Mammary development and milk secretion in transgenic mice expressing the sheep β -lactoglobulin gene

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Mammary development and milk secretion were studied in transgenic mice which exhibited mammary tissue-specific expression of the sheep β -lactoglobulin gene, and secreted significant quantities of the foreign protein in milk. Mammary development was unaffected by transgenesis. Tissue DNA content and the activities of several key enzyme markers of cell differentiation were similar in transgenic mice and non-transgenic controls. Milk yield, whether estimated by pup weight gain or measured by a $^3\text{H}_2\text{O}$ -dilution method, was unchanged by foreign gene expression. Gross milk composition, including milk protein concentration, was also similar in transgenic and non-transgenic animals, even though β -lactoglobulin accounted for 29 % of total milk protein. Therefore the foreign gene product was synthesized at the expense of endogenous milk proteins. However, transgenic mammary tissue *in vitro* exhibited a significantly higher rate of total protein synthesis than did control tissue. This suggested that a factor limiting milk protein synthesis or secretion in transgenic mice *in vivo* may have been removed by short-term explant culture of mammary tissue. The results emphasize that the use of transgenesis for manipulating milk composition may depend not only on high-level mammary-specific expression of the foreign gene, but also on the biosynthetic capacity of the mammary gland itself.

INTRODUCTION

Production of transgenic animals is an attractive method for studying gene expression *in vivo*, and offers new possibilities for the control of animal performance. One possible application of this technology is in the manipulation of mammary-gland function, either for large-scale production of non-milk proteins (Pittius *et al.*, 1988; Clark *et al.*, 1989b; Archibald *et al.*, 1990) or for insertion of heterologous milk proteins (Simons *et al.*, 1987; Lee *et al.*, 1988; Wall *et al.*, 1991). It should also be possible to use the same techniques to modify the concentration or structure of endogenous milk proteins (Clark *et al.*, 1989a). The DNA regulatory elements required for expression of milk proteins are as yet not well characterized. Consequently, gene constructs designed for mammary-specific expression have so far elicited levels of expression which range between < 1 % and in excess of 4 times that of the native gene (Hennighausen, 1990). However, the usefulness of transgenic techniques also depends on another factor which has received rather less attention, that is, the effect of gene insertion on normal lactational performance.

In this study we have investigated the effect of high-level expression of a heterologous milk protein gene on mammary development and lactation in transgenic mice containing the entire sheep β -lactoglobulin (β LG) structural gene. β LG transgene expression in these animals is restricted to the secretory epithelium of the mammary gland, and transcription is initiated at the correct position within the β LG promoter (Simons *et al.*, 1987; Harris *et al.*, 1991). Moreover, induction of β LG expression coincides with that of β -casein in the tissue, indicating that the pattern of expression is determined by the differentiated state of the mammary cells (Harris *et al.*, 1991). Initial measurements suggested that β LG constituted a substantial proportion of total milk protein in these animals (Simons *et al.*, 1987). Thus any effect of high-level expression of an inserted gene on mammary function should be apparent in these animals, and can be attributed to a direct effect within the gland. Part of this work

has been presented in a preliminary communication (Wilde *et al.*, 1991a).

MATERIALS AND METHODS

Animals

Transgenic mice were hemizygous animals of a line designated BLG45 (Simons *et al.*, 1987), which contained a 10.8 kb *SalI*–*XbaI* fragment comprising the 4.9 kb β LG transcription unit, 4 kb of 5'-flanking sequences and 1.9 kb of 3'-flanking sequences. They were fed *ad libitum* (CRM diet from Special Diet Services, Witham, Essex, U.K.) and housed at 21 °C with a daily photoperiod of 14 h (05:00–19:00 h). Transgenic mice were identified by PCR analysis of tail DNA samples (Whitelaw *et al.*, 1991), after biopsy of weanling animals under general anaesthesia (Hypnorm, 2.5 ml/kg, Janssen, Oxford, U.K.; Hypnovel, 2.5 ml/kg, Roche, Dunstable, U.K.). Animals with litters of 8–11 pups were studied in their first lactation, and compared with non-transgenic siblings fed and housed under the same conditions.

Mammary development and milk yield

Mammary DNA content and enzymic indices of cell differentiation were assayed on days 9–14 of lactation as described previously (Shipman *et al.*, 1987). Milk yield was measured by a $^3\text{H}_2\text{O}$ -dilution method on days 9–13 (Knight *et al.*, 1986), and by pup growth rate.

Milk composition

Milk samples were collected under Hypnorm/Hypnovel anaesthesia after intraperitoneal injection of 300 m-units of oxytocin (Intervet, Cambridge, U.K.), and assayed for fat content (Fleet & Linzell, 1964) and protein concentration (Bradford, 1976). Lactose concentration was measured by an enzymic method adapted from that of Kuhn (1972).

For analysis of milk proteins, milk was defatted by centri-

Abbreviations used: β LG, β -lactoglobulin; α LA, α -lactalbumin.

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fugation (2000 g, 15 min, 15 °C) and dialysed extensively against water. The dialysis residue was freeze-dried, reconstituted in 10 mM-imidazole, pH 7.0, and a sample (4.0 mg) was subjected to anion-exchange chromatography on an f.p.l.c. Mono-Q HR 10/10 column (Pharmacia, Milton Keynes, U.K.). Protein fractions eluted by a linear gradient of 0–1.0 M-NaCl in 10 mM-imidazole, pH 7.0, were dialysed against water, freeze-dried and assayed for protein (Bradford, 1976). Fractions containing caseins, β LG and α -lactalbumin (α LA) were identified by co-migration with purified mouse and sheep milk proteins on SDS/PAGE.

Tissue culture

Mammary tissue explants were prepared rapidly (within 10 min) from glands of transgenic and non-transgenic mice killed by cervical dislocation on days 9–14 of lactation, and equilibrated for 5 min in medium 199 containing 12.5 mM-Hepes, 5 μ g of insulin/ml, 100 ng of cortisol/ml and 1 μ g of prolactin/ml. Immediately after equilibration, the rate of total protein synthesis was measured by culture [37 °C, in air/CO₂ (19:1)] for 15 min in the same medium containing L-[4,5-³H]leucine at 50 μ Ci/ml, and synthesis of caseins, β LG and α LA was measured over 1 h in medium containing 20 μ Ci of L-[4,5-³H]leucine/ml.

Explants were homogenized at 4 °C in 10 mM-Tris/HCl, pH 7.0, containing 5 mM-EGTA and 2 mM-phenylmethanesulphonyl fluoride by 10 strokes with a glass/Teflon homogenizer, followed by sonication for 30 s (setting 30, Kontes KT50 cell disrupter). Total radiolabel incorporated in protein was measured by precipitation with trichloroacetic acid (final concn. 5%, w/v). ³H-labelled milk proteins were isolated by SDS/PAGE (Laemmli, 1970), and bands corresponding to 43 kDa, 39 kDa and 26 kDa caseins, β LG and α LA were excised and counted for radioactivity (Wilde *et al.*, 1984).

Materials

The sources of radiochemicals, hormones and enzyme assay reagents were as described previously (Wilde *et al.*, 1986). Culture medium was from Northumbria Biologicals, Cramlington, U.K. Dialysis membrane was Spectropor-1 from Pierce and Warriner, Chester, U.K.

RESULTS

Mammary tissue weight, DNA concentration and total DNA content on day 12 of lactation were similar in transgenic mice and non-transgenic controls (Table 1). The activities of several mammary enzymes previously identified as markers of secretory-cell differentiation (Shipman *et al.*, 1987) were also similar in transgenic and non-transgenic mammary tissue (Table 1). These results indicated that expression of the foreign gene affected neither mammary cell number nor the degree of differentiation of the secretory epithelial cells. Pup weight gain in litters from transgenic dams also did not differ from that of controls (Fig. 1), indicating that the rate of milk secretion was unaffected by insertion of foreign DNA. This was confirmed by direct measurement of milk yield by ³H₂O dilution on days 9–13 of lactation (Table 2).

β LG expression did not alter the fat or lactose content of mouse milk (Table 2), nor its ionic composition (results not shown). Milk protein concentration was also similar in transgenic and non-transgenic mice, even though chromatographic separation of the major protein species confirmed that β LG was secreted in transgenic-mouse milk (Fig. 2). The fraction containing β LG accounted for a large proportion (29.4 $\pm$ 8.8%) of total milk protein in these animals (Table 3). This indicated that the foreign protein was being produced at the expense of

endogenous milk proteins. Comparison of the proportion of proteins in other fractions obtained from transgenic and non-transgenic-mouse milk supported this conclusion, and indicated that β LG secretion was at the expense not of one specific protein, but of endogenous milk proteins in general (Table 3). The depletion of native milk proteins in transgenic-mouse milk was shown by protein fractions which together constituted 98.7% of total milk protein in non-transgenic mice (Table 3).

Although transgenic-mouse milk had a similar milk protein concentration to control milk, the rate of [³H]leucine incorporation into total protein was 41% higher ($P < 0.05$) in explants

Table 1. Mammary cell number and differentiation in transgenic and non-transgenic mice

Mammary tissue weight, DNA content and enzyme activities were measured on days 9–14 of lactation. Enzyme activities are expressed as μ mol/min per mg of DNA or *nmol/min per mg of DNA. Values are means $\pm$ S.E.M. for the numbers of determinations in parentheses.

	Control	Transgenic
Tissue weight (g)	4.67 $\pm$ 0.28 (8)	4.44 $\pm$ 0.38 (9)
DNA (mg/g of tissue)	4.21 $\pm$ 0.18 (10)	4.22 $\pm$ 0.13 (9)
Total DNA (mg)	19.46 $\pm$ 0.99 (8)	18.77 $\pm$ 1.73 (9)
Acetyl-CoA carboxylase*	0.61 $\pm$ 0.09 (10)	0.69 $\pm$ 0.03 (10)
Fatty acid synthetase	0.43 $\pm$ 0.10 (10)	0.60 $\pm$ 0.11 (10)
Glucose-6-phosphate dehydrogenase	3.81 $\pm$ 0.37 (9)	4.51 $\pm$ 0.62 (10)
Galactosyltransferase	0.42 $\pm$ 0.04 (10)	0.48 $\pm$ 0.05 (10)
Arylesterase	0.66 $\pm$ 0.04 (10)	0.61 $\pm$ 0.05 (10)

Fig. 1. Pup growth of transgenic and non-transgenic mice

Values are means $\pm$ S.E.M. of 10 (transgenic, ●) or 5 (non-transgenic, ○) determinations.

Table 2. Milk yield and composition in transgenic mice

Milk yield was measured by ³H₂O dilution on days 9–13 of lactation. Milk composition was assayed on days 9–14 as described in the Materials and methods section. Values are means $\pm$ S.E.M. for the numbers of determinations in parentheses.

	Control	Transgenic
Milk yield (g/day)	13.80 $\pm$ 2.50 (5)	15.47 $\pm$ 0.75 (12)
Protein (mg/ml)	105.6 $\pm$ 3.5 (10)	94.6 $\pm$ 6.3 (12)
Fat (% v/v)	28.3 $\pm$ 2.7 (8)	30.8 $\pm$ 2.7 (9)
Lactose (mm)	75.1 $\pm$ 5.6 (7)	62.0 $\pm$ 3.5 (7)

Fig. 2. Anion-exchange chromatography of transgenic and non-transgenic mouse milk proteins

Milk proteins were prepared from transgenic- and non-transgenic-mouse milk and separated by f.p.l.c. anion-exchange chromatography as described in the Materials and methods section. Fractions designated a–e were collected for determination of protein content and composition. An arrow indicates the elution position of β LG. Each profile is a representative example from one animal.

prepared from transgenic mice than from controls (Table 4). Measurement of [3 H]leucine incorporation into specific milk protein components under conditions designed to measure the synthetic capacity of the tissue (Shipman *et al.*, 1987) showed no significant difference between transgenic and control explants (Table 4).

DISCUSSION

The sheep β LG structural gene has previously been shown to be expressed specifically in the mammary glands of transgenic mice during pregnancy and lactation (Simons *et al.*, 1987; Harris *et al.*, 1991). This construct is also one of the few found to elicit secretion of significant quantities of a foreign protein in milk: β LG secretion by the hemizygous transgenic line used in the present study constituted 29% of total milk protein, a value similar to that determined by densitometry of Coomassie Blue staining after SDS/PAGE of total milk proteins

Table 3. Relative concentration of mouse milk proteins in fractions obtained by anion-exchange chromatography

Proteins were separated and assayed as described in the Materials and methods section. Fractions containing caseins, β LG and α LA were identified by comparison with the migration of purified proteins on SDS/PAGE. Values are means $\pm$ S.E.M. for five animals: ** $P < 0.01$ compared with non-transgenic mice.

Fraction	Protein constituent	Protein (% of total)	
		Non-transgenic	Transgenic
a	–	1.3 $\pm$ 0.5	1.9 $\pm$ 0.8
b	α LA, WAP†	30.2 $\pm$ 9.8	16.9 $\pm$ 3.7
c	β LG	1.7 $\pm$ 0.4	29.4 $\pm$ 8.8**
d	–	18.0 $\pm$ 4.6	13.6 $\pm$ 1.7
e	Casein	44.9 $\pm$ 5.7	37.1 $\pm$ 5.7
f	–	3.0 $\pm$ 1.0	0.9 $\pm$ 0.2

† Fraction b also contains a protein of approx. 16 kDa, which is thought to be whey acidic protein (WAP).

Table 4. Rates of protein synthesis in explants freshly prepared from transgenic and non-transgenic mice

Total protein synthesis was measured over 15 min by incorporation of L-[4,5- 3 H]leucine (25 mCi/mmol), and is expressed as d.p.m./min per μ g of DNA. Incorporation into individual proteins was measured over 1 h by using 10 mCi of [3 H]leucine/mmol, and is expressed as d.p.m./h per μ g of DNA. Values are means $\pm$ S.E.M. for the numbers of determinations in parentheses: * $P < 0.05$, ** $P < 0.01$ compared with non-transgenic controls.

Protein	Control	Transgenic
Total protein	111 $\pm$ 6 (6)	157 $\pm$ 16 (6)*
Casein		
43 kDa	241 $\pm$ 50 (6)	273 $\pm$ 55 (6)
39 kDa	300 $\pm$ 89 (6)	331 $\pm$ 89 (6)
26 kDa	471 $\pm$ 64 (6)	547 $\pm$ 53 (6)
Total	1012 $\pm$ 112 (6)	1152 $\pm$ 171 (6)
α LA	111 $\pm$ 22 (6)	195 $\pm$ 44 (6)
β LG	76 $\pm$ 7 (5)	227 $\pm$ 30 (6)**

(M. McClenaghan, A. J. Clark & J. P. Simons, unpublished work). High-level expression and secretion of β LG had no adverse effect on mammary development or the tissue's rate of milk secretion. On the other hand, expression of the additional milk protein gene did not produce an increase in milk protein concentration, and β LG secretion was accompanied by a general decrease in the concentrations of endogenous milk proteins. This suggested that *in vivo* total milk protein production was limited by a factor common to all the endogenous proteins.

Mammary explants from transgenic mice incorporated [3 H]leucine at a greater rate than did explants from controls. This increase is not due to a bias in the amino acid composition of transgenic milk protein, since the leucine content of β LG is similar to that of mouse caseins. Nor is the effect likely to be due to a change in amino acid pool size, since under the conditions employed free amino acids are rapidly equilibrated (Mayer, 1979; A. Gillespie, V. N. Pain, R. G. Vernon & C. J. Wilde, unpublished work). Rather, the data suggest that there is a significant increase in the rate of total protein synthesis in transgenic tissue explants. This increase is not reflected by the net rate of milk protein synthesis *in vivo*, as measured by the protein content and yield of control and transgenic-mouse milks. One possible explanation for the difference is that there is a finite limit

to the post-translational processing capacity (i.e. secretory capacity) of the gland. If this is the case, then an increase in the gross level of protein synthesis owing to β LG expression must be compensated by an increase in the rate of protein degradation, although a high level of protein turnover is not normally observed in the lactating gland. Nevertheless, increased intracellular degradation of newly synthesized caseins by a lysosomal mechanism has been observed in certain circumstances, for example when prolactin was removed or when milk removal was impaired (Razooki Hasan *et al.*, 1982; Wilde & Knight, 1986; Wilde *et al.*, 1989, 1991b).

Alternatively, the increased rate of protein synthesis observed *in vitro*, despite an unchanged yield of milk protein *in vivo*, may reflect a limit on milk protein synthesis that operates in the transgenic animal, perhaps through limitation of intracellular amino acid (or aminoacyl-tRNA) availability. Translational control of mammary protein synthesis, albeit during gland development rather than established lactation, has been reported in other species: a prolactin-induced increase in casein mRNA in pseudopregnant rabbits was not accompanied by an equivalent stimulation of casein synthesis (Houdebine, 1976), indicating that the response was limited by availability of translational machinery, such as aminoacyl-tRNA synthetases (Devinoy *et al.*, 1978). Changes in rat mammary polysome profiles during cellular differentiation *in vivo* and *in vitro* are also consistent with hormonal regulation of translation (A. Gillespie, V. N. Pain, R. G. Vernon & C. J. Wilde, unpublished work). Neither of these alternatives exclude the possibility that transcription or post-transcriptional mRNA processing may also limit endogenous milk protein production *in vivo* when β LG is expressed at high levels. Indeed, preliminary measurements suggest that the steady-state levels of specific milk protein mRNAs are lower in transgenic mice than in non-transgenic controls (M. McClenaghan, J. P. Simons, C. J. Wilde & A. J. Clark, unpublished work).

In summary, the study shows that insertion and mammary-specific expression of a foreign gene does not itself guarantee an increase in milk protein concentration. Indeed, since β LG synthesis produced an equivalent decrease in endogenous milk protein production, such that there was no significant difference in the protein content of transgenic and non-transgenic mouse milk, this suggests that mammary tissue has little or no spare capacity for protein synthesis. In a wider context, the study emphasizes that transgenic technology for manipulating milk composition cannot be applied in isolation, since its outcome is inextricably linked with the biochemical mechanisms regulating mammary-gland function.

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REFERENCES

- Archibald, A. L., McClenaghan, M., Hornsey, V., Simons, J. P. & Clark, A. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5178–5182
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Clark, A. J., Ali, S., Archibald, A. L., Bessos, H., Brown, P., Harris, S., McClenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A. & Wilmut, I. (1989a) *Genome* **31**, 950–955
- Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A. & Wilmut, I. (1989b) *Bio Technology* **7**, 487–492
- Devinoy, E., Houdebine, L.-M. & Delouis, C. (1978) *Biochim. Biophys. Acta* **517**, 360–366
- Fleet, I. R. & Linzell, J. L. (1964) *J. Physiol. (London)* **175**, 15P–17P
- Harris, S., McClenaghan, M., Simons, J. P., Ali, S. & Clark, A. J. (1991) *Dev. Genet. (NY)* **12**, 299–307
- Hennighausen, L. (1990) *Protein Expression Purif.* **1**, 3–8
- Houdebine, L.-M. (1976) *Eur. J. Biochem.* **68**, 219–225
- Knight, C. H., Maltz, E. & Docherty, A. H. (1986) *Comp. Biochem. Physiol.* **84A**, 127–133
- Kuhn, N. J. (1972) *Biochem. J.* **130**, 177–180
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Lee, K.-F., Demayo, F. J., Aitee, S. H. & Rosen, J. M. (1988) *Nucleic Acids Res.* **16**, 1027–1041
- Mayer, R. J. (1979) *Vitam. Horm. (NY)* **36**, 101–163
- Pittius, C. W., Hennighausen, L., Lee, E., Westphal, H., Nichols, E., Vitale, J. & Gordon, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5874–5878
- Razooki Hasan, H., White, D. A. & Mayer, R. J. (1982) *Biochem. J.* **202**, 133–138
- Shipman, L. J., Docherty, A. H., Knight, C. H. & Wilde, C. J. (1987) *Q. J. Exp. Physiol.* **72**, 303–311
- Simons, J. P., McClenaghan, M. & Clark, A. J. (1987) *Nature (London)* **328**, 530–532
- Wall, R. J., Pursel, V. G., Shamay, A., McKnight, R. A., Pittius, C. W. & Hennighausen, L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1696–1700
- Whitelaw, C. B. A., Archibald, A. L., Harris, S., McClenaghan, M., Simons, J. P. & Clark, A. J. (1991) *Transgen. Res.* **1**, 3–13
- Wilde, C. J. & Knight, C. H. (1986) *Comp. Biochem. Physiol.* **84B**, 197–201
- Wilde, C. J., Razooki Hasan, H. & Mayer, R. J. (1984) *Exp. Cell Res.* **141**, 519–532
- Wilde, C. J., Henderson, A. J. & Knight, C. H. (1986) *J. Reprod. Fertil.* **76**, 289–298
- Wilde, C. J., Addey, C. V. P. & Knight, C. H. (1989) *Biochim. Biophys. Acta* **992**, 315–319
- Wilde, C. J., Clark, A. J., Kerr, M. A., Knight, C. H., McClenaghan, M. & Simons, J. P. (1991a) *Proc. NZ Soc. Anim. Prod.* **51**, 225–226
- Wilde, C. J., Kerr, M. A. & Calvert, D. T. (1991b) *Exp. Physiol.* **76**, 533–537

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