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Biochemistry

Effect of estradiol-17β on the synthesis of specific uterine nonhistone chromosomal proteins

(estrogen action/chromatin/nuclear acidic proteins/actinomycin D/estrogen receptor)

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ABSTRACT **The synthesis of specific nonhistone chromosomal proteins in the uterus of the ovariectomized rat was examined as a function of time after treatment with estradiol-17β. Sequential stimulations in the rates of synthesis of at least five nonhistone chromosomal proteins having molecular weights of 96,000, 70,500, 29,400, 20,700, and 16,400, respectively, were observed. The rate of synthesis of the nonhistone chromosomal protein having a molecular weight of 70,500 was increased at 1 hr after hormone treatment. This was the first nonhistone chromosomal protein to be induced by estrogen, and its induction was blocked by pretreatment with actinomycin D. The data reported suggest but do not prove that this protein is the 4.5 S estrogen receptor, and that it is the induced nuclear acidic protein described earlier by Teng and Hamilton. The rates of synthesis of the nonhistone proteins with molecular weights of 96,000, 29,400, 20,700, and 16,400 were increased at 3, 5, 24, and 24 hr, respectively, after hormone treatment.**

Stimulation of RNA synthesis is an early and dramatic feature in the response of the uterus of the ovariectomized rat to estradiol-17β (1, 2). This enhancement of genetic transcription has been shown by studies of RNA polymerase activity in isolated uterine nuclei (3) and the template capacity of isolated chromatin (4). Recently, evidence obtained in several laboratories has indicated that nonhistone chromosomal proteins may play an important role in the regulation of transcription (5). Hormonal stimulation of RNA polymerase activity in the uterus is blocked by pretreatment with cycloheximide (3, 6), indicating the need for protein synthesis prior to, or concomitant with, stimulated RNA synthesis. It has been shown that there is an increase in the content of nonhistone protein in uterine chromatin soon after administration of estrogen (7). Teng and Hamilton (8) observed by electrophoresis an increase in the synthesis of a single nuclear acidic protein at 12 hr after hormone treatment. We now report the results of a more detailed electrophoretic analysis of the effects of estrogen on the synthesis of specific nonhistone chromosomal proteins in the uterus of the overiectomized rat.

MATERIALS AND METHODS

Animals and Their Treatment. Female Sprague-Dawley rats, ovariectomized at least 3 weeks, were used. Estradiol-17β (20 μg), dissolved in 0.2 ml of propylene glycol, was injected intraperitoneally, and control animals received the carrier only.

In certain experiments with actinomycin D, the inhibitor was dissolved in 0.9% NaCl adjusted to 2% ethanol. Animals received intraperitoneally 800 μg of actinomycin D at 1 hr before treatment with estrogen. The dose of actinomycin D used inhibited RNA synthesis by 70% and protein synthesis by 30%, as indicated by incorporation of labeled precursors.

Incubation Conditions and Isotopic Labeling of Uterine Components. At the indicated times after treatment, animals (6 per group) were killed by cervical dislocation. The uteri were removed, stripped of fat and mesentery, slit lengthwise, and then rinsed thoroughly in ice-cold Krebs improved Ringer-phosphate buffer II (9). The tissue was then transferred to Erlenmeyer flasks (25 ml) containing 1 ml of the Krebs-Ringer buffer per uterus and either 15 μCi/ml of [^{14}C]algal hydrolysate or 130 μCi/ml of [^{3}H]algal hydrolysate (Schwarz Bioresearch). In most experiments the estrogen-treated uteri were labeled with [^{14}C]aminoacids, and the control uteri were labeled with [^{3}H]aminoacids. In several experiments the isotopes were reversed, and the estrogen-treated uteri were labeled with [^{3}H]aminoacids. Control and experimental uteri were incubated at 37° with gentle shaking for 1 hr. At the end of the incubation period, the two groups of uteri were pooled, rinsed thoroughly with ice-cold 0.9% NaCl, and rapidly frozen on dry ice. Under these conditions the incorporation of labeled amino acids into uterine protein was linear for incubation periods of at least 3 hr.

Cell Fractionation. Uterine chromatin was prepared by a modification of the method of Bonner *et al.* (10). All procedures were carried out at 0–4° unless otherwise indicated. Frozen uteri were placed in a stainless steel tissue pulverizer chilled to −80° and crushed to a powder. The frozen powder was homogenized in 20 ml of saline-EDTA (0.075 M NaCl, 0.025 M EDTA, pH 8) with a polytron PT 20 tissue disintegrator run at 60 V for 30 sec, and the homogenate was filtered through four layers of nylon bolting cloth. A pellet was collected by centrifugation at 1500 × *g* for 15 min in the Sorvall RC-2 centrifuge. The pellet was washed once with the saline-EDTA containing 0.5% Triton X-100, and then once with the saline-EDTA alone. It was then washed three times with 0.01 M Tris·HCl (pH 8) by centrifugation at 4300 × *g*, 10,000 × *g*, and 17,000 × *g*. The gelatinous pellet was resuspended in 15 ml of 0.01 M Tris·HCl (pH 8) and stirred for 30 min. Aliquots of the chromatin suspension were layered over 25 ml of 1.7 M sucrose, 0.01 M Tris·HCl (pH 8) in Beckman SW 25 centrifuge tubes. The upper two-thirds of each tube was thoroughly mixed, and the resulting discontinuous gradients were centrifuged for 3 hr at 22,500 rpm in a Beckman L3 ultracentrifuge. The translucent pellets were resuspended in 20 ml of 0.01 M Tris·HCl (pH 8) and washed twice with the same buffer, being collected the first time by centrifugation at 12,000 × *g* for 15 min and the second time at 27,000 × *g* for 15 min. The pellet was sheared in 8 ml of 0.001 M Tris·HCl (pH 8) by 400 strokes of the Dounce homogenizer (B pestle). The chromatin suspen-

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

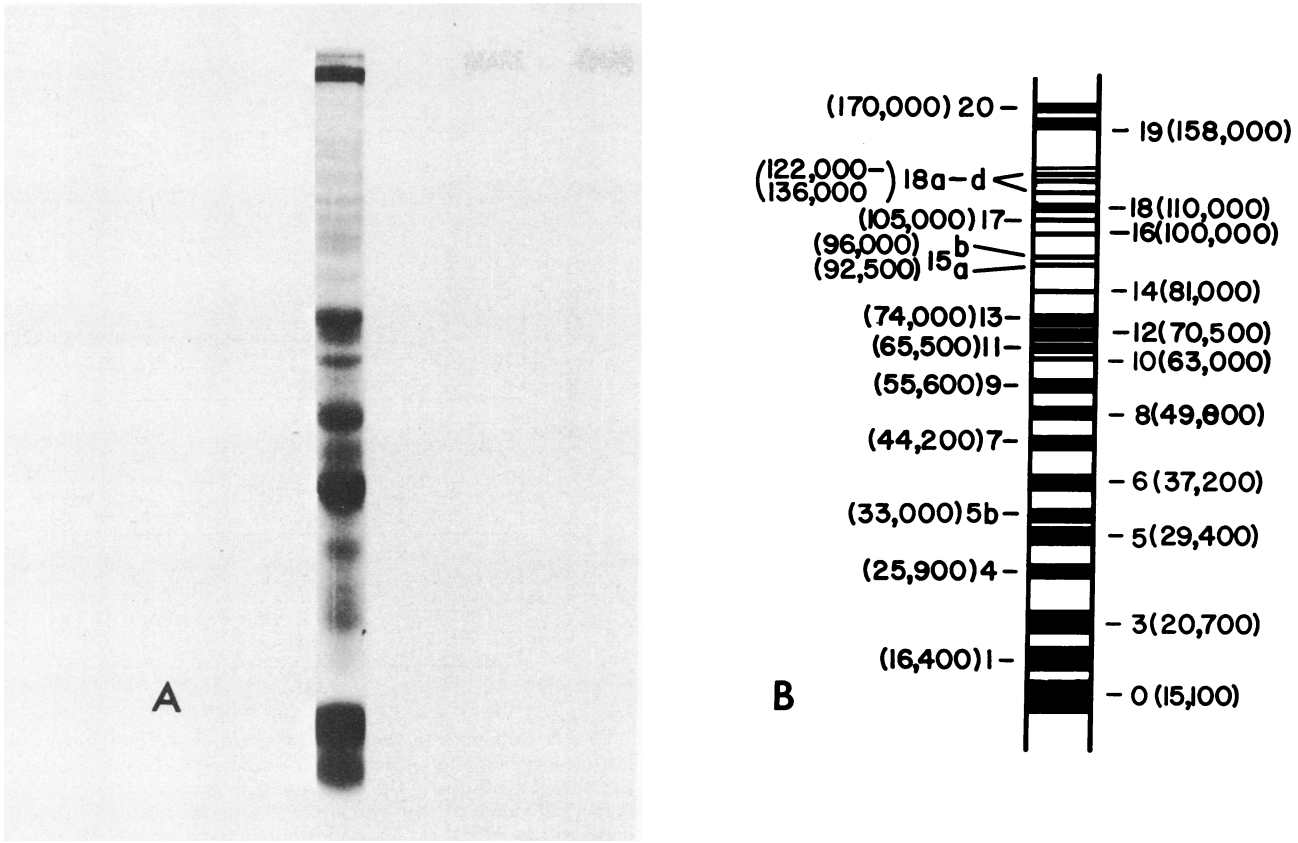

FIG. 1. Reproductions of the band pattern of uterine nonhistone chromosomal proteins separated in 5% acrylamide gels containing 0.1% $NaDodSO_4$ by (A) the gel photograph and (B) a drawing of the gel to exact scale. The numbering system used to identify the bands is an arbitrary one. The numbers in parentheses are the molecular weights of the individual bands.

sion was centrifuged at 800 × *g* for 2.5 min, the pellet was discarded, and the supernatant fraction was used immediately for the extraction of chromosomal proteins.

Isolation of Chromosomal Protein Fractions. The method of Elgin and Bonner was used primarily (11). Chromatin was extracted twice with 0.2 M H_2SO_4 for removal of histones. The acid-washed chromatin was dissolved at room temperature in 0.05 M Tris·HCl (pH 8), containing 1% sodium dodecyl sulfate ($NaDodSO_4$), and stirred at 37° for at least 4 hr. It was then dialyzed against 500 ml of 0.001 M Tris·HCl (pH 8), containing 0.1% $NaDodSO_4$, for 12 hr at 37° with three changes of buffer. The nucleic acids were then removed by centrifugation at 36,000 rpm at 25° for 18 hr in the Beckman SW 65 Ti rotor. The top three-fourths to four-fifths of the supernatant was taken as the nonhistone chromosomal protein fraction. This supernatant was dialyzed for 4 hr against 500 ml of 0.01 M phosphate buffer (pH 7.1) adjusted to 0.1% $NaDodSO_4$, 0.1% 2-mercaptoethanol, and 10% glycerol.

Gel Electrophoresis. Gels (12.5 × 0.6 cm) containing 5% acrylamide and 0.1% $NaDodSO_4$ were prepared and run by the method of Shapiro *et al.* (12). After electrophoresis, gels were stained with Coomassie brilliant blue R-250. For determination of radioactivity the individual bands were sliced from the gels, placed in scintillation vials, and dried at 80°. Then 1 ml of NCS (Nuclear Chicago Solubilizer) and 0.25 ml of H_2O were added to each vial. The capped vials were incubated at 50° for 8 hr. Scintillation solution (10 ml) was then added and the radioactivity determined. In some cases the entire gel was sliced into sections of 2 mm, and the radioactivity determined as described. The recovery of radioactivity from the gel slices was greater than 90%. There was no difference in the recovery of ^3H and ^{14}C.

Molecular weights of the individual nonhistone chromosomal proteins were determined by the method of Weber and Osborn (13). The molecular weights of the protein standards used for calibration were as follows: thyroglobulin, 160,000; bovine serum albumin, 68,000; pyruvate kinase, 57,000; ovalbumin, 43,000; pepsin, 35,000; chymotrypsinogen, 25,500; hemoglobin, 15,500; and cytochrome *c*, 12,700. The molecular weights cited are the subunit values since $NaDodSO_4$ dissociates proteins into their subunits.

RESULTS

Characterization of uterine nonhistone chromosomal proteins

Uterine chromatin prepared according to our procedures had the following ratios: histone to DNA, 1.01; nonhistone chromosomal protein to DNA, 0.77. Fig. 1 shows a wide variation in the molecular weights of the uterine non-histone chromosomal proteins, with weights ranging from 15,100 to greater than 170,000. Bands containing very little protein or radioactivity were not analyzed in the present work. The pattern of bands on the gels was very similar to that reported by Elgin and Bonner (11) for the nonhistone chromosomal proteins of other tissues.

Fig. 2 indicates that estrogen had little effect on synthesis of total cytosol and nonhistone chromosomal proteins during the first hour after treatment. The rate of synthesis increased rapidly after 1 hr, and declined after 12 hr. Histone synthesis was depressed during the first hour after hormone treat-

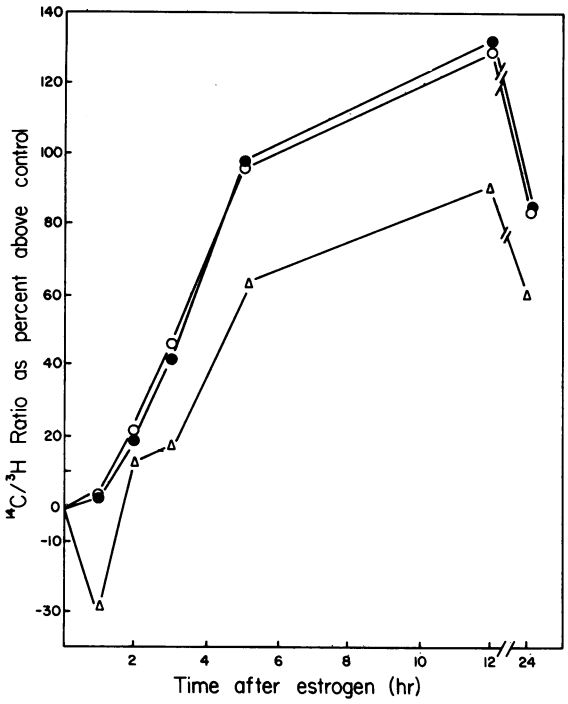

FIG. 2. Changes in the $^{14}C/^{3}H$ ratio of various uterine protein fractions at different time periods after estrogen treatment. Estrogen-treated uteri were labeled with [^{14}C]aminoacid hydrolysate and control uteri were labeled with [^{3}H]aminoacid hydrolysate. (●) Cytosol protein; (○) nonhistone protein; (△) histone.

ment, and then increased, but not to the level of synthesis observed for the cytosol or nonhistone chromosomal proteins.

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Fig. 3 shows a typical electrophoretic separation and the $^{14}C/^{3}H$ ratios of nonhistone chromosomal proteins extracted

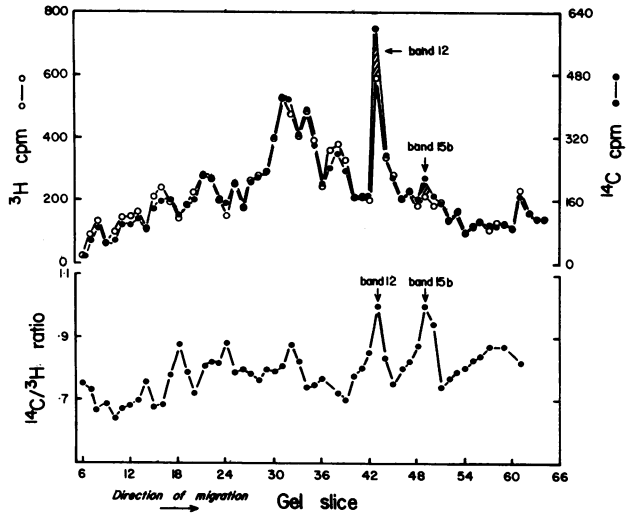

FIG. 3. (*Top*) Electrophoretic separation of nonhistone proteins extracted from chromatin of uteri treated for 3 hr with estrogen. Estrogen-treated uteri were labeled with [^{14}C]aminoacids and controls were labeled with [^{3}H]aminoacids. The shaded areas represent the regions of increased synthesis of protein. (●) ^{14}C cpm; (○) ^{3}H cpm. (*Bottom*) Ratios of $^{14}C/^{3}H$ for the gel slices of the same separation.

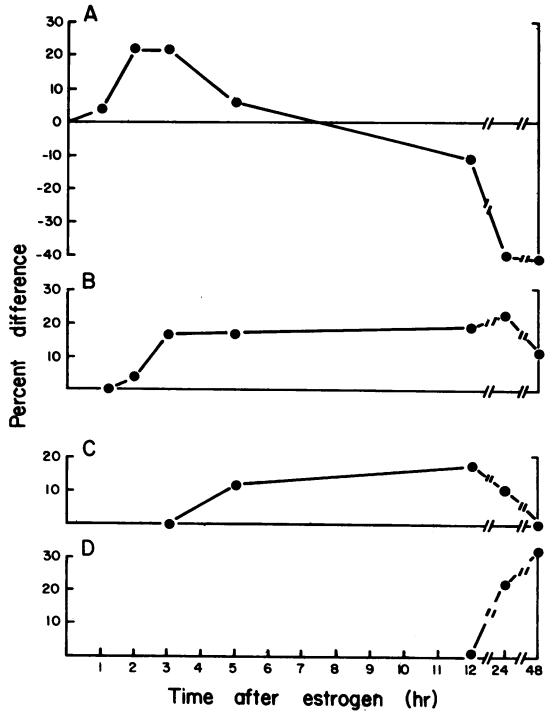

FIG. 4. Changes in the rates of synthesis of individual nonhistone chromosomal proteins after estrogen treatment, relative to the rate of synthesis of the total nonhistone protein fraction. The numerical values of the ordinate represent the percent difference between the $^{14}C/^{3}H$ ratio for a given band and the $^{14}C/^{3}H$ ratio of the total nonhistone protein fraction. (A) Protein in band 12; (B) protein in band 15b; (C) protein in band 5; (D) protein in band 1 and in band 3.

from uterine chromatin of ovariectomized rats treated for 3 hr with estrogen. Reversal of the isotopes of the precursors used to label control and experimental uteri gave comparable results. Only those peptide bands of Fig. 1 with relatively high rates of synthesis possessed sufficient specific activity to produce the 12 to 13 peaks of radioactivity shown in Fig. 3.

The changes in the relative rates of synthesis of five specific nonhistone chromosomal proteins are described in Fig. 4. The rate of synthesis of the nonhistone chromosomal protein in band 12 (molecular weight = 70,500) was increased at 1 hr after hormone treatment, and this was the first nonhistone protein to be stimulated by the hormone. This increase in rate of synthesis reached a peak between 2 and 3 hr, and decreased at 5 hr. By 12 hr the relative rate of synthesis of this protein was below that of the other nonhistone chromosomal proteins, and at 24 hr was less than those of the nonhistone chromosomal proteins from control uteri. The relative rates of synthesis of the proteins in bands 5 and 15b increased in a parallel fashion, except that the rate of synthesis of the protein in band 15b increased and plateaued before that of the protein in band 5. The relative rates of synthesis of the proteins in bands 1 and 3 were not increased until 24 hr after hormone treatment, and remained above control levels at 48 hr after treatment. Some of the proteins having a molecular weight greater than 120,000 appeared to be synthesized more rapidly at later time periods, but since there was little radioactivity in these bands, the ratios of $^{14}C/^{3}H$ determined could not be considered accurate. With exception of the protein of band 12, the other nonhistone proteins induced by estrogen were not detected in the cytoplasm (14). This observation indicates that these proteins

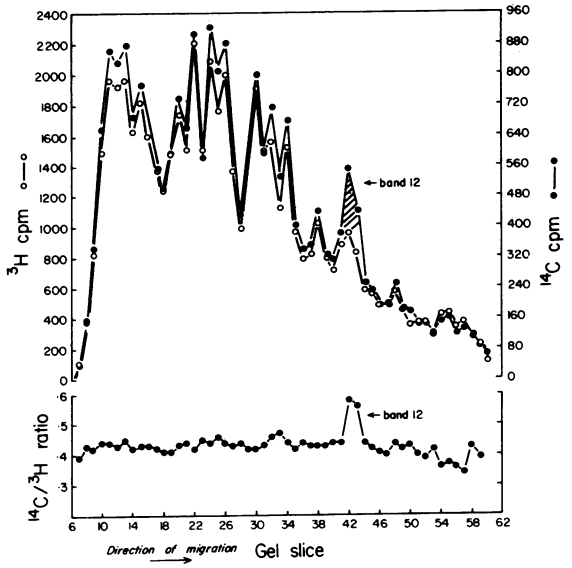

FIG. 5. (*Top*) Electrophoretic separation of the 0.4 M NaCl-soluble nonhistone proteins extracted from chromatin of uteri treated for 3 hr with estrogen. Chromatin was prepared as already described and then extracted, first with 0.14 M NaCl and then with 0.4 M NaCl. The shaded area represents the region of increased protein synthesis. (*Bottom*) Ratios of ^{14}C/^{3}H ratio for the gel slices of the same preparation.

newly synthesized in the cytoplasm are rapidly recruited into the nucleus, and argues strongly that the variations in incorporation observed reflect rates of synthesis rather than rate-limiting transport and processing. The possible significance of the dual location of the peptide of band 12 in the cytoplasm and nucleus is discussed below (see *Discussion*).

Since it has been reported (15) that the estrogen-protein receptor complex can be extracted with 0.3–0.4 M salt, an attempt was made to determine the extent to which protein in the nonhistone protein fraction could be extracted from chromatin with 0.4 M NaCl. Fig. 5 shows that the induced protein in band 12 (molecular weight = 70,500) was among the polypeptides extracted.

The effects of actinomycin D *in vivo* on the stimulation by estrogen of synthesis of nonhistone chromosomal proteins in the uterus were also examined. Hormonal induction of the synthesis of protein in band 12 was abolished completely by actinomycin D, whereas that of band 15b was inhibited by only 8%. This suggests that the appearance of new protein in band 12 requires a preceding stimulation of RNA synthesis. Interpretation of the data observed for the nonhistone protein in band 15b is difficult since RNA synthesis was not completely inhibited, as described under *Materials and Methods*. Either stimulation of the synthesis of the protein in band 15b takes place at a post-transcriptional level, or synthesis of the messenger for this protein was not blocked. We also recognize that the inhibitor may have effects on translation independent of the inhibition of DNA-dependent RNA synthesis (16).

DISCUSSION

The data presented above demonstrate that, after injection of estradiol-17β into the ovariectomized rat, a sequential increase in the relative rates of synthesis of at least five nonhistone chromosomal proteins occurs in the uterus. The five proteins separated in NaDodSO$_4$-acrylamide gels are localized in bands 15b, 12, 5, 3, and 1 (Fig. 1), and have molecular weights of 96,000, 70,500, 29,400, 20,700, and 16,400, respectively. Using a similar approach, King *et al.* (25) have also demonstrated an estrogen-induced synthesis of specific chromosomal proteins in the rat uterus.

It is likely that the nonhistone chromosomal protein of band 12 is identical to the acidic nuclear protein observed by Teng and Hamilton (8). In this earlier investigation, we measured the accumulation of protein for a period of 12 hr after the simultaneous injection of radioisotope and hormone. However, small amounts of radioactive amino acids, when injected into rats, disappear from the blood 10 min after injection (17). It appears unlikely therefore that the incorporation of labeled precursor into any proteins other than those synthesized very early after hormone treatment could have been detected. Furthermore, we have observed that when acidic proteins prepared by the method of Teng and Hamilton (8) are separated in NaDodSO$_4$-acrylamide gels, the induced protein of the nuclear acidic protein fraction also has a molecular weight of 70,500.

The data presented above suggest but do not prove that the nonhistone chromosomal protein in band 12 may be the 4.5S nuclear estrogen receptor. The protein of band 12 has a molecular weight of 70,500, which is the approximate weight of a 4.5S protein. The molecular weight of the isolated nuclear receptor has been calculated to be 61,000–75,000 (15). The receptor protein can be extracted from chromatin with 0.3–0.4 M NaCl (15), and a protein with the same molecular weight as that in band 12 is extractable with 0.4 M NaCl (Fig. 5). Our electrophoretic separations of the cytosol proteins were in the presence of 0.1% NaDodSO$_4$, which would have reduced the presumed 8S cytoplasmic receptors to single peptide chains. Stancel *et al.* (18) have recently presented evidence that the uterine cytoplasmic and nuclear receptors for estrogen are similar, if not the same, in molecular weight (3.8–4.5 S). Other workers have shown that the level of estrogen receptor decreases during the long-term absence of the hormone, and that hormone treatment causes an increase in the ability of the uterus to bind radioactive estradiol-17β (18–21). It is interesting that both the uptake of estrogen in the hormone-primed uterus (19) and the increase in synthesis of the nonhistone protein in band 12 (Fig. 4) are maximal at 2–3 hr after hormone treatment. We emphasize that the conditions of protein extraction and of electrophoresis made it impossible to measure the estrogen binding capacity of the protein of band 12.

We have recently found a specific estrogen-induced protein in the cytoplasmic fraction of the uterus of the ovariectomized rat (14). This cytoplasmic protein is similar if not identical to the nonhistone chromosomal protein (band 12) described above. Both proteins have identical electrophoretic mobilities and molecular weights and similar temporal patterns of induction during early estrogen action. These common features suggest that a single nonhistone protein, probably the estrogen receptor, exhibits an early cytoplasmic synthesis and translocation to the nucleus during the early action of estrogen when the uterine cells are continuing to sequester the hormone. It is known that when estrogen binds to receptor molecules in the cytoplasm, it is translocated to the nucleus by an energy-dependent reaction (22, 23).

The findings described above would seem to indicate the following model for estrogen action. The hormone enters the uterine cell and is transported by receptor to the nucleus, where it binds to chromatin in the form of a hormone-recep-

tor complex. The binding of this complex to the chromatin causes the derepression of a few genes coding for regulatory molecules. Among the regulatory proteins induced is the hormone receptor itself and the nonhistone proteins of bands 1, 3, 5, and 15b. These regulatory proteins synthesized in the cytoplasm then move into the nucleus and bind to the chromatin, causing a further increase in RNA synthesis. The general features of this model are similar to those proposed for estrogen action by O'Malley, Jensen, and Gorski, and their respective coworkers (1, 15, 24). Our model, however, has as an additional feature an emphasis on an early induction of specific regulatory proteins necessary for the complete genomic response to the hormone.

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