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The Utilization of Bacterial Genes to Modify Domestic Animal Biochemistry

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The ability to transfer genes between organisms in a way that maintains their inheritance and their function makes it possible to modify the biochemistry of domestic animals to improve productivity. One example of this is the modification of sheep biochemistry to provide an increased supply of the amino acid cysteine which is the first limiting amino acid in wool growth. The *cysE* and *cysK* genes were isolated from *Escherichia coli* and modified for expression in animals. In transgenic mice, the genes were transcribed and translated to produce two functional enzymes, serine transacetylase and *O*-acetylserine sulphydrylase which together were able to catalyse the biosynthesis of cysteine in transgenic mice provided that the mice were fed a diet containing a small amount of sodium sulphide. When placed on a diet that was deficient in sulphur-containing amino acids, non-transgenic control mice lost substantial amounts of hair and also lost body weight while the transgenic mice showed no hair loss and continued to grow at normal rates.

The modified genes have been transferred to sheep where they have been shown to integrate with the host genome and can be passed to progeny in normal ratios of Mendelian inheritance. However, the genes were only expressed in one animal and this expression was very low. The results are similar to those obtained in parallel experiments carried out in another laboratory and suggest that a functional cysteine biosynthesis pathway might interfere with the intermediary metabolism of sheep whereas apparently it can operate effectively in transgenic mice.

Introduction

While scientists and farmers generally agree that genetic engineering has the potential to improve domestic animal productivity, few projects have yet achieved commercial viability. Many laboratories have for some time been

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engaged in commercially oriented transgenic animal projects and this poor strike rate suggests that there is some underlying factor, so far largely ignored, that is influencing the process in domestic animals. Technical difficulties provide part of the explanation because the procedure of gene transfer by pronuclear microinjection in domestic animals is less efficient than in laboratory mice, thus reducing the number of transgenic animals available. However, with sufficient effort it is possible to produce enough transgenic domestic animals to adequately test for appropriate phenotypes, so the difficulty of achieving commercial goals is due to more than problems of technique.

A more insidious problem may be associated with the actual goals of the research to date. Most domestic animal-oriented genetic engineering research projects are directed towards improved animal productivity, either by increasing growth rates, altering body composition or the composition of an animal product, or by improving the health or husbandry requirements of the animals. To achieve this it is usually necessary to modify some component of the animal's physiology, thus potentially altering the existing delicate balance of nutrition, endocrinology and metabolism. Since this balance has been established through many generations of selection for superior performance and environmental compatibility, it represents a wide range of optimized gene combinations that are difficult to perturb without causing unexpected deleterious effects on animal phenotype. This suggests that the foreign genes that are introduced in domestic animal genetic engineering projects should be designed to make small changes to animal homeostasis so that the existing equilibrium is only slightly altered. The possibility exists that most projects so far attempted in this new and rapidly developing field of endeavour have attempted changes of a magnitude too great for assimilation by the optimized target animal genotype, thus reducing productivity and, in consequence, commercial viability of the resulting animals. Genetic manipulations that have as their goal the modification of target animal biochemistry might be expected to be particularly sensitive to this problem because they have the potential to alter the complex balance of substrates and co-enzymes that are integral to the smooth operation of intermediary metabolism. However, there are cogent reasons for attempting such biochemical alterations in some of our domestic animals and hence it is worth the effort to examine whether the problems of homeostatic imbalance can be overcome.

During the long evolutionary history of each of our major domestic species there has been a significant loss of biochemical capacity when compared with simple auxotrophs, resulting in the inability of animals to synthesize a wide range of enzyme substrates and cofactors. Whenever these are crucial to animal survival, they must be supplied in the diet as essential nutrients and vitamins. It can be argued that the loss of biosynthetic capacity provides an overall advantage to the animal as long as the nutrients in question are freely available in the diet, because the energy associated with

de novo synthesis is available for other purposes. Thus during any prolonged selection regime, be it natural or artificial, animals would, in general, perform better and hence be preferred for selection when their available energy was directed towards those compounds that could not be adequately provided by diet, with any excess being utilized for growth and survival in the wild or for production when domesticated. If this is correct, it may be necessary to trade some growth or fitness qualities for a gain in a particular production characteristic if specific biochemical pathways are replaced, provided that this trade-off is not to the detriment of the animal's health and welfare. Until recently, this concept has only been of theoretical interest, but with the advent of genetic engineering techniques, the restoration of lost biochemistry in animals is a genuine possibility. The inability to synthesize nutrients *de novo* normally results from the loss of the genes encoding critical enzymes of the relevant biosynthetic pathways, and functional counterparts to these genes can be readily identified in most auxotrophs. By modifying the functional genes for expression in a target domestic animal species, the missing enzymes can in theory be made available to catalyse the appropriate intermediary metabolism.

The Application of Biochemical Manipulation to Wool Production

The Australian wool industry might be helped significantly by the addition of a new biochemical pathway to the Merino sheep. The primitive breeds of sheep from which the Merino has been derived were poor wool producers. Their coats were characterized by short fibres which underwent cyclic annual growth and were generally shed each year. From this stock has been bred the modern Merino sheep which grows wool continuously and shows little evidence of annual cyclic growth. Under average conditions Merino strains in use today can produce about 3.5 kg of wool each year. However, this imposes a substantial demand on the supply of the amino acid cysteine, because wool fibres are composed of a complex mixture of keratin proteins which are characterized by a high cysteine content. For example, to produce 3–3.5 kg of wool each year requires a supply of 0.7–0.9 g day^{-1} of cysteine for wool growth alone and this represents about 50% of the total cysteine utilized by the sheep (Lee *et al*., 1993). This cysteine requirement must be satisfied either by transsulphuration from methionine or from the diet of the sheep (Black and Reis, 1979; Lee *et al*., 1993). Normal pastures are able to satisfy this cysteine demand but under conditions where nutrient supply is poor, cysteine is the first limiting substrate for wool growth (Reis, 1979).

A possible solution to this substrate limitation is to provide sheep with the enzymes necessary for cysteine biosynthesis, provided that the enzymes can be synthesized in tissues which also contain the appropriate substrates

for the operation of this pathway. These substrates consist of the amino acid serine, the enzyme cofactor acetyl-coenzyme A (acetyl-CoA) and a source of inorganic sulphur. In the ruminal and intestinal epithelia, all three of these substrates are present, the sulphur source being in the form of sulphide produced by bacterial fermentation in the rumen.

Isolation and Modification of Bacterial Genes Encoding Cysteine Biosynthesis

The genes encoding the enzymes for cysteine biosynthesis are functional in the bacterium *Escherichia coli* and it is therefore theoretically possible to isolate the relevant coding sequences from this organism and modify them for expression in eukaryotes. The cysteine biosynthetic pathway in *E. coli* is a complex pathway in which inorganic sulphur is reduced to sulphide (Umbarger, 1997) and then combined with the amino acid serine in two biosynthetic steps to produce cysteine. To avoid the pathway being constitutively active, it is regulated at the enzyme level by cysteine and *O*-acetylserine concentrations and at the transcriptional level by cysteine repression and *O*-acetylserine induction (Umbarger, 1997).

Since the sheep already has a sulphide source available, the portion of the pathway that is required is the component that catalyses the conversion of serine to *O*-acetylserine and *O*-acetylserine to cysteine. The enzymes required are serine transacetylase (SAT) and *O*-acetylserine sulphydrylase (OAS), encoded by the *cysE* and *cysK* genes in *E. coli*. Their isolation and characterization have been described in detail elsewhere (Denk and Bock, 1987; Byrne *et al*., 1988) as has the modification of the genes for expression in eukaryotes (Sivaprasad *et al*., 1989; Leish *et al*., 1993; Bawden *et al*., 1995). In summary, the coding sequence for the *cysE* gene and for the *cysK* gene were each fused to sheep metallothionein-Ia (Mt-Ia) promoter sequences and exon 5 of the sheep growth hormone gene then fused 3′ to each coding sequence. The two genes were then joined to provide a single piece of DNA (*MTCEK1*) which encoded both the SAT and OAS enzymes as shown in Fig. 12.1a.

In parallel experiments in another laboratory, similar gene constructions were also prepared using the equivalent genes isolated from the bacterium *Salmonella typhimurium* (Bawden *et al*., 1995). In some experiments, the *cysM* gene, which encodes a slightly different OAS enzyme in *E. coli* and *S. typhimurium*, was used in place of the *cysK* gene. A range of gene constructions have been made with these *Salmonella* genes (Bawden *et al*., 1995), the major difference between these and the gene *MTCEK1* being in the use of different eukaryotic promoter sequences.

In order to establish that these genes are able to direct the synthesis of the enzymes needed to catalyse cysteine biosynthesis in eukaryotic cells, they were used to transfect cells in culture. The results obtained with the

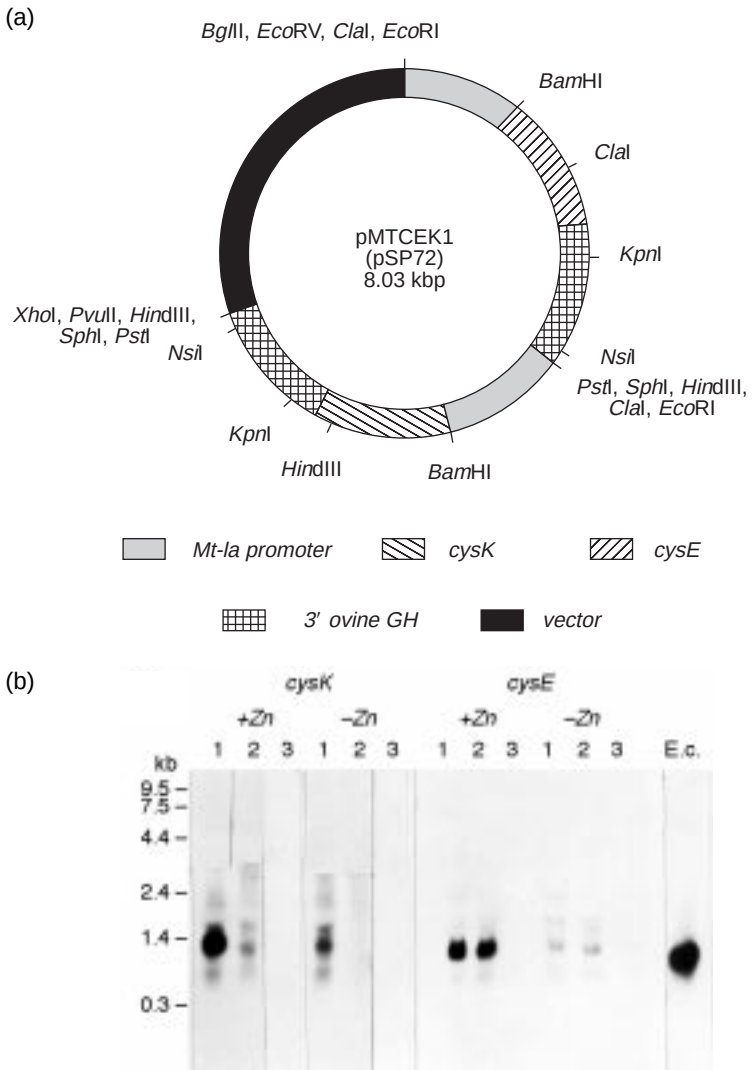

Fig. 12.1. (a) Structure of the plasmid MTCEK1. (b) Northern analysis of RNA from cells transfected with the gene *MTCEK1* (lane 1), with a mixture of two plasmids encoding *cysE* and *cysK* (Leish *et al*., 1993) (lane 2) or with vector pSP72 (lane 3). All transfections included the plasmid pSV2neo. RNA (40 μg) from transfected L-cells was fractionated on a 1.5% agarose formamide gel, transferred to a nylon membrane and hybridized with a *cysK* or *cysE* ^{32}P-labelled probe. RNA from *E. coli* induced for cysteine biosynthesis (lane E.c.) was used as a control.

gene *MTCEK1* inserted stably into mouse L-cells is shown in Fig. 12.1b and Table 12.1 (Leish *et al*., 1993). In Fig. 12.1b, the mRNAs specific for the *cysE* and *cysK* genes are readily detected in Northern blots of RNA preparations

Table 12.1. Enzyme activities of serine transacetylase and *O*-acetylserine sulphydrylase in mouse L-cells containing the gene *MTCEK1* (nmol of substrate (acetyl-CoA) degraded or of product (cysteine) formed per mg of protein in 30 min).

+ Zinc		− Zinc	
Serine transacetylase	*O*-acetylserine sulphydrylase	Serine transacetylase	*O*-acetylserine sulphydrylase
268 +/− 92	6960 +/− 829	86 +/− 33	1242 +/− 160

made from zinc-induced cell cultures. In Table 12.1, both enzyme activities are readily detected in extracts of the cells; these data are summarized from Leish *et al.* (1993). Results are expressed as nanomoles of the substrate degraded (acetyl-CoA) or the product formed (cysteine) per milligram of protein in 30 min. The values represent the means from two experiments corrected for the endogenous rates of substrate degradation with standard errors included.

This demonstrates that the bacterial genes can be regulated in eukaryotic cells by the sheep Mt-Ia promoter and, furthermore, that the bacterial codons enable efficient translation of the mRNA and that the bacterial proteins are stable in the cytoplasm of these cells. Similar results have also been obtained with the various gene combinations isolated from *S. typhimurium* (Sivaprasad *et al.*, 1992), indicating that the source of coding sequence is not limited to *E. coli*.

In vivo Studies in Transgenic Mice

Once it had been established that bacterial genes modified in this manner were able to function effectively in eukaryotic cells, it was possible to test their activities in transgenic mice. All genes were inserted by standard embryo pronuclear microinjection procedures (Hogan *et al.*, 1986; Palmiter and Brinster, 1986) and the progeny of founder animals examined for expression in various organs. The results for the gene *MTCEK1* are reported here but similar results have been obtained for some of the *S. typhimurium* gene constructions (Bawden *et al.*, 1995). For the gene *MTCEK1*, nine primary transgenic mice were produced and four were bred to stable lines. Figure 12.2 shows the Southern blot analysis of three of these lines, showing the expected *Bam*H1 fragments of 2.69 kb for the *cysE*-containing fragment and 2.85 kb for the *cysK*-containing fragment. The *MTCEK1* gene was stable in these animals from generation to generation, showing no signs of rearrangement despite the significant stretches of identical sequence in each copy of the inserted gene.

Transcription of the gene *MTCEK1* was measured in intestinal epithelium by Northern blot analysis of total RNA (Fig. 12.3a) and in intestinal

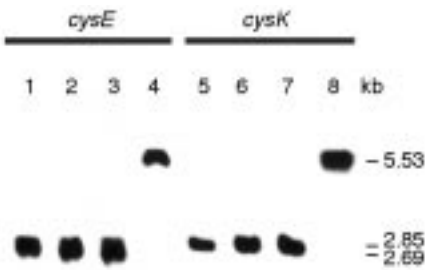

Fig. 12.2. Southern blot identification of transgenic mice carrying the insert from plasmid pMTCEK1. DNA was extracted from tail tissue by conventional techniques and digested with *Bam*H1. Membrane filters were probed with ^{32}P-labelled coding sequence for *cysE* (lanes 1–4) or *cysK* (lanes 5–8) genes. Lanes 1 and 5, mouse line EK8; lanes 2 and 6, mouse line EK28; lanes 3 and 7, mouse line EK46; lanes 4 and 8, pMTCEK1 insert DNA.

epithelium, skin, liver and kidney by reverse transcriptase (RT)-PCR analysis (Fig. 12.3b). The level of expression of the gene was different in the different lines, with EK28 showing the highest levels of mRNA. In Northern blots, the sizes of the mRNA transcripts were measured and shown to be 1.25 kb and 1.3 kb for the *cysE* and *cysK* genes. These sizes are slightly larger than the corresponding transcripts in *E. coli* (0.9 kb and 0.95 kb) and are the sizes predicted on the basis of the sizes of the bacterial coding sequences in combination with the 30 bp of Mt-Ia untranslated sequence, the ~250 bp derived from exon 5 of the sheep growth hormone gene and a poly(A) tract added post-transcriptionally. Thus, the gene appeared to be faithfully transcribed *in vivo* in a fashion analogous to that observed in cells in culture.

The demonstration that the mRNA transcripts from *MTCEK1* could also be detected by RT-PCR (Fig. 12.3b) provides a useful additional method for measurement because of the greatly increased sensitivity of this method compared with that of Northern blot analysis when expression is low. The primers CE1/CE3 and CK1/CK3 were designed for the analysis of the cDNAs synthesised from *cysE* and *cysK* mRNA transcripts. An additional primer, MT6, in combination with CE3 or CK3, provided a check for genomic DNA contamination of the mRNA preparations by amplifying a product from within the Mt-Ia promoter region of the gene *MTCEK1* (results not shown). The results in Fig. 12.3b confirm the Northern blot analyses and demonstrate the increased sensitivity of the RT-PCR method.

The translation of the *cysE* and *cysK* mRNAs in the mouse line EK8 is shown by the presence of the serine transacetylase and the *O*-acetylserine sulphydrylase in tissue extracts from zinc-induced mice (Table 12.2).

The highest enzyme levels were usually found in the intestinal epithelium, followed by kidney, liver and the skin. The enzymes were not detectable in non-transgenic animals, consistent with the proposal that they

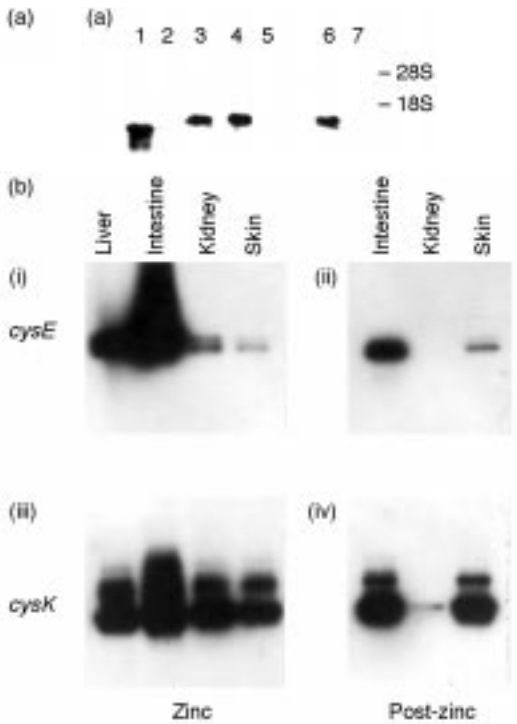

Fig. 12.3. (a) Northern blot analysis of the RNA isolated from intestinal tissue of zinc-fed transgenic mice containing the insert of plasmid pMTCEK1. Total cytoplasmic RNA was isolated from intestinal epithelium by the acid guanidinium thiocyanate–phenol–chloroform method (Chomznski and Sacchi, 1987), separated by electrophoresis on agarose gels containing 14% formamide and transferred to nylon membrane. Probes for *cysE* and *cysK* transcripts were antisense RNA sequences constructed from the coding sequences of the two bacterial genes. Lanes 1–5 were hybridized with a *cysK* probe and lanes 6 and 7 with a *cysE* probe. Lane 1, 4 μg *E. coli* C600 bacterial RNA; lane 2, non-transgenic mouse; lanes 3, 4, EK28 mouse, zinc treated for 3 months (lane 3) or 3 days (lane 4); lane 6, EK28 mouse, zinc treated; lane 7, EK28 mouse, minus zinc. (b) RT-PCR analysis of RNA isolated from EK28 mouse tissues zinc treated (i), (iii), or 3 days after removal of zinc treatment (ii), (iv). RNA was isolated as described above and 10 μg treated with ribonuclease-free deoxyribonuclease and incubated with Superscript reverse transcriptase (Gibco-BRL) to convert it to complementary DNA. The *cysE* and *cysK* transcripts were amplified by PCR in 50 μl volumes using the following primers; *cysE*; (CE1) 5′ATGTCGTGTGAAGAACTGGAA3′ and (CE3) 5′TTAGATCCCATCCCCATACAC3′. *CysK*; (CK1) 5′ATGAGTAAGATTTTTGAAGAT3′ and (CK3) 5′CTGTTGCAATTCTTTCTCAGT3′. The PCR programme consisted of 94°C for 3 min, 30 cycles of 94°C for 1 min, annealing temperature (65°C initially, decreasing by 0.6°C per cycle) for 2 min, 72°C for 1 min, 20 cycles of 94°C for 1 min, 45°C for 2 min, 72°C for 1 min, followed by a soak of 72°C for 7 min. Samples (10 μl) were separated by electrophoresis on 1.5% agarose gels, transferred to nylon membrane and hybridized with *cysE* or *cysK* ^{32}P-labelled probes.

Table 12.2. The specific activities of serine transacetylase and *O*-acetylserine sulphydrylase in tissue homogenates of zinc-induced transgenic mice containing the gene *MTCEK1*. Enzyme assays were performed as described in Leish *et al*. (1993) and results are expressed as in Table 12.1.

Organ	Serine transacetylase	*O*-acetylserine sulphydrylase
Intestine	2152	12,778
Kidney	105	128
Liver	9	3
Skin	3	312

are produced from transcripts of the *MTCEK1* gene and not due to bacterial contamination, an ever-present concern when working with tissues such as intestinal epithelium and skin. However, in order to provide a second line of evidence for the expression of the genes being derived from the transgenic mouse tissues and not from bacterial contamination, the *cysE* and *cysK* mRNAs were identified by *in situ* hybridization to sections of intestinal epithelium (Ward *et al*., 1994). A strong signal for expression of both mRNA species was obtained in tissues obtained from transgenic animals (Fig. 12.4), with the localization of the signal clearly within a subset of the intestinal epithelial cells themselves. No signal was detected in tissues from non-transgenic animals.

Cysteine biosynthesis in the intestinal epithelium of these animals was demonstrated as follows. The biosynthesis of the enzymes was induced by feeding the mice zinc-supplemented water, after which they were sacrificed and intestinal tissue dissected, rinsed and incubated *in vitro* with a small amount of radioactive $Na_2{}^{35}S$. The incubation medium was then analysed for the presence of radioactive cysteic acid. The results clearly showed the presence of radioactive cysteine in the incubations of intestinal tissue obtained from the transgenic mice but not in those from non-transgenic mice (Fig. 12.5, from Ward *et al*., 1994).

The Effect of *MTCEK1* Expression on Cysteine-deprived Mice

The level of expression of the SAT and OAS enzymes and the ability of these enzymes to catalyse cysteine from orally administered Na_2S in the transgenic mouse line EK28 suggested that these animals may be able to produce cysteine at levels that might be of dietary significance if they could be provided with a suitable source of sulphide. Accordingly, they were placed on an artificial diet (Leveille *et al*., 1961; Huovinen and Gustafsson, 1967) that contained only trace amounts of cysteine and methionine but which was supplemented with Na_2S as a sulphur source. The animals were approximately 5 months of age at the start of the experiment. After 2 weeks

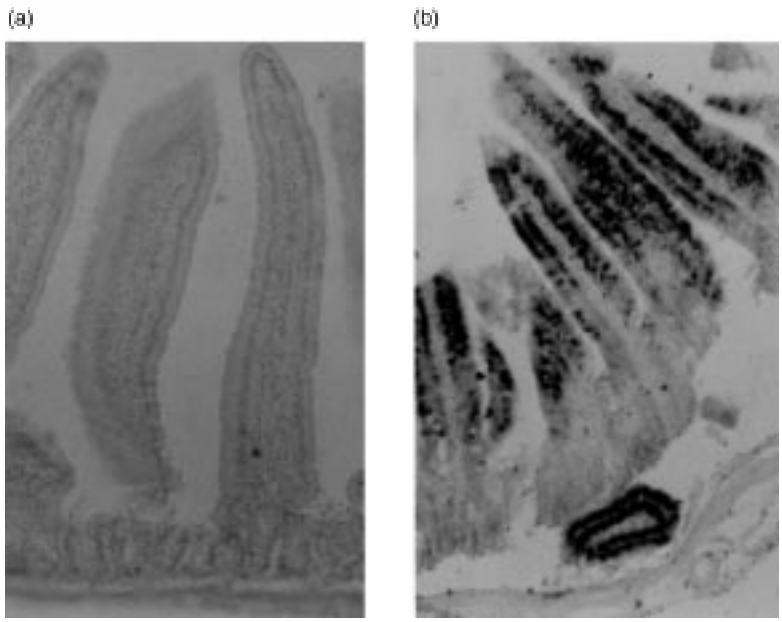

Fig. 12.4. *In situ* analysis of the expression of the gene *MTCEK1* in intestinal epithelia of transgenic mice. Small intestine of zinc-fed normal (a) and transgenic (b) mice was fixed in 4% paraformaldehyde–0.25% formaldehyde and sectioned at a thickness of 8 μm. An antisense *cysK* RNA probe was labelled with DIG (Boehringer Mannheim) and was hybridized and detected according to manufacturer's instructions using nitroblue tetrazolium (NBT).

on the sulphur-deficient diet, the most noticeable phenotypic response was a marked loss of hair in the control animals, while the transgenics showed a normal hair coat (Ward *et al*., 1994). The hair loss was most noticeable on the regions of the animals which were in the anagen phase of the hair cycle (Fig. 12.6). The experiments were repeated in more detail with a second line of transgenic mice, EK8, with detailed body weight measurements made during the time the animals were on a cysteine-restricted diet. The results of the hair growth measurements were generally consistent with the results obtained with the line EK28 and were also consistent with the observed weight changes (Table 12.3).

Thus, the control animals varied in appearance from overt hair loss to a severely dishevelled appearance and all lost weight during the period of cysteine deprivation. The transgenic animals in both groups I and II generally showed minimal disturbance to hair growth and increased in body weight during the same period, although some hair dishevelment was observed in a few of the transgenic males of group I. When this was observed, it was also correlated with a weight loss on the restricted diet.

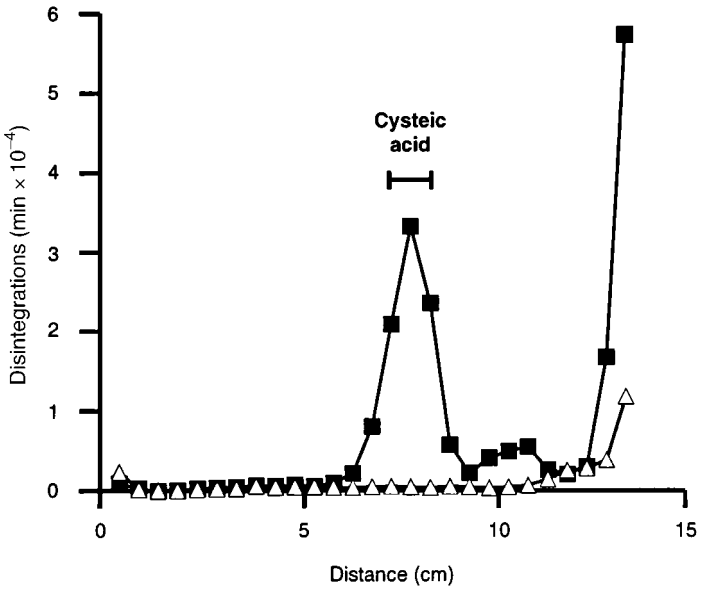

Fig. 12.5. The biosynthesis of cysteine from H_2S *in vitro* in intestinal tissue isolated from transgenic mice containing the gene *MTCEK1*. Small intestine (200 mg) from transgenic and non-transgenic mice was dissected, rinsed with 50 mM Tris buffer, pH 7.4 and incubated in 1 ml of Krebs–Ringer phosphate buffer, pH 7.4 at 37°C for 5 min. $Na_2{}^{35}S$ (5 μCi) was added and incubation continued for a further 15 min. The tissue was then removed and the incubation medium treated with 100 μl of dithiothreitol (100 mM) and 3 ml of performic acid at 4°C overnight. The solution was extracted with chloroform/mehanol (1:1) and the aqueous phase lyophilized and dissolved in 0.5 ml water. Amino acids were separated by paper electrophoresis at 10 V cm^{-1} in pyridine:acetic acid:water (1:10:90) (Huovinen and Gustafsson, 1967). The paper was cut into 1 cm strips and radioactivity determined.

Table 12.3. Weight changes associated with dietary deprivation of sulphur amino acids in transgenic mice containing the gene *MTCEK1*. Dietary details are as described in Fig. 12.6.

	Body weight change (g) (no. of animals)	
Experiment	Transgenic	Non-transgenic
I	+ 1.08 (10)	− 8.7 (14)
II	+ 1.9 (15)	− 7.6 (16)

Insertion of the Cysteine Biosynthetic Pathway into Sheep

The results obtained with the various cysteine gene constructions when inserted into transgenic mice supported the concept that functional

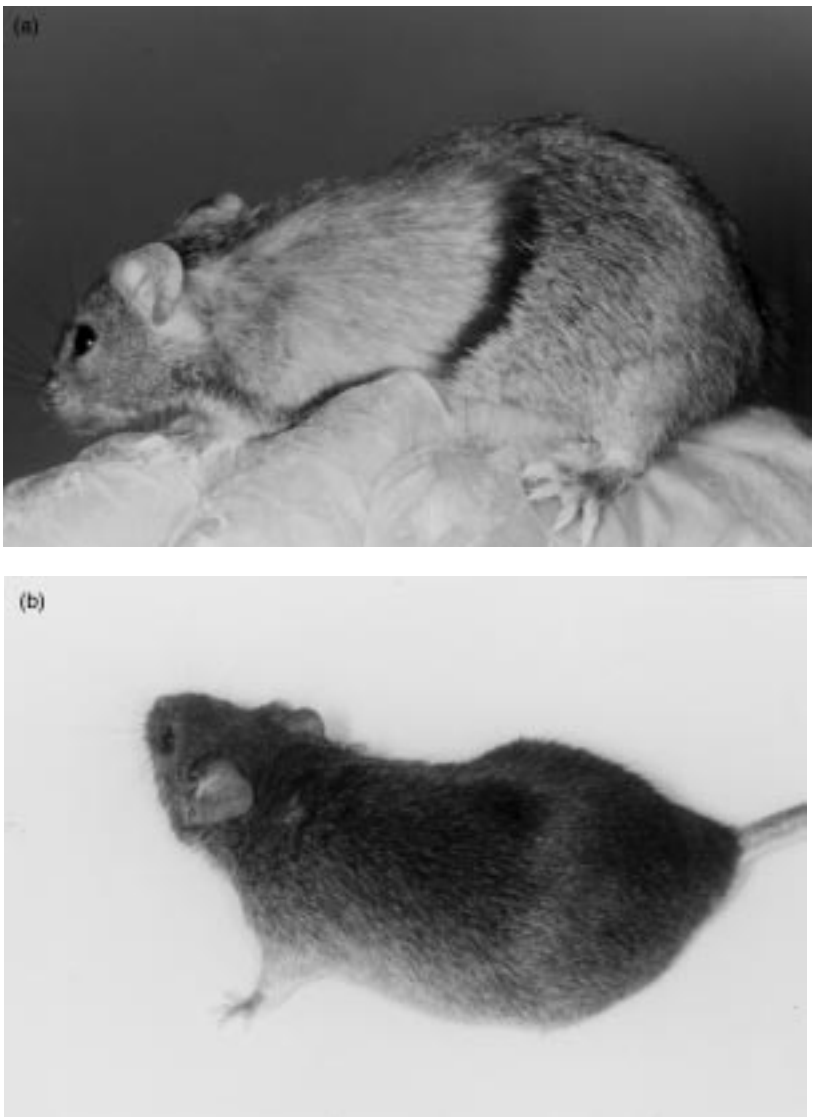

Fig. 12.6. The protective effect of the cysteine biosynthetic pathway on dietary-induced hair loss in mice. Age-matched mice, (a) non-transgenic control mice and (b) transgenic line EK28 were placed on a synthetic diet prepared essentially as described (Huovinen and Gustafsson, 1967). Initially, the mice were accustomed to the synthetic diet supplemented with the sulphur amino acids cysteine and methionine. The animals were then placed on a diet in which the combined sulphur amino acids were reduced to 0.1% (w/w) of the total diet (Leveille *et al*., 1961), but supplemented with 0.12% (w/w) Na_2S. Throughout the experiment, the drinking water of the mice was supplemented with 25 mM $ZnSO_4$. Photographs were taken after 7 days. Hair loss was observed in the non-transgenic mice but not in the transgenic animals.

metabolic pathways could be transferred from bacteria to animals. The work was then extended to the main aim of the research, namely the transfer of the cysteine biosynthetic pathway to sheep. This has proved to be more difficult than for mice.

In Table 12.4 is provided a list of transgenic sheep that have been produced containing various constructions encoding the SAT and OAS enzymes of *E. coli* or *S. typhimurium*.

The copy number in these various primary animals varies from 2 to >200 and there appears to be no unusual rearrangement of the genes compared with the insertions seen in transgenic mice. A Southern blot of some of the progeny bred from two transgenic sheep containing the gene *MTCEK1* is shown in Fig. 12.7. The results shown are those obtained with a *cysE* probe and are identical to those obtained with a *cysK* probe except that the *Bam*H1 fragment is then 2.8 kb in size. In the progeny of sheep no. 1830 (Fig. 12.7a), the copy number is estimated at about 20 and the predominant 2.65 kb band observed in *Bam*H1 digests indicates that most of the genes in the tandem array are arranged head-to-tail. However, there is also a prominent band at approx. 4 kb indicating that some of the array is arranged tail-to-tail. There is no evidence of rearrangement of the gene array during breeding, which supports the extensive body of evidence in the transgenic mouse lines showing that the gene is stable during prolonged breeding. The other animal in Fig. 12.7 shows evidence of two insertions in the F0 animal. The primary animal, no. 1290, contains a dominant tandem array of approximately 200 gene copies. *Bam*H1 digests of progeny from this animal (Fig. 12.7b) show that some animals retain this copy number but that an equal number of animals have a tandem array of about ten copies which is hard to detect in the presence of the larger insertion. While one explanation for this change in copy number could be that the large gene array in the F0 animal is unstable during breeding, this is unlikely since the ratio of transgenic to non-transgenic progeny is about 2:1. Final proof will be the demonstration that

Table 12.4. A summary of transgenic sheep containing various DNA constructs encoding the cysteine biosynthetic pathway. Much of the information in this table has been produced in the laboratory of Professor G.E. Rogers and these data have been summarized from Bawden *et al.* (1995).

Laboratory	Gene	TG Sheep (live)	Live TGs (% lambs born)	Animals expressing	Genes expressed
Prospect	*MTCEK1*	9	4	No. 1830	*cysE*, *cysK* (low)
Adelaide	RSVLTR–*cysE*M	2	3	No. 208	c*ysE*
Adelaide	RSVLTR–cysME	8	17	No. 196	*cysE* (low)
				No. 199	*cysE* (low)
Adelaide	mPgk–*cysK*E	6	1.0	No. 34	*cysE* (low)
Adelaide	mPgk–cysME	3	2.4	None	

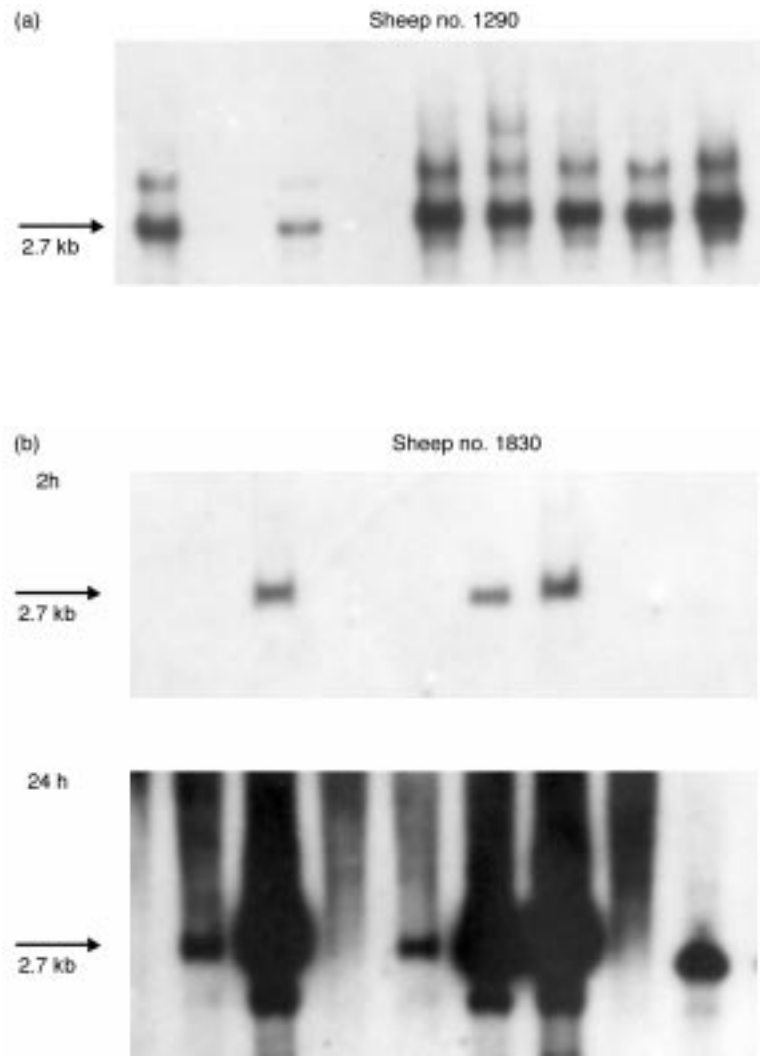

Fig. 12.7. Southern blot analysis of the progeny of (a) sheep no. 1290 and (b) sheep no. 1830 containing the gene *MTCEK1*. DNA was extracted from tail tissue, digested with *Bam*H1, separated on agarose gels and transferred to nylon membrane. Hybridization was with a ^{32}P-labelled *cysE* probe. Identical results were obtained with a *cysK* probe except that the *Bam*H1 band was 2.8 kb instead of 2.65 kb because of the slightly longer *cysK* coding sequence. Longer exposure of the autoradiograph identifies some progeny of no. 1830 with a lower copy number than the primary animal.

low-copy F1 and high-copy F1 generation lambs breed true to copy number in the F2 generation, and these experiments are in progress.

The expression of the genes in the transgenic sheep produced so far is summarized in Table 12.4. This expression is disappointingly low in comparison with that obtained in transgenic mice. Only five sheep have shown any expression of the genes and in each animal, at least one of the genes is expressed at a very low level. One of the sheep containing the gene *MTCEK1* has been shown to express both the *cysE* and *cysK* genes using the highly sensitive technique of RT-PCR to detect specific mRNA transcripts. As shown earlier, RT-PCR can detect the *cysE* and *cysK* gene transcripts in transgenic mice with great sensitivity and can readily detect expression in the skin of these animals. Accordingly, skin biopsies from the transgenic sheep containing the gene *MTCEK1* were analysed for the presence of the two transcripts. Animal no. 1830 contained a readily detectable *cysE* transcript in RNA from skin but the *cysK* transcript was barely detectable (Fig. 12.8). This is probably a real transcript, since no hybridization was detected in the appropriate control samples and genomic contamination is unlikely because the primer pair MT4/CE3, which is designed to amplify a piece of DNA starting in the sheep metallothionein promoter and finishing in the *cysE* coding sequence, failed to amplify any product. Enzyme assays of skin extracts from this animal failed to detect any SAT or OAS activities. In animals containing the *Salmonella* genes, four sheep expressed the *cysE* gene at low levels but not the *cysK* gene, although fibroblast cell cultures from two of the animals were reported to express low levels of *cysK* in addition to the *cysE* gene (Table 12.4).

Discussion of Results in Sheep and Mice

The results described above demonstrate that it is possible to introduce new biochemical pathways into animals by the judicious use of functional bacterial genes. Since genes required for the synthesis of most metabolites are to be found somewhere in the plethora of bacterial species that have been identified, the ability to use these pieces of DNA in transgenic animals provides the opportunity to remove many substrate limitations to specific production characteristics of domestic animals. However, it is equally clear that a new biochemical pathway that operates well in one species may not do so in a second species. In the example given above, the cysteine biosynthetic pathway appears to operate smoothly in transgenic mice but appears to interfere with the embryonic development of sheep. We can only speculate on the possible causes of these observations. One obvious explanation that cannot easily be ignored is that in the sheep the new pathway may be disturbing the concentration of an essential substrate or coenzyme crucial to embryonic development.

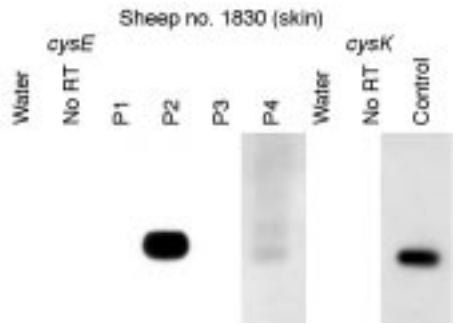

Fig. 12.8. RT-PCR analysis of RNA extracted from skin biopsies of transgenic sheep no. 1830. RNA extraction and RT-PCR analysis were as described for Fig. 3b. Lane 1, water control with primer pair CE1/CE3; lane 2, no reverse transcriptase control with primer pair CE1/CE3; lane 3, cDNA template with primer pair MT4/CE3; lane 4, cDNA template with primer pair CE1/CE3; lane 5, cDNA template with primer pair MT4/CK3; lane 6, cDNA template with primer pair CK1/CK3; lane 7, water control with primer pair CK1/CK3; lane 8, no reverse transcriptase control with primer pair CK1/CK3; lane 9, genomic DNA from sheep no. 1830 with primer pair CK1/CK3.

The substrate acetyl-CoA is one possible candidate. Sheep and mice differ in the way they utilize carbohydrates for the provision of cellular energy. The sheep is a ruminant and hence much of its metabolizable energy is supplied in the form of volatile acids such as acetate, propionate and butyrate, while mice utilize carbohydrates such as glucose (Armstrong *et al*., 1957, 1960). This results from the biota of the rumen that converts essentially all fermentable carbohydrate into the volatile acids, with acetate accounting for more than 90% in the peripheral circulation (Van Soest, 1982). The enzymes for intermediary metabolism in the sheep are adapted to the utilization of these volatile acids instead of glucose as a source of energy (Van Soest, 1982) with glucose being used only in those reactions which are not replaceable by volatile acids.

Acetyl-CoA holds a crucial position in this volatile fatty acid-oriented metabolism, since it provides the point of entry for acetate into intermediary metabolism where it can be oxidized for energy in the tricarboxylic acid (TCA) cycle. Its levels are also crucial to early embryonic development both in ruminants and non-ruminants, providing 80–95% of the embryo's ATP via oxidative phosphorylation (Thompson *et al*., 1996). It is apparent that in mice, the insertion of the gene *MTCEK1* does not result in a deleterious alteration to the supply of this vital substrate during development, since

transgenic mice are produced in expected numbers. The explanation for this may be as simple as a lack of expression of the cysteine-encoding transgene in the early mouse embryo. However, earlier work has shown that a MT-Ia–growth hormone transgene is expressed in the developing mouse (Shanahan *et al.*, 1989) and it is therefore probable that the *MTCEK1* gene is also expressed at this time. In sheep, it has proved very difficult to produce transgenic animals expressing *MTCEK1* or any other of the cysteine-encoding genes that have been constructed. The development of ruminant embryos differs significantly from that of the mouse at the time of blastulation. At this stage, the energy required for the development of the mouse is supplied almost entirely by glucose catabolism through glycolysis to lactate while that of the ruminant is mainly derived from externally absorbed pyruvate which is converted to acetyl-CoA and oxidized in the TCA cycle (Reiger and Guay, 1988; Waugh and Wales, 1993; Thompson *et al.*, 1996). Clearly, any decrease in the level of acetyl-CoA would have the potential to interfere significantly with the energy supply of the sheep embryo but not with that of the mouse.

The levels of cytosolic acetyl-CoA are unlikely to fall in adult ruminants because it can be readily synthesized from acetate in the presence of the enzyme acetyl-CoA synthase. This may not be the case, however, for the ruminant embryo. Like most embryos, the ruminant at this stage utilizes exogenous acetate very poorly and the substrate has been calculated to make no significant contribution to the embryo's energy supply (Waugh and Wales, 1993). This has been ascribed to a very low level of acetyl-CoA synthase in embryos. It follows that any extensive depletion of cytosolic acetyl-CoA in ruminant embryos would cause a flow of acetyl-CoA from the mitochondrion, reducing the potential energy supply. The transfer of acetyl-CoA from mitochondria to cytosol is possible in ruminant embryos because ATP–citrate lyase, essentially non-functional in adult ruminant tissues, is active during development. Cytosolic depletion of acetyl-CoA would be a consequence of the unregulated action of the enzyme SAT in the embryo cytoplasm. This enzyme is allosterically regulated in bacteria by the concentration of cysteine, with complete inhibition of activity at approximately 100 μM (Kredich and Tomkins, 1966). Since the introduced cysteine biosynthetic pathway would be unable to synthesize cysteine in the embryo because there is no available source of sulphide, the conversion of serine and acetyl-CoA to *O*-acetyl-serine could proceed normally unless the SAT was inhibited by the normal intracellular levels of cysteine. These are not known in the developing sheep embryo, but circulating cysteine levels in the adult sheep are very low, with measurements varying between 12 μM and 37 μM being reported (Lee *et al.*, 1993). In addition, the glutathione levels in sheep are also very low in comparison with monogastric animals (Lee *et al.*, 1993). These data suggest that overall cysteine supply in the

sheep is low and that the intracellular levels in the embryo are unlikely to reach those required for the inhibition of the enzyme SAT. This being so, the possibility exists that in any embryo expressing the SAT at high levels, acetyl-CoA concentrations might be reduced below the level needed for embryo survival. This would result in most transgenic sheep being animals with no or very low levels of expression of the SAT and hence by default the OAS, since both are linked in a common gene construct.

One way of determining this is to produce transgenic animals containing either the *cysE* gene or *cysK* genes and to mate such animals to produce progeny with an intact pathway. A second approach is to modify the gene constructs so that expression is confined to the adult animal and to those tissues where a source of sulphide might be expected. These experiments would benefit greatly from an improved method of producing transgenic sheep since they would require many animals for appropriate testing of the various genes. The recent advances in the production of viable embryos from the fusion of enucleated embryos and cultured cells (Wilmut *et al*., 1997) might be advantageous in pursuit of this goal.

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