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Persistent Changes in Gene Expression Induced by Estrogen and Progesterone in the Rat Mammary Gland

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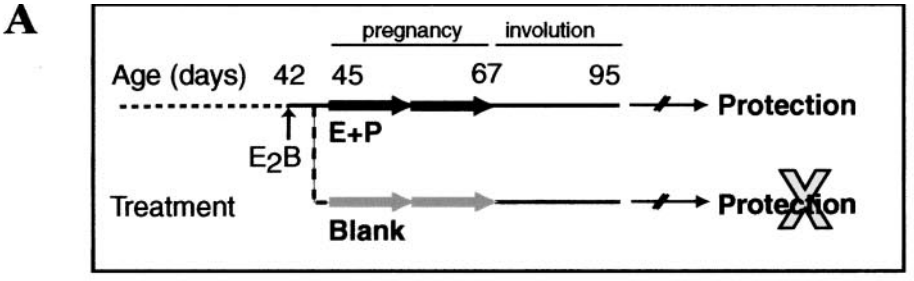
Epidemiological studies have consistently shown that an early full-term pregnancy is protective against breast cancer. We hypothesize that the hormonal milieu that is present during pregnancy results in persistent changes in the pattern of gene expression in the mammary gland, leading to permanent changes in cell fate that determine the subsequent proliferative response of the gland. To investigate this hypothesis, we have used suppression subtractive hybridization to identify genes that are persistently up-regulated in the glands of E- and progesterone (P)-treated Wistar-Furth rats 28 d after steroid hormone treatment compared with age-matched virgins. Using this approach, a number of genes displaying persistent altered expression in response to previous treatment with E and P were identified. Two markers have been characterized in greater detail: RbAp46 and a novel gene that specifies a noncoding RNA (designated G.B7). Both were persistently up-regulated in the lobules of the regressed gland and required previous treatment with both E and P for maximal persistent expression. RbAp46 has been implicated in a number of complexes involving chromatin remodeling, suggesting a mechanism whereby epigenetic factors responsible for persistent changes in gene expression may be related to the determination of cell fate. These results provide the first support at the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland. (*Molecular Endocrinology* 15: 1993–2009, 2001)

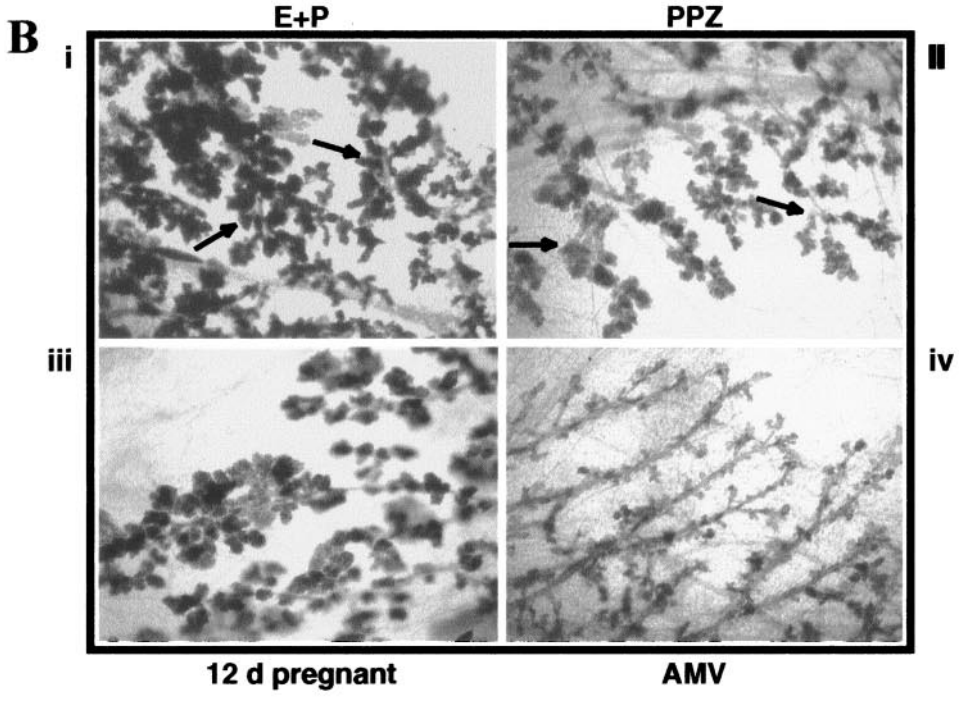
BREAST CANCER IS one of the most frequently diagnosed cancers affecting women in the Western world. Despite advances in the technologies for the diagnosis and treatment of breast cancer (1), our understanding of the etiological factors contributing to the development of this disease is limited (2). However, one fundamental concept arising from studies of mammary gland biology is the understanding that the endocrine processes involved in normal mammary development and carcinogenesis are intrinsically related (3). One of the most frequently cited examples of this principle is the role of reproductive history and breast cancer risk. There is significant evidence that the timing of normal developmental events such as menarche, menopause, and parity and the duration of lactation have a significant impact on an individual's susceptibility to breast cancer (4, 5). In particular, there is strong epidemiological evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer (5–7). This is substantiated by studies in several rodent models that demonstrate that early pregnancy confers resistance to tumorigenesis in animals treated with chemical carcinogens (8–11). These studies also revealed a strong age-related component to the timing of developmental events and exposure to carcinogens (12–14). Thus, the regulation of normal developmental events governing the timing and period of exposure to ovarian hormones appears to be a critical factor in determining the risk of developing breast cancer. Furthermore, there appears to be a functional interplay between the endocrine processes controlling mammary development and carcinogenesis.

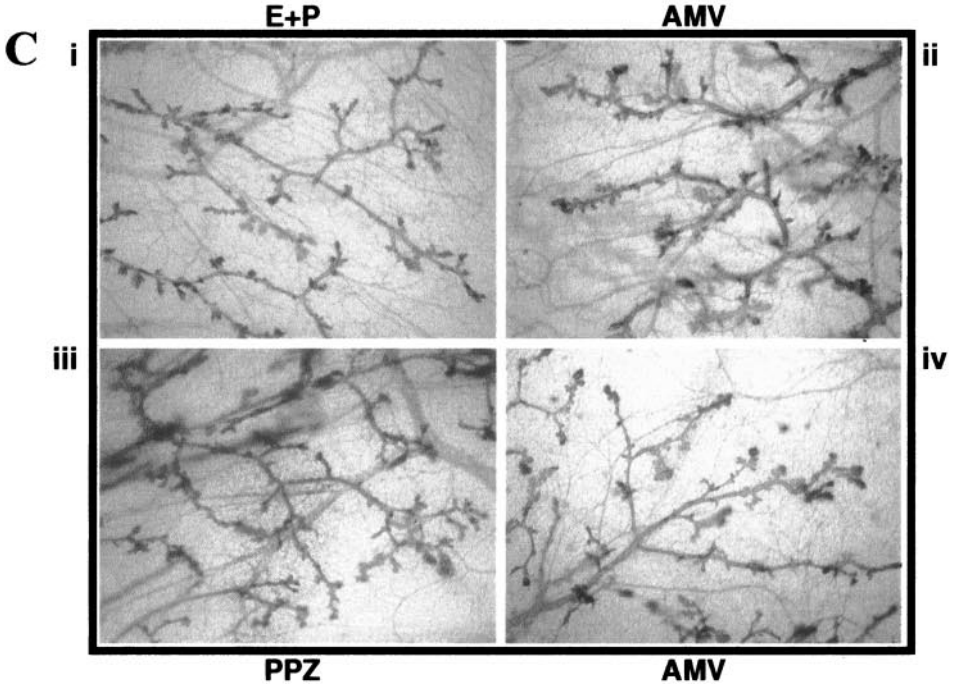
The observation that the protective effect of an early full-term pregnancy can be accurately reproduced in rodents has led to the development of defined animal models for studying this parity-related phenomenon. Huggins *et al.* (15) initially demonstrated that treatment with the ovarian hormones E and progesterone (P) could inhibit tumorigenesis in rats after previous exposure to chemical carcinogens. Since then, numerous investigators have extended these observations to show that hormonal manipulation (by treatment with E and P or human CG), either before or immediately after carcinogen exposure, can inhibit carcinogenesis by inducing a refractory state. These studies have been performed in a variety of rat and mouse strains (11, 16–23). Together, these findings provide strong support for the utility of the rodent model to define the molecular mechanisms by which a defined hormonal regimen can mimic the protective effect of pregnancy.

Despite the wealth of literature supporting the role of endocrine-mediated processes in parity-related re-

Abbreviations: AMV, Age-matched virgin; CoA, coenzyme A; P, progesterone; PPZ, perphenazine; Rb, retinoblastoma; SSC, standard saline citrate; SSH, subtractive suppressive hybridization.







fractoriness, little is known about the molecular mechanisms governing pregnancy-specific developmental changes in the mammary gland. Mammary gland development is mediated by a complex interaction between systemic hormones and local growth factors (24), which is in turn modulated by the topography of the cells receiving the stimuli (2). E and P appear to be key players in these processes (25). However, although distinct morphogenic functions have been associated with these hormones, the molecular pathways that are elicited in response to the combined effect of E and P signaling remain to be elucidated fully (24). Furthermore, it seems likely that the signaling pathways induced by these hormones vary depending on the context of the population of cells receiving the stimuli. Therefore, we hypothesize that the normal hormonal milieu that is present during pregnancy results in persistent changes in the molecular pathways governing cell fate in a defined population of cells in the mammary gland. Accordingly, these changes dictate the type of response that is elicited by subsequent exposure to hormones or chemical carcinogens. A critical aspect in understanding these processes is the elucidation of target genes for E and P in the mammary gland. Such information is necessary to determine how the molecular pathways involved in normal mammary development and tumorigenesis converge with systemic hormones to mediate parity-specific protection.

Previous studies, using a rational approach to study known targets associated with proliferation and differentiation in the mammary gland, have met with limited success in revealing the molecular pathways involved in conferring the refractory state (11, 21). Therefore, we used an alternative strategy to identify novel targets for E and P action in the rat mammary gland. Conditions for the hormonal regimen were based on previous studies, which defined the minimal doses of E and P required to induce a level of morphological differentiation equivalent to that observed during a full-term pregnancy (11). These studies have shown that previous treatment with E and P confers resistance to chemical carcinogen-induced tumorigenesis in Wistar-Furth rats in a reproducible and statistically significant manner. By using an experimental paradigm involving the administration of E and P, we have been able to circumvent the difficulties that are likely to be encountered by attempting to induce synchronized pregnancies within an age-matched cohort of animals. Furthermore, this approach is likely to give a clearer picture of the molecular pathways that are influenced by E and P during normal development and tumorigenesis. The 45-d-old rat was used as model for the initiation of hormonal treatment because it represents a stage of development that is analogous to that of humans both in the rapid development of the mammary epithelium during puberty and in the maximum susceptibility to carcinogenesis (25). Using this Wistar-Furth rat model, in conjunction with subtractive suppressive hybridization (SSH) methodologies, we have identified several markers that are persistently upregulated in response to previous exposure to E and P in the mammary gland. The expression of two of these markers, RbAp46 and a novel gene that specifies a noncoding RNA (G.B7), has been characterized in further detail.

RESULTS

Morphological Effects of Hormonal Treatments

To simulate the protective effect of a full-term pregnancy, 45-d-old Wistar-Furth rats were treated with a defined dose of E and P as described in *Materials and Methods* (Fig. 1A). In the expression studies presented below, we used perphenazine (PPZ) treatment to distinguish between the processes involved in differentiation and protection attributable to E+P or pregnancy. Using the experimental paradigm described previously (22), 45-d-old Wistar-Furth rats were treated with PPZ for a period of 3 wk. This treatment results in an increased serum level of PRL and P, but not E, which permits differentiation but does not confer protection against carcinogenesis (22). Hormonal stimulation (by either E+P or PPZ treatment) was verified by whole-mount analysis of mammary glands at the conclusion of the 21-d treatment period. In both cases, mammary glands from 6-, 12-, and 18-d pregnant animals were used as positive controls for pregnancy-specific differentiation of the gland. Figure 1B shows representative whole-mount analyses of mammary

Fig. 1. Mammary Gland Morphology After Different Hormonal Paradigms

A, Experimental paradigm for studying the protective effect of E and P treatment. Forty-two-day-old Wistar-Furth rats were administered E2 benzoate (E_2B) by sc injection and then a sc pellet of E+P 3 d later. The pellets were replaced after 10 d to provide hormone treatment for a total of 21 d. AMV animals were given blank pellets. After 21 d, the pellet was removed and the mammary gland was allowed to return to a resting stage, approximating a 28-d cycle of involution. At the end of the 28-d involution period, mammary glands were removed for investigation. B, Morphological analysis after hormonal stimulation. Whole-mount preparations of inguinal number 4 mammary glands of 66-d-old rats after 21 d of treatment with E+P (i) or PPZ (ii). A mammary gland from a 12-d pregnant animal was used as a positive control (iii) to assess to level of morphological development achieved by the hormonal regimen. A 66-d-old AMV animal was used as a negative control for mammary development in the absence of exogenous hormones (iv). *Arrows* indicate morphological differences between the E+P and PPZ treatments. C, Morphological analysis of mammary glands after hormonal stimulation and involution. Whole-mount preparations of number 4 mammary glands from 95-d-old animals treated with either E+P (i) or PPZ (iii) for 21 d and after a 28-d cycle of involution. AMV animals received either a blank pellet containing no hormones (ii) or vehicle alone (as a control for the PPZ treatment; iv).

glands from 12-d pregnant, 21-d E+P- and PPZ-treated rats, and age-matched virgin (AMV) rats. In the E+P- and PPZ-treated gland (Fig. 1B, i and ii), we observed a level of differentiation that was comparable to that of a 12-d pregnant animal (Fig. 1B, iii). At the gross structural level, these glands appear to be very similar, although a few minor differences were observed. In particular, the E+P-treated mammary gland (Fig. 1B, i) appeared to exhibit a slightly greater degree of secondary and tertiary branching than the PPZ-treated animals (Fig. 1B, b) but slightly reduced lobuloalveolar development compared with the mammary glands isolated at d 12 of pregnancy (Fig. 1B, iii) or from the PPZ-treated animal (Fig. 1B, ii). The mammary glands from 21-d PPZ-treated rats exhibited a level of lobuloalveolar development that was similar to the mammary glands from 12-d pregnant animals (Fig. 1B, ii and iii, respectively) although with less side branching than the glands from 12-d pregnant animals. In addition, 21-d E+P-stimulated rats expressed β-casein at a level that was readily detectable by Northern analysis using total RNA (data not shown). In contrast, mammary glands from AMV control animals receiving blank pellets showed no significant alveolar differentiation, suggesting that alveolar development in the E+P-treated and PPZ-treated groups was a result of hormonal stimulation. Previous studies demonstrated that treatment with either E+P or PPZ confers a high level of proliferation and lobuloalveolar differentiation to the mammary glands of treated animals (22). However, hormonal regulation has not been examined beyond the gross morphological level to determine whether the level of development is the same in both cases.

At the end of the 21-d treatment period, hormonal stimuli were withdrawn and the remaining animals were allowed to undergo involution for 28 d. This resting phase mimics the period of involution that occurs after a normal pregnancy and lactation and represents a period of extensive tissue remodeling. At the end of involution, the gland generally reaches a quiescent state, resembling that of the mature virgin animal (2). Figure 1C shows whole-mount analysis of 28-d involuted mammary glands from 95-d-old animals after previous treatment with either E+P (i) or PPZ (iii) and their age-matched controls (ii and iv, respectively). As reported in previous studies (11), similar morphological characteristics were observed in all of the treatment groups, indicating that there were no persistent structural differences as a consequence of hormonal stimulation of the gland.

Differentially Expressed Genes Identified by SSH

To help elucidate the mechanisms governing this hormone-dependent, parity-related phenomenon, we initially screened for molecular markers of an altered cell population or for changes in the subset of genes that were persistently expressed as a result of previous hormonal stimulation. Using the experimental paradigm described above, poly(A^+) RNA was isolated from pooled total RNA samples corresponding to either E+P-treated or AMV control glands after a 28-d involution period. SSH was then used to generate an E+P-subtracted library (as described in *Materials and Methods*). The library was screened by differential reverse Northern blot analysis (26) to identify genes that are differentially induced by this hormone treatment. An example of the screening method is presented in Fig. 2, illustrating the utility of this approach for identifying differentially expressed markers by a difference in hybridization signal intensity on duplicate filters. Figure 2A shows one set of 96 clones, amplified by PCR, arrayed by high-density gel electrophoresis, and photographed to ensure even loading between duplicates. Figure 2, B and C, shows duplicate membranes, each containing DNA corresponding to the complete set of markers shown in Fig. 2A. Duplicate membranes were screened by reverse Northern analysis using probes corresponding to either the E+P-subtracted (Fig. 2B) or virgin-subtracted (Fig. 2C) amplified cDNA used in the construction of the SSH library (as described in *Materials and Methods*). This procedure resulted in the identification of several markers that were characterized in greater detail in Fig. 3. These markers are indicated by boxes and include both low-abundance genes, such as RbAp46, Prl-1, PP1δ, and hnRNP A1 (Fig. 2, B and C, i, ii, iii, and v, respectively), as well as more highly expressed markers, such as transferrin and α-casein (Fig. 2, B and C, iv and vi, respectively).

Despite the morphological similarity of the tissues used in this procedure, 24% of the 864 randomly selected amplified markers appeared to be differentially expressed, based on the results of the high-density differential reverse Northern blot analysis (Table 1). Selected differentially expressed clones (n = 203) were sequenced, and their identities were determined using the BLAST homology search algorithms and the public sequence databases available through the National Center for Biotechnology Information. Clones were classified as known, homologous, unknown, or novel depending on the stringency of the search criteria and the databases from which the sequence matches were found. A summary of this analysis is presented in Table 1. Thus, according to the criteria detailed in the footnotes to Table 1, 61.5% of the markers sequenced corresponded to known genes, 10.8% corresponded to homologous genes, and 27.5% corresponded to genes of unknown function.

A summary of 50 known genes identified using this strategy is presented in Table 2. These genes fall into several distinct categories: those involved in normal metabolism and homeostasis of the gland (metabolic enzymes, transport molecules); those involved in cell-cell contact and the extracellular matrix; and regulatory factors (signaling molecules, transcription factors, etc.). A number of these genes (such as RbAp46, Nap1, and CDC42) encode molecules of known rele-

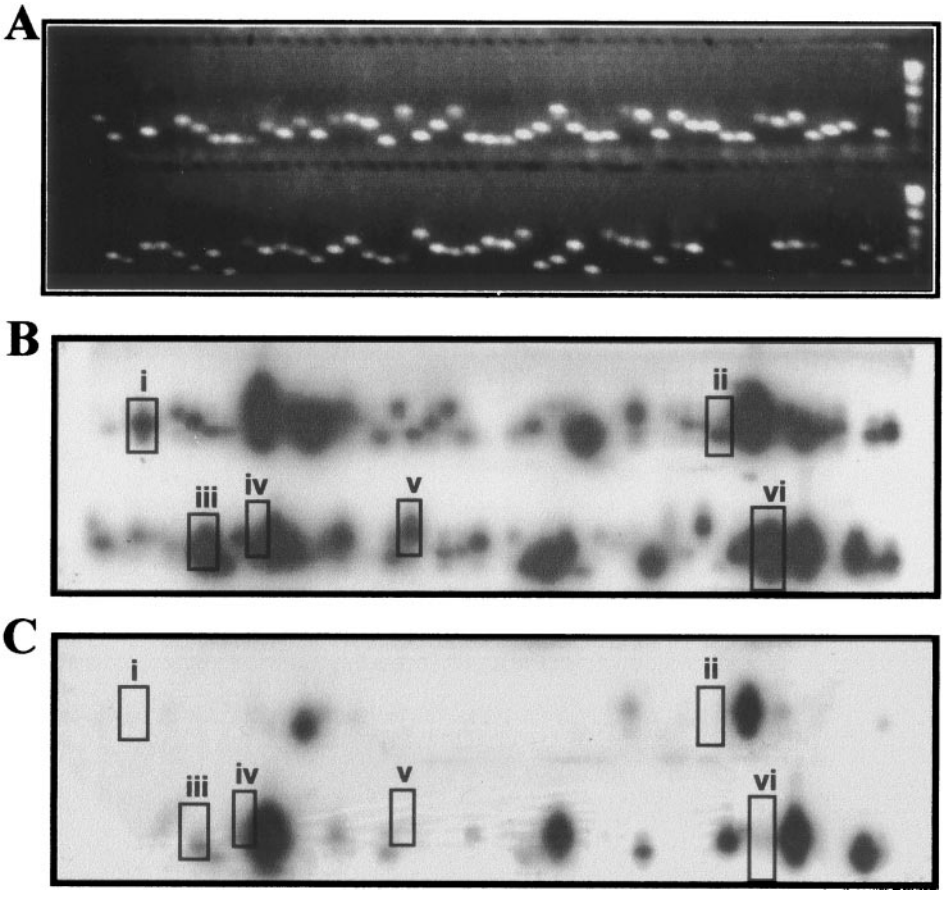


Fig. 2. Differential Screening of the E+P SSH Library

An illustration of the screening procedure used to select markers that are up-regulated in response to previous exposure to E+P. Amplified inserts from 96 randomly selected clones were arrayed in duplicate using high-density gel electrophoresis (A). Gels were stained with ethidium bromide to ensure equal loading and then transferred onto a charged nylon membrane. Duplicate filters were screened using differential reverse Northern analysis, in which [^{32}P]dATP-labeled probes of equal specific activity, generated from either the E+P subtracted (virgin driver) cDNA (B) or virgin subtracted (E+P driver) cDNA (C), was hybridized under stringent conditions. The resulting signal was detected by exposing the filters to BioMAX MR film for 6 h to 2 d. Six differentially expressed markers, identified using this method, are highlighted by boxes: i) RbAp46; ii) Prl-1; iii) PP1δ; iv) transferrin; v) hnRNP A1; vi) α-casein. The relative positions of these cDNAs, in the absence of a hybridization signal, are indicated in Fig. 2C.

vance to cellular proliferation pathways (27–29). Surprisingly, several well characterized markers of mammary differentiation (such as the milk protein genes, transferrin, α-casein, and κ-casein) were also identified using this screen and displayed persistent expression in E+P-treated glands. These markers were highly represented in the subset of clones that were selected for sequencing and accounted for 18.2%, 3.9%, and 2.9%, respectively, of the total number of clones sequenced. These observations suggest that there is some degree of persistent differentiation in the mammary epithelium after parity (or treatment with E+P). However, whether this differentiation persists as a consequence of direct effects on the mammary gland or is a consequence of an altered hormonal axis in the E+P-treated animal has not been determined. Similar findings involving sustained expression of these markers have also been reported from studies in the parous mouse (3).

Another marker that was highly represented was the mRNA for the stearyl-coenzyme A (CoA) desaturase, an enzyme that catalyzes the rate-limiting step in the biosynthesis of monounsaturated fatty acids (30). Stearyl-CoA desaturase is expressed in an isoform- and temporally specific manner during development, depending on the tissue of origin (31). The fact that it is so highly represented among the subset of clones sequenced suggests that there are also persistent developmental changes in the mammary stroma in response to E+P; however, the implications of this observation to the gland as a whole have not been determined.

SSH has been used in a variety of experimental contexts for the identification of differentially expressed genes. The efficiency of the subtraction procedure is validated by the fact that several markers isolated using this approach (such as α-casein) have been isolated in an independent study using quite

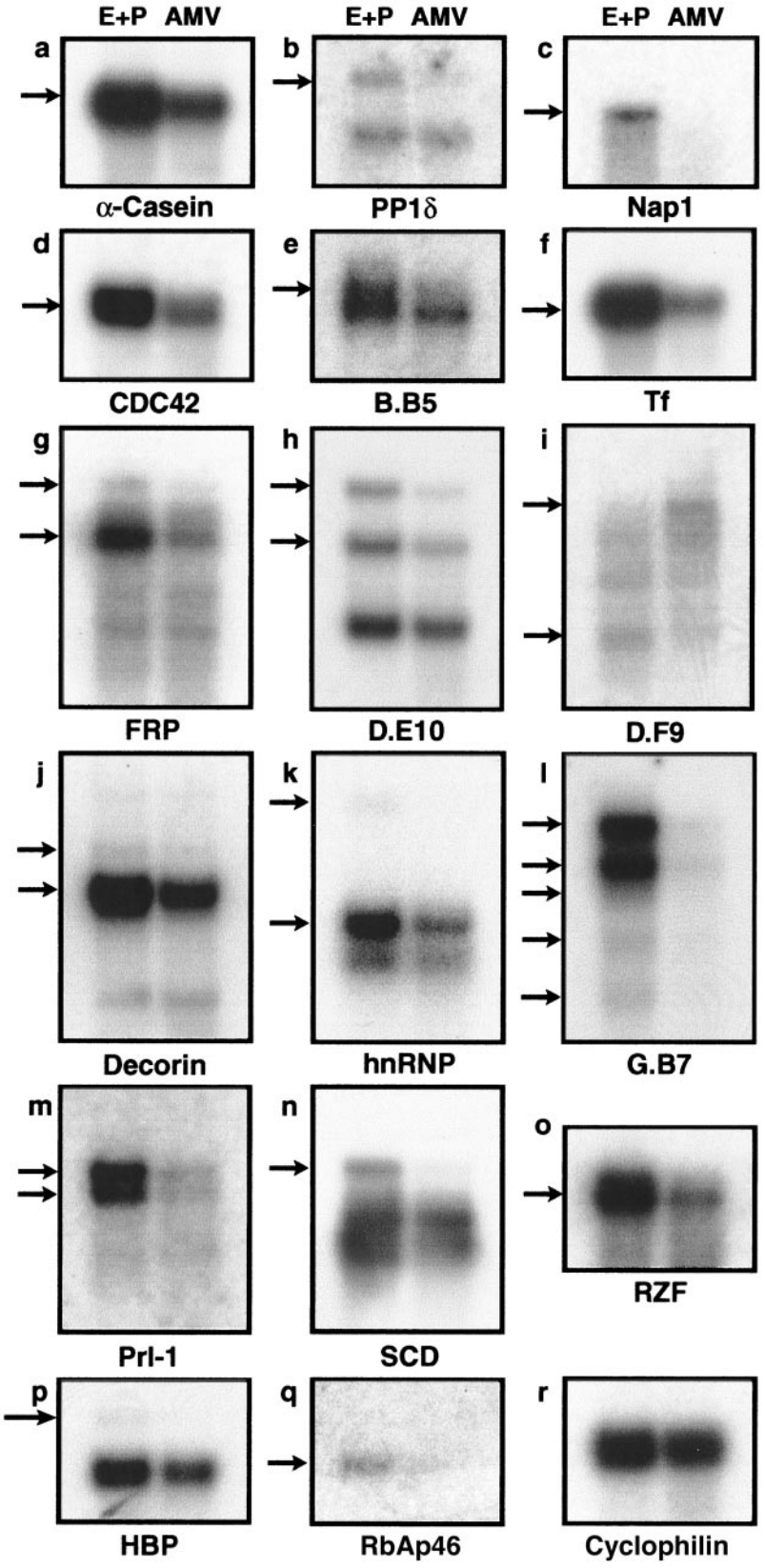


Fig. 3. Northern Validation of the Differential Screening Method

Selected clones, identified by the SSH library screen (described in Fig. 2), were used in Northern blot analysis to verify differential expression in response to previous exposure to E+P. Northern analysis was performed with 2 μg of poly(A^+) RNA from either E+P-treated mammary gland or AMV after 28 d of involution. Blots were hybridized with cDNA inserts prepared from the

Table 1. Summary of Rat E+P SSH Library Screening

Screening Step	No. of Clones Examined
Screened by PCR and reverse northern blotting	874
Identification of differentially expressed markers	213
Sequencing	203
Confirm expression by Northern blot analysis	21
cDNA Clones Sequenced	**Percent of Total Clones Sequenced**
Known genes[a]	61.5
Transferrin	18.2
α-Casein	3.9
κ-Casein	2.9
Stearyl-CoA desaturase	2.9
Homologous genes[b]	10.8
Unknown function[c]	27.5
ESTs	23.6
Novel genes[d]	3.9

[a] Clones classified as known genes exhibited ≥98% identity with the corresponding database entry.
[b] Clones corresponding to known genes for which the identity was not complete (because the relevant rat sequence was not available) were classified as homologous if they demonstrated an expected value of $<1^{-5}$.
[c] Clones of unknown function were classified as such if they matched sequences in the EST databases but their function was otherwise unknown.
[d] This subset also included a number of novel genes that displayed no significant homology with any sequences in the publicly available databases after extensive analysis using the BLAST, TBLASTN, and BEAUTY algorithms.

different approaches to identify genes that are differentially expressed in response to parity. In addition, parallel studies using SSH to identify markers that are down-regulated by E+P have isolated a quite different set of markers from those described in the present study (Ginger, M.R., and J.M. Rosen, unpublished data).

Validation by Northern Analysis

To validate the SSH screen and reverse Northern analysis (described above), a selected subset of markers were characterized further by Northern blot analysis to confirm their pattern of expression in the E+P-pretreated, involuted gland compared with the AMV control tissue. A pooled RNA fraction was used in these analyses to control for the effects of variation in estrous cycle within an age-matched cohort of animals (as described in *Materials and Methods*). Northern blots were hybridized with radiolabeled probes prepared from the partial cDNA fragments isolated from the SSH library. The expression of individual markers was determined by quantitative analysis of the subsequent hybridization signals using cyclophilin as a normalizing control. Twenty-one markers were examined in this manner, 18 of which were confirmed as differentially expressed based on quantitative Northern analysis of their expression in the E+P-treated gland *vs.* AMV. Because the SSH strategy uses an amplification step to permit the detection of low-abundance markers, it is possible that some sequences will be preferentially amplified, resulting in false-positives results. The observation that 85% of the markers examined by Northern analysis were true positives suggests that the screening criteria described above resulted in the stringent selection of differentially expressed markers.

The results of the Northern analysis are summarized in Table 3 and highlight several genes that might be putative candidates involved in the molecular pathways that are targets for E/P-mediated changes associated with parity-related protection of the gland. Representative Northern blots of markers demonstrating quantitative changes in E+P-treated glands are shown in Fig. 3. Several interesting observations accounting for the differential gene expression were revealed by these Northern analyses. Some of these genes were expressed as a single transcript and showed a small quantitative change, *e.g.* α-casein (Fig. 3a, 1.8-fold difference), Nap 1 (Fig. 3c, 8.4-fold change), cdc42 (Fig. 3d, 2.1-fold change), and RbAp46 (Fig. 3q, 3.5-fold change). Others were detected as multiple transcripts, and one or more of these transcripts were differentially expressed, *e.g*. follistatin-related protein (Fig. 3g, 2.1- to 2.3-fold change), D.E10 (Fig. 3h, 2.9- to 3.4-fold change), hnRNP A1 (Fig. 3k, 3.8-fold change). In the third case, several transcripts were observed and all of the transcripts appeared to be differentially expressed, *e.g*. G.B7 (Fig. 3l, 5- to 7.2-fold change). Whether these transcripts arise as differently spliced forms of the same gene product or closely related members of a gene family remains to be determined. Furthermore, the abundance of these transcripts identified by SSH and reverse Northern blotting varied considerably from highly abundant mRNAs, such as those encoding α-

following SSH clones: a, α-casein; b, protein phosphatase 1, subunit δ; c, Nck-associated protein 1; d, CDC42; e, clone BB5, which resembles an EST; f, transferrin; g, follistatin-related protein; h, clone D.E10, which resembles an EST; i, clone D.F9, which resembles an EST; j, decorin; k, hnRNP A1; l) G.B7; m, protein phosphatase, Prl-1; n, stearyl-CoA desaturase; o, ring zinc finger protein; p, heme-binding protein; q, RbAp46. Exposure times were as follows: 4 h (a and f), 12 h (j), 18 h (n), 2 d (k and p), 3 d (o), 4 d (c, d, and g), 5 d (e and q), 7–8 d (b, h, i, l, and m). Cyclophilin was used as a normalizing control to ensure even loading (r). Differentially expressed transcripts are indicated by *arrows*.

Table 2. Summary of Known Genes Isolated using SSH

Function	Marker	Accession Number	
		Known	Homologous
Metabolic	Stearyl CoA desaturase	J02585.1	
	Cysteine dioxygenase	D83481	
	GTP-specific succinyl CoA synthetase		AF058956
	2,4-Dienoyl CoA reductase	D00569	
Nucleic acid metabolism	5′ Nucleotidase	J05214	
	Guanine deaminase	AF026472.2	
Milk protein	α-Casein	J00710.1	
	κ-Casein	K02598	
	β-Casein	NM_017120	
Mineral transport/metabolism	Transferrin	D38380	
	Heme-binding protein	D30035	
	Ceruloplasmin	L33869	
Protein synthesis	Ribosomal protein L18	NM_031102.1	
	Ribosomal protein S20	X51537.1	
	40-kDa Ribosomal protein	D25224	
	Eukaryotic protein translation initiation factor-5	NM_020075.1	
Protein transport	Sec61		U11027
Transcription factor	Zinc finger protein, Pzf		U05343.1
	Ring zinc finger protein, Rzf		AF037205.1
	Zinc finger protein		U90919
	Sp3		AF062567.1
	SHARP-2	AF009330	
Chromatin remodeling	RbAp46	AF090306	
RNA splicing	hnRNP A1	M12156	
	Argine/serine-rich splicing factor 11		XM_001835.1
Cell cycle control	CDC42		NM_009861.1
	Retinoblastoma	D25233.1	
Signal transduction	Krit-1		AF310134.1
	Nap-1	D84346	
	Collibistin I	NM_023957	
Translational repressor	NAT-1		U76112
Kinase	JAK1		S63728.1
Phosphatase	Protein phosphatase type 1 δ	D90164	
	Tyrosine phosphatase Prl-1	L27843	
	Protein tyrosine phosphatase-like protein, PTPLB		AF169286
Extracellular matrix	Decorin	Z12298.1	
	Follistatin-related protein	U06864	
	Heparin sulfate proteoglycan core protein, Sdc2	NM_013082.1	
	Neuralin-1	AF305714	
Modulation of cell growth	Mast cell growth factor		U44725
	EGF-like protein, S1-5/T16	D89730.1	
Cell adhesion	Podocalyxin	AF109393.1	
	Neural cell adhesion		AF00246
	Cell adhesion molecule		XM_003064.2
Membrane transporter	PMP70, ATP-binding cassette family	NM_012804.1	
	KCl cotransporter	U55815	
Cell-surface marker	Epithelial glycoprotein antigen, EGP-314	AJ001044.1	
	Interferon γ receptor	U68272	
Apoptosis	E1B 19K/Bcl-2-binding protein, Nip3	AF243515.1	
Protease	YME1-like ATP-dependent metalloprotease		NM_013771.1

casein, which are easily detected after only a few hours of exposure (Fig. 3a), to low abundance mRNAs, such as RbAp46, which required more than 1 wk of exposure (Fig. 3q). On the basis of this preliminary screen of markers up-regulated by treatment with E+P, two genes were selected for further characterization in greater detail: RbAp46 and a novel clone (designated G.B7).

Table 3. Genes Up-Regulated by E+P

Marker	Identity	Accession number		Transcript[b] Size (kb)	Fold Difference[c]
		Known	Homologous[a]		
A.F10	Zinc finger protein, Pzf		U05343.1		—
B.A6	Transferrin	D38380		2.3	2.0
B.B5	EST		AI712974.1	4.2	1.9
				3.7	—
B.B8	Podocalyxin	AF109393.1		1.6	
B.D2	Sp3	AF062567.1			—
B.E3	Stearyl-CoA desaturase	J02585.1		6.0	4.4
				4.8	—
				3.8	—
B.E4	Decorin	Z12298.1		4.2	1.8
				3.1	1.4
				2.0	—
				0.9	—
B.F2	α-Casein	J00710.1		1.3	1.8
B.G10	Zinc finger protein		U90919		—
C.B2	RbAp46	AF090306		1.9	3.5
C.C6	Tyrosine phosphatase Prl-1	L27843		3.2	—
				2.8	6.5
				2.0	4.7
C.F7	Protein phosphatase-1, δ	D90164		2.1	1.4
				1.5	4.3
C.B10	hnRNP A1	M12156		3.2	3.8
				1.8	—
				1.3	—
D.B4	Ring zinc finger protein		AF037205.1	2.2	4.5
D.C6	Nap 1	NM_023957		5.0	8.4
D.F6	Heme-binding protein	D30035		1.7	5.3
				1.1	—
D.F9	EST		BF289700.1	2.9	0.27
				2.2	—
				1.6	—
				1.1	2.2
D.G2	Follistatin-related protein	U06864		3	2.3
				2.3	2.1
				1.6	—
				1.3	—
D.E10	EST		BF560064.1	4	3.4
				2.5	2.9
				1.7	—
G.B7	Unknown			6.0	7.2
				4.0	5.6
				2.8	7.2
				2.3	5.0
				1.5	6.1
I.E1	CDC42		NM_009861.1	2.2	2.1

[a] For ESTs, the accession number of the closest homologous sequence is shown.
[b] Estimated from Northern analysis.
[c] Fold difference in E+P *vs.* AMV is from quantitative analysis of Northern blots presented in Fig. 3 using cyclophilin as a normalizing control.

Cloning of Full-Length G.B7

Clone G.B7 corresponds to a 764-bp fragment isolated in the SSH screen described above. Analysis of the 764-nucleotide of sequence available for this cDNA revealed no significant homology with any sequences in the publicly accessible nucleotide databases; hence, it represented a potentially novel gene product. To further elucidate the function of this cDNA, we constructed and screened an E+P-treated mammary gland cDNA library and isolated several full-length clones corresponding to the different sized transcripts observed by Northern analysis (Fig. 3l). Three of these clones (6.3, 2.4, and 2.2 kb) were sequenced in their entirety. Analysis of the resultant sequences suggested that the different sized transcripts observed by Northern blot analysis arose as a result of differential splicing of a single gene product. The full-

length sequence of the largest of these clones (6.3 kb) was deposited in GenBank with the accession number AY035343.

In an attempt to determine the function of this gene, we submitted the full-length, 6.3-kb sequence to database searching using BLAST and TBLASTN search algorithms (with six-frame translation and BEAUTY postprocessing). The results of this analysis did not reveal significant homology with any known gene or protein motif. However, we found that a short section of the 5′- and 3′-regions of this gene (nucleotides 1,125–1,490 and 5,920–6,203 of the full-length sequence) exhibited homology with several rat and mouse expressed sequence tag (EST) clones. The best matches were to accession numbers BG079981, BE119249, and BG079981 and revealed an identity of 85–97% to these regions of approximately 300 bp in the full-length G.B7 sequence. In addition, we performed homology searching using the recently assembled human genome sequence database and found four regions of homology with human chromosome 2. These homologous regions corresponded to nucleotides 2,334–2,419, 2,435–2,601, 2,745–2,789, and 5,157–5,437 of the full-length G.B7 sequence, with identities of 93%, 83%, 95%, and 80%, respectively (expected value $< 1^{-9}$). This sequence maps to region 2q33 of the human genome and spans a known chromosomal breakpoint that is associated with a number of human cancers, including breast adenocarcinoma (32). No open reading frames or ESTs have been identified in the human sequence encompassing the region of homology with G.B7. However, this may be a consequence of incomplete annotation of the genome database or the failure of the search paradigms to detect a nontranslated RNA. Indeed, we have been unable to detect any significant open reading frames longer than approximately 200 nucleotides in the full-length G.B7 cDNA. *In vitro* translation experiments, with appropriate positive controls run in parallel to validate the assay, also failed to detect any translation product for the 6.3-, 2.4-, or 2.2-kb forms of this RNA (data not shown).

Expression of G.B7

Northern analysis demonstrated a 4- to 8-fold induction (depending on transcript) of G.B7 transcripts in the glands of hormone-pretreated rats compared with those in AMV controls. Multiple-tissue Northern analysis suggested that this gene is also expressed in the testes, liver, and heart of rats (data not shown), but its hormonal regulation in these tissues has not been determined. Because the mammary gland contains a heterogeneous population of cells (33), including fibroblasts, adipocytes, and several types of epithelial cells, it was important to determine in which cell types this RNA transcript was expressed. To investigate the spatial pattern of G.B7 expression in response to treatment with E+P and PPZ, we used *in situ* hybridization analysis with a ^{33}P-labeled probe generated from the 764-bp fragment isolated from the SSH library. As shown in Fig. 4a, G.B7 mRNA was highly expressed in the epithelium, but not in the stroma, of 12-d pregnant mammary glands. Furthermore, its expression appeared to be localized to the differentiated lobuloalveoli. In addition, G.B7 was persistently expressed in residual alveoli of the regressed gland after E+P treatment and 28 d of involution (Fig. 4c). By comparison, only a very faint hybridization signal was detected in the PPZ-treated gland after 28 d of involution (Fig. 4e). No signal was detected in the 95-d-old AMV gland (Fig. 4g) or with the sense control (data not shown). Interestingly, the expression of G.B7 in the E+P-treated and 12-d pregnant animal was primarily confined to the lobuloalveolar structures of the gland, and not in the ducts. Furthermore, persistent expression of G.B7 required previous exposure to E and P, because PPZ treatment alone did not induce expression. We have also examined the expression of G.B7 by *in situ* hybridization using mammary glands from parous *vs.* nonparous animals after 28 d of involution and have observed persistent changes in the expression of this marker (data not shown). This pattern of expression suggests that it represents a potentially interesting and biologically relevant marker of parity-related protection.

Expression of RbAp46

Northern analysis demonstrated that RbAp46 is persistently up-regulated in mammary glands of E+P-treated rats compared with AMV controls. To investigate the spatial pattern of its expression, we again used *in situ* hybridization analysis (Fig. 5). This analysis showed that RbAp46 was expressed at a high level in the lobuloalveoli at midpregnancy (Fig. 5, a and c). In addition, RbAp46 was persistently expressed in the alveolar structures of the E+P-treated gland after 28 d of involution (Fig. 5d). By contrast, RbAp46 expression was barely detectable in the PPZ-treated, 28-d involuted gland (Fig. 5e) or the 95-d-old AMV control gland (Fig. 5f). RbAp46 is also persistently expressed in the mammary glands of parous animals after 28 d of involution (data not shown). These results indicate that parity, or previous exposure to both E and P, is required for the maximal persistent expression of RbAp46.

DISCUSSION

The E+P-treated rat is a well established model for the study of the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. Two prevailing models have been proposed to account for this phenomenon. In the first model proposed by Russo and Russo (34), the refrac-

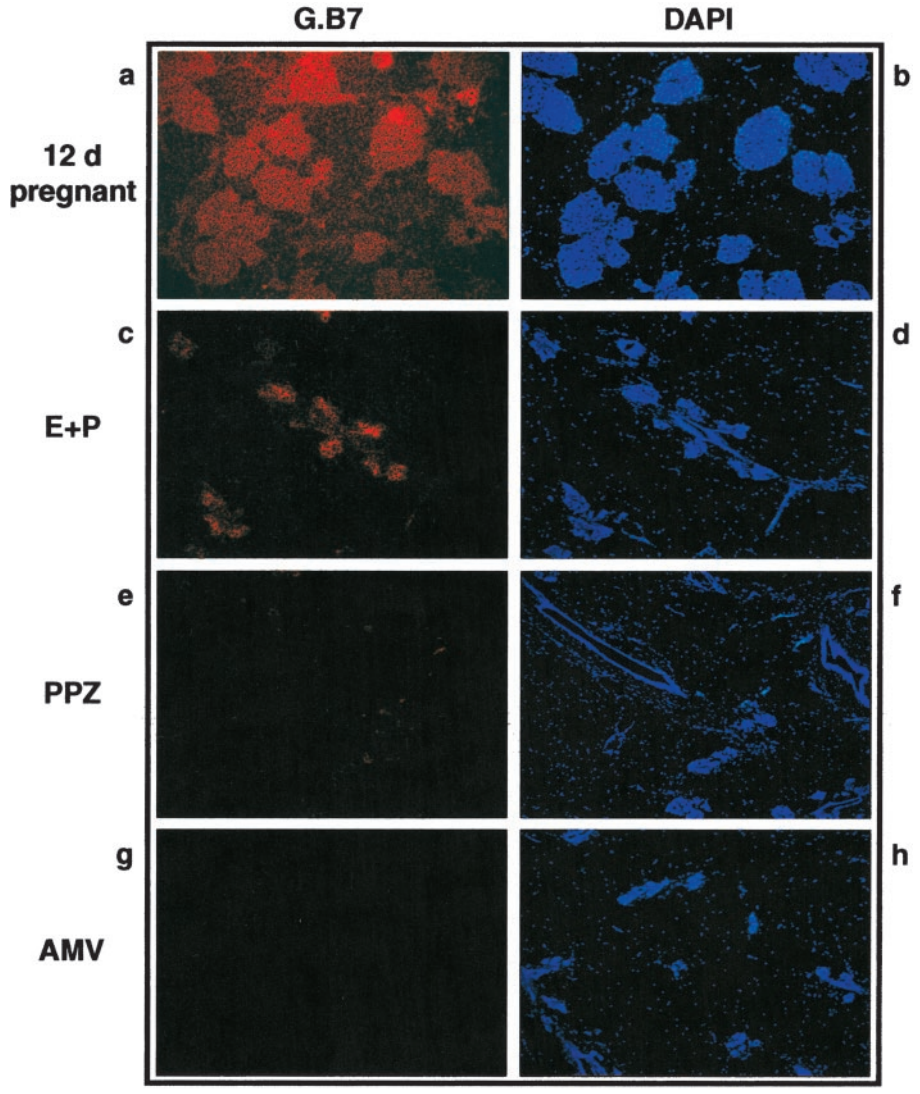


Fig. 4. A Novel Marker, G.B7, Is Expressed in the Lobuloalveolar Structures of the Rat Mammary Gland in Response to E and P

In situ hybridization analysis showing the localization of G.B7 mRNA in the lobuloalveoli of mammary glands from mid pregnancy (12 d pregnant) (a) and residual alveolar structures of E+P-treated rats (c) after 28 d of involution. As a comparison, expression was barely detectable after treatment with PPZ and 28 d of involution (e) and in the 95-d AMV glands (g). Hybridization signals were captured in the dark field. 4′,6-Diamidino-2-phenylindole (DAPI) staining of the same field is shown in parallel (b, d, f, and h). No hybridization was observed with the sense control (not shown).

tory state results from terminal differentiation of the mammary epithelia during pregnancy. According to this hypothesis, terminal differentiation rids the gland of a specific population of susceptible cells (present in the terminal end bud and terminal ducts) that are considered targets for tumorigenesis. Protection is manifested as a change in cellular kinetics, an alteration of the properties associated with carcinogen uptake, binding, and metabolism, and an enhanced capacity for DNA repair (14, 25, 34–36).

According to the second model, the refractory state is intrinsic to the host and is mediated by changes in the levels of systemic hormones (such as GH and PRL), which may subsequently down-regulate the expression of receptors for hormones and growth factors (21). These models may not be mutually exclusive, and although both are attractive, neither has gained sufficient mechanistic evidence to confirm its validity. In addition, comparison of these hypotheses is hampered by the fact that although one uses a pretreatment model of hormonal protection, the other uses a posttreatment paradigm. In the first case, differentiation is proposed as the mechanism for the protection, whereas in the posttreatment model, an agent that causes morphological differentiation of the gland does not confer refractoriness against cancer (22). Furthermore, several published studies have implied that the protection is independent of the level of differentiation achieved by parity or hormonal stimulation (10, 11, 37), whereas other studies have contradicted the finding of

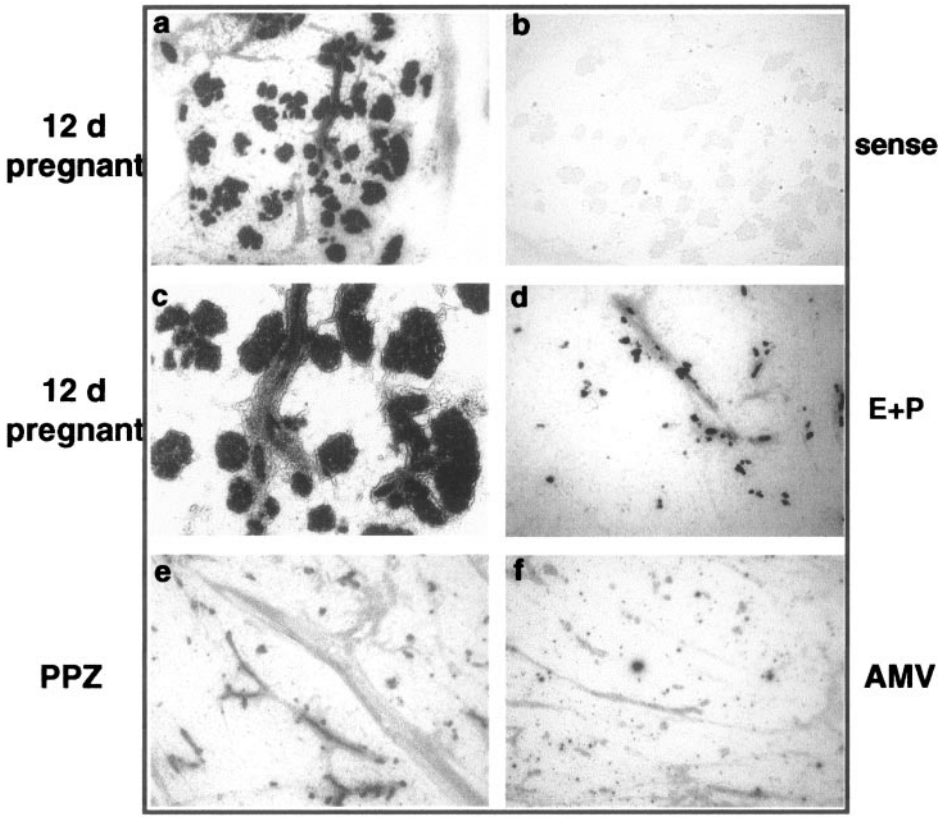


Fig. 5. Persistent Expression of RbAp46 Requires Previous Exposure to Both E and P

In situ hybridization analysis showing the localization of RbAp46 mRNA in the mammary gland at 12 d of pregnancy (a and c) and in E+P-treated (d) and PPZ-treated (e) rats and AMV (f) after 28 d of involution. Magnification, ×4 (a, d, e, and f) and ×10 (c). No hybridization was observed with the sense control (b).

a difference in cellular kinetics between parous and nonparous animals (10). Clearly, this is a complex problem that cannot be fully explained on the basis of existing evidence.

To overcome the limitations of the aforementioned models, we and others (11) have proposed an alternative hypothesis, *i.e.* that the normal hormonal milieu that is present during pregnancy results in persistent changes in the molecular pathways governing cell fate in a defined population of cells in the mammary gland. One possible way that this might occur is through epigenetic changes, which would subsequently determine the pattern of gene expression in a specific population of cells and their descendants. Epigenetic changes, such as an alteration in the methylation status of promoter sequences, the recruitment of methylation binding proteins, and remodeling of chromatin structure, would thus alter the transcriptional profile of those cells. Such epigenetic changes frequently accompany developmental processes and serve to provide a lasting signal that restricts the pattern of gene expression in those cells long after the inductive event (such as hormone or growth factor signaling) has been removed (38). In this respect, the elucidation of novel targets for E and P action in the mammary gland is crucial to our understanding of how an aspect of normal development might mediate the response of the organ to future proliferative signals. We have selected the 28-d involuted gland as the model for the molecular studies presented here because it is morphologically similar to that of mature virgin animals of the same age. Thus, we are provided with an appropriate control to examine molecular differences in two tissues that are very similar morphologically.

In the expression studies presented in Figs. 4 and 5, we used PPZ treatment to distinguish between the molecular pathways induced by either E and P or P alone. PPZ is a dopamine receptor agonist that alters the hormonal axis to the extent that both serum PRL and P levels are increased but E is not significantly increased. As stated above, PPZ caused differentiation of the gland, but not protection, in a posttreatment model (22). Whether it confers protection in the pretreatment paradigm described here remains to be tested, but in previous studies performed in the Wistar-Furth rat model, both E and P were required for subsequent protection from carcinogenesis (37). As we have shown in the present study, both E+P and PPZ cause differentiation of the gland. However, whether differentiation proceeds to the same extent after both treatment regimens has not been examined beyond a morphological level. The fact that we have

observed slight morphological differences between the PPZ- and E+P-treated gland (after 21 d of stimulation) suggests that different molecular pathways are invoked by E+P- or PPZ-mediated development of the gland. Although it remains controversial whether differentiation is causal to, or independent of, protection, treatment with PPZ provides an important control to distinguish between the signaling pathways induced by E and P or P alone.

SSH has been used in a variety of experimental contexts for the identification of differentially expressed genes. It has certain advantages over many conventional methods of gene discovery in that it is capable of identifying both known and novel genes as well as low-abundance genes. The last point is of particular interest because alternative methods, such as microarray analysis, often fail to detect quantitative changes in low-abundance transcripts that are detected by more sensitive techniques such as SSH (39). In many cases, it is necessary to use poly(A^+) Northern analysis to verify the expression of markers identified by SSH. In the present study, we have demonstrated the utility of this approach for isolating markers that are persistently up-regulated by treatment with E and P in the mammary gland. This has highlighted a number of markers of prospective importance to E- and P-dependent pathways in the mammary gland, potentially leading to the determination of cell fate. Although some of these markers represent genes of unknown function, others encode molecules of known relevance to the pathways involved in cellular proliferation and differentiation. It is difficult to reconcile such a diverse group of markers with the molecular events that might be involved in conferring protection to the gland. However, it is possible that some contribute in an independent but stochastic fashion to influence the processes controlling cell fate. Consideration of their functions may lead to a greater understanding of the significance of their persistent expression in the E+P-treated gland. For example, carcinogen treatment of the AMV adult gland leads to a proliferative burst. This proliferative burst is attenuated in the parous and E/P-treated gland (11). Several of the genes up-regulated in the E/P-treated gland could play a role in this proliferative block. For example, phosphatases play a critical role in the regulation of diverse cellular processes, including the modulation of gene expression, cell-cycle progression, and intracellular transport. Follistatin-related protein is a member of a wider family of proteins that have been ascribed the function of modulating the effects of cytokine and growth factor signaling (40). In addition, splicing factors such as hnRNP A1 may be important for maintaining the appropriate expression of certain regulators of cell growth.

We have characterized two of these markers (G.B7 and RbAp46) in greater detail and found that both are persistently expressed in a specific population of epithelial cells after treatment with E+P. The first of these markers encodes a gene of as yet unknown function that shows a 5- to7-fold induction in the glands of hormone-treated rats compared with those in AMV controls. The observation that it is homologous with sequences in a region of human chromosome 2 and spans an area that is known as a chromosomal breakpoint in a number of human cancers is tantalizing and merits further investigation. However, we have been unable to identify a putative translation product. Because this gene is clearly expressed as an mRNA transcript (based on the Northern analysis shown above and the presence of related sequences in the EST database) and was persistently induced after treatment with E+P, we speculate that G.B7 may instead function as a nontranslated regulatory RNA. There is some precedence for this hypothesis: several studies in a range of organisms (including *Caenorhabditis elegans*, *Drosophila*, and mouse) have shown that RNA molecules may function in a regulatory context without themselves being translated. In *C. elegans*, two short noncoding RNA species (*lin-4* and *let-7*) have been identified that repress the function of several genes that mediate developmental control pathways (41). These RNAs appear to exert their repressive effects by binding to complementary sequences in the 3′-untranslated region of their mRNA targets, thus preventing translation. In *Drosophila*, a family of noncoding RNAs encoded by the roX genes is involved in chromatin remodeling, leading to dosage compensation of the male X chromosome (42). In yet another example, the RNA molecule SRA acts as a steroid receptor coactivator in the SRC-1 complex (43). Recent studies suggest that many other examples of RNAs with regulatory functions will be revealed (44).

The Retinoblastoma (Rb)-associated proteins (RbAp46 and the closely related molecule RbAp48) constitute part of a small gene family with homology with the yeast molecule MSI1 (a negative regulator of the Ras-cAMP signaling pathway) (27, 45). Although originally isolated as protein components that bound to Rb by affinity chromatography (45), preliminary studies suggest that these molecules might play a broader function in the regulation of such processes as cellular proliferation and differentiation. Overexpression of either RbAp46 or RbAp48 can substitute for the activity of MSI1 in mutant yeast strains. In addition, RbAp46 appears to be a downstream target of the Wilms's tumor suppressor protein WT1, and overexpression of this gene can suppress the growth of transfected cells in culture (46). More recent studies have shown that these proteins also interact with the breast cancer tumor-suppressor protein BRCA1 (47). Furthermore, RbAp46 and RbAp48 interact directly with histones H3 and H4 (48) and are components of multisubunit complexes that are involved in histone deacetylation, histone acetylation, nucleosome disruption, and nucleosome assembly (49–51). In this capacity, RbAp46 appears to be involved in both *de novo* acetylation/deacetylation of the nascent chromatin (possibly leading to permanent imprinting of specific gene expression) and the targeted repression of

gene activation through its association with the Sin3/HDAC and NuRD complexes (50). Together, these studies imply a model in which RbAp46, through its actions as an adapter protein, might serve as a means of recruiting these chromosome-remodeling activities, leading to persistent changes in gene expression in the glands of parous animals.

Thus, epigenetic changes can provide an enduring memory that predetermines cell fate and prevents cell-lineage aberrations, leading to cancer (38). This offers a highly plausible explanation for the persistent changes in gene expression that have been observed in the mammary glands of parous animals. It is clear that further studies are necessary to fully elucidate the function of these markers in the parous mammary gland. However, the elucidation of markers that show persistent changes in gene expression in response to exposure to E and P is critical for understanding the molecular pathways that are altered in the parous gland and modulate the response of the gland to further proliferative stimuli. In this study, we have identified a number of such markers that warrant further study. These results provide the first support at the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland and may contribute to the response of mammary epithelial cells to future carcinogenic insults.

MATERIALS AND METHODS

Animals

Thirty-five-day-old virgin and 5-d pregnant Wistar-Furth rats were purchased from Harlan Sprague Dawley, Inc. (Chicago, IL). Animals were housed using approved American Association for Laboratory Animal Care guidelines in plastic cages containing wood-chip bedding under a 12-h light/dark cycle and were permitted *ad libitum* access to food and water. All experiments were performed in accordance with the NIH guidelines for the care and use of experimental animals.

Hormonal Manipulation

Forty-two-day-old Wistar-Furth rats were treated with a defined hormonal regimen to mimic the protective effects of an early full-term pregnancy using the experimental paradigm described previously by Sivaraman *et al.* (11). Rats were divided into two groups (n = 20 per group): those receiving the hormonal stimulus and those serving as AMV control subjects. In both cases, the rats were treated with a priming dose of E2 benzoate dissolved in sesame oil (2.5 μg in 0.1 ml; sc) to synchronize estrus both within and between the two groups. Three days subsequent to the E2 boost, animals in the experimental group were treated with E and P by sc implantation of a pellet containing 20 μg of E and 20 mg of P in a beeswax medium in the dorsal region of the back. Conditions for the preparation of the E/P pellets have been described previously (11). AMV control animals received blank pellets containing the vehicle alone. Pellets were replaced after 10 d to provide hormonal stimulation for a total of 21 d. The hormonal stimulus was continued for 21 d to mimic the period of a full-term pregnancy. After this phase, the beeswax pellets were removed and the rats were subjected to a resting period of 28 d to allow the mammary glands to involute (Fig. 1A).

To study the effects of differentiation in the absence of the hormonal changes conferred by pregnancy or E+P treatment, 45-d-old Wistar-Furth rats were treated with PPZ using a modification of the experimental paradigm presented above. Rats were divided into two groups, with 10 animals per group: those receiving the hormonal stimulus and those serving as AMV control subjects. Rats in the treatment group then received a sc injection of PPZ (Sigma; 5 mg/kg in 0.03 M HCl) five times per wk for a period of 3 wk. Controls received the vehicle alone. At the end of the treatment period, the rats were again subjected to a resting period of 28 d to allow the mammary glands to involute.

Tissue Collection

Animals were killed by an intracardial injection of 0.05 ml of ketamine/xyalazine/acepromazine. Inguinal number 4 mammary glands were harvested from 12-d pregnant, 21-d E+P-treated, 21-d PPZ-treated, 28-d involuted (E+P, PPZ), and AMV control subjects. Tissues were either flash frozen in liquid nitrogen for Western and Northern blot analyses or fixed in 4% paraformaldehyde in PBS at 4 C for 18 h for *in situ* hybridization analysis. For the 12-d pregnant samples, pregnancy was confirmed by dissection of the uterus and by the presence of a normal conceptus. For E+P- and PPZ-treated animals, hormonal stimulation of the gland was routinely confirmed by removing the left number 4 mammary gland immediately after the 21-d treatment period or after a 28-d involution period and subjecting it to whole-mount analysis as described previously (11). In this case, mammary glands were fixed in 10% neutral buffered formalin for 24 h and stained as described previously (52). Stained glands were examined to ensure that they displayed morphological development consistent with the particular regimen.

RNA Isolation

Total RNA was isolated from frozen tissues by homogenization in RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RNA fractions from different animals from the same group were combined to minimize variation between individuals and the poly(A) fraction was isolated from this pooled RNA sample using the PolyATtract mRNA purification system (Promega Corp., Madison, WI). RNA quality and yield were determined by spectrophotometric measurement, and the RNA was stored at −70 C until used.

SSH

SSH was performed using the PCR-Select cDNA Subtraction Kit (CLONTECH Laboratories, Inc., Palo Alto, CA) in accordance with the manufacturer's instructions but with the following modifications. Poly(A^+) RNA was isolated from pooled total RNA samples (n = 18 each) corresponding to either E+P-treated or AMV control glands after a 28-d involution period. cDNA fractions were synthesized from 2 μg of poly(A) RNA from each tissue pool, and then the AMV cDNA was used as a driver to subtract molecules common to both populations of RNA from the E+P tester cDNA. The efficiency of the subtraction procedure was monitored by gel analysis of the amplified products before and after subtraction and by depletion of glyceraldehyde-3-phosphate dehydrogenase in the subtracted *vs.* the nonsubtracted cDNA. Subtracted products were subjected to PCR-based amplification in which the primary and secondary PCR conditions were altered as follows to optimize product formation: in the primary PCR, the annealing temperature was reduced to 64 C and the

number of cycles was increased to 30; in the secondary PCR, a final extension cycle of 7 min was added and the amplified products were "A tailed" by incubation at 72 C for 15 min in the presence of 0.5 U of *Taq* polymerase (Life Technologies, Inc., Gaithersburg, MD). The resultant subtracted amplified cDNA products were purified and cloned into a pGEM-T Easy TA cloning vector (Promega Corp.). The ensuing E+P SSH library was propagated by transformation into Epicurian Coli XL2-Blue ultracompetent cells (Stratagene, La Jolla, CA) according to the recommendations of the supplier. Recombinant E+P SSH clones were selected by plating onto 150-mm-diameter plates supplemented with isopropyl-1-thio-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Differential Screening of the E+P SSH Library

Colonies (n = 864) from the E+P SSH library were selected at random and inoculated into 100 μl of Luria-Bertani medium supplemented with ampicillin (100 μg/ml) on 96-well round-bottomed microtiter plates. Bacteria were incubated at 37 C overnight on a shaking platform, and then 3 μl of the subsequent bacterial culture were transferred to individual wells of a 96-well PCR plate (Perkin-Elmer Corp., Foster City, CA). Bacteria were lysed by heating to 95 C for 5 min, and the recombinant DNA inserts were amplified using nested primers complementary to the adapter fragments used in the library construction, as described in the PCR-Select cDNA Subtraction Kit manual. PCR reactions typically contained 0.2 mM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.3 μM of each primer, and 1 U of *Taq* DNA polymerase (Life Technologies, Inc.) in a total volume of 20 μl in the presence 1× standard PCR buffer. *Taq* polymerase was preincubated with Taqstart antibody (CLONTECH Laboratories, Inc.) for 30 min at room temperature before addition to the reaction mix. Amplification was performed using 23 cycles each of 94 C for 30 sec and 68 C for 3.5 min. Equal volumes of the amplified products were arrayed, in duplicate, on high-density-format 1.5% agarose gels (Centipede gel electrophoresis chambers; Owl Scientific, Woburn, MA), as described by von Stein *et al.* (26), and then transferred onto charged nylon membranes (Hybond N+; Amersham Pharmacia Biotech, Uppsala, Sweden) using standard protocols.

The resulting duplicate filters were screened with double-stranded cDNA probes corresponding to either reverse or forward subtracted cDNA prepared during the library construction process. Hybridizations were performed under stringent conditions as described in the PCR-Select Differential Screening Kit users manual (CLONTECH Laboratories, Inc.). Hybridization signals were visualized by exposing the hybridized filters to BioMAX MR film in the presence of a BioMAX MS intensifying screen (Eastman Kodak Co., Rochester, NY), and the signals of replicate clones were compared. Clones displaying a differential pattern of expression were selected for further analysis, and plasmid DNA was isolated using a Quantum miniprep kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Isolation of Full-Length G.B7

A rat E+P cDNA library was prepared from poly(A^+) RNA isolated from 28-d involuted E+P-treated mammary glands and ligated into a λZAP Express bacteriophage vector (Stratagene) in accordance with the manufacturer's instructions. A total of 5×10^5 recombinant plaques from the resulting amplified library were screened by standard protocols using a random primed [α-^{32}P]dCTP-labeled cDNA probe corresponding to the 764-bp G.B7 fragment isolated from the E+P SSH library.

DNA Sequencing and Analysis

Purified plasmid clones were subjected to dideoxy sequencing using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA) at the DNA Sequencing Facility of the Child Health Research Center, Baylor College of Medicine. Clones from the SSH library were sequenced using the T7 universal primer. Full-length G.B7 clones were sequenced using sequence-specific internal oligonucleotide primers obtained from Integrated DNA Technologies (Coralville, IA). The resulting sequence data were analyzed using the homology analysis software (BLAST and BEAUTY) available through the National Center for Biotechnology Information (NIH, Bethesda, MD) and the BCM Search Launcher (Human Genome Sequencing Center, Baylor College of Medicine), respectively.

Northern Analysis

The poly(A^+) RNA fraction from E+P-treated 28-d involuted and AMV control mammary glands was prepared from a pooled total RNA sample as described above. Poly(A^+) RNA (2 μg/lane) was resolved by electrophoresis on a 1.2% agarose/0.66 M formaldehyde gel and subsequently transferred onto a charged nylon membrane. The blots were hybridized with [α-^{32}P]dATP-labeled cDNA probes prepared from selected clones isolated from the E+P SSH library, stripped, and reprobed with a cyclophilin probe. Hybridization signals were detected by exposing the filters to BioMAX MR film (Eastman Kodak Co.) in the presence of intensifying screens. Several exposure times were used to ensure that the signals from individual hybridizations were in the linear range for the film. Films were scanned using densitometry (Molecular Dynamics, Inc., Sunnyvale, CA), and quantitation was performed using ImageQuant 1.1 software (Molecular Dynamics, Inc.). The fold induction of individual markers was determined by normalizing the quantitative data to that obtained from the cyclophilin probe.

In Situ Hybridization

Paraffin-embedded sections (7 μm) from paraformaldehyde-fixed tissue were cut and mounted onto Probe-On Plus-charged slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized, rehydrated, treated with proteinase K (20 μg/ml) for 15 min, postfixed in 4% paraformaldehyde, and prehybridized for 1 h in hybridization buffer [50% formamide, 5× standard saline citrate (SSC; 20× SSC = 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0), 10% dextran sulfate, 5× Denhardt's solution, 2% SDS, and 100 μg/ml denatured salmon sperm DNA] at 38 C. [α-^{33}P]UTP-Labeled riboprobes for G.B7 and RbAp46 were transcribed from 764- and 429-bp fragments, respectively, isolated from the E+P SSH library cloned into pGEM-T Easy (Promega Corp.). The RbAp46 fragment corresponded to nucleotides 1,279–1,708 of the full-length rat RbAp46 cDNA (GenBank accession number AF090306). Hybridization was performed at 42 C overnight in the presence of 1×10^5 cpm/μl radiolabeled cRNA probe. Coverslips were removed in the presence of 4× SSC (55 C), and sections were washed in 2× SSC/50% β-mercaptoethanol for 20 min at room temperature and then digested with RNAse A (40 μg/ml in 2× SSC) for 15 min at 37 C. Stringency washes were performed in 0.1 × SSC for 15 min at 42 C and 0.1× SSC for 15 min at room temperature. Sections were exposed to emulsion (Eastman Kodak Co.) for 3–5 d and then mounted in Vectashield plus 4′,6-diamidino-2-phenylindole medium (Vector Laboratories, Inc., Burlingame, CA).

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