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Estrogen Action: An Inroad to Cell Biology[1]

GERALD C. MUELLER, BARBARA VONDERHAAR,[2] UH HEE KIM, AND MARY LE MAHIEU

McArdle Laboratory, University of Wisconsin, Madison, Wisconsin

I. Introduction

A description of the mechanism of estrogen action must account for the rapid and diverse responses which this hormone can incite in a range of tissues. To list just a few examples, estrogens have been shown to effect: the suppression of gonadotropin production and release by an action on the hypothalamic–pituitary axis (DaLage, 1966; Flerkó, 1957; Schwartz, 1969), the growth and differentiation of mammary tissues when combined with appropriate adrenal and pituitary hormones (Lyons *et al.*, 1958; Hilf *et al.*, 1967), accelerated growth and cornification of vaginal epithelium (Bigger and Claringbold, 1954; Ladinsky *et al.*, 1968), the synthesis of phosphoproteins in the liver (Greengard *et al.*, 1965), the synthesis and accumulation of fat in adipose cells (Gassner *et al.*, 1958), and the growth and differentiation of uterine tissue (O'Malley *et al.*, 1969; Mueller *et al.*, 1958; Oka and Schimke, 1969). Looking for common denominators among the many studies dealing with a broad spectrum of responses two observations stand out: (1) Cells, which are responsive to estrogens, contain a receptor protein (or receptor aggregate) that interacts specifically with certain structural features of the estrogen molecule to form a high affinity, noncovalent complex (Table I). (2) Many of the hormone responses (but not all) involve and are highly dependent on a hormonal acceleration of the genetic expression mechanisms in the sensitive cells (Table II). In the analysis of estrogen action which follows, an attempt is made to relate these two observations in molecular and biophysical terms. A concept of estrogen action is proposed in which: (a) the receptor protein is characterized by a remarkable propensity to enter into distinctive associations or aggregations with certain classes of macromolecules of the cell; (b) the character, composition, or stability of protein components in these complexes is modified through some function of the receptor protein which is activated by the binding of estrogen; (c) the character of the metabolic response which ensues is determined by the extent to which a

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[1] The Gregory Pincus Memorial Lecture.

[2] *Present address:* National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

limited cellular process is affected by the availability, state, or activity of the specific altered component of such receptor complexes, either catalytically or genetically. In this concept the hormone is visualized as a modulator in the class expression of multiple genes through controlling the availability or state of specific proteins which are essential in the nuclear scene for the expression of these genes. It is also suggested that the same mechanism may as well regulate certain extragenomic processes.

TABLE I
Evidence for Estrogen Receptor Mechanism

Target tissue	References
Rat uterus	See Table III
Mammary gland	Sander (1968), Sander and Attramadal (1968), Puca and Bresciani (1969a)
Hormone-dependent mammary tumors	Jensen *et al.* (1967c), Mobbs (1968), Puca and Bresciani (1968b), Terenius (1968a), Stumpf (1969), Korenman and Dukes (1970), Lemon (1970)
Hypothalamus	Eisenfeld and Axelrod (1965), Kato and Villee (1967), Pfaff (1968), Stumpf (1968b), Anderson and Greenwald (1969), Kahwanago *et al.* (1969), Whalen and Maurer (1969), Woolley *et al.* (1969), Chader and Villee (1970), Eisenfeld (1970)
Pituitary	Eisenfeld and Axelrod (1966), Eisenfeld (1967, 1970), Kato and Villee (1967), Stumpf (1968c), Anderson and Greenwald (1969), Kahwanago *et al.* (1969), Kato *et al.* (1969, 1970), Leavitt *et al.* (1969)
Mouse uterus and vagina	Martin and Stone (1965), Stone and Baggett (1965a,b), Terenius (1966, 1968b), King *et al.* (1968), Folman and Pope (1969a,b), Harris (1971)
Chick oviduct	Jonsson and Terenius (1965), Terenius (1969)
Chicken liver	Arias and Warren (1971)

II. Some General Aspects of Nuclear—Cytoplasmic Interactions in Living Cells

Before taking up experiments dealing with estrogen action let us first consider some general properties of nuclei and nucleochromatin—particularly the manner in which the chromatin interacts with its environment in the control of gene expression. This subject is important in our analysis of estrogen action since the hormone appears to act chiefly by modifying the function of chromatin.

As revealed in the electron microscopic studies of Dr. Hans Ris, nucleochromatin is a highly complex molecular aggregate of DNA and proteins (Ris and Chandler, 1963; Ris, 1967). Using Kleinschmidt's tech-

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nique to disrupt and gently spread nuclei on a water–air interface (Kleinschmidt *et al.*, 1961) he demonstrated that native chromatin consists of a complicated web of 200–250 Å nucleoprotein fibers (Fig. 1A–D). Treatment of the chromatin with chelating agents separates certain of these proteins and unravels the structure of the chromatin webs yielding 100 Å fibers with a "beaded" appearance. Progressive digestion with pronase removes the protein "beads" and reveals a core of 25 Å double-stranded DNA fibers in each branch of the web. It is apparent that

TABLE II
The Acceleration of Genetic Expression by Estrogens

Observation	References
Early general stimulation of uterine RNA synthesis	Mueller *et al.* (1958), Jervell *et al.* (1958), Gorski and Nicolette (1963), Wilson (1963), Hamilton *et al.* (1965), Means and Hamilton (1966a), Unhjem *et al.* (1968), Barry and Gorski (1971), Kapadia *et al.* (1971)
Estrogen induction of RNA polymerase	Gorski (1964), Nicolette and Mueller (1966b), Nicolette *et al.* (1968), Barker and Anderson (1968), Raynaud-Jammet and Baulieu (1969), Arnaud *et al.* (1971)
Inhibition of estrogen action by the inhibition of RNA synthesis	Ui and Mueller (1963), Szego and Lawson (1964), Nicolette and Mueller (1966a), DeAngelo and Gorski (1970)
Early general stimulation of uterine protein synthesis	Mueller (1953), Mueller *et al.* (1958), McCorquodale and Mueller (1958), Noteboom and Gorski (1963), Greenman and Kenney (1964), Szego and Lawson (1964)
Induced synthesis of uterine specific proteins by estrogens	Notides and Gorski (1966), Cecil and Bitman (1967), DeAngelo and Gorski (1970), Teng and Hamilton (1970), Cohen *et al.* (1970), Barker (1971)
Inhibition of estrogen action by inhibitors of protein synthesis	Mueller *et al.* (1961), Gorski and Axman (1964), Gorski and Morgan (1967), Cecil and Bitman (1967)

the webs of the native chromatin arise by the sticking together of protein-coated DNA structures and that in many cases a double-stranded chain of DNA may course its way into an arc of the web only to double back in the same arc, yielding what appears to be blind loops and blunt ends of nucleochromatin.

From these studies it is obvious that proteins play an important role in the structure of chromatin. In the analogy of the repressors of bacterial systems it appears quite likely that some proteins are highly specific in recognizing nucleotide sequences in DNA. An example of a transitory recognition of this type in bacterial systems is the initiation of RNA synthesis by RNA polymerase and the modifications of the tem-

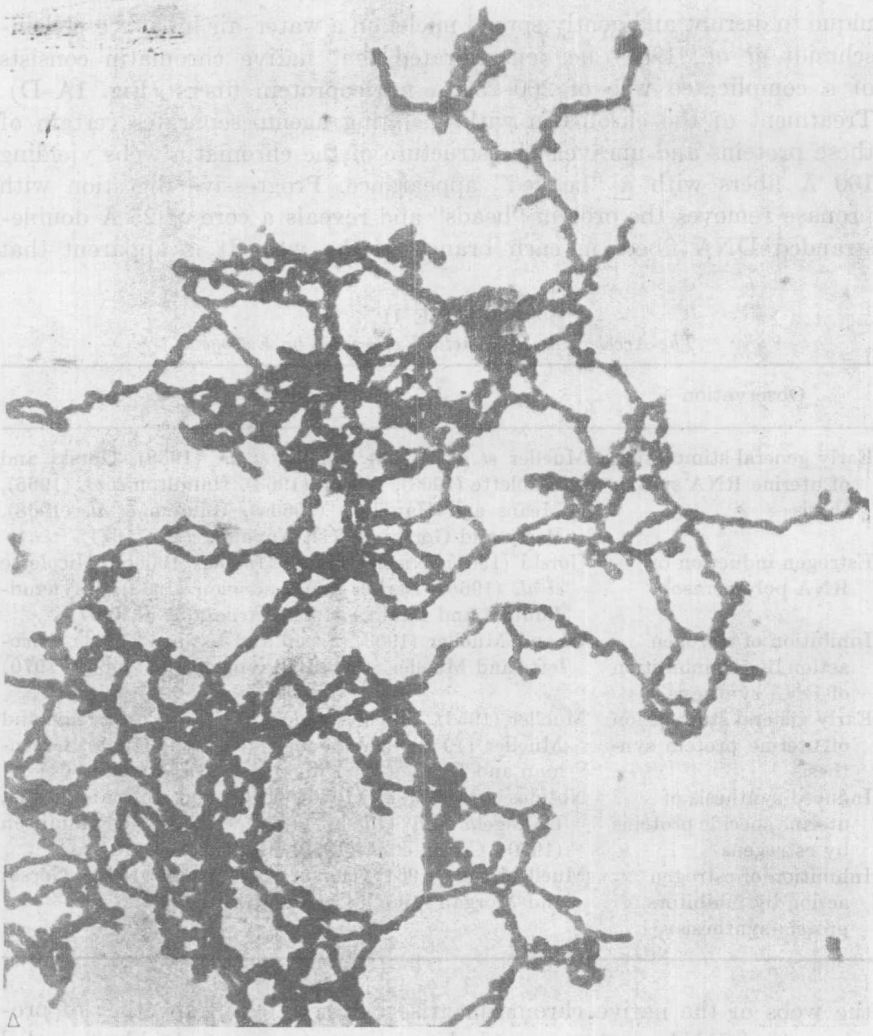

FIG. 1. The macromolecular character of nucleochromatin. Erythrocyte nuclei from the salamander *Triturus viridescens* have been spread with the indicated treatments at an air–water interface and examined electron microscopically.

(A) Nuclei have been spread directly at air–water interface, fixed in ethanol, dried from amyl acetate. Note that the webs are made up of 250 Å fibers. ×40,500.

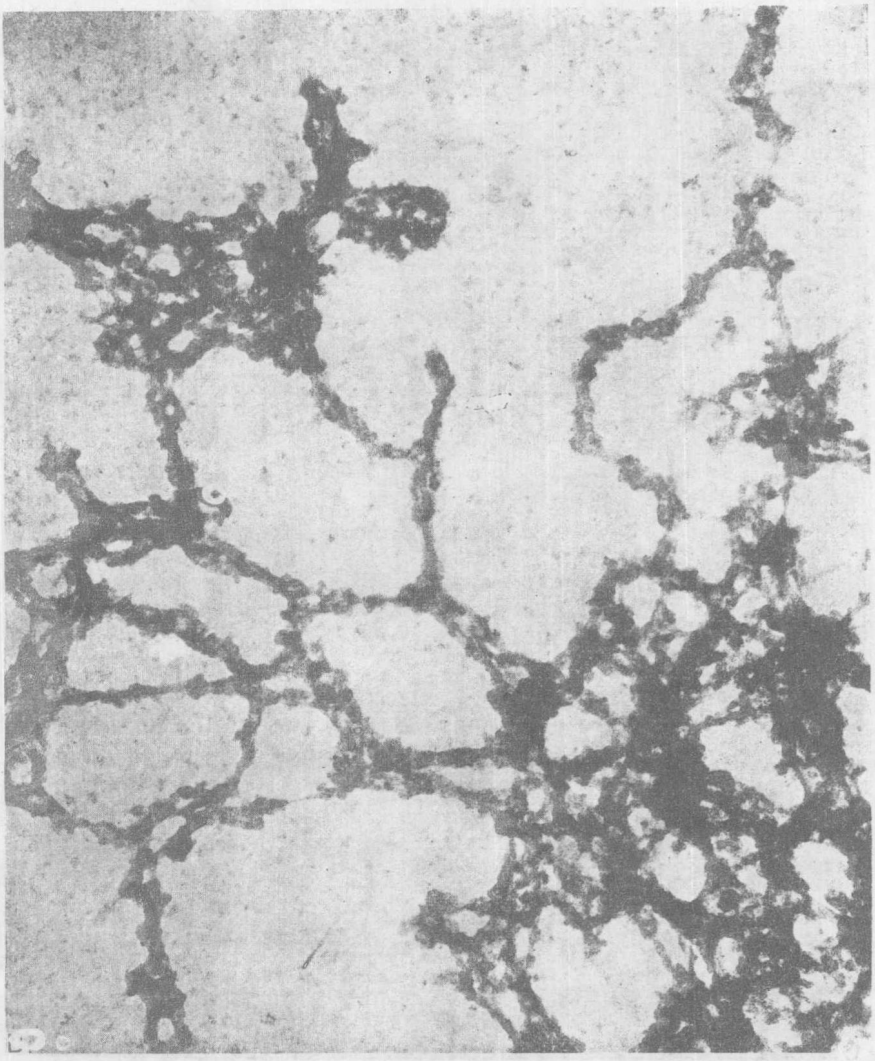

FIG. 1B. Nuclei have been treated for 10 seconds with 5 m*M* sodium citrate, fixed with 10% formalin, stained with 1% uranyl acetate, and dried from amyl acetate. Note that the 250 Å fibers consist of two 100 Å fibers which separate when treated briefly with chelating agents. ×96,000. See caption Fig. 1A.

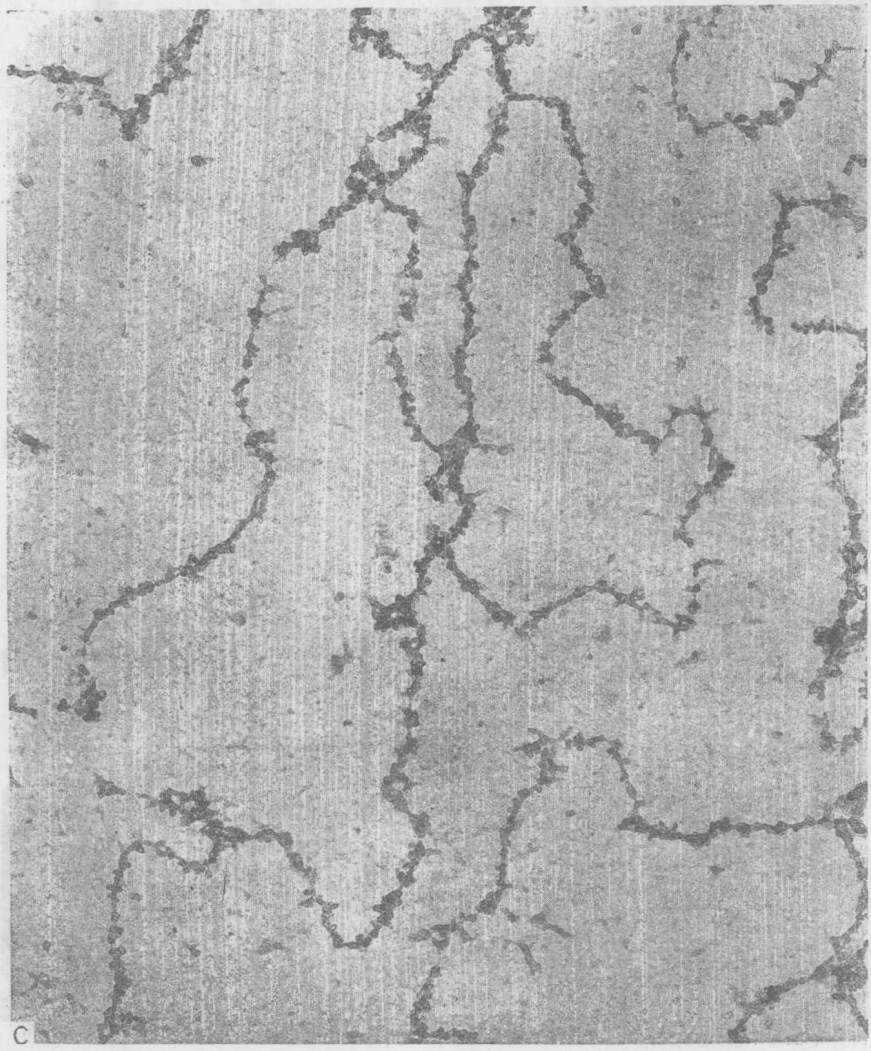

FIG. 1C. Nuclei have been spread on 5 m*M* sodium citrate, stained with 1% uranyl acetate, fixed in ethanol, and dried from amyl acetate. Note the knobby projections and "moth-eaten" character of the 100 Å fibers after more extensive treatment with the chelating agents. ×96,000. See caption Fig. 1A.

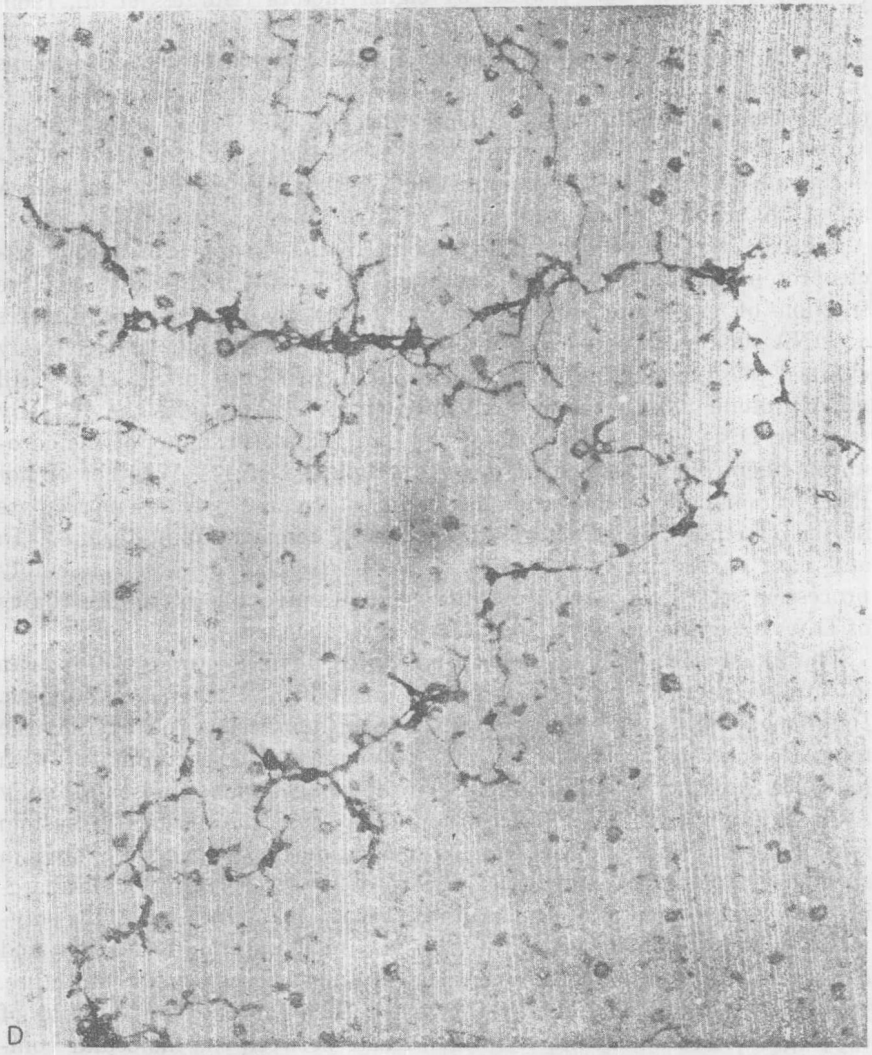

FIG. 1D. Nucleochromatin has been digested with pronase before fixation and staining as in (C). Note that digestion of the protein releases 20 Å double-stranded DNA. In many instances, the DNA folds back on itself to yield projections which appear to be residuals of the original web structure of the nucleochromatin. ×96,000. See caption Fig. 1A.

The author is very grateful to Professor Hans Ris, Department of Zoology, University of Wisconsin, for these electron microscopical studies and the permission to include the prints in this chapter. Further details of this study are present in his publication (Ris, 1967).

plate specificity of this enzyme by sigma factors (Burgess *et al.*, 1969; Travers and Burgess, 1969; Summers and Siegel, 1969; Crouch *et al.*, 1969). In eucaryotic nuclei a nonrandom distribution of certain proteins in chromatin is revealed in the distinctive nuclear distributions of antinuclear immunoglobulins from patients with systemic lupus erythematosis (Beck, 1961, 1963; Casals *et al.*, 1963). Similarly the reassociation experiments of Paul and Gilmour (1968) with acidic proteins and DNA attest to ordered relationships for some of these proteins with DNA.

Whereas the above observations point to the existence of unique nucleoprotein complexes within chromatin, it is quite likely that a large fraction of the proteins of chromatin recognize only general features of DNA such as the helical structure of the exposed chains of negatively charged phosphate groups. Histones appear to belong to this class. Still other proteins must recognize and interact with the proteins that already coat the DNA core to account for the bridging and webbing of the chromatin fibers seen in the electron-microscopical pictures. Whether or not RNA, ribonucleoproteins, complex lipids, and carbohydrates participate in the assembly or structure of chromatin remains to be studied. In any case chromatin is best viewed as the product of a self-assembly process in which the various protein subunits assemble along the chains of DNA according to their individual complementarities.

The importance of the complex and varied ultrastructure of chromatin in hormone action studies arises from its influence on gene expression. In order for given segments of DNA to be transcribed into RNA, the template must be accessible to RNA polymerase. Nucleochromatin, with its DNA existing as chains of DNA-protein aggregates, presents highly restricted templates for transcription. In a sense the chromatin system is a *molecular capacitor* in which information of the gene is stored in specific protein–DNA aggregates. The problem of releasing or discharging this information (i.e., the transcription of DNA into RNA by polymerase) is one of molecular mechanics involving the formation and dissolution of macromolecular aggregates amid the chromatin structure. It can be anticipated that the stability of each complex is a function of the degree of complementarity which exists between the molecular components of a particular aggregate. To change a molecular aggregate in preparation for transcription of the contained DNA it may be necessary to alter the existing complementarities in a specific manner; this requires the intervention of some outside force such as an approaching protein molecule. It is proposed that when an incoming molecule interacts with one of the components of the existing aggregate so as to induce a conformational change in this component, the bonding of the component to other units of the aggregate will be disturbed. A new comple-

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mentarity will arise which in certain cases will expose the underlying template DNA or facilitate the transcription process.

In this field of molecular mechanics the incoming or perturbing molecules may be large or small, stable or unstable, and belong to any compositional class; the only important requirements are that they exhibit some complementarity to a component of the existing aggregate and induce some change in it through the interaction. The changes may be implemented simply through the mass of the incoming molecule in the case of large molecules or may result from localized enzyme catalysis. Selectivity of the response can be expected to be greater and more efficient as the degree of complementarity increases between the entering molecule and the interacting component of the chromosomal site.

In this picture of nucleochromatin it is obvious that structure and function are inseparable. It is also obvious that the control of gene expression is a function of the availability of the specific inducing or perturbing molecules in the extranuclear environment. To regulate this availability Nature has invented a second set of *molecular capacitors* which oppose the operation of the *nuclear molecular capacitor;* these are the membrane systems of the cell. The latter, like the nucleochromatin, are molecular aggregates, and also guided in their self-assembly by the complementarity of the constituent macromolecules. The stability and perturbability of the associations are subject to the same physical bonding principles which operate in the nucleochromatin or, for that matter, in any macromolecular aggregation. Existing in direct contact with both the cytoplasm and extracellular compartment, however, the membranes provide a bridge for moment to moment sensing and buffering of molecular changes in the environment. Through processes of sequestration, metabolism, and transport, the membranes act as a second molecular capacitor to control the availability of certain molecules that can interact with chromatin to regulate gene expression. Distinct equilibrium states are expected to arise between these two opposing systems; situations in which the molecules needed for the induction of specific gene expressions in the chromatin system are retained in specific molecular associations amid the membrane structures and are only released or transported in response to the entry of some molecule from outside the affected cell, such as a hormone.

Thus the living cell is portrayed as an interaction between two molecular capacitor systems: the nucleochromatin and the membrane of the cell. Since both capacitors record moment-to-moment changes in the cell, the interaction is of a cascading nature as depicted in Fig. 2. Accordingly, both systems are modulated by factors that may affect intermediately the translational steps in protein synthesis, nucleic acid matu-

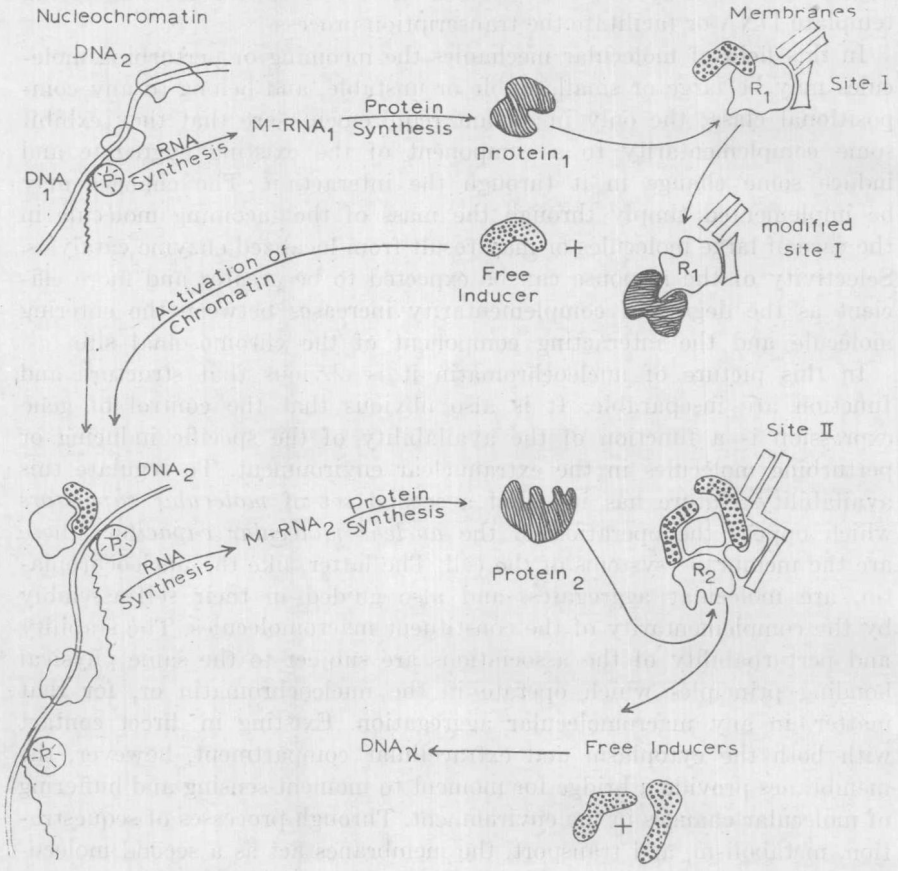

FIG. 2. A diagrammatic view of the interaction between nucleochromatin and the membrane systems of a cell during the sequential ordering of gene expressions. Nucleochromatin and membranes are visualized as two types of molecular capacitor systems which interact through the exchange of macromolecular components. In the diagram the synthesis of messenger RNA ($M\text{-}RNA_1$) gives rise to the synthesis of $Protein_1$ which in turn recognizes and associates with some specific site or component in the membrane (Site I). It is proposed that the perturbation which results leads to a localized activation of membrane metabolism and the release of a restrained inducer. By transport or diffusion the free inducer enters the nucleochromatin to react with a specific component of this structure to make the DNA available for transcription (messenger RNA, $M\text{-}RNA_2$). Carried through successive stages, a type of cascading control of gene expression ensures. The particular ordering rests in the character and stability of the macromolecular associations which can form in a given cell. Both the cell phenotype and the extracellular environment, i.e., factors from adjacent cells, influence the progression of gene expressions.

ration or metabolism, and any other processes that modify the character of existing macromolecules.

III. The Activation of Genetic Expression Mechanisms by Estrogens

In the framework of these concepts of cell biology, let us now examine and analyze the effects of estrogens in a highly responsive system, the rat uterus. Administration of a single physiological dose of a natural estrogen rapidly converts the atrophic uterus of the immature or ovariectomized female rat into an actively growing organ. Within minutes histamine levels decline (Szego, 1965) and cyclic AMP levels increase (Szego and Davis, 1967). As early as 1 hour after the administration of the hormone there is a generalized hyperemia of the tissue. (McLeod and Reynolds, 1938), followed by a general imbibition of fluid throughout the different cellular layers of this tissue. The water uptake reaches a maximum 4–6 hours after hormonal treatment (Astwood, 1938; Szego and Roberts, 1953). After 12 hours the increased dry weight of the organ becomes measurable and a second surge of water imbibition occurs which correlates with the striking acceleration of polymeric growth of the tissue (Mueller *et al.*, 1958; Telfer, 1953).

Compositional changes which attend this mobilization of growth are shown in Fig. 3. Where as the composition changes infer certain sequential effects of the hormone on the different synthetic pathways, incoporation studies with radioactive precursors demonstrate that each of these pathways (i.e., lipid, protein, and RNA synthesis) was accelerated with little or no lag period after the administration of estradiol (Mueller *et al.*, 1958). In confirmation of these findings, Hamilton (1968) and Means and Hamilton (1966b) have reported an increased labeling of nuclear RNA as early as 2 minutes. Similarly Barnea and Gorski (1970) have documented the estrogen-induced synthesis of a single electrophoretically resolvable protein as early as 40 minutes after an injection of 17β-estradiol. In this case evidence for the synthesis of some RNA, the presumptive messenger for this protein, was obtained as early as 15 minutes (DeAngelo and Gorski, 1970).

Early experiments using puromycin to block protein synthesis (Mueller *et al.*, 1961) and using actinomycin D to block RNA synthesis (Ui and Mueller, 1963) demonstrated very clearly that the early estrogen response was highly dependent on the synthesis of both new RNA and protein. These results have been confirmed and extended by many laboratories. For example, Gorski and Axman (1964) obtained similar results using cycloheximide, which inhibits protein synthesis by a completely different mechanism This group also showed that early estrogen effects on carbohydrate metabolism was prevented by cycloheximide (Nicolette

and Gorski, 1964; Gorski and Morgan, 1967). They demonstrated further that the delayed administration of inhibitors of protein synthesis caused a rapid loss of the accelerated transport and phosphorylation of deoxyglucose which was achieved by prior estrogen treatment. This result, showing that the perpetuation of the hormonal response requires the continuous synthesis of some essential protein, suggests that the synthesis, metabolism, or transport of certain proteins are important targets

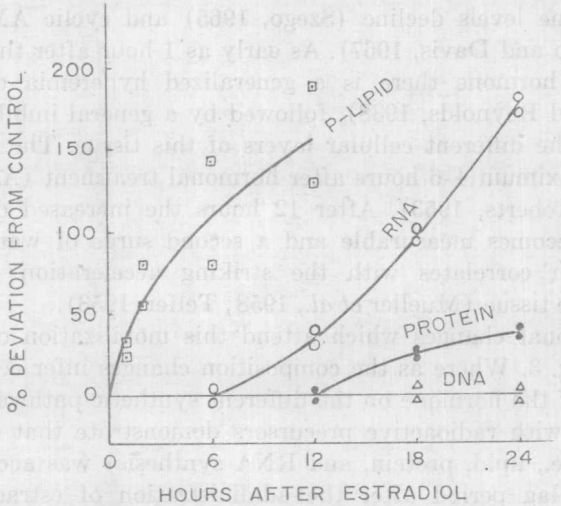

FIG. 3. Alterations in the composition of rat uteri following a single dose of estradiol (10 μg) injected at zero time. DNA was measured in micromoles of thymine per uterus. RNA was measured as micromoles of uridine and calculated as the ratio of uridine to thymine. Phospholipid (P-LIPID) was measured as micromoles of ethanolamine phosphate. All data are expressed as the percent deviation from the control during the first 24 hours after hormone treatment. Reprinted from Aizawa and Mueller (1961).

of the hormone action. Therefore, even though the hormones do exert extragenomic effects (Ui and Mueller, 1963; Lippe and Szego, 1965; Nicolette and Mueller, 1966a) a primary problem in elucidating estrogen action is to explain the role of protein synthesis in the control of genetic expression and the manner in which the hormone modulates this role.

IV. A Role for Protein Synthesis in the Estrogen Induction of and Maintenance of RNA Polymerase Activity

After the administration of estradiol, the DNA-dependent RNA polymerase activity of rat uterine nuclei rises rapidly (Gorski, 1964)

(Fig. 4). As in the case of the other *in vivo* estrogenic responses both the induction of this enzyme activity, as well as the maintenance of the induced activity, was abolished by levels of puromycin or cycloheximide which block protein synthesis in the intact rat (Gorski and Morgan, 1967; Gorski *et al.*, 1965). Thus protein synthesis appears to be required for both the induction and maintenance of this enzyme activity.

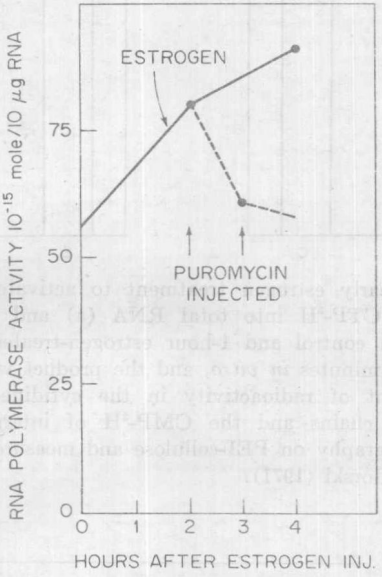

FIG. 4. The reversal by puromycin of the increase in RNA polymerase activity caused by estrogen. Estradiol injected at zero time into 21-day-old female rats. Puromycin (5 mg) was injected at 2 and in some groups again at 3 hours after estrogen. Rats were killed at times indicated in the figure and RNA polymerase assayed in uteri. Uteri from five rats were pooled for assays at each time point. Data are expressed as 10^{-15} mole cytidine-triphosphate-^{3}H incorporated per 10 μg of RNA released by ribonuclease treatment. Reprinted from Gorski (1964).

Analysis of the RNA products synthesized by isolated uterine nuclei has in turn revealed that the number of RNA chains in the process of synthesis per unit of DNA during the first hour of estrogen treatment is the same for both control and estrogen-treated rats; however, the rate of chain elongation is significantly enhanced (Barry and Gorski, 1971) (Fig. 5). These data, when added to the similarity of the product by nearest-neighbor frequency determinations (Mueller and LeMahieu, 1971) an hybridization studies (O'Malley *et al.*, 1969), suggests that the major initial effect of the hormone is to accelerate the rate of tran-

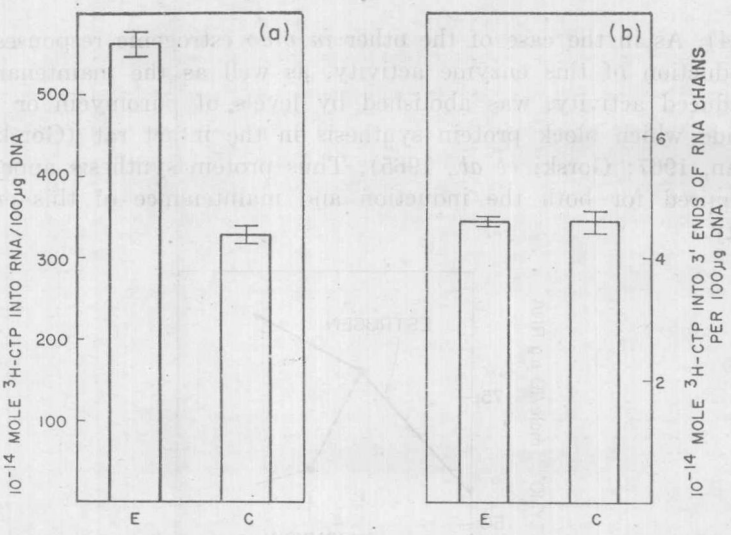

FIG. 5. Failure of early estrogen treatment to activate new polymerase sites. The incorporation of CTP-^{3}H into total RNA (a) and into 3′ chain ends (b) by uterine nuclei from control and 1-hour estrogen-treated uteri. RNA synthesis was carried out for 10 minutes *in vitro*, and the product was subjected to alkaline hydrolysis. The amount of radioactivity in the cytidine-^{3}H liberated from the 3′ ends of the RNA chains and the CMP-^{3}H of internucleotide linkages was separated by chromatography on PEI-cellulose and measured. Data are taken from a paper by Barry and Gorski (1971).

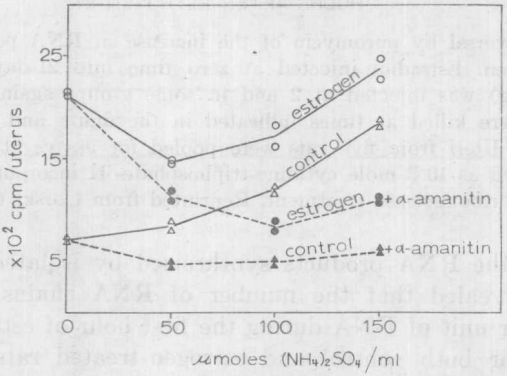

FIG. 6. The insensitivity of the estrogen-induced RNA polymerase activity to α-amanitin. Uterine nuclei from control (△) and estrogen-pretreated (○) (4 hour) rats were tested for their ability to synthesize RNA *in vitro* in the presence or absence of 5 μg/ml of α-amanitin at the specified concentration of $(NH_4)_2SO_4$ (Mueller and LeMahieu, (1971).

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