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Genetic Engineering of Livestock

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Genetic engineering of livestock is expected to have a major effect on the agricultural industry. However, accurate assessment of the consequences of transgene expression is impossible without multigenerational studies. A systematic study of the beneficial and adverse consequences of long-term elevations in the plasma levels of bovine growth hormone (bGH) was conducted on two lines of transgenic pigs. Two successive generations of pigs expressing the bGH gene showed significant improvements in both daily weight gain and feed efficiency and exhibited changes in carcass composition that included a marked reduction in subcutaneous fat. However, long-term elevation of bGH was generally detrimental to health: the pigs had a high incidence of gastric ulcers, arthritis, cardiomegaly, dermatitis, and renal disease. The ability to produce pigs exhibiting only the beneficial, growth-promoting effects of growth hormone by a transgenic approach may require better control of transgene expression, a different genetic background, or a modified husbandry regimen.

THE ABILITY TO INTRODUCE NEW GENES INTO THE GERM line of an animal and thereby produce proteins outside their normal environment and separated from their usual physiological control mechanism has been extremely valuable for studying numerous aspects of gene expression as well as other questions in biology (*1*). Most investigations of gene expression in animals have utilized the mouse as an experimental animal. However, gene transfer has recently been extended to domestic animals (Table 1).

Although the list of animal species and the number of genes that have been introduced and expressed is small, such a strategy will undoubtedly be important in agricultural improvement.

One of the first questions investigated by transgenic technology was the regulation of growth (*2*). The general strategy has been to introduce growth-regulating genes under the control of heterologous promoters into the germ line to allow long-term production of peptides in ectopic tissues. Successful expression of these proteins then creates a system in which the effects of a single hormone can be assessed in an otherwise normal animal.

The regulation of postnatal growth is an exceedingly complex process that involves an interplay between circulating hormones, genetic potential, and the prevailing nutritional status of the animal. The hormonal cascade controlling growth consists of an array of hormones produced principally in the hypothalamus, pituitary gland, and peripheral tissue. Growth hormone (GH), an intermediate in this cascade, is produced in somatotrophs of the pituitary and is under the neurohumoral regulation of two hypothalamic peptides, somatostatin and growth hormone–releasing factor (GRF). Somatostatin inhibits the release of GH, whereas GRF stimulates both GH synthesis and release. GH is thought to mediate growth by stimulating the synthesis and secretion of insulin-like growth factor I (IGF-I), which acts in concert with GH on peripheral tissues (*3*).

In mice, the most dramatic effect of expression of bovine (b), ovine (o), rat (r), or human (h) GH has been a stimulation of growth that commences at about 3 weeks of age and reaches a plateau at about 12 weeks when the mice are as much as twice their normal size (*2, 4–7*). During the maximum growth phase (5 to 11 weeks of age), the growth rate in transgenic mice is four times that in control mice (*8*). Postnatal growth can also be increased by ectopically expressing hGRF, which stimulates somatotroph cells to produce more endogenous GH (*9*). Similarly, production of hIGF-I in transgenic mice increases somatic growth rates, although less dramatically than GH or hGRF expression (*10*).

More recently, gene transfer technology has been extended to commercially important livestock (Table 1). The purpose of many such experiments was to test the feasibility of introducing foreign growth-promoting genes into the livestock genomes and thereby enhancing growth performance. The annual gross receipts for the sale of livestock are about $60 billion in the United States, and the gross receipts for pork amount to about $9.5 billion annually. Any strategy to improve the rate and efficiency of body weight gain has obvious interest to producers. Strategies to alter the composition of pork toward a leaner, less fat product are consistent with biomedical advice that people reduce their consumption of animal fat.

The first attempt at applying this technology to domestic animals involved introduction of a fusion gene consisting of the mouse metallothionein-I (MT) regulatory sequences linked to the hGH gene into the genome of pigs (*11, 12*). The effects of expression of hGH in pigs have been difficult to assess for several reasons. First, the growth performance data obtained on founder (G0) animals indicated that expression of hGH exerted a pronounced antilipogenic and lipolytic effect and stimulated IGF-I production, but body size was not dramatically altered (*13, 14*). Second, many expressing founder animals exhibited infirmities, including peptic ulcers and pericarditis, that precluded the possibility of making accurate evaluations of growth performance. Third, most founders that expressed GH displayed impaired fertility, which made propagation of lines of animals difficult.

To continue investigating the growth-promoting potential of these peptides in pigs, we introduced genes encoding bGH, hGRF, or hIGF-I into the pig genome under the control of the mouse MT promoter. We report the successful introduction and expression of these chimeric genes in founder pigs. Because definitive answers regarding growth effects in farm animals are most efficiently obtained by direct sibling comparisons, we compared the progeny in several generations of pigs expressing bGH.

Production of Animals

Several methodologies are currently being used to produce transgenic mice (including microinjection of eggs, retrovirus infection of embryos, and embryonic stem cell transfer into blastocysts); however, only microinjection of DNA has been successfully used for transferring genes into sheep and pigs (Table 1). Although microinjection of mouse egg nuclei has become relatively common, the application of this technique to domestic animal eggs was impeded by the opacity of the eggs. In rabbits and sheep, egg nuclei can be identified by means of differential interference-contrast (DIC) microscopy. The opacity of the cytoplasm of pig and cow ova makes identification of nuclei, even with DIC microscopy, virtually impossible. To overcome this impediment, pig and cow ova can be centrifuged at 15,000*g* for 5 min, which stratifies the cytoplasm and leaves the pronuclei or nuclei in a clear equatorial layer of the egg (*11, 15*). Egg nuclei can then be easily seen by DIC microscopy and injected.

The efficiency of production of transgenic pigs is still low compared to that of the production of transgenic mice. In mice, approximately 10 to 15% of the microinjected eggs develop to newborns, and, of those that are born and weaned, approximately 25% are transgenic (*16*). In contrast, during 3 years of gene transfer studies in pigs involving MThGH, MTbGH, MThGRF, and MTIGF-I genes, only 8% of the 7000 injected eggs developed to birth and about 7% of those born were transgenic (Table 2). This resulted in an integration efficiency of about 0.6% for pigs as compared to the 2.5 to 6% achieved in mice (*16, 17*). Similarly, low integration efficiencies for gene transfer into pigs have been reported for the introduction of a Moloney murine leukemia virus (MLV) rGH gene and a hMTpGH gene (*18, 19*). Although great strides have been made in applying gene transfer to domestic animals, the methodology is still inefficient. In mice, the frequency of integration of foreign DNA is affected by such factors as buffer composition, conformation of the DNA, concentration of DNA used for injection, and the skill and experience of the microinjector (*16*). In addition, the quality of the egg influences both its viability after injection and DNA integration frequency. The conditions used for gene transfer into pigs and sheep are those that are optimal for mice, and they may not be optimal for eggs of domestic animals.

Transgene Expression

A summary of the data on transgenic pigs that expressed the injected gene after birth is shown in Table 3. Of 29 founder MThGH and MTbGH transgenic pigs born, 19 (66%) produced

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detectable levels of hGH or bGH in plasma at birth (as measured by immunoassay), which is similar to the frequency of expression in transgenic mice (*4, 6*).

The concentrations of foreign GH in these two groups of pigs ranged from 3 to 949 ng/ml for hGH pigs and 5 to 944 ng/ml for MTbGH pigs. Mean concentrations of GH in expressing founder animals from 30 to 180 days of age ranged from 14 to 4000 ng/ml for hGH and 23 to 1600 ng/ml for bGH (*20*). The variability among founder animals is presumably due to the influence of chromosomal position on tissue specificity and to the activity of the MT promoter (*21*). Another factor may be mosaicism, which occurs in approximately 30% of transgenic mice (*22*). Individual pigs tended to maintain characteristic levels of expression (*20, 23*). The presence of either hGH or bGH in the plasma of pigs was accompanied by as much as a three- to fourfold increase in IGF-I concentrations (Table 3), as well as a decrease in the levels of pig GH (pGH) (*20*). A similar elevation in IGF-I concentrations has been reported for a pig that expressed rGH (*19*). The increase in endogenous IGF-I probably reflects the activity of transgenic GH on GH hepatocyte receptors (*24*), whereas the decrease in endogenous GH is due to feedback inhibition by transgenic GH and endogenous IGF-I on the hypothalamus and pituitary (*25*).

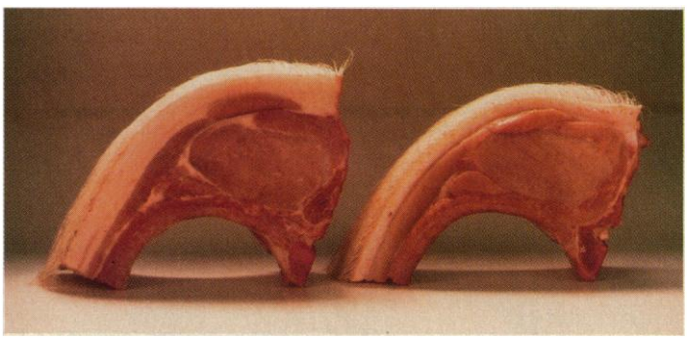

Fig. 1. Cross section through the loin at the 8th rib of a G2 MTbGH boar (right) from line 31-04 and a control (left) half-sibling male. Both animals weighed approximately 90 kg at the time when they were killed.

Of the seven founder pigs with the MThGRF minigene, two had high concentrations of GRF in plasma as detected by immunoassay (Table 3). The plasma levels of GRF were at least ten times the plasma GRF concentrations found in the littermate controls. Despite elevated plasma levels of GRF, the levels of plasma pGH were normal. In transgenic mice expressing this same construct, 100% of the mice with immunodetectable hGRF had elevated levels of mouse GH (mGH) (*9*). Most of the hGRF in the plasma is present in a biologically inactive form; the biologically active form is found principally in the pituitary, hypothalamus, and pancreas (*26*). The absence of an effect of hGRF in transgenic pigs may be attributable to lack of synthesis in cells capable of proper proteolytic processing of the GRF precursor peptide. This issue can be addressed in progeny of line 86-04.

Four transgenic pigs were produced containing an MThIGF-I gene, but only one of these animals (111-06) had elevated levels of

Table 1. Production of transgenic farm animals. Listed are genes that have been introduced into six species of animals. The slash separates the promoter or enhancer of one gene from the structural gene. The species is indicated by a lower case letter before the abbreviation of the gene: b, bovine; c, chicken; h, human; m, mouse; o, ovine; p, porcine; r, rat; rb, rabbit. Gene abbreviations: ALV, avian leukosis virus; α1AT, α1 anti-trypsin; BPV, bovine papilloma virus; Eμ, immunoglobulin heavy chain; FIX, factor IX; GH, growth hormone; βGal, galactosidase; hygro, hygromycin; βLG, β-lactoglobulin; MT, metallothionein; MLV, Moloney murine leukemia virus; REV, reticuloendotheliosis; PRL, prolactin; SV, SV40; TK, thymidine kinase. Integration means the gene became part of the DNA complement; expression indicates that the gene was integrated, and either transgene mRNA or protein (or both) was detectable.

Gene	Results	Reference
	Chicken	
ALV	Integration	(*48*)
REV	Integration	(*49*)
ALV	Expression	(*50*)
	Cow	
BPV	Integration	(*51*)
	Fish	
mMT/hGH	Expression	(*52*)
cδ-Crystallin	Expression	(*53*)
hGH	Integration	(*54*)
mMT/hGH	Integration	(*55*)
mMT/hGH	Integration	(*56*)
mMT/βGal	Expression	(*57*)
SV/hygro	Integration	(*58*)
	Pig	
mMT/hGH	Expression	(*11*)
mMT/hGH	Integration	(*12*)
mMT/bGH	Expression	(*14*)
hMT/pGH	Expression	(*18*)
MLV/rGH	Expression	(*19*)
bPRL/bGH	Expression	(*59*)
	Rabbit	
mMT/hGH	Expression	(*11*)
mMT/hGH	Integration	(*12*)
hMT/hGH	Expression	(*4*)
rbEμ/rb*c-myc*	Expression	(*60*)
	Sheep	
mMT/hGH	Integration	(*11*)
mMT/TK	Integration	(*61*)
oβLG/hFIX	Integration	(*61*)
oβLG/hα1AT	Integration	(*61*)
mMT/bGH	Expression	(*62*)
mMT/hGRF	Expression	(*62*)
oMT/oGH	Expression	(*63*)
oβLG/hFIX	Expression	(*47*)

Table 2. Production of transgenic pigs. Mature gilts were superovulated and bred as described (*10*). At 58 to 77 hours after human chorionic gonadotropin injection, ova were surgically collected from anesthetized donor females by flushing with modified Brinster medium for ovum culture (BMOC) (*64*) from the uterotubal junction through the cannulated infundibular end of each oviduct. Ova were maintained in modified BMOC. Centrifuged eggs were microinjected as described (*11*). The injected genes are described in the legend to Table 3. After microinjection, eggs were transferred to the oviducts of recipient gilts (*11*). Some pregnant recipients received control eggs in MThGRF and MThIGF-I experiments to aid in the maintenance of pregnancy. We estimated that 55% of control eggs develop to live piglets (*11*); to compensate for the control egg contributions, the number of pigs born has been reduced by 72 for the MThGRF experiment and 8 for the MThIGF-I experiment. Transgenic animals were identified by DNA dot hybridization to nucleic acids isolated from tail or ear biopsies (*11*). Expression indicates the presence of immunoassayable foreign protein in plasma.

Group	MThGH*	MTbGH*	MThGRF	MThIGF-I
Recipients	64	49	66	13
Injected ova transferred	2035	2330	2236	387
Pregnant recipients	37	24	35	5
Ova in pregnant recipients	1174	1255	1105	152
Pigs born	192	150	177	34
Transgenic pigs	20 (0.98)†	9 (0.39)	7 (0.31)	4 (1.0)
Expression	11	8	2	1

*Previously published (*11, 20*). †Percentage of injected eggs.

plasma IGF-I (Table 3). Unfortunately, it died before a line of transgenic MTIGF-I pigs could be established for an analysis of the biological effects of IGF-I expression. Relatively high mortality in normal young pigs is a complicating factor in transgenic experiments. Approximately 30% of pigs do not survive to market weight (90 kg, 6 months of age); about 6% are stillborn and 20% die before weaning (3 to 4 weeks) (*27*).

Transgene Expression in Successive Generations

Concentrations of transgene-derived GH in plasma varied widely from one founder pig to another (Table 3), in agreement with earlier results in the mouse (*4, 6*). In contrast, within successive generations of two lines of pigs the mean bGH levels were maintained at characteristic levels (Table 4). Line 31-04 maintained plasma levels of bGH of about 1300 ng/ml over three generations compared to plasma levels of endogenous pGH of about 5 ng/ml in controls (see legend to Table 3). Line 37-06 maintained plasma bGH levels of about 85 ng/ml, ranging from 10 to 30 times the level of endogenous pGH over several generations. Circulating levels of bGH were increased about twofold by including 1000 to 3000 ppm of zinc in the diet (*23*), a regimen that increases plasma transgenic GH concentrations in MTGH mice (*28*).

To ascertain the source of the bGH in plasma, bGH mRNA was measured in eight organs of 6 to 11 pigs from each generation by a solution hybridization assay (*29*). Although variation among pigs was considerable, the average bGH mRNA levels in line 31-04 ranged from 700 to 1000 molecules per cell in liver, kidney, adrenal gland, and pancreas and 60 to 200 molecules per cell in duodenum, lung, and gonads. By comparison, in line 37-06, which had 1/15 the level of bGH in plasma, bGH mRNA averaged from 15 to 50 molecules per cell in liver, kidney, heart, gonad, and pancreas and 5 to 10 molecules per cell in lung and adrenal gland. These are the same organs that have been shown to express MTGH genes in transgenic mice (*4, 6*).

Pathology

In transgenic mice, expression of rGH, hGH, or bGH genes is associated with several notable pathologic changes including hepatomegaly and glomerular sclerosis, which shorten life span (*30, 31*). In addition, female mice expressing transgenic rGH, bGH, and hGH genes are commonly infertile (*4, 32*); in the case of hGH mice, the infertility is due to an impaired release of prolactin after mating, which alters luteal function (*33*). Many founding MThGH and bGH transgenic pigs exhibited infirmities similar to those in transgenic mice and died prematurely (Table 3). To assess the deleterious consequences of the long-term presence of bGH, we clinically examined, killed, and carried out necropsies on transgenic and control pigs from the G1 and G2 generations of lines 31-04 and 37-06 (Table 5). The most common clinical signs of disease associated with transgene expression included lethargy, lameness, uncoordinated gait, exopthalmos, and thickened skin. The following gross and histopathologic changes were noted in some of the transgenic pigs: gastric ulceration, severe synovitis, degenerative joint disease, pericarditis and endocarditis, cardiomegaly, parakeratosis, nephritis, and pneumonia. In addition, gilts were anestrus and boars lacked libido. The joint pathology known as osteochondritis dissecans was also observed in a MLVrGH-expressing transgenic pig (*19*) and in groups of pigs subjected to long-term treatment with exogenous pGH (*34*).

As GH is diabetogenic in humans and animals, plasma insulin and glucose levels were measured in overnight fasted, transgenic MTbGH and control sibling pigs that were G3 offspring of line 37-06. The bGH pigs were slightly hyperglycemic [plasma glucose levels: 109 ± 13 (mean ± SEM) mg/dl, $n = 10$; control, 72 ± 5 mg/dl, $n = 10$; $P = 0.01$] and were hyperinsulinemic [plasma insu-

Table 3. Expression of MThGH, MTbGH, MThGRF, and MThIGF-I in transgenic pigs. MThGH (2.6 kb) consists of the mMT promoter and 5′ flanking DNA sequences to the Bst EII site (−350) fused to the hGH gene (*11*). MTbGH (2.6 kb) consists of the mMT promoter and 5′ flanking sequences to Bst EII fused to the bGH gene (*4*). MThGRF (2.5 kb) consists of the mMT promoter and 5′ flanking sequences to Stu I (−750) fused to hGRF minigene (*9*). MThIGF-I (3.8 kb) consists of the mMT promoter and 5′ flanking sequences to Stu I (−750) fused to a human insulin-like growth factor, I(hIGF-I) cDNA (*10*). DNA fragments were excised from bacterial plasmids, purified by agarose gel electrophoresis, eluted, and microinjected into fertilized eggs (*16*). NA, not assayed.

Animal*	DNA† copy per cell	Plasma‡			Life span
		GH (ng/ml)	hGRF (pg/ml)	IGF-I (ng/ml)	
		MThGH			
3-06 M	490	22		NA	7 months
7-03 F	90	104		NA	24 days
11-02 M	1	52		NA	11 months
16-09 M	1	85		NA	9 months
20-02 M	2	52		NA	2 months
20-08 M	110	3		465 ± 106	9 months
23-08 F	7	949		478 ± 141	11 months§
25-02 F	17	140		350 ± 47	8 months
Control				158 ± 13	
		MTbGH			
26-01 F	5	302		NA	5 days
29-01 M	5	884		378 ± 18	6 months
31-04 M	28	944		105 ± 30	21 months
32-04 F	2	94		350 ± 75	7 months
37-06 M	3	59		622 ± 43	6 months
39-02 F	1	5		516 ± 58	13 months§
41-03 F	1	70		322 ± 74	9 months
58-01 F	5	260		NA	4 days
Control				134 ± 13	
		MThGRF			
86-04 M	100	11 ± 2	380	NA	2 years
93-01 F	30	16 ± 5	220	NA	2 months§
Control		14 ± 5	<20	NA	
		MThIGF-I			
111-06 F	10	NA		311 ± 86	3 months
Control		NA		122 ± 13	

*Only the transgenic animals (G0) that survived more than 1 day and expressed the foreign genes are listed. Three hGH piglets that did not live 1 day also expressed the foreign gene. The total number of transgenic animals produced in each group is indicated in Table 2. †We estimated the number of copies of MThGH, MTbGH, MThGRF, and MThIGF-I per cell by extracting total nucleic acids from a piece of ear or tail sample and performing quantitative DNA dot hybridization, using probes specific for the various transgenes (*16*). ‡Concentrations of plasma hGH were determined at birth as described (*11*). Concentrations of bGH were determined at birth using USDA-bGH-B-1 as a standard USDA-bGH-I-1 for radiolabeled ligand and HS-T83-anti-bGH antiserum (*65*); bGH was not detected in plasma from control pigs. Concentrations of plasma pGH in control and MThGRF pigs were determined in samples collected weekly, using USDA-pGH-B-1 as standard, USDA-pGH-I-1 for radiolabeled ligand, and DM-2027 anti-pGH antiserum (*66*). The mean plasma pGH concentration in two control littermates of MThGRF pigs were 13 ± 22 and 15 ± 4 ng/ml (mean ± SEM). The plasma pGH concentration in control pigs between 7 and 30 days of age was 9.7 ± 2.7 ng/ml (mean ± SEM; $n = 64$) and between 90 and 120 days of age was 2.9 ± 0.3 ng/ml ($n = 117$). The plasma pGH concentration in pigs expressing hGH was <0.75 ng/ml. Concentrations of GRF were determined in plasma collected at 2 to 3 months of age as described (*67*). Concentrations of IGF-I were determined using recombinant human IGF-I (Amgen, Thousand Oaks, California) as the standard and radiolabeled ligand and CH549/805C anti-human IGF-1 serum (*68*). Plasma samples were extracted with acid-ethanol before assay (*69*). IFG-I levels (mean ± SEM, $n = 3$ or 4) for hGH and bGH transgenic and littermate controls ($n = 8$ or 23) are from samples collected from between 90 and 150 days of age. IGF-I levels ($n = 2$) for hIGF-I transgenic and littermate control are from samples collected at 2 and 2.5 months of age. §Animals that were killed to obtain tissues for assay. MThGRF pig 86-04 is alive and is approximately 2 years old.

lin levels: transgenic, 480 ± 118 pg/ml, n = 10; control, 24 ± 4 pg/ml, n = 10; P = 0.001]. A similar degree of hyperglycemia, but less pronounced hyperinsulinemia, was observed in pigs injected with pGH (*34, 35*). Similarly, one transgenic pig expressing rGH had severe hyperglycemia with glucosuria (*19*).

Several of the most prevalent health problems of the hGH and bGH transgenic pigs are widespread in the general swine population, although at a lower frequency and with less severity. Necropsy surveys conducted on market hogs at slaughter indicate that 10 to 30% have gastric ulcers (*35*), 30 to 80% have lesions typical of enzootic pneumonia (*36*), and up to 90% have osteochondrosis (*37*), which leads to synovitis and degenerative joint disease, the major cause of lameness in breeding-age swine.

Table 4. Plasma bGH in successive generations from two lines of transgenic pigs. Two founder (G0) MTbGH animals, lines 31-04 and 37-06 (Table 3), sired transgenic progeny (G1, G2, and G3) that were identified by DNA dot hybridization to nucleic acids isolated from tail samples. In both lines, the foreign genes were inherited as an autosomal dominant trait. The bGH levels were measured in plasma samples by radioimmunoassay (see Table 2). Values are means ± SEM; NA, not assayed. In control pigs pGH levels are 9.7 ± 2.7 ng/ml (mean ± SEM, n = 64) for pigs between 7 and 30 days of age and 2.9 ± 0.3 ng/ml (n = 117) for pigs between 90 and 120 days of age.

Generation	Plasma bGH (ng/ml)	
	Line 31-04	Line 37-06
G0	1345 ± 114 (1, 22)*	45 ± 4 (1, 19)
G1	1318 ± 88 (2, 34)	139 ± 25 (6, 60)
G2	1217 ± 134 (21, 29)	63 ± 9 (11, 43)
G3	NA	94 ± 13 (28, 45)

*(Total number of pigs, total number of samples).

Growth Performance

Many transgenic mice that express MTrGH, MThGH, or MTbGH genes grow at four times the rate of growth of control mice during the maximum growth phase (*4, 6, 8*). These results coupled with the observation that injections of pGH into pigs stimulate growth and improve feed efficiency suggested that expression of GH transgenes would also improve growth in pigs (*38*). This expectation, however, was not realized in the founder population of MThGH pigs (*13*) or MTbGH pigs (Table 6). Single pigs expressing rGH or pGH showed increased growth (*18, 19*); however, one must be cautious in not overinterpreting the significance of single-animal observations because daily weight gains in both transgenic and control animals have differed from the mean by as much as 30%. Although the lack of a dramatic growth effect in hGH and bGH founder animals was surprising, we believed it was possible that bGH transgenics might respond with enhanced growth performance if the protein, and particularly the lysine, content of the diet was elevated to levels that conform to those used in recent studies with exogenous pGH administration in pigs (*39*).

To systematically assess growth of transgenic pigs, we measured the average daily weight gain and the feed efficiency of animals from two successive generations of transgenic and control littermates of line 37-06 and the G2 generation of line 31-04 (Table 6). When G2 progeny of line 37-06 were placed on a diet with elevated crude protein and additional lysine, transgenic animals grew 15% faster than littermate controls. In combined growth data from G2 and G3 progeny, transgenic animals grew 11% faster than control siblings (P = 0.002). Similarly, G2 progeny from line 31-04 gained weight 14% faster than control siblings, but this difference was not significant. Perhaps the fact that this line of pigs had much higher levels of circulating GH (Table 4) adversely influenced the relationship of the beneficial to detrimental effects of GH. One of the detrimental effects is a suppression of appetite. In pigs injected with pGH, feed intake was suppressed between 14 and 17% when compared to controls (*34, 39*). In comparison to littermate controls, the feed intake of founder bGH pigs was depressed 20% and, in G2 progeny of line 37-06 that were given ad libitum feed, intake was depressed 17%. Therefore, depression of appetite limits the availability of essential nutrients for accretion of tissues, and, as a consequence, low feed intake prevents pigs with GH transgenes and pigs injected with pGH from attaining their maximum genetic potential for growth rate.

Although the founder group of expressing MTbGH pigs did not exhibit increased daily weight gain, they were 16% more efficient at converting feed into body weight than controls (Table 6). The G2 generation of line 37-06 was 18% more efficient than control siblings. Similar improvements in feed efficiency have been reported for pigs injected with exogenous pGH (*34, 39*). Because feed constitutes 60 to 70% of the noncapitalized cost of pig production, a 15% improvement in feed efficiency has the potential for an enormous impact if this technology becomes practical.

We also evaluated the effects of long-term exposure to elevated GH on visceral and skeletal growth. In transgenic pigs of line 37-06, the liver, heart, kidney, adrenal glands, and thyroid were significantly larger than in sibling controls (Table 7). Even more pronounced selective visceromegaly has been noted in transgenic mice expressing foreign GH (*4, 30, 40*). In addition to exhibiting a selective increase in organ weight, MTbGH animals from line 37-06 also had increased long bone weight and circumference (Table 8). In contrast, linear long bone growth was similar to that of sibling controls. These observations contrast with those made on a single transgenic boar expressing the MLVrGH gene. In this animal, the linear bone growth of fore and hind limbs was greater than for littermate controls (*19*). Reasons for this may be related to the nature of rGH function in pigs or the fact that only one animal was studied.

The presence of high levels of GH in the plasma of MThGH (*14*) and MTbGH transgenic pigs prevented subcutaneous fat accretion and enhanced the utilization of nutrients for other carcass components. Mean back fat thickness at the tenth rib of eight G1 and G2 bGH transgenic pigs from line 31-04 was 7.5 ± 2.3 mm, whereas for eight littermate controls the thickness averaged 21 ± 1.7 mm (mean ± SEM). This measurement is an underestimate of subcutaneous fat because it is a reflection of both skin thickness and subcutaneous fat over the loin. This dramatic difference is evident on a cross section through the loin of a transgenic and sibling control (Fig. 1). The decrease in carcass fat is of considerable importance in the production of leaner meat.

Conclusions and Prospects

Although we have been able to stimulate pig growth and enhance food conversion to protein, it is clear that detrimental effects on the general health of the pigs were also observed. It is also clear that multigenerational studies are essential to evaluate the physiological effects of transgenes specifically in pigs and perhaps in all livestock animals. These factors, coupled with the long reproductive interval in some of these species, will pose an obstacle to the rapid introduction of transgenic animals into the general agricultural community. However, several aspects of our study suggest avenues for improvement. Growth control is a complicated process governed by a number of hormones acting on a background of genes that are largely unknown. The hormones and other gene products that are rate-limiting for growth are not necessarily the same from one

Table 5. Histopathology of MTbGH transgenic pigs. Five transgenic and three control G1 and G2 pigs (age 4.5 to 10 months) from lines 31-04 and 37-06 were used. Animals were killed, a necropsy was performed, and tissues were processed for light microscopic examination. The number of pigs showing symptoms out of the number of pigs examined is indicated.

Diagnosis*	Number of animals	
	Transgenic	Control
Gastric ulcers	5/5	0/3
Synovitis	4/5	0/3
Cardiac myocyte nuclear hypertrophy	4/5	0/3
Dermatitis	4/5	1/3
Nephritis	3/5	0/3
Pneumonia	3/5	1/3

*See (*70*).

species to another and have undoubtedly been affected by natural or deliberate selection processes. Hence, centuries of selection for growth and body composition may limit the ability of the pig to respond to GH, that is, in contrast to the mouse. Although the 10 to 15% increase in daily weight gain and 16 to 18% increase in feed efficiency obtained in transgenic pigs are modest improvements in growth (when compared to the degree of enchanced growth in transgenic mice), the results are quite similar to those reported for pigs injected daily with pGH and could have a significant impact on the $9.5-billion annual pig industry. In swine, appetite depression is a major factor that limits the growth response from elevated GH, and therefore the alleviation of this side effect could further enhance the economic potential. Elevated GH in rats stimulates hyperphagia, which provides them with the necessary nutrients to support the rapid rate of protein accretion (*41*). A comparison of the appetite control mechanisms in the two species may suggest strategies to increase appetite and feed consumption in transgenic pigs.

Accurate assessment of the growth rate of pigs harboring GH transgenes was impossible until enough animals could be reared under controlled conditions for proper statistical analysis. The variable genetic background, environmental conditions, and biased attention that founder animals receive preclude accurate assessment of many characteristics. Initially, we thought that the failure of pigs to show more dramatic growth might be due to poor binding of the heterologous hGH or bGH to endogenous GH receptors. This now seems less likely because it is clear that IGF-I levels are elevated in pigs to a degree similar to that observed in transgenic mice, indicating that GH receptors in the pig liver are probably maximally stimulated. The pathological consequences of transgene expression might be ascribed to inappropriate binding of foreign GH to some receptors. Similar disorders were also observed when endogenous mGH was elevated by ectopic expression of hGRF (*30*). Thus, we believe that the infirmities observed in transgenic pigs are a consequence of the long-term exposure to elevated GH rather than the heterologous nature of the GH.

Most market hogs are crossbreeds of several purebred strains, and therefore our experiments were conducted on this type of animal. In addition, in commercial hog operations livestock are generally reared on concrete in an indoor environment within a confined space, and our experimental environment was similar. We believe that the infirmities observed in the pigs expressing GH transgenes would have been less frequent and less severe if the genetic base used for our experiments had been selected for structural soundness of legs and the ability to withstand these commercial rearing conditions. Nevertheless, elimination of side effects can probably only be achieved by rigorous regulation of transgene expression to a duration of 1 to 2 months during the rapid growth phase in domestic swine. Thus, tightly regulated GH transgene expression would be analogous to a short-term duration of exogenous pGH injections, which generally have not caused severe adverse health problems in treated pigs. This degree of transgene regulation has not yet been achieved, but the rapid pace of discovery regarding gene regulation makes such a requirement a possibility within a few years.

In the examples described here, we focused on enhancing the growth rate of pigs and a side effect was the reduction in subcutaneous fat. Perhaps other approaches could be used to more directly change the body composition. One example is that the body fat characteristics might be altered or muscle mass might be selectively increased by introducing appropriate genes. Likewise, the composition of milk could be altered to make it more nutritious or better suited for certain commercial processes.

Another possible mechanism for improving domestic farm animals involves introducing genes that would increase the resistance of these animals to infectious diseases or parasites. For example, novel immunoglobulins with specificities for particular antigens can be isolated from hybridomas and introduced into the germline. This has been accomplished in mice that expressed large amounts of antibodies to nitrophenyl, trinitrophenyl, and phosphorylcholine residues without prior immunization (*42–44*). A disadvantage of this approach is that the rearranged transgenes inhibit the rearrangement of endogenous immunoglobulin genes (*45*). Although this has not had a profound effect on the health of mice reared in the laboratory, it might compromise animals in a natural environment. It might be possible to redirect immunoglobulin production to

Table 6. Growth performance of MTbGH transgenic pigs. Values are least-squares means ± SEM; the number of animals is indicated in parentheses. Sample means were compared for significance by an analysis of variance. ND, not determined.

Group	Average daily weight gain (g)	Feed efficiency (kg feed/ kg gain)
Founder animals*		
Control	743 ± 32 (6)	3.12 ± 0.15
Transgenic	690 ± 65 (6)	2.62 ± 0.12
	$P = 0.480$	$P = 0.026$
37-06 G2 progeny†		
Control	760 ± 24 (8)	2.99 ± 0.12 (8)
Transgenic	874 ± 30 (5)	2.46 ± 0.16 (5)
	$P = 0.016$	$P = 0.026$
37-06 G3 progeny‡		
Control	867 ± 21 (15)	ND
Transgenic	933 ± 31 (8)	
	$P = 0.098$	
31-04 G2 progeny§		
Control	869 ± 44 (7)	ND
Transgenic	988 ± 62 (7)	
	$P = 0.15$	
Combined progeny		
Control	815 ± 17 (30)	ND
Transgenic	905 ± 21 (20)	
	$P = 0.001$	

*Six transgenic pigs (G0) that express MTbGH (Table 3) and six control nontransgenic littermates had free access to a corn-soybean diet (16% protein) at a body weight between 30 and 60 kg. †Transgenic and control pigs are G2 generation progeny of MTbGH founder 37-06 (Table 3). The experimental period began at an initial weight of 30 kg and continued to 90 kg. Pigs had either restricted (90% of ad libitum consumption) or ad libitum access to a corn-soybean diet containing approximately 18% crude protein supplemented with 0.25% lysine (*39*). Average daily weight gain and feed efficiency did not differ between pigs fed a restricted diet or pigs fed ad libitum, and therefore values are pooled means. ‡Transgenic and control G3 generation progeny of MTbGH founder 37-06 had free access to corn-soybean diet (18% protein) supplemented with 0.25% lysine (*39*) at a body weight between 30 and 90 kg. §Transgenic and control G2 progeny of MTbGH founder 31-04 had free access to a corn-soybean diet (18% protein) supplemented with 0.25% lysine (*39*) at a body weight between 30 and 90 kg.

Table 7. Effects of MTbGH expression on relative organ weight in transgenic pigs. Eight sex- and age-matched (range, 4.5 to 10 months) transgenic and control G2 animals from line 37-06 were used. Values are adjusted mean organ weights as a percentage of body weight. Sample means from control and transgenic groups were compared for significance by covariance analysis to adjust for age.

Organ	Control	MTbGH	SEM	*P*
Heart	0.35	0.44	0.02	0.013
Liver	1.69	2.53	0.15	0.002
Kidneys	0.37	0.61	0.03	0.0001
Adrenals	0.0048	0.0101	0.0007	0.0002
Thyroid	0.011	0.016	0.001	0.016

Table 8. Effects of MTbGH expression on bone measurements in transgenic pigs. Bone weight is a percentage of total body weight. Values are least-squares means and SEM for four control and four transgenic pigs except for femur weight where data are for eight control and eight transgenic pigs.

Value	Weight	Length (cm)	Circumference (cm)
		Humerus	
Control	0.31	18.75	8.90
MTbGH	0.41	18.25	10.40
SEM	0.02	0.58	0.23
P	0.025	0.57	0.006
		Femur	
Control	0.34	20.93	8.75
MTbGH	0.44	20.13	10.38
SEM	0.02	0.60	0.41
P	0.003	0.39	0.04

other cell types, thereby not interfering with the B cell function. Although expression of immunoglobulin genes of defined specificity would represent a general strategy, other approaches that would take advantage of pathogen receptors, mode of replication, or production of specialized gene products can be envisaged.

The third approach that has received attention is that of using animals to synthesize proteins of medical value. This would be particularly valuable for the production of proteins that cannot be synthesized in their active form by microorganisms, such as blood-clotting enzymes that require covalent modifications for activity. These proteins might be produced either in blood or milk under the control of regulatory elements from genes that are expressed in liver or mammary gland, respectively. Low levels of human tissue plasminogen activator and coagulation factor IX have been secreted into the milk or blood of transgenic mice (*46*) and most recently into the milk of transgenic sheep (*47*). Further improvements in the gene constructs could result in improved levels of gene expression that might make this a viable proposition. One problem will be the purification of the human protein from contaminating animal proteins: this could be particularly difficult if the human protein needs to be separated from the animal equivalent. Another potential problem is that the human protein produced in animal cells might not have exactly the same covalent modifications, such as glycosylation, as those produced in human cells. These differences might render the human protein immunogenic and thus restrict its prolonged use. These problems are not unique to protein production in transgenic animals and may be circumvented by appropriate engineering of the transgene. If high levels of protein production can be achieved in transgenic animals, this could be a very cost-effective means of producing medically important proteins.

Each of these ideas has inherent problems, and our experience to date is that in the process of making transgenic animals many other problems will become apparent. However, from the point of view of basic research, we anticipate that many aspects of genetic control and animal physiology will be unraveled as a consequence of trying to understand the results obtained when expressing novel gene constructs in transgenic animals. In addition, the continued extrapolation of these techniques to farm animals will ultimately result in more productive and healthier livestock.

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70. Gastric ulceration: focal loss of gastric mucosa with local inflammation in the pars esophagus and variable evidence of granulation tissue in healing lesions. Synovitis: inflammation of joint synovium, ranging from mild edema with focal aggregates of inflammatory cells to necrosis and ulceration of thickened synovium with erosion of underlying cartilage. Dermatitis: focal perivascular accumulation of inflammatory cells in subcutis and superficial dermis and mild epidermal thickening. Nephritis: mild, multifocal accumulation of lymphocytes in tubulointerstitial space. Pneumonia: inflammation of lung, ranging from mild interstitial accumulation of lymphocytes to multifocal perivascular and peribronchiolar accumulation of lymphocytes and plasma cells consistent with enzootic pneumonia. Cardiac myocyte nuclear hypertrophy: enlargement of numerous cardiac myocyte nuclei of unknown significance.
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Altering the Genome by Homologous Recombination

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Homologous recombination between DNA sequences residing in the chromosome and newly introduced, cloned DNA sequences (gene targeting) allows the transfer of any modification of the cloned gene into the genome of a living cell. This article discusses the current status of gene targeting with particular emphasis on germ line modification of the mouse genome, and describes the different methods so far employed to identify those rare embryonic stem cells in which the desired targeting event has occurred.

THE IMPLICATIONS OF THE NEW GENE TARGETING TECHnology are far-reaching. If the recipient cell is a pluripotent, embryo-derived stem (ES) cell, it is possible to transfer a modification of a cloned gene, created in a test tube, to the germ line of a living organism (*1–3*). The potential now exists for modifying any gene, in a defined manner, in any species from which functional ES cells can be obtained. ES cells have been isolated from mouse and hamster embryos (*4*) and major efforts are currently under way to isolate equivalent cells from domestic animals including sheep, pigs, and cattle. In addition, because many plant cells are intrinsically pluripotent and the means exist for generating whole plants from these cultured cells, we can anticipate the application of gene targeting to the modification of plant genomes as well (*5*).

The discussion need not, however, be limited to experiments directed only at germ line modifications. In specific cases it may be advantageous to modify only certain somatic tissues of an organism. For example, as the means to propagate a variety of human somatic stem cells (such as hematopoietic, epithelial, liver, or lung stem cells) become available, protocols based on gene targeting could be used to correct defective genes in the appropriate human tissue. This scenario of human somatic gene therapy has some obvious advantages over the random insertion of a nondefective gene: for example, the corrected endogenous gene is much more likely to be expressed in the appropriate tissue at appropriate levels. Further, it should be possible to use this approach to correct dominant mutations.

In addition to its implication for in vivo manipulations, gene targeting technology has broad potential for fundamental research with cells cultured in vitro. Many biological questions can be answered directly and more simply with tissue culture systems. In such cells, both alleles of an autosomal gene could be modified by the sequential application of gene targeting. Cell-lethal phenotypes could be maintained and analyzed by a variety of techniques, including the introduction of a transgene under the control of an inducible promoter.

However, in this article I will emphasize experiments involving mouse embryo-derived stem cells. This choice is based on the interest and potential of using targeted, modified ES cells as a vehicle to generate mice of any desired genotype. Unfortunately, this choice precludes reviewing the gene targeting literature leading

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