Tumor Necrosis Factor-α Regulation of Glucose Transporter (GLUT1) mRNA Turnover

CONTRIBUTION OF THE 3’-UNTRANSLATED REGION OF THE GLUT1 MESSAGE*

(Received for publication, May 24, 1996, and in revised form, September 20, 1996)

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Glucose transport across the plasma membrane is the rate-limiting step for subsequent glucose metabolism and energy production within the cell (1). This process is catalyzed by a family of tissue-specific integral membrane proteins known as the glucose transporters (GLUTs) (reviewed by Bell et al. (2)). Transporter activity can be regulated by hormones and growth factors through the redistribution of transporter proteins from intracellular vesicles to the plasma membrane (translocation), modulation of transporter intrinsic activity, and stimulation of new protein synthesis through increased transcription and/or alteration in transcript stability (reviewed by McGowan et al. (3)).

Previously, we have demonstrated that treatment of quiescent 3T3-L1 preadipocytes with the cytokine tumor necrosis factor-α (TNF) produces a 3–4-fold stabilization of the GLUT1 (HepG2/brain/erythrocyte glucose transporter) mRNA that leads to a parallel increase in glucose transport activity within these cells (4). Coincident with the stabilization of the message was the up-regulation of an RNA binding protein(s) exhibiting specificity for an in vitro synthesized 80-base transcript containing four back to back copies of the destabilizing motif, AUUUA. During this same period of message stabilization, proteins with molecular masses of 37 and 40 kDa, with apparent binding specificity for the GLUT1 3’-UTR were also up-regulated (5). While the GLUT1 3’-UTR is AU-rich, the actual sequence to which these proteins bound was not localized, and identity between the AU-rich binding protein and the GLUT1 3’-UTR binding proteins could not be established. However, their binding activity increased coordinately with the t₅₀ of the message, consistent with a hypothesis of involvement in stabilization of the GLUT1 transcript (5).

To determine if sequences within the GLUT1 3’-UTR regulate decay, we stably transfected 3T3-L1 preadipocytes with a set of chimeric reporter constructs. These constructs contained the 5’-UTR and coding region of preproinsulin as a reporter gene, attached to the normal and various truncated versions of the GLUT1 3’-UTR. The preproinsulin gene was selected as a reporter for the specific reason that, similar to the GLUT4 gene, the resultant transcript would be translated on ribosomes associated with the endoplasmic reticulum. We wished to preserve this interaction, since it provides a critical structure that may influence stability. Our results indicated that sequences within bases 2246 and 2347 mediate the rapid decay of this message in the quiescent fibroblasts. This analysis also suggested that the same region controlled the TNF-induced accumulation of the GLUT1 mRNA.

RNA gel mobility shift assays utilizing the full-length 3’-UTR and the various truncated forms as binding probes have detailed the occupancy of the GLUT1 3’-UTR by RNA-binding proteins. This analysis identified two RNA-binding proteins that bound to the stability-determining region of the GLUT1 3’-UTR. The RNA binding activity of these proteins increased coordinately with the stabilization of the GLUT1 message following TNF stimulation.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human TNF-α was provided by Biogen (Cambridge, MA). The specific activity was 9.6 × 10⁶ units/mg of protein, based on a cytotoxicity assay using L929 cells. The endotoxin contamination was 0.12 ng/mg of protein, based on a Limulus amoebocyte lysate assay (Sigma). Dulbecco’s modified Eagle’s medium was purchased from Hazelton Research Products (Lenexa, KS). Fetal calf serum was purchased from Hyclone (Logan, UT). All radiolabeled compounds were purchased from DuPont NEN. Hybond-N nylon filter paper was purchased from Amersham Corp. All restriction enzymes, DNA polymerases, and molecular biology enzymes were purchased from Life Technologies, Inc. Actinomycin D was purchased from Calbiochem. Protogel premixed acrylamide solution was purchased from National Diagnostics (Atlanta, GA).

3T3-L1 Fibroblast Cell Culture—Murine 3T3-L1 fibroblasts used in

*This work was supported in part National Institutes of Health Grant GM32892 and North Carolina Biotechnology Center Grant 9413-ARG-0082 (to P. H. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
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1The abbreviations used are: GLUT, glucose transporter; GLUT1, erythrocyte/HepG2/brain GLUT isofrom; GLUT4, insulin responsive muscle and adipose GLUT isofrom; TNF, tumor necrosis factor-α; UTR, untranslated region; HepG2, human hepatoma cell line.
these studies were the gift of the late Dr. Ora Rosen (Memorial Sloan-Kettering Cancer Center, New York, NY). These cells were plated and grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% calf serum as described previously (4). Four days postconfluence, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin. Cells were characterized as quiescent 24 h after this final medium change when the aphidicolin-sensitive component of DNA synthesis was at a minimum. All experiments were initiated within 24 h after reaching quiescence. TNF was dissolved in Krebs-Ringer-Hepes buffer containing 0.1% bovine serum albumin, and the polysome yields were buffer A. Once in solution, the polysomes were transferred to a microconcentration of 5.0 nM. The duration of all treatments was 10 h.

1:100 dilution was added to the cell culture medium, producing a final Ringer-Hepes buffer containing 0.1% bovine serum albumin. Cells were characterized as quiescent 24 h after Eagle's medium supplemented with 0.5% essentially fatty acid-free bovineserumalbumin.Cellswerecharacterizedasquiescent24hafter this final medium change when the aphidicolin-sensitive component of DNA synthesis was at a minimum. All experiments were initiated with these studies were the gift of the late Dr. Ora Rosen (Memorial Sloan-Kettering Cancer Center, New York, NY). These cells were plated and grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% calf serum as described previously (4). Four days postconfluence, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin. Cells were characterized as quiescent 24 h after this final medium change when the aphidicolin-sensitive component of DNA synthesis was at a minimum. All experiments were initiated within 24 h after reaching quiescence. TNF was dissolved in Krebs-Ringer-Hepes buffer containing 0.1% bovine serum albumin, and the polysome yields were buffer A. Once in solution, the polysomes were transferred to a microconcentration of 5.0 nM. The duration of all treatments was 10 h.

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FIG. 1. GLUT1 3'-UTR confers lability and TNF-responsive stabilization to the preproinsulin reporter construct. A, autoradiogram of mRNA half-lives for endogenous GLUT1, the PBC:GLUT1 construct, and the PBC12BI vector alone. Total RNA was isolated from control and TNF-treated (5 nM for 10 h) 3T3-L1 cells at 0, 1, 3, and 5 h following treatment with actinomycin D (6 μg/ml). Twenty micrograms of total RNA from each time point were analyzed by Northern blot with radiolabeled cDNA (a random primed probe made from a template corresponding to the full-length GLUT1 3'-UTR for GLUT1 and PBC:GLUT1) or cRNA (prepared from a 292-base SmaI/EcoRI fragment generated from the coding sequence of the preproinsulin clone for PBC12BI) probes as described under "Experimental Procedures." Quantification and half-life determinations were performed as described under "Experimental Procedures."

RESULTS

GLUT1 3’-UTR Contains a Destabilizing and a TNF-responsive Element—3T3-L1 preadipocytes were stably transfected with an expression vector containing the preproinsulin gene or a construct where the 3’-UTR of the preproinsulin gene has been replaced with the GLUT1 3’-UTR. All transfectants required cotransfection with the vector pSV2-neo that contained a neomycin resistance gene to allow for antibiotic selection. Following transfection, G418-resistant colonies were pooled to ensure a heterogeneity of integration sites, and permanent transfected cell lines were established. These transfectants along with a transfection control, which contained only the pSV2-neo neomycin resistance vector, were analyzed for GLUT1 mRNA turnover under control and TNF-treated (5 nM for 10 h) conditions; a typical analysis is shown in Fig. 1. The following summary half-life data represent the mean ± S.E. from a total of four independent experiments performed on each of two cell lines derived from two independent transfections. The results of the study demonstrate that the cells transfected with parental preproinsulin vector produce preproinsulin mRNA that is extremely stable under control conditions and does not respond to TNF (t₁/₂: values: control, 675 ± 20 min; TNF-treated, 682 ± 20 min). Replacing the preproinsulin 3’-UTR with the GLUT1 3’-UTR results in a transcript with a much shorter half-life (control t₁/₂ = 51 ± 9 min) that is stabilized in response to TNF treatment (TNF t₁/₂ = 181 ± 18 min). The turnover rate for this chimeric transcript resembled those of the GLUT1 mRNA, suggesting that the GLUT1 3’-UTR conferred mRNA decay characteristics consistent with the GLUT1 message to the normally stable preproinsulin reporter gene. Control transfectants that contained only the pSV2-neo vector were also analyzed for GLUT1 mRNA turnover. The half-life of GLUT1 within these cells was calculated to be 39 ± 8 min under control conditions and 168 ± 20 min following TNF treatment, indicating that the transfection procedure does not alter the behavior of the GLUT1 transcript.

Truncation Analysis of the GLUT1 3’-UTR—The initial findings suggested that the GLUT1 3’-UTR contained elements that could regulate the turnover of the normally stable preproinsulin transcript. Exonuclease III digestion was used to produce a series of constructs that were truncated from the 5’-end of the 3’-UTR (Fig. 2A). These constructs were transfected into 3T3-L1 preadipocytes, selected for resistance to G418, and pooled as described above. The expression of these constructs following transfection was verified by Northern blot analysis using a cRNA (riboprobe) generated against the terminal 250 bases of the GLUT1 message (Fig. 2B).

To investigate whether the removal of any portion of the GLUT1 3’-UTR altered the behavior of the pBC:GLUT1 chimera, the transfectants containing the truncation deletions were analyzed for the decay of the chimeric transcripts under control and TNF-treated conditions (Fig. 3). Under control conditions, transcripts encoded by PBC:TD1902, PBC:TD2088, and PBC:TD2242 possessed decay rates of 56 ± 11, 51 ± 10, and 52 ± 8 min, respectively. Treatment of the cells with TNF resulted in a significant (p < 0.05, Student’s t test) stabilization of the chimeric mRNA with values determined to be 176 ± 20 min for PBC:TD1902; 184 ± 9 min for PBC:TD2088; and 180 ± 9 min for PBC:TD2242. These values were comparable with that observed for the half-life for endogenous GLUT1 in control and TNF-treated cells. However, the construct PBC:TD2347 was remarkably stable in untreated quiescent cells with a half-life of 477 ± 30 min, a value that approximated the t₁/₂ of the preproinsulin mRNA (675 ± 20 min) encoded by the parental PBC12BI vector more than the PBC:GLUT1 chimera.
Deletions. The half-lives of the truncation deletion reporter gene constructs (described in Fig. 2) were determined as described under "Experimental Procedures." TNF treatment was for 10 h at a concentration of 5 nM. For this analysis, 40 μg of control RNA and 20 μg of RNA from TNF-treated cells were utilized.

(51 ± 8 min). TNF treatment yielded a half-life of 497 ± 35 min for the PBC:TD2347 chimera, a value that was not statistically different from the value obtained under control conditions (p > 0.05, Student's t test). These data indicate that the ability to destabilize the PBC:GLUT1 transcript resides within the region defined by bases 2242 and 2347 of the GLUT1 3'-UTR. Transcripts that contained this segment of the 3'-UTR remained unstable, while those that do not have this segment are inherently stable. The dramatic increase in stability produced by the removal of this region (2242–2347) complicated the identification of a TNF-responsive element. Prior to the removal of this portion of the GLUT1 3'-UTR, all of the chimeras exhibited significant stabilization in response to TNF (Fig. 3 and see above). This suggests that the TNF-responsive element resides somewhere between base 2242 and the 3' terminus.

Stabilization of the Message and Loss of TNF Response upon Deletion of the Region between 2246 and 2347 of the PBC:GLUT1 Chimera—The findings described above were consistent with the hypothesis that the region between 2242 and 2347 contained an element essential for the determination of the stability of this message. In addition, the region potentially contained a TNF-responsive element. Restriction sites at positions 2246 and 2347 of the construct permitted removal of the region to examine these possibilities (detailed under "Experimental Procedures"). The deletion mutant PBC:GLUT1 Δ2246–2347 was prepared (Scheme I) and stably transfected into the 3T3-L1 cells as described above.

| Construct                  | Control t½ (min) | TNF-treated t½ (min) |
|----------------------------|------------------|----------------------|
| GLUT1                      | 56 ± 18          | 226 ± 22             |
| PBC:GLUT1Δ2246–2347        | 121 ± 13         | 130 ± 15             |
| PBC:GLUT1Δ1304–2246        | 51 ± 9           | 190 ± 10             |

The data for endogenous GLUT1 and PBC:GLUT1Δ2246–2347 were obtained from RNA extracted from the same cell lines at the same time and visualized on the same Northern blots. The data for PBC:GLUT1 mRNA was obtained from a different stably transfected cell line.

Expression and TNF regulation of the mutant chimeric RNA were then examined. As shown in Table I, deletion of this region of the GLUT1 3'-UTR resulted in a significant stabilization of the message in control cells (2.4-fold over the PBC:GLUT1 mRNA, p < 0.05). These data were consistent with the deleted region containing an element essential for the rapid turnover of the mRNA. Moreover, the half-life of the message did not change when the cells were exposed to TNF, supporting a role for the region in mediating the stabilizing effects of this cytokine.

RNA Gel Mobility Shift Analysis with S10, S100, and Polysomal Protein Fractions—The occupancy of the GLUT1 3'-UTR by RNA-binding proteins was assessed through gel mobility shift analysis. Postnuclear (S10), cytosolic (S100), and polysomal protein extracts were prepared from control and TNF-treated quiescent 3T3-L1 cells. These proteins were incubated with a radiolabeled transcript corresponding to the full-length GLUT1 3'-UTR. A total of five RNA-protein complexes from the S10, S100, and polysomal protein extracts with molecular masses of approximately 67, 45, 40, 37, and 26 kDa were detected by the label transfer (Fig. 4). The postnuclear S10 fraction displayed all five bands, and the RNA binding activities at 67, 40, and 37 kDa appeared to increase with TNF treatment. The 67-, 46-, and 37-kDa proteins partitioned into the postmitochondrial S100 fraction, but no differences in their RNA binding activities between control and TNF-treated extracts were observed. The TNF-treated polysomal protein fraction contained all five RNA binding activities including the 40- and 37-kDa bands that were not observed within the polysomal preparations from control cells.

Comparison of Gel Mobility Shifts with the Full-length 3'UTR and the TD2347 Truncation Deletion—A comparison study was carried out with the S10, S100, and polysomal fractions using radiolabeled transcripts prepared from the full-length GLUT1 3'-UTR and the various truncation deletions. The primary goal of this study was to determine if any of the RNA-protein interactions observed in Fig. 4 were specific for the destabilizing region located between bases 2242 and 2347 of the GLUT1 transcript. The sequential removal of regions within the GLUT1 3'-UTR did not alter the binding pattern within any of the control extracts (Fig. 5, A and C). However, significant alterations in the RNA binding activity were found within the postnuclear (S10) and polysomal protein fractions obtained from TNF-treated cells. Removing the portion of the GLUT1 3'-UTR located between bases 2242 and 2347, the stability-determining element, reduced the label transfer to both the 37- and 40-kDa binding activities found within these extracts (Fig. 5, A and C). The use of S100 fraction demonstrated no alteration in binding activity when either transcript was used in the assay.

Protein Binding to the Region Located between Bases 2242 and 2347 of the GLUT1 3'-UTR—As described above, deletion of the region between 2246 and 2347 of the GLUT1 3'-UTR resulted in stabilization of the message and loss of the TNF response. Moreover, it has been identified by deletion analysis (Figs. 4 and 5) as the site of TNF-inducible protein binding. Thus, it was of interest to determine if this region upon isolation remained a ligand for the TNF-inducible protein. A riboprobe corresponding to the region was prepared as described under "Experimental Procedures," and RNA gel mobility shift

### Table I

| Construct                  | Control t½ (min) | TNF-treated t½ (min) |
|----------------------------|------------------|----------------------|
| GLUT1                      | 56 ± 18          | 226 ± 22             |
| PBC:GLUT1Δ2246–2347        | 121 ± 13         | 130 ± 15             |
| PBC:GLUT1Δ1304–2246        | 51 ± 9           | 190 ± 10             |

*The data for endogenous GLUT1 and PBC:GLUT1Δ2246–2347 were obtained from RNA extracted from the same cell lines at the same time and visualized on the same Northern blots. The data for PBC:GLUT1 mRNA was obtained from a different stably transfected cell line.*

### Scheme I

![Scheme I](image-url)
assays were performed. The data displayed in Fig. 6 indicate that TNF treatment resulted in the up-regulation of binding activity in the S10/polysome-containing fraction of proteins of 37 and 40 kDa as well as a doublet at 67 kDa. These data, similar to those obtained with the 3′-UTR truncations described in Figs. 4 and 5A, support the identification of the region between 2246 and 2347 as the site for TNF-inducible protein binding. Interestingly, use of this “binding site-specific riboprobe” demonstrated decreased binding in both the 67- and 37-kDa regions of the gel when the TNF-treated S100 fraction was used as a source of ligand. This was not evident in the data presented in Figs. 4 and 5, where larger portions of the 3′-UTR were used as probes and may reflect an alteration in affinity of the proteins for the smaller probe after TNF treatment or the ability to detect the partitioning of the binding proteins from soluble to particulate fractions of the cell. These possibilities are currently under investigation. However, the ability to use the specific binding site probe to demonstrate TNF-inducible binding provides a convenient assay for the purification and characterization of the proteins involved.

**DISCUSSION**

Regulation of transcript stability plays a major role in controlling the expression of the HepG2/erythrocyte/brain glucose transporter, GLUT1 (3). GLUT1 is the predominant glucose transporter within several biologically important tissues including blood-tissue barriers such as placenta and the blood-brain barrier (16, 17). Several laboratories have reported that conditions such as glucose deprivation (18, 19), experimental diabetes (20), and inhibition of oxidative phosphorylation (21) as well as cytokine and hormone stimulation (4, 22–24) alter the stability of the GLUT1 transcript. The mechanisms that produce this effect, including the role of cis-acting sequence elements and trans-acting factors, have remained unclear.

The current study provides evidence that the GLUT1 3′-UTR regulates the decay of the GLUT1 message. Insertion of the GLUT1 3′-UTR into the otherwise long lived preproinsulin transcript is sufficient to destabilize the resulting mRNA. Additionally, this chimeric transcript can also be stabilized by treatment with TNF, a characteristic of the endogenous GLUT1 mRNA. The 3′-UTR of a number of short-lived mRNAs such as oncogenes and growth factors has also been shown to promote the degradation of heterologous RNAs (25, 26). The 3′-UTRs of these early response genes contain a destabilizing motif known as an adenylate-uridylate-rich element that mediates their rapid decay. The destabilizing element within the GLUT1 3′-UTR was mapped to a portion located between bases 2242 and 2347. Importantly, when this region was deleted, the message was markedly stabilized (Table I). The -fold stabilization was not as large as we would have predicted based on the truncation mutations. As opposed to those experiments, the effect was observed in the context of the nearly intact 3′-UTR (Scheme I), suggesting that other regions may facilitate the stabilization of the message on their own or interact with the 2242–2347 region to affect stability. This region is predominantly GC-rich (~60%) and was not homologous to either a GC-rich stability element reported for the transforming growth factor-β1 gene (27) or to a TNF-responsive stability element identified within the surfactant-B protein mRNA (28).

A comparison of this 105-base sequence against sequences within GenBank revealed conservation of this element among GLUT1 isoforms across species lines (human, mouse, and chicken) conferring a degree of evolutionary significance to the reported observations. In addition to the homology found within 3′-UTRs, significant homology was also found to a portion of the GLUT1 coding region for both mice and humans beginning at base 290. The redundant expression of destabilizing sequences has been observed within the c-myc and c-fos protooncogene mRNAs. These mRNAs have been demonstrated to contain destabilizing sequences within their open reading frames in addition to elements within their 3′-UTRs (29–31). These observations have led to the suggestion that multiple destabilizing elements may be employed in order to prevent the overexpression of a protein within normal cells. It is also possible that this sequence may affect other aspects of the post-translational processing of the GLUT1 message such as translational initiation (32). This said, we did not observe any TNF-inducible binding to probes prepared from this coding region element and have not as yet investigated whether this element actually functions as a stability-determining sequence.

Our chimeric transcript analysis did not directly identify sequences that mediate the stabilizing effect of TNF that was observed with several of the chimeric transcripts as well as the endogenous GLUT1 message. However, when the region from 2246 to 2347 was deleted, the ability of TNF to stabilize the message was lost. These data suggest that the same region responsible for the inherent instability of the GLUT1 transcript also functions to stabilize this message in response to TNF.

The production of hybrid transcripts demonstrated that specific sequences within the GLUT1 3′-UTR regulate the decay of this message. To gain some insight into how these sequences may function to control GLUT1 mRNA turnover, we searched for trans-acting factors, such as RNA-binding proteins, using gel mobility shift analysis. We observed several binding activities within extracts prepared from control and TNF-treated cells (Fig. 4). Two of these proteins, with apparent molecular masses of 37 and 40 kDa, demonstrated enhanced RNA-binding activity following TNF treatment. These proteins were contained within the polysomal protein fraction and were also detected within the postnuclear S10 fraction (Figs. 4 and 5). The involvement of these RNA-binding activities in regulating the turnover of the hybrid constructs was investigated by comparing the RNA binding patterns obtained with the full-length

2 K. M. McGowan, S. Police, J. B. Winslow, and P. H. Pekala, unpublished observation.
GLUT1 3'-UTR with the various truncated versions used for the *invivo* analysis (Fig. 2A). Two RNA binding proteins, one of 37 kDa and the other of 40 kDa, previously detected within the TNF-treated extracts using the full-length GLUT1 3'-UTR, could not be detected when the TD2347 probe was used (Fig. 5). We demonstrated that these two proteins bound all forms of the GLUT1 3'-UTR except the one in which the stability-determining region had been removed (TD2347). This suggests that the RNA binding activity of these proteins is specific for the stability-determining region (2242–2347) and can be activated by TNF. This finding further supports the hypothesis that the destabilizing element located between bases 2242 and 2347 functions to stabilize the GLUT1 message through TNF-controlled RNA-protein interactions. Interestingly, the data displayed in Fig. 5B demonstrate that a 37-kDa protein present in the S100 (cytosolic fraction) bound all forms of the 3'-UTR and did not appear to be regulated by TNF. One possible explanation of this observation is that the regulated binding activities measured in the S10 and polysomal fractions are not derived from the same protein found in the S100. The S100 37-kDa protein that binds to the 2347 mutant may be a nonspecific RNA binding activity, many of which coincidentally are in this molecular mass range. This hypothesis is reinforced by our recent study of dehydrogenase binding to the GLUT1 3'-UTR, which indicated that both lactate and glyceraldehyde 3-phosphate dehydrogenases bind to this region of the UTR (33). Additionally, both enzymes have subunit molecular sizes in the appropriate range and are particularly abundant in the cytosolic fraction of these cells. A second explanation addresses the issue that only the S10 and polysomal fractions contain the translational machinery and that specific interactions mediated by the presence of these components may be necessary to observe the regulation. These issues are currently under investigation.

Our work has documented that in the GLUT1 3'-UTR, a GC-rich region (bases 2242–2347) contributes significantly to the post-transcriptional regulation of this gene. The interaction between this region and two RNA-binding proteins following TNF treatment resembles a similar mechanism through which the expression of the transferrin receptor mRNA is regulated.
post-transcriptionally (34). A destabilizing element within the transferrin receptor 3’-UTR serves as a nuclease recognition site that normally allows for the rapid decay of this message (35). In response to changes in cellular iron concentration, the iron-responsive binding protein binds to this destabilizing sequence, blocks the activity of the nuclease, and prevents the decay of the transferrin receptor mRNA (36). The similarities between the two systems have led us to propose the model shown in Fig. 7, where in control cells, the destabilizing region is unprotected and the message exhibits a rapid turnover. Treatment of the cells with TNF leads to occupation of the region by a complex composed of at least two proteins (37 and 40 kDa), protection against nucleolytic attack, and stabilization of the mRNA. The model presented in Fig. 7 represents the simplest of all models and a good place to start. However, the data presented in this study do not rule out more complex models where the proteins may bind at the defined site but interact with accessory proteins and/or other regions of the message. Further definition of the system requires the purification and cloning of these binding activities, a task that is currently under way. However, the results presented in the current study represent a unique contribution to the understanding of glucose transporter gene expression. Additionally, these findings support previous studies suggesting that TNF works through inducible RNA-binding proteins to influence gene expression at the post-transcriptional level (5, 32), a result that has important implications for the understanding of TNF involvement in numerous biological systems.

Acknowledgments—We are grateful for the expert technical assistance of Amy Heffner and Donna Hardee in the performance of these studies. In addition, we thank Drs. Sheree Long, James McCubrey, and Mike McIntosh for critically reading the manuscript; Drs. Richard Hanson, Ashok Aiyar, and Parvin Hakimi (Case Western Reserve University, Cleveland, OH) and Dr. Tom Kunkel (NIHES, National Institutes of Health, Raleigh, NC) for assistance with site-directed mutagenesis; and Drs. Gary Brewer and Belinda Wagner (Wake Forest University, Winston-Salem, NC) for useful discussions. Finally, we thank Renee McGowan for the artwork presented in Fig. 7.