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DOWN REGULATION OF C-MYC, C-FOS AND ERB-B DURING ESTROGEN INDUCED PROLIFERATION OF THE CHICK OVIDUCT

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Oncogenes c-myc, H-ras, c-fos and erb-B were constitutively expressed in immature chick oviduct withdrawn from estrogen administration for 2.5 weeks after 10 d of primary estrogen stimulation. Following secondary estrogen stimulation of the withdrawn chicks, synthesis of egg white proteins is rapidly induced and remaining non-functioning tubular gland cells are stimulated to proliferate with a doubling time of 24 h. During first 12 h of secondary estrogen stimulation, H-ras mRNA levels doubled and did not increase further at 24 h and 48 h. The steady state levels of c-myc, erb-B and c-fos mRNA decreased 24 h following secondary estrogen stimulation. The levels of these oncogene RNAs in oviduct were similar at 48 h following secondary estrogen stimulation to those from immature chicks administered 10 d of primary estrogen stimulation. Thus elevated expression of c-myc and c-fos mRNA does not appear to be necessary components for sustained estrogen induced cell proliferation in the chick oviduct.

Administration of steroid hormones to immature chicks induces differentiation and growth of the oviduct (1, 2). Estrogens are required for the initial induction of the primitive oviduct. Its administration results in the cytodifferentiation and proliferation of tubular gland cells in the magnum of the oviduct. The oviduct increase in weight from 20-30 mg to 500-750 mg after 10 d of daily estrogen stimulation and synthesis of egg white proteins is induced in the tubular gland cells. Following estrogen administration, ovalbumin constitutes 50-65% of the oviduct protein synthesis. Discontinuation of estrogen administration results in a gradual decline in ovalbumin synthesis and tubular gland cells, and regression of the oviduct over a period of 2-3 weeks. The synthesis of ovalbumin can be reinduced (secondary stimulation) by estrogens, progesterone (1, 2) and several carcinogens (3-5) or tumor promoter (6). Following secondary estrogen stimulation, synthesis of egg white proteins is rapidly induced and remaining non-functioning tubular gland cells in the withdrawn oviduct are stimulated to proliferate. Ovalbumin synthesis rapidly approaches to the levels observed in the primary estrogen stimulated oviduct. Again, discontinuation of the secondary stimulant results in the cessation of egg white protein synthesis, rapid degradation of the induced mRNAs, ribosomes and regression of the oviduct. Thus estrogens not only induce transcription of specific mRNA (7, 8), but also confer stability

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to the induced RNAs (7, 9). The gene products of specific cellular oncogenes have been implicated to play a pivotal role in the regulation of cell proliferation and differentiation (Reviewed in Ref. 10). Induction of c-fos and c-myc oncogene expression is one of the early transcriptional events which occurs during stimulation of cells in culture by many growth factors and differentiating agents. Oncogene expression is modulated during embryonic and fetal development (10, 11). The controlled response of the chick oviduct to steroid hormones provides a good system for studying the molecular events leading to cellular differentiation and proliferation.

We describe here that during estrogen induced rapid increase in ovalbumin mRNA and proliferation of the estrogen primed chick oviduct, levels of c-myc, c-fos and erb-B are down regulated, whereas levels of H-ras RNA increase during estrogen stimulation.

MATERIALS AND METHODS

Primary estrogen stimulation was accomplished in 4-5 d old female white leghorn chicks by daily injecting intramuscularly 1 mg 17-β-estradiol (Sigma) in sesame oil for 10 d. After 2.5 weeks following discontinuation of estrogen stimulation, a period defined as chronic estrogen withdrawal, at which time ovalbumin mRNA in oviduct was not detectable, chicks were given secondary estrogen stimulation by a single intramuscular injection of 2 mg 17-β-estradiol in sesame oil. At various times following secondary stimulation, chicks were sacrificed. The magnum portion of the oviduct was freed from connective tissue, weighed, quick frozen in liquid nitrogen and stored at -80°C.

Oviduct tissue from 5-6 animals was powdered under liquid nitrogen and used for RNA isolation according to Chirgwin et al. (12).

Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose (13).

10 μg of poly(A)$^+$ RNA was denatured in 5 mM methyl mercuric hydroxide and electrophoresed on 1.8% agarose gels containing 5 mM methyl mercuric hydroxide. The resolved RNA was transferred to Zeta probe membranes (Bio-Rad Laboratories, Richmond, CA) by electroblotting in TAE (10 mM Tris-HCl, pH 7.8, 5 mM sodium acetate and 0.5 mM EDTA) at 150 mA for 17 to 24 h. The membranes were washed in TAE and dried in a vacuum oven at 80°C for 2 h. For prehybridization, the membranes were washed in 0.5% SDS, 0.1x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) for 1 h at 65°C, prehybridized overnight at 65°C in 5x SSC, 50 mM sodium phosphate, pH 6.5, 10x Denhardt's reagent (1x Denhardt's reagent contained bovine serum albumin, Ficoll and polyvinylpyrrolidone each at a concentration of 0.02%) and 1 mg/ml yeast tRNA. Hybridization was carried out at 65°C for 18-24 h in 5x SSC, 20 mM sodium phosphate, pH 6.5, 2x Denhardt's reagent, 0.1 mg/ml yeast tRNA and 2.5 x 10^6 cpm/ml of heat denatured probe DNA in vitro radiolabeled with [α-^{32}P]dCTP by nick translation (14). The membranes were washed in 2x SSC, 0.1% SDS for 2 h at room temperature and 0.1 x SSC, 0.1% SDS for 1 h at 55°C. The membrane was exposed to Kodak XAR-5 x-ray film at -80°C using Cronex lightning plus intensifying screens (Dupont). Following autoradiography, the membranes were stripped of labeled probe by two changes of 0.1x SSC, 0.1% SDS at 95°C for 20 min each and autoradiographed to depict the extent of probe removal. In some cases, it was necessary to store the membranes until the residual isotope had decayed sufficiently so as not to interfere with subsequent hybridizations. Molecularly cloned DNA probe pOV230 for ovalbumin (15) and PA 1 for β-actin (16) were kindly provided by Drs. B. W. O'Malley and M. J. Tsai, and Dr. D. W.

Cleveland, respectively. Probes for oncogenes were obtained from Oncor (Gaithersburg, MD).

RESULTS AND DISCUSSION

Primary estrogen stimulation of immature chicks for 10 d resulted in an increase in oviduct weight from 20 mg to 600 mg. On discontinuation of estrogen administration, the oviduct weight diminished to 250 mg after 2.5 weeks (chronic estrogen withdrawal). Following secondary estrogen stimulation by a single injection of 2 mg of estradiol to chicks which had been withdrawn from primary estrogen stimulation for 2.5 weeks, there was a gradual increase in the weight of the oviduct: 2-fold at 24 h and over 3-fold at 48 h (Table 1). The enhancement in the weight of the oviduct by estrogen administration is not simply due to increased uptake of water and amino acids since secondary estrogen stimulation is known to result in significant rise in the mitotic index between 12 and 24 h (17) and increase in DNA synthesis and rapid proliferation of the tubular gland cells in the oviduct with a doubling time of approximately 24 h (18, 19). Ovalbumin mRNA was barely detected in withdrawn chicks. After secondary estrogen stimulation, rapid accumulation of

Table 1. mRNA Levels in the Chick Oviduct During Estrogen Stimulation

Estrogen Treatment	Weight of Oviduct Magnum (mg)	mRNA Levels, % of Control					
		Ovalbumin	β-Actin	H-ras	c-myc	c-fos	erb-B
Secondary Stimulation (h)							
0	255 ± 66[a]	0	29	100	100	100	100
6	295 ± 54	5	64	95	94	110	89
12	343 ± 61	29	83	184	107	96	96
24	514 ± 71	85	100	189	73	15	5
48	820 ± 143	100	100	176	57	5	14
Primary Stimulation (10 d)	573 ± 100	200	56	240	6	0	19

[a] mean ± SD

Immature chicks were given primary estrogen stimulation (daily intramuscular injection of 1 mg 17-β-estradiol for 10 d) or secondary estrogen stimulation (one injection of 2 mg of 17-β-estradiol). Northern blots of oviduct poly(A)$^+$ RNA (10 μg) were probed for ovalbumin, β-actin (Fig. 1) or oncogene transcripts (Fig. 2). The autoradiograms were quantitated using a laser densitometer. Ovalbumin and β-actin mRNA levels at 48 h served as controls and were assumed to be 100%. The levels of oncogene specific mRNA at 0 h following secondary estrogen stimulation served as controls, and were assumed to be 100%. The two fos and erb-B related transcripts were quantitated together.

ovalbumin mRNA was evident at 6 h and rose by 6-fold at 12 h and 20-fold at 48 h (Fig. 1, Table 1). At 48 h following secondary estrogen stimulation, ovalbumin mRNA represented approximately 50% of the levels detected in chicks given 10 days of primary estrogen stimulation. The increased proliferative state of the oviduct was also seen by the increase in the relative amounts of β-actin transcripts following secondary estrogen stimulation. The steady state levels of oviduct β-actin mRNA increased over 2-fold at 6 h and 3.4-fold after 24 h of secondary estrogen stimulation compared to chicks withdrawn from primary estrogen stimulation for 2.5 weeks. Although the weight of oviduct increased further at 48 h, β-actin mRNA content did not increase after 24 h. However, as opposed to ovalbumin mRNA, β-actin mRNA levels at 24 and 48 h following secondary estrogen stimulation were 1.8 fold greater when compared to chicks receiving 10 d of primary estrogen stimulation.

Screening of oviduct poly(A)$^+$ RNA from chicks stimulated by estrogen administration with DNA probes for v-mos, v-myb, v-sis, erb-A, v-src, v-fos, erb-B, H-ras and c-myc indicated that significant levels of only v-fos, erb-B, H-ras and c-myc related transcripts were present in the oviduct. These oncogenes were constitutively expressed in the oviduct of chicks withdrawn from primary estrogen stimulation for 2.5 weeks (Fig. 2 and Table 1). The steady state levels of H-ras mRNA almost doubled by 12 h after estrogen stimulation and did not further increase at 24 and 48 h (Fig. 2, Panel A) although significant proliferation of the oviduct took place after 12 h (Table 1 and Ref. 19). The expression of c-myc, erb-B and c-fos mRNA was higher in the estrogen withdrawn chick oviduct and diminished during the course of secondary estrogen stimulation (Fig. 2, Panels B, C and D). The amount of c-fos tran-

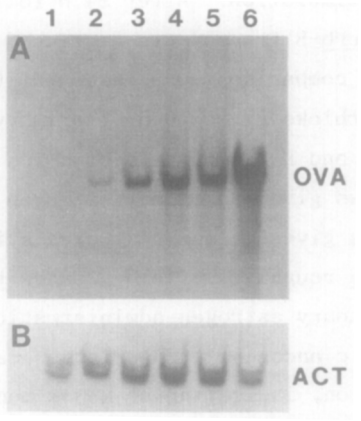

Figure 1. Increase in Ovalbumin and β-Actin mRNA Levels in the Chick Oviduct During Secondary Estrogen Stimulation. Poly(A)$^+$ RNA (10 μg) from chick oviduct was electrophoresed in denaturing agarose gels and transferred to Zeta probe membranes. The immobilized RNA was probed for: Panel A, ovalbumin using pOV 230 (15); Panel B, PA 1 for β-actin mRNA (16), and visualized by autoradiography. Lanes 1 through 5; 0, 6, 12, 24 and 48 h following secondary estrogen stimulation. Lane 6, 10 d of primary estrogen stimulation.

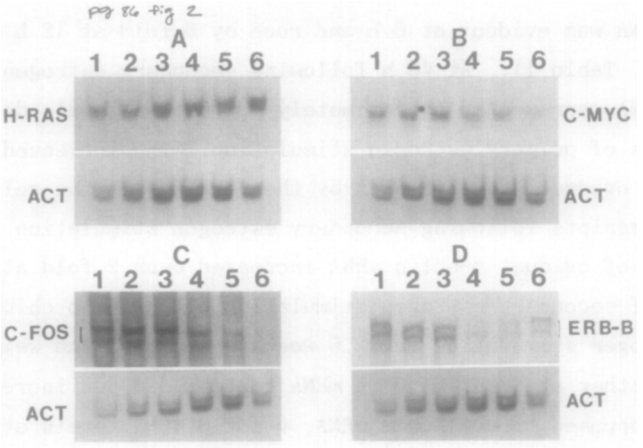

Figure 2. Steady State Levels of Oncogene mRNAs in Chick Oviduct During Secondary Estrogen Stimulation. Northern blots of poly(A)$^+$ RNA (10 µg) from estrogen stimulated chick oviducts were prepared as described in Fig. 1. The filters were probed for specific RNA using ^{32}P-labeled cloned DNA probes, and the transcripts visualized by autoradiography. Each filter was then stripped of the oncogene probe and rehybridized to β-actin DNA probe. Panel A, H-ras (20); Panel B, c-myc (21); Panel C, c-fos (22) and Panel D, erb-B (23). Lanes 1 through 5, chick oviduct poly(A)$^+$ RNA prepared at 0, 6, 12, 24 and 48 h, respectively, following secondary estrogen stimulation administered by a single injection. Lane 6, poly(A)$^+$ oviduct RNA from chicks given 10 d of primary estrogen stimulation.

scripts remained unchanged until 12 h, then decreased dramatically by 24 h; at 48 h post stimulation, barely detectable c-fos transcripts were evident (Fig. 2, Panel C). The amount of c-myc RNA also remained unaltered until 12 h post stimulation (Fig. 2, Panel B). Thereafter, the amount of c-myc RNA decreased approximately 40% of the amount present in the chick oviduct withdrawn from primary estrogen stimulation for 2.5 weeks. erb-B related mRNA also remained unchanged until 12 h post stimulation. After 24 h following secondary estrogen stimulation, the amount of erb-B transcripts decreased by 95% and then slightly increased 15% at 48 h. For comparison, expression of these oncogenes was also determined in oviduct from chicks given 10 d of primary estrogen stimulation. With the exception of c-myc and H-ras, levels of c-fos and erb-B RNA were similar to those detected from chicks given secondary estrogen stimulation for 48 h. The c-myc RNA levels from chicks given primary estrogen stimulation for 10 d were almost 10-fold lower and H-ras roughly 1.4-fold greater than from animals stimulated for 48 h by secondary estrogen administration.

The size of the specific oncogene transcripts remained unchanged during secondary estrogen stimulation, indicating no major modification in the processing of these RNA species had taken place. The size of the oncogene transcripts was estimated from their mobility in the agarose gel relative to the mobility of 28S (5.3 kb) and 18S RNA (2.1 kb). The estimated size of c-myc (2.4 kb) and H-ras (1.4 kb) agreed with the size of these transcripts previously reported (24). However, c-fos (3.6 and 2.9 kb) and erb-B (15.5 and

13.0 kb) transcripts were greater in size than those reported in literature (2.3 and 2.2 kb; 8.6 and 5.1 kb; respectively). In the case of c-fos, this discrepancy may be due to species specificity between chick (this report) and mouse (24). The discrepancy in the size of the chick erb-B RNA transcripts is most probably due to the inability of the 1.8% agarose gels used in this study to accurately estimate the size of RNA species migrating more slowly than the largest RNA marker.

Our results on the constitutive expression of c-myc and H-ras RNA in the oviduct of chicks withdrawn from primary estrogen stimulation and their alteration following estrogen induced proliferation of tubular gland cells differ from those reported for estrogen induced oncogene expression in the mammalian uterus (25, 26). Administration of estradiol to ovariectomized adult mice results in over 8-fold increase in H-ras specific RNA at the peak of DNA synthesis (12 h post hormone stimulation) in uterine epithelium cells (25). Progesterone inhibits estrogen induced surge in DNA synthesis but induces increase in H-ras RNA similar to estrogen. Stimulation of immature female rats with estrogen results in a transient increase in the levels of c-myc RNA in uterus at 4 and 28 h following hormone administration corresponding to the period of stimulated RNA and DNA synthesis, respectively (26). The H-ras specific RNA showed a transient increase at 8 h following estrogen administration, a time when the hormone induced increase in protein synthesis was maximum. The reason for the differences in expression of oncogenes between chick oviduct and mammalian uterus remains unclear. However, the two tissues are known to elicit other notable differences in their response to steroid hormones, including estrogens (27).

Increase in c-fos and c-myc expression in cells in culture stimulated by growth factors is suggested to be correlated with the transition of cells from G_0 to G_1 (10, 28). On the contrary, during estrogen induced proliferation of the chick oviduct expression of c-myc and c-fos specific RNA decreased. Thus elevated expression of c-myc and c-fos mRNA does not appear to be necessary component for sustained cell proliferation in the estrogen primed chick oviduct. Whereas elevated expression of H-ras specific RNA may be related to the estrogen induced proliferation of the oviduct. Estrogen dependency of a human breast cancer cell line (MCF-7) on growth *in vitro* and tumorigenicity *in vivo* can be circumvented by the expression of integrated viral H-ras sequences (29). While estrogen stimulation results in the induction of egg white mRNAs, a general increase in RNA synthesis along with proliferation of the oviduct, estrogen notably down regulated expression of c-myc, c-fos and erb-B specific RNAs. The mechanism of altered expression of oncogene specific RNAs in the chick oviduct by estrogen remains to be elucidated. It could stem from altered transcription and/or altered stability of oncogene transcripts.

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