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Genomic Structure of the Promoters of the Human Estrogen Receptor-α Gene Demonstrate Changes in Chromatin Structure Induced by AP2γ*

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Expression of human estrogen receptor-α (ERα) involves the activity from several promoters that give rise to alternate untranslated 5′ exons. However, the genomic locations of the alternate 5′ exons have not been reported previously. We have developed a contig map of the human ERα gene that includes all of the known alternate 5′ exons. By using S1 nuclease and 5′-rapid amplification of cDNA ends, the cap sites for the alternate ERα transcripts E and H were identified. DNase I-hypersensitive sites specific to ERα-positive cells were associated with each of the cap sites. A DNase I-hypersensitive site, HS1, was localized to binding sites for AP2 in the untranslated region of exon 1 and was invariably present in the chromatin structure of ERα-positive cells. Overexpression of AP2γ in human mammary epithelial cells generated the HS1-hypersensitive site. The ERα promoter was induced by AP2γ in mammary epithelial cells, and trans-activation was dependent upon the region of the promoter containing the HS1 site. These results demonstrate that AP2γ trans-activates the ERα gene in hormone-responsive tumors by inducing changes in the chromatin structure of the ERα promoter. These data are further evidence for a critical role for AP2 in the oncogenesis of hormone-responsive breast cancers.

There are at least two nuclear receptors for estrogen receptor, ERα[1] (1, 2) and ERβ (3). Most breast cancers that occur in post-menopausal women overexpress ERα (4). Patients with breast cancers that express ERα are more likely to respond to hormonal therapy (4, 5) and have an improved prognosis compared with patients with ERα-negative tumors (4, 6, 7). Studies of breast cancer cell lines (8) and primary tumors (9, 10) have indicated that transcription of the ERα gene plays an important role in regulating the expression of ERα. Thus, under-

standing transcriptional regulation of the ERα gene will likely provide critical insights into the pathogenesis of hormone-responsive breast cancers.

Transcription of the ERα gene is complex and involves activity of several distinct promoters (11–14). Functional promoter studies have concluded that ERα expression in breast cancer cell lines and various tissues is likely to involve trans-acting factors that have a specific cell or tissue distribution pattern (15–19). There appear to be a variety of factors that interact with the ERα promoter with trans-activating (15–17) or trans-repressing (20) functions. There is also evidence that ERα can autoregulate its own transcription (21, 22). Other studies suggest that the lack of ERα expression in ERα-negative breast cancer cell lines and tumors may be controlled by methylation of CpG islands in the 5′ end of the ERα gene (23, 24).

The main ERα promoter, P1, initiates transcription at a cap site previously mapped at the start of exon 1 (1). Exon 1 has a 233-base 5′-untranslated region preceding the AUG codon that initiates translation of the ERα protein. Studies in ERα-positive breast cancer cell lines have shown that transcription initiated at exon 1 accounts for 50–90% of all ERα mRNAs (18, 25). A functional analysis of the main ERα promoter identified a factor, ERF-1, that binds to high affinity sites in the untranslated region of exon 1 and can trans-activate the cloned ERα promoter (15, 26). ERF-1 was found to be a member of the AP2 family of transcription factors and has been renamed AP2γ (27).

All other ERα transcripts initiate at cap sites upstream of exon 1 and splice into a splice acceptor site at +163 in exon 1 (14). Although some of these upstream exons have open reading frames, there is no evidence that these are translated, and it appears that all alternate 5′ exons are non-coding. Exon 1′ has been reported to have two main cap sites giving rise to alternate 5′ exons of 110 bases or 1206 bases (12). The short and long forms of exon 1′ both utilize the splice donor site at −1884 (location relative to cap site of P1). We had previously described two additional alternate ERα transcripts called E and H (14). Both the E and H transcripts are expressed in ERα-positive breast cancer cell lines, primary breast cancers, ERα-positive endometrial carcinoma cell lines, and normal endometrium. The existence of the E and H transcripts of ERα were subsequently confirmed by other investigators, and these exons were reported to be expressed in a variety of tissues (25). The splice donor site of exon E was found to be at −169. The H transcript was found to utilize two upstream exons, Ha and Hb, separated by an intron of 9 kbp (14). The genomic location of the H exons was not determined but was concluded to be at least 20 kbp 5′ to exon 1 (14). An additional liver-specific exon, called exon C, has also been described (13). Exon C was reported to be spliced to an exon with sequence matching exon Hb, and it was concluded that exon C is farther 5′ than exon Ha (14).

In order to define the location of the ERα promoters active in

[1] The abbreviations used are: ERα, estrogen receptor α; kbp, kilobase pair; kb, kilobase; RACE, rapid amplification of cDNA ends; HMECs, human mammary epithelial cells; MEM, minimal essential media; FCS, fetal calf serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GST, glutathione S-transferase; CMV, cytomegalovirus; m.o.i., multiplicity of infection; bp, base pair; GFP, green fluorescent protein.

breast cancer, a detailed analysis of the genomic structure of the 5′ end of the ERα gene including the alternate ERα transcripts E and H was performed. Overlapping BAC clones have been isolated that generated a contig map that includes all known ERα exons. The exon Ha was found to be 124 kbp upstream of exon 1, and exon C was located 30–40 kbp 5′ to exon Ha. Together with the previously known exons of the ERα gene that span a region of over 160 kb, the ERα locus was found to encompass a genomic region of ~300 kbp. By using S1 nuclease and 5′-RACE, the cap sites for exons E and H have been mapped. Each of the alternate upstream exons was found to be associated with DNase I-hypersensitive sites specific to cells expressing ERα. The DNase I-hypersensitive site, HS1, was mapped to the binding sites for AP2γ in the untranslated leader of exon 1 and was invariably found in the chromatin structure of ERα-positive cells. In human mammary epithelial cells (HMECs), AP2γ expression induced the HS1-hypersensitive site and trans-activated the ERα promoter, which was dependent upon the region of the promoter containing the HS1 site. These findings provide additional evidence for a critical role for AP2γ in the oncogenesis of hormone-responsive breast cancer.

EXPERIMENTAL PROCEDURES

Cell Lines—All cell lines were obtained from American Type Culture Collection, Manassas, VA. Cells were maintained in minimal essential media (MEM, Life Technologies, Inc.) supplemented with 10% fetal bovine sera (FCS, Gemini BioProducts, Calabasas, CA), 25 mM HEPES, 26 mM sodium bicarbonate, 5000 units/ml penicillin G (Life Technologies, Inc.), 5000 μg/ml streptomycin (Life Technologies, Inc.), and 6 ng/ml bovine insulin (Sigma). HMECs were obtained from reduction mammoplasty and were a gift from Dr. J. Dirk Iglehart, Boston. HMECs were maintained in DFCI-1 media (28). All cells were incubated at 37 °C in 5% CO_2.

BAC Library Screening—BAC clones containing genomic DNA from the 5′ region of the human ERα gene were identified by hybridization of DNA arrays with probes corresponding to the human ERα exons 1′, Ha and Hb. Nylon membranes arrayed with DNA from a human BAC library were purchased from Research Genetics (Huntsville, AL). Gel-purified insert DNA from clones of ERα exons Ha (pHa2.5) and Hb (pHb4.1) were used for hybridization. Exon 1′ sequences between −3090 and −2670 were PCR-amplified from MCF-7 genomic DNA using primers ERSEQ1 (TCTAGAGCATGGGTGGCCAT) and ERSEQ2 (GTGCTCCTAGAGTGCCCACG) and TAQ polymerase. The cycle profile included an initial denaturation step of 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 56 °C for 15 s, and 72 °C for 2 min and terminated with a final extension step of 72 °C for 5 min. All DNAs used for hybridization were gel-purified and labeled by random priming with [α-32P]dCTP to specific activities greater than 7×10^8 dpm/μg.

Library membranes were prehybridized in 50% formamide, 5× SSC, 7% SDS, 1% polyethylene glycol, 25 mM sodium phosphate buffer, pH 6.7, and 0.5% non-fat dried milk at 42 °C for 1 h. Twenty ml of hybridization solution per membrane were used. For hybridization, the volume of hybridization solution was reduced to 6 ml per membrane, and each probe was added to 5×10^5 dpm/ml and hybridized for 12–18 h at 42 °C. Following hybridization the membranes were washed twice in 2× SSC, 1% SDS at 42 °C and then twice in 0.1× SSC, 0.1% SDS at 65 °C. Positive signals were identified by overnight exposure to film.

Cultures of *Escherichia coli* harboring the BACs identified in the initial screen were obtained from Research Genetics. A small amount of DNA was obtained from each culture using the vendor's protocols for secondary screening by PCR. DNA from 19 BACs were PCR-amplified with primers oEXON0INT and oEXON0–3′ (14) for the presence of ERα exon Hb sequences and with primers ERSEQ1 and ERSEQ2 for the presence of ERα exon 1′ sequences as described above.

Mapping BAC Clones—DNA from BACs containing portions of the ERα upstream region was prepared from 1-liter cultures using a Maxiprep kit from Qiagen (Valencia, CA) as described by the manufacturer. For restriction enzyme digest analysis, 1–2 μg of BAC DNA was digested in 20 μl with the appropriate enzyme and then subjected to pulse field gel electrophoresis in 0.5× TBE at 140 V with field switches increasing from 1 to 12 s over 20 h. Bands were visualized by ethidium bromide staining and photographed. Band sizes were calculated from standard curves constructed from molecular weight markers.

For Southern blot analysis, DNAs were transferred to positively charged nylon membranes (Hybond N+, Amersham Pharmacia Biotech) using protocols provided by the manufacturer. The blots were hybridized with various probes to identify the locations of the ERα exons relative to the restriction sites mapped in each BAC. Probes used were the ERα exon 1′, Ha and Hb probes described above. In addition, oligonucleotides corresponding to ERα exon C (TTCACAATCAAAAG-GATTGG) (13) and to the ends of the BAC genomic DNA inserts were used. The sequences of the ends of the BACs were determined by direct sequencing of BAC DNA.

S1 Nuclease Analysis—S1 nuclease analyses were performed essentially as described (18) with modifications of the protocols for probe synthesis. Messenger RNA was isolated from MCF-7 cells using a Fast Track mRNA isolation System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The probe for analysis of the ERα transcripts originating at the exon Ha promoter was synthesized by single-sided PCR from a template that encompassed exon Ha plus ~850 bases of upstream sequences. The template was PCR-amplified from MCF-7 genomic DNA using the primer H199.4 (GAGAAGATTAT-CACTCAGAGAC) as the 3′ primer and H853.3 (CGCCCCATTCTAC-CATTTTC) as the 5′ primer. PCR conditions were as described above except that the annealing temperature was 50 °C. To synthesize a single-stranded probe complementary to the expected mRNA sequence, PCR was performed using only H199.4 in the presence of [α-32P]dCTP. After PCR the probe DNA was desalted on a spin column to remove unincorporated label.

The single-stranded probe for analysis of the ERα transcripts originating from the promoter associated with exon E was synthesized by primer extension using Klenow enzyme. The primer used was ERPRO30 (GTGCAGACCGTGTCCCCGCA) and was annealed to the denatured ER724–210LUC plasmid (15) as a template. The 3′ end of the probe was defined by cleavage with *Pvu*II, and the single-stranded probe was isolated from the template by electrophoresis through a 1.5% alkaline agarose gel. The purified probe was eluted from the gel using a Millipore Ultrafree DA spin cartridge (Millipore Corp., New Bedford, MA).

Either the Ha or E probe (5×10^5 cpm) was hybridized to 2 μg of MCF-7 mRNA or yeast tRNA overnight at 45 °C. Nuclease S1 (500–2000 units/ml; Amersham Pharmacia Biotech) was added, and the samples were incubated at 37 °C for 1 h. The reactions were stopped, and the samples were extracted once with phenol/chloroform (1:1) before analysis on a 6% acrylamide sequencing gel. Signals were detected by overnight autoradiography.

5′-RACE Analysis—Messenger RNA from MCF-7 cells was analyzed by 5′-RACE using the 5′-RACE System from Life Technologies, Inc., as recommended by the manufacturer. Initial reverse transcription was primed with ERPRO22 (CCCTTGGATCTGATGCAGTA), which is located in exon 1 at approximately +300. The gene-specific primer for the first round of PCR was ERPRO30, which anneals to sequences in exon 1 upstream of ERPRO22. A second round of PCR amplification was performed using the gene-specific primers ERPRO94 (GCTGGATA-GAGGCTGAGTTT) and H199.4 for exons E and Ha, respectively. RACE PCR products were cloned into pCR2.1 using a TA Cloning Kit (Invitrogen) as recommended. The location of the 5′ end of each clone was determined by sequencing.

DNase I-hypersensitive Site Assay—DNase I-hypersensitivity was analyzed in a variety of ERα-positive and ERα-negative cell lines. Cells were harvested during exponential growth, and then washed once with cold phosphate-buffered saline (PBS). The cells were then washed once with cold buffer A (15 mM Tris, pH 7.4, 60 mM KCl, 15 mM NaCl, 0.2 mM EGTA, 0.2 mM EDTA, 0.25 M sucrose, 1 mM dithiothreitol, 0.15 mM spermine, 0.5 mM spermidine) and then resuspended in 5 ml of cold buffer A with 0.2% Nonidet P-40. The cells were lysed in a Dounce homogenizer using 10 strokes of a B pestle on ice; lysis was checked by trypan blue exclusion. Nuclei were pelleted and resuspended in 2.5 ml of buffer B (buffer A minus sucrose). To 0.5-ml aliquots of nuclei varying quantities of DNase I (0, 200, 400, 600, 800, 1200, 1600 units/ml; Roche Molecular Biochemicals) were added, and then $MgCl_2$ was added to 5 mM, and the samples were incubated on ice for 15 min. The reactions were stopped by addition of EDTA to 50 mM. Following addition of SDS to 0.5% and proteinase K to 1 mg/ml, the samples were incubated overnight at 37 °C. Residual protein was removed by extraction once each with phenol, phenol/chloroform (1:1), and chloroform. Following ethanol precipitation the nucleic acid pellets were dissolved in 10 mM Tris, pH 8.0, 0.5 mM EDTA, and the absorbance at A_{260} was measured.

Ten micrograms of each sample were cleaved with the appropriate restriction enzyme overnight at 37 °C. For analysis of hypersensitive sites near exon 1, the samples were then electrophoresed through 1% agarose (SeaKem GTG, BioWhittaker Molecular Applications, Rock-

land, ME) using 0.5× Tris/acetate/EDTA buffer and transferred to nylon membranes for hybridization as described above. The resulting blots were hybridized with the exon 1′ probe. For analysis of hypersensitive sites near exons Ha/Hb, samples were subjected to pulse field gel electrophoresis following restriction enzyme cleavage. DNA was immobilized on nylon membranes and hybridized with the same exon Ha probe used to screen the BAC library.

AP2γ Antibody Production—Antigen for the production of an AP2γ-specific polyclonal antibody (AP) was generated by cloning a fragment of AP2γ in frame with glutathione *S*-transferase (GST) to create a fusion protein. A fragment corresponding to nucleotides 474–607, which encodes amino acids 150–187, was generated by PCR and cloned in frame in the pGEX-4T3 vector (Amersham Pharmacia Biotech). The identity of the clone was confirmed by sequencing the entire insert from both directions. The clone was transformed into XL-1 Blue cells (Stratagene), and the production of a fusion protein of the proper size was confirmed by SDS-polyacrylamide gel electrophoresis. Large scale production of the fusion protein was induced with growth of the transformed cells in the presence of isopropyl-1-thio-β-D-galactopyranoside. Bacterial lysates were incubated with glutathione-agarose beads, washed with PBS, and the fusion protein eluted with the addition of 5 mM glutathione. The fusion protein was injected into rabbits, and antisera were generated by CalTag Laboratory (Healdsburg, CA). Following production of a polyclonal antisera, affinity purification was performed by passing antisera first over a GST affinity column to bind selectively antibodies directed to the GST portion of the fusion protein, and then over a GST-AP2γ affinity column with subsequent elution of the affinity-purified antibody. Affinity columns were prepared using Affi-Gel 10 supports (Bio-Rad). Gel shift assays were performed as described previously (15). In supershift assays, 2 μl of AP antisera was used. The rabbit polyclonal antibody to AP2, SC-184, was obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

Construction of AP2γ/pAdTrack-CMV and AP2α/pAdTrack-CMV Shuttle Vector—In order to generate AP2γ and AP2α adenoviral constructs, the AP2γ and AP2α cDNAs were first cloned into a shuttle vector. By using a previously described AP2γ clone retrieved from a MCF7 expression library (27) as template, AP2γ cDNA was PCR-amplified from the translation start site at +167 to the stop codon at +1519 using a 5′ primer (GACGCAGATCTCCATGTTGTGGAAAATA-AC) containing a *Bgl*II site and a 3′ primer (TCGTTCTCGAGTTATT-TCCTGTGTTTCTCC) containing an *Xho*I site. AP2α cDNA was PCR-amplified from the translational start site to the stop codon using an AP2α cDNA clone (26) as template. PCR was performed using a 5′ primer AP2α5′ *Sal*I (CGATCCGTCGACATGCTTTGGAAATTGACG) containing a *Sal*I site and a 3′ primer AP2α3′ *Xba*I (GGGAGGTCTA-GATCACTTTCTGTGCTTCTC) containing an *Xba*I site. The AP2γ and AP2α cDNA fragments were ligated into the pAdTrack-CMV shuttle vector (gift of Dr. Burt Vogelstein, The Johns Hopkins University) (29) after digestion with appropriate restriction enzymes to create AP2γ/pAdTrack-CMV and AP2α/pAdTrack-CMV. pAdTrack-CMV also encodes the GFP protein so that viral production can be monitored by fluorescence.

Generation of Recombinant AdAP2γ, AdAP2α, and AdWT Adenoviral Plasmids—To generate AdAP2γ, AdAP2α, and AdWT adenoviral recombinants, 1 μg of AP2γ/pAdTrack-CMV, AP2α/pAdTrack-CMV, or pAdTrack-CMV plasmid DNA was linearized overnight at 37 °C with *Pme*I, extracted two times with phenol/chloroform and once with chloroform, and followed by ethanol precipitation. Cotransformation of linearized DNA and 100 ng of pAdEasy-1 adenoviral backbone vector (gift of Dr. Vogelstein) (29) into electrocompetent *E. coli* BJ5183 cells was performed in 2.0-mm cuvettes at 2.5 kV, 200 ohms, and 25 microfarads using a Bio-Rad Gene Pulser electroporator (Bio-Rad). Recombinants were selected for kanamycin resistance, and recombination was confirmed by restriction digestion with *Pac*I, *Bam*HI, *Bst*XI, and *Nde*I. True recombinants were retransformed into electrocompetent *E. coli* DH10B (Life Technologies, Inc.) as described above, and DNA was purified using the Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol.

Production of AdAP2γ, AdAP2α, and AdWT Adenoviruses in 293 Cells—AdAP2γ, AdAP2α, and AdWT adenoviruses were produced as described previously (29) with several modifications. Briefly, 2 × 10⁶ 293 cells were plated in 25-cm² flasks 24 h before transfection in MEM with 10% FCS so that cells had reached 70–80% confluence by 24 h. On the day of transfection, 4 μg each of AdAP2γ, AdAP2α, and AdWT DNA were linearized by *Pac*I digestion. Each linearized DNA was mixed with 20 μl of LipofectAMINE (Life Technologies, Inc.) in 500 μl of Opti-MEM (Life Technologies, Inc.) and incubated at room temperature for 15–30 min according to the manufacturer's protocol. Meanwhile, the cells were

washed once with 3 ml of Opti-MEM. After incubation, the lipid/DNA mixes were brought up to 2 ml with Opti-MEM and overlaid onto the 293 cells. After incubation at 37 °C, 5% CO₂ for 4 h, the transfection mix was removed, replaced with 6 ml of MEM + 10% FCS, and returned to the incubator. Cells were monitored by fluorescence microscopy for GFP expression over 7–9 days at which time most of the cells were fluorescent and had detached from the flask. Cells were collected, pelleted, resuspended in 2 ml of 1× PBS buffer, and subjected to 4 cycles of freeze/thaw/vortex (dry ice/37 °C). AdAP2γ, AdAP2α, and AdWT adenoviruses were then plaque-purified by infecting 5 × 10⁵ 293 cells in 35-mm plates with 100 μl of serial dilutions of viral supernatants from 10⁻¹ to 10⁻⁴ made in Opti-MEM. After a 1-h incubation at 37 °C, 5% CO₂, cells were overlaid with 3 ml of 0.8% agarose in MEM + 10% FCS and returned to the incubator. Plates were monitored for plaque formation and GFP expression over 9 days at which time plaques were isolated as agarose plugs into 200 μl of MEM + 10% FCS and subjected to 3 freeze/thaw (dry ice/37 °C) cycles. Fifty μl of viral lysate was used to infect 2 × 10⁶ 293 cells in a 25-cm² flask, and the cells were harvested as described above at 3–4 days when the cells were at least 50% detached. Virus was then titered by GFP expression, and 3 more rounds of infection were performed at an m.o.i. of 0.1 to generate higher titer viral stocks. A final round of infection was performed at an m.o.i. of 5 using 5 × 10⁸ 293 cells in six 175-cm² flasks. Cells were harvested at 60 h post-infection and after 4 cycles of freeze/thaw in 8 ml of 1× PBS, the virus was purified by CsCl banding using a density of 1.35 g/ml CsCl in a SW41Ti rotor at 32,000 rpm, 10 °C, 18–24 h. Virus was collected in ~1 ml with an 18-gauge needle and dialyzed against 1 liter of Storage Buffer (5 mM Tris, pH 8.0, 50 mM sodium chloride, 0.05% bovine serum albumin, and 25% glycerol) at 4 °C for 6 h. Viruses were titered by GFP expression in both 293 cells, and HMECs generally resulted in titers of 10¹¹ plaque-forming units/ml in 293 cells and 10⁸ plaque-forming units/ml in HMECs.

Confirmation of AdAP2γ, AdAP2α, and AdWT Identity—In order to confirm the identities of AdAP2γ, AdAP2α, and AdWT viruses, DNA was isolated from the viruses using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol for non-nucleated blood. Viral DNA was subjected to PCR amplification using 3 sets of primer pairs for AP2γ and AP2α and one set for AdWT. Primer pairs for AdAP2γ were composed either of one primer derived from the pAdTrackCMV vector sequence and one primer from AP2γ (primer pair 1, GFP-AdTrack/APseq6; primer pair 2, Rt. Arm-AdTrack/Apseq2) or of two primers from the pAdTrack-CMV sequence (primer pair 3, GFP-AdTrack/Rt. Arm-AdTrack). For AdAP2α, primer pair 1 was composed of GFP-AdTrack and AP2α5′ *Sal*I, and primer pair 2 was composed of Rt. Arm-AdTrack and AP2α3′ *Xba*I. Primer pair 3 remained the same. Viral DNA for AdWT was amplified using only primer pair 3. Primer sequences are as follows: GFP-AdTrack (GCCGTCCTCGATGTTGTG-GCGGATC); APseq6 (CATCAAAGAAGCCCTGATT G); Rt. Arm-AdTrack (CATCAAACGAGTTGGTGCTCATGGC); and Apseq2 (GTGCTGCCCGGCGGAGGAGA).

Infection of HMEC with Adenoviruses—The day before infection, a total of 3 × 10⁷ HMEC cells for each virus was plated in three 175-cm² flasks. The next day, media were removed from the flasks, and the cells were washed with 5 ml of Opti-MEM. Cells were infected at an m.o.i. of 10 for 24 h at which time they were analyzed for DNase I hypersensitivity as described above.

Trans-activation of the ERα Promoter—The ERα promoter constructs used have been described previously (15). The constructs ER3794–230LUC and ER3794–0LUC were previously called ER3500–230LUC and ER3500–0LUC, respectively. However, sequence analysis has shown that the 5′ end of the constructs are at −3794 bp relative to the P1 cap site. Transfections in HMECs were performed in triplicate using FuGene 6, and luciferase expression was normalized using β-galactosidase expression as described previously (26).

RESULTS

Genomic Mapping of the Alternate ERα Exons—We had previously identified alternate 5′ exons of the human ERα gene that were expressed in breast cancer cell lines and primary breast tumors (14). However, genomic mapping with genomic lambda clones failed to provide a contig spanning the entire region. It remained to be determined how large the intron was between exon Hb and the splice acceptor site in exon 1. The location of the liver-specific exon C (13) also had not previously been determined. In order to locate the 5′ alternative exons Ha, Hb, and C, a BAC library was screened using probes represent-
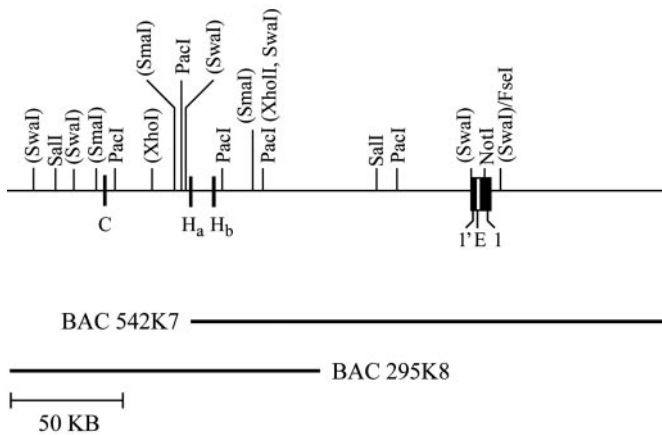
FIG. 1. **Genomic map of 5′ region of the ERα gene.** A genomic map of the 5′ end of the ERα gene is shown with restriction sites. Sites in *parentheses* indicate that not all sites of that enzyme are shown. The location of the ERα exons *C, Ha, Hb, 1′, E,* and *1* are shown. The positions of BAC clones BAC 542K7 and BAC 295K8 are shown *below* the map.

FIG. 2. **S1 nuclease and 5′-RACE for exons Ha and E.** *A,* mapping for exon Ha. Results of S1 nuclease are shown that demonstrate 10 start sites over a 66-base region. The main S1 fragments are labeled *A–J.* RNA used in the experiments is from MCF-7 cells (*lanes 1* and *2*) or tRNA (*lanes 3* and *4*). Increasing concentration of S1 nuclease was used as indicated. To the *right* of the gel is the sequence locating the approximate position of the S1 nuclease products. *Closed circles* represent the 5′-terminal nucleotide from the 5′-RACE clones. *Arrow* indicates the position of the longest cDNA previously reported for ERα H transcripts. *B,* mapping for exon E. S1 nuclease results shown for exon E presented in a manner similar to exon Ha. Five main S1 nuclease fragments are labeled *A–E.* Fragment A is ~120 bp upstream of the cluster represented by *B–E.* *Closed circles* represent the 5′-terminal nucleotide of 5′-RACE clones. *Arrow* indicates the position of the longest cDNA previously reported for ERα E transcripts.

ing exons 1′, Ha and Hb. Of the BAC clones identified in the initial screen only BAC 542K7 encompassed sequences from both exon 1′ and exon Hb and therefore was likely to span the intron separating these two exons. This BAC clone also hybridized to a probe specific for exon Ha. Three other BACs including BAC 295K8 were isolated that contained sequences from exons Ha and Hb but not exon 1′. BAC 295K8 also hybridized to a probe for the liver-specific exon C.

Southern blot analysis was used to generate a restriction map, to locate the positions of the ERα exons, and to determine the overlap in the two BAC clones (data summarized in Fig. 1). Previous results using lambda genomic clones had demonstrated that exon Hb was at least 20 kb upstream of exon 1′ (14). The results from mapping BAC 542K7 and 295K8 revealed that exon Hb was more than 110 kb upstream of exon 1′ and that the distance between the cap site for exon Ha and the cap site for exon 1 was ~124 kb (Fig. 1). Southern blot analysis revealed that the liver-specific exon C was located on a *Sma*I/*Pac*I fragment between 30 and 40 kb upstream of exon Ha.

Mapping the Cap Sites for the Alternate ERα Transcripts— The location of the alternate 5′ exons of the human ERα gene suggests that there are separate ERα promoters that may be independently regulated. Functional promoter studies have been performed for promoters initiating transcription at exons 1 (15) and 1′ (17), but no studies have been performed on promoters that may be involved in expression of exons E or Ha. In order to further localize possible promoters controlling expression of these two alternate exons, experiments to locate the cap sites of the E and H transcripts were performed using S1 nuclease and 5′-RACE. The results of these findings are shown in Fig. 2. For the S1 analysis, MCF-7 mRNA was hybridized to a probe extending several hundred bases upstream of the end of the longest cDNA previously identified for ERα transcripts initiating with exon Ha (14). The analysis revealed multiple species of protected fragments with 5′ ends between 15 bases (Fig. 2*A, fragment J*) and 80 bases (Fig. 2*A, fragment A*) upstream of the 5′ end of the longest cDNA clone previously obtained for Ha.

To validate that the cap sites of Ha had been correctly identified by S1 nuclease analysis, 5′-RACE was performed on MCF-7 mRNA. A clear single PCR product was seen after the second round of PCR using a gene-specific primer in exon Ha (data not shown). Twelve clones with inserts were sequenced to determine the 5′-most base, and the results were correlated with the S1 nuclease analysis data (see Fig. 2*A*). The *circles*
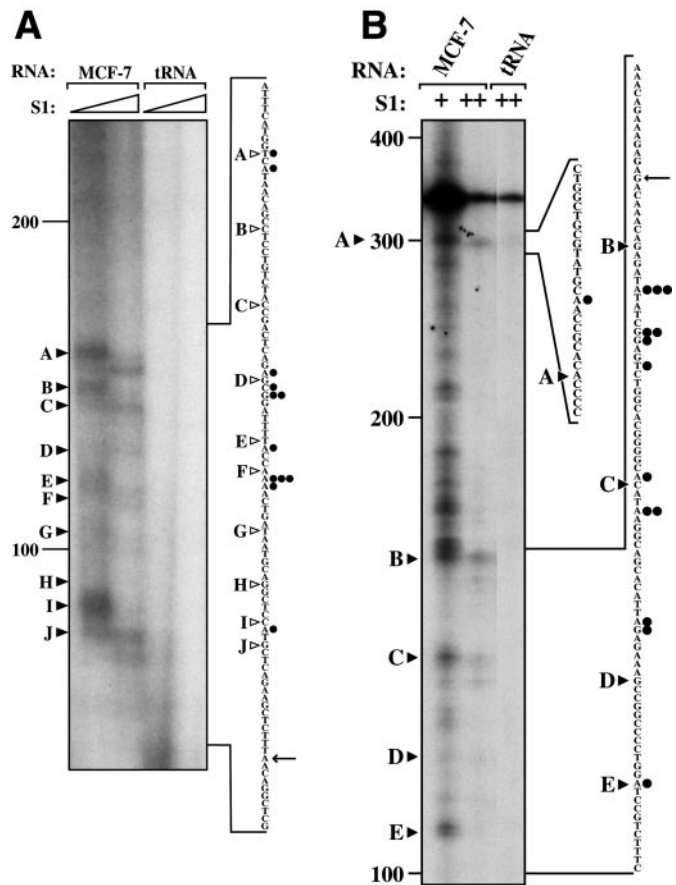
plotted on the *right side* of the vertical sequence represent the locations of 5′-most bases from these 12 clones. Similar to the S1 analysis, RACE clones displayed a 66-base range in length, which coincided with the mRNA ends identified by S1 analysis. Two clusters of clones were observed that terminated ~45 bases and 57 bases upstream of the previous end noted from ER cDNA clones. Two clones mapped to within 2 bases of the longest S1 product (*fragment A*), and one clone corresponded to the smallest S1 product (*fragment J*). These results demonstrate that exon Ha represents a genuine ERα cap site with multiple start sites scattered over ~60 bp.

Exon E was previously identified as an alternative 5′ exon from screening a cDNA library and was localized between exon 1′ and exon 1 (14). The 3′ end of exon E was identified 169 bp upstream of the main transcriptional start site for the ERα gene. In order to confirm that ERα transcripts have a cap site associated with transcription initiated at exon E, a similar analysis to that for exon Ha was undertaken. A single strand DNA probe extending from the 3′ end of exon E sequences to several hundred bases upstream was hybridized to MCF-7 mRNA. S1 nuclease analysis of the hybrids revealed several

protected species as was observed for exon Ha (Fig. 2*B*). A prominent protected band was observed that corresponded to an mRNA end ~120 bases upstream of the end previously identified from the longest cDNA clone for exon E (Fig. 2*B, fragment A*). Four additional protected species were identified, the 5′-most of which (*fragment B*) was 8 bp from the previously identified cDNA 5′ end. The other three protected species (*fragments C–E*) corresponded to shorter mRNAs.

The 5′-RACE analysis of ER mRNA from MCF-7 cells using exon E-specific primers produced two prominent bands of ~200 and 300 bp (data not shown). Fourteen of the RACE clones were sequenced, and the 5′-most base correlated with the S1 analysis data (Fig. 2*B*). One clone extended several bases beyond the length of S1 fragment A, and the rest extended over an 80-base range with lengths similar to fragment B–E identified by S1 analysis. A cluster of 7 clones had 5′ ends within an 11-base range just 3′ to the fragment corresponding to S1 product B. Since the S1 nuclease fragment sizes are estimated from the mobility in gel electrophoreses, the S1 nuclease results and 5′-RACE results are in excellent agreement.

DNase I-hypersensitive Sites—The location of DNase I-hypersensitive sites has been shown to correspond to the position of binding by transcription factors involved in transcriptional regulation of a gene (30). Therefore, mapping DNase I-hypersensitive sites can provide important information about the location of the transcriptional regulatory regions. Previously published data (31) had identified three DNase I-hypersensitive sites near exon 1 that were specific to ERα-positive cells, HS1 located near the cap site for exon 1, HS2 at approximately −350 bp, and HS3 at approximately −2000 bp (positions relative to the cap site for exon 1). A repeat of this experiment confirmed these results and localized the sites with more precision (Fig. 3*A*). The first hypersensitive site (HS1) was observed within the 5′-untranslated region of exon 1 at approximately +200 bp. A second hypersensitive site was observed at −800 bp (HS2), and a third hypersensitive site (HS3) was seen at −2000 bp. In agreement with previous work, each of these three hypersensitive sites was specific to ERα-positive cells, and we have adopted the earlier nomenclature. The location of HS1 coincides with binding sites for AP2γ, which were previously identified in a functional promoter analysis of the main ERα promoter (15). HS3 appears to be associated with exon 1′ and may be functionally related to the P0 promoter (also known as the B promoter) (17) for exon 1′. Exon 1′ may be transcribed as a 110-base or 1206-base exon both of which end at a splice donor site at −1884 (12) indicating that there may be two alternate transcriptional start sites. If the smaller exon is expressed, HS3 would be mapped just upstream of the start of exon 1′. HS2 is located adjacent to exon E and may relate to the promoter controlling transcription starting at this cap site.

DNase I-hypersensitive site analysis was performed using an exon Ha probe on Southern blots of DNA from MCF-7 and MDA-MB-231 cells to locate potential regulatory elements involved in transcription of the H transcript (Fig. 3*B*). Southern analysis of DNA digested with *Swa*I revealed a 32-kb fragment in both MCF-7 and MDA-MB-231 DNA, which is consistent with the size of the *Swa*I fragment determined from the BAC mapping experiments. An additional band migrating at 15 kb was observed in MCF-7 DNA treated with DNase I (Fig. 3*B, top panel, HS4*). This band mapped a hypersensitive site that is specific to ERα-positive cells to a location ~5 kb downstream of exon Hb. Southern analysis of DNA digested with *Xho*I demonstrated a band at 48 kb in both cell lines. A prominent band of lower mobility (*bottom panel, HS4*) was observed in MCF-7 DNA treated with DNase I but not in similarly treated MDA-MB-231 DNA. This band maps to the same location as HS4 in
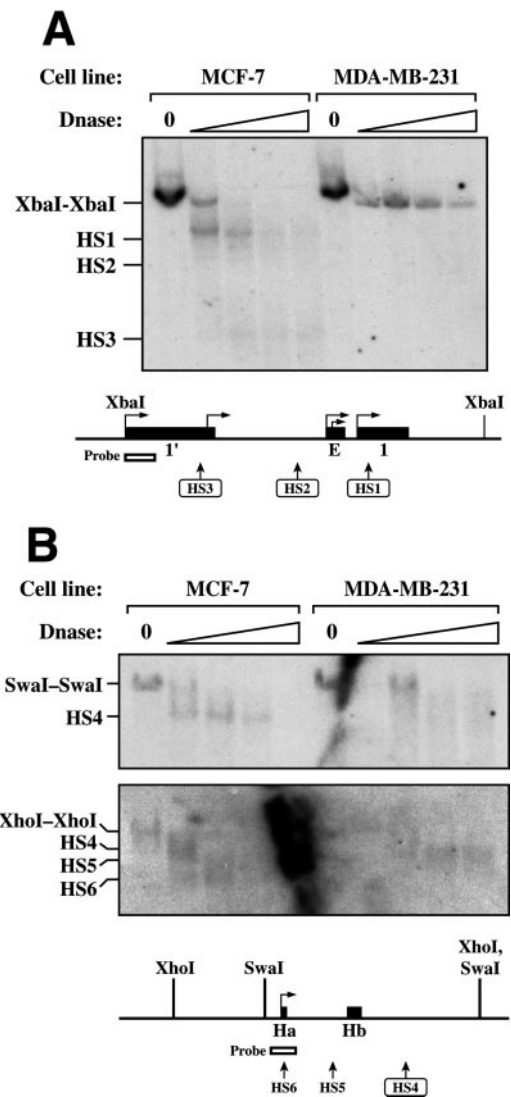

FIG. 3. **DNase I-hypersensitive sites in region of ERα cap sites.** *A,* hypersensitive sites in region of exons 1, E, and 1′. Hypersensitive sites were determined using chromatin extracted from MCF-7 (ERα-positive) and MDA-MB-231 (ERα-negative) cells. Chromatin was untreated (*0*) or treated with increasing amounts of DNase I as indicated. Hypersensitive sites HS1, HS2, and HS3 correspond to sites specific to ERα-positive cells as reported previously (30). Map *below* shows schematic location of exons *1, E,* and *1′* relative to location of hypersensitive sites and probe used in the hybridization. *Arrows* indicate major cap sites identified. *B,* hypersensitive sites in region of exons Ha and Hb. Results for mapping DNase I-hypersensitive sites are presented in a manner similar to exons 1, E, and 1′. Data demonstrate hypersensitive site HS4, which was specific to ERα-positive cells. Location of site was confirmed in two experiments with digestion with *Swa*I or *Xho*I. Hypersensitive sites HS5 and HS6 were identified in both cell lines.

the *top panel*. Additional faster migrating bands were observed in both sets of DNA (*lower panel, HS5* and *HS6*) and are, therefore, not specific to ERα-positive cells. These bands mapped hypersensitive sites to the locations indicated on the diagram *below* the autoradiographs. HS5 mapped to a location ~2 kb upstream of exon Hb, and HS6 mapped to a location near or within exon Ha.

The HS1-hypersensitive Site Is Present in ERα-positive Cells—The location of the HS1-hypersensitive site suggests that this alteration of chromatin structure is required for transcription from the main ERα promoter. If this hypothesis were correct, the HS1 site should be found invariably in cells that express ERα. The chromatin structure of a panel of ERα-positive and ERα-negative cell lines was previously examined for

FIG. 4. **Analysis of HS1 in a panel of cell lines.** A panel of ERα-positive breast carcinoma (T47-D, ZR75-1, BT20, MDA-MB-361), ERα-positive endometrial carcinoma (ECC-1), ERα-negative endometrial carcinoma (HEC1A), ERα-negative breast carcinoma (HBL-100), and an ERα-negative cervical carcinoma cell line (HeLa) were analyzed for the presence of the HS1-hypersensitive site. All ERα-positive lines demonstrated the presence of HS1, whereas none of the ERα-negative lines had the site.
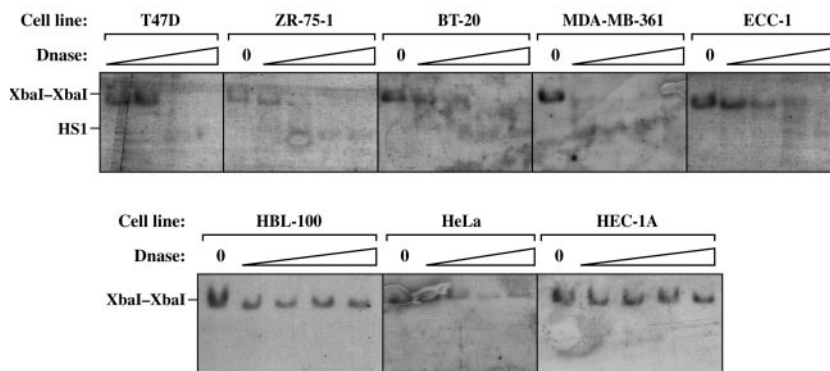
the presence of the HS1 site (31). Fig. 4 shows the results of an analysis of the HS1 site in an additional panel of cell lines. The ERα-positive breast carcinoma cell lines T47-D, ZR75-1, BT20, and MDA-MB-361 all demonstrated the HS1 site. The ERα-positive endometrial cancer cell line ECC-1 also demonstrated the HS1-hypersensitive site. In distinct contrast, the HS1 site was not found to be present in an analysis of the chromatin structure of the ERα-negative cell lines HEC1A, HBL-100, or HeLa (see Fig. 4). Together with the results in Fig. 3A, this analysis of the HS1 site in 10 cell lines demonstrated a striking correlation between the expression of ERα and the presence of the HS1 site. Previous studies in these cell lines have shown that each of the cell lines that demonstrate the HS1 site (MCF7, T47-D, ZR75-1, BT20, MDA-MB-361, and ECC-1) had been found to overexpress AP2γ, whereas those cell lines that lack the HS1 site (MDA-MB-231, HEC1A, HBL-100, and HeLa) express negligible amounts of the AP2 factors (15, 32). This result suggests that the HS1 site may be generated by AP2 binding to chromatin in the ERα promoter.

AP2γ Induced the HS1-hypersensitive Site in HMECs—The location of HS1 mapped to the untranslated region of exon 1 and coincides with the location of high affinity binding sites for the AP2 transcription factors (15). Functional analysis of the main ERα promoter indicated that AP2γ as well as AP2α are able to induce transcription from the main ERα promoter by binding to the AP2 sites found in the untranslated leader of exon 1 (15, 26). To define better which AP2 proteins may be involved in regulation of the ERα gene in MCF7 cells, an AP2γ-specific antisera was generated. As seen in Fig. 5, the commercially available polyclonal antibody, SC-184, is able to supershift purified AP2α and AP2γ. The antisera, AP, is specific for AP2γ and does not supershift purified AP2α (Fig. 5). An analysis of MCF-7 nuclear extracts indicates that the majority of AP2 activity in MCF-7 is supershifted with the AP antisera. Densitometer analysis indicated that ~75% of the AP2 activity in MCF-7 nuclear extract is supershifted by the AP2γ-specific antisera. Assuming that homo- and heterodimers of AP2γ will supershift, it is estimated that ~50% of the AP2 activity in MCF-7 cells is AP2γ.

Functional promoter analysis established an important role for AP2γ in regulating the expression of the main ERα promoter in ERα-positive cells (15). HS1 was specific to the chromatin of ERα-positive cells, and the location of HS1 coincided with the location of the AP2-binding sites. These two findings led us to hypothesize that binding of AP2γ to high affinity sites in the ERα promoter induces the HS1 site. HMECs express minimal amounts of ERα mRNA or protein and are generally considered to be ERα-negative. An adenovirus was engineered that expressed AP2γ (AdAP2γ) and was used to induce overexpression of AP2γ in HMECs. Infection of HMECs with AdAP2γ induced high levels of AP2 expression, which was supershifted with AP2 polyclonal antibody, SC-184 (Fig. 6A). However, no
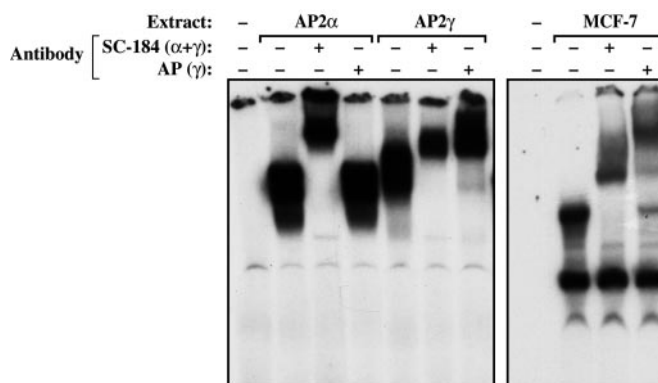

FIG. 5. **Gel shift with AP2γ-specific antisera.** Gel shift using AP2-binding site probe and purified AP2α, AP2γ-GST fusion protein (26), or MCF-7 nuclear extract. Antibody used in supershift is either SC-184, which shifts both AP2α or AP2γ, and antisera AP, which is AP2γ-specific. Free probe is not shown. The panel on the *right* shows that the majority of AP2 activity in MCF-7 cells is supershifted with AP antisera.
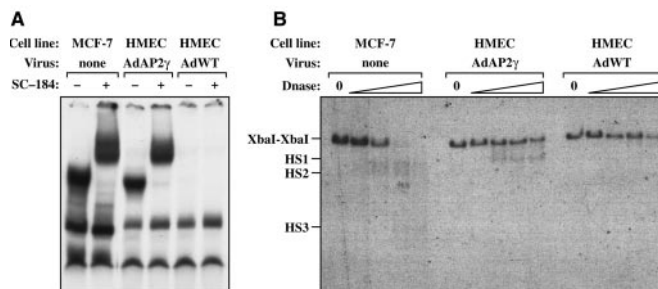

FIG. 6. **HS1 DNase I-hypersensitive site is induced in HMECs by AP2γ.** *A,* AP2 activity. *A* demonstrates that infection of HMECs with AdAP2γ virus generated AP2 activity that co-migrates with activity in MCF-7 nuclear extract and is supershifted with the AP2 antibody SC-184. *B,* hypersensitive site in HMECs. *B,* demonstrates that the AdAP2γ virus induced the HS1-hypersensitive site in HMECs. Infection of HMECs with AdWT had no effect.

AP2 activity was detected in HMECs infected with wild-type adenovirus. AP2γ expression was able to induce the HS1-hypersensitive site in ERα exon 1 (Fig. 6B). However, HS2 and HS3 were not induced by AP2γ expression. Infection of HMECs with AdWT did not induce formation of any hypersensitive sites in the ERα exon 1 region (Fig. 6B). These experiments were repeated using an adenoviral construct that expressed the AP2α protein. Identical results were obtained (data not shown) indicating that binding of either AP2α or AP2γ is capable of altering the chromatin structure of the ERα promoter and inducing the HS1 site which is a characteristic of the chromatin in ERα-positive cells.
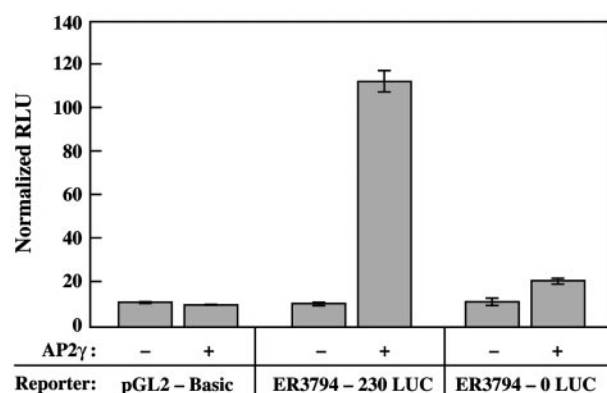
AP2γ Induces Transcription of the ERα Promoter in

FIG. 7. **ERα promoter analysis in HMECs.** HMECs were transfected with the reporter plasmids pGL2-Basic, ER3794-230LUC, or ER3794-0LUC. The AP2γ expression plasmid, AP2γ/pcDNA3.1 (26), or vector alone, pcDNA3.1, was co-transfected. Luciferase expression was normalized with a β-galactosidase control vector. Results are the average and standard deviation from three transfections.

HMECs—Expression of the endogenous ERα gene was examined in HMECs in which AP2 expression was induced by viral infection. After viral infection with AdAP2α, AdAP2γ, or AdWT, no changes in the level of endogenous ERα expression were detected in HMECs (data not shown). This result suggests that either additional factors or additional changes in chromatin structure are required to induce endogenous ERα expression in mammary epithelial cells. To investigate these possibilities further, HMECs were transfected with a reporter construct in which the ERα promoter was inserted upstream of luciferase. Fig. 7 shows the results of experiments in which an AP2 expression construct was co-transfected into HMECs with ERα promoter reporter plasmids. As seen in Fig. 7, the pGL2-Basic reporter has relatively low basal activity in HMECs. The activity of pGL2-Basic was not altered by co-transfection of an AP2γ expression construct. An ERα promoter reporter containing the untranslated leader to +230 (ER3794–230LUC) had low levels of basal expression in HMECs that was identical to the promoterless pGL2-Basic construct. However, AP2γ was able to trans-activate the ERα promoter in HMECs and induced expression from the promoter by approximately 10-fold. An ERα promoter truncation that deletes the untranslated leader (ER3794–0LUC) lacks the region of the promoter containing the HS1-hypersensitive site. ER3794–0LUC has identical basal expression in HMECs as the larger construct containing the untranslated leader. However, this construct is profoundly reduced in its ability to be trans-activated by AP2γ. These results clearly demonstrate that AP2γ can trans-activate the ERα promoter in HMECs by interacting with the region of the promoter encompassing the HS1-hypersensitive site.

DISCUSSION

Breast cancers that express ERα are more likely to occur in postmenopausal women (4), are associated with a better prognosis (6, 7), and are more likely to respond to hormonal therapy (4, 5) than tumors that do not express the receptor. ERα-positive breast cancers overexpress ERα protein and have 10–100-fold more ERα than normal mammary epithelial cells (33). Studies of breast cancer cell lines (8) and primary carcinomas (9, 10) indicate that transcriptional regulation is a critical level of control of ERα expression. The genomic map of the 5′ region of the ERα locus described in this study provides a physical map of the ERα cap sites and the location of regulatory regions involved in transcriptional control. Thus, these data provide the basis for a functional analysis of the alternate ERα promoters in breast cancer. The hypersensitive site, HS1, is invariably

found to be a feature of chromatin in cell lines that express ERα. The location of the HS1 site corresponds to binding sites for the AP2 transcription factors in the ERα promoter, and the HS1 site can be generated in HMECs by expression of the AP2γ transcription factor. We have further shown that AP2γ can trans-activate the ERα promoter in HMECs by interacting with the region of the promoter encompassing the HS1 site. Since ERα expression is necessary for hormone response, these results provide further evidence that overexpression of AP2γ is a critical mechanism in the oncogenesis of hormone-responsive breast cancer.

The exons 1–8 of the ERα gene encode the ERα protein and span a region of ~160 kbp of genomic DNA (34). We have developed a contig map that includes all of the known 5′ non-coding exons of the ERα gene that adds an additional 160–170 kbp to the ERα gene locus, which brings the total size of the ERα locus to ~300 kbp. We were surprised to find that exon Ha is ~124 kbp upstream of the coding region of the ERα gene and that the intron between exon Hb and the splice acceptor site in exon 1 is over 110 kb. Exon C has been described as a liver-specific ERα exon (13). Exon C has been reported to splice to exon Hb (14), and it was fortuitous that this exon was also in the contig. Exon C is located ~30–40 kbp upstream of exon Ha, contrary to the genomic location for exon C in a previous report (25).

The two alternate ERα transcripts, E and H, are both initiated with genuine cap sites indicating that there are separate ERα promoters controlling expression of the ERα gene. In addition, there are multiple cap sites for exons Ha and exon E, as demonstrated by the S1 nuclease analysis and 5′-RACE results (Fig. 2). In the case of exon Ha, these cap sites are clustered over ~70 bp, whereas for exon E, there appears to be two separate clusters of cap sites separated by 120 bp. Multiple cap sites are a common feature of TATA-less promoters, which is consistent with the lack of clear TATA elements associated with either the exon Ha or E 5′-flanking sequence.

It is interesting that the ERα gene is controlled by several different promoters. The biologic basis for a gene having multiple promoters that encode the same protein is not entirely clear. A recent paper (35) described the expression of an isoform of ERα that lacked the amino terminus of the protein. The truncated ERα isoform repressed trans-activation of the full-length ERα protein. This isoform was encoded by an H-type transcript that skipped exon 1 and spliced directly to exon 2. It seems likely that one purpose of these alternate cap sites may be to regulate expression of ERα proteins with alternate function. There may be additional factors involving tissue-specific expression that requires the existence of alternate cap sites. Defining the genomic organization, cap sites, and hypersensitive sites associated with these exons is an important step toward determining the regulation of these alternate ERα promoters.

DNase I-hypersensitive sites are regions of chromatin that are open and accessible to DNase digestion (30). These sites correspond to the location of important regulatory regions of eukaryotic gene promoters. Indeed, hypersensitive sites specific to active transcription of ERα are located near each of the ERα cap sites. However, eukaryotic regulatory signals can be far removed from a cap site, and often a hypersensitive site may identify the location of a regulatory sequence relevant to regulation of one or more promoters. Our results are in agreement with other studies that have described hypersensitive sites of the ERα gene near exon 1 (31). In addition, a new hypersensitive site, HS4, has been identified that is specific to ERα-positive cells. The HS4 site is just downstream of exon Hb and may represent a regulatory element controlling expression of ERα transcription initiated at exon Ha.

The HS1 site has been localized to the untranslated leader of the main ERα promoter in exon 1 and is found in all ERα-positive breast and endometrial carcinoma cell lines examined ((31) Figs. 3 and 4) but is not a characteristic of the chromatin structure of HMECs (Fig. 6) or ERα-negative cell lines ((31) Figs. 3 and 4). The location of the HS1 site corresponds to the region of the ERα promoter that was defined in a functional assay to be responsible for ERα-specific transcription (15, 26). This region of DNA bound a factor, initially called ERF-1 (15), that was found to be overexpressed in ERα-positive breast and ERα-positive endometrial cancer cell lines. The ERF-1 factor was cloned and found to be identical to AP2γ (27). ERF-1 in MCF-7 nuclear extract is composed of AP2γ but contains other AP2 factors as well, presumably AP2α (Fig. 5). These two AP2 factors have identical binding specificity and both are able to induce expression from the cloned ERα promoter (26). The data herein show that AP2α and AP2γ are able to generate the HS1-hypersensitive site in HMECs, indicating that these factors are able to induce changes in the chromatin structure that is known to be associated with transcription of the ERα gene. We have further shown that AP2γ can trans-activate the ERα promoter in HMECs and that trans-activation requires the region of the promoter that contains the HS1 site. These results provide compelling evidence that AP2γ has an important role in regulating transcription of the ERα gene by altering chromatin structure of the promoter.

Comparing the genomic structure and expression of the ERα gene in HMECs to hormone-responsive breast cancer can be a useful cell culture model to dissect the oncogenic processes that leads to a hormone-responsive tumor. Overexpression of AP2 factors appears to be one important step. This conclusion is supported by data that demonstrated overexpression of AP2γ in breast cancer compared with normal mammary epithelial cells (36). This report also demonstrated a significant correlation between AP2α expression and the ERα-positive breast cancer phenotype (36). However, we were not able to detect an increase in ERα mRNA in HMECs infected with the AdAP2γ or AdAP2α viruses (data not shown). This result is not surprising since other investigators (20) have reported the existence of factors in ERα-negative cells that repress expression of ERα. In addition, other HS1 sites are present within the promoter region of the ERα gene that were not generated by expression of AP2 factors alone (Fig. 6) indicating that other trans-activating factors may also be necessary to induce overexpression of the ERα gene in HMECs. Identifying other factors involved in the regulation of ERα gene transcription will be an area for further investigation. In addition, there may be other changes of chromatin structure that are needed to induce overexpression of ERα. Determining what other changes, in addition to AP2, are required to induce overexpression of ERα will help to

define the mechanisms of oncogenesis of hormone-responsive breast cancer.

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Genomic Structure of the Promoters of the Human Estrogen Receptor-α Gene Demonstrate Changes in Chromatin Structure Induced by AP2 γ

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