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UNIVERSITY OF ALBERTA

GLUCOSE TRANSPORTERS IN BOVINE MAMMARY GLAND

BY

FENG-QI ZHAO 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

in

Animal Biochemistry

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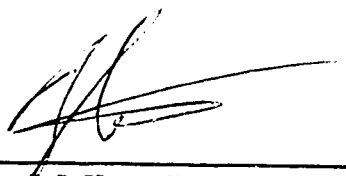

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **GLUCOSE TRANSPORTERS IN BOVINE MAMMARY GLAND** submitted by **FENG-QI ZHAO** in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOCHEMISTRY**.

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Dedicated to All People Who Educated Me

ABSTRACT

The epithelial cells of the mammary gland take up glucose by the passive process of facilitated diffusion. In mammalian cells, facilitated glucose diffusion is mediated by a family of structurally related glucose transporter proteins. To study the glucose transporters in bovine mammary gland, the glucose transporters were identified and localized. The gene expression of GLUT1 during different physiological states was studied as was the effect of administration of exogenous bGH and bGHRF on the gene expression of glucose transporters.

The complementary DNA for five human facilitative glucose transporters (GLUT1, GLUT2, GLUT3, GLUT4, and GLUT5) were used to determine the distribution of mammalian facilitative glucose transporter mRNA in bovine tissues by Northern blotting. Under high stringency hybridization conditions, a single 2.8 kb transcript of GLUT1 was the only messenger RNA species detected in the lactating bovine mammary gland at an appreciable level. The mRNA of the other four transporters were either expressed at very low levels or undetectable.

GLUT1 and GLUT4 proteins were assessed by Western blotting and immunohistochemical staining, using polyclonal antibodies against the C-terminal peptide of rat GLUT1 and GLUT4. Bovine mammary gland expressed a relatively high level of GLUT1 protein, whereas GLUT4 protein was not detected in the mammary gland of either lactating or dry cows. The absence of GLUT4 may indicate that glucose transport is not regulated by insulin in the lactating and dry cow mammary gland. The anti-GLUT1 antibody strongly stained the single layer of epithelial cells of mammary alveoli. The expression of GLUT1

mRNA was similar in the mammary gland of late lactation and non-lactating cows. However, a smaller molecular weight species (38 kDa) of GLUT1 protein was detected in the mammary gland of non-lactating cows where its expression was 80% higher than in lactating animals. There were no significant differences in GLUT1 mRNA in bovine mammary gland at 118 d and 181 d postpartum, however, GLUT1 protein tended to be greater at 118 d postpartum ($P = 0.17$).

To investigate the effect of administration of exogenous bGH and bGHRF on the gene expression of glucose transporters, primiparous cows received 29 mg bGH/d or 12 mg bGHRF/d by continuous intravenous infusion or no treatment (controls) from 118 to 181 d postpartum. Compared with controls, bGHRF and bGH did not influence GLUT1 protein level in the mammary gland, however, GLUT1 mRNA was increased by bGHRF, but not by bGH. GLUT4 mRNA in skeletal muscle was decreased by 44% as a result of bGH treatment, but there was no significant effect of bGHRF. GLUT4 mRNA was barely detectable in the omental fat of control cows and became undetectable in bGH and bGHRF treated cows. These results indicate that bGH and bGHRF may increase glucose availability to the mammary gland by regulating glucose transporter expression in skeletal muscle and omental fat.

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LIST OF ABBREVIATIONS NOT DEFINED IN THE TEXT

Abbreviation	Definition
ANOVA	Analysis of variance
A-V	Arterio-Venous
bGH	Bovine growth hormone
bGHRF	Bovine growth hormone-releasing factor
bp	Base pair(s)
BSA	Bovine serum albumin
°C	Degree Celsius
d	Day(s)
EDTA	Ethylenediaminetetra-acetate
g	Gram(s)
GLUT1	The erythrocyte-type glucose transporter
GLUT2	The liver-type glucose transporter
GLUT3	The brain-type glucose transporter
GLUT4	The muscle/fat-type glucose transporter
GLUT5	The small intestine-type glucose transporter
GLUT7	The hepatic microsomal glucose transporter
GT	Galactosyl transferase
h	Hour(s)
kb	Kilobases

kDa	Kilodaltons
kg	Kilograms
α-LA	α-Lactalbumin
3-*O*-MG	3-*O*-methyl-*D*-glucose
min	Minute
M_r	Relative molecular mass
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NEFA	Non-esterified fatty acids
P	Probability
PMSF	Phenylmethanesulphonyl fluoride
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulphate
SGLT1	Sodium-dependent glucose transporter 1
TBS	Tris-buffered saline
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Glucose is a major source of energy for mammalian cells as well as a substrate for macromolecules, such as glycoproteins, proteoglycans, glycolipids, and nucleic acids. In the epithelial cells of bovine mammary gland, glucose is the main precursor of lactose synthesis and is used in providing NADPH for fatty acid synthesis. Since lactose maintains the osmolarity of the milk in the formation and secretion process, the rate of lactose synthesis serves as the major control of the volume of milk produced (Neville *et al.*, 1983). As mammary gland cannot synthesize glucose from other precursors because of the absence of glucose 6-phosphatase (Threadgold and Kuhn, 1979), the glucose supply to mammary gland can be obtained only from the blood. Therefore, mammary glucose uptake is a rate-limiting factor for milk production (Kronfeld, 1982). It has been estimated that in the lactating cows 72 g of glucose uptake by the mammary gland is required to produce 1 kg of milk (Kronfeld, 1982). Mammary uptake can be 60-85% of the total glucose entering the blood (Chaiyabutr *et al.*, 1980; Annison and Linzell, 1964).

The transport of glucose across cell membranes occurs by the passive process of facilitated diffusion in most tissues. Recent cDNA cloning studies have shown that facilitated glucose diffusion is mediated by a family of structurally related glucose transporter proteins encoded by distinct genes. So far, six different facilitative glucose transporters have been described. Each transporter has different kinetics and is tissue- and cell-type-specific (Burant *et al.*, 1991; Waddell *et al.*, 1992, Gould and Holman, 1993). Except for the most recently

cloned rat microsomal glucose transporter (GLUT7) (Waddell *et al.*, 1992), the expression of the other five facilitative glucose transporter genes have been extensively examined in human tissues. GLUT1 and GLUT3 are expressed in many tissues, including the brain, placenta, and kidney (Burant *et al.*, 1991). The ubiquitous distribution of GLUT1 and GLUT3 in human tissues suggests that these two transporters may play a function in basal uptake of glucose by cells. GLUT2 is expressed only in the liver, small intestine, kidney, and pancreatic ß-cells (Thorens *et al.*, 1988). The tissue distribution and high K_m of GLUT2 suggest that it mediates the uptake and release of glucose by hepatocytes and release of absorbed glucose across the basolateral surface of epithelial cells of the kidney and small intestine. GLUT4 is the transporter isoform primarily responsible for insulin-stimulated glucose transport and is therefore found mainly in insulin-sensitive tissues, such as fat and muscle (James *et al.*, 1989). GLUT5 is expressed predominantly in the jejunal region of the small intestine (Kayano *et al.*, 1990). Although the role of GLUT5 in glucose transport has not yet been determined, it has recently been reported to also be a fructose transporter (Burant *et al.*, 1992).

As in most mammalian tissues, the epithelial cells of the mammary gland take up glucose by the passive process of facilitated diffusion (Delaquis *et al.*, 1993). However, only a few studies have been carried out to examine glucose transporters in the rat mammary gland (Burnol *et al.*, 1990; Madon *et al.*, 1990; Camps *et al.*, 1994) and it remains necessary to extend these studies in the bovine mammary gland. Hence, the primary focus of this project was to identify and localize the glucose transporter(s) in the bovine mammary gland. Further efforts were made to investigate glucose transporter gene expression during different

physiological stages and its possible regulation by growth hormone.

Literature cited in this chapter is listed in the following chapter **Review of Literature**.

CHAPTER II

REVIEW OF LITERATURE

This review is divided into three sections: glucose metabolism in the mammary gland, the role of glucose in regulating milk secretion, and the recent advances in our knowledge of the mammalian glucose transporter.

1 Glucose Metabolism in the Lactating Mammary Gland

In the lactating mammary gland of the nonruminant animal, glucose is a precursor for lactose synthesis as well as supplying α-glycerol-phosphate and acetyl coenzyme-A for triacylglycerol esterification (Kuhn, 1978). Furthermore, glucose is also used as an oxidative substrate to supply the energy required for milk synthesis. In the lactating mammary gland of the ruminant animal, glucose is primarily used for lactose synthesis and NADPH generation for fatty acid synthesis (Chaiyabutr *et al.*, 1980).

1.1 Lactose Biosynthesis

The biosynthesis of milk lactose was first firmly established by Watkins and Hassid (1962). They found that an enzyme prepared from guinea pig and bovine mammary glands could convert UDP-[^{14}C]galactose plus glucose into lactose. The biochemical pathways for lactose synthesis are outlined below (Faulkner and Peaker, 1987).

1) Glucose + ATP $\xrightarrow{\text{hexokinase}}$ Glucose-6-phosphate + ADP

2) Glucose-6-phosphate <—phosphoglucomutase—> Glucose-1-phosphate

3) Glucose-1-phosphate + UTP <—UDP-glucose pyrophosphorylase—>

UDP-glucose + pyrophosphate

4) UDP-glucose <—UDP-galactose 4-epimerase—> UDP-galactose

5) UDP-galactose + Glucose —lactose synthetase—> Lactose

Steps one to four of lactose synthesis occur within the cytosol of the mammary gland. Step one is thought to be an irreversible reaction and the following three steps are equilibrium reactions. In the rat and guinea pig, the UDP-galactose 4-epimerase, UDP-glucose pyrophosphorylase, and phosphoglucomutase activities increase markedly after the onset of lactation. In contrast, these increases are not shown in the cow's mammary gland as the enzymes appear to be already present prior to lactation (Schmidt, 1971).

The final step in lactose synthesis is probably the rate limiting step and occurs in the lumen of the Golgi apparatus. UDP-galactose and glucose are transported into the lumen of the Golgi vesicle and form lactose catalyzed by the enzyme lactose synthetase. This enzyme consists of two components, a membrane-bound enzyme galactosyl transferase and a regulatory protein α-lactalbumin. Galactosyl transferase is found in the inner membranes of Golgi vesicles in many tissues where it is involved in the transfer of galactosyl groups from UDP-galactose to carbohydrate groups of glycoproteins. Only in the mammary gland is free glucose the acceptor. This specificity of galactosyl transferase in the mammary gland is mediated by α-lactalbumin which increases the affinity of galactosyl transferase for glucose from $K_m \approx 1$ M to $K_m \approx 1$ mM (Neville *et al.*, 1983).

Lactose synthetase is specific for the β-anomer of glucose (Kuhn *et al.*, 1980). The

final reaction also has a bivalent cation requirement and is activated by metal ions which bind to two sites on galactosyl transferase (Kuhn, 1983). Site one has a high affinity for Mn^{+}, Co^{2+}, Zn^{2+} and Cd^{2+}. The binding of one of these metal ions to Site one stabilizes the active conformation of the enzyme. Ca^{2+} binds to Site two and this binding probably forms a bridge between the enzyme and the substrate.

The activity of lactose synthetase increases markedly during lactation and there is a diurnal variation such that lactose synthesis is greatest during the night and least in the late afternoon (Hartmann, 1973). Factors regulating lactose synthetase include α-lactalbumin, galactosyltransferase, UDP-galactose, glucose, and Ca^{+}(Kuhn *et al.*, 1980).

For an overview of lactose synthesis, refer to Fig. II-1.

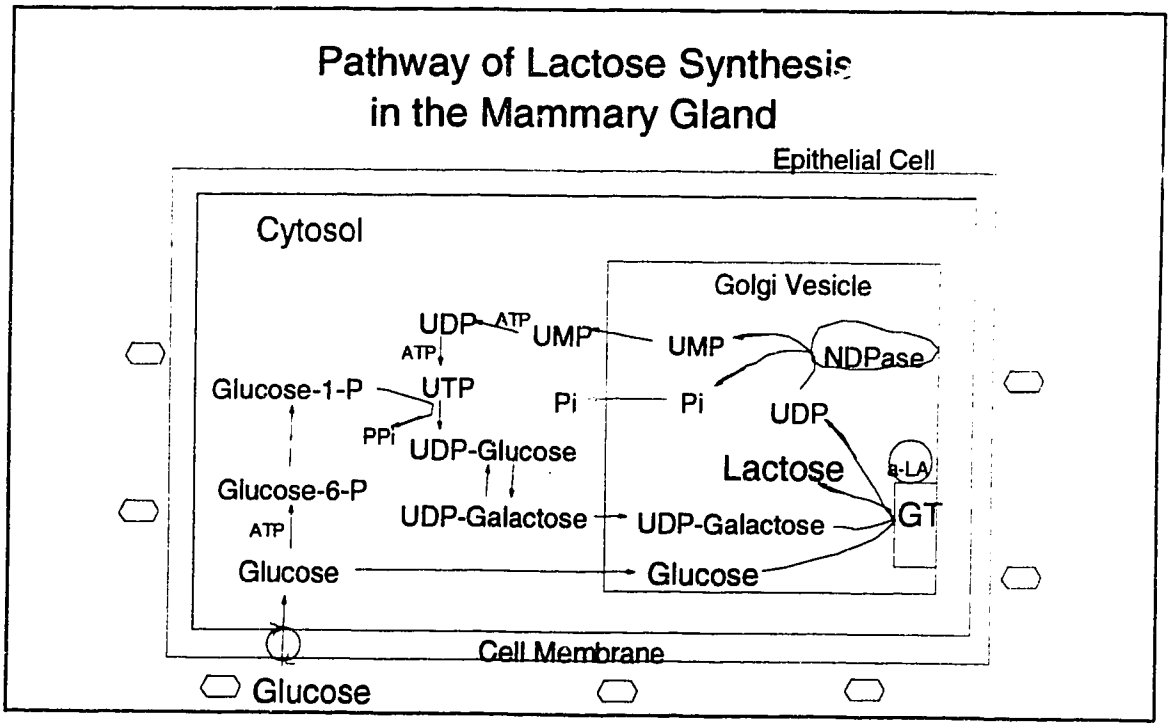

Fig. II-1. Lactose synthesis in mammary epithelial cell (modified from Kuhn, 1983).

1.2 Pentose Phosphate Pathway

The lactating mammary gland has a considerable requirement for reducing equivalents in the form of NADPH for fatty acid synthesis. The oxidative decarboxylation of glucose-6-phosphate to ribulose-5-phosphate in the pentose phosphate pathway is an important source of NADPH. In the mammary gland of the lactating goat, 20% of the glucose taken up from the blood can be metabolized via the pentose phosphate pathway and thus provides about 25% of the total NADPH for *de novo* fatty acid synthesis (Chaiyabutr *et al.*, 1980). The pentose phosphate pathway is more important in nonruminant species where glucose supply is less likely to be limiting. In the isolated acini of the lactating rat mammary gland, 25 to 50% of glucose taken up by the acini can be metabolized via the pentose phosphate pathway and thus provides 70 to 100% of the NADPH required for fatty acid synthesis (Katz *et al.*, 1974; Robinson and Williamson, 1977).

The lactating ruminant mammary gland cannot effectively use glucose for *de novo* fatty acid synthesis as the activity of one of the enzymes linking glucose metabolism to fatty acid synthesis, ATP-citrate lyase, is very low in ruminant tissues (Hanson and Ballard, 1967). Acetate is the predominant precursor for fatty acid synthesis and for NADPH generation via the isocitrate dehydrogenase pathway (Bauman and Davis, 1974).

2 Role of Glucose in Regulating Milk Secretion

2.1 Glucose as the Sole Precursor of Lactose

Lactose is the predominant carbohydrate in the milk of most mammalian species. The

concentration of lactose within a species tends to be relatively constant and thus is considered to maintain the osmolarity of milk in the formation and secretion process. In practice, glucose is the sole precursor of lactose. Decisive evidence for the essential role of glucose in the lactating mammary gland was provided by Hardwick *et al.* (1961) who showed that omission of glucose from the perfusion medium stopped milk lactose and water secretion completely and reintroduction of glucose into the perfusate reestablished lactose secretion in the perfused goat udder. Earlier, Kleiber *et al.* (1955) using radioactive glucose injections into the jugular vein showed that about 80% of milk lactose *in vivo* was derived from the plasma glucose pool in the cow. Advances in our understanding of the unique and essential role of glucose in the synthesis of lactose and hence in the maintenance of milk production in the lactating mammary gland were largely achieved by the development of methodology to quantify substrate uptake (Linzell, 1974). Using these improved methods with lactating cows (Bickerstaffe *et al.*, 1974) and goats (Linzell, 1974), Linzell *et al.* reported a mean uptake of 8.0 g glucose per 100 ml milk secreted. Glucose uptake by the mammary gland accounts for 50-80% of total glucose entry rate and about 85% of lactose carbon is derived from blood glucose. These results were confirmed by several other observations (Chaiyabutr *et al.*, 1980; Kronfeld, 1982).

2.2 Glucose Availability and Milk Synthesis

Mammary cells cannot synthesize free glucose because of the absence of glucose-6-phosphatase (Threadgold and Kuhn, 1979). Hence, glucose for mammary metabolism is derived from plasma and its supply is determined by blood flow, blood glucose concentration,

and glucose uptake by the mammary gland.

2.2.1 Glucose Supply in the Ruminant

Blood glucose is derived from exogenous and endogenous glucose (Gluconeogenesis). In the nonruminant, glucose is primarily derived from the digestion of carbohydrates in the intestine. In the ruminant, most dietary carbohydrate is fermented to volatile fatty acids (mainly acetate, propionate, and butyrate) in the rumen and thus the amount of starch entering the duodenum is relatively low (Sutton 1985; Owens *et al.*, 1986). It has also been suggested that the capacity for digestion of starch in the small intestine of ruminants is limited (Ørskov, 1986). Furthermore, there is extensive metabolism of glucose in the epithelial cells of the bovine small intestine (Okine *et al.*, 1994). The result is that as little as 15% of blood glucose is derived from dietary sources (Lomax and Baird, 1983; Reynolds *et al.*, 1988) with the remainder being provided by hepatic gluconeogenesis (Bergman *et al.*, 1974; Bauman, 1984) which can contribute in excess of 3 kg glucose/d in the lactating cow (Reynolds *et al.*, 1988). The liver also fulfills an important role during lactation in altering patterns of nutrient availability to adipose, muscle, and mammary tissues. Blood flow rate through the liver is 52% greater in lactating compared with non-lactating cows (Lomax and Baird, 1983) and hepatic glucose output is linearly related to milk yield (Lomax and Baird, 1983). The kidney is also an important organ in whole-body glucose metabolism with gluconeogenesis in the kidney accounting for approximately 10% of the glucose produced by nonpregnant sheep (Gans and Mercer, 1984). In addition, the kidney plays a key role in the reabsorption of glucose filtered in the glomerulus (Gans and Mercer, 1984).

In lactation, glucose utilization in nonmammary tissues may be depressed, notably in bovine adipose tissue (Metz and Van den Bergh, 1977) and skeletal muscle (Pethick and Lindsay, 1982). Body tissues other than the mammary gland preferentially use other substrates as energy sources which spares glucose for lactose synthesis. At day 30 prepartum 34% of total glucose turnover is oxidized to CO_2, whereas this decreases to only 8 to 9% by day 7 of lactation (Bennink *et al.*, 1972).

In both ruminant and nonruminant species, glucose availability is under the control of insulin and glucagon (Weekes, 1991). Insulin stimulates glucose transport in skeletal muscle and adipose tissue by accelerating the translocation of glucose transporters to the plasma membrane. Insulin inhibits hepatic glucose output (Brockman, 1983; Weekes *et al.*, 1983; Debras *et al.*, 1989), but GH decreases the ability of insulin to inhibit gluconeogenesis (Boisclair *et al.*, 1989; Gopinath and Etherton, 1989). Intravenous infusion of glucagon into lactating cows rapidly increases blood glucose concentration (Davis and Collier, 1985).

2.2.2 Arterial Glucose Concentration and Milk Synthesis

An elaborate homeostatic mechanism exists in mammals to maintain constant blood glucose concentration. However, glucose concentration can be altered by insulin, infusion of glucose, and short-term starvation. The relationship between mammary glucose uptake and arterial glucose concentration in the bovine has not been clearly established. Davis and Collier (1985) extrapolated mean glucose A-V differences and arterial concentrations from a series of separate studies and demonstrated a linear relationship between these two variables. However, Miller *et al.* (1991) showed that glucose uptake in the mammary gland was not

influenced by arterial concentrations in the range of 2.2 to 4.7 mM and attributed the apparent dependence of glucose A-V difference on plasma arterial concentration reported by Davis and Collier to the limited number of observations. Data from Miller *et al.* suggest that physiological glucose concentrations are not limiting for glucose utilization by the mammary gland. In accordance with Miller *et al.*'s observation, Rook and Hopwood (1970) showed a direct relationship between plasma glucose concentration and lactose synthesis at concentrations of up to 2.2 mM with little further increase in lactose synthesis at higher glucose concentrations. A value of 2.2 mM is at the lower limit of the normal range for lactating cows. Kronfeld *et al.* reported that the relationship between milk production and plasma glucose concentration was linear below a plasma glucose concentration of about 3.0 mM (see Kronfeld, 1982). Further increases in plasma glucose concentration did not influence milk production. Increasing glucose availability from 1107 to 1622 μmol/min in fed and 317 to 862 μmol/min in starved goats by infusing glucose did not change glucose uptake in the mammary gland (see Faulkner and Peaker, 1987). Milk yield was not altered by hyperglycaemia (> 5.5 mM) induced by intravenous infusion of glucagon (Davis and Collier, 1985).

2.2.3 Blood Flow and Milk Synthesis

Blood flow plays an important role in regulating glucose supply to the mammary gland. A marked increase in mammary blood flow and cardiac output occurs at parturition (Burd *et al.*, 1978). The galactopoietic effect of exogenous bGH is associated with increased mammary blood flow in lactating cows (McDowell *et al.* 1987; Davis *et al.* 1988b). Short-

term fasting resulted in a rapid fall in blood flow to the mammary gland in lactating goats which was accompanied by a corresponding decline in glucose uptake (Faulkner and Peaker, 1987). In the cow and goat, a positive correlation between changes in mammary blood flow and milk yield has been established; 400 to 500 litres of blood passing through the mammary gland for each litre of milk produced (Linzell, 1974; Davis and Collier, 1985). The control of blood flow to the mammary gland of lactating cows (15 to 16% of cardiac output in fed animals) is proposed to play a role in the regulation of nutrient partitioning between the mammary gland and other body tissues (Davis and Collier, 1985). However, the control mechanisms involved in the regulation of mammary blood flow are still unclear.

2.2.4 Mammary Glucose Uptake

Lactose is synthesized in the Golgi lumen. The rate of lactose synthesis is dependent on the intra-Golgi glucose concentrations. However, isolated Golgi vesicles are readily penetrated by monosaccharides of $M_r < 300$, but not by disaccharides (White *et al.*, 1981). Also isolated Golgi membranes display transport properties consistent with the presence of water-filled pores of approximately 1.1 nm diameter (see Leong *et al.*, 1990). Thus glucose can enter the Golgi vesicles freely from the cytoplasm. The Golgi membrane is not permeable to lactose. Thus lactose remains within the Golgi lumina and exerts an osmotic effect.

As there is very little restriction on glucose entry into Golgi vesicles, lactose synthesis is more dependent on the intracellular glucose concentration. The exposure of Golgi membrane vesicles to varying concentrations of glucose showed that apart from inhibition at high concentration, the rates of lactose synthesis follow classical Michaelis-Menten kinetics

with a K_m of 1.5 mM (Kuhn *et al.*, 1980), which exceeds the glucose concentration within the cell. Thus the intracellular glucose concentration may be a critical factor determining the rate of lactose synthesis and milk secretion.

Intracellular glucose concentrations directly affect the rate of the reactions catalyzed by hexokinase and lactose synthetase which utilize free glucose in the cells. Ruminant tissues lack glucokinase; the phosphorylation of glucose in ruminant tissues is accomplished by hexokinase, the low k_m glucose-ATP phosphotransferase enzyme (Bartley *et al.*, 1966). The hexokinases of the mammary tissues of the rat are mainly types I and II which have reported K_m values for glucose of 0.045 mM and 0.23 mM, respectively. The type II hexokinase is the predominant form (Faulkner and Peaker, 1987). Lactose synthetase has a much higher K_m for glucose than hexokinase. A K_m value of around 3 mM has been reported for bovine lactose synthetase (Schanbacker and Ebner, 1970). Thus, the intracellular glucose concentrations (0.1-0.3 mM) would not saturate the type II hexokinase and lactose synthetase.

Direct measurements of intracellular glucose in the mammary gland are difficult to obtain due to the overwhelming amount of extracelluar glucose and the problem of correcting for the milk space of the tissue. It is assumed that the concentration of glucose in the aqueous phase of milk can reflect its intracellular concentration (see Faulkner and Peaker, 1987). This assumption is supported by two lines of evidence. Firstly, it seems that glucose carriers are present in the apical membrane of mammary secretory cells which could equilibrate milk glucose with cytosolic glucose (Faulkner *et al.*, 1985b). Investigation of hexose transport in the apical membrane is possible by infusion of hexose via the teat canal and lumen of the mammary gland in vivo. The injection of [^{14}C] 3-O-MG into the mammary teat canal resulted

in its appearance in the mammary venous blood (Kuhn *et al.*, 1980). Infusion of different hexose solutions via the teat canal into the udder showed that glucose crossed the apical membranes readily, but other hexoses (such as galactose and fructose) were slower to cross (Faulkner *et al.*, 1985a). This specificity indicates that the properties of the glucose transporter in the apical membrane are similar to those described for the plasma membrane of the mammary epithelial cell of the rat (Threadgold and Kuhn, 1984). Secondly, infusion of 3-O-MG into the blood resulted in its appearance in the milk in a concentration similar to that within the mammary cells (Kuhn and White, 1975). Direct and indirect techniques demonstrate that a steep concentration gradient of glucose occurs across the plasma membrane, from 3.0 to 3.5 mM in plasma to 0.1 to 0.3 mM in the cell (Faulkner *et al.*, 1981). Furthermore there is no correlation between arterial glucose concentrations and milk glucose concentrations (Faulkner and Peaker, 1987). Therefore, glucose transport into the mammary epithelial cells may be the rate-limiting step in lactose synthesis and milk production. Tracer studies have shown a linear relationship between the rate of glucose transport and milk yield in cows (Kronfeld, 1982). The galactopoietic effect elicited by administration of exogenous bGH requires increased glucose uptake by mammary cells (Mepham *et al.*, 1990). Fasting of lactating goats resulted in a sharp decrease in milk yield which was associated with a reduction in the concentration of glucose in milk of a similar magnitude (Faulkner and Peaker, 1987). The theory that transport is rate-limiting is consistent with the lack of correlation observed between blood glucose concentration and milk synthesis.

Glucose uptake in most tissues is under the hormonal control of insulin. However, mammary glucose transport does not appear to be regulated by insulin (Hove, 1978a,b;

Laarveld *et al*, 1981). Changes in plasma insulin concentration had no effect on milk yield and milk composition in lactating goats (Hove, 1978a,b). Dexamethasone inhibits glucose uptake and milk output (Hartmann and Kronfeld, 1973). Glucose uptake is partially dependent on acetate uptake (Miller *et al.*, 1991). Therefore, animal variation in glucose uptake can be potentially explained by the contribution of alternative energy sources and milk precursors and by the requirement of glucose for lactose synthesis. Mammary glucose uptake can also be reduced by treatment with adrenocorticotrophic hormone (ACTH) in goats (Stewart and Thompson, 1981) and by cold exposure in sheep (Thompson, 1980).

Glucose transport in the epithelial cells of the mammary gland is specific, saturable, Na^+-independent, and inhibitable by cytochalasin-B or phloretin in the rat, mouse, and bovine (Threadgold *et al.*, 1982; Prosser, 1988; Delaquis *et al.*, 1993). In the lactating rat mammary acini, the apparent K_m for 2-deoxy-D-[^{3}H]glucose is 16 mM and V_{max} approx. 56 nmol/min per mg of protein (Threadgold *et al.*, 1982). In mammary gland of lactating rats, overnight starvation results in a 90% decrease in the transport of 2-deoxy-D-[^{3}H]glucose and the transport can be restored by 2 h refeeding and insulin (Threadgold and Kuhn, 1984). The basal rate of carrier-mediated glucose transport increases about 40-fold in mouse mammary epithelial cells isolated from the virgin to midlactating state and declines during involution (Prosser and Topper, 1986). Insulin does not acutely stimulate glucose transport in cells isolated from virgin or lactating mice, but evokes a 50% increase in the transport rate in cells from pregnant and early postlactational animals. The V_{max}, but not K_m, for 3-*O*-MG is markedly increased during ontogeny which is associated with an increase in the number of functional transporters. Fasting mice for 16 h resulted in a 70% decrease in cytochalasin B-

binding sites of the plasma membranes and the carrier-mediated uptake of 3-*O*-MG by mammary epithelial cells (Prosser, 1988). Refeeding fasted mice for 3 h can restore both the 3-*O*-MG uptake and cytochalasin B-binding sites. As the synthesis of new protein seems not to be involved in the restoration of transport activity after refeeding, the effect of fasting probably involves a redistribution of glucose transporters within the cell.

2.3 Administration of GH and Glucose Metabolism

Treatment of dairy cows with exogenous bGH can increase milk yield by as much as 40% (Peel and Bauman, 1987). Although the lactogenic effects of growth hormone have been known for several decades, the physiological mechanisms underlying this phenomenon are not well understood. The absence of a GH effect on casein, alpha-lactalbumin, and lipid synthesis in cultured mammary tissue (Akers, 1985) and the failure to identify GH receptors in the bovine mammary gland (Kazmer *et al.,* 1986; Keys & Djiane, 1988) has led to the generally accepted view that the effect of GH on lactogenesis is indirect through intermediates such as IGF-I. However, the presence of growth hormone receptor mRNA in mammary cells raises the possibility of a direct action of GH on the mammary tissue (Glimm *et al.*, 1990).

Administration of bGH in dairy cows does not alter the digestibilities of dietary dry matter, energy, and nitrogen (Bauman *et al.*, 1988). In short-term bGH treatment (1-3 wk), milk production increased in the absence of changes in feed intake (Bauman and McCutcheon, 1986). Thus, at least within this period, nutrients needed to provide additional milk precursors and energy substrates for GH-induced increases in milk production are derived primarily from the mobilization of adipose tissue and skeletal muscle. In adipose tissue, GH decreases

glucose uptake, inhibits lipogenesis, and stimulates lipolysis (Davidson, 1987). The combination of decreased lipogenesis and increased lipolysis contributes to decreased body stores and elevated concentrations of NEFA and glycerol in the blood (Bauman *et al.*, 1988; Cisse *et al.*, 1991; Dahl *et al.*, 1993). Thus, NEFA can be used as an alternative energy source to glucose (Peel *et al.*, 1982; Tyrrell *et al.*, 1988) and the liver can use glycerol for increased gluconeogenesis (Bauman *et al.*, 1988). GH also decreases glucose uptake in muscle (McDowell *et al.*, 1987) and increases mammary blood flow (McDowell *et al.*, 1987; Davis *et al.*, 1988b). The net result of these changes is increased glucose availability for lactose synthesis in GH-treated animals. It has been reported that the mammary gland increased glucose uptake during GH treatment (Davis *et al.*, 1988a; Hart, 1988; Fullerton *et al.*, 1989).

3 Mammalian Glucose Transporters

Solutes enter a cell in several ways which are classified as simple diffusion, facilitated diffusion, and active transport (Fig. II-2). Simple diffusion is accomplished by the random movement of molecules (such as O_2, CO_2, NH_3, and water) through membranes. Facilitated diffusion is a process by which certain otherwise impermeable compounds can diffuse through a membrane. For example some proteins can form a channel or ion pore through which Cl^- and HCO_3^- can pass. A second facilitated diffusion process is accomplished by stereospecific carriers (transmembrane proteins) which can provide a mechanism to allow a particular substrate into a cell down a concentration gradient. Active transport differs from the

facilitated diffusion in that transport is against a concentration gradient. When the active transport is directly coupled with ATP hydrolysis, the transport is termed primary active

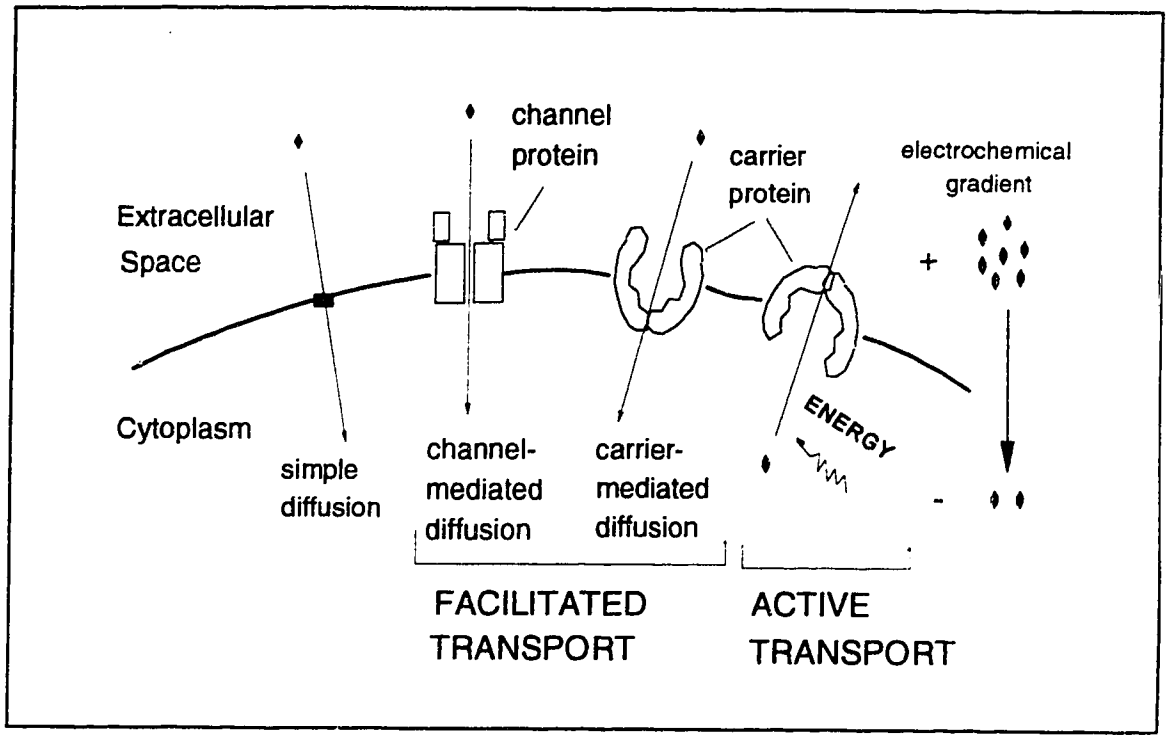

Fig. II-2. The molecular mechanism of memebrane transport in mammalian cell

transport. The transport of a molecule across the plasma membrane at the expense of a previously established ion gradient is called secondary active transport. Active transport is also accomplished by stereospecific carriers. In most mammalian cells glucose uptake occurs by carrier-mediated facilitated diffusion. In a few sites, including the kidney and small intestine, glucose is taken up by the active, sodium-linked transport process (Baldwin, 1990). Recent cDNA cloning studies have shown that the facilitated and sodium-dependent glucose transport are mediated by two different families of glucose transporters (Burant *et al.*, 1991).

3.1 The Sodium-Dependent Glucose Transporter Family

Glucose absorption from the small intestine and kidney is a two-step process. The Na^+-dependent transporter on the apical brush borders of intestinal and kidney epithelial cells actively accumulates glucose against its concentration gradient by coupling the transport of glucose with the down gradient transport of sodium, the sodium gradient is maintained by the active transport of sodium across the basolateral surface of the epithelial cells by the membrane-bound Na^+/K^+/ATPase. The accumulated glucose is subsequently released into the capillaries via the facilitated transporter localized on the basolateral surface of the cells. To isolate cDNA clones encoding the sodium-dependent glucose transporter expressed in the small intestinal mucosa, Wright and co-workers used a novel expression cloning strategy (Hediger *et al.*, 1987). They injected the poly(A)$^+$-RNA from the rabbit intestine into *Xenopus laevis* oocytes and measured phlorizin (a reversible competitive inhibitor of Na^+/D-glucose cotransport system)-sensitive α-methyl-D-glucopyranoside (a specific substrate for the sodium-dependent transporter) uptake into oocytes. After size selection of poly(A)$^+$-RNA, the mRNA coding for the transporter was found to be highly enriched in a 2.3-kilobase fraction. A complementary DNA library was prepared from this fraction using an *in vitro* expression vector and screened by injecting mRNA synthesized *in vitro* into oocytes and then assaying for sodium-dependent α-methyl-D-glucopyranoside uptake. The resulting DNA sequence indicated that the rabbit small intestine sodium-dependent transporter, designated SGLT1, was a 662-amino acid polypeptide with a molecular weight of 73,080 Da.

The cDNA encoding the human SGLT1 (Hediger *et al.*, 1989) and cDNA encoding part of the pig SGLT1 (Ohta *et al.*, 1990) have been isolated using the rabbit cDNA clone as

a probe. The human SGLT1 is 664 amino acids in length and possesses 85% identity with rabbit SGLT1 and 86% with pig SGLT1. SGLT1 has also been recently cloned from a ruminant species, sheep (Wood *et al.*, 1994). Sequencing of ovine SGLT1 has revealed a protein of 664 amino acids and the sequence exhibits a homology of 85% identity and 93% similarity to the rabbit SGLT1 sequence. SGLT1 is a member of a superfamily of proteins that also includes sodium-dependent amino acid transporters and the bacterial Na^+/proline cotransporter (Hediger *et al.*, 1989).

Computer analysis of the sequence of the rabbit SGLT1 suggests that it has 12 transmembrane domains (Hediger *et al.*, 1987). The protein is glycosylated at an asparagine residue in the small exofacial loop connecting M5 to M6. Radiation inactivation studies indicate that SGLT1 may function *in situ* as a tetramer of four identical monomer subunits (Stevens *et al.*, 1990). SGLT1 cannot mediate the bidirectional transport of glucose.

In rabbit, SGLT1 mRNA is expressed with highest abundance in the small intestine, very low abundance in the colon and undetectable levels in gastric mucosa (Coady *et al.*, 1990). The relative abundance within the small intestine from greatest to least is jejunum, ileum and duodenum. In humans, the abundance of SGLT1 mRNA is developmentally modulated with highest levels in the adult small intestine (Davidson *et al.*, 1992). In ruminants, most dietary carbohydrate is fermented to volatile fatty acids (mainly acetate, propionate, and butyrate) in the rumen. Thus, the amount of non-structural carbohydrate entering the duodenum is relatively low. SGLT1 is present on the luminal membrane of the intestine of pre-ruminant lambs, but both SGLT1 function and the levels of SGLT1 protein decline in the apical plasma membrane of enterocytes of ruminant sheep. There is also a

significant decline in the SGLT1 mRNA (Shirazi-Beechey *et al.*, 1991, Lescale-Matys *et al.*, 1993). Delaying rumen development by extending the normal weaning period can prevent the loss of SGLT1 protein and function. Introduction of various sugars into the duodenal lumen of sheep stimulates the expression of functional SGLT1 in the brush-border membrane (Shirazi-Beechey *et al.*, 1991; Lescale-Matys *et al.*, 1993). Infusion of glucose into the distal intestinal lumen of sheep can induce the expression of SGLT1 in the distal intestinal brush border membrane, but not in the proximal intestinal brush border membrane (Shirazi-Beechey *et al.*, 1994). Intravenous infusion of glucose in sheep did not induce SGLT1 expression, although an increased abundance of GLUT2 was shown in the basolateral membrane of enterocytes (Shirazi-Beechey *et al.*, 1994).

SGLT1 is also expressed in the renal cortex and is responsible, at least in part, for reabsorption of filtered glucose in the kidney (Coady *et al.*, 1990). However, the active transport of glucose in the kidney may involve two proteins. Kinetic and physical studies in the rabbit indicate that the outer medulla contains a high-affinity transporter with a K_m for glucose of 0.35 mM whereas the outer portion of the renal cortex contains a low-affinity transporter with a apparent K_m of 6 mM. The vesicles prepared from whole cortex contains about 80% low-affinity and 20% high-affinity Na^+/glucose cotransporters (Turner and Moran, 1982). Sequencing of a partial cDNA clone that likely represents the cortical (i.e., high-affinity) renal SGLT isoform revealed that there was 100% identity between the amino acid sequences of the rabbit renal and small intestinal cDNA clones (Coady *et al.*, 1990). Immunogold studies in the rat showed that SGLT1 was localized in the microvillous plasma membrane in the apical brush borders of the cells of all three proximal tubule segments

(Takata *et al.*, 1991). Thus, SGLT1 represents the major Na^+/glucose cotransporters in the kidney and the low-affinity renal SGLT isoform remains to be identified.

3.2 The Facilitative Glucose Transporter Family

Except for the active uptake of glucose from the lumen of the small intestine and proximal tubule of the kidney, the transport of glucose across the cell membrane in most tissues occurs by facilitated diffusion. Facilitative transport of glucose can be specifically inhibited by the fungal toxin cytochalasin B, which provides a means of distinguishing facilitative from sodium-dependent glucose transport. Recent cDNA cloning studies have shown that the facilitated diffusion is mediated by another family of structurally related proteins which show little homology to SGLT1 (Gould and Holman, 1993). These facilitative glucose transporters are expressed in a tissue-specific manner and mediate a saturable, stereoselective, bidirectional, and energy-independent process of glucose transport. So far, six functional facilitative glucose transporter isoforms have been cloned and characterized (Mueckler *et al.*, 1985; Fukumoto *et al.*, 1988, 1989; Kayano *et al.*, 1988, 1990; James *et al.*, 1989; Waddell *et al.*, 1992). These six transporters have been designated GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, and GLUT7, respectively based on their chronological orders of publications of the cDNA sequences. Based on their major sites of expression, these transporters have also been designated in some publications as erythrocyte-type, liver-type, brain-type, muscle/fat-type, small intestine-type, and hepatic microsomal isoform, respectively. In addition, a facilitative glucose transporter pseudogene (GLUT6) has been identified in human tissues (Kayano *et al.*, 1990).

3.2.1 GLUT1/Erythrocyte-type isoform

Glucose transporters comprise about 3-5% of the membrane protein in erythrocytes. In the early 1980s, Baldwin *et al.* (1982) isolated the glucose transporter from erythrocytes and identified it as a 55-kDa glycoprotein. After removing N-linked oligosaccharides by N-glycanase, the 55-kDa glycoprotein can be resolved to a single 46-kDa polypeptide (reviewed by Wheeler and Henkle, 1985). Using antibodies raised against the purified erythrocyte glucose transporter, Mueckler *et al.* (1985) first isolated clones encoding the GLUT1 isoform from hepG2 hepatoblatoma cell line. GLUT1 is 492 amino acids in length and its amino acid sequence deduced from the cDNA clone is in good agreement with that obtained from partial sequence data of the purified erythrocyte glucose transporter (Baldwin *et al.*,1982). Subsequently, GLUT1 cDNAs were isolated from rat, rabbit, mouse, pig, and bovine tissues (reviewed by Burant *et al.*, 1991; Boado and Pardridge, 1990). The amino acid sequence of GLUT1 is highly conserved and there is more than 97% identity between the sequences of human and rat, rabbit, mouse, pig, and bovine GLUT1.

Based on the analysis of hydrophobicity plots and secondary-structure determinations, Mueckler *et al.* (1985) have constructed a model for the topology of GLUT1 in the plasma membrane. In this model, GLUT1 spans the plasma membrane 12 times with the N and C termini located on the cytoplasmic side. There is a large and highly hydrophilic intracelluar segment of 65 amino acids and a smaller 33-amino-acid extracellular segment (between M1 and M2) which contains a single potential N-linked glycosylation site at Asn-45. Several topological features of the model have been confirmed by vectorial proteolytic digestion and sequence-specific antibody studies (reviewed by Gould and Holman. 1993). In its native state,

GLUT1 may exist in the plasma membrane as a homotetramer (Carruthers, 1990).

GLUT1 is the only facilitative glucose transporter isoform which has been isolated from bovine tissue. Although phylogenetic analysis of the GLUT1 cDNA sequences in the bovine and human revealed that there is 97.0 and 89.3% identity in the amino acid and cDNA sequences, respectively, a unique Pro^{55}-Ile^{56}-Pro^{57}-Pro^{58} sequence is found in bovine GLUT1 (Boado and Pardridge, 1990). The proline-rich region may confer a different conformation in the N-terminus of the bovine transporter.

Although GLUT1 mediates a bidirectional process, the kinetic property of this transporter is asymmetrical (Diamond and Carruthers, 1993). GLUT1 has a net influx K_m for glucose of 1.6 mM which is significantly lower than either its net efflux K_m value or equilibrium exchange K_m value of approximately 20 mM. The asymmetry would allow this transporter to transport glucose into cells more effectively and unidirectionally under the conditions where extracelluar glucose is relatively high and intracellular glucose is low. The kinetic asymmetrical property of GLUT1 appears to be inhibited by intracellular ATP and is regulated by binding of some intracellular metabolites (Diamond and Carruthers, 1993).

The GLUT1 transporter has been detected in brain, fetal tissue, placenta, heart, kidney, fibroblast, adipose tissue, mammary gland, and every transformed cell line tested (Burant *et al.*, 1991; Madon *et al.* 1990). In many tissues in which it is expressed, GLUT1 is concentrated in cells of blood-tissue barriers (Cornford *et al.*, 1994). Because of its ubiquitous distribution and its cellular localization, GLUT1 is thought to be the primary transporter responsible for basal glucose uptake.

3.2.2 GLUT2/Liver-type isoform

Biochemical studies revealed that the hepatocyte glucose transporter had ~10-fold higher K_m for glucose and a 10-fold lower affinity for cytochalasin B compared with the erythrocyte glucose transporter (Axelrod and Pilch, 1983), which suggests that the glucose transporter in liver is distinct from the erythrocyte-type isoform described in the previous section. Using low-stringency hybridization screening of human liver cDNA library with human GLUT1 cDNA as a probe, Fukumoto *et al.* (1988) isolated the cDNA of GLUT2, a novel facilitative glucose transporter. Human GLUT2 is 524 amino acids in length and has 55% amino acid identity with GLUT1. Computer analysis of the predicted primary structure of GLUT2 indicated that its overall topological structure in the plasma membrane was very similar to that of GLUT1. There is a potential site for N-linked glycosylation in GLUT2. In addition to human GLUT2, GLUT2 cDNA clones have also been isolated from the rat and mouse (Burant *et al.*, 1991). The sequence of GLUT2 is not as highly conserved as GLUT1 with only 81% amino acid sequence identity between human GLUT2 and both rat and mouse GLUT2.

Analysis of mRNA and protein distribution indicates that GLUT2 is preferentially expressed in the liver, small intestine, kidney, and insulin-secreting β-cells of the pancreas (Fukumoto *et al.*, 1988; Thorens *et al.*, 1988). However, Western blotting studies have shown different sizes of GLUT2 protein expressed in these tissues which may be due to the differences in glycosylation (Thorens *et al.*, 1988, 1990a). Immunohistochemical studies have shown that GLUT2 is localized to the basolateral surface of the kidney and intestinal epithelium and the sinusoidal membrane of hepatocytes (Thorens *et al.*, 1990a,c). The tissue

distribution and subcellular localization of GLUT2 suggest that it is involved in the uptake and release of glucose in liver, in the release of absorbed and reabsorbed glucose in the small intestine and kidney, respectively, and in the regulation of insulin secretion from β-cells.

Characterization of the kinetics of GLUT2 protein expressed in *Xenopus* oocytes revealed a K_m for equilibrium exchange of 3-O-MG of 42 mM, considerably higher than the value measured for GLUT1 (Gould *et al.*, 1991). Thus glucose flux through this transporter, at physiological glucose concentrations, would be virtually linear with extracellular/intracellular glucose concentrations and transporter saturation by glucose is not likely to be rate limiting. The observed kinetics are consistent with the proposed physiological function of GLUT2.

GLUT2 has recently been found to be responsible for fructose transport across the intestinal basolateral membrane (BLM) (Cheeseman, 1993). Fructose can completely inhibit the transport of glucose in isolated rat jejunal BLM vesicles. The K_i for fructose inhibition of glucose transport is 16 mM. There is a clear difference in the affinity for fructose when GLUT2 is present in liver and intestine.

3.2.3 GLUT3/Brain-type isoform

The skeletal muscle is responsible for disposal of about 90% of glucose after a meal, however, Northern blotting analysis showed that it contained a very low level of GLUT1 mRNA and no GLUT2 mRNA. Screening of a human fetal skeletal muscle cDNA library using low-stringency-hybridization strategy with a human GLUT1 cDNA probe identified GLUT3, the third member of the facilitative glucose transporter family (Kayano *et al.*, 1988).

GLUT3 is a 496 amino-acid protein having 64% and 52% identity with human GLUT1 and GLUT2, respectively. As with GLUT2, GLUT3 is not as highly conserved as GLUT1 with an 83% identity between the amino acid sequences of human and mouse GLUT3.

Analysis of the equilibrium-exchange K_m of GLUT3 for 3-O-MG, when expressed in *Xenopus* oocytes, revealed that this transporter exhibited a K_m of about 10 mM, considerably less than GLUT1 and GLUT2 (Gould *et al.*, 1991). Although GLUT3 is ubiquitously distributed in human tissues, it is most abundant in adult brain, kidney, and placenta (Yano *et al.*, 1991; Shepherd *et al.*, 1992). However, in rodents GLUT3 seems to be restricted to the brain (Yano *et al.*, 1991). Thus, a major role of GLUT3 is as the brain neuronal glucose transporter. The presence of a high affinity glucose transporter in the brain may be required to efficiently utilize the low blood concentrations of glucose. Surprisingly, although GLUT3 cDNA was isolated from the fetal skeletal muscle cDNA library, GLUT3 is barely detectable in adult skeletal muscle (Shepherd *et al.*, 1992), suggesting that another facilitative glucose transporter may be responsible for glucose uptake by this tissue.

3.2.4 GLUT4/Muscle/Fat-type isoform

Insulin causes an approximately 20-30-fold increase in the rate of glucose transport across the plasma membrane of adipocytes (Simpson and Cushman, 1986). However, upon insulin challenge, the amount of GLUT1 protein at the plasma membrane increases only about threefold in adipocytes, which is insufficient to account for the full stimulation of transport by insulin (Calderhead and Lienhard, 1988). Simultaneously, four independent groups identified the cDNA clones encoding the insulin-responsive facilitative glucose transporter,

GLUT4, using the same cloning strategy as used in the cloning of GLUT2 and GLUT3 (reviewed by Burant *et al.*, 1991). Human GLUT4 is a 509-amino-acid protein and has 65%, 54%, and 58% identity with human GLUT1, GLUT2, and GLUT3, respectively. The sequence of GLUT4 is as highly conserved as GLUT1.

GLUT4 exhibits a K_m for 3-O-MG exchange transport of 1.8 mM (Gould and Holman, 1993). The low K_m value suggests that this transporter may operate close to its V_{max} at the normal physiological range of blood glucose concentrations. The increase in GLUT4 protein abundance in the plasma membrane should proportionally increase cell uptake of glucose. GLUT4 has kinetically symmetrical affinities for 3-O-MG influx and efflux (Gould and Holman, 1993).

GLUT4 transporter is expressed only in tissues where glucose uptake is insulin-regulative (muscle and fat) and it is predominantly located in the intracellular compartment in the basal state (James *et al.*, 1989; Slot *et al.*, 1991). In rat adipocytes, GLUT4 accounts for ~90% of the total glucose transporter detected. Insulin promotes the translocation of GLUT4 transporter from the intracellular location to the plasma membrane (Slot *et al.*, 1991). Thus GLUT4 mediates insulin-stimulated glucose uptake.

Tissue distribution of GLUT4 has been examined in Holstein cattle using rabbit antiserum raised against a synthesized peptide corresponding to the C-terminal region of rat GLUT4 (Abe *et al.*, 1994). The pattern is similar to that in rodent animals, but the contents of GLUT4 in ruminant tissues are much lower compared with nonruminant tissues. GLUT4 content in the skeletal muscle and adipose tissue of cattle is 26-35% and 14-22%, respectively, of that observed in the rat. The low levels of GLUT4 in ruminant tissues is

consistent with the low basal rates of glucose transport observed in these tissues (Sasaki, 1994). In addition, insulin-induced translocation of GLUT4 in sheep skeletal muscle is lower compared to that observed in rats, which may explain why the maximal insulin-stimulated transport of 3-O-MG in ovine skeletal muscle is less than 50% of that in rats (Sasaki, 1994).

3.2.5 GLUT5/Small intestine-type isoform

Bell and his colleagues isolated the cDNA of another glucose transporter (GLUT5) from a human jejunal cDNA library by low-stringency hybridization (Kayano *et al.*, 1990). This 501-residue protein exhibits only 42%, 40%, 39%, and 42% sequence identity to human GLUT1, 2, 3, and 4, respectively. In the human, it is expressed at highest levels in the small intestine and spermatozoa and at much lower levels in kidney, skeletal muscle, and adipose tissue. In the small intestine, GLUT5 is localized to the luminal surface of mature absorptive epithelial cells (Davidson *et al.*, 1992). Thus, it has been suggested that GLUT5 participates in the uptake of dietary glucose from the lumen of the small intestine. As the absorption of glucose in the small intestine is a process of active transport and mediated by the sodium-dependent glucose transporter, it is difficult to explain how GLUT5 is involved in this process. However, GLUT5 has been found to also function as a fructose transporter, with a high affinity for fructose (K_m=6 mM) and a poor ability to transport glucose (Burant *et al.*, 1992). Thus, its primary role on the luminal surface of the small intestine would be to facilitate the transport of dietary fructose. The presence of GLUT5 on the mature spermatozoa is also consistent with the idea of GLUT5 being a fructose transporter, because human spermatozoa mainly use fructose in seminal fluid.

Rat GLUT5 cDNA has recently been isolated by Rand and his coworkers (1993). It shares an identity of 81.5% in amino acid sequence with human GLUT5. The tissue distribution of rat GLUT5 is different from human GLUT5 in that rat GLUT5 is not detectable in rat adipose, skeletal muscle, and testicular tissues. In addition, rat GLUT5 can transport glucose as well as fructose when expressed in *Xenopus* oocytes.

3.2.6 GLUT7/Hepatic microsomal isoform

The most recent member of the facilitative glucose transporter family to be identified is GLUT7 (Waddell *et al.*, 1992). cDNA clones encoding GLUT7 were isolated by screening a rat liver cDNA library using the antibodies raised against a 52 kDa rat liver microsomal glucose-transport protein. Sequence analysis revealed that GLUT7 clone is 2161 bp in length and encodes a 528 amino-acid protein which exhibits a close relation to GLUT2, there being 68% identity at the amino acid level. One important difference between GLUT2 and GLUT7 is the presence of six extra amino acids at the C-terminal end of GLUT7 which contains a consensus motif for retention of membrane-spanning proteins in the endoplasmic reticulum (KKMKND). Waddell *et al.* were able to confirm, by expression in COS 7 cells, that the isolated clone indeed encodes a functional glucose transporter. Interestingly, in the COS 7 cells the expressed GLUT7 protein is only localized in the endoplasmic reticulum and nuclear membrane, but not in the plasma membrane where five other members of facilitative glucose transporter are localized. Thus, it seems that GLUT7 is an endoplasmic-reticulum protein.

In the liver, glucose is produced by glycogenolysis and gluconeogenesis. The final step of both processes is the removal of phosphate from glucose-6-phosphate in the lumen of the

endoplasmic-reticulum by glucose-6-phosphatase, a multicomponent enzyme. The released glucose must cross the endoplasmic-reticulum membrane before it is released into the blood. It is proposed that GLUT7 is all or part of the T_3 glucose-transport protein of the glucose-6-phosphatase system (Waddell *et al.*, 1992).

3.3 Regulation of Glucose Transporter Gene Expression

3.3.1 *In Vitro*

In many cell lines, the rate of glucose transport is influenced by the stage of growth of these cells and by transformation. As GLUT1 is expressed in nearly all cultured cell lines and is the predominant, if not only, isoform expressed in many cell cultures, the studies of glucose transporter regulation *in vitro* have focused primarily on the expression of GLUT1 (reviewed by Kahn and Flier, 1990). The expression of GLUT1 mRNA and protein is induced by factors that stimulate cellular growth and division, such as growth factors (Hiraki *et al.*, 1988; Rollins *et al.*, 1988; Garcia de Herreros & Birnbaum, 1989), oncogenes, and tumour promoters (Flier *et al.*, 1987). Addition of platelet-derived growth factor (PDGF), fibroblast growth factor, epidermal growth factor, insulin-like growth factor I (IGF-I), insulin, or serum to quiescent cells leads to a rapid and transient increase in GLUT1 expression. However, the response to individual factors is cell-type specific. In the basal state, GH suppresses adipocyte GLUT1 expression at a pretranslational level with no effect on GLUT4 (Ku-Tai *et al.*, 1990). However, acute exposure of adipocytes to GH leads to a rapid translocation of GLUT1 and GLUT4 proteins from a low-density microsomal fraction to the plasma membrane (Tanner *et al.*, 1992). The signalling systems used may involve tyrosine phosphorylation. The gene

expression of glucose transporters can be increased by agents other than insulin that are therapeutic for diabetes, such as sulfonylureas and vanadate. Exposure of cultured cells to dexamethasone decreases GLUT1 mRNA levels. In vitro glucose starvation or high glucose concentrations affect GLUT1 gene expression. Cellular differentiation not only changes the gene expression of the glucose transporter isoform present in the precursor cell, but also induce the expression of the other species which are not present in the precursor cell.

3.3.2 Insulin-sensitive tissue

The major insulin-sensitive tissues in both ruminant and nonruminant animals are adipose tissue, skeletal muscle, and heart. Glucose transport appears to be the rate-limiting step in glucose utilization by these tissues. In these tissues, glucose transport increases rapidly after brief exposure to insulin (Simpson and Cushman, 1986; Weekes, 1991). The major mechanism for this increase is that insulin stimulates the translocation of glucose transporters from an intracellular pool to the plasma membrane (Simpson and Cushman, 1986). GLUT1 and GLUT4 are major glucose transporter isoforms present in muscle and adipose cells (Kahn, 1994). In the basal state, GLUT1 transporters in the plasma membrane are responsible for basal glucose uptake, whereas GLUT4 transporters are associated with low-density microsomes (Kahn *et al.*, 1989). With insulin exposure, GLUT4 content in the plasma membrane of rat adipocytes increases by 15-20-fold, whereas GLUT1 content only increases by 3-5-fold (Gould and Holman, 1993). The insulin-induced redistribution of these transporters to the plasma membrane is also shown in skeletal muscle. As GLUT4 is much more abundant in these tissues than GLUT1, GLUT4 is the major transporter responsible for

insulin regulation. The intrinsic activity of GLUT4 may increase in parallel to translocation, although its contribution to increased transport activity is still uncertain. In addition to its acute effect on the redistribution of glucose transporters, insulin stimulates transporter gene expression after prolonged exposure of insulin-sensitive cells.

In adipose tissue, the levels of GLUT4 mRNA and protein are reduced by as much as 90% during fasting and in diabetes mellitus (see Burant *et al.*, 1991). Refeeding or insulin treatment can restore the mRNA and protein levels. A chronic depletion of GLUT4 is also associated with the insulin resistance in adipocytes in obese human subjects.

In skeletal muscle, fibre types differ in insulin sensitivity. The difference may be dependent on the amount of GLUT4 protein. Kong *et al.* (1994) reported that GLUT4 protein levels varied among fibres by a factor of 20 (slow oxidative > fast oxidative glycolytic > fast glycolytic). In addition, a strong correlation was observed between GLUT4 and hexokinase activity in these fibers. In contrast to adipose tissue, skeletal muscle has increased GLUT1 and GLUT4 levels in fasted animals and both transporters appear to be coordinately regulated (Charron and Kahn, 1990). Acute and chronic exercise training increases skeletal muscle GLUT4 protein levels in the rat and human and induces the translocation of GLUT4 (see Devaskar and Mueckler, 1992). Thyroxine also induces increased GLUT4 expression in skeletal muscle.

Skeletal muscle and adipose tissue play an important role in maintenance of glucose homeostasis in the fed state. Glucose uptake by these tissues is affected by the quantity and composition of the diet. Recent studies show that the diet strongly influences the gene expression and activity of GLUT1 and GLUT4 in both the absence and presence of insulin

and this dietary regulation is tissue specific (Kahn, 1994). High fat feeding results in decreased GLUT4 and GLUT1 protein levels, these alterations being much greater in adipose cells than in skeletal muscle. Thus, dietary effects on glucose transport are due in large part to alterations of GLUT1 and GLUT4 gene expression in adipose tissue and to alterations of functional activities of the transporters in the skeletal muscle. Complete energy restriction results in a profound down regulation of GLUT1 and GLUT4 expression in adipose cells whereas the gene expression of the same transporters increases in the skeletal muscle. The divergent regulation in both tissues may adjust nutrient partitioning when nutrient supply is limiting.

3.3.3 Liver and pancreatic β-cells

The liver and pancreatic β-cells play a central role in the regulation of glucose homeostasis. In nonruminants, the liver can store glucose as glycogen or break down these stores and release glucose into blood. In ruminants, the liver is the major supplier of glucose through gluconeogenesis. The liver is a metabolically unique tissue as the intracellular concentration of glucose in the hepatocyte may exceed that in the circulation. β-cells control the blood glucose level by varying their rates of insulin biosynthesis and secretion. GLUT2 is the predominant glucose transporter isoform expressed in the liver and β-cells. It is responsible for glucose uptake and release in the liver and may function as a component of the glucose-sensing apparatus of the β-cells.

GLUT2 expression is not under insulin regulatory control. The effect of chronic hypoglycaemia and hyperinsulinemia on liver GLUT2 expression is the subject of controversy

(see Burant *et al.*, 1991). GLUT2 expression may be mainly regulated posttranscriptionally in rat liver, as GLUT2 mRNA levels are depressed by fasting and diabetes and are elevated by refeeding with minimal alteration in GLUT2 protein levels (Thorens *et al.*, 1990b). In β-cells, the levels of GLUT2 mRNA are reported to vary directly with blood glucose levels in glucose- or insulin-infused rats (Chen *et al.*, 1990).

3.3.4 Mammary Gland

The study of glucose transporters in the mammary gland has been limited to a few reports in the rat. Burnol *et al.* (1990) and Camps *et al.* (1994) reported that GLUT1 and GLUT4 were present in the rat mammary gland before conception. However, the expression of GLUT4 decreased progressively during pregnancy and became undetectable during lactation, whereas the levels of GLUT1 increased during pregnancy and reached the highest levels in the lactation period. The pattern of GLUT1 and GLUT4 expression during the reproductive cycle reflects differences in the cell composition of the mammary gland. The adipocytes predominate before pregnancy, whereas epithelial cells proliferate and become the predominant cell type during pregnancy and lactation. GLUT4 is expressed in adipocytes but not in epithelial cells in the mammary gland, whereas GLUT1 is expressed in both cell types. Madon *et al.* (1990) used quantitative Western blotting and cytochalasin B binding studies and demonstrated that GLUT1 represented the major glucose transporter species in plasma membranes and about half of the glucose transporters in the Golgi membranes of lactating mammary epithelial cells. The presence of glucose transporter on Golgi vesicle membranes raises new questions on the presence of pores in Golgi vesicle membranes and the role of the

transporters in lactose synthesis. As only about half the sites on Golgi vesicle membranes could be accounted for by GLUT1, it may indicate the existence of a second glucose transporter species in the Golgi fraction. In a study of hormonal regulation of GLUT1 in the rat mammary gland, GLUT1 content in the plasma membranes decreased by 37% when prolactin secretion was suppressed by bromocryptine but did not change significantly when GH was specifically inhibited with the anti-rat GH serum (Fawcett *et al.*, 1991). Prolactin alone could maintain GLUT1 levels, whereas GH could only maintain about half of normal GLUT1 levels. The suppression of both prolactin and GH led to sharply lower GLUT1 levels. Insulin and IGF-I were not involved in the stimulation of GLUT1 expression in the rat mammary gland. GLUT1 protein is overexpressed in human breast cancer which may contribute to the increased glucose uptake by the tumour cells (Brown and Wahl, 1993). Interestingly, GLUT2 protein is not expressed in the rat mammary gland (Burnol *et al.*, 1990; Madon *et al.*, 1990) but is present in the human breast (Brown and Wahl, 1993). Neither GLUT3 nor GLUT5 is expressed in human or rat mammary tissues (Brown and Wahl, 1993; Camps *et al.*, 1994). More recently, Camps *et al.* (1994) showed that GLUT1 expression was acutely regulated in the epithelial cells of the rat mammary gland, probably at the posttranslational level. Abrupt weaning resulted in a 92% decrease in GLUT1 protein with a 30% decrease in GLUT1 mRNA. In addition, no small molecular weight fragments of GLUT1 were detected in the weaned group, which indicated that partial proteolysis was not involved in this process. The fall of GLUT1 protein levels in the plasma membranes was not due to the decreased plasma concentration of prolactin, as bromocryptine treatment could not mimic the effect. Furthermore, GLUT1 protein content in the lactating mammary gland was

not affected by 24-h fasting.

4 Summary

Glucose transport across the plasma membrane of mammary epithelial cells occurs by facilitative diffusion and appears to be a rate-limiting step in milk synthesis. Facilitative glucose transport is mediated by a family of structurally related facilitative glucose transporter proteins encoded by distinct genes. So far, six facilitative glucose transporters have been identified and each transporter has different kinetics and is tissue- and cell-type specific. A few studies have been carried out to examine glucose transporters in the rat mammary gland. In the research reported herein these studies have been extended to the bovine mammary gland.

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CHAPTER III

DISTRIBUTION OF MAMMALIAN FACILITATIVE GLUCOSE TRANSPORTER MESSENGER RNA IN BOVINE TISSUES[1]

INTRODUCTION

Glucose is a major source of energy for mammalian cells as well as a substrate for macromolecules, such as glycoproteins, proteoglycans, glycolipids, and nucleic acids. Glucose is also the sole precursor of lactose, the main carbohydrate in milk. In the lactating ruminant, up to 85% of the total glucose entering the blood is utilised by the mammary gland (Chaiyabutr *et al.*, 1980).

The transport of glucose across cell membranes occurs by the passive process of facilitated diffusion in most tissues. Recent cDNA cloning studies have shown that facilitated glucose diffusion is mediated by a family of structurally related glucose transporter proteins encoded by distinct genes. So far, six different facilitative glucose transporters have been described. Each transporter has different kinetics and is tissue- and cell-type-specific (Pessin and Bell, 1992; Waddell *et al.*, 1992). Except for the most recently cloned rat microsomal glucose transporter (GLUT7) (Waddell *et al.*, 1992), the expression of the other five facilitative glucose transporter genes has been extensively examined in human tissues. GLUT1 and GLUT3 are expressed in many tissues, including the brain, placenta, and kidney (Burant

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et al., 1991). The ubiquitous distribution of GLUT1 and GLUT3 in human tissues suggests that these two transporters may play a function in basal uptake of glucose by cells. GLUT2 is expressed only in the liver, small intestine, kidney, and pancreatic ß-cells (Thorens *et al.*, 1988). The tissue distribution and high K_m of GLUT2 suggest that it mediates the uptake and release of glucose by hepatocytes and release of absorbed glucose across the basolateral surface of epithelial cells of the kidney and small intestine. GLUT4 is the transporter isoform primarily responsible for insulin-stimulated glucose transport and is therefore found mainly in insulin-sensitive tissues, such as fat and muscle (James *et al.*, 1989). GLUT5 is expressed predominantly in the jejunal region of the small intestine (Kayano *et al.*, 1990). Although the role of GLUT5 in glucose transport has not yet been determined, it has recently been reported to also be a fructose transporter (Burant *et al.*, 1992).

The major difference in glucose metabolism between ruminants and nonruminants is that in ruminants the majority of dietary carbohydrate is fermented to volatile fatty acids in the rumen. Thus, gluconeogensis represents a major metabolic activity required to maintain glucose homeostasis in ruminants, and is the primary source of glucose for maintenance and productive processes. Reasoning that the transport of glucose by cells in ruminants and nonruminants is probably accomplished by similar sets of structurally related glucose transporter proteins, Northern blotting analyses have been carried out to evaluate the expression of GLUT1, 2, 3, 4, and 5 in various bovine tissues. The results of this study indicate that several differences exist in the tissue pattern of glucose transporter gene expression between ruminants and nonruminants.

MATERIALS AND METHODS

Chemicals:

Riboprobe labelling kit and pGEM3 plasmid were purchased from Promega (Madison, WI, U.S.A.). [α-^{32}P]CTP was purchased from DuPont-NEN (Boston, MA, U.S.A). RNA ladder and yeast tRNA were purchased from BRL (Gaithersburg, MD, U.S.A.). Salmon testes DNA and dextran sulfate were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Low-fat milk power was purchased from Carnation Ltd. (Toronto, ON, Canada). All other molecular biology grade reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

Animals and Tissues:

The tissues of mammary gland, liver, kidney, duodenal epithelia, skeletal muscle (*Pectoralis profundis*), and omental fat were obtained from two first lactation (160 days postpartum) Holstein cows, *Bos Taurus*, immediately after slaughter, frozen in liquid N_2, and stored at -70°C until used for RNA isolation.

RNA Isolation:

Total RNA was isolated from different tissues by a guanidinium thiocyanate-CsCl procedure (Chirgwin *et al.*, 1979). Polyadenylated RNA was isolated from total RNA using oligodeoxythymidylate cellulose chromatography (Jacobson, 1987).

Northern Blotting:

RNA was electrophoresed on denaturing 1% agarose/0.66 M formaldehyde gels and

transferred to nylon membranes (BIO-RAD, Richmond, CA, U.S.A.) by capillary diffusion. The integrity and relative amounts of RNA were assessed by UV-light visualization of ethidium bromide-stained RNA. Membranes were prehybridized for 2 h at 50°C in a medium containing 60% (v/v) formamide, 1 x SSPE (0.18 M NaCl, 0.01 M sodium phosphate at pH 7.4, 1 mM EDTA), 0.5% (w/v) nonfat dried milk, 10% (w/v) dextran sulfate, 1% (w/v) sodium dodecyl sulfate, 500 µg/ml salmon testes DNA and 200 µg/ml yeast tRNA. Following prehybridization, hybridization was carried out for 16-18 h at 50°C in fresh buffer containing [^{32}P]-labelled antisence RNA probes (10^6 cpm/ml). Membranes were then rinsed briefly in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate at pH 7.0), washed for 15 min at 22°C in 2 x SSC containing 0.1% sodium dodecyl sulfate, washed at 70°C in 0.2 x SSC containing 1% sodium dodecyl sulfate, and rinsed briefly in 0.2 x SSC. Autoradiography was performed at -70°C with an intensifying screen.

RNA Probes:

A radiolabeled GLUT1 antisense riboprobe was generated from Hind III-linearized plasmid DNA [pGEM4Z-hGLUT1-2 (Shows *et al.*, 1987)] using [^{32}P]cytidine triphosphate and SP6 RNA polymerase. A radiolabeled GLUT2 antisense riboprobe was generated from Xba I-linearized plasmid DNA [pGEM4Z-HTL-3 (Fukumoto *et al.*, 1988)] and T7 RNA polymerase. A radiolabeled GLUT3 antisense riboprobe was generated from Xho I-linearized plasmid DNA [pBS-MGT-3 (Kayano *et al.*, 1988)] and T3 RNA polymerase. A radiolabeled GLUT4 antisense riboprobe was generated from Bam HI-linearized plasmid DNA [pGEM4Z-

AMT-7 (Fukumoto *et al.*, 1989)] and T7 RNA polymerase. A GLUT5 cDNA insert from plasmid pUC13-phJHT-5 (Kayano *et al.*, 1990) was subcloned into the Bam HI and Eco RI sites of pGEM3 plasmid and antisense RNA was synthesized using Bam HI-linearized plasmid and T7 RNA polymerase. The five human glucose transporter cDNA plasmids [pGEM4Z-hGLUT1-2], pGEM4Z-HTL-3, pBS-MGT-3, pGEM4Z-AMT-7, pUC13-phJHT-5] were kindly donated by Dr. G. I. Bell (Howard Hughes Medical Institute, University of Chicago). The specific activities of the five antisense riboprobes ranged from 1.1 to 1.4 x 10^9 cpm/μg of RNA.

RESULTS

Northern blotting analysis of GLUT1 mRNA

Northern blotting of total RNA under stringent conditions with the GLUT1 antisence riboprobe yielded a single transcript of 2.8 kb (Fig. III-1). This transcript was detected in blots of total RNA prepared from mammary gland, kidney, duodenum, skeletal muscle, and omental fat. GLUT1 mRNA was most abundant in the mammary gland and kidney and only very faint hybridization signals were observed in duodenum, skeletal muscle, and omental fat. No GLUT1 mRNA was detected in total RNA isolated from liver.

Northern blotting analysis of GLUT2 mRNA

The GLUT2 riboprobe hybridized to transcripts of 6.3, 3.8, 2.2, and 1.6 kb in RNA prepared from liver, kidney, and duodenum (Fig. III-2). However, kidney and duodenum

contained much less GLUT2 mRNA than liver. No GLUT2 hybridization signals were detected in either 10 μg polyadenylated RNA from mammary gland or 30 μg total RNA from skeletal muscle and omental fat.

Northern blotting analysis of GLUT3 mRNA

Even after an extraordinarily long (13d) autoradiographic exposure, only faint hybridization signals of the GLUT3 probe to three transcripts (6.7, 2.8, and 1.8 kb) were detected in polyadenylated RNA isolated from mammary gland and in total RNA from skeletal muscle (Fig. III-3). A single transcript (1.8 kb) was observed in total RNA from duodenum. The approximately 4.8 kb band was consistently observed in all tissues and is considered to be the result of nonspecific interaction of the GLUT3 riboprobe with 28S ribosomal RNA.

Northern blotting analysis of GLUT4 mRNA

The GLUT4 riboprobe hybridized to transcripts of 9.3, 6.0, and 2.8 kb in polyadenylated RNA prepared from mammary gland, and to a single transcript of 2.8 kb in total RNA from kidney, skeletal muscle, and omental fat (Fig. III-4). With the GLUT4 riboprobe an approximately 4.8 kb band was also observed in all tissues and considered to be the result of nonspecific interaction with 28S ribosomal RNA. Skeletal muscle contained the highest abundance of GLUT4 mRNA, whereas omental fat, kidney, and mammary gland contained a barely detectable amount of GLUT4 mRNA.

Northern blotting analysis of GLUT5 mRNA

Two GLUT5 mRNA transcripts of 2.2 and 1.5 kb were present at relatively high abundance in liver and kidney (Fig. III-5). The 2.2 kb transcript was also detected in duodenal tissue, but at much lower relative abundance. A low abundance of GLUT5 mRNA was also observed in polyadenylated RNA isolated from mammary gland; however, the GLUT5 probe hybridized to transcripts of different sizes (3.2, 2.1, and 0.9 kb) in the mammary gland.

DISCUSSION

cDNA cloning studies have shown that the facilitated diffusion of glucose in mammalian tissues is mediated by a family of structurally related glucose transporter proteins. So far, only one facilitative glucose transporter, GLUT1, has been cloned from the bovine (Boado and Pardridge, 1990). Phylogenetic analysis of the GLUT1 cDNA sequences in the bovine and human revealed that there is 97.0 and 89.3% identity in the amino acid and cDNA sequences, respectively (Boado and Pardridge, 1990). To assess the expression of homologous facilitated glucose transporter genes in various bovine tissues, Northern blotting analyses were carried out under high stringency conditions using antisence riboprobes generated from five human glucose transporter cDNA clones.

As in human tissues the presence and extent of expression of glucose transporter mRNA varies markedly depending on the tissue. With the exception of the mammary gland, for which human data have not been reported, the distributions of GLUT1, 2, and 4 mRNA for the bovine tissues examined are similar to that reported for humans (Burant *et al.*, 1991),

On the other hand, the distributions of GLUT3 and 5 mRNA in the bovine differ from those reported for humans (Burant *et al.*, 1991). Hybridization with the human GLUT3 probe to various bovine RNA preparations detected GLUT3 mRNA at a very low level only in the mammary gland, skeletal muscle, and duodenal tissue. This pattern of GLUT3 gene expression differs from the ubiquitous distribution observed in human tissues (Burant *et al.*, 1991). Thus, the GLUT3 gene is either not expressed or expressed at a very low level in the bovine tissues examined. This observation is consistent with that of GLUT3 gene expression in rabbits, rats, and mice. In these species GLUT3 mRNA is very abundant in the brain, but is present at low abundance or undetectable in other tissues (Yano *et al.*, 1991). An alternative explanation is that there is limited cross-hybridization between bovine GLUT3 mRNA and the human GLUT3 probe. In support of this possibility is the observation that the sequence of GLUT3 is not as highly conserved as GLUT1 and GLUT4 (Burant *et al.*, 1991). There is only 83% amino acid sequence identity between human and mouse GLUT3. The pattern of expression of the GLUT5 gene in the bovine also differs from that observed in human tissues, where it is expressed predominantly in the jejunal region of the small intestine (Burant *et al.*, 1991). In the bovine the highest abundance of GLUT5 mRNA was observed in the liver and kidney.

In ruminants, most dietary carbohydrate is fermented to volatile fatty acids (mainly acetate, propionate, and butyrate) in the rumen. Thus the amount of soluble carbohydrate entering the duodenum is generally low (Sutton, 1985; Owens *et al.*, 1986). It has also been suggested that the capacity for digestion of raw starch in the small intestine of ruminants is

limited (Ørskov, 1986). Thus, the uptake of glucose from the small intestine of ruminants is generally low compared to nonruminants (Gross *et al.*, 1988). Furthermore, there is extensive metabolism of glucose in the epithelial cells of the bovine small intestine (Okine *et al.*, 1994). Consistent with these facts, the present studies show that the bovine duodenum does not contain appreciable amounts of the mRNA of any glucose transporter examined, including GLUT2 which functions in the release of absorbed glucose across the basolateral surface of epithelial cells of the small intestine in nonruminants (Burant *et al.*, 1991). Although the initial entry of glucose across the brush-border membrane of the small intestine is mediated by a sodium-dependent glucose transporter, the exit of glucose across the basolateral membrane is a process of facilitated transport (Cheeseman, 1992). Alternatively, other regions (ie. jejunum and ileum) of the bovine gastrointestinal tract may be more important sites of glucose absorption.

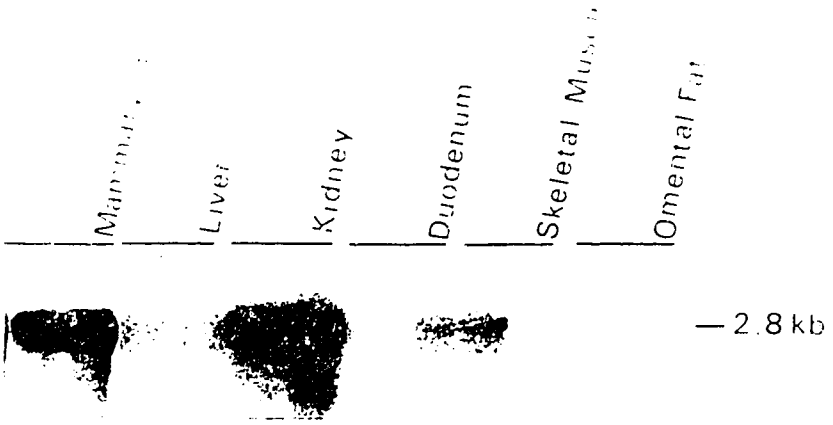
In ruminants, glucose is derived primarily from gluconeogensis in the liver. In human, rat, and mouse liver it has been demonstrated that GLUT2 is the primary transporter mediating glucose uptake and release by hepatocytes. This study did not reveal a high abundance of GLUT2 mRNA in the bovine liver. This may be due to limited cross-hybridization between the bovine GLUT2 mRNA and the human GLUT2 probe. In support of this possibility is the observation that, as with GLUT3, the sequence of GLUT2 is not as highly conserved as GLUT1 and GLUT4 (Burant *et al.*, 1991). There is only 81% amino acid sequence identity between human GLUT2 and both rat and mouse GLUT2. However, a high abundance of GLUT5 mRNA was observed, implying that in the bovine liver GLUT5 may be actively involved in the uptake and release of glucose.

Analysis of bovine kidney RNA revealed not only a high level of GLUT1 mRNA, but also a high level of GLUT5 mRNA. In human, GLUT5 is expressed predominantly in the jejunal region of the small intestine (Burant *et al.*, 1991), with a localization to the luminal surface of mature absorptive epithelial cells (Davidson *et al.*, 1992). Thus, it has been suggested that GLUT5 participates in the uptake of dietary glucose from the lumen of the small intestine. In the bovine kidney, GLUT5 may play a similar function in the reabsorption of glucose.

As in nonruminants, insulin is the major hormone in ruminants controlling glucose utilization by extrahepatic tissues, such as skeletal muscle and adipose tissue (Weeks, 1991). As observed in other species, the present study demonstrated that in the bovine these tissues have the highest abundance of GLUT4 mRNA, which is considered to be the insulin-regulatable glucose transporter.

The mammary gland is the major glucose utilizing tissue in the lactating ruminant (Chaiyabutr *et al.*, 1980). The results of the present study suggest that in the lactating bovine mammary gland GLUT1 represents the major glucose transporter isoform of the five glucose transporters examined. This finding is consistent with previous studies on lactating rat mammary gland by quantitative Western blotting and cytochalasin B-binding, which revealed that GLUT1 constitutes the major glucose transporter species in the plasma membranes of mammary epithelial cells (Madon *et al.*, 1990). The K_m of human GLUT1 has been estimated to be about 1-3 mM (Elsas and Longo, 1992). If bovine GLUT1 has a similar K_m value for glucose, then physiological glucose concentrations (3.0 to 3.5 mM) (Faulkner *et al.*, 1981)

would not be limiting for glucose utilization in the mammary gland of the lactating cow. By showing that the GLUT4 gene is expressed at only a very low level in lactating bovine mammary tissue, the results of our study support the theory that during lactation mammary glucose transport is not regulated by insulin (Laarveld *et al.*, 1981).



GLUT 1

Fig. III-1. Northern blotting analysis of the tissue distribution of GLUT1 mRNA in two lactating cows. The size of the hybridizing transcript is indicated. The amount of RNA loaded in each lane was 30 μg total RNA, except in the lanes of mammary gland (20 μg total RNA). The autoradiogram was exposed for 49 h.

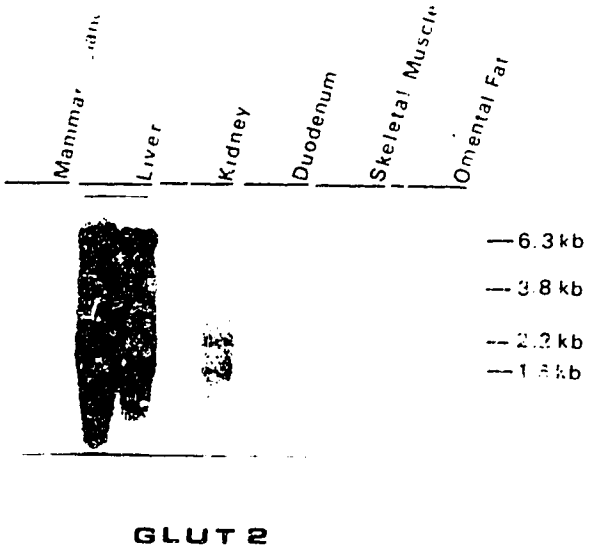


Fig. III-2. Northern blotting analysis of the tissue distribution of GLUT2 mRNA in two lactating cows. The sizes of the hybridizing transcripts are indicated. The amount of RNA in each lane was 30 µg total RNA, except in the lanes of mammary gland, which were 10 µg polyadenylated RNA. The autoradiogram was exposed for 13 d.

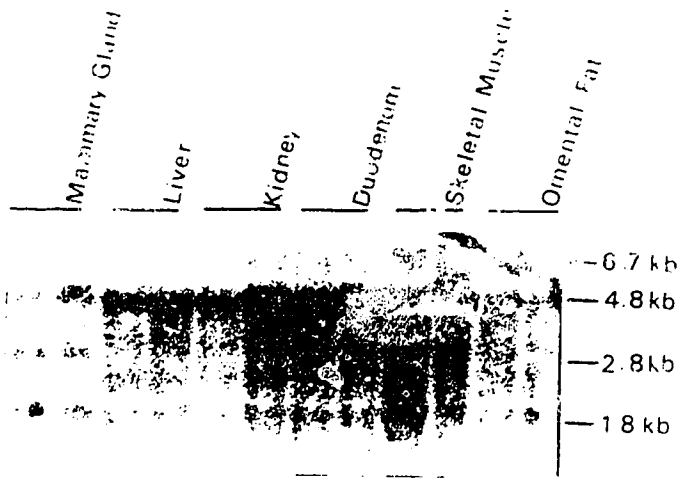


Fig. III-3. Northern blotting analysis of the tissue distribution of GLUT3 mRNA in two lactating cows. The sizes of the hybridizing transcripts are indicated. The amount of RNA in each lane was 30 µg total RNA, except in the lanes of mammary gland, which were 10 µg polyadenylated RNA. The autoradiogram was exposed for 13 d.

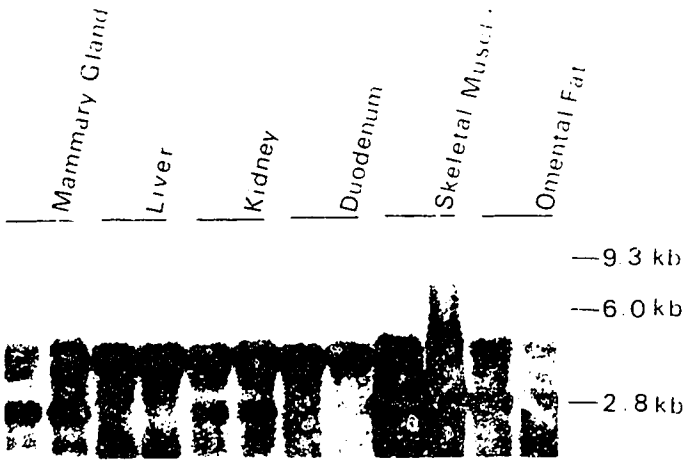
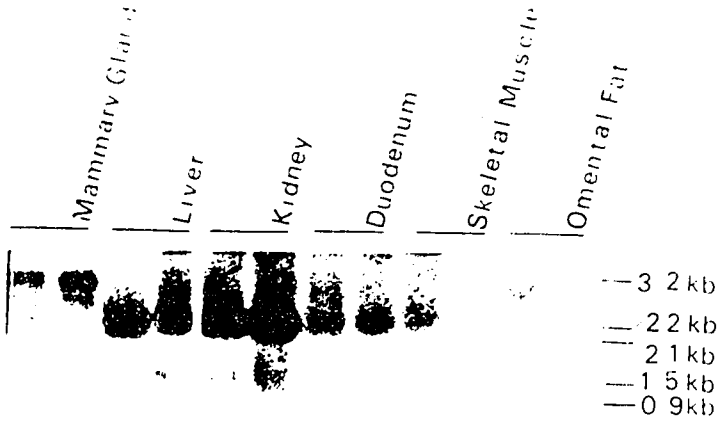


Fig. III-4. Northern blotting analysis of the tissue distribution of GLUT4 mRNA in two lactating cows. The sizes of the hybridizing transcripts are indicated except 4.8 kb transcripts shown in all tissues. The amount of RNA in each lane was 30 μg total RNA, except in the lanes of mammary gland, which were 10 μg polyadenylated RNA. The autoradiogram was exposed for 66.5 h.



GLUT 5

Fig. III-5. Northern blotting analysis of the tissue distribution of GLUT5 mRNA in two lactating cows. The sizes of the hybridizing transcripts are indicated. All lanes contained 30 μg total RNA, except liver and mammary gland, which contained 20 μg total RNA and 10 μg polyadenylated RNA, respectively. The autoradiogram was exposed for 99 h.

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CHAPTER IV

IDENTIFICATION AND GENE EXPRESSION OF GLUCOSE TRANSPORTERS IN BOVINE MAMMARY GLAND

INTRODUCTION

Lactose constitutes about 40% of total solids in bovine milk. As lactose maintains the osmolarity of milk, the rate of lactose synthesis serves as a major control of the volume of milk produced (Neville *et al.*, 1983). Glucose is the main precursor of lactose synthesis in the epithelial cell of the mammary gland, however, the mammary gland cannot synthesize glucose from other precursors due to the lack of glucose 6-phosphatase (Threadgold and Kuhn, 1979). Therefore, the mammary gland is dependent on the blood supply for its glucose needs and as a consequence, mammary glucose uptake is a rate-limiting factor for milk production (Kronfeld, 1982). It has been estimated that in a lactating cow, 72 g of glucose is required to produce 1 kg of milk (Kronfeld, 1982) and mammary uptake can account for as much as 60-85% of the total glucose entering the blood (Chaiyabutr *et al.*, 1980; Annison and Linzell, 1964).

The epithelial cells of the mammary gland take up glucose by a passive process of facilitated diffusion (Delaquis *et al.*, 1993) which is mediated by a family of structurally related glucose transporter proteins (Gould and Holman, 1993). To identify the relevant glucose transporter(s) in bovine mammary gland, Zhao *et al.* (1993) used five human glucose

transporter cDNAs in Northern blotting analysis of bovine tissues and found that only GLUT1 mRNA was present at high levels in the mammary gland of lactating cows. This finding is consistent with previous studies on lactating rat mammary gland which used quantitative Western blotting and cytochalasin B-binding and revealed that GLUT1 constitutes the major glucose transporter species in the plasma membranes of rat mammary gland epithelial cells (Madon *et al.*, 1990).

The objective of this study was to identify the GLUT1 isoform present in bovine mammary gland and investigate its expression during different physiological states at the protein and mRNA level. The GLUT4 was also measured to investigate the possible role of insulin regulation on glucose transport in the mammary gland of the lactating cow.

MATERIALS AND METHODS

Animals and Tissues

Experiment I: Two late lactation and two dry (non-lactating) Holstein cows, *Bos taurus*, were selected for this experiment. Animals were fed a late-lactation total mixed ration *ad libitum* for a minimum of 14 days prior to slaughter. Animals were shipped to a slaughter-house and tissues of mammary gland, skeletal muscle (*Masseter*) and liver were collected immediately post-slaughter, frozen in liquid nitrogen, and stored at -75°C for future analysis. Experiment II: Six primiparous Holstein cows were used in a randomized block design. Within each block two cows were started on the experiment at 118 d and 55 d after parturition, respectively. Cows were fed a total mixed ration *ad libitum*. After 9 weeks two

cows within a block were slaughtered each week. Mammary gland tissues were obtained immediately after slaughter, frozen in liquid nitrogen, and stored at -75°C. Rat brain and leg muscle (*gastrocnemius*) were taken from three adult Sprague-Dawley rats and used as controls.

Preparation of Crude Membrane Fractions

Membrane fractions were prepared by homogenization of the tissues in 10 volumes of ice-cold 0.25 M sucrose, 1 mM EDTA, 10 mM Tris·HCl (pH 7.4), 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, using a Brinkman Polytron homogenizer. Homogenates were centrifuged for 10 min at 4°C at 1000 g (3000 rpm in a Beckman J2-21 centrifuge using a JA-14 rotor), and the supernatants were centrifuged for 45 min at 4°C at 13,000 g (9,500 rpm with the same rotor). The cytosols were then centrifuged for 90 min at 4°C at 100,000 g (37,000 rpm with a Sorvall T647.5 rotor), and the membrane pellets were resuspended in the same buffer as above. Protein concentrations were measured by the Bradford dye-binding assay (Bradford, 1976) using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA, U.S.A.) and BSA as a standard.

Primary Antibodies

The primary antibodies were purchased from East Acres Biologicals (Southbridge, MA. U.S.A.). The anti-GLUT1 was raised against a synthetic peptide corresponding to the 11 amino acids in the carboxyl terminus of the rat brain glucose transporter. The same 11 amino acid peptide sequence is also present in bovine GLUT1 (Boado and Pardridge, 1990).

An anti-GLUT4 was also raised against a synthetic peptide specific to an 11 amino acid sequence of the carboxyl terminus of rat GLUT4. Although GLUT4 has not been cloned from bovine tissues, the same peptide sequence is conserved in the GLUT4 proteins reported for several different species (Burant *et al.*, 1991).

Western Blotting

Membranes were resuspended in Laemmli sample buffer (Laemmli, 1970), and resolved on 10% or 12% (w/v) SDS-polyacrylamide gel using a Bio-Rad Mini-protein II Electrophoresis Cell (Bio Rad, Richmond, CA, U.S.A.). The proteins were electrophoretically transferred to nitrocellulose filters (BA 85, Schleicher and Schuell, Dassel, Germany). Protein markers (Rainbow markers, Amersham, Arlington Heights, IL, U.S.A.) were used as molecular mass standards and also to assess the efficiency of the transfer. The blots were blocked overnight at 4°C in TBS (20 mM Tris [pH 7.4], 137 mM NaCl) containing 5% nonfat dried milk (Carnation Ltd., ON, Canada), and incubated for 1.5 h at room temperature in TBS, 0.5% (w/v) non-fat dry milk, containing a 1:200 dilution of either GLUT1 antiserum or GLUT4 antiserum. The filters were then washed twice at room temperature for 15 min in and incubated for 1 h at room temperature in TBS, 0.5% nonfat dried milk, with a dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Calbiochem, La Jolla, CA, U.S.A.). The immune complex was detected using the Amersham ECL Western blotting system (Amersham, Arlington Heights, IL, U.S.A.) following the manufacturer's instructions. The resulting X-ray film images were quantified by scanning densitometry (Imaging Densitometer GS-670, Bio-Rad, Richmond, CA, U.S.A.).

Light Microscopy immunolabelling:

Tissues were removed from a lactating cow immediately after slaughter. Tissue samples were fixed in 4% paraformaldehyde in PBS for 4 h at 4°C, washed in PBS, and immersed in 0.5 M sucrose in PBS overnight. Tissue blocks were mounted on specimen holders and frozen in liquid nitrogen. Sections (10 μm) were cut and thaw-mounted on the surface of gelatin-coated slides. Immunocytochemical staining was performed according to the peroxidase-antiperoxidase procedure of Sternberger (1986), with 3',3-diaminobenzidine (DAB) as cosubstrate. In brief, the endogenous peroxidase activity of samples was blocked at 22°C for 30 min in 0.25% hydrogen peroxidase in PBS. Tissue sections were then washed and incubated at 22°C for 1 h with a 1:500 dilution [in 1% (w/v) BSA in PBS] either with the anti-GLUT1 antibody or with the anti-GLUT4 antibody. After three washes with PBS, samples were incubated for 1 h at 22°C with a 1:50 dilution (in 1% BSA in PBS) of goat anti-rabbit IgG secondary bridging antibody (Calbiochem, La Jolla, CA, U.S.A.). After several washes with PBS, samples were incubated at 22°C for 1 h with 1:200 dilution (in 1% BSA in PBS) of horseradish peroxidase-anti-peroxidase complex (Calbiochem, La Jolla, CA, U.S.A.). The sections were finally washed three times for 5 min in PBS, developed 2-5 min in DAB (Sigma, St Louis, MO, U.S.A.) substrate solution and rinsed in water. Some of the sections were stained with hematoxylin for 1 min and all sections were mounted in Crystal/Mount (Fisher, Pittsburgh, PA, U.S.A.). Sections were visualized using a Leitz Dialux 20 (Midland, ON, Canada) and photographed with Kodak Ektachrome 400 film.

Northern Blotting

Total RNA was isolated from different tissues by a guanidinium thiocyanate-CsCl procedure (Chirgwin *et al.*, 1979). 30 µg of total RNA were electrophoresed on denaturing 1% (w/v) agarose/0.66 M formaldehyde gels and transferred to nylon membranes (Bio-Rad, Richmond, CA, U.S.A.) by capillary diffusion. The integrity and relative amounts of RNA were assessed by UV-light visualization of ethidium bromide-stained RNA. Membranes were prehybridized for 2 h at 50°C in a medium containing 60% (v/v) formamide, 1 x SSPE (0.18 M NaCl, 0.01 M sodium phosphate at pH 7.4, 1 mM EDTA), 0.5% (w/v) nonfat dried milk, 10% (w/v) dextran sulfate, 1% (w/v) sodium dodecyl sulfate, 500 µg/ml salmon testes DNA and 200 µg/ml yeast tRNA. Following prehybridization, hybridization was carried out for 16-18 h at 50°C in fresh buffer containing [^{32}P]-labelled antisense RNA probes (10^6 cpm/ml). Membranes were then rinsed briefly in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate at pH 7.0), washed for 15 min at 22°C in 2 x SSC containing 0.1% sodium dodecyl sulfate, washed at 70°C in 0.2 x SSC containing 1% sodium dodecyl sulfate, and rinsed briefly in 0.2 x SSC. Autoradiography was performed at -70°C with an intensifying screen and quantified by scanning densitometry (Imaging Densitometer GS-670, Bio-Rad, Richmond, CA, U.S.A.). RNA probes were generated as previously described (Zhao *et al.*, 1993).

Statistical Analysis

The significance of differences between mean values for parameters measured in Experiment II were assessed using ANOVA for paired data.

RESULTS

Characterization of the antibodies and distribution of GLUT1 and GLUT4

Immunoblot analysis using 10% (w/v) polyacrylamide gels showed that the anti-GLUT1 antibody and the anti-GLUT4 antibody detected a 42 kDa protein and a 43 kDa protein, respectively, in both bovine and rat tissues (Fig. IV-1 and Fig. IV-2). These observations are in good agreement with the observed M_r of GLUT1 (Burnol *et al.*, 1990) and GLUT4 (James *et al.*, 1989) and indicate that both primary antibodies used can specifically recognize GLUT1 or GLUT4 in bovine tissues. However, when 12% (w/v) polyacrylamide gels were used and the gels were run for longer times, both the 42 kDa band of GLUT1 and the 43 kDa band of GLUT4 could be resolved as two separate bands: 45 kDa and 42 KDa for GLUT1, 43 kDa and 41 kDa for GLUT4, respectively. The 42 kDa band of GLUT1 is 2.6 fold stronger than the 45 kDa band while both bands of GLUT4 have almost the same intensity (Fig. IV-1 and Fig. IV-2). To date, it is not clear whether these different bands result from differential glycosylation of the proteins or other post-translational protein processing.

The anti-GLUT1 antiserum detected strong bands in rat brain and bovine mammary gland and weak bands in rat skeletal muscle with slightly different sizes (Fig. IV-1). The GLUT1 protein level detected in the crude membrane of lactating bovine mammary gland was 1.5 fold less than in rat brain, unlike the situation in lactating rat mammary gland which expresses more GLUT1 than rat brain (Burnol *et al.*, 1990; Camps *et al.*, 1994). GLUT1 protein was essentially undetectable in the liver, skeletal muscle and omental fat of lactating

cow (Fig. IV-1). The anti-GLUT4 antiserum detected strong bands in both rat and bovine skeletal muscle and a weak band with a slightly different size in bovine omental fat (Fig. IV-2). The GLUT4 level in the crude membrane of bovine skeletal muscle was 1.7 fold less than in rat skeletal muscle and 1.2 fold higher than in bovine omental fat. GLUT4 protein was undetectable in the mammary gland and liver in dry animals (Fig. IV-2) and lactating animals (data not shown). Although the different signal intensity could reflect different affinities of the antisera for these tissues, it is more likely to indicate the relative abundance of glucose transporters in these tissues.

Light microscopy immunohistochemical localization of GLUT1 and GLUT4

Based on the high expression of GLUT1 substantiated in the mammary gland during lactation, the histochemical localization of GLUT1 in bovine mammary gland was studied in tissue sections using an antibody directed against the COOH-terminus of this protein. A strong anti-GLUT1 staining, and no anti-GLUT4 staining, were observed in lactating bovine mammary gland (Fig. IV-3). The strong anti-GLUT1 staining was found mainly in the single layer of epithelial cells of alveoli, the functional unit of the mammary gland. Staining of GLUT1 was hardly seen in connective tissue. In fact, epithelial cells accounted for the majority of cells visualized in the mammary gland sections from the lactating bovine (Fig. IV-3). In histochemical controls, when the primary antibodies were omitted, positive staining was essentially absent in the mammary gland. The anti-GLUT1 staining in bovine liver was only found on the cells surrounding the hepatic vein as reported previously (Tal *et al.*, 1991) and the sinusoidal membrane of hepatocytes.

Expression of GLUT1 mRNA and protein during different lactation stages

In Experiment I, the expression of GLUT1 mRNA and protein was assessed in bovine mammary gland at different stages of lactation. To this end, total RNA and membrane fractions were isolated from lactating and non-lactating bovine mammary gland. Northern blotting analysis showed that the expression of GLUT1 mRNA was similar in mammary gland of late lactation and non-lactating bovine (Fig. IV-4). However, Western blotting analysis demonstrated that anti-GLUT1 detected a smaller molecular weight band (38 kDa) in the mammary gland of dry cows and the protein level was 80% higher than that in the lactating cow. Although differences were detected in the yield of membrane proteins per g tissue in dry (2.6 ± 0.2 mg protein/g tissue) and lactating (3.8 ± 0.8 mg protein/g tissue) animals, the content of GLUT1 protein in the mammary gland of the dry cow, expressed as arbitrary densitometric units per g tissue, was still higher (385) than that in the lactating cow (312).

In Experiment II, the expression of GLUT1 mRNA and protein in bovine mammary gland was examined during different lactation periods. Total RNA and membrane fractions were isolated from bovine mammary gland at 118 d and 181 d post-partum and mRNA and protein levels measured. No difference in the tissue content of GLUT1 mRNA was detected between the two groups (Fig. IV-5). Although the average value of GLUT1 protein level in 118 d post-partum mammary gland was 73% higher than that in 181 d post-partum mammary gland, there was no statistically significant difference ($P = 0.17$) between the two groups due to the large individual difference observed within the 118 d group (Fig. IV-5).

DISCUSSION

Glucose is the major precursor of lactose synthesis. Because lactose draws water osmotically into the milk space, the rate of lactose synthesis is considered to be an important factor in the control of milk yield (Neville *et al.*, 1983). The exposure of Golgi membrane vesicles to varying concentrations of glucose showed that apart from inhibition at high concentration, the rates of lactose synthesis follow classical Michaelis-Menten kinetics with a K_m of 1.5 mM (Kuhn *et al.*, 1980), which exceeds the glucose concentration within the cell. Thus, the intracellular glucose concentration may be a critical factor determining the rate of lactose synthesis and milk secretion. Because a steep concentration gradient of glucose occurs across the plasma membrane, from 3.0 to 3.5 mM in plasma to 0.1 to 0.3 mM in the cell (Faulkner *et al.*, 1981), the transport of glucose across the plasma membrane may be the rate-limiting step. Tracer studies have shown a linear relationship between the rate of glucose transport and milk yield in cows (Kronfeld, 1982). The epithelial cells of the mammary gland take up glucose by a passive process of facilitated diffusion (Delaquis *et al.*, 1993). In the present study, the Western blot and immunostaining data showed that bovine mammary gland expresses a relatively high level of GLUT1. As only GLUT1 mRNA was detected with a high abundance in the mammary gland of lactating cows (Zhao *et al.*, 1993) and GLUT1 constitutes the major glucose transporter species in the plasma membranes of mammary gland epithelial cells of lactating rats (Madon *et al.*, 1990), GLUT1 may also represent the major glucose transporter species in the lactating bovine mammary gland.

The expression of GLUT1 in the epithelial cells of mammary gland is consistent with

the physiological behaviour of glucose metabolism in bovine mammary gland. GLUT1 has a high affinity for glucose and its K_m has been reported to be from 2 to 20 mM (Kahn, 1992). Bovine GLUT1 may have a low K_m value for glucose (1-3 mM) and is probably nearly saturated by physiological plasma concentrations of glucose, since the physiological plasma glucose concentrations seem not to be limiting for glucose utilization in the mammary gland of the lactating cow (Forsberg *et al.*, 1985). Uptake of glucose is not influenced by its arterial concentration of 2.2 to 4.7 mM (Miller *et al.*, 1991). In accordance with this observation, Rook and Hopwood (1970) showed a direct relation between plasma glucose concentration and lactose synthesis at concentrations up to 2.2 mM. However, there is little further increase in lactose synthesis at higher concentrations. A value of 2.2 mM is at the lower limit of the normal range for lactating cows. Kronfeld *et al.* found that the relationship between milk production and plasma glucose concentration was linear below a plasma glucose concentration of about 3.0 mM. Raising the plasma glucose concentration above this did not increase milk production (Kronfeld, 1982).

Interestingly, bovine mammary gland expresses two different species of GLUT1 protein during dry and lactation periods. This result is not consistent with previous studies on the rat mammary gland, which revealed that no additional molecular weight species of GLUT1 protein were expressed in the weaned rat (Camps *et al.*, 1994). As the Northern blot analysis of GLUT1 mRNA in the mammary gland of lactating cow and dry cow showed that a similar size mRNA is expressed in both mammary tissues, the difference in the apparent molecular masses of the transporter protein present in both mammary tissues probably results from posttranslational modifications. It is not known whether differences in the single *N*-

linked oligosaccharide side chain could account for this observation or whether proteolysis is involved. Proteolysis occurring during the homogenization of the tissues was unlikely in the presence of the cocktail of proteinase inhibitors used. However, since the transporter was detected with an antiserum specific for the COOH-terminus, any proteolytic cleavage must be restricted to the NH_2-terminus. Further studies should be carried out using the antibody specific for the NH_2-terminus and assessing the molecular mass of the transporter polypeptide itself by removing quantitatively the single *N*-linked oligosaccharide from the transporter.

The expression of GLUT1 protein in the mammary gland of dry cows was surprisingly higher than in the lactating mammary gland. During the reproductive cycle, the relative proportions of epithelial and adipose cells in the mammary gland change dramatically. During lactation, epithelial cells proliferate and become the predominant cell type, whereas adipocytes are a minor component. In contrast, during the dry period, mammary adipocytes become the predominant cell type and epithelial cells are interdispersed among them. The Western blotting analysis and immunohistochemical staining in this study showed that the epithelial cells express a high level of GLUT1, while the adipocytes hardly express any GLUT1. In rat mammary gland, there is a large reduction in the content of GLUT1 glucose transporter found after weaning (Camps *et al.*, 1994). Explanations for the paradoxical result are unknown. However, previous research indicates that the expression of GLUT1 mRNA and protein is induced by factors that stimulate cellular growth and division, such as growth factors (Hiraki *et al.*, 1988; Rollins *et al.*, 1988; Garcia de Herreros and Birnbaum, 1989), oncogenes, and tumour promoters (Flier *et al.*, 1987). Therefore it is likely that the high expression of GLUT1 in the mammary gland during the dry period could be related to increased cellular

division during the involution of mammary cells. Linked with the observation is the question of the physiological significance of the high expression of GLUT1 protein in the dry bovine mammary gland. It is speculated that the mammary gland has a high demand for glucose to provide energy or NADPH for cell involution after drying-off and perhaps the smaller molecular weight species of GLUT1 expressed during this period may have different kinetic properties. As the expression of GLUT1 mRNA was similar in the mammary gland of late lactation and dry cows, the enhanced GLUT1 expression in bovine mammary gland during the dry period may be due to either translational or posttranslational steps.

Generally, milk production declines from 118 d to 183 d of lactation. GLUT1 protein levels detected in the mammary gland of 118 d lactating cows were not statistically different to those observed for cows at 181 d of lactation. This lack of significance is mainly due to the large individual variation in GLUT1 protein within the 118 d group. Even so, our data show a trend of higher GLUT1 protein in earlier lactation cows. Higher glucose transporter levels in mammary gland is not necessarily a prerequisite for higher milk production. Administration of bovine growth hormone (bGH) or bGH-releasing factor (bGHRF) to lactating cows for 63 days increases milk yield, but does not change GLUT1 protein levels in the mammary gland (Chapter 5). The primary action of bGH and bGHRF in increasing mammary glucose availability may result from increased blood flow rate to the mammary gland (McDowell *et al.* 1987; Davis *et al.*, 1988) and decreased glucose utilization in providing NADPH or α-glycerol-phosphate for triacylglycerol esterification (Bauman *et al.* 1988).

In the present study GLUT4 protein was not detected in lactating bovine mammary gland. GLUT4 is known to be the transporter isoform primarily responsible for insulin-

stimulated glucose transport (James *et al.*, 1989). The result indicates that glucose transport is not regulated by insulin in the mammary gland of the lactating cow. As in nonruminants, insulin is the major hormone controlling glucose utilization by extrahepatic tissues, such as skeletal muscle and adipose tissue in ruminants (Weeks, 1991). However, in the lactating bovine mammary gland there is little evidence to suggest that insulin controls the entry of glucose into the mammary epithelial cells (Hove, 1978a,b; Laarveld *et al.* 1981). The non-insulin-sensitive character of mammary glucose uptake may play an important role in lactation. During fasting, lactation or pregnancy, when plasma insulin concentrations are low (Athanasiou and Phillips, 1978; Lomax *et al.*, 1979) and less glucose is taken up by adipose tissue and muscle (Metz and Van den Bergh, 1977; Pethick and Lindsay, 1982), a greater proportion of glucose is available for the non-insulin-responsive mammary tissues.

Surprisingly, GLUT4 protein was also not detected in the mammary gland of dry cows. During the dry period, involution of mammary secretory cells accelerates when milk removal is stopped, the space previously occupied by the degenerating alveoli is replaced with adipose cells. GLUT4 is a major glucose transporter isoform in adipose tissues. A possible explanation for our results is that GLUT4 content in ruminant adipose tissues is much lower than observed in nonruminant animals. It has been reported that GLUT4 protein content in cattle adipose tissue is only 14-22% of rat (Abe *et al.* 1994). In our Northern blotting (Zhao *et al.*, 1993) and Western blotting analysis, GLUT4 mRNA and protein are only barely detectable in omental fat using 30 μg total RNA and 50 μg crude membrane protein, respectively.

In conclusion, GLUT1 appears to be the major glucose transporter isoform in bovine

mammary gland. GLUT4 protein was not detected in the mammary gland of both lactating and dry cows which would suggest that glucose uptake is not regulated by insulin in the bovine mammary gland during dry and lactation periods.

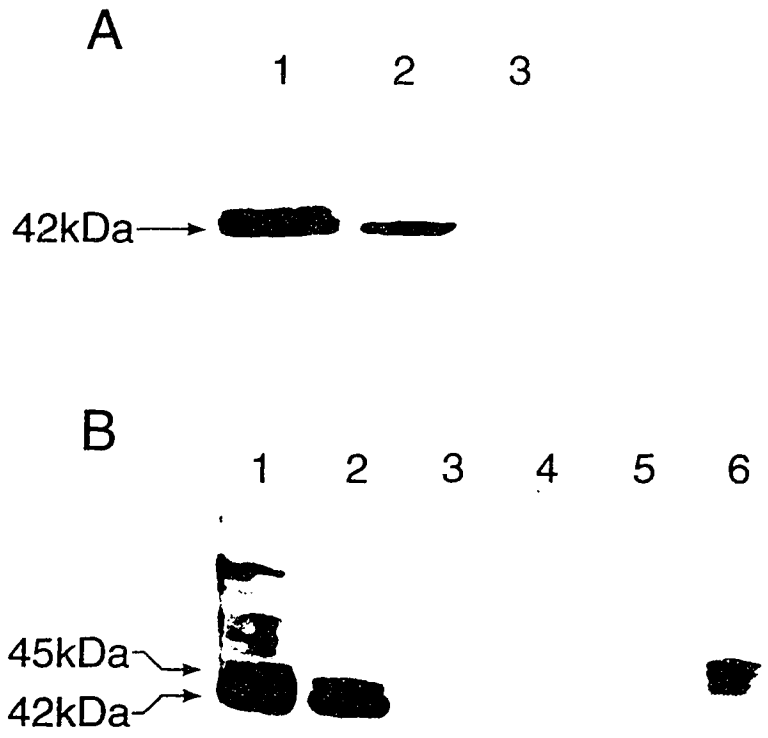


Fig. IV-1. Immunoblotting of rat and bovine tissues using an antibody to GLUT1. A: Membrane proteins (50 μg) of rat brain (1), 118 day lactating bovine mammary gland (2) and liver (3) were resolved on 10% (w/v) SDS-polyacrylamide gel at 150 V for 1 h. B: Membrane proteins (50 μg for bovine tissues and rat skeletal muscle and 25 μg for rat brain) of the mammary gland (2), liver (3), skeletal muscle (*Masseter*, 4), and omental fat (5) from a late lactating bovine, rat brain (1) and skeletal muscle (6) were resolved on 12% (w/v) SDS-polyacrylamide gel at 100 V for 2 h.

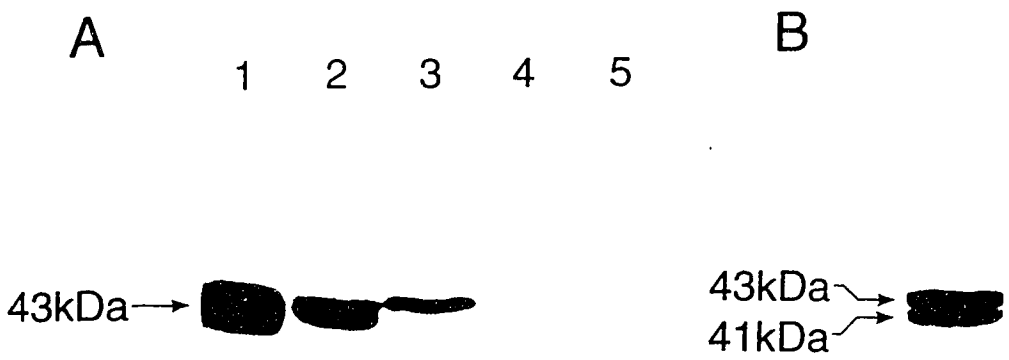
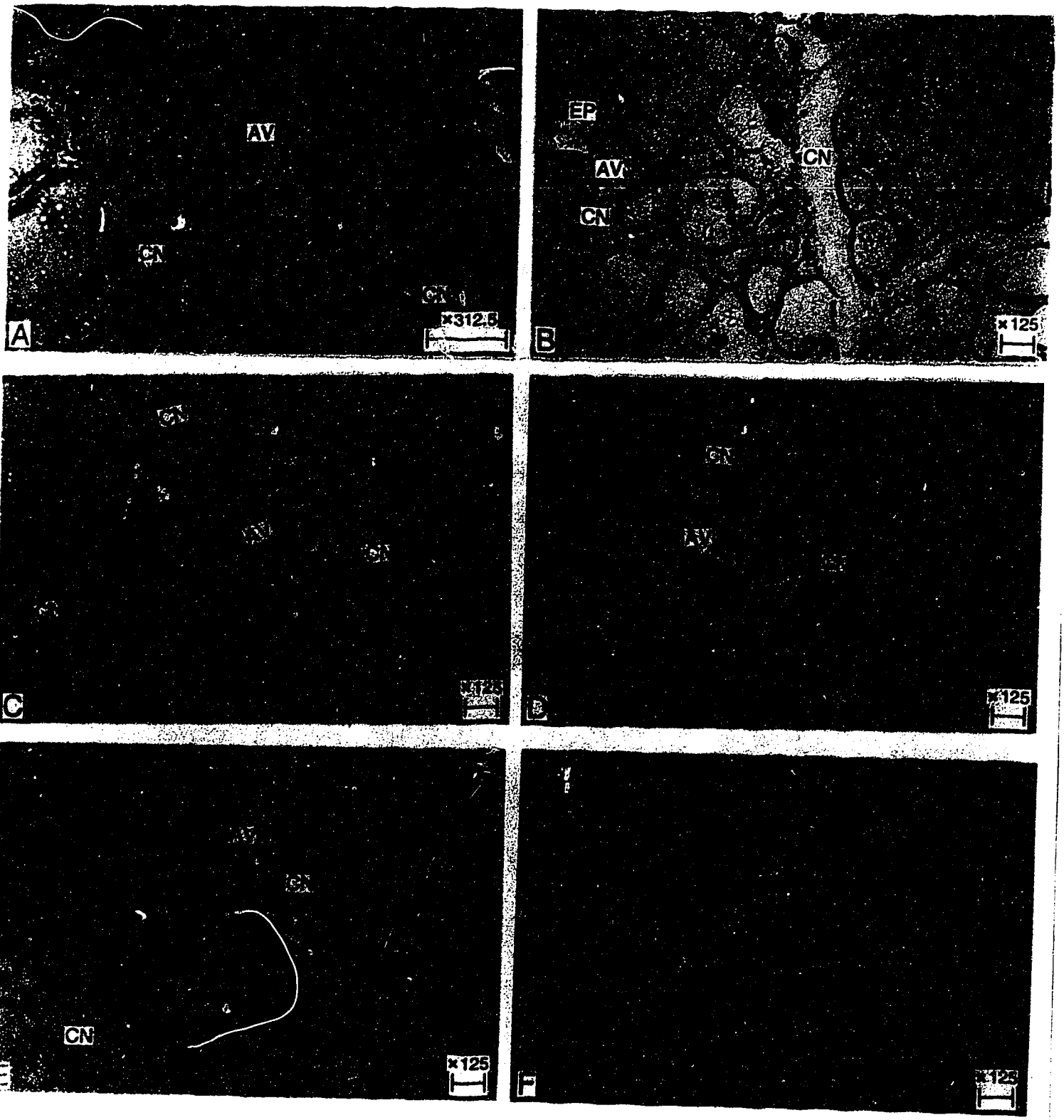


Fig. IV-2. Immunoblotting of rat and bovine tissues using an antibody to GLUT4. A: Membrane proteins (50 μg for bovine tissues and 25 μg for rat skeletal muscle) of the skeletal muscle (*Masseter*, 2), omental fat (3), mammary gland (4), and liver (5) from a non-lactating bovine and rat skeletal muscle (1), were resolved on 10% (w/v) SDS-polyacrylamide gel at 100 V for 2 h. B: Membrane proteins (50 μg) of the skeletal muscle (*Masseter*) from a cow were resolved on 12% (w/v) SDS-polyacrylamide gel at 150 V for 1.5 h.

Fig. IV-3. Light microscopy immunohistochemical localization of GLUT1 and GLUT4 in bovine mammary gland and liver sections. Immunolocolization on cryosections from fixed bovine tissues was performed as described in *Materials and Methods*. (A,C) Photomicrographs of GLUT1-positive staining of the mammary sections from a lactating bovine. The strong anti-GLUT1 staining was found mainly in the single layer of epithelial cells (EP) of alveoli (AV). Positive staining for GLUT1 was hardly seen in connective tissue (CN). (B) Section stained with hematoxylin. (D) Photomicrograph of GLUT4-negative staining of the mammary section from the lactating bovine. (E) A control section from the bovine mammary gland incubated without the first antibodies. (F) A control section from the bovine liver incubated with anti-GLUT1. The anti-GLUT1 staining was only found on the cells surrounding the hepatic vein (V) and the sinusoidal membrane of hepatocytes (S: sinusoid). (A) 312.5x; (B-F) 125x.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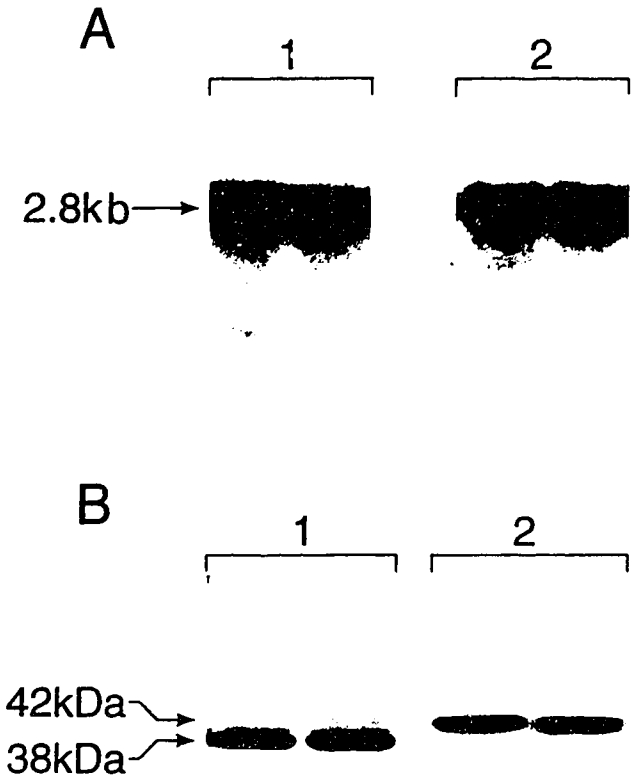

Fig. IV-4. Northern blotting analysis of GLUT1 mRNA (A) and Western blotting analysis of GLUT1 protein (B) in the mammary gland from dry (1) and late lactating (2) cows. 50 μg crude membrane fractions were used in each lane in Western blotting analysis.

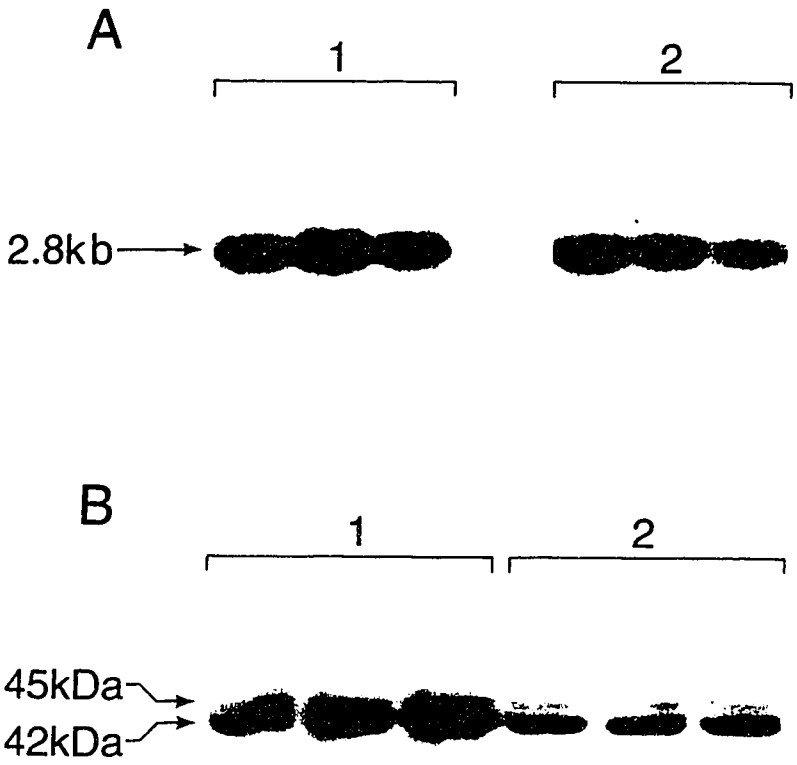

Fig. IV-5. Northern blotting analysis of GLUT1 mRNA (A) and Western blotting analysis of GLUT1 protein (B) in the mammary gland at 118 (1) and 181 (2) days of lactation. 50 μg crude membrane fractions were used in each lane in Western blotting analysis.

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CHAPTER V

REGULATION OF GLUCOSE TRANSPORTER GENE EXPRESSION IN MAMMARY GLAND, MUSCLE AND FAT OF LACTATING COWS BY ADMINISTRATION OF bGH AND bGHRF

INTRODUCTION

Administration of bGH and bGHRF to dairy cows increases milk yield (Dahl *et al.*, 1990; Bauman & Vernon, 1993; Burton *et al.*, 1994; Binelli *et al.*, 1995). The augmented production associated with exogenous bGH and bGHRF administration increases the animal's requirement for milk precursors and energy substrates. However, bGH treatment does not alter the digestibilities of dietary dry matter, energy and nitrogen in lactating ruminants (Eisemann *et al.*, 1986; Tyrrell *et al.*, 1988). Short-term bGH treatment (1-3 weeks) increases milk production without a concomitant increase in feed intake (Bauman and McCutcheon, 1986). Thus, increased milk yield in response to bGH treatment requires repartitioning of nutrients to accommodate the increase in rates of milk synthesis, perhaps at the expense of maintenance and(or) growth of other tissues. For instance, GH decreases glucose uptake in the skeletal muscle and adipose tissues (Davidson, 1987; McDowell *et al.*, 1989), whereas it increases glucose uptake in the mammary gland (Davis *et al.*, 1988a; Fullerton *et al.*, 1989; Mepham *et al.*, 1990).

During lactation, lactose synthesis from glucose in the mammary epithelial cells

appears to be the rate limiting step in milk synthesis (Neville *et al.*, 1983). In many mammalian cells a major point of metabolic regulation of glucose utilization is the transport of glucose across cell membranes which is mediated by a family of tissue-specific facilitative glucose transporters (Kahn, 1992). The mammary epithelial cells have GLUT1 glucose transporter protein present in their plasma membranes (Madon *et al.*, 1990; Zhao *et al.*, 1993; Chapter 4); whereas muscle and fat primarily express GLUT4 (Madon *et al.*, 1990; Zhao *et al.*, 1993).

To address the hypothesis that GH may regulate glucose partitioning by influencing the amount of glucose transporters in various tissues, this study examined the gene expression of glucose transporters in the mammary gland, skeletal muscle and omental fat of lactating cows receiving continuous intravenous infusion of bGH and bGHRF. Data on lactation performance and serum hormone concentrations have been reported by Benelli *et al.* (1995) and Vanderkooi *et al.* (1995).

MATERIALS AND METHODS

Animals and Tissues

Nine primiparous Holstein cows, *Bos taurus*, were used in a completely randomized block design with repeated measurement. Within each block, three cows were randomly assigned to receive continuous intravenous infusions of bGH (29 mg/d), bGHRF (12 mg/d) or no treatment (controls) for 63 days, commencing at day 118 after parturition. Doses selected were based on previous experiments (Dahl *et al.*, 1993), where these same doses

elicited similar increases in serum GH concentrations. Cows were fed a total mixed ration *ad libitum*. On day 63 of treatment, three cows in a block were slaughtered each week. Samples of mammary gland, skeletal muscle (*Pectoralis prefundis*) and omental fat were obtained immediately after slaughter, frozen in liquid nitrogen, and stored at -75°C as described previously (Zhao *et al.*, 1993).

Northern Blotting

Total RNA was isolated from tissue samples by a guanidinium thiocyanate-CsCl procedure (Chirgwin *et al.*, 1979). Total RNA (30 µg) was electrophoresed on denaturing 1% (w/v) agarose/0.66 M formaldehyde gels and transferred to nylon membranes (Bio-Rad, Richmond, CA, U.S.A.) by capillary diffusion. The integrity and relative amounts of RNA were assessed by UV-light visualization of ethidium bromide-stained RNA. Membranes were prehybridized for 2 h at 50°C in a medium containing 60% (v/v) formamide, 1 x SSPE (0.18 M NaCl, 0.01 M sodium phosphate at pH 7.4, 1 mM EDTA), 0.5% (w/v) nonfat dried milk, 10% (w/v) dextran sulfate, 1% (w/v) sodium dodecyl sulfate, 500 µg/ml salmon testes DNA and 200 µg/ml yeast tRNA. Following prehybridization, hybridization was carried out for 16-18 h at 50°C in fresh buffer containing [^{32}P]-labelled RNA probes (10^6 cpm/ml). Membranes were then rinsed briefly in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate at pH 7.0), washed for 15 min at 22°C in 2 x SSC containing 0.1% sodium dodecyl sulfate, washed at 70°C in 0.2 x SSC containing 1% sodium dodecyl sulfate, and rinsed briefly in 0.2 x SSC. Autoradiography was performed at -70°C with an intensifying screen and quantified by scanning densitometry (Imaging Densitometer GS-670, Bio-Rad, Richmond, CA, U.S.A.).

RNA probes were generated as previously described (Zhao *et al.*, 1993).

Preparation of Crude Membrane Fractions

Membrane fractions were prepared by homogenization of the tissue in 10 volumes of ice-cold 0.25 M sucrose, 1 mM EDTA, 10 mM Tris·HCl (pH 7.4), 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, using a Brinkman Polytron homogenizer. Homogenates were centrifuged for 10 min at 1000 g (3000 rpm in a Beckman J2-21 centrifuge with a JA-14 rotor), and the supernatant was centrifuged for 45 min at 13,000 g (9,500 rpm with the same rotor). The cytosol was then centrifuged for 1 h at 100,000 g (37,000 rpm with a Sorvall T647.5 rotor), and the membrane pellet was resuspended in the same buffer as above. Protein concentration was measured by the Bradford dye-binding assay using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA, U.S.A.), using BSA as a standard.

Primary Antibodies

The primary antibody was purchased from East Acres Biologicals (Southbridge, MA, U.S.A.). The anti-GLUT1 was raised against a synthetic peptide corresponding to the 11 amino acids in the carboxyl terminus of the rat brain glucose transporter. The same 11 amino acid peptide sequence is also present in bovine GLUT1 (Boado & Pardridge, 1990). The GLUT1 antiserum can specifically recognize GLUT1 in the bovine tissues (Chapter 4).

Western Blotting

Membranes were resuspended in Laemmli sample buffer (Laemmli, 1970), and resolved on 12% (w/v) SDS-polyacrylamide gel using a Bio-Rad Mini-protein II Electrophoresis Cell (Bio-Rad, Richmond, CA, U.S.A.). The proteins were electrophoretically transferred to nitrocellulose filter (BA 85, Schleicher and Schuell, Dassel, Germany). Protein markers (Rainbow markers; Amersham, Arlington Heights, IL, U.S.A.) were used as molecular mass standards and also to assess the efficiency of the transfer. The blot was blocked overnight at 4°C in TBS (20 mM Tris [pH 7.4], 137 mM NaCl) containing 5% nonfat dried milk (Carnation Ltd., ON), and incubated for 1.5 h at room temperature in TBS, 0.5% non-fat dry milk, containing a 1:200 dilution of GLUT1 antiserum. The filter was then washed twice at room temperature for 15 min in TBS and incubated for 1 h at room temperature in TBS, 0.5% nonfat dried milk, with a 1:2000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Calbiochem, La Jolla, CA, U.S.A.). The immune complex was detected using the Amersham ECL Western blotting system (Amersham, Arlington Heights, IL, U.S.A.) following the manufacturer's instructions. The resulting x-ray film images were quantified by scanning densitometry (Imaging Densitometer GS-670, Bio-Rad, Richmond, CA, U.S.A.).

Statistical Analysis

The significance of differences between mean values for parameters measured were assessed using ANOVA for paired data.

RESULTS AND DISCUSSION

Administration of bGHRF for a 63 d period resulted in a 21% increase in GLUT1 mRNA level ($P < 0.01$) in the mammary gland (Fig. V-1 and Table V-1) as judged by quantitative densitometry. However, GLUT1 protein level in the crude mammary membrane preparation was not affected by bGHRF treatment (Fig. V-2 and Table V-1). Neither GLUT1 mRNA level nor GLUT1 protein concentration was changed by 63 d bGH treatment (Fig. V-1, Fig. V-2, and Table V-1). Administration of bGH and bGHRF increased the milk yield of cows in this study by 17% and 14%, respectively (Binelli *et al.*, 1995) and thus substrate requirements for milk precursors and energy were elevated. Our data indicate that the increased glucose supply to the mammary gland in bGHRF- and bGH-treated cows does not result from changes in glucose transporter level in the mammary cells, although bGHRF increased GLUT1 mRNA level. This is consistent with the observation that bGH does not alter extraction rates of glucose in lactating mammary gland (McDowell *et al.*, 1987; Davis *et al.*, 1988a; Miller *et al.*, 1991).

Glucose is the major precursor of lactose synthesis. Because lactose draws water osmotically into the milk space, the rate of lactose synthesis is considered to be an important factor in the control of milk yield (Neville *et al.*, 1983). The exposure of Golgi membrane vesicles to varying concentrations of glucose showed that apart from inhibition at high concentration, the rates of lactose synthesis follow classical Michaelis-Menten kinetics with a K_m of 1.5 mM (Kuhn *et al.*, 1980), which exceeds the glucose concentration within the cell. Thus, the intracellular glucose concentration may be a critical factor determining the rate of

lactose synthesis and milk secretion. Because a steep concentration gradient of glucose occurs across the plasma membrane, from 3.0 to 3.5 mM in plasma to 0.1 to 0.3 mM in the cell (Faulkner *et al.*, 1981), the transport of glucose across the plasma membrane may be the rate-limiting step. Tracer studies have shown a linear relationship between the rate of glucose transport and milk yield in cows (Kronfeld, 1982). In the lactating mammary gland, the transport of glucose is mainly mediated by the facilitative glucose transporter GLUT1 (Madon *et al.*, 1990; Zhao *et al.*, 1993; Chapter 4). Results of the current study indicate that bGH and bGHRF do not alter GLUT1 level in mammary cells. Since previous data also showed that bGH does not change plasma glucose concentration in lactating cows (Bauman *et al.*, 1988), the primary action of bGH in increasing mammary glucose availability may result from increased blood flow rate to the mammary gland (McDowell *et al.*, 1987; Davis *et al.*, 1988b) and decreased glucose utilization as an energy source or glycerol moiety for triacylglycerol synthesis (Bauman *et al.*, 1988). Mammary blood flow has been correlated to milk yield, 400 to 500 liters of blood passing through the mammary gland for each liter of milk produced in the cow and goat (Linzell, 1974). The K_m of GLUT1 has been reported to range from 2 to 20 mM (Kahn, 1992). If bovine GLUT1 has a similar K_m for glucose, the physiological blood glucose concentration (3.0 to 3.5mM) should not completely saturate GLUT1. Thus, increased blood flow would enhance glucose uptake by GLUT1. The control mechanisms involved in regulating mammary blood flow and milk synthesis in response to bGH treatment are unknown. Direct action of bGH on the bovine mammary gland during lactation has been discounted due to the failure to identify specific GH receptors in mammary tissue (Kazmer *et al.*, 1986; Keys and Djiane, 1988), although GH receptor mRNA is present in mammary

tissue (Glimm *et al.,* 1990). An alternative to direct action of bGH at the mammary gland is an indirect mediation of bGH action by IGF-I. Elevated GH is associated with increased liver secretion of IGF-I (Gluckman *et al.,* 1987). Intra-arterial infusion of IGF-I into the goat mammary gland increased mammary blood flow and milk secretion (Prosser *et al,.* 1990). However, IGF-I cannot replace GH in maintaining GLUT1 level in the lactating rat mammary gland (Fawcett *et al.,* 1992). Thus, GH effects on milk production are not mediated solely through IGF-I.

GLUT1 mRNA level was significantly higher in the mammary gland from the cows treated with bGHRF compared with those treated with bGH ($P < 0.02$) (Fig. V-1 and Table V-1). As both groups showed a similar serum GH concentration (Binelli *et al.,* 1995; Vanderkooi *et al.,* 1995), the observed difference in the effect of bGHRF and bGH on GLUT1 mRNA expression in the mammary gland indicates that the galactopoietic action of bGHRF may not be mediated solely through GH. This view is supported by observations made in several other studies. Dahl *et al.* (1993) reported that bGHRF-treated cows had approximately 10% greater milk yield than bGH treated cows, although bGH and bGHRF resulted in similar concentrations of serum GH and IGF-I. In addition, milk yield declined relatively slowly after withdrawal of bGHRF but rapidly after withdrawal of bGH (Hart *et al.,* 1985; Dahl *et al.,* 1993). GHRF, initially isolated from a pancreatic tumor (Rivier *et al.,* 1982), stimulates insulin secretion from pancreatic islet cells of rats *in vitro* (Green *et al.,* 1990) and can also bind to vasoactive intestinal polypeptide receptors thereby stimulating adenylate cyclase activity in epithelial cells of the intestine (Laburthe *et al.,* 1983). Thus, a direct action of GHRF on the gastrointestinal tract, liver or mammary gland cannot be

discounted.

Administration of bGH decreased GLUT4 mRNA by 44% ($P < 0.03$) in the skeletal muscle (Pectoralis prefundis), but the effect of bGHRF was not significant (Fig. V-3 and Table V-1). GLUT4 mRNA level was barely detectable in the omental fat of control cows, but became undectable in bGH- and bGHRF-treated cows (Fig. V-4). Our results are consistent with observations on the effects of GH on glucose transporter proteins in rat adipocyte plasma membrane (Kilgour *et al.*, 1993) and the effects of porcine GH on GLUT4 mRNA and protein levels in pig adipose tissue and skeletal muscle, in which there were 20 to 65% decreases in mRNA and 30 to 40% decreases in GLUT4 protein (Etherton and Louveau, 1992; Liu *et al.*, 1992). However, *in vitro*, GH treatment decreased the mRNA level and the number of GLUT1 transporter but not GLUT4 in adipocytes (Tai *et al.*, 1990). In skeletal muscle and fat, glucose transport regulates glucose utilization (Crofford and Renold, 1965; Berger *et al.*, 1975). Thus, bGH and bGHRF may decrease glucose utilization in the skeletal muscle and fat by decreasing glucose transporter expression in these tissues. The physiological impact of these effects may be to shift glucose from these tissues towards the mammary gland.

It has long been known that the effects of GH on adipocytes and muscle are acute insulin-like and chronic anti-insulin-like effects (Davidson, 1987). Insulin-like activities occur soon after the tissue is exposed to GH and include increased glucose uptake and utilization as well as antilipolysis. In this case, GH rapidly stimulates translocation of GLUT1 and GLUT4 proteins from a low-density microsomal fraction to the plasma membrane (Tanner *et al.*, 1992). The physiological significance of these insulin-like effects is uncertain. The anti-

insulin-like effects of GH in intact animals can be seen as early as several hours after the first injection as well as after daily injections. The effects include inhibiting glucose uptake and glucose utilization, producing insulin resistance and stimulating lipolysis (Davidson, 1987). Results from the present study indicate that the decreased glucose transporter expression in muscle and fat tissues is involved in the anti-insulin effect of GH. GLUT4 transporter is the predominant form of glucose transporter expressed in skeletal muscle and omental fat (Zhao *et al.*, 1993; Chapter 4). Its expression and translocation are regulated by insulin in intact animals (Kahn, 1992). As the concentration of insulin was not changed in the serum of bGHRF-treated cows and even increased in the serum of bGH-treated cows in the present study (Vanderkooi *et al.*, 1995), GH and GHRF administration obviously not only impairs the ability of insulin to stimulate glucose transporter synthesis, but also suppresses transporter expression. The intracellular GH signalling pathway may impede the insulin signalling pathway(s). It is also possible that GH acts directly, e.g. via GH response elements in the GLUT4 gene. Indeed, a GH response element 5' of the serine protease inhibitor (Spi) 2.1 gene has been identified in rat liver (Yoon *et al.*, 1990).

There is an increasing body of data to support the hypothesis that the galactopoietic response of exogenous GH and GHRF is strongly influenced by their ability to direct nutrient partitioning and(or) utilization in various tissues (Bauman & Vernon, 1993). In adipose tissues, GH decreases glucose uptake, inhibiting lipogenesis and stimulating lipolysis. The combination of decreased lipogenesis and increased lipolysis contributes to decreased body stores and elevated concentrations of NEFA and glycerol in the blood (Bauman *et al.*, 1988; Cisse *et al.*, 1991; Dahl *et al.*, 1993). Peripheral tissues can then use NEFA as an alternative

energy source to glucose (Peel *et al.*, 1982; Tyrrell *et al.*, 1988) and the liver can use glycerol for increased gluconeogenesis (Bauman *et al.*, 1988). GH treatment also decreases glucose uptake in muscle (McDowell *et al.*, 1987). All these GH effects result in more glucose being available to the mammary gland. Therefore, the mammary gland can increase glucose uptake (Davis *et al.*, 1988a; Fullerton *et al.*, 1989; Mepham *et al.*, 1990) and use NEFA as precursors for the synthesis of milk fat (Hart, 1988) which also enables sparing of glucose resulting in greater availability of glucose for lactose synthesis during GH treatment. The results presented here provide cellular evidence for this hypothesis.

In summary, the results of the present study provide insights into the physiological action of exogenous GH and GHRF in lactating cows. Evidence was obtained for effects of GH and GHRF on nutrient repartitioning and(or) utilization in the bovine, the increased glucose supply for mammary metabolism associated with bGH and bGHRF treatments may result from the decreasing glucose transporter expression in muscle and fat tissues.

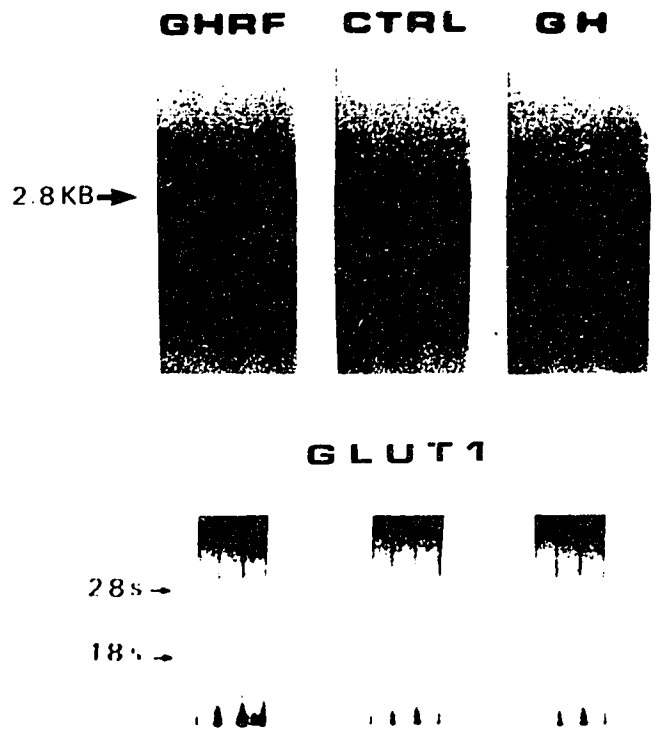

Fig. V-1. Northern blotting analysis of GLUT1 mRNA from the mammary gland of bGH- and bGHRF-treated cows and control cows. *The upper panels* are photographs of autoradiograph produced by hybridizing Northern blotting with a radiolabled GLUT1 riboprobe. *The lower panels* are photographs of the UV light visualization of the gel with ethidium bromide-stained RNA to demonstrate that equivalent amounts of total RNA were loaded in each lane. GH, RNA from bGH-treated cows. CTRL, RNA from control cows. GHRF, RNA from bGHRF-treated cows.

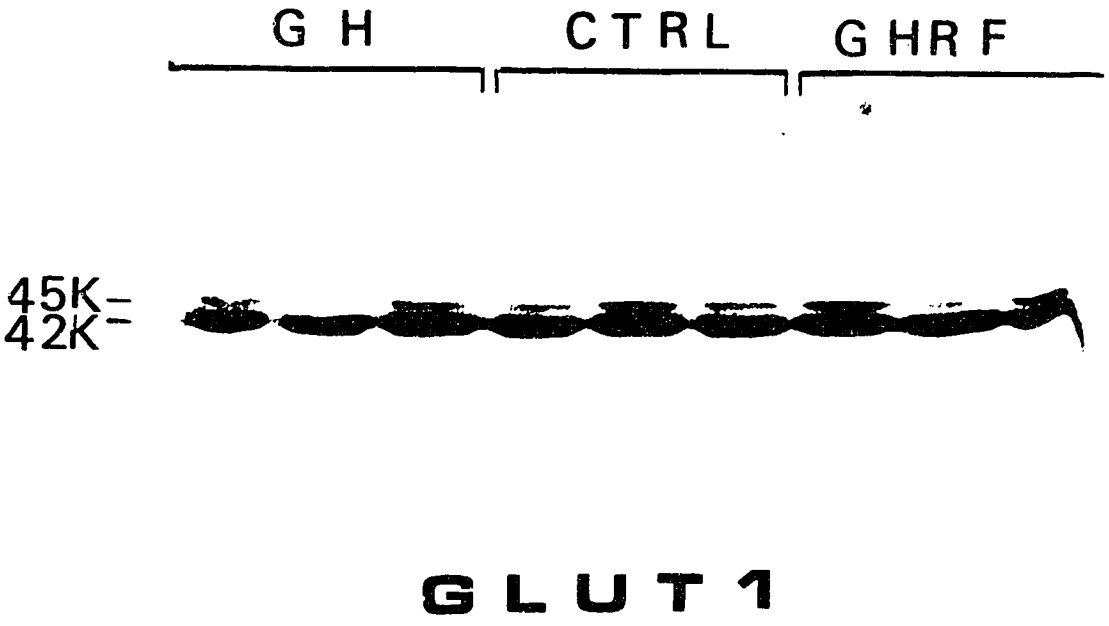

Fig. V-2. Western blotting analysis of GLUT1 protein from the mammary gland of bGH- and bGHRF-treated cows and control cows. GH, membrane protein from bGH-treated cows. CTRL, membrane protein from control cows. GHRF, membrane protein from bGHRF-treated cows.

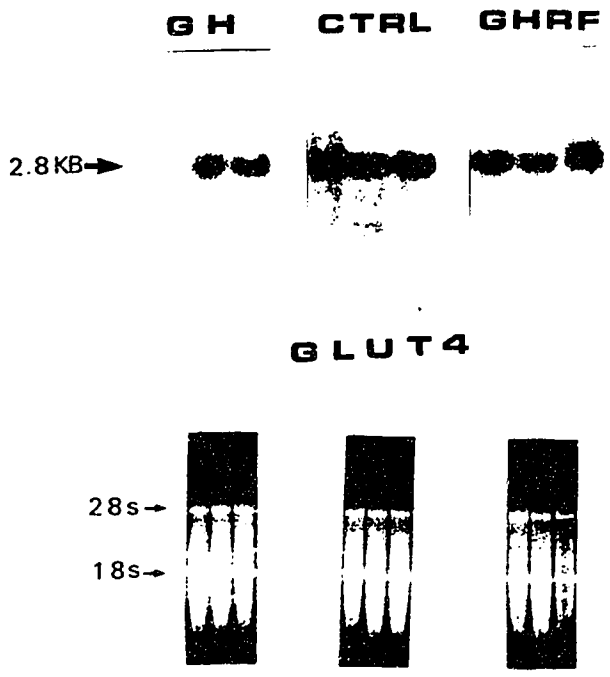

Fig. V-3. Northern blotting analysis of GLUT4 mRNA from the skeletal muscle of bGH- and bGHRF-treated cows and control cows. *The upper panels* are photographs of autoradiograph produced by hybridizing Northern blotting with a radiolabled GLUT4 riboprobe. *The lower panels* are photographs of the UV light visualization of the gel with ethidium bromide-stained RNA to demonstrate that equivalent amounts of total RNA were loaded in each lane. GH, RNA from bGH-treated cows. CTRL, RNA from control cows. GHRF, RNA from bGHRF-treated cows.

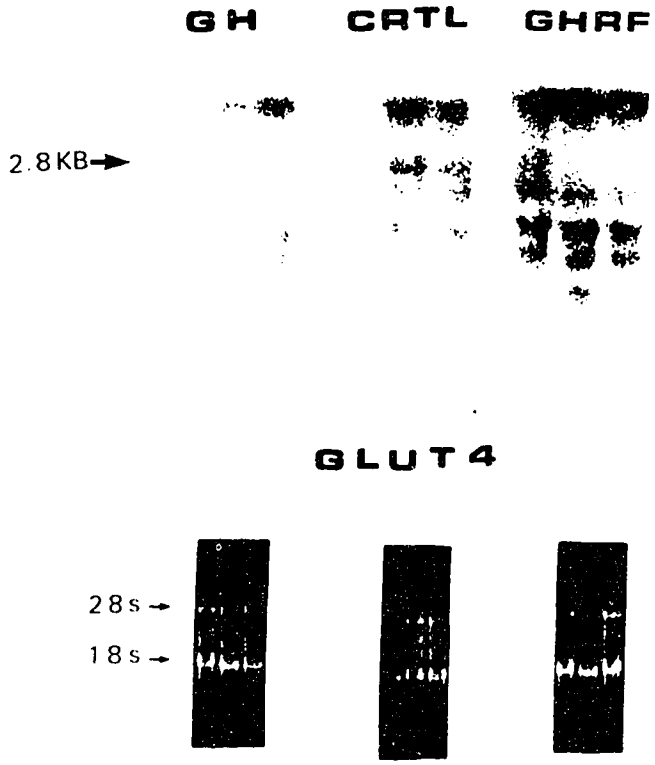

Fig. V-4. Northern blotting analysis of GLUT4 mRNA from the omental fat of bGH- and bGHRF-treated cows and control cows. *The upper panels* are photographs of autoradiograph produced by hybridizing Northern blotting with a radiolabled GLUT4 riboprobe. *The lower panels* are photographs of the UV light visualization of the gel with ethidium bromide-stained RNA to demonstrate that equivalent amounts of total RNA were loaded in each lane. GH, RNA from bGH-treated cows. CTRL, RNA from control cows. GHRF, RNA from bGHRF-treated cows.

Table V-1. Mean abundance (densitometric unit) of GLUT1 mRNA in mammary gland, GLUT1 protein in mammary gland and GLUT4 mRNA in skeletal muscle and fat of cows receiving no treatment (Ctrl), 29 mg bGH/d, or 12 mg bGHRF/d.

					P values		
					GH vs.	GHRF	GH vs.
Abundance	Ctrl	GH	GHRF	SEM	Ctrl	vs. Ctrl	GHRF
GLUT1 mRNA in mammary gland	15.7[a]	16.7[a]	19.0[b]	0.4	.17	.01	.02
GLUT1 protein in mammary gland	8.8[a]	8.0[a]	9.5[a]	1.1	.60	.67	.37
GLUT4 mRNA in skeletal muscle	12.4[b]	7.0[a]	9.7[ab]	1.2	.03	.18	.19

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CHAPTER VI

GENERAL DISCUSSION

Glucose uptake in the mammary gland appears to be the rate-limiting step in milk synthesis. Glucose transport across the plasma membrane of mammary epithelial cells is carried out by a facilitative diffusion process which is specific, saturable, Na^+-independent, and inhibitable by cytochalasin-B or phloretin (Delaquis *et al.*, 1993). Recent cDNA cloning studies demonstrate that the facilitative diffusion of glucose is mediated by a family of the facilitative glucose transporters which are expressed in a tissue- and cell-specific manner. The objective of this research program was to determine the glucose transporters in the bovine mammary gland and investigate their gene expression during different physiological stages and by hormonal regulation.

The first section of this final chapter is to integrate all the results presented in this thesis and to gain further insight into the regulation of glucose uptake in the bovine mammary gland. The second section is devoted to the experimental controls for various techniques used. Based on the results of this thesis, I list a few future directions to extend the study of glucose transporter in bovine mammary gland in the final section.

1 GLUT1: Glucose Transporter in the Bovine Mammary Gland

Several lines of evidence in this thesis demonstrate that GLUT1 is an important glucose transporter isoform in bovine mammary gland. 1) GLUT1 mRNA was expressed at

the highest level in the lactating bovine mammary gland (Chapter 3). 2) Western blotting analysis detected relatively high levels of GLUT1 protein in the mammary gland of cows at different physiological stages as well as those treated with bGH or bGHRF (Chapters 4 and 5). 3) Immunocytochemistry study demonstrated that anti-GLUT1 antibody strongly stained bovine mammary epithelial cells (Chapter 4).

GLUT1 may represent the major glucose transporter in the lactating bovine mammary gland. Of the five facilitative glucose transporters studied, only GLUT1 mRNA was detected at the appreciable level in the bovine mammary gland (chapter 3). Although the cDNAs of GLUT2, 3, 4, and 5 have not been isolated from bovine tissue, it is unlikely that the low or no mRNA expression of these transporters was due to low homologies between the cDNAs of these transporters of bovine and human species as relatively high levels of mRNA of each these transporters were detected in other bovine tissues, except for GLUT3. GLUT3 mRNA is ubiquitously distributed in human tissues but restricted to the brain in rodents (Yano *et al.*, 1991). Thus, the distribution of GLUT3 mRNA in bovine tissues may be similar to that in rodent animals. GLUT1 protein was also present in the bovine mammary gland at a high level (Chapter 4 and 5). GLUT4 protein was undetectable in the bovine mammary gland using the anti-GLUT4 antibody raised against a synthetic peptide specific for 11 amino acids of the carboxyl terminus of rat GLUT4, although this antibody can be used to detect GLUT4 protein in bovine skeletal muscle and omental fat (Chapter 4). The carboxyl terminus of GLUT4 is conserved in all GLUT4 proteins reported for several different species (Burant *et al.*, 1991). The proteins of GLUT2, 3 and 5 cannot be assessed in bovine mammary gland due to the lack of antibodies against these transporters for bovine tissues. The finding that GLUT1 is the

major glucose transporter in bovine mammary gland is consistent with data on glucose transporters in rat mammary tissue. Quantitative Western blotting and cytochalasin B binding studies demonstrated that GLUT1 was the major glucose transporter in the plasma membranes of lactating rat mammary gland (Madon *et al.,* 1990). GLUT1 and GLUT4 are present in the rat mammary gland before conception. However, the expression of GLUT4 decreases progressively during pregnancy and becomes undetectable during lactation, whereas the levels of GLUT1 increase during pregnancy and reach the highest level in lactation (Burnol *et al.*, 1990; Camps *et al.*, 1994). GLUT2, 3 and 5 are not expressed in the rat mammary gland (Burnol *et al.*, 1990; Madon *et al.*, 1990; Camps *et al.*, 1994).

The expression of the facilitative GLUT1 transporter in the epithelial cells of the mammary gland is consistent with the physiology of glucose metabolism in bovine mammary gland as discussed in Chapter 4. The lactating mammary gland has a high requirement for glucose which could easily be met if the mammary gland expressed GLUT2, a facilitative glucose transporter with a high K_m for glucose. Glucose transport mediated by GLUT2 never becomes rate-limiting under physiological glucose concentrations. However, the lactating mammary gland does not express GLUT2, which indicates that glucose transport across the plasma membrane of mammary epithelial cells is controlled and provides a means of regulating glucose utilization in the mammary gland. The expression of GLUT1 is compatible with tissues which have very high metabolic rates and with regulatory activities such as barrier functions. One example is the very high expression of GLUT1 in the brain capillaries that comprise the blood-brain barrier (Pardridge *et al.*, 1990; Cornford *et al.*, 1994), brain parenchymal cells are completely dependent on blood glucose as an energy source under

normal conditions. Glucose uptake is dependent on glucose availability in the plasma and glucose transporter activity in the cell membrane. Modulation of glucose transporter activity at the mammary gland may be achieved by an alteration in the total mammary GLUT1, in the functional kinetics of GLUT1, or acutely mediated through rapid translocation of cytoplasmic GLUT1 to the plasma membrane. Thus, increased mammary glucose uptake in GH-treated lactating animals (Davis *et al.* 1988; Fullerton *et al.*, 1989; Mepham *et al.*, 1990) can occur in the absence of increased GLUT1 gene expression (Chapter 5). The mammary gland of non-lactating cows appeared to express more GLUT1 (Chapter 4) compared to the lactating cows.

The control of gene expression can be transcriptional, translational, or post-translational. The transcriptional and translational controls are the controls of the copying rate of genetic information from the form of a linear sequence of nucleotides in DNA into a complementary strand of mRNA within the nucleus or from the mRNA sequence into an amino acid sequence of a specific protein within the cytoplasm, respectively. The post-translational control alters the rates of protein secretion and/or protein degradation. Activation of transcription are excellent regulatory devices for relatively long-term control (hour to days), but the relatively slow response makes it unsuitable for short-term control. GHRF increased the mRNA levels of GLUT1 but did not change GLUT1 protein levels in the lactating mammary tissue (Chapter 5). The levels of GLUT1 mRNA were similar in both lactating and dry animals, but the levels of GLUT1 protein were higher in the dry cows (Chapter 4). Thus, the gene expression of GLUT1 in bovine mammary gland seems to be regulated mainly post-transcriptionally.

The absence of GLUT4 in the lactating bovine mammary gland (Chapter 4) indicates

that glucose transport in this tissue is not regulated by insulin as in adipose tissue and skeletal muscle. Although insulin has been shown to acutely stimulate several enzyme activities involved in glucose metabolism in the mammary tissue, such as glucose-6-phosphate dehydrogenase, phosphofructokinase I, pyruvate dehydrogenase, and acetyl-CoA carboxylase (Burnol *et al.*, 1990) and stimulate a variety of other processes, such as α-aminoisobutyric acid uptake, casein gene expression, DNA synthesis, lipogenesis (Camps *et al.*, 1994), the presence of a stimulatory effect of insulin on glucose transport in the mammary gland remains controversial. It has been reported that mammary gland from fasted rats, but not that from fed rats, is sensitive to insulin by acutely stimulating glucose transport (Threadgold and Kuhn, 1984; Burnol *et al.*, 1987). With prolactin and glucocorticoids, chronic insulin treatment can cause marked stimulation of glucose transport activity (Prosser and Topper, 1986). However, other evidence indicates that insulin has no acute effect on glucose transport in the lactating mammary epithelial cells (Prosser and Topper, 1986). Thus, it is possible that in some physiological states, insulin may acutely influence glucose transport in the mammary gland by triggering GLUT1 translocation from the intracellular pool (Madon *et al.*, 1990) to cell surface as it does in fat and muscle tissues.

2 Experimental Controls

2.1 Controls for Northern Blotting Analysis

Northern blotting analysis is a quantitative technique to measure the specific mRNA concentration in a tissue. mRNA concentrations can, in theory, be controlled by the rate of

specific transcription, processing, or turnover and is considered to be constant unless a new stimulus increases or decreases the rate. To maximize accuracy and precision of Northern blotting analysis, it is essential to ensure the integrity of the purified RNA and that equal amounts of RNA are loaded in each lane. Actin and other housekeeping genes have been used as an internal control in many hybridization studies in the past to normalize Northern blotting results. However, there is increasing evidence that the expression of these housekeeping genes have biological variability, thus, 28S ribosomal RNA levels may be more appropriate as an accurate indicator of the relative amounts of RNA loaded (De Leeuw *et al.*, 1989). The level of 28S ribosomal RNA is a constant fraction of total sample in comparison to other internal controls. 28S and 18S rRNA bands can be directly visualized and photographed from the ethidium bromide-stained gel. Quantitative comparison of the 28S rRNA bands can then be carried out by scanning the negative film and measuring the densities of each band which avoids an extra step of rehybridization. In addition, the ratio of 28S/18S eukaryotic rRNAs is an indicator of the integrity of the RNA, which should exhibit a near 2:1 ratio. Since the 28S ribosomal RNA is characteristically degraded to an 18S-like species, the ratio will be reversed in RNA samples that have been degraded (Correa-Rotter *et al.*, 1992).

2.2 Controls for Western Blotting Analysis

Western blotting analysis were employed to identify and quantitate GLUT1 and GLUT4 proteins in the bovine tissues. To maximize the sensitivity of Western blotting, crude nuclear membrane fractions were prepared and used from different bovine and rat tissues. The homogenate of the tissues was first centrifuged at 1000 g to remove any unbroken cells and

then at 13,000 g to remove mitochondrial and nuclear fractions. The resulting supernatant was centrifuged at 100,000 g and the pellet contained a crude membrane fraction and cytosolic fraction (Clegg, 1981; Kanno *et al.*, 1982; Akers and Keys, 1984; Prosser, 1988). Similar crude membrane preparations have been used previously in the mammary gland from different species at different physiological states (Djiane *et al.*, 1977; Clegg, 1981; Kanno *et al.*, 1982; Akers and keys, 1984; Prosser, 1988; Burnol *et al.*, 1990; Madon *et al.*, 1990) as well as other tissues from rat, human, rodent, and pig (Thorens *et al.*, 1988; Shepherd *et al.*, 1992; Pinches *et al.*, 1993). To inhibit the proteolysis of membrane proteins during the crude membrane preparation, phenylmethylsulphonyl fluoride (PMSF), aprotinin and EDTA were used in all buffer solutions as proteinase inhibitors (Evans, 1987). PMSF is an inhibitor of serine proteases ; EDTA is the proteolytic inhibitor of metalloproteases; Aprotinin is an inhibitor of trypsin, chymotrypsin, kallikrein and plasmin. To evaluate transfer efficiency and homogeneity of the protein binding to the nitrocellulose matrix, proteins immobilized on the nitrocellulose filter were stained with ponceau S and SDS-polyacrylamide gels were stained with coomassie brilliant blue. Well known GLUT1-enriched tissue rat brain and GLUT4-enriched rat skeletal muscle were used as positive controls for anti-GLUT1 and anti-GLUT4 antibodies, respectively.

2.3 Controls for Immunocytochemistry

Appropriate controls were included in the immunohistochemical experiments to correct for background and nonspecific labelling inherent in the immunohistochemical analysis of the cells and tissues:

1). Immunocytochemistry was performed without the primary antibodies to test for non-specific background staining.

2). A dilution series for the primary antibody was used in each experiment to determine the specificity of the antibodies.

3). GLUT1 and GLUT4 provide positive and negative controls for each other.

4). Western blotting analysis of GLUT1 and GLUT4 in bovine mammary gland provide direct information for the immunohistochemistry studies.

5). Immunostaining of GLUT1 in bovine liver is consistent with that reported for rat liver (Tal *et al.*, 1991).

Another useful control for the specificity of the antibody is to block specific binding of the primary antibody by adding the purified antigen. This control was not carried out due to the lack of the glucose transporter antigen.

Four percent paraformaldehyde in PBS was successfully used as the fixative in preparing the lactating mammary gland for immunocytochemistry. Paraformaldehyde is a conventional fixative which primarily target protein and lipid moieties (Larsson, 1988). The concentration of paraformaldehyde must be high enough to achieve some stabilization of proteins and lipids, but low enough to preserve the antigenicity.

3. Future Directions

1) Isolate the cDNAs of GLUT2, GLUT3, GLUT4, GLUT5 and GLUT7 from bovine tissues and develop the antibodies for each transporter isoform. Use these antibodies to

confirm the absence of these isoforms in bovine mammary gland and to study the control of glucose transporter isoform during different physiological states.

2) Purify the plasma membranes and subcellular membrane fractions from the bovine mammary gland and use electron microscopy immuno-gold staining techniques, study the subcellular distribution of glucose transporter isoforms and investigate the possible translocation of the transporters in the regulation of mammary glucose uptake.

3) Study the functional kinetics of glucose transporters in the bovine mammary gland. Isolate the cis-acting elements and trans-acting factors of genetical control of the transporters. Identify the intracellular signalling pathways which regulate glucose transporter gene expression.

4) Identify the smaller GLUT1 fragment detected in the dry cow mammary gland by using the antibody specific for the NH_2-terminus and by removing quantitatively the single *N*-linked oligosaccharide from the transporter. Additional information can be obtained by multi-enzyme digestion of the immunoprecipitated proteins from the lactating and dry cow mammary gland. Localize the GLUT1 expression in dry cow mammary gland by immunocytochemistry.

5) Measure glucose uptake in the mammary gland and skeletal muscle from control and GH-treated cows by measuring the blood flow and A-V differences across these organs. Perform radiotracer experiments to study the patterns of glucose utilization in these tissues (Baldwin and Kim, 1993). These data should provide direct evidence for our hypothesis (Chapter 5) that glucose is spared in skeletal muscle and adipose tissues to meet the increased requirement of the mammary gland associated with GH treatment.

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APPENDIX A

REGULATION OF GLUCOSE TRANSPORTER GENE EXPRESSION IN LIVER AND KIDNEY OF LACTATING COWS BY ADMINISTRATION OF bGH AND bGHRF

INTRODUCTION

Glucose availability in the mammary gland is a critical factor in milk synthesis. However, unlike monogastric animals most dietary carbohydrate in ruminants is fermented to volatile fatty acids (mainly acetate, propionate, and butyrate) in the rumen so that the amount of soluble carbohydrate entering the duodenum is relatively low. It has also been suggested that the capacity for digestion of starch in the small intestine of ruminants is limited (Ørskov, 1986). Furthermore, there is extensive metabolism of glucose in the epithelial cells of the bovine small intestine (Okine *et al.*, 1994). The result is that as little as 15% of blood glucose may be derived directly from dietary sources (Lomax and Baird, 1983; Reynolds *et al.*, 1988). About 85% of the glucose taken up by the mammary gland is provided by hepatic gluconeogenesis (Bergman *et al.*, 1974) with the net hepatic glucose production being more than 3 kg/d in lactating cows (Reynolds *et al.*, 1988). The kidney is also an important organ in whole-body glucose metabolism with gluconeogenesis in the kidney accounting for approximately 10% of the glucose produced by nonpregnant sheep (Gans and Mercer, 1984). In addition, the kidney plays a key role in the reabsorption of

glucose filtered in the glomerulus (Gans and Mercer, 1984).

An important first step in gluconeogenesis and glucose reabsorption is transport of glucose across the plasma membranes. This process is carried out by two families of membrane carrier proteins called glucose transporters (Gould and Holman, 1993). The sodium-dependent glucose transporter is responsible for transfer of glucose across the apical membranes of polarized intestinal and renal epithelial cells. The facilitative glucose transporter family is involved in the net uptake of blood glucose for cellular metabolism in most cell types and in the net efflux of glucose from the cells of liver, kidney and intestine to plasma. To date, SGLT1 is the only sodium-dependent glucose transporter that has been cloned (Hedige *et al.*, 1987). However, at least six other glucose transporters (GLUT1, 2, 3, 4, 5, and 7) belonging to the facilitative glucose transporter family exist (Gould and Holman, 1993). Each transporter has different kinetics and is tissue- and cell-type-specific. GLUT2 is the major transporter isoform expressed in hepatocytes and kidney (Gould and Holman, 1993) and is involved in the net release of endogenous glucose in the liver and in the reabsorption of glucose in the kidney. In the lactating cow, liver and kidney also express relatively high levels of GLUT5 (Zhao *et al.*, 1993). The function of GLUT5 in these tissues is unclear. The mRNA of GLUT1 is also expressed in the kidney of lactating cows (Zhao *et al.*, 1993). GLUT1 in the kidney may be required mainly for glucose uptake from the blood as a source of metabolizable energy for acidification or alkalinization in the formation of urine.

Administration of bGH and bGHRF to dairy cows increases milk yield (Burton *et al.*, 1994, Binelli *et al., 1995*). The underlying mechanisms involved are not fully

understood. GH and GHRF alter glucose transporter gene expression in the mammary gland, skeletal muscle, and omental fat which would facilitate the repartitioning of glucose to the mammary gland (Chapter 5). This chapter reports the effects of bGH and bGHRF on gene expression of the facilitative glucose transporters in the liver and kidney. Production data and serum hormone concentrations were reported by Binelli *et al.* (1995) and Vanderkooi *et al.* (1995).

MATERIALS AND METHODS

Animals and Tissues

Twelve primiparous Holstein cows, *Bos taurus*, were used in a completely randomized block design with repeated measurement. Within each block, three cows were randomly assigned to receive continuous intravenous infusions of either bGH (29 mg/d), bGHRF (12 mg/d), or no treatment (controls) for 63 d, commencing at 118 d after parturition. Doses selected were based on previous experiments (Dahl *et al.*, 1993), where these same doses elicited similar increases in serum GH concentrations. Cows were fed a total mixed ration *ad libitum*. On d 63 of treatment cows were slaughtered. Samples of liver and kidney were obtained immediately after slaughter, frozen in liquid nitrogen, and stored at -75°C as described previously (Zhao *et al.*, 1993).

Northern Blotting

Total RNA was isolated from different tissues using a guanidinium thiocyanate-CsCl

procedure (Chirgwin *et al.*, 1979). 30 µg of total RNA were electrophoresed on denaturing 1% (w/v) agarose/0.66 M formaldehyde gels and transferred to nylon membranes (Bio-Rad, Richmond, CA, U.S.A.) by capillary diffusion. The integrity and relative amounts of RNA were assessed by UV-light visualization of ethidium bromide-stained RNA. Membranes were prehybridized for 2 h at 50°C in a medium containing 60% (v/v) formamide, 1 x SSPE (0.18 M NaCl, 0.01 M sodium phosphate at pH 7.4, 1 mM EDTA), 0.5% (w/v) nonfat dried milk, 10% (w/v) dextran sulfate, 1% (w/v) sodium dodecyl sulfate, 500 µg/ml salmon testes DNA and 200 µg/ml yeast tRNA. Following prehybridization, hybridization was carried out for 16-18 h at 50°C in fresh buffer containing [^{32}P]-labelled antisense RNA probes (10^6 cpm/ml) prepared as previously described (Zhao *et al.*, 1993). Membranes were then rinsed briefly in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate at pH 7.0), washed for 15 min at 22°C in 2 x SSC containing 0.1% sodium dodecyl sulfate, washed at 70°C in 0.2 x SSC containing 1% sodium dodecyl sulfate, and rinsed briefly in 0.2 x SSC. Autoradiography was performed at -70°C with an intensifying screen and quantified by scanning densitometry (Imaging Densitometer GS-670, Bio-Rad, Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

Administration of bGHRF for 63 d increased GLUT2 mRNA levels in the liver and kidney (43%, $P < 0.01$; and 39%, $P < 0.05$, respectively) (Fig. A-1, Fig. A-2, and Table A-1). In contrast, bGH did not significantly alter GLUT2 mRNA levels in these tissues ($P = 0.16$ and $P = 0.16$, respectively) (Fig. A-1, Fig. A-2 and Table A-1). Cows treated with

bGHRF tended to have greater liver GLUT2 mRNA levels than bGH-treated cows (P = 0.11).

Liver is a target tissue for GH. It expresses GH receptor (Waters *et al.*, 1989), and GH participates in the regulation of expression of several hepatic proteins (Berry *et al.*, 1993; Chirino *et al.*, 1994). Liver is metabolically unique in that the intracellular concentration of glucose in the hepatocyte may exceed circulating levels. The predominant glucose transporter isoform expressed in the livers is GLUT2. In ruminants, hepatic uptake of blood glucose is negligible due to the absence of glucokinase, a relatively low blood glucose level, and the low K_m of hexokinase (Stangassinger and Giesecke, 1986), GLUT2 is primarily responsible for the release of glucose from hepatocytes into blood. Previous studies showed that hepatic rates of gluconeogenesis in the lactating cow increased with GH treatment in vivo (Cohick *et al.*, 1989) and in vitro (Knapp *et al.*, 1992; Pocius and Herbein, 1986). Also, insulin inhibits hepatic glucose output (Brockman, 1983, Debras, *et al.*, 1989) and GH inhibits the effect of insulin on hepatic gluconeogenesis (Boisclair *et al.*, 1989; Gopinath and Etherton, 1989). Thus, GH should increase liver glucose output. In the present study, bGH did not significantly change GLUT2 mRNA level in the liver. This is consistent with the fact that GLUT2 has a high K_m and high V_{max} for glucose (Could and Holman, 1993). Thus, glucose flux through this transporter, at physiological concentrations of glucose, would be virtually linear with extracellular/intracellular glucose concentrations and transporter saturation by glucose is unlikely to be rate limiting. However, GLUT2 mRNA level in the liver was increased by bGHRF treatment. Gene expression of GLUT2 in bovine liver may be regulated posttranscriptionally as in rat liver where GLUT2 mRNA levels are

depressed by fasting and diabetes and are elevated by refeeding with minimal alteration in GLUT2 protein levels (Thorens, *et al.*, 1990b). Because both bGH- and bGHRF-treated animals used in this experiment had similar serum GH concentration (Binelli *et al.*, 1995; Vanderkooi *et al.*, 1995), bGHRF may increase liver gluconeogenesis by a mechanism other than through GH. Consistent with this possibility, Dahl *et al.* (1993) reported that bGHRF-treated cows had approximately 10% greater milk yield than bGH- treated cows, although both groups had similar concentrations of serum GH and IGF-I.

Glucose reabsorption from the tubular filtrate of kidney is a two-step process and occurs in the proximal convoluted tubules. The Na^+-dependent transporter on the apical brush border of the proximal tubule cells actively accumulates glucose against its concentration gradient (Takata *et al.*, 1991). The accumulated glucose is subsequently released into the capillaries via GLUT2 localized on the basolateral surface of proximal tubule cells (Thorens *et al.*, 1990a, c). The proximal convoluted tubule is the only gluconeogenic segment of the nephron. Although GH is an important hormone required for renal function (O'Shea and Layish, 1992), I am not aware of any report concerning the effect of GH and GHRF on glucose reabsorption and gluconeogenesis in kidney. I hypothesised that GHRF and GH treatment may increase both processes. In lactating cows, GH treatment increased cardiac output and mammary blood flow (Davis *et al.*, 1988) and tended to increase blood flow to leg muscle (McDowell *et al.*, 1987). GH also increased blood volume in sheep (Wheatley *et al.*, 1966). These findings suggest that GH may increase blood flow to the kidney which may result in increased glucose reabsorption. In this study, bGHRF increased GLUT2 mRNA level in kidney, but bGH did not. These results are consistent with

our data on the effects of GH and GHRF on GLUT2 mRNA expression in liver. GLUT2 protein levels in bovine kidney may also be regulated posttranscriptionally.

It was previously demonstrated that a relatively high level of GLUT1 mRNA can be detected in bovine kidney (Zhao *et al.*, 1993). Northern blotting analysis in this experiment indicated that both bGH and bGHRF tended to increase GLUT1 mRNA level in the kidney (21%, $P = 0.13$; and 24%, $P = 0.09$, respectively) (Fig. A-3 and Table A-1).

The kidney is both gluconeogenic and glycolytic and these activities take place in different parts of the kidney. Glycolysis is restricted to the straight proximal tubule, the thick ascending limb of Henle, the distal convoluted tubule, and the collecting duct. The GLUT1 transporter isoform has been detected in the basolateral membrane of cells from these segments of rat kidney nephron and its level of expression is related to the glycolytic activity of these segments (Thorens *et al.*, 1990c). Thus, GLUT1 in kidney may be required for glucose uptake from the blood as a source of metabolizable energy for acidification or alkalinization in the formation of urine. Under physiological conditions, kidney does not utilize plasma glucose to any considerable extent for its energy requirements (Gans and Mercer, 1984). Because GHRF and GH may increase blood flow to the kidney and therefore increase the kidney's activity, the numerically increased GLUT1 mRNA associated with bGH and bGHRF treatment in this study may have increased GLUT1 synthesis which would contribute to increased glucose uptake.

Previous work in our laboratory has also shown that unlike humans, the liver and kidney of lactating cows express a high level of GLUT5 mRNA (Zhao *et al.*, 1993). Surprisingly, in the present study GLUT5 mRNA could only be detected in the liver from

two of the four animals in each group (data not shown). Neither bGH nor bGHRF treatment significantly changed GLUT5 mRNA level in the kidney (Fig. A-4 and Table A-1), although a trend for increased GLUT5 mRNA was found in bGH-treated cows ($P = 0.11$).

The physiological role of GLUT5 is still a matter of conjecture. In humans, GLUT5 is expressed predominantly in the jejunal region of the small intestine, with localization to the luminal surface of mature absorptive epithelial cells (Davidson *et al.*, 1992). It has been suggested that GLUT5 participates in the uptake of dietary glucose from the lumen of the small intestine. Unlike humans, the liver and kidney of the bovine express relatively high levels of GLUT5 mRNA (Zhao *et al.*, 1993). GLUT5 in ruminant kidney may play a similar function in the reabsorption of glucose as in human intestine. Results of the present study show that there are large individual differences in the expression of GLUT5 mRNA in bovine liver. The physiological significance of these differences are unknown as is the physiological function of GLUT5 in bovine liver.

Administration of bGH and bGHRF increased milk yield in this study by 17% and 14%, respectively (Binelli *et al.*, 1995). The mechanism underlying the galactopoietic effect of bGH and bGHRF is still under investigation. There is an increasing body of data to support the hypothesis that the galactopoietic response of exogenous GH and GHRF is strongly influenced by their ability to direct nutrients to the mammary gland by influencing nutrient utilization in other tissues. It was previously reported that GH and GHRF alter glucose transporter gene expression in the mammary gland, skeletal muscle, and omental fat which could facilitate the repartitioning of glucose to the mammary gland (Chapter 5). The data presented here indicate that bGH and bGHRF may also change glucose transporter

expression in the liver and kidney to support changes in glucose metabolism in these tissues.

In summary, bGHRF, but not bGH, significantly increased GLUT2 mRNA levels in liver and kidney which may be associated with increased hepatic gluconeogenesis and possibly increased kidney glucose reabsorption. Both bGHRF and bGH tended to increase GLUT1 mRNA levels and GH tended to increase GLUT5 mRNA level in kidney. Large individual differences were observed in the expression of GLUT5 mRNA in liver. Further studies will be necessary to determine transcriptional and posttranscriptional aspects of glucose transporter expre: ne liver and kidney.

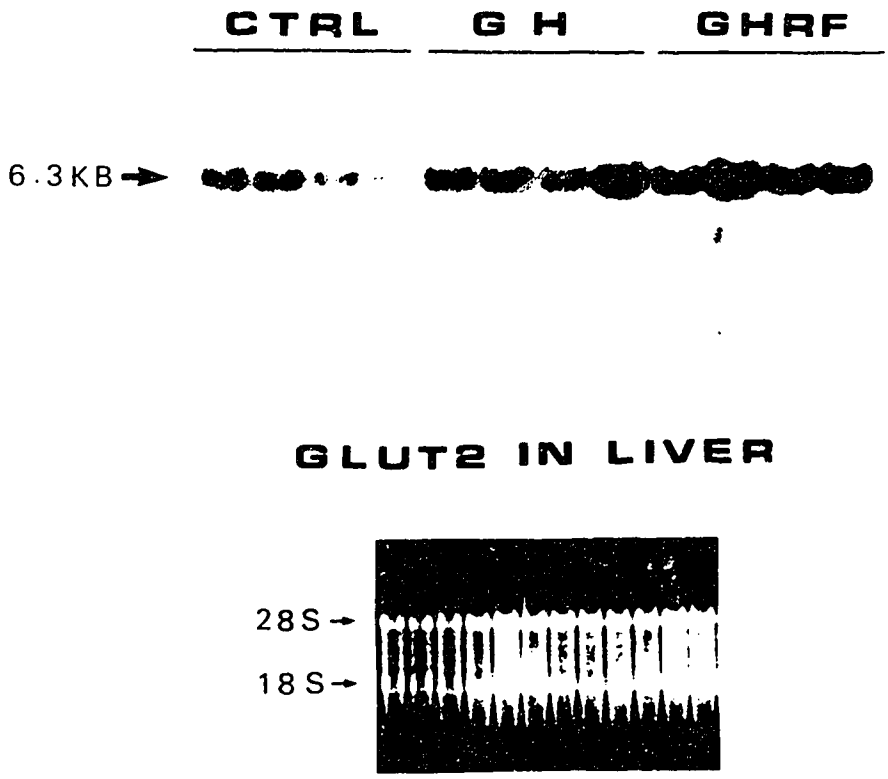

Fig. A-1. Northern blotting analysis of GLUT2 mRNA from the liver of control (CTRL), bGH- and bGHRF-treated cows. *The upper panel* is photograph of autoradiograph produced by hybridizing Northern blotting with a radiolabled GLUT2 riboprobe. *The lower panel* is photograph of the UV light visualization of the gel with ethidium bromide-stained RNA to demonstrate that equivalent amounts of total RNA were loaded in each lane.

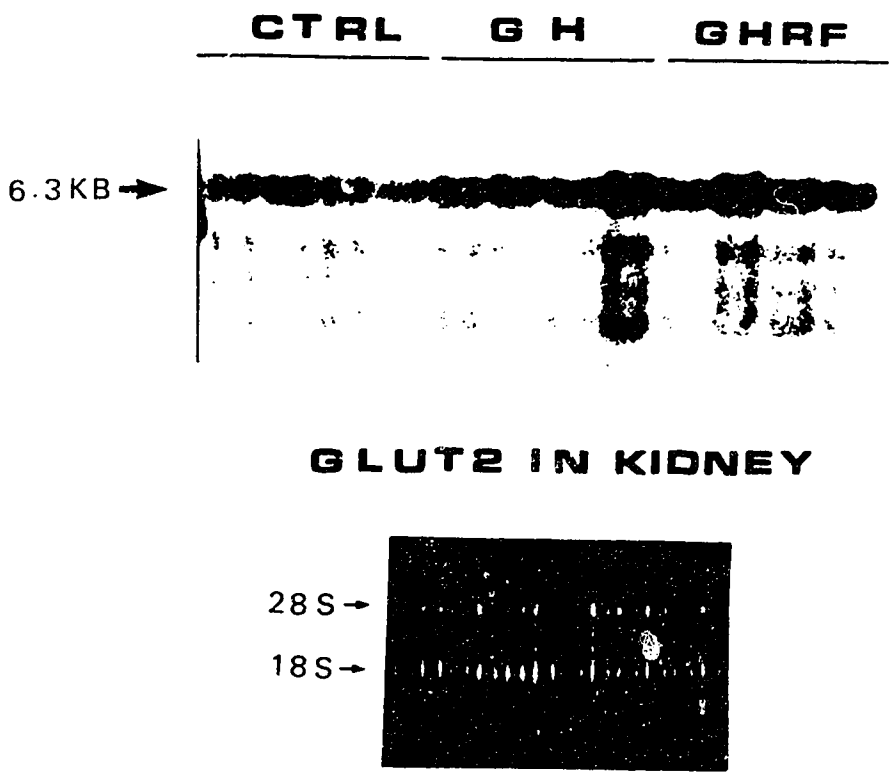

Fig. A-2. Northern blotting analysis of GLUT2 mRNA from the kidney of control (CTRL), bGH- and bGHRF-treated cows. The *upper* and *lower panels* are as described in Fig. A-1.

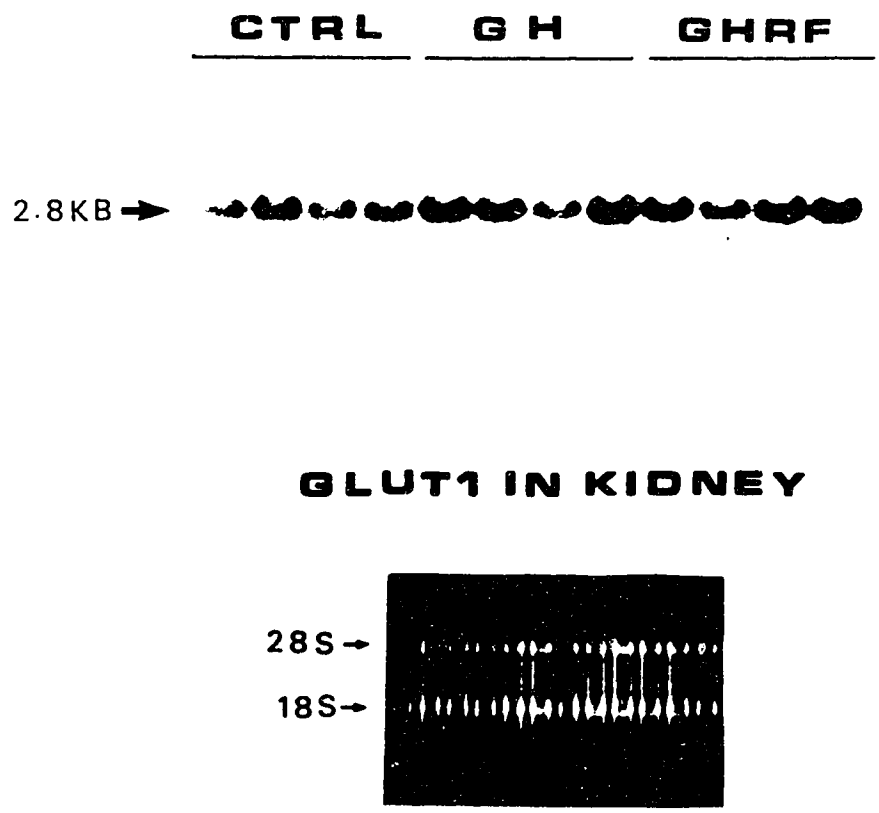

Fig. A-3. Northern blotting analysis of GLUT1 mRNA from the kidney of control (CTRL), bGH- and bGHRF-treated cows. *The upper and lower panels* are as described in Fig. A-1.

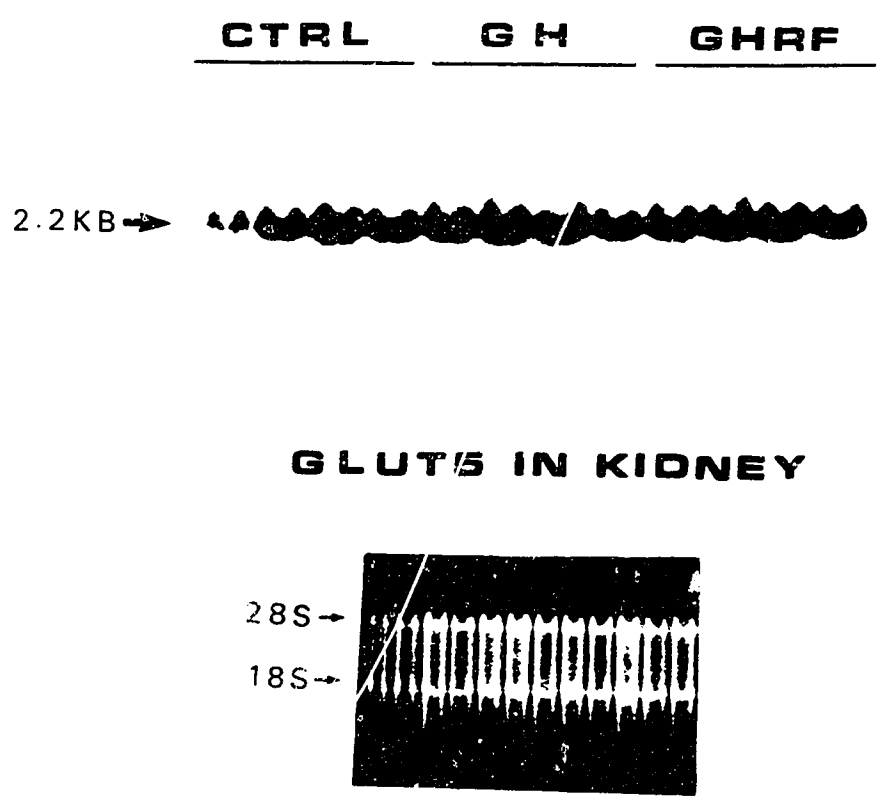

Fig. A-4. Northern blotting analysis of GLUT5 mRNA from the kidney of control (CTRL), bGH- and bGHRF-treated cows. *The upper and lower panels* are as described in Fig. A-1.

Table A-1. Mean abundance (arbitrary densitometric unit) of GLUT2 mRNA in liver and kidney and GLUT1 and GLUT5 mRNA in the kidney of cows receiving no treatment (Ctrl), 29 mg bGH/d, or 12 mg bGHRF/d

					P values		
mRNA abundance	Ctrl	GH	GHRF	SEM	GH vs. Ctrl	GHRF vs. Ctrl	GH vs. GHRF
GLUT2 in liver	27.5[a]	33.0[ab]	39.2[b]	2.4	.16	.01	.11
GLUT2 in kidney	22.9[a]	28.9[ab]	31.7[b]	2.7	.16	.05	.48
GLUT1 in kidney	14.9[a]	18.0[a]	18.4[a]	1.3	.13	.09	.83
GLUT5 in kidney	19.7[a]	24.8[a]	24.1[a]	1.9	.11	.15	.81

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