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Regulation of Hexose Transport in a Respiration-Deficient Human Fibroblast Cell Strain

by

Lia Continelli

A Thesis in

The Department of Biology

Submitted in Partial Fulfillment of the Requirements for

the Master of Science Degree in Biology

at Concordia University

Montreal, Québec, Canada

May 1994

ISBN 0-315-97603-9

Canada

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ABSTRACT

Regulation of Hexose Transport in a Respriation - Deficient Human Fibroblast Cell Strain

Lia Continelli

The regulation of hexose transport was examined in a human fibroblast cell strain deficient in NADH CoQ reductase. Hexose transport was monitored by 2 - deoxy - D - glucose uptake, and was found to have a 6-fold increase in 2 - DG transport in serum-deprived WG 750 cells when compared to MCH 55 cells. Hexose utilization was also 3-fold higher in WG 750 cells. Lactate and CO_2 production were elevated similarly (3-fold) in the WG 750 cells when compared to the normal (MCH 55) cell strain. Kinetic characterization of the two cell strains indicated that the increased 2 - DG transport in WG 750 cells was the result of an elevation in the V_{max} for transport with no change in the K_{in} for transport. Insulin stimulated hexose transport by ~2-fold in the MCH 55 cells, but to a smaller degree in the WG 750 cells. Moreover, insulin-specific binding was similar in both cell strains. Refeeding of serum deprived WG 750 cells led to a time delayed increase in 2 - DG transport; however, both cell strains responded to serum re-exposure by increasing 2 - DG transport.

Western blot analysis indicated that whole cell extracts contained 2-fold more transporter protein in the WG 750 cells when compared to MCH 55 cells. Subcellular membrane fractionation demonstrated that 3-fold more transporter protein was localized specifically in the plasma membrane of the WG 750 cells; however, transporter protein in the MCH 55 appeared to be evenly distributed in the plasma membrane and the intracellular pool.

Steady-state transcript RNA levels were analysed in both cell strains under various conditions. Glucose transporter mRNA (Glut1 mRNA) was similar in both cell strains under basal conditions (0% serum); however, in insulin-stimulated state and in glucose deprivation, a 2-fold increase was observed in the WG 750 cells.

The data in this study clearly show that glucose transport is permanently upregulated in these mutant cells and that this increase in glucose transport is regulated by a composite of two controls — a translational (or post-translational) and a transcriptional control. The observations that emanate from this study provide a basis in understanding the molecular mechanism(s) of transport regulation in human cells.

To my husband,
Stephen Daniel Scalzetti

ACKNOWLEDGEMENTS

I would like to acknowledge several individuals who have been instrumental in the work presented in this thesis.

To Dr. Ralph J. Germinario, my research advisor, I am greatly in debt for his continual support and guidance.

To Dr. Barry E. Knox I owe my sincere appreciation for helping me work out the molecular aspect of this investigation.

Dr. Dorine Starace I cannot thank enough for her superb help with the riboprobe.

Susannia Manuel provided crack technical assistance.

My father-in-law, Matthew R. Scalzetti, toiled countless tedious hours transposing handwritten notes into a polished electronic document.

My family has always been there to encourage me to finish this thesis! I thank each of them — Mom, Dad, Ann, Frank, Ron, Anthony, and Mark Steven.

Finally, this thesis would not have been possible without the constant support and encouragement of my husband, Stephen D. Scalzetti, especially during my two pregnancies. He never lost confidence in my ability to complete this work.

LIST OF ABBREVIATIONS

DME:	Dulbecco's modified Eagle's medium
PBS:	phosphate buffered saline
CB:	cytochalasin B
TCA:	trichloroacetic acid
TLC:	thin layer chromatography
2 - DG:	2 - deoxy - D - glucose
3 - OMG:	3 - O - methyl - glucose
RNA:	ribonucleic acid
DNA:	deoxyribonucleic acid
DEPC:	diethylpyrocarbonate
PEI - Cellulose:	polyethylenimine - cellulose
ATB - BMPA:	2 - N - 4 - (1 - azi - 2,2,2 - trifluoroethyl) benzoyl - 1,3 - bis - (D - mannos - 4 - deoxy) - 2 - propylamine
IGF - I:	insulin-like growth factor I
Glut1:	brain/HepG2/erythrocyte - type glucose transporter
Glut4:	muscle/adipocyte - type, insulin-responsive glucose transporter

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INTRODUCTION

Cultured Fibroblasts as a Model System for Studying the Regulation of Hexose Transport.

There have been a number of studies in recent years on the regulation of hexose transport in various animal cell cultures (Kalckar and Ullrey, 1973, Kletzien and Perdue, 1973, Kletzien and Perdue, 1974, Franchi *et al.*, 1978). A large number of investigators have employed the fibroblasts as a model system in an attempt to differentiate the normal and the diabetic cell (Germinario *et al.*, 1987). Studies on the regulation and properties of hexose transport in eukaryotic cells also reveal several mechanisms by which hexose transport can be affected. Glucose starvation (Christopher *et al.*, 1976, Franchi *et al.*, 1978, Kalchar and Ullrey, 1984, Haspel *et al.*, 1986), insulin (Simpson and Cushman, 1986, Garvey *et al.*, 1987), cell density (Gay and Hilf, 1980), and glucocorticoids (Horner *et al.*, 1987) have been shown to affect the hexose transport system. The hexose transport system of mammalian fibroblasts is thus subjected to many metabolic regulators. It is well established that animal cells in culture deprived of glucose develop greatly enhanced hexose uptake rates (Germinario *et al.*, 1982, Gay and Hilf, 1980, Christopher, 1977, Kletzien and Perdue, 1975, Kalckar and Ullrey, 1973). Previous kinetic studies indicated that this response to glucose starvation is due to an increase in the number of functioning carriers in the plasma membrane (Kletzien and Perdue, 1975, Franchi *et al.*, 1978, Germinario *et al.*, 1982) and this enhancement of transport from glucose starvation can be largely blocked by treatment with inhibitors of protein and RNA synthesis (Martineau *et al.*, 1972, Kletzien and Perdue, 1975). Since the starvation effect seems to depend in part on protein synthesis, Christopher has proposed that regulation of hexose carriers results from ongoing processes of carrier synthesis and inactivation, both of which are dependent of protein synthesis (Christopher *et al.*, 1976, Christopher, 1978). Tillotson has also shown that when chicken embryo fibroblasts are exposed to a glucose-free culture medium, they develop enhanced hexose transport activity 4- to 10-fold greater then the basal level (Tillotson *et al.*, 1984), whereas refeeding the cells with glucose decreased hexose transport activity. During the decline in hexose transport activity from glucose refeeding of previously glucose-starved cells, it is likely that hexose transporter turnover process appears to predominate. Conversely, when hexose transport activity increases, such as during glucose starvation, glucose transporter synthesis seems to predominate. It is not yet feasible to determine absolute activities of glucose transporter synthesis and

turnover; however, these studies provide an indication of the relative balance between these two processes.

Activation of hexose transport can also be brought about by insulin. Insulin stimulation of glucose entry is a well established parameter of insulin's action in mammalian physiology (Crofford and Renold, 1965).

A cell culture system responsive to insulin's glucoregulatory action would be a very useful tool for the study of the mechanism of insulin's glucoregulatory role on hexose transport, because of the availability of large quantities of material and the potential for long term studies. The human diploid fibroblast is a readily available source of human tissue for in vitro study, but there has been some ambivalence concerning its relevance as an insulin target organ. It is clear that the fibroblast has an insulin receptor (Rechler and Podskalny, 1976), and that this receptor is regulated by physiologic levels of insulin (Mott *et al.*, 1979). Insulin also has a mitogenic action on fibroblasts as evidenced by studies on thymidine incorporation (Hollenberg and Cuatrecasas, 1975, Rechler *et al.*, 1974). In addition, insulin has been shown to stimulate amino acid transport (Hollenberg and Cuatrecasas, 1975) synthesis of protein, RNA (Fujimoto and Williams, 1974), glycogen (DiMauro and Mellman, 1973) and lipid (Bhathena *et al.*, 1974). There have been conflicting reports on the action of insulin on glucose metabolism in human diploid fibroblasts. Fujimoto has reported that insulin stimulates glucose uptake in human fibroblasts (Fujimoto and Williams, 1974). However, others (Hollenberg and Cuatrecasas, 1974) have been unable to show insulin-stimulated hexose transport under conditions where insulin was stimulating other cell functions. However, Germinario and Oliveira demonstrated that insulin did indeed stimulate sugar transport in human fibroblasts (Germinario and Oliviera, 1979).

Hexose transport has been well characterized in other cell culture systems such as the chick embryo fibroblast (Christopher *et al.*, 1976, Weber, 1973), the Novikoff hepatoma (Plagemann and Richey, 1974) and other tumor lines (Hatanaka, 1974). Insulin action on hexose transport has also been studied in the chick embryo systems (Shaw and Amos, 1973, Rubin, 1976, Raizada and Perdue, 1976), where observation of its influence has required long term incubation and nonphysiological concentrations, and its action is sometimes correlated with its mitogenic function (Raizada and Perdue, 1976).

The action of insulin on glucose uptake in the cultured fibroblast is extremely sensitive to experimental conditions. It is enhanced by glucose starvation, a condition which paradoxically leads to increased basal rates of transport. The enhancement of hexose uptake after glucose starvation has been thoroughly studied in chick embryo fibroblasts (Christopher *et al.*, 1976, Weber, 1973) and has been proposed to be a derepression of

numbers of transport sites (Kletzein and Perdue, 1975). The kinetic data from Howard (Howard *et al.*, 1979) in the diploid fibroblast indicates an increase in V_{max} of 2-DG transport after glucose starvation and is consistent with the observation in chick embryo system. It is possible that this enhancement of insulin action by glucose starvation simply reflects this increase in number of transport carriers. However, Germinario clearly demonstrated that glucose starvation which increased basal sugar transport led to an inability of insulin to further stimulate transport in these cells (Germinario and Oliviera, 1984).

CELLULAR MECHANISMS FOR STIMULATION OF GLUCOSE TRANSPORT

Translocation of glucose transporters and/or insulin-intrinsic activity changes

One of the basic biological actions of insulin is its ability to stimulate glucose uptake in various tissues. The first step in the intracellular metabolism of glucose is its entry across the plasma membrane into the cell. In adipose tissue and muscle this process is under acute regulation by insulin. The extreme sensitivity of adipose cells to insulin, in particular, has made this the tissue of choice for examining the mechanism of action of the hormone on glucose metabolism. The stimulation of glucose transport in adipose cells by insulin could occur either via an increase in the affinity or in the maximum transport velocity of the carrier. Some investigators have suggested that insulin stimulates glucose transport by inducing changes in the affinity of the transporter (Blok *et al.*, 1988, Baly and Horuk, 1987, Joost *et al.*, 1988, Simpson and Cushman, 1986), while the major body of evidence supports the idea that this process occurs through an increase in the maximum transport velocity of the carrier (Cushman and Wardzala, 1980, Suzuki and Kono, 1980, Kono *et al.*, 1981).

At least two distinctly different mechanisms could give rise to the observed increases in V_{max} of glucose transport in response to insulin. The increased transport rate could result either from increases in the number of glucose transporters in the plasma membrane or from changes in the intrinsic activity of the plasma membrane glucose transporter such that each carrier transports glucose at a more rapid rate. The studies of Cushman and Kono support the former mechanism (Cushman and Wardzala, 1980, Suzuki and Kono, 1980, Kono *et al.*, 1981). Kono has proposed that insulin stimulates glucose transport by the translocation of recruitment of hexose transport carriers from an intracellular pool to the plasma membrane (Kono *et al.*, 1981). Cushman screened for the presence of transporters in subcellular membrane fractions of adipocyte using the D-glucose-inhibitable cytochalasin B-binding assay (Cushman and Wardzala, 1980). Two insulin-sensitive pools (plasma membrane and low-density microsomes) of glucose transport

activity were found in fat cell subcellular membrane fractions. In membranes prepared from cells not treated with insulin, the number of transporters in plasma membranes was low, while the number of transporters in low-density microsomes was about 10-fold higher. Insulin dramatically alters this distribution, increasing the number of glucose transporters in plasma membranes 5-fold, while decreasing the number of glucose transporters in the low-density microsomes by around 50%. Other independent studies have led to similar findings (Suzuki and Kono, 1980, Kono *et al.*, 1981). In these studies the distribution of glucose transporters in the rat adipocyte was measured using a reconstitution assay. Detergent-solubilized glucose transport activity was incorporated into an artificial phospholipid bilayer and the reconstituted vesicles were assayed for transport activity by D - [^{3}H] glucose uptake. In the presence of insulin, glucose transport activity was decreased in the low-density microsomes, while an increase in glucose transport activity was observed in the plasma membrane pool.

Karnielli has proposed a model for insulin action on glucose transport (Karnielli *et al.*, 1981). As insulin binds to cell surface receptors, it results in the generation of an intracellular signal. This insulin-mediated signal leads to the mobilization of glucose transporters from an intracellular vesicular pool to the plasma membrane exposing transporters to the extracellular environment when they can mediate an increase in glucose flux. The dissociation of insulin from its receptor terminates the signal and leads to a rapid reversal of the process, which ultimately results in the translocation of glucose transporters from the plasma membrane to an intracellular site (Figure 1). Though the translocation hypothesis has now been largely accepted, many difficulties still remain to be resolved. For example, insulin stimulation of 3T3 - L1 adipocytes resulted in a 2.5-fold increase in transporter at the cell surface, while the rate of 2 - DG uptake was increased 11-fold. Intrinsic activity of the transporter was proposed to be the factor responsible for this discrepancy (Calderhead and Lienhard, 1988).

Studies from several other laboratories have brought into question the idea that the effects of insulin on glucose transport are mediated solely via the translocation of carriers (Whitesell and Abumrad, 1985, Whitesell and Abumrad, 1986). They reported that insulin induced an affinity change in the carrier for glucose and that this explains the stimulatory effect of insulin on glucose transport in adipose cells. They suggest that insulin may activate basal carriers or cause basal carriers to be internalized as new carriers are added to the plasma membrane. Consistent with this idea, Hyslop used a membrane-impermeant probe and has obtained data suggesting that insulin stimulates transport either by direct activation of inactive carriers already in the plasma membrane or by activating nascent transporters. Taken together, these studies suggest that the stimulation of glucose

transport activity by insulin might involve more than one mechanism (i.e., translocation) (Hyslop *et al.*, 1985). Two independent studies reported that cycloheximide-treated rat adipocytes exposed to insulin show no change in the amount of glucose transporters in the plasma membrane fraction compared to plasma membranes from control cells. Nevertheless, insulin was still able to elicit a maximal increase in glucose transport activity in intact rat adipocytes. Thus, is was concluded that the insulin-stimulated increase in glucose transport activity and glucose transporter redistribution to the plasma membrane fraction are dissociable events (Baly and Horuk, 1987, Matthaei *et al.*, 1988). These data suggest that insulin may increase the intrinsic activity of cell-surface transporters. However, these results were contradicted by James and Cushman, who reported that cycloheximide (CHX) does not change the insulin-stimulated glucose transport activity or the distribution of glucose transporter proteins in isolated rat adipocytes. Therefore, they concluded that new protein synthesis is not required for the redistribution of glucose transporters to the cell surface or the associated increase in glucose transport activity in cells exposed to insulin (James and Cushman, 1989). Recent findings differ from all earlier studies in isolated rat adipocytes (Clancy *et al.*, 1991). Surprisingly, their data show that 3T3 - L1 adipocytes exposed to either anisomycin or CHX displayed a large increase in glucose transport activity that is not associated with an increase in the amounts of either Glut-1 and Glut-4 (insulin-regulatable isoform) protein in the plasma membrane fraction. These observations suggest that an apparent several-fold increase in the intrinsic catalytic activity of one or both transporter proteins accounts for the effect of these protein synthesis inhibitors on glucose transport in differentiated 3T3-L1 cells.

Germinario showed that various inhibitors of protein synthesis with different mechanisms of action (i.e., puromycin, pactamycin, and CHX) increased hexose transport in human skin fibroblasts by 2-fold. Nucleotidase enriched (i.e., plasma membrane) fractions of control and CHX-exposed cells showed no differences in D-glucose inhibitable cytochalasin B binding activity. The data indicate that inhibitors of protein synthesis can cause a significant elevation in hexose transport and that the hexose transporter mass in the isolated plasma membrane fractions does not reflect the whole cell transport change. It is suggested that a mechanism other than glucose transporter translocation to the plasma membrane may be involved in causing this sugar transport increase (Germinario *et al.*, 1992).

The mechanism(s) for the changes in intrinsic activity remains unknown. This activation could be achieved by direct modulation of the intrinsic activity of the carrier via covalent modification or by the activation of one of the steps involved in the recruitment of the carrier protein. Intrinsic properties of the transporter are often reflected by the

alteration in the transport activity (moles of glucose transported per transporter per unit of time). Both biochemical and kinetic analyses suggest that the intrinsic properties of the hexose transporter can be modulated by reagents such as Cytochalasin B (Jung *et al.*, 1986), phloretin (Krupka and Deves, 1986), ATP (Carruthers, 1986), insulin (Whitesell and Abumrad, 1985, Baly and Horuk, 1987, Kahn and Cushman, 1987, Toyoda *et al.*, 1987, Joost *et al.*, 1988), adenosine and catecholamine (Smith *et al.*, 1984, Joost *et al.*, 1986). Also, several studies suggest that intrinsic activity may result from changes in the phosphorylation state of the transporter (James, 1989, Begum *et al.*, 1993).

THE EFFECT OF INSULIN ON HEXOSE TRANSPORT/TRANSPORTER PROTEIN/TRANSPORTER MESSAGE

It is generally accepted, then, that the acute insulin stimulation of glucose transport activity in adipocytes (Simpson and Cushman, 1986, Kono *et al.*, 1981), heart (Watanabe *et al.*, 1984), and skeletal muscle (Klip *et al.*, 1987) occurs primarily via redistribution or recruitment of intracellular glucose transporter proteins into the plasma membrane and/or changes in intrinsic activity of the transporter in response to insulin. This mechanism has also been demonstrated in the murine 3T3-L1 adipocyte and the rat L6 skeletal muscle cell lines (Ramlal *et al.*, 1988, Calderhead and Lienhard, 1988). In addition, several studies have indicated that the glucose transport system may also be regulated at the level of the intrinsic activity of the cell surface glucose transporters, in both adipocytes (Kahn and Cushman, 1987) and skeletal muscle (Sternlicht *et al.*, 1988). In contrast to these rapid protein synthesis-independent mechanisms, cells can regulate their glucose transport activity over prolonged periods of time in response to a variety of stimuli, such as oncogenic transformation (Martin *et al.*, 1971, Isselbacher, 1972, Kletzien and Perdue, 1974), growth factors (Kletzien and Perdue, 1974), chronic exposure to insulin (Rosen *et al.*, 1978), and glucose starvation (Kletzien and Perdue, 1975). The regulation of glucose transport activity in response to these conditions is apparently complex, and evidence has accumulated for regulation at the transcriptional and translational levels in several culture cell lines (Christopher *et al.*, 1976, Shawver *et al.*, 1987)..

Of the hormones that influence glucose transport activity, insulin's effect on glucose transport in cardiac, skeletal muscles and adipose tissues is the most extensively studied. The insulin-stimulated increase in glucose transport in fat and muscle cells is associated with a rapid redistribution of glucose transporter proteins from an intracellular pool to the plasma membrane, and these effects are complete within minutes of hormone addition (Simpson and Cushman, 1986). Insulin stimulates glucose transport in adipocytes via at

least two temporally and mechanistically distinct pathways. Virtually immediately after addition of the hormone, the uptake of glucose increases 10-20-fold in a process which is not dependent of protein synthesis and is due, at least in part, to the distribution of glucose transporter molecules from an intracellular pool to the plasma membrane (Simpson and Cushman, 1986, Cushman and Wardzala, 1980, Suzuki and Kono, 1980). After 5-10h of treatment with insulin, there is a further increase in hexose uptake which, in contrast, requires ongoing protein synthesis (Rosen *et al.*, 1978). This long term effect might well be mediated by an increase in expression of the glucose transporter gene.

It is not yet understood how insulin regulates the membrane distribution of glucose transporters, nor is it clear whether the intrinsic activity of the translocated glucose transporter protein is also increased in response to insulin. A major advance in the understanding of this process has been the recognition that there is a family of glucose transporters (Gould and Bell, 1990). This family includes the HepG2 brain/erythrocyte-type transporter (Glut1), the predominant species expressed in mammalian brain, erythrocyte and fibroblasts and appears to be expressed at low levels in fat and muscle. The insulin-regulatable glucose transporter (Glut4) is the major species and is expressed exclusively in fat and muscle. Glucose transport across the plasma membrane of muscle and fat insulin-sensitive tissues appears to occur by means of at least these 2 distinct facilitated diffusion transport proteins, namely Glut1 and Glut4 (insulin regulatable isoform).

In rat adipocyte and 3T3 - L1 adipocytes, both the Glut4 and the Glut1 undergo insulin-dependent translocation (Piper *et al.*, 1991), and thus both presumably contribute to the acute increase in glucose transport observed in the presence of insulin. Piper has shown that Glut4 and Glut1 glucose transporters are expressed together in insulin-responsive 3T3-L1 adipocyte, and the ratio of the amount of transporter per unit protein in the plasma membrane fraction versus the intracellular membrane fraction was 1:2 for the Glut1 protein and 1:30 for the Glut4 protein. Insulin treatment increased the plasma membrane concentration of the Glut4 by 10-fold and the Glut1 by 3.5-fold (Piper *et al.*, 1991). Both Glut4 and Glut1 undergo insulin-dependent translocation in 3T3-L1 adipocytes, and presumably contribute to the acute increase in glucose transport in the presence of insulin.

An investigation of the long-term effect of insulin on these two glucose transport system was done using adipocytes. Chronic treatment of adipocyte with insulin, tolbutamide or both agents, increased 2 - DG uptake, Glut1 protein and Glut1 mRNA levels. In contrast, these treatments either had no significant effect or decreased the levels of Glut4 protein and mRNA. They proposed that a major component of chronic insulin

treatment of 3T3 - L1 adipocytes with insulin or tolbutamide increases glucose uptake primarily by means of a selective increase in the expression of the Glut1 mRNA (Tordjman *et al.*, 1989).

Calderhead has also shown that differentiation of 3T3 - L1 adipocyte from fibroblasts and rat adipocyte are associated with an increase in cellular mRNA and protein for the Glut4 isoform. Cell-surface labeling of Glut4 increased 15-fold above basal levels and the increase was similar to the stimulation of glucose transport activity in response to chronic insulin treatment. The Glut1 isoform is also abundant in differentiated 3T3 - L1 cells (Calderhead and Lienhard, 1988, Calderhead *et al.*, 1990, Tordjman *et al.*, 1989) and increases 3-fold-to-5-fold above basal levels in response to acute insulin. In contrast to this, chronic-insulin treatment has been shown to produce a 4-fold further increase in the mRNA and total protein for the Glut1 isoform in 3T3 - L1 cells. However, no change was observed in the total cellular mRNA or protein for the Glut4 isoform.

Walker has reported that chronic insulin treatment transiently induced the rat Glut1 mRNA by 3-fold (Walker *et al.*, 1989). Consistent with this data, the combination of chronic insulin treatment with glucose deprivation resulted in a more persistent 3- to 4-fold increase in transcription rate than either treatment alone. These data demonstrate that prolonged insulin and glucose-dependent regulation of glucose transporter function occurs by a complex mechanism which includes enhanced Glut1 mRNA transcription and glucose transporter synthesis, as well as changes in the subcellular distribution of the glucose transporter proteins (Walker *et al.*, 1990).

THE EFFECT OF GLUCOSE ON HEXOSE TRANSPORT AND TRANSPORTER MESSAGE

Recently, the effect of glucose deprivation on glucose transporter mRNA has been studied in many cell lines. Kosaki has demonstrated that glucose deprivation (36 hours) resulted in approximately 4-fold increase in the mRNA and 3-fold increase in the protein and basal 2-deoxyglucose uptake in cultured human skin fibroblasts (Kosaki *et al.*, 1991). In the presence of various concentrations of glucose, no differences were found in the Glut1 mRNA protein levels and basal and insulin-stimulated 2-DG uptake. Others have also demonstrated that Glut1 mRNA levels in tissue culture are regulated by glucose (Wertheimer *et al.*, 1991). In the absence of other factors like hormones, hexose concentrations above the normal range down-regulated the Glut1 mRNA levels, whereas glucose starvation up-regulated the mRNA in tissue culture (Wertheimer *et al.*, 1989, Walker *et al.*, 1988, Walker *et al.*, 1989). Glucose starvation increased transporter mRNA

and protein levels 2.2- and 3.5-fold in undifferentiated preadipocytes and 1.8- and 3.1-fold in differentiated adipocytes (Reed *et al.*, 1990).

The recent finding that Glut1 mRNA levels in tissue culture are regulated by glucose, has motivated the comparison of the regulation of Glut1 expression to that of the stress proteins, glucose-regulated proteins (GRP's) (Wertheimer *et al.*, 1991). This family of proteins is ubiquitously expressed and stimulated by glucose deprivation and other cellular stresses. GRP's are induced by the presence of malfolded proteins in the ER and are bound to prefolded and malfolded protein that accumulate in the ER (Pelham, 1986, Ellis, 1987, Kozutsumi *et al.*, 1988). It was suggested that GRP's may act as chaperones in the assembly of exocytotic proteins, both during normal synthesis and under conditions that promote protein denaturation and aggregation. Wertheimer hypothesized that Glut1 may be a glucose-regulated stress protein. Various stimuli known to induce GRP transcription were used to assess whether Glut1 expression and GRP expression are regulated co-ordinately. L8 myocytes and NIL-3T3 fibroblasts were subjected to glucose starvation, calcium ionophore A23187 or tunicamycin, all known to increase GRP levels. The mRNA for Glut1 was increased by 50-300% in a time-dependent manner, similar to the changes in GRP-78. When another major stress, heat shock, was applied, Glut1 was not induced. Thus, Glut1 expression seems to be specifically induced by factors that regulate GRP expression. Interestingly, the induction of GRP and Glut1 expression seems to be coordinated during cellular transformation by oncogenes (Stoeckle *et al.*, 1988, White and Weber, 1988, Birnbaum *et al.*, 1987, Hiraki *et al.*, 1989). Thus, transformation of chicken embryo fibroblasts by rous sarcoma virus results in src-dependent induction of GRP-78 (Stoeckle *et al.*, 1988), and increased levels of Glut1 protein (Birnbaum *et al.*, 1987). Likewise, mouse fibroblasts transformed by various oncogenes such as src, ras, fps show an increased glucose uptake that is correlated with elevated levels of the Glut1 mRNA (White and Weber, 1988, Birnbaum *et al.*, 1987, Hiraki *et al.*, 1989). It is proposed that Glut1 belongs to the GRP family of stress proteins and that its ubiquitous expression may serve a specific purpose during cellular stress.

A Family of Facilitative Glucose Transporters: Molecular Structure, Tissue Distribution and Their Properties

The uptake of Glucose into most eukaryotic cells is accompanied by a carrier-mediated transport system, facilitative diffusion, which transports glucose down its chemical gradient in a stereospecific manner. Recent studies have shown that facilitative transport of glucose across the plasma membrane is mediated by a family of structurally related proteins (Mueckler, 1994). These species exhibit considerable homology in their primary

sequences, but display a marked tissue-specific pattern of expression. Given that glucose transport in certain tissues is under both acute and chronic hormonal regulation, it would seem reasonable that this tissue specificity reflects the differing transport needs of certain tissues. For example, muscle and fat exhibit a dramatically increased rate of glucose transport when circulating levels of the hormone insulin are high, such as after a meal. The mechanisms by which insulin and other hormones are able to regulate the rate of glucose transport into cells, and the role of these different glucose transporters, may have important implications in the understanding of diseases such as diabetes mellitus and in metabolism. Molecular cloning of cDNA encoding the human erythrocyte facilitated-diffusion glucose transporter has elucidated its structure and has permitted a careful study of its tissue distribution and of its involvement in processes such as insulin-stimulated glucose uptake by adipose cells or transformation-induced increase in glucose metabolism. An important outcome of these studies was the discovery that additional isoforms of this transporter were expressed in a tissue-specific manner; these comprise a family of structurally and functionally related molecules. Their tissue distribution, differences in kinetic properties, and differential regulation by ambient glucose and insulin levels suggest that they play specific roles in the control of glucose homeostasis (Thorens *et al.*, 1990).

Currently, cDNAs encoding seven structurally-related proteins with the properties of facilitative glucose transporters have been isolated and characterized. Each glucose transporter (Glut) is numbered in the order of its discovery. The first, Glut1, is expressed in high levels in the endothelial cells that line the blood vessels, the placenta, and kidney. Smaller amounts of Glut1 are found in many other tissues, such as the colon, HepG2 cells, and fibroblasts. It is the transporter isoform (Glut1) responsible for the basal uptake of glucose. In addition, Glut1 is the predominant facilitative glucose transporter expressed by cultured cells and is present in many tumors. The amino acid sequence of Glut1 is highly conserved, and there is 97 - 98% identity between the human (Mueckler *et al.*, 1985), rat (Birnbaum *et al.*, 1986), rabbit (Asano *et al.*, 1988) and mouse (Kaestner *et al.*, 1989) sequences, thus implying that all domains of this protein are functionally important, either for transport of glucose or for the regulation of transporter activity. On the basis of an analysis of the primary structure of the Glut1 protein, Mueckler proposed a model for the two-dimensional orientation of this protein in the plasma membrane, in which the protein spans the lipid bilayer twelve times and the amino and carboxyl termini are located on the cytoplasmic side of the plasma membrane (Mueckler *et al.*, 1985) (Figure 2). Studies using peptide-specific antibodies to various regions of the protein have confirmed many of the topological features of this model. An extracellular loop of 33 amino acids are located between transmembrane segments M1 and M2, and an intracellular hydophilic segment of

65 amino acids connects M6 and M7. Interestingly, all the glucose transporter isoforms have twelve membrane-spanning domains, a relatively large middle cytoplasmic loop, a long extracellular loop with one glycosylation site, and the amino- and carboxy- ends oriented toward the cytoplasm (Mueckler *et al.*, 1985, Gould and Bell, 1990).

The role of the carboxy-terminal domain of the Glut1 glucose transporter has been implicated in the regulation of glucose uptake. Antipeptide antibody to the C-terminal part of the Glut1 transporter were loaded into fibroblasts by electroporation, and a 60% increase in basal 2 - DG uptake was observed (Tanti *et al.*, 1992). The interaction between the anti-Glut1 antibody and the carboxy-tail of the transporter seems to lead to an increase in the intrinsic activity of the transporter, since there was no increase in the total amount of transporters, and suggests that this part of the Glut1 transporter might be responsible for the regulation of glucose uptake activity.

Glut1 transporter has also been suggested to belong to the glucose-regulated protein (GRP) family of stress protein and that it is ubiqitously expressed and stimulated by cellular stresses (Wertheimer *et al.*, 1991). Glut1 was known to be increased under various conditions that induce other known GRPs, such as GRP-78 (Stoeckle, 1988, Birnbaum *et al.*, 1987). However, when a major stress, such as heat shock, was applied, Glut1 was not induced. Thus, Glut1 expression seems to be specifically induced by factors that regulate GRP expression. Interestingly, the induction of GRP and Glut1 expression seems to be coordinated during cellular transformation by oncogenes (Stoeckle *et al.*, 1988, White and Weber, 1988, Birnbaum *et al.*, 1987, Hiraki *et al.*, 1989). Thus, transformation of chicken embryo fibroblasts by Rous sarcoma virus results in src-dependent induction of GRP-78 (Stoeckle *et al.*, 1988), and increased levels of the Glut1 protein (Birnbaum *et al.*, 1987). Likewise, mouse fibroblasts transformed by various oncogenes such as src, ras, and fps show an increased glucose uptake that is correlated with elevated levels of the Glut1 mRNA (White and Weber, 1988, Birnbaum *et al.*, 1987, Hiraki *et al.*, 1989). It is believed that GRPs act as chaperones by binding to misfolded proteins to prevent aggregation (Pelham, 1986, Ellis, 1987, Kozutsumi *et al.*, 1988). The role for Glut1 transporter appears to function also in the recovery from cellular stress.

Glut2 appears in organs that release glucose into the blood, such as the intestine, the liver, and the kidney, and in the beta cells of the pancreas which secrete insulin. The sequence of this liver-type glucose transporter (Glut2) possesses 55% identity with that of Glut1, and 82% identity between human and rat Glut2.

Glut3 is found in the neuronal cells of the brain. Because it has a higher affinity for glucose than Glut1 does, this transporter ensures a constant movement of glucose into these cells. Human Glut3 has 64 and 52% identity with human Glut1 and Glut2,

respectively, and with an 83% amino acid sequence identity between the sequences of human and mouse Glut3. Thus, as with Glut2, the sequence of Glut3 is not as highly conserved among species as that of Glut1. The greatest degree of sequence divergence in Glut3 occurs in the extracellular loop and intracellular carboxy-terminal domains. These are the same regions that are the most divergent among the different isoforms.

Glut4 is the major transporter in muscle and adipose tissue. Insulin causes a rapid and reversible increase in glucose transport activity in both these tissues. The isolation and characterization of a monoclonal antibody that specifically recognized the insulin-regulatable glucose transporter indicated that it was a unique isoform, different from the transporters present in erythocytes, brain, kidney, and liver (James *et al.*, 1988).

Human Glut4 has 65, 54, and 58% identity with human Glut1, Glut2, Glut3, respectively. The sequence of Glut4 is highly conserved, and there is 95 and 96% identity between the sequences of human and rat or mouse Glut4, respectively. The Glut4 is the isoform expressed in insulin-responsive tissues which is localized predominantly in an intracellular location in the basal response to insulin (Gould and Bell, 1990). Recently, the amino terminus of Glut4 was found to be necessary and sufficient for intracellular sequestration (Piper *et al.*, 1992). In addition, the insulin-regulated translocation of Glut4 is conferred by cell-specific factors. The insulin-dependent movement of Glut4 to the plasma membrane does not occur in fibroblasts, and it is likely that insulin-sensitive cells express a unique machinery that acts to recruit transporters from the intracellular compartment to the plasma membrane (Haney *et al.*, 1991). Glut5 is a recent member of the facilitative glucose transporter gene family to be identified (Kayano *et al.*, 1990). Glut5 is found mainly in the small intestine and kidney. Its function has not yet been described in detail, except that it might transport glucose or it could be a carrier for another sugar or other small polar molecule. Human Glut5 shares 42, 40, 39, and 42% identity with Glut1, Glut2, Glut3, and Glut4, respectively.

Glut6 is an expressed facilitative glucose transporter pseudo-like sequence that has been identified in human tissues (Kayano *et al.*, 1990). This sequence is part of a ubiquitously expressed mRNA, but does not encode a functional protein.

Glut7 is thought to be located in hepatocytes and other gluconeogenic tissue. It is thought to mediate glucose flux across the endoplasmic reticulum membrane after the action of glucose-6-phosphatase on glucose-6-phosphate (Waddell *et al.*, 1992).

Objectives

The main objective of this thesis was to compare the hexose transport regulation in two cell strains, namely, the human diploid fibroblast (MCH 55) and respiration-deficient

mutant (WG 750). The WG 750 cell strain is a cultured skin fibroblast taken from a patient with severe lacticacidemia (Darley-Usmar *et al.*, 1983, Frerman and Goodman 1985, Randle and Smith, 1958). This strain has been characterized as having low rates of [1 - ^{14}C] pyruvate oxidation, lactate-to-pyruvate ratios 3-fold greater than control, and low rates of ATP and oxygen consumption with NAD-linked substrates (Robinson *et al.*, 1986). Robinson has shown that these WG 750 cells have a defect in NADH-Coenzyme Q reductase (Robinson *et al.*, 1986). Thus, the inability to oxidize mitochondrial NADH and generate energy via the electron transport chain necessitates a substantial increase in glucose utilization and anaerobic glycolysis to maintain cellular homeostasis. The elevation of hexose transport response has also been observed in avian erythrocyte (Wood and Morgan, 1969), rat skeletal muscle (Randle and Smith, 1958) and respiration deficient (i.e., NADH CoQ reductase deficient) Chinese hamster lung fibroblasts (Germinario *et al.*, 1990). The high requirement for glucose led to the question of whether the enzyme defect in the WG 750 cells would affect the normal regulatory controls governing hexose transport. Several factors known to modulate hexose transport were used to address this question, such as the presence or absence of serum or insulin. Kinetic studies were also performed to further characterize any difference between the two cell strains by determining the Michaelis-Menton constants K_m and V_{max} for 2 - DG transport.

Furthermore, the increase in hexose transport in WG 750 cells led to the question of whether more glucose transporter protein might exist in the cell. Whole cell extracts and subcellular membrane fractionation were analyzed by Western blots to quantitate the transporter protein and determine its distribution within the cell.

The final objective of this thesis was to determine if hexose transport was regulated at the transcriptional level. Steady-state transcript RNA levels were analyzed in both cell strains under various conditions, such as the presence or absence of insulin or glucose. Kosaki has shown that insulin increased the Glut1 gene expression in cultured human skin fibroblasts (Kosaki *et al.*, 1988). Kosaki has also demonstrated that glucose deprivation resulted in approximately 4-fold increase in Glut1 mRNA, 3-fold increase in Glut1 protein and basal 2 - DG uptake (Kosaki *et al.*, 1988). It was of interest to determine if the molecular mechanism(s) for the increased hexose transport regulation in the respiration-deficient mutant was transcriptional.

MATERIALS AND METHODS

Cell Culture

Human Diploid Fibroblasts

The cell lines employed in all the experiments were a normal human diploid fibroblast which was established from the skin (inguinal area) of a two-month-old female patient. The mutant cell line was a respiration deficient human fibroblast which was established from the skin (deltoid) of a two-week-old male patient. Both cell types were supplied by the Repository for Mutant Human Cell Strains (Montreal Children's Hospital, Montreal). It is a characteristic of the respiratory chain-deficient cell strain that cell death occurs very rapidly after the glucose in the culture medium has been used up. Therefore the culture was fed with Dulbecco's Modified Eagle's Medium (DME) three times each week. All cells were cultured in antibiotic-free DME supplemented with 1 mM pyruvate and 10% (v/v) fetal calf serum (flow). The cells were incubated at 37° C in an atmosphere of 5% CO_2 and 95% air. When the cells had reached confluence, they were harvested from the culture vessels (150-cm^2 plastic flasks) (Falcon Co. or Corning Co.) following an incubation of 2 min at RT with 0.04% (w/v) trypsin (Difco Labs, Detroit, Michigan) and 5 min with 0.02% (w/v) EDTA at 37° C. The cell suspension was centrifuged at 800 x g for approximately 5 min, the supernatnat was discarded, and the pellet resuspended in DME. The cells were counted with a hemocytometer and subcultured in 150-cm^2 plastic flasks (1:2 split ratio). The cell number at confluence was used in order to determine the number of population doublings (PD) accrued by the cells. When calculating PD, we made the assumption that 50% of the cells would not attach and grow after subcultivation (Good, 1972). That is, if there were 2×10^6 cells present at confluence and only 5×10^5 cells were placed in the flasks to begin with, the number of PD would be equal to 3, as calculated from the equation:

$$[\log_{10} (\text{final number of cells}) - \log_{10} (\text{initial number of cells}/2)] / \log_{10} 2.$$

In all experiments, unless otherwise stated, the cells had not completed more than 50% of their in-vitro life span.

GENERAL EXPERIMENTAL PROCEDURE

Human Diploid Fibroblasts

Cells were plated at a density of approximately 1×10^5 cells per 35 mm plastic petri dishes (Corning Co.) and grown to confluence (usually one week). When confluent, the medium was removed from the plates and the monolayers were rinsed once with serum-

free medium (0% MEM) containing 1 mg/ml bovine serum albumin (BSA; Sigma Chemical Co.) and 4 mg (22.2 mM) glucose/ml and incubated in 0% MEM for 24h, unless otherwise stated.

2-deoxy-D-Glucose Uptake Procedure

In all experiments, when measuring hexose transport, zero-time controls were subtracted to correct for non-specific diffusion. Sugar transport determinations were performed in triplicate monolayers. The uptake time used for 2-deoxy-D-glucose (2-DG) transport studies varied from 0.5, 2 or 5 min at 37° C. Hexose transport was linear and rate-limiting for the time interval employed for 2-DG at a 1.0 mM concentration. The specific activity of a 1.0 mM solution was 4.5 μCi/μmole 2-DG. The cell monolayers were rinsed twice with 2 ml glucose-free phosphate-buffered saline (PBS) at pH 7.4 (37° C). Then 0.8 ml of PBS containing the ^{3}H-labeled 2-DG was added to the cells and incubated for appropriate time intervals at 37° C. After incubation, the radioactive medium was removed and the cell monolayers were washed four times with 2 ml (each time) of cold PBS (pH 7.4). Zero-time control plates were rinsed twice with 2 ml cold PBS (pH 7.4) at 4° C, and 0.8 ml of PBS containing ^{3}H-labeled 2-DG was added. The radioactive medium was then rapidly removed and the cell monolayers were washed four times with 2 ml (each time) of cold PBS (pH 7.4) at 4° C. The monolayers were dissolved in IN NaOH and aliquots were taken for liquid scintillation counting and protein determination (Lowry *et al.*, 1951).

3-O-Methyl Glucose Uptake Procedure

Hexose transport was assessed by measuring the transport of ^{3}H-3-O-Methyl-glucose (3-O-MG) after 30 sec incubation time. When performing the assay for sugar uptake, the cell monolayers were rinsed twice with 2 ml of glucose-free PBS (pH 7.4) at 23 ° C. Then 0.8 ml of PBS containing the ^{3}H-labeled 3-O-MG was added to the cells for the appropriate time interval at 23° C. The specific activity of 1 mM labeled 3-O-MG was 4.5 μCi/μmole. After incubation, the radioactive medium was removed and the cell monolayers were washed four times with 2 ml (each time) of cold PBS (pH 7.4) containing 0.3 mM phloretin to prevent the efflux of free sugar. Zero-time control plates were rinsed twice with 2 ml cold PBS (pH 7.4) containing phloretin (4° C), then 0.8 ml of PBS containing ^{3}H-labeled 3-O-MG was added. The radioactive medium was rapidly removed and the cell monolayers were washed four times (2 ml each time) with cold PBS (pH 7.4) containing phloretin (4° C). The cell monolayers were dissolved in IN NaOH and

aliquots were taken for liquid scintillation counting and protein determination (Lowry *et al.*, 1951).

Determination of Lactate and CO_2 Production

For CO2 production, cells were grown in 25-cm_2 tissue culture flasks in the presence of 0% Dulbecco's Modified Eagle's medium (DME), glucose free, containing 0.5 mM D - [U - ^{14}C] - glucose. Stopper-sealed flasks were used, into which a plastic scoop with a center well had been placed and into this well a piece of filter paper had been inserted. One ml of IN H_2SO_4 was added to the medium to evolve the soluble CO_2, followed by the addition of 0.2 ml Protosol (NEN) into the center well. The flasks were then shaken for 30 min at room temperature (RT). The filter paper was then removed from the center well and transferred to a counting vial.

For lactate production, 200 µl of the above medium was used to run onto a Dowex 1 - X8 (Formate form) column to separate glucose and lactate. Glucose and lactate were separated by sequential elution with distilled water and 0.4 M Formate. D - [^3H] glucose and [U - ^{14}C] lactic acid standards were used to calculate recovery. For glucose and lactate, 89 to 97% and 108 to 122% were recovered, respectively.

Hexokinase Assay Procedure

Cells were plated at a density of approximately 1 x 10^6 cells per 100-mm petri dishes (Lux Scientific Corporation, Calif.) and grown to confluence at 37° C in CO_2 and air (5:95) atmosphere. The cell monolayers were rinsed once with 0% MEM containing 1 mg/ml borine serum albumin and 4 mg/ml glucose and incubated in 0% MEM for 24h. The cells were then rinsed twice with 5 ml of cold glucose-free PBS at pH 7.4 (4° C). To harvest the cells, 3 ml of cold 0.02 M tris-HCl in 0.32 M sucrose was added to the plates and scraped with a rubber policeman, pooled into centrifuge tube and centrifuged at 200 x g for 5 min. The cell pellets were then resuspended in 3 ml homogenizing buffer containing 0.4 M KCl, 0.02 M Tris-HCl, 1.5 mM EDTA and 2 mM dithiothreitol and homogenized with a cold-equilibrated hand glass homogenizer at 4° C. The homogenates were then centrifuged at 500 x g for 5 min and the supernatants assayed for hexokinase activity. The hexokinase assay employed selectively precipitates the phosphorylated ^3H - labeled 2 - DG by barium sulfate (Germinario *et al.*, 1978). The hexokinase reaction mixture (1.0 ml) contained 10 mM HEPES buffer (pH 7.4), 10 mM $MgCl_2$, 10 mM ATP, 10 mM KCl, 0.5 mM ^3H - labeled 2 - DG (4.5 µCi/µmole 2 - DG) and 0.2 ml aliquots of cellular homogenate (2 mg/ml). The reaction mixture was incubated at 37° C for 0.5, 10, 20 min time intervals. At the appropriate time, the phosphorylation of 2 - DG was stopped

by the addition of 0.5 ml of 0.3 N $Ba(OH)_2$ and 0.3 ml of 5% (w/v) $ZnSO_4$. The free 2 - DG remaining in the supernatant was determined by liquid scintillation. The amount of phosphorylated sugar was determined by subtraction of the amount of free 2 - DG in the control samples.

Insulin Binding Studies

Cells were plated at a density of 1 x 10^5 cells per 35-mm diameter petri dish and grown to confluence (usually one week). ^{125}I-insulin binding was determined following a 48 h period of serum starvation using serum-free MEM containing 1 mg/ml bovine serum albumin and 4 mg/ml glucose. After 48 h, the medium was removed and the confluent monolayers were washed twice with 3 ml of room temperature (22° C) Hank's—HEPES (20 mM) buffer (pH 7.4), containing 0.2% (w/v) BSA. Cells were incubated with 1 ml/plate of the above Hank's-HEPES (20 mM) buffer, containing 1.67 x 10^{-10} M I^{125} - insulin (specific activity = 80 - 90 μCi/μg) (New England Nuclear Corp., Boston, Mass.) alone or with 40 μg of unlabeled insulin (Insulin-Toronto, 100 μ/cm^3; Connaught Laboratories Ltd., Ontario, Canada) to allow determination of nonspecific binding.

The binding assay was performed at 22° C with gentle shaking. Preliminary experiments indicated that binding achieved its maximum level after 90 - 120 min. A 120 - min incubation time was used in all experiments. The equilibrium reaction was terminated by washing the cell monolayers with cold Hank's—HEPES buffer and solubilizing them in 1.2 ml of IN NaOH. Specific binding was determined by subtracting the nonspecific binding from the total binding.

Quantitation of Glucose Transporter Protein Using Whole Cell Extracts

Cells were plated at a density of approximately 1 x 10^6 cells/100 mm diameter petri dish (Falcon Co.) and grown to confluence. For each cell line 5 — 100 mm petri dish was used. The cells were rinsed once in serum-free MEM containing 1 mg/ml bovine serum albumin (BSA, Sigma Chemical Co.) and 4 mg/ml (22.2 mM) glucose, and incubated in serum-free MEM for 24h.

The subsequent procedure was performed at 4° C. The medium was aspirated and the monolayers were rinsed three times with 5 ml of ice-cold glucose-free PBS (pH 7.4). The PBS was thoroughly aspirated from all plates and the monolayers were scraped off the dish with a rubber policeman in approximately 3 ml PBS/100 mm petri dish. The cells from the plates were pooled in a centrifuge tube and centrifuged at 800 x g for 5 min. The supernatant was discarded and the pellet resuspended in 500 μl of glucose-free ice-cold PBS (pH 7.4), and the protein concentration was determined by Lowry assay (Lowry *et*

al., 1951). Protease inhibitors, pepstatin, and phenylmethanesulfonyl fluoride (PMSF) were added to the cell extracts at a final concentration of 1 μg/ml and 1 mM, respectively. Extracts were stored at -70° C and subsequently thawed and diluted in SDS sample buffer to a final protein concentration of 2 μg. Final concentrations in sample buffer for SDS polyacrylamide gel electrophoresis were 2% (w/v) SDS, 25 mM dithiothreitol, 10% (v/v) glycerol, 1 mM EDTA, 95 mM Tris-HCL (pH 6.8) and 100 μg/ml bromphenol blue.

Sodium Dodecyl Sulfate (SDS) — Gel Electrophoresis and Immunoblotting

Aliquots of whole cell extracts and various subcellular fractions were analyzed for glucose transporter protein by SDS-polyacrylamide gels according to the procedure of Laemmli (1970). A 5% stacking gel and a 10% resolving gel were used. Proteins (25 μg) in the gels were electrophoretically transferred in 25 mM sodium phosphate buffer (pH 6.5) onto a nitrocellulose membrane at 300 mA for 2h at room temperature. After transblotting, the nitrocellulose membrane was soaked in 3% BSA in a wash buffer (WB) for 1 hr at room temperature for nonspecific binding. It was then incubated overnight at 4° C with the primary monoclonal antibody raised against the human HepG2 erythrocyte-type glucose transporter (M ab g3) (provided by Dr. Gustav Lienhard of Dartmouth Medical School, Hanover, NH) in the wash buffer (WB) containing 0.3% BSA, under gentle shaking. The filter was then extensively washed five times (15 min each time) in 3% BSA/WB and then incubated with a secondary antibody, ^{125}I sheep anti-mouse (Fab)(DuPont, NEN)(1.2×10^6 dpm/10 ml) in 3% BSA/WB for 3h at room temperature. It was then washed four times in wash buffer containing 1M NaCl, then washed once in wash buffer for 15 min. The filter was finally wrapped in saran wrap and exposed to Kodak XAR-5 at -70° C for 1-4 days. The audioradiogram was scanned using densitometry.

Subcellular Fractionation and Plasma Membrane Isolation Using Sucrose Gradient

Fibroblasts were subcultured into polystyrene 850-cm^2 roller bottles (Corning Co.) and grown to confluence (usually two weeks). Prior to experimental procedure, the roller bottles were serum starved for 48h in serum-free MEM containing 1 mg/ml bovine serum albumin and 4 mg/ml glucose. After serum starvation, the plasma membranes were isolated at 4° C according to the modified method of Horner (Horner *et al.*, 1987). The culture medium was removed from the six roller bottles (three roller bottles/cell line) and the monolayers were washed four times with ice-cold PBS (pH 7.4). The roller bottles were then washed free of PBS with 20 ml/roller of ice-cold hypotonic lysis medium, consisting of 1 mM $NaHCO_3$ buffer, pH 8 (LM buffer). The roller bottles were placed in

an ice bath and were rotated slowly for 1 min. The cells were harvested using 20 ml Buffer B (300 mM Mannitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) containing 0.5 mM PMSF as a final concentration. The roller bottles were then shaken vigorously and the cells were scraped off using a rubber policeman. All the bottles were kept on ice until all of the cells were detached from the bottles. The lysates were then pooled and centrifuged in two polycarbonate tubes at 27,000 x g for 20 min at 4° C in a Beckman ultracentrifuge (Model L350). The supernatants (S^1) were centrifuged at 150,000 x g for 90 min at 4° C (yielding the putative microsomal pellet - P_3). The pellets (P1) from the first centrifugation were pooled and resuspended in 10% sucrose (w/v, final volume 7 ml) and layered onto a discontinuous gradient consisting of 60%, 48%, and 30% sucrose layers (w/v). The gradient was centrifuged at 76,000 x g for 2 h at 4° C in a Beckman SW-27 rotor. Bands A (10/30%) and B (30/48%) were carefully removed with a Pasteur pipet and resuspended in Buffer B and centrifuged at 65,000 x g for 25 min at 4° C. The resulting pellets were finally resuspended in 1-2 ml Buffer B. For all the fractions, protein content was determined by the method of Lowry (Lowry *et al.*, 1951) with bovine serum albumin as the standard. 5' Nucleotidase Assay was performed before fractions were frozen, and then stored at -70° C.

RNA Transcription in Vitro (Riboprobe): DNA Template Preparation for Transcription/Template Linearization

The Glut1 cDNA was originally subcloned in the pGEM vector. The plasmid was then linearized with restriction endonuclease Sal1 (NEB) to produce runoff transcripts derived from the insert sequence only, such that only the cDNA sequence downstream of the T7 promoter would be transcribed. The T7 promoter of the clone primes the transcription of one strand of the insert and the T3 promoter primes transcription of the complementary strand. Sal1 restriction enzyme digestion consisted of 1.4 µg plasmid, 1 X Sal1 buffer (2 µl) (NEB), 1 X BSA, 13.5 µL RNA-grade DEPC-H_2O and 10 units Sal1 enzyme in a final volume of 20 µL. The digestion was carried out at 37° C for 2h. After ascertaining complete digestion by gel analysis, the linearized plasmid was phenol and chloroform extracted. RNA-grade phenol (20 µL) was added, vortexed and centrifuged at 12,000 rpm for 5 min. Aqueous phase was transferred to a new tube and chloroform (20 µL) was added, vortexed, and centrifuged at 12,000 rpm for 5 min. Aqueous phase was transferred to a new microfuge tube and the DNA was precipitated with 1/10 volume of 3M sodium acetate (pH 6) and 2.5 volume of 100% ethanol, at -20° C overnight. The precipitated DNA was centrifuged at 14,000 rpm at 4° C for 20 min., the pellet was washed with RNA-grade 70% ethanol, and finally resuspended in DEPC-H_2O to a final concentration

of 0.2 mg/ml. Sal1 digestion must be complete before proceeding, since small amounts of indigested plasmid DNA would yield long transcripts and would incorporate substantial radiolabeled rNTP.

RNA Transcription Reaction and Labeling of Riboprobe

In a 0.5-ml microfuge tube, the following components were added: 1.5 µl DEPC-H_2O, 1X Transcription buffer (Promega), 10 mM DTT (Promega), 1 µl rRNasin (Promega), 0.5 mM CTP (Promega), 0.5 mM GTP (Promega), 0.5 mM UTP (Promega), 18 µM ATP (Promega), 70 µCi (1.16 µM) [α - ^{32}p] ATP (NEN), 0.2 µg linearized DNA template and 1 u/µl T7 RNA polymerase (Promega). The reaction mixture was incubated at 37° C for 1 hr. Prior to the addition of the T7 RNA polymerase, 0.5 µL of the reaction mixture was added to 0.1 ml 10% TCA and 0.5 µl was spotted onto a PEI-cellulose for the 0 min time point of TCA precipitation and thin layer chromatography (TLC) analysis. After additiion of T7 polymerase, the transcription reaction proceeded at 37° C for 1 hr. Again, 0.5 µl was removed for the 35-min time point for TCA precipitation and TLC analysis. To the TCA precipitate samples, 1.5 µL (10 mg/ml) salmon sperm DNA was added, and placed on ice for 2 hr. The samples were centrifuged at 14,000 rpm at 4° C and the pellets were resuspended in 125 µl protosol (NEN) overnight for specific activity counts. The TLC cellulose was run in 0.7 M KPi pH 3.5 to verify RNA integrity. TLC cellulose was dried and exposed to Kodak XAR-5 for 7 min at room temperature.

Removal of the DNA Template:

The DNA template was removed by digestion with DNAse I (RNase-free). One microliter of 0.25 u/µl RQl DNase (Promega) was added to the reaction tube, and incubated at 37° C for 10 min. After digestion of the DNA template, 0.5 µl was spotted onto the cellulose for the DNase point of the TLC analysis.

Phenol/Chloroform Extraction:

30 µl RNA-grade TE (10 mM Tris-HCl, 1 mM EDTA, pH8) was added to the transcript to bring volume up to 50 µl. The RNA was then extracted once with 50 µl TE-saturated phenol:chloroform (1:1). After organic extraction, 0.5 µl of the transcript mixture was spotted onto the TLC cellulose for the Phenol/Chloroform point of TLC analysis. The TLC cellulose was run in 0.7 M KPi, pH 3.5, to check for RNA integrity.

Purification of RNA Transcript from Unincorporated Nucleotides:

Unincorporated nucleotides were separated from the RNA transcript by size exclusion chromatography on a Sephadex G50 Nick Column (Pharmacia). The column was equilibrated with 10 ml RNA-grade TE, and the RNA transcript was eluted with 0.2 ml of the same buffer (TE). Ten fractions were collected (0.2 ml fractions) and analyzed by TLC to determine the peak of the purified RNA transcript.

Determination of Specific Activity:

50 µl out of the 125 µl of resuspended TCA precipitates (0 and 35 min) were counted in a total volume of 0.5 ml protosol. The specific activity of the RNA probe was determined from the 35 min sample as follows:

ng RNA transcript = 70 µCi x 1 nmol/3000 µCi x 4 NTPs x 300 ng/nmol

where 70 µCi is the amount of radioactivity used in the transcription reaction,

1 nmol/3000 µCi is the specific activity of the [α - ^{32}P], ATP and

300 ng/nmol is the molecular weight oligonucleotide.

The specific activity, S.A., of the probe is the total incorporated cpm/µg RNA transcript synthesized. Then,

$$S.A. = \frac{(35 \text{ min TCA cpm})\ (100 \text{ dilution})}{(\mu g \text{ RNA transcript})\ (\%\ ^{32}P \text{ incorporation})}$$

Gel Analysis of RNA Probe:

The purified RNA probe was resolved by electrophoresis to ascertain the integrity and molecular weight of the probe. If the RNA is intact and transcription went to completion, resolution of the probe by electrophoresis should reveal a single sharp band at the expected molecular weight. If RNA is degraded and transcription did not go to completion, resolution of the probe should reveal a smear.

Maxi Preparation for Purification of Plasmid DNA Using Cesium Chloride Gradient

Large-scale preparation of high-purity plasmid DNA was obtained by alkaline lysis and equilibrium centrifugation in cesium chloride-ethidium bromide gradients (Sambrook *et al.*, 1989). The Glut1 cDNA subcloned into the pGEM plasmid was transformed into *E. coli.*

Preparation of Large Culture of Transformant:

A 50-ml flask of LB + 50 µg/ml ampicillin was innoculated with one colony from a stock of transformant *E. coli* plates. The flask was shaken overnight at 37° C at 210 - 250 rpm. A 400-ml TB + 50 µg/ml ampicillin flask was then innoculated with 1 ml of the above culture. The 400-ml culture was shaken at 37° C at 210 - 250 rpm overnight.

The culture was centrifuged at 6,000 x g for 30 min at RT. The pellet was well resuspended in 36 ml of glucose solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). After cells were well resuspended, 4 ml lysosyme (4 mg/ml) in glucose solution was added for 10 min at RT to allow lysis. A denaturing solution (0.2 N NaOH and 1 % SDS) was added for 5 min on ice and gently mixed by inversion. Cold potassium acetate, pH 5.0 (40 ml), was added, and the tubes were quickly vortexed and placed on ice for 15 min. Potassium acetate renatures plasmid DNA only, while chromosomal DNA stays denatured and forms aggregates. The mixture was centrifuged at 8,000 rpm for 10 min at 4° C to pellet chromosomal DNA. The supernatant was gently poured into 250-ml centrifuge tubes through several layers of cheesecloth. The cheesecloth captures chromosomal clumps. To precipitate the plasmid DNA, 100 ml isopropanol was added to the supernatant and placed on ice for 20 min. It was then centrifuged at 8,000 rpm for 10 min (4° C). The supernatant was discarded and the pellet was resuspended gently in 28 ml 10X TE (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0). Once resuspended, it was transferred to a 50-ml falcon tube and 40 µl of RNAase A (10 mg/ml) was added to degrade RNA. It was incubated at 37° C for 60 min.

Cesium Chloride Gradient Purification of Plasmid DNA:

To the DNA solution, 31 g of CsCl was added and 0.3 ml of ethidium bromide (10 mg/ml) was also added. The density was then calculated. The density should not exceed 1.59. This range will allow the plasmid DNA to collect in the center of the tube. Adjust density, if necessary with 10X TE. Quick-Seal polyallomer tubes (38 ml) were filled with DNA/CsCl/ethidium bromide solution by using a 12-gauge needle attached to a 60-cc syringe. Air bubbles were removed by flicking the tube, and mineral oil was added to fill the tube. Quick-Seal tubes were sealed using a heating electric sealer. DNA was

centrifuged using ultracentrifugation at 45,000 rpm at 15° C for 16 hr. using no brake. The plasmid DNA band was removed by inserting a 12-gauge needle at the top of the Quick-Seal tube to form a vent. The actual plasmid band was removed by using a 10-ml syringe and a 23-gauge needle. The 10 ml syringe was inserted slightly below the band and gently pulling out on the plunger, making sure not to aspirate the neighboring bands inside the syringe. Once the band was collected, a piece of tape was placed on the hole where the needle was inserted. The band was placed into a 15-ml falcon tube. The ethidium bromide from the plasmid DNA was extracted using CsCl saturated butanol solution. To a 500-ml bottle, 150 ml H_2O was added and the rest of the bottle was filled with butanol. CsCl was added until saturation occurred. The bottle was shaken vigorously. If saturated, CsCl should form a precipitate. If no saturation occurs, more CsCl should be added.

Equal volume of CsCl butanol solution was added to the plasmid DNA band. The butanol/plasmid DNA was vigorously vortexed for 1 min, allowing phases to separate (takes 30 sec) and the top phase was then aspirated and discarded. The plasmid DNA was dialysed using a spectropor dialysis tubing (25,000 mol. wt. cutoff). The DNA was dialysed at RT with gentle stirring in 4 L TE (pH 7.5) and 0.5 M NaCl, overnight. Plasmid DNA was removed from dialysis tubing and transferred to Corex tube. To precipitate the DNA, two volumes of 100% ethanol were added and incubated overnight. Tubes were centrifuged at 11,000 rpm for 20 min. at 4° C. The supernatant was discarded and the pellet was washed once with 70% ethanol, air dried, and resuspended in 500 µl TE (pH 8.0).

Acid-Guanidinium-Phenol RNA Extraction Method

The method used is as described elsewhere (Chomczynski and Sacchi, 1987).

All solutions were made in baked glassware (180°C oven). Before using solutions, all water was treated with diethylpyrocarbonate (DEPC) (0.1% v/v, Sigma) and autoclaved for 60 min.

Cells were plated at a density of 1 x 10^6 cells/100-mm petri dish and grown to confluence. Cell monolayers were rinsed once with 0% MEM and serum starved in 0% MEM for 24h. Cells were rinsed once with cold PBS (pH 7.5), and were subsequently harvested by using 3 ml PBS (pH 7.5) and scraped with a sterile rubber policeman. Cells were pooled into a glass Corex tube and placed on ice until all the other plates were harvested. 4M Guanidinium thiocyanate (3 ml) was added to each tube, followed by 0.3 ml of 2M sodium acetate, (pH 4.0) vortexed and placed on ice. An equal volume (6 ml) of water-saturated phenol was added and vortexed. Only one phase should appear at this step. Equal volume (6 ml) of chloroform:iso-amyl alcohol (24:1) was then added,

vortexed, and placed on ice for 30 min or until 2 phases appear. The mixture was then centrifuged in Beckman (JA-21 rotor) for 20 min at 9,000 rpm at 4° C.

The aqueous (top) phase was removed with a P-1000 pipetteman and tranferred to a fresh corex tube. An equal volume of isopropanol was added, and the tubes were parafilmed and stored overnight at -20° C. RNA was collected by centrifugation for 30 min at 9,000 rpm at 4° C. The supernatant was removed and the pellet was resuspended after air drying, with 500 µL 4M GuSCN extraction buffer. The solution was then transferred to a sterile microfuge tube and 500 µl isopropanol was added and stored at -20° C overnight. RNA was centrifuged in a microfuge for 10 min at 4° C at 14,000 rpm. The supernatant was removed and 900 µl of 80% ethanol (stored at -20° C in RNA grade H_2O) was added. Immediately, it was centrifuged (without vortex) for 5 min at 14,000 rpm at 4° C. The supernatant was removed and the ethanol wash was repeated. The ethanol supernatant was removed and pellets were dried by inverting the tubes on a clean Kimwipe. The pellet was resuspended in 200 µl sterile RNA-grade TE. Samples were heated at 65° C for about 15 min until dissolved. UV spectra was taken to quantitate the nucleic acid and an agarose gel was run to determine the extent of DNA contamination and RNA integrity.

RNA FORMALDEHYDE GEL ELECTROPHORESIS (DENATURING GELS)

The method used is as described elsewhere (Sambrook *et al.*, 1989, Lehrach *et al.*, 1977). All solutions were made in baked glassware (180° C oven). Before using solutions, all water was treated with DEPC (0.1% v/v, Sigma) and autoclaved for 60 min.

Preparation of the agarose gel:

For a medium gel apparatus (100 ml), a final concentration of 2.2 M formaldehyde (6%) and 1X MOPS buffer was added (5X MOPS Buffer: 0.2 M MOPS pH 7.2, 50 mM sodium acetate, 5 mM EDTA). Agarose (1%) was added to a baked flask. DEPC-treated water (64 ml) was then added and microwaved to dissolve the agarose. When cooled, 20 ml of 5X MOPS buffer and 16 ml formaldehyde (37%) were added under the fume hood. The flask was quickly mixed and poured in the gel appartus, and allowed to solidify for 30 min.

Preparation of the samples:

To denature RNA: 10 µg RNA was added to DEPC-treated water to yield a total volume of 6 µl. 5X MOPS buffer (2.5 µL) and deionized formamide (12.5 µL) were added to yield final concentrations of 0.5X MOPS buffer and 50% formamide,

respectively. Finally, 4µl of formaldehyde was added to give final concentration of 6% (2.2 M). The samples were heated at 65° C for 5 min, and 0.5 µl of ethidium bromide (1 mg/ml) and 0.5 µl of saturated dye solution (bromophenol blue and xylene cyanol) were added to the samples before electrophoresis.

Electrophoresis:

The gel apparatus was filled with 1X MOPS buffer. The gel was pre-electrophoresed at 50 V for 5 - 10 min. The samples were loaded and high molecular weight RNA ladder (BRL) was used as RNA marker. The gel was electrophoresed at 70 - 80 volts. After 2 hr, the power supply was turned off, and the running buffer was changed with fresh running buffer (to maintain stable pH). The gel was electrophoresed for another 2 - 3 hr.

NORTHERN BLOT ANALYSIS

The method used is as described elsewhere (Sambrook *et al.*, 1989, Meinkoth and Wahl, 1984). For the riboprobe and the northern blots, all materials and solutions were treated with DEPC water (0.1% v/v), Sigma. After gel electrophoresis, the gel was soaked in double distilled water for 15 min to remove the formaldehyde, and then soaked in 10X SSPE (1.8 M NaCl, 100 mM NaPi, 10 mM EDTA) for 15 min. The gel was transferred using capillary transfer in 10X SSPE reservoir, to a Whatman 3MM paper and Nytran nylon membrane. Parafilm was placed around the gel to stop any "short-circuit" to the wick. Dry paper towels were cut to the appropriate size of gel and stacked on top of the gel. A light weight (~250-300 g) was placed on top of the paper towels to start capillary transfer. Gels were transferred overnight at RT. The Nytran membrane was removed and rinsed in 6X SSPE, air dried and baked in oven at 80° C for 2h using vacuum pump.

Prehybridization:

The membrane was wet in 6X SSPE and placed in a sealable bag. About 5 - 10 ml of prehybridization solution was added, which consisted of 50% deionized formamide, 2X Denhardt's, 0.5% SDS, 650 mM NaCl and 100 mM NaH_2PO_4, pH 6.8. Sonicated salmon sperm DNA at a final concentration of 100 µg/ml was then added by boiling the DNA for 8 min, then quickly chilling it on ice. Before sealing the bag, all air bubbles were removed and the bag was incubated at 60°C water bath overnight. For double-stranded DNA probe, the prehybridization solution was 5% deionized formamide, 10% dextran sulfate, 10X denhardt's, 0.4% SDS and 6X SSDE, and prehybridization was at 42° C overnight.

Hybridization:

A small piece of the prehybridization bag was cut and the prehybridization solution was replaced with 2 - 5 ml of fresh hybridization solution (same as prehybridization solution). Fresh sonicated salmon sperm DNA was added as in the prehybridization step. The riboprobe was not boiled, since it was single-stranded; however, the double-stranded DNA probe was boiled for 7 min and quickly chilled on ice. Approximately 5 x 10^6 cpm were added for the riboprobe and 2 x 10^6 cpm for the DNA probe. The bag was sealed and incubated at 60° C (riboprobe) and 42° C for the DNA probe for 16 - 24 hr. After incubation, the radioactive hybridization was carefully removed, the blot was rinsed twice in 6X SSPE and 0.5% SDS at 42° C for 20 min each, and finally, two high stringency washes with 1X SSPE and 0.5% SDS at 60 ° C for 20 min each.

The washes for the DNA probe were different. The blot was washed twice with 6X SSPE and 0.5% SDS at 42° C for 15 min each and finally, the higher stringency wash was done twice with 1X SSPE and 0.5% SDS at 60 ° C for 15 min each.

The blot was wrapped in saran wrap and exposed to a Kodak XAR-5 film at - 20° C.

Random Primer Labeling Method

The method used is as described elsewhere (Smith and Summers, 1980).

Random oligonucleotides have been used as primers in this method for labeling DNA to high specific activity. The procedure relies on the ability of random hexanucleotides to anneal to multiple sites along the length of a DNA template. The enzyme synthesizes new DNA by incorporating nucleotide monophosphates at the free 3' - OH groups provided by the primer. The newly synthesized DNA is made radioactive by substituting a radiolabeled nucleotide for a nonradioactive one in the reaction mixture. The resulting labeled DNA can then be used as a sensitive hybridization probe for probing Northern blots.

Glut1 cDNA (25 µg) was added to a microfuge tube. Random oligonucleotide primers (10µl) (Promega) were added and the reaction mixture was heated at 100° C for 5 min to denature the DNA strands. The reaction mixture was then incubated at RT for 5 min to anneal the primers. The reaction mixture was briefly centrifuged at RT and the following components were added: 10 µl 5X Primer buffer containing dCTP, dGTP and dTTP (0.1 mM/each), 5 µl (50µCi, 3000 Ci/nmole) [α^{32}P] dATP, and 1 µl (2u/µl) T7 DNA polymerase. The mixture was quickly incubated at 37° C for 20 min. To determine the progression of the probe, two time points were taken. Prior to the addition of the enzyme, 0.5 µl of the reaction mixture was added to 0.2 ml of 10% TCA and 0.5 µl was spotted onto a PEI-Cellulose for the 0 min time of TCA precipitation and thin layer chromotography (TLC) analysis. After addition of T7 polymerase, the labeling reaction

proceeded at 37° C for 20 min. Again, 0.5 µl was removed from the mixture at the 20-min time point for TCA precipitation and TLC analysis. To the TCA samples, 20 µg salmon sperm DNA was added and placed on ice for 2 hr. The TCA samples were centrifuged at 14,000 rpm for 15 min at 4° C and the pellet was washed with 0.8 ml cold 10% TCA. The TCA wash was repeated twice. The pellets were finally resuspended in 500 µl protosol (NEN), overnight at RT. The TLC cellulose was run in 0.7 M KPi, pH 3.5, to verify the extent of the labeling reaction. TLC cellulose was dried and exposed to Kodak XAR-5 for 7 min at RT. To purify the probe, unincorporated nucleotides were separated by size exclusion chromatography on a sephadex G50 Nick column (Pharmacia). The column was equilibrated with TE and the probe was eluted with 0.2 ml TE. The fractions were collected (10 fractions) and analyzed by TLC to determine the peak of the DNA probe.

Radioactive Materials

2 - D - [2,6 - ^{3}H] glucose - (50 Ci/mmole) and [methyl - ^{3}H] 3 - OMG - (50 Ci/mmole) were purchased from ICN Radiochemicals, California. [^{32}P] - α - rATP and [^{32}P - α - dATP] were obtained from New England Nuclear Corp., Boston, Massachussetts. D - [^{14}C] glucose and D - [^{3}H] glucose were purchased from ICN Radiochemical Division, Inc. ^{125}I - labeled sheep anti-mouse Fab was purchased from Amersham Chemicals.

Chemicals

2 - deoxy - D - glucose and insulin (bovine pancreas crystalline) were obtained from Sigma Chemical Co., (St. Louis, MO). Phoretin was obtained from ICN Biomedicals Inc. (Plainview, NY). Cytochalasin B was obtained from Aldrich Chemical Co. (Milwaukee, WI). L - glucose and Bovine Serum Albumin were obtained from ICN Pharmaceuticals Inc. (Cleveland, OH). Random Prime Kit was obtained from Promega. RNA Invitro Transcription Kit was obtained from Stratagene, California. Pepstatin and Phenylmethylsulfonyl fluoride were purchased from Sigma.

Scintillation Counting

The counting solutions used were Formula 963 and Eqoscint, purchased from New England Nuclear Corp., Boston, Massachussetts. Samples were added to 5 ml of counting solution (Formula 963 or Eqoscint) in 7-ml vials. Vials were counted in a liquid scintillation spectrophotometer (LKB Rackbeta) with a counting efficiency of approximately 30% for ^{3}H. ^{3}H-toluene was used to generate a "calibration curve." They were quenched to varying degrees with diluted saturated pycric acid, IN NaOH, H_2O and

the cells were finally solubilized in IN NaOH. A standard quench correction curve was generated by the Automatic External Standardization channels' ratio method.

Statistics

Statistical analysis was performed using the Student's T test for paired data, 5% being the level chosen for significance unless otherwise stated.

RESULTS

Respiration-Deficient and Normal Human Skin Fibroblasts

The block in respiration of the mutant cells (WG 750) necessitating an ample amount of glucose in the media for the WG 750 cells led us to question whether glucose metabolism in those cells was regulated differently from the MCH 55 control cells. Several experiments were performed to monitor glucose metabolism in both cell strains. First, the rate of glucose utilization was monitored by measuring the glucose content in the media. The data in Table I clearly indicate that the rate of glucose utilization was elevated in the WG 750 cells by more than 2-fold. Cell monolayers were incubated in serum-free glucose containing media for 24h before samples from the media were analyzed for glucose content. Surprisingly, the two cell strains displayed identical doubling times of 48 h, indicating that the WG 750 cells double and grow as well as the normal cells in the presence of adequate amounts of glucose.

The mutant's inability of the NADH-Coenzyme Q reductase to synthesize ATP through the oxidative phosphoryation pathway led to the question of whether the defect affected the glycolytic pathway as well. The data in Table II show that the production of $^{14}CO_2$ and ^{14}C-lactate from U - D - ^{14}C - glucose in WG 750 cells were 3- and 4-fold greater, respectively, than the control cell line. It appears that the WG 750 cells undergo anaerobic glycolysis at a much greater rate to compensate for their respiratory block.

To determine whether it was the carrier-mediated transport that contributed to the elevated uptake or whether subsequent step(s) in the glycolytic pathway were affected, the first enzyme involved in the glycolytic pathway, namely hexokinase, was examined. Hexokinase activity was assayed in cellular homogenates of serum-fed cultures. The results of these studies demonstrated that the level of hexokinase activity was similar (Figure 3). This suggests that hexokinase activity is not affected and that up-regulation of glucose utilization is occurring specifically at the transport level.

We next questioned whether the defect in NADH-Coenzyme Q reductase affected the normal regulatory controls governing hexose transport. A glucose analog, 2-DG, was used to assay hexose uptake. This substance is transported by the glucose carrier and is phosphorylated inside the cell and effectively trapped therein (Germinario *et al.*, 1978). The basal transport level in both cell strains were examined using 2-DG. The data in Table III show that the basal 2-DG transport is indeed significantly elevated (6-fold) in serum-deprived WG 750 cells ($p < 0.01$, two-tailed t test, n=11). Cell monolayers were incubated in serum-free glucose containing media for 24h before samples were assayed for 2-DG uptake. Another glucose analog, 3-O-methyl glucose (3 - OMG), was used to confirm the observation made using 2-DG, and to determine whether the enhanced uptake of 2-DG in

WG 750 cells can be attributed to an increase in transport and not phosphorylation, since 3-OMG is transported into the cell via the glucose carrier, but it is not phosphorylated (Christopher, 1977). The data in Table IV indicate that 3-OMG is 3-fold greater in WG 750 cells. These data, taken together, suggest that hexose transport and not phosphorylation is up-regulated in WG 750 cells.

Cytochalasin B (CB) is known to specifically inhibit carrier-mediated transport, without affecting passive diffusion (Jung and Rampal, 1977, Lin *et al.*, 1974, Dolberg *et al.*, 1975). To determine how much hexose transport was carrier-mediated in both cell strains, the uptake of 2 - DG in the presence and absence of CB was determined. As shown in Table V, the addition of 10 μM CB inhibited 2-DG transport by 75 - 80% in both cell strains. Thus, the increase observed in transport is predominately via a carrier-mediated process.

L-glucose uptake was employed as a measure of the non-saturable component of hexose uptake, and was compared with 2-DG uptake to determine the amount of passive diffusion to total sugar uptake. L-glucose uptake accounted for approximately 1% of 2-DG uptake in both cell strains.

The kinetic constants for glucose transport in WG 750 cells and MCH 55 cells were investigated using the Lineweaver-Burk double reciprocal analysis (Lineweaver and Burk, 1934). The data in Figure 4 show the results of measurements of initial rates of 2-DG uptake at substrate concentrations between 0.025 mM and 10 mM plotted on a double reciprocal plot. Analysis of the intercepts indicate that differences exist in the V_{max} for 2-DG transport and not the transport K_m. The mutant WG 750 displayed a dramatic increase (6-fold) in the transport V_{max} when compared with the control cell strain (18.0 ± 1.6 vs. 3.2 ± 1.6 nmol 2-DG/mg protein/2 min; $p < 0.001$, $n = 5$) (Table VI). Further, the K_m (affinity) for 2 - DG was essentially the same in the two cell strains (1.97 ± 1.7 vs. 1.87 ± 1.1 mM). These results suggest an increase in the number of functional carriers at the plasma membrane in the WG 750 cells.

The Effect of Serum on Hexose Transport in MCH 55 and WG 750 Cells

To further characterize any differences that might exist in transport regulation, both cell types were exposed to serum, which is known to modulate sugar transport (Kletzien and Perdue, 1974a, Perdue, 1976, Klip *et al.*, 1984). The data in Figure 5 (transport ratios) demonstrate that serum refeeding of serum-starved MCH 55 and WG 750 cells behaved differently in their response. Serum refeeding stimulated 2 - DG uptake in MCH 55 to a greater degree than in WG 750 cells. The MCH 55 cells showed a 150% and 162% increase in 2 - DG uptake by 30 and 60 min of serum refeeding, respectively. By 120 min

of serum addition, 200% increase was observed in the MCH 55 cell strain. Interestingly, the WG 750 cells showed no increase in 2 - DG uptake by 30 min when compared to the MCH 55 strain. Only after 60 min of serum refeeding was a small increase in 2 - DG uptake observed (~25%) ($P < 0.05$, $n = 4$). Serum addition after 120 min resulted in a significant increase in 2 - DG transport in the mutant cells (175%) ($P < 0.05$, $n = 4$). The data in Figure 6 represent the same data as in Figure 5 but the actual rates of 2 - DG transport are plotted. While both cell types exhibit 175-200% increases in transport ratios after 120 min, there is clearly a dramatic quantitative increase in 2 - DG transport in the WG 750 vs. MCH 55 cell strains (i.e., the uptake at 120 min minus the uptake at 0 min). For WG 750, this would be 15 nmoles/mg protein (35 nmoles/mg protein - 20 nmoles/mg protein), while for MCH 55, the net increase would be 5 nmoles/mg protein (10 nmoles/mg protein - 5 nmoles/mg protein).

The Effect of Insulin on Hexose Transport in MCH 55 and WG 750 Cells

The data in Table VII show the stimulatory effects of 67 and 670 nM insulin on the 2 - DG transport in MCH 55 and WG 750 cells. Confluent monolayers were serum starved for 24 h and insulin was then added for 2 h. The magnitude of response for the MCH 55 cells was higher than the WG 750 cells at both concentrations of insulin. The addition of 67 or 670 nM insulin to the MCH 55 cells led to increases in 2 - DG transport ratios (1.99 ± 0.25 and 2.33 ± 0.26, respectively) over basal 2 - DG transport. The WG 750 cell's response to insulin, however, was significantly lower at both insulin concentrations (1.23 ± 0.06 and 1.34 ± 0.1, respectively; $P < 0.005$, $n = 6$). Basal 2 - DG transport for unstimulated cells (control) was 3.2 ± 1.0 nmol 2 - DG/mg protein/2 min and 17.9 ± 2.9 nmol 2 - DG /mg protein/2 min for MCH 55 and WG 750, respectively. The results concur with the previous data on serum refeeding which showed that the degree of response was not as great as the control (MCH 55) cells. Although the magnitude of response of the WG 750 cells to the presence of serum or insulin might appear to be smaller than the control cells, one must keep in mind that the basal transport is 6-fold higher in the WG 750 cells, and this might have some bearing on the lack of a dramatic insulin response. The number of insulin receptors were determined to see if the observed increase in 2 - DG uptake in the WG 750 cells might be attributed to an increase in the number of insulin receptors on the plasma membrane. The insulin binding data in Table VII indicate that the mutant and control cell strains have similar levels of specific insulin binding.

Dexamethasone is known to inhibit 2 - DG transport by reversing the translocation of the carrier process from an intracellular pool to the plasma membrane (Horner *et al.*,

1987). The increase in basal 2 - DG uptake observed in the WG 750 cells prompted the question of whether there might be a defect in this reversal process. The data in Figure 7 show that in the presence of 1 mM dexamethasone, 2 - DG transport was inhibited by 50% in both WG 750 and MCH 55 cell strains. Confluent cell monolayers were serum starved for 48 h and then exposed to the hormone for 6 h. This result indicates that there is no difference in the reversal process. The mobility of the glucose transporters to translocate from the plasma membrane to the intracellular pool does not appear to be affected.

Quantitation of the Glucose Transporter Protein Levels Using Total Cell Extracts

To characterize and quantify the glucose transporter protein levels in the MCH 55 and WG 750 cell strains, total cellular Glut1 transporter levels were determined by Western blot analysis using a monoclonal antibody raised against the purified human erythrocyte transporter (a gift from Dr. G. Lienhard). The Glut1 transporter is the only transporter expressed in these cell strains. The data in Figure 8 is a Western blot using whole cell extracts from MCH 55 and WG 750 cells. Varying amounts of protein were added in each lane to show the consistency in the 2-fold increase in total protein in the WG 750 cells. Lanes 1, 3, and 5 are the MCH 55 cells with 25 μg, 50 μg, and 100 μg protein, respectively. Lanes 2, 4, and 6 are the WG 750 cells with 25 μg, 50 μg, and 100 μg protein, respectively. Lane 7 is the purified Glut1 glucose transporter (200 ng). The data indicate a 2-fold increase in total protein across all the concentrations in the mutant cells. The autoradiograph of the Western blot reveals a 55 - kDa band which corresponds to the size of the glucose transporter protein. Densitometric analysis of this autoradiograph confirms this finding (Figure 9).

Distribution of the Glucose Transporter Protein in Plasma Membrane and Microsome Fractions

This 2-fold increase in total protein content in the WG 750 cells could account for the 6-fold increase in basal transport. To ascertain the distribution of the Glut1 protein, subcellular membrane fractionation was employed. 5' nucleotidase, a membrane marker enzyme for plasma membrane, was performed to determine the activity in each fraction (Figure 10). It is apparent that some cross-contamination of the marker enzyme activities occurred in all fractions. However, band B had the highest nucleotidase activity in both cell strains (~ 3-fold greater). This implies an enriched plasma membrane fraction in band PM-B for MCH 55 and WG 750 cell strains. Bands A and B have been fully characterized using the same method (Horner *et al.*, 1987, Germinario *et al.*, 1992). A Western blot

analysis of the isolated membrane fractions was performed using the monoclonal antibody raised against the Glut1 transporter protein (Figure 11). Glut1 transporter was detected by the antibody as a broad 55 - kDa band in all the fractions. Lanes 1, 3, 5, and 7 are the MCH 55 cell strain corresponding to the crude pellets, plasma membrane A, plasma membrane B, and microsome fraction, respectively. Lane 9 is the purified glucose transporter. Lanes 2, 4, 6, and 8 are the WG 750 cell strain corresponding to crude pellets, plasma membrane A, plasma membrane B, and microsome fraction, respectively. All the lanes contain 25 μg total protein except the purified glucose transporter, which contains 200 ng protein. The data in Figure 12 is a densitometric analysis of this Western blot. When the filter was scanned, the amount of glucose transporter was normalized to lane 2. Clearly, the mutant strain indicates a 3.2-fold more protein in the nucleotidase enriched PM-B membrane fraction when compared to the PM-B control strain (Figure 12). Although the MCH 55 PM-A shows 50% more protein when compared to the PM-A of the mutant strain (Figure 12), this fraction is not a nucleotidase enriched membrane fraction. The % distribution of the glucose transporter protein within the plasma membrane and microsome fractions varies in the cell strains. In the WG 750 cells, 60.9% of the protein is found within the plasma membrane, and 19% is found in the microsome fraction. For the MCH 55 cells, however, 28% and 29% are found in the plasma membrane and microsome fraction, respectively (Table VIII). It appears that in the mutant strain, the distribution of the glucose transporter protein within the cell is localized mainly on the plasma membrane. This clearly coincides with the observed increases in 2 - DG transport in the WG 750 cells.

The Effect of Insulin and Glucose on Glut1 Transporter Gene Expression

Steady-state transcript levels were examined to ascertain if transport was regulated at the transcriptional level. Total RNA from cultured fibroblasts was isolated using the Guanidinium Thiocyanate Phenol-Chloroform extraction method. Figure 13 is a Northern blot which demonstrates the Glut1 transporter mRNA levels under basal conditions. As indicated by the figure, the gene size of the Glut1 message corresponds to a 2.4 Kb fragment which is the reported size of the mRNA for the Glut1 transporter (Mueckler *et al.*, 1985). A single-stranded RNA probe was used to detect the Glut1 mRNA level. Lane 1 represents the MCH 55 cell strain and lane 2 represents the WG 750 cell strain. In both lanes, 10 μg total RNA was added. No difference was evident in Glut1 mRNA levels under basal conditions (0% serum) for both cell strains. Insulin was then added to determine if the hormone had any effect on the Glut1 message. Cells were incubated with 100 nM Insulin for 0, 4, and 8 h. Total RNA was then isolated and a Northern blot was

performed as shown in Figure 14. Panel A corresponds to the formaldehyde RNA gel and panel B corresponds to the Northern blot. Lanes 1, 2, and 3 correspond to the MCH 55 cell strain with 100 nM Insulin at 0, 4, and 8 h, respectively. The Northern blot was probed with a single-stranded RNA probe, and a 2.4 Kb message was detected and found to be the Glut1 mRNA. Below panel B is the same blot but stripped of its radioactivity and reprobed with a housekeeping gene — β-actin cDNA — which is constitutively expressed in these fibroblasts. It is evident in the WG 750 cell strain that insulin had an effect on Glut1 mRNA (lanes 5 and 6). Densitometric analysis of the autoradiogram (Figure 15) shows that by 8 h, Glut1 mRNA increased 2-fold. Insulin did not appear to increase Glut1 mRNA of the MCH 55 cell strain. Furthermore, lanes 1 and 4 of Figure 15 reconfirm the previous finding - that there is no difference in Glut1 mRNA under basal conditions (0% serum) (Figure 13).

It has been shown that glucose-deprived cells in cultured skin fibroblasts manifest increases in 2 - DG transport (3-fold), glucose transporter protein content (3-fold), and glucose transporter mRNA (4-fold) (Kosaki *et al.*, 1991). The question was raised as to whether glucose starvation might modulate Glut1 transporter synthesis in the mutant cells. Cells were serum-starved for 24 h and then either glucose-starved or glucose-fed (25 mM) for 8 h. Total RNA was then isolated and a Northern blot was performed (Figure 16). Panel A corresponds to the formaldehyde gel and panel B is the corresponding Northern blot. Lanes 1 and 2 are the WG 750 cell strain, corresponding to 0% glucose and 25 mM glucose (8 h), respectively. Lanes 3 and 4 are the MCH 55 cell strain, corresponding to 0% glucose and 25 mM glucose (8 h), respectively. The Northern blot was probed with a double-stranded DNA probe. In all the lanes, 20 μg total RNA was added. Clearly, glucose deprivation had an effect on Glut1 mRNA in the WG 750 cell strain. Densitometric analysis indicates a 2-fold increase in Glut1 mRNA in the WG 750 cells (lane 1) when compared to the MCH 55 cells (lane 3) in the presence of 0% glucose (Figure 17). The presence of 25 mM glucose in the WG 750 cells (lane 2) appear to have decreased Glut1 mRNA levels. However, the presence or absence of glucose (lanes 3 and 4) did not appear to have an effect on Glut1 mRNA levels in the MCH 55. β-actin probe used to control for RNA loading showed no differences in all lanes (data not shown).

Table I Glucose Utilization in MCH 55 and WG 750 Cells

Cell Strain	Glucose Utilized µmoles/mg protein/h
MCH 55	310 ± 74
WG 750	667 ± 139

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h. The samples from the media were analyzed at this time for glucose content using Glu-cinet (Sclavo Inc., Wayne, N.J.). Data represent average ± SEM of 3 different experiments (triplicate plates in each experiment).

Table II Metabolism of D - [U - ^{14}C] Glucose to $^{14}CO_2$ and [^{14}C] Lactate in MCH 55 (Normal) and WG 750 (Mutant) Cells

Cell Strain	$^{14}CO_2$ dpm x 10^{-3}/mg protein	[^{14}C] Lactate dpm x 10^{-3}/mg protein
MCH 55	7.97 ± 2.2	56.7 ± 12.5
WG 750	21.2 ± 6.5	230.8 ± 86.0

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h. The serum-deprived confluent monolayers were exposed to 0.5 mM D-glucose containing 4.5 μCi D - [U - ^{14}C] glucose/ml DME for 1h. Significant difference between groups using Student's t test, $P < 0.05$.

Table III 2 - Deoxyglucose Transport in Serum-Deprived Confluent Monolayers in MCH 55 (Normal) and WG 750 (Mutant) Cells

Cell Strain	2 - DG Transport nmol/mg protein/5 min
MCH 55	3.6 ± 1.80
WG 750	21.6 ± 10.5

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h. Concentration of 2 - DG employed was 1.0 mM. Data represent average ± SEM of 11 separate experiments (triplicate plates in each experiment). Significant difference between 2 groups using Students t test, $P < 0.01$.

Table IV 3-OMG Transport in Serum-Deprived Confluent Monolayers in MCH 55 (Normal) and WG 750 (Mutant) Cells

Cell Strain	3-OMG Transport nmol/mg protein/30 sec
MCH 55	0.67 ± 0.20
WG 750	1.97 ± 0.45

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h. Data represent average ± SEM of 5 separate experiments (triplicate plates in each experiment). Significant difference between 2 groups using Student's t test, $P < 0.01$.

Table V The Effect of the Presence and Absence of Cytochalasin B on 2 - DG Uptake by MCH 55 and WG 750 Cells

Cell Strain	2 - DG Transport nmoles/mg protein/2 min		L - glucose Uptake nmoles/mg prot/2 min
	- CB	+ CB	
MCH 55	2.8 ± 0.93	0.69 ± 0.08	0.71 ± 0.57
WG 750	27.9 ± 1.0	7.78 ± 3.22	0.31 ± 0.29

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h. 2 - DG uptake was determined in the presence of CB (10 μM) and L-Glucose in the absence of CB. The concentration of 2 - DG employed was 0.5 mM. Data represent average ± SEM of 3 separate experiments (triplicate plates in each experiment).

Table VI K_m and V_{max} of Hexose Transport for MCH 55 and WG 750 Cells

Cell Strain	K_m mM	V_{max} nmoles/mg protein/2 min
MCH 55	1.87 ± 1.10	3.2 ± 1.6
WG 750	1.97 ± 1.70	18.0 ± 1.6

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h and then monitored for 2 - DG uptake employing 7 concentrations of 2 - DG (0.025 to 10 mM) corrected for L-glucose uptake. Data represent average ± SEM of 5 separate experiments (triplicate plates in each experiment at all concentrations).

Table VII Specific [^{125}I] Insulin Binding and Insulin Stimulated 2-Deoxyglucose Transport in MCH 55 (Normal) and WG 750 (Mutant) Cell Strains

Cell Strain	[^{125}I] Insulin Bound fmol/mg protein	2 - DG Transport Ratio (Insulin/Basal)	
		+67 nM Insulin	+670 nM Insulin
MCH 55	5.0 ± 2.2	1.99 ± 0.03	2.33 ± 0.03
WG 750	5.4 ± 0.9	1.23 ± 0.06	1.34 ± 0.10

Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 24h. Insulin exposure time was 2h. Specific insulin binding was 35.5 ± 12.6 and 39.5 ± 0.25% for MCH 55 and WG 750 cell strains, respectively. For 2 - DG transport ratio data, basal rate of 2 - DG for MCH 55 was 3.2 ± 1.0 nmol/mg protein/2 min; basal rate for WG 750 cells was 17.9 ± 2.9 nmol/mg protein/2 min. Data represent average ± SEM of 4 separate experiments for insulin binding and average of 6 experiments ± SEM for transport ratio (triplicate plates in all experiments). Significance difference, $P < 0.005$.

Table VIII Distribution of Glucose Transporter Protein in Total Plasma and Microsomal Membranes in MCH 55 and WG 750

Membrane Fractions	% Glucose Transporter Protein	
	MCH 55	WG750
Plasma membrane (Band A)	35.0	10.2
Plasma membrane (Band B)	28.0	60.9
Microsomal membrane (P3)	29.0	19.0
Crude membranes (P1)	8.0	6.1

Densitometric analysis was determined (Figure 12) and the % distribution of the glucose transporter protein was determined for all the cellular fractions in the MCH 55 and WG 750 cells.

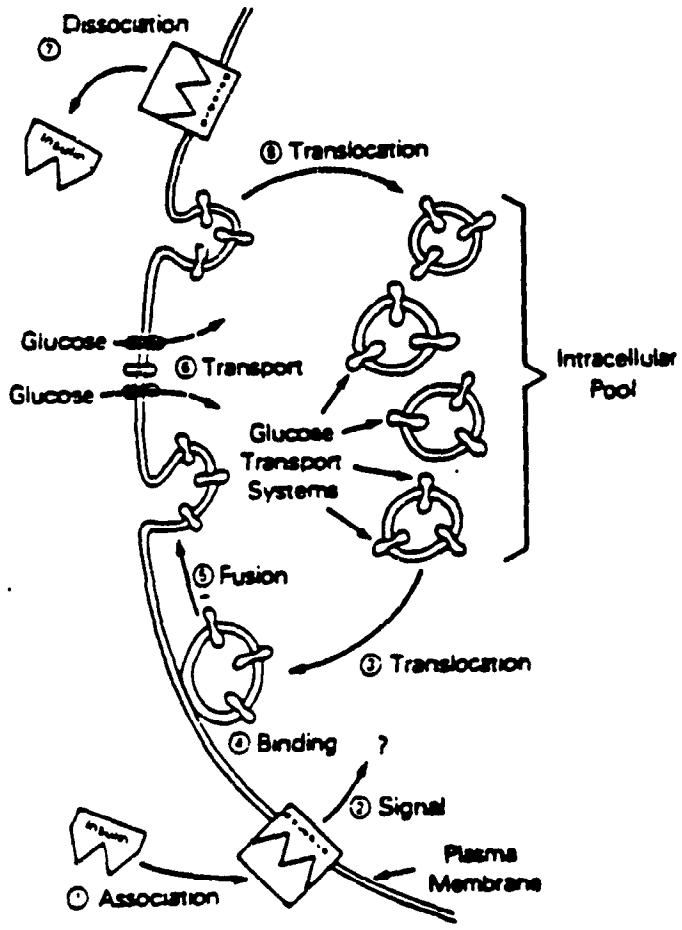


Figure 1. Model of insulin action on glucose transport. As insulin binds to the cell surface receptors, it generates an intracellular signal. This signal leads to the mobilization of glucose transporters from an intracellular pool to the plasma membrane. The dissociation of insulin from its receptors terminates the signal and leads to a rapid reversal of the process, which ultimately results in the translocation of the glucose transporters from the plasma membrane to an intracellular site.

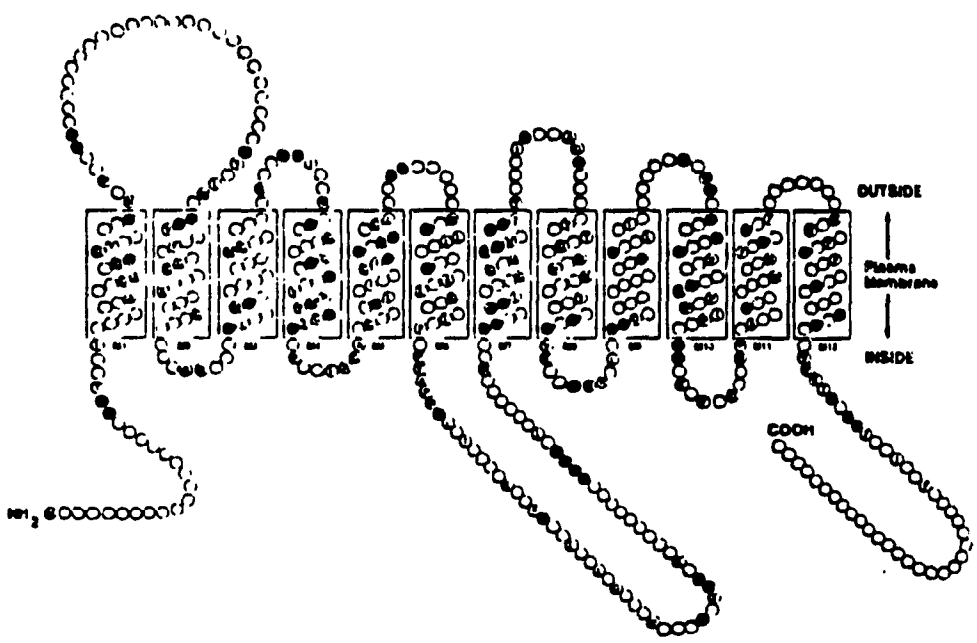


Figure 2. Model for the orientation of the glucose transporter protein in the plasma membrane. The 12 membrane-spanning segments are shown as boxes and are numbered M1 to M12. The lengths of the NH_2- and COOH-terminal domains as well as that of the extracellular loop between M1 and M2 may differ between transporter isoforms. Both the NH_2- and COOH-termini are internally oriented (in the cytoplasm). 50% of the protein lies within the lipid bilayer.

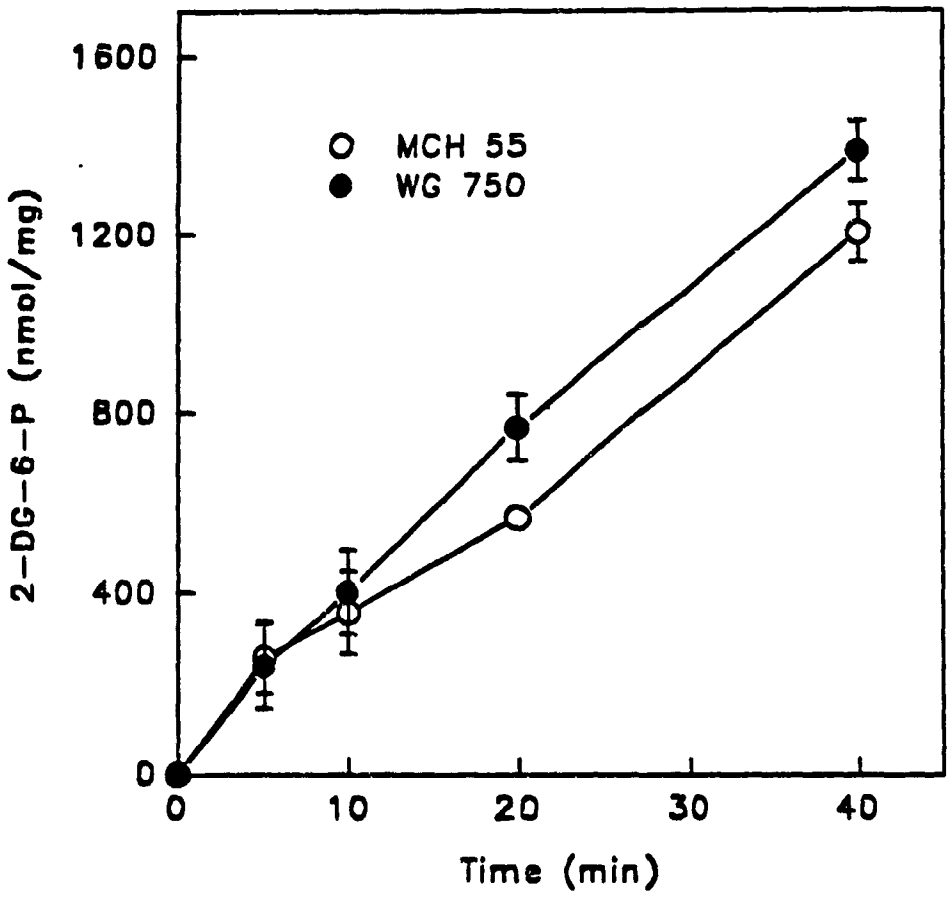


Figure 3. Hexokinase activity in cellular homogenate of serum-fed MCH 55 and WG 750 cultures. Phosphorylation of 2 - DG for serum-fed (o) MCH 55 and (•) WG 750 cells over different periods of time. Points represent the average ± SEM of 2 separate experiments (triplicate plates in each experiment).

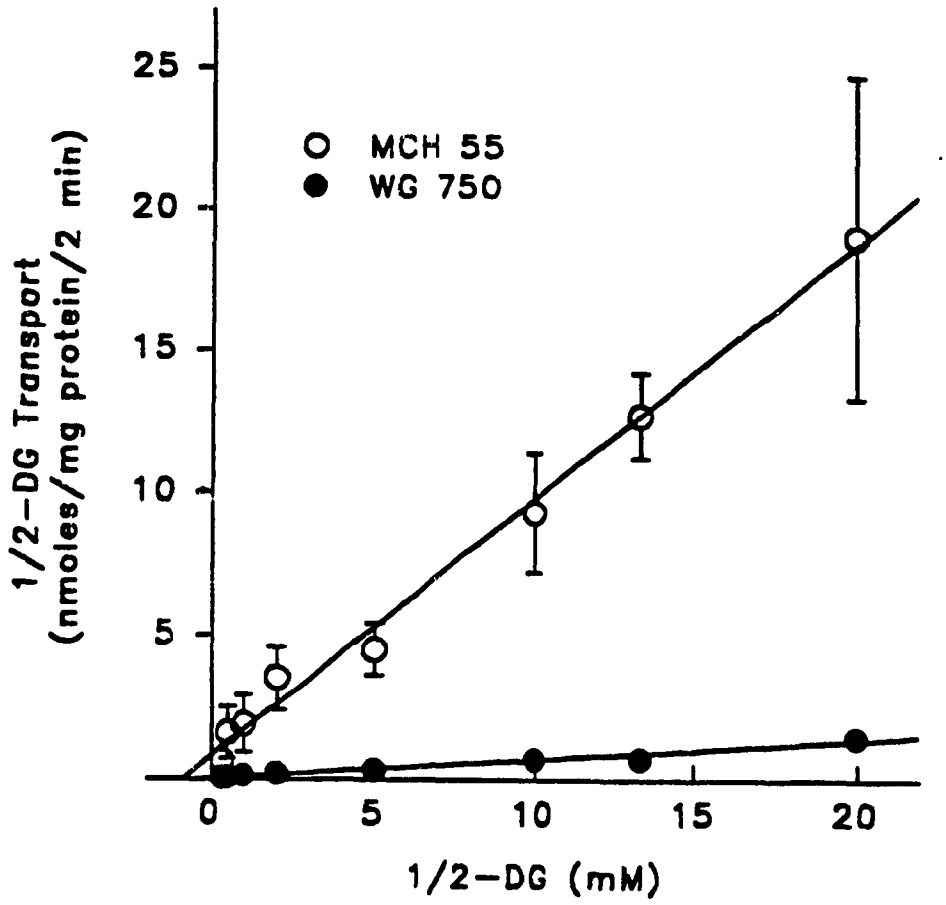


Figure 4. Kinetic characterization of 2 - DG transport by confluent serum-starved MCH 55 (o) and WG 750 (•) cells using Lineweaver-Burk double reciprocal plot. Data represent average ± SEM of 5 separate experiments (triplicate plates in each experiment at all concentrations).

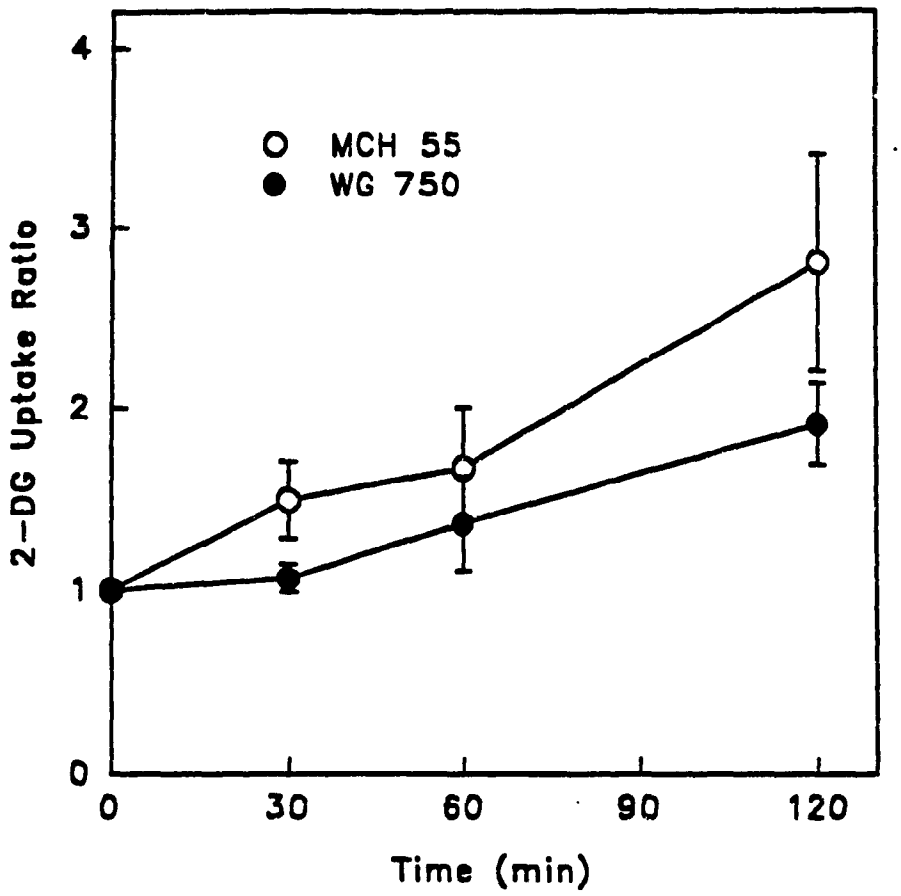


Figure 5. Effect of serum refeeding on hexose transport in MCH 55 and WG 750 cells. Hexose transport was measured in serum-deprived (0% MEM, 48 h) MCH 55 (o) and WG 750 (•) cells after short-term re-exposure to serum. 2 - DG transport ratio determined as the ratio of increase of 2 - DG transport in serum-fed cells versus (baseline) serum-starved cells. Data represent the average ± SEM of 4 separate experiments (triplicate plates in each experiment).

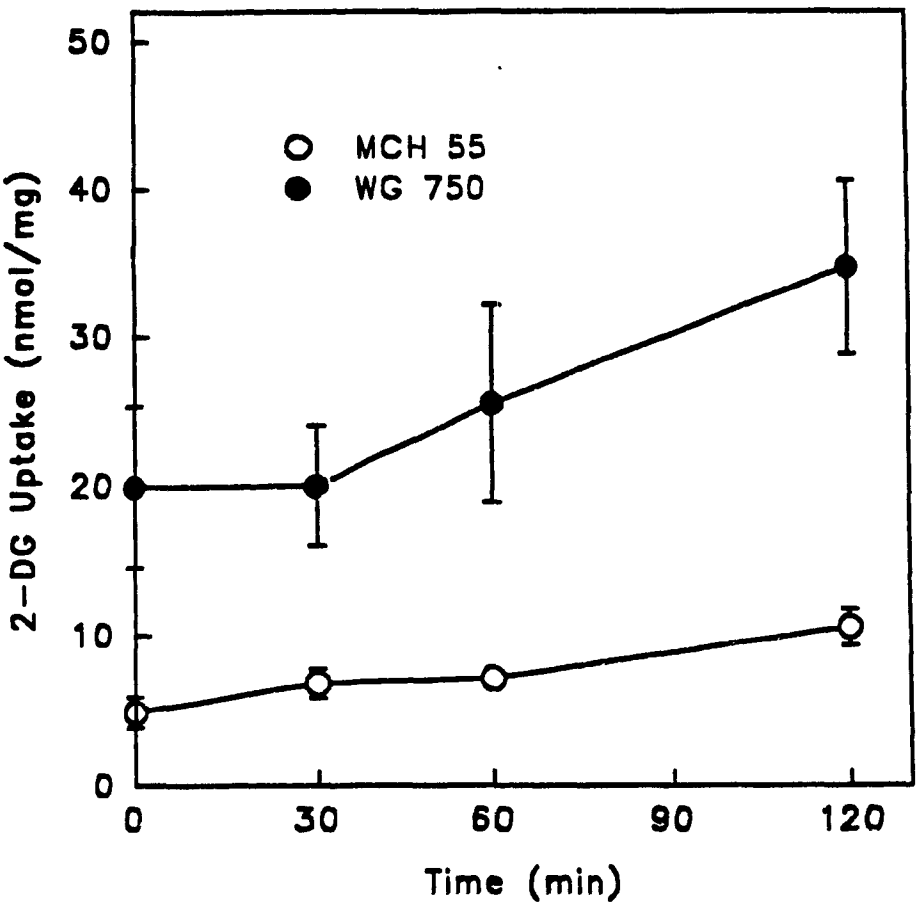


Figure 6. Effect of serum refeeding on hexose transport in MCH 55 and WG 750 cells. Hexose transport was measured in serum-deprived (0% MEM, 48 h) MCH 55 (o) and WG 750 (•) cells after short-term re-exposure to serum. Demonstrates differences in basal uptake. Data represent the average ± SEM of 4 separate experiments (triplicate plates in each experiment).

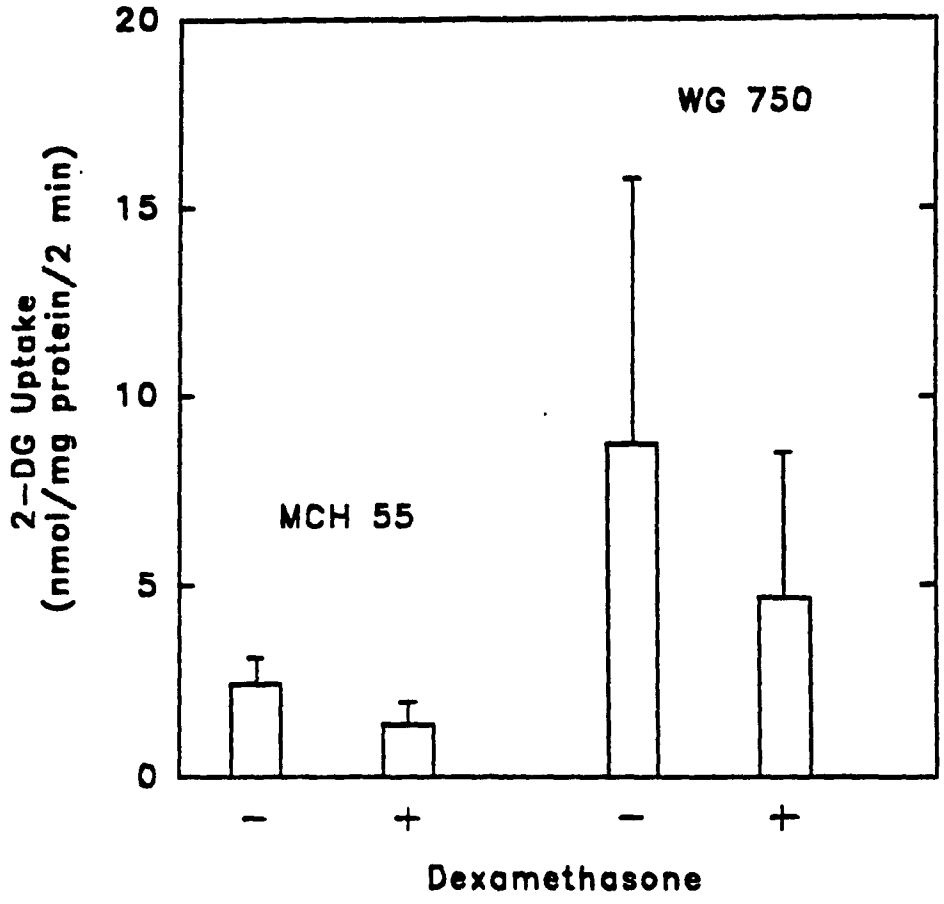
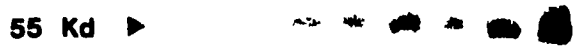


Figure 7. Effect of dexamethasone on hexose transport in MCH 55 and WG 750 cells. Cell monolayers were incubated in serum-free MEM containing 4 mg/ml glucose for 48h. The cells were either untreated or treated with 100 nM dexamethasone for 6h. Data represent average ± SEM of 4 separate experiments (triplicate plates in each experiment).



1 2 3 4 5 6 7

Figure 8. Western blot analysis for Glut1 transporter content of the MCH 55 and WG 750 cells. Total whole cell extracts were prepared and separated by SDS-PAGE and subjected to a Western blot procedure. The primary monoclonal antibody used was raised against the purified human erythrocyte transporter. Lanes 1, 3 and 5 are the MCH 55 cells with 25 μg, 50 μg and 100 μg protein, respectively. Lanes 2, 4 and 6 are the WG 750 cells with 25 μg, 50 μg and 100 μg protein, respectively. Lane 7 represents the purified Glut1 transporter (200 ng). Number to the left indicates the size of the Glut1 transporter protein.

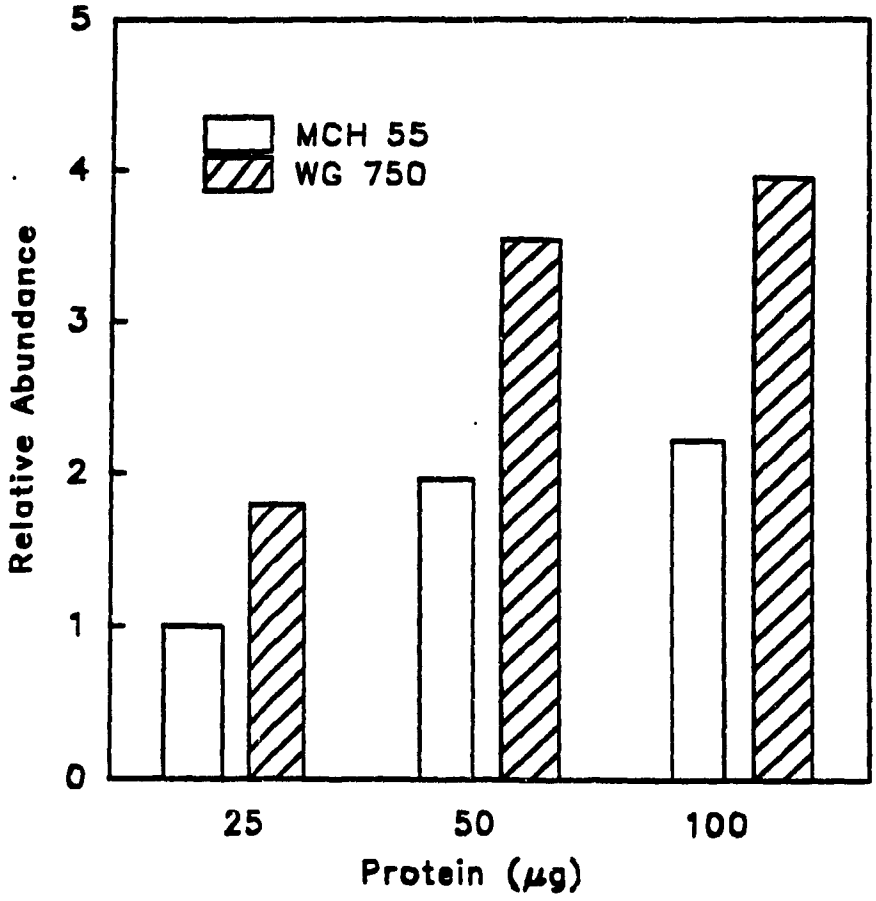


Figure 9. Relative Glut1 transporter content of MCH 55 and WG 750 cell strains as determined by densitometric analysis. Data was normalized to the MCH 55 lane (25 μg).

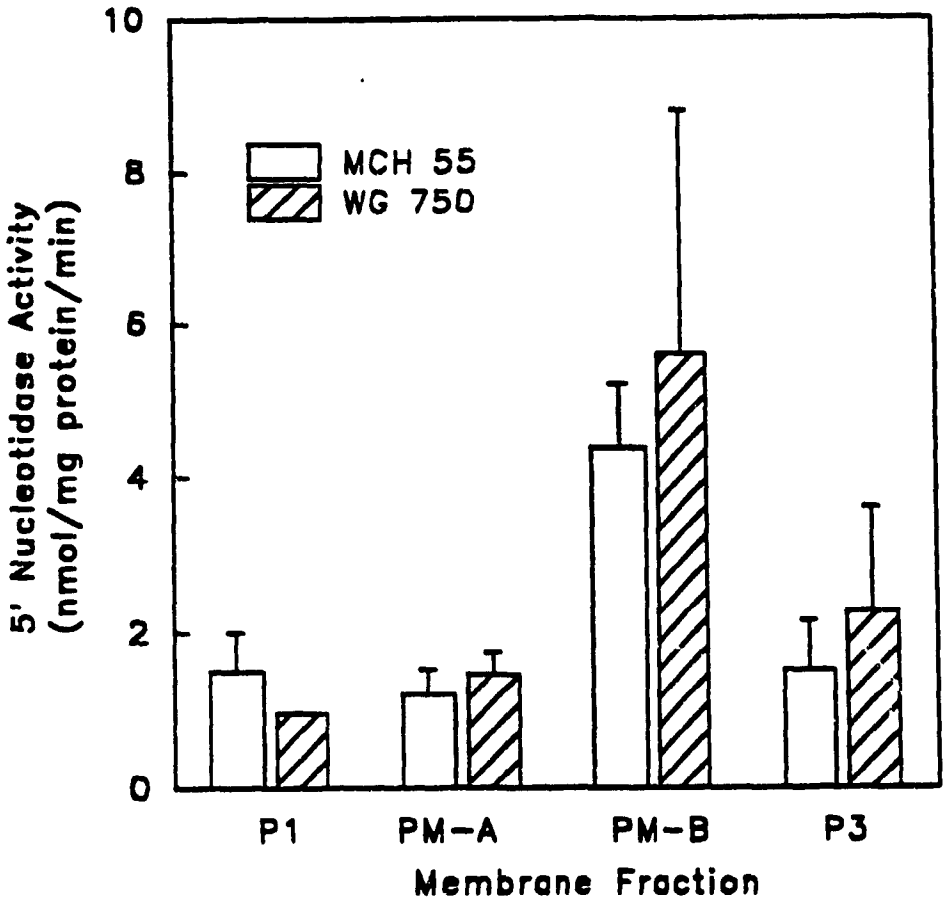


Figure 10. Nucleotidase activity in cellular fractions derived from MCH 55 and WG 750 cell strains. Total cellular homogenates from both cell strains were fractionated and layered onto a sucrose gradient. 5' nucleotidase, a membrane marker enzyme for plasma membrane, was then performed to determine the activity in each fraction. PI = crude pellet; PM-A = plasma membrane A at 10/30% sucrose interface; PM-B = plasma membrane B at 30/48% sucrose interface; P3 = high speed microsomal pellet. Data represent average ± SEM of 4 separate experiments.

1 2 3 4 5 6 7 8 9

Figure 11. Western blot analysis for Glut1 transporter content of the MCH 55 and WG 750 cell strains, in plasma membrane and microsome fractions. The primary monoclonal antibody used was raised against the purified human erythrocyte transporter. Lanes 1, 3, 5 and 7 are the MCH 55 cells corresponding to the crude pellets, plasma membrane A, plasma membrane B, and microsome fraction, respectively. Lanes 2, 4, 6 and 8 are the WG 750 cells corresponding to crude pellets, plasma membrane A, plasma membrane B, and microsome fraction, respectively. Lane 9 is the purified glucose transporter (200 ng). Number to the left indicates size of the Glut1 transporter protein.

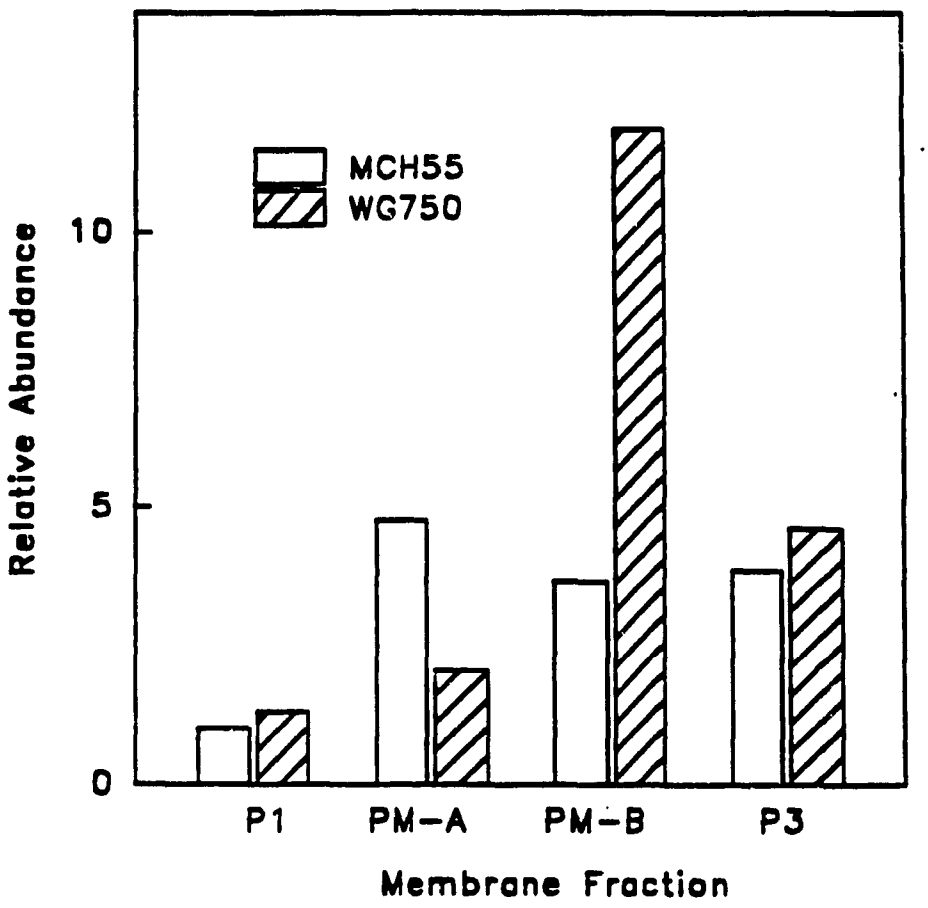


Figure 12. Relative Glut1 transporter content of MCH 55 and WG 750 cell strains in plasma membrane and microsome fractions, as determined by densitometric analysis. PI = crude pellets; PM-A = plasma membrane A at 10/30% sucrose interface; PM-B = plasma membrane B at 30/48% sucrose interface; P3 = high speed microsomal pellet. Data was normalized to the MCH 55 lane (PI).

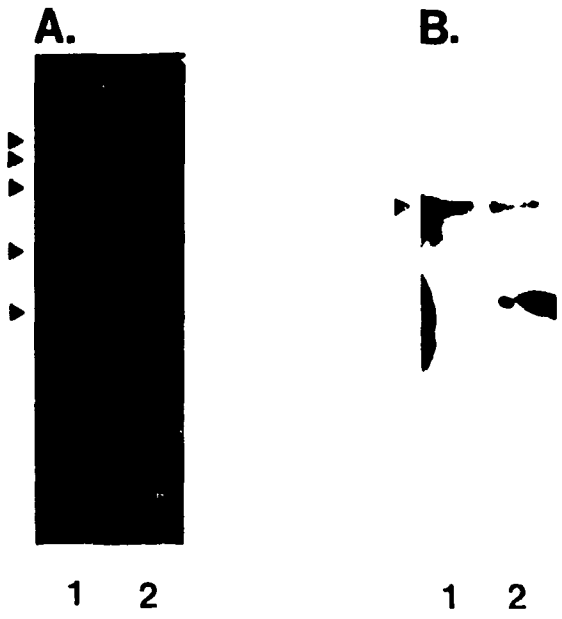


Figure 13. Northern blot analysis showing the effect of 0% serum (basal condition) on Glut1 mRNA levels in MCH 55 and WG 750 cells. Total RNA was isolated using the Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction method, and then subjected to a Northern blot procedure using an RNA probe. Panel A represents the RNA formaldehyde gel. Lane 1 is the MCH 55 cell strain (10 μg total RNA/lane) and lane 2 is the WG 750 cell strain (10μg total RNA/lane). Arrows to the left indicate the position of the molecular size markers (9.4, 7.5, 4.4, 2.4 and 1.4 Kb). Panel B. The corresponding Northern blot showing the effect of 0% serum on Glut1 mRNA levels. Lane 1 is the MCH 55 cell strain (10 μg total RNA/lane) and lane 2 is the WG 750 cell strain (10μg total RNA/lane). The arrow to the left indicates the size of the Glut1 mRNA (∠.4 Kb).

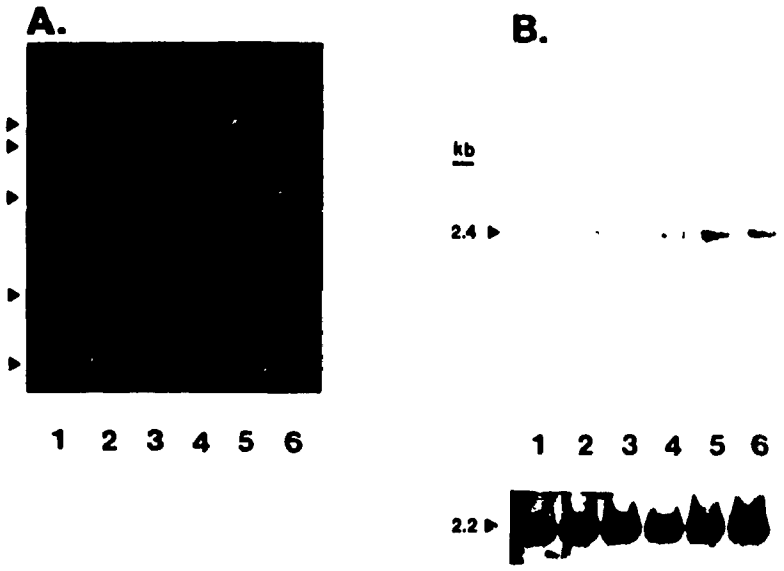


Figure 14. Northern blot analysis showing the effect of insulin on Glut1 mRNA levels in MCH 55 and WG 750 cells. Total RNA was isolated using the Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction method, and then subjected to a Northern blot procedure using an RNA probe. Panel A is the RNA Formaldehyde gel. Lanes 1, 2 and 3 are the MCH 55 cell strain at 0h, 4h and 8h with 100 nM insulin, respectively (10 μg total RNA/lane). Lanes 4, 5 and 6 are the WG 750 cell strain at 0h, 4h and 8h with 100 nM insulin, respectively (10 μg total RNA/lane). Arrows to the left indicate molecular size markers (9.5, 7.5, 4.4, 2.4 and 1.4 Kb). Panel B. The corresponding Northern blot analysis. Arrow to the left indicates the size of the Glut1 mRNA (2.4 Kb). Below panel B is the same Northern blot, but reprobed with human β-actin to ensure even loading of RNA. Arrow to the left indicates the size of the β-actin gene (2.2 Kb).

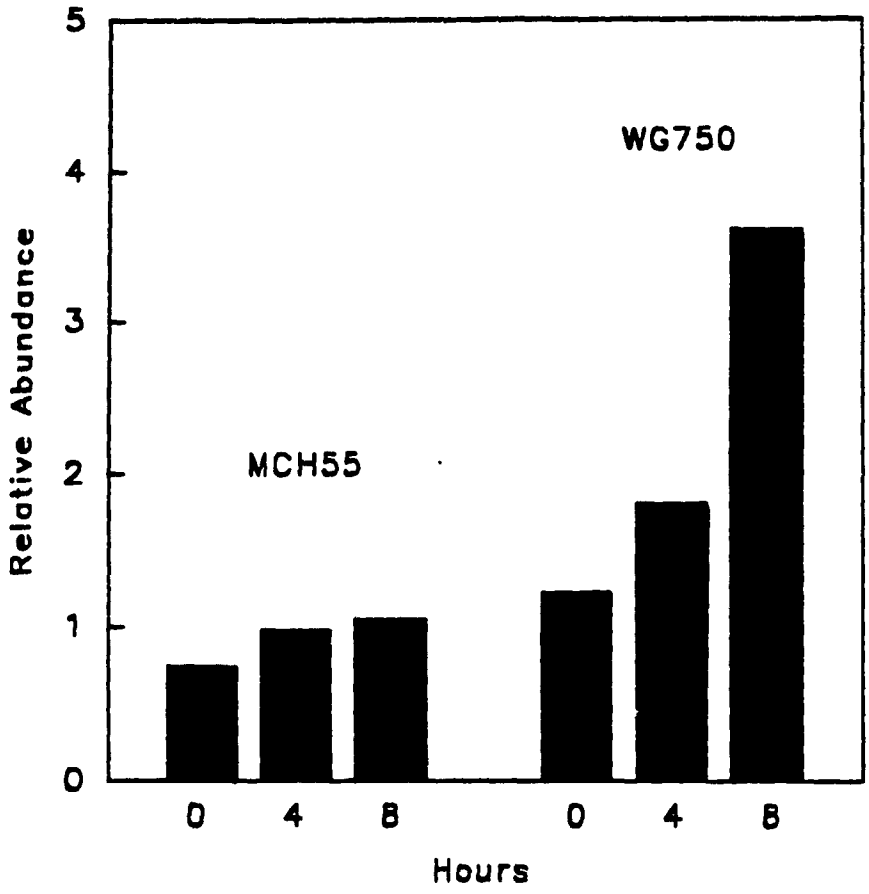


Figure 15. Relative Glut1 mRNA levels of MCH 55 and WG 750 cell strains during 0 h, 4 h and 8 h of insulin (100 nM) exposure, as determined by densitometric analysis. Data was normalized to the MCH 55 lane (0 h).

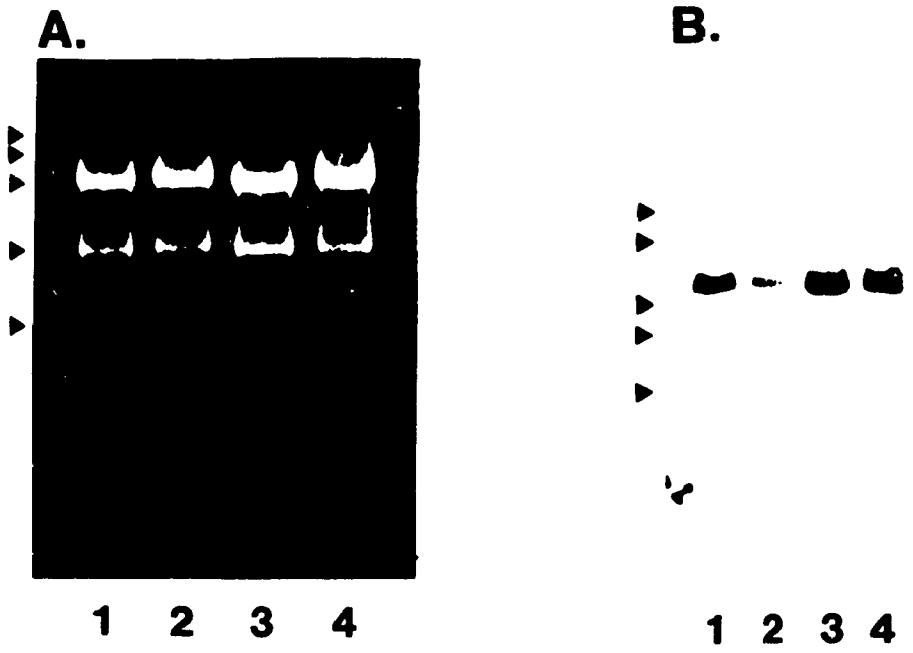


Figure 16. Northern blot analysis showing the effect of glucose deprivation on Glut1 mRNA levels in MCH 55 and WG 750 cell strains. Total RNA was isolated using the Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction method, and then subjected to a Northern blot procedure using a DNA probe. Panel A is the RNA formaldehyde gel. Lanes 1 and 2 are the WG 750 cell strain with 25 mM glucose and 0% glucose, respectively (20 µg total RNA/lane). Lanes 3 and 4 are the MCH 55 cell strain with 25 mM glucose and 0% glucose, respectively (20 µg total RNA/lane). Arrows to the left indicate molecular size markers (9.5, 7.5, 4.4, 2.4, and 1.4 Kb). Panel B is the corresponding Northern blot probed with a DNA probe. Arrow to the left indicates the size of Glut1 mRNA (2.4 Kb).

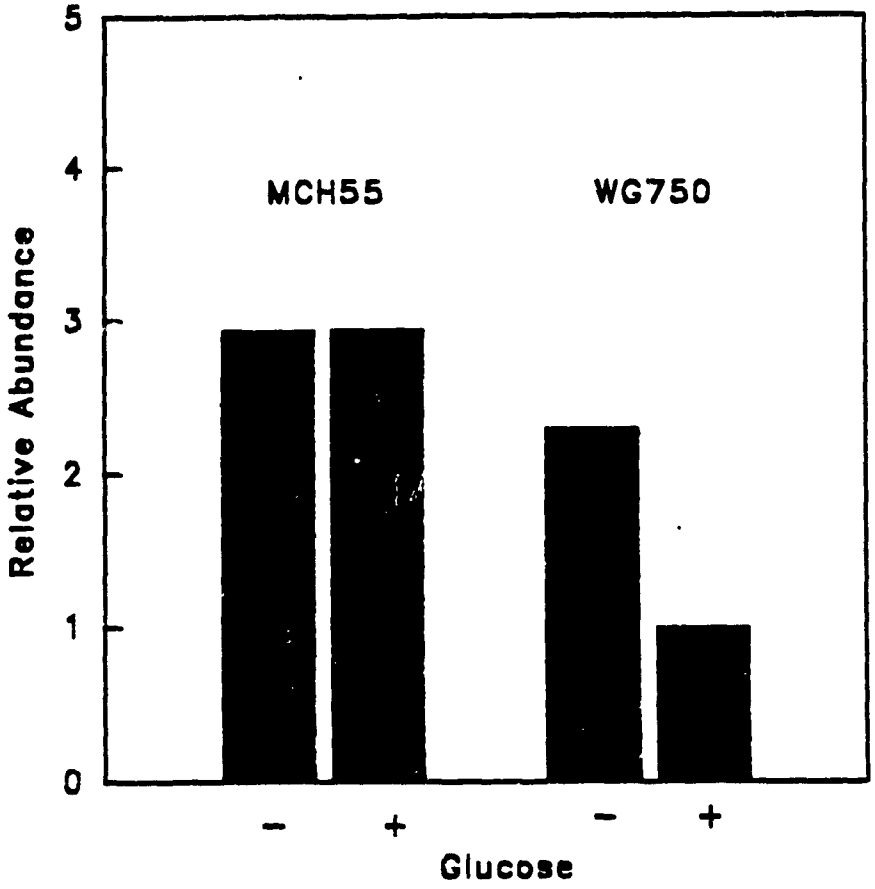


Figure 17. Relative Glut1 mRNA levels of MCH 55 and WG 750 cell strains in the presence and absence of 25 mM glucose, as determined by densitometric analysis. Data was normalized to the WG 750 lane (25 mM glucose).

DISCUSSION

Respiration-Deficient and Normal Human Skin Fibroblasts

The data presented herein clearly indicate that the respiration-deficient mutant cell strain WG 750 displayed altered responses to several factors governing glucose transport. Consistently, the mutant has upregulated its glucose transport to meet metabolic demands due to the gene defect affecting NADH CoQ reductase. The high requirement for glucose utilization (Table I) by the WG 750 cells was expressed by their great need to maintain cellular energy supply. Thus, glucose metabolism was increased significantly; CO_2 and lactate production was increased 3-fold and 4-fold, respectively (Table II). The WG 750 cells are unable to oxidize mitochondrial NADH and generate energy (ATP) via the electron transport chain; therefore, the cells undergo anaerobic glycosis at a much greater rate to compensate for the anomaly in respiration. Interestingly, the two cell strains displayed identical doubling times (48 h), indicating that the WG 750 cells double and grow as well as the normal cells in the presence of adequate amounts of glucose.

The increased rate of glucose uptake in WG 750 cells was questioned as to whether it was the result of increased transport or due to an elevation in the subsequent step in the glycolytic pathway. It was important, then, to determine whether there were significant differences in hexokinase activity in the WG 750 and MCH 55 cells. Hexokinase phosphorylates 2 - DG to 2 - DG - 6-phosphate and is the first enzyme in the glycolytic pathway. The rate of accumulation of glucose by the intact cell is dependent both on the rate at which it can be transported into the cell and the extent to which the transported glucose is phosphorylated, a reaction which lowers the intracellular glucose concentration and favors further net glucose entry (Kosow and Rose, 1972). Either of these steps may be rate-limiting under specific conditions. The total flux of 2 - DG into the cell is then a result of both transport and hexokinase action, and the measurement of total 2 - DG incorporation is a valid measurement of transport only if 2 - DG is phosphorylated more rapidly than its entry via transport into the cell. It has been shown that greater than 85% of transported 2 - DG that enters the cell was rapidly phosphorylated after incubation for 2 min or less at the substrate concentrations employed (0.1 to 3.0 mM) (Germinario *et al.*, 1978). No intracellular free sugar pool was found at early times (Renner *et al.*, 1972, Kletzien and Perdue, 1974). Moreover, experiments with CB demonstrated that an inhibition of transport was always paralleled by an equal inhibition of sugar phosphorylation (Germinario *et al.*, 1978). Under these conditions, transport is the rate-limiting step. Germinario has shown in cultured human skin fibroblasts that sugar transport is the rate-limiting step in 2 - DG metabolism and that phosphorylation is distinct from transport (Germinario *et al.*, 1978).

Hexokinase activity was measured in cell-free homogenates of glucose-fed, serum-fed, cultures in MCH 55 and WG 750 cells. The data in Figure 3 indicate that no significant differences were found between hexokinase activity in both cell strains. Differences in transport would then explain the differences observed in the uptake rates between MCH 55 and WG 750 cells. It has been asserted that normal and transformed cells do not have differing hexokinase specific activities, and that hexokinase activity is in excess of transport. Transport would then be the rate-limiting step in the total uptake of 2 - DG (Kletzien and Perdue, 1974). In chick fibroblasts, little if any change in hexokinase activity was detected; whether cells were fed or starved or in soluble or membrane-bound fractions (Kletzien and Perdue, 1975).

One of the questions asked regarding transport was whether the defect in NADH-Coenzyme Q Reductase affected the normal regulatory controls governing hexose transport. Much of the literature on the uptake of glucose by animal cells is based on experiments carried out with 2 - DG. Basal 2 - DG uptake was assayed in glucose-fed, serum-starved cultures of MCH 55 and WG 750 cells and shown to be 6-fold higher in WG 750 cells when compared to the control cells (Table III).

Furthermore, to further confirm the question of whether the increased rate of 2 - DG uptake in the WG 750 cells was due to an increase in transport and not phosphorylation, 3-O-methyl-D-glucose (3-OMG) was employed. 3 - OMG has been shown to use the same carrier system as glucose in chick fibroblasts and in mammalian cells (Venuta and Rubin, 1973, Cuatrecases and Tell, 1973). The data in Table IV demonstrates that the rate of 3-OMG uptake in the WG 750 cells is 3-fold higher than the control cells. These transport data, taken together with the 2 - DG data suggest that hexose transport is specifically up-regulated, independent of phosphorylation in the WG 750 cell strain.

To further characterize any differences that might exist in transport between the two cell lines, studies were performed in the presence and absence of a known potent competitive inhibitor of glucose transport in many cell types (Kletzien *et al.*, 1972, Mizel and Wilson, 1972, Estensen and Plagemann, 1972, Wardzala *et al.*, 1978). An essential step toward the understanding of the transport carrier at the molecular level is to chemically identify and isolate the carrier molecule. Cytochalasin B has been used as a molecular marker for the identification of the glucose carrier, since a close correlation was established between CB binding and glucose carrier inhibition (Jung and Rampel, 1977). It is important to note that CB selectively inhibits the facilitated mode of glucose uptake (saturable uptake) and does not affect simple diffusion (non-saturable uptake) (Germinario *et al.*, 1978, Dolberg *et al.*, 1975). When performing experiments with CB, L-glucose was used as the substrate to estimate the non-saturable component relative to the total glucose

uptake. Kletzien and Perdue found that when using L-glucose, they estimated that even at 40 mM sugar concentration, less than 3% of total uptake was due to simple diffusion (Kletzien and Perdue, 1974). The data in Table V indicate that in the presence of 10 μM CB, 2-DG transport was inhibited by 80% in both cell lines. L-glucose uptake represented less than 3% of the total uptake in both cell strains. The data indicate that the increase in transport is predominantly carrier-mediated. Thus, CB substantially obliterated the saturable component of glucose uptake (facilitated mode), without affecting the non-saturable component (simple-diffusion).

Kinetic studies were performed to determine the V_{max} and K_m for 2 - DG transport in the WG 750 and MCH 55 cell lines. Previous 2-DG transport analysis indicated an increase (6-fold) in hexose uptake. The question was raised as to whether this increase was due to an increase in V_{max} or K_m. Kinetic analysis of the initial rates of 2 - DG transport by Lineweaver-Burk plot (Lineweaver and Burk, 1934) (Figure 4) showed that the V_{max} for 2 - DG transport was increased in the WG 750 cells. Clearly, the mutant WG 750 cells displayed a significant increase (6-fold) in the V_{max} for transport, compared to the control MCH 55 cell strain. The K_m for 2-DG was essentially the same in both cell strains (Table VI). The K_m and V_{max} for saturable sugar uptake were calculated after subtracting the contribution of non-saturable sugar uptake (i.e., L-glucose uptake). It appears that the 6-fold increase in V_{max} for transport in the WG 750 cells is consistent with the 2-DG uptake in WG 750 cells shown in Table III. The increase in V_{max} for 2 - DG transport suggests an increase in the number of functional carriers at the plasma membrane for the WG 750 cells. Kinetic analysis of the hexose transport using L8 myocytes show that the V_{max} of 2 - DG transport is also modulated without a significant change in the K_m value; they suggest that glucose controls the number of glucose transporters in the plasma membrane (Sasson and Cerasi, 1986). Treatment of serum-deprived fibroblasts with serum or transformation of fibroblasts by the Rous sarcoma virus also resulted in an increase in sugar uptake which was associated with a V_{max} increase in the transport system (Kletzien and Perdue, 1974a Kletzien and Perdue, 1974c). Kletzien and Perdue have also shown that initial rates of 2 - DG transport by Lineweaver-Burk plot increased the V_{max} for sugar transport in chick embryo fibroblasts while the K_m remained unchanged (Kletzien and Perdue, 1975).

To further characterize any differences that might exist in transport regulation, both cell types were exposed to conditions known to modulate transport. The rate of growth of uninfected cells in culture is influenced by the density of the cultures and the presence of serum factors (mitogens) in the medium. The removal of serum from the medium results in a rapid decrease in the rate of 2 - DG transport (Kletzien and Perdue, 1974c). Whereas the

addition of serum to serum-deprived cultures of cells stimulates the rate of glucose uptake and its analogues (Sefton and Rubin, 1971, Bradley and Culp, 1974, Kletzien and Perdue, 1974c). The rapid response in the rate of transport following serum addition is designated post-translational; since the stimulation occurred even in the presence of the inhibitor of protein synthesis, cycloheximide (Perdue, 1976). Kinetic analysis of the serum-induced increases has established them as changes in V_{max} with no change in K_m and they are not the consequence of alterations in the levels of hexokinase (Kletzien and Perdue, 1974c). The stimulation of sugar transport in chick embryo fibroblasts by serum was biphasic with the first increase insensitive to cycloheximide, whereas the second increase was completely blocked by the drug. Actinomycin D had no effect on either increase. Therefore, the serum stimulation of sugar transport in chick fibroblasts is regulated by a composite of two controls, a post-transcriptional and a post-translational control.

That this dynamic function of the plasma membrane should be controlled at two levels suggests that the two transport increases occur for different reasons. The early increase in transport, which could reflect the activation of pre-existing transport sites, may be a manifestation of the requirement of cells *in situ* to respond to signals from the milieu that are necessary to maintain homeostasis. The later increase in transport, which is dependent upon protein synthesis, may be a reflection of an increased need of the cells for metabolites essential for energy production and macromolecular synthesis (Kletzien and Perdue, 1974). Recent further evidence for post-transcriptional regulation of transport following serum stimulation of chick embryo fibroblasts, was shown by the drug cordycepin which had no effect on the first increase in transport, but blocked the second increase as effectively as cycloheximide. This drug is thought to interfere with post-transcriptional processing of messenger RNA by inhibiting polyadenylation of this RNA species (Darnell *et al.*, 1973). The data suggest that messenger RNA which codes for proteins involved in sugar transport resides in the nucleus as stable information and is probably polyadenylated as a consequence of serum stimulation (Kletzien and Perdue, 1974).

Serum refeeding of glucose-fed serum-deprived MCH 55 and WG 750 cells showed different patterns of response. The response to serum refeeding in WG 750 cells differed not in terms of its magnitude but rather in the time of response. The delay in serum refeeding response in the mutant (WG 750) cells might be attributed to the high basal rate of 2 - DG transport (Figures 5, 6), the signal-to-noise ratio being higher in the WG 750 cells. The effect of serum (or insulin) on sugar transport and the modification of the serum response by environmental factors depend on the basal level of sugar transport. For example, if the basal level of sugar transport is elevated, leading to depletion of the

intracellular sugar carriers, exposure to serum (or insulin) would not result in the expected sugar carrier translocation to the cell surface (Suzuki and Kono, 1980). Alternatively, the delay in serum response in the WG 750 cells could be due to the fact that more glucose transporters are present on the plasma membrane, so that the capacity to translocate stored carriers is low. It has been shown that in 3T3 cells and rat fibroblasts, short-term exposure (30 min) involves translocation of glucose transporters to the plasma membrane (Allen *et al.*, 1981, Kahn *et al.*, 1989). Whereas long-term exposure (greater than 2 h) may involve increased transporter expression as a result of increased gene transcription (Kitagawa *et al.*, 1989). The expression of the gene encoding the facilitated glucose transporter protein (Glut1) showed that with 15% serum feeding to confluent Balb/c3T3, NIH3T3 or rat-2 cells rapidly induced a 5-to-10-fold increase in Glut1 mRNA. The rise in Glut1 mRNA was maximal at 3-4 h after stimulation and then returned to basal level by 16 h. This serum-stimulated increase was not blocked by protein synthesis inhibitors cycloheximide or anisomycin. Interestingly, NIH3T3 cells increased gene transcription at least 10-to-20-fold by 30 min and returned to near basal levels by 2 h (Hiraki *et al.*, 1988). Stimulation of quiescent fibroblasts with serum also increased transcription of the glucose transporter gene. Mitsumoto has demonstrated that serum factors potently elevate the amount of Glut1 transporters in L6 myotubes. In contrast, the elevation of serum levels did not alter the amount of Glut4 protein. Interestingly, when Glut1 transporter levels were raised by high serum exposure, the response of the cells to short exposure to insulin was obliterated, in spite of unaltered levels of Glut4 transporters. In fact, a loss of insulin response was observed, possibly due to the high basal transport rate (Mitsumoto *et al.*, 1991).

Recently, insulin has been shown to stimulate hexose transport in cultured human fibroblasts (Germinario and Oliveira, 1979, Howard *et al.*, 1979, Berhanu and Olefsky, 1981, Germinario *et al.*, 1984). The data in the literature, however, present large discrepancies with regard to the sensitivity of fibroblasts to insulin; in some cases (Berhanu and Olefsky, 1981, Howard *et al.*, 1979), fibroblasts are as sensitive to insulin as adipocytes or myocytes, while in others (Hollenberg and Cuatrecasas, 1975, Germinario and Oliviera, 1979) they are markedly less sensitive to the hormone. In general, fibroblasts are less sensitive to insulin than adipocytes in the stimulation of sugar transport. This might be due to a smaller number of insulin receptors in fibroblasts or to a poor affinity for the hormone (Cynober *et al.*, 1986). Cynober found that the response to insulin depends greatly on culture conditions (confluency, serum or glucose starvation) and that this cellular model must be used carefully in studies concerning insulin action on glucose uptake (Cynober *et al.*, 1986). It has been reported that insulin stimulates glucose uptake

by both promoting the translocation from an intracellular pool to the plasma membrane (Cushman and Wardzala, 1980, Kono *et al.*, 1982, James *et al.*, 1988, Gould *et al.*, 1989) and enhancing the intrinsic activity of the molecule (Kahn and Cushman, 1987). Kosaki has shown that insulin increased the Glut1 gene expression in cultured human skin fibroblasts (Kosaki *et al.*, 1988). Moreover, long-term treatment of these cells with insulin not only increased Glut1 mRNA, but glucose transporter protein which was accompanied by an increase in glucose uptake. These findings are in agreement with those previously reported in 3T3 - L1 adipocytes (Tordjman *et al.*, 1989) and in rat L6 skeletal muscle cell lines (Walker *et al.*, 1989).

The data in Table VII also show that insulin stimulated glucose transport in glucose-fed serum-deprived cultures of normal and mutant cell strains, but to different degrees. The addition of 67 or 670 nM insulin to control cells led to increases in 2 - DG transport ratios (1.99 ± 0.25- and 2.33 ± 0.26-fold, respectively) over basal 2 - DG transport, whereas the mutant cells response to insulin was significantly lower at both insulin concentrations. The response to insulin in the WG 750 cells differed in terms of its magnitude of response. The mechanism responsible for the decreased insulin response in the mutant cells appears to be similar to the delay in the serum refeeding response in these mutant cells in that there could be a decrease in internal membranes containing the glucose transporters. This is likely due to an increase in the basal 2 - DG transport which leads to a loss of insulin sensitivity. Recent data show that the human glucose transporter protein (Glut1), which is not responsive to insulin in HepG2 cells, is regulated by insulin when expressed at low levels in Chinese hamster ovary cells. Insulin stimulated sugar uptake only by 10% in cells expressing human Glut1 protein, and no insulin stimulation of sugar uptake was detected in several cell lines expressing very high levels (12-to-17-fold) of human Glut1 protein (Harrison *et al.*, 1990). This loss of insulin-regulated sugar transport in cells expressing very high levels of glucose transporter protein suggests that the cell-specific machinery responsible for insulin stimulation of glucose transport may be down-regulated or inhibited by chronically high levels of intracellular glucose, glucose metabolites or glucose transporter proteins. The intracellular glucose concentration in both WG 750 and MCH 55 cells was found to be similar (data not shown); therefore, it is more likely that the insensitivity observed in the mutant cells to insulin might be attributed to the glucose metabolites or to the high levels of glucose transporter protein Glut1 in the plasma membrane.

The high basal 2 - DG levels in the mutant cells led to the question of whether there might be a defect in the reversal process of the glucose transporter protein, in that the mobility of the carriers to translocate from the plasma membrane to the intracellular pool

might be inhibited. Dexamethasone is a glucocorticoid known to down-regulate glucose uptake by reversing the translocation process — redistributing the carrier protein from the plasma membrane to an intracellular pool. Kinetic studies have established that the inhibition of glucose transport by dexamethasone is due to a decrease in the maximal velocity of transport (V_{max}) (Yorke, 1967, Livingston and Lockwood, 1975, Zydkowski and Munck, 1979, Madar and Felig, 1983). Furthermore, it has been shown that the dexamethasone effect was retained in isolated plasma membrane from rat adipocytes, as indicated by both reduced D-glucose transport into vesicles and reduced photolabelling of the transporter with CB (Carter-Su and Okamoto, 1985). These findings suggest that the inhibitory action of glucocorticoids on glucose transport is mediated by a specific glucocorticoid-induced protein(s) whose effect is to decrease either the number of functional glucose transporters in the plasma membrane or to decrease the intrinsic activity of these transporters. The former situation could be the result of either inactivation of transporters that remain in the plasma membrane, the net translocation of transporters from the plasma membrane to intracellular membranes or the net loss of transporters through protein degradation. Horner has shown evidence that glucocorticoids inhibit glucose transport by causing the translocation of transporters from the plasma membrane to an intracellular membrane fraction, and the addition of insulin reversed the dexamethasone-induced decrease in glucose transport and the dexamethasone-induced increase in the intracellular pool (Horner *et al.*, 1987). The data in Figure 7 illustrates that in the presence of 100 nM dexamethasone, glucose transport was inhibited by 50% in both the WG 750 and MCH 55 cells. The mobility of the carriers to translocate from the plasma membrane to the intracellular pool does not appear to be defective. The cellular machinery involved in the reversal process of glucose transport is then functional.

Quantitation and Distribution of the Glucose Transporter Protein Levels Using Total Cell Extracts and Subcellular Membrane Fractions

The following studies have been directed at elucidating the mechanism(s) responsible for the compensatory adaptations of hexose transport regulation in the mutant cell strain. The augmentation in glucose transport in WG 750 cells is most likely due to an increased number of hexose transporters at the cell membrane. This notion is supported by the enhanced 2 - DG transport uptake and the increased V_{max} for transport in the WG 750 cells. Alternatively, there may be altered rates in steady-state RNA transcript such that an increase in mRNA for Glut1 protein might be evident in the WG 750 cells. Both these approaches were examined in the mutant (WG 750) and control (MCH 55) cells.

One approach to determine if the enhanced transport was due to an increase in glucose transporter protein was to use solubilized whole cell extracts and quantitate total cellular glucose transporters. Analysis by SDS polyacrylamide/immunoblotting using a monocional antibody specific for the human glucose transporter (Mueckler *et al.*, 1985) was used in Figure 8. Varying amounts of protein were added for both WG 750 and MCH 55 cells. For all three concentrations, a 2-fold increase in glucose transporter protein was detected in the WG 750 cells. Autoradiograph of the Western blot was scanned using a densitometer to determine the relative intensity of each band corresponding to the 55 Kdal Glut1 protein (Figure 9). The scanning confirms the observation that there appears to be a 2-fold increase in protein in the mutant cells (Figure 8). Specifically, it was of interest to determine the distribution of the glucose transporter protein within the cell. Is the protein mainly distributed at the plasma membrane or in the intracellular compartment? To address this question, one approach was to isolate subcellular membrane fractions from MCH 55 and WG 750 cells. Plasma membrane and microsomes fractions were isolated by the modified methods of Buchanan and Horner (Buchanan *et al.*, 1985, Horner *et al.*, 1987). The quantitation of the number of glucose transporters in the subcellular fractions indicate whether there is an intracellular redistribution of hexose transporters in WG 750 cells. Prior to performing a Western blot, a nucleotidase assay (membrane marker enzyme for plasma membrane) was done to determine the activity in each fraction. The data in Figure 10 indicate that band B had the highest nucleotidase activity in both MCH 55 and WG 750 cells. Analysis by SDS polyacrylamide/immunoblotting using a monoclonal antibody specific for the human glucose transporter (Mueckler *et al.*, 1985) was used in Figure 11 to quantitate and determine the distribution of the Glut1 protein. Clearly, there was a 3-fold increase in Glut1 protein in the plasma membrane of the WG 750 cells (lane 6) when compared to the control MCH 55 cells (lane 5). The scanning of the autoradiograph confirms this observation in Figure 12. The % distribution of the glucose transporter protein from each subcellular fraction was determined in both cell strains. Interestingly, the distribution of the glucose transporters in the WG 750 cells was mainly localized on the plasma membrane (Table VIII). This is not surprising, since repeatedly, an increased basal 2 - DG uptake in the WG 750 cells was observed. Moreover, Western analysis indicated an increase (2- to 3-fold) in glucose transporter protein within the plasma membrane in the mutant cells. The % distribution of transporter protein in the control MCH 55 cells, however, appears to be evenly distributed between the plasma membrane and the microsome (intracellular pool) (Table VIII). The physiological significance in having an increased amount of transporter protein on the plasma membrane in the mutant cells is probably related to the compensatory mechanism(s) of hexose transport regulation. The

adaptive response of the cell to its inability to oxidize mitochondrial NADH and generate energy (ATP), might explain the need to respond to the cell's demands quickly by having available these glucose carriers on the plasma membrane or in close proximity to the plasma membrane and being accessible to extracellular substrates. In relation to this, perhaps a low concentration (non-optimal condition) of ATP in the cell could be a factor in slowing the transporter recycling process (exocytotic/endocytotic) which is ATP dependent, such that the glucose carriers have a longer half-life on the plasma membrane. The removal of ATP caused a significant increase in cell surface levels of Glut4 in 3T3 - L1 adipocytes, suggesting that ATP may be required for intracellular sequestration of these transporters (Robinson *et al.*, 1992). It is possible that this mechanism may exist also in the mutant WG 750 cells. Stress, such as hyperthermia, treatment with arsenite, infection with vesicular stomatitis virus or Semliki forest virus, and treatment with insulin, cause the glucose transporter to move from an intracellular site to the plasma membrane. Widnell concluded that stress induced an insulin-like distribution of certain membrane proteins (Widnell *et al.*, 1990).

The discrepancy between the glucose transporter number (2- to 3-fold) and hexose transport activity (6-fold) in the mutant cells has also been observed in other systems. A modulation of glucose transporter intrinsic activity has been invoked to explain those alterations in glucose transport activity which appear to occur without major alterations in the subcellular distribution of the glucose transporter proteins. Insulin stimulates glucose transport activity in rat adipose cells primarily by promoting the translocation of the two glucose transporter isoforms, Glut1 and Glut4 (i.e., the insulin regulatable isoform), from intracellular membranous compartments to the plasma membrane (Simpson and Cushman, 1986, James *et al.*, 1988). However, the extent of this translocation, as determined by either CB binding to, or Western blotting of, the plasma membranes, does not fully account for the increase in glucose transport activity measured in the intact cells (Simpson *et al.*, 1983). Vannucci recently used a technique for studying glucose transporters in the plasma membrane of intact adipose cells which involved surface labeling of the transporter with the impermeant photolabel, ATB-BMPA. By using this ligand, which only binds to those transporters (Glut1 and Glut4) which are appropriately exposed to the extracellular fluid (Clark and Holman, 1990), a more accurate correlation between transport activity and transporter number can be obtained (Vannucci, 1992). The mechanism(s) responsible for modulating the intrinsic activity of the glucose transporters are unclear. Phosphorylation of the transporter and covalent modification are known to modulate the intrinsic activity. Both Glut1 and Glut4 can be phosphorylated by protein Kinase C (Gibbs *et al.*, 1986) and protein Kinase A (Lawrence *et al.*, 1990), respectively. To determine if

intrinsic activity modulates WG 750 cells, one would have to perform further studies using subcellular membrane fractions grown under various conditions, for example, in the presence or absence of insulin. Thus, it cannot be determined whether the discrepancy in transport number and transport activity in the WG 750 cells is due to intrinsic activity of the glucose transporter. We cannot exclude the fact that the mechanism involved in the regulation of hexose transport is, in part, post-translational. This view is supported by the 6-fold increase in both 2 - DG uptake and V_{max} for transport, and the 3-fold increase in Glut1 protein in the WG 750 cells.

The Effect of Insulin and Glucose on Glut1 Transporter Gene Expression

To rule out whether transport was regulated at the transcriptional level, steady-state RNA transcript was analyzed under various conditions. Total RNA was isolated from both the mutant and control cells under basal conditions. Analysis by Northern blot using a RNA probe revealed that the level of Glut1 mRNA was the same in both cell strains (Figure 13). This observation was surprising, since 2 - DG uptake under basal conditions in the mutant cells was significantly higher (6-fold) than in the control cells (Table III) and glucose transporter protein was elevated in the plasma membrane and whole cells (Figures 8 and 11). The expectation was that Glut1 mRNA would be higher in the mutant. It is conceivable that under basal conditions, the cell's mechanism for transport regulation is translational or post-translational. The cell responds to the basal state by redistributing the carriers such that more are available on the plasma membrane and thus are accessible to extracellular substrates. Moreover, Glut1 is the transporter isoform responsible for basal glucose uptake (Mueckler, 1990). It appears that insulin is able to modulate Glut1 mRNA, since a 2-fold increase was observed at 8h of insulin exposure in the mutant cells (Figure 14). The scanning of the autoradiograph confirms this observation in Figure 15. Human β-actin gene was used as the housekeeping gene, since it is constitutively expressed. Kosaki has also reported that insulin increased Glut1 gene expression using cultured human skin fibroblasts (Kosaki *et al.*, 1988). Chronic exposure of the cells with insulin also resulted in parallel increases in the cellular levels of Glut1 mRNA and protein, accompanied by increases in insulin-stimulated glucose uptake. These findings are in agreement with those previously reported in 3T3-L1 adipocytes (Tordjman *et al.*, 1989) and in rat L6 skeletal muscle cell lines (Walker *et al.*, 1989). Interestingly, the Glut1 isoform which is abundant in differentiated 3T3 - L1 cells (Calderhead and Lienhard, 1988, Calderhead *et al.*, 1990, Tordjman *et al.*, 1989) increased 3-fold to 5-fold above basal levels in response to acute insulin exposure. In contrast to this, chronic-insulin treatment has been shown to produce a 4-fold further increase in the mRNA and total protein for the Glut1 isoform (Tordjman

et al., 1989, Hanique *et al.*, 1990). However, no change was observed in total cellular mRNA or protein for the Glut4 isoform. The availability of Glut4 at the cell-surface was down-regulated to one-half the level found in the acute treatment but with no change in the total cellular level. It appears that chronic treatment with insulin (500 nM for 24 h) selectively down-regulates cell-surface Glut4 transporters in 3T3 - L1 adipocytes, while levels of Glut1 at the surface are markedly increased (Kozka *et al.*, 1991). Reed has also shown that differentiation of 3T3-L1 adipocytes in the continued presence of insulin leads to a 19- and 23-fold increase in the amount of Glut1 mRNA and protein present in an individual cell (Reed *et al.*, 1990). The large elevation in Glut1 protein content in 3T3 - L1 adipocytes when differentiated in the presence of insulin does not arise from a single regulatory event, but from the effects of general message and protein pool expansion, combined with specific regulatory increases from differentiation and insulin exposure (Reed *et al.*, 1990). The level of transporter expression therefore might vary depending upon the time at which insulin is removed during the process of differentiation. Garcia de Herreros, using a different but related 442A preadipocyte cell line which does not require insulin to induce differentiation, analyzed message changes in serum-starved cells before and after differentiation and observed no increase in the relative mRNA content of Glut1 transporter after differentiation (Garcia de Herreros and Birnbaum, 1989). In the same study, these investigators demonstrated that only differentiated 442A and not undifferentiated cells exhibit the ability to elevate Glut1 transporter message during chronic insulin exposure.

It is evident that the expression of mRNA can be altered by various stimuli. Acute and chronic exposure of cells to insulin or other growth factors, differentiation, transformation and glucose starvation all can modulate Glut1 mRNA and protein. Insulin increased transport and mRNA levels 60% in human fibroblasts (Kosaki *et al.*, 1988) and PDGF, FGF, EGF and serum increased Glut1 mRNA levels 5- to 7-fold in Balb 3T3 fibroblasts (Rollins *et al.*, 1988, Hiraki *et al.*, 1988). Transformation by oncogenes decreased transporter turnover in chick fibroblasts (Shawver *et al.*, 1987), and increased the rate of transcription and concentration of transporter mRNA in rat fibroblasts (Williams and Birnbaum, 1988). Mouse 3T3 - C2 cells after starvation elevated only the amount of transporter protein and not the level of translatable mRNA (Hasple *et al.*, 1986), indicating that starvation enhances transport activity by decreasing the rate of transporter turnover. Rat glial cells, however, showed a 4- to 6-fold increase in mRNA and protein concentration (Walker *et al.*, 1988), consistent with starvation increasing the rate of transporter synthesis.

In addition to insulin, glucose was employed to ascertain if it can modulate Glut1 mRNA in the WG 750 cells. Glucose starvation has been shown to alter the expression of the Glut1 mRNA and protein in the 3T3-L1 cell line. Incubation of undifferentiated 3T3-L1 preadipocytes in glucose-free media for two days increased transporter mRNA and protein content by a factor of 2.2 and 3.5, respectively, concomitant with the loss of the fully glycosylated form of the transporter. Perhaps full glycosylation of the transporter is not required to produce a functionally active transporter (Reed *et al.*, 1990). In L6 muscle cells in culture, increased levels of the Glut1 mRNA and Glut1 transporter protein are observed in response to prolonged hypoglycemia. Elevations in the glucose concentration in the medium depressed the levels of Glut1 mRNA, Glut1 protein and of Glut1 transporter present in the plasma membrane in the L6 muscle cells (Klip and Pâquet, 1990).

Kosaki has also demonstrated in cultured human skin fibroblasts that glucose deprivation resulted in approximately a 4-fold increase in Glut1 mRNA, a 3-fold increase in Glut1 protein and basal 2 - DG uptake. However, when cells were incubated with various concentrations of glucose (11.1 to 44.4 mM), no differences were found in Glut1 mRNA, protein levels, and basal or insulin-stimulated 2 - DG uptake (Kosaki *et al.*, 1991). Kahn has shown that changes in ambient glucose did not alter Glut1 mRNA nor the translocation of the protein to the plasma membrane in response to insulin. Thus, it may result from changes in Glut1 functional activity (Kahn *et al.*, 1991).

Analysis by Northern blot using a DNA probe revealed that glucose deprivation increased the level of Glut1 mRNA by approximately a factor of 2 in the WG 750 cells, whereas 25 mM glucose down-regulated Glut1 mRNA (Figure 16, lanes 1 and 2). Densitometric analysis indicates a 2-fold increase in Glut1 mRNA in the WG 750 cells in the presence of 0% glucose (Figure 17, lane 1). Surprisingly, the level of Glut1 mRNA in both glucose-deprived and glucose-fed cases was the same in the MCH 55 cells (Figure 16, lanes 3 and 4). This lack of response in the MCH 55 cells could be attributed to the DNA probe, in that its sensitivity in detecting small amounts of steady-state transcripts my be reduced. This experiment would have to be repeated using an RNA probe, if any comparison is to be made with the previous Northerns.

In summary, the data in this study clearly show that glucose transport is permanently upregulated in these mutant cells and that this increase in glucose transport is regulated by a composite of two controls — a translational (or post-translational) and a transcriptional control. That this dynamic function of the glucose transport system should be controlled at two levels suggests that the mechanism in transport regulation in the mutant cells is highly complex and is the result of the cell's great need to respond to metabolic demands and

other signals that are necessary to maintain homeostasis. Evidence to support a translational regulation was shown by an increase in V_{max} for transport and 2 - DG uptake and an increase in the number of glucose transporters on the plasma membrane in the WG 750 cells. Transcriptional regulation was substantiated by an increase in Glut1 mRNA in both glucose deprivation and insulin-stimulated state. Furthermore, the molecular control(s) in transport regulation in these mutant cells regarding Glut1 mRNA respond more rapidly to the modulating conditions, i.e., insulin or glucose starvation, while the MCH 55 (control) do not appear to be responding. The reason for the sensitive response in the mutant is due to the cell's inability to generate energy via the electron transport chain and as part of the adaptation to the enzyme defect, the cell responds more quickly than the control cells by increasing Glut1 mRNA levels. Interestingly, Germinario showed that respiration-deficient Chinese hamster fibroblasts (G14) derived from a transformed parental cell line (V79) did not respond to any normal regulatory signals affecting transport, i.e., demonstrating no responses to serum deprivation, serum refeeding, insulin exposure or glucose deprivation (Germinario *et al.*, 1990). Both the mutant WG 750 cells and the mutant hamster cell line (G14) increase their sugar transport to compensate for the enzyme defect, but their responses to regulatory signals are very different, suggesting that different mechanisms for the increased transport could be involved. The understanding of the molecular mechanism(s) responsible for the increased hexose transport in WG 750 cells need to be confirmed with further studies.

Although much progress has been made in our understanding of glucose transport in human fibroblasts, the molecular signals and protein(s) involved in the translocation of glucose transporters to the plasma membrane remain a black box. Further studies are needed to define the characteristics of the insulin-recruitable intracellular pools and the signals that target them to the plasma membrane. The effects of post-translational modification, if any, such as phosphorylation and glycosylation on glucose transporter function also have to be determined. The regulation of glucose transport activity through changes in intrinsic activity need to be demonstrated and the underlying molecular events defined. The observations that emanate from this study provide a basis in understanding the molecular mechanism(s) of transport regulation in the mutant cells. The use of these respiration-deficient fibroblasts will be an excellent model system for studies in understanding how glucose uptake and metabolism are controlled in normal and altered metabolic states in human cells.

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Appendix I List of Substrates and Chemicals Used in Sugar Transport Studies

1.	2 - DG:	Non-metabolizable substrate used in transport studies which becomes phosphorylated as 2 - DG - 6 - PO_4 and remains trapped inside the cell.
2.	3 - OMG:	Non-metabolizable substrate which does not become phosphorylated and rapidly reaches equilibrium.
3.	CB:	Inhibitor of the saturable component of sugar transport (carrier mediated transport). Inhibits transport by specifically binding to the exofacial side of the glucose carrier (cytoplasmic side).
4.	L - glucose:	Measure of the non-saturable component of sugar transport (simple-diffusion).
5.	Phoretin:	Inhibitor of sugar transport, inhibiting efflux of 3 - OMG.
6.	DEX:	Inhibitor of sugar transport; inhibiting transport by redistributing the glucose carrier from the plasma membrane to the intracellular pool.