Oncogene (2001) 20, 3247–3257

www.nature.com/onc

Protein kinase CK2 in mammary gland tumorigenesis

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Protein kinase CK2 is a ubiquitous and evolutionarily conserved serine/threonine kinase that is upregulated in many human cancers and can serve as an oncogene in lymphocytes. Recently, we have demonstrated that CK2 potentiates Wnt/β-catenin signaling in mammary epithelial cells. To determine whether CK2 overexpression contributes to mammary tumorigenesis, we have performed comparative studies of human and rat breast cancer specimens and we have engineered transgenic mice with dysregulated expression of CK2α in the mammary gland. We find that CK2 is highly expressed in human breast tumor specimens and in carcinogen-induced rat mammary tumors. Overexpression of CK2α in the mammary gland of transgenic mice, under control of the MMTV-LTR, causes hyperplasia and dysplasia of the female mammary gland. Thirty per cent of the female MMTV-CK2α transgenic mice develop mammary adenocarcinomas at a median of 23 months of age, often associated with Wnt pathway activation, as evidenced by upregulation of β-catenin protein. NF-κB activation and upregulation of c-Myc also occur frequently. Thus, in mice, rats, and humans, dysregulated expression of CK2 is associated with and is capable of contributing to mammary tumorigenesis. Targeted inhibition of CK2 could be useful in the treatment of breast cancer. *Oncogene* (2001) **20,** 3247–3257.

Keywords: casein kinase II; CK2; transgenic mice; breast cancer; NF-κB; β-catenin

Introduction

Protein kinase CK2 (formerly known as casein kinase II), is a tetrameric serine-threonine kinase constituted of two α or α′ catalytic subunits of 42–44 and 38 kDa, respectively, and two β regulatory subunits of 28 kDa. It is remarkably conserved throughout evolution (Heller-Harrison *et al*., 1989; Seldin and Leder, 1995) and ubiquitously found in all eukaryotic cells, indicative of a vital cellular role for this protein. CK2 is required for cell cycle progression in yeast (Glover, 1998), and may have a role in G_2 checkpoint regulation (Toczyski *et al*., 1997). CK2 is also involved in cell cycle progression in mammalian cells (Orlandini *et al*., 1998; Pepperkok *et al*., 1994); we have shown that CK2 phosphorylates HSIX1, a homeobox protein with a G_2 cell cycle checkpoint function in human breast cancer cells (Ford *et al*., 2000). Targeted disruption of the CK2α′ subunit in mice reveals an indispensable role for this subunit in male germ cells where it is preferentially expressed (Xu *et al*., 1999). In general, CK2 is elevated in proliferative tissues: its expression is high in embryonic tissues (Issinger, 1993; Mestres *et al*., 1994), regenerating liver (Pancetti *et al*., 1996; Perez *et al*., 1988), and also in the proliferative cell layer at the base of the crypt in colonic mucosa (Munstermann *et al*., 1990). The mechanisms by which CK2 regulates cell proliferation remain unclear, as CK2 has hundreds of substrates in cells and affects many critical cellular growth pathways (reviewed in Allende and Allende, 1995; Guerra and Issinger, 1999; Pinna and Meggio, 1997). Like HSIX1, many other transcription factors can be regulated by CK2 phosphorylation, as has been demonstrated for c-Myc, c-Myb, AP-1, UBF, homeodomain proteins, steroid hormone receptors, and others. The p65 subunit of NF-κB is a direct CK2 substrate (Bird *et al*., 1997), and CK2 also regulates NF-κB through phosphorylation of its inhibitory subunit IκB (Janosch *et al*., 1996; Lin *et al*., 1996; Pando and Verma, 2000; Schwarz *et al*., 1996). In *Drosophila*, phosphorylation of the IκB homolog cactus by CK2 is required for wild type axis formation (Liu *et al*., 1997). Recently, CK2 was shown to interact with elements of the wingless/Wnt signaling pathway in insect cells (Willert *et al*., 1997) and we have demonstrated that CK2 is a positive regulator of Wnt signaling in mouse mammary epithelial cells (Song *et al*., 2000). Activation of this pathway by the mouse mammary tumor virus

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Received 25 October 2000; revised 14 February 2001; accepted 21 February 2001

(MMTV) leads to breast cancer in mice (Nusse and Varmus, 1982). In many human cancers, activation of the Wnt signaling pathway occurs through inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene or mutation of the transcriptional cofactor β-catenin (Morin *et al*., 1997; Polakis, 1999; Roose and Clevers, 1999).

CK2 is also upregulated in cancer. This was reported in lymphoblastoid cells in bovine tropical theileriosis, a parasite-induced pseudoleukemia (ole-MoiYoi *et al*., 1993). In human malignancies, CK2 is elevated in lung, prostate, head and neck cancers (Ahmed, 1994; Daya-Makin *et al*., 1994; Faust *et al*., 1996; Gapany *et al*., 1995; Munstermann *et al*., 1990), malignant melanoma (Mitev *et al*., 1994), and in leukemias (Pena *et al*., 1983; Roig *et al*., 1999). CK2 overexpression may be a contributor to the molecular pathogenesis of these malignancies, as overexpression of CK2 in lymphocytes in transgenic mice leads to lymphoma (Seldin and Leder, 1995). CK2 transformation requires the participation of other oncogenic events, as lymphoma develops slowly in clonal cells in transgenic mice. Lymphomagenesis is accelerated by the presence of c-*myc* or *tal-1* transgenes or by loss of the *p53* tumor suppressor gene (Kelliher *et al*., 1996; Landesman-Bollag *et al*., 1998; Seldin and Leder, 1995). In human breast cancer, immunohistochemistry has demonstrated strong nuclear staining of the CK2α subunit in breast tumor compared to normal tissue (Munstermann *et al*., 1990). This finding, along with the functional role in the Wnt/β-catenin pathway and in regulation of HSIX1 in breast cancer cells, suggested that CK2 might play a causative role in mammary tumorigenesis. To investigate this hypothesis, we examined CK2 expression in human and rat breast tumors. We then engineered transgenic mice overexpressing the α subunit of CK2 in the mammary gland and studied the consequences of dysregulated CK2 expression in that tissue.

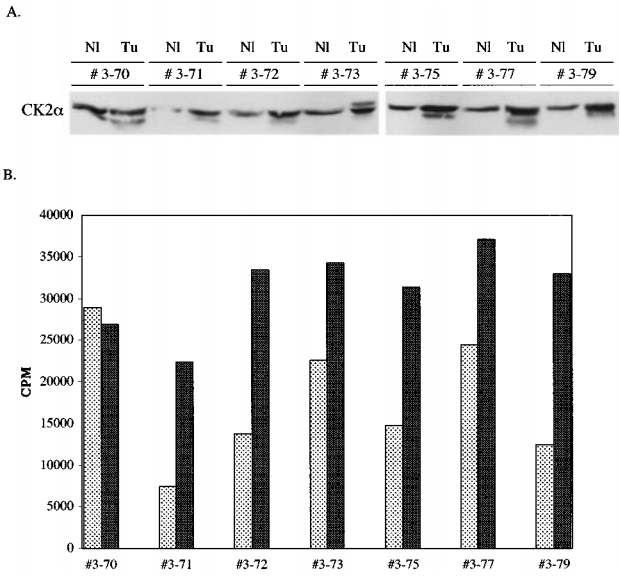

Figure 1 Protein kinase CK2 expression and activity in carcinogen-induced mammary tumors in rats. (**a**) One hundred micrograms of protein extracted from paired normal mammary glands (N1) and mammary tumors (Tu) from rats treated with DMBA were subjected to Western blotting for CK2; equal loading was confirmed by Ponceau S staining (not shown). CK2 expression is elevated in six out of seven tumor samples. (**b**) CK2 kinase activity: equal amounts of protein lysates were assayed in duplicate for phosphorylation of a specific CK2 substrate peptide (Kuenzel *et al*., 1987). Data are expressed as the difference of the mean counts of the kinase reaction carried out without the specific peptide subtracted from the mean with peptide; error bars cannot be displayed for the difference of means but were generally $<10\%$ for the means themselves. In six out of seven pairs, a marked increase in CK2 kinase activity in tumors (dark bars) is seen compared to normal mammary tissue (light bars). The overall difference in kinase activity between mammary glands and tumors is statistically significant ($P<0.003$)

Results

CK2 is highly expressed in rat and human breast tumors

As a first step in determining whether protein kinase CK2 plays a role in the pathogenesis of mammary tumorigenesis, we measured CK2 catalytic protein subunit expression and activity in rat carcinogen-induced breast tumors. Rats treated with a single oral dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) develop mammary tumors 7–20 weeks later (Huggins *et al*., 1961; Rogers and Wetsel, 1981). Proteins extracted from snap frozen tumors or control pooled normal mammary tissue from the same rats were analysed as matched pairs of samples. In six out of seven pairs, CK2 protein is upregulated in the tumors; densitometry analysis showed an average increase of 2.7-fold (Figure 1a). The differing mobility of the bands corresponds to the 42 kDa CK2α and the 38 kDa CK2α' subunits, both of which are recognized by the polyclonal rabbit antibody. Recombinant CK2 holoenzyme was run in parallel, and the recombinant α and α' bands comigrated with the rat CK2 bands (not shown). Western blot analysis of the CK2β subunit revealed no significant change in band intensity between normal and malignant tissue (not shown).

We measured kinase activity in these samples using an assay based on phosphorylation of a specific CK2 substrate peptide, RRREEETEEE (Kuenzel *et al*., 1987). Samples were assayed in duplicate, and [γ-^{32}P]GTP was employed as a phosphate donor to further ensure specificity of the assay, since CK2 is one of the few enzymes capable of utilizing GTP. The same six out of seven pairs exhibited increased kinase activity in the tumors, with a mean increase of twofold (Figure 1b). Thus both CK2 protein levels and kinase activity are comparably increased in rat mammary tumors.

We then examined CK2 levels in human breast cancer. Ten anonymous human breast tumor specimens and a non-malignant control reduction mammoplasty specimen were obtained from a tumor bank at Boston University School of Medicine, with approval of the Institutional Review Board. All breast tumor specimens exhibited abundant expression of the catalytic

subunits compared to the non-malignant mammoplasty specimen (Figure 2a). CK2 kinase activity was determined in the seven samples in which there was adequate material for duplicate measurements. All of these seven samples had more CK2 activity than the control, and on average the increase was more than 10-fold (Figure 2b). Again, the varying mobility of these bands may reflect the relative amounts of CK2α and CK2α'; furthermore, in transformed cells, a faster migrating form of CK2α has been observed (Roig *et al*., 1999). Thus, in both carcinogen-induced breast tumors in a rodent model system and in human breast tumors, CK2 protein levels and activity are elevated, suggesting that CK2 may play a role in mammary tumorigenesis.

Transgenic expression of CK2α in the mouse mammary gland

To determine whether CK2 overexpression plays a causative role in mammary tumorigenesis, we engineered transgenic mice to overexpress the α catalytic subunit of CK2 in the mammary gland using an MMTV-LTR transgene vector (Stewart *et al*., 1984). FVB/N mice were utilized. Integration of the construct into the genome of potential founders was assessed by Southern blot using the CK2α cDNA as a probe. Four founders successfully passed the transgene through the germline and their progeny were used for this study. A transgene-specific RT–PCR assay, using a 5′ CK2α cDNA sense primer and a 3′ SV40 poly(A)$^+$ antisense primer, was employed to assess the expression of the transgene in mouse organs. Virgin, pregnant, and involuting mammary glands all express the transgene (Figure 3). Weak bands are also seen in thymus, small intestine, and salivary gland; this glandular pattern of expression has been seen with other MMTV transgenes (Sinn *et al*., 1987). Both spliced and unspliced transgenic CK2α PCR products (arrows, Figure 3) were gel-purified and sequenced to confirm their identity.

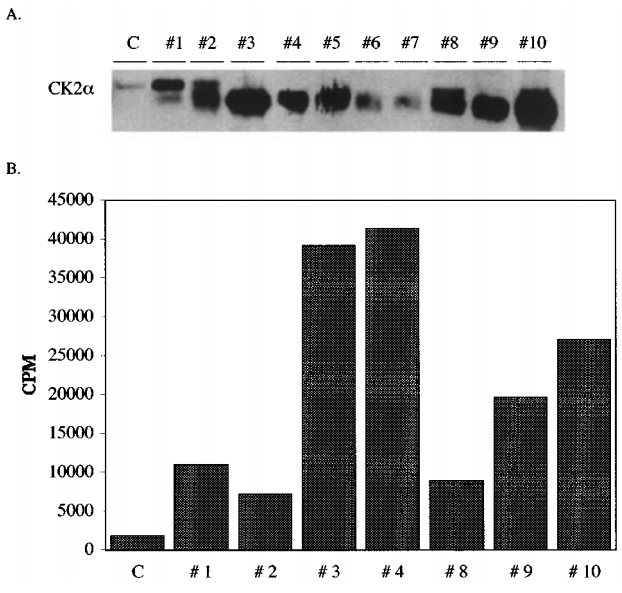

Figure 2 Protein kinase CK2 expression and activity in human breast tumors. (**a**) Representative Western blot of tissue extracts with 50 μg of protein lysate in each lane. Breast tissue obtained from a reduction mammoplasty was used as a control and compared with 10 breast cancer specimens; increased CK2 protein as seen in all 10 specimens. Equal loading was obtained as in Figure 1. (**b**) CK2 kinase activity: equal amounts of protein lysates were assayed for phosphorylation of the specific CK2 peptide as described in Figure 1b; insufficient material was available for analysis from samples 5–7. All breast tumor specimens assayed have increased levels of CK2 activity

MMTV-CK2 mice develop hyperplasia and dysplasia in the post-lactating mammary gland

MMTV-CK2α transgenic mice develop and breed normally. To promote transgene expression from the hormone-dependent MMTV-LTR, female mice were continuously bred and pups were removed after 7 days of lactation. Mammary glands from female mice at several stages of mammary gland development were taken for histological analysis. Histological abnormalities were observed in half of the MMTV-CK2α female transgenic mice (14 out of 27 mice examined). In three mice, pubertal mammary gland development was incomplete or retarded, with less than 20% of the mammary fat pad being filled with glandular tissue, although ultimately the females were able to produce adequate milk. Incomplete mammary gland regression was observed in eight mice whose mammary glands were harvested 2–12 months after the last pregnancy. Importantly, seven mice developed a variety of dysplastic lesions that are rarely seen in wild type FVB/N females, and are considered to be premalignant (Cardiff, 1996). These included areas of dysplastic alveoli and squamous metaplasia, commonly seen with areas of inflammation in the stroma and the epithelium

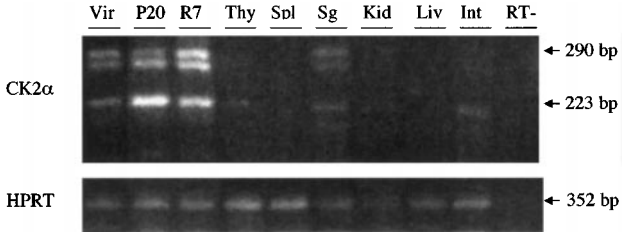

Figure 3 MMTV-LTR-driven CK2α transgene expression in transgene mice. Five to 10 micrograms of total RNA derived from organs obtained from virgin, full-term pregnant and 7 days post-lactation transgenic female mice were subjected to DNAse treatment, followed by RT–PCR. Transgene-specific oligonucleotides encompass a 67 bp splicing region in the SV40 polyA tail. Thus, unspliced mRNA yields a 290 bp band while the spliced mRNA yields a 223 bp band. Virgin, pregnant and post-lactational mammary glands express the transgene. CK2α transgenic message is also detected in thymus, salivary glands and small intestine. *hprt* amplification confirms the integrity of the reverse transcription reaction (Johnson *et al*., 1988). Vir: virgin, P20: 20th day of gestation, R7: 7 days post-lactation, thy: thymus, spl: spleen, sg: salivary gland, kid: kidney, liv: liver, int: small intestine, RT–: no reverse transcriptase added

(Figure 4). Ductal ectasia was frequently seen in regressed mammary glands, as has been reported with other oncogenic transgenes (Webster *et al*., 1998). Thus, mice with dysregulated expression of CK2α in the mammary gland frequently exhibit abnormalities of mammary gland development and regression, and inflammatory and preneoplastic lesions.

MMTV-CK2α mice develop late-onset mammary adenocarcinomas

A cohort of 56 transgenic female mice was observed for over 2 years. Thirty per cent of the mice developed mammary tumors at a median age of 23 months. In addition, over 35% of the mice developed non-mammary malignancies, including lung tumors and lymphomas. The incidence of non-mammary tumors was very similar to that reported for wild type mice of the same FVB/N strain (Mahler *et al*., 1996), and thus most likely does not result from an effect of the transgene (Table 1). No spontaneous mammary tumors were reported in the wild type cohort (Mahler *et al*., 1996), and while spontaneous mammary tumors do occur with a very low incidence (<1%) in FVB/N mice (RD Cardiff, unpublished observation), all four of our founder lines developed breast tumors. Thus, mammary tumor development in the transgenic cohort is likely due to transgene CK2α expression rather than an insertional event. The tumors generally arose as solitary masses in single mammary glands, although multiple tumors developed synchronously in separate glands in six mice. Tumors and other mammary glands and organs were harvested for histological and molecular analyses. All the tumors were adenocarcinomas, which could be further classified into a variety of subtypes. The most common histologic subtypes were glandular and papillary adenocarcinomas, in seven tumors each (Figure 5). Four were classified as adenosquamous carcinomas (keratoacanthomas) and two as scirrhous carcinomas. One tumor consisted of large cells similar to those seen with expression of a c-*myc* transgene in the mammary gland, and one had the solid cord-like pattern of tumors as seen arising through transgenic expression of *int-2* (Cardiff and Wellings, 1999). Some tumors exhibited mixed patterns with classical adenocarcinoma in some regions and the cellular connective tissue stroma characteristic of scirrhous carcinomas in others.

Six tumors from four mice had histologic features of spindle cell carcinomas. The spindle cell pattern suggested that these may have arisen from the mammary stroma or mesenchyme rather than from the epithelium. To identify the cell of origin, immunohistochemical staining was carried out using antibodies to vimentin as a marker for mesenchymal cells and antibodies to cytokeratins 8 and 14 and smooth muscle actin to identify cells of epithelial or myoepithelial origin. The spindle cell tumors stained negligibly for vimentin but were strongly positive for the cytokeratins and smooth muscle actin, identifying these tumors to be of epithelial origin (Figure 6). This

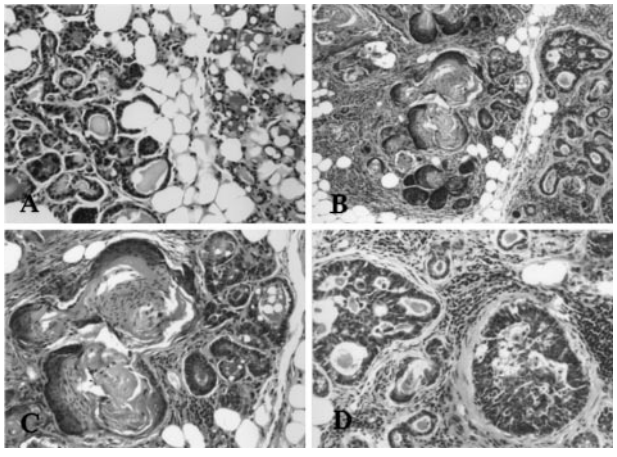

Figure 4 Mammary gland dysplasia in regressed, multiparous MMTV-CK2α transgenic mice. Hematoxylin and eosin-stained sections reveal that mammary glands from female transgenics frequently present with foci of dysplasia. (**a**) This field contains a dysplastic region on the left hand side and normal-appearing regressing mammary gland on the right hand side of the image. Compare the size and density of the nuclei in the dysplastic alveolar nodules on the left with the normal epithelium on the right. (**b**) This field includes both alveolar and squamous nodules, with the squamous nodules enlarged in (**c**) and the alveolar nodules enlarged in (**d**). Note that all fields of dysplasia are associated with extensive fibrosis and inflammation

Table 1 Tumor incidence in female MMTV-CK2α transgenic mice compared with wild type FVB/N at 24 months of age

	MMTV-CK2α transgenic mice[a] (%)	*Wild type*[b] (%)
Breast tumors	30	0
Lung tumors	21	28
Lymphomas	14	13
Other tumors or undiagnosed	17	21

[a]Fifty-six females from the MMTV-CK2α transgenic mouse colony (FVB/N background strain) were observed for tumor incidence. [b]Seventy-one wild type FVB/N female mice were monitored for tumor incidence as reported by the National Institute for Environmental Health Sciences (Mahler *et al*., 1996). For this comparison, only histological malignant lesions were scored as tumors; adenomas and other benign lesions were not included

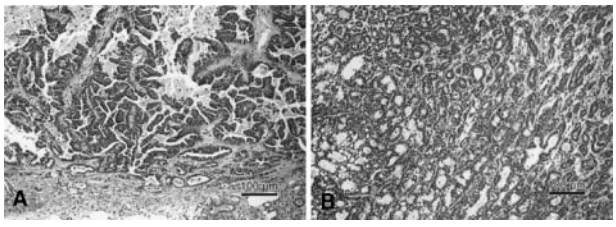

Figure 5 Representative histopathologies of breast tumors developing in aging MMTV-CK2α transgenic female mice after multiple cycles of pregnancy and regression. All the breast tumors are adenocarcinomas, presenting with a variety of patterns, including papillary adenocarcinomas, characterized by a frond-like pattern with central fibrovascular stalks covered by neoplastic epithelium (**a**), or pure glandular adenocarcinomas. (**b**). Hematoxylin and eosin staining (100×)

result was further confirmed by immunofluorescence staining of two cell lines derived from the spindle cell tumors, which stained positively with a broad specificity anti-keratin antibody, similar to control MCF-7 human mammary epithelial breast tumor cells (not shown). Thus, these spindle cell adenocarcinomas have arisen through the transformation of epithelial cells into cells with histological features of mesenchymal cells; this phenomenon has been described as an epithelial to mesenchymal transition (EMT). Tumors with features of EMT have been reported to have increased invasiveness (Birchmeier *et al*., 1996). Indeed, the spindle cell transgenic breast tumors behave more aggressively than other MMTV-CK2α mammary tumors, growing rapidly in culture or upon transplantation into subcutaneous tissues of syngeneic recipients.

Expression of CK2 and candidate downstream targets in transgenic mammary tumors

CK2 protein expression and kinase activity in the tumors was determined as previously done in the rat and human breast tumor specimens; these assays measure the sum of transgenic and non-transgenic catalytic CK2. Grossly normal mammary gland from transgenic mice that developed mammary tumors were paired with the mammary tumors as controls. Twelve pairs of tumors and controls were analysed, and all exhibit higher levels of CK2α protein in the tumors. Four pairs are depicted in a representative Western blot (Figure 7a); CK2 expression can be seen in the normal glands with longer exposures (not shown). We also find upregulation of CK2 in transformed cell lines derived from these tumors, indicating that this is a property of the tumor cells themselves. CK2 kinase activity was also elevated in the tumors, averaging sixfold in six pairs assayed (Figure 7b). Similarly to rat tissues, no significant change in CK2β expression was detected (not shown).

The mammary tumors in the transgenic mice occurred in older mice, suggesting a requirement for multiple additional molecular events for complete mammary epithelial cell transformation. Some molecular alterations may be direct consequences of CK2α misexpression, while others may occur independently. Our recent observations implicate CK2 as a positive regulator of the Wnt/β-catenin pathway in mammary epithelial cells (Song *et al*., 2000). To determine whether the CK2α transgenic tumors harbor an activated Wnt/β-catenin signaling pathway, β-catenin levels were assessed by Western blot analysis of tumor tissue extracts; a representative Western blot is shown (Figure 8a). Overall, six of 11 breast tumors tested exhibited elevated levels of β-catenin compared to non-malignant transgenic mammary tissues, indicative of an activated Wnt/β-catenin pathway.

CK2 phosphorylates IκB and promotes its degradation, regulating basal as weel as inducible levels of nuclear NF-κB (Janosch *et al*., 1996; McElhinny *et al*., 1996; Pando and Verma, 2000; Schwarz *et al*., 1996).

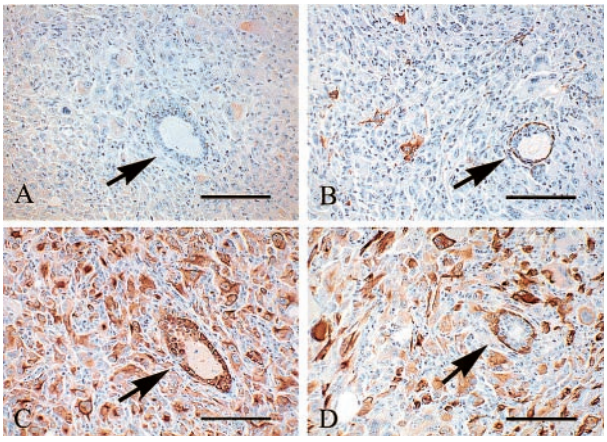

Figure 6 Immunohistochemistry of a transgenic spindle cell carcinoma. Comparable fields are shown to illustrate the mixture of tumor giant cells, fusiform spindle cells and scattered glands (arrows). Note that the tumor cells show relatively low levels of staining for vimentin (**a**). Anti-smooth muscle actin stains a well-defined myoepithelial layer in the glands (**b**, arrow) and scattered tumor cells. Anti-cytokeratin 8 stains the luminal epithelium in the glands (**c**, arrow) and many of the spindle cells and tumor giant cells. Anti-cytokeratin 14, a marker of myoepithelial cells, identifies both the myoepithelium and the spindle cell population but not the luminal cells of the glands (**d**). These results indicate that the spindle cell tumors are derived from cells of epithelial origin. Scale bars = 100 μm

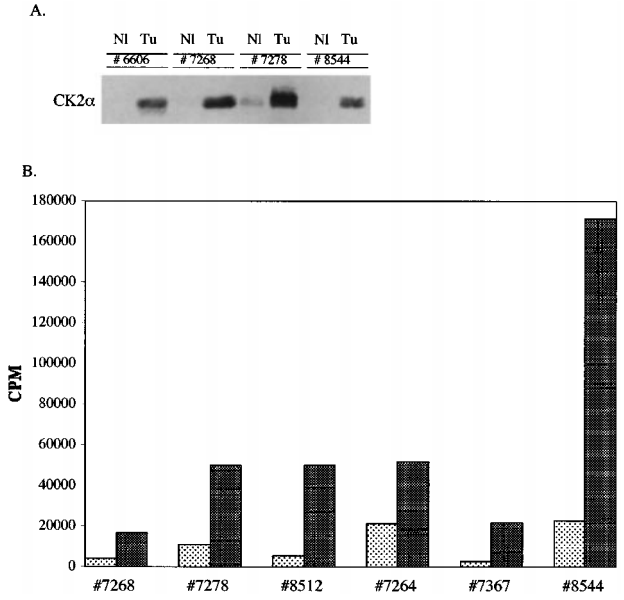

Figure 7 Protein kinase CK2 expression and activity in transgenic female MMTV-CK2α breast tumors. (**a**) One hundred micrograms of protein extracted from paired normal mammary glands (N1) and breast tumors (Tu) of transgenic mice were subjected to Western blotting for CK2. A representative blot demonstrates that the tumors have increased levels of CK2 protein. (**b**) CK2 kinase activity of the protein extracts was measured with the CK2 peptide kinase asssay as in Figure 1. Although kinase activity in the tumors was variable, the overall difference in kinase activity between mammary glands and tumors was statistically significant ($P < 0.023$). Normal mammary tissue: light bars; breast tumors: dark bars

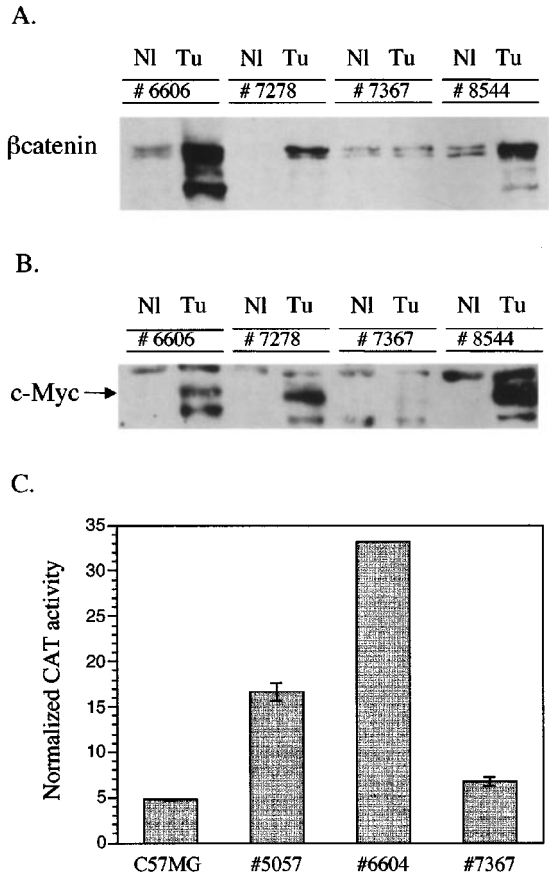

Figure 8 Expression and activity of β-catenin, NF-κB and c-*myc* in transgenic female MMTV-CK2α breast tumors. Forty micrograms of nuclear extracts obtained from paired normal mammary glands (Nl) and breast tumors (Tu) of transgenic mice were subjected to Western blotting with β-catenin (**a**) and c-Myc antibodies (**b**). Note the increase in β-catenin and c-*myc* levels in three out of four breast tumors. Equal loading was verified with Ponceau S staining. (**c**) Transgenic breast tumor and C57MG cell lines were transfected with an NF-κB-CAT reporter construct and mutant control to evaluate NF-κB transcriptional activity. Transfection efficiency was normalized using an SV40βgal reporter construct; data is expressed as mean and standard deviation of duplicate transfections. The increase in NF-κB transcriptional activity in the tumor cell lines averages fourfold over the control cell line

We and others have demonstrated constitutive high levels of NF-κB in rat and human breast cancer (Foo and Nolan, 1999; Kim *et al*., 2000; Nakshatri *et al*., 1997; Romieu-Mourez *et al*., 2001; Sovak *et al*., 1997). To determine whether this is also the case in the CK2α transgenic mammary tumors, we first examined nuclear NF-κB in primary CK2α transgenic tumors. While all four specimens examined contained constitutive nuclear NF-κB (data not shown), some of the specimens derived from the primary tumors also proved to be contaminated with lymphocytes. Thus, to determine whether there is transcriptionally active NF-κB in the malignant mammary epithelial cells, three cell lines derived from the primary tumors were transfected *in vitro* with an NF-κB-dependent chloramphenicol acetyl transferase (CAT) reporter. As a control, we used C57MG normal mouse mammary epithelial cell line (Howard *et al*., 1983). To normalize for transfection efficiency, CMV-βgal was transfected in parallel. All three cell lines exhibited higher CAT activity than the C57MG control cell line, with an average fourfold activation, indicating that the transgenic tumor lines contain transcriptionally active NF-κB (Figure 8c).

Overexpression of the c-*myc* oncogene (Seldin and Leder, 1995) or loss of *p53* (Landesman-Bollag *et al*., 1998) are collaborating events with CK2α in lymphomagenesis, and we hypothesized c-*myc* overexpression or *p53* loss might occur in CK2α transgenic mammary tumorigenesis. In particular, we expected to find elevated c-Myc expression, as c-*myc* is reported to be a transcriptional target of both NF-κB and β-catenin (Duyao *et al*., 1990; He *et al*., 1998; Kessler *et al*., 1992). Levels of c-Myc were determined by Western blot analysis of tumor tissue extracts. Three out of four tumors analysed had evidence of elevated c-Myc protein levels (Figure 8b). On the other hand, in the majority of primary tumors, *p53* mRNA was expressed and appeared normal (not shown). Thus, *p53* loss does not appear to be a frequent event in these transgenic mammary tumors.

Discussion

This study demonstrates a pathogenic relationship between protein kinase CK2 expression and mammary tumorigenesis. In carcinogen-induced rat mammary tumors and spontaneous human breast cancers, an association between CK2 levels and breast cancer was found; a transgenic mouse model demonstrated causality, as dysregulated expression of CK2α in the mammary gland promotes dysplasia and eventually cancer.

Carcinogen-induced mammary tumors produced in rats by administration of the polycyclic aromatic hydrocarbon (PAH) DMBA is a well-studied model system that has revealed molecular pathways of breast carcinogenesis (Kim *et al*., 2000; Ohi and Yoshida, 1992). This model is very relevant to human breast cancer because of the postulated role of environmental carcinogens in human mammary tumorigenesis (Morris and Seifter, 1992). A complex cellular metabolic response is initiated by the administration of DMBA, leading to DNA damage (Nebert *et al*., 1987; Randerath *et al*., 1985; Safe, 1984). Our data indicate that upregulation of CK2 protein and activity is also a component of this response, and suggest that there is a requirement for upregulation of this kinase in the development and/or maintenance of DMBA-induced mammary tumorigenesis. The relevance of CK2 to human mammary tumorigenesis is supported by our demonstration of elevated CK2 expression in human breast cancer specimens; at this time we cannot determine whether this occurs through the action of environmental pollutants or alternative molecular mechanisms.

These data are consistent with CK2 upregulation as either a consequence or a cause of mammary

gland transformation. To test the hypothesis that the overexpression of CK2 may directly contribute to mammary tumorigenesis, we used the MMTV-LTR to misexpress CK2α in the mammary epithelium of transgenic mice. Females from four independent transgenic lines exhibited histological abnormalities of the mammary gland, such as defects in involution and dysplasia. Several laboratories have observed incomplete involution in multiparous wild type FVB/N mice (Nieto *et al*., submitted); thus, this phenotype in our mice may be related to a background strain effect rather than to an effect of transgene expression. However, the dysplasic lesions observed in our transgenics are uncommon in wild type FVB/N mice and can be attributed to transgene expression; such lesions are thought to be precancerous (Cardiff and Wellings, 1999). Thirty per cent of the female transgenics developed mammary adenocarcinomas. In FVB/N mice these are rare as spontaneous malignancies (Mahler *et al*., 1996; RD Cardiff, unpublished observation). Furthermore, the bulk of the histological subtypes in our transgenics are glandular and papillary adenocarcinomas, and spindle cell carcinomas, while the rare spontaneous tumors in FVB/N are ketatoacanthomas or squamous cell carcinomas. This phenotypic difference implies a transgene-specific effect in the formation of MMTV-CK breast tumors.

The tumors had very high levels of CK2 protein and activity, suggesting that CK2 activity was required for ongoing tumor growth. This is supported by experiments with a selective pharmacologic inhibitor of CK2, the flavonoid apigenin, which inhibits growth of the CK2α transgenic breast cancer cell lines *in vitro* (not shown). Upregulation of the CK2α transgenic mRNA was only detected in some tumors (not shown); thus, we presume that the CK2 protein is derived from both transgene and endogenous CK2 expression. Variable transgene expression in tumors has been reported by other investigators and has been attributed in some cases to a hit and run mechanism in which the transgene mediates early transformation events but is not required in later steps of the process. EMT occurring in some of our transgenics could be another factor accounting for downregulation of transgene expression; as MMTV-LTR expression is restricted to epithelial and lymphoid cells, we would not expect mesenchymal tissue to express the transgene. Thus we predict that transgene expression would be shut off in our transgenic spindle cell carcinomas, since they have undergone EMT.

The variety of histologic patterns of the transgenic tumors is consistent with a variable and presumably stochastic activation of cooperating or downstream molecular pathways. The requirement for multiple additional steps to mammary transformation was also suggested by the long latency of tumor onset. We screened for such events based upon prior data about CK2-dependent pathways in growth control and cancer. CK2 participates in Wnt/β-catenin signaling (Song *et al*., 2000) and in the regulation of IκB turnover and NF-κB activity in mammary epithelial cells (Romieu-Mourez *et al*., 2001). In the CK2α transgenic breast tumors, upregulation of the Wnt/β-catenin pathway and of NF-κB occurred frequently. The role of the Wnt/β-catenin pathway is well-established in mouse mammary tumors (Barker *et al*., 1999; Bui *et al*., 1997; Dale *et al*., 1996; Huguet *et al*., 1994; Tsukamoto *et al*., 1988), and in humans it is emerging as an increasingly important pathway to breast cancer (Schlosshauer *et al*., 2000; Kuhl *et al*., 2000). CK2 phosphorylates the dishevelled signaling intermediates (Song *et al*., 2000; Willert *et al*., 1997) and β-catenin itself (Song *et al*., 2000). The NF-κB pathway has recently been documented to also play an important role in early mammary tumorigenesis (Kim *et al*., 2000; Sovak *et al*., 1997). Signal-dependent activation of NF-κB occurs in the cytoplasm via phosphorylation and degradation of IκBα which free the NF-κB subunits, allowing them to translocate to the nucleus and be transcriptionally active (reviewed in Israel, 2000). CK2 phosphorylates the C-terminal PEST domain of IκBα constitutively, and this has been found to also play a significant role in regulating IκB turnover, particularly with respect to regulation of basal levels of nuclear NF-κB (Bren *et al*., 2000; Heilker *et al*., 1999; Janosch *et al*., 1996; Lin *et al*., 1996; McElhinny *et al*., 1996; Pando and Verma, 2000; Schwarz *et al*., 1996; Tran *et al*., 1997). CK2 phosphorylates the p65 subunit of NF-κB directly (Bird *et al*., 1997). The transgenic CK2α mammary tumors have active nuclear NF-κB, suggesting cooperation between CK2 and NF-κB in mammary tumorigenesis.

c-*myc* is an important oncogene regulated by both Wnt/β-catenin signaling and by NF-κB (Duyao *et al*., 1990; He *et al*., 1998; Kessler *et al*., 1992). Bitransgenic expression of CK2α and c-*myc* in lymphocytes has demonstrated the potent transforming potential of co-expression of these genes (Seldin and Leder, 1995). c-Myc was well-expressed in the CK2α mammary tumors. On the other hand, although CK2α and *p53* loss are also collaborative in lymphoid transformation, we found evidence of *p53* loss in only two of the transgenic mammary tumors (data not shown).

In summary, we find increased expression and activity of protein kinase CK2 in breast tumors of human and rodent origin. Enforced overexpression of CK2α in the mammary gland of transgenic mice substantiates a model in which dysregulation of CK2 promotes hyperplasia and neoplasia of the mammary gland, although the latency of tumor formation points to a multistep pathway of tumorigenesis. Secondary events that may synergize with CK2 in mammary tumorigenesis include activation of the NF-κB and Wnt/β-catenin pathways, with c-*myc* as critical downstream transcription factor target. CK2 inhibitors may have a potential role in the treatment or prevention of mammary tumorigenesis.

Materials and methods

Human and rat tissues

Breast tumors were induced in rats by a single gavage with 15 mg/kg of DMBA at 8 weeks of age. The rats were sacrificed 17 weeks later. Tumors and pooled normal mammary glands were snap frozen at the time of necropsy; portions of these tissues were used for other experiments on NF-κB and breast cancer (Kim *et al*., 2000). All animal experimentation was carried out in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care and with approval of the Boston University School of Medicine I.A.C.U.C. committee. Unneeded portions of anonymous frozen human breast tissue samples and breast tumor specimens were obtained with approval of the Institutional Review Board, Boston Medical Center.

Transgenic animals

Mice from the FVB/N strain were utilized to generate transgenic animals. The murine CK2α catalytic subunit cDNA was subcloned into a vector in which the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) directs expression chiefly to the mammary epithelium, with ras 5′ untranslated sequences provided upstream of the cDNA and an SV40 intron and polyadenylation signal downstream (Sinn *et al*., 1987). Plasmid sequences were removed by restriction digestion at the *Sal*I and *Spe*I sites, and the excised transgene construct was gel purified and microinjected into pronuclei of fertilized one-cell zygotes. There were reimplanted into pseudopregnant foster mothers and the offspring were screened for presence of the transgene. Carriers were bred to establish four independent transgenic lines. Female transgenic mice were continuously bred to induce transgene expression through activation of the hormone-dependent MMTV-LTR. Mice were monitored weekly for the appearance of tumors. Ill mice were sacrificed and tissues collected for histopathological analysis, cell culture, RNA and protein analyses. To assess expression levels of the transgene in the mouse organs, tissues were collected from 6–8 week-old virgin females, from females that were pregnant, and from females at day 7 of mammary gland involution. Mice were housed in a 2-way barrier at the Boston University School of Medicine Core Transgenic Mouse Facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

Genotyping and expression analyses

Genomic DNA was extracted from 1 cm tail tip samples by digestion with proteinase K, high salt extraction of proteins, and ethanol precipitation of DNA. Genotyping was performed by Southern blot analysis or by PCR. For Southern analysis, DNA was digested with *Bam*HI, electrophoresed on 1% agarose gels (FMC Rockland, ME, USA), capillary blotted with 0.4 N NaOH onto nylon membranes (Gene Screen Plus, NEN, Boston, MA, USA), and hybridized with a α-^{32}P-radiolabeled 1.1 Kb *Bam*HI fragment comprising the entire mouse CK2α cDNA (Seldin and Leder, 1995). For PCR, a 290 bp fragment of the transgene was amplified using a sense oligonucleotide primer from the mouse CK2α cDNA coding sequence (5′-GGGATTTCTTCAGTGCCATC-3′) and an antisense oligonucleotide from the SV40 poly(A)$^+$ signal of the vector construct (5′-CCCATTCATAAGTTCCATAG-3′). PCR was performed in a thermal cycler (MJ Research, Watertown, MA, USA) by denaturing at 95°C for 30 s, annealing at 52°C for 40 s, and extending at 72°C for 40 s for 30 cycles.

For expression analysis, total RNA was extracted from mouse tissues (Chirgwin *et al*., 1979). After DNase treatment (Roche, Indianapolis, IN, USA), RNA was re-extracted and ethanol precipitated. Five to 10 micrograms of RNA were then reverse transcribed using the ProSTAR™ First Strand RT–PCR kit (Stratagene, La Jolla, CA, USA). PCR was performed with CK2α and SV40 primers described above, for 35 cycles, to detect the transgene mRNA. Both spliced and unspliced transgene mRNA could be amplified, as there is a splice donor and acceptor in the amplified region of the SV40 polyA tail. The quality of first strand synthesis was verified with *hprt* amplification. PCR was performed by denaturing at 95°C for 30 s, annealing at 55°C for 40 s, and extending at 72°C for 40 s for 30 cycles using the sense oligonucleotide (5′-GTTGGATACAGGCCAGACTTTGT-TG-3′) and the antisense oligonucleotide (5′-GAGGGTAGGCTGGCCTA-TAGGCT-3′) (Johnson *et al*., 1988).

Western blot analyses

Protein extracts were prepared by homogenizing frozen tumors or mammary gland specimens in lysis buffer containing a cocktail of protease and phosphatase inhibitors (40 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 125 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM Na_3VO_4, and 10 mM sodium pyrophosphate) followed by gentle centrifugation to remove debris. The same method was used for whole tissue culture cell extracts, after directly lysing the cells in lysis buffer. Nuclear extracts from breast tissue samples or mammary tumor cell lines were prepared as described (Sovak *et al*., 1997). Lysate protein content was quantified by BCA protein assay (Pierce, Rockford, IL, USA). Samples (40–100 μg) were electrophoresed on 7.5% or 10% SDS polyacrylamide gels (Laemmli, 1970) and transferred onto nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Filters were blocked in 5% milk, 1 × PBS, 0.05% Tween, incubated with primary antibody, washed, incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed again and visualized by ECL (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Primary antibodies were rabbit α-human-CK2α (Stressgen, Victoria, BC), rabbit α-CK2β (a generous gift from N Chester and D Marshak), MAb anti-β-catenin (Signal Transduction, Lexington, KY, USA), and rabbit anti-c-myc (UBI). Densitometry analysis was performed using Quantity One software (BioRad, Hercules, CA, USA). Verification of equal loading was done by staining the nitrocellulose filters with Ponceau S (Sigma, St Louis, MO, USA). This was found to be more accurate than quantitation of selected cytoskeletal proteins such as actin or tubulin or of cytokeratins, since the tumors clearly exhibited variable expression of these proteins than did not correlate with the non-selective BCA or Ponceau S techniques, as has been reported by others (Adam *et al*., 1998; Perfetti *et al*., 1991). For immunofluorescence, we used the broad spectrum anti-keratin antibody, clone C-11, (NeoMarkers, Union City, CA, USA).

Immunohistochemistry

The tumor samples were sectioned and placed on glass slides. They were analysed using immunohistochemical stains for

vimentin (PH 514, diluted 1 : 2000, Binding Site, San Diego, CA, USA), smooth muscle actin (A2547 diluted 1 : 1000, Sigma), cytokeratin 8 (PH 192 diluted 1 : 200, Binding Site San Diego, CA, USA) and cytokeratin 14 (PH 503 diluted 1 : 200, Binding Site). The anti-smooth muscle actin, a mouse monoclonal antibody, was amplified and detected using the 'mouse on mouse' kit K3954 (Dako Corp., Carpinteria, CA, USA). The other antibodies, all sheep anti-peptide polyclonal antibodies (Binding Site), were detected and amplified using biotinylated rabbit anti-sheep IgG, diluted 1 : 1000 (BA 6000 Vector Labs, Burlingame, CA, USA). Microwaving of the sections in citrate buffer was used for antigen retrieval. The general conditions and times were according to manufacturer's instructions. However, all antibodies and detection systems were optimized using a prepared panel of mouse control tissues. Positive and negative controls were included with each experimental run as quality controls. The sections were lightly counter-stained with hematoxylin to provide contrast. Each slide contained sufficient normal tissue to provide internal controls for the observer. Digital images were captured using a Kontron digital camera with ProgRes software and processed in PhotoShop.

Histology

Upon necropsy, tumors and organs were removed and immediately fixed in Optimal Fix (American Histology Reagent Company, Inc) and shipped in alcohol. The tissues were processed, embedded in paraffin and sectioned at 7 microns. The sections were mounted on glass slides and stained with hematoxylin and eosin using routine laboratory procedures in the Transgenic Core Pathology Laboratory at the University of California, Davis. Sections were compared with other specimens in the extensive mouse mammary tumor database (http://www-mp.ucdavis.edu/tgmice/firststop.html).

Cell culture

Transgenic breast tumor cell lines were generated by mincing the tumor in DMEM under sterile conditions followed by culture in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Mediatech Inc. Cellgro, Herndon, VA, USA) in a 5% CO_2 incubator at 37°C to establish immortalized cell lines. In some cases, the tumorigenic nature of these cell lines was confirmed by transplantation of approximately 10^6 cells subcutaneously into syngeneic recipients.

CK2 peptide kinase assay

Samples (2–5 μg) of protein lysate were incubated in a 1 mM solution of the specific protein kinase CK2 substrate peptide RRREEETEEE (Sigma-Genosys, The Woodlands, TX, USA) in CK2 kinase buffer (100 mM Tris pH 8.0, 20 mM $MgCl_2$, 100 mM NaCl, 50 mM KCl, 0.1 μg/μl BSA and 100 μM Na_3VO_4) and 5 μCi of [γ-^{32}P]GTP (6000 Ci/mmol) at 30°C for 10 min (Kuenzel *et al*., 1987). The reaction was stopped by adding 25 μl of 100 mM ATP in 0.4 N HCl. Samples were spotted onto a P81 Whatman filter and washed in 150 mM H_3PO_4, four times, 5 min each, to remove unincorporated [γ-^{32}P]GTP. Phosphorylated peptides were quantified in an automatic scintillation counter. The samples were assayed in duplicate and background kinase activity in the absence of the specific peptide substrate was subtracted. The statistical significance of differences in activity between pairs was determined by the *t*-test for Paired Two Sample for Means.

NF-κB reporter assay

To evaluate NF-κB transcriptional activity, wild type (E8-CAT) and mutant (dmE8-CAT) NF-κB element-thymidine kinase (TK) promoter-CAT reporter vectors, containing two copies of either the wild type or mutant NF-κB element from upstream of the c-*myc* promoter were employed (Duyao *et al*., 1990). Cells were transfected, in duplicate, using Fugene reagent (Boehringer Mannheim) according to the manufacturer's directions. CAT assays were performed, and the pSVβ-galactosidase (SV40βgal) reporter vector was used to normalize transfection efficiency, as previously described (Arsura *et al*., 1997). The mean and standard deviation of duplicates were calculated.

Acknowledgments

We thank Drs Adrianne Rogers, Carol Rosenberg, and Abdul Traish in the Departments of Pathology and Medicine at Boston University School of Medicine for providing breast tumor and tissue specimens from rats and from human patients; Anne Harrington and Philip Leder in the Genetics Department at Harvard Medical School for transgenic injections; Judy E Walls in the Transgenic Mouse Core Histopathology Laboratory at UC. Davis School of Medicine for outstanding technical work on the histology and immunohistochemistry; and members of the Seldin and Sonenshein laboratories for helpful comments and review of the manuscript. This work was supported by the Massachusetts Department of Public Health Breast Cancer Training Program (E Landesman-Bollag, DH Song and DC Seldin); by the Association pour la Recherche sur le Cancer (R Romieu-Mourez) and the Fondation Bettencourt-Schueller (R Romieu-Mourez); the Department of Defense DAMD 17-98-1 (GE Sonenschein, DC Seldin); NIH RO1 CA 82742 (GE Sonenshein, DC Seldin); and by the University of California Breast Cancer Research Program grant #JB-0014 (RD Cardiff).

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