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Production and Analysis of Transgenic Pigs Containing a Metallothionein Porcine Growth Hormone Gene Construct

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While initial studies with growth hormone (GH) fusion genes demonstrated that transgenesis could be used to enhance growth performance in the pig, they also highlighted the need to be able to control expression in order to avoid pathological problems associated with high-level expression. We have produced transgenic pigs containing a GH construct consisting of a modified human metallothionein IIA (MT) promoter fused to the cDNA sequence for the porcine growth hormone gene. A total of 289 pigs were born live of which 88 (2.8% of embryos injected) were transgenic. Founders were reared on diets containing 100 ppm of zinc. Induction of transgene expression was assessed by feeding 1000 ppm of zinc in the diet (high zinc) for 3 weeks and measuring plasma IGF-I as a marker of GH production, before, during and after the high zinc diet. Evidence to suggest that transgene expression could be induced was obtained in 12/36 founders tested. Founders were mated to non-transgenic animals to produce transgenic progeny. Twenty-two per cent (4/18) of male founders did not transmit the transgene and 39% (7/18) transmitted the transgene at frequencies of less than 30%. The effect of transgene expression on growth performance was evaluated by feeding transgenic and non-transgenic progeny the high zinc diet from 20–100 kg liveweight. Rate of gain, feed intake and estimates of carcass fat and muscle were compared between the two groups of progeny of 60–100 kg liveweight. Analysis of transgenic progeny growth performance was confounded by considerable individual variation between transgenic progeny and

 Transgenic Animals in Agriculture
(eds J.D. Murray, G.B. Anderson, A.M. Oberbauer and M.M. McGloughlin)

the relatively few transgenics available for evaluation from mosaic founders. A number of transgenic progeny exhibited enhanced growth performance and have been selected for further breeding and analysis.

Introduction

The ability to manipulate the genome of domestic livestock has the potential to revolutionize animal production in the coming decades (reviewed by Brem and Muller, 1994; Wall, 1996). The mouse experiments of Palmiter *et al.* (1982) were the first to demonstrate that growth hormone (GH) fusion genes could dramatically improve animal growth. Since these initial studies, a number of groups have examined the potential of transgenesis to improve growth performance in the pig (reviewed by Pursel *et al.*, Chapter 10, this volume, 1990a,b; Brem and Muller, 1994; Table 11.1). While many of these studies demonstrated that transgenesis could be used to enhance growth performance, they also highlighted the need to be able to control transgene expression to avoid pathological problems associated with high level expression, including lameness and infertility (Pursel *et al.*, 1987; Ebert *et al.*, 1988; Wieghart *et al.*, 1990). Our own experience in producing GH transgenic pigs is discussed in this chapter.

Production of Transgenic Founders

Transgenic pigs are currently produced by injecting hundreds to thousands of copies of a transgene into the pronucleus of a recently fertilized egg. The injected DNA then becomes incorporated at random, normally in head-to-tail arrays at a single genomic site (Palmiter *et al.*, 1982; Hammer *et al.*, 1985a; Burdon and Wall, 1992). A number of GH constructs and growth-related constructs have been used in pigs (reviewed by Pursel *et al.*, Chapter 10, this volume, 1990a,b; Brem and Muller, 1994; Table 11.1). The majority of these have used elements from the mouse or human metallothionein (MT) promoter fused to genomic or cDNA clones of the pig, bovine or human GH gene. The MT promoter appears to have been used because of earlier evidence obtained in mice suggesting that expression of thymidine kinase and GH transgenes could be induced by the addition of zinc to the diet (Brinster *et al.*, 1981; Palmiter *et al.*, 1982; reviewed by Seamark and Wells, 1993).

The initial aim of our study was to produce transgenic pigs in which GH expression could be regulated by manipulating the level of zinc in the diet, with the overall goal of producing commercial lines of transgenic pigs with enhanced growth performance. The transgene used in our studies consisted of a modified human MT II-A promoter fused to the cDNA sequence for the porcine growth hormone gene (Fig. 11.1). Transgenic

Table 11.1. Growth hormone transgenic pig studies.

Fusion gene	Embryos transferred	Piglets born	Number of transgenics	Reference
mMT-hGH	286	15 (5.2)	1 (0.4)	Brem *et al*. (1985)
mMT-hGH	2035	192 (9.4)	20 (1)	Hammer *et al*. (1985a)
mMT-bGH	2330	150 (6.4)	9 (0.4)	Pursel *et al*. (1987)
mMT-hGH	1014	21 (2.1)	4 (0.4)	Brem *et al*. (1988)
MLV-rGH	59	15 (25.4)	1 (1.7)	Ebert *et al*. (1988)
hMT-pGH	423	17 (4.0)	6 (1.4)	Vize *et al*. (1988)
WAP-hGH	1028	51 (5.0)	7 (0.7)	Brem *et al*. (1988)
bPRL-bGH	289	20 (6.9)	5 (1.7)	Polge *et al*. (1989)
MLV-pGH	410	59 (14.4)	6 (1.5)	Ebert *et al*. (1990)
CMV-pGH	372	32 (8.6)	15 (4.0)	Ebert *et al*. (1990)
PEPCK-bGH	1057	124 (11.7)	7 (0.7)	Wieghart *et al*. (1990)
hMT-pGH	1327	148 (11.1)	43 (3.2)	Nottle *et al*. (1994)
hMT-pGH	1835	141 (7.6)	45 (2.5)	Nottle *et al*. (1994)
Total	12465	985 (7.9)	169 (1.3)	

Numbers in brackets are values expressed as a percentage of embryos injected.

founders were produced by pronuclear microinjection using procedures described previously (Nottle *et al.*, 1994). Two groups of transgenic founders were produced (Table 11.1). Previously reported studies have shown that 0.3–4.3% of injected embryos resulted in the birth of a transgenic pig (Pursel and Rexroad, 1993). In our study, the number of live-born piglets that were transgenic was 2.8% of embryos injected. In the mouse, the concentration at which the DNA is injected does not appear to influence the integration rate between 1 and 10 ng μl^{-1} (Brinster *et al.*, 1985). However, our experience over several years with a variety of constructs suggests that the concentration at which DNA is injected in this range may influence integration rates in the pig (Nottle *et al.*, 1997). In particular, we have found

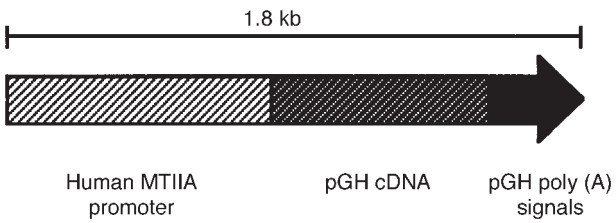


Fig. 11.1. The construct used consisted of a 1.8 kb insert, containing approximately 840 bp of the human metallothionein IIA promoter (MTIIA; including the metal response elements) 5′ to a pig GH cDNA (containing the entire protein coding region), followed by a portion of the pig GH genomic DNA containing polyadenylation signals.

that DNA injected at 10 ng μl^{-1} consistently results in 2–4% of embryos injected or 20–30% of live-born pigs being transgenic.

Transgene expression has been shown to vary depending on where in the genome the transgene becomes incorporated (so called 'position effects'; reviewed by Bishop, 1997). In the majority of reported studies with GH transgenic pigs, GH was constitutively expressed, albeit at variable levels, in sufficient amounts to have a number of deleterious side-effects including lameness and infertility (Pursel *et al.*, 1987; Ebert *et al.*, 1988). Attempts to obtain better control over GH transgene expression using different promoters such as phosphoenolpyruvate carboxykinase (Wieghart *et al.*, 1990) have also proven to be unsatisfactory. To avoid any deleterious effects associated with high-level constitutive expression, we measured plasma GH in our transgenic founders prior to weaning and culled animals with GH levels outside the range of those measured in the non-transgenics. Eight of the 88 founders produced were identified as having high-level constitutive expression and were euthanased.

Induction of Transgene Expression in Founder Populations

The metallothionein promoter contains a complex array of metal responsive elements (Lee *et al.*, 1987) and can be induced by metals such as zinc. In MT-bGH transgenic mice, concentrations of bGH were elevated more than tenfold after zinc was added to their drinking water (Hammer *et al.*, 1985b). In pigs containing the same constructs the addition of 1000–3000 ppm of zinc to the feed approximately doubled bGH expression (Pursel *et al.*, 1990a).

Because we produced a relatively large number of founder transgenics (approximately half of all GH transgenic founders reported; Table 11.1) we decided to screen our founder populations for animals in which expression could be induced. However, it was apparent from earlier work that animals maintained on high zinc diets for long periods may develop pathological problems as a result of chronic overexpression of GH (Pursel *et al.*, 1987; Ebert *et al.*, 1988; Wieghart *et al.*, 1990). We reasoned that exposure to increased amounts of zinc for a relatively short period might allow us to identify founders in which the transgene could be induced without the risk of animals developing any pathological problems. For the first group of founders, induction was tested by feeding animals a diet containing 1000 ppm of zinc (as zinc sulphate; high zinc) for 10 days. Plasma IGF-I was measured as a marker of GH production, the day before, 7 days after the start of and 7 days after the end of the high zinc diet. Plasma IGF-I has been shown previously to be increased in response to daily GH injection (Owens *et al.*, 1990), in animals implanted with slow release GH (Buonomo *et al.*, 1995) and in transgenic pigs expressing GH (Miller *et al.*, 1989). No increase in IGF concentration was detected in this experiment. As a consequence of

this finding, the period over which the high zinc diet was fed was increased to 3 weeks for the second group of founders. Increases in plasma IGF-I, of 25% or more above the concentration of plasma IGF-I measured prior to induction, were demonstrated in five of the 24 founders.

On the basis of this finding we retested the group 1 founder males for evidence of transgene induction. Seven of the 12 founders exhibited evidence of being inducible when tested at around 70 weeks of age (Fig. 11.2; Table 11.2). In the majority of these founders, IGF-I had returned to pre-induction levels when measured 6 weeks after the end of the high zinc diet. These findings suggest that transgene expression could be regulated by manipulating the level of zinc in the diet.

Transgene Transmission by Founders

Each transgenic founder produced by pronuclear microinjection is unique in terms of its expression. Assessment of growth performance of GH transgenic pigs has been limited mostly to comparisons between transgenic founders

Table 11.2. Induction status and transgene transmission frequency for group 1 and 2 male founders.

Founder	Inducible	Transgenic/ total progeny	% Transgenic
Group 1			
50402	No	22/53	42
50403	Yes	23/45	51
50404	No	0/36	0
50405	No	24/63	38
50406	Yes	19/103	18
50408	Yes	15/62	24
50409	Yes	0/72	0
50410	Yes	6/122	5
50411	No	13/26	50
50413	Yes	0/52	0
50414	No	0/55	0
50415	Yes	21/56	38
Group 2			
51201	Yes	Not mated	
51202	No	6/67	9
51203	No	30/94	32
51204	No	6/91	7
51205	No	11/62	18
51206	No	26/98	27
51207	Yes	Not mated	
51208	Yes	30/71	42

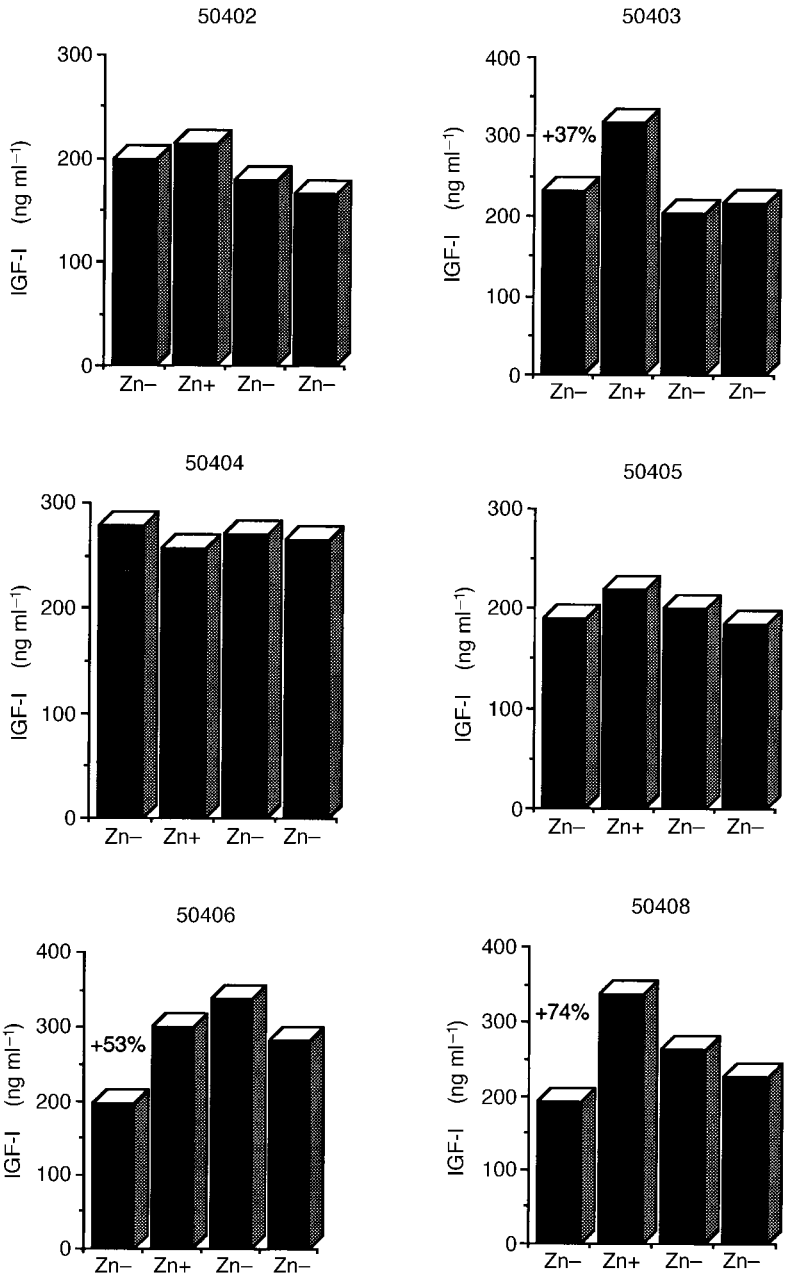
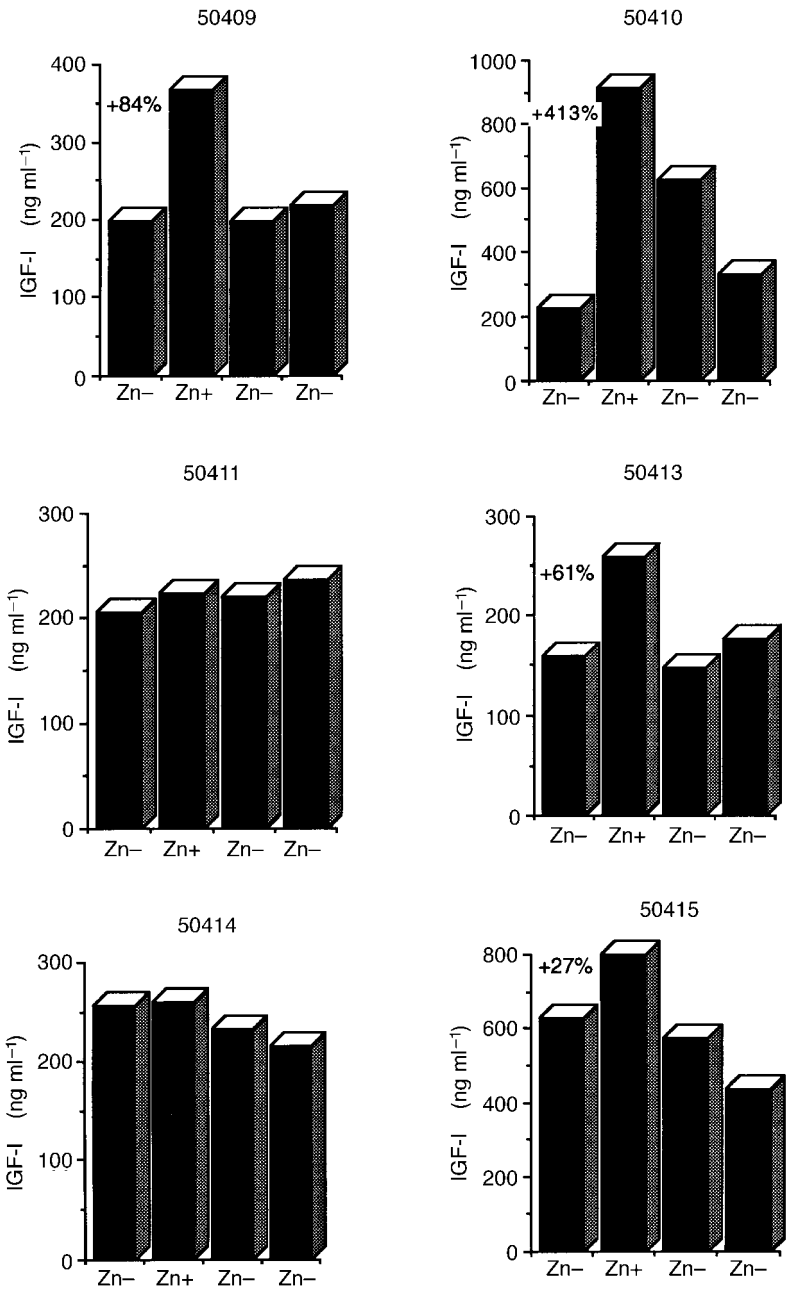


Fig. 11.2. Plasma IGF-I concentration in 12 group 1 founder males (continued opposite). Animals were tested for induction at approximately 70 weeks of age. A diet containing 1000 ppm of zinc (as zinc sulphate) was fed for 3 weeks. Animals were bled twice daily (a.m. and p.m.) on the day before, last day of, and 3 and 6 weeks after the high-zinc diet. Plasma IGF-I was measured according to methods described by Owens *et al.* (1990).


50409
+84%
IGF-I (ng ml⁻¹)
400
300
200
100
0
Zn– Zn+ Zn– Zn–
50410
+413%
1000
800
600
400
200
0
50411
300
200
100
0
50413
+61%
50414
50415
+27%
800
600
400
200

and non-transgenic littermates (reviewed by Pursel *et al.*, 1990a). To evaluate the effect of the transgene used in our study on growth performance, transgenic founders were mated to non-transgenic animals to produce transgenic and non-transgenic progeny whose growth performance could be compared when fed the high zinc diet. Transgenes are normally inherited in a Mendelian fashion if they have been integrated at a single site. Mating of hemizygous transgenics theoretically results in 50% of the progeny being transgenic. Of the male founders mated in our study (group 1 and group 2), 22% (4/18) did not transmit the transgene to their progeny while 39% (7/18) transmitted the transgene at frequencies less than 30% (Nottle *et al.*, 1996). In mice the incidence of germline mosaicism has been reported to be 30% (Wilkie *et al.*, 1986). Our results, together with those of other workers (Pursel *et al.*, 1990a,b; Brem and Muller, 1994), suggest that the incidence of germline mosaicism may be higher in pigs than in mice.

Effect of GH Transgene Expression on Growth Performance

Daily administration of GH results in increased growth rate, a decrease in feed intake, increased muscle mass and a reduction in carcass fat (Campbell *et al.*, 1989). Similar improvements have been demonstrated in GH transgenic pigs which constitutively express GH (Pursel *et al.*, 1990a,b).

To evaluate the effect of the transgene on growth performance in our studies transgenic and non-transgenic progeny were fed high zinc diets from 20 to 100 kg liveweight. Rate of gain and feed intake were measured between 60 and 100 kg liveweight and estimates of carcass fat and meat content were obtained at 100 kg liveweight. The results for these evaluations were confounded by the relatively low numbers of transgenic progeny available for the majority of founders due to the relatively high incidence of germline mosaicism. This was exacerbated by large variations in growth performance observed between the transgenic progeny. These factors made any comparison within founders between progeny of the same sex virtually impossible. In mice, variation in expression is often seen between transgenic progeny from the one founder possibly due to differences in DNA methylation (Mehtali *et al.*, 1990) and heterochromatin formation (Martin and Whitelaw, 1996). In such cases, selection needs to be carried out for more than one generation to generate a transgenic line. A number of transgenic progeny exhibited enhanced growth performance during the period they were fed a high zinc diet in these evaluations. These animals have been selected for further breeding and analysis. Growth performance and induction data for three male progeny selected from two inducible group 1 founders are shown in Table 11.3.

Table 11.3. Growth performance data for six male F1 progeny selected from two inducible group 1 founder males.

Founder	Founder IGF-I (% increase)	Progeny	Liveweight gain (g day^{-1})[a]	Feed conversion[a]	P2[b] (mm)	Muscle depth[c] (mm)	Progeny IGF-I[d] (% increase)
50406	52	2774	820	2.14	11	39	42
		2968	890	2.26	8	35	3
		2951	790	2.41	8	38	44
Non-transgenic male littermates (mean ± SEM)		745 ± 36	2.68 ± 0.14	10 ± 1	34 ± 1		
50408	74	2296	880	1.86	8	35	15
		2990	900	1.87	6	29	7
		2844	950	2.04	10	35	26
Non-transgenic male littermates (mean ± SEM)		836 ± 32	2.21 ± 0.07	13 ± 7	36 ± 1		

[a] Progeny were fed high-zinc diets from approximately 25 to 100 kg liveweight. Liveweight gain and feed conversion (feed consumed/liveweight gain) was measured from 60 to 100 kg liveweight.
[b] P2 is fat depth measured over the last rib 6.5 cm off the midline at 100 kg liveweight.
[c] Muscle depth is the depth of the longissimus dorsi measured over the last rib, 6.5 cm off the midline at 100 kg liveweight.
[d] Plasma IGF-I was measured using the induction protocol described in the text.

Conclusions

The major finding from our study was that MT–pGH transgenic pigs can be produced using pronuclear microinjection in which expression can be regulated by manipulating the level of zinc in the diet. While we have been able to generate potentially useful genotypes using this technique, our experience (and that of other groups) demonstrates that this is a major undertaking. The low efficiency with which founder transgenics are produced, the variation in the level of expression between founders and the relatively high degree of mosaicism, are all major drawbacks. Furthermore, the large variation between transgenic progeny in their growth performance suggests that the production of commercial lines of transgenic animals may require a number of generations of selection. In order for the full potential of transgenesis to be realized in pigs as well as in other livestock species, methods will be required which allow a single copy of the transgene to be inserted at high efficiency at a predetermined site in the genome which does not interfere with expression.

Acknowledgements

We are grateful to the numerous BresaGen and Bunge staff who made invaluable contributions during the course of this study.

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