*Proc. Natl. Acad. Sci. USA*
Vol. 90, pp. 512–516, January 1993
Biochemistry

# Insulin down-regulates expression of the insulin-responsive glucose transporter (GLUT4) gene: Effects on transcription and mRNA turnover

[3T3-L1 adipocyte, non-insulin-dependent (type 2) diabetes mellitus/glucose uptake/insulin resistance]

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*Contributed by M. Daniel Lane, October 13, 1992*

ABSTRACT **Insulin rapidly represses expression of the gene encoding the insulin-responsive glucose transporter (GLUT4) in 3T3-L1 mouse adipocytes. Upon exposure to the hormone the cellular level of GLUT4 mRNA falls ($t_{1/2} \approx 2.5$ hr) to 20–30% of its initial level within 10 hr. This is followed by a similar decrease in the level of GLUT4 protein. Down-regulation of GLUT4 mRNA is a result of both rapid repression of transcription of the GLUT4 gene and an increased rate of turnover of the GLUT4 message. As a consequence of prolonged exposure to insulin, 3T3-L1 adipocytes lose their capacity for acute stimulation of hexose uptake by insulin. These findings provide an explanation for the resistance of glucose uptake to insulin in adipose tissue observed in non-insulin-dependent (type 2) diabetes mellitus, particularly that associated with hyperinsulinemia and obesity.**

In mammalian cells the facilitated diffusion of glucose across the plasma membrane is mediated by a diverse group of transporters, each with different properties (1–3). The five facilitative glucose transporters identified and sequenced to date exhibit a unique pattern of expression among different cell types (3). One of these transporters, GLUT4, which is expressed only in adipocytes, heart, and skeletal muscle, is responsible for the acute stimulation of glucose uptake by insulin that is observed only in these cell types (3–6). It has been established (7, 8) that this acute regulation of GLUT4 by insulin involves rapid translocation of the transporter from intracellular vesicles to the plasma membrane, although changes in the transporter's intrinsic activity may also occur (9, 10).

In addition to the acute regulation of GLUT4 activity by insulin, expression of the GLUT4 gene appears to be hormonally and metabolically regulated. For example, both fasting and insulin-dependent (including streptozotocin-induced) diabetes, which alter blood levels of insulin and its counterregulatory hormones, drastically lower GLUT4 mRNA and protein levels in adipose tissue (11–14). Recent findings suggest that this effect may be mediated, at least in part, by cAMP, which was shown to repress transcription of the GLUT4 gene in 3T3-L1 adipocytes (15). This view is supported by the fact that the level of cAMP in adipose tissue increases in the starvation-induced state (16). Non-insulin-dependent (type 2) diabetes, which is usually accompanied by hyperinsulinemia and peripheral insulin resistance, also leads to reduced levels of GLUT4 message and protein in adipose tissue (17), most likely by a different mechanism (see *Discussion*). To gain insight into the mechanism of insulin resistance in adipose tissue in type 2 diabetes accompanied by hyperinsulinemia, we investigated the possibility that insulin itself might repress expression of the GLUT4 gene. Previous results from other laboratories (18–21), although not in complete agreement, suggested that the level of GLUT4 protein in adipocytes falls upon prolonged exposure to insulin. Using 3T3-L1 adipocytes as a model system, we demonstrate that insulin rapidly induces down-regulation of GLUT4 mRNA and protein by a dual mechanism involving changes in transcription and mRNA turnover rates.

## EXPERIMENTAL PROCEDURES

3T3-L1 preadipocytes were induced to differentiate into adipocytes essentially as described (22); however, on day 4 insulin was withdrawn from the culture medium and on day 9 the adipocytes were subjected to treatment with insulin, insulin-like growth factor I (IGF-I), or 8-bromo-cAMP. Total cellular GLUT4 content was determined by immunoblotting membrane extracts prepared (in the presence of protease inhibitors; ref. 23) from 3.5-cm 3T3-L1 adipocyte monolayers, treated or not with 1 $\mu$M insulin as described (15). Hexose uptake assays were carried out essentially as described (24).

**Isolation and Analysis of RNA.** Total cellular RNA was isolated by the guanidine thiocyanate method (25). GLUT1 and GLUT4 mRNAs were analyzed by RNase protection (26) employing antisense RNA probes transcribed from appropriate cDNA templates with T7 RNA polymerase in the presence of [$\alpha$-$^{32}$P]CTP (800 Ci/mmol; DuPont; 1 Ci = 37 GBq). The GLUT4 template was obtained by subcloning into pGEM-3Z (Promega) a segment of the GLUT4 cDNA (4) which protects a 182-base fragment extending from nucleotide 2007 (*Eco*RI site) to nucleotide 2189 (*Sma* I site). The GLUT1 template in the same vector corresponds to an exonuclease III-derived segment between nucleotides 878 and 1073 of the GLUT1 cDNA (15), which protects a 195-base fragment of the message. mRNA turnover was assessed at different times after treating cells with an RNA polymerase inhibitor [5,6-dichloro-1-$\beta$-D-ribofuranosylbenzimidazole (DRB); ref. 27] at 100 $\mu$M, as described (15). Yields of total RNA obtained from one 10-cm dish of insulin-treated cells were comparable to those from untreated cells (152.0 ± 6.3 $\mu$g and 149.4 ± 4.3 $\mu$g, respectively).

**Isolation of Nuclei and Run-On Transcription Assays.** After the indicated treatments of 3T3-L1 adipocytes (day 9), nuclei were isolated for transcriptional run-on assays as described by Cornelius *et al.* (28). Briefly, equal amounts of nuclei (based on $A_{260}$ and $A_{230}$ in 0.1% SDS) were incubated for 30 min at 25°C in the presence of [$\alpha$-$^{32}$P]UTP (3000 Ci/mmol; DuPont). Assays were terminated by addition of RNase-free DNase, and then $^{32}$P-labeled RNA was isolated by guanidine thiocyanate extraction and cesium chloride density gradient

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Abbreviations: IGF-I, insulin-like growth factor I; DRB, 5,6-dichloro-1-$\beta$-D-ribofuranosylbenzimidazole.

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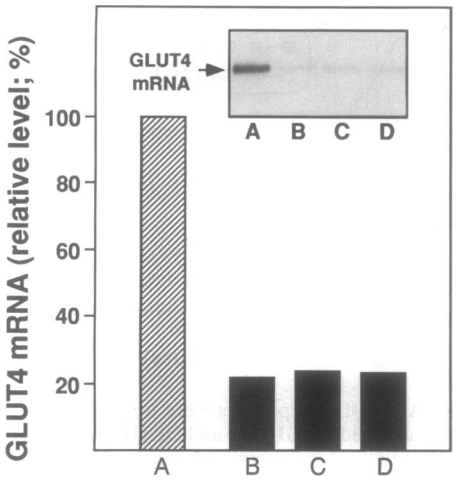


FIG. 1. Effect of insulin on cellular GLUT4 glucose transporter mRNA level. 3T3-L1 adipocytes either were not treated (A) or were treated with 1 mM 8-bromo-cAMP for 16 hr (B), with 1 $\mu$M insulin for 6 hr (C), or with 20 $\mu$M cycloheximide for 20 min followed by 6 hr with cycloheximide and 1 $\mu$M insulin (D). Total cellular RNA was isolated, and RNase protection assays were carried out with 10 $\mu$g of RNA and a $^{32}$P-labeled GLUT4-specific antisense RNA probe. The relative intensities of bands in the autoradiogram (*Inset*) were determined by densitometry normalized with GLUT4 mRNA from untreated cells.

centrifugation. After partial hydrolysis in 0.2 M NaOH, $^{32}$P-labeled RNA was recovered by ethanol precipitation. Hybridization of identical amounts (based on $^{32}$P activity) of the labeled transcripts was performed using nylon membranes (Hybond-N; Amersham) containing the appropriate cDNAs (GLUT1 and GLUT4) covalently attached by UV crosslinking. Genomic DNA was obtained from 3T3-L1 preadipocytes (generously provided by P. Cornelius). After the blots were exposed for autoradiography, the intensity of the bands was quantitated by laser scanning densitometry and normalized to $^{32}$P-labeled transcripts hybridized to total genomic DNA.

## RESULTS

Preliminary experiments to determine whether expression of the GLUT4 gene is altered by insulin revealed that exposure of fully differentiated 3T3-L1 adipocytes to the hormone caused an ≈80% decrease in the steady-state level of GLUT4 mRNA within 6 hr (Fig. 1). The magnitude of this effect is comparable to that induced by 8-bromo-cAMP (Fig. 1), which was shown previously (15) to suppress transcription of the GLUT4 gene. Under the conditions of these experiments insulin has no effect on cellular cAMP level (results not shown). Down-regulation of the GLUT4 message by insulin (or cAMP; ref. 15) does not require new protein synthesis, since the process is unaffected by a level of cycloheximide (Fig. 1) that inhibits cellular protein synthesis by 97% (results not shown).

Down-regulation of GLUT4 mRNA by insulin occurred at relatively low concentrations of the hormone (Fig. 2). Exposure of the cells to ≈20 nM insulin for 24 hr caused a 50% decrease in message abundance, and insulin concentrations as low as 5–10 nM caused a 25–30% decrease (Fig. 2). Moreover, the actual insulin concentration required for these effects is probably much lower, since 3T3-L1 adipocytes rapidly degrade insulin (18). This is consistent with the finding that the extent of down-regulation is greater at shorter times of insulin treatment (see below). IGF-I, which binds to the insulin receptor with much lower affinity than insulin, is far less potent than insulin in causing down-regulation of GLUT4 mRNA (Fig. 2*A*). Thus, it is likely that the small IGF-I effect is mediated by the insulin receptor.

The insulin-induced decrease in GLUT4 mRNA was surprisingly rapid, approaching completion within 4–5 hr and achieving a new steady-state level of 20–30% that of untreated controls by 10–12 hr (Fig. 3). No change in GLUT4 mRNA level was detected in untreated (control) cells within the 24-hr time frame of the experiment (results not shown). Insulin-induced down-regulation of the GLUT4 message was rapidly and completely reversed upon removal of the hormone. Within 4 hr of removal of insulin from the culture medium, the level of GLUT4 mRNA returned to that of untreated controls (results not shown).

In contrast to its down-regulating effect on GLUT4 message level, insulin induced an immediate, albeit transient, increase in GLUT1 message. This effect of insulin on GLUT1 mRNA abundance has been observed in fibroblasts and 3T3-L1 adipocytes (18, 29). As shown in Fig. 3, GLUT1 mRNA level increased ≈12-fold within 4 hr and then rapidly decreased by 40–50% during the next 8 hr, reaching a new steady-state level ≈6-fold higher than the initial level. Thus, the rise in GLUT1 message was accompanied by a reciprocal fall in GLUT4 message. Consequently, the acute responsiveness of hexose uptake to insulin was decreased (see below).

Down-regulation of GLUT4 mRNA by insulin would be expected to lead to depletion of GLUT4 transporter protein.

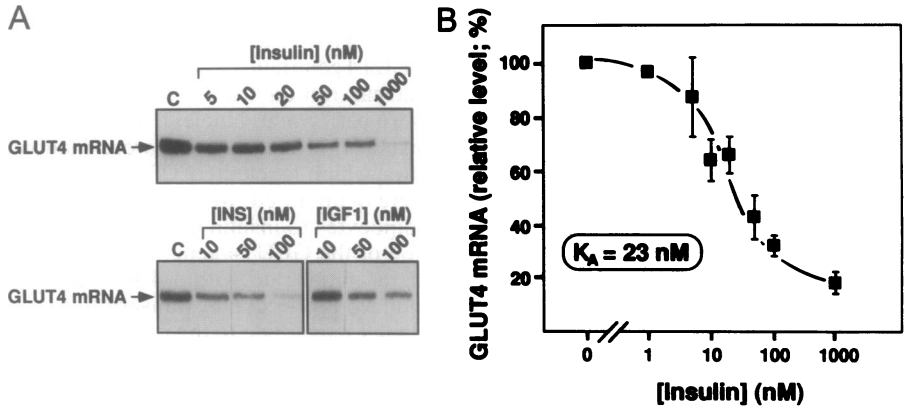


FIG. 2. Dependence of cellular GLUT4 mRNA level on insulin and IGF-I concentration. 3T3-L1 adipocytes were treated with the indicated concentrations of insulin or IGF-I for 24 hr, after which total cellular RNA was isolated. The level of GLUT4 mRNA was determined by RNase protection as described in Fig. 1. Autoradiograms of representative experiments are shown in *A*, and the averages of normalized (with respect to untreated cells) intensities of the autoradiographic bands (determined as in Fig. 1) from three independent experiments are plotted in *B*. The $K_A$ value for down-regulation induced by insulin was 23 nM.

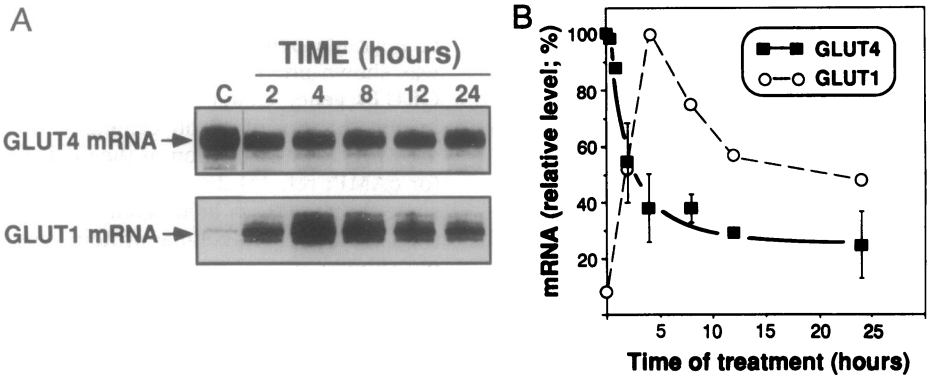


FIG. 3. Time course of insulin-induced down-regulation of glucose transporter mRNA. 3T3-L1 adipocytes were treated with 1 $\mu$M insulin for the indicated times, after which total RNA was isolated. (*A*) RNA (10 $\mu$g per lane) was assayed for GLUT4 and GLUT1 message abundance by RNase protection analysis. (*B*) Band intensity was determined by densitometry and normalized with respect to untreated cells (GLUT4, ■) or maximal induction of GLUT1 mRNA after 4 hr of insulin treatment (GLUT1, ○). Results are from three independent experiments for GLUT4, and for GLUT1, from the experiment shown in *A*.

To follow the kinetics of loss of GLUT4 protein, total cellular membranes were isolated from 3T3-L1 adipocytes exposed to insulin for various periods of time. The GLUT4 transporter content of the membranes was determined by immunoblotting with an affinity-purified anti-GLUT4 polyclonal antibody after deglycosylation of the transporter. Treatment of 3T3-L1 adipocytes with insulin caused an ≈80% reduction in the total cellular level of GLUT4 protein within 36 hr (Fig. 4). This rate of decay of GLUT4 protein corresponds to a $t_{1/2}$ of ≈18 hr. After reaching a minimum at 36 hr the level of GLUT4 protein appeared to rise despite insulin treatment (Fig. 4). This increase was probably due, at least in part, to degradation of insulin. In experiments in which insulin was added every 24 hr, although the decrease in GLUT4 protein was more pronounced and remained lower at all times, the level of GLUT4 protein increased slightly after 36–40 hr (results not shown).

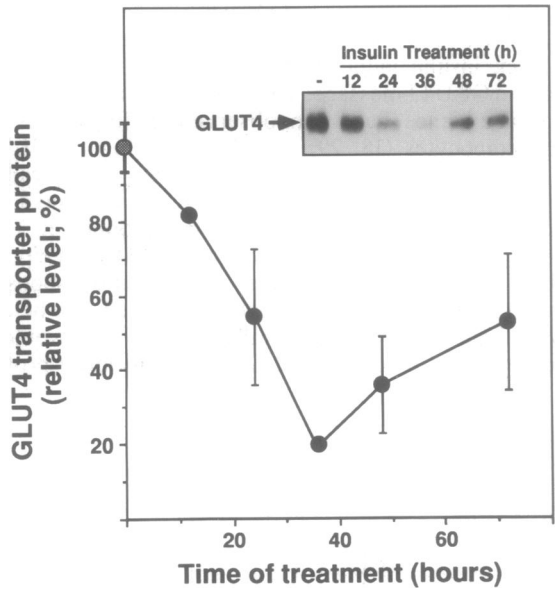


FIG. 4. Time course of insulin-induced down-regulation of GLUT4 protein. 3T3-L1 adipocytes were treated with 1 $\mu$M insulin for the indicated times and total cellular membranes were prepared. Equivalent amounts of the cellular membranes were extensively deglycosylated by protein-N-glycosidase F digestion, subjected to electrophoresis in an SDS/8% polyacrylamide gel, and transferred to a nitrocellulose filter. GLUT4 protein was immunodetected with GLUT4 antibody and the blots were developed by enhanced chemiluminescence. Inset shows a representative experiment, and the averaged results from two independent experiment are presented in the bar graph after quantitation of band intensities by laser scanning densitometry. Results are normalized with respect to untreated cells, which were prepared for this analysis at the end of the longest incubation with insulin (72 hr).

To assess possible changes in GLUT4 function following down-regulation, the ability of 3T3-L1 adipocytes to exhibit acute activation of hexose uptake by insulin was determined before and after down-regulation of GLUT4 (Table 1). Although this analysis is complicated somewhat by the fact that GLUT1 increases during chronic insulin treatment (Fig. 1), hexose uptake by GLUT1 is feebly activated by insulin, whereas hexose uptake by GLUT4 is strongly activated (15- to 20-fold) by insulin (10, 30). As shown in Table 1, insulin acutely (within 10 min) activated 2-deoxyglucose uptake by 15-fold with adipocytes not previously exposed to insulin. However, after exposure to insulin for 24 hr (following complete wash-out of the hormone prior to the hexose uptake assay†) the cells completely lost their capacity to undergo insulin-stimulated hexose uptake. The large increase in basal hexose uptake caused by chronic exposure to insulin (Table 1) appears to be due entirely to insulin-induced expression of GLUT1 (Fig. 3 and refs. 18 and 20). Furthermore, the insulin-induced expression of GLUT1 and increased basal hexose uptake rate are abrogated by cycloheximide treatment (unpublished results). The fact that there is no further increase in hexose uptake upon subsequent brief exposure to insulin is consistent with the finding (Fig. 4) that most of the GLUT4 protein has been down-regulated during the prior 24-hr exposure of the cells to insulin.

To determine whether insulin-induced down-regulation of GLUT4 message is the result of repressed transcription of the GLUT4 gene and/or accelerated turnover of GLUT4 mRNA, nuclear run-on transcription and mRNA turnover studies were performed. Nuclei from 3T3-L1 adipocytes exposed to insulin for various times were incubated with [$\alpha$-$^{32}$P]UTP, after which labeled GLUT4 and GLUT1 run-on transcripts were quantitated. Relatively brief (2 hr) exposure of 3T3-L1 adipocytes to insulin markedly suppressed run-on transcription of the GLUT4 gene (Fig. 5*A*), decreasing to about 25% of its initial rate within 4 hr (Fig. 5*B*). This rapid insulin-induced repression of transcription of the GLUT4 gene is of similar magnitude to the previously observed effect of cAMP (15), which is shown here for comparison (Fig. 5*A*). In contrast to the repressive effect of insulin on run-on tran-

†The possibility that the increased rate of basal 2-deoxyglucose transport was due to residual insulin remaining after the chronic exposure of the cells to insulin was ruled out. After brief exposure of the cells to insulin followed by wash-out, the 2-deoxyglucose uptake rate returned essentially to the basal level within 2 hr.

Table 1. Effect of prolonged insulin treatment on acute stimulation of hexose uptake by insulin

| Prior treatment | 2-Deoxyglucose uptake, nmol/min per $10^6$ cells: Control | + insulin (10 min) | Fold stimulation |
|---|---|---|---|
| None | 0.13 ± 0.01 | 1.9 ± 0.06 | 15 |
| Insulin (24 hr) | 2.8 ± 0.02 | 3.1 ± 0.1 | 1.1 |

3T3-L1 adipocytes (day 10) were treated or not with 100 nM insulin for 24 hr in medium containing 10% fetal bovine serum. To remove insulin, cell monolayers were washed three times with serum-free medium lacking insulin. After a 2-hr incubation in the insulin-free medium, the cell monolayers were treated or not with 500 nM insulin for 10 min, after which 2-deoxy-D-[1-$^{14}$C]glucose uptake was measured.

scription of the GLUT4 gene, insulin rapidly activates (within 2 hr; Fig. 5) transcription of the GLUT1 gene. The reciprocal transcriptional activation of the GLUT1 gene and transcriptional repression of the GLUT4 gene by insulin are similar in magnitude (3- to 4-fold) and persist for at least 8 hr. It is evident that down-regulation of GLUT4 mRNA and protein caused by insulin results, at least in part, from repression of transcription of the GLUT4 gene.

In view of our previous finding that the $t_{1/2}$ of GLUT4 mRNA in 3T3-L1 adipocytes is about 9 hr (15), repression of transcription alone could not alone account for the rapid decrease in GLUT4 mRNA caused by insulin (Fig. 3*B*). Therefore, to ascertain whether insulin might also affect the $t_{1/2}$ of the GLUT4 message, the rate of turnover of GLUT4 mRNA was determined in cells exposed to an inhibitor (DRB) of transcription and treated or not with insulin. The $t_{1/2}$ of the GLUT4 message was shortened from 8.6 hr to 4.9 hr following exposure of 3T3-L1 adipocytes to insulin for 24 hr (Fig. 6). Furthermore, the onset of this increased rate of message turnover was rapid, occurring within 2–4 hr and reaching a maximal rate (≈2.5 times faster than untreated controls) following exposure of the cells to insulin for only 4 hr (Fig. 6*B*). Thus, it can be concluded that insulin not only represses transcription of the GLUT4 gene but also accelerates turnover of the GLUT4 message in 3T3-L1 adipocytes.

## DISCUSSION

Hyperinsulinemia and the resistance of glucose uptake to insulin in peripheral tissues, notably muscle and adipose, are hallmarks of non-insulin-dependent (type 2) diabetes. Our findings that insulin is capable of repressing transcription of the GLUT4 gene and of shortening the half-life of GLUT4 mRNA in 3T3-L1 adipocytes suggest that this mechanism may underlie and/or exacerbate the insulin-resistant state.

While the major site of glucose disposal in nonobese subjects is believed to be skeletal muscle (31), compelling evidence (32–34) now suggests that adipose tissue contributes substantially to glucose disposal, particularly in obese subjects. Excessive accumulation of adipose tissue is a well-known predisposing factor for type 2 diabetes which ultimately leads to impaired glucose utilization. The contribution of adipose tissue to global glucose disposal *in vivo* may previously have been grossly underestimated, especially when this tissue was abundant. Much higher estimates of this contribution are based on measurements of the rate of conversion of glucose to lactate by adipose tissue (34), coupled with the large fraction of total body mass as adipose tissue in obese individuals. Furthermore, measurements of glucose disposal rates following oral administration of [$^{14}$C]-glucose (the normal route of glucose intake), rather than intravenously (the route of administration used in most other studies), revealed a greater contribution of adipose tissue to total glucose disposal (32). If these findings provide accurate estimates of glucose utilization by adipose tissue, the role of this tissue in the development of the insulin-resistant diabetic state needs to be reassessed.

Alteration of expression of the GLUT4 gene is one possible mechanism underlying peripheral insulin resistance. This has been shown to be the case in adipose tissue in diabetic/insulin-resistant states (11–14) and probably accounts for the attendant reduction in insulin-stimulated glucose uptake. Likewise, decreased levels of GLUT4 mRNA and protein in skeletal muscle, which occur late in the progression of streptozotocin-induced diabetes (12, 35, 36), account for the impaired glucose disposal observed in this tissue. Early in the development of streptozotocin-induced diabetes, however, at a stage when glucose uptake has already been impaired, the level of GLUT4 protein in skeletal muscle has not changed significantly (35). Thus, the impairment of glucose uptake at the onset of the diabetic state is most likely due to defective GLUT4 function [i.e., translocation to the plasma membrane or intrinsic activity (35)] rather than a lack of GLUT4 protein. The fact that the cellular level of GLUT4 mRNA has fallen appreciably at this stage (35) suggests that the turnover of GLUT4 protein is slower in skeletal muscle than in adipose tissue.

It is of interest that both cAMP and insulin, which have counterregulatory effects on glucose metabolism in the nor-

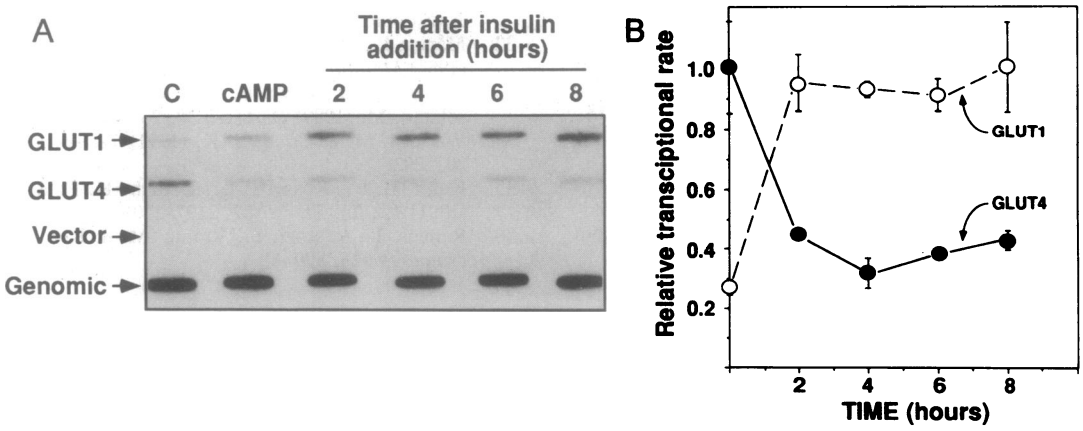


FIG. 5. Effect of insulin on nuclear run-on transcription of the GLUT4 and GLUT1 genes. (*A*) 3T3-L1 adipocytes were either not treated (control, C) or treated with 1 mM 8-bromo-cAMP (cAMP), or treated with 1 μM insulin for the indicated periods of time. Nuclei were then isolated and transcriptional run-on assays were carried out. (*B*) Averaged results from two independent experiments after densitometric quantitation of band intensity normalized with respect to untreated cells. ●, GLUT4; ○, GLUT1.

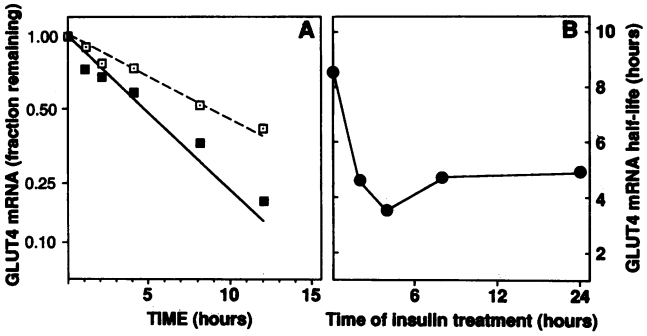


FIG. 6. Effect of insulin on the turnover rate of GLUT4 mRNA. 3T3-L1 adipocytes were treated with 1 $\mu$M insulin (■) or left untreated (□) for 24 hr, at which time cells were treated with 100 $\mu$M DRB (time 0), after which total RNA was isolated at the indicated times. GLUT4 mRNA abundance was determined by RNase protection and the results were quantitated by densitometry, which are shown in *A*, normalized with respect to time 0. The first-order decay rate constants were derived and used to calculate the half-life values [$t_{1/2}$ of 8.6 hr (− insulin) or 4.9 hr (+ insulin)]. An identical approach was used for the results shown in *B*, in which cells pretreated with 1 $\mu$M insulin for the indicated times subsequently received 100 $\mu$M DRB. Total RNA was isolated after 2, 4, 8, and 12 hr. The $t_{1/2}$ of GLUT4 mRNA in each case was then determined as shown in *A*.

mal physiological state, repress expression of GLUT4 in 3T3-L1 adipocytes. We suggest that in the insulin-resistant state *in vivo*, insulin may be incapable of exerting its normal counterregulatory effect of lowering the cellular level of cAMP. Thus, an elevated level of cAMP may occur in adipose tissue despite hyperinsulinemia, the combination of which would lead to down-regulation of GLUT4, exacerbating insulin resistance. Preliminary evidence (unpublished results) indicates that cAMP and insulin, when added together at saturating concentrations, exert an additive effect in repressing expression of GLUT4 mRNA in 3T3-L1 adipocytes. Thus, in the presence of both 8-Br-cAMP and insulin, the GLUT4 mRNA level falls to an undetectable level. The additive nature of these effects suggests that cAMP and insulin repress transcription through different cis-regulatory elements. Indeed, preliminary studies with various 5′ truncations of the GLUT4 promoter in promoter–reporter gene constructs (unpublished results) also indicate that different regulatory elements of the promoter are responsible for transcriptional repression by each of these agents.

We thank Dr. P. Cornelius for critically reviewing the manuscript and Ms. Natalie Tumminia for expert secretarial assistance. This work was supported by a research grant from the National Institutes of Health and a National Research Service Award to J.R.F.-R.

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