Biochem. J. (1990) 267, 815–819 (Printed in Great Britain)

Transferrin-gene expression in the rat mammary gland

Independence of maternal iron status

Murray R. GRIGOR, Fiona J. McDONALD, Nigel LATTA, Carol L. RICHARDSON and Warren P. TATE
Department of Biochemistry, University of Otago, Dunedin, New Zealand

Transferrin mRNA concentrations were measured in total RNA isolated from liver and mammary tissue of lactating rats at different stages of lactation. The mammary transferrin mRNA concentration varied in a biphasic manner, increasing up to parturition and then decreasing to undetectable levels at days 5 and 10 of lactation before increasing again markedly in late lactation. The values obtained at day 20 of lactation were double those found in livers of lactating rats. The concentrations of total RNA and mRNA for α-casein and α-lactalbumin did not change between days 5 and 20 of lactation. Transferrin concentrations were measured in milk from rats fed on an iron-free, a control and an iron-supplemented diet. Although there was a 5-fold difference in the transferrin concentration of samples taken between day 5 and day 20 of lactation, the dietary treatments did not result in significant changes. Maternal serum transferrin concentrations were, however, elevated, and pup haemoglobin concentrations were suppressed for the rats receiving the iron-free diet, indicating an alteration of the iron status of these rats.

INTRODUCTION

Rat milk contains the iron-binding protein transferrin at concentrations that vary markedly in a biphasic manner during the course of lactation (Grigor *et al.*, 1988). In colostrum, the transferrin concentration is approx. 1.5 mg/ml, falling to less than 0.5 mg/ml at days 5 and 10 of lactation and thereafter increasing for the remainder of lactation to reach values of greater than 4 mg/ml, or 4% of the total milk proteins, by day 20 of lactation. This pattern is quite unlike that for any other milk protein, and also differs from the pattern of expression of many mammary enzymes involved in lactose and lipid synthesis (Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1969; Grigor *et al.*, 1982). We have previously shown that the transferrin concentrations in rat milk can be manipulated by diet, being significantly lower in the milk of rats fed on either a low-protein diet or restricted amounts of a protein-adequate diet (Grigor *et al.*, 1988). It has been suggested that the milk transferrin in the rat may either be synthesized *de novo* within the mammary epithelial cells or be synthesized in the liver and transferred across the mammary epithelium into the milk (Jordan & Morgan, 1967). Evidence for both situations has been presented, with the proportion of the mammary-derived transferrin increasing as lactation progresses (Jordan & Morgan, 1967). These studies did not include the early colostrum transferrin, however, and the primary aim of the present study was to determine the origin of early-phase transferrin detected in the colostrum. This has been addressed by measuring the concentration of transferrin mRNA in samples of total mammary RNA prepared from rats at different stages of lactation. Specifically, we wished to see whether the concentrations of mammary transferrin mRNA changed in a pattern to match the changes in milk transferrin concentrations.

Transferrin synthesis in liver is modulated by the iron status of the animal and enhanced by iron deficiency (Morgan, 1969; Idzerda *et al.*, 1986). The second aim of the present study was to investigate whether changes in the iron status of lactating rats might modify the pattern of expression of transferrin in the milk. Here the effect of iron deficiency or iron overload on milk transferrin concentrations in lactating rats has been investigated.

EXPERIMENTAL

Materials

Plasmids containing the coding sequences for transferrin (Aldred *et al.*, 1984), α-casein (pαC16; Hobbs *et al.*, 1982) and α-lactalbumin (pLA35; Qasba & Safaya, 1984) were made available by Dr. G. Schreiber, Dr. J. Rosen and Dr. P. Qasba respectively. The isolation of rat whey transferrin and the production of rabbit anti-(rat transferrin) antibodies are described elsewhere (Grigor *et al.*, 1988). Restriction enzymes, [^{32}P]dATP and a Multiprime kit for DNA labelling were obtained from Amersham International, Amersham, Bucks., U.K. Desferrioxamine (Desferal) was obtained from Ciba–Geigy, Basel, Switzerland, sodium pentabarbitone (Sagatal) from May and Baker, Dagenham, Essex, U.K., and oxytocin (Syntocinon) from Sandoz, Basel, Switzerland.

Animals

Rats of the Wistar strain were purchased from the University of Otago Animal Breeding Station and mated at an age of 3–4 months. They were maintained on a commercial pelleted diet (A. E. Reeves, Dunedin, New Zealand) and kept in rooms at 20 °C on a 12 h-light/12 h-dark cycle. To test the effect of alterations in maternal iron status, one of three semi-synthetic diets was fed from day 17 of gestation. These were an iron-free diet containing no added iron, a low-iron diet containing 300 mg of $FeSO_4$/kg and a high-iron diet containing 2 g of $FeSO_4$/kg. The major components of the diet were (by wt.) sucrose (50%), casein (20%), starch (10%), salt mix minus iron (4%) and vitamin mix (1%). The detailed compositions of the vitamin and salt mixes are published elsewhere (Grigor *et al.*, 1987). Rats receiving the zero-iron diet were also injected three times with 50 mg of desferrioxamine, on days 1, 3 and 5 of lactation (day 1 of lactation is defined as the day of parturition). Milk and blood samples were obtained on days 5, 10, 15 and 20 of lactation. For this, rats were anaesthetized with pentobarbitone and milked as previously described (Grigor *et al.*, 1986). Approx. 0.5 ml of milk was obtained at each sampling. Blood samples (1 ml) were obtained from the tail vein, and both milk and blood samples were obtained within 30 min of removal of the dam from the pups.

Assays

Transferrin was measured in milk samples that had been diluted 20-fold and in serum samples diluted 100-fold, by an immunochemical assay described previously (Grigor *et al.*, 1988). Haemoglobin was measured in whole blood (0.075 ml) diluted into 3 ml of 1% (w/v) Triton X-100, spectrophotometrically as cyanomethaemoglobin (Zijlstra & Van Kampen, 1960).

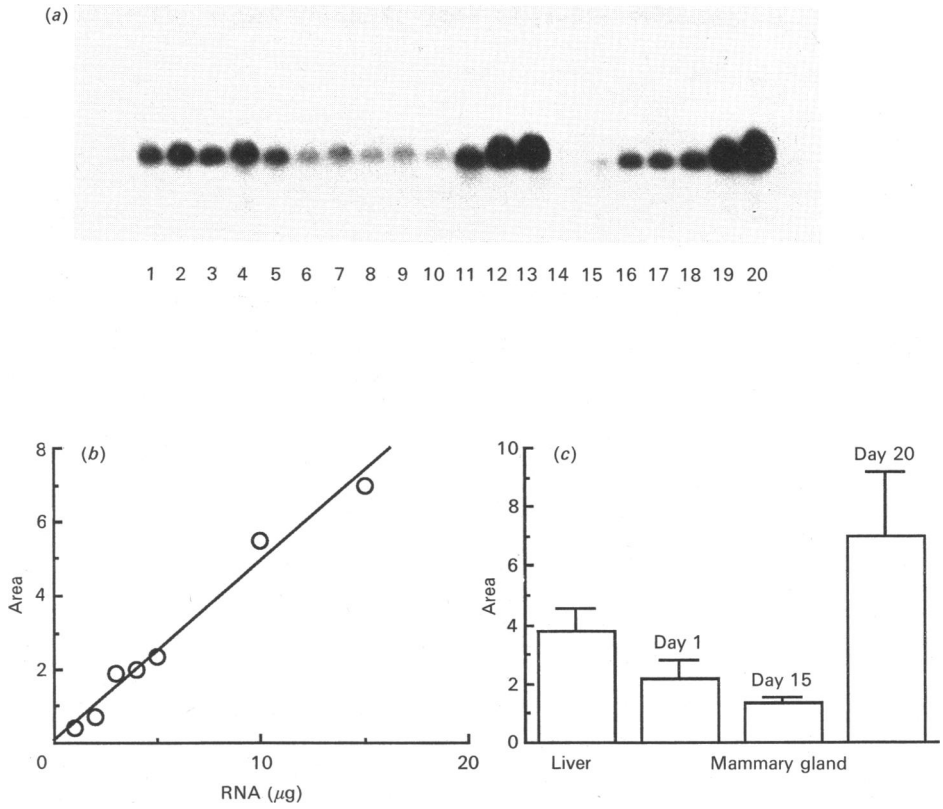

Fig. 1. Northern blots of mammary and hepatic transferrin mRNA

(*a*) Total liver RNA (lanes 1–4) and mammary RNA prepared from rats lactating for 1 (lanes 5–7), 15 (lanes 8–10) and 20 days (lanes 11–13) (all 5 μg per lane) and samples from a single rat at day 20, loaded at 1, 2, 3, 4, 5, 10 or 15 μg per lane (lanes 14–20), were electrophoresed, transferred to Hybond N, and hybridized with radioactive transferrin cDNA as described in the text. (*b*) Densitometer areas corresponding to lanes 14–20. (*c*) Comparison of liver and mammary transferrin mRNA concentrations determined by densitometric scanning of lanes 1–13. Values plotted are means ± S.D. for samples from three or four rats.

RNA preparation and hybridizations

Total RNA was prepared from mammary tissue and liver of rats at different stages of lactation as described by Chomozynski & Sacchi (1987) and quantified spectrophotometrically. RNA (5 μg) was separated by agarose-gel electrophoresis in the presence of glyoxal (Maniatis *et al.*, 1982) and transferred to Hybond N membranes in 3 M-NaCl/0.3 M-sodium citrate and fixed by baking at 80 °C under vacuum. Inserts containing the coding sequences of transferrin, α-casein and α-lactalbumin were isolated from plasmids that were grown in *Escherichia coli* (strain HB-101), amplified by treatment with chloramphenicol and recovered by the alkaline-lysis method of Maniatis *et al.* (1982). The cDNAs were purified by agarose-gel electrophoresis and labelled with ^{32}P by random oligo-labelling and used to hybridize against RNA on the filters. Hybridizations were done at 50 °C in the presence of 0.36 M-NaCl / 20 mM-sodium phosphate / 2 mM-EDTA/10 × Denhardt's solution/0.5 % SDS containing 100 μg of denatured salmon sperm DNA/ml (Maniatis *et al.*, 1982). Autoradiograms of the filters were made by using preflashed Cronex film at −70 °C with intensifying screens, and were scanned densitometrically with a LKB Ultrascan XL densitometer. To test the effect of stage of lactation on the milk-protein mRNA concentrations, the same filter was probed sequentially with the transferrin, α-casein and α-lactalbumin cDNAs, with the radioactive probes being removed by treating twice with boiling 20 mM-Tris/1 mM-EDTA, pH 8.0.

Statistics

Where appropriate, the results were subjected to analysis of variance followed by Student–Newman–Keuls' test to test significance between individual means (Zar, 1974).

RESULTS

Transferrin-gene expression in normal lactation in the rat

When total RNA from rat mammary or liver tissue was probed by Northern analysis with the transferrin cDNA, only a single species of approx. 2800 bases was detected (Fig. 1*a*). When scanned densitometrically, the peak areas obtained varied linearly with total RNA applied from 1 to 15 μg (Fig. 1*b*). Figs. 1(*c*) and 2(*a*) also show that the concentration of the transferrin mRNA varied considerably during the course of lactation, increasing in RNA samples prepared 5 and 2 days *pre partum* and on the day of parturition (day 1 of lactation). However, no transferrin mRNA could be detected at day 5 of lactation, and only very small amounts at day 10. The concentration thereafter increased as lactation progressed, and the greatest concentrations were observed in samples obtained at day 20. The concentration of the transferrin mRNA in the mammary gland at days 1 and 15 of lactation was approximately half that measured in total RNA from livers of lactating rats and, at day 20 of lactation, was double that found in liver (Fig. 1*c*).

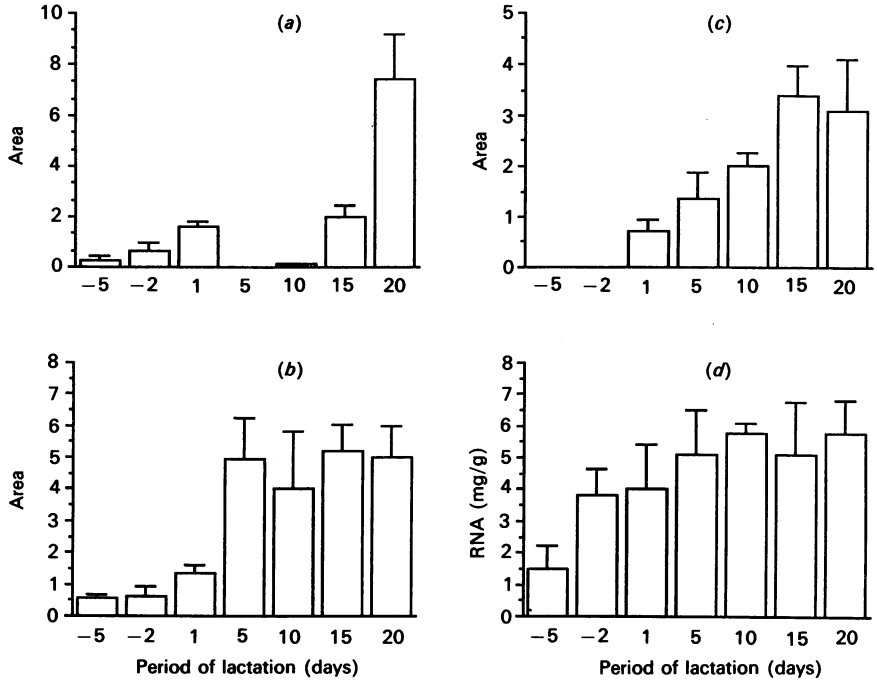

Fig. 2. Milk-protein mRNA concentrations during lactation

Total mammary RNA prepared from rats 5 and 2 days *pre partum* and at various stages of lactation (5 μg) was electrophoresed and probed sequentially for transferrin mRNA (*a*), α-casein mRNA (*b*) and α-lactalbumin mRNA (*c*), and autoradiographed as described in the text. Values plotted are densitometer areas, and represent means $\pm$ S.D. for samples from three rats (days −2, 1, 5, 15, 20) and means $\pm$ half the range for values from two rats (days −5 and 10). (*d*) Recovery of total RNA as mg of RNA/g wet wt. of tissue.

Table 1. Effect of dietary iron on maternal and pup body and tissue weights

	Weight (g)		
Diet...	Iron-free	300 mg of $FeSO_4$/kg	2 g of $FeSO_4$/kg
Maternal wt. (day 1)	275±26	269±19	265±16
Maternal wt. (day 20)	269±20	264±11	251±17
Litter wt. (day 20)	334±19	366±25	321±46
Maternal liver wt.	17.8±3.2	15.8±2.3	14.6±1.6
Pup liver wt. (day 20)	1.53±0.17	1.53±0.14	1.49±0.16

In contrast with the transferrin mRNA, the concentration of total RNA (mg/g of mammary tissue) or of the α-casein and α-lactalbumin mRNAs showed quite different patterns. Total RNA increased in the samples obtained *pre partum* to those at day 5 of lactation, after which no significant changes could be detected (Fig. 2*d*). Only single mRNA species were detected for the α-casein and α-lactalbumin mRNAs, of approx. 2000 and 1350 bases respectively. Casein mRNA was detectable *pre partum*, and increased to reach a plateau in samples obtained at day 5 of lactation and thereafter (Fig. 2*b*). α-Lactalbumin mRNA was barely detectable *pre partum*, but the concentration increased *post partum* to reach plateau values from day 10 of lactation (Fig. 2*c*).

These results clearly show that mammary transferrin-gene expression is regulated differently from that of other milk proteins. Furthermore, the pattern observed for the transferrin mRNA concentrations matches closely that observed for milk transferrin concentrations in the rat (Grigor *et al.*, 1988). The changes that we report for the α-casein and α-lactalbumin mRNA concentrations through lactation differ from those reported previously (Hobbs *et al.*, 1982; Nakhasi & Qasba, 1979). Hobbs *et al.* (1982) showed that the concentrations of each of the three major casein mRNAs increased throughout lactation, whereas the data of Nakhasi & Qasba (1979) suggest that both the α-lactalbumin mRNA and a 16 S casein mRNA reach maximum values around day 12 of lactation and fall to *pre partum* values by day 20 of lactation. Our results match more closely the changes in milk production and protein concentration observed throughout lactation (Knight *et al.*, 1984; Nicholas *et al.*, 1981; Grigor *et al.*, 1987, 1988).

Effect of dietary iron on mammary transferrin-gene expression

No significant differences could be detected in either the maternal body-weight changes during lactation or the total litter weight at day 20 among the three dietary groups (Table 1). The values obtained for pup weight gains are similar to those previously obtained in this laboratory (Grigor *et al.*, 1987). These data suggest that milk production was not being impaired by the treatments. Several observations, however, confirm that the maternal iron status was modified by the treatments used. Whereas there were no significant differences in the maternal haemoglobin concentrations measured at the four time points

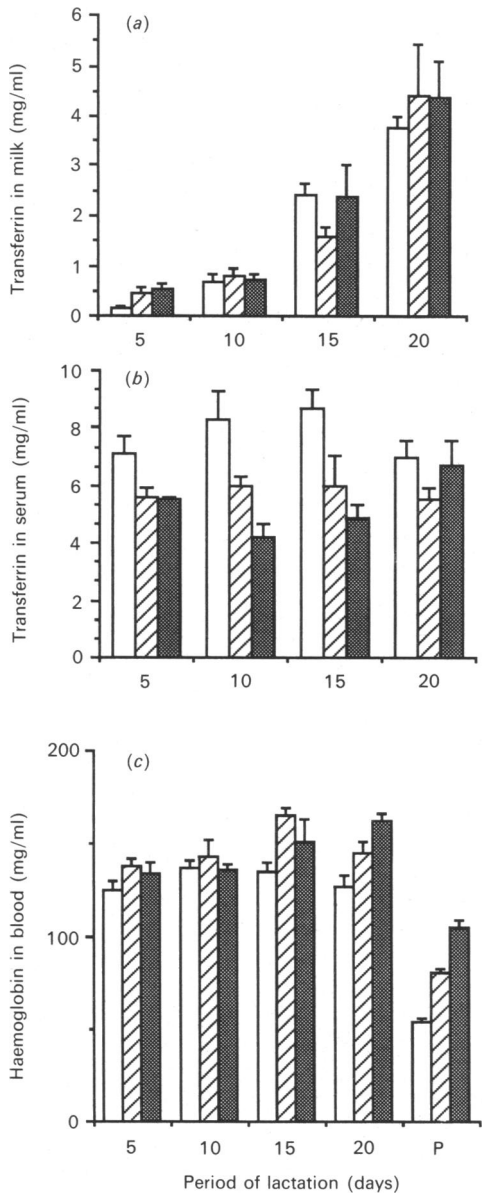

Fig. 3. Effect of maternal iron status on milk and serum transferrin and haemoglobin concentrations

Transferrin concentrations in milk (*a*) and serum (*b*) and haemoglobin concentrations in whole blood (*c*) were determined at the days of lactation indicated. Values represent means ± S.D. for samples from four to six rats: P, pups at day 20 of lactation, Dietary treatments were as described in the text. Diets: □, iron-free; ▨, low-iron (0.3 g of $FeSO_4$/kg); ■, high-iron (2 g of $FeSO_4$/kg).

that blood was sampled, the values obtained for the pup haemoglobins measured at day 20 of lactation show that pups of the dams receiving the zero-iron diet were severely anaemic as compared with both other groups (Fig. 3*c*). In all groups the pups had significantly lower haemoglobin concentrations than their mothers.

When the transferrin concentrations of the milk were measured, a significant increase was observed between days 5 and 20 of lactation for rats on all three treatments (Fig. 3*a*). This confirms the delayed induction of transferrin synthesis and secretion observed previously (Jordan & Morgan, 1967; Grigor *et al.*, 1988). However, no significant differences could be detected among the three groups for any time period except for the samples obtained at day 5, where the transferrin concentration in the milk of the zero-iron rats was significantly lower than that of the other two groups. By contrast, there were no consistent trends with time in the concentrations of serum transferrin for any of the groups (Fig. 3*b*), except that the values obtained for the rats on the zero-iron diet were significantly higher than those of the other rats at days 5, 10 and 15. This is further evidence of these rats becoming iron-deficient. Taken together, these results are consistent with the milk transferrin concentrations and mammary transferrin-gene expression being independent of the iron status of the lactating rat.

DISCUSSION

The liver has long been recognized as the major site of transferrin synthesis in the rat and other animals (Morgan, 1969; Idzerda *et al.*, 1986). However, several other tissues of the rat also make transferrin. These include the Sertoli cells of the testes (Skinner *et al.*, 1984; Lee *et al.*, 1986), the brain, particularly the cells of the choroid plexus (Aldred *et al.*, 1987), and, in fetal rats, various tissues, including muscle, kidney, small intestine, heart, lung and spleen (Levin *et al.*, 1984). The levels of expression in these fetal tissues are between 5 and 20% of those found in liver at the comparable stage of development, whereas, in the adult tissues examined, only the brain retained the ability to express transferrin at an appreciable rate (Levin *et al.*, 1984).

Our results have shown that the mammary transferrin-mRNA concentrations at parturition are approx. 50% of those in liver at the same stage of lactation, and in late lactation (day 20) are double the liver concentration. These results suggest that the mammary gland has a capacity for transferrin synthesis of a similar order to that found in liver. Other calculations show that, at day 15 of lactation, the rate of mammary transferrin secretion of 100 mg/day [calculated from a transferrin concentration of 2 mg/ml (Grigor *et al.*, 1988) and a daily milk production of 50 ml/day (Knight *et al.*, 1984)] exceeds the estimates of liver transferrin synthesis, based on turnover studies, of 55 mg/day (Schreiber *et al.*, 1982). Other animals, including rabbits (Jordan & Morgan, 1970) and mice (Lee *et al.*, 1987), also have transferrin in their milk. Transferrin synthesis has been detected in explants of rabbit mammary tissue (Bradshaw *et al.*, 1985) and in mammary epithelial-cell cultures from mice (Lee *et al.*, 1985). Transferrin mRNA has been detected in mouse mammary-epithelial cells and mammary tissue, with its concentration increasing during pregnancy and into lactation (Lee *et al.*, 1987; Chen & Bissell, 1987). In contrast with the rat, however, the transferrin concentrations in the milk of rabbits (Jordan & Morgan, 1970) and mice (M. R. Grigor & C. H. Knight, unpublished work) appear to increase steadily during the course of lactation, with no evidence of a decrease in the transferrin concentration in the early-mid phase of lactation being observed.

At present the factors regulating the biphasic expression of transferrin in the rat mammary gland remain to be elucidated. Transferrin synthesis in rat liver is markedly dependent on the iron status of the animal and is enhanced by iron deficiency (Morgan, 1969), and more recent results have shown that this regulation is effected at the level of gene transcription (Idzerda *et al.*, 1986). The results of the present study suggest that iron is unlikely to regulate mammary transferrin synthesis. The treatments that we used clearly altered the flux of iron across the mammary epithelium, yet did not alter significantly the concentrations of milk transferrin observed.

REFERENCES

Aldred, A. R., Howlett, G. J. & Schreiber, G. (1984) Biochem. Biophys. Res. Commun. **122**, 960–965

Aldred, A. R., Dickson, P. W., Marley, P. D. & Schreiber, G. (1987) J. Biol. Chem. **262**, 5293–5297
Baldwin, R. L. & Milligan, L. P. (1966) J. Biol. Chem. **241**, 2058–2066
Bradshaw, J. P., Hatton, J. & White, D. A. (1985) Biochim. Biophys. Acta **847**, 344–351
Chen, L.-H. & Bissell, M. J. (1987) J. Biol. Chem. **262**, 17247–17250
Chomozynski, P. & Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
Grigor, M. R., Geursen, A., Sneyd, M. J. & Warren, S. M. (1982) Biochem. J. **208**, 611–618
Grigor, M. R., Allan, J. E., Carrington, J. M., Carne, A. & Geursen, A. (1986) Biochem. J. **133**, 917–919
Grigor, M. R., Allan, J. E., Carrington, J. M., Carne, A., Geursen, A., Young, D., Thompson, M. P., Haynes, E. B. & Coleman, R. A. (1987) J. Nutr. **117**, 1247–1258
Grigor, M. R., Carne, A., Geursen, A. & Flint, D. J. (1988) J. Nutr. **118**, 669–674
Hobbs, A. A., Richards, D. A., Kessler, D. J. & Rosen, J. M. (1982) J. Biol. Chem. **257**, 3598–3605
Idzerda, R. L., Huebers, H., Finch, A. C. & McKnight, G. S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 3723–3727
Jordan, S. M. & Morgan, E. H. (1967) Q. J. Exp. Physiol. **52**, 422–429
Jordan, S. M. & Morgan, E. H. (1970) Am. J. Physiol. **219**, 1549–1554
Knight, C. H., Docherty, A. H. & Peaker, M. (1984) J. Dairy Res. **51**, 29–35
Kuhn, N. J. & Lowenstein, J. M. (1969) Biochem. J. **105**, 995–1002
Lee, E. Y.-H., Lee, W.-H., Kaetzel, C. S., Parry, G. & Bissell, M. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1419–1423
Lee, E. Y.-H., Barcellos-Hoff, M. E., Chen. L.-H., Parry, G. & Bissell, M. J. (1987) In Vitro **23**, 221–226
Lee, N. T., Chae, C.-B. & Kierszenbaum, A. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8177–8181
Levin, M. J., Tuil, D., Uzan, G., Dreyfus, J.-C. & Kahn, A. (1984) Biochem. Biophys. Res. Commun. **122**, 212–217
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Morgan, E. H. (1969) J. Biol. Chem. **244**, 4193–4199
Nakhasi, H. L. & Qasba, P. K. (1979) J. Biol. Chem. **254**, 6016–6025
Nicholas, K. R., Hartmann, P. E. & McDonald, B. L. (1981) Biochem. J. **194**, 149–154
Qasba, P. K. & Safaya, S. K. (1984) Nature (London) **308**, 377–380
Schreiber, G., Howlett, G., Nagashima, M., Millership, A., Martin, H., Urban, J. & Kotler, L. (1982). J. Biol. Chem. **257**, 10271–10277
Skinner, M. K., Cosland, W. L. & Griswold, M. D. (1984) Biochem. J. **218**, 313–320
Zar, J. H. (1974) Biostatistical Analysis, pp. 151–162, Prentice-Hall, Englewood Cliffs, NJ
Zijlstra, W. G. & Van Kampen, E. J. (1960) Clin. Chim. Acta **5**, 719–726

Received 28 December 1989; accepted 2 February 1990