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Stimulation of c-*myc* Oncogene Expression Associated with Estrogen-induced Proliferation of Human Breast Cancer Cells[1]

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ABSTRACT

Regulation of c-*myc* expression is known to be sensitive to a variety of mitogenic stimuli in various cell types. Since estrogen is a well documented mitogen of estrogen-responsive human breast cancer (HBC) cells, we studied the influence of estradiol and its antagonist tamoxifen on the expression of c-*myc* in HBC cell lines. Using Northern hybridization analysis, we monitored the accumulation of c-*myc* mRNA in a number of HBC cell lines. The cell lines studied included the estrogen-responsive, estrogen receptor positive (ER+) MCF-7, T-47D, the nonresponsive, estrogen receptor negative (ER−) MDA-MB-231, BT-20, and a nontumorous breast cell line, HBL-100. The effects of endogenous estrogen were minimized by culturing the cells in medium containing 10% (v/v) charcoal-treated fetal bovine serum and tamoxifen (10^{-6} M) for 48 h prior to estradiol (10^{-7} M) treatment. In the ER+ cell lines the addition of estradiol resulted in a noticeable increase in c-*myc* expression after 15 min with a maximal (>10-fold) induction in 1–2 h. In the ER− cell lines the level of c-*myc* mRNA was high and was unaffected by estrogen or tamoxifen; in the ER− cancer cell lines, neither amplification nor rearrangement of the c-*myc* gene was observed. In contrast, the expression of another oncogene, c-H-*ras*, remained constant in both ER+ and ER− cell lines and was insensitive to estrogen and antiestrogen. These results suggest that regulation of c-*myc* expression may be an important step in estrogen-induced proliferation of HBC cells.

INTRODUCTION

A group of genes, known as protooncogenes, has been recognized as being important in cancerous proliferation and transformation (1–3). Of these protooncogenes the importance and function of c-*myc* is among the most studied. This is due to the fact that amplification, rearrangement, and translocation of the c-*myc* gene or variations in c-*myc* mRNA levels have repeatedly been observed in various tumors, differentiating cell lines, and mitogenically stimulated cell lines (4–9). In breast carcinomas amplification and rearrangement of c-*myc* has been recognized (5). In some of these carcinomas these changes have been associated with enhanced c-*myc* mRNA expression. Clinically, about one-third of all breast tumors are estrogen responsive, in that estrogen stimulates their proliferation (10). To better understand the action of estrogen in HBC[5] we therefore chose to investigate the effects of this steroid hormone on c-*myc* mRNA expression in several HBC cell lines.

MATERIALS AND METHODS

Tissue Culture. The sources of all HBC cell lines were described previously (11). Stock cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with L-glutamine (4 mM), glucose (4.5 g/liter), penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively), bovine insulin (10 μg/ml), and 10% (v/v) fetal bovine serum. Trypsin/EDTA in Hanks' balanced salt solution was used for cell passage. All reagents were obtained from Flow or Gibco Laboratories. The cells were kept in a humidified atmosphere of 95% air:5% CO_2 at 37°C.

Hormones. 17β-Estradiol was purchased from Sigma Chemical Co., St. Louis, MO. Tamoxifen base was generously provided by Imperial Chemical Industries Pharma, Missassauga, Ontario. Stock solutions in ethanol were prepared each month for both hormones. Aqueous solution prepared from these stocks were used to treat the cells. The stock solutions were stored in the dark at −20°C.

Growth Response Studies. For growth studies, cells were plated at a density of 1×10^3 cells/cm^2 in insulin-free medium containing FBS (C) and left for 48 h. The medium was then changed to either C, C′ (containing cFBS) (12), or T′ (C′ with 10^{-6} M tamoxifen). After 48 h, estradiol (final concentration, 10^{-7} M) was added to C′ and T′. These conditions were then denoted as E′ and TE′, respectively. This concentration of tamoxifen (10^{-6} M) has previously been shown to achieve inhibition of cell growth without cytotoxicity, and the concentration of estradiol (10^{-7} M) used was appropriate for the stimulation of cell growth in the presence of tamoxifen (13). A corresponding volume of ethanol was added to dishes not receiving estradiol; the final concentration of ethanol was 0.007% in all dishes. The cells were then allowed to grow for a further 8–10 days without medium change. After this time the cells were trypsinized, dispersed through an 18-gauge needle, and the cell number determined using an electronic cell (Coulter) counter.

Preparation of Cells for RNA Extraction. Stock cells were plated at 0.7×10^4 cells/cm^2 in medium C and were left to attach for 48 h. This medium was then replaced with either insulin-free medium containing 10% (v/v) cFBS with (T′) or without (C′) 10^{-6} M tamoxifen for a further 48 h. Estradiol (10^{-7} M) was then added and at various times after estradiol addition, cells were harvested for RNA isolation.

RNA Isolation and Northern Hybridization Analysis. Total RNA was isolated by the guanidinium isothiocyanate/cesium chloride method (14). For Northern blot analysis this RNA was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, fractionated on a 1% (w/v) agarose-2.2 M formaldehyde denaturing gel, and then transferred onto nitrocellulose paper as described by Maniatis *et al.* (15). The RNA was fixed onto the nitrocellulose by baking for 2 h at 80°C. The blot was then prehybridized in hybridization solution containing 50% (v/v) formamide (16) at 42°C for at least 3 h. Nick-translated cDNA probe ($1–5 \times 10^8$ cpm/μg DNA) was then added. Hybridization was carried out at 42°C for 16–20 h. Following hybridization the blots were washed twice in 1× standard saline citrate-0.1% sodium dodecyl sulfate for 15 min and once in 0.1× standard saline citrate-0.1% sodium dodecyl sulfate at 65°C for 1 h. Blots were then exposed to Kodak XAR film at −70°C with an intensifying screen. When nitrocellulose blots were reused, they were first boiled in water for 3 min.

For dot blot analysis RNA isolated as described above was denatured according to the protocol of White and Bancroft (17) and spotted in varying concentrations (5–0.156 μg) onto nitrocellulose filters using a BRL dot-blot manifold. Filters were then hybridized as previously described.

Quantitation of the relative amounts of specific RNA transcripts was performed by densitometric scanning of the hybridization signals.

DNA Isolation and Southern Hybridization Analysis. DNA was isolated from appropriate cells, cut with the restriction endonuclease *Pst*I, fractionated on a 0.8% agarose Tris-borate-EDTA buffer gel, and transferred to nitrocellulose according to the protocol of Davis *et al.*

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[5] The abbreviations used are: HBC, human breast cancer; ER, estrogen receptor; FBS, fetal bovine serum; cFBS, charcoal-treated, heat-inactivated fetal bovine serum; cDNA, complementary DNA; kb, kilobase; SCP, sodium chloride phosphate.

(18). After baking for 2 h, the nitrocellulose blot was prehybridized for 3 h in SCP prehybridization buffer composed of 6× SCP (20× SCP = 2 M NaCl, 0.6 M Na_2HPO_4, and 0.02 M EDTA, pH 6.2), 0.4% *N*-lauroyl sarcosine, 200 μg/ml sheared salmon sperm DNA, 4× Denhardt's solution, and 50% formamide. The hybridization was carried out at 42°C for 24 h in SCP prehybridization buffer containing 10% dextran sulfate using a ^{32}P-labeled (by nick translation) genomic c-*myc* probe. Following hybridization, the blot was washed twice in 6× SCP/1% sarcosyl for 15 min, twice in 1× SCP/1% sarcosyl for 90 min, and once in 0.2× SCP/1% sarcosyl for 30 min, all at 65°C. The blot was then exposed to Kodak XAR film at −70°C with an intensifying screen.

cDNA Probes. The c-*myc* probe used for Northern analysis is a 0.5-kb cDNA probe corresponding to the *Pst*I fragment of the second exon of the human c-*myc* gene and was generously provided by Dr. W. S. Hayward (19). The genomic c-*myc* probe used for the Southern hybridization analysis is a 8.2-kb *Hind*III-*Eco*RI fragment containing the entire human c-*myc* gene and was described previously by Favera *et al.* (20). The c-H-*ras* probe is a 0.7-kb cDNA probe corresponding to the *Sst*I-*Pst*I fragment of the v-H-*ras* gene and was purchased from ONCOR, Inc., Gaithersburg, MD.

RESULTS

Cell Response to Estrogen and Tamoxifen. In order to maximize the effects of estradiol on HBC cells it was necessary to deplete the endogenous levels of estrogen present in both cells and growth medium. This was done by using cFBS in the medium and by adding a growth inhibitory, nontoxic, concentration of tamoxifen (10^{-6} M) to offset the effects of any residual estrogens. The growth response data for T-47D and MCF-7 are summarized in Fig. 1. Partial growth inhibition was observed in estrogen-depleted medium (C′) and was further suppressed on addition of tamoxifen (T′) when compared with cells growing in the presence of untreated FBS (C) which contains endogenous estrogens. This growth inhibition was reversed by estradiol (10^{-7} M). Although Fig. 1 shows cell growth 10 days after various treatments, we have also measured the growth response in the first 48 h, the time interval where we subsequently examined c-*myc* expression. At 48 h in T-47D the fold increase in cell number for the five groups was C, 2.0; C′, 1.3; T′, 1.2; E′, 1.8; and TE′, 1.6. These data correspond well with the results in Fig. 1.

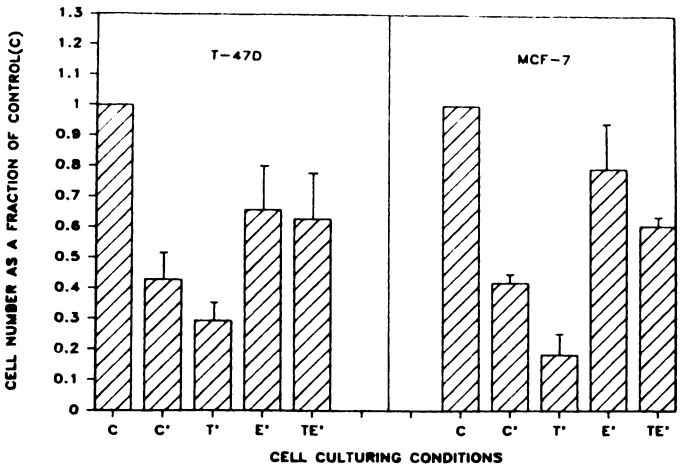

Fig. 1. Growth response of ER+ cells T-47D and MCF-7 to tamoxifen and estradiol. Cells were cultured and hormone treated as described in "Materials and Methods." Conditions *E′* and *TE′*, cells grown in C′ and T′ medium for 48 h, then treated with estradiol (10^{-7} M). This delayed addition of estradiol accounts for the apparent incomplete rescue of cell growth when compared to cells grown continuously in untreated FBS (medium C). Cell number was determined 10 days after estradiol addition and in no instance were the cells confluent. Results are mean values with SD (*bars*) of three experiments normalized to cell growth in medium C.

c-*myc* Expression in ER+ Cells. Fig. 2 depicts the time course of c-*myc* and c-H-*ras* mRNA accumulation in tamoxifen-inhibited MCF-7 cells "rescued" by estradiol. A significant increase in c-*myc* mRNA accumulation was observed after 15 min of estradiol rescue, with maximal levels (>10-fold) achieved between 60 and 90 min. The level of c-*myc* expression gradually declined for the next 10 h and remained at a level 3-fold above tamoxifen-treated cells for at least 54 h thereafter (Fig. 3). Fig. 3 also reveals a similar pattern of induction in another ER+ cell line, T-47D, although the maximal accumulation of c-*myc* mRNA occurred around 2 h after estradiol rescue. In contrast, the expression of c-H-*ras* was unaffected by either estradiol or tamoxifen (Fig. 2*c*).

Comparison of c-*myc* and c-H-*ras* Expression in ER+ and ER− HBC Cells. The patterns of expression of c-*myc* and c-H-*ras*, 60 min after estradiol rescue, in MCF-7 (ER+) and MDA-MB-231 (ER−) cells are depicted in Fig. 4. Whereas tamoxifen inhibited and estradiol stimulated c-*myc* expression in MCF-7, they had no effect on c-*myc* expression in MDA-MB-231 cells. It is of interest to note that the MDA-MB-231 cell line expressed a higher level of c-H-*ras* mRNA which was unaffected by tamoxifen and estradiol.

Using dot blot analysis, the estrogen nonresponsive BT-20 tumor line and the nontumorous HBL-100 line showed no regulation of c-*myc* expression by either estradiol or tamoxifen in contrast to MCF-7 and T-47D cells (Fig. 5). These estrogen nonresponsive cell lines, similar to the MDA-MB-231 cell line,

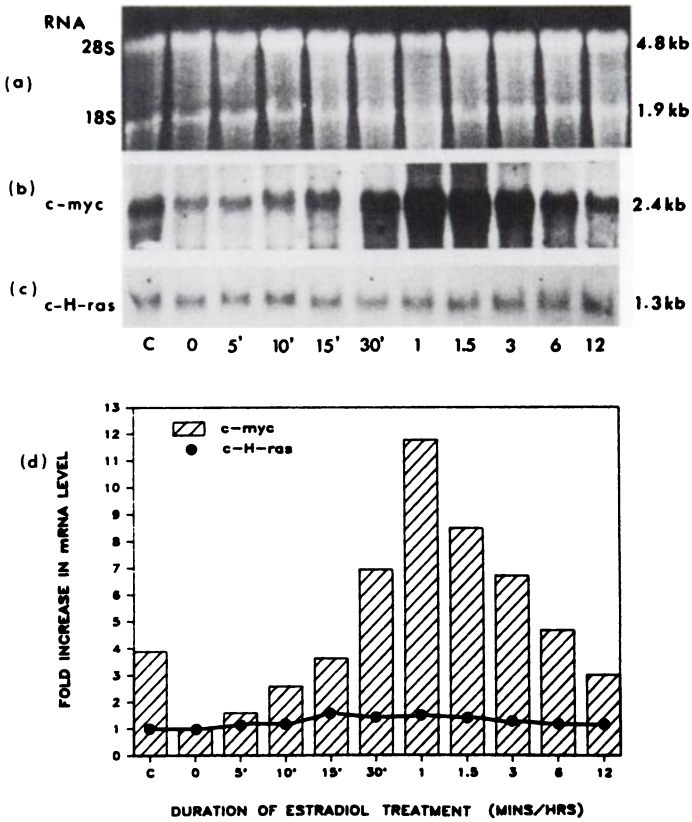

Fig. 2. Effects of estradiol on the accumulation of c-*myc* and c-H-*ras* mRNA in MCF-7 cells. Tamoxifen-inhibited cells were exposed to estradiol (10^{-7} M) for 0–12 h. At the times indicated cells were harvested and total RNA isolated for Northern analysis. Cells in medium C were harvested at time 0 h. For each sample, 50 μg of RNA were analyzed. *A*, ethidium bromide staining of total RNA; *b*, hybridization of nitrocellulose blot to c-*myc* insert probe (3×10^6 cpm/ml; 1-day film exposure); *c*, reprobing with v-H-*ras* insert probe (3×10^6 cpm/ml; 5-day exposure); *d*, quantitation of relative amounts of c-*myc* and c-H-*ras* normalized to levels present in tamoxifen-inhibited cells prior to estradiol addition (t = 0). *C*, oncogene expression in cells growing in FBS-containing medium.

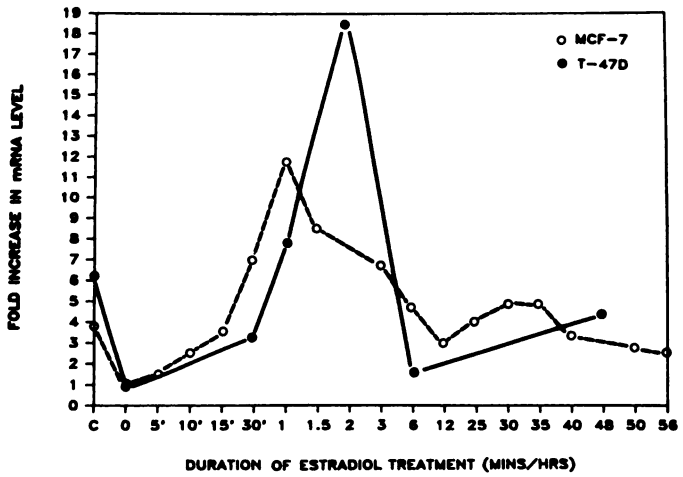

Fig. 3. Time course of the effect of estradiol on the accumulation of c-*myc* mRNA in ER+ MCF-7 and T-47D cells. Results are normalized in relation to tamoxifen growth-arrested cells prior to addition of estradiol ($t = 0$).

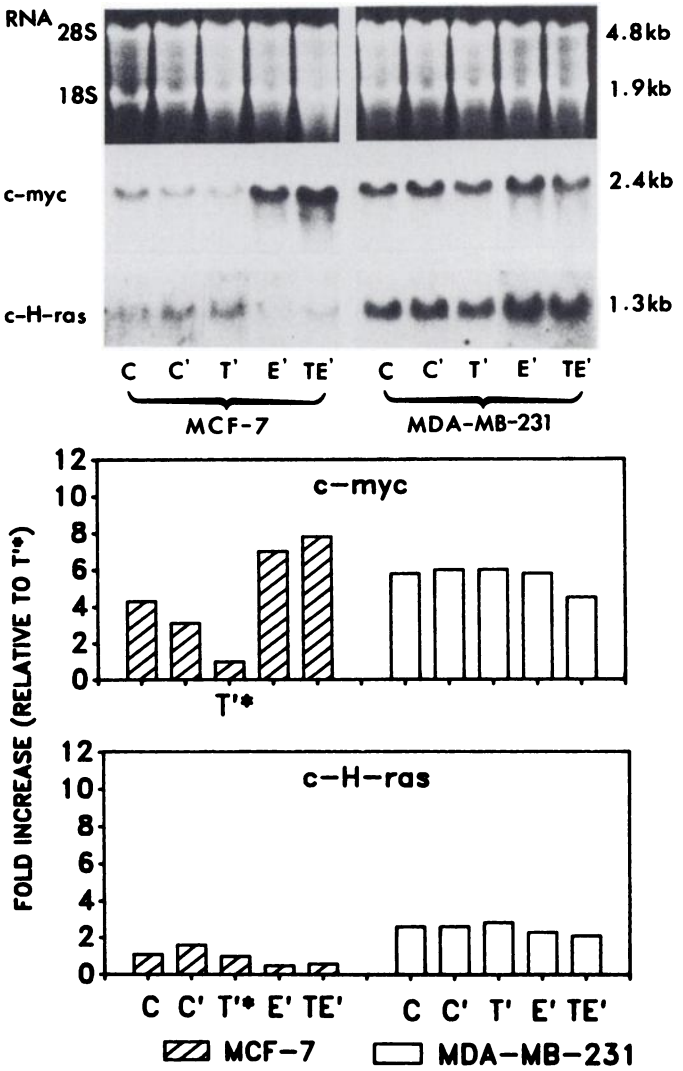

Fig. 4. Comparison of c-*myc* and c-H-*ras* expression in ER+ MCF-7 and ER− MDA-MB-231 cells. Total RNA (50 μg) was applied to each lane. Conditions *C*, *C'*, *T'*, *E'*, and *TE'* are as described in the legend to Fig. 2. Exposure to estradiol (10^{-7} M) was 1 h. The blot was hybridized to c-*myc* and v-H-*ras* probes and exposed to film as described in the legend to Fig. 2. Also shown is the quantitation of the relative amounts of c-*myc* and c-H-*ras*, normalized to levels present in tamoxifen-inhibited cells (*T'**).

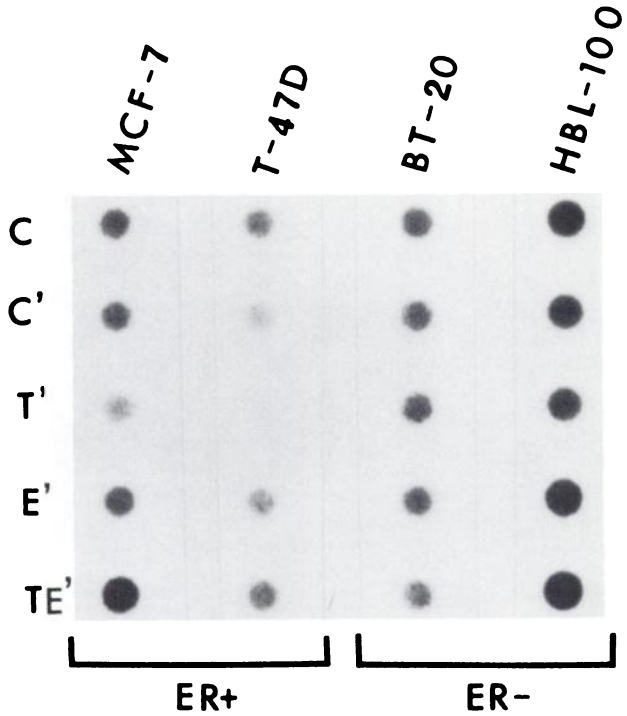

Fig. 5. Dot blot analysis of regulation of c-*myc* mRNA expression in human breast cell lines. Cells were grown and RNA isolated as described in "Materials and Methods." Growth conditions and estradiol treatment were identical to those in Fig. 4. The blot was hybridized to c-*myc* insert probe (1×10^6 cpm/ml; film exposure was 14 h). For clarity, hybridizations to only one concentration of RNA (0.625 μg) is shown.

also exhibited high, estrogen-independent, constitutive expression of c-*myc* mRNA. To determine whether this high level of c-*myc* expression in the estrogen nonresponsive cells was a result of gene rearrangement or amplification, Southern analysis of the c-*myc* gene was performed. The result of this study, shown in Fig. 6, indicates no rearrangement or amplification of the c-*myc* gene in any of the breast cancer cell lines. However, the nontumorous breast cell line HBL-100 has approximately 4-fold amplification, but no rearrangement of the c-*myc* gene.

DISCUSSION

We have shown that an induction (>10-fold) of c-*myc* mRNA occurs between 1 and 2 h after addition of estradiol to ER+ HBC cells growth retarded by the use of tamoxifen and cFBS. This time course is similar to c-*myc* induction by estradiol in the rat uterus (21) or by serum in hamster lung fibroblasts (4) and aortic smooth muscle cells (22). The higher level of c-*myc* expression in cells grown in medium containing untreated FBS (*C*, Figs. 2 and 3) is due to endogenous estrogens present in the FBS. Besides the estrogens present in FBS, phenol red, a pH indicator used in the growth medium, has been reported to have a weak estrogenic effect on ER+ cells (23). To offset the effects of phenol red and the residual estrogens associated with the cells, we added tamoxifen to our culture medium. This resulted in a 30–50% decrease in cell growth, when compared to that of cells grown in medium supplemented with cFBS alone (Fig. 1). We had also observed that c-*myc* induction was less marked if the cells were plated at more than 2.1×10^4 cells/cm^2 (data not shown). One possible explanation is that ER+ HBC cells normally, or as a result of cell-cell contact, produce internal or external factors that when present and at a sufficiently high concentration can overcome the effects of estrogen depletion. Therefore each dish was plated with no more than 0.7×10^4 cells/cm^2.

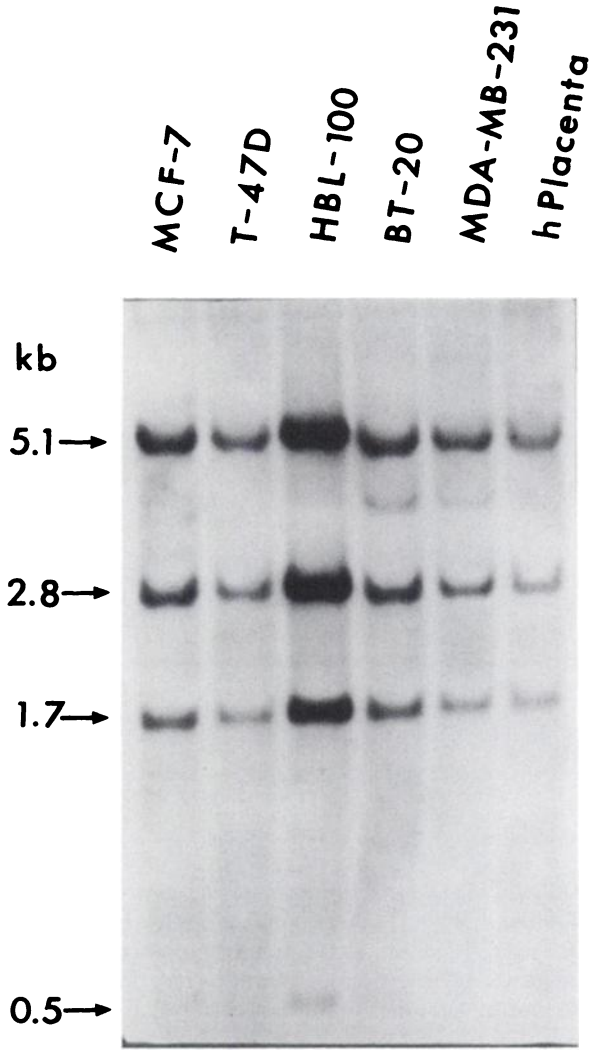

Fig. 6. Southern genomic DNA analysis of c-*myc*. Each *lane* contains approximately 15 μg of DNA cut with *Pst*I. The blot was hybridized to a genomic c-*myc* probe (3×10^6 cpm/ml; film exposure, 16 h). Using densitometric scanning, the slight variations of the hybridization intensities in the 5.1-, 2.8-, 1.7-, and 0.5-kb bands among the human placenta (*hPlacenta*) and all the breast cancer cell lines were less than 2-fold. These data together with a previous report of a lack of gene amplification in MDA-MB-231 (29), would indicate that the variation in band intensity is a result of slight differences in the amount of DNA loaded into each *lane*. Only the nontumorous cell line HBL-100 shows significant amplification of the c-*myc* gene (approximately 4 × that of the breast cancer cell lines). The faint 3.8-kb band in BT-20 and MDA-MB-231 is an artifact of hybridization.

Kasid *et al.* (24) reported that estrogen had no regulatory affect on a number of oncogenes, including c-*myc*, in MCF-7. Clearly our data do not support this conclusion. It is difficult to comment on this inconsistency because data on specific oncogenes were not provided by Kasid *et al.* (24). However, possible explanations include variation between the MCF-7 cell lines used and different culture conditions.

At present whether the induction of c-*myc* is a direct effect of estradiol on the gene or a consequence of cell proliferation is unresolved. Also, it is unknown whether the estradiol-induced increase in c-*myc* mRNA accumulation is due to the activation of transcription or the stabilization of mRNA transcripts. Alterations in c-*myc* expression by growth stimulating and inhibiting factors have involved both mechanisms (4, 6, 25, 26). Irrespective of the mechanism of activation, estradiol-induced gene expression in HBC cells appears relatively specific for c-*myc*, inasmuch as expression of c-H-*ras* (Ref. 24; Figs. 2 and 4) and c-*sis* (data not shown) was not similarly affected.

The results of our studies together with previously reported data offer a probable role for the enhanced expression of c-*myc* in estradiol-stimulated growth of HBC cells. Recently Studzinski *et al.* (27) have reported that the c-*myc* gene product regulates cell proliferation at the level of DNA synthesis, affecting the activity of DNA polymerase α. Also Edwards *et al.* (28) showed that antiestrogens decreased DNA polymerase α activity in MCF-7 while addition of estradiol restored the activity. These findings together with ours would indicate that estradiol increases the accumulation of the c-*myc* mRNA the product of which in turn interacts with DNA polymerase α to activate DNA synthesis.

Two interesting observations were made by comparing c-*myc* expression in ER+ *versus* ER− cell lines (Figs. 4–6): (*a*) neither the removal nor addition of estradiol nor the presence of tamoxifen has an effect on c-*myc* expression in ER− cells; (*b*) high constitutive expression of c-*myc* mRNA occurred in all three ER− cell lines. In BT-20 and MDA-MB-231, this expression is not due to amplification of the c-*myc* gene, whereas gene amplification appears to be able to account for the increased c-*myc* mRNA in the HBL-100 cells. These observations suggest the possibility that in HBC cells the ability to express unregulated high levels of c-*myc* may be a prerequisite for estrogen-independent cell growth. Thus, estradiol regulation of c-*myc* expression may represent a necessary step in the mechanism by which estrogen enhances the growth of ER+ human breast cancer cells.

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