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Occurrence of 2-5A and RNA Degradation in the Chick Oviduct during Rapid Estrogen Withdrawal†

Randall J. Cohrs, Biswendu B. Goswami, and Opendra K. Sharma*
Department of Molecular Biology, AMC Cancer Research Center, Denver, Colorado 80214
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ABSTRACT: Rapid withdrawal of estrogen from immature chicks, previously stimulated with the hormone, results in the inhibition of transcription of mRNAs of egg white proteins, rapid degradation of existing estrogen-induced mRNAs of egg white proteins, and decline in ribosomes and weight of the oviduct. On rapid withdrawal of estrogen, ovalbumin mRNA decreased to 65% after 3 h and was not detected after 24 h. In contrast to ovalbumin mRNA, cellular RNA content remained unchanged at 3 h and subsequently decreased to 51% of the stimulated value by 48 h. To study the mechanism of rapid degradation of RNA during estrogen withdrawal, the role of 2-5A- $[p_x(A2'p)_nA; x = 2 \text{ or } 3, n \geq 2]$ dependent RNase was investigated. The effect of 2-5A-dependent RNase on the stability of RNA in vitro was determined by incubating oviduct polysomes with 2-5A-dependent RNase and exogenous 2-5A. Ovalbumin mRNA was degraded more rapidly than β-actin mRNA and rRNA, and the kinetics of RNA degradation were very similar to those observed in vivo. Levels of 2-5A in the chick oviduct increased shortly after estrogen withdrawal. Analysis of the oviduct RNA revealed that a distinct 18S rRNA derived fragment, 450 nucleotides in length, increased at 6 h after withdrawal and at subsequent time points when significant degradation of total cellular RNA was occurring. The 18S rRNA derived degradation product observed in vivo from the chick oviduct had the same mobility in denaturing agarose gels as the 18S rRNA cleavage product liberated on incubation of isolated oviduct ribosomes with purified 2-5A-dependent RNase and exogenous 2-5A. These results indicate that in the chick oviduct 2-5A-dependent RNase is activated and may be involved in the degradation of mRNA and rRNA during estrogen withdrawal.

Estrogen administration to immature chicks results in cytodifferentiation and proliferation of tubular gland cells in the magnum portion of the oviduct. In the tubular gland cells, egg white proteins, of which ovalbumin is the major constituent, are synthesized (Schimke et al., 1975; O'Malley et al., 1977; Palmiter et al., 1977). The oviduct increases steadily in weight from 20-30 mg to 0.5-0.75 g after 10 days of estrogen administration at which time ovalbumin synthesis constitutes 50-65% of protein synthesis in the oviduct. Continuous presence of estrogen is needed for sustained synthesis of egg white proteins. Discontinuation of estrogen administration (chronic withdrawal) leads to a gradual decline in

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ovalbumin synthesis and regression of the oviduct to 50–100 mg over a period of 2–3 weeks. synthesis of egg white proteins and growth of the oviduct can be reinduced (secondary stimulation) by the administration of not only estrogens but also progesterone (Schimke et al., 1975; O'Malley et al., 1977; Palmiter et al., 1977), glucocorticoids (Hager et al., 1980), carcinogens (Sharma et al., 1976; Sharma & Borek, 1977; Sharma, 1978), or tumor promoter (Sharma & Kerr, 1979). However, the chick oviduct responds only to estrogens as a primary stimulant.

Rapid (acute) withdrawal of estrogens from circulation by experimental manipulation results in the inhibition of transcription of mRNAs of egg white proteins (McKnight & Palmiter, 1979; Swaneck et al., 1979), rapid degradation of existing hormone-induced mRNAs (Palmiter & Carey, 1974; Cox, 1977; Hynes et al., 1979; Shepherd et al., 1980), and decline in ribosomes and weight of the oviduct. Ovalbumin mRNA is barely detectable following 24 h of acute estrogen withdrawal. The half-life of ovalbumin mRNA of at least 24 h in hormonally stimulated chicks (Palmiter, 1973; Harris et al., 1975) is reduced to 2–6 h during acute estrogen withdrawal (Palmiter & Carey, 1974; Cox, 1977; Hynes et al., 1979; Shepherd et al., 1980). Thus, estrogens not only induce transcription of specific mRNAs but also confer stability to the induced mRNAs (Palmiter & Carey, 1974; Cox, 1977; Hynes et al., 1979; Shepherd et al., 1980; Wiskocil et al., 1980; Brock & Sharipo, 1983). In spite of the rapid degradation of estrogen-induced mRNAs, the tubular gland cells in the withdrawn chick oviduct are functionally stable. Restimulation with estrogen induces rapid transcription of mRNA for ovalbumin and other egg white proteins. In other systems, glucocorticoid (Vannice et al., 1984) and prolactin (Guyette et al., 1979) also regulate mRNA stability.

How the rapid removal of estrogen results in the accelerated degradation of RNA and tissue regression is not understood. We investigated whether the rapid degradation of RNA during acute estrogen withdrawal is due to the 2–5A[1]-dependent RNase which is known to mediate the effect of 2–5A on RNA stability [for a recent review, see Johnston and Torrence (1984)]. The enzyme which synthesizes 2–5A, 2–5A synthetase, is low or undetectable in the oviduct after stimulation with estrogen but increases dramatically during estrogen withdrawal (Stark et al., 1979). The 2–5A-dependent RNase is, therefore, a likely candidate to account for the rapid degradation of estrogen-induced mRNAs. 2–5A synthetase requires double-stranded RNA for activation and converts ATP into a series of 2′–5′-linked oligoadenylates (2–5A). At nanomolar concentration of the 5′-di- or 5′-triphosphorylated forms, the 2–5A molecules having three or more adenylate residues activate a latent RNase (Kerr & Brown, 1978). The activated RNase degrades RNA and consequently inhibits protein synthesis. 2–5A is rapidly degraded by cellular enzymes (Johnston & Torrence, 1984); therefore, its activation of the nuclease is transient.

The 2–5A system is an important component of the antiviral activity of interferon (Williams et al., 1979; Nilsen et al., 1982; Benavente et al., 1984; Goswami & Sharma, 1984; Watling et al., 1985). Changes in the level of 2–5A synthetase and 2–5A-dependent RNase under altered hormonal status (Stark et al., 1979; Krishnan & Baglioni, 1980), cell growth (Stark et al., 1979), and differentiation (Krause et al., 1985) have led to the suggestion that 2–5A has a wider role in hormone action and cell differentiation. However, direct evidence for the involvement of 2–5A-mediated RNA degradation during altered hormonal status or growth conditions is lacking. Degradation of rRNA and viral RNA by 2–5A-dependent RNase occurs during interferon-induced growth inhibition of both RNA- and DNA-containing viruses (Williams et al., 1979; Nilsen et al., 1982; Benavente et al., 1984; Goswami & Sharma, 1984; Watling et al., 1985). rRNA from interferon treated virus infected cells is degraded into characteristic products. A similar characteristic pattern of rRNA degradation is produced in vitro on incubation of ribosomes with cell-free extracts from interferon-treated cells in the presence of exogenous 2–5A. The characteristic cleavage of rRNA has been used as an indicator of the activation of 2–5A-dependent RNase (Silverman et al., 1983). However, cleavage of rRNA into characteristic products has been previously demonstrated for RNA isolated from cells in culture and not from tissues.

Experimental Procedures

Chicks and Hormone Treatment. Four to five day old female white leghorn chicks were given primary estrogen stimulation by injection intramuscularly of 1 mg of 17β-estradiol in sesame oil daily for 10 days (Sharma et al., 1976). After 3–4 weeks, a period defined as chronic estrogen withdrawal, chicks were given secondary estrogen stimulation by implanting a silastic tube containing 50 mg of diethylstilbestrol (Research Plus, Inc., Bayonne, NJ). Acute withdrawal of estrogen was accomplished by surgically removing the implant. At specified times after acute estrogen withdrawal, animals were sacrificed, and the oviduct was excised. The magnum portion of the oviduct was freed from connective tissue, quickly frozen in liquid nitrogen, and stored at −80 °C. Subsequently, frozen oviduct tissue from five to six animals was powdered under liquid nitrogen and used for the isolation of RNA and 2–5A.

Isolation of RNA. RNA was isolated from 0.5 g of powdered tissue according to Chirgwin et al. (1979) with some modification. Briefly, tissue was homogenized at 4 °C in 8 mL of guanidinium thiocyanate buffer solution with a Polytron homogenizer (speed setting of 6). Homogenate was layered on 4.0 mL of 5.7 M cesium chloride in 0.025 M sodium acetate (pH 5.0) and centrifuged at 20 °C for 20 h at 198000g in a Beckman SW41 rotor. The supernatant was aspirated to the interphase, and the sides of the tube were rinsed twice with ethanol. The cesium chloride solution was carefully aspirated without disturbing the pellet. To avoid contamination from nuclease, the bottom of the tube was cut and the pellet suspended in 3.0 ml of 6.5 M buffered guanidine hydrochloride solution. RNA was precipitated by the addition of 75 μL of 1 N acetic acid and 0.5 volume of ethanol. The pellet was extracted 3 times with 0.5 mL of water. The supernatant fractions were pooled, mixed with 0.1 volume of 2 M potassium acetate (pH 5.0) and 2.5 volumes of ethanol, and stored overnight at −20 °C. The precipitated RNA was collected by centrifugation.

Electrophoresis of RNA and Northern Analysis. A total of 10 μg of total RNA was denatured with methylmercuric hydroxide (5 mM) and electrophoresed on a 1.8% agarose slab gel under denaturing conditions with 5 mM methylmercuric hydroxide (Bailey & Davidson, 1976). The gel was stained with 0.5 μg/mL ethidium bromide and photographed under UV light. The resolved RNA was transferred to Zeta probe membrane (Bio-Rad Laboratories, Richmond, CA) by electroblotting in TAE (10 mM Tris-HCl, pH 7.8, 5 mM sodium acetate, 0.5 mM EDTA) at 150 mA for 17–24 h. The mem-

[1] Abbreviations: 2–5A, $p_x(A2'p)_nA$ ($x = 2$ or 3; $n \geq 2$); 2–5A (core), $(A2'p)_nA$; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

brane was washed in TAE and dried in a vacuum oven at 80 °C for 24 h. The membrane was washed in 0.5% SDS–0.1× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 1 h at 65 °C and prehybridized overnight at 65 °C in 5× SSC, 50 mM sodium phosphate, pH 6.5, 10× Denhardt's solution [1× Denhardt's solution contained bovine serum albumin, Ficoll, and poly(vinylpyrrolidone) at a concentration of 0.02% each], and 1 mg/mL yeast tRNA. Hybridization was carried out at 65 °C for 18–24 h in 5× SSC, 20 mM sodium phosphate, pH 6.5, 2× Denhardt's solution, 0.1 mg/mL yeast tRNA, and 2.5×10^6 cpm/mL heat-denatured DNA probe made radioactive with [α-^{32}P]dCTP by nick translation (Rigby et al., 1977). The membranes were washed in 2× SSC–0.1% SDS for 2 h at room temperature and in 0.1× SSC–0.1% SDS for 1 h at 55 °C and exposed to Kodak XAR-5 X-ray film at −80 °C with Cronex lightning plus intensifying screens (Du Pont).

Isolation and Quantitation of 2–5A. Powdered tissue (0.5 g) was homogenized in 10 volumes of 10% trichloroacetic acid in a Polytron homogenizer and centrifuged at 10000*g* for 10 min. The supernatant was extracted twice with an equal volume of a mixture containing 11 parts of tri-*n*-octylamine and 39 parts of 1,1,2-trichlorotrifluoroethane (Hersch et al., 1984) and desalted with a SEP-PAK C_{18} (Waters Assoc., Milford, MA) cartridge. 2–5A was eluted with 50% methanol. The eluate after lyophilization was dissolved in water. 2–5A was quantitated by a sensitive and specific functional assay (Silverman, 1985). 2–5A-dependent nuclease was isolated by affinity binding onto 2–5A (core)–cellulose. 2–5A is the only known activator of this enzyme. Activation of RNase by exogenous 2–5A was measured by hydrolysis of radioactive poly(U). We routinely observed 50% hydrolysis of poly(U) by 0.2–0.25 nM exogenous 2–5A tetramer [p_3(A2′p)$_3$A, P-L Biochemicals]. Incubation of sample with poly(U)–[5′-^{32}P]Cp in the absence of enzyme did not result in significant degradation of poly(U). Degradation of poly(U) was solely dependent on the addition of exogenous 2–5A tetramer. Recovery of exogenous 2–5A tetramer was 35–40%.

Isolation of Oviduct Ribosomes (Polysomes). Ribosomes were isolated by a magnesium precipitation technique (Palmiter, 1974) from frozen oviduct tissue from chicks given secondary estrogen stimulation for 3 days. The ribosomes were pelleted through a cushion of 1 M sucrose in 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, and 5 mM $MgCl_2$ (1.0 PB) by centrifugation in a SW41 rotor at 27000*g* for 10 min. The supernatant was removed by aspiration along with one-fourth of the sucrose pad. The upper portion of the tube was washed with water and the remaining sucrose pad removed by aspiration. The tube was cut close to the bottom, and the pellet was suspended in 4 mL of 20 mM Hepes (pH 7.5) and centrifuged at 13000*g* for 10 min to remove aggregated material. The ribosomes were reprecipitated from the supernatant by the addition of $MgCl_2$ to 0.2 M, pelleted through 0.2 M sucrose cushion (0.2 PB), suspended in 0.25 M sucrose, 0.05 mM Tris-HCl, 1 mM dithiothreitol, and 0.1 mM EDTA (pH 7.5), and stored frozen in aliquots at −20 °C.

Cleavage of RNA in Vitro by 2–5A-Dependent RNase. Oviduct tissue from 3-day secondary estrogen stimulated chicks was homogenized in 10 volumes of lysis buffer (Silverman, 1985) with a Polytron homogenizer and centrifuged at 15000*g* for 10 min. The supernatant was centrifuged at 100000*g* for 60 min, and the high-speed supernatant was used as the source of 2–5A-dependent RNase. Supernatant (1 mg of protein) was incubated at 0–4 °C for 60 min with 6.25 nmol (AMP) equiv of 2–5A (core)–cellulose in 2.6 mL of 11.5 mM Hepes (pH 7.6), 104 mM KCl, 5.8 mM magnesium acetate, 8.8 mM 2-mercaptoethanol, 1.2 mM ATP, and 100 μg/mL leupeptin (buffer A). After four washings with buffer A, 2–5A (core)–cellulose-bound enzyme was suspended in 500 μL of buffer A containing 1.15 A_{260} units of ribosomes and incubated at 30 °C. At specified time intervals, an 80-μL aliquot was withdrawn and treated with 3.2 μL of 10% SDS and 5.3 μL of proteinase K (10 mg/mL). After incubation at 30 °C for 30 min, the suspension was clarified by centrifugation at 10000*g* for 5 min. Unless specified, 10 μL of the supernatant was analyzed for specific RNA sequences by Northern blot hybridization technique.

Determination of RNA and DNA Content. Nucleic acid content was determined by precipitating aliquots from a 4% homogenate of oviduct tissue in water with equal volumes of 1 N perchloric acid at 0–4 °C. The precipitate was washed 3 times with 0.5 N perchloric acid at 0–4 °C, hydrolyzed in 0.1 N NaOH for 10 min in a boiling water bath, and chilled in ice, and perchloric acid was added to a final concentration of 0.5 N. The pellet was collected by centrifugation, and the supernatant was assayed for RNA content by the orcinol method (Schneider, 1957). The pellet was extracted in boiling water with 1 N perchloric acid for 20 min, clarified by centrifugation at 1500*g* for 5 min, and the supernatant was analyzed for DNA content by the diphenylamine procedure (Schneider, 1957).

Results and Discussion

Three to four weeks after primary estrogen stimulation, at which time synthesis of ovalbumin mRNA in oviduct was not detectable, chicks were given secondary estrogen stimulation for 3 days. After the secondary estrogen stimulation, the implants were removed, and the progress of withdrawal was determined by incubating oviduct explants in medium containing ^{14}C-labeled amino acids and measuring ovalbumin synthesis (Sharma et al., 1976). On removal of the estrogen implant from stimulated animals (acute estrogen withdrawal), as observed by others (Palmiter & Carey, 1974; Cox, 1977; Hynes et al., 1979; Shepherd et al., 1980), ovalbumin synthesis declined rapidly from 50–56% of the total protein synthesis at 0 h to less than 5% in 12 h and was undetectable by 24 h (data not shown). Concomitant with the decline in ovalbumin synthesis following acute estrogen withdrawal, the weight of the oviduct decreased. By 48 h of estrogen withdrawal, oviduct weight declined to 43% of the stimulated value. However, it is known from earlier studies that the number of cells in the oviduct does not significantly change during the first 3 days of estrogen withdrawal as evidenced by the constant levels of DNA content (Shepherd et al., 1980). Therefore, changes in the RNA/DNA ratio can be used to determine the loss of total cellular RNA during estrogen withdrawal. The RNA/DNA ratio of 3.94 from 3-day secondary estrogen stimulated chick oviduct remained unchanged at 3 h and thereafter declined to 3.78, 2.84, 2.88, and 2.0 at 6, 12, 24, and 4, h, respectively, following estrogen withdrawal. On the basis of these results, Figure 1 shows loss of cellular RNA from the oviduct. Cellular RNA content remained unchanged at 3 h and subsequently decreased to 51% of the stimulated value by 48 h.

To analyze for the changes in steady-state levels of cellular mRNA during acute estrogen withdrawal, Northern blots of oviduct RNA were probed with cloned DNA sequences specific for ovalbumin mRNA and β-actin mRNA (Figure 1). In contrast to total cellular RNA, ovalbumin mRNA decreased to 65% after 3 h of estrogen withdrawal and further reduced to 20% at 6 h and to 3% after 12 h and was not detected after 24 h of acute estrogen withdrawal. The ap-

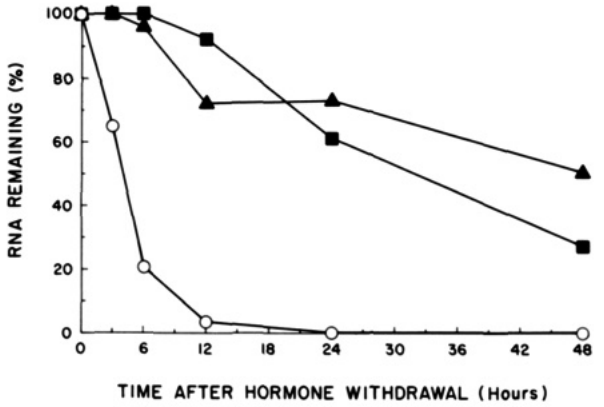

FIGURE 1: Decrease in oviduct RNA following acute estrogen withdrawal. After 10 days of primary estrogen stimulation, chicks withdrawn from estrogen for 3 weeks were given secondary estrogen stimulation for 3 days by implanting a silastic tube containing 50 mg of diethylstilbesterol. Acute estrogen withdrawal was accomplished by surgically removing the implant. RNA was isolated from pooled oviduct tissue from four to five animals. The steady-state levels of oviduct mRNA (○) and β-actin mRNA (■) were determined by Northern blot hybridization using excess of ^{32}P-labeled DNA probe pOV230 for ovalbumin (McReynolds et al., 1977) and PA 1 for β-actin (Cleveland et al., 1980). Autoradiograms were quantitatively evaluated by scanning with a soft laser densitometer. Loss in total RNA (▲) was calculated from RNA/DNA ratio. All values were normalized to 0 h of estrogen withdrawal.

Table I: Changes in Oviduct Magnum Weight and 2-5A Levels during Acute Estrogen Withdrawal[a]

acute estrogen withdrawal (h)	weight of oviduct magnum (g)	2-5A levels (nM)
0	1.5	0.7
6	1.02	1.3
12	0.79	2.5
24	0.89	3.0
48	0.64	1.5

[a] At the hours indicated, the animals were sacrificed, and the level of 2-5A was determined in oviduct with the 2-5A (core)-cellulose assay (Silverman, 1985). 2-5A was isolated from pooled tissue from four to seven animals for each time point, and 2-5A levels represent the mean of duplicate determinations. The recovery of exogenous $p_3(A2'p)_3A$ was 35-40%. The 2-5A concentrations are not corrected for the losses during isolation and are based on the weight of tissue from which extracts were made. The oviduct weight represents the mean of three to eight animals.

parent half-life of ovalbumin mRNA during acute estrogen withdrawal was 4 h. Ovalbumin mRNA migrated as a single band, and no distinct ovalbumin-specific RNA bands migrating below the region of ovalbumin mRNA were detected in RNA from the oviduct of withdrawn chicks.

Decay of actin mRNA was markedly different from that of ovalbumin mRNA and somewhat similar to that for the total cellular RNA content for the first 3 h after withdrawal. Unlike cellular RNA; however, actin mRNA remained unchanged until 6 h after hormone withdrawal and thereafter gradually declined (Figure 1). At 12 h, 30% of the total RNA was degraded whereas actin mRNA decreased by 8%. At 48 h, the decrease in actin mRNA content was 50% greater than that for total cellular RNA.

2-5A in the chick oviduct was quantitated by a sensitive and specific functional assay (Silverman, 1985). Low levels of 2-5A (Table I) were detected, in oviduct from chicks given secondary estrogen stimulation for 3 days. 2-5A content increased approximately 2-fold at 6 h and 4-fold by 24 h and then decreased at 48 h after acute estrogen withdrawal. Kerr et al. (1983) have detected 2-5A in the chick oviduct using HPLC fractionation, radioimmune, radiobinding, and RNase

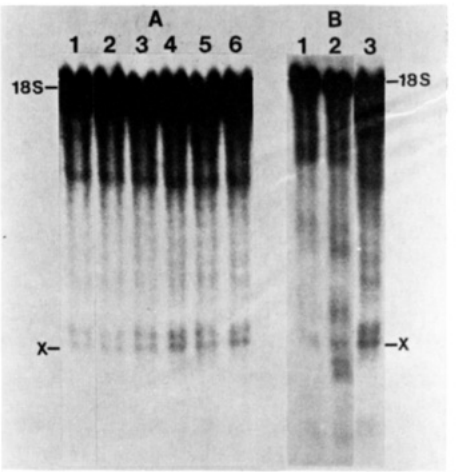

FIGURE 2: Accumulation of 2-5A-dependent RNase specific breakdown product during acute estrogen withdrawal. A total of 10 μg of oviduct RNA was electrophoresed on a 1.8% vertical agarose slab gel containing 5 mM methylmercuric hydroxide (Bailey & Davidson, 1976). RNA was electrophoresed at 50 V until the dye front migrated 30 cm. After electrophoresis, the gel was stained with ethidium bromide and photographed. The top part of the gel slightly above the 18S rRNA was excised, and from the remaining gel, the RNA was electroblotted on to Zeta probe membrane and probed with mouse 18S rRNA specific DNA, 25-46 (Arnheim, 1979). Panel A: oviduct RNA from 3-day secondary stimulated chicks (lane 1); RNA from chicks withdrawn from estrogen at 3 (lane 2), 6 (lane 3), 12 (lane 4), 24 (lane 5), and 48 h (lane 6). Panel B: purified ribosomes from 3-day estrogen-stimulated chicks were incubated with 2-5A (core)-cellulose-bound 2-5A-dependent RNase in the absence of 2-5A (lane 1) and in the presence of 25 nM $p_3(A2'p)_3A$ (lane 2) for 30 min. Oviduct RNA from chicks withdrawn from estrogen for 12 h (lane 3; same RNA as in panel A, lane 4).

activation assays. The levels of 2-5A peaked shortly after estrogen withdrawal.

Low levels of 2-5A occur in interferon-treated cells infected with reovirus (Nilsen et al., 1982) or vaccinia virus (Goswami & Sharma, 1984) under conditions when cleavage of rRNA into characteristic products is observed. Extended exposure of the Northern blots of oviduct RNA probed with 18S-specific DNA revealed that, in addition to a major 18S rRNA band, 18S rRNA fragments migrating below the 18S rRNA band were present (Figure 2A). A distinct band labeled X, 450 nucleotides in length, was present in RNA from chicks withdrawn from estrogen. The band X, faint though distinct, was similar in intensity in oviduct RNAs from 3-day estrogen-stimulated chicks (Figure 2A, lane 1) and chicks withdrawn from estrogen for 3 h (Figure 2A, lane 2). However, it increased in intensity at 6 h (lane 3) and at subsequent time points following estrogen withdrawal (lanes 4-6). The increase in intensity of band X correlated with the degradation of total RNA measured with the RNA/DNA ratio of chick oviduct. Although decrease in ovalbumin mRNA occurred soon after estrogen withdrawal (3 h), measurable increase in the degradation of total RNA, of which rRNA is a major constituent, was first observed at 6 h following estrogen withdrawal. The band X was not visible when the gel was stained with ethidium bromide and could only be detected with a ^{32}P-labeled 18S probe. This suggests that band X is an unstable intermediate product formed during RNA degradation which does not accumulate to significant concentrations. To ascertain whether this band X is cleaved from 18S rRNA by 2-5A-dependent RNase, the following experiment was conducted.

Oviduct polysomes and 2-5A-dependent RNase were isolated from chronic withdrawn chicks given secondary estrogen stimulation for 3 days. For these experiments, it was necessary to use polysome preparations devoid of endogenous nucleases.

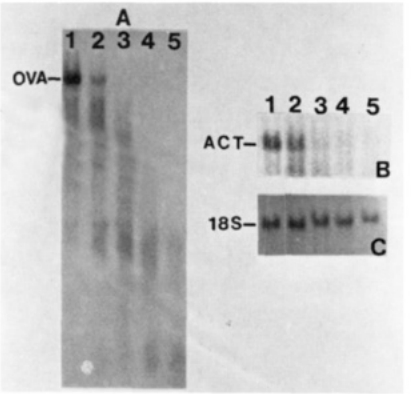

FIGURE 3: Degradation of RNA in vitro by 2–5A-dependent RNase. Purified oviduct polysomes from 3-day estrogen-stimulated chicks were incubated at 30 °C with 2–5A (core)–cellulose-bound 2–5A-dependent RNase and 25 nM $p_3(A2'p)_3A$; 10-μL aliquots (panels A and B) at 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), and 2 h (lane 5) were analyzed by Northern hybridization after digestion with proteinase K. Panel A, ovalbumin mRNA; panel B, β-actin mRNA. For 18S rRNA, panel C, 1-μL aliquots were analyzed to ensure an excess of 18S-specific probe DNA.

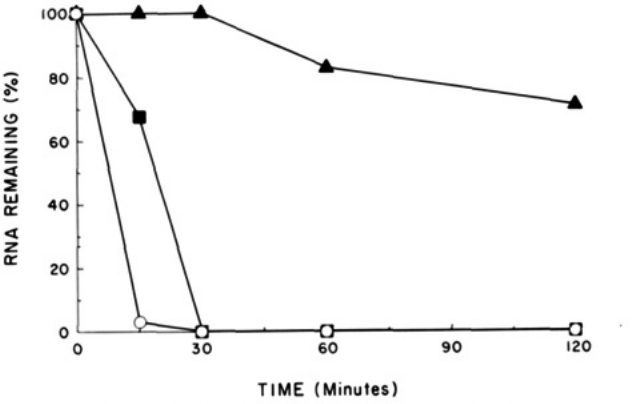

FIGURE 4: Kinetics of RNA degradation in vitro. RNA was quantitated from Figure 3 and was normalized to controls [ribosomes and 2–5A (core)–cellulose-bound enzyme incubated in the absence of exogenous 2–5A]. Ovalbumin mRNA (○), β-actin mRNA (■), and 18S rRNA (▲). RNA content at 0 min used as 100%.

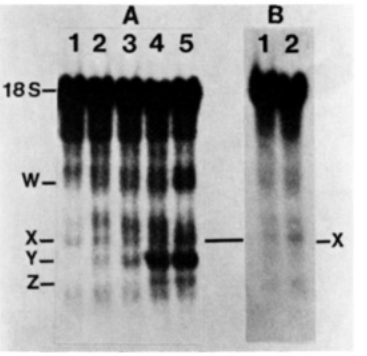

FIGURE 5: Generation of 18S rRNA degradation products in vitro by 2–5A-dependent RNase. Panel A: Details are the same as for Figure 3 (panel C), except that the autoradiograph was exposed for a longer period to show the appearance of 18S rRNA specific breakdown products with an increase in time of incubation with activated 2–5A-dependent RNase. For comparison, RNA isolated from chick oviduct from 3-day stimulated chicks (panel B, lane 1) and 12 h acute estrogen withdrawn chicks (panel B, lane 2) is included. Increase in the 2–5A-dependent RNase specific cleavage product observed in vivo is indicated by X. The gel in panel B is similar to the gel in Figure 2 (panel B) except that RNA was resolved on a 13-cm gel. Additional 18S rRNA specific degradation products W, Y, and Z are evident in lanes 2–5 (panel A).

The presence of contaminating nucleases was determined by incubating polysomes containing ovalbumin mRNA in buffer A for 2 h at 30 °C and probing for the integrity of mRNA. Preparations of oviduct polysomes with minimal degradation of ovalbumin mRNA were used. Polysomes and the 2–5A (core)–cellulose-bound enzyme were incubated with or without exogenous 2–5A. At the end of the incubation, RNA was resolved by electrophoresis on denaturing agarose gels and probed for 18S rRNA specific sequences (Figure 2B). The RNA from incubation containing polysomes and 2–5A-dependent RNase in the absence of exogenous 2–5A showed few minor bands in addition to the major 18S band (Figure 2B, lane 1). When exogenous 2–5A was added to the incubation, there was an increase in the intensity of faint bands and distinct additional bands were detected (lane 2) indicating that they are derived from 18S rRNA by 2–5A-dependent RNAse. A similar degradation of 18S rRNA was observed when rRNA instead of polysomes was incubated with 2–5A-dependent RNase and 2–5A (data not shown). The band X in Figure 2B (lane 2) had the same mobility on the gel as the band X present in chick oviduct RNA, Figure 2B (lane 3) and Figure 2A. The inability to detect RNA fragments corresponding to other bands derived from 18S rRNA in vitro in the presence of exogenous 2–5A (Figure 2B, lane 2) with mobilities similar to 18S rRNA fragments generated in vivo could be due to their degradation by the 2–5A-independent nucleases. Alternately, these fragments could be the 2–5A-dependent RNase cleavage products liberated from purified polysomes which are masked in vivo. The increase in the 2–5A-dependent nuclease specific product of 18S rRNA at a time when degradation of cellular RNA (of which 18S rRNA is a major component) was occurring together with an increase in 2–5A levels indicates that rRNA turnover may be mediated by the 2–5A system during acute estrogen withdrawal.

The effect of 2–5A-dependent RNase on the stability of RNA in vitro was determined by incubating purified oviduct polysomes from 3-day estrogen-stimulated chicks with 2–5A-dependent nuclease and 2–5A. Intact ovalbumin, β-actin, and 18S rRNA were quantitated by the Northern blot hybridization technique. For the quantitation of 18S rRNA, the amount of RNA resolved by gel electrophoresis was reduced by $^1/_{10}$ to ensure an excess of 18S probe DNA. Over 95% of the ovalbumin mRNA (Figure 3A) and 30% of actin mRNA (Figure 3B) were degraded during the first 15 min of incubation (compared to control samples incubated with 2–5A-dependent RNase but in the absence of exogenous 2–5A). Neither ovalbumin nor β-actin mRNA was detected after 30 min of incubation whereas significant degradation of 18S rRNA occurred only after 1 h of incubation (Figures 3C and 4). Prolonged exposure of the Northern blot hybridized with 18S rRNA specific DNA (Figure 3C) revealed the appearance of 18S-rRNA breakdown products as early as 15 min of incubation, which increased with incubation time (Figure 5A). These breakdown products at early time points (15 and 30 min) account for less than 1% of the intact 18S rRNA; therefore, the degradation of 18S rRNA was not apparent when it was assayed by quantitation of the major 18S-rRNA band. It is clear that in the in vitro system estrogen-induced ovalbumin mRNA is degraded more rapidly than β-actin mRNA transcripts by the 2–5A-dependent RNase. The kinetics of degradation of ovalbumin mRNA and actin mRNA observed in vitro are similar to those seen in vivo in that ovalbumin mRNA is degraded more rapidly than actin mRNA. The difference in the decay of ovalbumin mRNA, actin mRNA, and total cellular RNA on acute estrogen withdrawal could be due to the earlier breakdown of ovalbumin mRNA and the associated ribosomes followed by generalized

RNA degradation. Different rates of RNA synthesis could also contribute to this phenomenon.

Little is known about the enzymes and factors involved in the degradation of RNA (Brawerman, 1987; Raghow, 1987; Ross et al., 1987). Unique sequences in mRNAs have been implicated as determinants of their stability (Shaw & Kamen, 1986). Increased accumulation of the 2-5A-dependent RNase specific product at 6 h and at later times when degradation of cellular RNA was occurring indicates that in the chick oviduct 2-5A-dependent RNase may be involved in the degradation of rRNA induced by estrogen withdrawal. The presence of low but detectable levels of 2-5A and the 2-5A-dependent RNase specific cleavage product in estrogen-stimulated oviduct RNA suggests that this nuclease may be involved in the normal turnover of rRNA. It is tempting to implicate 2-5A-dependent RNase in rapid and preferential degradation of ovalbumin mRNA; however, in the absence of a characteristic degradation product of ovalbumin mRNA and any increase in the 2-5A-dependent RNase specific breakdown product of 18S rRNA (band X) in vivo at 3 h following estrogen withdrawal when 35% of ovalbumin mRNA was degraded, convincing evidence for the involvement of 2-5A-dependent RNase in the rapid degradation of ovalbumin mRNA is lacking. It would be of great value to be able to selectively activate or shut off the functioning of the 2-5A system in the oviduct. A potentially useful approach is the use of activators or inhibitors of the 2-5A-dependent RNase pathway, which is currently under investigation in our laboratory.

Localized activation of RNase (Baglioni et al., 1984) has been proposed for the selective degradation of RNA by 2-5A-dependent RNase. Two distinct species of 2-5A synthetase have been found in nuclei and cytoplasm from various sources (Johnston & Torrence, 1984) including chick erythrocytes (Sokawa, 1984). 2-5A synthetase (Dougherty et al., 1980; Yang et al., 1981) and 2-5A-dependent RNase (Nilsen et al., 1981) are also associated with ribosomes. Unlike the interferon-treated cells, where the viral RNA is the most likely activator of 2-5A synthetase, the nature of the activator in chicks is not known.

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A Monomer–Dimer Model Explains the Results of Radiation Inactivation: Binding Characteristics of Insulin Receptor Purified from Human Placenta†

Yoko Fujita-Yamaguchi* and Joan T. Harmon‡

Department of Molecular Genetics, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010, and Molecular Biology Laboratories, Jerome H. Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, Maryland 20855

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ABSTRACT: The technique of radiation inactivation has been used on highly purified human placental insulin receptor in order to determine the functional molecular size responsible for the insulin binding and to evaluate the "affinity regulator" hypothesis, which has been proposed to explain the increase in specific insulin binding to rat liver membranes observed at low radiation doses [Harmon, J. T., Hedo, J. A., & Kahn, C. R. (1983) *J. Biol. Chem. 258*, 6875–6881]. Three different types of inactivation curves were observed: (1) biphasic with an enhanced binding activity after exposure to low radiation doses, (2) nonlinear with no change in binding activity after exposure to low radiation doses, and (3) linear with a loss in the binding activity with increasing radiation exposures. A monomer–dimer model was the simplest model that best described the three types of radiation inactivation curves observed. The model predicts that an increase in insulin binding activity would result after exposure to low radiation doses when the initial dimer/monomer ratio is equal to or greater than 1 and a monomer is more active than a dimer. The monomer size of the binding activity was estimated to be 227 000 daltons by this model. This value most likely reflects the size of the monomeric $\alpha\beta$ form. To substantiate this model, the purified receptor was fractionated by Sepharose CL-6B chromatography. The insulin binding profile of this column indicated two peaks. Further studies revealed the following: (i) peak I ($\alpha_2\beta_2$-rich) and peak II ($\alpha\beta$-rich) receptors showed curvilinear Scatchard plots and straight Scatchard plots, respectively, and (ii) specific activity of the peak I receptors was estimated to be 26% of that of the peak II receptors under our standard conditions [50 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4]. These studies suggest that the affinity regulator does not exist as a separate structural protein but is due to the dimeric form of the receptor. The dimeric form ($\alpha_2\beta_2$) possesses a much lower specific activity for insulin binding than does the monomeric $\alpha\beta$ form (under the standard conditions), but the dimeric structure is necessary to observe the negative cooperative binding isotherm.

The insulin receptor is a membrane glycoprotein that is responsible for transferring the signal from the exterior to the interior of target cells and leading to insulin-dependent biological actions (Kahn et al., 1981). The structure of the receptor has been studied by a variety of techniques. These studies have indicated that the basic insulin receptor unit is composed of two α subunits and two β subunits in a β–α–α–β form (Jacobs et al., 1979; Maturo & Hollenberg, 1978; Yip et al., 1980; Pilch & Czech, 1980; Czech et al., 1981; Baron & Sonksen, 1983).

The technique of radiation inactivation can be used to ascertain the size of the functional unit, for example, the insulin binding site, corresponding to a given biological activity, insulin binding. When high-energy radiation causes ionization in a

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* Correspondence should be addressed to this author at the Beckman Research Institute of the City of Hope.
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