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Expression of the c-*erbB2* gene in the BT474 human mammary tumor cell line: measurement of c-*erbB2* mRNA half-life

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BT474 and SK-BR-3 mammary adenocarcinoma cells contain eight copies of the c-*erbB2* gene but overexpress the mRNA 80 times over the levels measured in normal breast or in the HBL-100 cell line. Using Northern blot analysis and molecular titration based on RNAase protection assay, the decrease in the c-*erbB2* mRNA level was monitored in BT474 cells treated with actinomycin D from 1 up to 24 h. The c-*erbB2* degradation rate during the first 12 h corresponds to a calculated c-*erbB2* mRNA half-life of approximately 7 h. Forty percent of the mRNA present in the cells before treatment remains undegraded after transcription has been blocked for 24 h. Pretreatment with cycloheximide results in complete mRNA degradation in 24 h, suggesting that labile proteins stabilize part of the c-*erbB2* mRNA population. Comparison with the c-*erbB2* mRNA turnover in HBL-100 'normal' cells indicated that the accumulation of the c-*erbB2* gene product in the tumor cells is not the result of stabilization of the messenger. Rather, it is correlated with an increased rate of c-*erbB2* mRNA transcription as indicated by run-on transcription assays. Both BT474 and SK-BR-3 tumor cell lines were found to synthesize 20–40 times more c-*erbB2* mRNA than HBL-100 cells.

Introduction

The c-*erbB2* proto-oncogene encodes a transmembrane tyrosine kinase glycoprotein, structurally related to the epidermal growth factor (EGF) receptor (Akiyama *et al.*, 1986). Several possible ligands for the c-*erbB2* receptor have recently been described, but none has been fully characterized (Yarden & Weinberg, 1989; Lupu *et al.*, 1990; Dobashi *et al.*, 1991; Tarakhovsky *et al.*, 1991; Yarden & Peles, 1991; Peles *et al.*, 1992).

Activation of the c-*erbB2* oncogene corresponds to the overexpression of an apparently non-mutated c-*erbB2* receptor (Lemoine *et al.*, 1990) and is correlated with poor prognosis in primary breast (Perren, 1991), ovarian (Berchuck *et al.*, 1990) and lung adenocarcinomas (Kern *et al.*, 1990; Weiner *et al.*, 1990). Abnormally elevated c-*erbB2* gene products are detected in primary breast tumors and derived mammary adenocarcinoma cell lines in the presence or absence of gene amplification, suggesting that different mechanisms are involved in the deregulation of c-*erbB2* gene expression (Kraus *et al.*, 1987; Slamon *et al.*, 1989). To characterize the underlying molecular alterations responsible for breast neoplasms, we compared different mammary epithelial cell lines for the synthesis of c-*erbB2* mRNA and gene amplification (Kraus *et al.*, 1987). The BT474 adenocarcinoma cell line contains eight copies of the c-*erbB2* gene and overexpresses the mRNA 80 times over the levels measured in the normal human breast, in MCF7 tumor cells or in the HBL-100 'normal' cell line derived from the milk of a nursing mother with no breast lesion and containing a single copy of the gene (Pasleau *et al.*, 1990). In BT474 cells, the overexpression of the transcripts is much more important than, and not proportional to, the gene copy number. Such elevated levels of c-*erbB2* gene products may be due either to an increase in the production or to a decrease in the decay of the mRNA. Besides an increase in c-*erbB2* mRNA synthesis, possibly correlated with the gene amplification, the stablization of the messenger can contribute to the accumulation of very high levels of mRNA.

Post-transcriptional modulations of mRNA stability have been reported to participate in the oncogenic activation of other proto-oncogenes such as c-*fos* (Raymond *et al.*, 1989), c-*myc* (Eick *et al.*, 1985; Lacy *et al.*, 1989) or c-*fms* (Weber *et al.*, 1989). To test this hypothesis, we compared the c-*erbB2* mRNA half-life in 'normal' HBL-100 and tumor BT474 cell lines.

We found that the decrease in c-*erbB2* mRNA in the BT474 cells treated with actinomycin D (Act D) evolves as a biphasic phenomenon. The degradation rate during the first 12 h of treatment corresponds to a calculated c-*erbB2* mRNA half-life of approximately 7 h. Surprisingly, 40% of the mRNA present in the cells before treatment remains undegraded after transcription has been blocked for 24 h. This proportion of undegraded messenger is reduced to 25% when BT474 cells are preincubated in the presence of cycloheximide (CHX) for 2 h prior to the addition of Act D.

Technical limitations did not allow an accurate determination of the c-*erbB2* mRNA half-life in HBL-100 cells, which synthesize very low amounts of this message. However, c-*erbB2* mRNA degradation rates of the same order of magnitude were measured in both cell lines, excluding a major contribution of post-transcriptional mechanisms. The nuclear run-on transcription assays confirmed the difference in the transcription level of the c-*erbB2* mRNA between the cell lines. In these experiments, the high-expressing SK-BR-3 tumor cells were included. Both tumor cell lines synthesize 20–40 times more c-*erbB2* mRNA than the normal cells.

In conclusion, the machinery involved in c-*erbB2* mRNA degradation was found to be complex and partially constituted by labile proteic factors that may contribute to the apparent stability of the c-*erbB2* mRNA, for example by protecting them from degradation by nucleases. The similar c-*erbB2* mRNA half-life measured in normal and tumor mammary epithelial cells indicates that the accumulation of the c-*erbB2*

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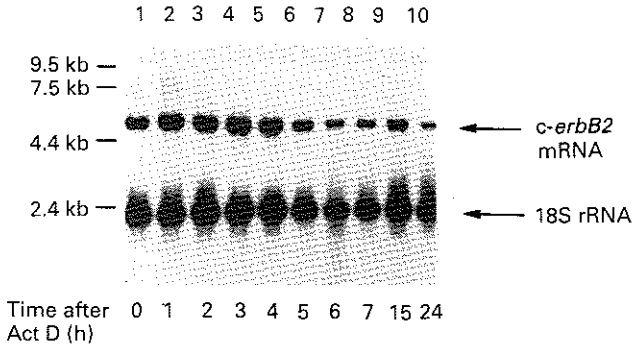


Figure 1 A 15-μg aliquot of total cytoplasmic RNA from BT474 cells treated with Act D for 1–24 h was analysed for c-*erbB2* mRNA and 18S rRNA levels by Northern blot hybridization. The positions of the c-*erbB2* mRNA (4.8 kb) and 18S rRNA are indicated relative to the standard RNA size markers (BRL). This result, obtained after 3-day-long autoradiography, is representative of six separate experiments using either pMAC-114 or pMAC-117 riboprobes

gene products in the cancer cells is not the result of the stabilization of the messenger. The transcriptional deregulation of the c-*erbB2* gene was confirmed by run-on assay, in which a 20–40 times higher rate of c-*erbB2* mRNA transcription was measured in the tumor cells.

Results

*Half-life of the c-*erbB2 *mRNA in Act D-treated BT474 cells*

BT474 cells were harvested at increasing times from 1 to 24 h after Act D treatment and total cellular RNA was extracted. After 15 h, the amount of recovered total RNA decreased gradually and the Act D toxic effects on the cell morphology became visible by phase-contrast microscopy.

The c-*erbB2* mRNA contents were measured by Northern blotting (Figure 1). The c-*erbB2*-specific autoradiographic signals appeared to be slightly increased during the first 4 h of Act D treatment (Figure 1, lanes 1–5).

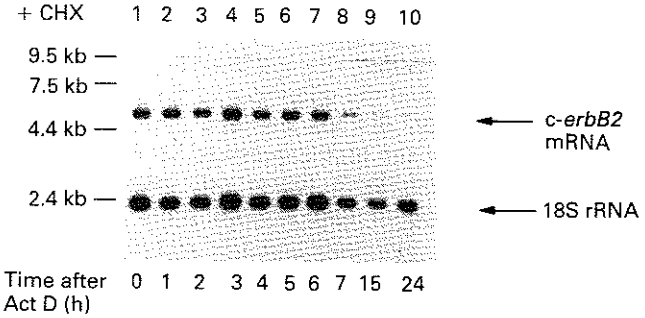


Figure 2 BT474 cells were treated with CHX for 2 h before addition of Act D and maintained in culture for 24 h. A 15-μg aliquot of total cellular RNA extracted from cells harvested at various times after Act D treatment was analysed by Northern blotting and hybridized with c-*erbB2* and 18S rRNA probes as described in the legend to Figure 1

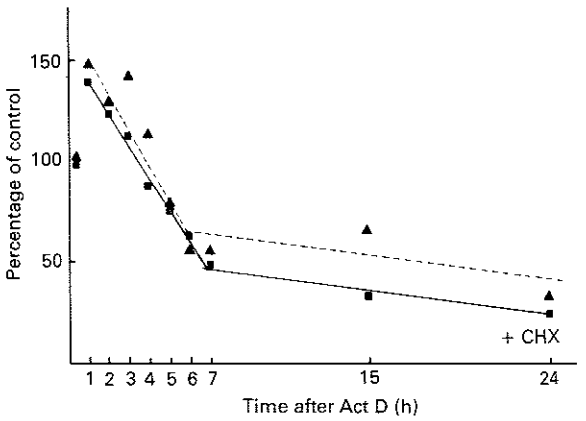


Figure 3 The c-*erbB2* mRNA and 18S rRNA autoradiographic signals obtained by Northern blot analysis of total cellular RNA from BT474 cells treated with Act D (▲---▲) or CHX + Act D (■---■) for 1–24 h (Figures 1 and 2) were quantitated by densitometric scanning. The relative c-*erbB2*/18S rRNA amounts were expressed as a percentage of the control value measured in untreated cells and plotted against the time (hours) following the addition of Act D

*Half-life of the c-*erbB2 *mRNA in the presence of CHX*

As the c-*erbB2* mRNA was not degraded during the first hours of Act D treatment, we searched for proteic factor(s) that would stabilize the messenger. Modulations of the mRNA half-life by inhibitors of protein synthesis have previously been described for c-*fos* (Greenberg *et al.*, 1986) or c-*fms* (Weber *et al.*, 1989) mRNA, the stability of which is increased or decreased respectively by CHX. BT474 cells were treated with CHX for 2 h prior to addition of Act D, and their c-*erbB2* mRNA content quantitated by Northern blotting as previously described (Figure 2). As a control of drug activities, the disappearance of c-*myc* mRNA after 30 min of Act D treatment (Dani *et al.*, 1984) and the stabilization of c-*fos* mRNA by CHX (Greenberg *et al.*, 1986) were verified (data not shown).

The relative c-*erbB2*/18S rRNA levels measured in the different samples (Figures 1 and 2) were compared with the corresponding value in untreated cells (100% at 0 h) and plotted against the time (hours) after Act D treatment in the presence or absence of CHX (Figure 3).

Similar kinetics of c-*erbB2* mRNA decrease is observed in the BT474 cells treated with Act D ± CHX, and corresponds to a calculated c-*erbB2* mRNA half-life of approximately 7 h. CHX has a destabilizing effect that potentiates the action of Act D, resulting in an almost complete degradation of the c-*erbB2* mRNA (75%) after 24 h of CHX + Act D treatment. This may indicate the presence of a proteic factor that would protect the c-*erbB2* mRNA from degradation by nucleases. However, this effect is not as strong as that described for c-*fms* mRNA, the half-life of which is reduced from 6.1 h to 30 min by CHX (Weber *et al.*, 1989).

*Measurement of c-*erbB2 *mRNA half-life by molecular titration assay*

These results were confirmed by RNAase protection assay using the pMAC117-NcoI antisense riboprobe

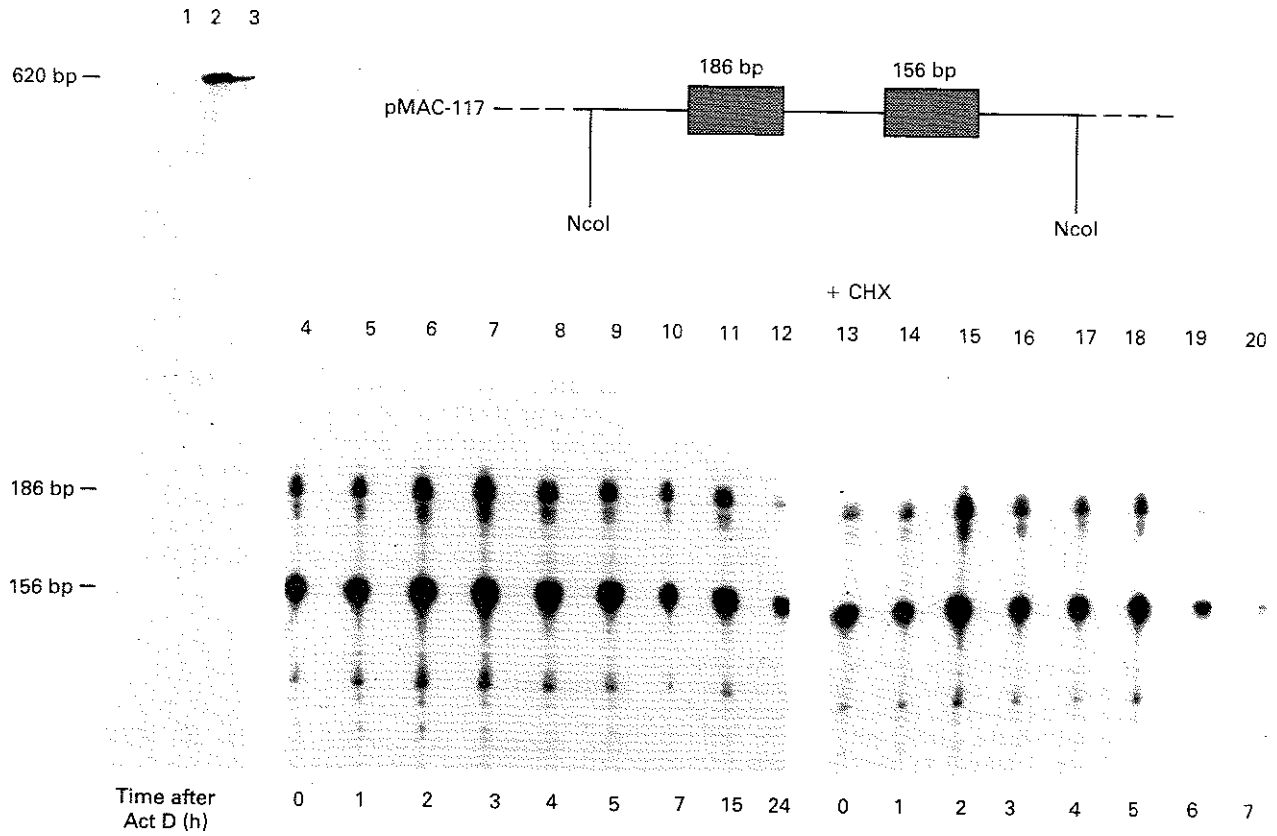


Figure 4 BT474 cells treated with Act D (lanes 4–12) or CHX + Act D (lanes 13–20) were analysed for the presence of c-*erbB2* mRNA by RNAase protection assay. A 20-μg aliquot of total cytoplasmic RNA extracted from cells harvested at the different time points was hybridized with the pMAC-117-derived riboprobe (10^5 c.p.m.) in the presence of 20 μg of yeast RNA. As indicated, the 620-bp c-*erbB2* single-stranded riboprobe (lane 3) contains two exons of 186 and 156 bp, separated by an intron; only the exon sequences were protected by the cellular c-*erbB2* mRNA (lanes 4–20). Control reactions performed in the presence of 40 μg of yeast RNA showed that the unhybridized riboprobe remained stable over 18 h incubation at 55°C (lane 2) and was completely digested by RNAases A and T1 when not protected by the c-*erbB2* mRNA (lane 1)

(Figure 4). Two major fragments of 186 and 156 bp, corresponding to the two exons encoded by the pMAC117-NcoI riboprobe, were protected when the hybridization reactions were performed in the presence of total cellular RNA from Act D- and Act D + CHX-treated BT474 cells (lanes 4–20).

In separate experiments, the protected c-*erbB2* double-stranded RNA hybrids were precipitated with trichloroacetic acid (TCA), and the radioactivity retained on a glassfibre filter was measured in a scintillation counter (Figure 5). This experiment confirmed the previous results (Figures 3), leading to similar conclusions:

(1) The c-*erbB2* mRNA is stable, even slightly induced, during the first hours of Act D treatment.

(2) The c-*erbB2* mRNA decreases in two phases: in the first phase the slope is not modified by CHX and corresponds to a calculated c-*erbB2* mRNA half-life of about 6–7 h. Thereafter, the degradation rate decreases so that approximately 40% of the initial c-*erbB2* mRNA is still detectable after 24 h of Act D treatment (Figure 1, lane 10, and Figure 4, lane 12); in

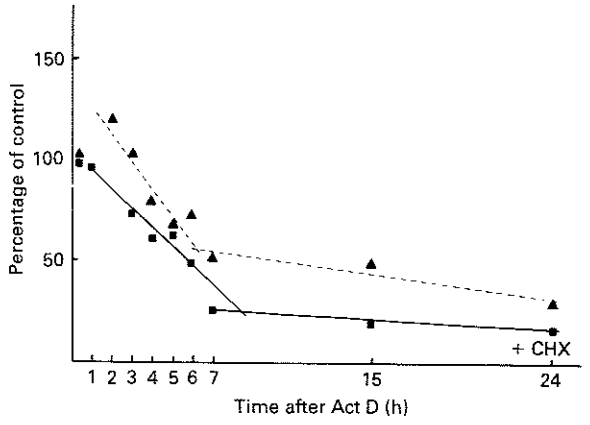


Figure 5 The c-*erbB2* mRNA amounts present in BT474 cells treated with Act D in the presence (■---■) or absence (▲---▲) of CHX were quantitated using a molecular titration assay and expressed as a percentage of the corresponding value measured in untreated cells (100% at 0 h). Total cellular RNA was incubated with a large excess of radioactive antisense c-*erbB2* riboprobe (10^5 c.p.m.) as described in the legend to Figure 4. The amount of probe protected in complementary RNA duplexes and quantitated by scintillation counting reflects the cellular mRNA content of the sample

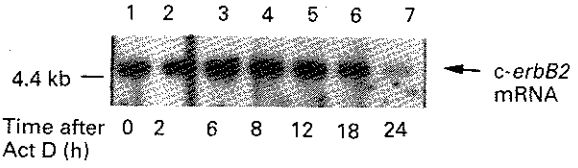
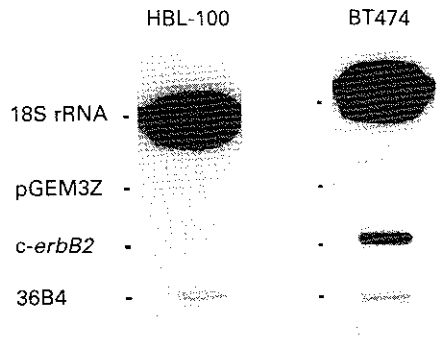


Figure 6 Northern blot anlaysis of 10 μg of polyadenylated mRNA purified from HBL-100 cells treated with Act D for varying lengths of time and hybridized with the pMAC-117-derived riboprobe

a



b

	HBL-100	*BT474*	*SK-BR-3*
c-*erB2*	0.2	4.4	7.8
GAPDH	1.4	1.1	0.9
36B4	1	1	1

Figure 7 Run-on transcription of the c-*erbB2* gene was compared in isolated nuclei from normal and tumor mammary epithelial cells. (**a**) Quantification of the relative concentrations of the 18S rRNA, 36B4 mRNA and c-*erbB2* mRNA in the HBL-100 and BT474 cell lines, estimated by autoradiography on a phosphor screen. (**b**) Quantitation of the intensity of the autoradiographic signals obtained in the HBL-100, BT474 and SK-BR-3 cell lines. The 36B4 mRNA, GAPDH mRNA and 18S rRNA were taken as controls for the amount of total RNA analysed in each assay. pGEM3Z DNA was used as a negative control. The pGEM3Z signal represented the background of non-specific hybridization. After deduction of the background, the intensity of the c-*erbB2* and GAPDH signals was compared with the 36B4 signal taken as a reference. This latter was given the value of 1

the presence of CHX, the c-*erbB2* mRNA is degraded almost to completion (>75% at 24 h) (Figure 2, lane 10).

Half-life of the c-erbB2 *mRNA in HBL-100 cells treated with Act D*

c-*erbB2* mRNA is barely detectable in HBL-100 cells by Northern blotting or RNAase protection assay (Pasleau *et al.*, 1990), as it is in normal human breast (Press *et al.*, 1990). No accurate quantitation of the autoradiographic signals was possible, for only very faint bands appeared after 2 or 3 weeks of exposure. Polyadenylated mRNA was therefore isolated from HBL-100 cells treated with Act D from 6 up to 24 h and analysed by Northern blotting (Figure 6).

Fluorescence of the samples in the presence of ethidium bromide indicated that equal amounts of nucleic acids were loaded on the gel. The c-*erbB2* content in Act D-treated HBL-100 cells was expressed as the percentage of the corresponding value measured in untreated cells (100% at 0 h). It increased by approximately 30% during the first 12 h of Act D treatment; a similar increase has previously been found in BT474 cells between the time points of 1 and 4 h. At the 18-h time point, it was still equal to 82% of the controls. It was finally reduced to 24% of the control value after 24 h. This result indicates that the c-*erbB2* mRNA turnover is certainly not faster in the normal cells than in tumor cells.

Nuclear run-on transcription assay

The accumulation of c-*erbB2* mRNA may result from an increased rate of transcription in tumor cells. This was investigated by run-on assays. HBL-100 and BT474 cells were compared first (Figure 7a). Equal amounts of total RNA (12×10^6 c.p.m.) synthesized *in vitro* in nuclei isolated from HBL-100 and BT474 cells contained similar amounts of 36B4 mRNA. While the c-*erbB2* mRNA was barely detectable in the HBL-100 nuclear extract, a strong c-*erbB2* hybridization appeared in the BT474 preparation. These results were confirmed in a second set of experiments, including SK-BR-3 cells as another high c-*erbB2* expressor with gene amplification (Kraus *et al.*, 1987) and glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal standard (Figure 7b). While similar amounts of the 36B4 and GAPDH mRNA were measured in the different cellular extracts, the c-*erbB2* mRNA content was 20–40 times higher in the BT474 and SK-BR-3 cells than in HBL-100 cells.

Discussion

The decrease in c-*erbB2* mRNA in BT474 breast adenocarcinoma cells treated with Act D in the presence or absence of CHX was followed by Northern blotting and RNAase protection assay.

The degradation of the c-*erbB2* mRNA proceeds in two phases. The first phase is represented by a progressive decrease in the messenger in Act D-treated BT474 cells, corresponding to a calculated c-*erbB2* mRNA half-life of 7 h. This value, which is not modified by CHX, is in the range of the c-*neu* protein half-life (9 h) (Gullick *et al.*, 1987). The second phase is characterized by a slow rate of degradation of the remaining c-*erbB2* mRNA so that about 40% of the starting population present in the cells before treatment is still detectable after a transcriptional block of 24 h. CHX accelerates the degradation of this apparently stable c-*erbB2* mRNA subpopulation, suggesting the presence of labile proteins that protect the mRNA from degradation. No major post-transcriptional regulation of the c-*erbB2* gene has been found, similar to the one described for the c-*fms* mRNA, which is stablized by a labile protein during monocytic differentiation from 30 min to 6 h (Weber *et al.*, 1989).

An accurate determination of the c-*erbB2* mRNA half-life in 'normal' HBL-100 cells was not possible because of the very low level of expression of the c-*erbB2* gene in these cells. Northern blot analysis of polyadenylated RNA purified from Act D-treated HBL-100 cells indicated that the c-*erbB2* mRNA levels remain contant for at least 12 h. The decrease in the c-*erbB2* autoradiographic signals was obvious after 24 h of Act D treatment only.

These results indicate that the c-*erbB2* mRNA half-life in 'normal' HBL-100 cells is similar to, if not longer than, that in BT474 tumor cells. A possible extension of this observation is that the normal situation would be represented by a stable c-*erbB2* mRNA. The tumor cells would contain a heterogeneous c-*erbB2* mRNA population, as indicated by the double shoulder in the messenger degradation curve. Besides the stable population, a less stable subpopulation

would appear with a half-life of approximately 7 h. These 'unstable' mRNAs would accumulate as a result of the increased rate of c-*erbB2* mRNA synthesis in tumor cells containing a limited concentration of the stabilizing proteic factors. The presence of labile proteic factors is supported by the almost complete disappearance of the c-*erbB2* signal in BT474 cells treated with CHX.

Moreover, these results indicate that accumulation of the c-*erbB2* gene product in the BT474 cells is caused not by an increased stability of the messenger but by an increase in its synthesis. This was confirmed by measuring the transcription of c-*erbB2* mRNA in isolated nuclei during *in vitro* transcription assays. c-*erbB2* mRNA synthesis was found to be 20–40 times higher in SK-BR-3 and BT474 tumor cells than in 'normal' HBL-100 cells. These results argue directly for a transcriptional deregulation of c-*erbB2* gene expression in these tumor cells.

Whenever c-*erbB2* mRNA was quantitated by Northern blotting or *in vitro* transcription assay in the tumor cells, it was found to be much higher than and not correlated to the gene copy number.

The c-*erbB2* protein is a member of a family of growth factor receptors (Kraus *et al.*, 1989) structurally related to the EGF receptor. In comparison with c-*erbB2* , the half-life of the EGF receptor mRNA is rather short, ranging from 0.9 to 2.2 h (Jinno *et al.*, 1988). The expression of the EGF receptor gene is regulated by multiple growth-stimulated factors such as retinoic acid, phorbol esters and EGF (Thompson & Rosner, 1989). EGF acts at both transcriptional and post-transcriptonal levels: it enhances the stability of its receptor mRNA by increasing its half-life to more than 6 h (Thompson & Rosner, 1989; Kesavan *et al.*, 1990). It will be interesting to test the effects of the c-*erbB2* ligand on the c-*erbB2* messenger and protein half-life.

Treating growth-arrested T47D and MCF-7 cells with estrogens has been found to dramatically down-modulate c-*erbB2* gene expression at both the mRNA and protein levels (Dati *et al.*, 1990; Read *et al.*, 1990). We found estrogen receptor and pS2 in the BT474 cells (Delvenne *et al.*, 1992); in contrast, HBL-100 cells are estrogen non-responsive. Both cell lines were grown in red phenol containing medium supplemented with 5% complete fetal calf serum (FCS). The slight difference in the measured c-*erbB2* mRNA half-life between the two cell lines may be linked to differences in estrogen responsiveness. If this is the case, we would expect an increase in the mRNA stability in BT474 cells cultured in estrogen-depleted medium.

In conclusion, the hypothesis of a post-transcriptional deregulation of c-*erbB2* gene expression in tumor cells can be excluded. We are currently testing the hypothesis of a transcriptional derepression of the c-*erbB2* gene in BT474 cells compared with HBL-100 cells.

Materials and methods

Cell culture

The BT474 and HBL-100 human mammary epithelial cell lines were purchased from ATCC and cultured according to the instructions in the presence of 5% FCS. Approximately, 3×10^6 cells were plated and grown in dishes for 36 h before the addition of 5 $\mu g\,ml^{-1}$ Act D (Sigma). Protein synthesis was blocked by the addition of 10 $\mu g\,ml^{-1}$ CHX (Sigma) 2 h before Act D.

Plasmids

A 500-bp NcoI–EcoRI fragment from pMAC114 (Kraus *et al.*, 1987) encoding the 5′ end of c-*erbB2* cDNA and a genomic 620-bp NcoI fragment from pMAC117 (King *et al.*, 1985) encoding a part of the intracellular domain of the c-*erbB2* protein were subcloned in pGEM-3Z (Promega) and used as templates for the synthesis of ^{32}P-labeled riboprobes by T7 or SP6 RNA polymerases (Riboprobe System, Promega; [α-^{32}P]dCTP, Dupont). Similarly, antisense c-*fos* and c-*myc* riboprobes were derived from pc-fos (human)-1 (ATCC) and pRyc7.4 (Marcu *et al.*, 1983) respectively. The 18S rRNA cDNA (from J. Sylvester) was labeled by random priming (Prime-It Random, Stratagene). pBS-GAPDH was cloned by G. Huez.

RNA purification and analysis

Total cellular RNA was extracted by RNAzol (Cinna/Biotecx) at various times after Act D treatment and quantitated by spectrophotometry. Samples of 10 and 20 μg were ethanol precipitated and stored at −70°C. Polyadenylated mRNA was purified by oligo-dT (Boehringer) chromatography. Total cellular RNA was analysed by Northern blotting on GeneScreen Plus membrane (Dupont) and hybridized at 60°C (Melton *et al.*, 1984) with the c-*erbB2* and 18S rRNA probes. As the c-*erbB2* mRNA co-migrated with the 28S rRNA, special care was taken to avoid even minor cross-hybridization of the c-*erbB2* riboprobe with 28S rRNA. For that purpose, the membrane was incubated for 30 min in 2 × SSC, in the presence of 10 $\mu g\,ml^{-1}$ RNAase A, between the hybridization and washing steps.

After autoradiography, the intensity of the signals corresponding to the c-*erbB2* mRNA (4.8 kb) and the 18S rRNA was monitored by densitometry using an LKB Gelscan. The c-*erbB2* mRNA amounts were calculated relative to the 18S rRNA content of each sample, which was representative of the total cellular RNA transferred to the membrane. The c-*erbB2* mRNA was further quantitated by a molecular titration assay (Lee & Costlow, 1987). A large excess of pMac117 antisense riboprobe (10^5 c.p.m.) was hybridized in solution to 20 μg of total cellular RNA in 30 μl at 55°C for 18 h (Pasleau *et al.*, 1990). Unhybridized probe and cellular RNA were digested by the RNAases A and T1. The c-*erbB2*-specific RNA duplexes were precipitated by the addition of an equal volume of 10% TCA and collected on GF/C filters. Radioactivity was determined by scintillation counting. Alternatively, the RNAases were removed by proteinase K; the duplexes were ethanol precipitated, redissolved in a denaturing loading buffer and submitted to electrophoresis on a 4% polyacrylamide sequencing gel containing 8 M urea.

Run-on transcription assay

Run-on assays were performed according to previously published procedures (Wathelet *et al.*, 1989). Briefly, nuclei were isolated from 2×10^8 growing cells lysed in 10 mM Tris-HCl pH 7.6, 10 mM sodium chloride, 10 mM magnesium chloride and 0.5% NP-40 for 1 min. Radiolabeled total RNA (12×10^6 c.p.m.) synthesized *in vitro* was purified by RNAzol and used for hybridization at 52°C for 72 h with 5 μg of unlabeled DNA probes immobilized on a nylon membrane. The intensity of the signals corresponding to the 18S rRNA and to the 36B4, GAPDH and c-*erbB2* mRNA was quantitated after autoradiography on a phosphorscreen (PhosporImager, Molecular Dynamics) using ImageQuant software.

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