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# STABILIZATION OF GLUCOSE TRANSPORTER mRNA BY INSULIN/IGF-1 AND GLUCOSE DEPRIVATION

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Chronic exposure of L6 myocytes to insulin/IGF-1 or glucose deprivation results in an increase in the level of brain-type glucose transporter (GLUT1) mRNA. We have investigated the effects of insulin and glucose deprivation on GLUT1 mRNA stability. The half-life of GLUT1 mRNA in control cells was 2-2.5 h. Insulin increased GLUT1 mRNA levels by 5- to 10-fold, and GLUT1 mRNA half-life and transcription by 2-fold. Glucose deprivation increased GLUT1 mRNA level by 2- to 4-fold and half-life by 2-fold. The effects of insulin and glucose deprivation on GLUT1 mRNA stability were additive. Cycloheximide partially blocked the induction of GLUT1 mRNA by insulin but not by glucose deprivation. GLUT1 mRNA was decreased to basal levels within 12h following insulin withdrawal or glucose refeeding. Cycloheximide did not block this de-induction, suggesting that insulin and glucose deprivation do not increase GLUT1 mRNA expression by inhibiting the synthesis of ribonucleases. These findings indicate that insulin/IGF-1 increases both GLUT1 mRNA stability and transcription by both protein synthesis-dependent and independent mechanisms, whereas glucose deprivation enhances GLUT1 mRNA stability by mechanisms independent of de novo protein synthesis. 

The facilitated glucose transporter is an integral membrane protein responsible for the transport of glucose across the plasma membrane. The glucose transporter was first cloned from HepG2 cells (1) and rat brain (2) (GLUT1) and is now known to belong to a family of related glucose transporter genes with differential tissue expression and possibly tissue specific function (3,4).

Transcriptional and post-transcriptional mechanisms are involved in the regulation of glucose transporter gene expression by hormones, growth factors and nutrients availability (5-12). GLUT1 mRNA levels have been shown to increase or decrease following exposure of cultured cells to insulin (5) or dexamethasone (6), respectively, without changes in mRNA stability, suggesting direct effects on GLUT1 gene transcription. Induction of GLUT1 transcription has been demonstrated in fibroblasts exposed to transforming oncogenes and fetal calf serum (7, 8) while both transcription and GLUT1 mRNA stability are enhanced by PDGF in 3T3 cells (9). The serum- and PDGF-induced increases in GLUT1 mRNA expression do not require protein synthesis (8, 9), suggesting that these factors interact directly with regulatory sequences in the gene or mRNA rather than via the synthesis of trans-acting proteins.

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We and others have previously shown that insulin/IGF-1[1] and glucose deprivation increase the levels of GLUT1 mRNA in L6 myocytes (10-12). Both insulin and glucose deprivation have been recently reported to induce transcription of the GLUT1 gene (13), however, their effects on the stability of GLUT1 mRNA have not been reported. We have investigated the effects of insulin/IGF-1 and glucose deprivation on GLUT1 mRNA stability and transcription in L6 myocytes and determined the requirement for protein synthesis in the induction and de-induction on GLUT1 mRNA expression.

## METHODS

**Cell Culture**: L6 myocytes were cultured as previously described (10). Culture media were glucose-free Dulbecco's modified Eagle medium (DME) or DME containing 5mM glucose, with and without 1μM insulin. Media contained 5% dialysed fetal calf serum.

**Northern Blot Hybridization**: Cytoplasmic total RNA was extracted as previously described (10). RNA (25μg) was electrophoresed in 1% agarose/formaldehyde gels, transferred to nitrocellulose and hybridized with an oligonucleotide probe (RGT-1) to the rat brain glucose transporter sequence (2) (nucleotides 904-942 encoding part of the cytoplasmic loop).

**In Vitro Transcription in Isolated Nuclei**: Nuclei were prepared by a modification of the method of Schibler et al (14). In vitro transcription was performed by modifications of previously decribed methods (15, 16). $10^7$ nuclei were incubated for 30min at 26°C in 100μl reaction buffer containing 100μCi $\alpha$-$^{32}$P-UTP (3000 Ci/mmol; Amersham International, UK.), 1mM each of ATP, GTP and CTP (Pharmacia), 30% [v/v] glycerol, 20-30 units RNasin (Pharmacia), 10mM creatine phosphate, 1.2mM dithiothreitol, 0.4mM EDTA, 0.2M NaCl, 4mM $MnCl_2$, 4mM $MgCl_2$, 0.3mM $(NH_4)_2SO_4$ and 100mM Tris.HCl, pH 7.9. Yeast tRNA (1mg/ml) and 50 units DNase I (Pharmacia) were added for 15min at 37°C. The reactions were deproteinized at 42°C for 10min by addition of 100μl NETS (20mM Tris.HCl, pH 7.9, 20mM EDTA, 1% SDS) and proteinase K (0.5mg/ml). RNA was purified by two extractions with phenol:chloroform:isoamylalcohol (50:50:1) and two ethanol precipitations of the aqueous phase in the presence of 2M ammonium acetate and 2 volumes ethanol at 4°C. The RNA pellet was resuspended in 500μl each of NETS, formamide and hybridization buffer (50% formamide, 5xSSPE, 0.5% SDS, 10xDenhardt's solution, 1mg/ml salmon testis DNA). Labelled transcripts were hybridized to DNA probes immobilized on nitrocellulose (prehybridized 2 days at 42°C) for 2-3 days at 42°C. Filters were washed in three changes of 2xSSPE/0.5% SDS at 35°C for 10min, 0.2xSSPE/0.5% SDS at 42°C for 30min and 0.1xSSPE/0.5% SDS at 42°C for 30min, then autoradiographed.

## RESULTS

### Effects of Insulin and Glucose Deprivation on GLUT1 mRNA Stability and Transcription

The half-life ($t_{1/2}$) of GLUT1 mRNA was determined in myocytes preincubated in medium containing 1μM insulin, glucose-free medium, or glucose-free medium with insulin. In control glucose-fed cells GLUT1 mRNA $t_{1/2}$ was 2-2.5h (Fig. 1). In cells incubated with insulin for 8h the GLUT1 mRNA level was increased 5-fold and the $t_{1/2}$ doubled to 4-5h. Transcription of the GLUT1 gene in nuclei isolated from insulin-treated cells was increased approximately 2-fold within 2h and remained elevated for 15h (Fig. 2). In cells incubated in glucose-free medium for 12-16h GLUT1 mRNA levels were maximally increased 2- to 4-fold and the mRNA stability was increased 2-fold ($t_{1/2}$ = 4-5h) (Fig. 1). We could not detect a change in glucose transporter gene

[1] In this L6 myocyte line insulin regulates GLUT1 glucose transporter expression via the IGF-1 receptor (10). Insulin (1μM) was used in all the experiments in the present report.

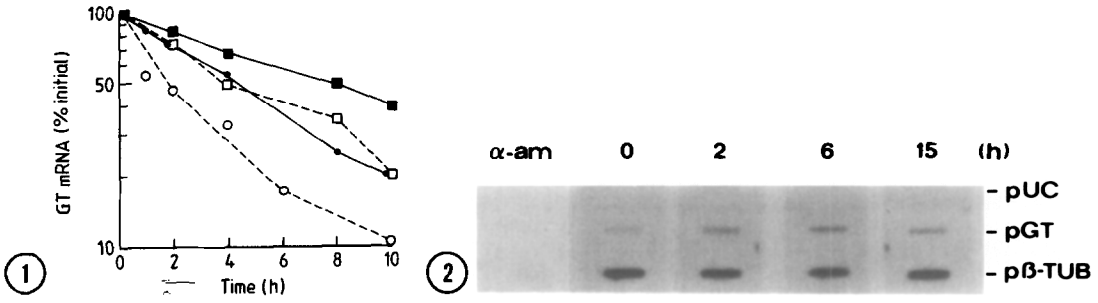


Figure 1. **Effect of Insulin and Glucose Deprivation on GLUT1 mRNA Half-Life.** Myocytes were preincubated for 12h prior to addition of 5μg/ml actinomycin-D (Time=0h). RNA was extracted after 0-12h and analysed by Northern blot hybridization with RGT-1. The mean values from densitometric analysis of three Northern blots (range=25%) are expressed as % of initial (time=0h) value. Medium contained 5mM glucose (O); 5mM glucose, 1μM insulin (●); no glucose (□); no glucose, 1μM insulin (■).

Figure 2. **Effect of Insulin on GLUT1 Transcription in vitro.** Nuclei were isolated from myocytes preincubated with 1μM insulin for 0-15h and assayed for transcription in vitro in the presence and absence of 1μg/ml α-amanitin (α-am). $^{32}$P-labelled transcripts were hybridized to control plasmid pUC19 DNA (pUC), pGT2-14, a full length rat GLUT1 glucose transporter cDNA inserted in pUC19 (pGT), and a ß-tubulin cDNA inserted in pBR322 (pß-TUB).

transcription in nuclei from cells deprived of glucose for 6 or 15h (data not shown). The effects of glucose deprivation and insulin on GLUT1 mRNA stability were additive; mRNA levels were maximally increased 10-fold and the $t_{1/2}$ increased to 8h (Fig. 1).

### Effect of Cycloheximide on the Induction and De-Induction of GLUT1 mRNA Expression

In order to determine whether protein synthesis is required for the induction and stabilization of GLUT1 mRNA by insulin and glucose deprivation, de novo protein synthesis was blocked with cycloheximide

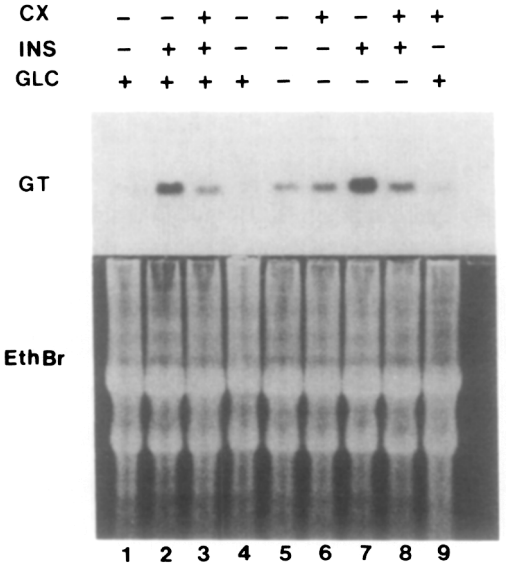


Figure 3. **Effect of Cycloheximide on Induction of GLUT1 mRNA by Insulin and Glucose Deprivation.** Myocytes were incubated for 18h in medium without (-) or with (+) 5mM glucose (GLC), 1μM insulin (INS) and 10μg/ml cycloheximide (CX). GT: GLUT1 mRNA, 2.8kb. EthBr: Ethidium bromide stain of the gel.

(10μg/ml) during the induction period. Incubation of glucose fed cells with cycloheximide caused a minor increase in GLUT1 mRNA levels (Fig. 3, lane 9). The increase in GLUT1 mRNA in insulin treated cells was partially blocked by cycloheximide (Fig. 3, lane 3) suggesting that insulin acts via both protein synthesis-dependent and -independent mechanisms. As insulin increases the amount of GLUT1 mRNA by both mRNA stabilization and transcriptional activation, de novo protein synthesis must be required for one or both mechanisms. In contrast to the effect of insulin, the increase in GLUT1 mRNA level in glucose deprived cells was not blocked by cycloheximide (Fig. 3, lane 6). Therefore, glucose deprivation increases GLUT1 mRNA expression by a protein synthesis-independent mechanism. The increased level of GLUT1 mRNA in response to glucose deprivation plus insulin was partially blocked by cycloheximide (Fig. 3, lane 8). GLUT1 mRNA was reduced to the level seen in glucose deprived cells, consistent with the previous result that protein synthesis is required in part for the insulin effect, but not the glucose deprivation effect.

An alternative mechanism by which insulin or glucose deprivation could increase GLUT1 mRNA stability is by inhibiting the synthesis of a specific ribonuclease. Such a mechanism may be relevant in the glucose deprived state as chronic (24h) glucose deprivation of L6 myocytes resulted in a 20-30% reduction in the recovery of cellular protein and RNA. If inhibition of ribonuclease synthesis was involved, removal of insulin or repletion of glucose would be expected to decrease GLUT1 mRNA levels by inducing the synthesis of ribonuclease(s) and may therefore be sensitive to cycloheximide. To test this hypothesis, L6 cells were

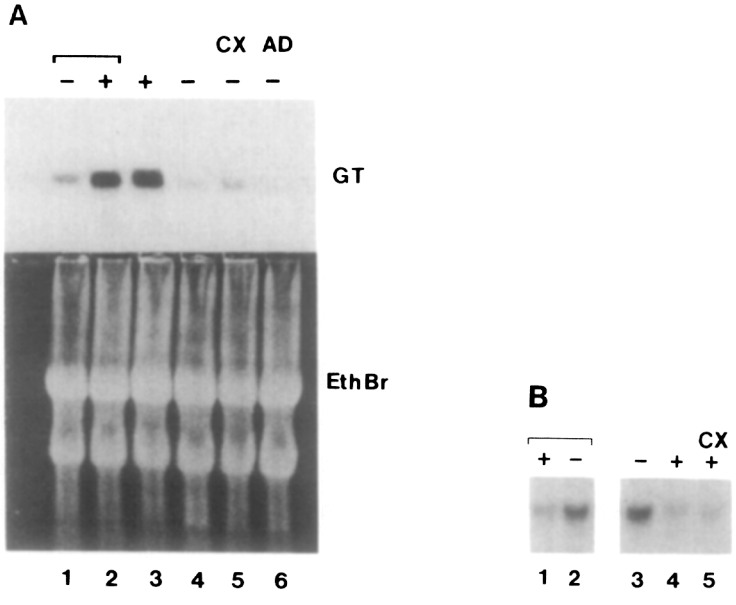


Figure 4. **Effect of Cycloheximide and Actinomycin-D on De-induction of GLUT1 mRNA after Insulin Withdrawal or Glucose Refeeding.** A: Myocytes were incubated in medium containing 5mM glucose. Lane 1: no insulin control, 12h. Lane 2: insulin, 12h. Lanes 3-6: insulin 12h followed by incubation for 12h with (+) or without (-) 1μM insulin, 10μg/ml cycloheximide (CX) or 5μg/ml actinomycin-D (AD). B: Lane 1: glucose fed control, 18h; Lane 2: glucose deprivation, 18h; Lanes 3-5: glucose deprivation for 18h followed by incubation for 12h with (+) or without (-) 10mM glucose and 10μg/ml cycloheximide (CX). GT: GLUT1 mRNA, 2.8kb. EthBr: ethidium bromide stain of the gel.

incubated in medium containing insulin or in glucose free medium to increase GLUT1 mRNA expression, then transferred to medium without insulin or with glucose, respectively, in the absence and presence of cycloheximide. Insulin withdrawal resulted in a decrease in GLUT1 mRNA to below control levels within 12h (Fig. 4A, lane 4). The explanation for this overshoot effect is not known. Addition of cycloheximide or the RNA synthesis inhibitor actinomycin-D at the time of insulin withdrawal did not prevent the decline in GLUT1 mRNA levels. This indicates that neither transcription nor de novo protein synthesis is required for degradation of GLUT1 mRNA following removal of insulin. Refeeding of glucose deprived cells with 10mM glucose also resulted in a decrease in GLUT1 mRNA to control levels within 12h (Fig. 4B, lane 4). This was not blocked by cycloheximide, indicating that the glucose-dependent decrease in GLUT1 mRNA does not require protein synthesis.

## DISCUSSION

Our results demonstrate that insulin/IGF-1 and glucose deprivation act by different pathways to increase the steady-state levels of GLUT1 mRNA in L6 myocytes. Enhancement of both transcription (2-fold) and mRNA stability (2-fold) by insulin contribute to the 5-fold increase in GLUT1 mRNA levels, whereas the stabilization of GLUT1 mRNA caused by glucose deprivation (2-fold) appears to account for the increase in GLUT1 mRNA levels. Walker et al (13) have recently demonstrated transcriptional activation of the GLUT1 gene in another subclone of L6 myocytes by both insulin and glucose deprivation, although they did not report the effects on mRNA stability. Our studies differ from theirs in that the induction of GLUT1 mRNA expression by insulin is sustained (10) and by glucose deprivation follows a slower time course (maximal induction at 12-16h) (Maher and Harrison, manuscript submitted), rather than being rapid and transient and probably reflect differences in the L6 subclones used.

Although insulin and glucose deprivation both enhance the stability of GLUT1 mRNA by the same degree, their effects are additive,suggesting the involvement of different pathways for mRNA stabilization. This is supported by the requirement for protein synthesis for the accumulation of GLUT1 mRNA in response to insulin, but not in response to glucose deprivation. However, as previously noted, whether protein synthesis is required for the insulin induced GLUT1 mRNA stabilization or the stimulation of transcription is not answered by these experiments. It is conceivable that glucose deprivation and insulin/IGF-1 independently regulate different pools of GLUT1 mRNA or distinct transporter mRNA species which are homologous in the cytoplasmic loop region of the transporter (the region recognized by the oligonucleotide probe used in these experiments). However, in view of the specificity of oligonucleotide probes it is likely that we are detecting only the GLUT1 glucose transporter.

Two lines of evidence suggest that rapidly synthesized or short-lived proteins are not involved in the degradation of GLUT1 mRNA in L6 myocytes. First, inhibition of de novo protein synthesis by cycloheximide does not cause a marked increase in GLUT1 mRNA levels. The small increase observed in cycloheximide-treated control cells could be attributed to inhibition of a constitutively synthesized protein

involved in GLUT1 mRNA degradation in these cells under normal growth conditions. Second, cycloheximide does not block the degradation of GLUT1 mRNA following insulin withdrawal or glucose refeeding. If de novo protein synthesis was required for degradation, the inclusion of cycloheximide during insulin withdrawal would have maintained the elevated level of GLUT1 mRNA. Therefore, by implication, insulin/IGF-1 does not stabilize GLUT1 mRNA by blocking the synthesis of a specific ribonuclease. Moreover, we can conclude that glucose deprivation does not stabilize GLUT1 mRNA by blocking the synthesis of a specific glucose-regulated ribonuclease because the decrease in GLUT1 mRNA following repletion of glucose does not require protein synthesis. Thus, degradation of GLUT1 mRNA in L6 myocytes is most likely effected by long-lived proteins.

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