BRIEF COMMUNICATION

Isolation of cDNA subclones encoding ovine glucose transporter proteins

B.L. BENNETT, M.R. GRIGOR[1] AND C.G. PROSSER

Dairying Research Corporation, Ruakura Agricultural Centre, Hamilton, New Zealand.

Keywords: glucose transporter; mammary; ovine; polymerase chain reaction.

INTRODUCTION

A physiological understanding of the factors influencing lactation must include a knowledge of the nutrient supply to the mammary tissues. Glucose is a fundamental substrate in the lactating gland being both a metabolic fuel and precursor of milk products. The lactating ruminant may utilise three quarters of body glucose (Mepham, 1987) for mammary processes, and, glucose (transport) is probably rate-limiting for milk production. The mammary secretory epithelial cell contains a host of membranous organelles which compartmentalise the synthetic reactions producing milk components. The lipid membrane is relatively impermeable to aqueous solutions so the transport of glucose across these intracellular membranes and the cell plasma membrane is facilitated by a transmembrane spanning glucose transporter protein. A family of proteins (Baldwin, 1993), named GLUT 1-7, display a characteristic tissue distribution in mammals reflecting the various physiological functions and requirements for glucose. The GLUT-1 isotype mRNA and protein has been identified in the mammary epithelium though other GLUT's may also be present (Burnol *et al.*, 1990). Research into mammary glucose transport has previously focused on the rat as a useful model but interest is now shifting to ruminant animals because of their obvious commercial significance. Our objective is to isolate the molecular "tools" (cDNA clones) that will allow the study of glucose transport in the sheep mammary gland.

RESULTS AND DISCUSSION

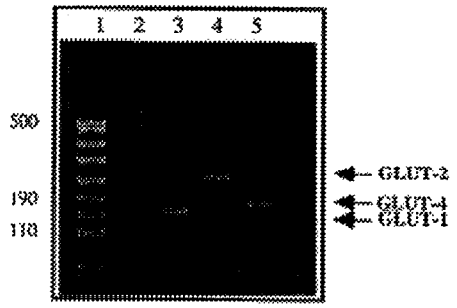
An alignment of available glucose transporter protein sequences from human, rat and mouse shows extensive regions of high homology especially in the centre of the molecule which is proposed to constitute the transmembrane spanning domains. Both termini and an extracellular domain between transmembrane domains 1 and 2 are regions of sequence variability among the different isotypes. Sivitz and Lee, 1991, observed that this extracellular domain not only varies in sequence but that the domain differs in length which is characteristic of each transporter isotype. We have exploited this feature in a polymerase chain reaction based strategy to rapidly isolate unique ovine glucose transporter subclones.

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985) allows a DNA sequence to be greatly amplified in copy number with the sequence specificity determined by the primers used in the reaction. We have constructed a primer pair (upstream and downstream) complimentary to the 5' and 3' boundaries of the extracellular domain respectively. These two primer binding regions code for portions of transmembrane spanning domains 1 and 2 and are thus highly conserved across the GLUT isotypes. As a control experiment we amplified this region from the full length rat cDNA clones we had available (Fig 1). The fragments were subcloned into pGEM3Z (Bennett and Molenaar, 1994) and sequenced to verify their identity and the expected lengths of GLUT-1 (137bp), GLUT-2 (233bp) and GLUT-4 (149bp). Therefore the assay shows that the expected fragment sizes are amplified and can be used as an indicator of which glucose transporter has been amplified by the PCR. To isolate novel subcones from the sheep it was necessary to use total RNA purifications from tissues characteristic of each isotype. Because the PCR reaction amplifies only DNA, an initial reverse transcription of the RNA to a single-stranded DNA copy is required (Ausubel *et al.*, 1992). The primer used for the reverse transcriptase enzyme is the downstream primer used in the PCR. Following this reaction the mixture is used as a template target in the PCR. We performed this reverse transcriptase-PCR (RT-PCR) assay on RNA prepared from rat brain, adipose, liver, (Fig 2) and ovine/bovine heart and liver (Fig 3), along with brain, adipose and mammary tissues (data not shown). Fragments corresponding in size to the rat cDNA clone controls were isolated to give ovine subclones for GLUT-1, GLUT-2 and GLUT-4. Sequence analysis demonstrated 84%, 79% and 94% conservation in nucleotide sequence with their human homologues. Similar homologies are found with the rat sequences.

Because tissues are composed of different cell types eg. epithelial, endothelial, fibroblast, adipocyte etc, it is typical for the RT-PCR analysis of tissue RNA to yield more than one GLUT isotype. In addition an artifact band(cloned and sequenced) of identical length to GLUT-2 fragments is routinely amplified in the RT-PCR assay. However the presence of GLUT-2 is obvious by an increased band intensity to background (Fig 3, lanes 4 and 6). Our procedure can dis-

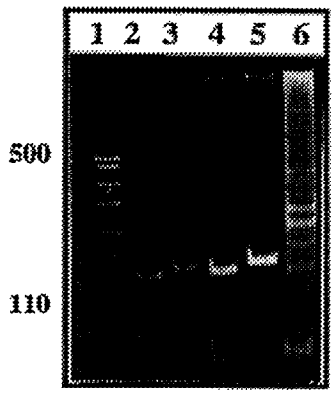
[1] Department of Biochemistry, University of Otago, Dunedin, New Zealand.

FIGURE 1: PCR amplification of control rat glucose transporter cDNA clones.



PCR was performed as described (Jeffreys *et al.*, 1990) and an 8ul aliquot from a 40ul reaction analysed on a 8% polyacrylamide TBE gel stained with ethidium bromide. Lanes contain: 1. pUC18 restricted with MspI to give markers of 500, 489, 404, 331, 242, 190, 147, 111/110 and 67 base pairs, 2.minus DNA control, 3. rat GLUT-1, 4. rat GLUT-2 and 5. rat GLUT-4.

FIGURE 2: RT-PCR of rat tissue RNA.



Lanes contain: 1. pUC18 restricted with MspI (see Fig. 1 legend), 2. rat GLUT-1 control cDNA, 3. rat GLUT-4 control cDNA, 4. brain RNA, 5. adipose RNA, 6. liver RNA. Samples (10ul of 50ul reaction) were separated on a 8% polyacrylamide TBE gel and stained with ethidium bromide.

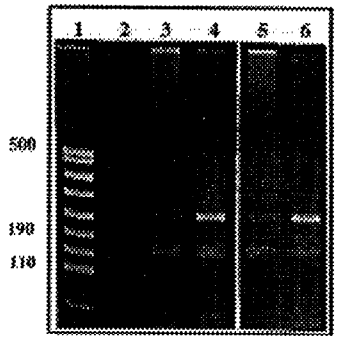
criminate between isotypes and increase the potential of isolating all possible isotypes present.

What of the other GLUT isotypes not subcloned? While GLUT-1 and GLUT-3 share a common extracellular domain length (137bp) it is proposed that a careful selection of tissue RNA will maximise the recovery of one isotype over the other. In addition differential screening of putative subclones and sequencing will distinguish these two isotypes. GLUT-5 is predominantly a fructose carrier and shows more divergent homology while GLUT-6 is a human pseudogene and GLUT-7 differs from GLUT-2 in only a few N-terminal amino acids.

CONCLUSIONS

We have designed a polymerase chain reaction based method to rapidly subclone at least three different glucose

FIGURE 3: RT-PCR of ovine and bovine RNA.



Lanes contain: 1. pUC18 restricted with MspI (see Fig.1 legend), 2. minus RNA control, 3. ovine heart RNA, 4. ovine liver RNA, 5. bovine heart RNA, 6. bovine liver RNA. Samples (10ul of 50ul reaction) were separated on a 8% polyacrylamide TBE gel and stained with ethidium bromide.

transporter isotypes. This procedure uses a "universal" GLUT primer pair to amplify a variable length fragment which defines a GLUT isotype. This approach should be useful in any mammalian species. We have used this technique to isolate and sequence the ovine subclones for GLUT-1, GLUT-2 and GLUT-4.

With the isolation of these subclones future research will focus on the recovery of full length clones from a cDNA library. The full length cDNA's and subclones shall be used as molecular probes for studying glucose transporter gene expression and potentially as synthetic templates for protein synthesis and subsequent antibody production.

REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., 1992. The Polymerase Chain Reaction, Chapter 15.4. *Current Protocols in Molecular Biology*, Vol 2. John Wiley and Sons, New York.

Baldwin, S.A., 1993. Mammalian Passive Glucose Transporters: *Biochimica et Biophysica Acta* **1154**: 17-49.

Bennett, B.L. and Molenaar, A.J., 1994. Cloning of PCR products can be inhibited by Taq polymerase carryover. *BioTechniques* **16**: 32-37.

Burnol, A-F., Leturque, A., Loizeau, M., Postic, C. and Girard, J., 1990. Glucose transporter expression in rat mammary gland. *Biochem. J.* **270**: 277-279.

Jeffreys, A.J., Neumann, R. and Wilson, V., 1990. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell* **60**: 473-485.

Mepham, T.B., 1987. Maternal Physiology During Lactation, *Physiology of Lactation*, Open University Press, Milton Keynes, UK, 165-167.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B.,Horn, G.T., Erlich, H.A. and Arnheim, N., 1985. Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* **230**: 1350-1354.

Sivitz, W.I. and Lee, E.C., 1991. Assessment of glucose transporter gene expression using the polymerase chain reaction. *Endocrinology* **128**: 2387-2394.