Identifying genes relevant to animal nutrition

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Summary

New techniques for mapping genes affecting quantitative traits or for measuring gene expression will permit identification of genes important to animal nutrition. Gene mapping will allow us to identify the genes causing animals to respond differently to similar diets and help us to explain differences in food conversion efficiency and partitioning of energy stores. Differential display and related techniques will allow identification of any gene whose expression is modified by nutritional manipulation or in response to other genetic changes, such as mutations of large effect or even selection. Ultimately this information will enable us to better match genotype and nutrition in the animal production systems of the future.

Introduction

Among animal scientists, nutrition and genetics are traditionally viewed as separate competing disciplines. Animal breeders are seen as modifying the intrinsic genetic and physiological properties of animals by identifying and selecting favourable genes to increase productivity and economic value. Nutritionists aim to improve productivity by modifying the environment to which the animals are exposed. These environmental changes include manipulation of feed processing, feed composition, including additives and supplements, and adjustments to the quality and quantity of feed relative to the life stage of the animal. Both genetic and nutritional changes have led to substantial improvements in animal productivity and quality of product over the past half century or so. Indeed there has been a continuing and generally friendly rivalry between the practitioners of animal genetics and animal nutrition as to who should claim the most credit for the quite spectacular improvements in productivity in the more intensive poultry, pig and dairy industries. One study in poultry has attempted to quantify rigorously the relative contributions of genetics and nutrition to improved performance. It compared highly selected broiler chickens from 1991 with a 1957 control strain, using a feed comparison in which each strain was fed either a 1957 dietary formulation or a 1991 diet (Haverstein *et al.* 1994). The answer, I hesitate to say at a nutrition meeting, indicated that about 80% of the improvement in broiler growth performance between 1957 and 1991 chickens was attributable to genetic improvement, with 20% attributable to better nutrition. For dairy cattle, the results are more encouraging for nutritionists. BLUP analysis of data from about 1975 till 1995 for the University of Sydney dairy herds, which permits measurement of genetic trends, indicates that about 50% of the improved performance is genetic with the remainder due to improved nutrition and management (Nicholas pers.comm).

However, this simple 'adversarial' view of these two disciplines is not very helpful and we need to ask some apparently simple but in reality profound questions. Why are there large differences between animals in their responses to the same nutritional environment that allow us to select, for example, for increased growth rate? What is the nature of the genetic differences between some animals, which become fat when fed a particular diet, and other animals, which remain lean when fed the same diet? Why do some animals have a genetic predisposition to eat a lot and others to eat less? Why is it that genetically improved strains will generally perform well only if they are maintained on an improved nutritional regime? Why do such genetically improved animals tend to have increased sensitivity to nutritional stresses, which could predispose them to metabolic or infectious diseases? The tools for gaining answers to these questions are at last becoming available to animal geneticists as we begin to make substantial inroads into understanding and cataloguing the entire genomes of mammals and birds. An especially important tool at the moment is genetic mapping as this allows us to recognise the existence of genes relevant to these questions and sets us on the trail to identifying them and exploiting them.

The interface between nutrition and genetics is rife with the phenomenon which quantitative geneticists term 'genotype x environment interaction'. Genes which

are advantageous in one environment, for example for efficient production of milk under pasture based conditions, may not be advantageous in a different environment, for example where cattle are fed concentrates. Genotype by environment interaction means that different genes will affect productivity in different ways in different environments. The best animals in one environment may not necessarily be the best in another. This causes an unpredictability of performance when animals are moved from one environment to another. In such circumstances, it will be important to match the genotype of the animals to the nutritional and management environment to which they will be exposed. Ideally we would like to be able to identify the genes which are responsible for these differences in performance in different environments. There is a continuing need for nutritionists and geneticists to work alongside each other to optimise animal productivity. An understanding of the genes involved in different responses to nutrition will help both.

Particularly good examples of this complex interface between nutrition and genetics can be found in studies of human obesity. The increasing incidence of obesity in human populations, which has reached 30% or more in North American populations and is still increasing, can be directly related to the ready and cheap availability of energy–dense food in western society. To that extent, obesity is a simple nutritional problem. The genetic complication is that not all people are equally likely to become obese in the face of the same availability of food. In fact, it has been hypothesised that thrifty genotypes, which may be advantageous in conditions of food shortage, will be quite disadvantageous in conditions of plenty, leading to obesity and related health problems. An enormous amount of human genetical research is setting out to identify genes for obesity. For the first time, genetic mapping is allowing us to get a glimpse of these genes. However, it should be realised that what we are seeing is still the result of a complex interaction between genetics and nutrition. In conditions of universal shortage, obesity would not exist. If current projections from North American populations continue, then it seems that eventually the entire human population will be obese. In neither extreme scenario would there be much opportunity for expression of obesity 'susceptibility' genes. In suitable circumstances, environmental factors can overrule genetic predispositions.

In the remainder of this review, I wish to consider the various genetic and molecular biological tools for asking genetic questions relevant to nutrition. First I will address strategies for mapping genes that relate in some way to nutrition. These include loci influencing appetite, food conversion efficiency, growth rate, fatness, fat distribution and so forth. Some of the apparent 'nutrition genes' may not be directly involved in digestive or metabolic processes. For example, it could be that genes involved in superior food conversion efficiency are loci for resistance/susceptibility to chronic, subclinical infection. I will also deal briefly with techniques for directly identifying genes which are switched on or whose expression is amplified in response to nutritional changes (or to selection for improved growth rate or food conversion efficiency).

The gene mapping approach

For many years, breeders and researchers have been estimating heritability for continuously distributed traits in domestic animals. These include traits like food conversion efficiency and various measures of fatness. These estimates show that a substantial proportion of the variation in a population is genetically determined. In the pig for example, heritability of back fat thickness is very high at about 70%, and even for food conversion efficiency, the heritability is 50%. These heritability estimates have been and continue to be utilised for predicting response to selection and designing breeding programs. More importantly, the estimates have been used in the calculation of Estimated Breeding Values (EBVs), which are the basis for ranking and selecting animals for breeding. Such breeding programs have been extremely successful and have substantially improved the productivity of animals for numerous economically important traits. However, until quite recently, it was not possible to investigate the genes underlying this genetically determined variation. Traditional quantitative genetics, as pioneered by R.A Fisher, was predicated on a model assuming that an infinitely large number of genes of infinitesimally small effect were responsible for the observed quantitative variation. The theory, developed from this model, predicted selection responses in breeding programs very well. As there were no tools available to test for the existence of genes with moderately large effect on performance until relatively recently, so there was no possibility of searching for them. Back in the 1960s, *Drosophila* researchers, most particularly Thoday's group in Cambridge, pioneered the use of mapped genetic markers in this model organism to search for genes or genetic regions responsible for observed quantitative variation. It soon became apparent that a relatively small number of genes or regions on chromosomes was responsible for a substantial proportion of the genetically determined continuous variation for many traits (Spickett and Thoday 1966). However it was to be another quarter of a century before a suitable genetic marker technology was developed for domestic animals and humans which permitted the detection and mapping of genes and genetic regions responsible for quantitative variation in these species. A new term, quantitative trait locus (QTL), was introduced to describe the loci or groups of loci responsible for this variation. With this gene mapping technology in hand, a search has begun for QTL affecting numerous traits, including nutrition–related traits like food conversion efficiency, growth and fatness. The critical breakthrough was the isolation and mapping of hyperpolymorphic microsatellite genetic

markers in many species, enabling the construction of dense genetic maps. These markers are so variable and so numerous that it is possible to choose a subset of the markers evenly distributed across all chromosomes to enable scanning of the whole genome for evidence of QTL. Crosses between outbred animals within commercial populations or human nuclear families can provide sufficient information to detect QTL influencing the trait of interest by testing for cosegregation of marker alleles and QTL alleles. Thus a completely systematic and universal procedure is now available to search for genes influencing nutritionally related traits in animals, or any other traits for that matter. A resource population is required in which the appropriate performance has been measured, as well as DNA samples from the entire pedigree (two or three generations) and a set of informative markers. Sophisticated and continually improving analytical procedures can then be used to analyse jointly the genotypic and performance data.

Candidate genes

An attempt to shortcut the gene mapping approach

Candidate genes are those whose role in a physiological, metabolic or regulatory processes suggest that their allelic variants could cause variation in a phenotype related to that process. As a crude example, one might wish to test genetic variants of a digestive enzyme, like alanyl aminopeptidase N, to see if they have any influence on a trait like food conversion efficiency. The problem with this approach is the enormous number of candidates, even among currently known genes. To make matters worse, we still know the identity and function of only about 15% or less of all genes in mammals. When the Human Genome Project is completed in about 5 years time, we will then know the identity of all the 70,000 to 100,000 genes in the human/ mammalian/ vertebrate genome. We will have an even bigger problem then in choosing among this surfeit of candidates for explaining inherited quantitative variation.

There are two approaches to refining the candidate gene approach. First we can confine our attention to candidate genes where mutations in at least one species have already been shown to have a large effect on the trait, easily observed segregating in families. A good example in the nutritional context of such a monogenic trait is the *obese (ob)* mutation of mice. This mutation causes severe obesity. Recognition that the locus encoded the gene product, *leptin*, caused a flurry of interest in testing any genetic variants of leptin for more subtle influence on fat deposition in many other species including humans and pigs. Interest is not restricted to large monogenic effects causing gross obesity, but to determining whether other variants at the locus might have minor to moderate effects which cannot be so easily observed. This extends even to non–coding variants located within introns or 5' or 3' untranslated regions, which might be associated with undetected coding variants.

The other way of refining the candidate gene approach is to combine the results of QTL mapping with detailed and comprehensive information on the position of all genes in the genome. For example, the Human Genome Project will soon provide the full DNA sequence of the human genome. From this, the existence and position of all genes, including many genes now unrecognised, can be deduced. Even if a human QTL is only inaccurately mapped to a particular chromosomal interval, this information will enable focussing on a subset of candidates located in that interval, excluding the vast majority of genes lying outside of this interval. Total genome sequencing is neither feasible nor necessary in animals. Fortunately very good comparative maps have been made between the human and most domestic animal genomes (for example, see Goureau *et al.* (1996) for a description of the human/pig comparative map). If a QTL is located in an animal species within a particular chromosomal region, it will be possible to focus on the relevant set of human positional candidates, since the corresponding human chromosomal region will be known.

Identifying genes for obesity in humans

Chagnon *et al.* (1998) have comprehensively reviewed the evidence for loci related to obesity from human, mouse, rat and domestic animal studies and have converted the results from all species into human chromosomal locations, using comparative maps. Some human chromosomes have at least three putative loci related to obesity on both arms (1, 2, 6, 8, 11, and 20) and several on one chromosome arm only (3p, 4q, 5q, 7q, 12q, 13q, 15q, 15p, 22q, and Xq). Clearly there are many genes influencing obesity and many more are likely to be discovered. Nevertheless it should be stressed that these genes will explain variation only in some families in some populations.

Comuzzie and Allison (1998) have listed candidate genes for human obesity and body composition identified from animal models and physiology. Table 1 summarises information on these candidates. Despite their physiological plausibility or clear importance in animal models, there is little convincing evidence for involvement of most of these in human obesity. This is an important lesson as it reflects both the difficulty of picking candidates and the different relative importance of variation in specific genes between species. There is also the problem that variation in these genes may be important in some populations or families of humans, but not in others. For example, human mutations at the POMC locus (Krude *et al.* 1998) have been identified that abolish its expression, thus preventing production of some or all of adrenocorticotrophin (ACTH),

melanocyte–stimulating hormones (MSH) alpha, beta and gamma, as well as the opioid–receptor ligand beta-endorphin which are derived from POMC. These mutations, found in a very small number of families, have defined a new monogenic endocrine disorder characterised by early–onset obesity, adrenal insufficiency and red hair pigmentation. The absence of alpha–MSH means that the brain melanocortin–4–receptor (MC4–R) is not activated to regulate food intake. Interestingly a QTL for human obesity has been detected independently on chromosome 2 in close proximity to the POMC locus suggesting that minor variants at this locus may have smaller effects on obesity.

While many genes cause obesity, mutation at the *mahogany* locus in the mouse is known to have an anti–obesity effect (Dinulescu *et al*. 1998). Homozygosity for the *mg* mutation blocks obesity, hyperinsulinemia and increased linear growth induced by other mutations, such as *agouti* (MC1R), by an increase in basal metabolic rate. Despite the fact that it induces hyperphagia, *mahogany* also can suppress diet–induced obesity. The *mahogany* protein is a single–transmembrane–domain protein expressed in pigment cells and the hypothalamus. The extracellular domain of the protein is orthologous to human attractin, which has been implicated in immune–cell interactions (Nagle *et al*. 1999; Gunn *et al*. 1999) but its precise role in regulating appetite and metabolic rate remains to be elucidated.

Fatness QTL in mice

Numerous monogenic conditions have been described in mice affecting obesity and feeding behaviour. Additionally, much effort has also been put into identifying genes of smaller effect usually by making crosses between strains differing considerably in the trait of interest. From analysis of progeny from a cross between strains of mice with approximately 8 and 18% body fat respectively, Mehrabian *et al.* (1998) found strong evidence for QTL for obesity–related traits (two for subcutaneous fat and one for percent lipid). These QTL were mapped to three different positions on chromosome 2 (Figure 1), with a single QTL for percent body fat mapping to chromosome 9. The fatness effects on chromosome 2 co–localise with effects on hepatic lipase activity, which is known to be associated with visceral obesity and lipoprotein levels in humans. The fatness QTL effects are probably mediated via this enzyme. Interestingly a QTL for plasma leptin level was identified on chromosome 4 but this had no effect on fatness. Sixteen QTL for fat pad weights or percent body weight as fat were reviewed in this study and were located on mouse chromosomes 1, 2, 4, 6, 7, 9,12, 15 and 17. QTL on chromosomes 2, 7 and 15 were confirmed in independent studies using different stains of mice. Numerous other potential QTL were detected in these studies but did not satisfy stringent significance criteria required to make strong claims for their existence. Again it is noteworthy that many chromosomal locations are

Table 1 Candidate genes for obesity (from Comuzzie and Allison 1998).

Gene	Phenotype	Human location
Agouti signalling protein (ASIP)	obesity	20q11.2–q12
Carboxypeptidase (CPE)	obesity	4q28
Leptin (LEP)	obesity	7q32
Leptin receptor (LEPR)	obesity	1p31
Tubby (TUB)	obesity	11p15.4–p15.5
Uncoupling protein 1 (UCP1)	energy balance	4q31
Uncoupling protein 2 (UCP2)	energy balance	11q13
Uncoupling protein 3 (UCP3)	energy balance	11q13
Melanocortin receptor 3 (MC3R)	feeding behaviour	20q13
Melanocortin receptor 4 (MC4R)	feeding behaviour	18q21.3–q22
Pro–opiomelanocortin (POMC)	obesity	2p23.2
Neuropeptide Y receptor 5 (NPYR5)	appetite regulation	4q31–q32
Myostatin (MSTN)	skeletal muscle growth	2q32.1
Cholecystokinin A receptor (CCKAR)	satiety	4p15.1
Tumor necrosis factor α (TNFA)	obesity	6p21.3
Peroxisome proliferator activated receptor γ (PPAR–γ)	adipocyte differentiation	3p25
Beta–3–adrenergic receptor (ADRB3)	adipocyte differentiation	8p11.1–p12

responsible for variation in these traits and also that the QTL variants are frequently strain or population specific. The gene(s) underlying these QTL effects remain to be identified. Until the genes are found and characterised, our nutritional and physiological understanding of the processes involved will remain unenlightened, despite the enormous genetic progress. Inevitably the genes will be identified and, quite likely in the short to medium term.

QTL studies in pigs

The first ever report of QTL in domestic animals originated from the laboratory of Leif Andersson in Sweden (Andersson *et al*. 1994). An F2 derived from crosses between Large White and Wild Boar revealed evidence for QTL on chromosome 4 for backfat, abdominal fat and intestine length. Interestingly, Large White have intestines which are several metres longer than in Wild Boar and the difference is suspected to influence food conversion efficiency. The first report of fatness QTL in pigs generated considerable interest in laboratories investigating human and domestic animal fatness.

Are these QTL found by crossing domestic pigs with Wild Boar found in other crosses or in economically relevant populations? Rohrer and Keele (1998) reported detection of QTL affecting fat deposition in a backcross resource pedigree from Chinese (Meishan) and European pigs. They detected significant QTL on chromosomes 1, 7 and X, but not on chromosome 4. Surprisingly the QTL allele from chromosome 7 inherited from the fat Meishan parent produced leaner pigs, corroborating results found earlier in German studies (Moser, pers. com). This has been termed cryptic QTL variation as the favourable 'lean' gene is hidden amongst many unfavourable genes for fatness in the Meishan parent. Although quantitative geneticists have predicted the existence of such cryptic variation for many years, this proof of principle in animals should provide additional impetus to conservation of rare breeds of domestic animals, as most breeds would be expected to harbour beneficial cryptic genes for some traits.

At the University of Sydney, my laboratory has been engaged in gene mapping and QTL studies in the pig for a number of years. Part of our work has involved an international collaboration with a group in Germany, which has bred a resource by making F2 crosses between Wild Boar, Meishan and Pietrain. Our results (Lee *et al*. 1998) from a preliminary scan of porcine chromosome 2 from the Pietrain by Meishan component of the German resource pedigree are shown in Figure 2. The three QTL illustrated all satisfy the most stringent genome–wide criteria for significance. However there are several important limitations of QTL mapping illustrated in this figure. First the peaks are broad and the position of the QTL is correspondingly inaccurately estimated. It is now known that increasing the number of markers genotyped or even increasing the sample size of the F2 or backcross rapidly runs into an information plateau, providing little improvement in

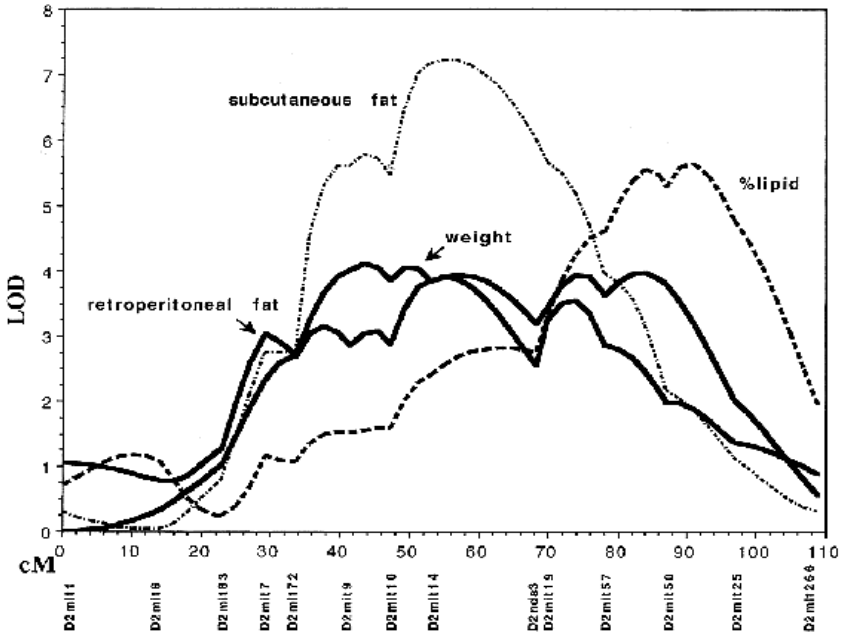

Figure 1 Results of a typical QTL scan in mice. Mice derived from a cross between fat and lean strains have been used in a scan of chromosome 2. There is evidence for QTL for weight of subcutaneous fat and peritoneal fat, % fat and bodyweight, with lod scores of 4.3 or greater interpreted as highly significant evidence for a QTL. The position of microsatellite markers is indicated on the X axis (from Mehrabian *et al.* 1998).

estimation of QTL position. Refinement of map position of QTL can only be obtained by breeding generations beyond the F2 or backcross generation. By gradually eliminating markers at the extremes of the estimated QTL position, it is possible to refine the map position. Leif Andersson's group in Sweden has been doing this for some years with the chromosome 4 fatness QTL. After five generations, they have refined the position of this QTL (Andersson pers. comm.). Another problem inherent in QTL mapping is that of pleiotropy, that is the tendency of the same genes or genetic variants to affect different traits. For example, are the three QTL illustrated in Figure 2 really three different genes or sets of genes or are they simply pleiotropic manifestations of the same underlying QTL, which happen to influence all three related traits? Once QTL are mapped, animal geneticists and breeders can exploit them directly in marker assisted selection (MAS). However nutritionists and physiologists will be much more interested in identifying the underlying genes responsible for the quantitative variation. In theory, this is possible via a strategy known as positional cloning. In practice, this is severely constrained by the inaccuracy with which QTL positions are estimated. A five to tenfold improvement in determination of QTL position, relative to that obtained from F2 and backcross resources, will be necessary before positional cloning becomes a reality for the genes underlying QTL. In the meanwhile, evaluation of positional candidates or comparative positional candidates is more likely to identify the genes involved.

In collaboration with colleagues at the Animal Genetics and Breeding Unit at the University of New England and at Bunge Meat Industries, my laboratory has also been searching for QTL for numerous traits in pigs using commercial populations of Large White and Landrace derivation in Australia (Kerr *et al*. 1999). While intellectual property constraints prevent disclosure of map positions, there is clear evidence in some sire families for the existence of QTL affecting backfat and other traits, which may correspond to QTL found in other studies employing wide crosses. Thus QTL for these nutritionally related traits can be found even in commercial populations.

Of course, interest is not restricted to mapping genes for fatness in pigs. QTL influencing other nutritionally relevant traits have been mapped. To cite but one recent example from many, Paszek *et al.* (1999) have reported a highly significant QTL for growth from weaning to 56 kg in a cross between European and Chinese pigs. This QTL which maps to chromosome 1 has an estimated additive effect of 31 grams per day. Arrest–Specific Gene–1, Transforming Growth Factor–beta Receptor, type 1 and Insulin–like growth Factor 1 Receptor are interesting candidates from the interval in which this QTL has been localised, although of course it is quite likely that some other possibly uncharacterised gene(s) is responsible for the effect observed.

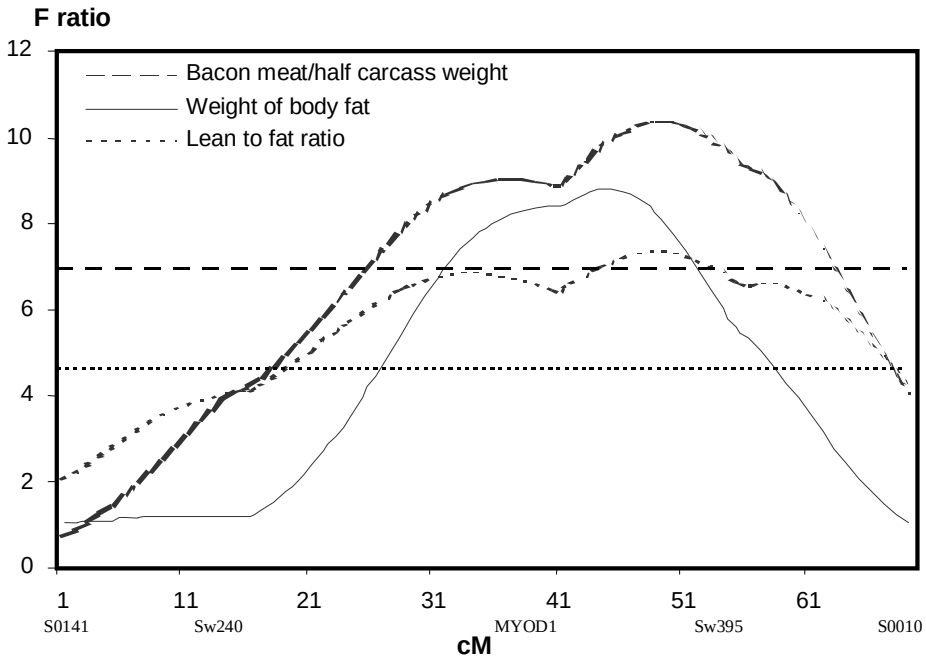

Figure 2 Scan of porcine chromosome 2 for fatness and body composition QTL using the F2 of a cross between German Pietrain and Chinese Meishan pigs. Peaks exceeding the dashed line are significant at the very stringent genome wide level.

Differential display

A rapid technique for identifying genes whose expression is changed by nutritional or genetic manipulation

Molecular geneticists have been assaying the effects of environmental, developmental or tissue–specific variables on the level of expression of genes for many years. Quantitative Northern analysis and quantitative RT–PCR have provided the tools for evaluating factors increasing or decreasing expression of specific loci. This means that in theory it is possible to test the effect of any nutritional variable on the expression of any gene. LeFebvre *et al.* (1998) describe analysis of gene expression in a nutritional context, comparing expression levels of 15 genes related to fat metabolism between omental and subcutaneous fat depots and between obese and non–obese humans. Four genes showed substantially different levels of expression between the fat depots, but the most interesting finding was that peroxisome proliferator activated receptor-gamma (PPAR–gamma) mRNA levels were significantly lower in visceral adipose tissue in non–obese, but not in obese subjects. PPAR–gamma expression is increased in omental fat in obesity and could be responsible for expansion of adipose tissue. Cheema and Cladinin (1996) describe a similar type of study in mice where the mice were fed either low fat or high fat diets and the dietary influences on expression of genes encoding lipogenic enzymes were measured in both lean and obese mice. Arrays are now being developed by many biotechnological supply companies, which allow screening of the expression of hundreds of genes simultaneously. Carefully controlled amounts of unique DNA fragments from these loci are arrayed on nylon membranes. Radioactively labelled probes are generated from RNA pools using carefully chosen gene–specific primers. The level of binding of the probe to the target on the membrane provides an index of the expression of the gene. However, until we have arrays representing all genes and methods for probing their expression, the biggest problem is choice of candidate genes for inclusion in the gene expression assays. Gene chip technology may provide the answer to this dilemma in the future, at least for humans, after all genes have been identified from genome sequencing projects. Gene chips have the potential to assay expression of thousands of loci simultaneously. In the meanwhile, choosing candidates for inclusion in expression assays is fraught with the same problems as choosing candidates for QTL: there are far too many loci to choose from and the most important loci may be ones of whose existence we are unaware.

Is there some systematic way for assaying variation in expression of any gene in response to such variables, even for unknown genes, analogous to genome scanning for searching for QTL? Fortunately methods exist for doing just this. It is possible to screen for variation in expression of all genes, remarkably even including totally unknown and uncharacterised genes. What is even better, this methodology also provides a means for identifying and characterising these previously unknown genes. The technique is differential display. RNA must be obtained from treatment and control samples. For example, we might wish to purify RNA from the liver or brains of animals fed a control diet or a special diet, for example high in saturated fat. This RNA is then used for reverse transcription and finally for quantitative PCR amplification using sets of small random primers, which have the capacity to amplify fragments from any gene. The PCR products from each primer set are usually radioactively labelled, and separated by electrophoresis. The resulting autoradiographs show a complex but reproducible pattern of bands, similar to an old style DNA fingerprint. The bands correspond to fragments derived from mRNAs of genes expressed in that tissue. Comparisons are made of band intensity between the control and treatment samples, and any bands which show a consistent difference in intensity (level of expression) between control and experimental samples are isolated, cloned (Wan *et al*. 1996) and sequenced. This may enable identification of the gene involved, if the gene has been previously discovered in any species, and will allow isolation of full–length clones, if it is a novel gene requiring further characterisation. There have been few reported applications of this technique in the nutritional context. Maratos–Flier *et al.* (1997) have reported its use to scan for differences in gene expression between the brains of obese and non–obese mice. It has enormous potential for identifying virtually any gene whose expression is altered by a nutritional modification of an animal's environment. It has wider usage than this though. For example, gene expression levels can be compared between lines of animals selected for obesity or leanness, for high food conversion efficiency or low or indeed for any divergent lines of animals. The technique works best when the genetic background of the animals being compared is similar but has potential even for divergently selected lines of outbred livestock species. Surprisingly, an important limitation of the differential display technique is that it is so efficient at detecting changes in expression caused by the environmental or genetic manipulation, even for genes well downstream of those directly affected. This means that a rigorous process of evaluation of the importance and causal involvement of the differentially expressed gene is still required. Of course, it will also be important to genetically map newly discovered, differentially expressed genes discovered by differential display to determine whether they are positional candidates for QTL.

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