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Differential Regulation of Glucose Transporter Isoforms by the *src* Oncogene in Chicken Embryo Fibroblasts

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The increase in glucose transport that occurs when chicken embryo fibroblasts (CEFs) are transformed by *src* is associated with an increase in the amount of type 1 glucose transporter protein, and we have previously shown that this effect is due to a decrease in the degradation rate of this protein. The rate of CEF type 1 glucose transporter biosynthesis and the level of its mRNA are unaffected by *src* transformation. To study the molecular basis of this phenomenon, we have been isolating chicken glucose transporter cDNAs by hybridization to a rat type 1 glucose transporter probe at low stringency. Surprisingly, these clones corresponded to a message encoding a protein which has most sequence similarity to the human type 3 glucose transporter and which we refer to as CEF-GT3. CEF-GT3 is clearly distinct from the CEF type 1 transporter that we have previously described. Northern (RNA) analysis of CEF RNA with CEF-GT3 cDNA revealed two messages of 1.7 and 3.3 kb which were both greatly induced by *src* transformation. When the CEF-GT3 cDNA was expressed in rat fibroblasts, a three- to fourfold enhancement of 2-deoxyglucose uptake was observed, indicating that CEF-GT3 is a functional glucose transporter. Northern analyses using a CEF-GT3 and a rat type 1 probe demonstrated that there is no hybridization between different isoforms but that there is cross-species hybridization between the rat type 1 probe and the chicken homolog. Southern blot analyses confirmed that the chicken genomic type 1 and type 3 transporters are encoded by distinct genes. We conclude that CEFs express two types of transporter, type 1 (which we have previously reported to be regulated posttranslationally by *src*) and a novel type 3 isoform which, unlike type 1, shows mRNA induction upon *src* transformation. We conclude that *src* regulates glucose transport in CEFs simultaneously by two different mechanisms.

The transport of glucose into mammalian cells is mediated by a family of proteins with a common structural motif that differ in tissue distribution and are encoded by discrete genes (reviewed in reference 11). There are at least six different types of glucose transporter genes that have been cloned from cDNA libraries. In humans, these have been designated GLUT1 to GLUT6 (11, 15). Type 1 transporters include the rat brain transporter (RAT-GT1) (2), which was used in some of the studies described in this report, and the HepG2/erythrocyte transporter (19), which is the human equivalent (GLUT1). This is the type of transporter that is expressed in rodent fibroblasts in culture (3, 8, 26). The type 2 transporter isoform is that found in the liver and pancreatic beta cell and has a high K_m for glucose (10, 24). The type 3 isoform (GLUT3), cloned from a human fetal skeletal tissue cDNA library (16), is expressed at variable levels in many adult human tissues and is especially abundant in brain, kidney, and placenta (11, 16). Type 4 transporters are regulated at the level of translocation to the plasma membrane by insulin and are found exclusively in those tissues that show an insulin sensitivity in glucose transport (1a, 6, 9, 13, 14); type 5 is expressed mainly in the small intestine (15). A sixth type (GLUT6) has been described but is not a functional glucose transporter (15).

The aim of this study was to further investigate the nature of the *src*-induced increase of glucose transport in chicken embryo fibroblasts (CEFs). Both CEFs and mammalian fibroblasts show an increased rate of glucose transport when they are transformed by the *src* oncogene, and this increase is due to an increase in the amount of glucose transporter protein (21, 22). However, we have previously shown that CEFs differ from rodent fibroblasts in the mechanism whereby this increase is induced (26). Transformation of mammalian fibroblasts by *src* results in a large induction of mRNA encoding type I glucose transporter (8, 26, 30) and a corresponding increase in the biosynthesis of the type 1 transporter protein (26). This is thought to be the major mechanism whereby *src* transformation enhances glucose transport into these cells. In CEFs, however, measurement of transporter biosynthesis with antibody raised against the human erythrocyte type 1 transporter revealed no change upon *src* transformation; rather, the increase in transporter protein was due to an inhibition of its turnover (23, 26). Similarly, *src* could modulate the turnover of human type 1 transporter which was expressed in CEFs from a retroviral vector (29). In agreement with the data showing no change in CEF type 1 transporter protein biosynthesis, Northern (RNA) analysis of CEF RNA with a human type 1 probe showed a single mRNA species (with an approximate size of around 3.4 kb), the abundance of which was not affected by *src* (26). Thus, in CEFs *src* modulates type 1 transporter protein turnover but not type 1 transporter biosynthesis or mRNA level (26).

In light of this finding, it was of interest to clone transporter cDNAs from CEFs. This has led to the unexpected discovery of a 1.7-kb cDNA encoding a functional glucose transporter protein which we refer to as CEF-GT3. This protein has the most degree of sequence similarity to the human GLUT3 transporter and is distinct from the type 1 chicken transporter that we described in an earlier report (26). Furthermore, CEF-GT3 is regulated differently from the GLUT1 homolog in that its mRNA level is increased by *src* transformation.

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MATERIALS AND METHODS

Cells and cell culture. The culture of CEFs and their transformation by Schmidt-Ruppin subgroup A Rous sarcoma virus (RSV) and the culture of Rat-1 fibroblasts and their transformation by the *ts*LA29 RSV mutant have been described elsewhere (26).

cDNA cloning and sequencing. The lambda GT10 cDNA library from CEFs transformed by Schmidt-Ruppin subgroup A RSV was a kind gift from J. T. Parsons. Plasmid prGT4-12 was a kind gift of M. J. Birnbaum and consists of the full-length rat brain type 1 glucose transporter (RAT-GT1) cDNA cloned into the *Eco*RI site of pUC19 (2). The library was screened under conditions of reduced stringency (40% formamide–6× SSC [1× SSC is 150 mM NaCl plus 15 mM sodium citrate] hybridization at 42°C; 2× SSC wash at 50°C) with this 2.6-kb *Eco*RI fragment labelled by nick translation to high specific activity. The cDNA inserts from positive clones were cloned into the *Eco*RI site of pUC19. None of the initial clones were full length. One such clone was used to rescreen the library for longer clones under identical conditions except that washes were conducted at 60°C. Sequencing of the inserts was done by the dideoxynucleotide chain termination method, using the protocol described for double-stranded DNA in the Sequenase manual (United States Biochemical). Complete sequencing of both strands was accomplished after cloning of appropriate subfragments into pBS(+/−).

Northern analysis. Total RNA was isolated from cells and subjected to Northern blot analysis, using methods that we have previously described (26) with the following modifications. Hybridizations were carried out at 42°C in 6× SSC with 50% formamide, and filters were washed in 1× SSC at 67°C. Blots were probed with DNA labelled by nick translation to high specific activity. The probes used were (i) the 1.7-kb *Eco*RI fragment containing CEF-GT3; (ii) the 2.7-kb *Eco*RI insert of plasmid prGT4-12, which contains the rat type 1 glucose transporter cDNA (RAT-GT1) (2); (iii) pBR322 total plasmid; and (iv) a 1.1-kb *Pst*I fragment from pGAD3, which is from the chicken glyceraldehyde-3-phosphate dehydrogenase cDNA (7). The same filter could be probed more than once by stripping it before reprobing. This was done by washing the filter at 100°C in 0.01× SSC–0.1% sodium dodecyl sulfate. The efficacy of the stripping was monitored by autoradiography before reprobing.

Southern analysis of chicken genomic DNA. DNA was extracted from confluent cultures of CEFs and digested to completion with either *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, or *Not*I. Each digest (10 μg per lane) was loaded in duplicate on a 0.8% agarose gel. After electrophoresis, the DNA was fragmented, denatured, and transferred to a nitrocellulose filter, which was then baked and cut in half. One half was probed with CEF-GT3, and the other was probed with RAT-GT1. Preparation of probes and hybridization and washing conditions were the same as for Northern analysis.

Construction of a vector expressing the chicken transporter. Plasmid pGTDOL (which has the human glucose transporter cloned into the *Bam*HI site of the multiple cloning region of the expression vector pDOL) was the kind gift of Mike Mueckler and has previously been described (12, 29). Plasmid pGTDOL was cut with *Bam*HI, and the backbone was purified and religated to regenerate empty pDOL. To clone the CEF-GT3 cDNA into pDOL, the following strategy was used. Plasmid pUC19 containing the CEF-GT3 cDNA inserted at the *Eco*RI site was cut with *Bam*HI and *Sal*I (which flank the pUC19 *Eco*RI site), and the cDNA-containing fragment was purified. Plasmid pDOL was cut with *Bam*HI and *Sal*I, which cut at the multiple cloning site. The cDNA-containing *Bam*HI-*Sal*I fragment was then ligated with the cut pDOL, giving plasmid pDOL-cGT3, in which the CEF-GT3 cDNA fragment is cloned into the expression site of pDOL in the correct orientation.

Introduction of plasmids into rat cells. Plasmids pDOL-cGT3 and pDOL (control) were introduced into Rat-1 cells and Rat-1 cells transformed by the *ts*LA29 *src* mutant (Rat-1-*ts*LA29) in the following manner. Note that both plasmids have the gene for neomycin resistance driven by the simian virus 40 promoter and a site for retroviral packaging. Plasmid was introduced into the packaging cell line PA317 (18) with use of lipofectin (Bethesda Research Laboratories) as described in the accompanying technical manual; 10 μg of DNA was used per lipofection. After 2 days, the cells were subcultured into medium containing G418 (750 μg/ml). The resulting G418-resistant colonies were grown, and cultures were grown overnight in the absence of G418. Viral supernatants from these overnight cultures were filtered through 0.22-μm-pore-size filter and put onto Rat-1 cells or Rat-1-*ts*LA29 cells in the presence of Polybrene (3 μg/ml). After 2 days, the cells were subcultured into medium containing G418 at 500 μg/ml. The resulting G418-resistant colonies were grown and used in subsequent experiments.

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession number M37785.

RESULTS

Isolation and sequences of CEF glucose transporter clones. Initially, four clones were isolated from an RSV-transformed CEF library by low-stringency cross-hybridization with a fragment containing the rat brain glucose transporter gene (2). The sequences of these clones indicated that none of them contained the complete protein-coding region of the mRNA. The library was rescreened by using one of these clones as a probe under more stringent hybridization conditions, and a full-length clone was subsequently isolated. This clone contains the complete protein-coding region and 85 nucleotides of the 5′ noncoding region of the mRNA (Fig. 1). The sequences of all clones agreed in overlapping regions, showing them to be derived from the same mRNA. Figure 1 shows the sequence of the 1,653-nucleotide cDNA which contains a 1,488-nucleotide open reading frame. The predicted amino acid sequence of the 496-residue CEF-GT3 protein (M_r = 54,117) has 72.6% sequence identity with the human GLUT3 glucose transporter protein, which also has 496 amino acids (Fig. 2). It also has sequence identity with the other types of human glucose transporters but to a lesser degree (68.5, 57.9, and 45.6% to GLUT1, GLUT4, and GLUT2, respectively). The CEF-GT3 protein is likely to have a structure similar to that proposed for the mammalian transporters (19). Hydropathic and secondary structure analyses suggest the presence of 12 hydrophobic transmembrane domains (designated M1 to M12 in Fig. 2) with a predominantly alpha-helical secondary structure, an extracellular loop between M1 and M2 with a potential N-glycosylation site, and a large intracellular hydrophilic loop in the center of the protein. Between amino acids 63 to 90 of CEF-GT3 is a leucine zipper motif also found in other glucose transporters (28) and other membrane proteins (5, 17), although the significance of such motifs in membrane proteins is thus far unknown (4).

Expression of CEF-GT3 in rat cells. CEF-GT3 cDNA was

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   1  GGCGGTGCGTGCGCGTGGGCGGAGTACCGTACCGTTCGGGTTCCGTTAGGCTGGGTGTCG

                                    MetAlaAspLysLysLysIleThrAlaSerLeuIle
  61  GTGCTGGAGCAGTTCGCCGCAGGAGATGGCTGACAAGAAGAAAATCACTGCATCCCTTAT

       TyrAlaValSerValAlaAlaIleGlySerLeuGlnPheGlyTyrAsnThrGlyValIle
 121  CTATGCTGTTTCTGTTGCTGCCATTGGATCTCTCCAGTTTGGGTACAACACTGGTGTCAT

       AsnAlaProGluLysIleIleGlnAlaPheTyrAsnArgThrLeuSerGlnArgSerGly
 181  TAATGCTCCTGAGAAGATCATCCAGGCCTTCTACAACAGGACTTTATCTCAGAGGAGTGG

       GluThrIleSerProGluLeuLeuThrSerLeuTrpSerLeuSerValAlaIlePheSer
 241  GGAGACTATCTCCCCAGAGCTTCTTACCTCACTATGGTCCCTTTCTGTGGCAATCTTCTC

       ValGlyGlyMetIleGlySerPheSerValSerLeuPheValAsnArgPheGlyArgArg
 301  AGTAGGAGGTATGATTGGCTCCTTCTCAGTCAGCCTTTTCGTCAATCGATTTGGCAGGAG

       AsnSerMetLeuLeuValAsnValLeuAlaPheAlaGlyGlyAlaLeuMetAlaLeuSer
 361  AAACTCCATGCTGTTGGTGAATGTCTTGGCTTTTGCTGGGGGTGCTCTCATGGCCTTATC

       LysIleAlaLysAlaValGluMetLeuIleIleGlyArgPheIleIleGlyLeuPheCys
 421  CAAGATTGCAAAGGCAGTGGAGATGCTGATAATTGGGCGCTTCATTATTGGCCTTTTCTG

       GlyLeuCysThrGlyPheValProMetTyrIleSerGluValSerProThrSerLeuArg
 481  TGGTCTGTGCACTGGCTTTGTTCCCATGTACATCAGTGAGGTCTCACCCACCAGCCTTCG

       GlyAlaPheGlyThrLeuAsnGlnLeuGlyIleValValGlyIleLeuValAlaGlnIle
 541  TGGAGCCTTTGGAACCCTCAACCAGCTGGGCATAGTTGTAGGCATCCTGGTGGCCCAGAT

       PheGlyLeuGluGlyIleMetGlyThrGluAlaLeuTrpProLeuLeuLeuGlyPheThr
 601  TTTTGGCCTCGAGGGGATAATGGGGACTGAAGCACTTTGGCCACTGCTGCTGGGGTTCAC

       IleValProAlaValLeuGlnCysValAlaLeuLeuPheCysProGluSerProArgPhe
 661  GATTGTTCCAGCAGTTCTGCAGTGTGTGGCTCTTCTTTTCTGCCCTGAGAGCCCCCGTTT

       LeuLeuIleAsnLysMetGluGluGluLysAlaGlnThrValLeuGlnLysLeuArgGly
 721  CCTGTTGATCAACAAGATGGAGGAAGAAAAAGCACAAACTGTTCTTCAAAAGCTCCGTGG

       ThrGlnAspValSerGlnAspIleSerGluMetLysGluGluSerAlaLysMetSerGln
 781  TACACAGGATGTATCTCAAGACATCTCAGAGATGAAAGAAGAGAGTGCTAAAATGTCTCA

       GluLysLysAlaThrValLeuGluLeuPheArgSerProAsnTyrArgGlnProIleIle
 841  GGAAAAGAAAGCCACTGTACTAGAGCTATTCCGCTCTCCAAACTATCGTCAACCCATTAT

       IleSerIleThrLeuGlnLeuSerGlnGlnLeuSerGlyIleAsnAlaValPheTyrTyr
 901  CATTTCCATCACACTGCAGCTCTCTCAGCAGCTCTCAGGCATCAATGCTGTATTCTATTA

       SerThrGlyIlePheGluArgAlaGlyIleThrGlnProValTyrAlaThrIleGlyAla
 961  TTCTACAGGGATTTTTGAAAGAGCTGGTATCACACAGCCTGTGTATGCCACCATTGGAGC

       GlyValValAsnThrValPheThrValValSerLeuPheLeuValGluArgAlaGlyArg
1021  TGGTGTGGTAAACACGGTCTTCACTGTTGTGTCACTGTTCCTGGTAGAGCGTGCAGGGCG

       ArgThrLeuHisLeuValGlyLeuGlyGlyMetAlaValCysAlaAlaValMetThrIle
1081  CAGGACCCTCCATTTAGTTGGTTTGGGTGGCATGGCTGTGTGTGCTGCTGTTATGACTAT

       AlaLeuAlaLeuLysGluLysTrpIleArgTyrIleSerIleValAlaThrPheGlyPhe
1141  TGCTTTGGCTCTGAAGGAAAAGTGGATCAGATATATCAGCATTGTTGCCACTTTTGGCTT

       ValAlaLeuPheGluIleGlyProGlyProIleProTrpPheIleValAlaGluLeuPhe
1201  TGTGGCCCTTTTTGAGATTGGCCCTGGACCTATCCCTTGGTTCATTGTGGCAGAACTTTT

       SerGlnGlyProArgProAlaAlaMetAlaValAlaGlyCysSerAsnTrpThrSerAsn
1261  CAGTCAAGGCCCACGGCCTGCAGCCATGGCGGTGGCTGGTTGTTCTAACTGGACCTCTAA

       PheLeuValGlyMetLeuPheProTyrAlaGluLysLeuCysGlyProTyrValPheLeu
1321  TTTCTTAGTGGGAATGCTCTTCCCCTATGCTGAGAAATTATGTGGCCCCTATGTCTTCCT

       IlePheLeuValPheLeuLeuIlePhePheIlePheThrTyrPheLysValProGluThr
1381  TATCTTCCTTGTTTTCCTGCTCATCTTCTTCATATTCACATACTTCAAAGTGCCAGAGAC

       LysGlyArgThrPheGluAspIleSerArgGlyPheGluGluGlnValGluThrSerSer
1441  CAAGGGCAGGACTTTTGAAGATATCTCCAGGGGCTTTGAAGAACAAGTAGAAACAAGCTC

       ProSerSerProProIleGluLysAsnProMetValGluMetAsnSerIleGluProAsp
1501  CCCATCCTCACCACCTATAGAGAAGAACCCCATGGTGGAGATGAACAGCATAGAACCTGA

       LysGluValAlaOC
1561  CAAAGAAGTTGCCTAAGATTCATGACACACCCTTTCTTTGACTCTTGCATATTCATAGAG

1621  TCATTAAAGGAATGAGCAAAGAAAAAAAAAAAA
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FIG. 1. Nucleotide sequence of CEF-GT3 and amino acid sequence of the predicted CEF-GT3 protein. The number of the nucleotide at the start of each line is indicated. The nucleotide sequence is that of the longest clone. This sequence is in agreement with the sequence of the incomplete clones.

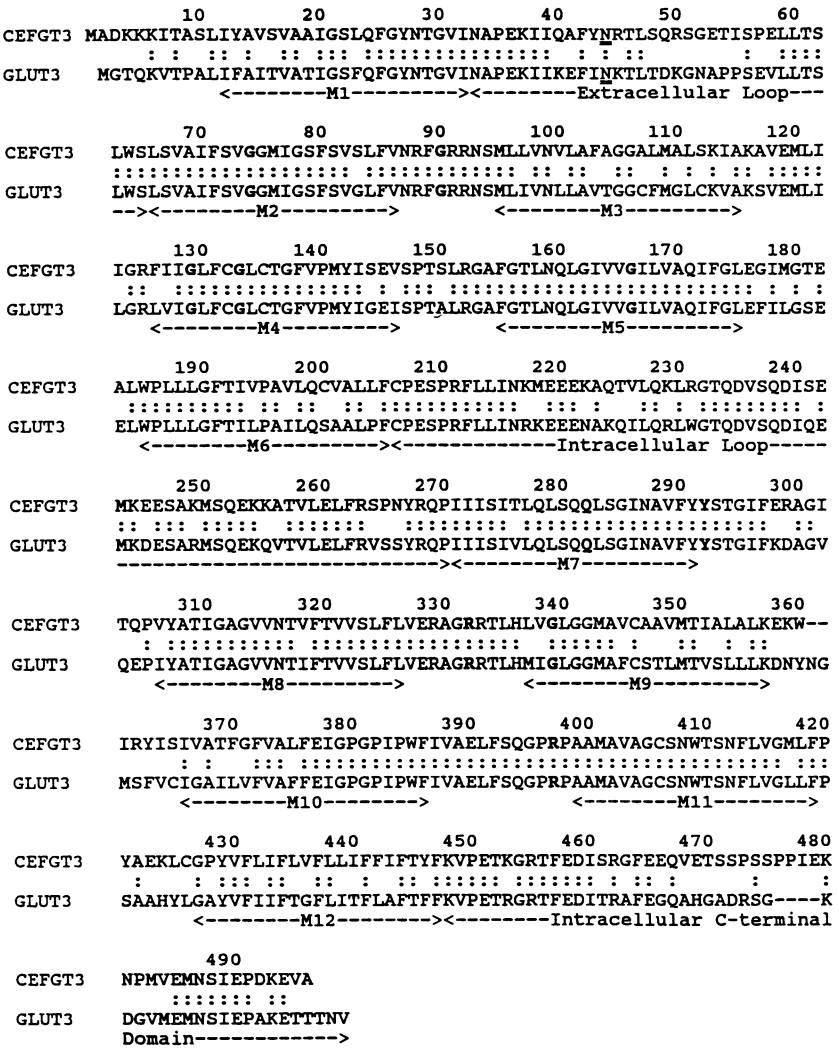

FIG. 2. Comparison of CEF-GT3 with human GLUT3. The amino acid sequences of CEF-GT3 and human GLUT3 (16) were aligned by the Fasta method of Pearson and Lipman (20). Colons connect the 360 identical residues. The 12 predicted transmembrane regions are labelled M1 to M12, and other domains are indicated. The asparagine residue which is a potential glycosylation site in the extracellular loop (between M1 and M2) is doubly underlined. Ten amino acid residues that are conserved in the sugar transporter superfamily are indicated in bold type (15).

ligated into the multiple cloning site of the pDOL retroviral expression vector to make plasmid pDOL-cGT3 (Fig. 3), and this plasmid was introduced into Rat-1 fibroblasts. Plasmid pDOL was also introduced into these cells as a control. Table 1 shows the relative uptakes of 2-deoxyglucose by the infected cells. Introduction of pDOL-cGT3 gave a 3.7-fold increase in uptake relative to the pDOL control in Rat-1 cells.

Both plasmids were also introduced into Rat-1 cells transformed by the temperature-sensitive *src* mutant *ts*LA29 (Rat-1-*ts*LA29). In these cells, expression of the endogenous type 1 transporter, RAT-GT1, is dependent on *src* activity and is thus itself temperature sensitive (26). At the nonpermissive temperature of 39.5°C, at which the level of endogenous RAT-GT1 message is low, there is a fourfold-higher rate for pDOL-cGT3 than for pDOL (26). At 35.5°C, the RAT-GT1 mRNA is induced (26) and both the pDOL and pDOL-cGT3 derivatives have high rates of 2-deoxyglucose uptake.

These data strongly indicate that CEF-GT3 is a functional facilitative glucose transporter. Similar results have been obtained when pDOL was used to express human GLUT1 in rat cells (29a).

Northern analyses of RNA from CEFs and Rat-1 derivatives. Figure 4 shows four Northern blot analyses, each represented by a separate panel. The same filter was used in each hybridization, and thus in each panel lanes represent the same RNA hybridized with a different probe. RNA samples were derived from CEFs, RSV-transformed CEFs, Rat-1 × pDOL, and Rat-1 × pDOL-cGT3. These samples were blotted with CEF-GT3, RAT-GT1, pBR322, and G3PDH.

The data demonstrate a number of important points. First, there are two messages corresponding to CEF-GT3 in CEFs. One is 1.7 kb (corresponding to the length of the CEF-GT3 cDNA), and the other is about 3.3 kb. Kayano et al. have demonstrated two mRNAs for human GLUT3, resulting from alternative sites of polyadenylation (16). Second, both messages are greatly induced by RSV. Third, there is no

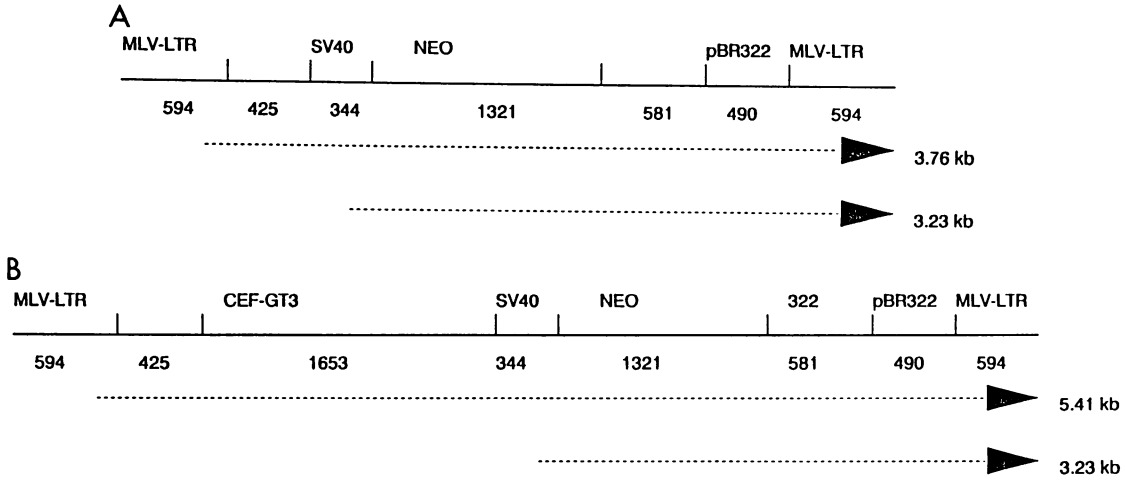

FIG. 3. Transcripts from plasmids pDOL and pDOL-cGT3. The maps of plasmids pDOL (A) and pDOL-cGT3 (B) and their transcripts are shown. The solid lines represent the plasmid DNAs, and the different regions are labelled. The numbers beneath the regions indicate their sizes in nucleotides. Both plasmids produce two transcripts, which are indicated by arrows. The shorter one begins at the simian virus 40 promoter and is 3.23 kb in length. The longer one begins at the Moloney leukemia virus long terminal repeat (MLV-LTR) and is 3.76 kb in pDOL and 5.41 kb in pDOL-cGT3 as a result of insertion of the CEF-GT3 cDNA at the multiple cloning site 425 nucleotides downstream of the Moloney leukemia virus long terminal repeat.

cross-hybridization of mRNAs of different isotype under the high-stringency conditions used. Figure 3 shows a schematic diagram of the transcripts expected in Rat-1 cells infected with pDOL or pDOL-cGT3. Figure 4 shows that the CEF-GT3 probe recognizes the expected 5.4-kb retroviral pDOL-cGT3 transcript. However, no signal is seen for the Rat-1 × pDOL control RNA, indicating that CEF-GT3 does not cross-hybridize with endogenous RAT1-GT1 mRNA. The converse is also true: RAT-GT1 probe recognizes the endogenous 2.7 kb RAT-GT1 mRNA in both rat cell derivatives but does not cross-hybridize to the 5.4-kb mRNA of Rat-1 × pDOL-cGT3. Even at long autoradiography exposure times, no cross-hybridization could be detected between isotypes. Fourth, the RAT-GT1 probe detects the 3.3-kb type 1 homolog in CEF DNA, and this mRNA is not substantially induced by RSV transformation. We have previously reported this result with use of a human GLUT1 probe (26).

Probing with pBR322 (which is downstream of both pDOL promoters; Fig. 3) demonstrated that transcripts of the predicted sizes (Fig. 3) were present in both of the Rat-1 derivatives. Fig. 4D shows the filter probed with chicken G3PDH as a loading control. Since rat G3PDH has 85% sequence identity to chicken G3PDH, the rat RNA lanes

TABLE 1. Uptake of 2-deoxyglucose by Rat-1 and Rat-1-*ts*LA29 derivatives[a]

Cell type	Relative uptake	
	39.5°C	35.5°C
Rat-1 derivatives		
Rat-1 × pDOL	1.0	
Rat-1 × pDOL-cGT3	3.7	
Rat-1-*ts*LA29 derivatives		
Rat-1-tsLA29 × pDOL	1.0	4.4
Rat-1-tsLA29 × pDOL-cGT3	4.0	5.5

[a] Measured as previously described (25); expressed as the amount of tritiated 2-deoxyglucose taken up per minute per milligram of cell protein relative to the values for controls, which are Rat-1 × pDOL in the case of the Rat-1 derivatives and Rat-1-*ts*LA29 × pDOL grown at 39.5°C in the case of the Rat-1-*ts*LA29 derivatives.

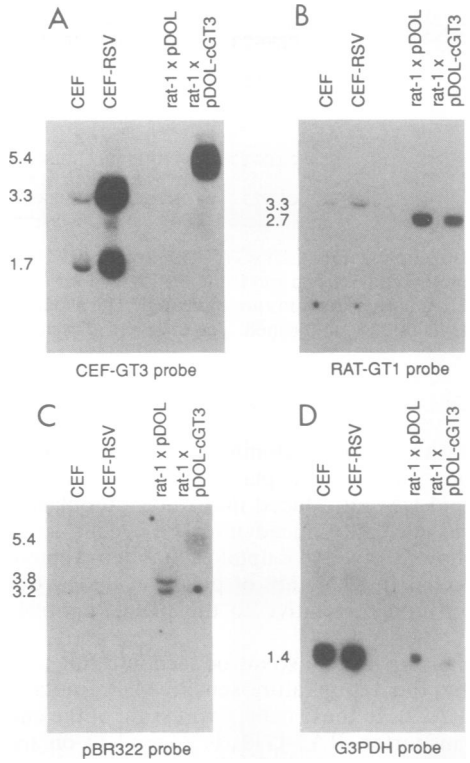

FIG. 4. Identification of mRNAs by Northern blot hybridization. RNA from the following cells was run on a gel and transferred to a nitrocellulose filter: CEFs, CEFs transformed by RSV (CEF-RSV), rat-1 × pDOL, and rat-1 × pDOL-cGT3. Northern hybridization of the filter was done sequentially with the probes indicated below the panels. Thus, each of the panels represent the same filter hybridized with a different probe. Sizes of the mRNAs are given in kilobases.

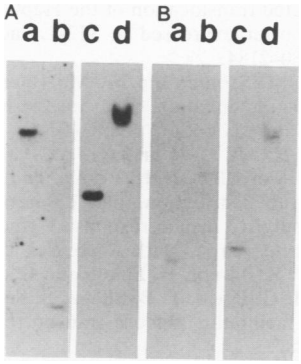

FIG. 5. Southern blot analysis of chicken genomic DNA. Genomic DNA was isolated from CEFs and digested with *Bam*HI (lane a), *Bgl*II (lane b), *Hin*dIII (lane c), and *Not*I (lane d). The digests were run in duplicate on an agarose gel and transferred to a nitrocellulose filter, which was then cut in half. One half was probed with CEF-GT3 (A), and the other was probed with RAT-GT1 (B). The autoradiography exposure time of panel B was twice that of panel A.

gave a lower signal than did the chicken RNA lanes. Loading of chicken RNA is comparable to that of the rat RNA. There is somewhat more signal for Rat-1 × pDOL than for Rat-1 × pDOL-cGT3, indicating higher loading in this lane. Thus, the absence of signal for the CEF-GT3 probe (Fig. 4A) is not due to Rat-1 × pDOL RNA being underloaded compared with Rat-1 × pDOL-cGT3 RNA.

Southern analysis. Figure 5 shows a Southern blot of chicken genomic DNA cut with five different restriction endonucleases. Each restriction digest was run in duplicate, and the filter was cut in half and probed with either CEF-GT3 (Fig. 5A) or RAT-GT1 (Fig. 5B). The patterns seen on the two halves of the filter are clearly different. For example, the CEF-GT3 gene is on a single *Bam*HI fragment (Fig. 5A, lane a) but is cut by *Bgl*II (lane b), whereas the chicken type 1 homolog is on a single *Bgl*II fragment (Fig. 5B, lane a) but is cut by *Bam*HI (lane b). This confirms the Northern assay data; i.e., the two probes are recognizing two distinct genes.

DISCUSSION

In earlier work, we investigated the regulation of glucose transporters in CEFs by using human GLUT1 cDNA to measure mRNA levels and antibody raised against the human erythrocyte (GLUT1) glucose transporter to measure transporter protein (26, 27). We observed that the mRNA level and protein biosynthetic rate were unaltered by *src* transformation, whereas turnover of protein was inhibited. This finding is in contrast to observations for rodent cells, in which *src* increases the level of the rat type 1 transporter (RAT-GT1) mRNA and the rate of RAT-GT1 protein biosynthesis. To further understand the molecular basis of the difference in regulation found in CEFs, it was necessary to clone chicken glucose transporters. This has led to the unexpected discovery of a transporter, CEF-GT3, which is distinct from the chicken type 1 homolog that we have previously described (26). The two types of chicken transporter can be clearly distinguished by both Northern blot analysis of cellular RNA and Southern blot analysis of genomic DNA with use of a type 1 (RAT-GT1) or type 3 (CEF-GT3) probe, and there is no question that they are distinct. Moreover, the two transporter isoforms differ in their mode of regulation by *src*. As discussed above, the type 1 transporter is regulated by *src* at the level of protein turnover, and its mRNA level is unaffected by transformation. Thus, the data in Fig. 4, which show the chicken type 1 transporter measured with RAT-GT1, confirm the data in our earlier study (26), in which we probed chicken RNA with a human hepatoma (GLUT1) cDNA probe. In contrast, the levels of the two CEF-GT3 mRNAs are greatly increased by *src*. Northern analyses of chicken RNAs with these two probes have been performed many times with both total and poly(A)-selected RNA preparations, and these results are completely reproducible with different preparations of CEFs.

Since the level of CEF-GT3 mRNAs is induced by *src*, it is therefore likely that CEF-GT3 protein biosynthesis is also increased. We are currently raising antisera against CEF-GT3 to measure this. Interestingly, CEF-GT3 gives two mRNAs in Northern blots: one corresponding to the length of the CEF-GT3 cDNA (1.7 kb) and a larger, 3.3-kb message. This is similar to what has been found for human GLUT3, in which 2.7- and 4.1-kb mRNAs arise from the same gene by alternative polyadenylation. In CEF-GT3, the two mRNAs arise from the same gene, since only one band is seen in the Southern blot of genomic DNA digested with some restriction endonucleases (Fig. 5) and thus alternative polyadenylation might also be the mechanism in chicken cells, although other mechanisms such as alternative splicing cannot be ruled out.

When RNA from rat cells is probed with CEF-GT3, no signal corresponding to rat type 3 mRNA is seen (Fig. 4). Recently we have measured the level of endogenous rat type 3 mRNA by using a mouse type 3 probe (1). The level of type 3 mRNA in Rat-1 cells is very low, and it is not induced by transformation by wild-type *src* or by shifting cells transformed by a temperature-sensitive *src* mutant (*ts*LA29) to the permissive temperature (29a).

The chicken type 1 homolog is 3.3 kb in size, but it is clearly distinct from the 3.3-kb type 3 mRNA in its specificity of probe binding in Northern blots and its lack of induction by *src* transformation. Furthermore, the gene from which it is transcribed can be distinguished from the chicken type 3 gene by Southern analysis of chicken genomic DNA (Fig. 5). The actual level of the chicken type 1 mRNA relative to type 3 is not known, since only mammalian type 1 cDNA probes are available to measure it and the degree of sequence conservation is not known. We are currently isolating cDNA clones corresponding to this mRNA by using higher-stringency hybridization to the rat type 1 probe and a lambda GT10 library derived from untransformed CEFs (in which the ratio of type 1 to type 3 mRNAs is much higher). Recently we have isolated a cDNA corresponding to the chicken type 1 isoform, and we are currently sequencing it. It is perhaps surprising that only type 3 clones were obtained in the first screenings with type 1 probe. However, the library that we screened was derived from transformed CEFs, and since *src* massively induces type 3 but not type 1 mRNA, the former would be much better represented in the library. Furthermore, we used lower-stringency conditions when screening these plaque lifts, and the RAT-GT1 probe recognizes chicken type 3 plaques under these conditions.

Thus, two mechanisms of control of glucose transporters by *src* exist in CEFs: (i) an inhibition of type 1 transporter turnover (23, 26, 29) and (ii) an induction of type 3 mRNA. The relative contributions of these two phenomena to the *src*-induced increase in glucose transport by CEFs is not yet known, since the relative expression of the two transporters is not known. We are currently attempting to generate

specific antisera to these transporter isoforms in order to further investigate their regulation. The control of glucose transport in CEFs is thus different from that in mammalian cells, in which *src* induces transcription of the type 1 isoform (8, 26, 30). The molecular basis for the differing forms of regulation and for the species-specific differences in regulation of the GLUT1 genes will require determination of the sequence of the CEF GLUT1 gene and a comparison of its promoter region with that of the rat GLUT1 gene (30). Finally, it can be generally concluded that an oncogene can regulate a single cellular process through an effect on more than one regulatory modality.

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