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The 6-Kilobase c-*erbB2* Promoter Contains Positive and Negative Regulatory Elements Functional in Human Mammary Cell Lines[1]

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ABSTRACT

A 6-kilobase fragment extending at the 5′-end of the c-*erbB2* proto-oncogene was isolated from a normal human lymphocyte genomic DNA library. The full-length fragment and five subfragments with identical 3′-ends were obtained by progressive unidirectional deletion from the 5′-end and were cloned in front of the luciferase reporter gene. The hybrid genes were analyzed for transcriptional activity in human mammary cell lines synthesizing low (HBL-100 and T-47D), moderate (MDA-MB-453), or high (BT-474) amounts of the c-*erbB2* mRNA and were also analyzed in HeLa cells. Gene-specific expression was observed, indicating the presence of multiple *cis*-acting sequences in the c-*erbB2* promoter. A major negative element is located in the −2- to −4-kilobase region. It is flanked on both sides by positive elements that display enhanced transcriptional activity in the BT-474 tumor cells only. While predominant in the low-expressing cells, the effect of the repressor appears to be overcome by the distal transactivator in the high-expressing BT-474 cells, resulting in a 15 to 50 times increase in luciferase activity relative to the HBL-100 and T-47D cells, respectively. Cell-specific expression relies on the *trans*-acting factors present in the different cell lines. The formation of cell-specific protein-DNA complexes was demonstrated by gel retardation assay.

INTRODUCTION

The c-*erbB2* gene codes for a transmembrane protein structurally related to the epidermal growth factor receptor (1). Different rat and human cell lines were found to synthesize possible ligands for the c-*erbB2* receptor (2–12). Interaction between estrogen and the c-*erbB2* receptor was also reported (13).

The oncogenic activation of the c-*erbB2* protooncogene in 20–30% of primary human breast (14, 15) and ovarian (15, 16) cancers results in abnormally elevated amounts of the nonmutated receptor on the cell surface. It appears through the amplification of the c-*erbB2* gene and/or the accumulation of the c-*erbB2* mRNA. In many primary beast cancers (15) and mammary adenocarcinoma cell lines (17), the overexpression of the messenger is much more elevated than and not proportional to the gene copy number, indicating that mechanisms other than gene amplification must be involved. Even though the c-*erbB2* overexpression is accepted today as a factor of poor prognosis (14, 15, 18, 19), the reasons for this overexpression remain unknown.

Previously, we have compared different mammary cell lines either overexpressing c-*erbB2* mRNA (BT-474, SK-BR-3, and MDA-MB-453) or not (HBL-100, MCF7, and T-47D; Refs. 20 and 21). We have shown that a posttranscriptional stabilization of the messenger in the BT-474 tumor cells is unlikely, since the c-*erbB2* mRNA half-life is similar in HBL-100 and BT-474 cells. Moreover, run-on analysis has indicated that the *in vitro* c-*erbB2* mRNA transcription rates are approximately 20–40 times higher in the BT-474 and SK-BR-3 compared to the HBL-100 cells, suggesting that the deregulatory events lie rather at the transcriptional level (21). Similar conclusions were drawn by others from a comparison of the ZR75–1 and HBL-100 cell lines (22).

The protein and DNA elements controlling the breast-specific expression of the human c-*erbB2* gene are not well known. Some regulatory sequences found to be active in the rat or mouse *neu* gene (23–26) are conserved in the human gene, but their effective role remains to be demonstrated. The available information concerns only the first 1 kilobase of the c-*erbB2* promoter and proceeds from transfection experiments performed in animal cell lines (27, 28) or in human cells which do not express the natural c-*erbB2* gene (29). A comparison of *erbB2-CAT* reporter genes in T-47D and ZR75-1 mammary cell lines was recently published by Hollywood and Hurst (22). An OB2–1 transcriptional activator binding to the 212-base pair proximal promoter was found more important in overexpressing tumor cell lines.

The originality of our approach has been to isolate a 6-kilobase fragment of the c-*erbB2* promoter and to compare its transcriptional activity in four mammary cell lines synthesizing elevated (BT-474), moderate (MDA-MB-453), or low (T-47D and HBL-100) amounts of the c-*erbB2* mRNA. The highest expression levels were measured in the overexpressing BT-474 cells. Five reporter vectors derived by progressive deletion of the c-*erbB2* promoter were also analyzed in order to delineate more precisely the transcriptionally active domains. Multiple positive and negative *cis*-acting elements were found along the 6-kilobase fragment of the c-*erbB2* promoter. Their contribution to the expression the *erbB2-LUC*[3] reporter genes varied depending on the cell lines, suggesting differences between the populations of *trans*-acting factors. This was confirmed by gel retardation experiments.

MATERIALS AND METHODS

Cell Lines. The HBL-100, T-47D, MDA-MB-453, and BT-474 human mammary epithelial cells and the HeLa human cervix epithelial cells were purchased from American Type Culture Collection and cultured in recommended media supplemented with 5% fetal calf serum, 2 mM glutamine, and 100 μg/ml penicillin/streptomycin. The HBL-100 cell line was established from the milk of a nursing mother with no breast lesion. The T-47D, MDA-MB-453, and BT-474 cell lines were derived from primary breast adenocarcinoma.

Reporter Vectors. A 150-base pair *Eco*RI-*Nco*I probe encoding the 5′ untranslated sequence of the c-*erbB2* mRNA was used to screen a normal human lymphocyte genomic DNA library constructed in the EMBL3 vector (Clontech). The λEB2 clone containing a 13.5-kilobase fragment was purified and characterized by restriction mapping. It encoded the c-*erbB2* promoter and the first four exons. By chromosome walking, an overlapping clone extending further upstream from the λEB2 5′-end was isolated from Maniatis's library and characterized (30). Six reporter vectors were constructed containing frag-

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[3] The abbreviations used are: LUC, luciferase; HSV, herpes simplex virus; tk, thymidine kinase; RLU, relative light units.

ments of increasing size (255 to 6047 base pairs) of the c-*erbB2* promoter cloned in pXP2 (31) in front of the *LUC* gene. In each *erbB2-LUC* reporter gene, the junction between the c-*erbB2* and *LUC* fragments was sequenced using the T7 Sequencing kit (Pharmacia).

To construct the p*tk-LUC* plasmid, a 198-base pair *Hin*dIII-*Xho*I fragment containing the HSV *tk* promoter isolated from the pBLCAT2 plasmid (32) was cloned between the corresponding sites in the pXP2 polylinker. The 1769-base pair *Xho*I-*Hin*dIII fragment of the c-*erbB2* gene extending −4 to −2 kilobases upstream from the transcription start site was inserted in both orientations in front of the *tk* promoter at the *Hin*dIII unique restriction site of the p*tk*-LUC plasmid. The resulting vectors were called pR2s *tk-LUC* and pR2as *tk-LUC*.

Transient Transfection Assay. One × 10^6 HBL-100, T-47D, and BT-474 cells, 1.5 × 10^6 MDA-MB-453 cells, and 5 × 10^5 HeLa cells were plated in 60-mm tissue culture dishes and grown in recommended medium supplemented with 5% fetal calf serum for 24 h. The parameters for transient transfection by the lipofection method using the DOTAP reagent (Boehringer) were optimized for every cell line separately. Briefly, the cells were incubated for 3 h (HBL-100), 17 h (BT-474, T-47D, and HeLa) or 24 h (MDA-MB-453) in 3 ml of media containing 15 μg DOTAP/8 μg DNA mixtures. After replacing the transfection media by fresh media, the HeLa cells were incubated further for 8 h and the mammary cells for 24 h. Cells were harvested, and the enzymatic activities were measured in the cell lysates. The *LUC* enzymatic activities were measured according to Nguyen *et al.* (33) using a luminometer (Berthold 9501) and were expressed in RLU. The protein contents of the cell extracts were quantitated using the Micro BCA Protein Assay Reagent (Pierce).

The *pXP2*, *pSV2-LUC*, and *pCMVIE-LUC* reporter genes were systematically transfected in separate dishes in each experiment. The promoterless *pXP2* plasmid was used as a negative control for *LUC* activity. The *pSV2-LUC* and *pCMVIE-LUC* plasmids are positive controls, allowing assessment of the reproducibility of the transfection.

Statistical Analysis. In a typical transfection experiment, each reporter vector was transfected in triplicate in the five cell lines. The transfection experiments were repeated at least three times.

LUC activities were transformed to a log-scale to estimate the skewness and normalize their distribution. All subsequent statistical calculations were performed on the log values. Two-way analysis of variance with repeated measurements was applied to assess the effect of genes and experimental conditions on *LUC* activity in each cell line. The interaction between the two factors was tested. We also compared the between and the within experiment variabilities. Results were considered statistically significant at $P < 0.05$ (34). The geometric mean of *LUC* activity (*i.e.*, the exponential of the mean of the log values) was computed for each cell line per gene combination.

Bandshift Assay. Nuclear proteins were extracted from the HBL-100, BT-474, and HeLa cells according to Dignam (35). The 2069-base pair long c-*erbB2* promoter was digested into 11 restriction fragments that were end-labeled with the Klenow fragment of DNA polymerase. Gel retardation assays were performed with the BandShift kit according to the instructions of the manufacturer (Pharmacia). Briefly, radiolabeled fragments of the c-*erbB2* promoter (10,000 cpm) were incubated with 5 μg of total nuclear protein, 1 μg of poly(dI-dC)-poly(dI-dC), and a 150–350 molar excess of DNA competitor. The specific competitor was the corresponding unlabeled c-*erbB2* fragment. The nonspecific competitor was a 277-base pair long fragment of the pBluescript plasmid. The reaction mixtures were incubated for 20 min at room temperature and then electrophoresed at room temperature through a 5% polyacrylamide gel, which was dried and analyzed by autoradiography.

RESULTS

Construction of the *erbB2-LUC* Reporter Genes. The partial restriction map of the genomic DNA fragment extending 6185 base pairs upstream from the c-*erbB2* translation initiation site is shown in Fig. 1*A*. The *Sma*I site located at the position −138 relative to the ATG was changed to a unique *Sac*I site in order to facilitate the isolation of c-*erbB2* promoter fragments sharing identical 3′-ends. These were inserted in the *pXP2* polylinker (31) in front of the *LUC* gene (Fig. 1*A*). The six *erbB2-LUC* reporter genes contain the *Eco*RI-*Sac*I (6047-base pair), *Xho*I-*Sac*I (3838-base pair), *Hin*dIII-*Sac*I (2069-base pair), *Pvu*II-*Sac*I (756-base pair), *Sma*I-*Sac*I (537-base pair), and *Pst*I-*Sac*I (255 base pair) fragments of the c-*erbB2* promoter, respectively (Fig. 1*B*). The *erbB2-LUC* junctions were sequenced and found identical in the different constructions. Assuming that the transcription of the *erbB2-LUC* reporter genes would take place at the major c-*erbB2* transcription start site described by Ishii *et al.* (27) and Tal *et al.* (28), the 5′ untranslated region of the hybrid messenger should contain 40 nucleotides from the c-*erbB2* gene and 37 nucleotides from the *LUC* gene.

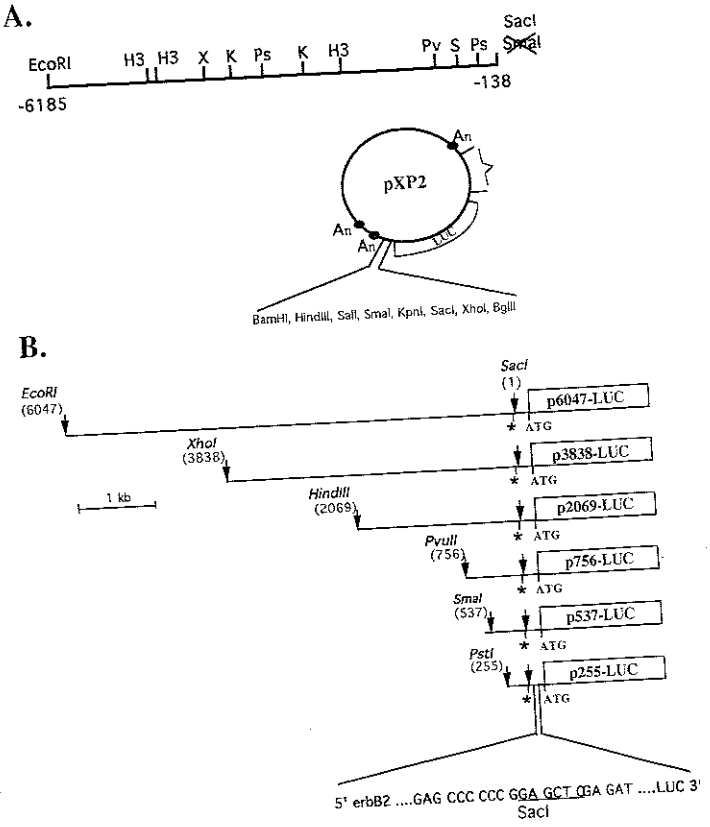

Fig. 1. *A*, the position of the *Eco*RI, *Hin*dIII (*H3*), *Pst*I (*Ps*), *Pvu*II (*Pv*), *Kpn*I (*K*), *Sac*I, *Sma*I (*S*), and *Xho*I (*X*) restriction sites are indicated in the c-*erbB2* promoter relative to the translation start site. In the *pXP2* plasmid, the *LUC* coding sequence is flanked by a polylinker, the SV40 intron (⌒) and three SV40 polyadenylation signals (*An*). *B*, the *erbB2-LUC* reporter genes contain fragments of increasing size of the c-*erbB2* promoter (*line*) subcloned into *pXP2* in front of the *LUC* coding sequence (*open box*). The length of the c-*erbB2* inserts is measured starting to the *Sac*I site (+1), which replaces the natural *Sma*I site located at the −138-base pair position. The major transcriptional start site (*), the *LUC* translation start site (ATG), and the restriction sites used for cloning are indicated. The sequence of the c-*erbB2/LUC* junction is reported.

The T-47D, MDA-MB-453, and BT-474 Tumor Mammary Cells Are Transfected with Similar Efficiency. The transcriptional activity of the promoter fragments inserted in the reporter vectors was estimated through the measure of LUC activities in the transfected cells. These activities are controlled by transcription factors present in the nuclei. However, they are also, depending from the amounts of reporter plasmids, effectively captured by the cells during the transfection experiment. As we were interested in comparing the expression of the different *erbB2-LUC* chimeric genes in different mammary cell lines, it was important to estimate the relative transfection efficiency of those cells. For that purpose, the *pSV2-LUC* and *pCMVIE-LUC* reporter genes were systematically included in each assay in order to control both the reproducibility and the efficiency of the transfection. Geometric mean values of *LUC* activities measured in the different *pSV2-LUC* and *pCMVIE-LUC* transfected cells are displayed in Table 1. Mean values (±SDs) of the log *LUC* activities are

Table 1 *Transient expression of the LUC reporter genes in human mammary and cervix cell lines*

Results are expressed as geometric means of *LUC* activities (RLU/mg of protein × pmol of reporter plasmid). Mean values of the log *LUC* activities ± SDs are indicated in parentheses.

	LUC activities				
Reporter gene	HBL-100	T-47D	MDA-MB-453	BT-474	HELA
pXP2	3,200 (8.06 ± 0.24)	2,200 (7.68 ± 0.28)	1,600 (7.37 ± 0.35)	3,700 (8.22 ± 0.23)	3,600 (8.20 ± 0.26)
pSV2-LUC	660,000 (13.4 ± 0.30)	1,330,000 (14.1 ± 0.42)	3,610,000 (15.1 ± 0.57)	1,200,000 (14.0 ± 0.60)	7,280,000 (15.8 ± 0.42)
pCMVIE-LUC	3,610,000 (15.1 ± 0.45)	14,700,000 (16.5 ± 0.43)	26,700,000 (17.1 ± 0.78)	21,900,000 (16.9 ± 0.64)	36,000,000 (17.4 ± 0.61)
p255-LUC	19,000 (9.85 ± 0.48)	4,300 (8.36 ± 0.31)	10,200 (9.23 ± 0.41)	26,900 (10.2 ± 0.49)	297,000 (12.6 ± 0.50)

also indicated in parentheses. Differences in the populations of nuclear *trans*-acting proteins are illustrated by the ratios of the *CMVIE* to SV40 activities, which vary from 5 to 11 according to the cell line. However, the *CMVIE* and SV40 promoters display transcriptional activities of the same order of magnitude in T-47D, MDA-MB-453, BT-474, and HeLa cells, suggesting similar transfection efficiencies of these cells. Lower expression levels of the viral promoters were measured in the HBL-100 cells.

The alternative approach for comparing the transfection efficiencies was the cotransfection of a *pRSVZ* reporter gene as an internal control. This was tested and found inappropriate, essentially because the weak expression levels of the *pRSVZ* plasmid varied, depending on the cotransfected plasmid. Normalizing the expression of the different reporter genes to the β-galactosidase internal standard would have resulted in experimental artifacts. The same observation had already been described (36).

A third approach was to quantitate by dot-blotting the amounts of reporter vector DNA introduced in the different cell lines. Lower signals were detected in the HBL-100 than in the other cells, confirming that the transfection efficiency of the HBL-100 cells was lower (data not shown).

The 255-Base Pair c-erbB2 Promoter Displays Similar Transcriptional Activity in the HBL-100, MDA-MB-453, and BT-474 Mammary Cell Lines. The expression of the *p255-LUC* reporter vector was compared in five human mammary cell lines synthesizing different amounts of the c-*erbB2* gene product and in HeLa cells. The *LUC* activities were compared to the levels obtained in parallel transfections with the promoterless *pXP2* plasmid to give a measure of promoter activity (Table 1).

The *p255-LUC* construct had similar activity to *pXP2* in T-47D cells. Comparable levels of *LUC* activities, six to seven times over the background values, were measured in the three other mammary cell lines independently of their c-*erbB2* mRNA contents. The highest *LUC* activity was measured in HeLa cells, where it was increased by a factor of 77 above the control. The c-*erbB2* mRNA in these cells is almost undetectable.

The 219-Base Pair *Pvu*II-*Sma*I Fragment Contains an Activating Sequence Particularly Active in BT-474 Cells. We have searched for additional regulatory elements located upstream from the *Pst*I site in the c-*erbB2* promoter. To facilitate their comparison, we have calculated, for every cell line, the ratios between the *LUC* activities induced by each reporter vector and the *p255-LUC* reporter gene. The results are presented in the Fig. 2. Positive elements were found in the *Pvu*II-*Pst*I fragment. The statistically significant stimulatory effects are marked by *asterisks*. The *Sma*I-*Pst*I fragment contains a transcriptional activator (*A1*), responsible for a doubling of the *LUC* activity in the *p537-LUC* transfected T-47D cells. The *Pvu*II-*Sma*I fragment contains an activator (*A2*) functional in all the cell lines. A striking difference appears between the BT-474 cells, where the *LUC* activity was induced by a factor of 29; in the other cells, a 2.6- to 4.9-fold induction was measured.

The −2- to −4-Kilobase Region Contains a Repressor Element Functional in All the Cell Lines. The *Hin*dIII-*Pvu*II fragment was the only one that displayed opposite transcriptional activities, depending on the cell type. It significantly increased the *LUC* activity in the HBL-100 cells. But to the contrary, it significantly reduced the transcription in the MDA-MB-453 mammary tumor cells. This suggests that this promoter fragment should contain two binding sites for an *A3* activator and an *R1* repressor.

The *Xho*I-*Hin*dIII fragment of the *p3838-LUC* vector may contain a negative element (*R2*), antagonizing the transactivation by the downstream regulatory sequences in the HBL-100, T-47D, BT-474 and HeLa cells. It is noteworthy that the *LUC* activities never decreased below the levels measured with the proximal promoter.

The ability of this 1769-base pair fragment to inhibit gene transcription was tested on the heterologous HSV *tk* promoter. Reporter vectors either containing the *R2* repressor in front of the HSV *tk* promoter or not were constructed and introduced in parallel in HeLa cells (Fig. 3). The HSV *tk* promoter is 10 and 100 times less active than the SV40 and *CMVIE* viral promoters, respectively (data not shown). A 70% inhibition of the HSV *tk* promoter activity was observed when the repressor was inserted in the 5′-3′ orientation in front of the HSV *tk* promoter. When inserted in the opposite orientation, the repressor did not modify markedly the HSV *tk* promoter activity.

A Distal Activator Is Functional in the BT-474 Cells. The repressing effect of the *Xho*I-*Hin*dIII fragment may partially be reversed by a regulatory element located upstream from the *Xho*I site. Interestingly, this occurred only in the BT-474 cells, where the *LUC* activity was increased by a factor of 14 compared to the *p255-LUC* control level. The BT-474 cells may contain an *A4* activator binding the −6- to −4-kilobase region of the c-*erbB2* promoter. As the *LUC* activity in the *p255-LUC* transfected BT-474 was 26,900 RLU/(mg protein × pmol of DNA) (Table 1), the stimulated *LUC* activity reached 376,600 (26,900 × 14) RLU/(mg protein × pmol of DNA) in the *p6047-LUC* transfected cells. The *A4* activator would be absent or inactive in the other cell lines, where the longest construct, *p6047-LUC*, had similar activity to the *p255-LUC* plasmid. Indeed, *LUC* activities of 7,740 (4,300 × 1.8), 11,220 (10,200 × 1.1) and 28,500 (19,000 × 1.5) RLU/(mg protein × pmol of DNA) were measured in the *p6047-LUC* transfected T-47D, MDA-MB-453, and HBL-100 cells, respectively. In conclusion, the expression of the *p6047-LUC* reporter gene appeared 13 to 50 times higher in the overexpressing BT-474 cells compared to the three other mammary cell lines.

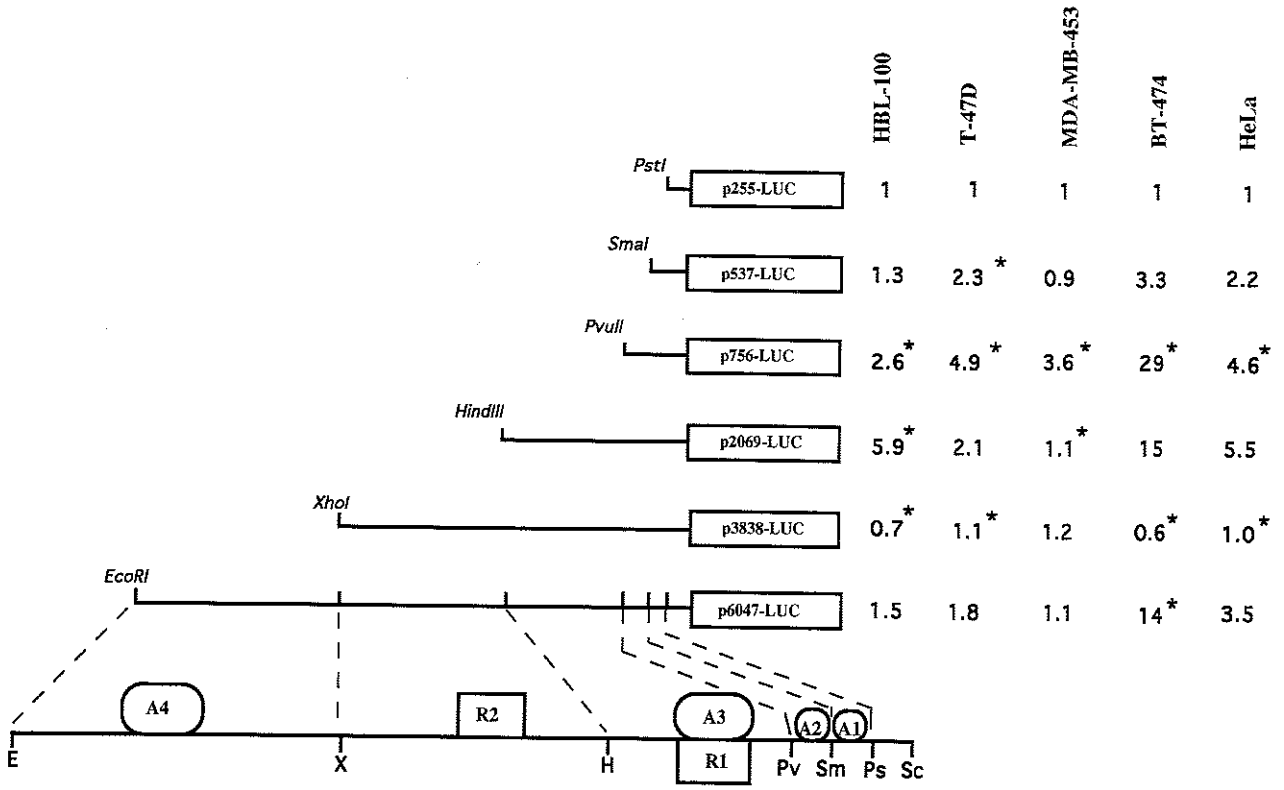

Fig. 2. The *LUC* activities measured in the different transfected cells were compared to the minimal promoter directed *LUC* activity. The *asterisks* indicate significant modulations of *LUC* activity. The results are summarized at the *bottom* of the figure; the regions where the activators (*A*) and repressors (*R*) are supposed to bind to the c-*erbB2* promoter are indicated.

The HBL-100, BT-474, and HeLa Nuclear Proteins Display Specific DNA Binding Activities. The 2069-base pair *Hin*dIII-*Sac*I fragment was analyzed by bandshift assays for its ability to form specific protein-DNA complexes. Eleven radiolabeled subfragments were incubated with crude nuclear extracts from exponentially growing HBL-100, BT-474, and HeLa cells (Fig. 4). Nuclear proteins bound to the four fragments covering the first 537 base pairs of the proximal promoter. An almost uniform pattern of retardation was observed that reflected the uniform *LUC* activities measured in the transfected cells. Representative retarded migrations are presented in Fig. 5. Two complexes migrating closely to each other were present in similar proportions in the incubation mixtures containing the *Pst*I-*Bssh*II fragment and nuclear extracts from the three cell lines. A third, slowly migrating complex of weaker intensity appeared only with the HeLa cells extracts (Fig. 5*A*).

Fig. 5*B* presents the results obtained with the *Pvu*II-*Acc*I located in the region responsible for the strong transactivation of the *LUC* gene in the *p756-LUC*-transfected BT-474 cells. Interestingly, different patterns of retarded complexes were observed in the three cell lines. Two slowly migrating complexes were detected with the HeLa cell extracts. The mammary cell extracts contained additional, faster migrating complexes. One complex seemed common to HBL-100 and BT-474 cells, but the second appeared predominant in the later cells. The size of the bound protein was probably small as the complex moved just behind the free radiolabeled DNA.

Among the six fragments located upstream from the *Pvu*II site, only three were able to bind nuclear proteins from the HeLa cells (Fig. 4). They formed complexes similar to those observed in the BT-474 cellular extracts. Retarded complexes specific for the HBL-100 cells were systematically observed. The *Hin*fI fragment was recognized only by proteins from the HBL-100 cells. The heterogeneity of the observed retarded complexes reflected the opposite transcriptional effects driven by the *Hin*dIII-*Pvu*II fragment in the HBL-100 cells and in the other mammary cells.

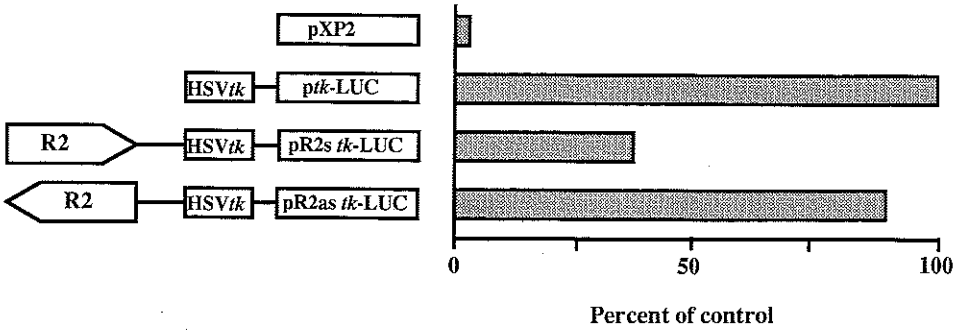

Fig. 3. The *Xho*I-*Hin*dIII fragment containing the *R2* negative element of the c-*erbB2* promoter was inserted in both orientations in front of the HSV *tk* promoter in the p*tk-LUC* reporter vector. The *LUC* activities were measured in the HeLa cells transfected with the different reporter vectors. The results are presented as the percentages of the control value measured in the p*tk-LUC*-transformed cells. They represent the average of three separate experiments.

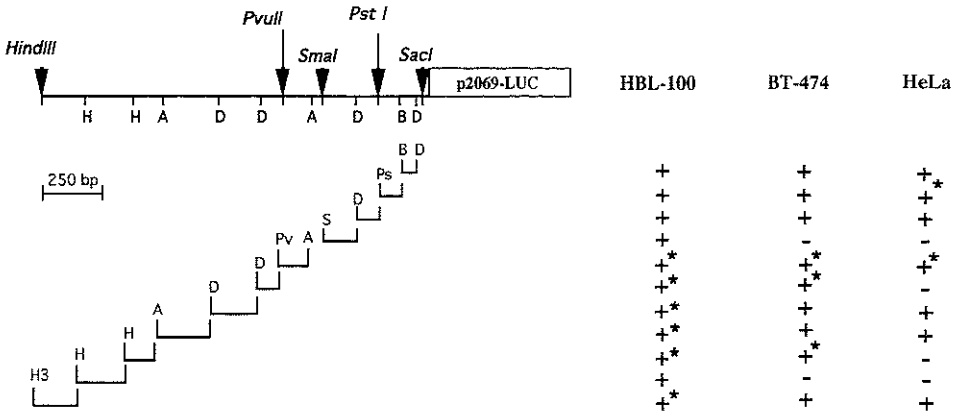

Fig. 4. Crude nuclear protein extracts from HBL-100, BT-474, and HeLa cells were compared for DNA-binding activity to the 2069-base pair *Hin*dIII-*Sac*I region of the c-*erbB2* promoter. Eleven subfragments were generated by digestion with the following restriction enzymes: *Acc*I (*A*; −594, −1401); *Bssh*II (*B*; −127), *Dde*I (*D*; −45, −379, −861, −1095); *Hin*dIII (*H3*; −2069); *Hin*fI (*H*; −1544, −1819); *Pst*I (*Ps*; −255); *Pvu*II (*Pv*; −756); and *Sma*I (*S*; −537). Their cleavage sites, indicated in parentheses, were calculated relative to the *Sac*I site located at the c-*erbB2-LUC* junction. + and − signs, the presence and absence of specific DNA-proteins retarded complexes, respectively. The *asterisks* signify that additional specific bandshifts were observed in the considered cell line.

DISCUSSION

Whereas the homologous rat *neu* gene is activated by point mutations in the transmembrane domain (37), the human c-*erbB2* gene does not seem mutated (15, 38). The oncogenic properties of the human c-*erbB2* gene are correlated by the presence of abnormally elevated amounts of the receptor on the cell surface. This seems to provide the tumor cells with some growth advantage (39–41) and is correlated with a poor clinical outcome (14, 18) and aggressive tumor behavior (42). The overexpressed receptor is constitutively hyperphosphorylated, suggesting that it might be active even in the absence of the ligand (43, 44).

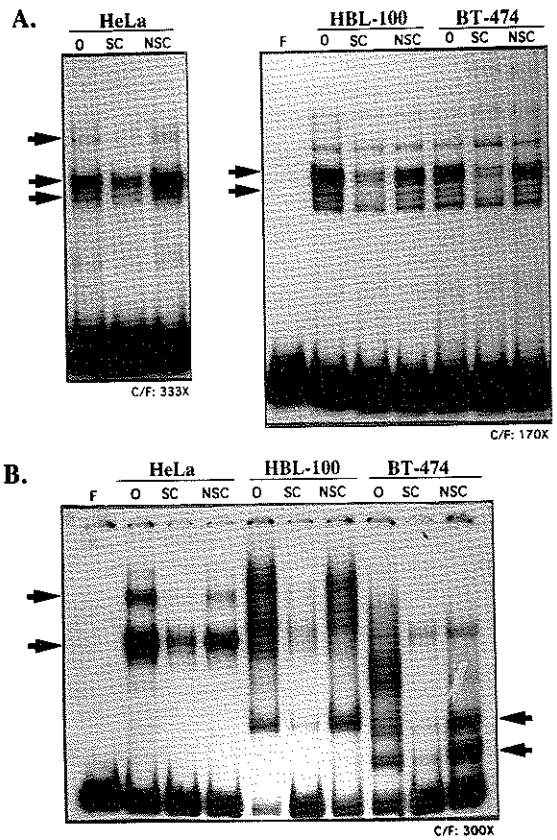

Fig. 5. The radiolabeled *Pst*I-*Bssh*II (*A*) and *Pvu*II-*Acc*I (*B*) fragments of the c-*erbB2* promoter were incubated with crude protein extracts from HBL-100, BT-474, and HeLa cells in the absence (0) or the presence of specific (*SC*) or nonspecific (*NSC*) competitor DNA. The ratio between the competitor and the radiolabeled fragment concentration (*C/F*) are reported. *Lane F*, migration of the uncomplexed DNA. *Arrows*, positions of specific protein-DNA retarded complexes.

Whereas the homologous rat *neu* gene is activated by point mutations in the transmembrane domain (37), the human c-*erbB2* gene does not seem mutated (15, 38). The oncogenic properties of the human c-*erbB2* gene are correlated by the presence of abnormally elevated amounts of the receptor on the cell surface. This seems to provide the tumor cells with some growth advantage (39–41) and is correlated with a poor clinical outcome (14, 18) and aggressive tumor behavior (42). The overexpressed receptor is constitutively hyperphosphorylated, suggesting that it might be active even in the absence of the ligand (43, 44).

Previous studies based on the comparison of the c-*erbB2* mRNA half-life and on run-on transcription assays have demonstrated that the accumulation of the c-*erbB2* mRNA in the BT-474, SK-BR-3, and ZR-75 cells resulted from the transcriptional deregulation of the c-*erbB2* gene expression (21, 22). The molecular alterations responsible for this overexpression remain unknown.

To address this question, a 6-kilobase full-length fragment and five subfragments of the c-*erbB2* promoter were used to construct *LUC* reporter genes. Their expression was compared in different human mammary cell lines synthesizing low (HBL-100, T-47D), moderate (MDA-MB-453), or high (BT-474) amounts of the c-*erbB2* mRNA and in HeLa cells.

Our shortest chimeric gene contains a 255-base pair *Pst*I-*Sac*I c-*erbB2* promoter that represents probably a minimal promoter. A similar construction, where the c-*erbB2* promoter was shortened to 100 base pairs, was inactive in the T47-D and ZR75-1 cells (22). The *p255-LUC* reporter gene was expressed in four mammary epithelial cell lines, similar to many other cells (27–29, 45). Multiple binding sites for ubiquitous transcription factors such as Sp1, E4TF1, AP2, and OTF-1 are located in this region (26). This could explain why this fragment is functional in a large variety of cell lines. The rather uniform pattern of retarded complexes observed in bandshift assays confirms that nuclear proteins are common to the different cell lines. Their concentrations may be variable, explaining the different levels of *LUC* activities between the five cell lines. The transcriptional activity of the 255-base pair promoter can be modulated under particular conditions. Negative autoregulation of the *neu* gene (46) and transcriptional repression by the c-*myc* (24) and adenovirus 5 EIA gene products (23) and by estrogen-stimulated estrogen receptor (45) are mediated by *cis*-acting elements located in the proximal promoter.

Our study has demonstrated that the c-*erbB2* upstream region contains positive and negative regulatory elements. A transcriptional activation directed by a sequence located in the −756- to −537-base pair region was observed in all of the cell lines. A 2.6-, 3.6-, and 4.9-fold induction of *LUC* activity was measured in the HBL-100, MDA-MB-453, and T47-D cells, respectively. Hollywood and Hurst (22), who have transfected similar erbB2-CAT chimeric genes, did not report any variation of the promoter activity in the T-47D cells. These differences might be due to the lower sensitivity of the *CAT* reporter system. The stimulatory effect is much higher in the overexpressing BT-474 cells. Footprinting analysis are currently performed to compare the DNA sequences recognized by the nuclear proteins in the different cell lines. The BT-474 cells may contain a

particular transactivating factor. Alternatively, a transcription factor common to the different cells could be either more abundant or more active in the BT-474 cells.

Two negative regulatory elements were located in the −756- to −4-kilobase region of the c-*erbB2* gene. Their presence may not be surprising, since the gene expression is very low in normal adult tissues. The synthesis of the c-*erbB2* mRNA is normally restricted to fetal epithelial cells of the kidney, lung, gastrointestinal tract, and placenta at term and tends to decrease in the corresponding normal adult tissues (47). Repressors may be necessary to inhibit the transcription of the c-*erbB2* gene in the low-expressing cells.

A first repressor (*R1*), located between −756 and −2069 base pairs was functional only in the MDA-MB-453 cells. A second repressor (*R2*), located in the −2069- to −3838-base pair region was functional in the other cell lines. Two negative elements were found at approximately the same positions by two independent studies. One was localized in the rat (position, −0.6 to 1.6 kilobases; Ref. 48) and the other in the mouse (position, −2 to −6 kilobases; Ref. 49) *neu* genes. The progressive decrease of *LUC* expression in the *p2069-LUC-* and *p3838-LUC*-transfected BT-474 cells suggests a cooperation between the *R1* and *R2* repressors. The *R2* repressor inserted in the normal orientation inhibited the HSV *tk* promoter activity in the HeLa cells. Thus, *R2* might not be a classical silencer. However, the presence of the *R1* repressor could be necessary for its full activity.

The inhibition of *LUC* activity by the repressors may be reversed by a distal element located in the −6 to −4-kilobase region. This occurs specifically in the BT-474 cells, where elevated expression levels of the full-length reporter gene were measured. In the other mammary cell lines, the *LUC* activities remain very low and similar to those expressed by the minimal promoter.

The expression levels of the chimeric genes represent the sum of all the regulatory elements whose transcriptional activities vary in the different cell lines. In the BT-474 cells, the higher activity of the transcriptional activators and particularly the distal A4 element results in elevated *LUC* activity. This corresponds to an induction of *LUC* activity by a factor of 105 over the background value measured in the promoterless *pXP2*-transfected cells.

To the contrary, the levels of *LUC* activity are rather stable in the overexpressing MDA-MB-453 tumor cells, where the slight transcriptional activation produced by the A2 element located in the *Pvu*II-*Sma*I fragment is abolished by the upstream repressor. The influence of the negative elements is also predominant in the low-expressing HBL-100 and T-47D cells, resulting in low expression of the full-length reporter gene.

In the case of the T-47D cells, an additional parameter must be considered, which is the hormonal status of the cell line. The T-47D cells are estrogen responsive and cultured in the presence of fetal calf serum. Transcriptional down-regulation of the p185erbB2 receptor by estrogen has been described (50–53) and is directed by a regulatory element located in the first 100-base pair sequence at the 5′-end of the c-*erbB2* gene (45). Since this element is present in each *erbB2-LUC* hybrid gene, it may be responsible for their systematically lower expression. Whatever the considered reporter gene, the *LUC* activities remain very low as well as the expression level of the natural c-*erbB2* gene.

The HeLa cells were included in the comparison to point out the tissue-specific regulatory elements. Surprisingly, very high expression of the minimal c-*erbB2* promoter was measured in these cells where the c-*erbB2* mRNA is almost undetectable. This may be due to the abundance of ubiquitous transcription factors in these cells. Many of them were indeed isolated from HeLa cells. We cannot exclude that additional elements located outside of the studied fragment are required to inhibit the synthesis of the c-*erbB2* mRNA in the low-expressing cells.

The present study suggests interesting differences in the content of regulatory proteins controlling the expression of the c-*erbB2* gene between the mammary cell lines. Gel retardation assays confirmed that the populations of transcription factors were different in the different cell lines. Cell-specific retarded complexes were observed for DNA fragments that display different promoting activity. Mainly, the *Pvu*II-*Acc*I fragment responsible for a strong induction of the *LUC* activity in the BT-474 cells forms three retarded complexes, one of which appears only in the BT-474 cellular extract. The *Hin*dIII-*Pvu*II fragment, which displays transactivating activity only in the HBL-100 cells, forms, with the HBL-100 proteins, particular complexes that are absent in the other cell lines.

In conclusion, important positive and negative regulatory elements are located several kilobases upstream of the 5′-end of the c-*erbB2* gene. Elevated expression of the full-length chimeric gene in the BT-474 tumor cells is apparently correlated to *trans*-acting nuclear proteins that would be either more abundant or more active in these cells compared to the low-expressing mammary cells. A similar conclusion has been reported by Hollywood and Hurst (22) that found a OB2–1 DNA binding protein much more abundant in the ZR75–1 tumor cells. It is tempting to consider that this represents a general mechanism for the overexpression of the c-*erbB2* gene in the mammary cancer cells. It is probably not the only mechanism, since we did not observe similar transactivating activity in the MDA-MB-453. In such cases, the genetic alterations responsible for the c-*erbB2* overexpression should rather be searched elsewhere, *i.e.,* in the genome of the cancer cells. Our study has indicated multiple possible target sites for mutation that would result in enhanced expression of the gene. Positive regulatory DNA sequences could be modified, resulting in increased DNA-binding activity of the transactivating factors, or the mutations could also disrupt the mechanism of negative control. Other regulatory mechanisms that modify gene conformation at the chromatin level may also be very important in controlling the access of regulatory proteins to transcriptionally active genes.

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REFERENCES

1. Schechter, A. L., Hung, M-C., Vaidynathan, L., Weinberg, R. A., Yang-Feng, T. L., Francke, U., Ullrich, A., and Coussens, L. The *neu* gene: an *erbB*-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science (Washington DC), *229:* 976–978, 1985.
2. Yarden, Y., and Weinberg, R. A. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc. Natl. Acad. Sci. USA, *86:* 3179–3183, 1989.
3. Lupu, R., Colomer, R., Zugmaier, G., Sarup, J., Shepard, M., Slamon, D., and Lippman, M. E. Direct interaction of a ligand for the *erbB2* oncogene product with the EGF receptor and $p185^{erbB2}$. Science (Washington DC), *249:* 1552–1555, 1990.
4. Davis, J. G., Hamuro, J., Shim, C. Y., Samanta, A., Greene, M. I., and Dobashi, K. Isolation and characterization of a neu protein-specific activating factor from human ATL-2 cell conditioned medium. Biochem. Biophys. Res. Commun., *179:* 1536–1542, 1991.
5. Dobashi, K., Davis, J. G., Mikami, Y., Freeman, J. K., Hamuro, J., and Greene, M. K. Characterization of a neu/c-erbB2 protein-specific activating factor. Proc. Natl. Acad. Sci. USA, *88:* 8582–8586, 1991.
6. Tarakhovsky, A., Zaichuk, T., Prassolov, V., and Butenko, Z. A. A 25 kDa polypeptide is the ligand for p185neu and is secreted by activated macrophages. Oncogene, *6:* 2179–2186, 1991.
7. Yarden, Y., and Peles, E. Biochemical analysis of the ligand for the neu oncogenic receptor. Biochemistry, *30:* 3543–3550, 1991.
8. Lupu, R., Colomer, R., Kannan, B., and Lippman, M. E. Characterization of a growth factor that binds exclusively to the erbB-2 receptor and induces cellular responses. Proc. Natl. Acad. Sci. USA, *892:* 287–2291, 1992.

9. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Odgen, S. G., Ben Levy, R., and Yarden, Y. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell, *69:* 205–216, 1992.
10. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. Neu differentiating factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell, *69:* 559–572, 1992.
11. Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggest complex ligand-receptor relationships. EMBO J., *12:* 961–971, 1993.
12. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W-J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. Identification of heregulin, a specific activator of $p185^{erbB2}$. Science (Washington DC), *256:* 1205–1210, 1992.
13. Matsuda, S., Kadowaki, Y., Ichino, M., Akiyama, T., Toyoshima, K., and Yamamoto, T. 17β-Estradiol mimics ligand activity of the c-*erbB2* protooncogene product. Proc. Natl. Acad. Sci., USA, *90:* 10803–10807, 1993.
14. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science (Washington DC), *235:* 177–182, 1987.
15. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. Science (Washington DC), *244:* 707–712, 1989.
16. Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J. T., Dodge, R., Clarke-Pearson, D. L., Marks, P., McKenzie, S., Yin, S., and Bast, R. C., Jr. Overexpression of *HER-2/neu* is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res., *50:* 4087–4091, 1990.
17. Kraus, M. H., Popescu, N. C., Amsbaugh, S. C., and King, C. R. Overexpression of the EGF receptor-related proto-oncogene *erbB-2* in human mammary tumor cell lines by different molecular mechanisms. EMBO J., *6:* 605–610, 1987.
18. Perren, T. J. c-*erbB-2* oncogene as a prognostic marker in breast cancer. Br. J. Cancer, *63:* 328–332, 1991.
19. Press, M. F., Pike, M. C., Chazin, V. R., Hung, G., Udove, J. A., Markowicz, M., Danyluk, J., Godolphin, W., Sliwkowski, M., Akita, R., Paterson, M. C., and Slamon, D. J. HER-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res., *53:* 4960–4970, 1993.
20. Pasleau, F., Grooteclaes, M., and Gol-Winkler, R. Using a molecular titration assay to quantitate and characterize the c-*erbB2* mRNA synthesized in human mammary adenocarcinoma cell lines. Arch. Int. Physiol. Biochem., *98:* 40, 1990.
21. Pasleau, F., Grooteclaes, M., and Gol-Winkler, R. Expression of the c-*erbB2* gene in the BT-474 human mammary tumor cell line: measurement of c-*erbB2* mRNA half-life. Oncogene, *8:* 849–854, 1993.
22. Hollywood, D. P., and Hurst, H. C. A novel transcription factor, OB2–1, is required for overexpression of the proto-oncogene c-*erbB-2* in mammary tumour lines. EMBO J., *12:* 2369–2375, 1993.
23. Yu, D., Suen, T-C., Yan, D-H., Chang, L-S., and Hung, M-C. Transcriptional repression of the *neu* protooncogene by the adenovirus 5 E1A gene products. Proc. Natl. Acad. Sci. USA, *87:* 4499–4503, 1990.
24. Suen, T-C., and Hung, M-C. c-myc reverses neu-induced transformed morphology by transcriptional repression. Mol. Cell. Biol., *11:* 354–362, 1991.
25. Yan, D-H., and Hung, M-C. Identification and characterization of a novel enhancer for the rat neu promoter. Mol. Cell. Biol., *11:* 1875–1882, 1991.
26. White, M. R-A., and Hung, M-C. Cloning and characterization of the mouse neu promoter. Oncogene, *7:* 677–683, 1992.
27. Ishii, S., Imamoto, F., Yamanashi, Y., Toyoshima, K., and Yamamoto, T. Characterization of the promoter region of the human c-*erbB-2* protooncogene. Proc. Natl. Acad. Sci. USA, *84:* 4374–4378, 1987.
28. Tal, M., King, C. R., Kraus, M. H., Ullrich, A., Schlessinger, J., and Givol, D. Human HER2 (neu) promoter: evidence for multiple mechanisms for transcriptional initiation. Mol. Cell. Biol., *7:* 2597–2601, 1987.
29. Hudson, L. G., Erlt, A. P., and Gill, G. N. Structure and inducible regulation of the human c-erbB2/neu promoter. J. Biol. Chem., *265:* 4389–4393, 1990.
30. Pasleau, F., Dijkmans, H., Bertrand, B., and Gol-Winkler, R. Cloning and sequencing the upstream regulatory region of the c-*erbB2* gene. Arch. Int. Physiol. Biochem., *98:* 2, 1990.
31. Nordeen, S. K. Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques, *6:* 454–456, 1988.
32. Luckow, B., and Schutz, G. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoter and regulatory elements. Nucleic Acids Res., *15:* 5490, 1987.
33. Nguyen, V. T., Morange, M., and Bensaude, O. Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. Anal. Biochem., *171:* 404–408, 1988.
34. Armitage, P., and Berry, G. Statistical Methods in Medical Research. London: Blackwell, 1989.
35. Dignam, G. D. Preparation of extracts from higher eukaryotes. Methods Enzymol., *182:* 194–203, 1990.
36. Farr, A., and Roman, A. A pitfall of using a second plasmid to determine transfection efficiency. Nucleic Acids Res., *20:* 920, 1992.
37. Bargmann, C. I., Hung, M-C., and Weinberg, R. A. Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion. Cell, *45:* 649–657, 1986.
38. Lemoine, N. R., Staddon, S., Dickson, C., Barnes, D. M., and Gullick, W. J. Absence of activating point mutations in the c-*erbB-2* proto-oncogene in human breast cancer. Oncogene, *5:* 237–239, 1990.
39. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, A. A. *erbB2* is a potent oncogene when overexpressed in NIH/3T3 cells. Science (Washington DC), *237:* 178–182, 1987.
40. Hudziak, R. M., Schlessinger, J., and Ullrich, A. Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH3T3 cells. Proc. Natl. Acad. Sci. USA, *84:* 7159–7163, 1987.
41. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D. and Muller, W. J. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA, *89:* 10578–10582, 1992.
42. Schroeter, C. A., De Potter, C. R., Rathsmann, K., Willighagen, R. G. J., and Greep, J. C. c-erbB-2 positive breast tumors behave more aggressively in the first years after diagnosis. Br. J. Cancer, *66:* 728–734, 1992.
43. Lonardo, F., Di Marco, E., King, C. R., Pierce, J. H., Segatto, O., Aaronson, S. A., and Di Fiore, P. P. The normal erbB2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biol., *2:* 992–1003, 1990.
44. Wildenhaim, Y., Pawson, T., Blackstein, M. E., and Andrulis, I. L. $p185^{neu}$ is phosphorylated on tyrosine in human breast tumors which overexpress *neu/erbB2*. Oncogene, *5:* 879–883, 1990.
45. Russell, K. S., and Hung, M-C. Transcriptional repression of the *neu* protooncogene by estrogen stimulated estrogen receptor. Cancer Res., *52:* 6624–6629, 1992.
46. Zhao, X-Y., and Hung, M-C. Negative autoregulation of the *neu* gene is mediated by a novel enhancer. Mol. Cell. Biol., *12:* 2739–2748, 1992.
47. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the *HER-2/neu* proto-oncogene in normal human adult and fetal tissues. Oncogene, *5:* 953–962, 1990.
48. Suen, T-C., and Hung, M-C. Multiple *cis*- and *trans*-acting elements involved in regulation of the *neu* gene. Mol. Cell. Biol., *10:* 6306–6315, 1990.
49. Yan, D-H., Marin, M. C., and Hung, M-C. Differential expression of the *neu* oncogene in mouse liver and pancreatic cell lines. Biochem. Biophys. Res. Commun., *186:* 363–370, 1992.
50. Antoniotti, S., Maggiora, P., Dati, C., and De Bortoli, M. Tamoxifen up-regulates c-erbB-2 expression in oestrogen-responsive breast cancer cells *in vitro*. Eur. J. Cancer, *28:* 318–321, 1992.
51. Dati, C., Antoniotti, S., Taverna, D., Perroteau, I., and De Bortoli, M. Inhibition of c-*erbB2* oncogene expression by estrogens in human breast cancer cells. Oncogene, *5:* 1001–1006, 1990.
52. De Bortoli, M., Dati, C., Antoniotti, S. Maggiora, P., and Sapei, L. Hormonal regulation of c-*erbB-2* oncogene expression in breast cancer cells. J. Steroid Biochem. Mol. Biol., *43:* 21–25, 1992.
53. Read, L. D., Keith, D., Slamon, D. J., and Katzenellenbogen, S. Hormonal modulation of *HER-2/neu* protooncogene messenger ribonucleic acid and p^{185} protein expression in human breast cancer cell lines. Cancer Res., *50:* 3947–3951, 1990.