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Rapid regulation of c-*myc* protooncogene expression by progesterone in the avian oviduct

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ABSTRACT **The mRNA levels of genes known to be regulated by sex steroids are not altered until 1 hr or longer after steroid treatment, although the steroid receptor complexes are bound to nuclear acceptor sites within 5 min. In a search for early regulation of gene transcription, total chick oviduct RNA was isolated at various times after injection (i.p.) of progesterone and analyzed for c-*myc* expression. Levels of c-myc mRNA began to decrease in response to progesterone by 10 min after injection. The mRNA levels continued to decrease, reached a 70% reduction at 30 min, and returned to control values by 8 hr after steroid injection. Changes in α-tubulin mRNA levels were markedly less in these same RNA preparations. The effect was dependent on the dose of the steroid and was target-tissue specific. These changes occurred much more rapidly than changes in egg-white protein mRNA levels. Vehicle alone did not alter c-myc mRNA levels. Early regulated genes such as c-*myc* may represent the initial site of action of steroid receptors in the genome.**

In many instances steroid hormones and vitamins regulate the expression of genes in target cells at the level of transcription. The regulated genes include enzymes, secretory products, and structural proteins (1, 2) as well as growth factors (3, 4) and protooncogenes (5–11). One dilemma associated with direct sex steroid receptor mediation of these effects is the time interval between the initial nuclear binding of steroid receptor complexes (5–10 min) and the steroid-induced changes in mRNA levels (2–3 hr). There is strong evidence that protein synthesis is required during this interval for the steroid-induced changes in mRNA levels to occur (2, 12). One possibility considered by our laboratory is that steroid-induced gene regulation might involve early "regulatory" genes whose transcription would be rapidly modulated by steroid receptor binding and whose protein products would in turn regulate the transcription (or expression) of the well-defined "late" structural genes of housekeeping or secretory proteins. This model would require rapid steroid regulation of the expression of these regulatory genes that might encode transcription factors or other nuclear proteins.

As a first step to test this model, we investigated whether there were any genes that were rapidly regulated by progesterone in the avian oviduct. We screened a panel of genes with ^{32}P-labeled mRNA isolated from the avian oviduct at early periods after progesterone injection (i.p.). Emphasis was given to protooncogenes whose protein products migrate to the cell nucleus. Analysis of mRNA from estrogen-primed chick oviducts revealed the expression of several protooncogenes, including c-*myc*. This type of analysis also revealed that c-myc mRNA levels were decreased 1 hr after progesterone administration. In this paper, RNA gel blot analyses of total cellular RNA probed with v-*myc* were used to define the time course, dose dependence, and tissue specificity of the progesterone-mediated down-regulation of c-*myc* expression. Evidence for an extremely rapid regulation by progesterone of a v-*myc*-related RNA transcript in the avian oviduct is presented. Preliminary results of some of these results have been presented (13).

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MATERIALS AND METHODS

Treatment of the Animals. Specifically, immature chicks were injected subcutaneously with 5 mg of diethylstilbestrol in sesame oil per day from 6 to 25 days of age. Progesterone was injected (i.p.) in 0.2 ml of propylene glycol 10 hr after the last diethylstilbestrol injection. Chicks were sacrificed at various times after progesterone injection, and the oviducts, livers, and spleens were excised and placed on dry ice. Tissues were frozen at −70°C and processed to total cellular RNA within 1–2 days. All studies described in this paper involved several experiments each with different groups of animals.

Isolation of Total Cellular RNA. Total cellular RNA was prepared from oviducts by a modification of the method of Chirgwin *et al.* (14). Up to 3 g of tissue was homogenized in 65 ml of 6.0 M guanidine hydrochloride/20 mM sodium acetate/10 mM dithiothreitol, pH 5.2, and centrifuged 30 min at 20,000 × *g*. The supernatant was layered over 8 ml of 5.7 M cesium chloride/0.1 M EDTA, pH 7.0, and centrifuged in a Beckman SW 28 rotor at 22°C for 22 hr at 22,000 rpm to sediment the RNA. The pellet was resuspended in H_2O and ethanol-precipitated twice by the addition of 0.1 vol of 3 M sodium acetate (pH 5.2) followed by 2.5 vol of 95% (vol/vol) ethanol at −20°C. The RNA was resuspended and stored in sterile distilled water at −70°C.

RNA Gel Blot Analyses with v-*myc* DNA as Probe. RNA preparations were fractionated by using glyoxal denaturation (15) and electrophoresis was performed in 1.1% agarose. Each lane contained 30 μg of total cellular RNA denatured by incubation for 1 hr at 52°C in a solution of 1 M glyoxal, 50% (vol/vol) dimethyl sulfoxide, and 10 mM sodium phosphate (pH 7.0). Electrophoresis was performed in 10 mM sodium phosphate (pH 7.0) at 70 V for ≈4 hr. The RNA was then transferred to Micron Separations (Westboro, MA) Magnagraph nylon filters by capillary action over 12–29 hr in 20× SCC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.). The filters were baked for 2 hr at 80°C and hybridized as described below.

The v-*myc* DNA from Oncor (Gaithersburg, MD) consisted of a 1500-base-pair *Pst* I–*Aha* III fragment of v-*myc* containing only c-*myc* protein-encoding sequences and was labeled with ^{32}P by random hexanucleotide primer extension by using the Multiprime DNA labeling system from Amersham. Specific activities of 1.5×10^9 dpm/μg of RNA were routinely obtained. The α-tubulin DNA probe was isolated and cloned by Cleveland *et al.* (16). It represents a 1900-base-pair cDNA to α-tubulin mRNA from chicken brain representing ≈75% of the full length of the mRNA. The

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cDNA was cloned into pBR322 by using poly(dG)·poly(dC) tailing. The probe was ^{32}P-labeled by using the New England Nuclear nick-translation system. The conditions for DNA·RNA hybridization were a modification of those reported by Woloschak (17). Baked filters with immobilized RNA were shaken 10 min in 20 mM Tris·HCl (pH 7.6) at 100°C. They were rinsed in warm 3× SSC/0.1% $NaDodSO_4$ and prehybridized in hybridization buffer [50% (vol/vol) deionized formamide, 5× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), poly(A) at 10 μg/ml, denatured salmon sperm DNA at 50 μg/μl, 0.01% $NaDodSO_4$, 3× SSC] for 2–12 hr at 43°C. ^{32}P-labeled probe was denatured by incubation at 90°C for 3 min, cooled to 4°C, and then added to fresh hybridization buffer. Hybridization proceeded for 16–48 hr at 43°C. High-stringency washes included 15 min in 3× SSC/0.1% $NaDodSO_4$ at room temperature, two 30-min washes in 1× SSC/0.1% $NaDodSO_4$ at 43°C, and two 15-min washes in 0.5× SSC/0.1% $NaDodSO_4$ at 65°C. Filters were then wrapped in polyethylene and exposed to x-ray film (Kodak AR X-Omat) with intensifying screens for 1–4 days at −70°C.

Scanning Densitometric Analyses of the RNA Gel Blots. Scanning densitometry was used to obtain quantitative estimations of the relative amounts of c-myc mRNA in each RNA preparation. Autoradiographs of RNA gel blots washed at high stringency were used. Three 15-mm scans were taken across each hybridization band. The peak of each scan was selected visually, and the area under the peak was calculated by the spectrometer (Hirshmann Escript 400). These areas were averaged from three separate scans to give a number representing the relative signal intensity at each band.

RESULTS

Fig. 1 *Upper* shows an RNA gel blot analysis of total cellular RNAs isolated from developed oviducts of diethylstilbestrol-treated chicks at various times after a 1-mg dose (i.p.) of progesterone and reveals rapid down-regulation of c-myc mRNA level (Fig. 1 *Upper*). Lane O′ (RNA from untreated animals) shows that c-myc mRNA is expressed in the oviducts of estrogen-primed animals. The 2.4-kilobase (kb) size of the major transcript is consistent with the reported size of c-myc mRNA in avian and other tissues (7, 8). The minor band at ≈4 kb was largely removed by high-stringency washes and did not show any regulation by progesterone.

The relative abundance of the 2.4-kb c-myc mRNA begins to decrease within 10 min after injection of progesterone, reaches a minimum between 30 min and 1 hr, and returns to control values by 4 hr. This effect has been observed in six groups of chicks at two different doses of progesterone (250 μg and 1.0 mg). This rapid effect of progesterone on the expression of c-*myc* represents one of the most rapid changes of mRNA levels induced by any steroid reported in the literature. As shown in Fig. 1 *Lower*, the same RNA gel blot reprobed with ^{32}P-labeled α-tubulin DNA shows that the injection of progesterone alters α-tubulin mRNA levels markedly less than the c-myc mRNA levels.

Fig. 2 shows the effects of injecting vehicle alone on c-myc mRNA levels in diethylstilbestrol-stimulated oviducts. No change in c-myc mRNA level is observed at any time point studied when vehicle without steroid is injected. The reduction in c-myc mRNA thus appears to be dependent on progesterone. The tissue specificity of progesterone-mediated down-regulation of c-myc mRNA was examined by isolating total cellular RNA from spleen and liver at various times after a 1.0-mg dose of progesterone. Fig. 3 shows that progesterone did not alter c-myc mRNA levels in spleen. Analyses of liver RNA in the same experiments

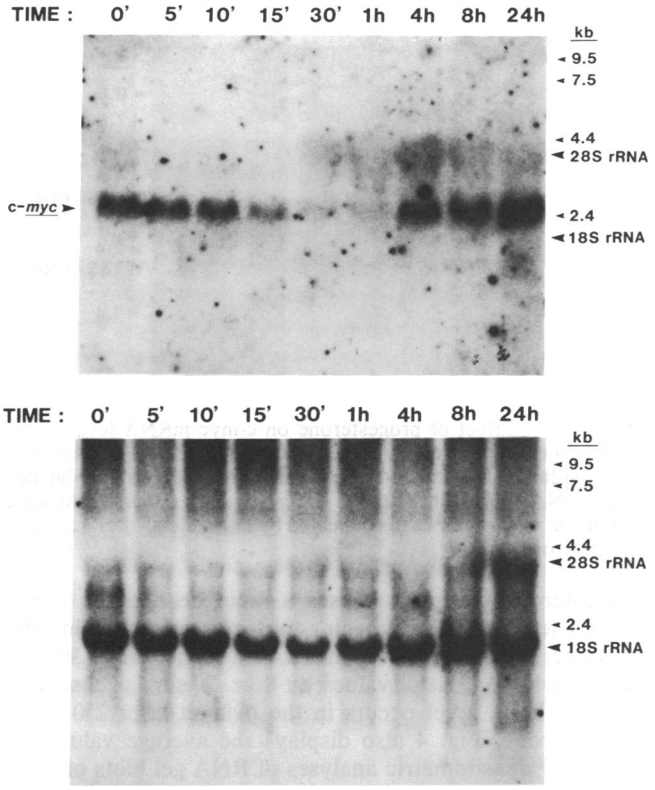

FIG. 1. Progesterone regulation of c-myc and α-tubulin mRNA levels in the diethylstilbestrol-stimulated avian oviduct. RNA gel blots of total cellular RNA (14) from oviducts of diethylstilbestrol-stimulated chicks given 1 mg of progesterone (i.p.) were hybridized with ^{32}P-labeled v-*myc* DNA or α-tubulin plasmid cDNA and washed at high stringency. RNA was denatured and electrophoresed in the presence of formaldehyde, as described (18). (*Upper*) RNA was isolated from oviducts at various times (as indicated by lane labels) after treatment with 1.0 mg of progesterone and hybridized with v-*myc*. (*Lower*) Same blot hybridized with α-tubulin cDNA.

revealed that progesterone also did not alter the low levels of c-myc mRNA in avian liver (data not shown). Thus the progesterone-induced changes in c-myc mRNA are tissue specific.

The results of densitometric tracings of the autoradiographs of RNA gel blots from two groups of chicks given 1.0 mg of progesterone and from one group given 250 μg of progesterone are graphed in Fig. 4. The average densitometer values from two 1-mg-dose experiments show that

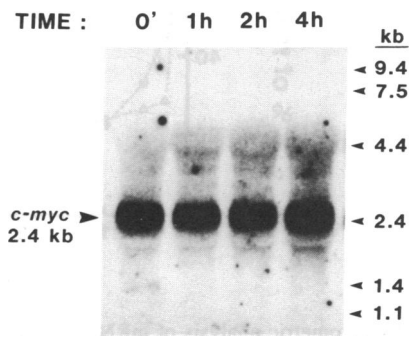

FIG. 2. Effect of vehicle on c-myc mRNA level in the diethylstilbestrol-stimulated avian oviduct. RNA gel blot of total cellular RNA (15) from the oviducts of diethylstilbestrol-stimulated chicks was hybridized with ^{32}P-labeled v-*myc* DNA and washed at high stringency. The oviducts were harvested at 0, 1, 2, and 4 hr after injection (i.p.) of 0.2 ml of vehicle (propylene glycol).

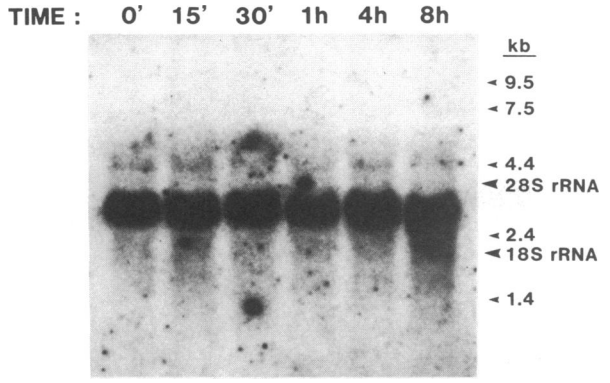

FIG. 3. Effect of progesterone on c-myc mRNA level in the avian spleen. RNA gel blot of total cellular RNAs from spleens of diethylstilbestrol-stimulated chicks was hybridized with ^{32}P-labeled v-*myc* DNA, and washed at high stringency. The spleen RNAs were isolated at various times (as indicated by lane labels) after 1.0 mg of progesterone.

progesterone treatment causes a rapid decrease in c-myc mRNA level. The level begins to decrease by 10 min after progesterone injection, reaches a 70% reduction by 30 min, and returns to control values by 8 hr. A similar change in c-myc mRNA level occurs in the oviduct after 250 μg of progesterone. Fig. 4 also displays the average values obtained by densitometric analyses of RNA gel blots of other experiments investigating mRNA levels in oviduct and spleen after progesterone or vehicle. As can be seen, the α-tubulin mRNA level in the oviducts changes only slightly after 1.0 mg of progesterone, the c-myc mRNA level in spleen does not change after an injection of 1.0 mg of progesterone, and vehicle alone causes no change in the c-myc mRNA level in oviduct.

Fig. 5 *Left* shows that the progesterone effect on c-myc mRNA level is dependent on the dose of the steroid. In this experiment, various doses of progesterone were administered in 0.2 ml of vehicle, and the RNA was isolated after 30 min, when c-myc mRNA levels are minimal. Doses as low as 20 μg appear to cause a decrease in c-myc mRNA level. Values obtained by densitometric tracing of the autoradiograph of Fig. 5 *Left* together with tracings from other similar experiments for the 250-μg and 1.0-mg doses of progesterone are shown in Fig. 5 *Right*. The values represent the means of multiple densitometric scans of several blots from two separate experiments, each involving a different group of animals. Changes in mRNA levels at 30 min are observed at doses of progesterone as low as 20 μg per animal followed by a plateau above doses of 250 μg of progesterone.

DISCUSSION

These studies demonstrate that a v-myc-related RNA species, termed c-myc based on its 2.4-kb size and hybridization properties, is present in the developed oviducts of estrogen-treated chicks and that the level of this c-myc mRNA is rapidly down-regulated by progesterone. The fact that the effect of progesterone on c-myc mRNA level requires steroid, is dose-dependent, and tissue-specific suggests that the decrease in c-myc mRNA levels by progesterone in the avian oviduct is a steroid receptor-dependent process. The changes in c-myc mRNA level should not be due to differential amounts of RNA in the gel lanes nor to the vehicle alone as demonstrated by α-tubulin reprobing and the vehicle time course studies. Further, the results of the α-tubulin analysis rule out the possibility that progesterone causes an equivalent generalized degradation of mRNAs. We cannot exclude the possibility that the vehicle or the steroid causes a small generalized effect on mRNA stability or rate of transcription, but the effect by progesterone on c-myc

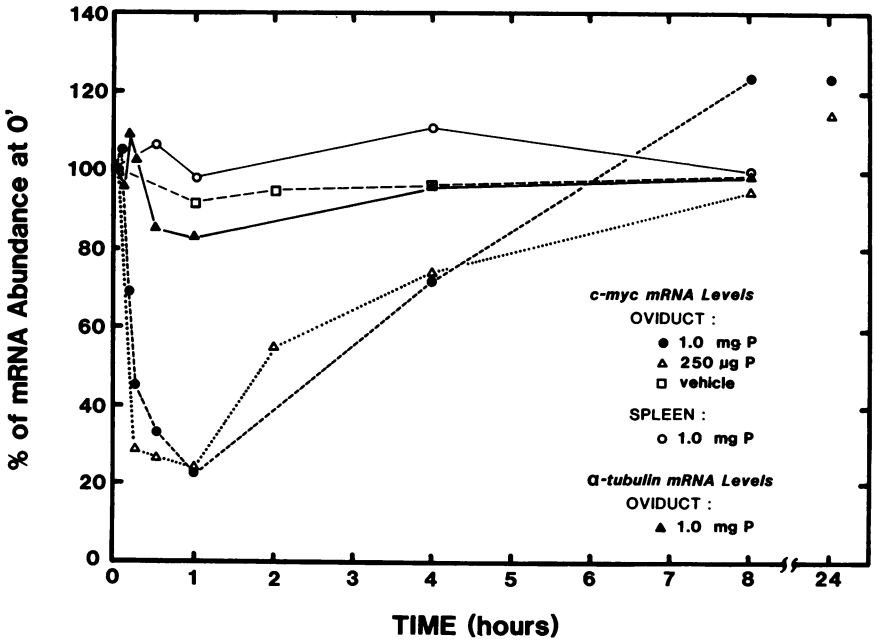

FIG. 4. Densitometric analysis of mRNA levels in avian oviduct and spleen after administration of progesterone or vehicle. Scanning densitometry analyses of autoradiographs of RNA gel blots show the effects of two doses (1.0 mg and 250 μg) of progesterone on c-myc mRNA levels (● and △, respectively) and the effect of a 1-mg dose of progesterone on α-tubulin mRNA levels (▲) in diethylstilbestrol-stimulated chick oviducts. The values for the c-myc and α-tubulin mRNA levels after 1.0 mg of progesterone represent the average of two separate experiments involving different groups of animals. The effects of vehicle on c-myc mRNA levels in the avian oviduct (□) and of progesterone on c-myc mRNA levels in the spleen (○) are included. The data are plotted as the percent c-myc mRNA level of the control value obtained at time zero. Data were gathered from the following numbers of experiments: one for oviduct at 250 μg of progesterone, two for oviduct at 1 mg of progesterone, one for vehicle, one for spleen at 1.0 mg of progesterone.

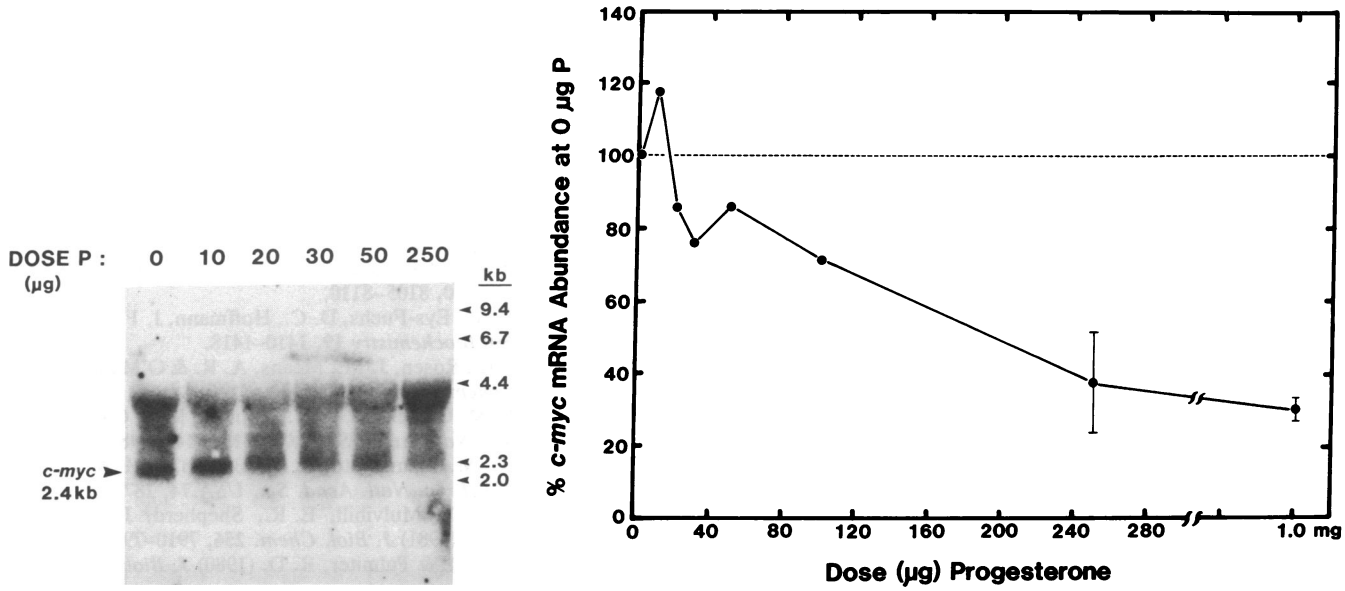

FIG. 5. Dose-dependent progesterone down-regulation of c-myc mRNA levels at 30 min in the diethylstilbestrol-stimulated avian oviduct. (*Left*) RNA gel blot of the total cellular RNAs from the oviducts of diethylstilbestrol-stimulated chicks given various doses of progesterone in 0.2 ml of propylene glycol (i.p.) for 30 min, hybridized with ^{32}P-labeled v-*myc* DNA, and washed at high stringency. (*Right*) Scanning densitometry of the autoradiographs from the experiment shown (*Left*) combined with data from replicate experiments. Error bars on these points reflect the standard deviation of multiple densitometric scans of several blots from two experiments involving different groups of animals.

mRNA abundance is clearly much greater than the small effect on α-tubulin mRNA level. The effect of progesterone, therefore, appears to be c-myc specific. It should be mentioned that the 4-kb band observed in some RNA gel blots may represent another myc-related RNA species. Alternatively, this band may represent cross-hybridization with 28S rRNA. This band disappears on higher stringency washes.

Although protooncogenes, including c-*myc*, have been shown to be regulated by vitamin D metabolites and glucocorticoids in cultured cells (5–9) and by estrogens in animals (10, 11), to the best of our knowledge, these studies represent the first report of (*i*) the action of progesterone in the regulation of c-*myc* gene expression in target tissues in whole animals and of (*ii*) an effect occurring within minutes (and not hours) after injection of any steroid. The expression of c-*myc* has been identified in many tissues (7, 19), and the level of c-myc mRNA appears to correlate with mitotic events occurring during growth and differentiation (7, 8, 20). It is well known that progesterone inhibits estrogen-induced mitotic events and cytodifferentiation in avian oviduct and mammalian uterus (2, 21–23), and progesterone also causes regression of some estrogen-dependent tumors (24). The reduction of c-*myc* gene expression by progesterone in target cells might represent one mechanism by which this inhibition occurs. It is interesting to note that c-myc mRNA levels have been found to be increased by another steroid, estrogen, in rat uterus, a tissue that responds to estrogen with marked growth and proliferation (10, 11). However, the estrogen action on c-myc mRNA levels in this system occurred at 1 hr (10) or 4 hr (11) after injection of the steroid.

This progesterone-induced reduction of c-myc mRNA expression in the avian oviduct within 5–10 min after injection *in vivo* represents one of the most rapid effects of any steroid on mRNA abundance of any gene in an animal model system and markedly precedes the action of steroids including progesterone on the mRNA levels of egg-white proteins as reported in several laboratories (2, 25–29). It should be mentioned, however, that one group has reported a somewhat shorter lag period of change in ovalbumin mRNA levels by progesterone in the avian oviduct (29). This rapid regulation of c-myc mRNA levels is similar to the primary regulation of the Ps-2 gene mRNA levels by estrogen in human MCF-7 breast cancer cells as reported by Chambon and co-workers (30) and to the rapid effects of glucocorticoids on murine mammary tumor virus transcription (31). It is of further interest that the effect of progesterone on c-myc mRNA occurs at doses (20 μg) less than that required to alter the mRNA levels of the egg-white proteins (32, 33).

The action of c-myc in normal cells is not entirely known, but it is interesting to note that the c-myc protein is localized to the nucleus (20, 34, 35) and might be involved in the regulation of other genes (20, 35). Further, a rapid turnover of c-myc mRNA and c-myc protein in other animal systems has been reported (36, 37). These properties make the c-*myc* gene a good candidate as a regulatory gene and possibly as the primary site of action of steroid hormones as described above. However, this early regulation of c-myc mRNA and its coding for a nuclear protein does not constitute proof of this regulatory gene model. Other models using different assumptions could explain the delayed regulation of many structural genes. To help elucidate the role of c-*myc* gene regulation, it could be determined whether the effect of progesterone on c-myc mRNA abundance occurs at the level of transcription or at the level of mRNA half-life, and the effects of cycloheximide could be assessed on progesterone regulation of c-*myc* expression in the oviduct.

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1. O'Malley, B. W., Roop, D. R., Lai, E. C., Nordstrom, J. L., Catterall, J. F., Swaneck, G. E., Colbert, D. A., Tsai, M. J., Dugaiczyk, A. & Woo, S. L. C. (1979) *Rec. Prog. Horm. Res.* **35**, 1–46.
2. Mulvihill, E. R. & Palmiter, R. D. (1980) *J. Biol. Chem.* **255**, 2085–2091.
3. Dickson, R. B., McManaway, M. E. & Lippman, M. E. (1986) *Science* **232**, 1540–1543.
4. Walker, P., Weichsel, M. E., Hoath, S. B., Poland, R. E. & Fisher, D. A. (1981) *Endocrinology* **109**, 582–587.
5. Reitsma, P. H., Rothberg, P. G., Astrin, S. M., Trial, J.,

Bar-Shavit, Z., Hall, A., Teitelbaum, S. L. & Kahn, A. J. (1983) *Nature (London)* **306,** 492–494.
6. Norris, J. S., Cornett, L. E., Hardin, J. W., Kohler, P. O., MacLeod, S. L., Srivastava, A., Syms, A. J. & Smith, R. G. (1984) *Biochem. Biophys. Res. Commun.* **122,** 124–128.
7. Brelvi, Z. S. & Studzinski, G. P. (1986) *J. Cell Physiol.* **128,** 171–179.
8. Eastman-Reks, S. B. & Vedeckis, W. V. (1986) *Cancer Res.* **46,** 2457–2462.
9. Chen, S. V. & Pollard, J. W. (1986) *FEBS Lett.* **196,** 309–314.
10. Murphy, L. J., Murphy, L. C. & Friesen, H. G. (1987) *Endocrinology* **120,** 1882–1888.
11. Travers, M. T. & Knowler, J. T. (1987) *FEBS Lett.* **211,** 27–30.
12. Chen, C. L. & Feigelson, P. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2669–2673.
13. Fink, K. L., Horton, M. J., Hora, J. F., Wieben, E. D., Woloschak, G. E. & Spelsberg, T. C. (1986) *J. Cell Biol.* **103,** 36 (abstr.).
14. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
15. McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4835–4838.
16. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. (1980) *Cell* **20,** 95–105.
17. Woloschak, G. E. (1986) *Mol. Immunol.* **23,** 581–591.
18. Lehrach, H., Diamond, D., Wogney, J. M. & Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751.
19. Gonda, T. J., Sheiness, D. K. & Bishop, J. M. (1982) *Mol. Cell Biol.* **2,** 617–624.
20. Kelly, K. & Siebenlist, U. (1986) *Annu. Rev. Immunol.* **4,** 317–338.
21. Oka, T. & Schimke, R. T. (1969) *Science* **163,** 83–85.
22. Socher, S. H. & O'Malley, B. W. (1973) *Dev. Biol.* **30,** 411–417.
23. Hseuh, A. J., Peck, E. J. & Clark, J. H. (1975) *Nature (London)* **254,** 337–339.
24. McGuire, W. L., Carbone, P. P., Sears, M. E. & Fisher, G. C. (1975) in *Estrogen Receptors in Human Breast Cancer*, eds. McGuire, W. L., Carbone P. P. & Vollmer, E. P. (Raven, New York), pp. 1–7.
25. Palmiter, R. D., Moore, P. B., Mulvihill, E. R. & Emtage, S. (1976) *Cell* **8,** 557–572.
26. Cox, R. F., Haines, M. E. & Emtage, J. S. (1974) *Eur. J. Biochem.* **49,** 225–236.
27. McKnight, G. S., Pennequin, P. & Schimke, R. T. (1975) *J. Biol. Chem.* **250,** 8105–8110.
28. Seaver, S. W., Eys-Fuchs, D. C., Hoffmann, J. F. & Coulsen, P. B. (1980) *Biochemistry* **19,** 1410–1418.
29. Harris, S. E., Rosen, J. M., Means, A. R. & O'Malley, R. W. (1975) *Biochemistry* **14,** 2072–2081.
30. Brown, A. M. C., Jeltsch, J.-M., Roberts, M. & Chambon, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6344–6348.
31. Ringold, G. M., Yamamoto, K. R., Bishop, J. M. & Varmus, H. E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2879–2883.
32. Palmiter, R. D., Mulvihill, E. R., Shepherd, J. H. & McKnight, G. S. (1981) *J. Biol. Chem.* **256,** 7910–7918.
33. Mulvihill, E. R. & Palmiter, R. D. (1980) *J. Biol. Chem.* **255,** 2085–2091.
34. Alitalo, K., Ramsey, G., Bishop, J. M., Pfeifer, S. O., Colby, W. W. & Levinson, A. D. (1983) *Nature (London)* **306,** 274–277.
35. Studzinski, G. P., Brelvi, Z. S., Feldman, S. C. & Watt, R. A. *Science* **234,** 467–470.
36. Ramsay, G., Evan, G. I. & Bishop, J. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7742–7746.
37. Dani, Ch., Blanchard, J. M., Piechaczyk, M., El Sabouty, S., Marty, L. & Jeanteur, Ph. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7046–7050.