Overexpression of transcription factor AP-2α suppresses mammary gland growth and morphogenesis

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Abstract

AP-2 transcription factors are key regulators of mouse embryonic development. Aberrant expression of these genes has also been linked to the progression of human breast cancer. Here, we have investigated the role of the AP-2 gene family in the postnatal maturation of the mouse mammary gland. Analysis of AP-2 RNA and protein levels demonstrates that these genes are expressed in the mammary glands of virgin and pregnant mice. Subsequently, AP-2 expression declines during lactation and then is reactivated during involution. The AP-2α and AP-2γ proteins are localized in the ductal epithelium, as well as in the terminal end buds, suggesting that they may influence growth of the ductal network. We have tested this hypothesis by targeting AP-2α expression to the mouse mammary gland using the MMTV promoter. Our studies indicate that overexpression of AP-2α inhibits mammary gland growth and morphogenesis, and this coincides with a rise in PTHrP expression. Alveolar budding is severely curtailed in transgenic virgin mice, while lobuloalveolar development and functional differentiation are inhibited during pregnancy and lactation, respectively. Our studies strongly support a role for the AP-2 proteins in regulating the proliferation and differentiation of mammary gland epithelial cells in both mouse and human.

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Introduction

The AP-2 family of transcription factors regulates important aspects of vertebrate embryogenesis and has also been linked to the control of cell proliferation and tumorigenesis. The AP-2 proteins constitute a distinct class of transcription factor, which is defined by the presence of a “basic–helix–span–helix” DNA binding and dimerization motif (Williams and Tjian, 1991a,b). To date, four AP-2 genes have been characterized in mammalian species, AP-2α, AP-2β, AP-2γ, and AP-2δ (Bosher et al., 1996; Chazaud et al., 1996; Imagawa et al., 1987; Mitchell et al., 1987; Moser et al., 1995; Williams et al., 1988; Zhao et al., 2001). Considerable sequence identity exists between the DNA binding domains of these four AP-2 proteins, and they can all bind as homo- or heterodimers to a GC-rich element typified by the consensus sequence 5’-GCCNNNGGC-3’ (Bosher et al., 1996; McPherson and Weigel, 1999; Mobhibullah et al., 1999). Many genes that mediate cell growth, cell shape, cell movement, and cell communication frequently possess important AP-2 binding sites as a component of their cis-regulatory sequences (Bosher et al., 1995; Gaubatz et al., 1995; Gille et al., 1997; McPherson et al., 1997; Zeng et al., 1997). Indeed, the finding that genes, including cytokeratins, E-cadherin, integrins, and matrix metalloproteinases, are regulated by AP-2 reflects an important role for these transcription factors in morphogenesis (Byrne et al., 1994; De Clerk et al., 1994; Fini et al., 1994;
Hennig et al., 1996; Mitchell et al., 1987; Somasundaram et al., 1996; Zutter et al., 1994). Currently, the AP-2α, AP-2β, and AP-2γ genes are the best characterized. During embryogenesis, all three genes are expressed in tissues undergoing complex morphogenetic changes, principally in the neural crest, neural tube, kidney, eye, facial prominences, and limb buds (Chazaud et al., 1996; Mitchell et al., 1991; Moser et al., 1997b). The expression of the individual AP-2 genes often coincides in particular tissues, but overall, each family member has a unique spatiotemporal pattern of expression. Mouse molecular genetic studies have demonstrated that there is a critical requirement for the AP-2 genes during mammalian development. A homozygous null mutation for any of the AP-2 genes results in lethality either during embryogenesis or shortly after birth. In particular, the disruption of the AP-2α gene leads to defects in the formation of the neural tube, face, eyes, heart, and limbs during embryogenesis (Brewer et al., 2002; Nottoli et al., 1998; Schorle et al., 1996; West-Mays et al., 1999; Zhang et al., 1996). Moreover, these AP-2α knockout mice lack a ventral body-wall, resulting in a severe thoracoabdominoschisis. Mice lacking the AP-2β gene die shortly after birth, due to inappropriate morphogenesis of the kidneys (Moser et al., 1997a), but do not display any other major developmental abnormalities. AP-2γ-null mice die around E7.5, shortly after implantation (Auman et al., 2002; Werling and Schorle, 2002). In this instance, the defects occur within the extraembryonic cell lineages and there is no requirement for AP-2γ in the embryo proper (Auman et al., 2002). The fact that none of the AP-2 knockout mice survive until weaning indicates the collective importance of the AP-2 genes, but at present, precludes an understanding of how these genes might regulate later developmental events, in particular mammary gland maturation.

Although much is known about the spatiotemporal regulation of the AP-2 genes during embryogenesis, little information exists concerning their expression in the postnatal mammary gland. Nevertheless, it is clear from studies of human tissue samples that the AP-2 proteins can be expressed in the adult breast (Turner et al., 1998). Moreover, several studies have indicated that the AP-2 proteins may regulate gene expression in breast cancer (Gee et al., 1999; Turner et al., 1998). The AP-2α, AP-2β, and AP-2γ proteins have been detected in breast cancer biopsies, and there is a significant up-regulation of AP-2γ expression in early-stage breast tumors compared with benign tissue (Turner et al., 1998). The presence of various combinations of the AP-2 proteins in tumor tissue also coincides with the expression of several genes involved in cell growth and proliferation. Significant correlations have been reported for the presence of the insulin-like growth factor I receptor (IGF-I-R) and AP-2γ expression, the estrogen receptor (ER) and AP-2α, and p21waf1/cip1 and AP-2 family members (Gee et al., 1999; Turner et al., 1998). However, the most striking finding was that the majority of tumors containing significant levels of ErbB2 (HER2/Neu) protein expressed both the AP-2α and AP-2γ proteins (Turner et al., 1998). The significance of these various correlations is strengthened by the finding that AP-2 binding sites are present within the promoters of the human IGF-I-R, ER, p21waf1/cip1, and ErbB2 genes (Bosher et al., 1995; McPherson et al., 1997; Turner et al., 1998; Zeng et al., 1997). Thus, AP-2 may regulate the transcription of these targets in human breast cancer. Evidence obtained in vitro concerning the role of AP-2 in cellular transformation suggests two alternative hypotheses for the presence of increased expression of AP-2 in breast cancer (Kannan et al., 1994; Zeng et al., 1997). One possibility is that the AP-2 proteins are proliferative signals that activate a variety of growth factor pathways. An alternative hypothesis is that the AP-2 proteins are more akin to tumor suppressors, and the activation of the AP-2 genes would represent a failed attempt to halt cell proliferation. However, it has not been possible to distinguish between these potential roles for AP-2 using the current strains of AP-2 knockout mice, due to their early lethality. Therefore, in the current analysis, we have generated and analyzed transgenic mice in which the AP-2α gene is overexpressed in the postnatal mammary gland. We have combined these transgenic studies with an analysis of AP-2 expression during normal mammary gland maturation. Taken together, our findings demonstrate that the AP-2 proteins can regulate the growth and morphogenesis of the mammary gland. In particular, the data support the hypothesis that the presence of exogenous AP-2α inhibits proliferation of the ductal epithelia, and we therefore infer that the endogenous AP-2 genes may be activated for the same purpose in human breast cancer.

Materials and methods

Tissue collection and histological analysis

Mammary gland tissue samples were obtained by removing the fourth inguinal gland from mice at various stages of postnatal development, i.e., from virgin, pregnant, and lactating mice, and from mice in which the mammary gland was regressing. In virgin wild-type and transgenic mice, mammary glands were removed at equivalent stages of the estrous cycle (Rugh, 1968). In lactating mice, the pups were removed from the mothers 5–6 h before sacrifice and mammary gland isolation. The tissue specimens were fixed with freshly made 10% neutral buffered formalin or 4% paraformaldehyde, dehydrated in a graded series of ethanol and xylene, then embedded in paraffin wax. Sections of 5 μm were cut and mounted on poly-L-lysine-coated slides. These sections were stained with hematoxylin and eosin or used for immunohistochemical analysis. Whole-mount analysis of mouse mammary tissue was performed as previously described (Wysolmerski et al., 1995). The detection of β-galactosidase activity in mouse mammary tissue was as described (Brisken et al., 1998), and slides were counter-
Fig. 1. Expression of the AP-2 gene family in the mouse mammary gland. (A) RNase protection analyses of AP-2α (upper panel), AP-2β (middle), and AP-2γ (lower) expression at various stages of mammary gland maturation (as indicated at top: V, virgin; Preg, pregnant; Lact, lactation; Reg, regressing). Samples were normalized to β-actin RNA (data not shown), except during lactation, when the RNA levels of the milk protein genes skewed the total RNA population. Note that two to three mice were used for the virgin and regression stage RNA samples, while one mouse was utilized for the other stages. (B–G) Detection of AP-2 protein expression in the mouse mammary gland. The presence of AP-2α (brown) was detected in cell nuclei by using the 3B5 monoclonal antibody.
Fig. 2. The expression of β-galactosidase in the mammary glands of mice heterozygous for the AP-2α lacZ knock-in allele. The detection of β-galactosidase activity was performed on whole-mount preparations (A, C, E, G), and subsequently visualized in cross-section (B, D, F, H). Mammary glands were derived from mice at 5 weeks of age (virgins; A and B); at 10 days of pregnancy (C and D); at 7 days of lactation (E and F); and during regression, 7 days after weaning (G and H).

(B–D). AP-2α protein was visualized by using the γ96 polyclonal antiserum (E–G). Sections shown are ductal tissue (B, E) and terminal end bud structures (C, F) from a 7-week virgin mouse; and alveolar tissue from a mouse at 14 days of pregnancy (D, G). Body cells (arrow) and cap cells (arrowhead) of the terminal end bud are marked in (C) and (F).
stained with nuclear fast red. The generation and characterization of mice containing the AP-2α lacZ knock-in allele have been described elsewhere (Brewer et al., 2002).

**Antibodies and immunohistochemistry**

Immunohistochemical analysis of endogenous AP-2 protein expression was performed by using either an AP-2α-specific monoclonal antibody (3B5) or an AP-2γ-specific polyclonal antiserum (γ96), as previously described (Turner et al., 1998; Zhang et al., 1996). The analysis of transgene expression was performed by using the polyclonal antiserum SC-807 (Santa Cruz), which recognizes the FLAG epitope tag. Sections were incubated with a 1:100 dilution of SC-807 overnight at 4°C and subsequently processed for reactivity by using the avidin–biotin peroxidase technique (Vectastain ABC Kit; Vector Laboratories). The assessment of cell proliferation in mammary tissue was performed by using the Phosphohistone H3 (Ser10) (6G3) (Cell Signaling Molecular Probes). Images were captured on a SPOT 2 digital camera by using a Leica microscope with fluorescence optics, and were manipulated in Adobe Photoshop. Cell proliferation was also measured by using the BrdU Cell Proliferation Kit (Boehringer Mannheim) or the Fluorescence-activated Cell Sorting Kit (Oncogene Research Products). Briefly, 13.5-day or 18.5-day pregnant transgenic or wild-type mice were injected with bromodeoxyuridine solution (50 mg/kg, i.p.) and euthanized 2 h later. The fourth inguinal mammary gland was isolated and fixed in 4% paraformaldehyde. Following immunological detection of BrdU on sectioned material, the number of BrdU-positive cells per 1000 epithelial cells was counted from 5 different randomly chosen areas in the mammary gland tissue sections, and the data were analyzed by using the Student’s t test.

For apoptotic cell analysis, frozen sections were cut at 12 μM and mounted on poly-l-lysine-coated slides. The TUNEL assay was performed by using the “In Situ Cell Detection Kit” (Bohringer Mannheim) or the “Fluorescent DNA Fragmentation Kit” (Oncogene Research Products), and slides were then counterstained with nuclear fast red. Data were quantitated as above for the BrdU-labeled tissue.

**Construction of transgenes**

Transgenes were constructed by using standard subcloning techniques. The plasmid SPRSV-AP2 (Williams and Tjian, 1991a) was used as a starting vector for attachment of a sequence encoding a FLAG epitope tag to the 3′ end of the human AP-2α cDNA. Specifically, the sequence 5′-GGC GAT TAC AAA GAC GAC GAT AAA TAG GAA TTC CTC GAG -3′ was used to replace the normal TGA stop codon as well as the 3′ flanking sequences up to the XhoI site, which is present in the adjacent polylinker. The new plasmid, termed SP(RSV)AP-2 FLAG, encodes a version of AP-2α in which the normal 437-amino-acid protein is extended by 10 residues (gly gly asp tyr lys asp asp asp lys Stop), of which the final 8 encode the FLAG epitope. This tag enables expression of the transgene to be distinguished from the endogenous gene at the level of RNA and protein expression. The AP-2 FLAG fusion construct was then placed under the control of mouse mammary tumor virus long terminal repeat (MMTV LTR). The SP(RSV)AP-2 FLAG plasmid possesses a second polyclonal sequence immediately upstream of the AP-2α cDNA open reading frame. A HindIII site is present at the 5′ end of this polylinker, and an initiator codon occupies the last three nucleotide positions, 5′-AAG CTT GAA TTC GGT ACC CGC CAT G-3′. The SP(RSV) AP-2 FLAG plasmid was digested with the restriction enzyme HindIII and subsequently repaired with T4 DNA polymerase in the presence of dNTPs. The AP-2α cDNA insert was then removed by digestion with XhoI and placed between the Smal and XhoI sites of the plasmid pMSG (Amersham Pharmacia) to generate the plasmid MSG AP-2 FLAG. The fidelity of the construct was confirmed by DNA sequence analysis. For microinjection, MSG AP-2 FLAG was digested with HindIII and XbaI restriction enzymes, and the fragment corresponding to the transgene was then separated from vector sequences by sucrose density gradient ultracentrifugation. The fractions that contained the transgene fragment were collected and dialyzed extensively against 10 mM Tris–Cl, 0.25 mM EDTA, pH 7.5, and the DNA concentration was adjusted to 2 μg/ml prior to microinjection.

**Generation of transgenic mice**

Transgenic mice were generated by injecting DNA into pronuclei of fertilized eggs of inbred FVB mice (Taconic). The embryos surviving the microinjection were transferred into the oviducts of pseudopregnant females (CD1 strain; Charles River). After birth, founders carrying the transgene were identified by Southern blot analysis of genomic DNA. The offspring of the founders carrying the transgene were identified by PCR. Transgene expression was monitored by RNase protection of total RNA isolated from mammary tissue of 14-day pregnant mice.

**Isolation of genomic DNA, southern blot, and PCR analysis**

Genomic DNA was isolated from tails of 3- to 4-week-old mice as described (Laird et al., 1991). A 12-μg aliquot of genomic DNA was digested with the restriction enzyme BglII, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose (Schleicher & Schuell) or Hybond N filters (Amersham). The filter was hybridized with a Smal–NcoI fragment corresponding to nucleotides 557 to 1290 of
the human AP-2α cDNA (Williams et al., 1988). This DNA fragment was either radioactively labeled by random-priming in the presence of 32P-dATP, or labeled nonradioactively with the “Genius Nonradioactive Nucleic Acid Labeling and Detection Kit” (Boehringer-Mannheim). The hybridized products were visualized by autoradiography or by light-emission, respectively. PCR was performed by using forward primer MMTV-5 (corresponding to MMVT LTR nucleotides 7591–7611 in the plasmid pMSG): 5’-TCA CAA GAG CGG AAC GGA CTC-3’, and reverse primer TWY3 (corresponding to positions 128–111 of the human AP-2α cDNA): 5’-GCT GGT GCC GTC GTC ACG-3’. PCR conditions were: 1 cycle at 94°C for 1 min 20 s; 32 cycles at 94°C for 45 s, 53°C for 45 s, 72°C for 2 min; and 1 cycle at 72°C for 10 min. A fragment of 320 bp was amplified from mice containing the transgene.

Isolation of RNA, RNase protection, and in situ hybridization

Mouse mammary tissue was homogenized in guanidine isothiocyanate buffer, and the total RNA was isolated as described (Chomczynski and Sacchi, 1987). RNase protection was performed essentially as published (Williams et al., 1988), except that 10 μg of total RNA was used for each protection assay. Riboprobes specific for transcripts corresponding to the human AP-2α transgene, or the endogenous mouse AP-2α, AP-2β, or AP-2γ genes, were generated from the following four plasmids. The plasmid TRIP1 contains the mouse AP-2α cDNA sequences corresponding to amino acids 97 to 437 inserted between the HindIII and EcoRI sites of pBS II KS— (Stratagene). The plasmid JSmuBS contains the mouse AP-2β cDNA sequences corresponding to amino acids 66 to 214 inserted between the HindIII and Acc65I sites of pBS II SK— (Stratagene). The plasmid mu-γ-3’ contains the mouse AP-2γ cDNA sequences corresponding to amino acids 364 to 449 inserted between the HindIII and KpnI sites of pBS II SK— (Stratagene). The plasmid P4 contains the human AP-2α cDNA corresponding to amino acids 1 to 437, and the sequences encoding the C-terminal FLAG epitope tag, inserted between the EcoRI and XhoI sites of pBluescript SK+ (Stratagene). TRIP1, JSmuBS”, mu-γ, and P4 were linearized with MluI, Xbal, HindIII, and NcoI, respectively, and the cRNA probes were synthesized with T7 RNA polymerase in the presence of α32P UTP. The protected fragments were 580 nt for AP-2α, 450 nt for AP-2β, 300 nt for AP-2γ, and 150 nt for the human transgene. The riboprobe specific for the transgene incorporated sequences corresponding to the FLAG epitope tag, as well as a 120-nt fragment of the 3’ end of human AP-2α cDNA (see Fig. 3). A 380-bp mouse PTHrP cDNA fragment, corresponding to nucleotide positions 121–501 of exon 5 (from GenBank Accession No. M60057), was derived from total cellular RNA of 12-day pregnant mammary tissue by RT-PCR using the primers 5’-TCT AGG TTC CAA GGA CAC GTT ACA GGA TT-3’ (forward) and 5’-CAT GAA TTC ATG CAC AGG AAA TCA GT-3’ (reverse). Subsequently, this fragment was subcloned into the EcoRI site of pBS II KS (Stratagene), and an antisense riboprobe was made by using T3 RNA polymerase after linearization with BamHI. A 231-bp p21 waf1/cip1 cDNA fragment, corresponding to nucleotides 20–241 (from GenBank Accession No. U24173), was obtained similarly, using the primers 5’-ATTCC GAA TTC GTC AGA GTC TAG GGG AAT TG-3’ (forward) and 5’-ATCC GGA TCC ACG AAG TCA AAG TTC CAC CG-3’. The PCR product was digested with EcoRI and BamHI and subcloned into the corresponding sites of pBS II KS (Stratagene). Subsequently, the plasmid was linearized with EcoRI, and an antisense transcript was obtained by using T7 RNA polymerase. The mouse K18 cDNA was a gift of Dr. Robert Oshima (the Burnham Institute), and a riboprobe was made by using SP6 RNA polymerase after the plasmid was linearized with EcoRI. The β-actin control riboprobe was obtained from Ambion, Inc (Austin, TX). The mouse EGF-R, ErbB2, ErbB3, whey acidic protein (WAP), β-casein, and α-lactalbumin cDNAs were obtained from Drs. Frank Jones and David Stern in the Department of Pathology at Yale University School of Medicine. The ErbB2 and ErbB3 plasmids were both linearized with BamHI, prior to transcription with T7 RNA polymerase, while the EGF-R plasmid was linearized with HindIII and transcribed with T3 RNA polymerase. In situ hybridization with the other plasmids was performed essentially as described (Jones et al., 1999), except that probes were radiolabeled by using α32P UTP.

Results

Expression of endogenous AP-2 genes in the mouse mammary gland

The majority of mammary gland development occurs during puberty and pregnancy, in response to changes in hormone levels (Daniel and Silverstein, 1987; Henighausen and Robinson, 1998). At birth, the mammary gland consists of a rudimentary network of ducts that occupies a small region of the fat pad in the vicinity of the nipple. Outgrowth and bifurcation of the ducts occur in response to estrogen during puberty. Expansion of the lobuloalveolar network and differentiation into a secretory organ occur in pregnancy and lactation, respectively. Weaning leads to involution, and the mammary gland is eventually remodeled to resemble the virgin state. To determine how the AP-2 genes are regulated during these various stages of postnatal mammary gland development, we examined both RNA and protein expression. Transcripts corresponding to all AP-2 family members, with the exception of AP-2δ, were readily detected in mammary tissue (Fig. 1A, and data not shown). RNase protection analysis indicated that the AP-2α, AP-2β, and AP-2γ genes were all expressed in a
Fig. 3. The MMTV-AP-2α transgene. (A) The structure of the MMTV-AP-2α construct. The locations of the PCR primers used to identify transgenic progeny (arrows), and of the cRNA probe used for the RNase protection analyses (underlined), are shown. (B) RNase protection analysis of transgene expression in the mammary tissue of transgenic founder offspring. The numbers refer to the identity of the founder mice. (C–F) Detection of transgene protein expression using a polyclonal antiserum specific for the FLAG epitope tag. Mammary tissues were derived from normal mice (C, E) or from siblings that possessed the MA44 transgene insertion (D, F). Mammary tissues were obtained from 6-month-old virgin mice (C, D) and mice at 14 days of pregnancy (E, F). The locations of ductal and alveolar epithelial cells are indicated, respectively, by arrows and arrowheads.
similar manner at various stages in the growth of the mammary gland (Fig. 1A, and data not shown). Transcripts from all three genes were readily detected in the early virgin mammary tissue, and expression levels continued to increase as the mammary gland matured. Higher levels of AP-2 expression were observed in pregnant mammary tissue. As gestation progressed, the expression of all three AP-2 genes continued to increase and reached a peak at 15 days of pregnancy, the latest pregnant mammary tissue analyzed. The expression of these genes was sharply reduced in lactating mammary tissue, but expression was reactivated during the stage of involution.

We next utilized immunological reagents specific for AP-2α and AP-2γ to examine the mammary gland cell types in which these two AP-2 proteins were expressed (Turner et al., 1998; Zhang et al., 1996). Immunohistochemical analysis with these reagents revealed that both the AP-2α and AP-2γ transcription factors were localized in the nuclei of the ductal epithelium of the virgin mouse mammary gland (Fig. 1B and E). Staining for both proteins was also apparent in the body cells of the terminal end buds (Fig. 1C and F). At later stages, the presence of AP-2α and AP-2γ could also be observed in the alveolar epithelium of pregnant mice (Fig. 1D and G). The AP-2 proteins were reduced to an undetectable level in the lactating mammary gland, but became evident again during involution (data not shown). The AP-2β protein was expressed in a similar developmental profile in the ductal and alveolar epithelia, although in this instance, detailed examination was not possible with the available immunological reagents (data not shown). Together, these data show an excellent correlation with the RNase protection analyses concerning the developmental profile of AP-2 expression in the mouse mammary gland. However, there were qualitative differences between the expression of AP-2α and AP-2γ. First, during the initial phase of lactation, AP-2γ transcripts were maintained at higher levels than AP-2α transcripts. Second, in both virgin and pregnant animals, the AP-2α protein was...
present in the majority of ductal and alveolar epithelial cells. In contrast, AP-2γ was expressed in approximately 70–80% of the virgin ductal epithelial cells and displayed variegated expression in the alveolar clusters during pregnancy. Further differences between AP-2α and AP-2γ expression were observed in the terminal end buds (TEBs). These structures are responsible for the outgrowth of the ductal network into the surrounding fat pad. Two cell populations, termed cap cells and body cells, have been identified within the TEB. Cap cells will form the myoepithelial layer, and body cells will form ductal epithelial tissue. The AP-2α protein was present in the majority of both the body (Fig. 1C, arrow) and cap cells (Fig. 1C, arrowhead), although the latter cells had lower expression levels. AP-2γ was also present in both body and cap cells. However, AP-2γ was present within fewer cells of the TEB, and the level of expression within an individual body cell (Fig. 1F, arrow) or cap cell (Fig. 1F, arrowhead) was equivalent.

We have recently generated a mouse strain in which the bacterial lacZ gene is inserted in-frame into the coding region of the AP-2α gene locus (Brewer et al., 2002). Mice that are heterozygous for this allele are viable and fertile and provide an additional means by which the expression profile of the AP-2α locus can be followed during postnatal development of the mammary gland. In these mice, β-galactosidase activity is detected in the ductal epithelium and TEBs of virgin animals, and continues to be expressed in the ducts and lobuloalveoli during pregnancy (Fig. 2A–D). Expression of the AP-2α lacZ allele is undetectable during lactation, but β-galactosidase activity is restored during regression (Fig. 2E–H). These results provide an independent confirmation of our previous findings concerning the spatial and temporal expression pattern of the AP-2α gene in the postnatal mammary gland.

**Generation and characterization of AP-2α transgenic mouse lines**

The dynamic expression profile of the AP-2 proteins in the mammary gland suggested that these transcription factors could be involved in regulating gene expression during mammary gland growth and morphogenesis. We utilized a transgenic approach to test this hypothesis. The human AP-2α cDNA was placed under the control of the MMTV LTR (Fig. 3A), and injected into one-cell mouse embryos. The human and mouse AP-2α proteins are almost identical at the protein level, differing by only 1 out of 437 amino acids. The utilization of the human transgene ensured that its expression would be readily distinguished from the endogenous mouse gene by RNase protection analysis. We also attached a FLAG epitope tag to the C terminus of the transgene, and this tag enables the protein derived from the transgene to be distinguished from the endogenous AP-2α protein. Ten transgenic founder mouse lines were generated as assessed by Southern blot analysis. To examine transgene expression, RNase protection assays were performed on offspring of the MMTV AP-2α founders by using total RNA isolated from mammary tissue of 14-day pregnant mice. Seven of these transgenic lines expressed significant levels of the human AP-2α transgene in the mammary glands (Fig. 3B), and three lines, MA7, MA14, and MA44, were kept for further analysis. We also determined that, in these three lines, lower levels of transgene expression occurred in the lung, salivary gland, and kidney, but no expression was detected in the ovaries (data not shown). With the exception of the mammary gland phenotype discussed below, no gross anatomical differences between transgenic mice and their wild-type littermates were detected in any tissue.

We next utilized immunohistochemistry to examine the relative expression levels of the transgene in the various mammary gland cell populations of the MA44 transgenic mice and nontransgenic siblings (Fig. 3C–F). Mammary tissues taken from both virgin and pregnant mice were probed with a polyclonal antiserum specific for the FLAG epitope tag. Transgene expression was clearly detected in both ductal (arrows) and alveolar (arrowheads) epithelial cells of the MA44 mouse line (Fig. 3D and F). In contrast, transgene product was not detected in these cell types in nontransgenic sibling mammary tissue (Fig. 3C and E). Note that expression of the transgene was found to be nonuniform in the ductal epithelia of the mammary gland at various stages of development (for example, Fig. 3D). Variegated expression is frequently seen for mammary gland transgenes driven by the MMTV, WAP, or β-lactoglobulin promoters (Barash et al., 1999; Deckard-Janatpour et al., 1997; Faerman et al., 1995; Jones and Stern, 1999; Jones et al., 1999; Robinson et al., 1995). There is also evidence that endogenous genes, such as the progesterone receptor and those encoding milk proteins, are not uniformly expressed in all cells of the ductal or lobuloalveolar epithelium of the mouse mammary gland (Robinson et al., 1995; Silverstein et al., 1996). Therefore, the differential transgene staining we observe is not atypical. Nevertheless, the mosaic nature of our transgene’s expression does preclude a straightforward morphometric analysis of the mammary gland as a whole.

**Overexpression of AP-2α inhibits alveolar budding in the virgin mouse mammary gland**

As shown above, the analysis of the FLAG-stained tissue from virgin and pregnant mice indicated that we had been successful in targeting the AP-2α transgene to the appropriate mammary epithelial layers. It was also evident from an examination of the sections at lower magnification that there was a reduced amount of epithelial tissue in the mice that overexpressed AP-2α compared with their wild-type siblings (data not shown, and see below). To determine further the effects of overexpression of AP-2α, mammary glands from virgin, pregnant, and lactating mice were examined by whole-mount analysis. All three transgenic lines
studied in our analysis (MA44, MA14, and MA7) generated a similar mammary gland phenotype (data not shown). Since the MA44 transgenic line produced the most pronounced alterations in morphology, mammary glands from these mice were studied in detail. The initial phase of postnatal mammary gland development, involving ductal outgrowth through the mammary fat pad, was not affected in the transgenic mice. Indeed, a comparison of whole-mount mammary gland tissues from a normal littermate (Fig. 4A) and a transgenic mouse (Fig. 4B) at 8 weeks of age indicated that the ductal network had filled the entire fat pad in both instances. In older virgin mice, however, there was a dramatic difference in the morphology of the ducts. At 6 months of age, the ductal system of a normal littermate had a highly branched organization that was associated with multiple alveolar bud structures (Fig. 4C and E, arrows). In contrast, mammary glands from the transgenic mice displayed a simpler, sparser, ductal network. While there was no alteration in the number of major side-branches, we noted a dramatic reduction in the frequency of the alveolar buds (Fig. 4D and F, arrows).

**Overexpression of AP-2α causes defects in lobuloalveolar development during pregnancy and lactation**

Subsequent examination of the transgenic mice during pregnancy demonstrated significant underdevelopment of the lobuloalveolar structures when compared with their non-transgenic littermates. By day 15 of pregnancy, the normal mammary gland had developed well-formed alveoli (Fig. 5A and C, arrows). In contrast, the transgenic gland had far fewer alveoli, and these were smaller and more compact (Fig. 5B and D, arrows). Strikingly, ducts were clearly visible in whole-mount preparations of transgenic mammary glands from a normal littermate (Fig. 5D, arrowheads), whereas these structures were obscured by lobuloalveolar expansion in normal control mice. Histological examination confirmed that transgenic mice had fewer clusters of lobuloalveoli, and showed that each individual alveolus was smaller and more condensed as compared with the normal littermate (Fig. 5E and F). Moreover, in contrast to the abundant presence of cellular and luminal lipids in normal littermates at this stage (Fig. 5E, red arrows), we did not observe the accumulation of such lipids in the lobuloalveoli of transgenic mice (Fig. 5F).

We next examined whether the decreased quantity of lobuloalveolar tissue in the transgenic mice resulted from alterations in cell death and/or cell proliferation. The TUNEL assay was used to examine the incidence of cell death in the mammary glands of wild type and transgenic mice at 13.5 and 18.5 days of pregnancy (Table 1, and data not shown). Few apoptotic cells were observed in the wild type mammary epithelial tissue at either time point (0.38 and 0.16% cells, respectively), but there was a significant increase in apoptosis at both stages of mammary gland development in the transgenic mice (0.96 and 0.5% cells, respectively). In the transgenic mammary glands, apoptotic cells were often found in regions that contained the characteristic compact alveolar tissue described above (data not shown). We next determined the distribution of proliferating cells after 2 h of BrdU labeling in wild type and transgenic mammary glands at 13.5 and 18.5 days of pregnancy (Table 2). In both strains of mice, the greatest numbers of labeled cells were observed at the 13.5-day time point, when the alveolar network is rapidly increasing in size. However, at this time point, nontransgenic mammary glands displayed two- to threefold greater levels of proliferating cells than their transgenic counterparts. Similar differences in proliferation between the two strains of mice were also observed at 18.5 days of pregnancy. We also examined cell proliferation in the wild type and transgenic animals using immunohistochemistry for the phosphohistone H3 cell proliferation marker. Again, we found that there were more labeled cells in wild-type than transgenic mammary glands at these two time points and the quiescent state tended to coincide with regions of high transgene expression (data not shown). Note that the differences seen in proliferation and apoptosis between wild type transgenic mice probably underestimate the influence of ectopic AP-2α expression on these cellular processes since the MA44 mammary glands are mosaic, not uniform, with respect to transgene expression. Taken together, these findings indicate that the reduction in lobuloalveolar tissue in the transgenic mice results from a combination of decreased proliferation and increased cell death.

Morphological differences between the mammary glands of transgenic and control mice were also apparent during lactation (Fig. 6). Whole-mount staining performed at 1 day postpartum revealed an extensive secretory network in normal mice, in which lobuloalveoli were expanded due to the presence of lactation products (Fig. 6A). In contrast, the lobuloalveoli of transgenic mice were sparser and remained more condensed (Fig. 6B). One consequence of the underdeveloped lobuloalveolar structures is that the ducts were still clearly visible in the transgenic mice compared with their normal siblings (Fig. 6B, blue arrows). The ducts themselves had a similar distended appearance in both wild-type and transgenic animals, consistent with milk secretion (see below). Histological analysis at 1 day postpartum revealed further differences between wild-type and transgenic mammary glands. First, more lobuloalveolar clusters were present in the normal mammary glands (Fig. 6C) than in sibling transgenic mice (Fig. 6D). Second, alterations were also apparent with respect to the overall architecture of the mammary gland and lobuloalveolar morphology. In control animals, the lobuloalveolar lumens were engorged with lactation products, and the alveolar epithelial cells had a flattened morphology, indicating that these cells had undergone secretory differentiation (Fig. 6C). Moreover, although there was a limited degree of heterogeneity in the control animals, the lobuloalveolar structures from these mice were generally uniform in appearance throughout the...
gland. In contrast, the mammary glands from transgenic mice displayed considerable heterogeneity in the morphology of the lobuloalveoli (Fig. 6D). A subset of the gland contained alveolar epithelial cells typical of this maturation stage (Fig. 6D, arrows), although sometimes the lumens in these regions could be more distended, a phenotype also noted for mice carrying a dominant negative FGFR2 transgene (Jackson et al., 1997). However, other regions of the gland appeared to be at an earlier stage in the maturation process, since alveolar cells were more cuboidal in shape, and the lumens contained lower amounts of lactation products (Fig. 6D, arrowheads).

Fig. 5. Morphology of transgenic mammary glands at 15 days of pregnancy. (A–D) Whole-mount preparations are shown of the fourth inguinal mammary gland from either a normal mouse (A, C) or a transgenic sibling (B, D). (C, D) Higher magnifications of the periphery of the glands shown in (A) and (B). Arrows indicate alveoli, and arrowheads show ducts. (E, F) Histology of mammary tissue taken from a normal mouse (E) or from a transgenic sibling (F). Red arrows indicate intracellular lipids; red arrowheads indicate condensed lobuloalveoli.
By 7 days postpartum, lobuloalveolar lumens were prominent throughout the whole mammary pad of nontransgenic mice (Fig. 6E). Histological sections of these normal mice indicated that stromal fat cells were rarely observed and that the lobuloalveolar lumens were engorged with secretory proteinaceous materials (Fig. 6E, red arrows). In contrast, fat cells were still readily apparent in the transgenic mammary glands at 7 days postpartum (Fig. 6F, red arrowheads). Pups were removed from the mothers 5–6 h before sacrifice and mammary gland isolation.

Fig. 6. Morphology of postpartum transgenic mammary glands. (A, B) Whole-mount preparations are shown of the fourth inguinal mammary gland of a normal mouse (A), or a transgenic sibling (B), at 1 day postpartum. Blue arrows indicate ducts. (C, D) Histology of the mammary tissue shown in (A) and (B), respectively. Arrows show tissue that has a normal appearance for this stage of mammary gland maturation, and arrowheads indicate tissue that is less well-developed. (E, F) Histology of mammary tissues taken from 7 days postpartum normal (E), or transgenic (F), sibling mice. Red arrows indicate secretory proteinaceous material; red arrowheads show lipids within the immature epithelial cells; and the asterisk identifies fat cells. Pups were removed from the mothers 5–6 h before sacrifice and mammary gland isolation.
Table 1
Comparison of apoptosis levels in mammary epithelial cells between transgenic and wild type mice

<table>
<thead>
<tr>
<th>No. apoptotic cells per 1000</th>
<th>at 13.5 days of pregnancy</th>
<th>at 18.5 days of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA44 transgenic</td>
<td>Nontransgenic</td>
<td>MA44 transgenic</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Average 9.6</td>
<td>Average 3.8</td>
<td>Average 5</td>
</tr>
<tr>
<td>0.96%</td>
<td>0.38%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Note. Student’s t test probability at 13.5 days: $P = 0.001$; at 18.5 days: $P = 0.0004$.

Table 2
Comparison of proliferation levels in mammary epithelial cells between transgenic and wild type mice

<table>
<thead>
<tr>
<th>No. proliferating cells per 1000</th>
<th>at 13.5 days of pregnancy</th>
<th>at 18.5 days of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA44 transgenic</td>
<td>Nontransgenic</td>
<td>MA44 transgenic</td>
</tr>
<tr>
<td>21</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>30</td>
<td>36</td>
<td>8</td>
</tr>
<tr>
<td>17</td>
<td>48</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>56</td>
<td>5</td>
</tr>
<tr>
<td>19</td>
<td>47</td>
<td>6</td>
</tr>
<tr>
<td>Average 22</td>
<td>Average 45.8</td>
<td>Average 5</td>
</tr>
<tr>
<td>2.2%</td>
<td>4.6%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Note. Student’s t test probability at 13.5 days: $P = 0.0003$; at 18.5 days: $P = 0.016$.

Interestingly, the mammary glands of transgenic mice that overexpressed dominant negative versions of ErbB2 or ErbB4 also contained regions in which alveolar clusters more typical of late pregnancy were present during lactation (Jones and Stern, 1999; Jones et al., 1999). However, the influence of these two transgenes on lactogenesis could be distinguished by their influence on milk protein gene expression. Therefore, we further investigated the effect of exogenous AP-2α on mammary gland development by examining the expression of three molecular markers of lactation: whey acidic protein, α-lactalbumin, and β-casein. In situ hybridization studies were performed on serial sections of wild-type and transgenic mammary tissue at 1 day postpartum, and the results were compared with the presence of the FLAG epitope on adjacent sections. FLAG epitope staining revealed that exogenous AP-2α protein was present throughout the transgenic gland, in both the cuboidal epithelial cells and the more flattened epithelial cells (Fig. 7, and data not shown). However, the level of transgene expression was variable within an individual gland, with a tendency for higher levels of FLAG reactivity to occur in the epithelial cells with the less mature, cuboidal, morphology. In situ hybridization studies were performed by using sense and anti-sense probes specific for the three milk protein genes. No significant hybridization was observed with the sense probes (data not shown). Data obtained using the anti-sense probes indicated that these three milk protein genes were expressed almost uniformly in both transgenic mice (Fig. 7) and wild-type mice (data not shown). Notably, in the transgenic mice, uniform expression of these genes occurred despite the distinct lobuloalveolar morphologies present in different regions of the gland. Taken together, these findings indicated that the overexpression of AP-2α could alter lobuloalveolar morphology but did not prevent transcription of the major milk protein genes.

Next, we investigated the mechanism by which the overexpression of AP-2α might influence the postnatal development of the mammary gland. The expression of several genes known to regulate the growth and morphogenesis of the mammary gland and/or postulated to be AP-2 targets were examined in 12-day pregnant wild-type and transgenic mice, using a combination of RNase protection and semi-quantitative RT-PCR analyses (Fig. 8, and data not shown). These studies revealed an ~3-fold increase in the RNA levels for the parathyroid hormone-related protein (PTHrP) in the mammary glands of transgenic mice compared with their wild-type littermates (Fig. 8). The increase in PTHrP expression was also observed by using semiquantitative RT-PCR (J.Z., data not shown). Previous studies have shown that the PTHrP promoter is directly activated by AP-2 in vitro (Prager et al., 1994). Moreover, mice that express a PTHrP transgene in the mammary gland also display an inhibition of development (Wysolmerski et al., 1995). Therefore, the increase in PTHrP expression seen in the AP-2α transgenic mice represents a plausible mechanism by which mammary gland morphology is altered in these animals. In contrast to the changes in PTHrP expression, we did not detect any differences in transcript levels between transgenic mice and wild-type littermates for three further potential AP-2 target genes, p21(waf1/cip1), ErbB2, and ErbB3 (Fig. 8). We also found no significant alteration in the expression of several additional genes which encode growth factor signaling molecules associated with mammary gland morphogenesis, including wnt4, the progesterone receptor, the prolactin receptor, EGF-R, and ErbB4 (Fig. 8, and data not shown).
Discussion

Previous studies have demonstrated that the AP-2 family of transcription factors regulates multiple aspects of mammalian development. Formation of the neural tube, face, eyes, limbs, heart, ventral body-wall, and kidneys all rely upon the appropriate expression of the AP-2 genes during mouse embryogenesis (Brewer et al., 2002; Moser et al., 1997a; Nottoli et al., 1998; Schorle et al., 1996; West-Mays et al., 1999; Zhang et al., 1996). The observation that the human AP-2 genes are also expressed in both benign mammary epithelial tissue and early stage breast cancer indicated that these transcription factors might also be important for regulating the growth and morphogenesis of the postnatal mammary gland (Gee et al., 1999; Turner et al., 1998). Our current studies, in which we have first examined the expression profile of the AP-2α, AP-2β, and AP-2γ genes in the postnatal mouse mammary gland, and subsequently manipulated the levels of the AP-2α protein in this organ, strongly support this hypothesis.

The spatiotemporal expression pattern of the AP-2 gene family throughout mouse mammary gland maturation and remodeling was determined by using a combination of RNase protection, immunohistochemistry, and lacZ gene knock-in technology. These complimentary techniques indicated that all three genes were present in the ductal epithelia of the postnatal mouse mammary gland, but showed that they were not expressed in the surrounding myoepithelium. These findings support our previous contention that the AP-2 proteins are a regular component of the gene network expressed in normal human breast epithelial tissue (Turner et al., 1998). However, in our previous studies using human tissue samples, it was not possible to assess the expression profile of the AP-2 genes at specific stages of development. Using the mouse model system, we now show that expression of all three AP-2 genes occurs in the virgin mammary gland. AP-2 expression increases in the virgin gland during the period of ductal outgrowth and continues to rise during pregnancy as lobuloalveolar development proceeds. Comparison with cytokeratin expression indicated that the general rise in AP-2 expression reflected the large increase in mammary ductal epithelial tissue during these growth periods and was not caused by increased AP-2 levels in individual cells (J.Z., unpublished observations). In contrast, it is clear that AP-2 expression drops off dramatically in the mammary epithelial cells during lactation, before it is reactivated with the remodeling of the mammary gland that occurs during involution. Detailed examination indicated that the AP-2α and AP-2γ proteins were not only present in the ductal epithelial tissue, but were also found within the TEBs. Significant AP-2α expression occurred in the majority of the TEB body cells, while much lower levels were apparent in cap cells. In contrast, AP-2γ expression was apparent in a subpopulation of both the body cells and cap cells. Differences in the distribution of AP-2α and AP-2γ proteins within these TEB regions indicate that they may perform distinct functions in the growth and morphogenesis of the ductal network. One possibility is that high levels of AP-2α are consistent with a body cell and ductal epithelial cell lineage, while the down-regulation and eventual loss of AP-2α would mark cap and myoepithelial cells. Similarly, the differential expression of AP-2γ in the TEB might indicate cells that have different fates with respect to gene expression, proliferation, or apoptosis (Humphreys, 1999; Seagroves et al., 2000).

The expression profile of the AP-2 genes is similar to that of several key regulators of mammary gland development and maturation. In other development processes, the AP-2 transcription factors, and in particular AP-2α, control multiple aspects of growth and morphogenesis. We therefore wished to determine whether this key regulatory molecule could also influence the morphology of the mammary ductal network. Moreover, since the levels of AP-2 are increased in breast cancer, we wished to ascertain whether increased levels of AP-2α might lead to mammary tumor formation. For these purposes, we generated transgenic mice that overexpressed the AP-2α gene under the control of the MMTV LTR. Our findings indicate that the overexpression of AP-2α causes a unique spatiotemporal sequence of changes in mammary gland morphology but does not result in increased tumorigenesis. In some instances, transgene expression in the endocrine organs, especially the ovaries, can affect mammary gland morphology by changing circulating hormone levels. However, two lines of evidence indicate that AP-2α is acting autonomously within the mammary glands of our transgenic mice. First, we did not detect transgene expression within the ovaries. Second, the mammary glands examined in our studies displayed morphological heterogeneity that coincides with transgene expression. Indeed, the presence of the epitope tag served as a powerful means by which transgene expression could be correlated with morphological differences in the gland.

In the transgenic mice, there were no obvious defects in the outgrowth of the ducts, nor in the bifurcation of the individual ducts, two processes that occur during puberty in response to estrogen. However, there was a significant impairment of alveolar bud formation in the mammary glands of virgin mice. Furthermore, during pregnancy, transgenic mice had a less extensive ductal network than was observed in control mice, and the lobuloalveolar cells also remained condensed and failed to accumulate lipids at the appropriate time points. One mechanism by which the overexpression of AP-2α might directly inhibit ductal proliferation is through increased expression of the p21^waf1/cip1 cell cycle inhibitor, since the human version of this gene is regulated by AP-2 in vitro (Zeng et al., 1997). However, we were unable to detect significant changes in the levels of mouse p21^waf1/cip1 in vivo between wild-type and transgenic mammary tissue. Nevertheless, we did note that there was a significant decrease in cell proliferation in the transgenic epithelial tissue as measured by BrdU incorporation and the analysis of markers of cell proliferation, including phospho-
histone H3. These findings indicate that a reduction in actively cycling cells in the mammary gland indeed accompanied the overexpression of AP-2α, and this may in part account for the phenotype of the transgenic tissue in the virgin and pregnant animals. Cell death, which is increased in the presence of the transgene, may act as a second mechanism by which the lobuloalveolar tissue is reduced in the transgenic mice. A link between altered levels of AP-2 expression and an increase in cell death has also been previously noted in both AP-2α and AP-2β knockout mice.
The loss of AP-2 inhibinatory molecules, some of which act as targets for these signaling pathways in the ductal epithelia (Robinson et al., 2000). The misexpression of several additional key regulatory molecules of the TGF-β, FGF, and parathyroid hormone-related protein (PTHrP) signaling pathways (Jackson et al., 1997; Jhappan et al., 1993; Pierce et al., 1993; Wysolmerski et al., 1995). We have examined several of these genes to determine whether their transcript levels were altered in response to the AP-2α transgene. We did not detect any differences between transgenic mice and their wild-type littermates with respect to the progesterone receptor, the prolactin receptor, or wnt4. However, we did observe a consistent ~3-fold increase in transcripts corresponding to the signaling molecule parathyroid hormone-related protein (PTHrP) in the transgenic mice. Intriguingly, previous studies have shown that the overexpression of PTHrP in the myoepithelial compartment of the mammary gland inhibits both side-branching in virgin mice and lobuloalveolar development during pregnancy (Wysolmerski et al., 1995). Therefore, it is possible that altered AP-2α levels cause changes in mammary gland development in part by affecting PTHrP expression, especially since the PTHrP promoter can be activated by AP-2 in vitro (Prager et al., 1994). In the future, it will be of interest to determine whether components of the PTHrP pathway are indeed downstream targets for the AP-2 transcription factors. The finding that transgenic mice show no change in the expression of other postulated AP-2 gene targets, such as p21waf1/cip1, could result from a number of factors. First, the results may reflect differences in promoter activity measured here in vivo, versus in vitro, where many of these human targets were characterized. Alternatively, these findings may indicate that there are sequence differences between the promoters of the human and mouse target genes that may alter their ability to bind and respond to AP-2 (Bates and Hurst, 1997). Finally, it is also possible that a different AP-2 protein is required for expression of these target genes or that AP-2α cannot act alone but needs to function in concert with other AP-2 proteins or other transcription factors that are limiting in vivo (Turner et al., 1998).

Following parturition, the sparser appearance of the ductal tree in AP-2α transgenic mice also persisted into lactation. Even at 7 days of lactation, some areas of the gland were still occupied by lobuloalveolar structures that were more appropriate for an earlier stage of the differentiation process, namely late pregnancy or parturition. Note that we did not detect transgene expression at 7 days of lactation by either immunohistochemistry or RNase protection in these regions (J.Z., data not shown), a result that has also been observed for at least one other MMTV transgene construct (Jones and Stern, 1999). Therefore, we postulate that the earlier presence of high levels of AP-2α during late pregnancy has prevented maturation in these areas. We also hypothesize that regions having lower levels of transgene expression during pregnancy would instead escape from the influence of AP-2α and would proliferate and differentiate appropriately. Different levels of transgene expression would therefore lead to the observed variegation in the morphology of the mammary glands. This hypothesis is supported by the observation that, at 1 day postpartum, there is a higher incidence of transgene expression in regions of the mammary gland that are delayed in differentiation compared with areas that are more mature.

We further addressed the differentiation state of the mammary epithelia by studying the expression of three molecular markers of lactation: whey acidic protein (WAP), β-casein, and α-lactalbumin. Using in situ hybridization, we found that all three genes were expressed in the transgenic
mammary gland at 1 day of lactation. Moreover, transcripts were present not only in the flattened epithelial cells, characteristic of this stage of lactation, but also in the atypical and morphologically distinct cuboidal epithelial cells. Thus, our data suggest that, although the MMTV AP-2α transgene may alter the morphology of the mammary ductal epithelium, it does not inhibit the expression of the major lactation proteins. A similar relationship between abnormal morphology and normal transcript levels of these milk protein genes is typical of inhibinβB knockout mice as well as mice expressing the dominant negative ErbB2 transgene (Jones and Stern, 1999; Robinson and Hennighausen, 1997). In other instances, notably dominant negative ErbB4 transgenic mice and mice lacking the STAT5a gene, altered morphology is accompanied by a decrease in expression of one or more of the milk protein genes (Jones et al., 1999; Liu et al., 1997; Taglialento et al., 1998). When taken together, the AP-2α transgenic phenotype has a distinctive combination of changes in morphology and in gene expression that distinguish it from other mutations affecting mammary gland development.

Despite the link between AP-2 protein expression and human breast cancer, to date we have not detected any mammary tumors in the MMTV-AP-2α transgenic mice, many of which have been kept for greater than 18 months. Indeed, the overexpression of AP-2α suppressed the growth and development of the ductal network. We therefore postulate that, in human breast cancer, the rise in AP-2α expression is not responsible for the inappropriate cell cycle progression. Instead, we suggest that the activation of the AP-2 genes in breast cancer represents a failed attempt to halt cell proliferation. The conclusion that the AP-2 genes may have an inhibitory role in tumor progression is supported by recent in vitro and in vivo studies. First, it has been shown that transfection of AP-2α into HepG2 hepatocarcinoma cells or SW480 adenocarcinoma cells can inhibit their growth and tumorigenicity, possibly by activating the transcription of p21waf1/cip1 (Zeng et al., 1997). Second, there is a tendency for AP-2 gene expression to be lost during the process of metastasis in both breast cancer and melanoma (Bar-Eli, 1999; Gee et al., 1999), while the ectopic expression of AP-2α in melanoma cell lines can reduce the tumorigenicity of these cells when they are transplanted into nude mice (Bar-Eli, 1999).

Considerable amino acid identity exists between the AP-2α, AP-2β, and AP-2γ proteins, and indeed these transcription factors can all bind to essentially the same recognition site (Boscher et al., 1996). Nevertheless, human breast cancer studies indicate that these transcription factors may regulate different target genes (Gee et al., 1999; Turner et al., 1998). Subtle differences in the spatiotemporal patterns of AP-2 expression also occur within the mammary epithelium. Therefore, while the overexpression of AP-2α may be equivalent to expressing any or all of the AP-2 genes, it is possible that each gene could perform a unique function within the mammary gland—a function that is subverted by the overexpression of one particular family member. Continued analysis of how these AP-2 genes might act alone or in concert within the mammary gland is clearly warranted. In the context of the whole organism, the demonstration that the AP-2α protein can profoundly influence postnatal mammary gland morphology again illustrates the importance of this gene family for regulating growth and morphogenesis. Furthermore, the ability of AP-2α to inhibit lobuloalveolar proliferation strongly suggests that this gene family can modulate cell growth in human breast cancer.

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References


