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Characterization of the Avian GLUT1 Glucose Transporter: Differential Regulation of GLUT1 and GLUT3 in Chicken Embryo Fibroblasts

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Vertebrate cells that are transformed by oncogenes such as v-*src* or are stimulated by mitogens have increased rates of glucose uptake. In rodent cells, the mechanisms whereby glucose transport is up-regulated are well understood. Stimulation of glucose transport involves an elevation in mRNA encoding the GLUT1 glucose transporter that is controlled at the levels of both transcription and mRNA stability. Cloning and sequencing of chicken GLUT1 cDNA showed that it shares 95% amino acid sequence similarity to mammalian GLUT1s. Nevertheless, unlike mammalian GLUT1 mRNA, it was not induced by v-*src*, serum addition, or treatment with the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate in chicken embryo fibroblasts. Rather, the induction of glucose transport in chicken embryo fibroblasts by v-*src*, serum, and 12-*O*-tetradecanoylphorbol 13-acetate was associated with induction of GLUT3 mRNA level and GLUT3 transcription. Rat fibroblasts were also found to express both GLUT1 and GLUT3 isoforms, but v-*src* induced GLUT1 and not GLUT3. This suggests that animal cells require both a basal and an upregulatable glucose transporter and that these functions have been subsumed by different GLUT isoforms in avian and mammalian cells.

INTRODUCTION

Transport of glucose into the cell is a major control point in the cellular metabolism of energy and carbon and it is regulated in many physiological and pathological situations including malignant transformation. The rate of glucose uptake into vertebrate cells is regulated by oncogenic transformation and by many other factors including mitogens, growth factors, and glucose deprivation. When chicken or rat fibroblasts are malignantly transformed by the v-*src* oncogene of Rous sarcoma virus there is a marked induction of hexose transport into the cell due to an increase in the number of glucose transporter protein molecules at the plasma membrane (Salter and Weber, 1979; Salter *et al.*, 1982). In addition, the glucose transport of untransformed fibroblasts is stimulated by factors that regulate their proliferation such as serum (Hiraki *et al.*, 1988; Williams and Birnbaum, 1988) and phorbol ester tumor promoters (Driedger and Blumberg, 1977; Hiraki *et al.*, 1988).

In mammals, there is a gene family designated GLUT1-GLUT7 (recently reviewed by Mueckler, 1994). This includes the four glucose transporter protein isoforms that mediate the passive diffusion of glucose across the plasma membrane, GLUT1-GLUT4, as well as a fructose transporter, GLUT5 (Kayano *et al.*, 1990; Burant *et al.*, 1992), a pseudogene, GLUT6 (Kayano *et al.*, 1990), and a protein found in the endoplasmic reticulum of the liver, GLUT7 (Waddell *et al.*, 1992). The GLUT proteins exhibit sequence similarity but differ in tissue distribution and regulation and are encoded by discrete genes (Gould and Bell, 1990; Kayano *et al.*, 1990). GLUTs 2, 4, and 5 appear to have specialized physio-

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logical functions confined to discrete differentiated tissues (Fukumoto *et al.*, 1988; Thorens *et al.*, 1988; Birnbaum, 1989; Charron *et al.*, 1989; Fukumoto *et al.*, 1989; James *et al.*, 1989; Kaestner *et al.*, 1989; Kayano *et al.*, 1990; Burant *et al.* 1992). Mammalian GLUT1 and GLUT3 have a more generalized tissue distribution and it is disregulation of these transporters that occurs in malignant transformation of mammalian cells both in vivo (Yamamoto *et al.*, 1990; Nagamatsu *et al.*, 1993; Mellanen *et al.*, 1994) and in tissue culture.

In cultured rodent fibroblasts, v-*src* transformation leads to an increase in the level of GLUT1 mRNA (Flier *et al.*, 1987; White and Weber, 1988) and in the rate of GLUT1 protein biosynthesis (White and Weber, 1988). Recently we have shown that GLUT1 is also inducible in an interleukin-3 (IL-3)-dependent hematopoietic cell line (FDC-P1) by v-*src* (White *et al.*, 1994). In this cell line, v-*src* also abrogates dependency on IL-3 (McCubrey *et al.*, 1993). Nuclear run-on analyses of GLUT1 transcription in both fibroblastic cells and FDC-P1 cells demonstrated that v-*src* enhanced its rate of transcription (Williams and Birnbaum, 1988; White *et al.*, 1994). Stimulation of rodent fibroblasts with growth factors also causes an induction in GLUT1 transcription and this is very rapid and transient (Hiraki *et al.*, 1988; Rollins *et al.*, 1988). Promoter and enhancer regions in the mouse GLUT1 gene that upregulate transcription have been described (Murakami *et al.*, 1992). In contrast, stimulation of FDC-P1 cells with IL-3 elevated GLUT1 mRNA levels by increasing the half-life of GLUT1 mRNA with no effect on transcription (White *et al.*, 1994). Thus in mammalian cells GLUT1 is regulated both at the transcriptional level and by altering mRNA stability.

In mammals, GLUT3 is expressed at variable levels in many tissues (Fukomoto *et al.*, 1988) with especially high levels occurring in the neuronal cells of the brain (reviewed by Maher *et al.*, 1994). GLUT3 is also expressed in some human malignancies (Yamamoto *et al.*, 1990; Nagamatsu *et al.*, 1993; Mellanen *et al.*, 1994). In tissue culture, GLUT3 is the major isoform of cultured neurons (Maher and Simpson, 1994) and is also expressed in the FDC-P1 myeloid cell line (White *et al.*, 1994). In the latter cell type, it displayed a basal level of expression that was not affected by v-*src* transformation or stimulation with IL-3 or 12-*O*-tetradecanoylphorbol 13-acetate (TPA). GLUT3 is regulated by insulin in L6 muscle cells, where it is also a basal glucose transporter (Bilan *et al.*, 1992). Here we report that GLUT3 is a basal glucose transporter in rat fibroblasts.

In contrast to mammalian cells, there has been relatively little study of the glucose transporter isoforms in chicken embryo fibroblasts (CEFs). Because CEFs have been studied in much detail as a model of malignant transformation by v-*src*, we have sought to characterize the molecular mechanisms of glucose transport regulation. Early studies focused on GLUT1 protein analysis and showed that it was elevated by v-*src* transformation through an inhibition of protein turnover (Shawver *et al.*, 1987; White and Weber, 1988). We then sought to isolate avian glucose transporter cDNAs and to characterize their pattern of expression. Surprisingly the first type of cDNA that we isolated was most closely related to mammalian GLUT3 (White *et al.*, 1991). This cDNA corresponded to a v-*src*–inducible mRNA and was isolated from a library derived from v-*src*–transformed CEFs. This cDNA clone was shown to encode a functional glucose transporter and it did not cross-hybridize with GLUT1 mRNA in Northern blots (White *et al.*, 1991). This GLUT3 isoform was expressed as two mRNAs with approximate sizes of 1.7 kb and 3.2 kb, which were transcribed from the same gene and may arise by alternative polyadenylation as is the case for the two GLUT3 mRNAs produced in human cells (Kayano *et al.*, 1988).

In addition to these two chicken GLUT3 mRNAs, another message was detected in CEFs with a size of 3.2 kb using the human or rat GLUT1 cDNA as a probe in low-stringency Northern blot hybridizations (White and Weber, 1988; White *et al.*, 1991). We now report the isolation and characterization of this GLUT1 cDNA and that its structure is very similar to mammalian GLUT1 but that it is very different in regulation. We suggest that vertebrate fibroblasts require two glucose transporters, one basal and one transcriptionally inducible. Furthermore, mammals and birds differ in the assignment of these roles between two transporter isoforms that have undergone a relatively recent evolutionary divergence, GLUT1 and GLUT3.

MATERIALS AND METHODS

Cells and Cell Culture

Cells were grown in high glucose DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% defined iron-supplemented bovine calf serum (HyClone Laboratories, Logan, UT). Experiments with chicken and rat embryo fibroblasts were conducted with cells at low passage that had been prepared by standard techniques. Growth of the rat-1 cell line and its transformation by v-*src* have been described previously (White and Weber, 1988). Chicken cells were transformed after infection with the wild-type Schmidt-Ruppin strain of Rous sarcoma virus subgroup A (CEF-src^{wt}) or with the tsNY68 (Kawai and Hanafusa, 1971) temperature-conditional derivative of this virus (CEF-src^{ts}). Rat cells were transformed by infection with an amphotropic murine leukemia virus carrying the v-*src* gene (Anderson and Scolnick, 1983). All cultures were subconfluent or just confluent at the time of experimentation. Glucose starvation (−glucose) was performed by placing cells in glucose-free DMEM-10% dialyzed fetal calf serum with control cultures (+glucose) being placed in the same medium supplemented with 4.5 g/l glucose.

Isolation and Sequencing of Chicken GLUT1

A lambda GT10 cDNA library from untransformed CEFs was screened at high stringency with rat GLUT1 cDNA (Birnbaum *et al.*,

1986) (50% formamide, 6× SSC at 42°C; washes: 1× SSC at 65°C). This library was used because GLUT3 is less represented compared with transformed CEF libraries (White *et al.*, 1991) and high stringency was used because lower stringency allows cross-hybridization between rat GLUT1 and chicken GLUT3 plaques (White *et al.*, 1991). The cDNA inserts were subcloned into the *Eco*RI site of pBluescript (Stratagene Cloning Systems, La Jolla, CA) for analysis. Sequencing was done by the dideoxynucleotide chain termination method, using the protocol described for double-stranded DNA in the Sequenase manual (United States Biochemical, Cleveland, OH). All sequence data were verified on both strands. Because the longest clones obtained from library screening were still about 0.5 kb smaller than the expected full-length cDNA, clones corresponding to the missing region were generated by the rapid amplification of cDNA ends (5′-RACE) method (Frohman *et al.*, 1988). The Life Technologies 5′-RACE kit was used and the cDNA PCR fragments were treated with Klenow to generate blunt-ended fragments, which were then cloned into the *Eco*RV site of pBluescript and sequenced as before.

Assay of Glucose Uptake

Uptake of tritiated 2-deoxyglucose (2DG) per minute per milligram of cell protein was measured as previously described (Weber, 1973; White *et al.*, 1981).

Northern Analysis

Total RNA was isolated from cells and analyzed by Northern blotting using methods described previously (White *et al.*, 1991). Hybridizations were carried out under stringent conditions (50% formamide, 6× SSC at 42°C). The final washes of filters were carried out at 67°C in 0.1× SSC. Blots were probed with the following cDNA probes labeled to high specific activity by nick translation: 1) a 2.7-kb *Eco*RI fragment containing nucleotides 531-3206 of the chicken GLUT1 cDNA described in this paper, 2) a 1.7-kb *Eco*RI fragment containing chicken GLUT3 (White *et al.*, 1991), 3) a 2.7-kb *Eco*RI fragment containing rat GLUT1 (Birnbaum *et al.*, 1986), 4) a 0.6-kb *Hin*cII fragment containing mouse GLUT3 (Nagamatsu *et al.*, 1992), and 5) a 1.1-kb *Pst*I fragment containing chicken glyceraldehyde-3-phosphate dehydrogenase (Dugaiczyk *et al.*, 1983).

Nuclear Run-on Transcription Assay

Nuclear run-on transcription assays were performed by a modification of the method that we have previously described (White *et al.*, 1994). For each experimental condition, 10 100-mm dishes of CEFs were grown and their nuclei were isolated, aliquoted, snap frozen, and stored at −70°C. The run-on transcription assays were performed with 250 μg of nuclei and 250 μCi [α-^{32}P]UTP (3000 Ci/mmol; NEN, Boston, MA) for 30 min at 25°C. RNA was isolated using triazol reagent (Life Technologies). ^{32}P-labeled RNA samples were incubated with nitrocellulose filters to which had been immobilized 2 μg of linearized cDNA-containing plasmids, 2 μg of plasmid vector control, and 0.1 μg of total genomic DNA. The amount of ^{32}P-labeled RNA binding to each DNA was quantitated using a phosphoimager (model 425E; Molecular Dynamics, Sunnyvale, CA) and normalized to the genomic DNA control in each sample.

Measurement of mRNA Stability

CEFs or CEF-*src*^{wt} were subcultured in normal medium and allowed to grow to subconfluency. At time zero, dishes were fluid-changed to growth medium containing 5 μg/ml actinomycin D to inhibit transcription (Sigma, St. Louis, MO). RNA was isolated at time points ranging between time zero and 16 h. The levels of GLUT1 mRNA and GLUT3 mRNAs were determined by Northern blot analysis (as described above).

Production of an Antiserum to Chicken GLUT3 and Western Blot Analysis of GLUT3 Protein Expression

A TrpE-GLUT3 fusion protein immunogen was produced as follows. A 200-bp *Eco*RV fragment from chicken GLUT3 cDNA (White *et al.*, 1991) containing 114 nucleotides at the 3′ end of the coding sequence and all of the 3′-untranslated region (3′-UTR) was ligated into pBluescript SK+. This allowed the directional cloning from this plasmid of a *Bam*HI/*Cla*I fragment into pATH11 (Koerner *et al.*, 1991) to produce a *trpE*::GLUT3 gene fusion. This construct encoded a TrpE-GLUT3 fusion protein that contained the N-terminus of TrpE followed by eight amino acids encoded by the polylinker region of pBluescript and then the C-terminal 38 amino acids of GLUT3. Starvation for tryptophan of *Escherichia coli* harboring this plasmid caused induction of a strong protein band at the expected molecular weight for TrpE-GLUT3, which was 37,000. Parallel control cultures harboring pATH11 produced a band at 33,000 corresponding to TrpE. TrpE-GLUT3 protein was purified by SDS-PAGE, dialyzed extensively, and used as an immunogen to generate antisera in New Zealand White rabbits by standard techniques. Antiserum was used in Western blotting experiments as follows. CEFs and CEF-*src*^{wt} cells were grown in the presence or absence of glucose (as described above) and a total membrane fraction was prepared by hypotonic treatment, cell disruption by Dounce homogenization, and removal of nuclei and large cell fragments by centrifugation at 1500 × *g* for 5 min followed by the harvesting of the membrane fraction by centrifugation at 30,000 × *g* for 20 min (Hay, 1974). One hundred micrograms of protein/lane was loaded in Laemmli sample buffer and separated by SDS-PAGE (10%). After transfer to polyvinylidene difluoride membrane (Sigma), the membrane was stained with Ponceau S to ascertain even transfer and then blocked with blotto (1% dry milk, 0.3% bovine serum albumin, 0.1% Tween-20, 0.1% sodium azide in phosphate-buffered saline) for 1–6 days at 4°C. The membrane was incubated with the antiserum diluted 1/100 in blotto overnight at 37°C, washed four times with blotto, four times with blotto + 3% Tween-20, and four times with blotto. For the secondary antibody, ^{125}I-labeled donkey anti-rabbit IgG (Amersham; Arlington Heights, IL) diluted to 0.8 μCi/ml in blotto was used for 3 h at 37°C followed by washing in the same manner as after the primary incubation.

Effects of TPA and Serum

Parallel subconfluent cultures of CEFs were incubated in DME without serum for 16 h and then shifted at time zero to DME supplemented either with 20% fetal calf serum or with 50 ng/ml TPA. Control cultures were processed at time zero. Measurements of tritiated 2DG uptake, GLUT mRNA levels, and GLUT gene transcription were measured as described above.

RESULTS

Isolation and Sequencing of Chicken GLUT1 cDNA

We first isolated clones by screening a CEF cDNA library with a rat GLUT1 cDNA. The longest clone that could be isolated by this method was 2660 nucleotides in length and thus lacked several hundred nucleotides at the 5′ end. Clones containing the missing region were isolated by 5′-RACE (Frohman *et al.*, 1988). The overall sequence of chicken GLUT1 derived from these clones is shown in Figure 1. There is an open reading frame stretching from nucleotide 53–1523. FASTA comparison (Pearson and Lipman, 1988) showed 79% DNA sequence identity with mammalian GLUT1s over a region from 1–1580 (stop codon is 1525). The longer length of chicken GLUT1 is due to

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                                                                M  E  S  G  S  K  M  T  A  R  L  M  L  A  V  G  G  A  V  L  G  S  L
CCGCGCGACATCGCTCTGCTGTCCCGCGCCGATCCGACGCCCCCGGAGCAACATGGAGAGCGGCAGCAAGATGACAGCTCGCCTGATGCTGGCCGTGGGAGGAGCGGTGCTGGGCTCGCT   120

 Q  F  G  Y  N  T  G  V  I  N  R  P  Q  K  V  I  E  D  F  Y  N  H  T  W  L  Y  R  Y  E  E  P  I  S  P  A  T  L  T  T  L
GCAGTTCGGCTACAACACCGGCGTCATCAATCGCCCGCAGAAGGTGATTGAAGACTTCTACAACCACACCTGGCTGTACCGATATGAGGAGCCCATCAGCCCTGCCACCCTCACCACACT   240

 W  S  L  S  V  A  I  F  S  V  G  G  M  I  G  S  F  S  V  G  L  F  V  N  R  F  G  R  R  N  S  M  L  M  S  N  I  L  A  F
GTGGTCCCTCTCTGTCGCCATCTTCTCGGTTGGAGGCATGATTGGATCCTTTTCCGTGGGACTCTTTGTCAACCGCTTTGGCCGGCGTAACTCCATGCTGATGTCAAACATCCTCGCCTT   360

 L  A  A  V  L  M  G  F  S  K  M  A  L  S  F  E  M  L  I  L  G  R  F  I  I  G  L  Y  S  G  L  T  T  G  F  V  P  M  Y  V
CCTGGCTGCTGTCCTCATGGGCTTCTCCAAGATGGCTTTGTCCTTTGAGATGCTCATTCTTGGCCGCTTCATCATCGGCCTGTATTCTGGCCTCACCACTGGCTTTGTGCCCATGTATGT   480

 G  E  V  S  P  T  A  L  R  G  A  L  G  T  F  H  Q  L  G  I  V  L  G  I  L  I  A  Q  V  F  G  L  D  L  I  M  G  N  D  S
GGGCGAGGTGTCACCAACGGCGCTGCGGGGTGCACTGGGGACCTTCCACCAGCTCGGCATTGTCTTGGGCATTCTCATTGCACAAGTGTTTGGCTTGGACTTGATCATGGGAAACGACTC   600

 L  W  P  L  L  L  G  F  I  F  V  P  A  L  L  Q  C  I  I  L  P  F  A  P  E  S  P  R  F  L  L  I  N  R  N  E  E  N  K  A
GCTCTGGCCACTGCTCCTGGGGTTCATCTTCGTCCCTGCCCTGCTGCAGTGCATCATCCTCCCCTTTGCCCCTGAGAGCCCCCGCTTCCTCCTGATCAACCGCAATGAGGAGAACAAAGC   720

 K  S  V  L  K  K  L  R  G  T  T  D  V  S  S  D  L  Q  E  M  K  E  E  S  R  Q  M  M  R  E  K  K  V  T  I  M  E  L  F  R
CAAGAGCGTCCTCAAGAAGCTCCGGGGCACAACAGATGTCAGCAGCGATCTGCAGGAGATGAAGGAGGAGAGCCGGCAAATGATGAGAGAGAAGAAGGTCACTATAATGGAGCTCTTCCG   840

 S  P  M  Y  R  Q  P  I  L  I  A  I  V  L  Q  L  S  Q  Q  L  S  G  I  N  A  V  F  Y  Y  S  T  S  I  F  E  K  S  G  V  E
CTCTCCCATGTACCGCCAGCCCATCCTCATCGCAATCGTCTTGCAGCTGTCCCAGCAGCTCTCAGGGATCAATGCGGTTTTCTACTACTCCACCAGCATCTTTGAGAAGTCAGGTGTGGA   960

 Q  P  V  Y  A  T  I  G  S  G  V  V  N  T  A  F  T  V  V  S  L  F  V  V  E  R  A  G  R  R  T  L  H  L  I  G  L  A  G  M
GCAGCCCGTCTACGCCACCATTGGCTCTGGGGTGGTGAATACAGCTTTCACTGTTGTTTCGCTCTTCGTGGTGGAGCGAGCTGGACGCAGGACCCTTCACCTCATTGGCCTGGCAGGGAT  1080

 A  G  C  A  I  L  M  T  I  A  L  T  L  L  D  Q  M  P  W  M  S  Y  L  S  I  V  A  I  F  G  F  V  A  F  F  E  I  G  P  G
GGCAGGGTGTGCAATACTCATGACCATTGCTCTGACGCTGCTGGACCAAATGCCCTGGATGTCCTACCTCAGCATCGTTGCCATCTTCGGGTTCGTGGCCTTCTTTGAGATTGGTCCAGG  1200

 P  I  P  W  F  I  V  A  E  L  F  S  Q  G  P  R  P  A  A  F  A  V  A  G  L  S  N  W  T  S  N  F  I  V  G  M  G  F  Q  Y
CCCCATCCCTTGGTTTATTGTGGCAGAACTGTTCAGCCAAGGCCCTCGTCCTGCTGCCTTCGCTGTTGCTGGGCTGTCTAACTGGACCTCCAACTTCATTGTGGGCATGGGCTTCCAGTA  1320

 I  A  Q  L  C  G  S  Y  V  F  I  I  F  T  V  L  L  V  L  F  F  I  F  T  Y  F  K  V  P  E  T  K  G  R  T  F  D  E  I  A
CATTGCGCAACTCTGTGGCTCCTACGTCTTCATCATCTTCACGGTGCTGCTAGTCCTCTTCTTCATCTTCACCTACTTCAAGGTGCCGGAGACAAAGGGCCGGACCTTCGATGAGATCGC  1440

 Y  R  F  R  Q  G  G  A  S  Q  S  D  K  T  P  D  E  F  H  S  L  G  A  D  S  Q  V
CTACCGCTTCCGCCAGGGGGGTGCGAGCCAGAGCGACAAGACCCCGGATGAGTTCCACAGCTTGGGTGCTGACTCCCAGGTCTAACCAGCTGCGGCACGTCCCGTCTCCTCCGAGCCTCC  1560

TAACTTCTCTCCTGAGTGTTTTTAATACTTGTACAGAAACGAGGAAGGAGGGAGCGGGGGGTCTGGCTCTTCTGCATACACTCCTTCTCCAGACTCCCTCACTGGCCGCCTGGTTCATCA  1680

GTGACTGACTGACGTATTTTTGCTAGGAACGATTTTAGCTGGTTTCACGTCCAGGATTTCAAAGCCGATAGATTCAGCAACAATGACTAATGTTTTAAAAACCAACCAAGCAGCATCCTC  1800

CAGCCTCCGCGAGCAGGAGGGTGGCTCCCTTTTTATTGAAGAGATAGGTTATTCCATGTGAAAAAATCACATAGAGGAAGTTTTTAAAGCTGGAAACTTTTTTTTTTAACATTCTAATCAT  1920

AGAGAAATCGTGTAATTTGACTAGTTCTAAACCAGGATTTTTTTTTTAAAGTTATTTTTTAAAGCAGTCTAAGATTTCAGCCCCCCCATTGGGTTTGTGGATGGGGATTCCCTGCCCTCC  2040

AGGAGCGCCATGGGGCAGCACTGAGGATGAGAGGTGCGCAGCCCTGCTCTTCTGGGGGTGCCAGTACGGAGAGCATTCCCCTGGAGCAGGTTGGGGAGGTCAGTAGTACTGGAGCAGGTG  2160

GCAGAGCACTGCTTCCCCTCGTTGCAGGCTCCTGGAACAGCTTCCCACCTTCCTGAGCAGAGGGCTGTGCGTGCTTCCTCACCTGGATTTCATCCATTCTTGTGCCGGCTGTGCGCTCGG  2280

GCGGGCTGAGATGGGAGGGGAGGGGATCTCTCGGTGTATTCTTGCAATATTTGCCTTATTGGTTAACCGTGGCACGAAGCGCTGGCGGAGAGGAGCGAGGGTTCAGCGGCAGCGCAGAGC  2400

TGTGGCCTCAGCCTTCCCCGGCATGATGCTGTCCCCTGGACGGCGGGGCCGGGAGCGGGCGGCGGTCCCACGCTGCAGCACACCTTCCCATCGCCACCACATGGATCCCAGAGTCACCAA  2520

AATTCACCACATGGATACATACATTTCCTGCTCTGTGTATAGACTGGAAGATATTTATATTATATATATTTTTGTTCAATGGTGTTAATAGTAGACGTTAAGTTATAGTGCTTCTGATTG  2640

ACAAGGAACTGTACTGTAAATATTCTTGGAGTATATTCACAGTGTATAATGTATACAAAAAGGTACAGATGCAAAGGTTTAGTCTCTATAGCAACTCTAAGGAAAGCTTGTACATGGTTA  2760

GGATGACTTTTGTATAGTTAATTACCTGCACTGTAGTTGTATCTATTGCACTGAATTTTAGTCCTTAGTATATTAAAAATGAGAAATATCTGTCCTTTTAAAACAAAACATAAAAACCAA  2880

CACAAAAAAGCCAGCCCTTCTCCCCCAGGTGCAACTGTTGTAGGATCATGGCACATTCCCAAAGCCATGTGCAGTTATTTAATAGTGGTTTTTGTTTTTTTTTAATTTATTTGTCTGTTTT  3000

GGAAGTGAGTACATTGAAAATAAAGGTACAAAAAACCATGACAAACTCTCGCAGTCTGTGCAGCTCCCAGAATCGCACACACTTTTTTTTTTCTTTTTTTTTCCTAATTGGCATAAACAT  3120

AAGTGCCAAGAGAATGGAGAAAGGCGTGTGGTTGGTCAACAATAAACAGGAATGTATGCAGCGACCCAAAAAAAAAAAAAAAAAAA  3206
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Figure 1.

the 3′-UTR being about 470 nucleotides longer than in mammals. The 490 amino acid translation product of the open reading frame (Figure 1) showed a high degree of sequence conservation with mammalian GLUT1 proteins, e.g., human GLUT1 has 88% sequence identity and 95% sequence similarity (Figure 2A).

Although most of the 1.7-kb 3′-UTR had no homology to the mammalian GLUT1s (or indeed to any other sequences in GenBank), a small subregion was found that did match mammalian GLUT1 3′-UTRs. Figure 2B shows the alignment of these subregions from chicken and the six mammalian GLUT1 mRNA sequences in GenBank 87.0 (BESTFIT; Devereux *et al.*, 1984). The length of this region is about 180 bases and the degree of sequence identity is about 80%. This region had no similarity to any of the other GLUT isoforms. Within this region is found the motif AUUUA and the overall AU content of the region in chick GLUT1 is about 70%.

Effect of v-src and Glucose Starvation on the Levels of GLUT1 and GLUT3 mRNAs in CEFs

Both of the isoforms of glucose transporter that are expressed in CEFs have been cloned (GLUT1 described here and GLUT3; White *et al.*, 1991). This allowed the investigation of the contribution of changes in the relative expression of their mRNAs to the induction of glucose transport observed when CEFs are transformed by the v-*src* oncogene and/or deprived of glucose. Three types of cell were investigated: CEFs, CEF-*src*^{wt}, and CEF-*src*^{ts}. In each experiment, Northern blots were run and probed for GLUT1 and GLUT3. The results are shown in Figure 3. In Figure 3A, the effect of growing the cells at either the permissive or nonpermissive temperature in the presence of glucose is shown. In Figure 3B, the effect of normal glucose levels or glucose deprivation on these cells at the permissive temperature was investigated. Figure 3C represents a similar set of experiments to Figure 3B but these experiments were performed at the nonpermissive temperature. These data were quantified by densitometry and each lane was normalized for the intensity of ethidium bromide staining of the 18S and 28S ribosomal bands, which are also shown in Figure 3, A–C. For the sake of clarity, these data are shown as histograms in Figure 4 in conjunction with 2-[^{3}H]-deoxyglucose (2DG) uptake assays done on the same cells under the same conditions (Figure 3, A–C). The panels are arranged such that experiments done under the same conditions are aligned horizontally. For example, comparison of Figure 4, A, D, and G, shows that active v-*src* (i.e., CEF-*src*^{ts} at 36°C and CEF-*src*^{wt} at both temperatures) is associated with an increase in 2DG uptake (Figure 4A) and GLUT3 (Figure 4D) with no change in GLUT1 (Figure 4G). The effects of glucose deprivation at 36°C and 41°C are shown in Figure 4, panels B, E, and H, and panels C, F, and I, respectively. In response to glucose deprivation, the v-*src*-transformed cells at 36°C (Figure 4, B, E, and H) showed a two to threefold increase in 2DG uptake and corresponding increases in GLUT3 mRNA. However in untransformed CEFs, glucose deprivation caused a more than threefold increase in 2DG uptake but no significant changes in GLUT1 or GLUT3 mRNA levels.

Effect of v-src on GLUT Gene Transcription

To determine whether the elevation in GLUT3 mRNA levels observed when CEFs were transformed by v-*src* was due to a change in the rate of GLUT3 transcription, nuclear run-on analyses were performed. A clear induction of the rate of GLUT3 transcription was seen in CEF-*src*^{wt} compared with untransformed CEFs (both grown at 37°C) and in CEF-*src*^{ts} at the permissive temperature compared with the nonpermissive temperature (Figure 5). The increase in GLUT1 transcription seen for CEF-*src*^{ts} at 36°C was not reproducible and was likely due to experimental variation.

Effect of v-src on GLUT mRNA Stability

To test whether v-*src* might have an effect on the stability of GLUT mRNAs, normal CEFs and CEF-*src*^{wt} were treated with actinomycin D to inhibit transcription and the time courses of turnover of the GLUT1 mRNA and the two GLUT3 mRNAs were analyzed by Northern blotting (Figure 6). Three experiments were performed (each with different batches of cells) and one such experiment is shown in Figure 6A. It can be seen that all three GLUT mRNAs are quite stable, with mRNA signal still being clearly visible after 16 h of actinomycin D treatment. The signals from these Northern blots were quantitated by phosphoimaging and normalized first for loading and then to the time zero level (100%). Graphs of the logarithms of the percentage of signal remaining versus time are shown in Figure 6B. In Figure 6C, linear regression was used to determine half-life values from these graphs (first figure in each column that is shown in bold) and from analogous graphs generated in the other two experiments done with different batches of cells. The numbers in parenthesis represent the corre-

Figure 1 (continued). Nucleotide sequence of the chicken GLUT1 cDNA clone and the predicted amino acid sequence. The nucleotide sequence of chicken GLUT1 cDNA is given as determined by sequencing of appropriate subclones on both strands. The number of the nucleotide at the end of each line is indicated. The sequence is 3185 nucleotides long excluding the poly(A) region. The predicted amino acid sequence of the long open reading frame from nucleotide 53–1523 is given on the line above. The putative mRNA destabilization element ATTTA is double underlined and the putative polyadenylation signal AATAAA is underlined. The GenBank accession number of this sequence is L07300.

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MESGSKMTARLMLAVGGAVLGSLQFGYNTGVINRPQKVIEDFYNHTWLYRYEEPISPATLTTLWSLSVAIF 71
MEPSSKKLTGRLMLAVGGAVLGSLQFGYNTGVINAPQKVIEEFYNQTWVHRYGESILPTTLTTLWSLSVAIF 72

SVGGMIGSFSVGLFVNRFGRRNSMLMSNILAFLAAVLMGFSKMALSFEMLILGRFIIGLYSGLTTGFVPM 141
SVGGMIGSFSVGLFVNRFGRRNSMLMMNLLAFVSAVLMGFSKLGKSFEMLILGRFIIGVYCGLTTGFVPM 142

YVGEVSPTALRGALGTFHQLGIVLGILIAQVFGLDLIMGNDSLWPLLLGFIFVPALLQCIILPFAPESPR 211
YVGEVSPTAFRGALGTLHQLGIVVGILIAQVFGLDSIMGNKDLWPLLLSIIFIPALLQCIVLPFCPESPR 212

FLLINRNEENKAKSVLKKLRGTTDVSSDLQEMKEESRQMMREKKVTIMELFRSPMYRQPILIAIVLQLSQ 281
FLLINRNEENRAKSVLKKLRGTADVTHDLQEMKEESRQMMREKKVTILELFRSPAYRQPILIAVVLQLSQ 282

QLSGINAVFYYSTSIFEKSGVEQPVYATIGSGVVNTAFTVVSLFVVERAGRRTLHLIGLAGMAGCAILMT 351
QLSGINAVFYYSTSIFEKAGVQQPVYATIGSGIVNTAFTVVSLFVVERAGRRTLHLIGLAGMAGCAILMT 352

IALTLLDQMPWMSYLSIVAIFGFVAFFEIGPGPIPWFIVAELFSQGPRPAAFAVAGLSNWTSNFIVGMGF 421
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QYIAQLCGSYVFIIFTVLLVLFFIFTYFKVPETKGRTFDEIAYRFRQGGASQSDKTPDE.FHSLGADSQV 490
QYVEQLCGPYVFIIFTVLLVLFFIFTYFKVPETKGRTFDEIASGFRQGGASQSDKTPEELFHPLGADSQV 492
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B

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mouse    .....GGACC UCGGAGGCUG AGAACUUA.. ...ACUGCUG AAGACACGGA
rat      ......GACC UCGGAGGCUG AGAACUU... ...ACUGCUG AAGACACGGA
bull     ......GCCA UGGGAAACCG AGAACUCACU AUU...CAAG AAGACCUGGA
rabbit   .......... ..CGAGGCUG AGAACUCACU GCU...CCAG AAGACAUGGA
human    .......... .......... .AAACUCACU GCU...CAAG AAGACAUGGA
pig      ......ACCA UGGGAGGCUG AGAACUCACU ACUACUCAAG AAGACACGGA
chicken  CCAUCGCCAC CACAUGGAUC CCAGAGUCAC CAAAAUUCAC CACAUGGAUA

mouse    CACUCCUGCC CUGC..UGUG UAUAGA.UGG AAGAUAUUUA UAUAU..UUU
rat      CACUCCUGCC CUGC..UGUG UAUAGA.UGG AAGAUAUUUA UAUAU..UUU
bull     CACUCCUGCC CUGC..UGUG UAUAGU.UGG AAGAUAUUUA UAUAUAUUUU
rabbit   CACUCCUGCC CUGC..UGUG UAUAGA.UGG AAGAUAUUUA UAUAUAUUUU
human    GACUCCUGCC CUGU..UGUG UAUAGA.UGC AAGAUAUUUA UAUAUA.UUU
pig      CACUCCUGCC CUGCUGUGUG UAUAGA.CGG AAGAUAUUUA UAUAUAUUUU
chicken  CAUACAUUUC CUGCUCUGUG UAUAGACUGG AAGAUAUUUA UAUUAUAUAU

mouse    UUGGUUGUCA A....UAUUA AAUACAGACA CUAAGUUAUA G..UAUAUCU
rat      UUGGUUGUC. .....UAUUA AAUACAGACA CUAAGUUAUA GUAUAUAUCU
bull     UUGGUUGUCA A....UAUUA AAUACAGACA CUAAGUUAUA G..UAUAUCU
rabbit   UUGGUUGUCA A....UAUUA AAUACAGACA CUAAGUUAUA G..UAUAUCU
human    UUGGUUGUCA A....UAUUA AAUACAGACA CUAAGUUAUA G..UAUAUCU
pig      UUGGUUGUCA A....UAUUA AAUACAGACA CUAAGUUAUA G..UAUAUCC
chicken  AUUUUUGUUC AAUGGUGUUA AUAGUAGACG UUAAGUUAUA GUGCUUCUGA

mouse    GGACAAACCC ACUUGUAAAU ACACCAACAA ACUCCUGUAA CU........
rat      GGACAAACCC ACUUGUAAAU ACACCAACAA ACUCCUGUAA CUUU......
bull     GGACAAACCA ACUUGUAAAU AUACCACCUU ACUCCCUCUG UAACUUACCU
rabbit   GGACAAACCA ACUUGUAAAU ACACCAACAC A.UCCUUCUG UAACUUACCU
human    GGACAAGCCA ACUUGUAAAU ACACCACCUC ACUC...CUG UUACUUACCU
pig      GGACAAACCA ACUUGUAAAU ACACCACCUU CCUC...... ..........
chicken  UUGACAAGGA ACUGUACUGU AAAUAUUCUU GGAGUAUAUU CACAGUGUAU
```

Figure 2. Comparisons of chicken GLUT1 to human GLUT1. (A) Comparison of the predicted 490 amino acid sequence of the chicken GLUT1 cDNA (upper line) to that of the 492 amino acid sequence of human GLUT1 (lower line; Mueckler *et al.*, 1985) is shown using the BESTFIT method (Devereux *et al.*, 1984). They are 88.1% identical (94.7% similarity) with one gap and a quality score of 676. (B) Comparison of conserved subregions of the 3′-UTR of chicken GLUT1 and mammalian GLUT1s. The comparison of the 3′-UTR of the chicken GLUT1 cDNA clone (upper line) and the mammalian GLUT1s present in GenBank Release 87 using the PILEUP program. The ATTTA elements are underlined.

lation coefficient of each regression. We conclude that all three GLUT mRNAs have relatively long half-lives (>2 h). Thus there is no significant stabilization of these mRNAs by v-*src* transformation. Indeed, if anything, the GLUT3 mRNAs seem to have a shorter half-life in the transformed cells.

Effect of v-src and Glucose Starvation on GLUT3 Protein Levels

To test whether the changes observed in GLUT3 mRNA level were reflected in changes in the level of GLUT3 protein, antiserum raised against a TrpE-GLUT3 fusion protein immunogen was used to quantitate the amounts of this protein in CEFs and CEF-*src*^{wt} that were grown either in the presence or absence of glucose. These results are shown in Figure 7. The chicken GLUT3 protein ran as a relatively broad band with an apparent molecular weight of 45,000. This is similar to the electrophoretic behavior of GLUT1 (Salter *et al.*, 1982; Haspel *et al.*, 1986; Shawver *et al.*, 1987; White and Weber, 1988) and is due to the hydrophobicity and heterogeneity of glycosylation of glucose transporters. Another band (NS) at 49,000 was used as a loading control. This represents nonspecific binding

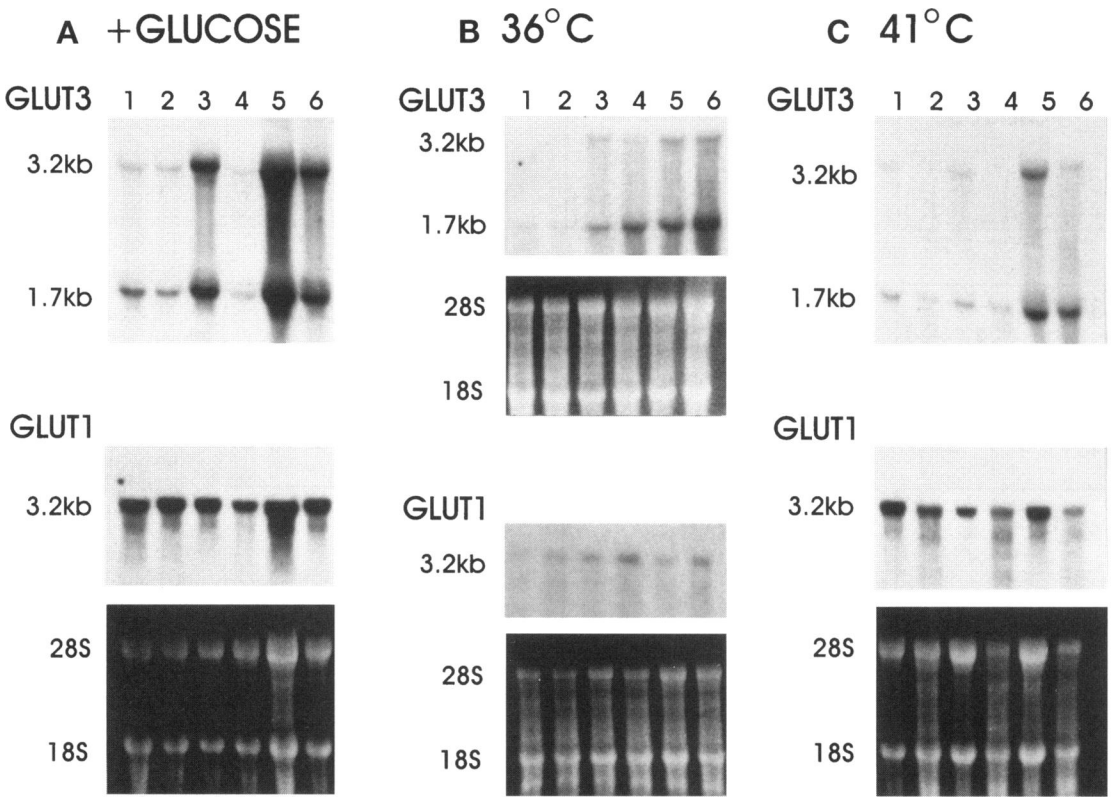


Figure 3. Northern blot analysis of GLUT expression in CEFs. In all panels, lanes 1 and 2 are CEFs, lanes 3 and 4 are CEF-*src*^{ts}, and lanes 5 and 6 are CEF-*src*^{wt}. (A) Cells were grown with glucose at either 36°C (lanes 1, 3, and 5) or 41°C (lanes 2, 4, and 6). (B) Cells were grown at 36°C with glucose (lanes 1, 3, and 5) or without glucose (lanes 2, 4, and 6). (C) Cells were grown at 41°C with glucose (lanes 1, 3, and 5) or without glucose (lanes 2, 4, and 6). This experiment was performed on three occasions using RNA derived from different cell cultures.

to a major Ponceau S-staining membrane protein and was also observed with nonimmune serum. The amount of GLUT3 protein in the two CEF-*src*^{wt} lanes was higher than in the CEF lanes and densitometric analysis of the autoradiograph showed that the difference was about twofold. There was no significant effect of glucose deprivation on GLUT3 protein levels in either the CEFs or the CEF-*src*^{wt} cells.

Effect of Serum and TPA on Glucose Transport, GLUT mRNA Levels, and Transcription Levels in CEFs

Figure 8 shows the effects of the addition of either serum or TPA to CEFs that had been cultured in the absence of serum for 12 h. Addition was made at time zero and uptake of tritiated 2DG was measured at 2, 4, and 8 h. The uptake of labeled sugar was normalized relative to cultures assayed at time zero. The results (Figure 8A) show that addition of serum to the CEF cultures induced a 4.9-fold increase in sugar uptake after 8 h of incubation, and that maximal uptake had almost been reached after 4 h of incubation (4.5-fold). Similar uptake results were also observed for the CEFs stimulated with TPA although maximal uptake was approximately one-half (2.6-fold) of that observed for the CEFs treated with serum. Again maximal stimulation of uptake had almost been reached after 4 h of incubation (2.2-fold). Figure 8B shows Northern analyses of RNA extracted from CEFs stimulated with either serum for 3 h (CEF+S) or TPA for 3 h (CEF+T) relative to RNA from control CEFs at time zero (CEF-S). RNA was also extracted from CEF-*src*^{wt} as a positive control. Both chicken GLUT3 mRNAs were induced by treatment with either serum or TPA. This induction was greater with serum than with TPA, which correlates with the relative effects of these treatments on glucose transport. In contrast, GLUT1 mRNA was unaffected by treatment with either TPA or serum. A probe to chicken glyceraldehyde-3-phosphate dehydrogenase was used as a loading control in this experiment.

To determine whether the changes observed in the GLUT mRNAs were due to an effect on transcription, nuclear run-on analyses were performed. Nuclei were isolated from serum-starved CEFs at time zero and from CEFs that had been treated for 1 h with serum or

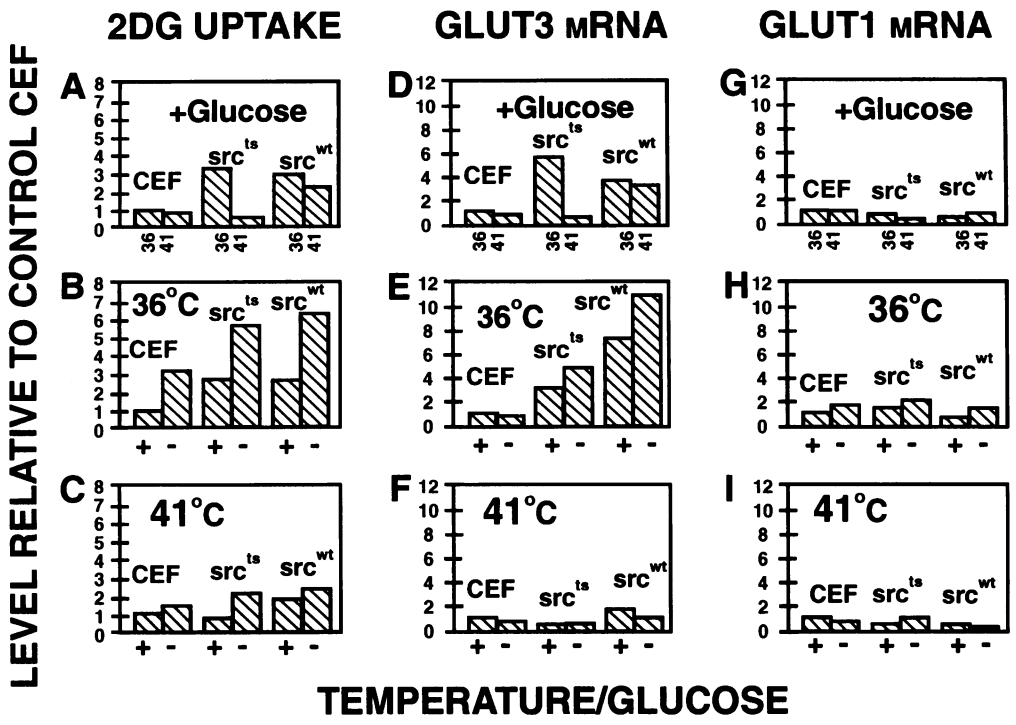


Figure 4. Quantitation of glucose transport and GLUT mRNA levels in CEFs. (A, B, and C) 2-[^{3}H]-deoxyglucose (2DG) uptake was measured in the same cells under the same conditions as Figure 3. Uptake data (tritium cpm per mg cell protein) were normalized to the control CEFs, which are the first bars in each histogram. (D–I) The data in Figure 3 were quantitated by densitometry, normalized first to the intensity of ethidium bromide staining of the ribosomal bands, and then to the control CEF RNA in lane 1 of each panel. In the case of GLUT3 both the 1.7-kb and 3.2-kb mRNAs were quantified and the values were added to give the amount of total GLUT3 mRNA. Note that the panels are arranged such that parallel experiments are aligned horizontally.

TPA, run-on reactions were performed, and the RNA was hybridized to filters on which DNA probes were immobilized (Figure 8C). These filters were subject to phosphoimager analysis with normalization first to total chicken genomic DNA and then to CEFs at time zero (Figure 8D). Treatment with TPA or serum induced GLUT3 transcription with no effect on GLUT1, and the effect of serum was greater than TPA. Nuclei from CEF-*src*^{wt} were again included as a control.

Expression of GLUT1 and GLUT3 in Rat Cells

To examine GLUT1 and GLUT3 expression in mammalian cells, Northern blot analyses were performed on two types of rat fibroblast. Figure 9 shows the expression of rat GLUT1 and GLUT3 in primary rat embryo fibroblasts, in the established rat fibroblast cell line rat-1, and in v-*src*-transformed derivatives of both of these cells. The 2.7-kb rat GLUT1 mRNA was induced by v-*src* in both cell types (Figure 9A). In contrast, all cells expressed 4.0-kb GLUT3 mRNA but this was independent of v-*src* (Figure 9B).

Phylogenetic Analysis of GLUT Isoforms

The phylogenetic relationship of the 24 vertebrate GLUT proteins present in GenBank 87.0 (April 1995) was determined using the PILEUP program (Devereux *et al.*, 1984). The dendrogram is shown in Figure 10. Each isoform is clustered on its own branch. The first branch point separates the fructose transporter (GLUT5). The next divergence is the liver GLUT isoforms (GLUT2 and GLUT7) from the non-liver isoforms. The insulin-regulated isoform (GLUT4) is the next to diverge and the final divergence is GLUT1 from GLUT3.

DISCUSSION

Since the initial cloning of a mammalian GLUT1 (Mueckler *et al.*, 1985), much research has focused on its regulation. This protein is expressed in a wide variety of tissues and by most if not all types of tissue culture cells (reviewed by Gould and Bell, 1990; reviewed by Mueckler, 1994). In cultured rodent fibroblasts, transformation by v-*src* or other oncogenes led to an induction in GLUT1 protein biosynthesis, GLUT1 mRNA level, and GLUT1 gene transcription (Birnbaum *et al.*, 1987; Flier *et al.*, 1987; White and Weber, 1988; Williams and Birnbaum, 1988; Figure 9A). Figure 9B shows that cultured rat fibroblasts also expressed the GLUT3 isoform but that it was *not* up-regulated by v-*src* transformation. Similar results have been obtained with FDC-P1 mouse myeloid cells that also expressed an inducible level of GLUT1 and a constant level of GLUT3 (White *et al.*, 1994). It has also been reported that GLUT3 is an abundant transporter in human mononuclear cells (Estrada *et al.*, 1994) and muscle cells where it is up-regulated by prolonged exposure to IGF-1 (Klip *et al.*, 1993).

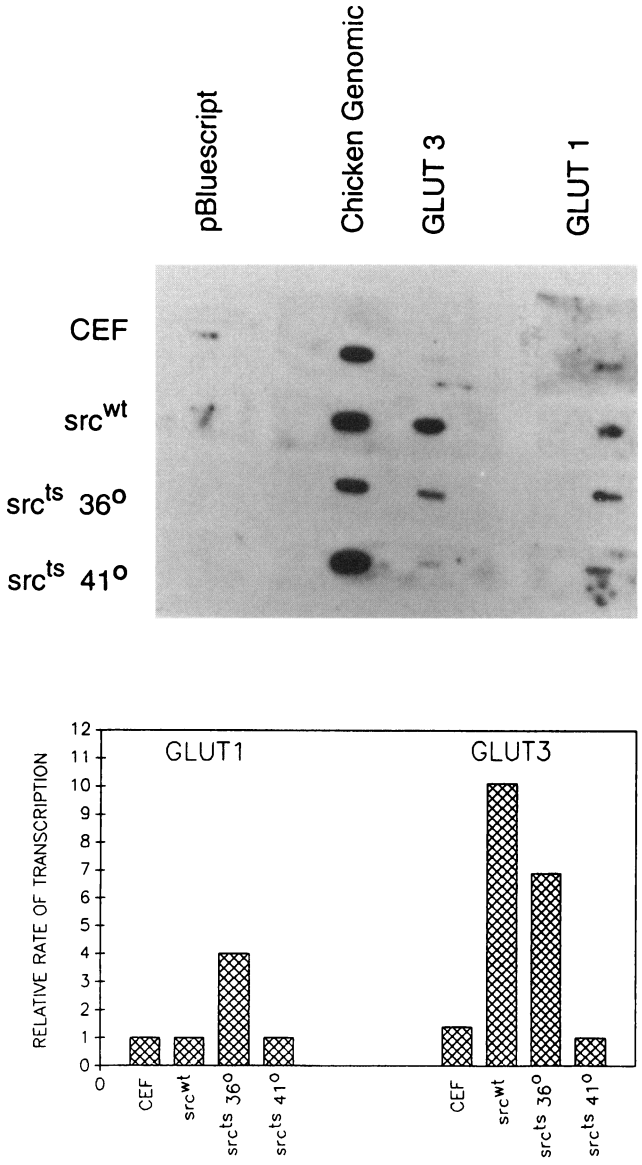
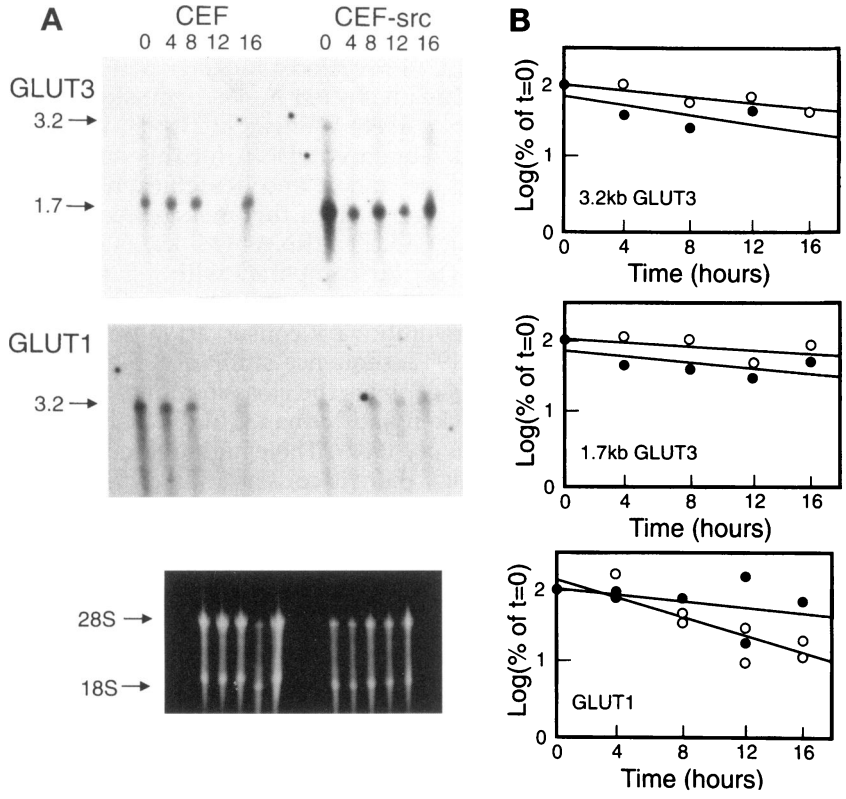


Figure 5. Nuclear run-on analysis of GLUT gene transcription. Nuclear run-on reactions were performed with nuclei isolated from CEFs (37°C), CEF-*src*^{wt} (37°C), and CEF-*src*^{ts} (36°C and 41°C). [^{32}P]-RNA preparations isolated from these reactions were hybridized with filters to which had been immobilized the following DNAs: pBluescript, total chicken genomic, chicken GLUT1, and chicken GLUT3. The filters were washed and autoradiographed (upper panel) and then quantitated by phosphoimaging. The rate of transcription was determined by dividing the intensity of each GLUT cDNA slot by the intensity obtained for total chicken genomic DNA and is shown as a histogram (lower panel).

In contrast, avian glucose transporters exhibited important differences compared with their mammalian counterparts. Initial investigations used antiserum against human GLUT1 to quantitate GLUT1 protein in CEFs and demonstrated that v-*src* inhibited chicken GLUT1 protein turnover with no effect on biosynthesis (Shawver *et al.*, 1987; White and Weber, 1988). Furthermore low stringency Northern blot hybridization with human or rat GLUT1 revealed a message of 3.2 kb, the level of which was unaffected by v-*src* transformation (White and Weber, 1988; White *et al.*, 1991). We have now cloned and sequenced cDNA for this noninducible 3.2-kb message and it encodes chicken GLUT1 (Figure 1). The larger size of this message compared with mammalian GLUT1 mRNAs (2.7 kb) is due to a longer 3′-UTR (1.7 kb compared with 1.2 kb). The primary structure of chicken GLUT1 protein shows a high degree of evolutionary conservation with mammalian GLUT1 (95% sequence similarity, Figure 2A). Incidentally, this explains the close immunological relatedness of chicken and human GLUT1 (Salter *et al.*, 1982; Shawver *et al.*, 1987). Their high degree of similarity suggests that there is conservation of regulatory regions and not just those involved in protein function (transporting glucose). For example, when CEFs were transformed by v-*src*, GLUT1 protein level was increased due to post-translational regulation resulting in a decrease in its rate of degradation (Shawver *et al.*, 1987; White and Weber 1988). Similarly, when human GLUT1 protein was expressed in CEFs from a retroviral promoter, its turnover was also regulated by v-*src* transformation (White and Weber, 1990). Taken together these data suggest that the human and chicken GLUT1 proteins share conserved motifs for post-translational regulation. However we have not ruled out the possibility that the regulation of human GLUT1 is via dimerization with the endogenous chicken GLUT1 (White and Weber, 1989, 1990; Pessino *et al.*, 1991).

Most of the large (1.7 kb) 3′-UTR of the chicken GLUT1 mRNA shows no significant sequence similarity to those of the mammalian GLUT1 genes. However there is a subregion about 600 nucleotides from the 3′ end in which there is a significant match. Figure 2B shows the result of a comparison of the chicken GLUT1 3′-UTR to the known mammalian GLUT1s using the PILEUP program (Devereux *et al.*, 1984). There is a clear region of sequence similarity (~80% identity) in an ~200-nucleotide region. The region is AU-rich (~70%) and contains a consensus AUUUA mRNA-destabilizing motif (Shaw and Kamen, 1986; Wilson and Treisman, 1988). These features and its conservation from chicken to mammals suggest that it may have a role in regulating mRNA turnover. However GLUT1 mRNA was not up-regulated under any conditions that we applied to CEFs and it had a relatively long half-life in CEFs (Figure 6). It may be that regulation of GLUT1 mRNA stability is predominantly a mechanism in hematopoietic cells. For example, in rat fibroblasts, TPA had no effect on GLUT1 mRNA stability (Mountjoy *et al.*, 1989) whereas TPA prolonged GLUT1 mRNA half-life by 3.5-fold in mouse myeloid cells (White *et al.*, 1994). Also, when a chicken erythroid cell line was induced to differenti-



C

mRNA	Cell	$t_{1/2}$ (hrs)
GLUT1	CEF	**7.1(-0.88)**, 7.7(-0.63), 6.5(-0.95)
	src^{wt}	**14.7(-0.49)**, 3.8(-0.90), 4.8(-0.66)
1.7kb GLUT3	CEF	**18.7(-0.68)**, 13.7(-0.70)
	src^{wt}	**6.9(-0.75)**, 4.2(-0.63)
3.3kb GLUT3	CEF	**11.7(-0.69)**, 12.4(-0.46), 10.8(-0.79)
	src^{wt}	**4.3(-0.96)**, 2.60(-0.94), 2.2(-0.85)

Figure 6. Turnover of GLUT mRNAs in CEFs. CEFs and CEF-*src*^{wt} were treated with actinomycin D to inhibit transcription and RNA was isolated at 0, 4, 8, 12, and 16 h. This was subject to northern blot analysis and probed for expression of GLUT3 and GLUT1 (A). Phosphoimaging was used to determine the amount of label at each time point, and this was normalized to ethidium bromide staining and calculated as a percentage of the value for t = 0. The logarithms of these values were plotted against time (B). Thus the origin of each graph is 2.0 ($\log_{10}$ 100). These graphs were analyzed by linear regression to calculate half-life values and correlation coefficients (C, bold type). Panel C also gives the results of other experiments performed in the same way. The calculated half-life from each experiment is given in hours followed by the correlation coefficient in parentheses.

ate, GLUT1 mRNA was rapidly down-regulated (Mathew *et al.*, 1994), suggesting that GLUT1 mRNA turnover regulation is important in this cell type. Recently it has been reported that the consensus AU-rich sequence conferring instability on c-*fos* mRNA is the nonamer UUAUUUA(U/A)(U/A) (Lagnado *et al.*, 1994) or UUAUUUAUU (Zubiaga *et al.*, 1995) with mismatches in the AUUUA sequence not being tolerated and mismatches in the first two and last two positions being partially tolerated. In mammalian and chicken GLUT1s, the nonamer AUAUUUAUA is found (Figure 2) that matches the consensus with one mismatch at the first nucleotide. GLUT1 mRNAs contain no other sequences of this type and so this is likely the destabilizing element.

As cDNA clones for both GLUT1 and GLUT3 were now available, it was possible to undertake a comprehensive analysis of the effects of transformation and glucose deprivation on GLUT mRNA expression in CEFs. These data are shown in Figures 3 and 4. The following conclusions can be drawn regarding v-*src* transformation: 1) CEF-*src*^{wt} showed elevated glucose transport and GLUT3 mRNA at all temperatures, 2) CEF-*src*^{ts} showed elevated glucose transport and GLUT3 mRNA at the permissive but not at the nonpermissive temperature, and 3) GLUT1 mRNA was unaffected by v-*src*. Nuclear run-on analyses showed that the up-regulation of GLUT3 by v-*src* was due to increased transcription (Figure 5) and not to stabilization of GLUT3 mRNA (Figure 6). Taken together with earlier work on GLUT1 protein expression in CEFs (Shawver *et al.*, 1987; White and Weber 1988, 1990), it can be concluded that v-*src* has two effects on glucose trans-

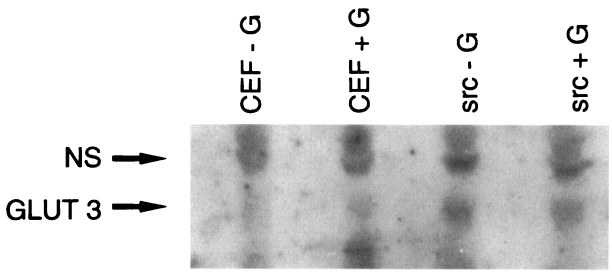


Figure 7. Immunoblot analysis of GLUT3 expression in CEFs. Total membrane proteins from CEFs grown in the absence or presence of glucose (CEF −G, CEF +G) and CEF-*src*^{wt} grown in the absence or presence of glucose (src −G, src +G) were immunoblotted with an antiserum raised against a TrpE-GLUT3 fusion protein. The two bands are as follows: 1) a nonspecific band of apparent molecular weight 49,000 (NS) serving as a loading control that corresponds to a major Ponceau-S-staining band also seen with nonimmune serum and 2) the chicken GLUT3 protein band (GLUT3) of apparent molecular weight 45,000.

port, i.e., transcriptional up-regulation of GLUT3 and post-translational up-regulation of GLUT1.

Figures 3 and 4 also show the effects of glucose starvation. Small changes in the level of GLUT mRNAs occurred in some cases but these changes could not account for the observed increases in glucose transport, e.g., depriving CEFs of glucose resulted in a more than threefold increase in 2DG uptake but no change in either GLUT1 or GLUT3 mRNA. Because GLUT1 protein is increased by glucose starvation (Shawver *et al.*, 1987), these results suggest the existence of a post-translational mechanism of action whereby glucose deprivation elevates glucose transport and GLUT1 protein in CEFs. The GLUT1 protein of rodent fibroblasts is also regulated post-translationally by glucose deprivation (Haspel *et al.*, 1986; reviewed in Klip *et al.* 1994). The mechanism of this regulation remains unclear.

Because this post-translational regulation of glucose transporter proteins can occur, the amount of protein product may not bear a direct relationship to the amount of its mRNA for a particular set of conditions. Therefore it was important to quantitate the effects of v-*src* transformation and glucose starvation on GLUT3 protein levels because, unlike GLUT1, it had not been previously quantitated by immunoblotting. Figure 7 shows the results of an immunoblot to measure the effects of transformation on GLUT3 protein levels.

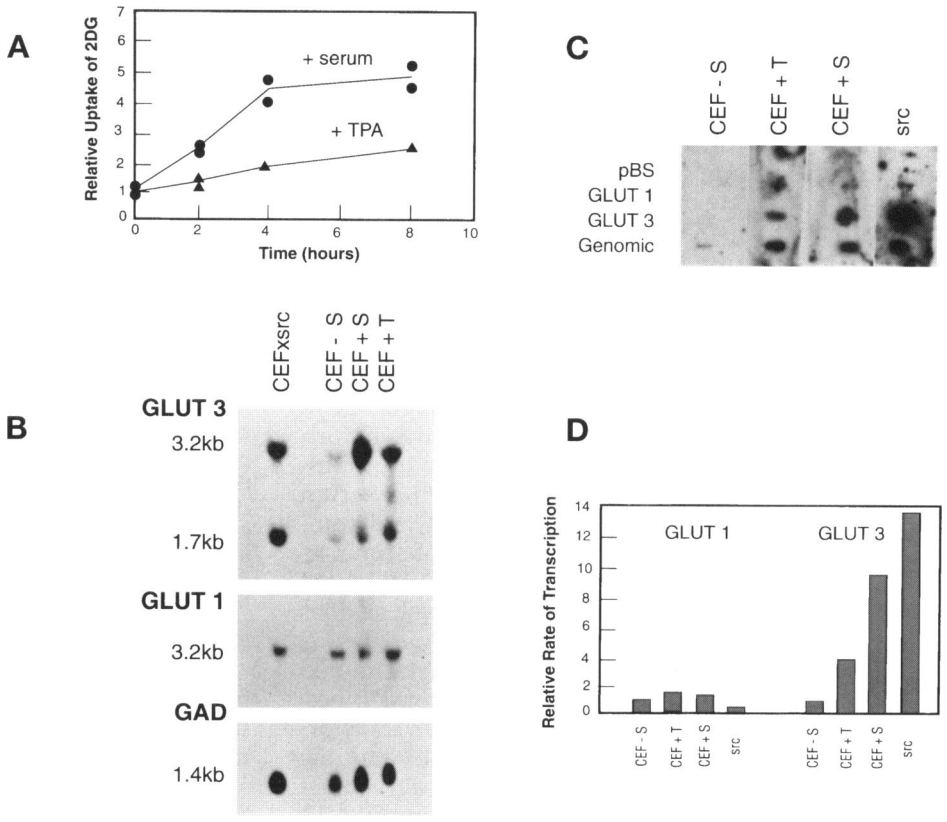


Figure 8. Effect of serum and TPA on glucose transport, GLUT mRNA levels, and GLUT transcription in CEFs. The effects of supplementing serum-starved cultures of CEFs for various times with medium containing either 20% serum (square symbols) or 50 ng/ml TPA (triangular symbols) were determined for the rate of uptake of 2DG (A), GLUT mRNA levels (B), and GLUT gene transcription (C and D). (A) Time course of stimulation of 2DG uptake. Uptake values (cpm/mg protein) were normalized relative to serum-starved cultures at time zero. (B) RNA samples from the following sources were analyzed by Northern blotting: CEF-*src*^{wt}, CEFs starved of serum for 12 h (CEF-S), serum-starved CEFs incubated with 20% serum for 3 h (CEF + S), and serum-starved CEFs incubated with 50 ng/ml TPA for 3 h (CEF + T). The following cDNA probes were used: chicken GLUT3, chicken GLUT1, and chicken glyceraldehyde-3-phosphate dehydrogenase (loading control). (C) Nuclear run-on assays were performed on nuclei from the following sources: CEF-*src*^{wt}, CEFs starved of serum for 12 h (CEF-S), serum-starved CEFs incubated with 20% serum for 1 h (CEF+S), and serum-starved CEFs incubated with 50 ng/ml TPA for 1 h (CEF + T). The following immobilized DNAs were used as targets for the labeled RNA produced by these reactions: pBluescript, chicken GLUT1, chicken GLUT3, and chicken genomic. (D) The relative rates of transcription of the GLUT genes were quantified and graphed from the data in panel C as described in the legend to Figure 5.

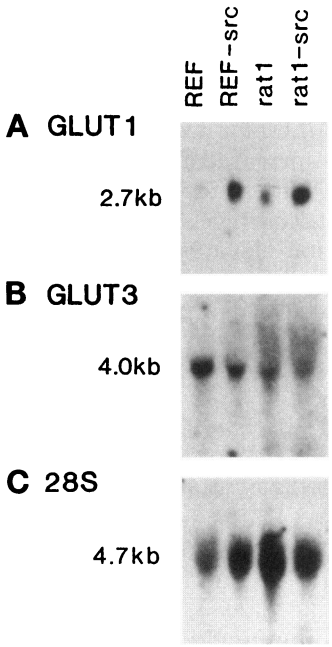


Figure 9. Northern blot analysis of rat cells. RNA samples extracted from the following cells were analyzed: primary rat embryo fibroblasts (REF), REFs transformed by *src* (REF-src), rat-1 cells (rat1), and rat-1 cells transformed by *src* (rat1-src). The cDNA probes were as follows: (A) rat GLUT1 and (B) mouse GLUT3. Panel C shows the 28S ribosomal RNA.

GLUT3 was induced about twofold by v-*src* transformation but glucose starvation had no effect. Thus the levels of GLUT3 protein correlated with the levels of GLUT3 mRNA. Because the amount of GLUT1 protein is also increased two- to threefold after v-*src* transformation due to a decrease in the rate of its degradation (Shawver *et al.*, 1987; White and Weber, 1988), up-regulation of the levels of both GLUT1 and GLUT3 proteins can account for the three- to fivefold increase observed in the rate of uptake of 2DG caused by v-*src* transformation.

The data in Figure 8 show the effects of serum and TPA on CEFs. Both of these stimulated 2DG uptake into CEFs that had been deprived of serum overnight and serum was more potent than TPA (Figure 8A). Northern analysis showed that this was associated with an induction of GLUT3 mRNA with no change in GLUT1 mRNA (Figure 8B). This induction was in turn associated with an increase in GLUT3 gene transcription (Figure 8, C and D). The relative effects of serum and TPA on both GLUT3 mRNA content and transcription matched their relative potencies at stimulating transport. We conclude that serum and TPA both act to induce GLUT3 at a transcriptional level, as does v-*src*.

To compare rat and chicken cells, we also examined GLUT1 and GLUT3 expression in two rat cell types. The data in Figure 9 show the effect of v-*src* transformation on primary rat embryo fibroblasts and the immortalized rat-1 fibroblast cell line. In agreement with previous reports of our own and others, GLUT1 mRNA was induced by v-*src* (Flier *et al.*, 1987; White and Weber, 1988). This data also provides the novel finding that GLUT3 is expressed in rat fibroblasts and that it is not regulated by v-*src*. This is also the case in mouse hematopoietic cells (White *et al.*, 1994).

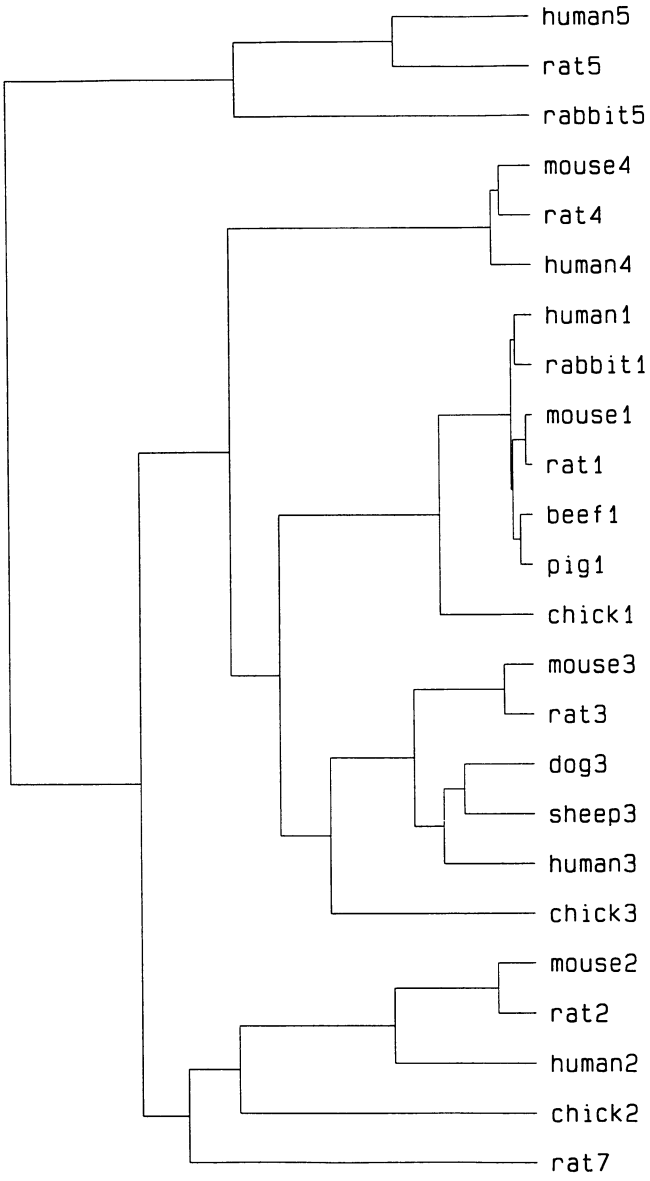


Figure 10. Phylogenetic analysis of the 24 known vertebrate GLUT proteins using the pileup program. Each protein is designated by the common name of the animal followed by isoform number, e.g., chick1 represents chicken GLUT1 (Figure 1). The sequences were extracted from GenBank 87.0 and the accession numbers are as follows (reading from bottom to top on the figure): X66031, Z22932, J03810, J03145, X15684, M37785, M20681, L39214, L35267, U17978, M75135, L07300, X17058, M60448, M13979, M22998, M21747, K03195, M20747, J04524, M23383, D26482, D13871, and M55531.

In conclusion, both mammalian and avian fibroblasts express two isoforms of glucose transporter: one is highly inducible by oncogenes and mitogens and the other is constant. Presumably the latter serves to

provide the cell with a basal supply of glucose regardless of its proliferative status and the former serves to provide the cell with the extra carbon and energy needed when the cell is transformed or mitogenically stimulated. Surprisingly, it is GLUT3 and not GLUT1 that is regulated by oncogenes and mitogens in CEFs. How did these two roles become subsumed by different isoforms in different classes of vertebrates? The GLUT family of isoforms is thought to have arisen by gene duplication events and, interestingly, phylogenetic analysis of GLUT protein sequences predicts that GLUT1 and GLUT3 arose by the most recent of these duplication events (Figure 10). Analysis of the 24 vertebrate GLUT proteins present in GenBank 87.0 showed that each isoform is clustered on its own branch, indicating that the gene duplication events that gave rise to each isoform occurred before the divergence of all of the species analyzed. The first branch point separates the fructose transporter (GLUT5) from the glucose transporters. The next divergence is that of the liver isoforms (GLUT2 and GLUT7) from the non-liver isoforms. The insulin-regulated isoform (GLUT4) is the next to diverge and finally the most recent divergence is that of GLUT1 and GLUT3. It is tempting to speculate that this last event occurred around the time that mammalian and avian classes diverged in evolution and that regulatory elements segregated with different structural genes. It is likely that enhancer and promoter elements present in the mammalian GLUT1 gene (Murakami *et al.*, 1992) are absent or altered in the chicken GLUT1 gene and that the chicken GLUT3 gene has gained elements allowing it to respond to v-*src* and mitogens. We are currently analyzing genomic clones of chicken GLUT1 and GLUT3 to test these hypotheses.

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