

A ROLE OF THE CELL CYCLE IN HORMONE-DEPENDENT DIFFERENTIATION

BARBARA K. VONDERHAAR and YALE J. TOPPER. From the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

An interesting concept in developmental biology, based on a proposal presented by Holtzer (1), concerns the necessary coupling of differentiation to a prior round of cell division. An extension of this concept states that critical events must occur during either the DNA synthetic (S) or the mitotic (M) phase of the cell cycle, or both, which result in developmentally competent daughter cells, qualitatively different from their relatively undifferentiated progenitors (2). These events having occurred, altered phenotypic expression (i.e., differentiation) can take place. A similar proposition

has been examined in several other systems including the estrogen-primed immature chick oviduct (3-6), erythropoietin-sensitive hemoglobin-producing cells (7, 8), and cultured pancreatic exocrine cells (9, 10). The model system we have used to investigate such a dependence on cell proliferation (which we termed "critical mitosis" [11]) is the terminal differentiation of the mouse mammary gland.

Epithelial cells in mammary gland explants are stimulated to undergo DNA synthesis and cell division, and subsequently to produce the milk

proteins, casein and α -lactalbumin (12, 13), during culture in a chemically defined medium supplemented with insulin (I), hydrocortisone (F), and either prolactin (P) or human placental lactogen (L) (14, 15). In our previous studies we examined the dependence of milk protein production on the previous round of cell division, using explants from glands of animals in three different stages of development (i.e., adult virgin [11], midpregnant [11], and nonpregnant primiparous [2, 16]). For these studies we employed two inhibitors of DNA synthesis, 1- β -D-arabinosylcytosine (ara-C) and fluorodeoxyuridine (FUdR), which produce their inhibitory effect by different mechanisms (17-19). It was observed (11) that the simultaneous addition of either ara-C or FUdR to explants from adult virgin glands cultured in the presence of I, F, and P, suppressed the hormonally induced increase in DNA synthesis and mitosis. The later emergence of α -lactalbumin and casein was also prevented, suggesting that DNA synthesis and/or mitosis are required before production of these proteins. However, neither ara-C nor FUdR prevented α -lactalbumin production by virgin cells which had previously divided *in vitro*. The results of this postmitotic test suggested that the inhibitors' effect on milk protein production was not the result of a general cellular toxicity. In addition, the insulin-mediated induction of the combined activities of the intracellular proteins glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase (20) was not prevented by ara-C or FUdR, demonstrating that these agents do not interfere with acquisition of insulin sensitivity.

Similar experiments revealed that the production of milk protein is not coupled to cell proliferation when tissue from midpregnant or postlactational animals is used (2, 11, 16). While the explants in the cultures containing the inhibitors had a diminished total capacity to make milk proteins, this is primarily a reflection of the smaller number of epithelial cells present; relative to the number of epithelial cells, the cultures containing inhibitor made nearly as much α -lactalbumin and casein as the cultures without inhibitor. This is true for both the insulin-sensitive, proliferative tissue from midpregnant (11), and the insulin-insensitive, nonproliferative, involuted tissue from nonpregnant primiparous animals (2, 16). In an attempt to elucidate the mechanism involved in such a critical mitosis, apparently required only for adult virgin mice, similar experiments were con-

ducted using tissue from immature animals. As a result of these studies, a new concept relating terminal differentiation of the mouse mammary gland to the cell cycle emerged.

MATERIALS AND METHODS

Chemicals

Crystalline pork insulin was a gift from the Eli Lilly Company, Indianapolis, Ind., hydrocortisone and human placental lactogen were obtained from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio, UDP-[14 C]galactose (298 mCi/mmol) and [*methyl*- 3 H]thymidine (8 Ci/mmol) from New England Nuclear Boston, Mass., fluorodeoxyuridine from CalBiochem, San Diego, Calif., 1- β -D-arabinosylcytosine from the Upjohn Co., Kalamazoo, Mich.

Methods

Explants were prepared from the abdominal mammary glands of C3H/HeN mice (21, 22) which were either 5-6 wk old (immature virgins) or whose tissues had been allowed to involute for at least 4 wk after a single pregnancy and a 10-12-day period of lactation (primiparous). Explants were cultured in medium 199 supplemented with the indicated hormones. Each hormone was used at a final concentration of 5 μ g/ml. Media were changed at least every 48 h. The accumulation of α -lactalbumin, the B protein of the lactose synthetase system (23), was measured in the presence of excess endogenous UDP-galactosyl transferase as described previously (13). Epithelial DNA synthesis was measured by the incorporation of [3 H]thymidine into an acid-insoluble fraction of the cultured tissue (20). Epithelial DNA content was determined by the method of Burton (24) as described previously (11).

RESULTS AND DISCUSSION

Our previous studies had shown that the quiescent cells in the mammary gland of the mature virgin mouse are themselves incompetent to produce milk proteins under the influence of I, F, and P. Competent daughter cells are formed *in vivo* during pregnancy and can be formed *in vitro*. The quiescent cells in primiparous involuted glands, although similar in some respects to those in the mature virgin animal (25), are, however, themselves competent to produce milk proteins in the presence of I, F, and P. It appeared that a necessary change occurs when cells from the mature virgin pass through the S, G₂, and/or M phase of the cell cycle *in vitro* or during pregnancy,

and that manifestation of this change is retained by the cells in the involuted gland.

Furthermore, since the incompetent cells in the mature virgin are products of the mitoses which occur during adolescence, it was concluded that the proliferation which occurs at that time was qualitatively different from that which occurs during pregnancy (2). On this basis, it was predicted that mammary cells in the adolescent mouse, like those in the mature virgin, are themselves incompetent, and that development of competency would require a round of proliferation *in vitro*. This prediction was not borne out. The data in Table I show that freshly explanted tissues from immature glands have little or no detectable α -lactalbumin *in vivo*. When explants from these glands are cultured in the presence of I, F, and L, this milk protein can be induced. Even under conditions where [³H]thymidine incorporation into DNA is inhibited by 95% with ara-C, α -lactalbumin can be formed. The lower level of this milk protein produced in the explants cultured in the presence of ara-C, compared to the explants from the contralateral glands cultured in the absence of the inhibitor, probably reflects primarily a lower total number of cells.

The combined data from all four stages of development show that the necessary coupling of development to proliferation is unique to the epithelial cells of the dormant, mature virgin gland. Thus, our previous suggestion that the cell proliferation which occurs during adolescence is qualitatively different from that which occurs during pregnancy (2) is not tenable.

One plausible interpretation consistent with all the observations is based on the possibility that hormone-dependent critical events necessary for the ultimate synthesis of milk proteins can occur only at a particular point in the G₁ phase of the cell cycle. This is best visualized with the aid of Fig. 1. The epithelial cells in the nonsecretory, nonproliferating mature virgin gland are depicted as arrested in G₁, distal to these events. On the other hand, the majority of the cells of the nonsecretory, dormant primiparous mouse mammary gland are depicted as arrested in G₁, proximal to these critical events. Thus, in order to become competent to ultimately make the unique products of the mammary gland, cells from glands of primiparous animals would only have to progress through G₁ under proper hormonal conditions, while cells

TABLE I
Effect of Inhibitors of DNA Synthesis on α -Lactalbumin Production in Explants of Mammary Glands from Immature Mice

Animal no.	Lactose synthetase activity (pmol lactose formed/10 mg wet wt tissue/30 min)		[³ H]Thymidine incorporation into DNA (cpm/mg wet wt tissue/4 h)	
	0 h	72 h in culture		
1	Not detectable	IFL	IFL ara-C	37.1
2	Not detectable	—	—	61.6
3	—	47.5	47.5	33.1
72 h in culture				
4		IFL	IFL ara-C	
		524	19	
5		552	29	

Explants from 5-wk old mice were cultured for 72 h in medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing I, F, L, and ara-C (7.5 μ g/ml), as indicated. Accumulation of α -lactalbumin, measured as described in Materials and Methods, is expressed as picomoles of lactose formed per 10 mg wet weight tissue per 30 min. Epithelial DNA synthesis was measured during the last 4 h of culture as described in Materials and Methods. Pooled tissue could not be employed in these experiments because of the nonuniform distribution of the epithelial cells within the gland. Therefore, each determination represents an entire abdominal gland, and only two determinations could be made per animal. Indicated paired determinations represent results obtained with contralateral glands. Data shown are representative of several experiments.

from the mature virgin animal would have to traverse the entire cycle and return to G₁. According to this hypothesis, the action of any mitogen *in vitro*, therefore, should convert the dormant mature virgin cells to their competent form simply by causing them to progress through the cell cycle. This is indeed the case with insulin as well as the mitogens present in insulin-free fetal calf or porcine serum (2, 16).

The mammary epithelial cells in immature and midpregnancy tissue are actively proliferating *in vivo* and are probably positioned randomly throughout the cell cycle at the time of explantation. When cultured in the presence of I, F, P (or L), and a DNA synthesis inhibitor, those cells

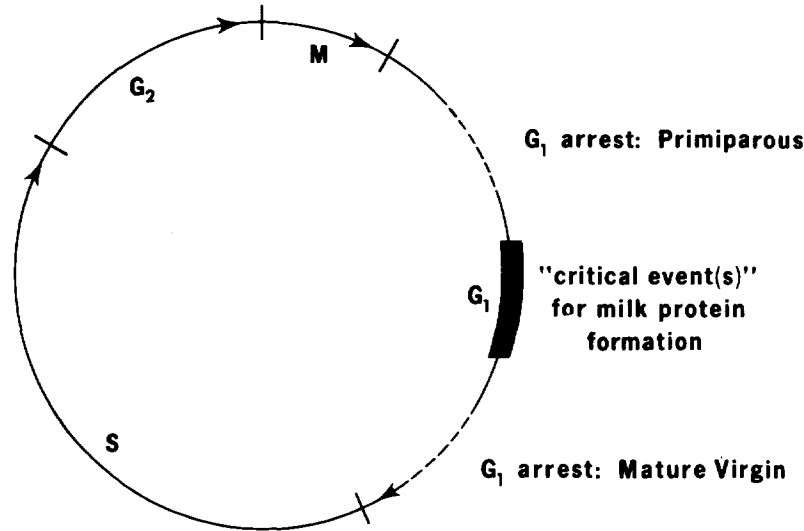


FIGURE 1 Terminal differentiation of mammary epithelial cells in relation to the cell cycle.

capable of continuing through the cell cycle and passing the hormone-dependent critical portion of G₁ gather at the G₁ → S interphase and make the milk proteins.

While the proliferating cells in the mammary gland of the immature animal become both insulin sensitive (25) and competent to make milk proteins *in vitro* in the presence of I, F, and P (or L), they do not make casein or α -lactalbumin *in vivo* even though they pass the site of the G₁ events. This inability *in vivo* is presumably due to low serum prolactin levels and/or insensitivity of the mammary cells to insulin (25).

Support for this interpretation is provided by the results recorded in Table II. In these experiments the cells in explants from primiparous, nonpregnant mice are allowed to traverse the critical portion of G₁ in the absence of hormone (NH). Under these circumstances the primiparous cells become virgin-like. They are able to make α -lactalbumin when allowed to continue through the cell cycle and re-enter the critical portion of G₁ in the presence of the hormones (NH₂₄ → IFL₂₄₋₇₂), but are unable to make α -lactalbumin when prevented from continuing through the cycle because of the addition of FUdR (NH₂₄ → IFL FUdR₂₄₋₇₂). In contrast, when the cells are allowed initially to pass through the critical portion of G₁ in the presence of hormones, the presence of FUdR (IFL FUdR₇₂) does not prevent the formation of α -lactalbumin. Similar results were obtained with

ara-C. Relative to the amount of epithelial DNA, the cells in the IFL FUdR₇₂ system make almost as much α -lactalbumin as those in the IFL₇₂ system. The cells in the NH₂₄ → IFL FUdR₂₄₋₇₂ system are viable, since they make more glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase in response to insulin (data not shown).

It appears, then, that although mature virgin mammary epithelial cells must proliferate in order to become competent to produce milk proteins, development of such competency is not related to S phase or M phase *per se*. Rather, traversal of the cell cycle is the necessary route by which these cells can re-enter the critical portion of G₁ in the presence of the requisite hormone(s). It is of interest that this hormone-dependent process occurs, apparently, only in a limited part of the cycle. However, the nature of the proposed G₁ events critical for milk protein production and the minimal hormone requirement are not known. The mechanism which controls the position of G₁ arrest for either mature virgin or primiparous cells is also unknown.

This hypothesis may also apply to other systems involving development of previously quiescent cells. While the response of immature oviduct to estrogen seems to require prior cell proliferation, restimulation of involuted oviduct does not (5). Here, again, the cells may be arrested at different points in G₁.

TABLE II
Conversion of Cells from Primiparous Nonpregnant
Glands into a Virgin-Like State

Culture conditions	Epithelial DNA (ng/mg wet wt tissue)	Lactose synthetase activity (pmol lactose formed/ mg wet wt tissue/ 30 min)	Activity per ng of DNA
0 h	50	<1	<0.02
NH ₂₄ → IFL ₂₄₋₇₂	85	15	0.18
NH ₂₄ → IFL FUDR ₂₄₋₇₂	50	<1	<0.02
IFL ₇₂	75	12	0.16
IFL FUDR ₇₂	47	6	0.13

Pooled explants from three to four primiparous animals were first cultured in medium 199 in the absence of hormones (NH) for 24 h. Fresh media containing I, F, L (cf. Materials and Methods), and FUDR (25 µg/ml) were then added as indicated. The cultures were continued for an additional 48 h. Simultaneously, some explants were exposed to IFL in the presence, or absence, of FUDR for the full 72 h of culture, with a medium change occurring after 24 h. Accumulation of α-lactalbumin was measured as described in Materials and Methods. Data are expressed as picomoles of lactose formed per milligram wet weight of tissue per 30 min. Epithelial DNA content was determined as described in Materials and Methods. Data shown are representative of several similar experiments.

SUMMARY

Terminal differentiation of some cells appears to require hormone-dependent critical events which can occur only during a limited portion of the G₁ phase of the cell cycle. As a consequence, certain cells previously arrested in the cell cycle must undergo proliferation as a necessary route to this portion of G₁. This is characteristic of terminal differentiation of mammary epithelial cells, and possibly other cell types.

Received for publication 6 May 1974.

REFERENCES

- HOLTZER, H. 1963. Mitosis and cell transformations. General Physiology of Cell Specialization. D. Mazia and A. Tyler, editors. McGraw-Hill Book Company, New York. 80-90.
- TOPPER, Y. J., and B. K. VONDERHAAR. 1974. Concerning the role of critical cell-proliferation in

- differentiation of mammary epithelial cells. Control of Proliferation in Animal Cells. B. Clarkson and R. Baserga, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 843-852.
- OKA, T., and R. T. SCHIMKE. 1969. Progesterone antagonism of estrogen-induced cytodifferentiation in chick oviduct. *Science (Wash. D. C.)*. **163**:83-85.
- OKA, T., and R. T. SCHIMKE. 1969. Interaction of estrogen and progesterone in chick oviduct development. I. Antagonistic effect of progesterone on estrogen-induced proliferation and differentiation of tubular gland cells. *J. Cell Biol.* **41**:816-831.
- OKA, T., and R. T. SCHIMKE. 1969. Interaction of estrogen and progesterone in chick oviduct development. II. Effects of estrogen and progesterone on tubular gland cell function. *J. Cell Biol.* **43**:123-137.
- PALMITER, R., and J. T. WRENN. 1971. Interaction of estrogen and progesterone in chick oviduct development. III. Tubular gland cell cytodifferentiation. *J. Cell Biol.* **50**:598-615.
- PAUL, J., and J. A. HUNTER. 1968. DNA synthesis is essential for increased haemoglobin synthesis in response to erythropoietin. *Nature (Lond.)*. **219**:1362-1363.
- GROSS, M., and E. GOLDWASSER. 1970. On the mechanism of erythropoietin-induced differentiation. VII. The relationship between stimulated deoxyribonucleic acid synthesis and ribonucleic acid synthesis. *J. Biol. Chem.* **245**:1632-1636.
- WESSELLS, N. K. 1968. Problems in the analysis of determination, mitosis, and differentiation. Epithelial-Mesenchymal Interactions. R. Fleischmajer and R. E. Billingham, editors. The Williams & Wilkins Company, Baltimore, Md. 132-151.
- WESSELLS, N. K., and W. J. RUTTER. 1969. Phases in cell differentiation. *Sci. Am.* **220**:36-44.
- OWENS, I. S., B. K. VONDERHAAR, and Y. J. TOPPER. 1973. Concerning the necessary coupling of development to proliferation of mouse mammary epithelial cells. *J. Biol. Chem.* **248**:472-477.
- STOCKDALE, F. E., and Y. J. TOPPER. 1966. The role of DNA synthesis and mitosis in hormone-dependent differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **56**:1283-1289.
- VONDERHAAR, B. K., I. S. OWENS, and Y. J. TOPPER. 1973. An early effect of prolactin on the formation of α-lactalbumin by mouse mammary epithelial cells. *J. Biol. Chem.* **248**:467-471.
- TURKINGTON, R. W., and Y. J. TOPPER. 1966. Stimulation of casein synthesis and histological development of mammary gland by human placental lactogen *in vitro*. *Endocrinology*. **79**:175-181.
- TURKINGTON, R. W., K. BREW, T. C. VANAMAN, and R. L. HILL. 1968. The hormonal control of lactose synthetase in the developing mouse mammary gland. *J. Biol. Chem.* **243**:3382-3387.
- VONDERHAAR, B. K., and Y. J. TOPPER. 1973.

- Critical cell proliferation as a prerequisite for differentiation of mammary epithelial cells. *Enzyme (Basel)*. **15**:340-350.
17. HEIDELBERGER, C. 1965. Fluorinated pyrimidines. *Prog. Nucl. Acid. Res. Mol. Biol.* **4**:1-50.
 18. FURTH, J. J., and S. S. COHEN. 1968. Inhibition of mammalian DNA polymerase by the 5'-triphosphate of 1- β -D-arabinofuranosylcytosine and the 5'-triphosphate of 9- β -D-arabinofuranosyladenine. *Cancer Res.* **28**:2061-2067.
 19. GRAHAM, F. L., and G. F. WHITMORE. 1970. The effect of 1- β -D-arabinofuranosylcytosine on growth, viability, and DNA synthesis of mouse L-cells. *Cancer Res.* **30**:2627-2635.
 20. FRIEDBERG, S. H., T. OKA, and Y. J. TOPPER. 1970. Development of insulin-sensitivity by mouse mammary gland *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **67**:1493-1500.
 21. ELIAS, J. J. 1957. Cultivation of adult mouse mammary gland in hormone-enriched synthetic medium. *Science (Wash. D. C.)*. **126**:842-843.
 22. JUERGENS, W. G., F. E. STOCKDALE, Y. J. TOPPER, and J. J. ELIAS. 1965. Hormonal-dependent differentiation of mammary gland *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **54**:629-634.
 23. BRODBECK, U., W. L. DENTON, N. TANAHASHI, and K. E. EBNER. 1967. The isolation and identification of the B-protein of lactose synthetase as α -lactalbumin. *J. Biol. Chem.* **242**:1391-1397.
 24. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
 25. OKA, T., J. W. PERRY, and Y. J. TOPPER. 1974. Changes in insulin responsiveness during development of mammary epithelium. *J. Cell Biol.* **62**:550-556.