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

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The *ERBB2* gene is overexpressed in 30 % of human breast cancers and this is correlated with poor prognosis. Overexpression of the *ERBB2* gene is due to increased transcription and gene amplification. Our previous studies have identified a new *cis* element in the *ERBB2* promoter which is involved in the gene's overexpression. This *cis* element, located 501 bp upstream from the main *ERBB2* transcription initiation site, binds a transcription factor called HTF (HER2 transcription factor). We report here the identification of HTF as an AP-2 (activator protein-2) transcription factor. The new *cis* element is bound by AP-2 with high affinity, compared with a previously described AP-2 binding site located 284 bp downstream. Co-transfection

of an AP-2 α expression vector with a reporter vector containing the newly identified AP-2 binding site in front of a minimal *ERBB2* promoter induced a dose-dependent increase in transcriptional activity. We examined the contribution of the new AP-2 binding site to *ERBB2* overexpression. For this purpose we abolished the new and/or the previously described AP-2 binding sequence by site-directed mutagenesis. The results show that the two functional AP-2 sites in the first 700 bp of the *ERBB2* promoter co-operate to achieve maximal transcriptional activity.

Key words: AP-2 binding site, *ERBB2* promoter, HER2 transcription factor, HTF, human, transcription regulation.

INTRODUCTION

The *ERBB2* gene, a member of the epidermal growth factor (EGF) receptor family of genes, is overexpressed in about 30 % of primary human breast cancers, and this represents a negative prognostic factor for the patient (reviewed in [1]). The *ERBB2* gene codes for a 185 kDa transmembrane tyrosine kinase (p185^{erbB-2}). Among the more than 20 growth factors of the EGF superfamily recognized by these receptors, none binds to p185^{erbB-2} with high affinity. This receptor is activated by hetero-oligomerization with one of the other ligand-activated receptors. p185^{erbB-2} is the preferred dimerization partner for all ErbB receptors. Dimers containing p185^{erbB-2} are more long-lived and are more active than all other dimers (reviewed in [2]).

The molecular mechanisms leading to *ERBB2* gene overexpression are gene amplification and increased rates of transcription (reviewed in [3]). Consequently, several groups have studied *ERBB2* promoter activity in breast cancer cells. Gene reporter constructs have allowed the identification of a number of positive and negative regulatory elements across the *ERBB2* promoter [4]. The *ERBB2* promoter is composite, containing two major transcription initiation sites. One is a classical TATA box, and the second is an initiator-like element located approx. 40 bp upstream from the TATA box. It is interesting to note that the rat *neu* gene promoter does not contain a TATA box (reviewed in [5]).

To date, the involvement of only two transcription factor families has been clearly established in *ERBB2* gene overexpression in breast cancer cell lines. An ets-family transcription

factor stimulates expression through a site located immediately upstream from the TATA box [4]. AP-2 (activator protein-2) transcription factors stimulate *ERBB2* expression and bind to a sequence located 217 bp upstream from the main transcription initiation site [6]. AP-2 also controls the expression of at least two other members of the erbB network, namely *ERBB3* [5] and *EGFR* (EGF receptor gene) [7]. In primary breast cancers, the role of AP-2 transcription factors is controversial [8,9].

AP-2 transcription factors form a family of four closely related and evolutionarily conserved transcription factors: AP-2 α , AP-2 β , AP-2 γ [10] and AP-2 δ [11]. AP-2 factors show a highly conserved, original, helix-span-helix dimerization domain preceded by a helical basic DNA binding domain [12]. AP-2 α , AP-2 β and AP-2 γ bind with similar affinities to GC-rich recognition sequences. However, when AP-2 is present in high concentrations, it can bind to other lower-affinity sites as well [13,14]. The binding sequence for AP-2 δ has not yet been well defined [15].

We have described several regions in a 6 kb fragment upstream from the *ERBB2* coding region which modulate the expression of the gene. Notably, a fragment extending between –497 and –716 bp upstream from the main initiation site strongly activates transcription in BT-474 cells overexpressing the endogenous *ERBB2* gene [16]. The activating sequence within this fragment is bound with high affinity by a transcription factor that we have called HTF (HER2 transcription factor). A good correlation was observed between HTF levels and *ERBB2* overexpression in a series of breast cancer cell lines [17].

We now show that HTF is identical to AP-2. AP-2 α , AP-2 β and AP-2 γ transcription factors bind the HTF binding site.

Abbreviations used: AP-2, activator protein-2; EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; HTF, HER2 transcription factor.

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Table 1 Sequences of oligonucleotides used in this study

The boxes denote the AP-2 binding sites. The substituted bases in the mutated oligonucleotides are given in bold and underlined. Missing nucleotides are replaced by hyphens. wt, wild type.

Name	Sequence (5' → 3')
HTF wt	5'-TCAAAGATTCCAGAAGATATGCCCCGGGGTCTTGAAGCC-3'
HTF (A)	5'-TCAAAGATTCCAGAAGATATGCACCGGGTCTTGAAGCC-3'
HTF (AA/T)	5'-TCAAAGATTCCAGAAGATATGAACCGTGGTCTTGAAGCC-3'
HTF (MCS)	5'-TCAAAGATTCCAGAAGATATGCC---GGTCTTGAAGCC-3'
HTF (M5)	5'-TCAAAGATTCCAGAAG---GCCCGGGGTCTTGAAGCC-3'
HTF (M3)	5'-TCAAAGATTCCAGAAGATATGCCCGGGG---TGAAGCC-3'
AP2 wt	5'-GAGAACGGCTGCAGGCAACCCAGGCGT-3'
AP2 (T/C)	5'-GAGAACGGCCGCAGGCAACCCAGGCGT-3'
A/H (T/C)	5'-GAGATATGCCGCAGGCAACCCAGGCGT-3'
AP2 (A/T)	5'-GAGAACGGCTGAATGCAACCCAGGCGT-3'
AP2 (AA/T)	5'-GAGAACGGCAAGCATGCAACCCAGGCGT-3'
Oct-1	5'-AGGTCGACTCTAGAGGATCCATGCAATGGATCCCCGGGTACC-3'

Moreover, mutation of either one or both binding sites induces a comparable decrease in the transcription rate, indicating that AP-2 has to be bound to both sites to achieve maximal transcriptional activity.

MATERIALS AND METHODS

Cell lines

BT-474 and ZR75.1 human mammary adenocarcinoma cells and HCT116 colon carcinoma cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.) and cultured in the recommended media supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and 100 µg/ml penicillin/streptomycin (BioWhittaker, Walkersville, MD, U.S.A.).

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from BT-474 cells were prepared as described elsewhere [18]. The sequences containing the HTF [17] and the AP-2 [19] binding sites, as well as the sequences of the mutant oligonucleotides (Eurogentec, Seraing, Belgium) used in EMSAs, are presented in Table 1.

EMSAs were performed as described previously [17]. The retarded complexes were analysed on a non-denaturing 5% (w/v) polyacrylamide gel in 0.25 × Tris/borate/EDTA buffer. For supershift assays, the proteins were incubated overnight at 4 °C in EMSA buffer with 2 µl of an anti-AP-2α antibody (sc-184; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Double-stranded oligonucleotides were end-labelled using the Klenow enzyme (Roche Diagnostic, Basel, Switzerland), incubated with the protein/antibody mixture and analysed as for EMSAs. The gels were dried and analysed using a PhosphorImager (Molecular Dynamics).

AP-2 expression vectors [12,20] were *in vitro* translated using the TⁿT kit (Promega, Madison, WI, U.S.A.).

DNase I footprinting

DNase I footprinting reactions were performed as described elsewhere [21] with minor modifications. Briefly, a 205 bp fragment was PCR-amplified from p756-HTF wt and HTF (AA/T) vectors with 5'-TCCTCCCAAATTTGTAGACCCTCT-3' as the 5' primer and 5'-AGCTTGATCCTACTCCATCCCAAG-3' as the 3' primer. The fragment was cut by the *AccI* restriction enzyme, which recognizes the underlined sequence in the 5'

primer. The resulting 192 bp fragment was purified on an agarose gel and was end-labelled by fill-in with the Klenow enzyme (Roche) in the presence of [α -³²P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.). The probe (2 ng) was diluted in a total volume of 25 µl of buffer D [30 mM Tris (pH 7.9), 100 mM KCl, 0.5 mM EDTA, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P40] containing 1 µg of poly(dI-dC). Samples of 20 µg of nuclear proteins in buffer D were added in a final volume of 25 µl. The mixture was incubated for 15 min on ice and then for 2 min at 20 °C. An equal volume of a solution containing 5 mM CaCl₂ and 10 mM MgCl₂ was added, followed by the addition of 1 µl (0.1–0.4 unit) of freshly diluted DNase I solution (Roche). The reactions were carried out for 40 s at 20 °C and were stopped by the addition of 100 µl of STOP solution [1% (w/v) SDS, 200 mM NaCl, 20 mM EDTA, 10 µg/ml salmon sperm DNA]. The DNA was extracted and analysed on an 8% (w/v) polyacrylamide/40% (w/v) urea gel.

Plasmids and constructs

The p756-LUC plasmid has been described previously [16]. The p86-LUC plasmid was constructed by introducing a *Bss*HII–*Hind*III *ERBB2* promoter fragment into the pGL3-basic reporter vector (Promega). p86-HTF wt-LUC and p86-HTF (AA/T)-LUC were made by inserting a double-stranded DNA oligonucleotide containing the wild-type or the mutant version of the AP-2 site into p86-LUC linearized with *SacI* and *Bss*HII. The AP-2α expression vector pSPRSV-AP-2 has been described elsewhere [12].

Site-directed mutagenesis

Site-directed mutagenesis of the p756-LUC vector was performed using the Chameleon kit (Stratagene, La Jolla, CA, U.S.A.). AP-2 binding was abolished by changing three bases in the binding sites (Table 1). The mutations were verified by sequencing.

Transient transfection assays

ZR75.1 and HCT116 cells were transfected using FuGENE 6 reagent (Roche). Cells (4 × 10⁵) were plated on 35 mm tissue culture dishes, treated with a FuGENE/DNA ratio of 3:1 and incubated for 48 h in complete medium. Cells were then harvested and lysed, and the luciferase enzymic activities were measured using the Luciferase Reporter Gene Assay kit (Roche).

Western blotting

Samples containing 10 μ g of HCT116 cell nuclear extracts were loaded per well, separated on an SDS/12% (w/v) polyacrylamide gel and transferred to a PVDF membrane (Millipore). An anti-AP-2 α antibody (sc-184; Santa Cruz) was used at a 1:700 dilution. The secondary antibody (DAKO Diagnostic, Glostrup, Denmark) was detected with the ECL[®] system (Amersham/Pharmacia Biotech).

RESULTS

AP-2 transcription factors bind the HTF *cis* sequence

We previously described a 44 bp fragment (C4), located –500 bp upstream from the main transcription initiation site, which induces strong activation of *ERBB2* transcription in BT-474 cells. We named the putative transcription factor HTF. By DNase I footprinting experiments, we were able to narrow down HTF binding to a 17 bp region containing a putative AP-2 binding site (GCCCCGGGG) [17].

To analyse further the identity of HTF, we performed gel retardation and supershift experiments with BT-474 nuclear extracts, the HTF probe and an AP-2 α -specific antibody (Figure

1A). As a control, we performed the same experiments using the previously identified proximal AP-2 recognition sequence as a probe [19]. The sequences of all oligonucleotides used in this work are shown in Table 1. For the sake of clarity, the newly identified recognition site will be called HTF, and the sequence identified by Hollywood and Hurst [19] will be called AP-2. Comparable retarded complexes were observed with the HTF (Figure 1A, lanes 1–6) and the AP-2 (Figure 1A, lanes 13–18) probes. Binding of transcription factors to the HTF and AP-2 sites was compared by competition with different unlabelled oligonucleotides. The 'HTF complex' was completely displaced by unlabelled wild-type HTF, but not by the HTF (A) oligonucleotide (Figure 1A, lanes 2 and 3). Interestingly, equal amounts of unlabelled wild-type AP-2 oligonucleotide only partially competed with HTF binding (lane 4), suggesting a lower affinity for this site. Competitors containing the AP-2 (A/T) mutant [19] or an Oct-1 binding site did not interfere with the HTF complex (lanes 5 and 6). The AP-2 complex was completely displaced by unlabelled wild-type AP-2 or HTF oligonucleotides (lanes 14 and 16), but not by the AP-2 (A/T) mutant (lane 15). It is interesting to note that the unlabelled HTF (A) oligonucleotide partially competed for the AP-2 complex (lane 17). This result, and our previous observation that the HTF (A) mutation led to a partial decrease

Figure 1 Binding of the HTF *cis* sequence by AP-2 transcription factors

(A) EMSA (lanes 1–6 and 13–18) and supershift with anti-AP-2 α antibody (lanes 7–12 and 19–24) of BT-474 nuclear protein extracts using HTF or AP-2 probes (see Table 1). The different competitors (Comp) were used at a 200-fold molar excess. The arrow indicates the supershift and the arrowhead indicates the specific protein–DNA complex. (B) EMSA using wild-type HTF and AP-2 oligonucleotides (lanes 1 and 3 respectively) and their (AA/T) mutant versions (lanes 2 and 4 respectively). The asterisk indicates the non-specific complex. (C) *In vitro* synthesized AP-2 α , AP-2 β and AP-2 γ were assayed for binding to the HTF probe by EMSA. (D) DNase I footprinting of a 190 bp PCR fragment containing the HTF binding site. BT-474 nuclear extracts were used in lanes 2 and 4. *In vitro* synthesized AP-2 α protein was used in lane 6. The wild-type HTF probe was used in lanes 1, 2, 5 and 6, and the (AA/T) mutant version was used in lanes 3 and 4. Lanes 1, 3, and 5 show the control reaction without added proteins. The lanes marked ATGC contain the sequence of the footprinted fragment. FP, footprint.

Figure 2 AP-2 transcription factors bind with higher affinity to the HTF sequence than to the AP-2 sequence

(A) Samples of 2 μ g of BT-474 crude nuclear proteins were tested by EMSA with HTF and AP-2 probes. The competitors (Comp) were used at a 100-fold molar excess. The arrowhead indicates specific protein–DNA complexes, and the asterisk indicates the non-specific complex. (B) HTF and mutant AP-2 sequences were used as probes in EMSA with 2 μ g of BT-474 crude nuclear proteins. Competitors were used at a 50-fold molar excess.

in the transcriptional activity of the enhancer [17], indicate that this mutation is not sufficient to abolish AP-2 binding. We thus designed mutants containing three point mutations (AA/T) in the HTF and AP-2 sequences. The HTF (AA/T) and AP-2 (AA/T) probes did not give rise to retarded complexes (Figure 1B, lanes 2 and 4).

To elucidate whether the transcription factor binding the HTF sequence is indeed AP-2, we performed supershift experiments with the HTF and AP-2 probes. The anti-AP-2 α antibody did supershift the HTF- and the AP-2-retarded complexes (Figure 1A, lanes 7–12 and 19–24 respectively), showing that AP-2 binds to the HTF sequence.

To confirm the identity of HTF, we carried out gel retardation experiments with the HTF probe and *in vitro* translated AP-2 α , AP-2 β and AP-2 γ proteins (Figure 1C). All three proteins bound specifically to the HTF probe, as shown by using specific and non-specific competitors (Figure 1C, lanes 2, 3, 5, 6, 8 and 9; see also below). As further proof, a 192 bp PCR fragment containing the HTF site was footprinted with *in vitro* translated AP-2 α protein and with crude nuclear extract from BT-474 cells. The nuclear proteins and the *in vitro* translated AP-2 α gave rise to similar footprints as the wild-type fragment (Figure 1D, lanes 2 and 6 respectively). In contrast, the HTF (AA/T) fragment was not footprinted by the BT-474 protein extract (Figure 1D, lane 4). Taken together, these results indicate that HTF belongs to the

AP-2 family of transcription factors. Moreover, AP-2 binds with higher affinity to the HTF site than to the AP-2 site.

Binding affinity of AP-2 for the HTF and AP-2 sites

AP-2 binds the HTF sequence with high affinity, and the C nucleotide at the third position of the core binding site is vital for this binding ([17], and see above). The sequences surrounding the core binding site have been shown to influence the binding of AP-2 to DNA [7]. Therefore, to better characterize the binding of AP-2 to the HTF and AP-2 sequences, we performed EMSAs with both probes and used oligonucleotides containing deletions of sequences 5' or 3' to the core binding site [HTF (M5) and HTF (M3) respectively] as competitors. An additional mutant oligonucleotide was synthesized that lacked four nucleotides inside the core binding site [HTF (MCS); see also Figure 1C]. The HTF complex was completely abolished in the presence of unlabelled HTF and HTF (M3) oligonucleotides (Figure 2A, lanes 2 and 6). In contrast, the HTF (M5) oligonucleotide was only a partial competitor of the HTF complex, indicating that sequences located upstream from the core DNA binding site contribute to the binding of AP-2 to the HTF sequence (Figure 2A, lane 5). Moreover, the competition with the HTF (A) and HTF (MCS) mutants did not attenuate the intensity of the HTF complex (Figure 2A, lanes 3 and 4). The AP-2 complex was completely displaced by the unlabelled HTF (M5) and HTF (M3) mutants (Figure 2A, lanes 11 and 12 respectively), but not by the AP-2 (A/T) and HTF (MCS) mutants (lanes 9 and 10). These results confirm that AP-2 binds to the HTF sequence with a higher affinity than to the AP-2 sequence (see also Figure 1A). In addition, they show that the nucleotides located upstream from the core AP-2 binding site contribute to the high-affinity binding to the HTF sequence.

Interestingly, the C nucleotide responsible for the high-affinity binding of the transcription factor to the HTF sequence is replaced by a T in the AP-2 sequence [19]. We thus tested if the different binding affinities are due to the differences in the core binding sites and/or in the flanking sequences. For this purpose, we replaced the T in the AP-2 sequence by a C, giving the AP-2 (T/C) mutant. In the A/H (T/C) mutant, the AP-2 site was modified further by replacing the three nucleotides located immediately upstream from the core binding site with the corresponding nucleotides from the HTF sequence. An additional modification was introduced in this oligonucleotide by the replacement of the final C in the core binding site by a G. This mutant sequence thus closely resembles the HTF sequence. In contrast with AP-2, the AP-2 (T/C) and A/H (T/C) mutants were efficient competitors of the HTF complex (Figure 2B, lanes 3 and 5). When used as probes, these mutants behave similarly to the HTF probe, being only partially competed by the unlabelled wild-type AP-2 probe (Figure 2B, lanes 9 and 14) and almost completely displaced by unlabelled HTF and by themselves (Figure 2B, lanes 7, 8, 10 and 12, 13, 15). These experiments further emphasize the importance of the third C in the core binding sequence of HTF for the high-affinity binding of AP-2 to the fragment.

AP-2 α activates the *ERBB2* proximal promoter through the HTF sequence

Next we examined the AP-2-dependent enhancer activity of HTF *in vivo*. The HTF and HTF (AA/T) mutant sequences were cloned in front of a 86 bp *ERBB2* minimal promoter in the

Figure 3 Transcription activation from the HTF site is AP-2-dose-dependent

(A) HCT116 cells were transiently transfected with 0.7 μ g of luciferase reporter vectors [p86/p86-HTF (AA/T)/p86-HTF wt] and 0, 0.075, 0.15 and 0.3 μ g of the AP-2 α expression vector (lanes 2–4, 6–8 and 10–12) in 35 mm tissue culture dishes. Transcriptional activity is expressed as fold induction compared with the level obtained with each vector in the absence of AP-2. The experiment was repeated three times in triplicate. Luciferase activity in cells transfected with the reporter vector alone was given a value of 1. Data are means $\pm$ S.D. of all experiments. (B) AP-2 levels were measured by Western blotting with 20 μ g of nuclear extract from the transfected HCT116 cells. The arrowhead indicates the 50 kDa AP-2 α protein. Molecular mass markers (kDa) are indicated on the left.

Figure 4 Co-operative effect of the two AP-2 sites on *ERBB2* promoter activity

ZR75.1 breast cancer cells were transfected with 1 μ g of the wild-type p756-LUC reporter vector, plus vectors bearing the HTF (AA/T) and AP-2 (AA/T) mutations alone or in combination (●). Transcriptional activity is expressed as a percentage relative to that with wild-type p756-LUC. The experiment was repeated three times in triplicate, and data are means $\pm$ S.D.

pGL3-basic reporter vector. These vectors were transfected along with increasing amounts of an AP-2 α expression vector into HCT116 cells expressing very low levels of the endogenous

transcription factor (Figure 3B). Neither the enhancerless promoter (Figure 3A, lanes 1–4) nor the mutant enhancer (Figure 3A, lanes 5–8) showed any significant variation in transcriptional activity with increasing levels of AP-2 α expression vector. In contrast, the construct containing the wild-type HTF sequence showed an increase in transcriptional activity which was dependent on AP-2 level (Figure 3A, lanes 9–12). AP-2 α protein amounts are presented in Figure 3(B). This result shows that the HTF sequence does respond dose-dependently to AP-2 *in vivo*.

Co-operation between the HTF and AP-2 sites on a 700 bp *ERBB2* promoter fragment

We have shown previously that the HTF (A) mutation leads to a 2-fold decrease in p756-LUC reporter vector transcriptional activity [17]. Therefore we examined more thoroughly the contribution of the HTF site, alone or in combination with the AP-2 site, to *ERBB2* overexpression. We introduced the AA/T mutation into the AP-2 and HTF sites in p756-LUC reporter vector. Three vectors were generated, containing the mutation in each AP-2 site alone, or in both of them. Figure 4 shows the transcriptional activity of these vectors in ZR75.1 cells. These cells overexpress *ERBB2* and endogenous AP-2 α and AP-2 γ [20]. The triple mutation of the HTF site or the AP-2 site decreased

transcriptional activity to approx. 20–25% of the wild-type level. This is in good agreement with previous results showing a decrease in transcriptional activity following mutation of each AP-2 binding site alone [17,19]. Interestingly, mutation of both AP-2 binding sites did not decrease further the transcriptional activity of the 700 bp promoter fragment.

The results presented here (see Figure 3) as well as previously published data [6] indicate that a single AP-2 binding site is enough to increase the transcriptional activity of a short promoter fragment. However, our observations indicate that, in the context of a 700 bp promoter fragment, both HTF and AP-2 sites must be functional to allow optimal transcriptional activity of the *ERBB2* promoter.

DISCUSSION

We show in the present paper that the AP-2 transcription factors bind to the HTF site that we have described previously in the *ERBB2* promoter, and thereby contribute to overexpression of this gene. Indeed, the HTF nuclear protein complex was supershifted by an anti-AP-2 antibody (Figure 1A). Moreover, this sequence was retarded by *in vitro* translated AP-2 α , AP-2 β and AP-2 γ proteins (Figure 1C). Finally, BT-474 cell nuclear proteins, as well as *in vitro* translated AP-2 α , protected the fragment containing the HTF site from digestion by DNase I (Figure 1D). In addition, transfection of a reporter vector containing the HTF site together with increasing amounts of an AP-2 α expression vector resulted in dose-dependent transcriptional activity (Figure 3). This new AP-2 binding site is thus located 284 nucleotides upstream from the previously identified AP-2 site [19].

AP-2 transcription factors bind with higher affinity to the HTF site than to the previously identified AP-2 site in the *ERBB2* promoter (Figure 2). The third C nucleotide in the HTF core binding site confers high affinity to the binding of AP-2, due to the low rate of dissociation of the factor from the DNA [17]. When the T present at this position of the AP-2 site was replaced by a C, the binding affinity of the transcription factor was greatly increased, as shown by EMSA using this mutant oligonucleotide as a probe or as a competitor (Figure 2B). This is in good agreement with the results of Mohibullah and co-workers [13], who systematically analysed the influence of sequence on AP-2 α binding affinity. These authors also noticed that the T in the third position reduces the binding of the transcription factor to the DNA. Moreover, we show that the sequence upstream from the core binding site also influences binding affinity i.e., the presence of an upstream G in the AP-2 sequence decreases binding affinity. The HTF core sequence is flanked by a T, which favours binding [13].

The contribution of the two AP-2 binding sites to transcriptional activity was analysed by transfecting reporter vectors containing wild-type or different combinations of mutant AP-2 binding sites in the *ERBB2* promoter. The results clearly show that mutation of either one or both binding sites results in a similarly low promoter activity, indicating co-operation between the transcription factor bound to both sites in ZR75.1 cells expressing high levels of the endogenous AP-2.

Co-operation between AP-2 factors bound to several sites in a promoter has been reported for several genes. The genes encoding sodium–potassium co-transporter type 2 [22], placental lactogen [23] and chorionic gonadotropin [24] are examples where such a co-operation between AP-2 factors occurs. Moreover, factors bound to several sites in the pro- α 1(I) collagen gene interact with each other to form loops that bring the enhancers closer to the transcription initiation site [25].

In conclusion, we show that AP-2 transcription factors present a significantly higher affinity for the HTF site than for the proximal AP-2 site in the *ERBB2* gene promoter. It is thus conceivable that low cellular levels of AP-2 will lead to preferential binding to the high-affinity HTF site, leaving the AP-2 site unoccupied and the promoter poorly active. Only when the factor is overexpressed will it also bind to the lower-affinity AP-2 site and, in co-operation with the factor bound to the HTF site, induce overexpression of the *ERBB2* gene. This hypothesis remains to be tested experimentally.

Recently, the clinical potential of *ERBB2* receptor-targeted therapeutics has been demonstrated [1]. However, receptor-linked resistance mechanisms now point to the need for additional therapeutic alternatives. Strategies that transcriptionally inhibit *ERBB2* promoter activity are particularly appealing, because *ERBB2* gene and transcript targets per cell are fewer in number than the expressed p185^{c-erbB-2} [4]. Alternatively, *ERBB2* promoter activity could be used to increase specifically the expression of suicide genes in cancer cells [3]. These emerging *ERBB2*-promoter-targeted strategies are dependent on a complete understanding of the architecture of the endogenous *ERBB2* promoter and the factors that specify *ERBB2* overexpression [4].

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