibiting the biosynthesis of one or more proteins, perhaps RNases, that are responsible for the normal degradation of mRNAs containing AUAUA instability motifs (16). 5) Both responses appear to occur as a result of insulin occupying and stimulating the protein-tyrosine kinase activity of its own receptor. 6) Both responses to insulin are meager in normal fibroblasts expressing relatively few insulin receptors, but are much more dramatic in a variety of cell types expressing relatively large numbers of receptors. 7) Both responses occur after exposure of the cells to active phorbol esters and other activators of protein kinase C, but the responses of both genes to insulin appear to be virtually normal in cells depleted of protein kinase C by overnight incubation in high concentrations of PMA. Despite these similarities, a comparison of the c-fos protein sequence with that of the putative protein TTP reveals no significant regions of sequence identity or similarity.

One possible difference between the responses of the two genes to insulin is that modest expression of the TTP gene appears to occur at substantially lower insulin concentrations than are needed to stimulate c-fos expression, even in the HIR 3.5 cells expressing ~3 x 10^6 insulin receptors/cell (4). We previously showed (6, 7) that insulin-stimulated c-fos transcription used sequences in the serum-response element of the c-fos promoter and that the dose-response relationship of insulin-stimulated c-fos expression was virtually superimposable upon the curve for insulin receptor occupancy. In contrast, the insulin response of the TTP gene begins to occur at concentrations well below full receptor occupancy (4). In addition, the response of the TTP gene to insulin is not only dramatic in cells representing normal tissues that express high numbers of insulin receptors, such as 3T3-L1...
adipocytes and H4 hepatoma cells, but is also readily apparent in normal 3T3-L1 fibroblasts (see, for example, Fig. 1). These data suggest that the TTP response to insulin may be more sensitive to insulin concentration than the c-fos response, making it more likely to be of physiological significance in the intact animal. Such conclusions will be testable by evaluation of putative insulin response elements in the promotor region of the TTP cellular gene, now underway in our laboratory.

The DU2 clone that we have sequenced appears to contain the entire coding region of the putative TTP protein, ~780 bp of 3′-untranslated region, terminating in a polyadenylation signal sequence and poly(A) tail. However, the DNA sequence of the genomic clone that we have recently isolated hybridizes specifically to probes complementary to residues 1-31, 1-842, and 1223 to the 3′-end of clone DU2 (see Fig. 1).5; in addition, we noted, which seemed to appear in parallel with the major 2.0-kb mRNA. It is possible that this represents a second, related mRNA species, a possibility supported by the presence of several minor hybridizing species on Southern blotting of mouse genomic DNA; however, it seems more likely that the larger species represents precursor or nuclear mRNA that contains an unspliced intron.

The sequence of the putative protein encoded by the DU2 insert, which we have tentatively called tris-tetraprolin, contains no obvious regions of biologically significant similarity to those of other proteins in the National Biomedical Research Foundation and SwissProt Data Bases, except for part of the sequence of the open reading frame encoded by clone TIS11, previously described by Varnum et al. (19). Comparison of the two protein sequences revealed complete amino acid identity between the sequence encoded by TIS11 from bases 853 to 1162 and our TTP amino acid sequence from positions 81 to 183 (Fig. 2). However, the DNA sequence of TIS11 was missing a C residue at position 1163 compared to our cDNA, resulting in different reading frames from that point to the carboxyl-terminal ends of the two proteins. There were other minor differences between the two DNA sequences at the extreme 3′-ends of the clones. More significantly, there was complete loss of both protein and DNA identity or similarity prior to their position 853. We are confident that our cDNA represents an intact mRNA species because a mouse genomic clone that we have sequenced appears to contain a further 100–300 bp of 5′-untranslated region. In some of the blastn, a still larger hybridizing species of ~3.3 kb was noted, which seemed to appear in parallel with the major 2.0-kb mRNA. It is possible that this represents a second, related mRNA species, a possibility supported by the presence of several minor hybridizing species on Southern blotting of mouse genomic DNA; however, it seems more likely that the larger species represents precursor or nuclear mRNA that contains an unspliced intron.

The explanation for this significant difference between the two clones remains unknown.

The TTP protein is very basic, with an overall pI of 11.2, and contains no regions of hydrophobicity characteristic of membrane-spanning domains. In addition, the TTP sequence does not contain a typical nuclear localization sequence (25, 26). Nonetheless, it is tempting to speculate that the TTP protein, like the c-fos protein and the products of other early response genes, might be a DNA-binding protein capable of trans-activating other genes that are expressed later in the response to insulin or serum. One bit of evidence in support of this speculation is that the TTP protein is very rich in proline (14.4 mol %) and contains several regions extremely high in proline content; for example, the regions between residues 61 and 90 and 190 and 220 each contain 37–40% proline. Such proline-rich regions have been shown recently to be characteristic of a new type of transcriptional activation domain (27). For example, the CCAAT-box-binding protein and transcriptional activator CTF/NF-I contains a proline-rich region (~25% proline) at its carboxyl terminus that is distinct from the DNA-binding and protein dimerization domains of the protein, which both occur near the amino terminus (27). This region of the protein, which, like the TTP protein, is also rich in serine residues, is capable of activating transcription of a heterologous promoter when fused to the DNA-binding domain of a protein that normally interacts with that promoter. Finally, Mermod et al. (27) have pointed out that similar proline-rich regions have been found in several other proteins involved in transcriptional activation, including the other members of the CTF/NF-I family, receptors for estrogen and progesterone, and proteins encoded by the AP-2, c-Jun, and OTF-2 genes as well as certain proteins involved in regulation of Droso phila gene expression (26). It should be possible to test the hypothesis that one of the proline-rich domains of the TTP protein is a transcriptional activating domain by performing experiments similar to those of Mermod et al. (27), in which the proline-rich domain is fused to, for example, the DNA-binding domain of a different protein.

If one of the proline-rich domains of the TTP protein is involved in transcriptional activation, then other regions of the molecule should be responsible for DNA binding, dimerization, or other activities. We have failed so far to find in the TTP protein sequence motifs common to other DNA-binding proteins, such as zinc finger or homeodomain motifs (28). However, the amino terminus of the protein contains an abbreviated form of the leucine zipper sequence motif, in which a region of α-helix contains 3 leucine residues spaced 7 amino acids apart. This does not fit the usual description of a leucine zipper, which generally requires at least 4 leucines in the helical region, preceded by a basic domain that is thought to be responsible for DNA binding (10). The possibility that the amino-terminal helical region of the TTP protein is involved in dimer or oligomer formation can readily be tested.

Whatever the function of the TTP protein is eventually determined to be, the gene itself joins the growing list of genes whose transcription is regulated by insulin, acting through its own receptor and through its intrinsic protein-tyrosine kinase activity. Given the extreme rapidity and large magnitude of the insulin response, we hope that the evaluation of the TTP gene promoter currently underway will allow us eventually to describe the mechanism of this response at the molecular level.

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Insulin Induction of TTP mRNA


Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein.

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