Development 104, 87–95 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

Steroid-induced cell proliferation *in vivo* is associated with increased c-*myc* proto-oncogene transcript abundance

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Summary

Enhanced c-*myc* transcript abundance has been observed in a variety of human malignancies, in normal liver tissue induced to proliferate *in vivo* by partial hepatectomy and in cells in culture induced to proliferate with the addition of protein hormones and growth factors. Little is known, however, about the expression of cellular proto-oncogenes in cells induced to proliferate *in vivo* by steroid hormones. Experiments reported here indicate that when cells of the immature chicken oviduct are induced to undergo rapid *in vivo* proliferation by application of the estrogen hormone 17β-estradiol, the onset of this proliferation is associated with a rapid, large, and transient increase in c-*myc* transcript abundance. When estrogen is administered to chickens in which the oviduct has already differentiated, neither massive cell proliferation nor large increases in c-*myc* transcript abundance are induced. We conclude that the abundance of c-*myc* transcripts *in vivo* correlates well with the degree of cell proliferation induced by steroid hormone.

Key words: proto-oncogene, c-*myc*, steroid hormone, estrogen, chick oviduct, *in vivo*, cell proliferation.

Introduction

Elevated expression of proto-oncogenes is found in proliferating cells both *in vivo* (Makino *et al.* 1984; Slamon *et al.* 1984) and *in vitro* (Müller *et al.* 1984). Numerous reports have established that the expression of some of these genes and, in turn, cell proliferation, may be regulated *in vitro* by protein hormones and growth factors (for a review see Adamson, 1987). For example, platelet-derived growth factor stimulates proliferation of quiescent fibroblasts *in vitro* by first binding to a specific cell surface receptor. This binding initiates a cascade of events that leads to activation of c-*fos* gene transcription within 5 min, followed later by transcription from the c-*myc* gene, and later still by cell division (Müller *et al.* 1984). We wished to determine whether a comparable sequence of events might also arise during steroid hormone activation of cell proliferation *in vivo*.

For our analyses, we chose the chicken oviduct, a tissue that is both simple in structure and highly sensitive to estrogen hormones. When administered to 5-day-old chicks, 17β-estradiol induces an initial phase of rapid mitoses within the oviduct followed over a two week period by cellular differentiation and secretion of egg white proteins. If this is followed by withdrawal of the hormone for 14 days and then by subsequent readministration of the steroid for a further 14 days, cell proliferation ceases during withdrawal and is followed by a second, much smaller, round of mitoses at the onset of hormone readministration (Palmiter, 1975). The immature mucosa of the oviduct, therefore, provides a suitable tissue for the study of proto-oncogene expression during controlled, steroid hormone-induced, proliferation *in vivo*.

The chicken c-*myc* gene encodes a 2·5 kb transcript (Gonda *et al.* 1982), and its protein product ($58–62\times10^3$ M_r) localizes in the nucleus and binds to chromatin (Hann & Eisenman, 1984; Ramsey *et al.* 1984). Although its precise role in the control of cell proliferation remains unclear, when c-*myc* protein is injected into quiescent NIH 3T3 cells, DNA synthesis in induced (Kaczmarek *et al.* 1985). Conversely, inhibition of c-*myc* expression by c-*myc* complementary oligodeoxynucleotide prevents S-phase entry in mitogen stimulated T-lymphocytes (Heikkila *et al.* 1987). These results are consistent with a role for the c-*myc* protein as a competence factor for cell prolifer-

ation. We therefore expected that enhanced c-*myc* transcript abundance would accompany the onset of proliferation in the oviduct and that decreased abundance would be observed in differentiated oviduct tissue. Experiments described here confirm our earlier report (Rempel & Johnston, 1987) and indicate that c-*myc* transcript abundance is indeed transiently elevated within 6 h of primary steroid hormone administration, providing evidence that c-*myc* expression may be regulated by hormone or growth factor levels *in vivo*, as is apparently the case *in vitro*. Furthermore, c-*myc* transcript abundance during primary and secondary hormone administration correlates well with the amount of proliferation induced during each of these phases.

Materials and methods

Materials

Nitrocellulose was obtained from Schleicher and Schuell. Yeast tRNA and restriction enzymes were obtained from Boehringer Mannheim and poly(A) from Sigma. Klenow fragment was purchased from Bethesda Research Laboratories and dNTP's from Pharmacia. Gene Clean was purchased from BIO 101 Inc. 17β-estradiol was obtained from ICN Biomedicals Inc. Radioisotopes were obtained from Amersham. All other reagents were reagent grade from Fisher or Sigma.

Chicks

5-day-old Shaver pullets were injected daily with 1 mg of 17β-estradiol for 14 days (Oka & Schimke, 1969). Chicks were killed by subluxation of the cervical vertebrae and the organs (oviduct, heart and liver) were removed, frozen directly in liquid nitrogen, and stored at −80°C until used to prepare RNA. For the secondary stimulation studies, chicks were injected daily for 14 days, withdrawn (i.e. no hormone) for 14 days and reinjected daily for another 14 days.

Histological sections

Oviducts were removed from unstimulated (day 0) and stimulated (day 14) chicks. The tissues were formalin-fixed and embedded in paraffin wax. Serial sections were mounted on albumin-coated slides, deparaffinized in xylene, dehydrated in alcohol and haematoxylin/eosin stained.

RNA isolation

Total RNA was extracted from oviduct or control tissues by LiCl precipitation (Auffray & Rougeon, 1980): tissues were homogenized in 4 ml of 3 M-LiCl, 6 M-urea, 0·5 % SDS, 70 mM-2-mercaptoethanol, 10 mM-sodium acetate, pH 5·0 using a Janke and Kunkel polytron. The homogenized tissues were stored at 4°C overnight, then RNA was pelleted by centrifugation in an HB4 rotor at 8000 revs min^{-1} for 20 min. RNA was resuspended in 0·2 % SDS, 100 mM-sodium acetate, pH 5·0 and extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1) and once with chloroform:isoamyl alcohol (24:1). The aqueous phase was adjusted to a final concentration of 0·3 M-sodium acetate pH 5·2 prior to overnight ethanol precipitation at −80°C. The RNA was recovered by centrifugation at 8000 revs min^{-1} for 20 min, the pellet washed twice with 70 % ethanol, once with 95 % ethanol and air dried. The RNA was then dissolved in sterile water and stored at −80°C until used for Northern blots.

Northern blots and hybridizations

RNA samples were quantified by measuring absorbance at 260 nm, electrophoresed on 1·2 % agarose–formaldehyde gels, and stained with ethidium bromide for photography. Gels were soaked for 2 h in 20×SSC (1×SSC = 0·15 M-NaCl/0·015 M-sodium citrate, pH 7·0) prior to transfer to nitrocellulose. After transfer (Maniatis *et al.* 1982), the nitrocellulose filter was baked under vacuum for 2 h at 80°C. The filter was then prehybridized overnight at 42°C in a 5×SSC, 3×Denhardt's (1× = 0·02 % Ficoll; 0·02 % polyvinylpyrrolidone; 0·02 % bovine serum albumin), 0·1 % SDS, 1 mg ml^{-1} yeast tRNA, 10 μg ml^{-1} poly(A), 0·5 M-sodium phosphate pH 6·5, 50 % or 40 % formamide solution. The filters were then hybridized overnight at 42°C in a filtered solution prepared as above with 10 % dextran sulphate and the appropriate denatured labelled probe. After hybridization, the filters were washed once with 1×SSC, 0·1 % SDS at 42°C followed by four to five washes with 0·1×SSC, 0·1 % SDS at 65°C. Filters were then exposed to Kodak XAR5 film with an intensifying screen for periods varying up to 2 days.

Molecular clones and hybridization probes

The chicken β-actin pA1 plasmid (Cleveland *et al.* 1980) was provided by Dr W. J. Rutter from which the *Pst*1/*Pst*1 insert was obtained for hybridization. The pOVE12 chicken ovalbumin plasmid (Wickens *et al.* 1979) was provided by Dr R. T. Schimke. The pSV10 chicken c-*myc* plasmid (Collins & Groudine, 1982) was obtained from Dr M. Groudine from which the 3·2 kb *Sst*1/*Sst*1 insert (second and third exon and second intron to minimize background hybridization to rRNA) was used in hybridization experiments. Double-stranded DNA probes were prepared by oligo-labelling (Feinberg & Vogelstein, 1984) plasmids or gel-purified, Gene-cleaned inserts to specific activities between 1×10^7–1×10^8 cts min^{-1} μg^{-1}. Labelled probes were denatured for 5 min with 0·1 vol. of 2 M-NaOH, added to hybridization buffer and filtered through a 0·22 μm Millipore filter.

Densitometry

The autoradiograms were scanned using a Joyce–Loebl Chromoscan 3 densitometer. All integrals were equalized to the maximum amount of RNA actually loaded in the lanes (as determined by densitometric scans of ethidium bromide profiles). Background-corrected integral values were expressed as a percent maximum integral value for each datum point for each mRNA. The percent maximum transcript abundance is relative within a particular autoradiograph.

Results

Beginning at 5 days of age, chicks were injected with 1 mg of 17β-estradiol daily for 14 days. Chicks were killed on various days, and the oviducts were removed along with the heart (estradiol nonresponsive) and liver (estradiol responsive, nonproliferative) (Tam *et al.* 1986). Within 2 days, oviduct wet weight had increased 2·8-fold; by 14 days, the increase in wet weight was approximately 100-fold, in agreement with data obtained by others (Oka & Schimke, 1969). No steroid-hormone-induced growth was seen in either control tissue, indicating that the growth response is specific to the oviduct. This dramatic oviductal growth and differentiation (already well documented by Kohler *et al.* 1969 and Socher & O'Malley, 1973) was visualized by haematoxylin/eosin-stained cross-sections of unstimulated (day 0) and estradiol-stimulated (day 14) oviduct tissues (Fig. 1).

Hormone-induced functional differentiation of the oviduct epithelium was monitored by measuring the abundance of mRNA encoding ovalbumin, the major egg white protein (Palmiter & Wrenn, 1971). A Northern blot of total RNA samples, isolated from oviducts after 0, 0·25, 1, 7 and 14 days of hormone administration, was probed with [^{32}P]dCTP-oligolabelled ovalbumin plasmid pOVE12 (Wickens *et al.* 1979). 2·1 kb ovalbumin transcripts (Schimke *et al.* 1975) were detected in day 7 and 14 tissue but not in day 0, 0·25 or 1 oviduct tissue (Fig. 2A), confirming that differentiation of the oviduct had occurred (Schimke *et al.* 1975). 100 % maximum transcript abundance was observed in day 7 oviduct tissue with 97 % maximum transcript abundance occurring in day 14 oviduct tissue (Fig. 4B). As expected, ovalbumin transcripts were not detected in either of the heart or liver control tissues (data not shown).

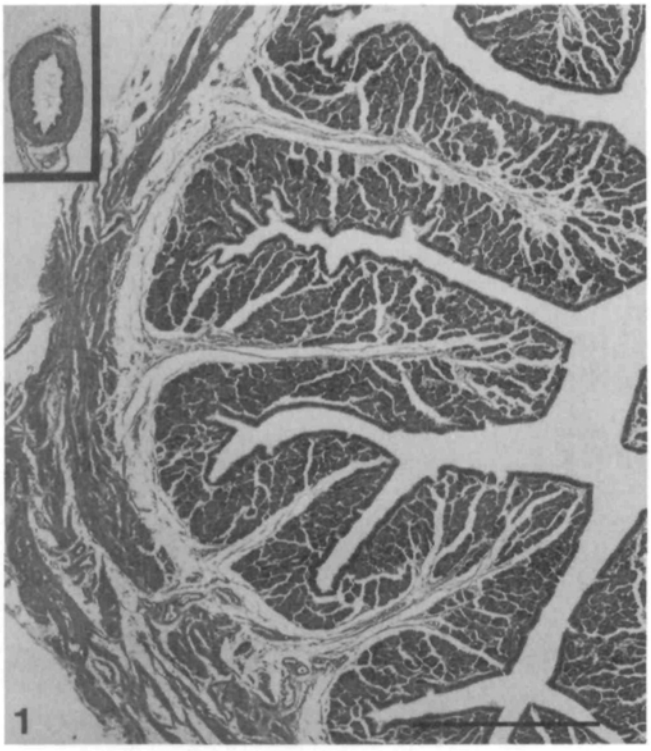


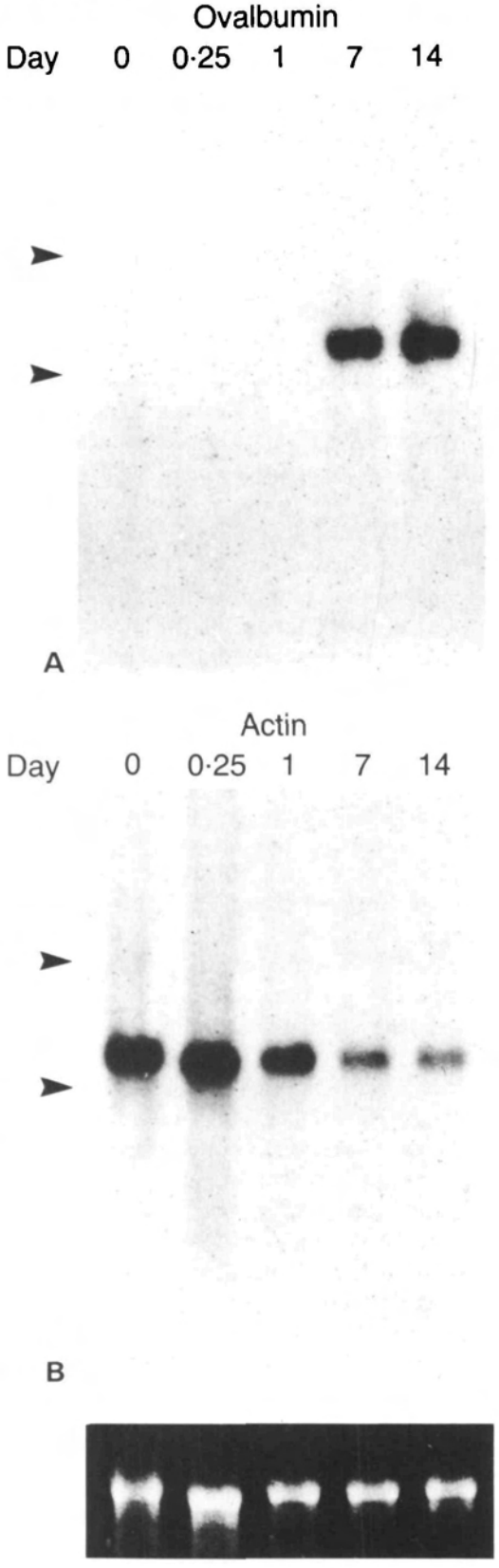
Fig. 1. Haematoxylin/eosin stained cross-sections of unstimulated (day 0; inset) and a portion of a stimulated (day 14) oviduct demonstrating both the growth and differentiation due to steroid hormone administration. Both micrographs are at the same magnification; bar indicates 0·5 mm.

We also examined the transcript abundance of the β-actin gene, the expression of which we anticipated would be detectable throughout the growth and differentiation of the oviduct. A Northern blot was hybridized to a [^{32}P]dCTP-oligolabelled β-actin probe and the abundance of the 2 kb actin transcripts (Cleveland *et al.* 1980) monitored over time (Fig. 2B). While actin transcript abundance in day 0 and 6 h tissue was high (98 % and 100 % of maximal values, respectively), the abundance declined approximately 10-fold over the ensuing 14 days (Fig. 4C). The control tissues, however, showed no change in the β-actin levels from day 0 to day 14 (data not shown). The decline in abundance of actin transcripts was unexpected and may reflect the onset of differentiation in the oviductal tissue.

A series of Northern blots prepared with total RNA extracted from day 0, 0·25, 1, 7 and 14 oviduct and control stimulated tissues was probed with [^{32}P]dCTP-oligolabelled pSV10 chicken c-*myc* insert (Collins & Groudine, 1982). Hybridization conditions were optimized to reduce nonspecific hybridization of the c-*myc* probe to 28S and 18S rRNA. 2·5 kb c-*myc* transcripts (Gonda *et al.* 1982) were detected at each stage in both control and oviduct tissues. Although c-*myc* transcript abundance did not change in control tissues during hormone administration (data not shown), its abundance in oviduct tissue was maximal within 6 h (Fig. 3), increasing fourfold (range threefold to sixfold in 4 experiments) over levels in unstimulated tissue. The abundance then declined to 44 % of maximal by day 1 of 17β-estradiol administration with further declines by day 14 (Fig. 4A). Even then, however, the abundance of the c-*myc* transcripts remained elevated (approximately 1·5-fold) above levels found in unstimulated oviduct tissues.

Furthermore, we examined whether c-*myc* transcript abundance would vary during secondary hormone stimulation of oviduct tissue. Chicks were injected daily with hormone for 14 days, withdrawn from hormone (i.e. not injected) for a further 14 days, then injected once again with hormone for another 14 days. Northern blot analysis of the with-

drawn and secondarily stimulated oviducts indicates that c-*myc* transcript abundance falls slightly during the withdrawal period, then increases slightly (1·5-fold) by day 1 during the secondary stimulation period (Fig. 5).



Discussion

When protein growth factors are administered to serum-starved cells *in vitro*, the cellular responses include the rapid activation of expression by such proto-oncogenes as c-*fos*, c-*myb* and c-*myc* (Greenberg & Ziff, 1984); expression from these genes precedes the onset of DNA synthesis and rapid cell proliferation. Cell differentiation generally results in diminished oncogene expression, but in some cases these genes are inducible even in differentiating or postmitotic cells *in vitro*. For example, c-*fos* is inducible by NGF in pheochromocytoma cells (Milbrandt, 1986; Curran & Morgan, 1987) and c-*myc* is inducible by serum or insulin in skeletal myotubes (Endo & Nadal-Grinard, 1986). While variations in c-*myc* expression among various tissues during embryonic development (Pfeifer-Ohlsson *et al.* 1984, 1985) or during hepatic regeneration (Makino *et al.* 1984) are well documented, the signals controlling this expression *in vivo* are not known. Two major questions that remain, therefore, are how these proto-oncogenes may be regulated *in vivo* and how their induced expression *in vivo* correlates with proliferation.

We have therefore analysed the initial responses (described also in a preliminary report; Rempel & Johnston, 1987) of a well-characterized tissue, the chicken oviduct, to the administration of a defined hormonal signal, 17β-estradiol. This hormone induces a program of oviductal responses that culminates in the intensively studied synthesis of egg white proteins. Here, our attention is focused instead on the initial responses of the oviduct to primary hormonal exposure. Less well characterized than the later responses, the onset of rapid growth in the oviduct following hormone exposure is nonetheless dramatic, resulting in a 100-fold increase in mass in 14 days (Fig. 1; Oka & Schimke, 1969).

To examine the *in vivo* expression of c-*myc*, we

Fig. 2. Steroid modulation of mRNA in chicken oviduct. (A) Hybridization to a [^{32}P]dCTP-oligolabelled ovalbumin-containing plasmid pOVE12 (Wickens *et al.* 1979). (B) Hybridization to a [^{32}P]dCTP-oligolabelled chicken β-actin *Pst*1/*Pst*1 insert of pA1 plasmid (Cleveland *et al.* 1980). 5-day-old Shaver pullets were injected daily with 1 mg 17β-estradiol for 14 days. Chicks were sacrificed at day 0 (unstimulated), 6 h, day 1, day 7 and day 14 (stimulated tissues) at constant times after estrogen administration. 5 μg samples of total cellular RNA were loaded per lane. After hybridization to the ovalbumin probe and exposure to X-ray film, the same blot was stripped and rehybridized to the actin probe. Arrowheads indicate the positions of the 28S and 18S rRNA. The lower panel shows the ethidium bromide staining pattern of 28S RNA in the formaldehyde-agarose gel used to prepare the Northern blot.

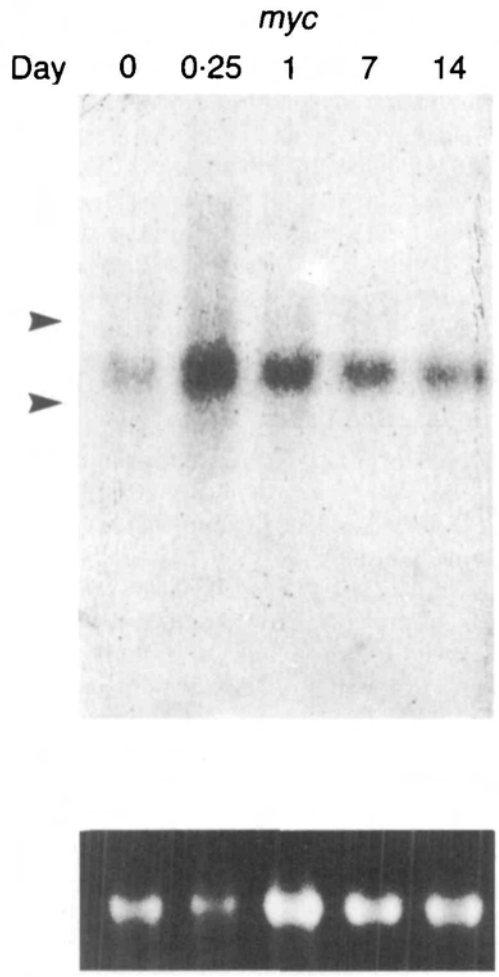


Fig. 3. C-*myc* mRNA expression during steroid-hormone-induced development of the chick oviduct. 5-day-old Shaver pullets were injected daily with 1 mg 17β-estradiol for 14 days. Chicks were sacrificed at day 0 (unstimulated), 6 h, day 1, day 7 and day 14 (stimulated tissues) at constant times after estrogen administration. A Northern blot of oviduct total RNA (20 μg per lane) was hybridized in a 50 % formamide solution to a [^{32}P]dCTP-oligolabelled 3·2 kb *Sst*1/*Sst*1 insert of the chicken c-*myc*-containing plasmid pSV10 (containing the second and third exon and second intron; Collins & Groudine, 1982). The filter was washed and exposed as detailed in experimental procedures. Arrowheads indicate the positions of the 28S and 18S rRNA. The lower panel shows the ethidium-bromide staining pattern of 28S RNA in the formaldehyde–agarose gel used to prepare the Northern blot.

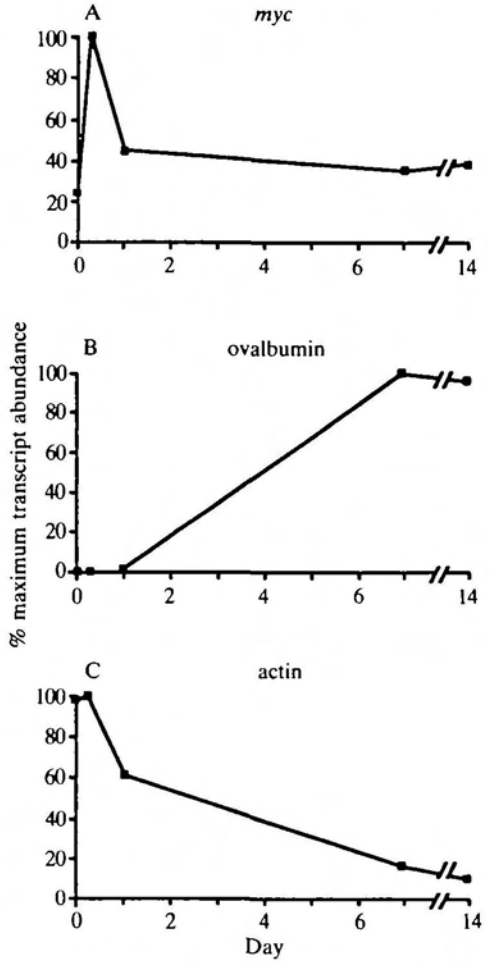


Fig. 4. Percent maximal transcript abundance of mRNAs during 17β-estradiol stimulation of the chick oviduct; (A) c-*myc*; (B) ovalbumin; (C) actin. Transcript abundance was determined by quantifying hybridization signals on the X-ray films illustrated in Figs 2 and 3, using a Joyce-Loebl Chromoscan 3 densitometer and correcting for variations in RNA loaded per lane. Background-corrected integral values were expressed as percent of the maximum integral value for each mRNA.

compared the abundance of its transcripts with the transcript abundance of β-actin and ovalbumin genes in oviduct, liver and heart tissues, before and after hormone administration. The different expression patterns we observe for ovalbumin (Fig. 2A), β-actin (Fig. 2B), and c-*myc* (Fig. 3) demonstrate the independent regulation of these three genes. The transient elevation in c-*myc* expression (Fig. 3) observed in primary hormone-stimulated chicken oviduct is similar to the elevation observed when quiescent fibroblasts are induced to proliferate *in vitro* with the addition of serum (Thompson *et al.* 1986). Thus, the increase in c-*myc* transcript abundance in 6 h stimulated oviduct tissue may reflect a synchronous activation of oviductal cells and their entry into the cell cycle (Thompson *et al.* 1985), while the subsequent decrease in abundance may reflect the onset of differentiation and consequent decline in cell proliferation during oviductal maturation.

After primary injection of 1 mg of 17β-estradiol, the mitotic index of the oviduct increases almost sevenfold, from 0·4 % in the unstimulated oviduct to a peak of 2·7 % by 18 h following hormone administration (Socher & O'Malley, 1973). The threefold to sixfold increase in c-*myc* transcript abundance we observe at 6 h is one of the earliest responses of the immature oviduct to steroid hormone administration,

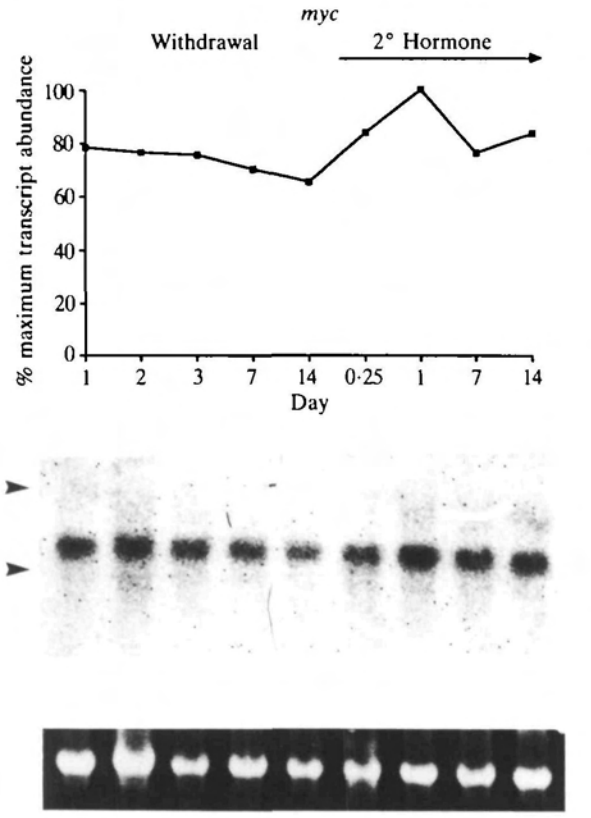


Fig. 5. Percent maximal transcript abundance of c-*myc* mRNA during withdrawal and secondary hormone administration. Upper panel: Transcript abundance was determined as in Fig. 4 using the X-ray film of the Northern blot in the middle panel. Middle panel: A Northern blot of total oviduct RNA (20 μg per lane) from chicks sacrificed at constant times after estrogen withdrawal and secondary estrogen injection on days indicated was hybridized to a [^{32}P]dCTP-oligolabelled c-*myc* probe as in Fig. 3. Arrowheads indicate the positions of 28S and 18S rRNA. Lower panel: The ethidium bromide staining pattern of 18S RNA in the formaldehyde-agarose gel used to prepare the Northern blot.

and precedes by 12 h the elevation in mitotic index as observed by Socher & O'Malley (1973). The initial increase and the subsequent decrease in transcript abundance in differentiating tissue during primary hormone stimulation provide further support for a role for this proto-oncogene in the regulation of normal cell proliferation. We are presently investigating the expression of c-*myc* at even shorter time intervals after hormone administration, in order to determine more precisely when c-*myc* transcript abundance is maximal. It should be noted that cells induced to proliferate *in vitro* with protein mitogens express maximal levels of c-*myc* transcripts as early as 1 h after mitogen administration (Müller *et al.* 1984).

After 10 days of primary hormone stimulation and 10 days of hormone withdrawal, steroid readministration induces a maximum mitotic index of 1·8 % in the surface epithelium and an index of only 0·5 % in the tubular gland cells (Socher & O'Malley, 1973). As the epithelial layer comprises less than 10 % of the cells present in the magnum of the differentiated oviduct (Palmiter, 1975), the net proliferative response in the tissue as a whole following secondary stimulation is actually comparable to that of the unstimulated primary oviduct. Therefore, the slight increase we detect in c-*myc* transcript abundance during secondary hormone administration after 14 days of primary steroid administration (Fig. 5) is consistent with the overall weak proliferative response induced in the oviduct tissue at this time (Socher & O'Malley, 1973), in marked contrast to the intense proliferation induced during primary stimulation, as above (Fig. 1). It should be noted that other investigators have already examined c-*myc* proto-oncogene expression during secondary (but not primary, as here) stimulation of the oviduct with steroid hormones (Hodgson, 1986; Fink *et al.* 1988). Hodgson observed no increase in c-*myc* transcript abundance due to secondary administration of diethylstilbestrol and concluded that this proto-oncogene was not activated during proliferation of the oviduct. Our assessment of c-*myc* activity in the hormone-stimulated oviduct differs from that of Hodgson. We argue instead that the small increase we observed in c-*myc* transcript abundance during secondary stimulation correlates with the minimal proliferation induced at this time, whereas the dramatic mitotic burst induced during primary stimulation corresponds well to the much larger increase in c-*myc* transcript abundance shown in Fig. 3. In a more recent report, Fink *et al.* (1988) described the reduction of c-*myc* expression during secondary hormone treatment with progesterone. Clearly, the expression of c-*myc* will depend both upon the state of differentiation of the tissue as well as on the hormone administered. The anti-proliferative effects of progesterone on the chicken oviduct, and the consequent reduction in c-*myc* expression, are consistent with the results reported here.

We do not know whether the elevation in c-*myc* transcript abundance is achieved primarily by steroid hormone enhancement of transcription of the c-*myc* proto-oncogene or by stabilization of the transcripts against degradation, as reported for ovalbumin and vitellogenin transcripts (Palmiter & Carey, 1974; Brock & Shapiro, 1983). However, preliminary results (not shown) indicate that transcription of the c-*myc* gene is indeed enhanced following estradiol administration.

The stimulation of the c-*myc* gene may be by direct binding of a steroid–receptor complex to the gene (Gorski *et al.* 1986), or it may be indirect and

mediated by other hormone-induced products (Laugier *et al.* 1983). To date, estrogen-induced proliferation seen *in vivo* has not been reproduced *in vitro* with secondarily hormone-treated chicken oviducts (Seaver *et al.* 1982) or in estrogen-dependant CH3/C14 pituitary or H301 hamster kidney cell lines, implying a role for other growth factors *in vivo* (Leland *et al.* 1981). However, we note that two regions in the chicken c-*myc* sequence (nucleotides 317–329 and 1146–1158) (Watson *et al.* 1983) are highly similar (77 % nucleotide identity) to the estrogen enhancer sequences reported for the chicken vitellogenin genes (Walker *et al.* 1984). In addition, high-affinity estrogen-receptor proteins have been identified in immature chicken oviduct (Best-Belpomme *et al.* 1975). These observations are consistent with the possibility that steroid-hormone–receptor complexes may indeed interact with the chromosomal c-*myc* gene.

Our results from the primary and secondary hormone administration experiments indicate that the c-*myc* gene is available for stimulation of expression only when a cell is proliferation competent and that this capacity diminishes once the cell has differentiated, since reexposure to hormone during secondary stimulation does not induce c-*myc* mRNA to levels seen during primary stimulation. A similar decrease in c-*myc* mRNA level is seen when mouse erythroleukemia cells (Lachman *et al.* 1986) or F9 teratocarcinoma cells (Griep & Deluca, 1986) are induced to differentiate *in vitro*.

In any case, the data presented here provide clear evidence of an elevation in proto-oncogene transcript abundance during stimulated cell proliferation *in vivo*. That c-*myc* transcript abundance is also enhanced by estrogen treatment in immature rat uterus (Travers & Knowler, 1987; Murphy *et al.* 1987) suggests that the ability of the steroid to enhance c-*myc* transcript abundance is not restricted to the chicken oviduct. Our observations may illuminate the relationship between steroid hormones and the proliferation of hormone-responsive malignancies in humans, such as those derived from mammary, endometrial or prostatic tissues (Huang & Cho-Chung, 1984; Kasid *et al.* 1985).

We thank Dr W. J. Rutter for the use of the pA1 plasmid, Dr M. Groudine for the use of the pSV10 plasmid, Dr L. Browder and Dr R. T. Schimke for helpful comments, and B. Kucey for technical assistance. This work is supported by the Alberta Heritage Foundation for Medical Research, by the Alberta Cancer Board, and by the National Cancer Institute of Canada.

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(*Accepted 16 May 1988*)