Distal *ERBB2* Promoter Fragment Displays Specific Transcriptional and Nuclear Binding Activities in *ERBB2* Overexpressing Breast Cancer Cells

LAURENCE DELACROIX, DOMINIQUE BEGON, GUILLAUME CHATEL, PASCALE JACKERS,
and ROSITA WINKLER

ABSTRACT

Overexpression of the *ERBB2* gene occurs in 30% of human breast cancers and is correlated with poor prognosis. The deregulation is the consequence of an increased transcription level and gene amplification. Several laboratories, including our own, have identified, in the proximal promoter, enhancers implicated in the gene overexpression. However, our previous studies of a 6-kb *ERBB2* promoter fragment revealed the presence of repressing fragments, which were able to overcome the effect of the proximal enhancers. These repressing elements were functional in all cell lines, regardless of their endogenous *ERBB2* expression level. Here, we show that a distal *ERBB2* promoter region restores high transcription rates specifically in *ERBB2* overexpressing breast cancer cells. This distal promoter region thus contains enhancers essential for the overexpression of the gene. By EMSA, performed with nuclear extract of cells overexpressing (BT-474) or not (MDA-MB-231) the *ERBB2* gene, we show that at least two sequences of the distal promoter region are bound exclusively by BT-474 extract. Further experiments reveal that AP-2 transcription factors contribute to this differential binding activity, by binding recognition sequences located 4500 bp and 4000 bp upstream of the transcription start site. These sites are occupied by AP2 *in vivo*, as demonstrated by ChIP assay. Inactivation of AP-2 proteins in *ERBB2* overexpressing cells reduces the distal promoter activity up to 70%, indicating the AP-2 factors are implicated in the strong distal enhancing effect. Moreover, we identified a 54-bp fragment that is bound specifically by BT-474 nuclear extract. Further experiments did not lead to the identification of the protein responsible for this binding. Our results thus highlight the importance of *ERBB2* distal promoter region and further implicate AP-2 in *ERBB2* overexpression in breast cancer cells.

INTRODUCTION

THE *ERBB2* GENE ENCODES a 185-kDa transmembrane tyrosine kinase receptor, p185^{c-erbB-2}, belonging to the epidermal growth factor receptor (EGFR) family (Akiyama *et al.*, 1986). Thus far, no ligand capable of binding p185^{c-erbB-2} with high affinity has been identified; the receptor is activated by heterodimerization with another ligand-bound receptor of its own family (reviewed in Yarden and Sliwkowski, 2001). The *ERBB2* gene is expressed at low levels in the majority of adult tissues (Press *et al.*, 1990), but is overexpressed in 30% of breast carcinomas, where it is a marker of poor prognosis (reviewed in Ross and Fletcher, 1999). The oncogenicity of *ERBB2* overexpression was demonstrated in cellular and animal models (Di

Fiore *et al.*, 1987; Bouchard *et al.*, 1989; D'Souza and Taylor-Papadimitriou, 1994) leading to the development of a p185^{c-erbB-2} targeted antibody, Trastuzumab (Herceptin), which is currently in therapeutic use for breast cancer (Cobleigh *et al.*, 1999; Slamon *et al.*, 2001).

In primary breast tumours strong *ERBB2* gene overexpression is associated with gene amplification and increased transcription (King *et al.*, 1985; Kraus *et al.*, 1987; Slamon *et al.*, 1987; Pasleau *et al.*, 1993). Most studies investigating the transcriptional mechanisms responsible for p185^{c-erbB-2} accumulation in breast cancers have focused on the *ERBB2* proximal promoter region. Indeed, the 500-bp fragment located upstream of the transcription start site, whose activity is increased in cells overexpressing *ERBB2* gene, is well characterized. Several ets factors, which bind a se-

quence lying immediately upstream of the TATA box, were shown to enhance promoter activity (Scott *et al.*, 1994; Benz *et al.*, 1997). These factors may act by inhibiting the formation of a DNA triple helix, which inhibits the transcription of the gene (Scott *et al.*, 2000). AP-2 transcription factors also stimulate the promoter activity in breast cancer cell lines by binding two sequences in the *ERBB2* proximal promoter, located respectively 213 bp (Bosher *et al.*, 1996) and 500 bp (Vernimmen *et al.*, 2003a) upstream of the transcription start site.

The AP-2 family of proteins, in addition to an essential role in normal growth and morphogenesis during mammalian development (Schorle *et al.*, 1996; Nottoli *et al.*, 1998) has been associated with neoplasia, primarily because of its involvement in the *ERBB2* proximal promoter activity. This was further supported by the correlation between AP-2 and p185^{c-erbB-2} levels observed in breast tumor-derived cell lines (Bosher *et al.*, 1996) and tumor samples (Turner *et al.*, 1998). Moreover, the AP-2 proteins regulate other genes involved in growth and differentiation, such as the preferred p185^{c-erbB-2} dimerization partner p185^{c-erbB-3} (Skinner and Hurst, 1993; Zhu and Domann, 2002), the cell-cycle inhibitor p21^{ciP} (Zeng *et al.*, 1997), the estrogen receptor (McPherson *et al.*, 1997), and E-cadherin (Faraldo *et al.*, 1997). The AP-2 family contains five conserved members, encoded by different genes: AP-2 α -, β -, γ -, δ -, and ϵ -. These helix-span-helix factors are highly conserved within the DNA-binding and dimerization domains but differ in their N-terminal transactivation domain. AP-2 α -, β -, and γ bind as homo- or hetero-dimers to the same GC-rich sequences that can diverge from the GCC (N)3/4 GGC consensus (Mohibullah *et al.*, 1999), while AP-2 δ has a distinct DNA binding preference (Zhao *et al.*, 2001).

Besides this proximal promoter fragment, our previous observations revealed that distal regulatory elements were implicated in *ERBB2* gene transcription regulation (Grooteclaes *et al.*, 1994). The 3-kb fragment upstream of the proximal promoter inhibited the transcriptional activity in all the tested cell lines, whether they expressed low or high levels of the *ERBB2* transcript. This indicated that the fragment contains repressing elements able to overcome the positive effects of the proximal enhancers. Interestingly, the 2.2-kb fragment further upstream was able to restore high transcriptional activity only in BT-474 cells, which strongly overexpress the gene.

In this study, we show that the distal *ERBB2* promoter region enhances the transcriptional activity in all *ERBB2* overexpressing breast cancer cells tested, and is thus required for the overexpression. This specific transcriptional activity is accompanied by a specific nuclear binding activity, underlying that *ERBB2* expression level is governed dominantly by the transcription factors population or activity in the cells. We also demonstrate that AP-2 transcription factors contribute to this cell-specific effect by binding two different recognition sequences of the distal *ERBB2* promoter, located respectively 4500 and 4000 bp upstream of the transcription start site.

MATERIALS AND METHODS

Cell lines

The human breast cancer cell lines (BT-474, ZR-75.1, SKBR3, and MDA-MB-231) were purchased from ATCC

(Rockville, MD) and cultured in the recommended media supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μ g/ml penicillin/streptomycin (Cambrex, MD).

Reporter and expression vectors

ERBB2 reporter vectors containing promoter fragments of increasing sizes were described previously (Grooteclaes *et al.*, 1994). The p3798pGEM vector was constructed by the insertion of the whole pGEM3Z sequence at the *Bam*HI site, upstream of the 3798-bp *ERBB2* promoter sequence, in the p3798-Luc reporter vector. The *Eco*RI/*Xho*ITK-Luc vector was obtained by the insertion of the *Eco*RI/*Xho*I fragment at the *Sal*I site, upstream of the HSV Thymidine Kinase promoter, in the pXP2 derived TK-Luc vector.

The p6007DR plasmid was obtained by religating the p6007-Luc vector, following *Xho*I and *Pvu*II restriction and subsequent end blunting using Klenow polymerase (Roche, Indianapolis, IN). The pSPRSV and pSPRSV-DNAP-2 (or Δ N278) expression vectors were generously provided by Dr H. Hurst, and were described elsewhere (Williams and Tjian, 1991). The AP-2 α cDNA was inserted in the pGEM3Z vector, downstream of the T7 promoter, for the *in vitro* translation assay.

Transfection experiments

Cells were transfected with the FuGENE 6 reagent (Roche). One to three hundred thousand cells were seeded in six-well plates and treated with a FuGENE:DNA ratio of 3 μ l:1 μ g for 48 h in complete medium. Cells were harvested, lysed, and luciferase activity was measured using the Luciferase Reporter Gene Assay Kit (Roche), according to the manufacturer.

Oligonucleotides and primers

All oligonucleotides and primers were purchased from Eurogentec (Liege, Belgium).

The following oligonucleotides (annealed to their complementary sequence) were used in EMSA experiments. The AP-2 binding sequences are shown in bold characters, nucleotide substitutions are underlined. -4500BS: 5'-AC-CTGTGCCTCAGCCTCAAGGCACTGTGGGGTA-3'; -4500mutBS: 5'-ACCTGTGCCTCAGACTCAAGTACTGTGGGGTA-3'; -4000BS: 5'-CCCTTCCCTTCCCTGAGGGTCCCCCTCGCTG-3'; -4000mutBS: 5'-CCCTTCCCTTCCCTGAAATATCCCCCTCGCTG-3'; NS (SP1 binding site): 5'-GATTCGATCGGGCGGGGCGAGC-3'; AP-2BS: 5'-TTCAAAGATTCCAGAAGATATGCCCCGGGGGTCTGGAAGCC-3' (described in Vernimmen *et al.*, 2003a).

The following primers were used in ChIP assays, their position relative to transcription start site is indicated in brackets. -4500BS forward (-4578 bp): 5'-CTCAAGCCTACTCTGAGGAA-3', -4500BS reverse (-4288 bp): 5'-TGGA-GACTGGGGGACACAGGA-3', -4000BS forward (-4127 bp): 5'-ATCTGCCCTGATCTGAGTTT-3', -4000BS reverse (-3907 bp): 5'-GCTGCTGATTCGATTTCTC-3', -500BS forward (-575 bp): 5'-GACTGTCTCTCCCAAATTT-3', -500BS reverse (-349 bp): 5'-CTTAACTTCTCTGGGAGC-3', -6900 forward (-6981 bp): 5'-GCAGTAG-

CAAGCATCGAGTT-3', -6900 reverse (-6780 bp): 5'-TG-GATCATCACAAGGTTTTC-3', ECAD forward (-171 bp): 5'-TAGAGGGTCACCGCTCTATG-3', ECAD reverse (-6 bp): 5'-GGGTGCGTGGCTGCAGCCAGG-3'.

The following primers were used to generate subfragments covering the 3'-extremity of the *EcoRI/XhoI* distal *ERBB2* promoter fragment. They were subsequently used as probes and competitors in EMSA experiments. Their position relative to transcription start site is indicated in brackets. A forward (-3985 bp): 5'-CCCTCAGACATCCTCTTTC-3', A reverse (-3907 bp): 5'-GCTGCTGATTTCGATTTCTC-3', B forward (-3927 bp): 5'-GGAGGAAATCGAATCAGCAG-3', B reverse (-3823 bp): 5'-GAGCCATTCAGAGCTGGGA-3', C forward (-3877 bp): 5'-TGTCAGACACCAGACGAGGT-3', C reverse (-3755 bp): 5'-GGAGGAGCTGGGGTCA-GAAC-3'.

Electrophoretic mobility shift assay

The double-stranded oligonucleotides were 3' end-labeled with the Klenow fragment of DNA polymerase (Roche) and [α^{32} P]dCTP (ICN, Aurora, OH), while PCR fragments were radiolabeled with T4 polynucleotide kinase (Roche) and [γ^{32} P] ATP (ICN). Nuclear proteins were extracted as described elsewhere (Vernimmen *et al.*, 2003a). In a total volume of 20 μ l, 10–50 $\times 10^3$ cpm of labeled oligonucleotides were mixed with 2–5 μ g nuclear extract in a solution consisting of 10 mM Tris, 20 mM NaCl, 2.5 mM DTT, 10% (v/v) glycerol, 0.5% (v/v) NP40, 5 mM MgCl₂, 2.5 mM EDTA, and 1 μ g polydG-dC. When necessary, a 100-fold molar excess of competitor DNA was added. After a 20-min incubation at room temperature, the sample was mixed with 1/10th its volume of loading buffer (250 mM Tris-HCl, 0.2% [w/v] Bromophenol Blue, 0.2% [w/v] Xylene Cyanol, 40% [v/v] glycerol) and the mixture was submitted to electrophoresis in a 5% acrylamide gel for 2–5 h in TBE buffer (0.025 M Tris base, 0.022 M boric acid, and 0.5 mM EDTA, pH8.3) at 140 volts.

Supershifts were performed by preincubating nuclear extracts with 2 μ l of anti-AP-2 antibody (SC-184 Santa Cruz, Santa Cruz, CA) or preimmune rabbit serum (Santa Cruz) overnight at 4°C.

In the variant EMSA, a mix of *AvaII* restricted fragments was 3' end-labeled with the Klenow fragment of DNA polymerase (Roche) and [α^{32} P]dCTP (ICN). Ten thousand cpm of the "probe mix" were incubated in the conditions described above with 2, 4, and 8 μ g of nuclear extracts.

ChIP assay

In brief, after crosslinking 15 min with 1% formaldehyde, glycine was added to a final concentration of 125 mM and incubated 5 min at room temperature. The cells were rinsed twice in PBS, trypsinized, and harvested in culture medium. After centrifugation, the cell pellet was washed in PBS and lysed 10 min on ice in cell lysis buffer (PIPES 5 mM pH 8.0, KCl 85 mM, IGEPAL [or NP-40] 0.5%, supplemented with proteases inhibitors cocktail; Roche). The lysate was washed in nuclei washing buffer (EDTA 1 mM pH 8.0, EGTA 0.5 mM, HEPES 10 mM pH 6.5, NaCl 200 mM), then incubated 10 min on ice in nuclei lysis buffer (SDS 1%, Tris-HCl, 50 mM pH 8.0, EDTA 10 mM pH 8.0, supplemented with proteases inhibitors cock-

tail; Roche). The chromatin was sheared by sonication to an average DNA length of 500–1000 bp. The sonicated chromatin was subjected to a 2-h preclear by incubation with protein A-sepharose or protein G-sepharose beads (equilibrated in Tris-HCl 50 mM pH 8.0 and blocked with salmon sperm DNA 0.2 mg/ml and BSA 0.5 mg/ml) at 4°C, under rotation. 100 μ g of precleared chromatin was then incubated overnight, under rotation at 4°C, with 2 μ g of specific antibodies (normal rabbit IgG, polyclonal rabbit AP-2 (SC-184) and normal mouse IgG were purchased from Santa Cruz Biotechnology, mouse RNA pol II (CTD4H8) was obtained from Covance Research Products, Richmond, CA). Immunocomplexes were collected by a 2–3 h incubation with protein A-Sepharose or protein G-sepharose (equilibrated and blocked as above), at 4°C under rotation. Precipitates were washed once, sequentially, in a low salt solution (Tris-HCl 20 mM pH 8.0, EDTA 2 mM, Triton X-100 1%, SDS 0.1%, NaCl 150 mM), high salt solution (Tris-HCl 20 mM pH 8.0, EDTA 2 mM, Triton X-100 1%, SDS 0.1%, NaCl 500 mM), low LiCl solution (Tris-HCl 10 mM pH 8.0, EDTA 1 mM, IGEPAL 1%, sodium deoxycholate 1%, LiCl 250 mM) and TE (Tris-HCl 10 mM pH 8.0, EDTA 1 mM). The Ab-protein-DNA complexes were eluted twice in 250 μ l elution buffer (NaHCO₃ 0.1 M, SDS 1%) by medium power vortexing and rotation, 15 min at room temperature. The combined eluates as well as the input sample (2% of the amount used in the IP procedure) were crosslink reversed by heating overnight at 65°C in the presence of 200 mM NaCl. After proteinase K digestion, the chromatin fragments were phenol-chloroform purified and precipitated in EtOH. PCR amplifications (see primers in the "oligonucleotides and primers" section) were performed on 10% of the DNA samples, the resulting products were electrophoresed on a 1.5% agarose gel, and quantified by densitometric analysis (MultiAnalyst software, Biorad, Hercules, CA). Data were statistically analyzed by an unpaired Student's *t*-test (STATISTICA 6.0).

RESULTS

The distal EcoRI/XhoI promoter fragment is required for ERBB2 gene overexpression in breast cancer cells

We have previously shown that the *EcoRI/XhoI* distal fragment of the *ERBB2* promoter strongly enhanced transcription rates in the *ERBB2* overexpressing breast cancer cell line BT-474, while it had no effect in cells expressing low levels of the gene (Grooteclaes *et al.*, 1994). To investigate if this enhancing effect was commonly observed in *ERBB2* overexpressing cells, we tested the promoter activity in ZR-75.1 and SKBR3 cells, carrying the deregulation. The MDA-MB-231 cell line, which expresses low levels of *ERBB2*, was used as a control. Luciferase reporter vectors containing *ERBB2* promoter fragments of increasing sizes (Fig. 1A) were transfected in the different cell lines and the luciferase activities were normalized to that induced by the 215-bp promoter. This 215-bp promoter directed similar activities in all the cell lines when compared to the empty pXP2 vector (data not shown), and was thus considered as the basal promoter. The results, presented in Figure 1B, show strong activity of the 716-bp promoter fragment in *ERBB2* overexpressing cells (10- to 30-fold over the basal pro-

FIG. 1. The distal *EcoRI/XhoI* promoter fragment is required for strong transcriptional activity in *ERBB2* overexpressing breast cancer cells. (A) Representation of the *ERBB2* endogenous promoter and the derived reporter vectors. The main transcription start site is represented by the arrow and defines position +1. (B) Luciferase activity of the *ERBB2* reporter vectors following transient transfection in *ERBB2* overexpressing BT-474, ZR-75.1, and SKBR3 cell lines and in the MDA-MB-231 cell line which expresses low levels of the gene. The results are presented as fold induction above the level induced by the p215–Luc vector. The experiments were repeated three times in triplicate with comparable results.

motor level). However, the activity was brought to basal in all the cell lines when longer promoter fragments were used (p2029–Luc and p3798–Luc). This result thus confirms our previous observations indicating the presence of strong repressing elements in the *XhoI/PvuII* region. The presence of the distal *EcoRI/XhoI* region in the 6007-bp promoter fragment counteracted this repressing effect and induced an 8- to 18-fold transcriptional activation in *ERBB2* overexpressing cells. In contrast, it had no effect in MDA-MB-231 cells. These results, together with previous observations, demonstrate that the ability of the distal promoter to restore strong transcriptional activity is restricted to *ERBB2* overexpressing cells. Hence, this distal *ERBB2* promoter region is required for the overexpression of the gene in breast cancer cells.

The activity of the EcoRI/XhoI fragment is maximal in the context of the natural ERBB2 promoter

To verify that the activity of the distal fragment was specific, the *EcoRI/XhoI* fragment of the p6007–Luc vector was replaced by a same sized but unrelated sequence, namely pGEM3Z, to create the p3798pGEM–Luc reporter vector (Fig. 2A). The activities of p3798pGEM–Luc and p3798–Luc vec-

tors in BT-474 and ZR-75.1 cells were similar (Fig. 2B), indicating that specific sequences in the distal *ERBB2* promoter are required for the increased expression levels in *ERBB2* overexpressing cells.

Further experiments were designed to investigate the activation potential of the *EcoRI/XhoI* fragment. First, we introduced the fragment directly upstream of the thymidine kinase (TK) promoter in a TK–Luc reporter vector (Fig. 2A). Transfection of the vectors into BT-474 and ZR-75.1 cells show that the distal fragment induced a significant transcriptional activation in both cell lines (about three- to fivefold), when compared to the enhancerless promoter (Fig. 2C). The *EcoRI/XhoI* fragment was thus active on a heterologous promoter, but not as potent as in the 6-kb *ERBB2* promoter. Next, the *XhoI/PvuII* fragment containing the repressing activity was deleted from the p6007–Luc vector (Fig. 2A). In the resulting vector the distal fragment was contiguous to the strong proximal enhancing elements. The activity induced by the deleted vector in both cell lines was comparable to that of the full-length promoter (Fig. 2D). This result shows that the elements contained in the distal fragment were unable to further increase the transcriptional activity induced by the strong proximal enhancers. Taken together, these results indicate that the activity of the distal

FIG. 2. The activity of the *EcoRI/XhoI* fragment is maximal in the context of the natural *ERBB2* promoter. (A) Schematic representation of the p3798pGEM, *EcoRI/XhoI* TK-Luc, and p6007DR reporter vectors used in (B). (B) Relative luciferase activity induced by p3798-Luc and p3798pGEM reporter vectors following transient transfection in BT-474 and ZR-75.1 cell lines. (C) Luciferase activity induced by TK-Luc and *EcoRI/XhoI* TK-Luc reporter vectors after transient transfection in BT-474 and ZR-75.1 cells. The results are presented as fold induction over the enhancerless promoter activity. (D) The p215-Luc, p6007-Luc, and p6007DR reporter vectors were transiently transfected in BT-474 and ZR-75.1 cells. Luciferase activities were normalized to that induced by the 215-bp promoter. All the experiments were repeated three times in triplicate.

EcoRI/XhoI fragment is maximal in the context of the natural *ERBB2* promoter.

The BT-474 and MDA-MB-231 nuclear proteins display different DNA binding activities towards the distal ERBB2 promoter fragment

The differential activity of the distal *EcoRI/XhoI* promoter fragment between breast cancer cells that do or do not overexpress the *ERBB2* gene probably reflects the differences in transcription factor populations or activities. To identify the sequences of the distal fragment recognized by cell-specific proteins, a variant Electrophoretic Mobility Shift Assay (EMSA) was performed (Kozmik and Paces, 1990). The *EcoRI/XhoI* fragment was first restricted by *SphI* enzyme, and the two resulting subfragments were individually digested with *AvaII* (Fig. 3A). The fragments of mix 1 and 2 were radiola-

beled in bulk and incubated with increasing amounts of BT-474 or MDA-MB-231 nuclear extracts. This assay uses limiting amounts of radioactive DNA, so that when a protein binds a specific fragment, the corresponding band disappears.

The results presented in Figure 3B show that the 657- and 716-bp fragments, present in mix 1, disappeared when incubated with small quantities of nuclear proteins from both BT-474 and MDA-MB-231 cells, indicating that they were bound by factors present in both cell lines. On the contrary, the 182-bp probe progressively disappeared with increasing amounts of BT-474, but not with MDA-MB-231 nuclear factors. The intensity of the 90-bp band did not vary upon protein addition, indicating that it was not bound by nuclear factors present in these cells.

In mix 2, the 356-bp probe bound a protein present in both cell lines. In contrast, while the 208-bp band disappeared completely with BT-474 extract, its intensity was attenuated by

FIG. 3. Sequences in the *EcoRI-XhoI* activating fragment are recognized by nuclear factors present in the *ERBB2* overexpressing cells, BT-474 but absent from MDA-MB-231 cells. **(A)** The *ERBB2* distal promoter fragment was first digested by *SphI* enzyme. The resulting 1645- and 554-bp fragments were individually digested by *AvaII*. The subfragments obtained were radiolabeled in bulk and used as "probe mix" in a variant Electrophoretic Mobility Shift Assay (EMSA). **(B)** EMSA performed with radiolabeled mix 1 and mix 2 (left and right panel of the figure, respectively) incubated with 2, 4, and 8 μ g of BT-474 or MDA-MB-231 nuclear extracts.

small amounts of MDA-MB-231 nuclear extract, but did not further vary when the amount of proteins was increased.

These results indicate that the *EcoRI/XhoI* fragment contains multiple protein binding sites, some of which interact with proteins present in both cell lines, while at least two, located in the 182-bp and 208-bp fragments, are recognized exclusively by proteins of BT-474 cells. These differences of binding activities might explain the differences in the distal promoter activities, and thus in the different *ERBB2* expression levels observed between the cells.

AP-2 transcription factors bind two recognition sequences on the distal *ERBB2* promoter

The TRANSFAC (Wingender *et al.*, 2000) analysis of the 182- and 208-bp distal subfragments revealed the presence of potential binding sites for several transcription factors. Among these, AP-2 recognition sequences were present in both of the differentially bound subfragments. These potential binding sites slightly differ from AP2 consensus; they consist in GCC TCAA GGC and CCC TGA GGG sequences, located respectively 4500 and 4000 bp upstream of the main transcription start site. Noteworthy, the -4000 bp binding site (present on the 208-bp frag-

ment) overlaps the *AvaII* restriction site, and was thus disrupted in the previous variant EMSA experiment. AP-2 transcription factors are appealing candidates to explain the differences in DNA binding and transcriptional activities between the two cell lines, since they are poorly expressed in MDA-MB-231 cells, but are abundant in BT-474 cells. AP-2 proteins are also highly expressed in other *ERBB2* overexpressing cells, such as ZR-75.1 and SKBR3, and their levels were found to be correlated to those of p185^{erbB-2} in breast tumours (Turner *et al.*, 1998). Furthermore, as already mentioned, these factors are implicated in the strong activity of the proximal *ERBB2* promoter (Bosher *et al.*, 1996; Vernimmen *et al.*, 2003a).

We thus tested, by regular EMSA experiments, the ability of AP-2 factors to bind the potential recognition sequences located on the distal promoter. As shown in Figure 4A, incubation of the radiolabeled oligonucleotides, containing the potential AP-2 binding sites (-4500BS and -4000BS; see Materials and Methods section), with *in vitro* translated AP-2 α induced a retarded complex (lanes 2 and 8), suggesting the transcription factor efficiently binds these DNA sequences. A similar complex was observed with BT-474 nuclear extract (lanes 3-4 and 9-10) but not with MDA-MB-231 nuclear proteins (lanes 5-6 and 11-12), while a faster migrating complex was present in both

A.

B.

FIG. 4. AP-2 transcription factors bind two different sites on the distal *ERBB2* promoter fragment. (A) EMSA carried out with the labeled oligonucleotides, containing the -4500 bp and -4000 bp AP-2 potential binding sites (left and right panel of the figure, respectively). The -4500 BS probe was incubated alone (lane 1) or with *in vitro* produced AP-2 α (lane 2), 2 and 4 μ g of BT-474 nuclear extract (lanes 3 and 4), 2 and 4 μ g of MDA-MB-231 nuclear extract (lanes 5 and 6). The -4000 BS probe was incubated alone (lane 7), with *in vitro* produced AP-2 α (lane 8), with 2 and 4 μ g of BT-474 nuclear extract (lanes 9 and 10) or with 2 and 4 μ g of MDA-MB-231 nuclear extract (lanes 11 and 12). (B) EMSA carried out with the -4500 BS or -4000 BS probes and 2 μ g of BT-474 nuclear extract in all lanes except from lanes 1 and 9. Competition towards the -4500 BS probe was tested with a 100-fold molar excess of unlabeled oligonucleotides -4500 BS (lane 3), nonspecific (lane 4), -4500 mutBS (lane 5) and AP-2 (lane 6). Competition towards the -4000 BS probe was tested with a 100-fold molar excess of unlabeled oligonucleotides -4000 BS (lane 11), nonspecific (lane 12), AP-2 (lane 13), and -4000 mutBS (lane 14). Supershifts were tested with AP-2 antibody (lanes 7 and 15) or preimmune rabbit serum (lanes 8 and 16).

cases. Several competition and supershift experiments were performed to further ascertain the presence of AP-2 in the retarded complexes obtained with BT-474 nuclear extract. Figure 4B shows that the upper complex contains AP-2 factor, since it is competed by a 100-fold excess of unlabeled AP-2 binding sequence (lanes 6 and 13), but is not affected by addition of an unrelated sequence (lanes 4 and 12). This was confirmed by the use of an AP-2 specific antibody that further retarded the upper complex (lanes 7 and 15). In contrast, the control rabbit preimmune IgG did not affect the migration profile (lanes 8 and 16). The lower complex was shown to be nonspecific for the -4500 BS probe, since it was competed by the unrelated sequence (lane 4). Noteworthy, the lower complex formed with -4000 BS was not affected by any of the competitors, including AP-2 binding sequence (lane 5). Competitors -4500 BS and -4000 BS totally abolished AP-2 fixation on the corresponding probes when the wild-type sequences were used (lanes 3 and 11). In contrast, sequences bearing mutations in the AP-2 potential recognition sites were unable to compete with the probe for AP-2 binding (lanes 5 and 14; see Materials and Methods section for -4500 mutBS and -4000 mutBS sequences). These results indicate that nucleotides comprised in the potential binding sites are effectively crucial for AP-2 binding. When the same experiments were performed with the MDA-MB-231 extract, no specific competitions or supershifts of the retarded complex could be observed. In contrast, strong AP-2 binding activities were observed in ZR-75.1 and SKBR3 cell lines (data not shown).

Taken together, these data demonstrate the presence of effective AP-2 binding sites on the distal *ERBB2* promoter fragment. The differential binding activity of BT-474 and MDA-MB-231 nuclear proteins towards the 182-bp fragment can thus be attributed to AP-2 binding on the -4500 bp site. In addition, we found that AP-2 could also bind a recognition sequence located around position -4000 bp. This binding site is incomplete in the 208-bp fragment, and cannot be responsible for its differential binding activity.

AP-2 binding sites on *ERBB2* promoter are occupied *in vivo*

Endogenous promoters may not be accessible to transcription factors because of the complex chromatin structure. The AP-2 sites, described above, were thus tested for *in vivo* occupancy by chromatin immunoprecipitation (ChIP) assay in BT-474 cells. Crosslinked and sonicated chromatin was immunoprecipitated by specific antibodies, and different regions of interest (depicted in Fig. 5A) were PCR amplified and quantified. The results were reported to that observed in the absence of precipitating antibody (no antibody sample). ECAD promoter, containing four distinct AP-2 sites within its $-171/-6$ bp region, was used as a positive control for *in vivo* AP-2/DNA association (Decary *et al.* 2002). An *ERBB2* promoter sequence, located further upstream the distal AP-2 sites (around position -6900 bp), was used as a DNA quantity control.

FIG. 5. AP-2 proteins are associated to *ERBB2* promoter in living BT-474 cells. (A) Representation of the *ERBB2* and *ECAD* promoter regions amplified by PCR in the ChIP assay. The black boxes represent AP-2 binding sites. (B–F) ChIP assays. The BT-474 sheared chromatin, immunoprecipitated with the indicated antibodies, was PCR amplified to detect the –4500BS (B), the –4000BS (C), the –500BS (D), the –6900 bp (E), and the proximal *ECAD* promoter (F) regions. The PCR were performed in duplicate, and quantification results were reported to that of the control sample (no antibody). The graph presents the mean of two individual ChIP experiments, and is accompanied by a representative agarose gel loaded with PCR products.

The distal –4500 bp site (Fig. 5B) was significantly immunoprecipitated by an AP-2 antibody ($P = 0.0006$), indicating that the transcription factor is associated to this recognition sequence in living BT-474 cells. The enrichment of the –4000 bp site (C) by AP-2 immunoprecipitation was very weak, but significant ($P = 0.0249$). These results suggest that although these two distal AP-2 sites display similar binding affinities *in vitro* (data not shown), their *in vivo* loading of AP-2 differ,

probably because of the nature of the *cis* sequences and/or the accessibility of these two sites. The proximal *ERBB2* and *ECAD* promoters (D and F) showed strong AP-2 binding, since the corresponding PCR products were enriched four- and six-fold by AP-2 immunoprecipitation compared to control (no antibody or rabbit IgG). The *ERBB2* promoter region covering position –6900 bp, containing no AP-2 recognition sequence, was equally detected in all samples (E). When the chromatin frag-

ments were immunoprecipitated by an RNA pol II antibody, a strong increase in *ERBB2* and *ECAD* proximal promoters was observed (D and F), reflecting the transcription machinery assembly on these genes. In these conditions, the amount of -4500 BS and -4000 BS PCR products was also higher, compared to control. This increase is weak (1.99- and 1.69-fold, respectively) but significant ($P = 0.0001$ and 0.0402 , respectively), suggesting that some RNA pol II-containing complexes are contacted by the distal AP-2-bound enhancers.

In summary, the ChIP experiments performed on BT-474 cells prove the *in vivo* association of AP-2 transcription factor with the distal and proximal recognition sequences described on *ERBB2* promoter.

Inactivation of AP-2 transcription factors reduces the activity of the distal as well as the full-length ERBB2 promoter

To estimate the transcriptional effect of AP-2 binding on the newly described sites within the distal promoter, we inactivated the factors by expressing a dominant negative AP-2 protein variant (DN-AP-2). This 18-kDa variant, lacking the transactivation domain, dimerizes with the endogenous AP-2 transcription factors and inhibits their activity (Williams and Tjian, 1991). The DN-AP-2 expression vector was cotransfected with *EcoRI/XhoI* TK-LUC or p6007-Luc reporter vectors in BT-474 and ZR-75.1 cells. The results, presented in Figure 6, show that the variant induced a dose-dependent repression of *EcoRI/XhoI* TK-Luc reporter vector activity (50 to 70%), while it had no effect on the enhancerless vector. DN-AP-2 also reduced the activity of the full-length promoter in both cell lines (about 50%), indicating an overall AP-2 effect on the 6-kb *ERBB2* promoter activity. Taken together, the results shown in Figures 4, 5, and 6 indicate that AP-2 proteins contribute to the distal *ERBB2* promoter activity by binding two recognition sequences present on the *EcoRI/XhoI* fragment.

Characterization of the 208-bp fragment

To precise the sequences of the 208-bp fragments that are bound specifically by BT-474 extract, we carried out EMSA experiments with three overlapping subfragments. The results summarized in Figure 7A indicated that fragment A did not bind BT-474 or MDA-MB-231 nuclear proteins. On the contrary, both the fragments B and C bound specifically proteins extracted from the *ERBB2* overexpressing BT-474 cells. A representative EMSA result obtained with the radiolabeled fragment B is shown in Figure 7B. Incubation of the probe with BT-474 nuclear extract induced the formation of two retarded complexes (lanes 2, 3, and 4). These complexes were specific, since they disappeared with a 50-fold excess of unlabeled fragment B (lane 5), but not with an unrelated sequence (lane 6). When the probe was incubated with MDA-MB-231 extract, only the faster migrating complex was weakly present (lanes 8, 9, and 10). These results show that the $-3927/-3823$ -bp fragment of the distal *ERBB2* promoter contains a sequence that binds a nuclear protein specific to the *ERBB2* overexpressing BT-474 cells. Interestingly, fragment C showed similar results when used as a probe; furthermore, it was able to compete for protein binding on the fragment B (lane 7). Taken together, these data indicate that the common region between fragments

FIG. 6. Inactivation of AP-2 transcription factors in BT-474 and ZR-75.1 cells represses the activity of the distal *EcoRI/XhoI* promoter fragment. (A) Luciferase activities induced by TK-Luc, *EcoRI/XhoI* TK-Luc and p6007-Luc reporter vectors following transient cotransfection with increasing amounts of the DN-AP-2 expression vector in BT-474 cells. Luciferase activities were normalized for each vector to that induced by the same vectors in the absence of DN-AP-2 (set at 100%). The result is representative of three different experiments performed in triplicate. (B) Cotransfection experiment performed as in (A), in ZR-75.1 cells. The result is representative of three different experiments performed in triplicate.

B and C is probably responsible for the differential binding activity of the 208-bp fragment, observed between cells expressing high or low *ERBB2* levels. This 54-bp sequence, presented in Figure 7C, comprises potential recognition sequences for several transcription factors. Among them, NMP4 and Pax5 were tested for DNA binding. We observed that individual mutations of the two recognition sequences had no effect on nuclear binding. Moreover, *in vitro* translated NMP4 was not able to form complexes with the 54-bp probe (data not shown). We thus exclude the contribution of NMP4 or Pax5 in the *ERBB2* distal promoter binding activity, and thus their contribution to the *ERBB2* transcriptional activity. We believe that the identity of the transcription factor binding the 54-bp fragment cannot be determined on the basis of potential binding sequences given by the TRANSFAC database. The identification of this transcription factor will require alternative approaches such as affinity chromatography purification and subsequent characterization.

FIG. 7. The differential binding activity of the 208-bp fragment is restricted to a 54-bp sequence. **(A)** Representation of the overlapping subfragments tested in EMSA experiment. The DNA binding activities of proteins from BT-474 and MDA-MB-231 cells are indicated. **(B)** EMSA carried out with the labeled fragment B and 2, 4, and 6 μ g of BT-474 (lanes 2, 3, and 4) or MDA-MB-231 nuclear extracts (lanes 8, 9, and 10). In lanes 6 and 11, a 50-fold excess of unrelated DNA sequence (SP1 binding site) was added. In lanes 5 and 7, a 50-fold excess of unlabeled fragments B and C were added, respectively. **(C)** Representation of the 54-bp sequence common to fragments B and C. The potential binding sites are underlined or boxed, and the corresponding transcription factors are indicated.

C. -3877 NMP4 PAX5 -3823
 TGTCAGACACCAGACGAGGTTTTTCTCTGGCTTCCCAGCTCTGAATGGGCTC

Most studies on *ERBB2* transcriptional regulation have focused on short promoter fragments. Our data show that the proximal promoter cannot by itself explain the extent of deregulation observed in breast cancer cells. Indeed, the 3.8-kb promoter fragment displays basal transcriptional activity in all the breast cancer cell lines we tested, regardless of their *ERBB2* mRNA level. Interestingly, the activity of the proximal *Neu* gene promoter, the rodent *ERBB2* ortholog, is also repressed by upstream elements (Suen and Hung, 1990; White and Hung,

In this study, we show that the repressing effect is counteracted in *ERBB2* overexpressing breast cancer cells by the activity of the *EcoRI/XhoI* distal promoter fragment (located between positions -6007 and -3798 bp with respect to the main transcription start site). In contrast, this distal fragment is inactive in the nonoverexpressing cell lines (Fig. 1B and (Grooteclaes *et al.*, 1994)). Furthermore, the activity of the 6-kb promoter, containing the distal fragment, is in good agreement with

the degree of *ERBB2* overexpression in the cells. Indeed, the *ERBB2* mRNA level per copy of the gene is increased 6–10-fold in BT-474, ZR-75.1, and SKBR3 cells when compared to nonoverexpressing breast cancer cells (Hollywood and Hurst, 1993, and personal unpublished data). Thus, our data highlight the importance of distal *ERBB2* regulatory elements, and strongly suggest that the distal *EcoRI/XhoI* promoter fragment is required for *ERBB2* overexpression in breast cancer cells. The promoter activities we describe in this work were not observed in other tumor types. Indeed, in colon and ovarian cancer cell lines, where moderate *ERBB2* overexpression has been described, neither the proximal nor the distal enhancers are active (Vernimmen *et al.*, 2003b). Hence, the implication of the distal promoter in *ERBB2* overexpressing cells is specific to breast cancer. Interestingly, tissue-specific expression of a great number of genes such as α -foetoprotein (Ramesh *et al.*, 1995) or GATA-3 (Lakshmanan *et al.*, 1999) is often governed by distal regulatory sequences that may lie several kilobases upstream of the transcription start site.

We demonstrated that specific sequences of the distal *EcoRI/XhoI* promoter fragment are required for the transcriptional activation (Fig. 2B) and also showed that the distal promoter activity is maximal in the context of the natural *ERBB2* promoter (Fig. 2C and D). There are several interpretations of these results. The enhancers present in the *EcoRI/XhoI* distal promoter fragment might be context-dependent, that is, their activity is reduced or nullified when their location relative to basal promoter is modified (Alvarez *et al.*, 2003). Improper location could impair the interactions between transcription factors bound on different *cis*-regulating sequences that need to interact to be fully functional. Alternatively, the inactivity of the distal fragment when the repressing fragment was deleted might indicate an indirect mechanism of activation. Elements in the *EcoRI/XhoI* fragment could inhibit the downstream repressing effect, and would thus be implicated in transcription derepression. According to this hypothesis, the distal fragment activity would thus result from two different effects, both leading to gene overexpression, that is, one driven by a classical enhancer (emphasized by its activity on the TK promoter) and the second directing transcription derepression.

The differences in transcription factor populations and/or activities between *ERBB2* overexpressing and nonoverexpressing cells are probably the main determinants for the specificity of the distal promoter effect. The variant EMSA experiment allowed us to rapidly point out sequences differentially bound by factors from BT-474 and MDA-MB-231 cells. In particular, a 182-bp subfragment was recognized by a protein expressed only in BT-474 cells. We demonstrated by regular EMSA and supershifts assays that this difference in binding activity could be attributed to AP-2 transcription factors, which are abundant in BT-474 compared to MDA-MB-231 cells. A second AP-2 binding site, overlapping an *AvaII* restriction site, was identified on the distal *EcoRI/XhoI* fragment. Additional studies performed with fragments covering the entire *EcoRI/XhoI* region did not reveal other AP-2 binding sites (data not shown).

We proved, by ChIP experiment, that AP-2 transcription factor associates with *ERBB2* promoter *in vivo*. The enrichment of the distal binding sites (Fig. 5B and C) by AP-2 immunoprecipitation was moderate compared to that of proximal binding site (5D), or *ECAD* promoter (5E). This reflects the presence of multiple AP-2 binding sites within *ERBB2* and *ECAD* prox-

imal promoters, while the distal *ERBB2* enhancers contain single AP-2 sites. Moreover, the dynamic of AP-2 loading on the enhancers might differ from that on promoter. The nature of the *cis* sequence could stabilize AP-2 interaction with proximal promoter, while binding could be transient on enhancers. This was evidenced for the androgen receptor on *PSA* promoter (Kang *et al.* 2004). Proximal *ERBB2* and *ECAD* promoters were efficiently immunoprecipitated by a RNA pol II antibody, while only a slight increase was observed for the distal *ERBB2* regions. These results indicate that pol II-containing complexes are assembled on proximal promoters, but that a moderate, but significant, amount of pol II is recruited to the distal sites, probably by DNA loop formation.

Functionally, our results show that inactivating all AP-2 family members present in BT-474 or ZR-75.1 cells results in the inhibition of the *EcoRI/XhoI* fragment activity (Fig. 6A and B). This clearly indicates that the AP-2 proteins, in addition to proximal promoter activation, contribute to the distal promoter enhancing effect in *ERBB2* overexpressing breast cancer cells.

The 6-kb *ERBB2* promoter activity showed a 50% reduction following inactivation of the AP-2 proteins, confirming they are not the only contributors to *ERBB2* overexpression. Indeed, other nuclear proteins have been shown to enhance the proximal promoter activity, such as the ets family of transcription factors (Scott *et al.*, 2000), as already mentioned. Moreover, we think that other transcription factors are implicated in *ERBB2* overexpression by acting on the distal promoter. Our results show that a 54-bp fragment, comprised in the 3' part of the distal promoter is bound by a nuclear factor that is specific to the *ERBB2* overexpressing cell line (Fig. 7). The identity of this factor could not be determined by classical competition and supershift assays, and will thus be the subject of future experiments.

The present study contributes to a better understanding of *ERBB2* transcription deregulation in breast cancer cells by demonstrating the implication of distal promoter elements and the additional contribution of the AP-2 transcription factors. Given the emerging *ERBB2* promoter-targeted therapeutic strategies (Hurst, 2001), designed to either transcriptionally inhibit *ERBB2* or exploit the breast cancer-specific potential of the *ERBB2* promoter to drive the expression of suicide genes, it is essential that the basic science of these processes are adequately defined.

ACKNOWLEDGMENTS

We are grateful to Dr. Helen C. Hurst for the DN-AP-2 expression vector, and to Dr. Michel Moutschen for critical reading of the manuscript. This work was supported by grants from the Belgian National Fund for Scientific Research (F.N.R.S.), Leon Fredericq Foundation, Anticancer Centre from the University of Liege and Federation against cancer. R.W. is a F.N.R.S. Senior Research Associate, and L.D. is the recipient of grants from Televie (F.N.R.S) and Leon Fredericq Foundation.

REFERENCES

- AKIYAMA, T., SUDO, C., OGAWARA, H., TOYOSHIMA, K., and YAMAMOTO, T. (1986). The product of the human *c-erbB-2* gene: A 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* **232**, 1644–1646.

- ALVAREZ, M., RHODES, S.J., and BIDWELL, J.P. (2003). Context-dependent transcription: All politics is local. *Gene* **313**, 43–57.
- BENZ, C.C., O'HAGAN, R.C., RICHTER, B., SCOTT, G.K., CHANG, C.H., XIONG, X., CHEW, K., LJUNG, B.M., EDGER-TON, S., THOR, A., *et al.* (1997). HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. *Oncogene* **15**, 1513–1525.
- BOSHER, J.M., TOTTY, N.F., HSUAN, J.J., WILLIAMS, T., and HURST, H.C. (1996). A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene* **13**, 1701–1707.
- BOUCHARD, L., LAMARRE, L., TREMBLAY, P.J., and JOLICOEUR, P. (1989). Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. *Cell* **57**, 931–936.
- COBLEIGH, M.A., VOGEL, C.L., TRIPATHY, D., ROBERT, N.J., SCHOLL, S., FEHRENBACHER, L., WOLTER, J.M., PATON, V., SHAK, S., LIEBERMAN, G., *et al.* (1999). Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* **17**, 2639–2648.
- DECARY, S., DECESSE, J.T., OGRYZKO, V., REED, J.C., NAGUIB-NEVA, I., HAREL-BELLAN, A., and CREMISI, C.E. (2002). The Retinoblastoma protein binds the promoter of survival gene bcl-2 and regulates its transcription in epithelial cells through transcription factor AP2. *Mol. Cell. Biol.* **22**, 7877–7888.
- DI FIORE, P.P., PIERCE, J.H., KRAUS, M.H., SEGATTO, O., KING, C.R., and AARONSON, S.A. (1987). erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* **237**, 178–182.
- D'SOUZA, B., and TAYLOR-PAPADIMITRIOU, J. (1994). Overexpression of ERBB2 in human mammary epithelial cells signals inhibition of transcription of the E-cadherin gene. *Proc. Natl. Acad. Sci. USA* **91**, 7202–7206.
- FARALDO, M.L., RODRIGO, I., BEHRENS, J., BIRCHMEIER, W., and CANO, A. (1997). Analysis of the E-cadherin and P-cadherin promoters in murine keratinocyte cell lines from different stages of mouse skin carcinogenesis. *Mol. Carcinog.* **20**, 33–47.
- GROOTECLAES, M., PASLEAU, F., DIJKMANS, H., BERZI, P., ALBERT, A., and WINKLER-GOL, R. (1994). The 6-kilobase c-erbB2 promoter contains positive and negative regulatory elements functional in human mammary cell lines. *Cancer Res.* **54**, 4193–4199.
- HOLLYWOOD, D.P., and HURST, H.C. (1993). 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.
- HURST, H.C. (2001). Update on HER-2 as a target for cancer therapy: The ERBB2 promoter and its exploitation for cancer treatment. *Breast Cancer Res.* **3**, 395–398.
- KANG, Z., JANNE, O.A., and PALVIMO, J.J. (2004). Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. *Mol. Endocrinol.* **18**, 2633–2648.
- KING, C.R., KRAUS, M.H., and AARONSON, S.A. (1985). Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* **229**, 974–976.
- KOZMIK, Z., and PACES, V. (1990). Multiple bandshift assay: Rapid identification and cloning of DNA fragments containing specific protein-binding sites. *Gene* **90**, 287–291.
- KRAUS, M.H., POPESCU, N.C., AMSBAUGH, S.C., and KING, C.R. (1987). 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.
- LAKSHMANAN, G., LIEUW, K.H., LIM, K.C., GU, Y., GROSVELD, F., ENGEL, J.D., and KARIS, A. (1999). Localization of distant urogenital system-, central nervous system-, and endocardium-specific transcriptional regulatory elements in the GATA-3 locus. *Mol. Cell. Biol.* **19**, 1558–1568.
- MCPHERSON, L.A., BAICHWAL, V.R., and WEIGEL, R.J. (1997). Identification of ERF-1 as a member of the AP2 transcription factor family. *Proc. Natl. Acad. Sci. USA* **94**, 4342–4347.
- MOHIBULLAH, N., DONNER, A., IPPOLITO, J.A., and WILLIAMS, T. (1999). SELEX and missing phosphate contact analyses reveal flexibility within the AP-2 α protein: DNA binding complex. *Nucleic Acids Res.* **27**, 2760–2769.
- NOTTOLI, T., HAGOPIAN-DONALDSON, S., ZHANG, J., PERKINS, A., and WILLIAMS, T. (1998). AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc. Natl. Acad. Sci. USA* **95**, 13714–13719.
- PASLEAU, F., GROOTECLAES, M., and GOL-WINKLER, R. (1993). Expression of the c-erbB2 gene in the BT474 human mammary tumor cell line: Measurement of c-erbB2 mRNA half-life. *Oncogene* **8**, 849–854.
- PRESS, M.F., CORDON-CARDO, C., and SLAMON, D.J. (1990). Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. *Oncogene* **5**, 953–962.
- RAMESH, T.M., ELLIS, A.W., and SPEAR, B.T. (1995). Individual mouse alpha-fetoprotein enhancer elements exhibit different patterns of tissue-specific and hepatic position-dependent activities. *Mol. Cell. Biol.* **15**, 4947–4955.
- ROSS, J.S., and FLETCHER, J.A. (1999). HER-2/neu (c-erbB-2) gene and protein in breast cancer. *Am. J. Clin. Pathol.* **112**, S53–S67.
- SCHORLE, H., MEIER, P., BUCHERT, M., JAENISCH, R., and MITCHELL, P.J. (1996). Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* **381**, 235–238.
- SCOTT, G.K., DANIEL, J.C., XIONG, X., MAKI, R.A., KABAT, D., and BENZ, C.C. (1994). Binding of an ETS-related protein within the DNase I hypersensitive site of the HER2/neu promoter in human breast cancer cells. *J. Biol. Chem.* **269**, 19848–19858.
- SCOTT, G.K., CHANG, C.H., ERNY, K.M., XU, F., FREDERICKS, W.J., RAUSCHER, F.J., 3RD, THOR, A.D., and BENZ, C.C. (2000). Ets regulation of the erbB2 promoter. *Oncogene* **19**, 6490–6502.
- SKINNER, A., and HURST, H.C. (1993). Transcriptional regulation of the c-erbB-3 gene in human breast carcinoma cell lines. *Oncogene* **8**, 3393–3401.
- SLAMON, D.J., CLARK, G.M., WONG, S.G., LEVIN, W.J., ULLRICH, A., and MCGUIRE, W.L. (1987). Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177–182.
- SLAMON, D.J., LEYLAND-JONES, B., SHAK, S., FUCHS, H., PATON, V., BAJAMONDE, A., FLEMING, T., EIERMANN, W., WOLTER, J., PEGRAM, M., *et al.* (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–792.
- SUEN, T.C., and HUNG, M.C. (1990). Multiple *cis*- and *trans*-acting elements involved in regulation of the neu gene. *Mol. Cell. Biol.* **10**, 6306–6315.
- TURNER, B.C., ZHANG, J., GUMBS, A.A., MAHER, M.G., KAPLAN, L., CARTER, D., GLAZER, P.M., HURST, H.C., HAFFTY, B.G., and WILLIAMS, T. (1998). Expression of AP-2 transcription factors in human breast cancer correlates with the regulation of multiple growth factor signalling pathways. *Cancer Res.* **58**, 5466–5472.
- VERNIMMEN, D., BEGON, D., SALVADOR, C., GOFFLOT, S., GROOTECLAES, M., and WINKLER, R. (2003a). Identification of HTF (HER2 transcription factor) as an AP-2 (activator protein-2) transcription factor and contribution of the HTF binding site to ERBB2 gene overexpression. *Biochem. J.* **370**, 323–329.
- VERNIMMEN, D., GUEDELS, M., PISVIN, S., DELVENNE, P., and WINKLER, R. (2003b). Different mechanisms are implicated in ERBB2 gene overexpression in breast and in other cancers. *Br. J. Cancer* **89**, 899–906.
- WHITE, M.R., and HUNG, M.C. (1992). Cloning and characterization of the mouse neu promoter. *Oncogene* **7**, 677–683.

- WILLIAMS, T., and TJIAN, R. (1991). Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev.* **5**, 670–682.
- WINGENDER, E., CHEN, X., HEHL, R., KARAS, H., LIEBICH, I., MATYS, V., MEINHARDT, T., PRUSS, M., REUTER, I., and SCHACHERER, F. (2000). TRANSFAC: An integrated system for gene expression regulation. *Nucleic Acids Res.* **28**, 316–319.
- YARDEN, Y., and SLIWKOWSKI, M.X. (2001). Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2**, 127–137.
- ZENG, Y.X., SOMASUNDARAM, K., and EL-DEIRY, W.S. (1997). AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nat. Genet.* **15**, 78–82.
- ZHAO, F., SATODA, M., LICHT, J.D., HAYASHIZAKI, Y., and GELB, B.D. (2001). Cloning and characterization of a novel mouse AP-2 transcription factor, AP-2delta, with unique DNA binding and transactivation properties. *J. Biol. Chem.* **276**, 40755–40760.
- ZHU, C.H., and DOMANN, F.E. (2002). Dominant negative interference of transcription factor AP-2 causes inhibition of ErbB-3 expression and suppresses malignant cell growth. *Breast Cancer Res. Treat.* **71**, 47–57.

Address reprint requests to:

Rosita Winkler, Ph.D.

Molecular Oncology Laboratory

GIGA/Experimental Cancer Research Centre

University of Liege

Sart-Tilman B23

4000 Liege, Belgium

E-mail: rwinkler@ulg.ac.be

Received for publication November 20, 2004; received in revised form March 3, 2005; accepted April 27, 2005.