YY1 COOPERATES WITH AP-2 TO STIMULATE *ERBB2* GENE EXPRESSION IN MAMMARY CANCER CELLS*.

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Overexpression of the *ERBB2* oncogene is observed in about 30% of breast cancers and is generally correlated with a poor prognosis. Previous results from our and other laboratories indicated that elevated transcriptional activity contributes significantly to the overexpression of *ERBB2* mRNA in mammary adenocarcinoma cell lines. AP-2 transcription factors account for this overexpression through two recognition sequences located 215bp and 500bp upstream from the transcription start site. Furthermore, AP-2 transcription factors are highly expressed in cancer cell lines overexpressing *ERBB2*. In this report, we examined the cooperative effect of YY1 on AP-2-induced activation of *ERBB2* promoter activity. We detected high levels of YY1 transcription factor in mammary cancer cell lines. Notably, we showed that YY1 enhances AP-2a transcriptional activation of the *ERBB2* promoter through an AP-2 site both in HepG2 and in HCT116 cells, whereas a C-terminal truncated form of YY1 can not. Moreover, we demonstrated the interaction between endogenous AP-2 and YY1 factors in the BT-474 mammary adenocarcinoma cell line. In addition, inhibition of endogenous YY1 protein by an antisense decreased the transcription of an AP-2 responsive *ERBB2* reporter plasmid in BT-474 breast cancer cells. Finally, we detected *in vivo* AP-2 and YY1 occupancy of the *ERBB2* proximal promoter in chromatin immunoprecipitation assays. Our data thus provide evidence that YY1 cooperates with AP-2 to stimulate *ERBB2* promoter activity through the AP-2 binding sites.

The *ERBB2* protooncogene belongs to the epidermal growth factor receptor (*EGFR*)[1] gene family and encodes a 185kDa receptor tyrosine kinase (1). The *ERBB2* gene is overexpressed in several human tumors, mostly in breast and ovary carcinomas, where the overexpression is a marker of a poor prognosis (2). *ERBB2* gene overexpression is able to transform several cell types in culture and to induce mammary tumors in transgenic mice (3). *ERBB2* gene overexpressing tumors are more aggressive due to increased invasive, metastatic and angiogenic phenotype (4). Thus, elucidating the mechanisms leading to *ERBB2* gene overexpression is an important step in understanding the pathogenesis of a particularly aggressive subset of tumors.

The overexpression of the gene is the consequence of increased transcription rates, frequently but not always associated with gene amplification (5). Several laboratories have thus undertaken the study of the mechanisms leading to the accumulation of high levels of erbB2 transcript and the corresponding protein in breast cancer cells. We and others demonstrated that the overexpression is due to an increased transcription rate and not to the stabilization of the messenger RNA (6;7). Subsequent experiments aimed to identify regulatory sequences in the *ERBB2* promoter responsible for the overexpression of the gene, and the transcription factors binding them. Among these, those belonging to the Ets and AP-2 families were shown to be associated with the overexpression of the *ERBB2* gene. An Ets family factor, not yet defined, stimulates *ERBB2* transcription through a sequence located just upstream of the TATA box. Overexpression of several Ets family factors, e.g. PEA3 and ESX/Elf-

3, correlates with elevated *ERBB2* mRNA levels in breast cancer cells (8;9).

Two AP-2 binding sequences were identified in the *ERBB2* proximal promoter at 215bp (10) and 500bp (11;12) upstream from the transcription start site, enhancing *ERBB2* gene transcription. Furthermore, AP-2 transcription factors are highly expressed in primary breast tumors (13) and in breast cancer cell lines overexpressing *ERBB2* (11;14). The AP-2 transcription factor family currently includes five related 50kDa proteins: AP-2α, β, γ (14), δ (15) and ε (16). AP-2 factors present a conserved helix-span-helix dimerisation domain preceded by a DNA binding and a transactivation domain (17).

The role of AP-2 transcription factors in cancer progression seems to depend on the tumor type. For instance, melanoma progression was associated with the loss of AP-2 expression (18). Progression of a teratocarcinoma cell line towards a metastatic phenotype was associated with overexpression of AP-2 protein and inhibition of its transcriptional activity by self-interference (19). Actually, AP-2 factors modulate transcription through interaction with several nuclear factors. PARP (20), PC4 (21), CITED2 (22) and CITED4 (23) have been identified as co-factors which stimulate AP-2 transcriptional activity. Furthermore, p300 and CBP co-activate AP-2α through CITED2 (24).

Yin Yang 1 (YY1) is a multifunctional transcription factor that modulates the expression of a wide variety of genes (25). The YY1 protein contains an activation domain and two repression domains as well as a DNA binding domain formed by four C_2H_2 zinc fingers (26). It binds to DNA on a CCAT, or less frequently ACAT, consensus core binding site (27;28). YY1 was shown to act as a transcriptional activator or repressor depending on the context of its binding site within a particular promoter (29) and on other cell type-specific factors (26). A wide variety of proteins associate with YY1, indicating that protein-protein interactions are important for its activity. YY1 interacting-proteins include basal transcription factors, such as TBP (30), transcriptional coregulators, such as p300/CBP, PARP, HDAC1, HDAC2 and HDAC3, and several transcription factors such as Sp1, c-MYC or C/EBPβ (26).

Recently, Wu and Lee have shown that YY1 interacts with AP-2 on the histone H3.2 promoter in K12 Chinese hamster fibroblasts and 293T human kidney cells without showing a functional impact for this interaction (31). This observation prompted us to ask whether YY1 could co-operate with AP-2 on the *ERBB2* promoter in mammary cancer cell lines. Here, we show that breast cancer cell lines express high levels of YY1 protein. By co-transfection experiments of AP-2 and YY1 expression vectors, we prove that YY1 enhances AP-2α transcriptional activity through an AP-2 site within the *ERBB2* promoter both in HepG2 and in HCT116 cells. In contrast, a C-terminal truncated form of YY1 is inactive in this assay. Next, the inhibition of the endogenous YY1 in BT-474 cell line reduces transcription from an AP-2 responsive reporter plasmid. Moreover, we demonstrate the interaction between endogenous AP-2 and YY1 proteins in BT-474 mammary adenocarcinoma cells. Finally, we detected *in vivo* AP-2 and YY1 occupancy of the *ERBB2* proximal promoter in ChIP assays. Our results thus show that YY1 cooperates with AP-2 for the stimulation of *ERBB2* transcription in breast cancer cells.

EXPERIMENTAL PROCEDURES

Cell lines — The mammary (BT-474, ZR-75.1, MDA-MB-231, MDA-MB-453, MCF-7, T47D and SK-BR-3), hepatic (HepG2) and colonic (HCT116) human carcinoma cells were purchased from American Tissue Culture Collection and cultured in the recommended media supplemented with 10% (v/v) foetal bovine serum, 2mM glutamine and 100μg/ml penicillin/streptomycin (BioWhittaker).

Plasmids and constructs — The p86-LUC and p86-HTF-LUC plasmids were described by Vernimmen et al (12). The CMV-AP-2α and CMV-0 plasmids were provided by Dr E. Holthuizen (32). The pMSV-YY1 and pTC21 plasmids were gifts from Dr. T.-C. Lee (33). The CMV-YY1(1-333) plasmid was a gift from Dr M. Atchison (34). The pCMV-asYY1 and pCMV-asGal4 plasmids were gifts from Dr. T.F. Osborne (35). The as-Vim plasmid was a gift from Dr. C. Gilles (36), where the antisense vimentin cDNA was cloned into pcDNA3.1 plasmid (Invitrogen).

Preparation of cell extracts — Nuclear extracts were prepared as described elsewhere (37). For the preparation of whole cell extracts, cells scraped off

the culture dishes were harvested in PBS, pelleted by centrifugation, resuspended in 1% SDS and boiled for 10 minutes.

Antibodies — Mouse anti-AP-2α antibody (3B5), rabbit anti-AP-2α antibody (C-18), mouse anti-YY1 antibody (H-10), rabbit anti-YY1 antibody (H-414), rabbit anti-Shh antibody (H-160) and control rabbit IgG were purchased from Santa Cruz Biotechnology. Anti-phosphorylated RNA Polymerase II (clone CTD4H8) antibody was obtained from Covance Research Products.

Western blotting — Samples were separated on an SDS-PAGE (9 or 12%) and transferred to a PVDF membrane (Millipore). The primary antibodies were used at a 1:500 dilution. The secondary antibodies coupled with peroxydase (DAKO) at a 1:1000 dilution were detected with the ECL system (Amersham Biosciences).

Transient transfection assays ¾ HepG2, HCT116 and BT-474 cells were transfected using FuGENE 6 reagent (Roche Molecular Biochemicals). The cells ($4X10^5$) were plated onto 35mm tissue culture dishes, treated with FuGENE 6/DNA (ratio of 3:1) and incubated for 48h in complete medium. Cells were then harvested and lysed, and luciferase enzymatic activities were measured using the Luciferase Reporter Gene Assay kit (Roche) and a LUMAT luminometer (Berthold Technologies). The data were normalized to total protein content.

AP-2 and YY1 co-immunoprecipitation — BT-474 or HCT116 nuclear extracts (120µg and 80µg, respectively) were incubated with 5µg of antibody in TNT buffer (50mM Tris pH8, 150mM NaCl, 0.1% Tween) in a total volume of 100µl for 3 hours at room temperature with slow agitation. Protein A Sepharose resin (50µl)(Amersham Biosciences) was then added and the mixture was further incubated for 30 min. The mix was centrifuged for 1 min at 200xg and the pellet was washed twice with TNT buffer. Bound proteins were eluted by incubating the pellet in SDS sample buffer, applied onto an SDS-PAGE, transferred and immunoblotted with an anti-YY1 antibody.

ChIP assays — ChIP assays were adapted from Jackers et al (38) with modifications. Subconfluent BT-474 or HepG2 cells were treated with formaldehyde at a final concentration of 0.5% for 5 min at 37°C. Chemical cross-linking was terminated by addition of glycine to a final concentration of 0.125M, followed by additional incubation for 5 min. Cells were then pelleted, washed with ice-cold phosphate-buffered saline and lysed in ChIP cell lysis buffer (5mM PIPES pH8, 85mM KCl, 0.5% IGEPAL). Nuclei were obtained by centrifugation at 3500xg, washed in ChIP nuclei washing buffer (10mM Hepes, 1mM EDTA, 0.5mM EGTA, 200mM NaCl) and lysed in ChIP nuclei lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH8, 50µl protease inhibitor cocktail, Roche) ($1ml/10^7$ cells). DNA was sheared by sonication to yield an average fragment size of 600bp. Chromatins were stored at -70°C. For immunoprecipitation, about 100µg of chromatin was diluted in IP buffer (16.7mM Tris pH8, 1.1% TritonX-100, 1.2mM EDTA, 167mM NaCl, 0.01% SDS, protease inhibitors) and pre-cleared with 50µl of a 50% protein A-Sepharose slurry (equilibrated in 50mM Tris pH8, blocked with 0.2mg/ml salmon sperm DNA, 0.5mg/ml bovine serum albumin) for 1h30 at 4°C. After centrifugation at 14,000xg for 2 min, specific antibodies (2µg) were added to the supernatants. Immunocomplexes were formed overnight at 4°C and collected with 50µl of 50% protein A-Sepharose (equilibrated and blocked as above) for 2h at 4°C. Beads were then washed for 5 min in buffer A (20mM Tris pH8, 2mM EDTA, 1% Triton X-100, 0.1% SDS, 150mM NaCl), buffer B (buffer A with 500mM NaCl), buffer C (10mM Tris pH8, 1mM EDTA, 1% IGEPAL, 1% sodium deoxycholate, 250mM LiCl), and TE buffer (10mM Tris pH8, 1mM EDTA). Immunocomplexes were eluted off the beads with 2X250µl of 1% SDS, 0.1M $NaHCO_3$ and cross-links were reversed by incubation for 4h at 65°C. Proteins were digested with proteinase K (40µg/ml) for 1h at 50°C. DNA samples were then purified by phenol-chloroform extraction, ethanol precipitated, and further analyzed by PCR. Gene-specific primer sequences are : –500bp ERBB2 primers, 5'-GACTGTCTCCTCCCAAATTT and 5'-CTTAAACTTTCCTGGGGAGC (fragment –575bp to –349bp); -5300bp ERBB2 primers, 5'-GCCAAAGGAAGAGAAGAATC and 5'-CAGGACATCACTTGCTCACTC (fragment –5485bp to –5265bp); E-cadherin primers, 5'-TAGAGGGTCACCGCGTCTATG and 5'-GGGTGCGTGGCTGCAGCCAGG (39)

(fragment –171bp to –6bp); GR primers 5'-CCCCCTGCTCTGACATCTT and 5'-CTTTTCCGAGGTGGCGAGTATC (40) (fragment –2333bp to –2018bp) (41). PCR amplification signals were quantified by densitometric scanning using Fluor-S MultiImager and analysis with the MultiAnalyst software (Bio-Rad). Fold enrichment in each immunoprecipitation was determined as ratio between immunoprecipitated DNA and no antibody control DNA.

RESULTS

Comparison of YY1 and AP-2α levels in mammary cancer cell lines. — High levels of transcriptionally active AP-2 factors are present in breast cancer cell lines overexpressing *ERBB2*. In this paper, we wanted to ascertain whether YY1 cooperates with AP-2 to stimulate *ERBB2* promoter activity in mammary cancer cell lines. First, we compared YY1 and AP-2 protein levels in several cancer cell lines expressing different levels of the *ERBB2* mRNA. Whole cell extracts from seven mammary and one liver cancer cell lines were analyzed by western blotting using antibodies specific for AP-2α (Fig. 1A) and YY1 (Fig. 1B). The mammary cancer cell lines overexpressing *ERBB2* (BT-474, ZR-75.1, MDA-MB-453 and SK-BR-3) and T47D cells, contained high levels of AP-2 protein. AP-2 protein level was low in MCF-7 cells, while no AP-2 was detected in MDA-MB-231 and HepG2 cells (Fig. 1A). These results are in agreement with previously published data. High levels of YY1 protein were detected in all the cell lines analyzed, whether or not they overexpressed the *ERBB2* gene (Fig. 1B).

YY1 enhances AP-2 transcriptional activity. — Wu and Lee described the interaction between YY1 and AP-2 but they were unable to show a functional significance for this interaction (31). YY1 being well expressed in breast cancer cells, we wanted to know whether YY1 modulates AP-2 transcriptional activity on the *ERBB2* promoter. In order to answer this question, we co-transfected AP-2 and YY1 expression vectors and different *ERBB2*-LUC reporter vectors containing or not an AP-2 binding site (Fig. 2A) in HepG2 cells, devoid of AP-2. In the first set of experiments, we tested the AP-2/YY1 cooperation on the p86-HTF-LUC reporter vector where the AP-2 binding site, naturally located 500bp upstream from the transcription start site (called the HTF site), was cloned in front of an 86bp fragment of the *ERBB2* minimal promoter (Fig. 2A) (12). Co-transfection of increasing amounts of the YY1 expression vector with p86-LUC or p86-HTF-LUC reporter vectors did not affect luciferase activity of either vector, indicating that overexpression of the YY1 factor alone does not modulate activity of *ERBB2* proximal promoter in HepG2 cells (Fig. 2B). In contrast, AP-2α expression vector induced a dose-dependent increase of luciferase activity when co-transfected with the p86-HTF-LUC but not with the p86-LUC reporter (Fig. 2C). This confirms that AP-2 factor specifically activates the transcription of the *ERBB2* promoter through the HTF site (12). Co-expression of increasing amounts of YY1 with a constant low amount of AP-2α induced a YY1-dose-dependent increase in activity of the AP-2 binding site containing reporter only, up to 2.3 fold with 500ng of YY1 expression vector transfected (Fig. 2D). These experiments were also performed with a version of the p86-HTF-LUC reporter where the AP-2 site was mutated (12). The mutant reporter behaved like the p86-LUC reporter, supporting the specificity of the functional effect on the intact AP-2 binding site (data not shown). These results show that YY1 enhances the transcriptional activity of AP-2α. However, in the absence of AP-2 factors, YY1 is inactive on the *ERBB2* proximal promoter. We obtained similar results with AP-2β and γ transcription factors (see supplementary data A).

In the p86-HTF-LUC vector, the AP-2 binding site was inserted close to the transcription start site. YY1 might thus stimulate AP-2 activity by interacting directly with the basal transcription complex. In order to investigate the cooperation between YY1 and AP-2 when AP-2 is bound in its natural context within the *ERBB2* promoter, we repeated the co-transfection experiments using the p278-LUC and p716-LUC reporter vectors (see supplementary data B). These constructs contain fragments of the *ERBB2* promoter including one (p278-LUC) or both (p716-LUC) of the AP-2 sites located 215bp and 500bp upstream from the transcription start site. As a control, we used a version of the p716-LUC plasmid where the two

AP-2 sites were inactivated by mutation (p716mut) (12). Similarly to the previous results, YY1 expression vector alone was inactive, while AP-2α expression vector induced a dose-dependent increase in activity of both wild type promoters. However, co-transfection of increasing amounts of YY1 expression vector with a constant low amount of AP-2α expression vector gave a similar significant activation of transcription with both wild type reporter vectors (see supplementary data B). In conclusion, these results demonstrate that YY1 is able to stimulate the activity of AP-2 transcription factors on two AP-2 sites of the *ERBB2* promoter.

The activity of YY1 is known to be cell line dependent. We thus decided to test whether YY1 also cooperates with AP-2 in HCT116 colon carcinoma cells, which contain minimal amounts of AP-2 factors but high amounts of YY1 (Fig. 3A). The cells were co-transfected with AP-2 and YY1 expression vectors and the p86-LUC and p86-HTF-LUC reporter vectors (Fig. 2A). Increasing amounts of the AP-2α expression vector induced a dose-dependent increase of luciferase activity when co-transfected with the p86-HTF-LUC but not with the p86-LUC reporter (Fig. 3A). This is in agreement with results obtained with hepatoma cells. Increasing amounts of the YY1 expression vector with p86-LUC or p86-HTF-LUC reporter vectors did not affect luciferase activity of either vector, indicating that the YY1 factor alone does not act on the *ERBB2* proximal promoter in HCT116 cells (Fig. 3B). Co-expression of increasing amounts of YY1 with a constant low amount of AP-2α induced a YY1-dose-dependent increase in activity of the AP-2 binding site containing reporter, reaching a 2 fold induction of AP-2 transcriptional activity for 500ng of YY1 expression vector (Fig. 3C). These findings show that in presence of a small amount of endogenous AP-2 factor (Fig. 3A), further increase in YY1 content has no effect on *ERBB2* proximal promoter activity. However, when we simultaneously increase the AP-2 protein content, YY1 enhances transcriptional activity of AP-2α. This observation underlines the importance of the balance between AP-2 and YY1 protein levels for the cooperation between the factors.

To delve deeper in the mechanism by which YY1 enhances AP-2 activity, we performed the same experiments with YY1 (1-333), a version of YY1 deleted of its C terminus. YY1 (1-333) can no longer bind DNA but should still be able to interact with AP-2 (30;31). Co-transfection of increasing amounts of the YY1 (1-333) expression vector with p86-LUC or p86-HTF-LUC reporter vectors did not affect luciferase activity of either vector (Fig. 3B). Co-expression of increasing amounts of YY1 (1-333) with a constant low amount of AP-2α did not increase transcriptional activity of AP-2, although YY1 (1-333) is well expressed (Fig. 3C, IB YY1, lowest band). These results indicate that the C-terminal domain of YY1 is important for increasing AP-2 transcriptional activity.

Endogenous YY1 and AP-2 interact in BT-474 breast cancer cells. — Wu and Lee have shown by GST pull down that the YY1 (1-333) truncated protein interacts with AP-2 (31). To make sure that the absence of activity of YY1 (1-333) on AP-2 transcriptional activity was not due to a lack of *in vivo* interaction between these proteins, we made co-immunoprecipitation experiments. Nuclear extracts were prepared from HCT116 cells transfected with AP-2α vector (0,25µg) and either YY1 wt or YY1 (1-333) vectors (0,5µg) as indicated (Fig. 4A). Proteins were immunoprecipitated with an antibody recognizing AP-2. Normal rabbit IgG was used as a negative control. The YY1 (1-333) truncated protein (Fig. 4A, lane 2, lower band) was detected in the AP-2 immunoprecipitate, as was the full-length YY1 protein (Fig. 4A, lanes 2 and 6, upper band). In contrast, no YY1 protein was detected in the negative controls (Fig. 4A, lanes 3, 4, 7 and 8). These results show that YY1 (1-333) and AP-2 proteins interact in HCT116 cells.

Interaction between endogenous YY1 and AP-2 proteins was never assessed previously. So, we next examined whether the interaction between endogenous YY1 and AP-2 factors occurs in mammary tumor cells. For this purpose, we performed AP-2 and YY1 co-immunoprecipitation experiments using extracts from BT-474 mammary cancer cells, which express high levels of both proteins (Fig. 1, lane 1). Nuclear proteins from BT-474 cells were immunoprecipitated with antibodies recognizing AP-2 or YY1. A sonic hedgehog (Shh) specific antibody was used as a negative control. YY1 was detected in both AP-2 and YY1 immunoprecipitates (Fig. 4B, lanes 2 and

5), whereas no YY1 was detected in the negative controls (Fig. 4B, lanes 3 and 4). This result clearly shows that endogenous YY1 and AP-2 proteins from BT-474 cells interact *in vivo*.

Down-regulation of YY1 decreases endogenous AP-2 transcriptional activity in breast cancer cells. — In the experiments described above, AP-2 and YY1 were overexpressed in cells containing minimal amounts or no AP-2 factors at all. To further prove functional significance of the cooperation between AP-2 and YY1 factors, we tested the consequence of YY1 down-regulation on p86-HTF-LUC activity in BT-474 mammary cancer cells, expressing high levels of both AP-2 and YY1 proteins (Fig. 1, lane 1) and where interaction between the endogenous proteins was demonstrated (Fig. 4B). The results presented in figure 5 show that the transfection of YY1 expression vector in BT-474 cells induced a 2.4 fold increase in p86-HTF-LUC activity (lane 3). In contrast, transfection of a vector expressing an antisense YY1 mRNA inhibited activity by 75% (Fig. 5, lanes 4 and 5). The as-Gal4 and as-Vim vectors were used as negative controls and did not affect luciferase activity of the reporter vector (Fig. 5, lanes 8 to 11). These results indicate that endogenous YY1 and AP-2 transcription factors cooperate to stimulate *ERBB2* promoter activity. We also transfected the YY1 (1-333) vector which induces an inhibition of transcriptional activity reaching 57% (Fig. 5, lane 7). This result indicates that YY1 (1-333) might compete with the endogenous full-length YY1 for the interaction with AP-2 in BT-474 cells.

YY1 is recruited to ERBB2 promoter when AP-2 is present — The YY1-mediated activation of *ERBB2* requires the binding of AP-2 on the AP-2 binding sites (Fig. 2 and 3). Because endogenous AP-2 and YY1 interact *in vivo* in mammary cancer cells (Fig. 4B), we sought to determine whether a complex containing these molecules exists on the endogenous *ERBB2* gene promoter sequence. Moreover, from results presented above, we assume that YY1 stimulates *ERBB2* promoter activity by interaction with AP-2 and the formation of a multi-protein complex, and not by YY1 binding directly to the *ERBB2* promoter. To test this hypothesis, we sought to analyze the *in vivo* occupancy of the –500bp locus of the *ERBB2* promoter by AP-2 and YY1 in chromatin immunoprecipitation (ChIP) assays using two different cell lines. BT-474 mammary cancer cells and HepG2 hepatocarcinoma cells both contain high levels of YY1 protein. BT-474 contain high levels of AP-2 protein but the HepG2 cells are devoid of AP-2, allowing us to analyze the presence of YY1 on the *ERBB2* promoter in the absence of AP-2. After immunoprecipitation with antibodies against AP-2 or YY1, enrichment of the endogenous promoter fragments in each sample was monitored by PCR amplification using primers specific for several promoters. These primers amplified our locus of interest, that is the –500bp AP-2 site (HTF site) of the *ERBB2* gene, the E-cadherin gene promoter as a positive control for AP-2 binding (39), the glucocorticoid receptor (GR) gene promoter as a positive control for YY1 binding (40), and finally the –5300bp locus of the *ERBB2* promoter as a negative control. As a general positive control, 2% of the input chromatin was amplified by PCR (Fig. 6, input). As a negative control, a reaction lacking precipitating antibody was also performed (Fig. 6, -). ChIP assays and PCR amplifications were performed in duplicate for both cell lines. Quantitative analyses of all results are shown in graphics as fold enrichment compared to the no antibody control. Representative agarose gel images of PCR amplification experiments are shown below the respective graphics (Fig. 6).

As shown in figure 6 A, the –500bp AP-2 site of the *ERBB2* promoter is enriched about 3 fold in the anti-AP-2 and anti-YY1 immunoprecipitates of the BT-474 cells chromatin. Consistently, the phosphorylated RNA polymerase II was recruited to the *ERBB2* promoter (Fig. 6A, Pol). As controls, AP-2 and the RNA polymerase II were shown to occupy the E-cadherin proximal promoter (Fig. 6B, 6 and 10 fold enrichments, respectively), and YY1 was shown to occupy the GR promoter (Fig. 6C, 16 fold enrichment). In contrast, the –5300bp *ERBB2* locus was not enriched neither in the anti-AP-2, the anti-YY1 nor the anti-RNA Polymerase II immunoprecipitates. This confirms the specificity of the AP-2 and YY1 recruitment *in vivo* to the HTF site of the *ERBB2* promoter in BT-474 mammary cancer cells. Next, we performed the same experiment using HepG2 chromatin. In this case, no enrichment can be seen for the –500bp HTF site in the anti-AP-2 immunoprecipitate, consistent with the absence of AP-2 protein in

these cells (Fig. 6E, AP-2). Notably, the –500bp *ERBB2* locus was not enriched in the anti-YY1 immunoprecipitate (Fig. 6E, YY1), in agreement with our hypothesis. Moreover, the important enrichment of the GR promoter fragments, as a positive control for YY1 binding, in the anti-YY1 immunoprecipitate of the same chromatin from these cells validates the results for the –500bp locus of the *ERBB2* promoter (Fig. 6G, YY1, 16 fold enrichment). In summary, results from ChIP assays with BT-474 and HepG2 chromatins show that AP-2 and YY1 are both recruited to the *ERBB2* proximal promoter in BT-474 cells whereas YY1 is not recruited when AP-2 is absent (HepG2 cells, fig. 6E).

DISCUSSION

In this study we show, for the first time, a physical and functional interaction between endogenous AP-2 and YY1 factors in breast cancer cells. YY1 protein was detected in breast, liver and colon carcinoma cells. The interaction between YY1 and AP-2 was proven by co-immunoprecipitation experiments of the endogenous factors from BT-474 breast cancer cells. We also show that YY1 increases the activity of AP-2 on *ERBB2* promoter fragments. Finally, we demonstrate the recruitment of AP-2 and YY1 on the endogenous proximal *ERBB2* promoter *in vivo*. Our results thus describe a new function for YY1, the enhancement of AP-2 transcription factors activity on the *ERBB2* promoter.

YY1 is considered to be an ubiquitous transcription factor (25), although few published data show the levels of this factor in healthy or cancerous cells. We have detected high levels of YY1 by western blotting in all cancer cell lines we have tested (Fig. 1B).

Wu and Lee were unable to demonstrate a function for the interaction between AP-2 and YY1 in their system (31). Here, we present evidence that YY1 stimulates AP-2 transcriptional activity on *ERBB2* promoter. This enhancing effect was observed on promoter fragments bearing AP-2 cis sequences located at increasing distances from the transcription start site, and with three AP-2 factors, namely AP-2α, β and γ.

Our results allow us to assume that the mechanism by which YY1 enhances AP-2 activity on the *ERBB2* promoter is by interaction with AP-2 and the formation of a multi-protein complex, and not by YY1 binding directly to the *ERBB2* promoter.

First, we demonstrate the interaction between endogenous AP-2 and YY1 factors in BT-474 breast cancer cells. From our experiments, it is thus likely that the interaction and activity seen *in vitro* occur in the cellular environment. We point out that most previous experiments showing the interaction between YY1 and other nuclear proteins were performed with purified proteins or extracts prepared from cells transfected with expression vectors. One notable exception, in addition to our work, is the demonstration of the interaction between endogenous YY1 and pRb proteins in smooth muscle cells (42).

Second, YY1 alone does not modulate activity of the *ERBB2* promoter fragments in HepG2 cells (Fig. 2B), nor does it in HCT116 cells containing minimal quantity of endogenous AP-2 protein (Fig. 3B). In contrast, expression of exogenous YY1 in BT-474 cells, containing high amounts of endogenous YY1 and AP-2 proteins, increases activity of the *ERBB2* promoter (Fig. 5). This indicates that the maximal activity of the AP-2/YY1 complex depends on the relative amounts of the proteins. BT-474 cells contain high levels of AP-2 factors and the endogenous YY1 levels free to interact with AP-2 might not be saturating. In support to this hypothesis, YY1 increased activity of exogenous AP-2 factor in HCT116 colon carcinoma cells (Fig. 3C).

Third, we were not able to detect YY1 binding on the longest *ERBB2* promoter fragment we have tested in this study. A computational analysis revealed four YY1 core binding sites (CCAT or ACAT, supplementary data, Table 1) within the 716bp fragment of the *ERBB2* promoter, fitting partially consensus sequences (27;28). We performed EMSA experiments with BT-474 nuclear extracts on a YY1 consensus probe and oligonucleotides containing the *ERBB2* promoter sites as competitors (Supplementary data, Table 1). Only the –644bp site showed weak competitor activity (data not shown). This site is present only in the p716 reporter construct while the effect of YY1 alone and on AP-2 activity was the same for all three AP-2 site-containing constructs. Moreover, the *in vitro* translated YY1 protein does not bind to the HTF oligonucleotide (12) (data not

shown), nor to an oligonucleotide comprising the 86 first bases of the *ERBB2* promoter (data not shown). It was documented that YY1 DNA-binding activity is regulated by phosphorylation (25) and O-GlcNAcylation (43). We thus tested the binding ability of YY1 from our nuclear extracts. We showed that endogenous YY1 protein from breast and liver cancer cells binds efficiently to a consensus YY1 probe in EMSA and supershift experiments (Supplementary data C).

Finally, the chromatin immunoprecipitation results show that YY1 is recruited *in vivo* to the –500bp locus of the endogenous *ERBB2* promoter only when AP-2 is present. Indeed, YY1 is recruited to this *ERBB2* site along with the AP-2 protein and the phosphorylated RNA Polymerase II in the BT-474 cells which express high levels of AP-2, YY1 and ERBB2 proteins. In contrast, YY1 is not recruited to the *ERBB2* promoter in the HepG2 cells which do not express the AP-2 gene.

We also tested a truncated form of YY1, YY1 (1-333), which binds AP-2 (Fig. 4A) (31), but is not able to bind DNA (30). YY1 (1-333) was unable to enhance AP-2 transcriptional activity in co-transfection experiments in HCT116 cells. Moreover, transfection of YY1(1-333) in BT-474 cells decreased endogenous AP-2 activity, indicating a competitor effect for this protein. These observations are not incompatible with our hypothesis that YY1 stimulates AP-2 activity through protein-protein interaction. Indeed, although the domain of YY1 interacting with AP-2 was not mapped precisely, it is interesting to note that it was localized to the amino-terminal half (31), while the majority of the other interacting factors do so through the carboxy moiety of the protein (26). Notably, some proteins such as Sp1 and MYC, were shown to interact with YY1 through the domain missing in YY1(1-333) (26). YY1 might thus act as a bridge between AP-2 and those proteins interacting with the carboxy moiety of YY1. Moreover, YY1 transcriptional activity independent of DNA binding has been described previously. Bushmeyer & Atchison described the maintenance of YY1 activity even when all the YY1 binding sites were suppressed or when the YY1 DNA binding domain was mutated (34). Raval-Pandya et al. found that YY1 repressed the vitamin D induced expression of the 25-hydroxyvitamin D_3 24-hydroxylase gene by interacting with CBP in the absence of DNA binding (44).

In conclusion, YY1 cooperates with AP-2 to stimulate *ERBB2* gene expression in breast cancer cells, probably as a link between AP-2 and other proteins, yet to be identified, required for interaction with the basal transcription machinery.

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REFERENCES

1. Yarden, Y., and Sliwkowski, M. X. (2001) *Nat.Rev.Mol.Cell Biol.* **2,** 127-137
2. Klapper, L. N., Kirschbaum, M. H., Sela, M., and Yarden, Y. (2000) *Adv.Cancer Res.* **77,** 25-79
3. Neve, R. M., Lane, H. A., and Hynes, N. E. (2001) *Ann.Oncol.* **12 Suppl 1,** S9-13
4. Eccles, S. A. (2001) *J.Mammary.Gland.Biol.Neoplasia.* **6,** 393-406
5. Menard, S., Casalini, P., Campiglio, M., Pupa, S., Agresti, R., and Tagliabue, E. (2001) *Ann.Oncol.* **12 Suppl 1,** S15-S19
6. Hollywood, D. P., and Hurst, H. C. (1993) *EMBO J.* **12,** 2369-2375
7. Pasleau, F., Grooteclaes, M., and Gol-Winkler, R. (1993) *Oncogene* **8,** 849-854
8. Benz, C. C., O'Hagan, R. C., Richter, B., Scott, G. K., Chang, C. H., Xiong, X., Chew, K., Ljung, B. M., Edgerton, S., Thor, A., and Hassell, J. A. (1997) *Oncogene* **15,** 1513-1525
9. Neve, R. M., Ylstra, B., Chang, C. H., Albertson, D. G., and Benz, C. C. (2002) *Oncogene* **21,** 3934-3938
10. Bosher, J. M., Williams, T., and Hurst, H. C. (1995) *Proc.Natl.Acad.Sci.U.S.A* **92,** 744-747
11. Grooteclaes, M., Vernimmen, D., Plaza, S., Pasleau, F., Hodzic, D., and Winkler-Gol, R. (1999) *Cancer Res.* **59,** 2527-2531
12. Vernimmen, D., Begon, D., Salvador, C., Gofflot, S., Grooteclaes, M., and Winkler, R. (2003) *Biochem.J.* **370,** 323-329

13. 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) *Cancer Res.* **58,** 5466-5472
14. Bosher, J. M., Totty, N. F., Hsuan, J. J., Williams, T., and Hurst, H. C. (1996) *Oncogene* **13,** 1701-1707
15. Cheng, C., Ying, K., Xu, M., Zhao, W., Zhou, Z., Huang, Y., Wang, W., Xu, J., Zeng, L., Xie, Y., and Mao, Y. (2002) *Int.J.Biochem.Cell Biol.* **34,** 78-86
16. Tummala, R., Romano, R. A., Fuchs, E., and Sinha, S. (2003) *Gene* **321,** 93-102
17. Hilger-Eversheim, K., Moser, M., Schorle, H., and Buettner, R. (2000) *Gene* **260,** 1-12
18. Bar-Eli, M. (2001) *Pigment Cell Res.* **14,** 78-85
19. Kannan, P., Buettner, R., Chiao, P. J., Yim, S. O., Sarkiss, M., and Tainsky, M. A. (1994) *Genes Dev.* **8,** 1258-1269
20. Kannan, P., Yu, Y., Wankhade, S., and Tainsky, M. A. (1999) *Nucleic Acids Res.* **27,** 866-874
21. Kannan, P., and Tainsky, M. A. (1999) *Mol.Cell Biol.* **19,** 899-908
22. Bamforth, S. D., Braganca, J., Eloranta, J. J., Murdoch, J. N., Marques, F. I., Kranc, K. R., Farza, H., Henderson, D. J., Hurst, H. C., and Bhattacharya, S. (2001) *Nat.Genet.* **29,** 469-474
23. Braganca, J., Swingler, T., Marques, F. I., Jones, T., Eloranta, J. J., Hurst, H. C., Shioda, T., and Bhattacharya, S. (2002) *J.Biol.Chem.* **277,** 8559-8565
24. Braganca, J., Eloranta, J. J., Bamforth, S. D., Ibbitt, J. C., Hurst, H. C., and Bhattacharya, S. (2003) *J.Biol.Chem.* **278,** 16021-16029
25. Shi, Y., Lee, J. S., and Galvin, K. M. (1997) *Biochim.Biophys.Acta* **1332,** F49-F66
26. Thomas, M. J., and Seto, E. (1999) *Gene* **236,** 197-208
27. Hyde-DeRuyscher, R. P., Jennings, E., and Shenk, T. (1995) *Nucleic Acids Res.* **23,** 4457-4465
28. Yant, S. R., Zhu, W., Millinoff, D., Slightom, J. L., Goodman, M., and Gumucio, D. L. (1995) *Nucleic Acids Res.* **23,** 4353-4362
29. Shrivastava, A., and Calame, K. (1994) *Nucleic Acids Res.* **22,** 5151-5155
30. Austen, M., Luscher, B., and Luscher-Firzlaff, J. M. (1997) *J.Biol.Chem.* **272,** 1709-1717
31. Wu, F., and Lee, A. S. (2001) *J.Biol.Chem.* **276,** 28-34
32. Gaubatz, S., Imhof, A., Dosch, R., Werner, O., Mitchell, P., Buettner, R., and Eilers, M. (1995) *EMBO J.* **14,** 1508-1519
33. Lee, T. C., Zhang, Y., and Schwartz, R. J. (1994) *Oncogene* **9,** 1047-1052
34. Bushmeyer, S. M., and Atchison, M. L. (1998) *J.Cell Biochem.* **68,** 484-499
35. Riquet, F. B., Tan, L., Choy, B. K., Osaki, M., Karsenty, G., Osborne, T. F., Auron, P. E., and Goldring, M. B. (2001) *J.Biol.Chem.* **276,** 38665-38672
36. Gilles, C., Polette, M., Zahm, J. M., Tournier, J. M., Volders, L., Foidart, J. M., and Birembaut, P. (1999) *J.Cell Sci.* **112 (Pt 24),** 4615-4625
37. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
38. Jackers, P., Szalai, G., Moussa, O., and Watson, D. K. (2004) *J.Biol.Chem.* **279,** 52183-52190
39. Decary, S., Decesse, J. T., Ogryzko, V., Reed, J. C., Naguibneva, I., Harel-Bellan, A., and Cremisi, C. E. (2002) *Mol.Cell Biol.* **22,** 7877-7888
40. Kim, J., Kollhoff, A., Bergmann, A., and Stubbs, L. (2003) *Hum.Mol.Genet.* **12,** 233-245
41. Breslin, M. B., and Vedeckis, W. V. (1998) *J.Steroid Biochem.Mol.Biol.* **67,** 369-381
42. Petkova, V., Romanowski, M. J., Sulijoadikusumo, I., Rohne, D., Kang, P., Shenk, T., and Usheva, A. (2001) *J.Biol.Chem.* **276,** 7932-7936
43. Hiromura, M., Choi, C. H., Sabourin, N. A., Jones, H., Bachvarov, D., and Usheva, A. (2003) *J.Biol.Chem.* **278,** 14046-14052
44. Raval-Pandya, M., Dhawan, P., Barletta, F., and Christakos, S. (2001) *Mol.Endocrinol.* **15,** 1035-1046

FOOTNOTES

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[1] The abbreviations used are : AP-2, Activator Protein 2; ChIP, chromatin immunoprecipitation; CITED, CREB-binding protein/p300 interacting transactivator with ED-rich tail; EMSA, electrophoretic mobility-shift assay; EGFR, Epidermal Growth Factor Receptor; HTF, HER2 Transcription Factor; IB, immunoblot; LUC, luciferase; PARP, Poly ADP ribosyl polymerase; PC4, positive cofactor 4; S.D., standard deviation; YY1, Yin Yang 1.

FIGURES LEGENDS

Fig. 1 : AP-2α and YY1 proteins are expressed at different levels in diverse cell lines. Expression levels of AP-2α and YY1 proteins were estimated by western blotting using 40µg of whole cell extracts from breast and liver cancer cell lines as shown. Immunoblots were performed with anti-AP-2α (3B5)(A) and anti-YY1(H-10)(B) antibodies, respectively. The 50 kDa AP-2 protein and ± 68kDa YY1 protein are shown.

Fig. 2 : YY1 cooperates with AP-2α on the *ERBB2* promoter in HepG2 cells. (A) : *ERBB2* proximal promoter and reporter constructs; black diamond, CAAT box; black triangle, TATA box. (B) to (D) : HepG2 cells were transiently transfected with 1µg of p86 or p86-HTF luciferase reporter vectors and the indicated amounts of AP-2α and YY1 expression vectors. Transcriptional activity is expressed as fold induction compared to the level obtained with each reporter vector in the absence of expression vectors. The experiments were repeated three times in triplicate. Data are means ± S.D. of one typical experiment. Bottom, IB : immunoblots for nuclear AP-2 and YY1 proteins from transfected cells.

Fig. 3 : YY1 cooperates with AP-2α on the *ERBB2* promoter in HCT116 cells. HCT116 cells were transiently transfected with 1µg of p86 or p86-HTF luciferase reporter vectors and the indicated amounts of AP-2α, YY1 wt or YY1(1-333) expression vectors. Transcriptional activity is expressed as fold induction compared to the level obtained with each reporter vector in the absence of expression vectors. The experiments were repeated three times in triplicate. Data are means ± S.D. of one typical experiment. Bottom, IB : immunoblots for nuclear AP-2 and YY1 proteins from transfected cells.

Fig. 4 : *In vivo* co-immunoprecipitation of AP-2 with YY1 in HCT116 and BT-474 cells. (A) : YY1(1-333) and AP-2 proteins interaction. HCT116 cells were transfected with AP-2α vector (0.25µg) and either YY1 wt (0.5µg) or YY1 (1-333) (0.5µg) vectors as indicated. Protein extracts were immunoprecipitated (IP) with anti-AP-2α (C-18)(αAP-2) or control rabbit IgG antibodies as indicated. Proteins were then resolved by SDS-PAGE and immunoblotted using an anti-YY1 antibody (H-10). In lanes 4 and 8, no antibody was added. YY1 wt and YY1(1-333) proteins are indicated by arrows. The input lanes represent 7.5% of the input proteins. (B) : Interaction between endogenous YY1 and AP-2 proteins in BT-474 mammary cancer cells. Nuclear protein extracts were immunoprecipitated (IP) with anti-AP-2α (C-18), anti-Shh or anti-YY1 (H-10) antibodies as indicated. Proteins were then resolved by SDS-PAGE and immunoblotted using an anti-YY1 antibody (H-10). In lane 4, no antibody was added. YY1 and IgG proteins are indicated by arrows. The input lanes represent 10% of the input proteins.

Fig. 5 : Antisense YY1 expression vector inhibits AP-2 transcriptional activity in BT-474 mammary cancer cell line. BT-474 cells were transfected with 1µg of p86-HTF luciferase reporter vector and the indicated amounts of YY1, antisense YY1 (as-YY1), YY1(1-333), antisense Gal4 (as-Gal4) or antisense vimentin (as-Vim) expression vectors. Transcriptional activity is expressed as fold induction compared to the level obtained with each reporter vector in the absence of expression vectors. The experiments were repeated twice in triplicate. Data are means ± S.D. of one typical experiment.

Fig. 6 : YY1 is recruited to *ERBB2* promoter when AP-2 is present. ChIP assays and PCR amplifications were performed in duplicate for two cell lines, BT-474 (A-D) and HepG2 (E-H). PCR was performed on chromatin fragments enriched by immunoprecipitation with or without the indicated antibodies (AP-2 (C-18), YY1 (H-414) or Pol (CTD4H8)). "Input" represents 2% total cross-linked, reversed chromatin before immunoprecipitation. Primers specific for the promoter regions of the genes indicated below each agarose gel were used in PCR. PCR amplification signals were quantified by densitometric scanning and analysis with the MultiAnalyst software. Results are presented in graphics as means ± S.D. of fold enrichment in each immunoprecipitation determined as ratio between immunoprecipitated DNA and no antibody control DNA (-). A representative agarose gel of PCR amplifications experiments is shown below respective graphic.

Supplementary data A and B : YY1 cooperates with AP-2 factors on the HTF and AP-2 sites within the *ERBB2* promoter. (A) : HepG2 cells were transiently transfected with 1µg of p86 or p86-HTF luciferase reporter vectors and the indicated amounts of AP-2β or γ and YY1 expression vectors. (B) : *ERBB2* proximal promoter and reporter constructs; black diamond, CAAT box; black triangle, TATA box. HepG2 cells were transiently transfected with 1µg of indicated luciferase reporter vectors and the indicated amounts of AP-2α and YY1 expression vectors. (A) and (B) : Transcriptional activity is expressed as fold induction compared to the level obtained with each reporter vector in the absence of expression vectors. The experiments were repeated three times in triplicate. Data are means ± S.D. of one typical experiment.

Supplementary data C and Table 1 : YY1 DNA-binding activity. EMSA (lanes 2-4, 6-8 and 11-16) and supershift with anti-YY1 antibody (α-YY1, lanes 5 and 9) of nuclear protein extracts (NE) from various cell lines using YY1 WT probe (see Table 1). The different competitors (Comp) were used at a 100-fold molar excess. The arrow indicates the specific YY1-DNA complex.

EXPERIMENTAL PROCEDURES for supplementary data :

Plasmids and constructs — The RSV-AP-2β and RSV-AP-2γ plasmids (14) were provided by Dr H.C. Hurst. The p716 (p756), and p716 mut (p756 double mutant (HTF(AA/T)+ AP-2 (AA/T))) plasmids were described by Vernimmen et al (12). The p278 plasmid was constructed by inserting a 278bp PCR fragment located between –243bp and +35bp of the *ERBB2* promoter and bearing *Bgl*II and *Hind*III ends, in the *Bgl*II / *Hind*III sites of the pGL3-basic reporter vector (Promega).

Electrophoretic mobility-shift assay (EMSA) — The sequences of the YY1 consensus WT and mutant oligonucleotides, as well as those of the oligonucleotides containing the putative YY1 binding sites from the *ERBB2* promoter are presented in Table 1. EMSA experiments were performed as described previously (12). For supershift experiments, 4µl of anti-YY1 antibody were incubated with nuclear extracts overnight, prior to addition of the probe.

Figure 1

breast liver

BT-474 ZR-75.1 MDA-MB-453 T47D SK-BR-3 MCF-7 MDA-MB-231 HepG2

A IB AP-2

B IB YY1

1 2 3 4 5 6 7 8

Figure 2

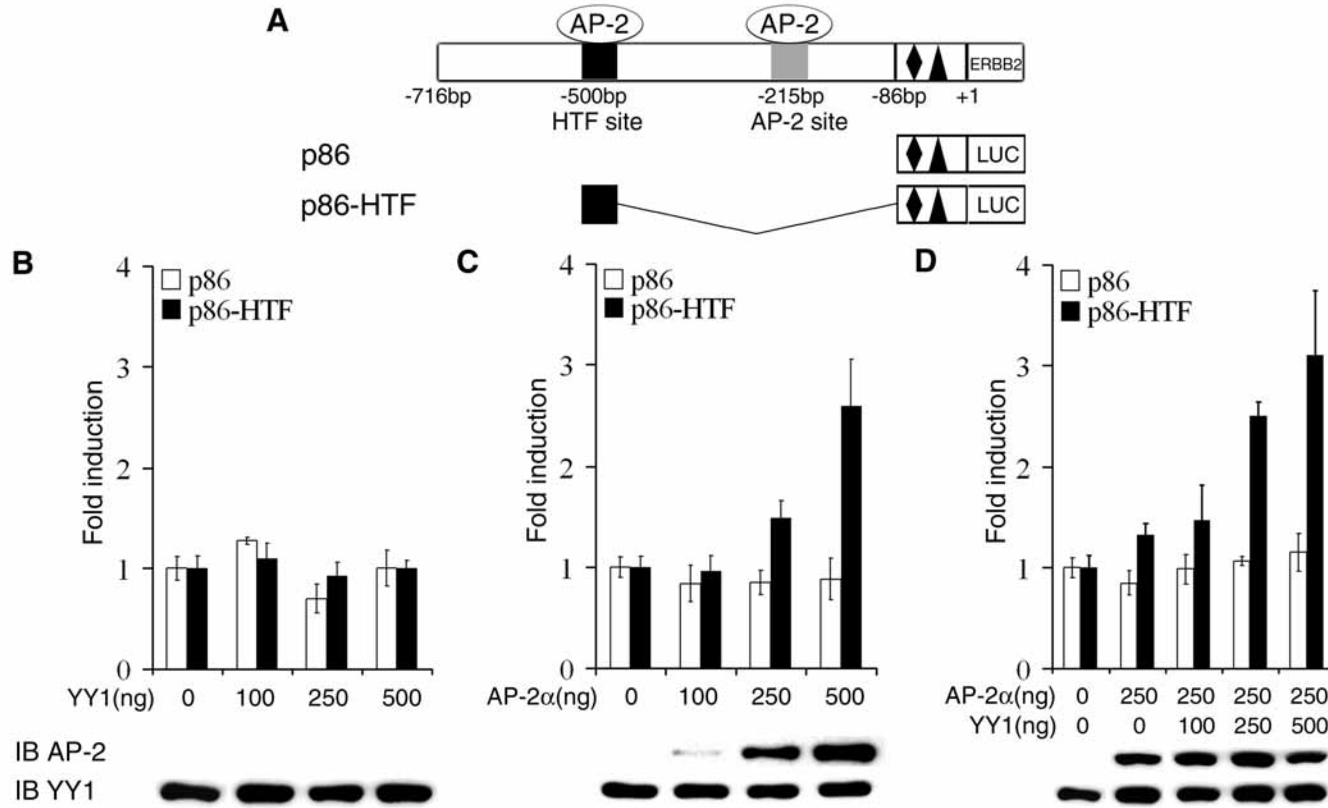


Figure 3

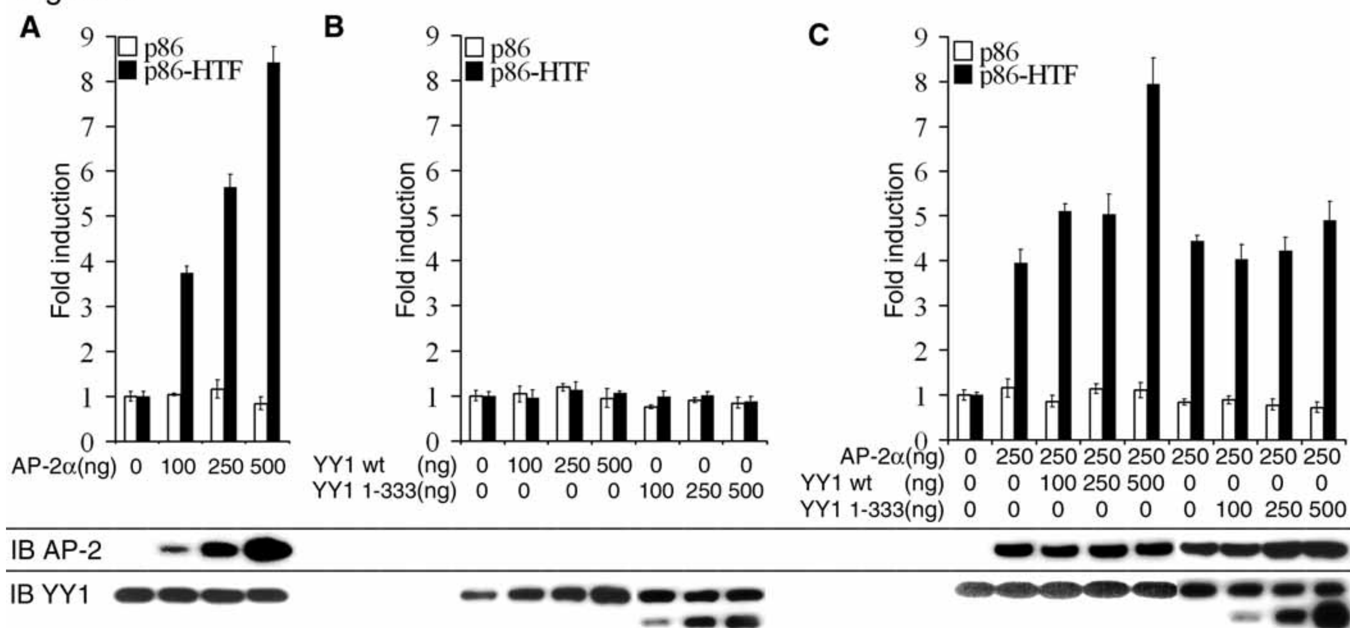
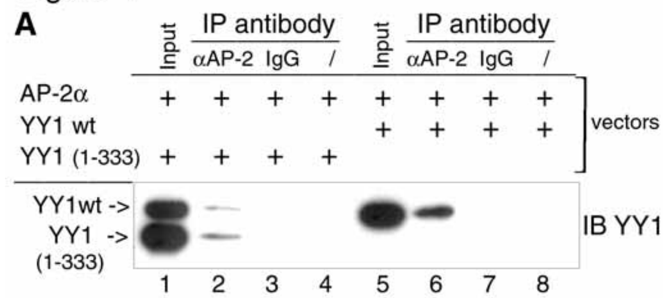


Figure 4



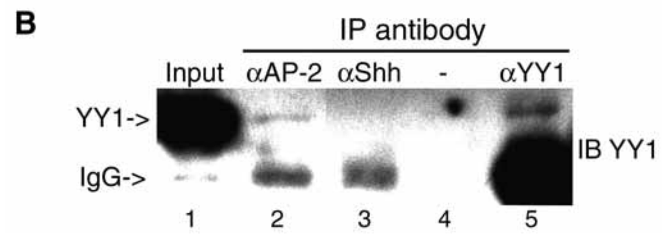


Figure 5

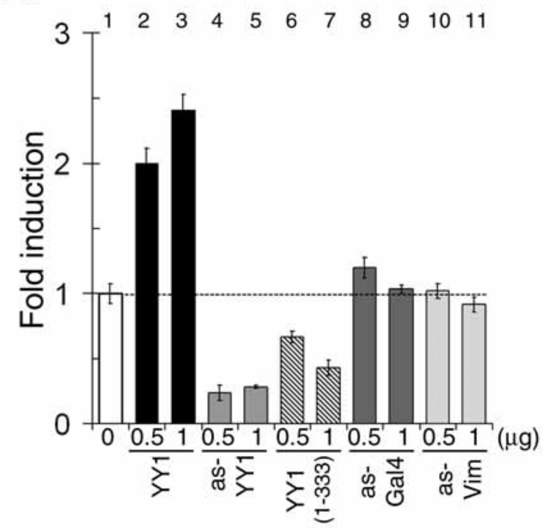
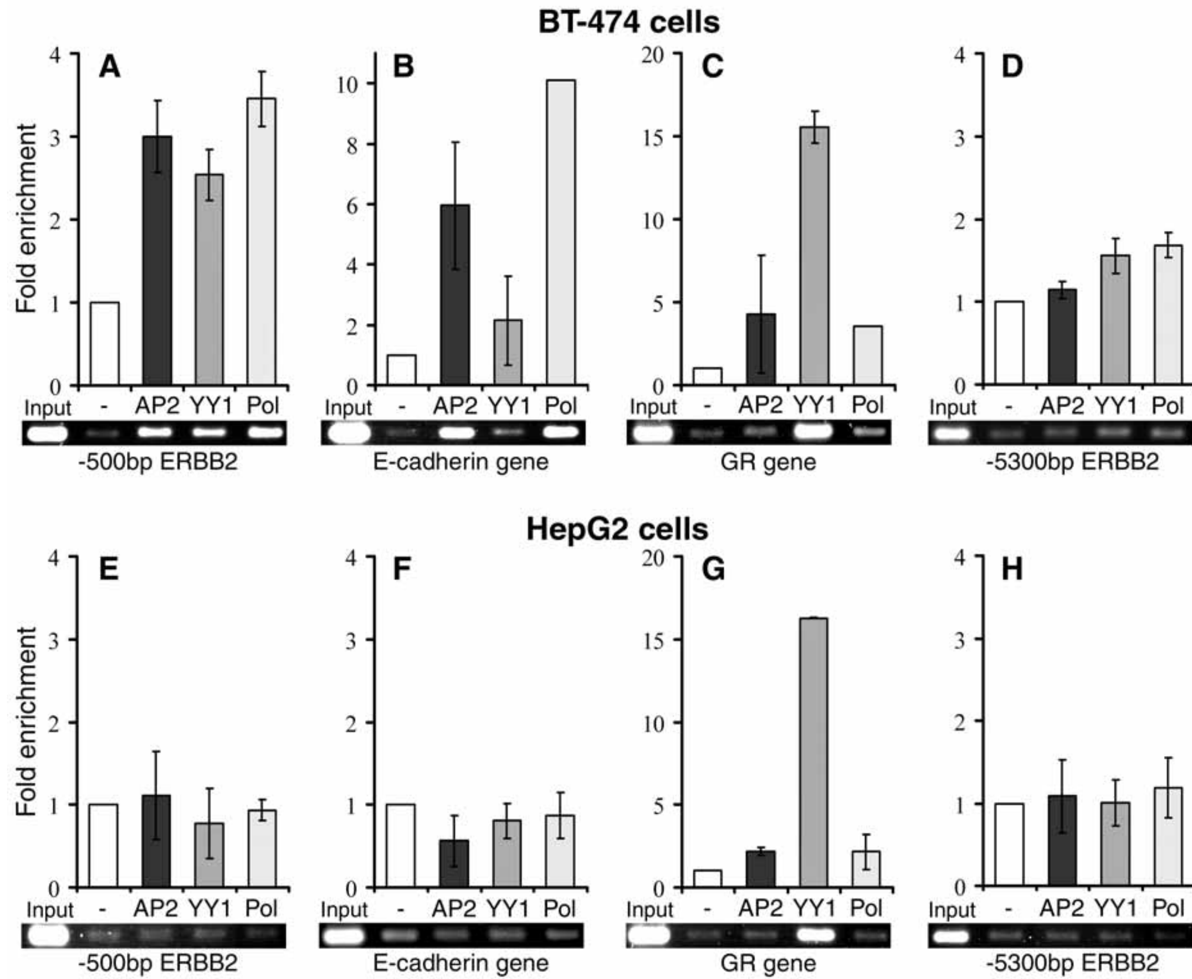
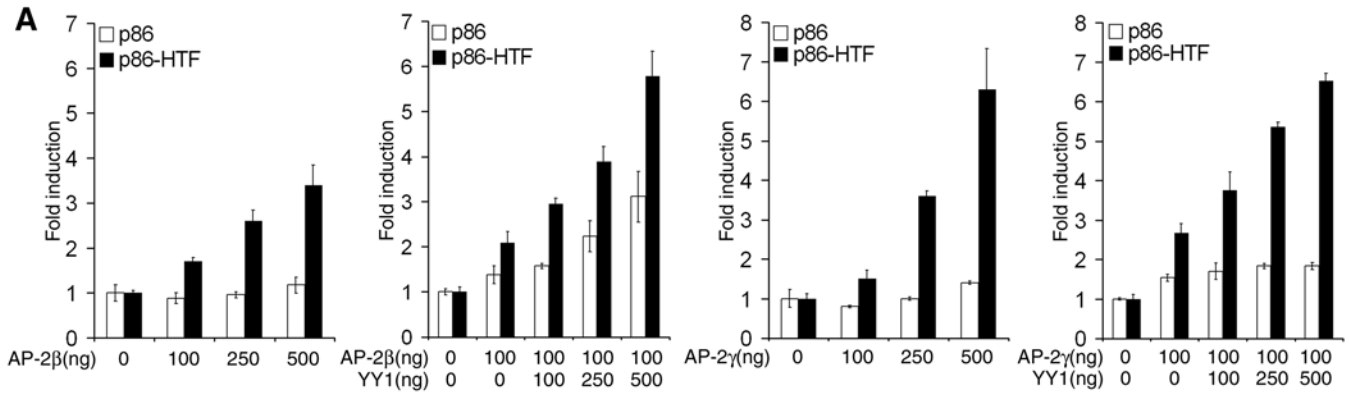
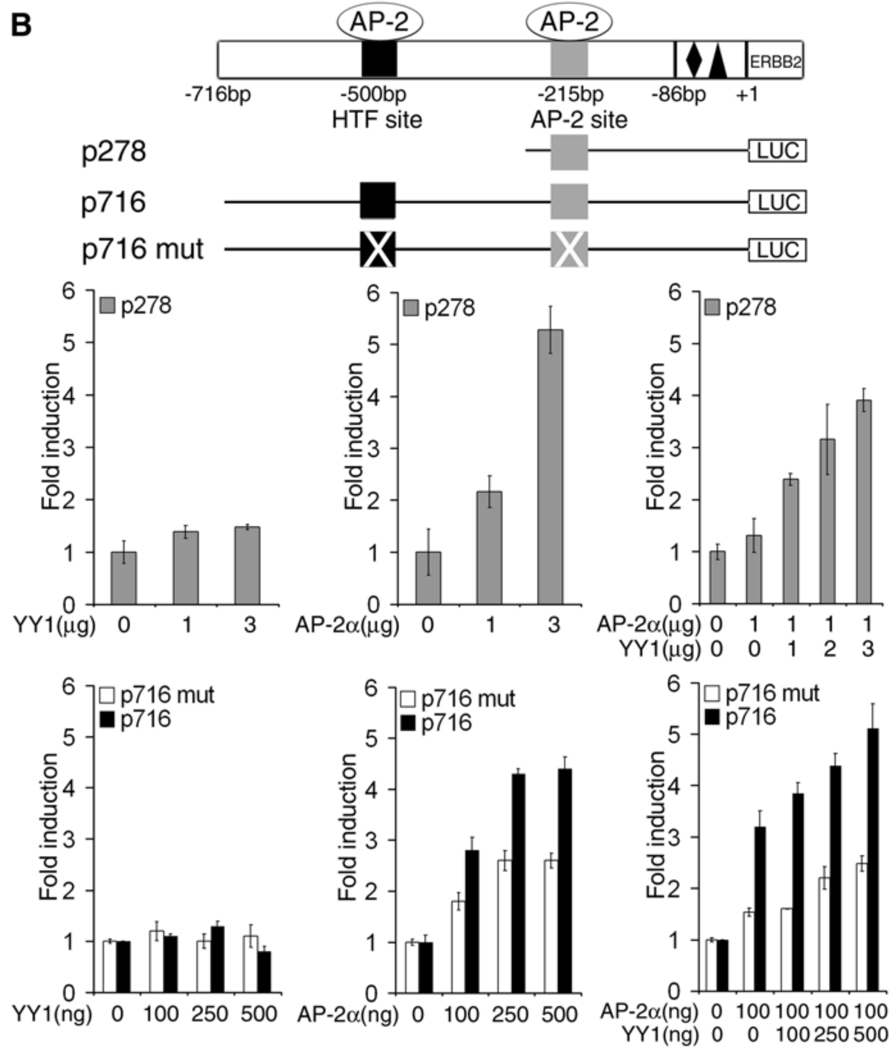


Figure 6



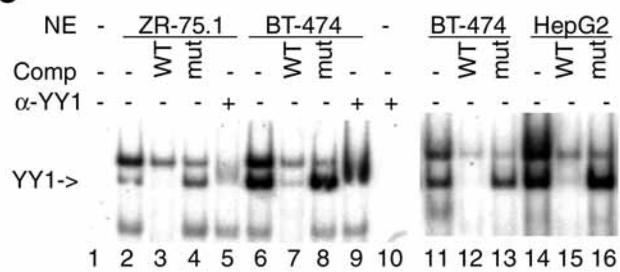
SUPPLEMENTARY DATA





SUPPLEMENTARY DATA

C



SUPPLEMENTARY DATA

Table 1. Oligonucleotide sequences.

Name	Sequence (5' -> 3')
YY1 WT	CGCTCCGCGG**CCAT**CTTGGCGGCTGGT GCGAGGCGCC**GGTA**GAACCGCCGACCAG
YY1 mut	CGCTCCGCG*ATT***AT**CTTGGCGGCTGGT GCGAGGCGC*TAA***TA**GAACCGCCGACCAG
YY1 +9	GCTGAGATTCCCCT**CCAT**TGGGACCGGAG GACTCTAAGGGGA**GGTA**ACCCTGGCCTC
YY1 -415	GTGGTGGGAAAA**CCAT**TATTTGATATTAA ACCACCCTTTT**GGTA**ATAAACTATAATT
YY1 -464	GGTAAACACAAC**ACAT**CCCCCTCCTTGA ATTTGTGTTG**TGTA**GGGGGAGGAACTG
YY1 -644	AGCAGAGATCGCG**CCAT**TGCTCTCCAG TCGTCTCTAGCGC**GGTA**ACGAGAGGTCGG

The YY1 core binding sites are indicated in bold. The mutated nucleotides are in italics. The numbers refer to position of the first base of the core YY1 binding site in the *ERBB2* promoter.