INTRODUCTION

Morphogenesis and biochemical differentiation of the female breast in vivo is regulated by a complex of hormones required at different stages of development. This is emphasized by the alveolar epithelial component during lactation, which represents a highly organized group of cells specifically differentiated under the influence of a number of hormones produced by the ovary, pituitary, and pancreas. The ovarian hormones include estrogen and progesterone; the pituitary provides prolactin and/or somatotropin; and the presumed requirement of insulin is provided by the pancreas.

Depending on the stage of duct and alveolar development, it is possible to identify some of the phases of hormone induction as: (1) an inactive resting phase, (2) a proliferative phase as pregnancy proceeds, (3) an active lactation phase immediately following pregnancy, (4) a postnatal regressive change with minor alterations evident during menses, and finally (5) a menopausal atrophy of the glandular tissue.

Experimental studies of mammogenesis in the mouse suggest that stages up to the 7th day of fetal life are independent of specific hormones required for development. Thus, Raynaud (51) demonstrated that gonadectomy of both sexes leads to exclusively female differentiation, indicating that hormones from the gonads at this stage are not essential for differentiation. Additional evidence that the testes are responsible for the normal course of male development is given credence by the fact that treatment of the gonads of the male fetus with 100 to 200,000 roentgens on the 13th day, shows a degree of development of the mammary gland primordia that corresponds with that of control x-irradiated females. The effect of testosterone on the dedifferentiation (duct degeneration) of the mammary gland begins in fetal life and may be reproduced in embryo culture experiments (26). Thus, at a specific time of mammary gland development testosterone receptor sites are evident, limited to the 7th day of development in the mouse mammary gland. Receptor sites are no longer detected after the 8th day of development.

Most experiments suggest that in vivo, estrogen provides an initial stimulus for development of the glandular duct system whereas progesterone from the corpus luteum stimulates together with the estrogens differentiation of the secretory alveoli. In later stages during pregnancy estrogen most probably is responsible for the production of prolactin by the pituitary required for the induction of milk protein synthesis.

HORMONE INDUCTION IN VITRO

It is extremely fortunate that some of these complex hormone interactions of both steroid and polypeptide hormones are amenable to studies in greater detail by explants in culture. In fact this technique was described almost 25 yrs. ago by Hardy (15), who presented the details of a technique for development of mouse mammary glands in vitro. In these early studies Prop (48 to 50) was one of the first to show evidence of hormone dependency of the mammary gland in vitro. More recently, the elegant work from the laboratory of Topper (10, 12, 30 to 32, 43, 45, 64 to 66, 68) and others (14, 19, 20) defined the hormone requirement of insulin, hydrocortisone, and prolactin as essential for the production of milk proteins by the culture system of mouse mammary gland. In these studies mammary gland explants prepared from midpregnant mice incubated in the presence of insulin and hydrocortisone, induced synthesis of DNA evident at 18 h and attained a maximum at 24 h. Synthesis of casein as well as the A and B proteins (galactosyl transferase and α-lactalbumin) of the lactose synthetase enzyme complex reached a maximum at 48 h in culture.

CONCEPT OF HORMONE INDUCED CRITICAL MITOSIS

One of the most important aspects of mammary gland alveolar epithelial cell differen-
tiation is the fact that an initial cell division is required. Thus, if the mitogenic response to insulin is blocked by a specific inhibitor of DNA synthesis as cytosine arabinoside, hydroxyurea or if the cells are blocked at metaphase by colchicine, mammary explants no longer demonstrate a capacity to respond to prolactin.

In contrast to midpregnant mammary tissue the production of casein and galactosyl transferase is delayed to 72 h in culture explants prepared from unprimed virgin mammary tissue. Not only is there also a delay in the production of casein, but in addition the α-lactalbumin fails to reach its maximum until 120 h. Thus, in the unprimed virgin mammary gland the coordinate synthesis of casein, galactosyl transferase, and α-lactalbumin characteristic of midpregnant explants is no longer evident. The most significant aspect of these studies is the fact that comparable tissue prepared from virgins that are primed with estrogen and/or prolactin resemble those of the midpregnant tissue.

Topper interprets these observations as follows: The temporal differentiation of the cell requires a "critical mitosis" which generates daughter cells (45). He suggests that in the case of midpregnancy, most mammary cells already have undergone this "critical mitosis" prior to the preparation of the in vitro explant. In agreement with this hypothesis is the fact that even in the presence of cytosine arabinoside, as a specific inhibitor of DNA synthesis, the midpregnant explant (on a per cell basis) responds to hormones in the induction of secretory proteins. In contrast, virgin tissue explants which have not passed through "critical mitosis" are not inducible in the presence of cytosine arabinoside. Presumably then, this "critical mitosis" is a result of the stimulation of DNA synthesis in the presence of hydrocortisone in vivo or during insulin induced mitosis in the presence of hydrocortisone in vitro is suggested by Topper to be essential. According to Topper's concept, prolactin then must serve two functions, the first is an in vivo synergism possibly with insulin to induce the critical mitosis and second, as has been demonstrated in vitro, to act as an inducer of synthesis of specific mRNA for milk protein synthesis. The study of this aspect is an important component of this review.

Hydrocortisone has exerted its effect on the accumulation of rough endoplasmic reticulum in the mouse mammary epithelial cell (38, 39, 40, 43). It is reasonable to speculate that in the in vitro experiments with insulin acting as a mitogen, hydrocortisone plays an essential role in the formation of rough endoplasmic reticulum to provide the necessary machinery to respond in a differentiated state to the prolac-tin.

**HORMONE CONTROL OF RNA TRANSCRIPTION**

Since molecular biology concerns itself primarily with the relationship between molecular structure in biological function, the mammary gland in vitro provides a remarkably good model system in that it responds to hormones in the production of new types of tissue-specific proteins. Thus, an important objective would be to determine how mammary gland cells acquire and maintain their differentiated characteristics in the control of mRNA transcription. There are few that would challenge the fact that selected transcription in eukaryotes represents an important role of gene expression and in particular those cells that respond to hormone-induced differentiation.

The culture system of mammary gland has been selected for our studies in that it represents a transient type of differentiation in which the alveolar epithelial cell has undergone a "critical mitosis" generating a derepressed
condition required for the synthesis of new mRNA's. We have initiated studies to determine the nature of the chromatin in the derepression of the DNA genome. These studies are based on the fact that both physiological and biochemical evidence indicate that synthesis of RNA is limited to selected loci in the interphase chromatin (27). There is also evidence that RNA synthesis occurs in the extended chromatin fibril (8, 9) whereas the condensed heterochromatin is less active. Indeed, the impetus for the development of biochemical procedures for fractionating chromatin and studying in vitro activity is based on the concept that active and inactive portions of the genome may differ in chemical and physical terms.

For example, one characteristic of the eukaryotic cell is the fact that only 4 to 5% of the genome is transcribed in some cells such as liver, kidney, and spleen whereas approximately 11% is transcribed for brain tissue (13). This, of course, is in sharp contrast to the extent of transcription in prokaryotic cells which constitute about 40 to 50% of the DNA genome. Even if one takes into account the genome size of eukaryotic cells as described by Baldwin et al. (3) must be packaged into each metaphase chromosome, on the order of several microns long.

2) Isolated chromatin usually manifests protein-DNA ratios in the range of 1.3:1 to 2:1 and RNA:DNA ratios of less than 2:1.

3) The chromatin complex contains two major classes of proteins: basic proteins, histones, and neutral to acidic proteins, referred to as “nonhistone chromosomal proteins.” It is essential that both of these classes of proteins be considered together, and they will be discussed later in this review.

4) Although there are conflicts in interpretations of physical studies on eukaryotic chromatin, the bulk of the evidence indicates that the DNA in chromatin is to a large extent covered by a protein matrix. Many of these studies favor figures in the range of 80 to 95% of the DNA being covered or masked by proteins, which is somewhat in conflict with studies of Clark and Felsenfeld, who suggested that only 50% of the DNA is covered (7). The higher figures are in basic agreement with other data from in vitro assays of chromatin template activity, as studies of RNA-DNA hybridization which indicate that usually less than 20% of the DNA present is available to act as a template for the transcription process as described by Bonner et al. (5).

5) Finally there is a great deal of evidence to indicate that the structure and function of chromatin is greatly influenced by interactions between DNA and its protein components.

A number of studies have attempted to visualize chromatin structure by electron microscopy. As a result of these studies, some important concepts are beginning to evolve. In the studies of Olins (44) linear array of spherical chromatin particles referred to as v bodies about 70 angstroms in diameter were observed in preparations of isolated eukaryotic chromatin. In many of these preparations, fibers were long, (approximately 8 μm) unbranched, and in parallel arrays revealing regularly distributed thick and thin regions. A number of studies suggest that the appearance of chromatin units on fibers somewhat like beads on a string is related to the native configuration and does not
represent an artifact of the preparation procedures.

Recent studies by Senior, Olins, and Olins (56, 57) show that it is possible to isolate chromatin fragments resembling \( \nu \) bodies. Thus, extensive sonication of water swollen formaldehyde fixed chromatin preparations do not destroy the \( \nu \) bodies but do obliterate the fibrillar arrangement of particles. Sucrose density and cesium chloride density gradients demonstrate that these \( \nu \) bodies represent molecular weights of approximately 295,000 ± 13,000 with a density of 1.414 which would represent a ratio of protein/DNA of approximately 1.22 (55). The DNA was examined after digestion with pronase and gave a molecular weight of 141,000. This suggests that approximately 210 base pairs pack into a volume of 70 to 80 angstroms. The expected linear DNA duplex would represent 726 angstroms and, thus, a packing ratio of 10:1.

Recent work by Baldwin et al. (3) based on data from neutron scattering studies of chromatin, also suggests a globular model in which polar segments of histones form a core surrounded by a DNA complex with basic segments of histones. Thus these studies imply that histones F2a1, F2a2 and F2b, F3 form an inner matrix on which the DNA supercoil is wound. Only in the case of histone F1 is there evidence that any histones are externally arranged. Additional detailed neutron scatter studies with \( \text{D}_2\text{O} \) will be required before conclusions can be made. Chromatin most probably consists of a string of such globular units referred to as \( \nu \) bodies with unknown orientations. The packing of such units in the chromosome is probably mediated through proteins, presumably F1 histone interactions (Fig. 1).

### POST SYNTHETIC MODIFICATIONS OF HISTONES

An attractive general hypothesis concerning the control of chromatin structure and, thus, the control of RNA transcription through the cell cycle, is that chemical modifications of histones are involved. With only five major histones, the chemical modifications of any one of these will modify its interaction with DNA throughout the genome. Further biochemical modifications which affect the state of charge of the basic residues (lysine and arginine, for example), or of serine, located in or close to the basic segments of the histone molecule, alter the degree of condensation. It is reasonable to suggest that only the basic segments of histones are complexed with DNA on the outside of the globular units and are thus accessible to enzyme modifications or enzyme attack. Phosphorylation of histone F1 has been implicated in the initial stages of the process of chromosome condensation and control of cell division (62).

In Table 1, our studies on mammary glands in culture have shown specific acetylation, and phosphorylation of histones and chromosomal proteins in response to hormone induction. Experiments with cell cultures of the mammary gland involve the fractionation of nuclei, fractionation of chromatin, association of chromosomal proteins, and their resolution and analysis. Our initial studies demonstrated that the amino acid compositions of mammary gland histones were not altered as a result of hormone induction (35). This seems to be an important

![FIG. 1. Schematic representation of a possible model for chromatin \( \nu \) body subunit structure. The \( \nu \) body represents a protein core in a complex with the apolar segment of the four histones (F3, F2b, F2a2, and F2a1) with the histone F1 possibly located on the outside of the globular subunits. In this way histone F1 may have a crosslinking role either between subunits of different chains or subunits of the same chain. The DNA supercoil possibly contains 1.5 to 2 turns of a DNA with a pitch of 5.5 nm. The DNA subunit length of approximately 205 base pairs would be accommodated in the model if the mean diameter of the DNA coil is 10.6 nm. Chromatin is believed to contain a string of such globular subunits with unknown orientations. This presents an attractive general hypothesis in the control of the chromatin structure throughout the cell cycle in which the chemical modifications as described in the text has the capacity to modify the interactions of that histone with DNA throughout the genome. This is particularly important since the basic segments of the histones that are modified are complexed with DNA on the outside of the globular subunit and would thus be accessible to enzyme modifications (3, 44, 55).](image)
TABLE 1. Hormone induction of phosphorylation of chromosomal proteins in mammary gland cultures.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Histones</th>
<th>Acidic chromosomal proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No hormones</td>
<td>10,040</td>
<td>35,400</td>
</tr>
<tr>
<td>2</td>
<td>Prolactin</td>
<td>5,510</td>
<td>46,300</td>
</tr>
<tr>
<td>3</td>
<td>Insulin</td>
<td>11,390</td>
<td>50,500</td>
</tr>
<tr>
<td>4</td>
<td>Hydrocortisone</td>
<td>12,630</td>
<td>66,600</td>
</tr>
<tr>
<td>5</td>
<td>Hydrocortisone + Prolactin</td>
<td>5,930</td>
<td>72,000</td>
</tr>
<tr>
<td>6</td>
<td>Insulin + Prolactin</td>
<td>30,610</td>
<td>141,100</td>
</tr>
<tr>
<td>7</td>
<td>Insulin + Hydrocortisone</td>
<td>41,510</td>
<td>185,400</td>
</tr>
<tr>
<td>8</td>
<td>Insulin + Hydrocortisone</td>
<td>39,230</td>
<td>135,300</td>
</tr>
<tr>
<td>9</td>
<td>Insulin + Dexamethasone + Prolactin</td>
<td>39,910</td>
<td>287,000</td>
</tr>
<tr>
<td>10</td>
<td>Insulin + Dexamethasone + Prolactin + 5% Fetal calf serum</td>
<td>173,190</td>
<td>506,000</td>
</tr>
</tbody>
</table>

(Total $^{32}$p CPM/100 mg)

and general conclusion for many systems that have been studied which include regenerating rat liver (63), phytohemagglutinin induced lymphocytes (24), step down and step up cultures of physarum polycephalum (29), as well as our studies on the developing reticulocyte versus the mature duck erythrocyte (54, 55). One possible exception to this general conclusion is that histone F1 has been altered as the result of hormone induction of the mammary gland (18, 60, 61).

Our recent investigations have concentrated on postsynthetic modifications of chromosomal proteins which include both histones and acidic chromosomal proteins. Observations on the acetylation of histones based on turnover of labeled acetate show that there are two types of acetylation (35). Thus, in the insulin induced mammary gland explant there is an immediate response in the incorporation of acetate which represents internal acetylation on the ε amino nitrogen of lysine of residues 14 and 23 in histone F3 (37), residue 16 in histone F2a1, and residue 36 in histone F2a2. Although histone F2b is acetylated in some cell systems, in the mammary gland it is not acetylated (36). These modifications are limited to the first 36 residues from the amino terminus.

Fig. 2 shows the amino acid terminal residues that are modified with a potential to react directly in the wide groove of the DNA in its supercoiled configuration. The acetyl CoA mediated acetylation is not subject to inhibition by protein synthetic inhibitors as cycloheximide or puromycin. In addition to the acetylation of the internal residues in response to hormones in the mammary gland, the histone F2a2 and F2a1 specifically are acetylated on the amino terminal serine residues, Fig. 2, only at the time of their synthesis. In contrast to the acetylation of internal lysine residues with a short half life, acetylated serine residues of F2a2 and F2a1 are extremely stable.

Comparison of postsynthetic phosphorylation of histones and acidic chromosomal proteins suggest different metabolic activities. As in Fig. 3a, for example, phosphorylation of histones is evident during late G1 and S phase of the cell cycle whereas the phosphorylation of acidic chromosomal proteins demonstrates an immediate response to hormone induction which continues throughout the cycle with a biphasic activity in early G1 and later during the S phase of the DNA synthesis.

In the mammary gland system it appears that histone F2a2 is the predominate species
FIG. 2. Represents a schematic diagram of histones F3, F2b, F2a2, and F2a1. The unit length is proportional to the total number of the amino acids in each histone molecule. The squares looking upward represent positively charged residues of either histidine, lysine, or arginine. The downward pointing circles represent negative charges of either aspartic or glutamic acid. The stars represent the hydroxyl groups of serine residues that have the capacity to be phosphorylated. Phosphorylation is represented by the letter P and acetylation by the letter A, methylation by the letter M. The numbers represent those residues that are modified. We have early suggestive evidence that residues either 18 or 19 are phosphorylated in F2a2. It should be noted that there are six serine residues available in F2b, three serine residues in F3, and three in F2a2, and one in F2a1. In the mammary gland the only evidence of phosphorylation to any appreciable extent is in residues 18, 19, and possibly the amino terminal serine.

that is phosphorylated during the S phase of the cell cycle. To demonstrate this, phosphorylated histone F2a2 was purified by Biorex 70, followed by Sephadex G100, after cyanogen bromide cleavage. The cyanogen bromide was useful in F2a2 since it is only histone devoid of methionine and, therefore, not subject to cleavage.

POST SYNTHETIC MODIFICATIONS OF ACIDIC CHROMOSOMAL PROTEINS

One of the most important unsolved problems facing biochemists today is the determination of the biological function of the neutral and acidic proteins associated with chromatin. Until recently, however, our knowledge of the biochemistry of acidic nucleoproteins has been obscured by technical difficulties in fractionation of these proteins (46). The importance of these proteins in hormonal stimulation, however, is already evident by the fact that insulin and prolactin stimulate their phosphorylation which is closely associated with increased RNA synthesis (38, 52, 67). The phosphorylation of these proteins in mammary explants resembles other hormone stimulations to include: (1) chorionic gonadotropin increase in protein phosphorylation and RNA synthesis in ovarian nuclei of immature rats (21, 22), (2) testosterone stimulation of phosphorylation of acidic proteins in the rat ventral prostate (Ahmed, 1),
and (3) cortisol induction of RNA synthesis and pattern of phosphorylation of acidic proteins in the rat liver within 5 min after administration (58). Even though these observed changes may represent merely the synthesis of acidic proteins that ultimately are destined to be phosphorylated, it does not exclude phosphorylation as a primary mechanism directly involved in hormone induction. In fact, some of our initial studies provide additional evidence that phosphorylation of specific proteins as a result of hormone induction is characteristic of the mammary gland, Table 1. When examined by acrylamide gel electrophoresis, the phosphorylation of specific chromosomal proteins appears to represent the effect of a specific hormone (Fig. 3b).

The foremost question is the molecular mechanism at the level of RNA transcription. In the case of steroid hormones there is some evidence of specificity of steroid protein receptor recognition of acidic chromosomal proteins. Thus, in a review of this area (59) Spelsberg has shown evidence of a progesterone receptor complex which binds specific acceptor AP3 chromosomal proteins. This implies that a potential mechanism of steroid action involves an initial association of the hormone with a cytoplasmic receptor for transport to the nucleus where it is modified and exhibits a capacity to bind to specific acidic chromosomal proteins with a potential to alter the charge interactions with DNA in the control of RNA transcription.

**PROPOSED MOLECULAR MECHANISM OF HORMONE INDUCTION OF RNA TRANSCRIPTION**

Although our knowledge of the molecular mechanism in peptide hormones is even more fragmentary than that of the steroids, review articles are given license to postulate mechanisms. Therefore, we take this opportunity to propose three possible mechanisms, all of which involve phosphorylation as a post synthetic protein modification.

The molecular mechanism of action of polypeptide hormones, as insulin and prolactin are mediated most likely as the result of membrane receptor hormone interactions to induce synthesis of a secondary message of cyclic nucleotides. Unfortunately, our knowledge of cyclic nucleotides in the mammary gland system will require additional studies. However, nuclear protein kinases are involved in phosphorylation of chromosomal proteins associated with chromatin DNA nuclear proteins, and at least some of these are stimulated by cyclic AMP. A proposed mechanism is diagrammed in Fig. 4a. To account for the effect of critical mitosis induced by insulin in the presence of hydrocortisone, we suggest that prolactin receptors are cryptic in Go phase mammary gland cells. The effect of insulin in the presence of hydrocortisone then would function to alter the cryptic state of the prolactin receptor as the cell passes from Go into G1, S, G2, and mitosis. The fact that prolactin can exert its effect on the mammary gland cell some time after the addition of insulin suggests that the prolactin receptor is available only after the cell has left the Go phase. If one assumes in addition, that the prolactin activity is mediated via cyclic AMP to stimulate a nuclear protein kinase, a mechanism of induced phosphorylation can be postulated. Either the chromosomal proteins could then interact directly with positively charged histones or indirectly with phosphorylated acidic chromosomal proteins in altering their charge interaction with DNA, ultimately

![Fig. 4a. A diagrammatic representation of a proposed model to explain hormone induction in the mammary gland. Insulin, bound to its receptor β may induce a secondary message as for example (cGMP) to interact with a regulatory site of a Protein Kinase, RC1. In the presence of ATP the activate Protein Kinase could then phosphorylate specific chromosomal proteins as a mechanism of derepression of mRNA transcription directed to proteins required for initiation of cell division. Hydrocortisone ϕ, binds with a cytoplasmic (8S) receptor β, which is then transported to the nucleus (4S) to induce the synthesis of rRNA. At Go, prolactin receptors β are cryptic. After a critical mitosis however the prolactin receptor becomes accessible β. At this stage of differentiation the prolactin may induce a secondary message (cAMP) in the activation of a Protein Kinase to phosphorylate specific chromosomal proteinsin the derepression of mRNA transcription directed to the synthesis of milk proteins (α-lactalbumin, casein, etc.).](image-url)
FIG. 4b. Schematic representation of the mode of action of phosphorylation of the acidic chromosomal proteins in the presence of protein kinase induced by cyclic AMP. One possible mechanism as shown is that the acidic chromosomal proteins are phosphorylated and therefore become negatively charged. The negatively charged protein species could then interact with the histones and be repelled from the DNA genome. The result of this action would be an unfolding of the chromatin as shown in the diagram.

FIG. 4c. An alternate hypothesis or perhaps in synergy with phosphorylation of acidic chromosomal proteins is the phosphorylation of acidic chromosomal proteins associated with the DNA dependent RNA polymerase in the eukaryotic cell. Thus in the presence of protein kinase the acidic chromosomal protein may be phosphorylated to alter the charge and thereby induce a steric modification of the polymerase and augment its activity.

to activate RNA transcription (Fig. 4b). An important corollary of this postulate is that phosphorylated chromosomal proteins may in turn interact with one of the five DNA dependent RNA polymerases found in eukaryotic cells (33). This is diagrammed in Fig. 4c. Nuclear protein kinases have been described by Langan (28) and are associated with chromatin, in which at least some of these enzymes are stimulated by cyclic AMP. One essential component of the phosphorylation model is the fact that phosphorylation represents a reversible process in the presence of chromatin bound dephosphorylation enzymes.

The third model is based on the fact that polyamines have the capacity to interact with chromatin in modifying the DNA dependent RNA polymerase activity. As shown in Fig. 5, synthesis of spermidine requires putrescine as a substrate and S-adenosyl methylmercapto propylamine. The synthesis of putrescine requires the enzyme arginase in the formation of ornithine, ornithine decarboxylase, and S-adenosyl methionine decarboxylase in the synthesis of putrescine from ornithine and S-adenosyl methylmercaptopropylamine from S-adenosyl methionine.

Spermidine has been shown by Oka and Perry (41, 42) to replace the hydrocortisone requirement in mammary gland epithelial cells. These investigators also have shown a close correlation between milk protein synthesis and spermidine accumulation as a function of time and hormone requirement. The effect of spermidine appears to be specific for the glucocorticoid since this polyamine does not replace the requirement for insulin or prolactin. In addition, Aisbitt and Barry (2) demonstrated that in cultured mammary tissues, insulin stimulates the activity of ornithine decarboxylase and S-adenosylmethionine decarboxylase is stimulated by putrescine. Spermidine is a basic polypeptide that undergoes phosphorylation and reacts with chromatin in the stimulation of RNA transcription.

There are many important features of this model not least of which is that polyamines are associated with gene activation (17, 47).
CONCLUSION

Although the mammary gland response to the sequential ordered effect of specific hormones is exceedingly complex and will require a large number of intensive investigations, some possible molecular mechanisms already are being formulated. These proposed mechanisms undoubtedly will form the basis of an exciting area for future studies.

The authors wish to emphasize that because of limited space, large segments of excellent research in hormone responses in mammary glands may have been omitted. Those selected were for emphasis rather than comprehensiveness.

In addition full responsibility is ours for speculations with the hope of stimulating interest. For further readings we suggest the following:


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