Transgenic Research (1996) **5**: 67–72

SHORT COMMUNICATION

Synthesis and secretion of the mouse whey acidic protein in transgenic sheep

ROBERT J. WALL[1]*, CAIRD E. REXROAD, Jr.[1], ANNE POWELL[1], AVI SHAMAY[2], ROBERT McKNIGHT[2] and LOTHAR HENNIGHAUSEN[2]

[1]*Gene Evaluation and Mapping Laboratory, Agricultural Research Service, USDA, Beltsville, MD 20705, USA (Fax: 1 301 504 8414)*
[2]*Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

Received 10 October 1994; revised 25 January 1995; accepted 16 February 1995

The synthesis of foreign proteins can be targeted to the mammary gland of transgenic animals, thus permitting commercial purification of otherwise unavailable proteins from milk. Genetic regulatory elements from the mouse whey acidic protein (WAP) gene have been used successfully to direct expression of transgenes to the mammary gland of mice, goats and pigs. To extend the practical usefulness of WAP promoter-driven fusion genes and further characterize WAP expression in heterologous species, we introduced a 6.8 kb DNA fragment containing the genomic form of the mouse WAP gene into sheep zygotes. Two lines of transgenic sheep were produced. The transgene was expressed in mammary tissue of both lines and intact WAP was secreted into milk at concentrations estimated to range from 100 to 500 mg/litre. Ectopic WAP gene expression was found in salivary gland, spleen, liver, lung, heart muscle, kidney and bone marrow of one founder ewe. WAP RNA was not detected in skeletal muscle and intestine. These data suggest that unlike pigs, sheep may possess nuclear factors in a variety of tissues that interact with WAP regulatory sequences. Though the data presented are based on only two lines, these findings suggest WAP regulatory sequences may not be suitable as control elements for transgenes in sheep bioreactors.

Keywords: whey acidic protein gene; transgenic sheep; bioreactor; mammary gland

Introduction

Whey acidic protein (WAP) is an abundant milk protein in mice, rats, rabbits and camels, but has not been found in pigs, sheep or cattle (for review see McKnight *et al.*, 1991). The WAP gene has been isolated from mice, rats and rabbits, and molecular mechanisms of its regulation are being analysed in transgenic animals. In mice, expression of the WAP gene is confined to mammary alveoli, and WAP RNA accumulates at the end of pregnancy. Steady-state levels remain high throughout lactation (Pittius *et al.*, 1988; Hennighausen *et al.*, 1991), with approximately 10% of the mRNA in lactating mammary tissue encoding WAP (Hennighausen and Sippel, 1982a,b). Because of its high-level tissue-specific expression, the WAP promoter has been used to direct expression of heterologous genes to alveolar epithelial cells for basic biological studies (Andres *et al.*, 1987; Schonenberger *et al.*, 1988; Jhappan *et al.*, 1993; Tzeng *et al.*, 1993) and to direct the synthesis of pharmacologically active human proteins to the mammary glands of goats and pigs, in the hope of producing commercial quantities of purified proteins (Ebert *et al.*, 1991; Velander *et al.*, 1993).

Although the mouse is frequently used to analyse the integrity and functionality of hybrid genes, ultimately commercial production of foreign proteins in milk will require the use of transgenic rabbits, pigs, sheep, goats or cows. This, however, requires that regulatory elements from mammary-gland-specific genes are functional across

*To whom correspondence should be addressed

species boundaries. Towards this end, we compared the expression of WAP transgenes in mice (Burdon *et al.*, 1991a) and pigs (Shamay *et al.*, 1991; Wall *et al.*, 1991). Whereas only about one half of the mouse lines expressed the WAP transgene (Burdon *et al.*, 1991a), the WAP gene was expressed in all lines of pigs and the WAP was secreted into the milk (Shamay *et al.*, 1991; Wall *et al.*, 1991).

To extend the range of our findings, we report here a study designed to test the feasibility of using genetic regulatory elements from the mouse WAP gene to target gene expression to the mammary glands of sheep.

Material and methods

Generation of transgenic sheep

Ova, zygotes and 2-cell embryos (referred to hereafter as "eggs") were harvested from superovulated parous Rambouillet ewes as previously described (Rexroad *et al.*, 1990). The eggs were transferred to a test tube containing 37 °C Dulbecco's PBS with 10% sheep serum and transported to the laboratory for gene injection. In the laboratory, the eggs were microinjected with a 6.8 kb *Eco* RI fragment isolated from mouse genomic DNA (Campbell *et al.*, 1984) containing 2.6 kb 5′ and 1.6 kb 3′ flanking sequences surrounding the entire WAP gene. After microinjections, surviving eggs were returned to the surgical facility and two to four injected eggs were transferred into oviducts of anaesthetized recipient ewes whose cycles had been regulated with progestin pessaries.

Indentification of transgenic sheep

To identify transgenic sheep, genomic DNA was isolated from tail tissue and analysed for the presence of the mouse WAP gene using PCR and Southern blot analysis by the same procedure previously reported (Shamay *et al.*, 1991).

Isolation of RNA and northern blot analysis

Mammary biopsies were taken a week prior to parturition from transgenic founder ewe 001 and again on days 2, 24 and 50 of lactation. Biopsied mammary tissue was immediately placed in liquid nitrogen, and total RNA was isolated by the acid-guanidinium-thiocycanate-phenol-chloroform extraction method, and fractionated on formaldehyde agarose gels (Chomczynski and Saachi, 1987). The RNA was then transferred onto GeneScreen Plus Nylon membranes (NEN Research Products, Boston, MA, USA), hybridized for 12 h at 65 °C with random-primed-labelled cDNA fragments encoding mouse WAP, pig β-casein and pig β-lactoglobulin, washed and exposed to X-ray films.

Loading of total RNA was quantified by hybridizing the blot with an antisense RNA to human 18S ribosomal RNA (Hillis and Dixon, 1991) which cross-hybridizes with the mouse 18S ribosomal RNA and sheep 18S ribosomal RNA. After hybridizing the blot for 4 h in 0.4 M NaCl at 60 °C, followed by washing with 0.2 × SSC (30 mM NaCl, 0.4 mM sodium citrate), and 0.1% sodium dodecyl sulfate (SDS), the filter was scanned with the aid of a Betascope (Betagen Inc., Boston, MA, USA). The WAP hybridization signals were normalized to the 18S signal.

Analysis of mouse WAP

Milk whey proteins were separated under denaturing conditions in SDS 16% polyacrylamide gels and either stained with Coomassie Blue or transferred to nitrocellulose filters. Filters were incubated overnight in TBS (Tris-buffered saline, 20 mM Tris-HCL at pH 7.5, 500 mM NaCl) containing 3% gelatin. The membranes were then probed for 90 min with a 1:200 dilution of rabbit anti-WAP serum, followed by washing and incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS containing 1% BSA for 1 h. The antigen-antibody complexes were stained with nitrobluetetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl at pH 9.5, 5 mM $MgCl_2$ and 100 mM NaCl.

Results and Discussion

Generation of transgenic sheep

Four hundred and forty-three eggs (26, 2-cell) were harvested from donor ewes and a genomic 6.8 kb *Eco* RI DNA fragment containing the mouse WAP gene was injected into pronuclei or nuclei of 281 eggs with visible nuclear structures. All 281 eggs survived injection, based on gross morphological appearance, and 2 to 4 were transferred to the oviducts of each of 118 recipients within 3 h of the time of injection. Thirty-one recipient ewes, into which 80 eggs had been transferred, maintained pregnancy (27%) and 29 lambs and 5 near-term foetuses (2 sets of twins and a single) were produced. The 2 sets of twin foetuses were recovered from recipient ewes that died within a month of their due dates. One ram foetus was transgenic. Three of the 29 live born lambs died within 24 h of birth. One of those lambs was transgenic. Of the remaining 26 healthy lambs (12 ewes, 14 rams) 2 ewe lambs were found to be transgenic. Based on the two surviving transgenic ewes, the overall experimental efficiency was 0.7% (2/281), similar to an efficiency of 0.8% achieved in a contemporaneous experiment (Clements *et al.*, 1994) and previous work (Rexroad *et al.*, 1990).

The founder ewe 001 (carrying approximately three copies of the WAP transgene) and founder ewe 029 (containing approximately seven copies of the WAP) were bred to non-transgenic rams. Ewe 001 produced a

transgenic ram during each of her first two pregnancies and twin full-term foetuses during her third pregnancy. Ewe 029 produced twin ewes lambs during her first pregnancy, one of which was transgenic, and twin full term foetuses during her second pregnancy.

Transcription of the WAP transgene

During lactation, mammary gland biopsies were taken from a non-transgenic ewe, both transgenic ewes, a mouse and a WAP transgenic pig. Total RNA was isolated and was analysed for the presence of WAP mRNA (Fig. 1a). The WAP RNA signal observed in mammary tissue of a mid-lactation (day 10) mouse (Fig. 1a, lane e) and a WAP transgenic pig (Fig. 1a, lane d) were of similar intensity as determined by Betascope analysis of the bands. Steady-state WAP RNA levels of ewes 001 and 029 were 7% and 6% respectively of the WAP RNA signal produced by the mouse band (Fig. 1a, lanes b and c compared to lane e). As expected, no signal was seen in mammary tissue of the non-transgenic ewe (Fig. 1a, lane a). DNA probes for porcine β-lactoglobulin and β-casein RNA cross-hybridized with the corresponding mRNAs in sheep mammary tissue, and were used as loading controls and to verify the integrity of sample RNA. Equal steady-state levels of β-lactoglobulin and β-casein were seen in both ewes. WAP RNA expression in mammary tissue of both ewes was similar to endogenous β-lactoglobulin RNA levels, by Betascope analysis.

Transcription of the endogenous WAP gene in mice is first seen in late pregnancy and is maintained throughout lactation (Pittius *et al.*, 1988). WAP transgenes are frequently not regulated in the same manner as the endogenous gene. Precocious transcription, aberrant hormonal regulation and silencing of the gene during the lactational period have been observed in both transgenic mice and pigs (Burdon *et al.*, 1991b; Shamay *et al.*, 1992). We therefore analysed steady-state levels of WAP RNA in mammary gland biopsies of both transgenic ewes throughout their lactational and dry periods and compared WAP RNA measurements to endogenous milk protein RNAs. Both ewes' profiles were similar. WAP-specific RNA data from ewe 001 is presented in Fig. 1b. Transcripts of WAP and endogenous β-lactoglobulin and β-casein were found one week prior to parturition (Fig. 1b, lane b). WAP RNA reached peak levels by day 24 of lactation (Fig. 1b, lane d) whereas RNA levels for β-lactoglobulin and β-casein had peaked at day 2 of lactation.

Efficient transcriptional activity of the mouse WAP gene in both of the transgenic ewes extends our earlier observation that genetic control elements of this gene are functional in species that do not have a recognizable WAP gene (Shamay *et al.*, 1991; Wall *et al.*, 1991). Clearly, the transcriptional machinery of the sheep alveolar epithelial cells recognizes the WAP gene. The

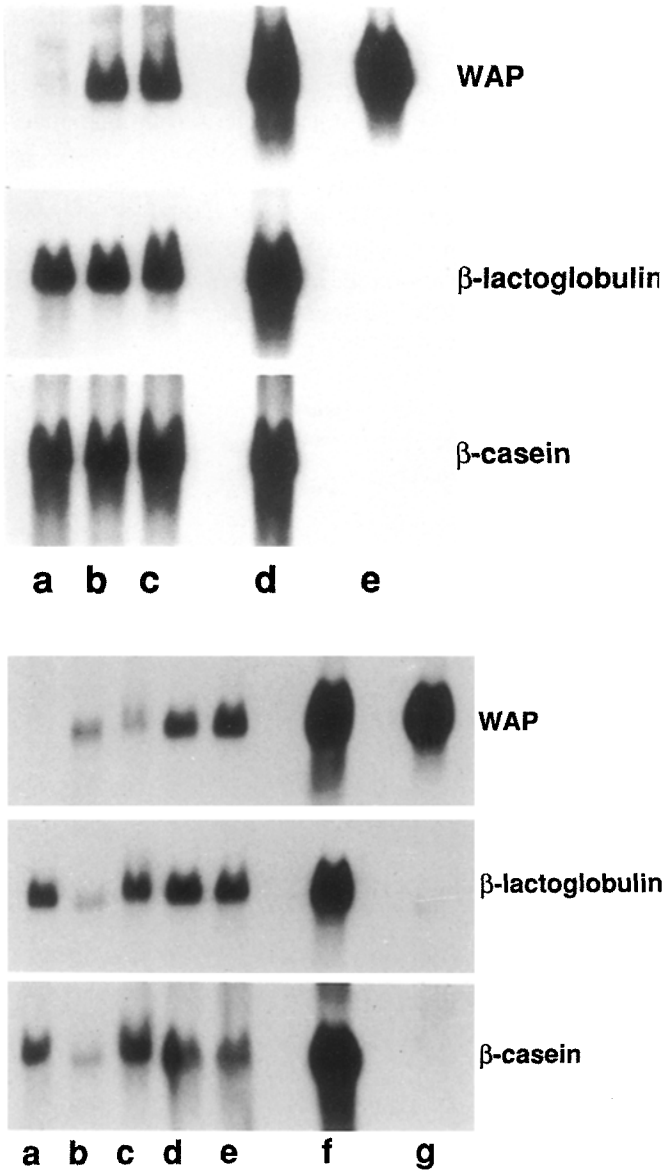

Fig. 1 (a) Expression of mouse WAP RNA in mammary of tissue lactating transgenic sheep. Total RNA was prepared from mammary gland biopsies taken during lactation from a non-transgenic sheep (lane a), ewe 001 (lane b), ewe 029 (lane c), a transgenic pig carrying the mouse WAP gene (lane d, Wall *et al.*, 1991) and from a mouse at day 10 (peak) of lactation (lane e). RNA (10 λg) was separated in a formaldehyde gel, transferred onto a nitrocellulose filter and hybridized with probes for mouse WAP, pig β-lactoglobulin and pig β-casein. (b) Levels of mouse WAP RNA in mammary tissue of a transgenic sheep prior to and during lactation. RNA was prepared from mammary biopsies taken from a lactating non-transgenic ewe (lane a), and from transgenic ewe 001 one week prior to parturition (lane b), at day 2 (lane c), day 24 (lane d) and day 50 (lane e) of lactation. RNA was also prepared from the mammary tissue of a transgenic pig (lane f, Wall *et al.*, 1991) and from a mouse (lane g). RNA (10 λg) was separated in a formaldehyde gel, transferred onto a nitrocellulose filter and subsequently hybridized with probes for mouse WAP, pig β-lactoglobulin and pig β-casein.

two WAP transgenic ewes experienced normal lactational cycles (each ewe produced 4 lambs and transmitted the transgene to their offspring), suggesting that synthesis and secretion of WAP did not interfere with mammary function. In contrast, sows from several lines of transgenic pigs and least two lines of transgenic mice carrying the identical WAP transgene showed aberrant mammary development which resulted in non-functional mammary glands (Burdon *et al.*, 1991b; Shamay *et al.*, 1992). It is not clear whether we have investigated a sufficient number of lines to detect mammary abnormalities, or whether WAP does not interfere with ovine mammary development.

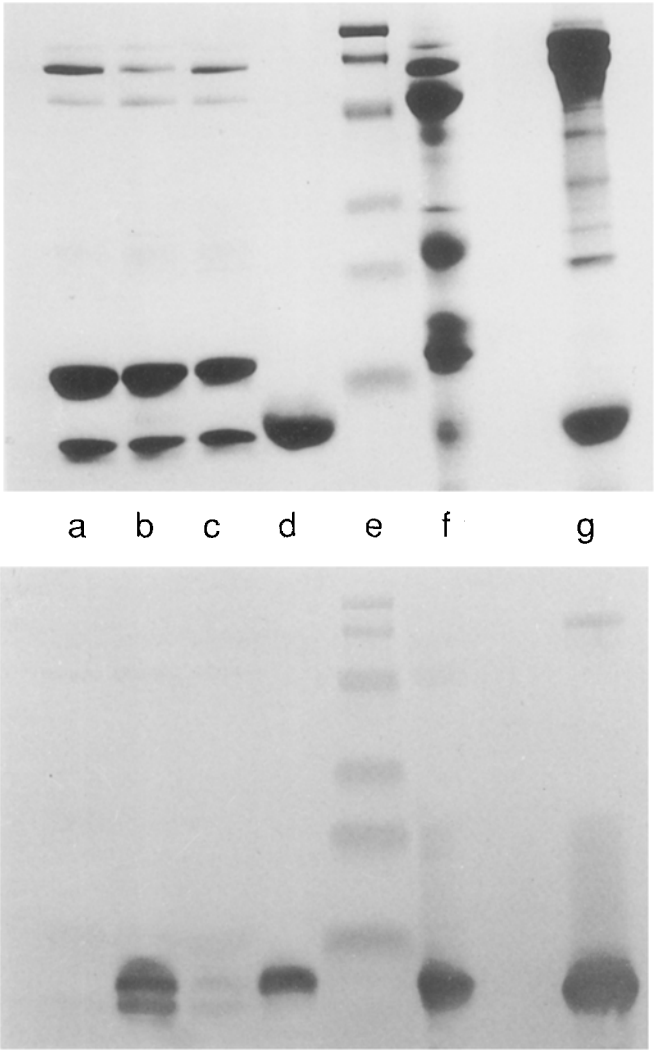

Fig. 2 Secretion of mouse WAP into sheep milk. Milk proteins from a non-transgenic ewe, two transgenic ewes, a transgenic pig, a non-transgenic mouse, and purified mouse WAP were separated in a 16% SDS-polyacrylamide gel, stained with Coomassie Blue (top panel) or transferred electrophoretically onto a nitrocellulose filter and analysed with rabbit anti-mouse WAP antibodies (bottom panel). Lane a, whey fraction from milk of a non-transgenic sheep; lane b, transgenic ewe 001; lane c, transgenic ewe 029; lane d, 5 mg of purified mouse WAP; lane e, prestained molecular weight standards (BRL); lane f, a pig transgenic for mouse WAP; lane g, a non-transgenic mouse.

Synthesis of WAP

Sheep milk whey was analysed for the presence of the mouse WAP on Coomassie-stained SDS-polyacrylamide gels (Fig. 2, upper panel) and with anti-mouse WAP antibodies (Fig. 2, lower panel). The major proteins in the whey fraction of non-transgenic ovine milk are lactalbumin (14 kDa), β-lactoglobulin (18 kDa) and lactoferrin (70 kDa) (Fig. 2, lane a). Since mouse WAP co-migrates with β-lactalbumin, its presence in transgenic ewes was determined immunologically (Fig. 2, lower panel). Whereas no WAP was detected in the whey of a non-transgenic ewe (Fig. 2, lane a), both transgenic ewes secreted mouse WAP into their milk (Fig. 2, lane b and c). The major immunoreactive bands from the sheep samples co-migrated with purified mouse WAP (Fig. 2, lane d), WAP in mouse milk (Fig. 2, lane g) and mouse WAP in the milk of transgenic pigs (Fig. 2, lane f). The lower molecular weight band cross-reacting with anti-WAP antibodies in the milk of transgenic ewes was probably a proteolytic product of WAP which has also been observed in the milk of WAP transgenic pigs (Shamay *et al.*, 1991).

No quantitative assays (e.g. RIA, ELISA) have been developed for measuring WAP concentration. Therefore it was necessary to rely on the more qualitative, western blot analysis, to estimate WAP concentrations in the milk of transgenic sheep. WAP band intensities (excluding the presumptive proteolytic products) of transgenic ewes 001 and 029 (Fig. 2, lanes b and c) relative to the 5 mg of purified mouse WAP band (lane d), were estimated to be 10% and 2% respectively. Therefore the milk samples from ewes 001 and 029 contained approximately 0.5 and 0.1 $mg\,ml^{-1}$ of WAP, respectively. These estimates of steady-state sheep milk WAP concentrations are in agreement with the WAP RNA levels detected in the tissue biopsies.

Ectopic expression of the WAP transgene

The endogenous WAP gene in mice and WAP transgenes in mice and pigs are tissue specific, expressed predominantly in mammary tissue and to a lesser extent in salivary glands (< 1% of mammary gland levels). To evaluate the tissue expression pattern of the WAP transgene in sheep, RNA was prepared from ten tissues for northern blot analysis. Tissues were recovered from founder ewe 029 following an unsuccessful attempt to rescue her twin lambs by caesarean section within a week of her expected parturition date. WAP gene expression was detected in a variety of tissues. The RNA samples were too degraded to quantitatively compare WAP expression in the various tissues with that of its expression in mammary gland tissue. Qualitatively, it appeared that

ectopic expression was highest in spleen and salivary gland, intermediate in liver, lung, kidney and heart muscle, lowest in bone marrow and not detectable in skeletal muscle or intestine.

This unexpected finding is based on tissues harvested under less than ideal conditions (the ewe had died) from one animal and therefore must be interpreted with caution. Tissues were also recovered from the other founder (001) which also died in the night following a caesarean section. However, the extracted RNA was degraded and could not be analysed. Though the poor quality of the RNA from ewe 029 precluded quantitative analysis, there is no question that WAP was expressed in tissues other than the mammary gland. It is possible that the ectopic expression observed was an artifact caused by the so-called transgene position effect. However, it is equally likely that sheep possess regulatory factors, in a variety of tissues that interact with the WAP gene. Additional lines of transgenic sheep will have to be analysed to distinguish between the two possibilities.

Health problems

There was an unusually high morbidity rate in this study. Founder 001 died near the end of her third gestation and ewe 029 died at the end of her second gestation. Dead full-term twin foetuses were recovered from both ewes the day before they died. No specific pathology was detected at the time of necropsy that could account for their deaths. It is not unreasonable to assume that the stress of carrying dead twin foetuses and the subsequent surgical trauma contributed to the founders' deaths. However, in a previous study, one of six founder pigs, carrying the same transgene also died at parturition (Shamay *et al.*, 1992).

As a result of her two successful pregnancies, ewe 001 produced 2 offspring, both of which were transgenic rams. One of the rams died at 7 months and the other 12 months of age. Necropsies revealed no gross organ abnormalities. Ewe 029 produced twin ewes, one of which is transgenic. That ewe, 211, is now two years old and appears to be in good health, and was recently bred.

Ectopic expression of the transgene was observed in tissues from founder ewe 029. Therefore, it is possible that expression of WAP in tissues other than the mammary gland contributed to the deaths of these transgenic animals. A recent study, in which we purposely expressed WAP in tissues other than the mammary gland, provides some support for that hypothesis (Hennighausen *et al.*, 1994). No increased morbidity or overt health problems were observed, but we detected hyperplasia and dysplasia of coagulation gland epithelium, one of the tissues that expressed a moderate amount of WAP RNA. However, because the function of WAP is unknown, it is difficult to speculate on a mechanism that might explain these observations.

In mouse and pig studies where the same transgene was used, we have observed a correlation between premature mammary gland expression of the WAP gene and the milchlos phenotype, a syndrome characterized by incomplete development of secretory epithelium (Burdon *et al.*, 1991b and Shamay *et al.*, 1992). We have inferred from these observations that WAP may play a role in mammary gland development. If that is the case, ectopic expression of the WAP gene may have deleterious developmental consequences in other organs.

Crossing species boundaries

Transgenic mice have been employed extensively to study regulatory features of genes from a variety of species, including humans, rats, cattle, pigs, sheep and goats. However, there have been only a few studies in which the regulation of a transgene has been compared in several species. Using WAP transgenes allows us to address fundamental issues of transgenesis and gene regulation across species boundaries. Surprisingly, expression of a mouse WAP transgene was more efficient in pigs (Shamay *et al.*, 1991; Wall *et al.*, 1991) than in mice (Burdon *et al.*, 1991a). Whereas only one half of the transgenic mouse lines expresses the WAP transgene, all six lines of pigs and both lines of sheep express the transgene. Mammary specificity was maintained in mice and pigs, suggesting that the molecular basis of mammary-specific gene expression has been conserved. However, the data presented here suggest that sheep and possibly other ruminants (cattle and goats) contain regulatory factor(s) that interact with the WAP gene in a variety of tissues.

The data presented here and in other work (Burdon *et al.*, 1991a; Shamay *et al.*, 1991; Jhappan *et al.*, 1993; Velander *et al.*, 1993) highlight some of the problems associated with the murine transgenic system as a predictive model. For the mouse, WAP transgenes behave somewhat erratically (precocious expression and lower than endogenous levels), but neither in mice nor pigs was tissue specificity lost. The one ewe from which RNA from a variety of tissues could be analysed, clearly demonstrated ectopic expression of the WAP transgene. Since only one animal was analysed, it is not clear whether the WAP gene generally loses its mammary-specific expression in the background of transgenic sheep, or whether the WAP transgene integrated within a chromosomal region that fosters widespread expression. Unfortunately, due to the high cost of producing transgenic sheep, it is unlikely that this question will be resolved until the efficiency of generating transgenic sheep is substantially improved.

Acknowledgements

The authors would like to thank Sherry Ogg and Paul

Graninger for their excellent assistance in producing the transgenic sheep.

References

Andres, A.C., Schonenberger, C.A., Groner, B., Hennighausen, L., LeMeur, M. and Gerlinger, P. (1987) Ha-ras oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation, and tumor induction in transgenic mice. *Proc. Natl Acad. Sci. USA* **84**, 1299–303.

Burdon, T., Sankaran, L., Wall, R.J., Spencer, M. and Hennighausen, L. (1991a) Expression of a whey acidic protein transgene during mammary development: evidence for different mechanisms of regulation during pregnancy and lactation. *J. Biol. Chem.* **266**, 6909–14.

Burdon, T., Wall, R.J., Shamay, A., Smith, G. and Hennighausen, L. (1991b) Over-expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype. *Mech. Devel.* **36**, 67–74.

Campbell, S.M., Rosen, J.M., Hennighausen, L., Strech-Jurk, U. and Sippel, A.E. (1984) Comparison of the whey acidic protein genes of the rat and mouse. *Nucl. Acids Res.* **12**, 8685–97.

Chomczynski, P. and Saachi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-pheonol-chloroform extraction. *Anal. Biochem.* **162**, 156–9.

Clements, J.E., Wall, R.J., Narayan, O., Hauer, D., Schoborg, R., Sheffer, D., Powell, A., Carruth, L.M., Zink, M.C. and Rexroad, C.E., Jr. (1994) Development of transgenic sheep that express the Visna virus envelope gene. *Virology* **200**, 370–80.

Ebert, K.M., Selgrath, J.P., DiTullio, P., Denman, J., Smith, T.E., Memon, M.A., Schindler, J.E., Monastersky, G.M., Vitale, J.A. and Gordon, K. (1991) Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Bio/Technology* **9**, 835–8.

Hennighausen, L.G. and Sippel, A.E. (1982a) The mouse whey acidic protein is a novel member of the family of 'four-disulfide core' proteins. *Nucl. Acid Res.* **10**, 2677–84.

Hennighausen, L.G. and Sippel, A.E. (1982b) Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. *Eur. J. Biochem.* **125**, 131–41.

Hennighausen, L., Westphal, C., Sankaran, L. and Pittius, C.W. (1991) Regulation of expression of milk protein. In First, N. and Haseltine F.P. eds, *Transgenic Animals* pp. 65–74. Stoneham, MA: Butterworth-Heinemann

Hennighausen, L., McKnight, R., Burdon, T., Baik, M., Wall, R.J. and Smith, G.H. (1994) Whey acidic protein extrinsically expressed from the mouse mammary tumor virus long terminal repeat results in hyperplasia of the coagulation gland epithelium and impaired mammary development. *Cell Growth Different* **5**, 607–13.

Hillis, D. and Dixon, T. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66**, 411–53.

Jhappan, C., Geiser, A.G., Kordon, E.C., Bagheri, D., Hennighausen, L., Roberts, A.B., Smith, G.H. and Merlino, G. (1993) Targeting expression of a transforming growth factor transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J.* **12**, 1835–45.

McKnight, R.A., Burdon, T., Pursel, V.G., Shamay, A., Wall, R.J. and Hennighausen, L. (1991) The whey acidic protein. In Dickson, R.B. and Lippman, M.E. eds *Genes, Oncogenes, Hormones: Cellular and Molecular Biology of Breast Cancer.* pp. 399–412. Boston: Kluwer Academic Publishers.

Pittius, C.W., Sankaran, S., Topper, Y. and Hennighausen, L. (1988) Comparison of the regulation of the whey acidic protein gene to a hybrid gene containing the whey acidic protein gene promoter in transgenic mice. *Mol. Endocrinol.* **2**, 1027–32.

Rexroad, C.E. Jr., Powell, A.M., Rohan, R. and Wall, R.J. (1990) Evaluation of co-culture as a method for selecting viable microinjected sheep embryos for transfer. *Anim. Biotech.* **1**, 1–10.

Schonenberger, C.A., Andres, A.C., Groner, B., Valk, N. van der, LeMeur, M. and Gerlinger, P. (1988) Targeted c-*myc* gene expression in mammary glands of transgenic mice induces mammary tumors with constitutive milk protein gene transcription. *EMBO J.* **7**, 169–75.

Shamay, A., Pursel, V.G., McKnight, R.A., Alexander, L., Beattie, C., Hennighausen, L. and Wall, R.J. (1991) Production of the mouse whey acidic protein in transgenic pigs during lactation. *J. Anim. Sci.* **69**, 4552–62.

Shamay, A., Pursel, V.G., Wilkinson, E., Wall, R.J. and Hennighausen, L. (1992) Expression of the whey acidic protein in transgenic pigs impairs mammary development. *Transgenic Res.* **1**, 124–32.

Tzeng, Y.J., Guhl, E., Graessmann, M. and Greassmann, A. (1993) Breast cancer formation in transgenic animals induced by the whey acidic protein SV40 T antigen (WAP-SV-T) hybrid gene. *Oncogene* **8**, 1965–71.

Velander, W.H., Johnson, J.L., Page, R.L., Russell, C.G., Subramanian, A., Wilkins, T.D., Gwazdauskas, F.C., Pittius, C. and Drohan, W.N. (1993) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc. Natl Acad. Sci. USA* **89**, 12003–7.

Wall, R.J., Pursel, V.G., Shamay, A., McKnight, R.A., Pittius, C.W. and Hennighausen, L. (1991) High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. *Proc. Natl Acad. Sci. USA* **88**, 1696–700.