

LACTOSE SYNTHETASE ACTIVITY IN MOUSE MAMMARY GLANDS IS CONTROLLED BY THYROID HORMONES

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ABSTRACT

Epithelial cells in explants from the mammary glands of euthyroid mature virgin mice are proliferatively dormant. They must undergo DNA synthesis and traverse the cell cycle *in vitro* before they are able to differentiate fully in response to insulin, hydrocortisone, and prolactin, and synthesize enzymatically active α -lactalbumin (measured as lactose synthetase activity). In contrast, glands from hyperthyroid mature virgin mice do not require DNA synthesis *in vitro* to differentiate. Explants from the euthyroid virgin tissue overcome their dependence on DNA synthesis when 10^{-9} M 3,5,3'-triiodo-L-thyronine is added directly to the cultures in addition to the other three hormones. Explants from involuted mammary glands from euthyroid primiparous mice do not require DNA synthesis *in vitro* to make the milk protein even though they, like explants from mature euthyroid virgin tissue, are proliferatively dormant and do not contain detectable lactose synthetase activity *in vivo*. Glands from primiparous animals made mildly hypothyroid by ingestion of 0.1% thiouracil in drinking water during 7 wk of involution remain morphologically indistinguishable from glands of their euthyroid counterparts. However, explants from the glands of these hypothyroid animals revert to a state of dependence on DNA synthesis to differentiate functionally. These observations suggest that the dependence on DNA synthesis and cell cycle traversal for hormonal induction of lactose synthetase activity in the mouse mammary gland is controlled by thyroid hormones.

KEY WORDS mammary glands ·
differentiation · thyroid hormones ·
 α -lactalbumin · DNA synthesis

The epithelial cells from mammary glands of the euthyroid mature female virgin mouse are proliferatively dormant (13, 17). With the onset of pregnancy, or by placing the tissue in explant culture in the presence of mitogens (3, 5, 13), active proliferation of the cells occurs. Ultimately, under the proper hormonal influences, functional differentiation occurs, resulting in the elaboration of the

milk proteins casein and α -lactalbumin (12, 17). In explants from glands of the euthyroid mature virgin mouse, the induction of these characteristic products of the secretory cells is necessarily coupled to the previous round of cell proliferation *in vitro* (10, 19). In involuted glands from primiparous animals and those in active cell division *in vivo* (i.e., from immature and mid-pregnant animals), milk proteins can be synthesized *in vitro*, even in the absence of DNA synthesis (10, 19).

Recently, thyroid hormones have been shown to play an important role in the morphological

development of the mouse mammary gland (16) and in the regulation of lactose synthetase activity (a measure of α -lactalbumin levels) in cultured explants from glands of both mature virgin and mid-pregnant animals (14, 15). Mammary tissue from either euthyroid or hypothyroid virgins has no detectable lactose synthetase activity *in vivo* (16, 17). Although growth of the glands of mildly hypothyroid animals is retarded, resulting in a sparse ductal system lacking alveoli, the epithelial cells retain full differentiative capability when cultured in the presence of the appropriate hormones (16). In contrast, tissue from hyperthyroid mature virgin mice contains many alveoli and frequently demonstrates significant lactose synthetase activity *in vivo* (16).

These observations led to a further examination of the role of thyroid hormones in terminal differentiation of the mouse mammary gland with emphasis on the coupling of this process to DNA synthesis. The studies reported in this paper show that this phenomenon is under the direct control of thyroid hormones.

MATERIALS AND METHODS

Chemicals

Porcine zinc insulin (I) was a gift from Eli Lilly and Co. (Indianapolis, Ind.). Ovine prolactin (PRL; NIH-P-S-12) was a gift from the Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases. Thyroxine (L-T₄); 3,5,3'-triiodo-L-thyronine (L-T₃); 2-thiouracil; and hydrocortisone (F) were purchased from ICN Pharmaceuticals, Inc., Life Sciences Group (Cleveland, Ohio). UDP-galactose, 1- β -D-arabinofuranosylcytosine (AraC), and ATP were purchased from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). UDP-[¹⁴C]galactose (298 mCi/mmol) and [methyl-³H]thymidine (8 Ci/mmol) were products of New England Nuclear (Boston, Mass.).

Mice

All mice were of the C3H/HeN strain. Virgins were 3-4 mo old. Mildly hyperthyroid virgins were prepared by placing 2-mo-old euthyroid females on drinking water containing 2 μ g T₄/ml for 5 wk (16). This treatment resulted in an increase in the total serum T₃ as well as T₄ (16). Primiparous mice were prepared by removing the pups from their nursing mothers 10 d after parturition. The mothers were then randomly selected to receive normal drinking water (euthyroid) or drinking water containing 0.1% of the antithyroid drug 2-thiouracil (hypothyroid) during 7 wk of involution (16). In all cases, only those animals whose mammary glands contained

no detectable lactose synthetase activity at the onset of culture were used.

Organ Culture Method

The abdominal mammary glands were removed with aseptic technique and cultured as described previously (2, 6). The pooled explants prepared from the tissue of several animals were cultured in serum-free Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing combinations of I, F, and PRL at a final concentration of 5 μ g/ml each. Where indicated, L-T₃ was added at a final concentration of 10⁻⁹ M and AraC at 15 μ g/ml. The culture medium was changed at least every 48 h.

Lactose Synthetase (EC 2.4.1.22) Activity

This enzyme activity in mammary tissue was determined in the presence or absence of purified galactosyl transferase as described previously (15). Production of lactose was determined using glucose as the acceptor for the reaction and was confirmed by paper chromatography (14). In all cases, results were corrected for nonspecific hydrolysis of the UDP-[¹⁴C]galactose using distilled water as the acceptor. All data are expressed as picomoles lactose formed per milligram wet tissue per 30 min.

Epithelial DNA Synthesis

The extent of DNA synthesis was measured by allowing explants to incorporate [methyl-³H]thymidine into TCA-precipitable material as described previously (3). That the incorporation of [³H]thymidine into TCA-insoluble material reflects epithelial DNA synthesis was established previously by mitotic index and autoradiography studies (11, 12).

RESULTS

When mammary tissue from euthyroid mature virgin mice is placed in explant culture in the presence of I, F, and PRL, the epithelial cells go through a round of replication and become functionally differentiated as characterized by the production of the milk proteins casein and α -lactalbumin (12, 17). Table I, in agreement with previously published data (10), shows that when DNA synthesis is blocked by >95% by the addition of AraC at the onset of culture, the emergence of lactose synthetase activity in explants from these glands is prevented. Increasing the hormone concentrations to 50 μ g/ml, more frequent medium changes, adding purified galactosyl transferase to the assay, or homogenizing the tissue in the presence of Triton X-100¹ did not overcome this block

¹ Vonderhaar, B. K., G. H. Smith, Y. J. Topper, J. M. Rosen, C. Waugh, and R. J. Pauley. Interrelationship of

TABLE I
Effect of AraC on Lactose Synthetase Activity in Explants from Euthyroid and Hyperthyroid Virgin Mouse Mammary Glands

Animals	Culture conditions	Lactose synthetase activity	
		DNA synthesis cpm/mg wet tissue/3 h	pmol lactose formed/mg wet tissue/30 min
Euthyroid	Uncultured	—	<1.0
	NH	551 ± 10	—
	IFPRL	1,817 ± 11	20 ± 1.2
	IFPRL + AraC	103 ± 6	<1.0
Hyperthyroid	Uncultured	—	<1.0
	NH	333 ± 16	—
	IFPRL	990 ± 20	53 ± 1.7
	IFPRL + AraC	51 ± 3	23 ± 0.5

Pooled explants from the mammary glands of three euthyroid or three hyperthyroid virgin mice were cultured in serum-free medium 199 alone (NH) or in the presence of insulin, hydrocortisone, and prolactin (IFPRL). Where indicated, 1-β-D-arabinofuranosylcytosine (IFPRL + AraC) was added at the time of explantation at a final concentration of 15 μg/ml. After culturing for 72 h (hyperthyroid) or 96 h (euthyroid), lactose synthetase activity in the tissue was measured as described in Materials and Methods. All enzyme assays were performed in duplicate. Epithelial DNA synthesis was measured by labeling the explants with [³H]thymidine for 3 h at the peak of DNA synthesis (45–48 h in culture). Data shown are representative of several similar experiments.

or prevent the fall in activity seen in this and previous studies (15, 17). In marked contrast, when tissue from mildly hyperthyroid virgins, which was selected for its increased growth but lack of detectable lactose synthetase activity *in vivo*, was placed in organ culture in the presence of I, F, and PRL, and DNA synthesis was blocked by >95% by the addition of AraC, emergence of lactose synthetase activity still occurred. Similar results were obtained when fluorodeoxyuridine was used to block DNA synthesis (data not shown).

Virgin mouse mammary tissue contains specific binding proteins for thyroid hormones (1). The presence of these receptors, coupled with the ele-

DNA synthesis, cytological development and functional differentiation in virgin mouse mammary gland. Manuscript submitted for publication.

vated levels of the thyroid hormones in the serum of the hyperthyroid animals (16), suggested that the tissue from these hyperthyroid mice may carry high levels of endogenous hormone into the culture system. The data in Table I would then represent a direct action of thyroid hormone on the explants rather than an intrinsic difference in the mammary epithelial cells from animals in different thyroid states. To test this possibility several experiments were performed, the results of which are presented in Table II. The hormonal induction of lactose synthetase activity in the presence and absence of DNA synthesis was examined in explants of mammary tissue from mature virgin mice

TABLE II
Induction of Lactose Synthetase Activity in Explants from Mammary Glands of Virgin Mice Given L-T₄ for Various Periods of Time

Animals	Culture conditions	Lactose synthetase activity	
		pmol lactose formed/mg wet tissue/30 min	
Hyperthyroid	Uncultured	<1.0	
	IFPRL	15 ± 1.0	
	IFPRL + AraC	7 ± 0.5	
Hyperthyroid withdrawn from T ₄ for 10 d	Uncultured	<1.0	
	IFPRL	8 ± 0.6	
	IFPRL + AraC	3 ± 0.4	
Euthyroid	Uncultured	<1.0	
	IFPRL	14 ± 0.7	
	IFPRL + AraC	<1.0	
Euthyroid given T ₄ for 3 d	Uncultured	<1.0	
	IFPRL	15 ± 0.4	
	IFPRL + AraC	6 ± 0.3	

All animals were 3-mo-old virgins. Mild hyperthyroidism was induced as described in Materials and Methods. 10 d before explantation, several hyperthyroid virgins were removed from the T₄-containing water and given normal drinking water. 3 d before explantation, several euthyroid virgins were placed on drinking water containing L-T₄. Pooled explants were prepared from three animals in each group and cultured in the presence of insulin, hydrocortisone, and prolactin (IFPRL) alone or in the presence of 15 μg/ml 1-β-D-arabinofuranosylcytosine (IFPRL + AraC). After 96 h in culture, lactose synthetase activity in the tissue was measured as described in Materials and Methods. All enzyme assays were performed in duplicate. Data shown are representative of several similar experiments. Similar results were obtained after 72 h in culture.

exposed to thyroid hormones *in vivo* for various periods of time. In the first case, mildly hyperthyroid animals were removed from T_4 for as long as 10 d to allow the serum thyroid hormone levels to return to the normal range (7). However, the mammary tissue from these animals retained the ability to express lactose synthetase activity even in the absence of DNA synthesis *in vitro*. In the second case, euthyroid animals were given thyroxine in their drinking water for only 3 d. It is clear that this short exposure to T_4 results in mammary tissue no longer dependent on DNA synthesis for hormonal induction of lactose synthetase activity *in vitro*.

Previous studies have shown that cells from glands actively growing *in vivo* are no longer dependent on DNA synthesis *in vitro* to express lactose synthetase activity (10, 19). Similarly, when glands from mature animals are stimulated to proliferate *in vivo* and then allowed to involute sufficiently to become proliferatively dormant, the induction of lactose synthetase activity *in vitro* remains independent of DNA synthesis (10, 18). Because thyroid hormones have been shown to increase mammary epithelial cell proliferation *in vivo* (16), it was not possible to entirely rule out this action in explaining the results shown in Table II. Therefore, $L-T_3$ was added directly to cultures of explants prepared from nonproliferating mammary glands of euthyroid virgin mice. Fig. 1 shows that while DNA synthesis is necessary in this tissue to induce lactose synthetase in the presence of I, F, and PRL, the dependence on cell proliferation is overcome by the simultaneous presence of 10^{-9} M $L-T_3$ in the cultures. That these findings represent an action of thyroid hormones independent of their proliferative effect is further shown in Fig. 2. Because the majority of DNA synthesis in mammary epithelial cells from euthyroid virgin mice occurs within the first 3 d of culture (9, 12), explants were initially cultured for 72 h in the presence of I, F, PRL, and AraC. This prevented both DNA synthesis and the induction of lactose synthetase. The subsequent addition of 10^{-9} M $L-T_3$ to these cultures, however, results in the emergence of this enzyme activity at 96 h. During this experiment, DNA synthesis was monitored by a [3 H]thymidine pulse every 6 h and found to be blocked by >95% at all times, even in the presence of $L-T_3$ (data not shown).

These data obtained with tissue from virgin animals suggest that the coupling of terminal differentiation in the mouse mammary gland to DNA

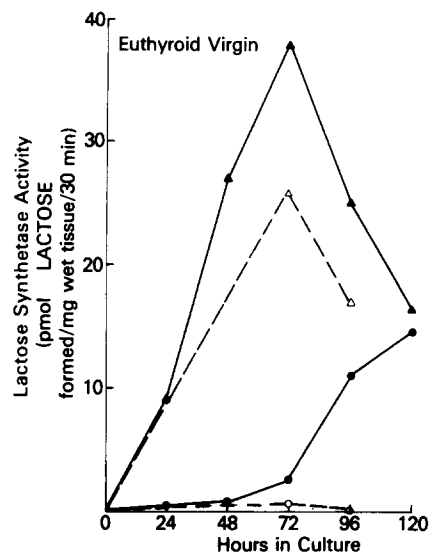


FIGURE 1 Time-course of the induction of lactose synthetase activity in explants from euthyroid virgin mice. Explants of mammary glands from eight euthyroid virgin mice were cultured as described in the legend to Table I. At the onset of culture and at 24-h intervals, the lactose synthetase activity in the tissue was determined as described in Materials and Methods. All enzyme assays were performed in duplicate. Data shown are representative of several similar experiments. Tissue cultured in the presence of insulin, hydrocortisone, and prolactin in the absence (IFPRL; ●) or presence of AraC (IFPRL + AraC; ○). Tissue cultured in the presence of insulin, hydrocortisone, prolactin, and $L-T_3$ in the absence (IFPRL- T_3 ; ▲) or presence of AraC (IFPRL- T_3 + AraC; △).

synthesis *in vitro* is overcome by the action of thyroid hormones. To test further this hypothesis, the impact of changes in the thyroid status of primiparous mice on the ability of mammary tissue to express lactose synthetase activity in the presence of AraC was examined. The involuted post-lactational gland from a euthyroid primiparous mouse reverts to a resting stage with several characteristics similar to those of tissue from euthyroid virgin animals. Even though the glands from primiparous C3H/HeN mice are morphologically more developed than those of virgins (16), both are biochemically quiescent as characterized by the low level of proliferation *in vivo* (9), the inability to respond to insulin within the first 12 h in culture (3, 9), and the lack of detectable lactose synthetase *in vivo* (17, 19). As with glands from virgin mice, tissue from euthyroid primiparous animals is able to express lactose synthetase activ-

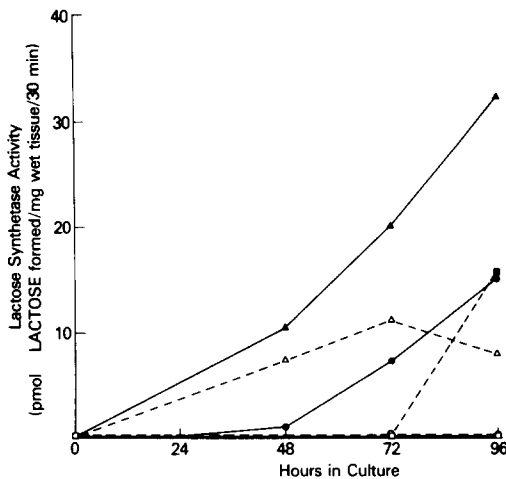


FIGURE 2 Effect of delayed addition of L-T₃ on the induction of lactose synthetase activity in explants from euthyroid virgin mice. Explants of mammary glands from six euthyroid virgin mice were cultured as described in the legend to Table I. At the times indicated, the lactose synthetase activity in the tissue was determined as described in Materials and Methods. All enzyme assays were performed in duplicate and data shown are representative of several similar experiments. Tissue cultured in the presence of insulin and hydrocortisone (IF; ○), or insulin, hydrocortisone, and prolactin in the absence (IFPRL; ●) or presence of AraC (IFPRL + AraC; □), or presence of insulin, hydrocortisone, prolactin, and L-T₃ in the absence (IFPRL-T₃; ▲) or presence of AraC (IFPRL-T₃ + AraC; ◊). Tissue cultured in the presence of IFPRL + AraC for 72 h followed by 24 h in the presence of IFPRL-T₃ + AraC (□--■).

ity when placed in explant culture in the presence of I, F, and PRL (19). Fig. 3 confirms this observation and again demonstrates that, in contrast to virgin tissue, elaboration of lactose synthetase activity in tissue from euthyroid primiparous mice is independent of DNA synthesis *in vitro*. However, Fig. 3 also shows that when the animals are made mildly hypothyroid during 7 wk of involution, the mammary tissue reverts to dependence on DNA synthesis, before emergence of lactose synthetase activity in the presence of the three hormones. This dependence on DNA synthesis *in vitro* did not develop in the glands of hypothyroid mice until the 6th wk of involution even though the hypothyroid state as well as insulin insensitivity had developed by the 3rd wk (data not shown).

DISCUSSION

Thyroid hormones appear to play a fundamental

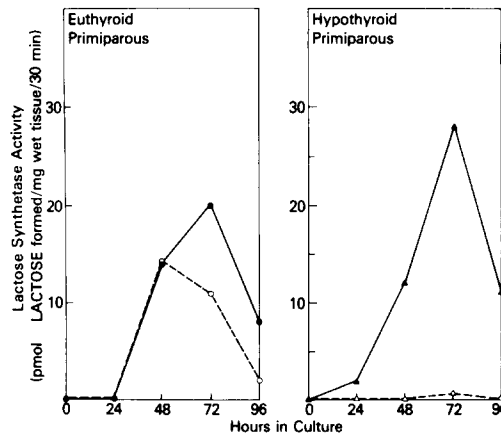


FIGURE 3 Time-course of the induction of lactose synthetase in explants from euthyroid and hypothyroid primiparous mice. Explants of mammary glands from five euthyroid and six hypothyroid animals were cultured as described in the legend to Table I. At the onset of culture and at 24-h intervals, the lactose synthetase activity in the tissue was determined as described in Materials and Methods. All enzyme assays were performed in duplicate and data shown are representative of several similar experiments. Tissue cultured in the presence of insulin, hydrocortisone, and prolactin in the absence (IFPRL; —) or presence of AraC (IFPRL + AraC; ---).

role in the regulation of lactose synthetase activity in the mouse mammary gland. Previous studies have shown that the presence of these agents in mammary cultures containing I, F, and PRL results in a three- to fivefold increase in this enzyme activity in the tissue (14, 15). The data presented in this paper suggest that the events controlling coupling of induction of lactose synthetase *in vitro* to exit from G₁ arrest and traversal of the cell cycle are overcome by thyroid hormones. In the absence of exogenous L-T₃, tissue from euthyroid virgin mice must undergo a round of replication *in vitro* to demonstrate lactose synthetase activity in the presence of I, F, and PRL. In contrast, tissue from hyperthyroid virgins or euthyroid primiparous animals are able to respond to these three hormones, and lactose synthetase activity is induced even when DNA synthesis is blocked. The mammary epithelial component from these latter animals is morphologically more developed (i.e., more alveolar structures) than the epithelium from the euthyroid virgin (16). However, this "ductal" vs. "alveolar" nature of the tissue alone is not sufficient to explain the difference in dependence on DNA synthesis. This is ruled out by the response of explants from hypothyroid primiparous mice.

Glands from these animals, after 7 wk of involution, are morphologically indistinguishable from those of their euthyroid counterparts (16), but unlike explants from the euthyroid animals' glands, tissue from these hypothyroid mice must traverse the cell cycle to demonstrate hormonally induced lactose synthetase activity.

It is unlikely that the difference between euthyroid virgin and primiparous animals in terms of dependence on DNA synthesis *in vitro* is caused by circulating levels of endogenous thyroid hormones. The concentrations of both T₃ and T₄ in serum from these animals are not significantly different (16; B. K. Vonderhaar and A. E. Greco, manuscript in preparation). However, this does not rule out differences in thyroid hormone receptor number and/or binding characteristics in the tissues of these animals. Such considerations are currently being investigated.

The mechanism by which thyroid hormones regulate terminal differentiation is unknown. Ultrastructural examination of explants of mammary glands from euthyroid virgin mice cultured in the presence of I, F, PRL and AraC demonstrates that these epithelial cells appear secretory (18, and footnote 1). These cells have been shown to contain casein mRNAs at levels similar to those in cells cultured in the presence of I, F, and PRL (18, and footnote 1). However, these messages are inefficiently translated in the blocked systems,¹ suggesting a post-transcriptional regulation of casein synthesis in virgin mouse mammary glands. Similarly, Houdebine et al. (4) have shown that the addition of thyroid hormone to cultures of pseudopregnant rabbit mammary glands has no effect on the level of casein mRNA, but appears to stimulate casein peptide synthesis post-transcriptionally. Although the mode of regulation of the synthesis of the major milk proteins, casein and α -lactalbumin, is not necessarily the same (8, 17), these alterations suggest that thyroid hormones may regulate differentiation post-transcriptionally. In mammary glands from euthyroid virgin animals, there may be one or more population of cells which require DNA synthesis *in vitro* or the presence of exogenously added L-T₃ to effectively translate the mRNA for α -lactalbumin, thus resulting in hormonal induction of lactose synthetase activity. These cells would predominate over DNA synthesis-independent cells in the gland. In involuted glands from euthyroid primiparous animals, the DNA synthesis-independent cells would be more predominant resulting in tissue which does

not require cell proliferation *in vitro* to express lactose synthetase activity. The presence of these cells *in vivo* would be dependent upon maintenance of the euthyroid state because involution under hypothyroid conditions appears to result in a selective loss of these cells.

The action of thyroid hormones in overcoming the need for DNA synthesis for induction of lactose synthetase activity is accomplished by addition of physiological levels of L-T₃ to serum-free medium (14, 16). This raises the question of whether a comparable situation exists *in vivo* in which DNA synthesis and traversal of the cell cycle are absolute requirements for terminal differentiation in the mouse mammary gland. Indeed, these observations may only reflect a situation which exists in the adult virgin gland in culture. Even if this is so, the observations may reflect characteristics of the state of arrest of these cells in the cell cycle (19) and, as such, afford a unique opportunity to study those events in the G₁ phase of the cell cycle which control the production and activity of α -lactalbumin.

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