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Estrogen-Induced Synthesis of Histones and a Specific Nonhistone Protein in the Uterus*

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ABSTRACT: Template capacity of uterine chromatin for DNA-dependent RNA polymerase is increased by estradiol given *in vivo*. Removal and replacement of certain histones on DNA is thought to control template capacity. Pulse labeling (30 min *in vivo*) of uterine proteins with [^{14}C]amino acids revealed a protein with increased specific activity associated with the arginine-rich histone fraction prepared by Amberlite IRC-50 chromatography from ovariectomized mature rats pulsed 15 min after estradiol. The total amount of the arginine-rich histone fraction in uterus was reduced to 58% of control levels by 15 min after estradiol. The radioactive complex was fractionated on Sephadex G-100, reduced with β-mercaptoethanol in urea and subjected to polyacrylamide gel electrophoresis. The radioactive protein was not a histone but it was an electrophoretically distinct protein associated with the F3 histone. The protein has a p*I* of 4–5 and its increased synthesis after estradiol is not inhibited by actinomycin D. This acidic nuclear protein is a likely candidate for an intermediate in the estradiol-induced increase in uterine RNA synthesis. It may function by complexing with the F3 histone through its SH groups to remove it or make it less inhibitory to the function of uterine DNA during transcription.

Administration of 17β-estradiol to either the immature or the ovariectomized mature female rat results in early stimulation of the incorporation of radioactive RNA precursors into uterine RNA followed by an increase in incorporation of labeled amino acids into protein (Ui and Mueller, 1963; Noteboom and Gorski, 1963; Hamilton, 1964; Means and Hamilton, 1966). Sequentially, incorporation of RNA precursors into rRNA appears to occur first followed by incorporation into tRNA and then DNA-like RNA (Billing *et al.*, 1969a; Hamilton *et al.*, 1968). The time course and magnitude of increases in uterine "RNA synthesis" measured *in vivo* remain open to question since the effect of estradiol on the specific activity of the immediate precursors of RNA, the nucleoside triphosphates, at the site of RNA synthesis has not been measured (Joel and Hagerman, 1969; Billing *et al.*, 1969b). The *in vitro* synthesis of RNA from radioactive nucleoside triphosphates by isolated whole uterine nuclei, where the effects of precursor pool dilution should be minimized, is elevated within 0.5 hr after *in vivo* treatment with estradiol (Gorski, 1964; Hamilton *et al.*, 1968). This increase in nuclear RNA synthesis has been shown to be due, at least in part, to the specific activation of the capacity of uterine chromatin to serve as a template for DNA-dependent RNA polymerase (Barker and Warren, 1966; Teng and Hamilton 1968; Church and McCarthy, 1970).

Several studies have shown that annealing of histones to DNA severely restricts the template efficiency of DNA in the DNA-dependent RNA polymerase reaction (Huang and Bonner, 1962; Huang *et al.*, 1964; Spelsberg *et al.*, 1969). Lysine-rich histones were observed to bind more tightly to DNA than the arginine-rich histones and could more effectively inhibit the DNA template capacity. In addition to its affinity for DNA, the arginine-rich histone had the capacity to bind to certain acidic proteins. At least one study suggested that synthesis of the arginine-rich histones in calf endometrium nuclei is associated with periods of enhanced RNA synthesis (Chalkley and Maurer, 1965). They did not determine which of the arginine-rich histones eluted from the Amberlite IRC-50 column (F3 or F2al) was synthesized and further, the possibility that the radioactivity was incorporated into a nonhistone protein with similar ion-exchange properties was not excluded (Stellwagen and Cole, 1968).

This investigation was originally undertaken to determine the effects of 17β-estradiol on the rate of synthesis of various classes of uterine histones during various phases of the uterine response to estradiol. The finding of a rapidly synthesized, nonhistone protein bound to the F3 arginine-rich histone led to its further preliminary characterization and determination of its possible relationship to the uterine response to estradiol. Since its synthesis is not inhibited by actinomycin D at doses capable of inhibiting 90% of the incorporation of [^{3}H]uridine into RNA, it must be either synthesized by a preexisting mRNA or a mRNA synthesized from actinomycin D insensitive regions of uterine DNA. This property suggests that it may be a specific protein, originally predicted by Mueller *et al.*

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(1961) to be synthesized during early estrogen action which was postulated to be responsible for the initiation of further uterine RNA synthesis. Its presence has been further substantiated by Gorski and Morgan (1967) using both puromycin and cycloheximide.

Materials and Methods

Animals. Female rats (Small Animal Supply Co., Omaha, Neb.), weighing 160–180 g, were ovariectomized by the dorsal approach under ether anesthesia at least 3 weeks prior to use. Feeding regime consisted of Purina Lab Chow and water *ad libitum* throughout the study. 17β-Estradiol (5 μg/animal) was routinely administered by a single tail vein injection in 0.5 ml of a vehicle composed of 5% ethanol in isotonic saline. Zero-time or control-treated animals received a sham injection of the vehicle immediately before or at the indicated times before sacrifice.

Intrauterine Application of [^{14}C]Amino Acids. A nonsurgical technique was used for the intraluminal uterine application of the radioactive amino acids. A Welch-Allen operating otoscope equipped with a 3-mm speculum and a specially prepared hypodermic needle (22 gauge × 5-in. blunt-ended needle with a 20-degree bend 0.25 in. from the tip) attached to a 0.5-ml tuberculin syringe were employed in this procedure. Under light ether anesthesia, the speculum was inserted deep into the vagina until the cervix was clearly visible and held firmly in place. The needle was inserted about $^3/_8$ in. into each uterine horn by aiming the needle in the direction of the horn to be injected and gently working the tip through the respective cervical canal. All compounds were administered in 50 μl of 1.8% NaCl with the total dose equally divided between the two uterine horns. This concentration of saline was employed to reduce edema caused by any trauma associated with the intraluminal application procedure.

The [^{14}C]amino acid mixture (New England Nuclear Corp.) obtained as a solution in 0.1 N HCl, was prepared for injection by neutralization (phenol red indicator) with 0.1 N NaOH with subsequent adjustment of salt concentration to 1.8% with NaCl. Final volume was adjusted with 1.8% NaCl such that the 50-μl dose contained 1.0 μCi (0.67 μg) of the radioactive amino acids. The mixture of 15 different [^{14}C]amino acids was employed to minimize possible hormone-induced gross changes in pool sizes that might occur with any one amino acid, to increase the extent of overall protein labeling and to eliminate the escape of any one protein from being labeled by being deficient in a single amino acid.

The specific radioactivity of the uterine free amino acid pool was determined at the end of each experiment. This was necessary for the following reasons: tracer doses of the amino acid mixture were used, the uterine free amino acid pool size increases after 4-hr estrogen treatment, the uptake of the label from the uterine lumen was possibly subject to estrogen influences on tissue permeability and a small portion of the label was occasionally lost through the vaginal opening depending on the manner in which the catheter was withdrawn after intraluminal application of the amino acid mixture. The free amino acid content and radioactivity was determined on an aliquot of the uterine homogenate after acidification to 0.2 N H_2SO_4 and addition of 0.2 ml of 10% Na_2WO_4/ml of acidified homogenate. After centrifugation, the amino acid content of the supernatant was determined by the ninhydrin reaction (Moore and Stein, 1948) and an aliquot was dried on a 1-in.^{2} Whatman No. 1 filter paper and counted in a liquid scintillation spectrometer. Estimates of the specific activity of the amino acid pool (disintegrations per minute per micromole of amino acids) are based on the specific ninhydrin color yield of a standard glycine solution.

Preparation of Acid-Soluble Uterine Proteins. At various times after administration of the [^{14}C]amino acids, usually 30 min, the animals were killed by cervical dislocation and the uteri were quickly removed and placed into ice-cold isotonic saline. The uteri were trimmed free of fat and connective tissue and each uterine horn was flushed with cold isotonic saline to remove any nonabsorbed radioactivity before isolation of nuclei, chromatin, or direct tissue extraction of histones. Depending on the experiment, acid-soluble proteins were extracted from isolated uterine nuclei, purified uterine chromatin or whole uteri by the following procedure. Chromatin or isolated nuclei were gently homogenized in an all-glass Duall homogenizer (Kontes) in 2.5 ml of 0.15 N H_2SO_4/mg of DNA followed by stirring for 1 hr at 2°. The acidic homogenate was centrifuged at 38,000g for 30 min, the supernatant was collected, and the pellet was rehomogenized and extracted in 0.4 N H_2SO_4. Following centrifugation, the two supernatants were pooled and filtered through a porous glass filter. The sulfates of the acid-soluble proteins were precipitated by addition of five volumes of cold 95% ethanol and storage for 18 hr at −20°. The precipitate was collected by centrifugation at 2000g for 30 min, washed two times with absolute ethanol, and stored in a desiccator at 2° until fractionated. In initial experiments, total uterine acid-soluble proteins were extracted by homogenization of whole uteri in 2.5 ml of 0.15 N H_2SO_4/uterus in a Duall homogenizer followed by extraction as indicated for chromatin and nuclei. Uterine chromatin was purified exactly as described by Barker and Warren (1966). Uterine nuclei were isolated by the method of Widnell *et al.* (1967) with the exception that they were gently suspended in a solution of 0.25 M sucrose–1 mM $MgCl_2$, which also contained 1% Triton X-100, by aspiration through a 5-ml serological pipet. This was then thoroughly mixed into nine volumes of 2.4 M sucrose–1 mM $MgCl_2$ which also contained 1% Triton X-100 and was centrifuged at 40,000g for 2 hr. The nuclear pellet was finally washed in 0.25 M sucrose–1 mM $MgCl_2$ without the detergent, suspended in a small volume of 0.15 M NaCl, assayed for DNA by method of Burton (1956), and extracted as indicated above. The yield of DNA in purified nuclei was 20–25% and appeared to be free of particulate contamination under the light microscope after staining with 0.1% methylene blue in 0.25 M sucrose.

Amberlite IRC-50 Fractionation of Acid-Soluble Uterine Proteins. The acid-soluble proteins were fractionated on a column (1.0 × 60 cm i.d.) of Amberlite IRC-50 by the use of a concentration gradient of guanidinium chloride by the method of Rasmussen *et al.* (1962). The acid-soluble protein fraction (6–10 mg) was dissolved in 4.0 ml of 7% guanidinium chloride in 0.1 M sodium phosphate buffer, pH 6.8 (7% GuCl-PO_4), and was centrifuged at 1000g for 30 min to remove any trace of insoluble material. An aliquot was assayed for protein and radioactivity and the remainder was applied to the column. The column was washed with 7% GuCl-PO_4 until the runoff peak began and was then developed with an increasing gradient of 7–14% GuCl-PO_4 while collecting 0.5-ml fractions. After the complete removal of the second major fraction after the runoff peak, the column was charged with 30% GuCl-PO_4 to remove the final fraction. Higher concentrations of GuCl-PO_4 (50%) would not remove additional protein from the column. The fractions after the runoff were labeled FI, FII and FIII in their order of elution. Subsequent analysis of these fractions by polyacrylamide gel electrophoresis, indi-

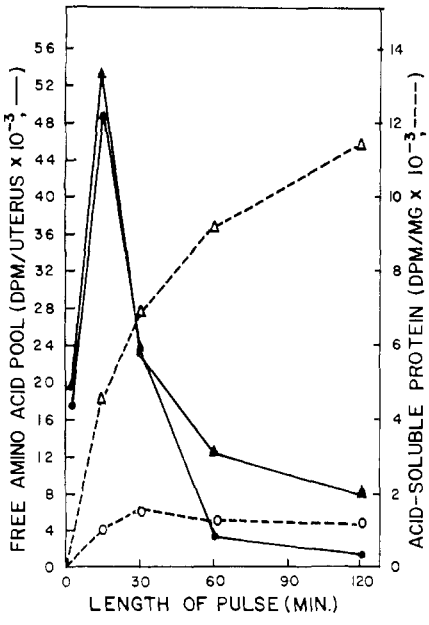

FIGURE 1: Incorporation of [^{14}C]amino acids into the free amino acid pool (closed symbols) and the acid-soluble proteins (open symbols) of uteri in ovariectomized mature rats receiving saline (circles) or estradiol (triangles), 12 hr before intrauterine application of the labeled amino acid mixture. Each point represents a single determination from a pooled sample obtained from eight identically treated animals.

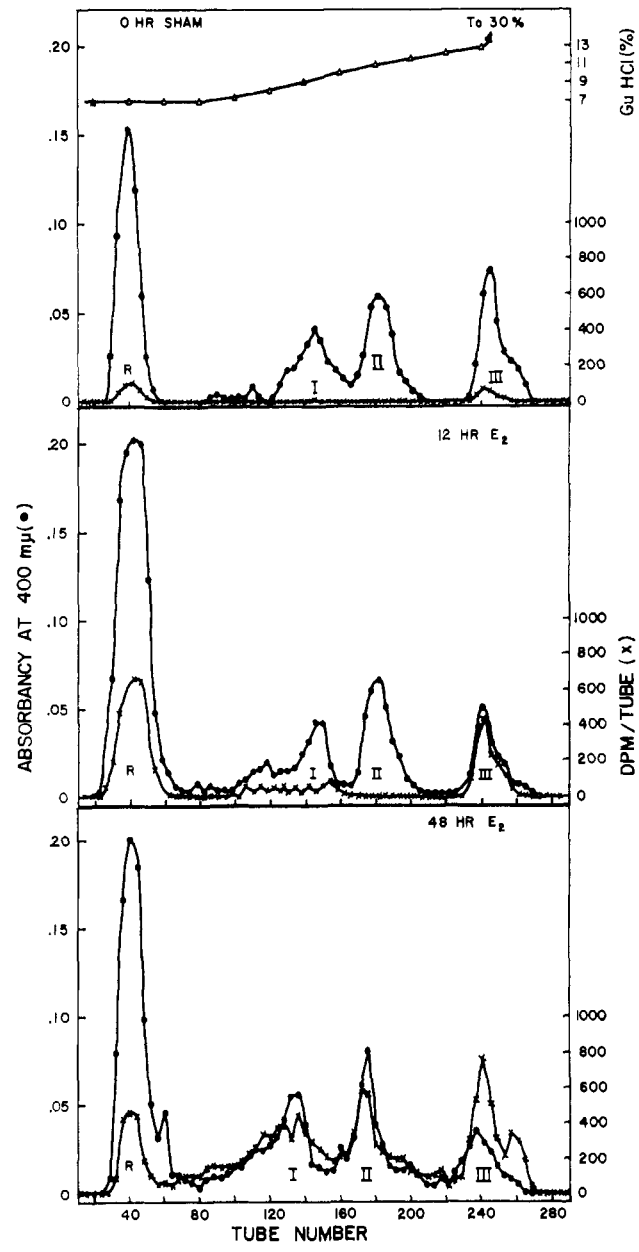

FIGURE 2: Incorporation of [^{14}C]amino acids into specific uterine histones as fractionated on Amberlite IRC-50 employing a gradient of guanidinum chloride. Groups of eight identically treated animals received a 1-hr pulse of a [^{14}C]amino acid mixture (intrauterine) beginning at 0, 12, and 48 hr after a single intravenous injection of estradiol. Acid-soluble proteins were prepared from whole uteri. Protein and radioactivity per tube are as indicated.

cated that FI contained primarily histone F1, FII contained primarily histone F2a2 + F2b, and FIII contained primarily F2a1 and F3 and a third protein we call Fx (nomenclature of Johns, 1964).

The protein content of each fraction was determined by an analysis of the turbidity resulting from the addition of 2.5 ml of 1.1 M trichloroacetic acid. Other conditions of this assay were as described by Luck *et al.* (1958). The assay was standardized with purified rat liver histone. The precipitate from each tube was collected and washed with 5% trichloroacetic acid on individual membrane filters (Schleicher & Schuell, B-6), dried, and placed in a scintillation vial with 10 ml of a toluene scintillator and counted in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency (*ca.* 65%) was estimated by the channels ratio method and all data were reported as disintegrations per minute per tube or disintegrations per minute per milligram of protein.

Polyacrylamide Gel Electrophoresis of Acid-Soluble Uterine Proteins. Polyacrylamide gel electrophoresis was initially performed by the method of Bonner *et al.* (1968), but was later changed to the method of Panyim and Chalkley (1969) due to the improved resolution of their system. With both methods the sample was reduced in a solution (1 mg/ml) of 0.5 M β-mercaptoethanol–8.0 M urea for 18 hr at 35° before electrophoresis of 50 μg of protein/gel. Other conditions, including preelectrophoresis, staining and band identity were as described by Panyim and Chalkley for the 8.5-cm, 15% polyacrylamide gels containing 2.5 M urea–0.9 N acetic acid (pH 2.8).

The relative amount of protein per band was determined by cutting the individual stained bands from two identical gels and extracting the dye into 3.0 ml of dimethyl sulfoxide by incubation at 50° for 6 hr. The amount of dye extracted was determined spectrophotometrically at 600 mμ. The dye in each band increased linearly with increasing amounts of protein applied to individual gels from 10 to 75 μg per gel. The relative amount of each protein was estimated assuming a constant Amido Black dye coefficient for each protein (Bonner *et al.*, 1968).

Radioactivity of proteins separated by polyacrylamide gel electrophoresis was determined by cutting the stained bands from six identical gels and hydrolyzing the protein at 90° in 6 N HCl for 4 hr. The supernatant from two successive extractions were pooled in a liquid scintillation vial and dried at 95°. NCS (2 ml, Nuclear-Chicago) was added followed by 10 ml of a toluene-based scintillation counting solution and the samples were counted in a liquid scintillation spectrometer. Recovery of total radioactivity applied to the gel was 80–85% when all visible bands were extracted.

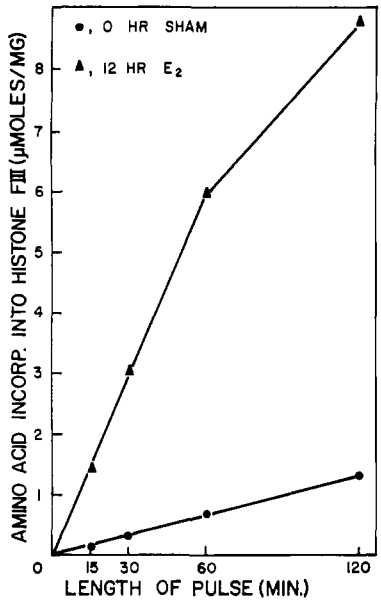

FIGURE 3: Effect of length of pulse on the incorporation of [^{14}C]-amino acids into uterine histone fraction, FIII. Each point represents a single determination from a pooled sample obtained from eight identically treated animals. Acid-soluble uterine proteins were extracted and fractionated as in Figure 2. Rate of incorporation of [^{14}C]amino acids into FIII have been converted into micromoles of amino acids incorporated per milligram of FIII based on the turbidity assay of protein and the specific radioactivity of the amino acid pool.

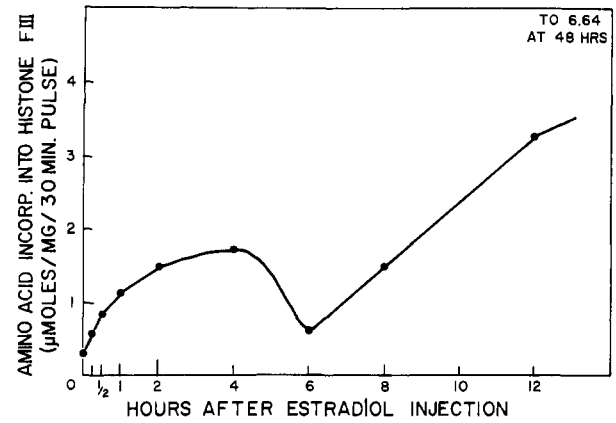

FIGURE 4: Effect of estradiol on the incorporation of [^{14}C]amino acids into uterine histone fraction, FIII. Groups of eight identically treated animals received a 30-min pulse of a [^{14}C]amino acid mixture beginning at the indicated interval after estradiol administration. Each point represents the average of two separate experiments at each time interval. Acid-soluble uterine proteins were extracted and fractionated as in Figure 2 and the results converted into micromoles of amino acids incorporated per milligram of FIII.

Isotope Dilution Analysis of Uterine Content of FIII. Acid-soluble uterine protein was prepared from groups of 100 to 150 ovariectomized female rats which had received a 48-hr pretreatment with 17β-estradiol and had been pulse labeled with [^{14}C]amino acids for 1 hr prior to sacrifice. The extract was prepared for analysis by dissolving in 7% GuCl-PO_4. Uteri were then removed from groups of eight similarly treated animals at various intervals after estrogen treatment, cooled in saline, trimmed, and pooled into 25 ml of cold 0.15 N H_2SO_4. Equal aliquots of the [^{14}C]acid-soluble protein extract were added to each group of uteri before homogenization. Histones were then isolated and analyzed as above using Amberlite IRC-50 column chromatography. An aliquot of the [^{14}C]acid-soluble protein extract equal to that added to each sample was analyzed for specific radioactivity (SA) and mass (M) of FIII. Using the specific activity of the unknown containing added [^{14}C]acid-soluble uterine proteins (SA*) the mass of the unknown FIII (M*) was determined as follows: $M^* = M[(SA^*/SA) - 1]$. The amount of FIII in purified chromatin was similarly assayed by this isotope dilution method.

Results

Labeling of Uterine Acid-Soluble Proteins in Vivo. The kinetics of labeling of the free amino acid pool and incorporation of labeled amino acids into uterine acid-soluble proteins following intrauterine application of 1 μCi of a [^{14}C]amino acid mixture are presented in Figure 1. Labeling of the total amino acid pool is observed to be maximal 15 min after injection and no difference is observed during the first 30 min between control and 12-hr estradiol-pretreated animals. After 30 min the estrogen-treated animals lose the label in this fraction at a somewhat slower rate. This is probably due to the greater size of the uterine amino acid pool in estrogen-treated animals (Kalman and Lombrozo, 1961). Pretreatment of animals for 12 hr enhances the rate of incorporation of [^{14}C]amino acid into the acid-soluble protein fraction of uterine tissue 4.5-fold during the first 30 min. Considering the free amino acid pool is enlarged 55% by this treatment, this would indicate a 7-fold increase in rate of incorporation of amino acids into total acid-soluble uterine proteins has occurred due to the estrogen pretreatment.

Synthesis of Specific Uterine Histone Fractions after Estradiol. The effects of 12- and 48-hr pretreatment of animals with estradiol on the rate of incorporation of [^{14}C]amino acids into specific histone fractions are presented in Figure 2. During the period of uterine hypertrophy (first 24 hr) there is stimulation of synthesis of proteins in the FIII (arginine-rich histones) peak only. After the onset of uterine hypertrophy between 24 and 48 hr (Telfer, 1953; Jervell *et al.*, 1958; Billing *et al.*, 1969a) there is an increase in the rate of synthesis of all classes of uterine histones.

The effect of duration of the labeling period on the amount of incorporation of [^{14}C]amino acids into the FIII fraction of the acid-soluble uterine protein is given in Figure 3. The incorporation rate, corrected for the amino acid pool specific activity observed at the end of the labeling period is observed to be linear for 1 hr in both control and 12-hr estradiol-pretreated animals. The rate of synthesis of FIII proteins is increased 10-fold by this treatment. In subsequent experiments a 30-min pulse interval was used to assess the rate of FIII synthesis since [^{14}C]amino acid incorporation was at a constant rate for at least this interval in these two extreme conditions.

The rate of synthesis of uterine FIII after various periods of estradiol pretreatment are given in Figure 4. During the 30 min following a 15-min pretreatment with estradiol, there is an 83% increase in the rate of synthesis of FIII proteins. The rate of FIII synthesis increases during the first 4-hr estradiol treatment, after which it decreases toward control rates at the 6th hr. Synthesis then increases during the next 6 hr and reaches the 10-fold greater rate by 12 hr after the estradiol injection. The rate of FIH synthesis at 48 hr after estradiol injection is increased 22-fold.

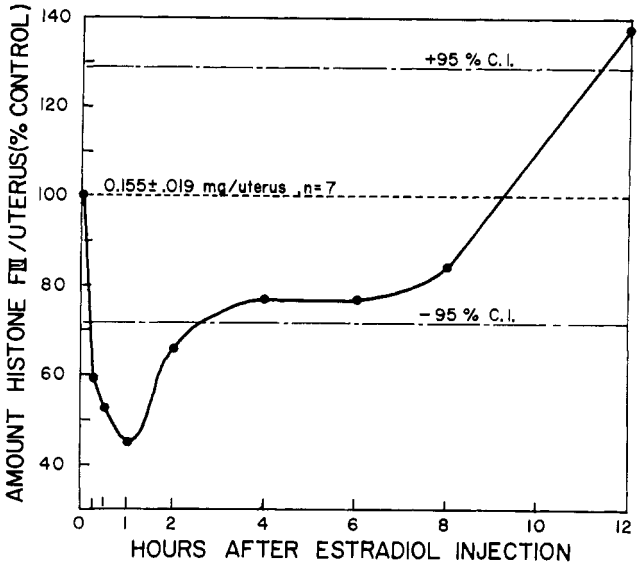

FIGURE 5: Isotope dilution analysis of the amount of histone fraction FIII per uterus at various intervals after administration of estradiol. Groups of eight identically treated animals were killed at the indicated times after estradiol injection. Aliquots of a radioactive acid-soluble uterine protein extract were added to each pooled sample and each sample was then extracted and fractionated as in Figure 2. The amount of FIII per uterus was then calculated based on the dilution of specific radioactivity of the added acid-soluble protein extract. Results represent the average of two separate experiments at each time interval with the exception of the zero-time period which is the average of seven determinations (three performed with the first experiment, four with the second). A 95% confidence inverval (CI) for control values was calculated and is indicated in the figure.

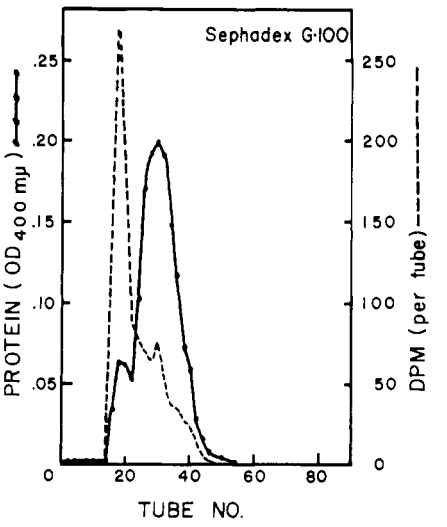

FIGURE 6: Fractionation of histone fraction FIII on Sephadex G-100. The Amberlite IRC-50 fraction FIII was collected, dialyzed against 0.1 N acetic acid, and lyophilized. The sample was then dissolved in an aliquot of 0.01 N HCl, stirred slowly for 18 hr at 2°, and fractionated on a column (0.8 × 50 cm) of Sephadex G-100 which had been preequiliibrated with 0.01 N HCl. Fractions (1 ml) were collected and 0.5-ml aliquots were assayed for protein and radioactivity as indicated in Materials and Methods for the Amberlite IRC-50 eluates. Uterine FIII was prepared from a group of 16 animals which had received estradiol for 1 hr before a 30-min pulse with the [^{14}C]amino acid mixture.

Quantitative Changes in the Amount of Uterine FIII Proteins after Estradiol. Isotope dilution analysis of the amount of chromatographically identifiable FIII per uterus at various intervals after estradiol injection is presented in Figure 5. Within 15 min after treatment the amount of this fraction is decreased to 58% of control levels and by 1 hr it is present at the minimum level of only 44% of control levels. The amount of this fraction in uterus is significantly depressed during the entire interval from 15 min to 2 hr ($P < 0.05$). At the 8th hr after hormone treatment the levels begin to increase and by the 12th hr they are 38% greater than control levels.

A similar analysis of the amount of FIII present in purified uterine chromatin is presented in Table I. The amount of FIII per unit of DNA in chromatin decreases 27% by 1 hr but unlike the pattern observed in whole uteri, the amount continues to decline through the second hour after estradiol treatment. This difference may be indicative of a nonchromatin pool of proteins which behave chromatographically as FIII protein. Considering the average DNA content per uterus of 1.17 mg of DNA the amount of FIII per uterus associated with chromatin would be 0.158 mg. There is close agreement between this value and the 0.155 mg/uterus measured when the entire uterine FIII is measured (Figure 5), suggesting that at least in the control uterus all of the FIII protein is associated with chromatin.

Fractionation of FIII Uterine Proteins. The FIII proteins were further fractionated on Sephadex G-100 (Figure 6). Two peaks of protein are eluted, the first of which coincides with the void volume of the column. The radioactivity of the FIII proteins is associated primarily with the runoff peak suggesting they are either aggregated F3 histone or nonhistone proteins.

Analysis of the two fractions obtained from the Sephadex G-100 column by polyacrylamide gel electrophoresis indicated that the runoff (gel A) contained three proteins: F2a1 histone, F3 histone and another distinct protein with a mobility that does not correspond to any known histone protein (Figure 7). In addition, it contained some proteins that did not enter the 15% acrylamide gels and remained at the origin. The second fraction (gel B) contained histones F2a1 and F3, and traces of histones F2a2 and F2b. Preliminary experiments using isoelectric focusing in polyacrylamide gels with a pH gradient of 3–10 indicates the presence of a single band

TABLE I: Effect of Estradiol on the Amount of FIII Proteins Associated with Uterine Chromatin.[a]

Time after Estradiol (hr)	FIII/DNA (μg/μg)	% of Control
0	0.185 (0.176–0.194)	100
1	0.135 (0.127–0.143)	73
2	0.119 (0.108–0.130)	64

[a] Chromatin was prepared from groups of 18 identically treated animals at 0, 1, and 2 hr after injection of estradiol. Aliquots of radioactive acid-soluble uterine proteins were added to samples of chromatin containing 5 mg of DNA and the amount of FIII per microgram of DNA in chromatin was determined by the isotope dilution method. Values represent average of two separate experiments with the individual values given in the parentheses.

TABLE II: Failure of Actinomycin D to Inhibit the Estradiol-Stimulated Incorporation of [^{14}C]Amino Acids into a Uterine F3 Histone-Associated Protein.[a]

	Specific Activity (dpm/mg)			
Fraction	Control	Estradiol	Actinomycin	Estradiol + Actinomycin
FIII	1,405	2,190	1,524	1,995
F3	814	913	953	702
Fx	10,380	17,220	14,570	22,100

[a] Acid-soluble uterine proteins were prepared from four groups of 25 identically treated animals. One group received 10.5 ml of saline (intravenously) for 1 hr before a 30-min pulse with intrauterine-applied [^{14}C]amino acids. The second group received 5 μg of estradiol (intravenously) for 1 hr before the 30-min pulse. The third and fourth groups received 10 μg of actinomycin D by the intrauterine route in 1.8% saline 5 min before injection of saline or estradiol and were pulse labeled 1 hr later. Acid-soluble uterine proteins were extracted and fractionated on Amberlite IRC-50. FIII was fractionated on Sephadex G-100 as described in Figure 6 and the first fraction was electrophoresed on eight individual gels as described for gel A in Figure 7. Protein was determined on the F3 and Fx bands eluted from two gels and radioactivity was determined on the bands eluted from the six remaining gels. Electrophoresis was by the method of Bonner *et al.* (1968). Incorporation of [^{3}H]uridine into total uterine RNA in parallel experiments was 9810 ± 1320 cpm (control), 13,259 ± 2440 cpm (estradiol), 1004 ± 552 cpm (actinomycin), and 1194 ± 694 cpm (estradiol + actinomycin) when 5 μCi of the [^{3}H]uridine was given by the intrauterine route instead of radioactive amino acids.

focusing at a pH between 4 and 4.5 in the Sephadex G-100 runoff peak.

Actinomycin D Sensitivity of the Synthesis of FIII Proteins. Groups of eight gels were electrophoresed from each of four preparations of histones prepared exactly as described for gel A of Figure 7, but differing in the pretreatment of the animal. The protein content and radioactivity of the F3 histone and Fx bands were determined and the specific activities of each band are reported in Table II. It is observed that injection of estradiol into the animals for 1 hr increases the rate of incorporation of [^{14}C]amino acids into FIII (based on assay of material applied to Sephadex). The increased incorporation into FIII is largely accounted for by the 66% increase in radioactivity of the Fx protein. Incorporation into histone F3 is only increased by 12%.

Direct intrauterine application of 10 μg of actinomycin D, a dose capable of inhibiting uterine RNA synthesis by 90% (see footnote to Table II) and which is twice that required to block estrogen induction of uterine glucose 6-phosphate dehydrogenase synthesis (Barker, 1967), was unable to inhibit the estrogen-induced increase in synthesis of the Fx protein but was able to inhibit the incorporation of [^{14}C]amino acids into F3 histone. In both groups receiving actinomycin D (control and estradiol) the total rate of incorporation of label into protein was slightly elevated relative to the respective control groups. This slight increase may be due to the effects of actinomycin D on the uptake of the intrauterine-applied

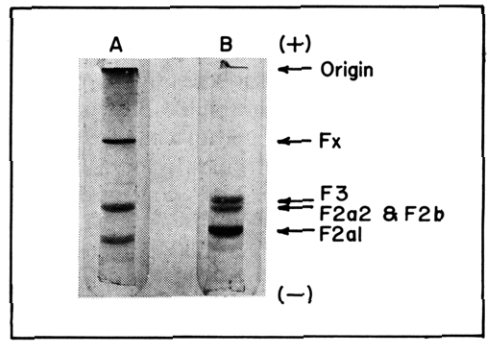

FIGURE 7: Polyacrylamide gel electrophoretic analysis of the two Sephadex G-100 fractions of uterine FIII. The first Sephadex fraction (gel A) and the second fraction (gel B) were collected, lyophilized, dissolved in 0.5 M β-mercaptoethanol in 8 M urea, and incubated for 18 hr at 35°. Each fraction (50 μg) was then electrophoresed as described by Bonner *et al.* (1968).

[^{14}C]amino acids into the tissue. Administration of 50 μg of cycloheximide intrauterine with the [^{14}C]amino acids completely blocks amino acid incorporation into acid-soluble uterine protein in both control and estradiol-treated animals.

Acid-Soluble Protein Composition of Uterine Nuclei. Acid-soluble protein was quantitatively extracted from nuclei prepared from uteri from control and 1-hr estradiol-pretreated groups of ovariectomized mature rats. Equal amounts of acid-soluble proteins were then separated electrophoretically and a comparison was made between the relative amounts of the seven protein bands in control and 1-hr estradiol-treated animals (Table III). F3 histone was the only protein which

TABLE III: Effect of Estradiol on the Amount of Specific Acid-Soluble Nuclear Proteins in the Ovariectomized Rat Uterus.[a]

			Estradiol	
Rel Mobility	Protein Identity	Control (μg/gel)	μg/Gel	% of Control
0.53	Fx	1.2	1.5	125
0.65	F1	9.7	9.6	99
0.72	F^{0}	1.9	2.1	110
0.82	F3	10.0	8.2	82
0.85	F2b	12.5	13.3	106
0.88	F2a2	7.5	8.0	107
1.00	F2a1	7.2	7.3	101

[a] Groups of 16 animals received either saline or 5 μg of estradiol for 1 hr before sacrifice. Nuclei were prepared and acid-soluble proteins were extracted as described in Materials and Methods. Electrophoresis and band identity were as described by Panyim and Chalkley (1969). Fx has the same mobility as the F3 histone-associated nuclear protein. Electrophoresis of 50-μg samples was for 3.25 hr at 125 V on 8.5 cm, 15% polyacrylamide gels containing 2.5 M urea–0.9 N acetic acid (pH 2.8). Samples were reduced in 0.5 M β-mercaptoethanol–8.0 M urea–0.9 N acetic acid (pH 2.8) for 18 hr before application to the prerun gels. Bands were cut from two identically prepared gels and protein was determined by elution of the bound dye into dimethyl sulfoxide and measurement of absorbancy at 600 mμ.

decreased in quantity following estradiol treatment. The fractional amount of F3 relative to the total histone content decreased from 0.213 to 0.176 after estradiol. When corrected for the total amount of acid-soluble protein extracted per milligram of DNA, it was found that the weight ratio of F3 to DNA in nuclei decreased from 0.16 to 0.10 after hormone stimulation. Assuming uterine DNA content does not change during the interval of treatment (Telfer, 1953; Jervell *et al.*, 1958; Billing *et al.*, 1969a) the total extractable uterine F3 content decreases to 63% of control levels 1 hr after estradiol injection. The F^0 band was described by Panyim and Chalkley (1969) to be a protein present only in nondividing cells. A preliminary analysis of nuclei prepared from uteri of 48-hr estradiol-treated animals indicated its absence, thus confirming that it is not present in tissue undergoing division.

Discussion

The data presented above suggest that the synthesis of histones occurs during periods of uterine hyperplasia. Synthesis of arginine-rich histones initially appeared to occur as a part of early estrogen action, but this apparent synthesis is due to the synthesis of a nonhistone protein that is coisolated with the arginine-rich histones. The amount of the arginine-rich histones associated with uterine chromatin is decreased dramatically during the first hour of estrogen action. Polyacrylamide gel electrophoresis of total acid-soluble uterine nuclear proteins suggests that this decrease is largely due to the F3 component of the histone complex in the FIII peak from Amberlite IRC-50 column. The present data do not indicate the fate of the F3 histone during early estrogen action. It may be lost through proteolysis or it could be complexed with nonhistone proteins in such a way that it is not extracted or chromatographed as a pure histone would be.

The exact relationship between the protein which is coprecipitated with the arginine-rich histone and the F3-histone is not indicated by the present data. The F3 histone from animal sources has been shown by Fambrough and Bonner (1968) to contain two cysteine residues per mole of histone. During isolation and presumably *in vivo*, these groups would have the potential of forming both intramolecular and intermolecular disulfide linkages. These could be either histone–histone linkages or presumably histone–nonhistone linkages. During the course of the present studies it was observed that electrophoresis of purified FIII proteins prior to reduction with β-mercaptoethanol yielded numerous bands between that of the F3 protein and the origin. There was a reduction in the amount of the F3 band and in addition the Fx band was not readily apparent suggesting that it may have been in an aggregate complex with the F3 histone. Whether this complex is an artifact of isolation or a true *in vivo* complex remains to be seen. Similarly its relationship to the loss of uterine F3 during early estrogen action remains to be seen. If its complexing with F3 histone were responsible for the initial loss of F3, then one would not expect to find any of the Fx protein with the arginine-rich peak on Amberlite IRC-50. If it is responsible for the loss of F3, then a higher degree of aggregation of the initial complex must occur to alter the chromatographic or acid extraction properties of the histone.

The relationship between the thiol content of chromatin and its physical and presumably its functional state has been studied by Ord and Stocken (1966). They find that diffuse active chromatin has a higher proportion of its sulfur residues as free thiol groups than does dense inactive chromatin. When they anneal purified F3 with DNA and evaluate its template efficiency with purified DNA-dependent RNA polymerase, they find that prior oxidation of the thiol group to the disulfide to produce histone–histone complexes yields histone with a greater inhibitory property (Hilton and Stocken, 1966). The state of the histone thiol group may therefore play an important role in the regulation of gene function, particularly when one includes the possibility of nonhistone–histone interaction.

Acidic nuclear proteins have been suggested as regulatory intermediates in the restriction of DNA–template activity by histones. They have been implicated as enzymes which modify histones through phosphorylation, acetylation, methylation, and as proteins which directly complex with histones through ionic bonding. They are capable of complexing with arginine-rich histones to prevent inhibition of RNA polymerase by direct binding to the enzyme of the histone (Spelsberg and Hnilica, 1969a,b; Wang, 1968). Specfic acidic nuclear proteins which are covalently linked to unique species of chromosomal RNA have been implicated in contributing tissue specificity to chromatin constructed from histones and DNA through *in vitro* reannealing processes (Bekhor *et al.*, 1969; Huang and Huang, 1969; Paul and Gilmore, 1968). The possibility that these nonhistone proteins could form disulfide interaction with the F3 histone rather than solely by ionic bonding further raises their potential as mechanisms for controlling RNA synthesis.

Teng and Hamilton (1969) have reported that nonhistone uterine nuclear proteins are capable of reversing the inhibition of RNA synthesis caused by addition of specific histones to a uterine chromatin—*Escherichia coli* RNA polymerase system. They did not show that this was a specific property of nuclear nonhistone proteins rather than acidic proteins in general, nor did they indicate the basis for using uterine chromatin rather than purified DNA in their system. Of particular interest in their study was the finding that a 12-hr estradiol pretreatment enhances the incorporation of [^{3}H]tryptophan into uterine nonhistone proteins and histones. Since tryptophan is not present in histones (Hnilica, 1967; Starbuck *et al.*, 1968), their histone preparation must also contain nonhistone protein, perhaps of the same type observed in the present report.

The specificity of the uterine response with regard to the estrogen induced synthesis of specific uterine proteins has been examined. Most studies reveal that alterations in the total uterine levels of many enzymes and proteins occur after estrogen administration (Barker and Warren, 1966; Barker *et al.*, 1966), but only recently has there been found proteins which are specifically synthesized under the influence of estradiol (Notides and Gorski, 1966; Barnea and Gorski, 1970; Mayol and Thayer, 1970). Interest in these proteins is due not only to their possible involvement in uterine function related to implantation of fertilized ova (Enders, 1967) but also because of their possible role in the early initiation of uterine RNA synthesis (Mueller *et al.*, 1961). Gorski and associates have detected a protein with unknown function that is synthesized and is present in uterine cytosol after 40-min estrogen treatment and continues to be synthesized until 4-hr post-estradiol treatment. Their initial report indicated that synthesis of this protein was only slightly sensitive to actinomycin D suggesting it may be the protein predicted by Mueller *et al.* (1961) to be responsible for uterine RNA synthesis. In later reports, however, they show that larger doses of actinomycin D (8 mg/kg) given intraperitoneally 15 min before estradiol holds synthesis of their induced protein to near control levels (De Angelo and Gorski, 1969, 1970). This inhibition was confirmed by Mayol and Thayer (1970).

The exact relationship between the Fx protein now reported

and that of the Gorski-induced protein remains to be seen. It appears to be acidic in nature, has similar kinetics of synthesis, but its synthesis is insensitive to actinomycin D inhibition. Even though function cannot be attached to the Fx protein, the curious finding of it in association with F3 histone places it in the right physical location to be considered as a further candidate for being an estrogen-induced derepressor of RNA synthesis.

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