family or to a differentially spliced single gene, and that there is a functional and structural relation between the low and high molecular weight BCGF moieties.

Note added in proof: Recent results from Caras *et al.* (*21*) indicate the presence of Alu-like sequences within the coding region of the nucleotide sequence for one form of decay-accelerating factor.

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9. Relative size determinations for BCGF mRNA were made by sizing total poly(A)$^+$ mRNA in association with in vitro translation. Normal human T cells (500×10^6), in the presence of monocytes (5%), were stimulated with PHA (0.75%) for 18 hours at a density of 2×10^6 cells per milliliter. Total RNA was isolated by CsCl gradient centrifugation in the presence of guanidinium isothiocyanate. Poly(A)$^+$ mRNA was isolated by elution of total RNA through an oligo-dT cellulose column. Total RNA (300 μg) routinely contributed 10 μg of poly(A)$^+$ RNA. Poly(A)$^+$ mRNA (60 μg) was size-fractionated on a 1% low-melting agarose gel in the presence of 10 m*M* methylmercuric hydroxide. The RNA was eluted from each sliced gel fraction and injected into *Xenopus laevis* oocytes. Supernatants conditioned by the injected oocytes were assayed for BCGF and TCGF (T-cell growth factor) activity at multiple dilutions.
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Elevated Levels of Glucose Transport and Transporter Messenger RNA Are Induced by *ras* or *src* Oncogenes

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An accelerated rate of glucose transport is among the most characteristic biochemical markers of cellular transformation. To study the molecular mechanism by which transporter activity is altered, cultured rodent fibroblasts transfected with activated *myc*, *ras*, or *src* oncogenes were used. In *myc*-transfected cells, the rate of 2-deoxy-D-glucose uptake was unchanged. However, in cells transfected with activated *ras* and *src* oncogenes, the rate of glucose uptake was markedly increased. The increased transport rate in *ras*- and *src*-transfected cells was paralleled by a marked increase in the amount of glucose transporter protein, as assessed by immunoblots, as well as by a markedly increased abundance of glucose transporter messenger RNA. Exposure of control cells to the tumor-promoting phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) for 18 hours had a similar effect of increasing the rate of glucose transport and the abundance of transporter messenger RNA. For *ras*, *src*, and TPA, the predominant mechanism responsible for activation of the transport system is increased expression of the structural gene encoding the glucose transport protein.

SINCE THE EARLY OBSERVATIONS OF Warburg (*1*), it has been widely appreciated that most tumor cells display increased rates of respiration, glucose uptake, and glucose metabolism as compared to untransformed cells. An accelerated rate of glucose transport is among the most characteristic biochemical markers of the transformed phenotype (*2, 3*). The molecular mechanisms responsible for the accelerated rate of glucose transport in transformed cells are unknown, but have been the subject of considerable inquiry.

When chicken embryo cells are infected with the Rous sarcoma virus (RSV), there is a marked increase both in the rate of glucose transport (*4, 5*) and in the abundance of glucose transport proteins in the plasma membrane (*6*). Substantial evidence indicates that these changes in glucose transport are independent of growth rate and are transformation specific (*5*). Although these changes are dependent on the activity of the viral transforming protein $pp60^{v\text{-}src}$ (*4, 7*), the molecular mechanism through which this transforming protein influences the number or activity (or both) of functional glucose transporters is unknown. In contrast to these findings with RSV, evidence obtained with another transforming virus, simian virus 40 (SV40), indicates that the increased transport rate may be caused, not by a change in the number of transporters, but by a functional change in transporters, including a redistribution to the plasma membrane (*8*).

Glucose transport in both normal and transformed fibroblasts is a facilitated diffusion, Na^+-independent process (*9*). These characteristics are similar to those of the erythrocyte glucose transporter (*9*); we have recently cloned the complementary DNA (cDNA) encoding this transporter (*10*) and have demonstrated its expression in many tissues and lines of cultured cells (*11*).

To explore the relation of transformation and glucose transport, we have examined

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Fisher rat 3T3 (FR3T3) fibroblasts into which were introduced by transfection one of three activated oncogenes (*myc*, *ras*, or *src*). Each of these oncogenes has a distinct gene product with unique biochemical properties responsible for transformation (*12*). In addition, we studied the effect of the tumor-promoting phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which activates protein kinase C (*13*).

Immortal FR3T3 cells were transfected with an activated *myc* oncogene using the vector pSV_c-myc-1, which contains the second and third exons of a translocated mouse c-*myc* gene under control of SV40 early promoter (*14*). The FR3T3 cells were also transfected with an activated *ras* oncogene, by means of pEJ6.6 (*15*). Rat embryo fibroblast (REF) cells were transfected with the v-*src* oncogene (*16*). The nonselected plasmids were added to cells in tenfold molar excess over pSV2neo, which contains a G418 resistance gene. G418-resistant clones carrying pSV2neo and the concomitantly introduced oncogene were selected and passaged as stable cell lines (*17*).

The cell clone *myc*-1 was a representative *myc*-transfected cell line, having epithelial morphology with well-defined cell margins and a polygonal shape (*16*). These cells were smaller than control fibroblasts. Both *ras*-3 cells and *src*-transfected cells displayed a characteristic refractile morphology. *Neo*-1 cells, having been transfected only with a G418 resistance marker, were morphologically similar to control cells.

Cells transfected with activated *ras* and *src* oncogenes displayed markedly increased rates of 2-deoxyglucose (2-DOG) uptake, whereas *myc*-transfected cells had an unchanged glucose transport rate (Fig. 1). Since glucose starvation is known to increase the rate of glucose transport into cultured cells (*18, 19*), we supplemented the media with glucose every 24 hours to avoid glucose depletion. By measuring the concentration of glucose in the medium, we determined that a glucose concentration sufficient to induce an increased glucose transport rate (less than 4 m*M*) was never reached.

We made immunoblots of membranes prepared from these cells by means of an antibody directed against the human erythrocyte transporter (*20*). A marked increase occurred in transporter protein in *ras*- and *src*-transfected cells (Fig. 2), but no change was observed in *myc*-transfected cells. The glucose transporter is known to be heterogeneously glycosylated and to exhibit a heterodisperse molecular weight distribution on sodium dodecyl sulfate (SDS) gels (*20*). This heterogeneity appears to be accentuated in *ras*- and *src*-transfected cells, although further investigation of this point is necessary. The prominent narrow band that we observed at approximately 20 kD has been observed by others in immunoblots of membranes from rat adipose cells under conditions where glucose transport has been stimulated by nutritional or hormonal perturbations (*21*), and we suspect that this represents a transporter degradation product.

When total cellular RNA from control and oncogene-transfected cells was probed on Northern blots with the human (Hep G2 cell) glucose transporter cDNA (*10*), results parallel to those obtained for transport and immunoblots were obtained (Fig. 1). Compared to control and *myc*-transfected cells, the *ras*- and *src*-transfected cells had a marked increase in the abundance of the single 2.8-kb messenger RNA (mRNA) transcript that encodes the human glucose transporter. The extent of this increase was of a magnitude similar to that observed for 2-DOG transport, suggesting that the predominant mechanism for transport activation in these cells is likely to reside at the level of transporter mRNA. Whether the increase in mRNA abundance is due to an increased rate of gene transcription or to increased mRNA stability has not yet been determined.

The *ras*- and *src*-transfected cells grew more rapidly and to a somewhat greater density than control or *myc* cells. We concluded that the increased transport rate in these cells was not dependent on higher cell density, however, since it was observed when cells were studied at equivalent density. Whether the transport system is affected by the rate of cell growth is a related and very interesting question. Since the normal cellular homologs of *ras*, *src*, and a variety of other oncogenes are thought to be intimately involved in growth control, it would not be surprising for cells whose growth is stimulated by serum or growth factors to have increased amounts of glucose transporter and transporter mRNA. In fact, our preliminary data suggest that this is the case.

Thus, the glucose transport system is affected by *ras* and *src* but not by the *myc* oncogene. *Ras* and *src* represent one class of oncogenes and *myc*, another class, with distinct effects on cell phenotype (*14, 22*).

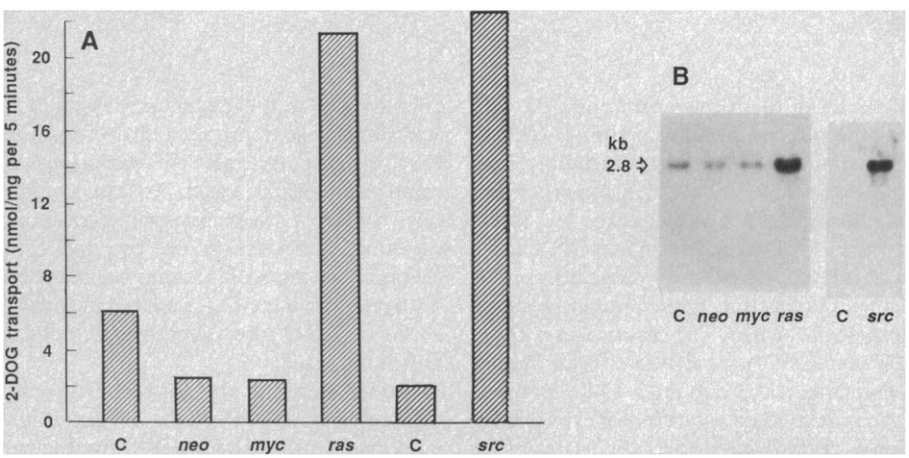

Fig. 1. The effect of transformation by *myc*, *ras*, or *src* oncogenes on glucose transport and transporter mRNA. Cells were transfected with oncogene-containing plasmids in tenfold molar excess over pSV2-neo, which contains a G418 resistance gene, as described (*17*). G418-resistant clones carrying pSV2-neo and the concomitantly introduced oncogene were selected and passaged as stable cell lines. Cells were grown to confluence in multiwell plates (Costar) in Dulbecco's minimum essential medium (DMEM) with 10% calf serum. For transport studies (**A**), cells were washed three times at room temperature with phosphate-buffered saline (PBS), and then incubated for 5 minutes at 37°C in glucose-free DMEM containing 0.1% fatty acid–free bovine serum albumin (Pentex) and 100 n*M* 2-DOG. After 5 minutes, 2×10^5 cpm of [^{3}H]2-DOG (specific activity, 5 mCi/mmol, New England Nuclear) was added. Uptake was stopped at 5 minutes by addition of cold PBS with 0.3 m*M* phloretin. Uptake was linear up to 10 minutes for all cell lines and was expressed as nanomoles of 2-DOG taken up per milligram protein per 5 minutes. C, control. Total cellular RNA was extracted by cesium chloride centrifugation after treatment with guanidinium isothiocyanate (*42*), and 10-μg aliquots were then separated on 1.2% formaldehyde agarose gels (*43*). After Northern transfer to a nylon filter, hybridization was carried out (**B**) to glucose transporter or, on a separate occasion, to a tubulin cDNA probe. The latter demonstrated an equivalent expression of this mRNA in the control and transfected cells. The glucose transporter cDNA probe is a mixture of two ^{32}P-labeled nick-translated glucose transporter (GT) cDNA fragments. These are 450-bp (pGT 25S) and 2400-bp (pGT 25L) Eco RI fragments that together contain nearly a full-length copy of the mRNA (*10*). Hybridization was carried out at 42°C in 50% formamide, 5× SSPE (0.9*M* NaCl, 50 m*M* NaH_2PO_4, 5 m*M* EDTA, *p*H 7.4), 0.2% SDS, 0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll and 200 μg/ml denatured, sheared salmon sperm DNA. The probes were included at 10^7 cpm/ml (glucose transporter) and 10^6 cpm/ml (tubulin). After being washed in 0.1× SSPE at 50°C (three washes of 30 minutes each), the blot was exposed to Kodak XAR-5 film at −70°C for 18 hours. C, control.

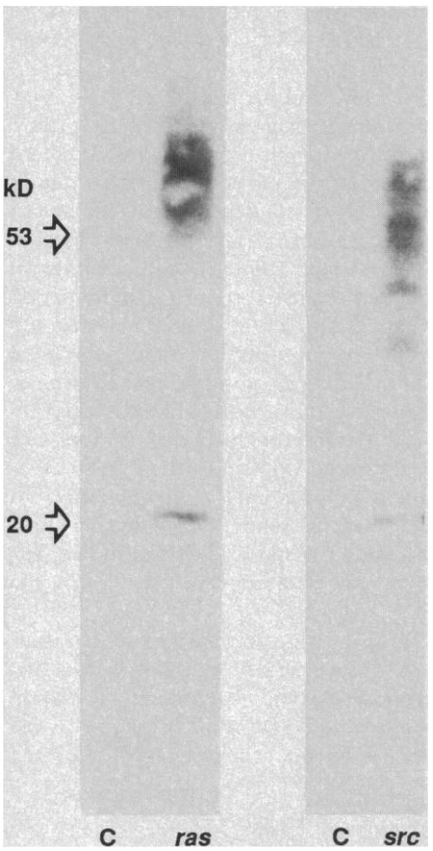

Fig. 2. Microsomal membranes were prepared from confluent cells that had been scraped from plates, washed with ice-cold PBS, and resuspended in 10 m*M* Hepes and 0.25*M* sucrose, *p*H 7.3. After 10 minutes at 4°C, the cells were disrupted with a Dounce homogenizer in the presence of aprotinin. The mixture was centrifuged at 600*g* for 10 minutes at 4°C, and the supernatant was then centrifuged at 200,000*g* at 4°C for 15 minutes in a Beckman TL-100 centrifuge. The membrane pellet was resuspended in the following solubilization cocktail for 1 hour at 4°C: 1% deoxycholate, 1% Triton X-100, 1 m*M* EDTA, 0.2 trypsin inhibitor units aprotinin and 150 m*M* NaCl, *p*H 7.4. The mixture was centrifuged at 12,000*g* at 4°C for 10 minutes, and the protein concentration of the solubilized membranes was determined. Membrane protein was diluted with sample buffer (8*M* urea, 5% SDS, 0.5*M* β-mercaptoethanol, 250 m*M* tris-HCl, *p*H 6.8) without boiling and subjected to SDS–polyacrylamide gel electrophoresis on a 10% gel (*44*). Control and *ras* lanes had 42 μg of protein, and control and *src* had 18 μg of protein. The proteins in the gels were electrophoretically transferred to nitrocellulose paper, reacted with a 1:200 dilution of antibody to the transporter protein (*21*), and then reacted with ^{125}I-labeled protein A. Dried blots were autoradiographed for 4 hours at −20°C with a Dupont Cronex intensifying screen.

Members of the former class cause classical morphological transformation, whereas members of the latter class (including *myc*, *myb*, polyoma large T, and adenovirus E1a) promote transformation weakly but facilitate the establishment of immortal cell lines.

These differences reflect differences in the nature and location of the protein products of these oncogenes. The products of both *src* (*23*) and *ras* (*24*) are associated with the cell membrane, and are thought to be involved in transduction or regulation of extracellular signals via tyrosine kinase (*25*) or guanosine 5′-triphosphate (GTP)–binding and regulatory activity (*26, 27*), respectively. Genes of the *myc* class specify nuclear proteins that are thought to act by regulating the expression of other genes (*28, 29*). On this basis, it could be viewed as surprising that *ras* and *src*, both of which encode proteins potentially poised to interact with the glucose transport system at the membrane level, each activate glucose transport, but do so at the level of transporter mRNA, whereas *myc* is completely without effect. At least as indicated by the glucose transport activation, the subcellular location and proximal biochemical function of proteins encoded by oncogenes are imperfect guides to the mechanism by which they alter specific cellular functions.

The transforming proteins encoded by the activated versions of cellular *ras* genes are implicated in the etiology of many human tumors (*30*). Thus, the present results may offer an explanation for the high level of glucose transporter mRNA in the human hepatoma cell Hep G2 (from which we cloned the transporter), whereas the mRNA is very low or absent in human and rat liver (*11*). Although the function of the *ras* protein in mammalian cells is not yet known, a role in transmembrane signal transduction is suggested by its functional and sequence homology to the family of GTP-binding proteins (*26*). Recent studies suggest a role for the *ras* and *src* proteins in the control of phosphatidylinositol turnover (*31–34*). Given these observations, we considered it possible that the tumor-promoting phorbol ester TPA, which mimics diacylglycerol in binding to and activating protein kinase C (*13, 35*), might mimic the action of *ras* to activate the glucose transport system. There are some reports that, in several cell types, TPA and diacylglycerol increase the rate of glucose transport within 2 minutes (*36–38*). The rapid nature of this action, together with the observation that protein kinase C activation may lead to rapid phosphorylation of the glucose transporter (*39*), have led to the suggestion that TPA-induced transport activation occurs via changes in the subcellular location or intrinsic activity of preexisting transporters.

When we treated FR3T3 cells with 10^{-6}*M* TPA for 15 minutes, the rate of 2-DOG transport increased only 15 to 30% above baseline, with no effect on the level of transporter mRNA (*40*). However, when these cells were exposed to TPA for 18 hours, the rate of glucose transport steadily increased over this period, reaching a maximum stimulation of three- to fivefold. This delayed stimulation of glucose transport was associated with a comparable increase in the abundance of glucose transporter mRNA on Northern blots (Fig. 3). Similar changes were caused by the synthetic diacylglycerol OAG, but not by the inactive analog 4β-phorbol. These data suggest that whereas protein kinase C activation by phorbol esters may play a role in the rapid regulation of glucose transport, as suggested by others (*36, 37*), a more potent effect of phorbol esters is exerted with a slower time course at the level of transporter mRNA induction. Further, these data lend additional support to the notion that *ras* and *src* induce some of their actions through changes in phosphatidylinositol turnover and subsequent activation of protein kinase C.

These studies demonstrate that the increased rate of glucose transport that characterizes many tumors and that is observed in cells transformed by a variety of means can be induced in 3T3 cells by transfection with cloned *ras* or *src* oncogenes. In addition, the accelerated rate of glucose transport that occurs in response to cell transformation by these two oncogenes is associated with, and is presumably caused by, marked increases in the expression of the structural gene encoding the glucose transporter protein. Finally, these studies strongly suggest that phorbol esters, probably through activation of pro-

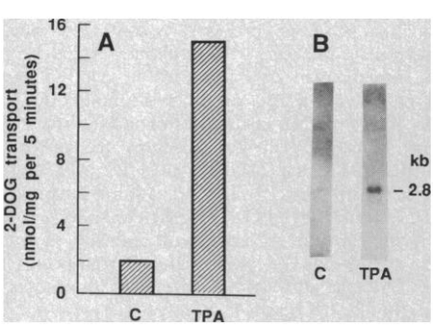

Fig. 3. The effect of TPA on glucose transport and glucose transporter mRNA levels. Control FR3T3 cells were grown to confluence in DMEM with 10% calf serum as in Fig. 1. Medium was aspirated, and monolayers were washed three times with iced PBS and replaced with serum-free DMEM with glucose (200 mg/dl) and 0.1% calf serum in the presence or absence of 10^{-6}*M* TPA. After 18 hours at 37°C, the rate of 2-DOG uptake was measured (**A**) and RNA was extracted for Northern blots (**B**) as described in the legend to Fig. 1.

tein kinase C, initiate events that alter the rate of synthesis and/or stability of glucose transporter mRNA. The implications of these findings for the control of glucose transporter gene expression in normal physiology and disease states, such as diabetes, in which glucose transport regulation is defective (*41*), will require further investigation.

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Transformation of Rat Fibroblasts by FSV Rapidly Increases Glucose Transporter Gene Transcription

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Elevation of glucose transport is an alteration common to most virally induced tumors. Rat fibroblasts transformed with wild-type or a temperature-sensitive Fujinami sarcoma virus (FSV) were studied in order to determine the mechanisms underlying the increased transport. Five- to tenfold increases in total cellular glucose transporter protein in response to transformation were accompanied by similar increases in transporter messenger RNA levels. This, in turn, was preceded by an absolute increase in the rate of glucose transporter gene transcription within 30 minutes after shift of the temperature-sensitive FSV-transformed cells to the permissive temperature. The transporter messenger RNA levels in transformed fibroblasts were higher than those found in proliferating cells maintained at the nonpermissive temperature. The activation of transporter gene transcription by transformation represents one of the earliest known effects of oncogenesis on the expression of a gene encoding a protein of well-defined function.

ONCOGENIC TRANSFORMATION BY viruses involves complex changes in the expression of numerous cellular genes (*1*). Critical to the understanding of the molecular mechanisms governing transformation is the identification of genes with products of known function whose transcription is altered directly as a consequence of oncogenesis. Such information will increase our understanding of the precise biochemical functions that account for the altered phenotype and provide "markers" essential for the experimental elucidation of the molecular pathways that produce the transformed state.

Elevation of glucose transport is a well-established feature of virally transformed cells (*2*). Sugar flux is accomplished by an integral membrane glycoprotein capable of transporting monosaccharides down a chemical gradient in the absence of additional metabolic energy. Transformation increases the number of immunologically detectable transporters in the cell membrane (*3*). Recently, the primary structures of the glucose transporter protein from a human hepatoma cell line (*4*) and from normal rat brain (*5*) have been determined and shown to be highly homologous. With the availability of molecular probes for the transporter messenger RNA (mRNA), it has become possible to determine whether the stimulation of hexose transport associated with transformation is a consequence of alterations in transporter gene expression.

Fujinami sarcoma virus (FSV) is a replication-incompetent avian virus capable of rapidly producing transformation both in vitro and in vivo (*6*). The transforming protein is formed by the fusion of viral *gag* sequences to the cell-derived *fps* gene, which together encode an enzyme with tyrosine protein kinase activity. Transformation of fibroblasts by FSV increases the rate of glucose transport approximately five- to sevenfold (*6, 7*). RNA was prepared from normal rat fibroblasts (3Y1 cells) and from fibroblasts transformed by wild-type FSV [FSV9 cells (*8*)] and assayed for glucose transporter mRNA by blot hybridization analysis. In both cases the complementary DNA (cDNA) probe identified a single RNA species with an apparent size of 2.8 kb (Fig. 1), identical to that found in a number of normal rat tissues (*5*). However, the virally transformed cells contained significantly more transporter mRNA than the untransformed fibroblasts. The difference was somewhat variable in Northern blots of polyadenylated [poly(A)$^+$]RNA, but generally represented an increase of five- to tenfold.

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