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AP2α and AP2γ: a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs

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ABSTRACT

The AP2 transcription factors exhibit a high degree of homology in the DNA binding and dimerization domains. In this study, we methodically compared the binding specificity of AP2α and AP2γ using PCR-assisted binding site selection and competitive gel shift assay and determined that the consensus binding site for both factors is $^{G}/_{C}CCNN^{A/}C_{/G}{}^{G}/_{A}G^{G/}C_{/T}$. The use of single site promoter constructs with either a high or low affinity site demonstrated a direct relationship between site affinity and transcriptional activation. Overexpression of AP2α and AP2γ resulted in the activation of a low affinity binding site construct to levels comparable to those seen with a high affinity site construct at lower amounts of protein expression. Both AP2α and AP2γ were able to trans-activate the cloned human estrogen receptor alpha promoter in ER-negative MDA-MB-231 cells through high affinity AP2 sites in the untranslated leader sequence. This provides a functional mechanism to explain the correlation between AP2 activity and estrogen receptor expression in breast cancer. Since there is overexpression of AP2 factors in breast cancer compared to normal breast epithelium, our results suggest that increased factor expression may activate a set of target genes containing lower affinity binding sites that would normally not be expressed in normal breast epithelium.

INTRODUCTION

Transcription factors play an important role in gene regulation by interacting with specific DNA sequences present in the promoter region of genes. Often transcription factors are members of larger families that exhibit structural similarities but are expressed in tissue-specific or spatial patterns or recognize different DNA binding sequences. AP2γ is a recently described member of the AP2 transcription factor family which also includes AP2α and AP2β (1–4). AP2α, first purified from HeLa cells, is a retinoic acid inducible transcription factor (2) that is expressed in neural crest cell lineages during mammalian embryogenesis and is involved in the morphogenesis of the peripheral nervous system, face, eyes, limbs, skin and nephric tissues (5–7). Functional AP2 binding sites have been identified in many viral and cellular enhancer elements including the SV40 (8,9), human T-cell leukemia virus-1 (10) and mouse mammary tumor virus (11) and the metallothionein II_A (8,12,13), proenkephalin (14), keratin K14 (15) and murine histocompatibility complex H-$2k^b$ genes (13). The number of genes identified to contain functional AP2 binding sites continues to increase. The AP2 family of proteins may also be involved in the development and progression of breast and endometrial cancers as they have been implicated in the transcriptional control of c-*erb*B-2 (16,17), E-cadherin (18,19), gelatinase A (20), heat shock protein 27 (21), insulin growth factor-1 receptor (22) and estrogen receptor (ER) (23) expression.

Patients with tumors that express ER alpha (herein referred to as ER) have an improved survival and longer disease-free interval than patients with tumors lacking ER expression (24,25). Functional mapping of the ER promoter identified a region of DNA between +210 and +135 (relative to the P1 cap site) that results in activation of the ER promoter in ER-positive breast carcinoma cells (23). Competitive gel shift analysis identified two binding sites within this region that bound a DNA-binding protein called estrogen receptor factor-1 (ERF-1). ERF-1 is present in ER-positive breast and endometrial carcinoma cells but absent in ER-negative cells. ERF-1 was purified from ER-positive MCF7 cells by ion-exchange and DNA-affinity chromatography and was identified by protein sequencing and subsequent cloning of the cDNA to be identical to AP2γ (1). Henceforth this nomenclature will be adopted.

AP2γ is highly homologous to AP2α, having an overall identity of 65% and a similarity of 83%. Homology is most striking within the C-terminal half of the proteins which contains the DNA binding and dimerization domains. Within the C-terminal half there is 76% identity and 90% similarity between these two proteins. Due to this high degree of homology within the DNA binding domains, it would be predicted that both AP2α and AP2γ would have similar or identical binding specificity. As determined by methylation interference assay and missing contact probing assays, the consensus site for AP2α is $GCCN_3GGC$ (26), although a number of sites that are specifically footprinted by AP2α have been shown to differ from this consensus sequence (13). AP2 proteins bind DNA as a dimer which forms through a helix–span–helix domain and the binding of this dimer to DNA is dependent on an adjacent region of net basic charge (27). Although its physiological

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relevance is unknown, gel shift assay has shown that *in vitro* synthesized AP2α, AP2β and AP2γ can bind indistinguishably as homo- or heterodimers to probes corresponding to the AP2α binding sites within the c-*erb*B-2 and human metallothionein II_A promoters (4,17). In addition, all three AP2 proteins are able to activate a reporter construct containing an artificial promoter with three tandem copies of a high affinity AP2α binding site as well as a reporter construct containing the native c-*erb*B-2 promoter between –300 and +40. However, AP2β demonstrates lower transcriptional activation compared to AP2α and AP2γ (17).

The identification of a new AP2 family member raises several interesting questions. Since AP2α and γ transcription factors play a central role in hormone responsive breast cancer, it is important to determine if these two factors have distinct or identical function. Binding site sequence specificity might be predicted to be similar based on homology between the two proteins. Although previous studies demonstrated that AP2α and AP2γ were able to bind the consensus DNA sequence, a rigorous study comparing the binding site specificity of each of these proteins has not been done. In addition, it has not been directly shown whether one or both of these proteins is able to activate the ER promoter through the proximal and distal AP2 binding sites within the untranslated leader sequence. Therefore, in this study we set out to characterize the binding specificity of AP2α and AP2γ and to examine the ability of these two proteins to activate AP2 responsive promoters in a sequence-specific manner.

MATERIALS AND METHODS

Cell lines

All cell lines were obtained from American Type Culture Collection, Rockville, MD. Cells were maintained as previously described (23).

AP2γ/pGEX-4T-1 plasmid construction and protein expression

To generate AP2γ protein for PCR-assisted binding site selection, the AP2γ cDNA was cloned into PGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ) to create a fusion protein with glutathione *S*-transferase (GST). Using a previously described AP2γ clone retrieved from a MCF7 expression library (1) as template, AP2γ cDNA from the translation start site at +167 to the stop site at +1519 was PCR amplified using a 5′ primer (GCCGCTCGAGATGTTGTGGAAAATAACCGA) and a 3′ primer (TCGTTCTCGAGTTATTTCCTGTGTTCTCC), each of which contained an *Xho*I site. After digestion with *Xho*I, this fragment was ligated into the *Xho*I site of pGEX-4T-1 to generate AP2γ/pGEX-4T-1. AP2γ–GST fusion protein was induced and purified using Glutathione Sepharose 4B according to the protocol provided with the GST Gene Fusion System (Pharmacia Biotech). For use as a negative control, GST protein was induced from pGEX-4T-1 and purified in the same manner.

PCR-assisted binding-site selection

The technique of PCR-assisted binding-site selection was used to determine the target specificity of AP2α and AP2γ. Random-sequence oligonucleotides to be used as a pool of potential binding sites were generated as described (28). Briefly, a 70 bp oligonucleotide containing a 20 bp random core (APbindSITE: CATTGATCTATTACAAGTCATACAGN_{20}GAATCATGTTATTCAACAGTAAGAC; Operon Technologies, Alameda, CA) was annealed to a 25 bp oligonucleotide (APbindREV: GTCTTACTGTTGAATAACATGATTC; Operon Technologies) complimentary to the 3′ end. Oligonucleotides were labeled with [α-^{32}P]dCTP (800 Ci/mmol; Amersham Life Science Inc., Arlington Heights, IL) using *Taq* DNA polymerase (Perkin-Elmer) in a single cycle as follows: 1 min at 94°C; 3 min at 62°C; and 9 min at 72°C. The extension reaction was chased with additional cold dCTP by incubating 9 min at 72°C. The double-stranded probe was purified on 8% non-denaturing PAGE, excised from the gel and eluted in elution buffer (0.5 M NH_4OAc, 1 mM EDTA and 0.1% SDS) for 2 h at 48°C. Eluate was removed to a fresh tube containing 20 µg glycogen and DNA was recovered by ethanol precipitation. The DNA pellet was resuspended in 10 µl dH_2O and 0.5 µl was counted in a scintillation counter to calculate recovery.

Twenty nanograms of GST protein, AP2γ–GST fusion protein, or affinity-purified AP2α (Promega, Madison, WI) were incubated with 0.4 ng of labeled oligonucleotide pool and 1 µg AP2 antibody (184X; Santa Cruz Biochem, Santa Cruz, CA) in 1× binding buffer [40 mM KCl, 20 mM HEPES pH 7.9, 1 mM $MgCl_2$, 0.08 mM EGTA, 0.4 mM DTT, 4% Ficoll, 12 µg/ml poly dI-dC (Pharmacia Biotech) and 1 mg/ml BSA (Sigma Chemical Company, St Louis, MO)]. After the first round, binding reactions contained only 0.2 ng of labeled oligonucleotide pool. Reactions were incubated at room temperature for 30 min to allow protein–DNA complexes to form. Complexes were immunoprecipitated with protein A agarose (Gibco BRL, Gaithersburg, MD) at 4°C overnight. Unbound oligonucleotides were removed by gently washing three times with 1× GSB buffer (40 mM KCl, 20 mM HEPES pH 7.9, 1 mM $MgCl_2$, 0.08 mM EGTA and 0.4 mM DTT). Bound oligonucleotides were incubated in 200 µl recovery buffer (50 mM Tris–HCl pH 8, 100 mM NaOAc, 5 mM EDTA, 0.5% SDS) for 1 h at 45°C, then extracted once each with phenol and chloroform. Specific binding site oligonucleotides were recovered by ethanol precipitation in the presence of 20 µg glycogen and one-fifth volume of 3 M sodium acetate. The pellet was resuspended in 5 µl of dH_2O and 0.5 µl was counted in a scintillation counter to calculate recovery.

One picogram of selected DNA was amplified by PCR in a reaction with 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3), 1 mM $MgCl_2$, 0.08 mM dGTP, 0.08 mM dATP, 0.08 mM dTTP, 4 µM dCTP, 0.01 mCi [α-^{32}P]dCTP (800 Ci/mmol), 160 ng each APbindFWD primer (CATTGATCTATTACAAGTCATACAG; Operon Technologies) and APbindRev primer, and 2.5 U *Taq* DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). Reactions were subjected to 18 PCR cycles as follows: 94°C, l min; 50°C, 1 min; and 72°C, 1 min. DNA was gel purified and recovered as described above. Amplified DNA (0.2 ng) was used as input DNA for the next round of DNA–protein binding. This cycle of DNA binding and amplification was performed a total of four times.

To further refine the AP2 binding site, a second series of binding reactions were performed using a 57mer oligonucleotide (APsite-1: CATTGATCTATTACAAGTCATACASCCNNNGGSAATCATGTTATTCAACAGTAAGAC; S = G/C)

as described above, except only two binding/amplification cycles were performed.

Selected binding site oligonucleotides obtained after the binding/amplification cycles were completed were ligated into pCR™II using the Original TA Cloning kit (Invitrogen Corp., San Diego, CA). Transformants were selected and plasmid DNA was prepared using the Wizard Plus Miniprep DNA Purification System (Promega). Plasmid DNA was sequenced using the T7 Sequenase v2.0 DNA Sequencing Kit (Amersham Life Science).

Competitive gel shift assay

Competitive gel shift assays were performed to determine the relative binding affinity of a series of mutations in the optimal binding site for AP2α and AP2γ. Twenty-seven double-stranded oligonucleotides were synthesized (Operon Technologies) with all possible point mutations in each position of the 9 bp optimal binding site, GCCTGAGGG. The mutant oligonucleotides were designed as 57mers to contain the identical flanking sequence used throughout the PCR-assisted binding-site selection (i.e. Mut1A = CATTGATCTATTACAAGTCATACA**A**CCTGAGGGAATCATGTTATTCAACAGTAAGAC; binding site in bold, point mutation in bold underlined). The 57mer containing the optimal binding site (OPT: GCCTGAGGG) was labeled as described for PCR-assisted binding-site selection and 0.2 ng was used as probe in each reaction. Competitive gel shift assays were performed as previously described (23) except that reactions were performed in DNA excess with protein amount limited (0.2 µl AP2α; 0.5 µl AP2γ/GST fusion). For each point mutation, competitor was added in increasing amounts from 10- to 5000-fold excess over labeled probe.

Images were obtained by standard autoradiography and by use of a Molecular Dynamics (Sunnyvale, CA) phoshorimager. Quantitative values for binding were obtained from the phosphorimager using ImageQuant software (Molecular Dynamics). The fold excess of competitor at which a 50% decrease in binding occurred was determined using linear regression analysis. Values were corrected by dividing the value at which a 50% decrease in binding was obtained by the value at which a 50% decrease in binding occurred when OPT was competed with itself. This corrected value is referred to as relative binding affinity.

Construction of AP2α/pcDNA3.1(+) and AP2γ/pcDNA3.1(+) expression plasmids

For use in transfection of cells, AP2α and AP2γ were cloned into identical vector backgrounds. To construct AP2α/pcDNA3.1(+), SP(RSV)AP2 (gift from Trevor Williams, New Haven, CT) was digested with *Kpn*I and *Xho*I to release a fragment containing the AP2α sequence from the translation start site at +62 through the 3′ untranslated region. This fragment was subcloned into pcDNA3.1(+) mammalian expression vector (Invitrogen) digested with *Kpn*I/*Xho*I. AP2γ/pcDNA3.1(+) was constructed by subcloning the *Xho*I fragment of AP2γ/pGEX-4T-1 into the *Xho*I site of pcDNA3.1(+). This *Xho*I fragment contains the AP2γ cDNA from the start site at +167 to the stop site at +1519. Plasmid DNA was prepared using the QIAGEN Plasmid Maxi Kit (Qiagen Inc., Valencia, CA).

Construction of OPT/IL-2, M3T/IL-2, ER3500-230LUC and ER3500-230p1d1 reporter plasmids

To determine the *in vivo* ability of AP2α and AP2γ to activate an optimal binding site versus a mutated binding site, two luciferase reporter constructs were made. Two 59 bp double-stranded, 5′-phosphorylated oligonucleotides with overhanging *Xho*I sites were synthesized (Operon Technologies) to contain either the optimal AP2 binding site (OPT: TCGAGATTGATCTATTACAAGTCATACA**GCCTGAGGG**AATCATGTTATTCAACAGTAAC; binding site in bold) or a mutated binding site (M3T: TCGAGATTGATCTATTACAAGTCATACA**GC**<u>**T**</u>**TGAGGG**AATCATGTTATTCAACAGTAAC; binding site in bold, point mutation in bold underlined). These oligonucleotides were ligated into *Xho*I-digested IL2-GL3 vector to create OPT/IL-2 and M3T/IL-2. IL2-GL3 (gift from Gerald Crabtree, Stanford, CA) is a luciferase reporter plasmid containing the IL2 minimal promoter. DNA was prepared using the QIAGEN Plasmid Maxi Kit and the insert was sequenced as described above to verify sequence and direction of insertion.

ER3500-230LUC and ER3500-230p1d1 reporter constructs were used to test the ability of AP2α and AP2γ to activate a native promoter *in vivo* and were prepared as described previously (23). Both ER3500-230LUC and ER3500-230p1d1 contain the native ER promoter from 3500 bp upstream of the P1 cap site to the translation start site except that ER3500-230p1d1 has inactivating mutations in both the proximal and distal AP2 binding sites.

Transfection

COS-1 cells were plated at 720 000 cells/60 mm diameter culture dish 1 day prior to transfection and were ≈80% confluent at the time of transfection. Cells were transfected with a total of 6 µg DNA using LIPOFECTAMINE™ Reagent (Gibco BRL). Each transfection contained 1 µg pβgal-Control vector (Clontech Laboratories, Palo Alto, CA) to measure transfection efficiency, 2 µg reporter plasmid (IL2-GL3, OPT/IL-2 or M3T/IL-2), 0–3 µg expression plasmid [pcDNA3.1(+), AP2α/pcDNA3.1(+) or AP2γ/pcDNA3.1(+)] and 0–3 µg salmon sperm DNA (Gibco BRL). All transfections were performed in triplicate. Cells were harvested 24 h post-transfection.

MDA-MB-231 cells were transfected using FuGENE™ 6 Transfection Reagent (Boehringer Mannheim Corp., IN). Cells were plated at 625 000 cells/60 mm diameter culture dish and were ≈80% confluent at the time of transfection. Each plate was transfected with 18 µl FuGENE™ 6 Transfection Reagent and a total of 5 µg DNA comprised of 1 µg pβgal-Control vector, 2 µg reporter plasmid (pGL2-Basic, ER3500-230LUC or ER3500-230p1d1) and 2 µg expression plasmid [pcDNA3.1(+), AP2α/pcDNA3.1(+) or AP2γ/pcDNA3.1(+)]. All transfections were performed in triplicate and cells were harvested 48 h post-transfection.

Whole cell extracts for gel shift assay or western blot were prepared and gel shift assays were performed as previously described (23). Cell extracts for luciferase assay or β-galactosidase assay were prepared using the Luciferase Assay System With Reporter Lysis Buffer (Promega). Luciferase assays were performed with 20 µl of cell extract and 100 µl of luciferase assay buffer. Luciferase activity was measured for 10 s in a Monolight® 2010 luminometer (Analytical Luminescence

Laboratory, San Diego, CA). β-Galactosidase activity was assayed in cell extracts using the β-Galactosidase Assay Kit (Stratagene, La Jolla, CA).

Western blot

To demonstrate AP2α and AP2γ expression in transfected cells, western blot analysis was performed. Whole cell extract (7.5 μg/lane) was resolved on 8% SDS–PAGE, blotted to PVDF membrane and reacted with 0.1 μg/ml AP2 antibody using the ECL Western Blotting Analysis System (Amersham Life Science) as described.

Data analysis

Luciferase data for AP2α and AP2γ activation of IL2-pGL3, OPT/IL2, M3T/IL2, ER3500-230LUC and ER3500-230p1d1 were corrected for transfection efficiency by dividing the relative luciferase units by β-Gal units for each transfection. Background activity of the reporter constructs in the presence of pcDNA3.1(+) vector was subtracted from the β-Gal corrected luciferase units to yield corrected luciferase units. Each transfection was performed in triplicate and data were analyzed for significance using ANOVA. Significance for two-group comparisons was tested using a Bonferroni *t*-test (29). Data are expressed as mean ± SD.

RESULTS

PCR-assisted binding site selection

PCR-assisted binding site selection using a 70 bp double-stranded DNA probe containing a 20 bp random core was used to determine the binding site specificity for AP2α and AP2γ. Sequences of selected clones were searched for a 9 bp binding site sequence beginning with GCC in positions 1, 2 and 3 ($GCCN_6$) as described previously for the AP2α consensus site (GCCNNNGGC) (26). Many clones contained more than one potential binding site including sites arranged as tandem arrays, partial overlap and palindromic sequences. All sequences beginning with GCC were included in the analysis. Of 112 selected clones sequenced for AP2α, 48 clones contained one site and 59 clones contained two or more binding sites beginning with GCC for a total of 185 potential binding sequences. Five clones contained no binding site sequence fitting these criteria. Of 100 clones sequenced for AP2γ, 48 clones contained a single site and 44 clones contained two or more binding sites for a total of 146 potential binding sequences. Eight clones contained no recognizable binding site. Preferred nucleotides at each position were determined using a chi-square goodness of fit test ($P < 0.05$). These results identified the consensus binding site for AP2α as $GCCC^{G/}C_{/T}{}^{G/}A_{/C}GG^{G}/_{C}$ (Table 1) and that for AP2γ as $GCC^{C/}T_{/G}{}^{G}/_{C}{}^{G/}A_{/C}GG^{G}/_{C}$ (Table 2) indicating that there may be some differences in binding site specificity in positions 4 and 5.

These data were also analyzed in several other ways including searching for a 9 bp binding site sequence containing a C in positions 2 and 3 ($NCCN_6$) and by identifying clones which contained only a single binding site sequence beginning with GCC. The first of these two alternative methods created a bias towards a G or a C in the internal positions due to long G and C stretches found in many of the clones. Analyzing only those clones that contained single binding sites dramatically reduced the number of sequences included in the analysis but only created subtle differences in the AP2α consensus site and no changes in the AP2γ consensus site (data not shown). Therefore, although the analysis method used included many

Table 1. Frequency of nucleotide occurrence by position in AP2α binding sites obtained by PCR-assisted binding site selection

Position	Nucleotide				Preferred nucleotide[a]
	G	A	T	C	
1	185	0	0	0	G
2	0	0	0	185	C
3	0	0	0	185	C
4	46	22	46	71	C
5	61	19	48	57	G/C/T
6	67	55	19	44	G/A/C
7	91	53	11	30	G
8	137	19	13	16	G
9	79	9	19	78	G/C

PCR-assisted binding site selection was performed using a probe containing a 20 bp random core as described in Materials and Methods to isolate 112 AP2α binding site clones. Sequences were analyzed for 9 bp binding sequences beginning with GCC according to the previously reported AP2α consensus site. Five clones contained no recognizable binding sequences while the remaining 107 clones contained one or more binding sequences for a total of 185 binding sequences reported above.

[a]Preferred nucleotide was determined using the chi-square goodness of fit test. $P < 0.05$.

Table 2. Frequency of nucleotide occurrence by position in AP2g binding sites obtained by PCR-assisted binding site selection

Position	Nucleotide				Preferred nucleotide[a]
	G	A	T	C	
1	146	0	0	0	G
2	0	0	0	146	C
3	0	0	0	146	C
4	31	4	46	65	C/T/G
5	58	14	28	46	G/C
6	57	46	8	35	G/A/C
7	93	28	12	13	G
8	116	12	9	9	G
9	63	11	16	56	G/C

PCR-assisted binding site selection was performed using a probe containing a 20 bp random core as described in Materials and Methods to isolate 100 AP2γ binding site clones. Sequences were analyzed for 9 bp binding sequences beginning with GCC according to the previously reported AP2α consensus site. Eight clones contained no recognizable binding sequences while the remaining 92 clones contained one or more binding sequences for a total of 146 binding sequences reported above.

[a]Preferred nucleotide was determined using the chi-square goodness of fit test. $P < 0.05$.

clones containing multiple potential binding sites, we felt that this method yielded the most useful data.

A second round of PCR-assisted binding site selection using the 57 bp APsite-1 probe containing the internal binding site SCCNNNGGS (S = G/C) was used to refine the consensus binding site. This second round was designed to further analyze the AP2α and AP2γ binding site preference at positions 4, 5, 6 and 9 which had shown the most variability in the previous round of binding site selection and to investigate the sequence preference of position 1 which had been held constant in the first round of analysis. In this round of selection, 27 AP2α and 25 AP2γ clones were isolated, sequenced and analyzed. The preferred nucleotide at each position was determined using the chi-square goodness of fit test ($P < 0.05$). These data refined the consensus binding site for both AP2α and AP2γ to be GCCNGNGG$^{G}/_{C}$ (Tables 3 and 4). From this analysis, a 57 bp probe (OPT) was designed to contain an optimized binding site sequence (GCCTGAGGG) which corresponds to the nucleotide that was most prevalent at each position in the set of AP2γ clones. This optimal binding site also corresponds to the nucleotide frequency observed in the set of AP2α clones except at position 4 in which C had a slightly higher but not statistically different frequency than T.

Table 3. Frequency of nucleotide occurrence by position in AP2α binding site clones selected using APsite-1 probe

Position	Nucleotide				Preferred nucleotide[a]
	G	A	T	C	
1	19	0	0	8	G
2	0	0	0	27	C
3	0	0	0	27	C
4	8	1	8	10	N
5	18	1	2	6	G
6	6	9	6	6	N
7	27	0	0	0	G
8	27	0	0	0	G
9	11	0	0	16	G/C

PCR-assisted binding site selection was repeated using the APsite-1 probe (SCCNNNGGS; S = G/C) as described in Materials and Methods to refine positions 1, 4, 5, 6 and 9 of the AP2α binding site.
[a]Preferred nucleotide was determined using the chi-square goodness of fit test. $P < 0.05$.

Competitive gel shift assay

To further examine the consensus binding site for AP2α and AP2γ at each position, competitive gel shift assays were performed using a series of 27 competitors containing all possible single point mutations in the optimal binding site. Relative binding affinity was determined by dividing the amount of competitor needed to reduce DNA binding by 50% by the amount of unlabeled OPT needed to obtain 50% competition against labeled OPT probe. Competitors with relative binding affinities of five or lower were considered to bind AP2α and AP2γ with equal affinity as OPT while competitors with higher relative binding affinities represent binding sites with only moderate or low affinity. This demonstrated that there were no significant differences in binding site specificity between AP2α and AP2γ (Figs 1 and 2) with the consensus site being $^{G}/_{C}$CCNN$^{A}/_{G}C_{/G}{}^{G}/_{A}G^{G}/_{T}C_{/T}$. There was a subtle difference observed in competitive gel shift analysis between AP2α and AP2γ at position 4. For AP2α, a T, C or G in position 4 has the same relative binding affinity whereas an A has ~5-fold lower relative binding affinity. For AP2γ, an A, C or G causes a 4–5-fold decrease in affinity compared to T, indicating that for AP2γ there may be a slight preference for T in position 4 that is not seen in AP2α binding.

Table 4. Frequency of nucleotide occurrence by position in AP2γ binding site clones selected using APsite-1 probe

Position	Nucleotide				Preferred nucleotide[a]
	G	A	T	C	
1	21	0	0	4	G
2	0	0	0	25	C
3	0	0	0	25	C
4	5	2	12	6	N
5	14	4	3	4	G
6	7	10	2	6	N
7	25	0	0	0	G
8	25	0	0	0	G
9	13	0	0	12	G/C

PCR-assisted binding site selection was repeated using the APsite-1 probe (SCCNNNGGS; S = G/C) as described in Materials and Methods to refine positions 1, 4, 5, 6 and 9 of the AP2γ binding site.
[a]Preferred nucleotide was determined using the chi-square goodness of fit test. $P < 0.05$.

Transcriptional activation of a single binding site by AP2α and AP2γ

It is important to determine if the DNA binding studies described above accurately reflect transcriptional activation directed by the site. We also wished to compare trans-activation for low and high affinity sites for these two transcription factors. For these studies, luciferase reporter plasmids were constructed using the IL-2 minimal promoter into which was cloned high or low affinity AP2 sites. COS-1 cells were cotransfected with AP2α or AP2γ and luciferase reporter constructs containing a single copy of either the optimal binding sequence (OPT/IL-2: GCCTGAGGG) or a mutated binding sequence (M3T/IL-2: GC**T**TGAGGG) with 20–40-fold lower affinity as determined by gel shift competition. Western blot analysis and gel shift assay confirmed that expression and DNA binding activity of AP2α and AP2γ increased as COS-1 cells were transfected with increasing amounts of input expression plasmid (Fig. 3A and B; Fig. 4A and B). The complex noted on gel shift corresponding to AP2 was also super-shifted with antibody to AP2 (data not shown). Gel shift analysis with these extracts was also tested using probes containing either an OPT or M3T sequence (data not shown). These results confirmed that functional AP2 activity increased with higher protein expression. Analysis of luciferase expression demonstrated

that both AP2α and AP2γ activated the construct containing the optimal binding site (OPT/IL-2) to a significantly higher level than the construct containing the mutated binding site (M3T/IL-2) over a range of protein expression (Figs 3C and 4C). For AP2α, activation of OPT/IL-2 was significantly higher than M3T/IL-2 when transfections were performed with 0.1, 0.5, 1 or 2 μg AP2α expression plasmid ($P < 0.01$). There was no statistically significant difference in activation at 0, 0.2 or 3 μg of expression plasmid, although the trend was lower at 0.2 and 3 μg. For AP2γ, activation of OPT/IL-2 was significantly higher than M3T/IL-2 when transfections were performed with AP2γ expression plasmid ≥0.2 μg ($P < 0.001$). Activation of both OPT/IL-2 and M3T/IL-2 was significantly higher ($P < 0.01$) than the baseline activation of a reporter construct containing only the IL-2 minimal promoter (IL-2) for all amounts of AP2α and AP2γ inputs >0 μg except for 1 μg for AP2α (M3T/IL-2 versus IL-2, $P > 0.05$). Overall, activation of OPT/IL-2 and M3T/IL-2 by AP2γ was 2.75 ± 0.81 (SD) and 3.8 ± 1.9 (SD) fold higher, respectively, than activation by AP2α.

Trans-activation of the ER promoter by AP2α and AP2γ

After demonstrating activation of an artificial promoter containing a single copy of a high affinity binding site, we were interested to see if AP2α and AP2γ could also activate a complex native promoter such is found in the ER gene. For these experiments, the ER-negative breast carcinoma cell line MDA-MB-231 was used since we have previously demonstrated that these cells have undetectable AP2 activity and do not demonstrate activation through the AP2 sites in the ER promoter (23). The native ER gene is methylated in these cells and, as expected, the native gene could not be activated by AP2 expression (data not shown). Instead, co-transfection with the cloned ER promoter was used in these experiments. MDA-MB-231 cells were co-transfected with pcDNA3.1(+) vector, AP2α or AP2γ expression plasmids and a luciferase reporter construct containing either no promoter (pGL2-Basic), the ER promoter between –3500 and +230 (ER3500-230LUC) or the ER promoter containing a double point mutation in both the proximal and distal AP2 binding sites (ER3500-230p1d1). Gel shift assay demonstrated that transfection of MDA-MB-231 cells with pcDNA3.1(+) vector resulted in no DNA binding as expected while transfection of either AP2α or AP2γ expression plasmids resulted in AP2 binding activity (Fig. 5A). Analysis of luciferase expression demonstrated that both proteins were able to trans-activate the native ER promoter to significantly higher levels than the promoterless construct, pGL2-Basic ($P < 0.001$) (Fig. 5B). The proximal mutation p1 and distal mutation d1 destroy binding of AP2 to each of these sites (23). Introduction of these point mutations in the proximal and distal AP2 binding sites (ER3500-230p1d1) resulted in a >5-fold ($P < 0.001$) decrease in the ability of AP2α and AP2γ to activate the ER promoter construct. For AP2α, activation of ER3500-230p1d1 was not significantly different than baseline activation of pGL2-Basic. Although it appears that AP2γ activated the ER promoter construct 1.8× higher than AP2α, quantitation of gel shift activity by phosphorimaging showed that DNA binding of AP2γ was also 1.8× higher than for AP2α, thus accounting for any differences seen. These results indicate that both AP2α and AP2γ are able to trans-activate the human ER promoter.

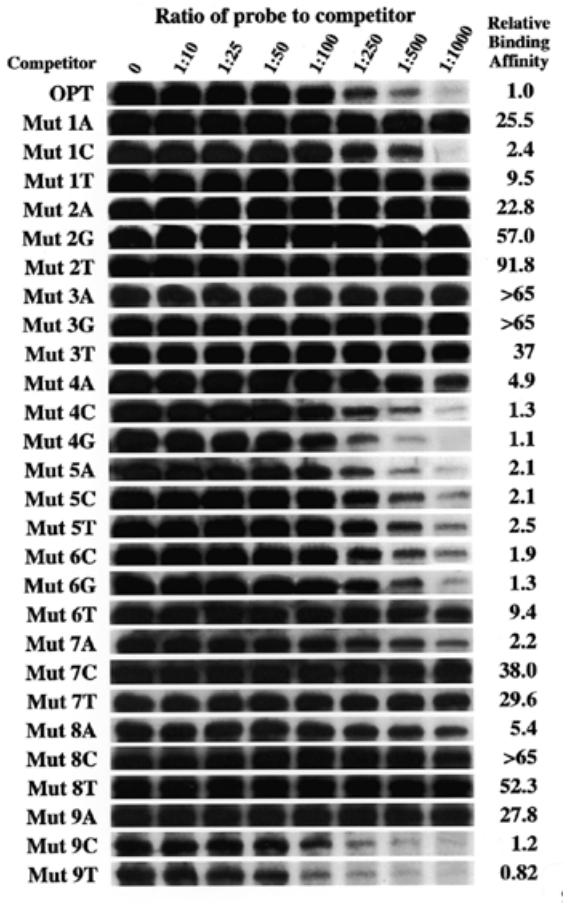

Figure 1. Comparison of relative binding affinities of single point mutants by competitive gel shift analysis for AP2α. Gel shifts were performed with a limiting amount of AP2α and between 0 and 5000-fold molar excess of competitor to labeled OPT probe (data shown out to 1000-fold molar excess). Probe and competitors were 57 bp double-stranded oligos containing either an optimal binding site (GCCTGAGGG) or a binding site containing a single point mutation. Each position of the binding site was mutated sequentially to all other possible nucleotides. Relative binding affinity was calculated by dividing the molar excess at which 50% competition was achieved by the molar excess of unlabeled OPT needed to compete labeled OPT by 50%.

DISCUSSION

The AP2 family of transcription factors has an important role in the regulation of genes during ectodermal development (6,7). The role of these proteins in the regulation of genes in breast cancer is also becoming evident (1,17,22,23). In this study we found that AP2α and AP2γ have identical binding specificity. Our results also demonstrate a greater diversity in the AP2 binding site than had been reported. It was previously determined that the consensus sequence for AP2 binding was GCCNNNGGC (26). However, our results demonstrate that high affinity AP2 sites may also contain a C in position 1, an A in position 7 and a G or T in position 9. A low affinity site generated by mutating the third nucleotide to a T reduces binding affinity by 20–40-fold. However, this site was able to direct trans-activation which approximated that of an optimal

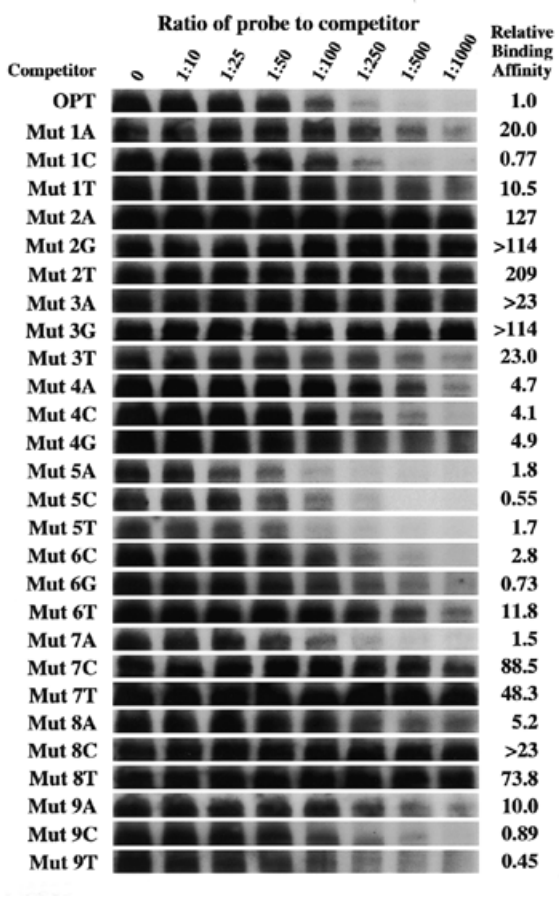

Figure 2. Comparison of relative binding affinities of single point mutants by competitive gel shift analysis for AP2γ. Gel shifts were performed with a limiting amount of AP2γ and between 0 and 5000-fold molar excess of competitor to labeled OPT probe (data shown out to 1000-fold molar excess). Probe and competitors were 57 bp double-stranded oligos containing either an optimal binding site (GCCTGAGGG) or a binding site containing a single point mutation. Each position of the binding site was mutated sequentially to all other possible nucleotides. Relative binding affinity was calculated by dividing the molar excess at which 50% competition was achieved by the molar excess of unlabeled OPT needed to compete labeled OPT by 50%.

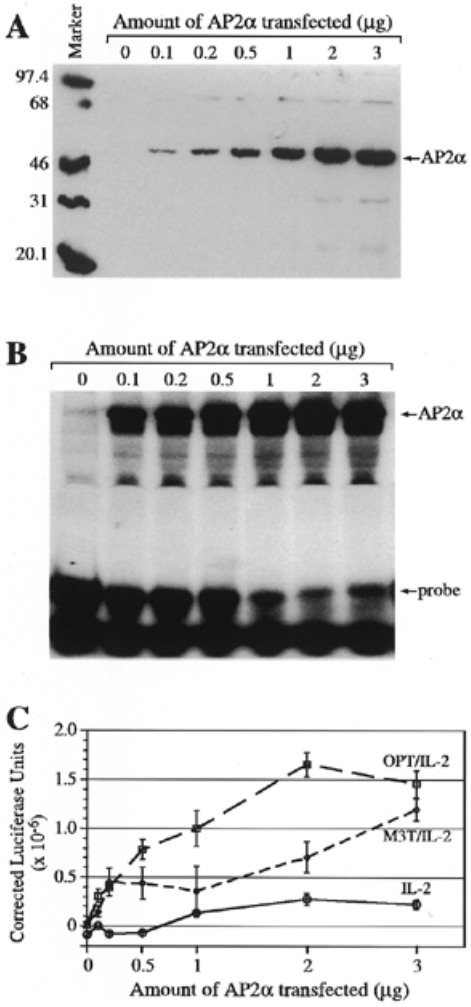

Figure 3. Effect of increasing amounts of input AP2α DNA in COS-1 cell transfections. COS-1 cells were cotransfected with 1 μg pβgal-Control vector, 2 μg of a luciferase reporter construct containing either the minimal IL-2 promoter (IL-2), a single high affinity binding site (OPT/IL-2) or a mutated low affinity binding site (M3T/IL-2) and increasing amounts of AP2α expression plasmid from 0 to 3 μg. (**A**) Western blots with whole cell extracts detected using AP2 antibody. There is an increase of AP2α protein expression with increasing amounts of input AP2α expression plasmid. (**B**) Gel shift assay with whole cell extract and probe corresponding to the distal AP2 binding site of ER. There is noted to be an increase of AP2α binding activity with increasing amounts of input AP2α expression plasmid. (**C**) Luciferase activity in cell lysates from transfected cells. There is an AP2-specific increase in luciferase activity with increasing AP2α expression. Values expressed as corrected luciferase units were corrected for transfection efficiency and background activity of reporter constructs. Values are means ± SD for three transfections.

site at lower protein levels. This finding suggests that overexpression of AP2 in tumors such as breast cancer may result in activation of target genes with low affinity binding sites. In addition, it was found that both AP2α and AP2γ are able to trans-activate the human ER promoter in breast cancer cells. Trans-activation by these factors appears to function through high affinity binding sites previously identified in the untranslated leader of the ER gene. This result provides a mechanism to explain the association between ER expression and AP2 activity in breast and endometrial cancer cell lines (23) and primary breast cancers (22).

The binding specificity of AP2α and AP2γ was characterized using two different methods, PCR-assisted binding site selection and competitive gel shift assay. PCR-assisted binding site selection has been used successfully by several groups to identify DNA target sites for a variety of proteins (30–32). In comparing the consensus sites derived from the second round of PCR-assisted binding site selection and competitive gel shift assay, the two methods yielded nearly identical results with only minor differences. These differences were most likely due to the fact that PCR-assisted binding site selection is done in

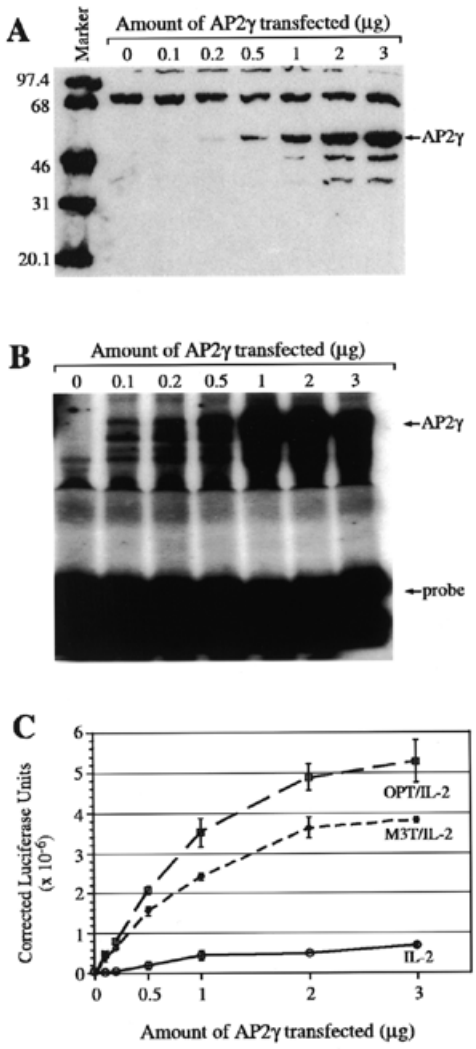

Figure 4. Effect of increasing amounts of input AP2γ DNA in COS-1 cell transfections. COS-1 cells were cotransfected with 1 μg pβgal-Control vector, 2 μg of a luciferase reporter construct containing either the minimal IL-2 promoter (IL-2), a single high affinity binding site (OPT/IL-2) or a mutated low affinity binding site (M3T/IL-2) and increasing amounts of AP2γ expression plasmid from 0 to 3 μg. (**A**) Western blots with whole cell extracts detected using AP2 antibody. There is an increase of AP2γ protein expression with increasing amounts of input AP2γ expression plasmid. (**B**) Gel shift assay with whole cell extract and probe corresponding to the distal AP2 binding site of ER. There is noted to be an increase of AP2γ binding activity with increasing amounts of input AP2γ expression plasmid. (**C**) Luciferase activity in cell lysates from transfected cells. There is an AP2-specific increase in luciferase activity with increasing AP2γ expression. Values expressed as corrected luciferase units were corrected for transfection efficiency and background activity of reporter constructs. Values are means ± SD for three transfections.

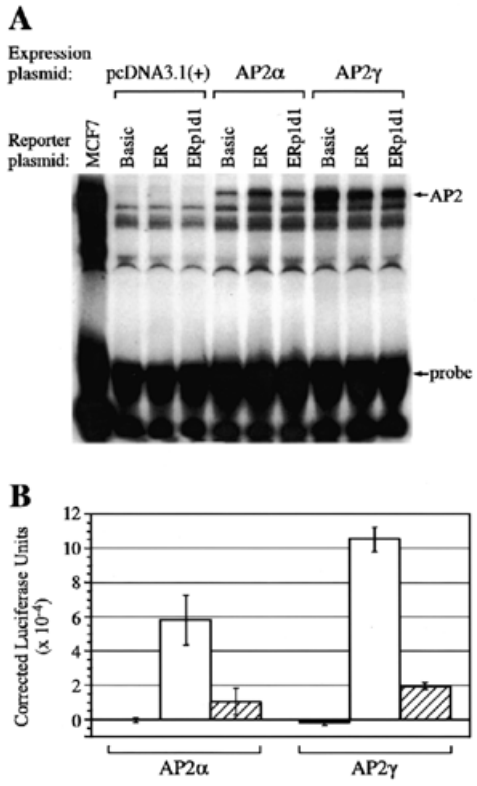

Figure 5. AP2α and AP2γ activate the ER promoter in a sequence-specific manner. MDA-MB-231 cells were cotransfected with 1 μg pβgal-Control vector, 2 μg expression plasmid [pcDNA3.1(+) vector, AP2α/pcDNA3.1(+) or AP2γ/pcDNA3.1(+)] and 2 μg of a luciferase reporter construct containing either no promoter (Basic), the ER promoter between –3500 and +230 bp (ER) or the same ER promoter containing double point mutations in the proximal and distal AP2 binding sites (ERp1d1). The ER promoter AP2 sites are: distal site, CCCTGCGGG; proximal site, GCCCGAGGT; mutation d1, CAATGCGGG; and mutation p1, GCCCGATTT. (**A**) AP2 binding activity in transfected cells. Gel shift assays were performed with probe corresponding to the distal AP2 binding site of ER and nuclear extract from MCF7 cells as a positive control (lane 1) or whole cell extracts from transfected MDA-MB-231 cells [lanes 2, 5 and 8 transfected with pGL2-Basic (Basic); lanes 3, 6 and 9 transfected with ER3500-230LUC (ER); and lanes 4, 7 and 10 transfected with ER3500-230p1d1 (ERp1d1)]. (**B**) Luciferase activity in transfected MDA-MB-231 cells. Luciferase activity was measured in cell lysates from transfected cells. Values expressed as corrected luciferase units were corrected for transfection efficiency and background activity of reporter constructs transfected with pcDNA3.1(+). Values are means ± SD for three transfections. Filled bar, pGL2-Basic; unfilled bar, ER3500-230LUC; hatched bar, ER3500-230p1d1.

the presence of excess protein while competitive gel shift assay is performed in the presence of excess DNA. Therefore, in early cycles of PCR-assisted binding site selection, an individual DNA species in the oligonucleotide pool may not be present in high enough concentration to saturate the DNA binding protein for subsequent amplification. This may lead to a bias in selected clones and consequently, the derived binding site may not be completely representative of the relative affinities of each nucleotide in each position. To within a factor of five, the relative binding affinity comparing AP2α and AP2γ for all point mutants was identical. Subtle differences in binding affinity reported for nucleotide position 4 is unlikely to have physiologic significance. However, it would be difficult to ascertain the effect mutations at this position have on trans-activation. Our *in vivo* transfection assays are not sensitive enough to

detect subtle differences that change the relative binding affinity by ≤5-fold. Nevertheless, it is possible that within the context of chromatin structure, a subtle difference in binding affinity such as this may lead to differences in the activation of target genes in cells where both AP2α and AP2γ are expressed.

A recent study utilizing a similar PCR-based site selection method identified a novel AP2α consensus site (TAGAAAG-NYCYNG) (33) that differs significantly from the consensus site identified in our present study as well as that previously described by Williams *et al*. (26). However, in that study only 17 sequences were recovered and of these, only eight were capable of binding AP2α in a gel shift assay. In addition, none of the clones contained a complete sequence corresponding to the previously described consensus site, $GCCN_3GGC$, suggesting that the number of selected clones was too small to be representative of the total population of AP2α binding site clones. This may be due to the fact that a probe containing a core of only 14 random nucleotides was used which may have reduced the number of potential sequences present in the oligonucleotide pool. In contrast, in our study we found 39 sites (21%) for AP2α and 22 sites (15%) for AP2γ that fit the previously reported AP2α consensus sequence ($GCCN_3GGC$) exactly.

A surprising finding from the competitive gel shift assays is that a T in position 9 has the same relative binding affinity as a site containing either a G or C. Examining other studies in which AP2 consensus sequences were determined (9,13,26), this is the first report of the occurrence of a T in this position. This suggests that there is an aspect of asymmetry to the binding site recognized by AP2 since an A in position 1 causes a 20–25-fold decrease in relative binding affinity in competitive gel shift assay. As another example, a T in position 3 has a greater effect on decreasing binding activity than an A in position 7. This asymmetry may be caused by the influence of nucleotides directly flanking the binding site in the oligonucleotide used in competitive gel shift analysis. However, this is not likely as we were careful in designing the oligonucleotide so that the flanking sequence would not bear any resemblance to an AP2 binding site. It is more likely that certain positions in the binding site can influence the effect of mutations found in other positions, thus resulting in asymmetry. In this study we only investigated the effect of single point mutations on relative binding affinity as it would have been impossible to assay by gel shift competition every possible combination of nucleotides in a 9 bp binding site. Therefore, an influence of one position on another would not have been detected by our methods. Previous work has proposed that AP2 proteins bind DNA using the scissors-grip model for bZIP proteins (27,34,35). This model is well suited to binding DNA sequences containing dyad symmetry. However, the inherent asymmetry which exists in the core of the previously described AP2 consensus site ($GCCN_3GGC$) can be accommodated by this model and therefore, additional asymmetry may also be acceptable. Consensus sites defined using *in vitro* methods such as PCR-assisted binding site selection and competitive gel shift assay are based on sequences which have the highest affinity. Although physiologically relevant binding sites are not always those with the highest affinity, the possibility that AP2 can bind with high affinity to an asymmetric site with a T in the last position may allow the identification of previously unknown targets of AP2α and AP2γ.

A recent study by Mohibullah *et al*. (36) concluded that AP2α can also bind sites containing a 4 nt core sequence. The possibility of a 4 nt core would further expand the repertoire of sites able to interact with AP2 factors. The three most common sequences reported with a 4 nt core were GCCTAAGGGC, GCCTGAGGGC and GCCTTAGGGC. These sites were identified based on the criteria that the AP2 site should begin with GCC and end with GGC. However, our data indicate that the first 9 nt of each of these sites represent high affinity AP2 sites. In fact the optimal site (OPT) used in our study was GCCT-GAGGG which matches the first 9 bp of the second reported sequence from the Mohibullah study. Therefore, it is not clear if AP2 is recognizing sequences with a 4 nt core or a 9 bp binding site containing a three base core and a G in position 9. Our data tend to support the latter conclusion.

In this study, transfection experiments indicate that overexpression of AP2 protein diminishes the distinction between higher and lower affinity binding sites. With increasing expression of AP2 protein, activation of a lower affinity site approaches that of a high affinity site achieved with much lower levels of protein expression. Previous studies have shown that AP2γ is overexpressed in ER-positive breast and endometrial carcinoma cell lines when compared to normal human mammary epithelial cells (17,23). In addition, a recent report shows that although both AP2α and AP2γ are also expressed in benign breast epithelium, AP2γ expression is significantly upregulated in primary breast tumor specimens (22). This suggests that while AP2 proteins may have a role in the growth and differentiation of normal breast, these proteins, in particular AP2γ, are also involved in the progression of breast cancer. It is possible that overexpression of AP2 in breast cancer may activate a set of genes containing lower affinity binding sites that would normally not be activated in normal breast epithelium, thus contributing to the breast cancer phenotype.

In addition to being able to activate an artificial promoter, both AP2α and AP2γ were also able to activate the cloned human ER promoter in the ER-negative breast carcinoma cell line, MDA-MB-231. This indicates that MDA-MB-231 cells contain all other necessary components needed for ER expression and that AP2 is sufficient to activate this promoter. Our data also show that AP2 trans-activation of the ER promoter is through the previously mapped AP2 binding sites present in the untranslated leader sequence of the ER gene. This region contains two binding sites for AP2, a distal site (CCCTGCGGG) and a proximal site (GCCCGAGGT) (23). Interestingly, this proximal site contains a T in position 9 which these data would predict would be a high affinity AP2 site. Recently, Turner *et al*. found a positive correlation between AP2α and ER expression in primary breast carcinoma (22). Our data provide a functional mechanism to explain the correlation between ER and AP2α expression in primary breast cancers. Although no correlation was found between AP2γ and ER expression in primary breast cancer, there may be other constraints that influence which AP2 protein will be overexpressed in a given tumor cell.

In conclusion, a methodical study of binding site specificity has shown that both AP2α and AP2γ recognize the same consensus DNA sequence and that transcriptional activation by AP2 proteins is directly related to the affinity of the binding site. Overexpression of AP2 proteins can lead to the increased trans-activation of promoters containing lower affinity binding sites that would not be activated in the presence of low levels

of AP2 expression. This suggests that the transition and progression of normal breast to a carcinogenic state may be due to a novel set of genes activated only in the presence of AP2 overexpression. In addition, the positive correlation between AP2 activity and ER expression observed in breast cancer is explained with our functional model of the ER promoter in which AP2 is able to activate ER expression by binding to two high affinity AP2 sites present in the ER leader sequence.

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