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Estradiol Stimulates c-*myc* Proto-oncogene Expression in Normal Human Breast Epithelial Cells in Culture

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The proto-oncogene c-*myc* is involved in the stimulation of cell proliferation, and its expression is known to be stimulated by estradiol (E2) in human breast cancer cell lines and various non-cancerous E2-dependent tissues. However, little information is currently available concerning its expression and regulation in normal human breast tissue. We therefore studied c-*myc* expression and hormone modulation in normal human breast epithelial (HBE) cells in culture, routinely obtained in our laboratory and which remain hormone-dependent. On these normal HBE cells, E2 induced a biphasic increase in c-*myc* mRNA level, with a first peak as early as 30 min, and a secondary increase after 2 h of treatment; this stimulation was dose-dependent, with an optimal concentration of 10 nM E2. Its primary action is probably at the transcriptional level since the half-life of c-*myc* mRNA measured in the presence of actinomycin D (12 ± 3 min) was not modified by E2 treatment. In addition, E2 stimulation of c-*myc* mRNA does not require protein synthesis since it was not suppressed by cycloheximide treatment. Western blot studies of c-*myc* protein in HBE cells revealed the same biphasic pattern of stimulation, with a first peak after 60 min and a second one after 2 h of E2 treatment. In conclusion, the c-*myc* proto-oncogene is expressed in normal HBE cells, as in breast cancer cells. Moreover, E2 stimulates c-*myc* expression which, therefore, may partly mediate the growth-promoting effect of E2.

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INTRODUCTION

Breast cancer affects 1 out of 8 women in Western countries [1]. In addition to genetic factors, hormones and particularly estrogens are considered to increase the risk of breast cancer [2–4]. Estradiol (E2) which is known to have a mitogenic effect on breast cells [5–7] apparently acts as a promoter of breast carcinogenesis through mechanisms not yet completely understood. Therefore, any complementary information on the mechanisms of E2 action upon normal cells would be beneficial.

Stimulation of the expression of proto-oncogenes and growth factors has been suggested as one of the mechanisms accounting for the mitogenic action of E2 [8–10]. In particular, the expression of the c-*myc* proto-oncogene, which encodes a nuclear protein essential for DNA replication, is stimulated by E2 in human breast cancer cell lines [11–13] and also in non-cancer E2-dependent tissues (endometrium, oviduct) in animals [10, 14, 15]. Moreover, its expression is inhibited by antiestrogens, which have been proven to slow down the growth of E2-dependent cancer cells [11, 16].

We therefore studied c-*myc* expression and hormone modulation in a culture system of normal human breast epithelial (HBE) cells, routinely obtained in our laboratory [17], that have been shown to remain hormone-dependent [7, 17–20]. We first identified c-*myc* mRNA expression and measured it as a function of time in the culture. We then studied E2 action on the expression

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of both c-*myc* mRNA and c-*myc* protein, after having defined the optimal culture conditions for these studies.

MATERIALS AND METHODS

Materials

Human serum was provided by the Centre National de Transfusion Sanguine (Les Ulis, France). Ham's F-10 (HAM) was obtained from Gibco (Cergy-Pontoise, France). Collagenase and epidermal growth factor (EGF) were acquired from Boehringer Manheim Chemical Corp. (Meylan, France). Hyaluronidase, triiodothyronine (T3), 11β,17α,21-trihydroxy-4-pregnene-3,20-dione (F), estradiol (E2), cholera toxin, transferrin, bovine pancreas crystalline insulin, and a protein assay kit were obtained from Sigma Chemical Corp. (St Louis, MO). The monoclonal mouse antibody directed against the c-*myc* protein was acquired from Oncogene Science, France. Anti-mouse IgG conjugated to peroxidase was obtained from Pasteur-Diagnostic (Marne la Coquette, France). The transcription kit used for the extension of riboprobe was obtained from Promega. The ECL chemiluminescence kit for detection of proteins was acquired from Amersham. Fuji X-ray films were used for autoradiography.

Culture procedures and media

The basal medium consisted of Ham's F-10 without phenol red, containing $NaHCO_3$ (0.24%), kanamycin (0.04%). Medium A was a basal medium enriched with 5% human serum, EGF (10 ng/ml), F (5 ng/ml), T3 (6.5 ng/ml), cholera toxin (10 ng/ml), transferrin (5 μg/ml) and insulin (0.12 U/ml).

Primary cultures of normal human breast epithelial (HBE) cells were obtained as previously described [17]. In brief, the normal breast tissue was first enzymatically digested with collagenase (0.15%) and hyaluronidase (0.05%) in Ham's F10, and then filtered consecutively through 500, 300, and 150 μm sieves in order to eliminate undigested tissue. The cell material retained on a final 60 μm sieve was used for epithelial cell culture. Primary cultures were grown in medium A. The number of cells per flask was determined throughout the culture as previously described [7].

Hormone treatment of HBE cells

Studies were carried out on cells after 7–10 days of primary culture.

For the Northern blot analysis, cells were then deprived of hormones and growth factors for 4 h by replacing medium A with the basal medium. E2 (10 nM) was then added, and RNAs were extracted at various times (0–2 h) during the E2 treatment.

For the Western blot analysis, cells were deprived of hormones and growth factors in the basal medium for 12 h. E2 (10 nM) was added, and proteins were extracted at various times (0–3 h) during E2 treatment.

c-myc mRNA studies

Probes used. Plasmid pRik, which was used to synthesize the c-*myc* riboprobe, consisted of pGem-3Zf (Promega) containing a 1080 bp human *myc* cDNA *Pst* I fragment (corresponding to exon 3 and a portion of exon 2). Plasmid pSPT18 containing a 700 bp human 36B4 cDNA *Pst* I fragment was generously provided by P. Chambon [21]. 36B4 mRNA, considered to be hormone-independent [21], was used as the reference to evaluate the RNA loaded on the gel. Labeled riboprobe was synthesized according to the manufacturer's instructions (Promega).

Northern blot analysis. Total RNAs were extracted with RNAzol (Bioprobe) and quantified by absorption at 260 nm. For Northern blot studies, total RNAs (30 μg/lane) were separated on 1% agarose, 2.2 M formaldehyde gel, and transferred onto a Genescreen membrane in 10 × SSC (0.75 M sodium chloride, 0.075 M sodium citrate). Prehybridization and hybridization were carried out at 65°C in a solution of: 50% formamide, 1% SDS, 1 mM EDTA, 0.05% Ficoll, 0.05% polyvinyl pyrrolidone, 0.05% BSA, 5 × SSPE (0.9 M NaCl, 0.05 M NaH_2PO_4, 0.005 M EDTA), and denatured herring sperm DNA (200 μg/ml). Hybridization with [^{32}P]-labeled riboprobes was carried out overnight at 65°C. Membranes were washed 4 times in 0.1 × SSC, 0.1% SDS at 60°C before being exposed, with an intensifying screen, to Fuji X-ray film at −80°C. Films were scanned with a Gelscan ultrascan (Pharmacia). Hybridization signals were quantified by densitometric scanning of multiple autoradiograms of various exposures, and were expressed relative to the control which was assigned a value of 1.

Effect of cycloheximide on c-myc mRNA level. The cells were grown in medium A. They were then deprived of hormones and growth factors by replacing medium A with the basal medium for 4 h. Cycloheximide (14 μg/ml) alone or together with E2 (10 nM) was then added. RNAs were extracted for Northern blot analysis 30 min after the addition of cycloheximide.

Half-life of c-myc mRNA. Cells maintained in medium A were treated for variable lengths of time (0–60 min) with the RNA polymerase inhibitor actinomycin D (5 μg/ml), alone or together with E2 (10 nM). The amount of c-*myc* mRNA was determined by Northern blot analysis as described above.

Western blot analysis of the c-myc protein

Western blots were performed on nuclear proteins. Cells were harvested with a rubber policeman and washed twice with ice-cold PBS. They were then lysed in a NP40 lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM sodium chloride, 3 mM magnesium chloride, 0.5% NP40) in order to isolate the nuclei. Proteins were extracted from the nuclei as described by Maniatis [22] in SDS loading buffer (50 mM Tris, 2% SDS,

10% glycerol, 100 mM dithiothreitol, pH 6.8). Nuclear proteins were measured in the supernatant and then separated on SDS–6% polyacrylamide gel. These proteins were transferred onto an Immobilon membrane (Millipore) for an immunoblot analysis. Nonspecific binding sites were blocked with 10% skimmed milk plus 1% sheep serum in TBS buffer (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.05% Tween-20. Membranes were then incubated: (1) with the c-*myc* antibody (0.1 μg/ml) for 18 h at 4°C; and (2) with the anti-mouse antibody conjugated with peroxidase (0.4 μg/ml) in TBS supplemented with 0.1% Tween-20, 1% skimmed milk and 1% sheep serum, for 30 min at room temperature. Each of these two steps was followed by an 8 min wash in TBS containing 0.2% Tween-20. Detection was performed with the ECL chemiluminescence kit (Amersham) following the manufacturer's instructions and with Fuji X-ray film. Membranes were stained with ponceau S. red in order to evaluate the nuclear proteins loaded in each lane.

Data analysis

The c-*myc* expression and regulation were measured within the series of cultures established from the same patient. Each measurement was carried out on parallel triplicate flasks and the results were expressed as the mean value ± SD. The intra-assay variation was less than 10%. All studies were carried out on breast cells from at least three patients and the results were compared in order to check reproducibility. The results obtained with cells from different patients sometimes varied up to 20%. The effects measured were always in the same proportion to one another, with less than 15% inter-assay variation.

RESULTS

Variations of c-myc mRNA expression during cell culture (Table 1)

Since cell confluency and the age of the culture can modulate c-*myc* expression [23, 24], we studied variations in c-*myc* mRNA throughout the duration of cell culture. The cells were grown in medium A and the expression of c-*myc* was measured by Northern blot analysis from the 5th to the 16th day during the primary culture. This expression was compared to the number of cells and the amount of RNAs per flask. The c-*myc* mRNA level remained stable until day 11 (Table 1); it did not vary by more than 15%. It decreased as of the 12th day, when cells reached confluency. On day 16, the c-*myc* mRNA level was 60% of its initial value. As a consequence, we carried out subsequent experiments on day 10 of the primary culture, before confluency.

Table 1. c-myc mRNA expression in HBE cells throughout the duration of primary culture

Days	5th	7th	9th	10th	11th	12th	14th	16th
Number of cells/flask	$6 \pm 0.5 \times 10^5$	$7 \pm 0.5 \times 10^5$	$1.2 \pm 0.1 \times 10^6$	$1.8 \pm 0.1 \times 10^6$	$2.2 \pm 0.2 \times 10^6$	$2.4 \pm 0.2 \times 10^6$	$2.5 \pm 0.2 \times 10^6$	$2.4 \pm 0.3 \times 10^6$
Total amount of RNA (μg/flask)	12 ± 2	15 ± 2	24 ± 3	37 ± 3	45 ± 4	48 ± 5	50 ± 5	48 ± 5
Relative c-*myc* mRNA level	1	1 ± 0.1	0.95 ± 0.05	1 ± 0.1	0.95 ± 0.1	0.80 ± 0.05	0.65 ± 0.10	0.55 ± 0.05

Cells were grown in T75 flasks and counted daily. Total RNAs were extracted from day 5 to 16 of the culture and Northern blot analysis performed (see Materials and Methods). This Table shows the number of cells, the corresponding amount of total RNAs and relative c-*myc* mRNA levels. The ratio of c-*myc* mRNA to total RNAs was calculated for each day, and the ratio observed on day 5 taken to be as 1. The results presented are means ± SD of 3 different experiments.

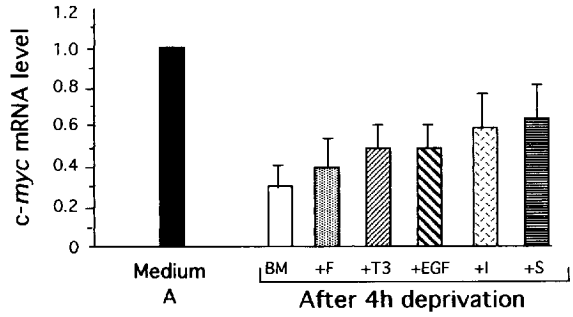

Fig. 1. Effect of growth factor deprivation on c-*myc* mRNA expression. Cells were grown in medium A as usual (see Materials and Methods). This medium was then replaced for 4 h by the basal medium, either alone (BM) or with various added hormones and growth factors: 10 nM, hydrocortisone (F); 10 nM, triiodothyronine (T3); EGF (10 ng/ml); insulin (I, 0.12 U/ml); 1% serum (S). Northern blots were performed. c-*myc* mRNA amounts are normalized on the basis of the level observed in cells before deprivation (medium A), considered as 1. Results are expressed as the mean values ± SD of 3 different experiments. In the basal medium, c-*myc* mRNA fell to 30% of its initial value.

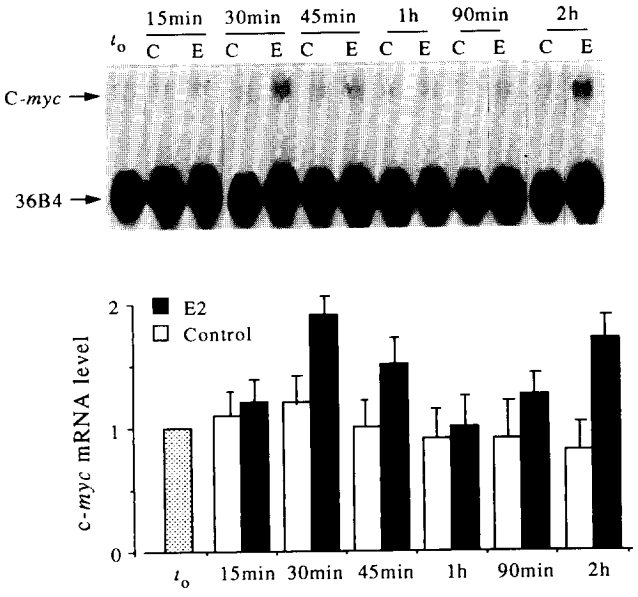

Fig. 2. E2 stimulation of c-*myc* mRNA in HBE cells. Cells were first deprived of serum and growth factors in the basal medium for 4 h. At that time (t_0), 10 nM E2 (E) or ethanol (0.1%, control = C) was added. c-*myc* mRNA was analyzed by Northern blot at various times. Upper panel: Autoradiography. Lower panel: Quantification of the c-*myc* mRNA, levels are normalized on the basis of the level observed before adding E2 (t_0), considered as 1. Results are expressed as the mean values ± SD of 3 different experiments.

Effect of growth factor deprivation on the c-myc mRNA expression (Fig. 1)

The first experiments carried out on cells grown in medium A showed a stimulatory effect of E2 on c-*myc* mRNA. However, in these conditions, results were not consistently reproducible. Similarly, the use of charcoal-stripped serum in medium A did not improve the reproducibility. We hypothesized that hormones and growth factors present in the medium might stimulate the expression of c-*myc* mRNA, and thus mask any further stimulation by E2. Medium A, in which the cells were grown, was then replaced by the basal medium devoid of any growth factor for up to 12 h. As a result the c-*myc* mRNA level fell to 30% of its initial value after 2 h, and remained low for the next 10 h (data not shown). When F, T3, EGF, insulin or serum (1%) was added to the basal medium, they partly prevented the c-*myc* mRNA level from decreasing (Fig. 1). All subsequent experiments of hormone treatments were therefore performed on cells grown in medium A and then deprived of growth factors in the basal medium for 4 h.

E2 stimulation of c-myc mRNA expression

As a function of time (Fig. 2). HBE cells were first deprived of hormones and growth factors in the basal medium for 4 h, and then treated with E2. c-*myc* mRNA levels were measured by Northern blotting at various times for up to 2 h. E2 induced a biphasic increase in c-*myc* mRNA level with a first peak (1.9 ± 0.3-fold) at 30 min and a secondary increase (1.7 ± 0.3-fold) at 2 h after treatment (Fig. 2). None of the factors, F, T3, EGF, insulin or 1% serum, modified this biphasic pattern of c-*myc* mRNA induction by E2 (data not shown).

As a function of dose (Fig. 3). HBE cells were treated for 30 min with various concentrations of E2 and mRNA analyzed by Northern blot. The stimulation of c-*myc* was E2 dose-dependent, the highest level being reached with the dose of 10 nM E2 (Fig. 3).

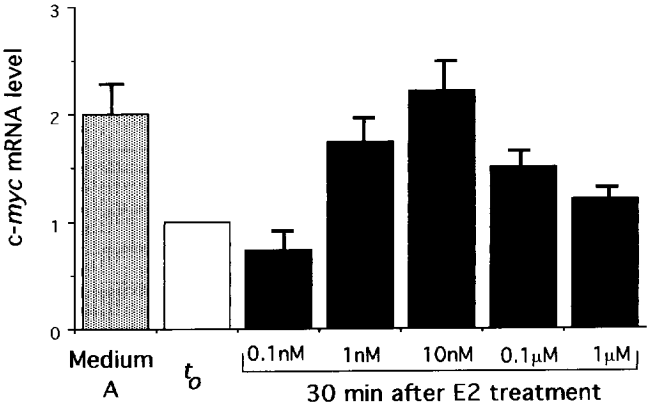

Fig. 3. Dose-dependence of E2 stimulation of c-*myc* mRNA level. Cells were grown in medium A. This medium was then replaced by basal medium for 4 h. Then (time t_0), E2 was added in various concentrations ranging from 0.1 nM to 1 μM and mRNA extracted 30 min later for Northern blot analysis. Results are expressed as the mean values ± SD of 3 different experiments.

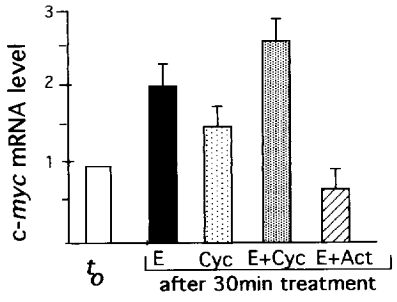

Fig. 4. Action of cycloheximide and actinomycin D on the E2 stimulation of c-*myc* mRNA in HBE cells. Cells grown in medium A were deprived of hormones and growth factors for 4 h in the basal medium. Then (time t_0), one of the following treatments was added: 10 nM E2 (E); cycloheximide (Cyc), E2 + cycloheximide (E + Cyc), or E2 + actinomycin D (E + Act). Total RNAs were extracted 30 min later for Northern blot analysis. Amounts of c-*myc* mRNA were normalized on the basis of the level observed at time t_0 and considered as 1. Results are expressed as the mean values ± SD of 3 different experiments.

Mechanisms of E2 stimulation of c-myc mRNA expression

To address the question of the mechanisms involved in this mRNA stimulation, we next studied the effects of protein synthesis and transcription inhibitors on E2 stimulation of c-*myc* mRNA level.

Effects of cycloheximide and actinomycin D on E2 action (Fig. 4). To determine whether protein synthesis was involved in E2 stimulation of c-*myc* mRNA, HBE cells were treated with the protein synthesis inhibitor, cycloheximide. When cycloheximide was added together with E2, the transcript level increased 150% (Fig. 4). This increase was higher than that observed with E2 alone. Therefore, cycloheximide does not prevent E2 from stimulating c-*myc* expression. These results suggest additive effects since cycloheximide alone induced a 50% increase in the c-*myc* mRNA level. This cycloheximide action could result from the inactivation of a labile regulatory protein, known to destabilize c-*myc* mRNA (25).

Actinomycin D, an inhibitor of transcription, suppressed the c-*myc* mRNA stimulation by E2 (Fig. 4). This also suggested that E2 acts primarily at the transcriptional level.

E2 and the half-life of c-myc mRNA. In order to verify whether E2 also has a stabilizing action on c-*myc* mRNA, the half-life of this transcript was studied in the presence and the absence of E2. The half-life of c-*myc* mRNA was measured to be about 12 ± 3 min. It did not vary regardless of the presence or absence of E2.

Estrogen stimulation of c-myc protein expression

To determine whether the E2 stimulation of c-*myc* mRNA was followed by an increase in the c-*myc* protein level, Western blot analyses were performed on HBE cells treated with E2 in the basal medium. E2 induced a biphasic stimulation of the c-*myc* protein, with a first peak 60 min, and a second one 2 h after treatment (Fig. 5).

DISCUSSION

c-*myc* proto-oncogene is involved in regulating cell proliferation [25–27], and seems to be one of the key genes required for a cell to progress through the cell cycle [28]. Its expression is activated by several mitogenic factors including E2. The stimulation of c-*myc* expression by E2 has been observed in MCF-7 and T47D human breast cancer cell lines [11–13], and in various non-cancerous E2-dependent tissues (uterus, oviduct) [10, 14, 15]. It seemed, therefore, worthwhile to study c-*myc* expression and its possible hormone modulation in normal breast cells.

Our laboratory has developed a system of culture for human breast cells originating from breast tissue samples obtained from surgical reduction mammoplasty [17]. These normal HBE cells remain hormone-dependent in culture, as shown by studies on cell growth, ultrastructure, enzyme activity and steroid receptor levels [7, 17–20]. It has been shown that E2 stimulates whereas antiestrogens inhibit the growth of these HBE cells [7, 19]. They therefore provide

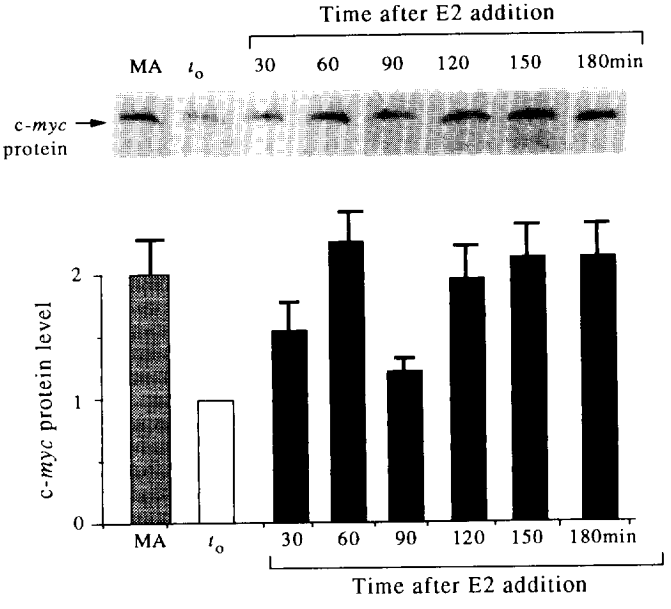

Fig. 5. E2 stimulation of the c-*myc* protein in HBE cells. Cells were grown in medium A (MA), which was then replaced by the basal medium for 12 h. Then (time t_0), the cells were treated with 10 nM E2 for various lengths of time. Nuclear proteins were extracted; 50 μg of the proteins were separated on polyacrylamide urea gel and transferred onto membranes before immunodetection of c-*myc* protein using chemiluminescence reaction. Upper panel: Western blot autoradiography. Lower panel: Quantification of c-*myc* protein. The levels were normalized on the basis of the level observed before E2 treatment (t_0) and considered as 1. Results are expressed as the mean values ± SD of 3 different experiments.

useful material for studying how E2 regulates c-*myc* expression.

Culture media routinely used are rich in hormones and growth factors. The stimulatory effects of these molecules could mask the E2 effects. In our previous experience with HBE cells, it was necessary to deprive the medium of these factors to demonstrate the growth promoting effect of E2 [17–19]. Similarly, only by depriving the HBE cells of hormones and growth factors before treating them with E2, could we obtain a reproducible stimulation of c-*myc* mRNA expression. The decrease in c-*myc* mRNA, observed in cells withdrawn from medium A to the basal medium, was partly prevented when F, T3, EGF, insulin or serum (1%) were added during deprivation. These factors seemed therefore to be potential stimulators of c-*myc* mRNA. The stimulation of c-*myc* mRNA by insulin, serum or EGF had already been observed in other cell types [13, 23, 29]; but no such stimulatory effect had been reported concerning T3 or F.

In the basal medium, which did not contain any growth-promoting substance, E2 stimulated the expression of c-*myc* mRNA. This stimulation was dose-dependent, the optimal concentration of E2 being 10 nM, which is similar to the optimal concentration for stimulating HBE cell growth [7].

This stimulation of c-*myc* mRNA is very rapid. When the basal medium was deprived of hormones and growth factors, the c-*myc* mRNA level decreased rapidly, thus corroborating the short half-life of the c-*myc* transcript. This half-life, as measured in HBE cells, was 12 ± 3 min. This is consistent with values observed in other cell types [12, 23]. The half-life of c-*myc* mRNA was not modified by E2. It therefore seems that the E2 action is not due to a stabilization of c-*myc* mRNA. Moreover, as described in the MCF-7 cancer cell line [12], this stimulation does not require protein synthesis, since it is not inhibited by cycloheximide.

All of these data suggest that E2 may act primarily at the transcriptional level. The classical mechanism of steroid hormone action involves binding of the hormone–receptor complex (E2–ER) on a regulatory sequence (ERE) located upstream from the gene. Dubik recently discovered a potential binding site for the E2–ER complex [30]. Though different from a classical ERE, the existence of this site, located upstream from the c-*myc* gene, supports the hypothesis of E2 action at the transcriptional level.

The biphasic pattern of c-*myc* mRNA stimulation by E2 has been previously observed in various models, in particular in breast cancer cells [11] and rat uterus tissue [10, 15]. It should be noted that the c-*myc* protein follows the same pattern as mRNA after E2 stimulation. This biphasic pattern can be due to a combination of several regulatory mechanisms that are known to modulate the expression of this proto-oncogene [31, 32], both at the transcriptional and post-transcriptional levels. A negative autoregulatory loop [33] in addition to the short half-life of the protein [34] may constitute such a combination.

In conclusion, c-*myc* proto-oncogene is expressed and stimulated by E2 in normal breast cells, as had been previously observed in breast cancer cells. To our knowledge, this is the first demonstration of c-*myc* proto-oncogene stimulation by E2 in normal HBE cells. In these cells, therefore, the growth-promoting effect of E2 may, at least partly, be mediated through c-*myc* stimulation. As a consequence, any abnormality in this process could result in cell growth disregulation. These data should be considered in the overall approach to a better understanding of the puzzling multistep process whereby normal cells become cancerous.

Further studies are underway both to understand the exact mechanisms of E2 action, at the transcriptional and/or post-transcriptional levels, and also to explore possible inhibitory effects of antiestrogens at these levels.

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