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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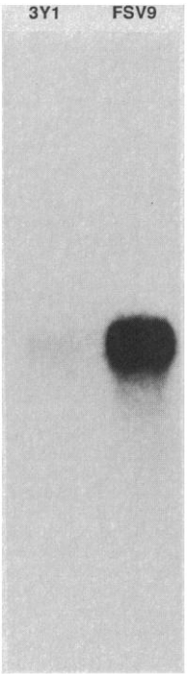

Fig. 1. Northern blot analysis of RNA from normal and FSV-transformed fibroblasts. Cells were grown at 39°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. Cells were fed at least 24 hours prior to the time of harvesting, and the nontransformed cells were confluent at that time. RNA was isolated by disruption with guanidinium isothiocyanate and cesium chloride centrifugation (*16*), and then subjected to oligo-(dT) affinity chromatography (*17*). Polyadenylated RNA (2 μg) was denatured with formaldehyde, separated by 1% agarose electrophoresis, transferred to a Nytran membrane (Schleicher & Schuell), and probed at high stringency with a nick-translated transporter cDNA (*5, 18*). The blot was washed and exposed to Kodak XAR-5 film with an intensifying screen at −70°C.

In spite of the marked difference in transporter mRNA levels in normal and transformed cells, it was necessary to determine that the changes were a direct consequence of transformation and not related to some other aspect of continuous growth in culture. Although the FSV9 cell line was originally isolated as a transformant of 3Y1, continuous passage might have resulted in phenotypic differences between the two cell lines unrelated to transformation. We have noted significant variability in the basal transporter mRNA levels as well as in the ratio of transporter protein to mRNA among various normal rodent fibroblast cell lines. Any conclusions concerning the immediate cause of changes in the expression of the glucose transporter gene, when based on the comparison of independently grown cell lines, must be regarded with extreme caution. Thus, the critical experiment is one in which measurements are made immediately after transformation.

To accomplish this, rat fibroblasts transformed with a temperature-sensitive (ts) variant of FSV (TS225) were utilized (*7, 8*). When chick fibroblasts transformed with a ts Rous sarcoma virus were grown at a nonpermissive temperature, increased glucose transport was apparent 2 to 4 hours after shift to the permissive temperature (*2*). Figure 2A shows an immunoblot of total cellular membranes prepared from 3Y1, FSV9, or TS225 cells, which had been grown for at least 2 days at either 39° or 32°C. As expected, there was more transporter in cells transformed with the wild-type FSV than in nontransformed cells. This effect was independent of the temperature at which the cells were grown. Glucose transporter protein was also increased in FSV9 cells refed with fresh media every 3 hours, indicating that the increase was not caused by more rapid depletion of glucose from the media of the transformed cells. Both normal and transformed cells had slightly more glucose transporter protein when grown at

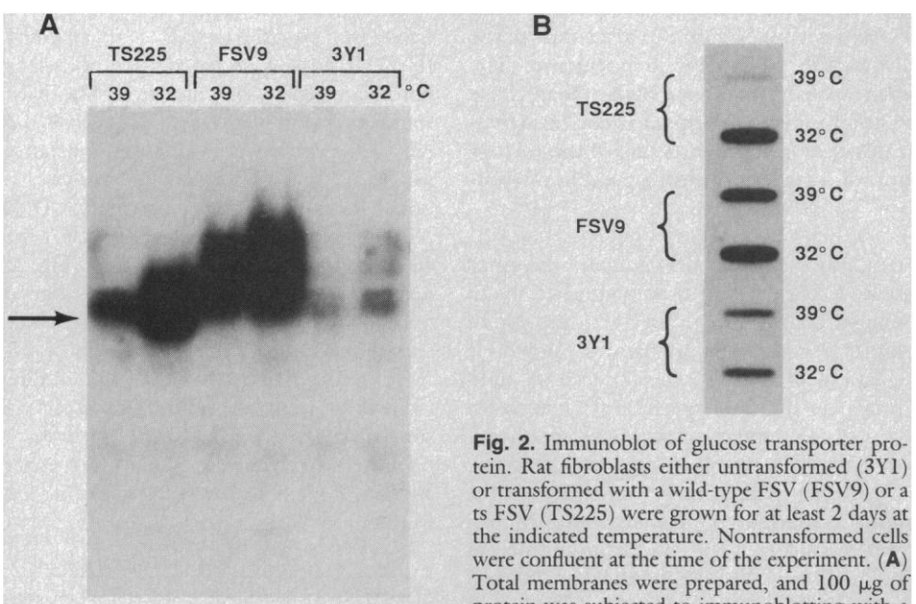

Fig. 2. Immunoblot of glucose transporter protein. Rat fibroblasts either untransformed (3Y1) or transformed with a wild-type FSV (FSV9) or a ts FSV (TS225) were grown for at least 2 days at the indicated temperature. Nontransformed cells were confluent at the time of the experiment. (**A**) Total membranes were prepared, and 100 μg of protein was subjected to immunoblotting with a polyclonal antibody prepared against the human erythrocyte transporter and known to recognize the rat transporter (*19*). (**B**) Total RNA (10 μg) was isolated and denatured as in Fig. 1, applied to nitrocellulose with a Schleicher & Schuell Minifold II slot blot apparatus, and probed with the rat glucose transporter cDNA (*5*).

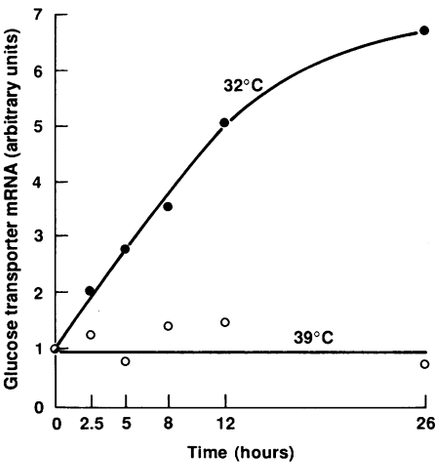

Fig. 3. Induction of glucose transporter mRNA by transformation. TS225 cells were grown to confluence at 39°C and either maintained at that temperature or shifted to 32°C. At the times indicated, cytoplasmic RNA was prepared from cells that had been washed and harvested by scraping (*18*). RNA (16 μg) was denatured with formaldehyde, applied to nitrocellulose paper, and probed as in Fig. 2B. The degree of hybridization was quantitated by scanning densitometry with an LKB 2202 ultrascan laser densitometer.

32°C than at 39°C. In contrast, fibroblasts transformed with the ts FSV showed a marked increase in transporter protein at 32°C in comparison to 39°C. The relative differences correlate closely with those observed for glucose transport measured in chick fibroblasts transformed with TS225 and treated under similar conditions (*7*). Cellular mRNA levels increased slightly in 3Y1 or FSV9 cells grown at 32°C in comparison to 39°C, whereas in TS225 cells the increase was five- to tenfold (Fig. 2B). Transporter mRNA concentrations were higher in the 3Y1 cells than in the nontransformed TS225 cells in comparison to the amount of immunodetectable protein in each of the cell lines. It is unclear whether this is attributable to differences in the rates of mRNA translation or protein turnover. However, as noted above, such variability has been frequently observed among fibroblast lines and serves to emphasize the need for performing these studies in temperature-sensitive transformants.

To ascertain the rate of induction of glucose transporter mRNA, TS225 rat fibroblasts were grown at 39°C for at least 4 days and transferred to 32°C; cytoplasmic RNA was prepared at various times (Fig. 3). The RNA was applied to nitrocellulose and hybridized to a transporter cDNA probe. Transporter mRNA levels were appreciably elevated 2.5 hours after shift to the permissive temperature and continued to rise over 26 hours. Measurement at later times indicated no further increase in transporter mRNA concentration.

Although the rapid response to transformation suggested a primary effect on transporter gene transcription, it was possible that the increase was mediated by the stabilization of an mRNA that was turning over rapidly. To resolve this, we shifted TS225 cells grown at 39°C to 32°C and at various times we prepared nuclei and subjected them to run-on transcriptional analysis (*9*). Transformation was accompanied by a rapid increase in transcription, which occurred in less than 30 minutes (Fig. 4). This correlates temporally with the reported increase in tyrosine protein kinase activity that occurs upon shift of ts FSV–transformed fibroblasts to a permissive temperature (*6*). The insert in Fig. 4 shows the autoradiogram of the 24-hour time point. There is an apparent three- to fourfold increase in glucose transporter gene transcription of nuclei from cells at 32°C, little or no hybridization to a vector plasmid control, and no change in actin gene transcription. However, since an equal amount of in vitro synthesized RNA was hybridized for each experimental point in the run-on transcriptional analysis, any increase in rates of total RNA transcription in nuclei from transformed cells would result in an apparent diminution of the increment in specific transporter gene activity. Ribosomal gene transcription, which accounts for more than half of the nuclear RNA synthesis, is significantly activated in proliferating fibroblasts (*10*). We have observed a consistent two- to fourfold increase in total nuclear transcription from transformed or normal, growing cells compared to quiescent, contact-inhibited fibroblasts. Therefore, when corrected for rates of total transcription, the increase in synthesis of glucose transporter mRNA correlates reasonably well with the change in steady-state levels.

It has been observed that glucose transport is elevated in growing cells as compared to contact-inhibited cells, but not to the extent caused by transformation (*11*). In all the experiments described thus far, nontransformed cells were harvested after they had reached confluence and ceased growth. Thus it was not possible to establish whether the increase in glucose transporter gene expression was a result of transformation per se or the induction of cell proliferation. To clarify this point, RNA was prepared from TS225 cells grown at 39°C to confluence, cells actively growing at 39°C, and cells grown at 32°C. In TS225 cells grown at the permissive temperature, transporter mRNA levels were higher than those of growing or contact-arrested normal fibroblasts (Fig. 5). This was associated with higher rates of gene transcription.

A number of observations are consistent with the conclusion that the increased glucose transport after viral transformation is the result of the transcriptional activation of the transporter gene. As stated above, the rapid onset of the changes in transport have been noted by several groups (*2*). Beug *et al.* (*12*) have studied transformation by Rous sarcoma virus in enucleated chick embryo fibroblasts and found that, while many morphological alterations associated with transformation remained intact, the increase in glucose transport was dependent on nuclear function. Thus, transformation appears to induce a complex program of nuclear changes, of which one of the most rapid is an increase in glucose transporter gene transcription. This activation of the transporter gene is most likely a component of a subset of transformation-induced responses related to the stimulation of cellular proliferation. Clearly, however, there is an effect of transformation on transporter mRNA levels independent of growth. In this context, it may be of interest that the brain transporter cDNA identifies low amounts of mRNA in both normal and growing (regenerating) rat liver, but abundant mRNA in rat and human hepatoma cell lines (*5*).

In addition to transformation, starvation of fibroblasts for glucose produces a marked increase in total glucose transporter protein as well as the rate of transport (*13*). However, in this case, the augmented transport is not associated with a concomitant rise in transporter mRNA levels (*13*). This is consistent with the slower time course of the activation of glucose transport stimulated by starvation as compared to transformation (*2, 14*). Insulin rapidly increases glucose transport in appropriate target tissues, but not in association with an increase in total cellular transporter protein (*15*). Thus, there are at least three independent mechanisms for activating glucose transport in mammalian cells: alteration of the half-life of the glucose transporter (*13*), activation of existing glucose transporters (*15*), and induction of glucose transporter biosynthesis.

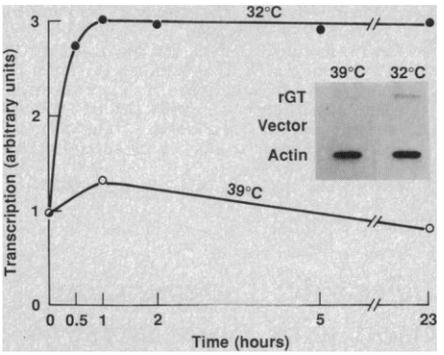

Fig. 4. Activation of glucose transporter gene transcription by transformation. Cells were grown as in Fig. 3. At the indicated times, nuclei were prepared, treated with ribonuclease A, and stored at −70°C until used for transcription (*9*). Approximately 4×10^7 nuclei were present per reaction yielding 1.5×10^6 to 3×10^6 cpm of incorporated [α-^{32}P]GTP, which was hybridized in a volume of 1 ml. Plasmid templates (5 μg), which were denatured by heating in alkali, were as follows: rGT, a 2.6-kb cDNA (*5*) cloned into the Eco RI site of pBlue M13+ (Stratagene, Inc.); vector, pBlue M13+ without insert; actin, pHF1, which contains a partial human actin cDNA (*20*). The extent of hybridization was quantitated as in Fig. 3 and corrected for the input radioactivity. The inset is the data from the 23-hour time point; it represents a 3-day exposure at −70°C with an intensifying screen. However, the 39°C points were slightly overexposed during reproduction of the autoradiogram to clearly show the hybridization to the transporter cDNA.

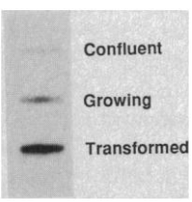

Fig. 5. Glucose transporter mRNA levels in growing fibroblasts. TS225 cells were grown as in Fig. 2, except that the cells grown at 39°C were harvested at confluence or at a density of approximately 15% confluence. RNA was prepared, and 20 μg were analyzed by slot blot analysis as described in Figs. 2 and 3.

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The Immunoglobulin Octanucleotide: Independent Activity and Selective Interaction with Enhancers

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The thymidine kinase (*tk*) promoter of herpes simplex virus includes an octanucleotide sequence motif (ATTTGCAT) that is also an essential component of immunoglobulin kappa gene promoters. In the absence of an enhancer, *tk* promoter derivatives that contain this element support a higher rate of transcription than those that lack it. The action of the kappa enhancer augments that of the octanucleotide in B lymphoid cells; when both elements are present, *tk* promoter activity is increased by more than an order of magnitude. In contrast, the presence of the octanucleotide in this promoter markedly reduces its response to a nonimmunoglobulin enhancer. These results suggest that the octanucleotide may mediate a selective interaction among promoters and enhancers.

AT LEAST TWO DISTINCT DNA sequence elements influence the transcription of immunoglobulin kappa light chain genes. One of these, occupying a 250–base pair (bp) region of an intron adjacent to the constant region exon (*1, 2*), is a transcriptional enhancer; it can act over distances of several kilobases to increase the activity of a promoter in cis regardless of its orientation relative to the promoter (*3*). The kappa enhancer is preferentially active in B lymphoid cells, undergoes a localized change in chromatin structure that correlates with the onset of kappa transcription (*2*), and is required for maximal expression of kappa genes transfected into B lymphocytes (*4, 5*). A second essential element is the octanucleotide motif ATTTGCAT, which is found 70 ± 10 bp upstream from the transcriptional initiation sites of all light chain genes, and in inverted orientation (ATGCAAAT) at the same location in heavy chain gene promoters (*6, 7*). The octanucleotide has been stringently conserved in vertebrate evolution, and deletions of DNA segments that encompass this element abolish immunoglobulin promoter activity (*5, 7, 8*).

Recent studies suggest that the octanucleotide locus is included in the binding site for a specific nuclear protein (*9*) and that similar or identical octameric motifs function in the promoters of the histone H2B (*10*) and *Xenopus* small nuclear RNA (snRNA) genes (*11*). Little is known, however, about the precise role of the octanucleotide in promoter function or the extent to which it might interact with the enhancer to influence the level of kappa gene expression. In this report we demonstrate that the same octanucleotide is also a functional component of the thymidine kinase promoter of herpes virus and is required for maximal promoter function in both lymphoid and nonlymphoid cells. The presence of the octanucleotide markedly reduces the responsiveness of this promoter to a nonimmunoglobulin enhancer, whereas the kappa enhancer and the octanucleotide element act in concert to produce a stimulatory effect far greater than that of either element alone.

The described sequence elements of the thymidine kinase (*tk*) promoter (Fig. 1A) include a TATA box at residues −21 through −26 relative to the transcriptional initiation site, as well as a cluster of distal sequences between residues −46 and −105 that encompasses at least three protein-binding domains. These elements alone are sufficient to establish promoter function either in vivo or in vitro (*12, 13*). Farther upstream, between residues −131 and −138, we noticed a perfect copy of the light chain octanucleotide. To determine whether this sequence might also contribute to the function of the *tk* promoter, we compared the activity of a DNA fragment termed promoter A, which comprised only sequences +55 through −109, to that of promoter B, which extended an additional 39 bp upstream to position −148 and so included the octanucleotide motif (Fig. 1A).

We constructed a series of recombinant plasmids (Fig. 1B), fusing each promoter fragment to the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene (*14*). In most instances, the plasmids also contained the polyoma virus early region and origin of replication, which enable plasmids to replicate in mouse cells and thereby greatly increase the sensitivity of the transient expression assay (*4*). The plasmids were transfected into the mouse myeloma cell lines P3X63Ag8 and SP2/0, and relative *tk* promoter activity was inferred by comparing the levels of CAT enzyme activity in lysates of the transfected cells. Plasmids containing promoter A yielded a relatively low basal level of enzyme expression, whereas those containing promoter B generated CAT activity at three to four times the basal level (Table 1). To determine whether this difference resulted from the light chain octanucleotide present in promoter B, we inserted a synthetic 12-bp DNA oligomer containing that sequence immediately upstream from promoter A (Fig. 1C); this modification (promoter AL) increased CAT expression to a level equivalent to that obtained with promoter B.

We next examined the effect of the kappa enhancer element on *tk* promoter derivatives that either contained or lacked the octanucleotide. When inserted 0.6 kb upstream from promoter A, the kappa enhancer increased CAT expression to three to five times the basal level (Table 1). In contrast, fusion of this enhancer with promoter B yielded CAT activity that averaged 15 times the basal value; similar levels (11 times basal) were achieved with a combination of the kappa enhancer and promoter AL. These values correspond to a three- to five-fold increase over the activity of promoters B or AL alone, implying that the kappa enhancer augments *tk* promoter activity by a fixed proportion regardless of the presence of the octanucleotide and that the combined action of both these sequence elements is approximately equal to the algebraic product of their individual effects. The results obtained were essentially identical for both of the myeloma lines tested and were not influenced by the presence of the polyoma sequences (*15*). Moreover, the octanucleotide produced similar quantitative effects on CAT expression in transfections of the mouse 3T3 or rat XC fibroblast lines (Table 1). Thus, the stimulatory activity of this

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