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Dehydrogenase Binding to the 3′-Untranslated Region of GLUT1 mRNA

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Employing RNA gel mobility shift assays we detected specific binding events between several dehydrogenases and various regions of the GLUT1 mRNA 3′-untranslated region. In particular, the enzymes glyceraldehyde 3-phosphate dehydrogenase (G3PDH), lactate dehydrogenase (LDH), and glucose 6-phosphate dehydrogenase (G6PDH) bound to the GLUT1 3′-UTR while isocitrate dehydrogenase (IDH) and glutamate dehydrogenase (GluDH) did not. Comparison of shifts obtained with purified dehydrogenases to those obtained using authentic cell extracts indicate that G3PDH and G6PDH may play a role in the intact cell.

We have previously demonstrated that the expression of the basal glucose transporter, GLUT1 in 3T3-L1 preadipocytes is regulated through alterations in mRNA turnover following treatment with the cytokine tumor necrosis factor-α (1, 2). The regulation of GLUT1 mRNA stability appears to be a central theme in control of GLUT1 gene expression as studies in the same cell line have demonstrated an identical alteration of GLUT1 mRNA half-life when the cells were exposed to a variety of stimuli (3). Our laboratory has further demonstrated that changes in GLUT1 mRNA turnover rates are accompanied by induction of a tumor necrosis factor-α responsive RNA-binding activity that recognizes a specific domain within the 3′-untranslated (UTR) region of the message (2, 3).

The discovery that NAD^+-dependent dehydrogenases could function as RNA binding proteins (4–6) with demonstrated regulatory function (5, 6) led us to hypothesize that control of GLUT1 mRNA half life could be achieved by the specific binding of one of the dehydrogenases involved in glucose metabolism. This would represent a rapid mechanism for controlling the rate limiting step of glucose utilization, glucose transport, through the action of enzymes involved in glucose metabolism.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies, Inc. Calf serum was purchased from HyClone (Logan, UT). Radiolabeled compounds were obtained from DuPont NEN. In vitro transcription kits were purchased from Ambion. Ribonucleotides were obtained from Pharmacia LKB Biotechnology, Inc. T7 RNA polymerase was obtained from Gibco BRL Life Technologies. Sequenase dideoxy sequencing kits were obtained from U.S. Biochemical. The dehydrogenases utilized in the binding studies and all other chemicals unless otherwise stated were of molecular biology grade and purchased from Sigma.

3T3-L1 Cell Culture. The murine 3T3-L1 cells used in this study were originally obtained from Dr. Howard Greene, Harvard University, Boston, MA. Cells were cultured, maintained, and prepared for experimentation as previously described (1).

cDNA constructs. The GLUT1 cDNA has been previously described (7). The polymerase chain reaction (PCR) was used to amplify specific regions of the GLUT1 3′-UTR based on U + A content. Primers were designed for each region such that the sense primer was a 41-mer oligonucleotide and contained at the 5′ end 20 bases encoding the T7 RNA polymerase start site (5′-GCCTAATACGACTCACTATA-3′) followed by 18 bases complementary to the noncoding strand. The antisense primer contained the final 18 bases of the region. All PCR products were verified by dideoxy sequencing using Sequenase.

In vitro transcription reactions. In vitro transcription was performed according to manufacturers procedures for the MAXI-script transcription kit (Ambion, Houston, Tx). These probes are radiolabeled through the inclusion of [α-$^{-32}$P]UTP (800 Ci/mmol) in the reaction. The resulting transcripts had a specific activity of approximately 1×10^8 cpm/μg as

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determined by TCA precipitation. We note that the number of uricil residues in Δs 1 through 5 were: 56, 46, 45, 23 and 53 respectively.

RNA gel mobility-shift assay. RNA band-shift analysis was performed as described by Nagy and Rigby (6). Radiolabeled RNA (50,000cpm) was prepared as described above and incubated with 2μg of commercially obtained dehydrogenase preparations or 25μg of a postmitochondrial soluble protein preparation designated S10. The incubations were performed at 37° for 30 minutes in a final buffer concentration of 10 mM HEPES, pH 7.9; 15 mM KCl; 3 mM $MgCl_2$; 0.2 mM dithiothreitol; 1μg/ml yeast tRNA; 25 g/ml poly(I) and 10% glycerol. The reactions were then placed on ice and cross-linked using a Stratalinker 1800 (Stratagene) for 5 minutes at 3000 W/cm2, followed by RNAse digestion (10 units RNAseT1, 10μg RNAse A) for 30 minutes at room temperature. The cross-linked complexes were analyzed by 12% SDS-PAGE, dried and subjected to autoradiography.

RESULTS AND DISCUSSION

We first examined the ability of five dehydrogenases to bind to a riboprobe corresponding to the full length (909 nucleotides) GLUT1 3′-UTR. The riboprobe was incubated with isocitrate (IDH), lactate (LDH), glutamate (GluDH), glyceraldehyde 3-phosphate (G3PDH), and glucose 6-phosphate (G6PDH) dehydrogenases. The binding reactions were then subjected to electrophoresis and analyzed by autoradiography (Fig. 1). Binding was observed in the reactions containing LDH, G3PDH and G6PDH (lanes 2, 4, and 5). Incubation of IDH with the riboprobe did not result in complex formation (lane 1) and while GluDH did bind, only a weak band shift was detected (lane 3). The dehydrogenases have been suggested to selectively bind RNA in the NAD^+ binding region (Rossman fold) (6), as bovine serum albumin (BSA) does not contain such a site it was used as a non-specific control (IDH serves as a specific control). As shown in lane 6, addition of BSA to the binding reaction did not result in complex formation.

Based on the observation that G3PDH bound to AU-rich regions of mRNA (6), the GLUT1 3′-UTR was divided into five regions (Fig. 2A) based on A+U content: Δ1: 1820–1950 (68.5% A+U); Δ2: 2400–2544 (69% A+U); Δ3: 1950–2150 (42.5% A+U); Δ4: 1660–1820 (37.5% A+U) and Δ5: 2150–2400 (44% A+U). Each region was isolated as described in Experimental Procedures, radiolabeled riboprobes prepared and RNA mobility gel shift assays performed to localize binding to specific regions of the 3′-UTR (Fig. 2B). LDH bound to all regions of the GLUT1 3′-UTR, exhibiting a significantly stronger shift when Δ3 was used as the probe. The ability of GluDH to bind and shift the probe was minimal but preference was displayed for Δ1 and Δ2. Interestingly, G3PDH, previously demonstrated to be an AU-rich binding protein, formed a major complex with Δ1, the region with highest A+U content. While G6PDH formed major complexes with both Δ1 and Δ2. The strength of the signal generated in these RNA mobility gel shift assays

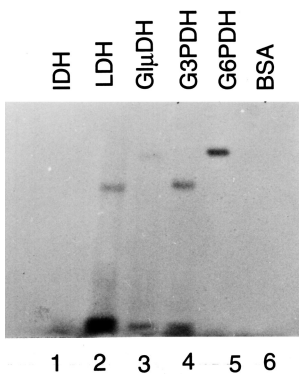

FIG. 1. Dehydrogenase binding to the GLUT1 3′UTR. RNA gel mobility-shift analysis was performed with 2μg of commercially obtained dehydrogenase preparations and 5×10^5 cpm of a radiolabelled riboprobe (approximately 2 ng) corresponding to the full length GLUT1 3′UTR. Analysis was carried out as described under Experimental Procedures.

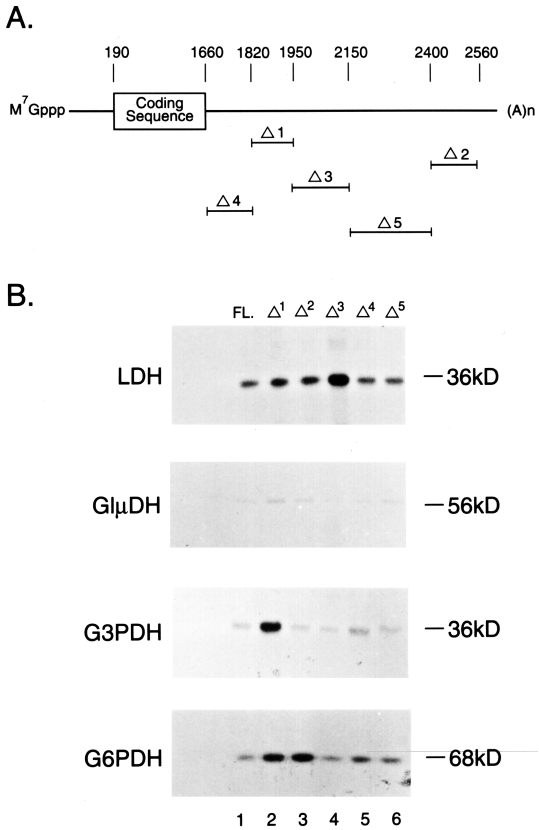

FIG. 2. (A) Uridylate rich regions of the GLUT1 3′UTR. The 876 base GLUT1 3′UTR was divided into regions based on uridylate content, with Δ1 having the highest content of uridylate residues. (B) Binding of the dehydrogenases to these regions of the GLUT1 3′UTR. Riboprobes were prepared corresponding to the five regions defined in A and RNA gel mobility shift analysis was performed as described in the legend to Fig. 1.

was dependent on the number of [^{32}P] labeled U-residues protected by the binding protein during RNase digestion. Interestingly, Δ4 and Δ5, which have nearly the same A+U content as Δ3 do not appear to be ligands for the dehydrogenases. This is particularly interesting when the longest runs of oligo-U and A+U in these constructs are compared to Δ3 and found to be nearly identical (8). Thus, while these data to not provide relative affinities of the dehydrogenases for the various constructs, they are consistent with recognition by the dehydrogenases of specific U-rich structural motifs.

We then determined whether extracts from control and TNF-treated cells contained proteins that shifted the probes in a manner similar to the dehydrogenases. As shown in Fig. 3, extracts from control cells exhibit a high molecular weigh binding complex that is increased when the cells are exposed to TNF (lanes 1 and 2). G6PDH (lane 5) yields a similar mobility in the assay system. Exposure of the cells to TNF results in the induction of a major binding activity (lane 2), which yields a mobility similar to G3PDH. Further identification of these binding proteins is currently in progress.

In conclusion, the data demonstrate that three dehydrogenases involved in glucose metabolism specifically bind to the 3′-UTR of the GLUT1 message. These observations prompt consideration of these enzymes as potential regulators of mRNA stability expanding the functional role of dehydrogenases with previously defined roles in glucose metabolism.

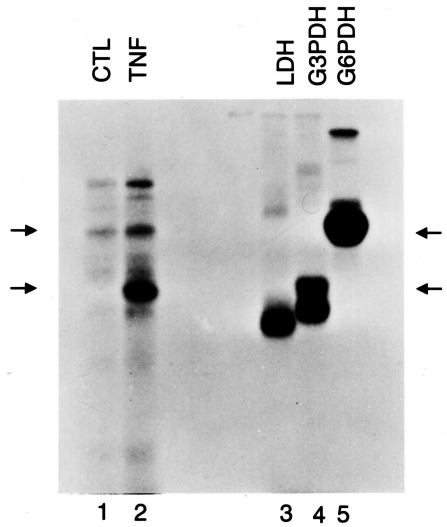

FIG. 3. RNA binding analysis for extracts prepared from control and TNF-treated 3T3-L1 preadipocytes. Postmitochondrial supernatants were prepared from control cells as well as those that had been exposed to 5 nM TNF for 17 h (a time selected due to maximum TNF-induced stabilization of the GLUT1 mRNA). RNA binding analysis was carried out using a riboprobe corresponding to the full length GLUT1 3′UTR and 25 μg of the cellular protein preparations (Lanes 1 and 2). For comparison, binding of the commercially obtained dehydrogenases was carried out as described in the legend to Fig. 1 (Lanes 3–5).

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