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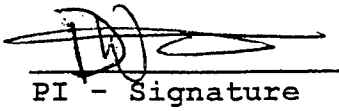

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Appendix: One paper

Please note: The new part of the report summary has been underlined.

ABSTRACT

The NF-κB/Rel family of dimeric transcription factors has been shown to promote cell survival, and increasing evidence suggests involvement in carcinogenesis. Recently, NF-κB/Rel was found to be constitutively active in the nuclei of human breast cancer cell lines, as well as in 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors from Sprague-Dawley rats (S-D). Malignantly transformed human mammary epithelial cells (HMEC), derived by carcinogen treatment of non-tumorigenic parental MCF-10F cells displayed increased constitutive NF-κB activation. In premalignant HMECs immortalized by carcinogen treatment *in vitro,* NF-κB activity was dysregulated in quiescence. Six founder lines of transgenic mice with targeted ectopic expression of the c-Rel subunit in the mammary gland were established, and studies are in progress to directly test the role of NF-κB/Rel in the mammary gland. Of note, one of the mice has developed a mammary tumor. AhR and RelA cooperatively transactivated the c-*myc* promoter in the MCF-10F HMECs, as well as in the Hs578T breast cancer cells. In the MCF-10F cells, AhR and RelA cooperatively led to increased expression of the c-Myc protein. These results suggest that the activation of NF-κB/Rel and AhR may be critically involved in proliferation and/or malignant transformation of the mammary gland and its functional target may be the c-*myc* proto-oncogene. Overall, these studies provide evidence for involvment of the AhR, NF-κB/Rel, and c-Myc proteins in a common pathway towards malignant progression of mammary epithelial cells.

I. Introduction

NF-κB/Rel is a family of transcription factors, which are expressed in all cells; however, in most non-B cells, they are sequestered in the cytoplasm in inactive complexes with specific inhibitory proteins, termed IκBs. We have recently shown that NF-κB/Rel factors are aberrantly activated in breast cancer, and function to promote tumor cell survival. Specifically, mammary tumors induced upon carcinogen treatment of Sprague-Dawley (S-D) rats, human breast tumor cell lines, and primary human breast tumor tissue samples were found to constitutively express high levels of nuclear NF-κB/Rel, whereas normal rat mammary glands and untransformed breast epithelial cells contained the expected low basal levels. Inhibition of this activity in breast cancer cells in culture via introduction of the specific inhibitory protein IκB-α led to apoptosis. Furthermore a time course study of induction of NF-κB/Rel factors upon carcinogen treatment of female S-D rats revealed that NF-κB/Rel activation was an early event, occurring prior to malignant transformation. In the first and second years of the research fellowship our lab has pursued the following objectives:

1. Quantitate and characterize the NF-κB/Rel subunits induced in the 7,12-dimethylbenz(*a*)anthracene (DMBA) and benzo[*a*]pyrene (BaP) transformed human mammary epithelial cell (HMEC) lines D3-1 and BP-1.
2. Determine the kinetics of NF-κB/Rel induction in the carcinogen transformation process by studying the activation/function of NF-κB/Rel in the premalignant BaP transformed 184A1 cells.
3. Establishment and characterization of MMTV-cRel transgenic mice.
4. Analysis of the interaction of aryl hydrocarbon receptor transcription factor (AhR) and NF-κB/Rel proteins in the human mammary epithelial cells.
5. Analysis of the functional role of AhR/RelA in human mammary epithelial cells.

II. Body

Projects in Progress

Project 1. Carcinogen Transformed Cell Line Analysis:

A. Malignant Lines Analysis:

MCF-10F cell line was established from mammary tissue from a patient with fibrocystic disease. As a collaboration with Dr. Russo, we have obtained the 7,12 dimethylbenz(a)anthracene (DMBA), and benzo[a]pyrene (BaP) transformed derivatives of this cell termed D3-1 and BP-1, respectively. BP-1 cells exhibit increased anchorage independent growth, increased chemotaxis and invasiveness. D3-1 cells exhibit similar traits but to a lesser extent than that of BP-1.

EMSA analysis revealed that D3-1 and BP-1 cells exhibit stronger binding of NF-κB oligonucleotide probe than the parental MCF-10F cells (Fig. 1A). Supershift analysis revealed that p65/p50 and p50/p50 dimers are present in the binding complexes of the D3-1 (Fig. 1B) similar to those found in the MCF-10F cells. Functionality of the NF–κB activity was assessed by performing transient transfection analysis (Fig. 1A). Wildtype (E8) and mutant (mutE8) NF-κB element-thymidine kinase (TK) promoter-chloramphenicol acetyltransferase (CAT) reporter vectors were used for the analysis. Briefly, these consisted of 2 copies of either the wild type NF-κB element from upstream of the c-*myc* promoter, or versions with the two internal G residues converted to C residues, which significantly reduces NF-κB binding and transactivation. MCF-10F cells displayed 1.6 to 2.6-fold wildtype E8 over mutant E8 activity. D3-1 cells displayed 4.1-fold higher levels of wildtype E8 activity over the mutant. Specificity of this activity was assessed by co-transfection with an IκB-α expression plasmid, and downregulation of the activity was noted (data not shown).

Similar analysis was also performed on the BP-1 cells. These cells also exhibited stronger binding of NF-κB oligonucleotide probe than the parental MCF-10F cells (Fig. 1A). Supershift analysis revealed that p65/p50 and p50/p50 dimers in the binding complexes (data not shown). Functionality of the NF-kB activity was again assessed by transient transfection analysis. The BP-1 cells displayed 11.6-fold higher E8 over the mutant activity (Fig. 1A). Specificity of this activity was again demonstrated by co-transfection with an IκB-α expression plasmid (data not shown).

Since the rate of IκB-α turnover controls NF-κB release, protein turnover assays were performed in these cells to determine if a decrease in IκB-α stability was responsible for this increased NF-κB activity. The results clearly indicate that the turnover rate of IκB-α is markedly increased in the D3-1 and BP-1 cells compared to its parental 10F cells (Fig. 2). Thus, it appears that a potential mechanism of NF-κB activation in these transformed cells may be due to dysregulation of the IκB-α stability.

B. Pre-malignant Lines Analysis

Mika Sovak, a former graduate student in the lab, and Greg Zanieski, a former technician, demonstrated that NF-κB/Rel binding is activated in the mammary glands of DMBA-administered rats prior to tumor formation. Thus, we hypothesized that the NF-κB activation might be an early event in the transformation process. Dr. Stampfer has recently established an

orderly staging of progression of human mammary epithelial cells (HMEC) from a mortal to an immortalized yet nonmalignant phenotype following BaP treatment. 184 HMEC lines were established from a reduction mammoplasty tissue and senesce after 22 passages. Extended lifespan 184Aa clone was isolated post in vitro treatment of 184 cells with BaP. Indefinite lifespan 184A1 cells appeared from 184Aa at p9. We initiated collaboration with Dr. Stampfer, and obtained nuclear extracts of fine lifespan 184 HMECs, and late passage (fully immortal) 184A1 HMECs. EMSA demonstrates that there is very little κB binding activity for the finite lifespan 184 HMECs at both quiescence (G0) and cycling stages. However, the fully immortal premalignant 184A1 cells at quiescence displayed a very high binding activity similar to that of the Hs578T human breast tumor cells (Fig. 3). The increased NF-κB binding of 184A1 cells only when G0 arrested, suggests a dysregulation of NF-κB activation in these cells at quiescence. Transient transfection analysis of E8 kappaB reporter construct confirmed this dysregulation in the fully immortal 184A1 cells. Supershift analysis revealed that p65 and p50 subunits are the primary binding subunits of NF-κB in both the finite lifespan 184 and the fully immortal 184A1 cells (data not shown). Thus, these results reveal that NF-κB activation is dysregulated in the premalignant cells in quiescence.

Project 2. MMTV c-Rel Transgenic Mice

A. Founder Copy Numbers and phenotypes:

Six lines of transgenic mice (lines 7,8,14,15, 16, & 18) have been established by the Core Transgenic Facility of Boston University Medical Center, and confirmed by Southern blot analysis. Each mice exhibits varying copy numbers of MMTV-c-Rel DNA.

Founder Number	Approx. Copy Number	Date of Birth
7	3.7	12/28/97
8	6.22	12/28/97
14	4.74	3/16/98
15	4.5	3/16/98
16	8.75	3/16/98
18	2.58	3/16/98

1. Founder 7 – We found an interesting trait for founder 7's progenies. The male mice are unable to produce more than 5 pups in a litter. Average pup size appears to be about 4. (4,5,3,5,5,5,3,2,4,4,5). These mice have been bred to F2's and homozygotes are being now being bred for tumor formation.
2. Founders 8, 14 – No remarkable phenotype as of yet. Homozygotes are being now being bred for tumor formation.
3. Founder 15 – Initially appeared to have small pup size, however, now appears to be breeding fine. A pregnant mouse from founder 15 developed a cystic lesion extending from the L1/2 mammary gland.
4. Founder 16 – Is the line with the highest copy number. Initially, there was noticeably a higher number of male to female ratio, but now appears to no longer have this discrepancy. A virgin mouse from founder 16 developed an ovarian cystic teratoma.

B. Analysis:

Wholemount was performed from a transgenic mouse from each of the lines. No apparent morphological abnormalities were detected upon analysis. Preliminary immunoblot and EMSA studies indicate enhanced c-Rel nuclear levels, but not binding to an NF-κB oligonucleotide. Hence, in future studies, experiments are planned to test whether they will display enhanced tumor formation upon carcinogen treatment.

C. Tumor

One of the mice has developed mammary tumors, histologically identified as being adenocarcinoma (Figure 6). EMSA analysis has revealed that c-Rel is functionally activated in these tumors cells, compared to adjacent normal mammary cells (Figure 7). Cells from this tumor has also been used to establish a cell line in culture.

D. Functional role of c-Rel

We have found that in majority of these mice, the c-Rel protein is highly expressed in the mammary cells, but functionally inactive. We hypothesize that this may be due to a potential factor X, yet unidentified which may be leading to sequestration of the c-Rel proteins to the cytoplasm, or in the nucleus in an inactive form. We are in the process of determining the identity of this factor, and determining methods of activation of the c-Rel proteins. Some experiments in progress involve treatment *in vitro* of cells derived from the MMTV-c-Rel mouse's mammary tumor with known inducers of NF-κB/Rel activity, such as carcinogens, EGF, TGF-α and overexpression of Her-2/neu. Once we determine the efficacy of the treatments *in vitro* for the activation of the c-Rel protein, we intend to similarly treat the MMTV-cRel mice *in vivo* or prepare appropriate bitransgenic mice and observe for phenotypic abnormalities.

Project 3: AhR and NF-κB cooperate to induce the c-*myc* promoter.

Recent studies have shown that p65 subunit and the aromatic hydrocarbon receptor/transcription factor (AhR) co-immunoprecipitate, and in some cases inhibit promoters driven by multimerized NF-κB elements. Dr. Sherr's lab (Boston University School of Public Health, Boston MA) has demonstrated that the p65 subunit co-immunoprecipitates with AhR in extracts from the MCF-10F cells and Hs578T cells, and that AhR seems to be involved in proliferation of these cells. Since our lab had previously demonstrated that the c-*myc* proto-oncogene has two NF-κB elements, we investigated the potential role of AhR and NF-κB in cooperating to induce the c-*myc* gene, in collaboration with Dr. Sherr's lab. I have performed transient transfection analysis and demonstrated that in the MCF-10F cells (Fig. 4) and Hs578T cells (data not shown), the p65 subunit and AhR can activate the c- *myc* promoter cooperatively. A c-*myc* promoter construct that has mutations in the URE and IRE (NF-κB elements), failed to be transactivated by the AhR and p65 (Fig. 4). RelB, and c-Rel subunits failed to cooperate with the AhR in transactivating the c- *myc* promoter (data not shown). Furthermore, EMSA analysis using the URE NF-κB oligonucleotide probe revealed that transfection of AhR and p65 subunit leads to an induction of a band that migrates more slowly than the classical p65/p50 subunit (Fig. 5A). This band is specific, as judged by cold oligonucleotide competition analysis (Fig 5B), and can be block shifted by both the p65 and AhR antibody suggesting that AhR and p65 subunits are in the binding complex (Fig. 5C and D). Cotransfection of AhR and RelA increased expression of the endogenous c-Myc protein in MCF-10F cells by immunoblot analysis (Figure

8) further supporting the potential role of AhR/RelA in transactivation of the c-*myc* gene. Thus, these preliminary findings suggest an exciting new role of AhR and p65 function.

III. Figures:

Fig. 1. Carcinogen-transformed D3-1 and BP-1 cells display higher constitutive levels of functional NF-κB than the parental MCF-10F cells. **A)** Comparison of lines. The MCF-10F cells (10F) and BP-1 cells were transiently transfected by lipofection, in triplicate or duplicate, respectively with 2 ug E8 or mutE8 reporter construct. Alternatively, D3-1 cells were transfected, in duplicate, using 20ug of either E8 or mutE8 by the calcium phosphate method. After 24 hours (for lipofectamine) or 72 hours (for calcium phosphate), extracts were prepared, normalized for protein, and assayed for CAT activity. The values for E8 CAT activity are represented as fold induction over mutE8 CAT activity which was set at 1.0 for each cell line. Shown is the representative data from a minimum of 2 experiments. (Inset) Equal amounts (5 ug) of nuclear extracts from exponentially growing parental MCF-10F cells or transformed D3-1 or BP-1 cells were subjected to EMSA with a radiolabeled oligonucleotide NF-κB element as probe. A representative experiment of two independent assays is shown.
B) Supershift analysis of D3-1 cells. Following a 30 min incubation of nuclear extracts (5 ug) with the probe, 1 ul of antibody against the p50 (sc-114), p65 (#1226, kindly provided by N. Rice) or c-Rel (sc-070) was added as indicated, incubated and subjected to EMSA.

Fig. 2. IκB-α protein in D3-1 and BP-1 cells has a shorter half life than in parental MCF-10F cells. **A)** MCF-10F, D3-1 and BP-1 cells were incubated in the absence or presence of 20 ug/ml emetine for the indicated periods of time. Cytoplasmic extracts (50 ug protein/lane) were separated by electrophoresis in a 10% polyacrylamide-SDS gel, and subjected to immunoblot analysis for IκB-α protein using SC-371 antibody. These blots are representative of two experiments. **B)** The immunoblot for IκB-α protein in part A was quantitated by densitometry, and the data plotted as percent of the original protein value in untreated control cells (0 hr). The decay curves were extrapolated by using an exponential best fit analysis.

Fig. 3. Dysregulated NF-κB/Rel expression in 184A1 immortalized HMECs. **A)** 184A1 cells display increased NF-κB binding in quiescence. EMSA was performed with nuclear extracts (5 ug) from finite lifespan 184 and fully immortal 184A1 cells following G_0 synchronization upon blockage of EGF receptor signal transduction for 48 hours (G0), or during exponential growth (CYC). Two distinct NF-κB binding complexes were detected.

Fig. 4. RelA and AhR cooperate to transactivate the wildtype p1.6Bgl, but not the p1.6Bgl dbl mut, c-*myc* promoter construct. Confluent MCF-10F cells (~ 200,000 cells in 35 mm^2 dishes) were transiently transfected, in duplicate, with either 1 μg p1.6 Bgl or p1.6 Bgl dbl mut, and 0, 2, or 4 μg *pcDNA3-AhR* (murine AhR) expression vector in the absence or presence of 0.125 μg *pEVRF-p65* (RelA expression) plasmid using 7 μl FUGENE reagent. In each transfection, 1 μg of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6 μg by addition *pcDNA3* plasmid (parent vector for *pcDNA3-AhR*). Transfected cells were harvested after 24 h in reporter lysis buffer, and analyzed for CAT and luciferase activity. CAT activities are presented normalized for transfection efficiency, using the luciferase activity.

Fig. 5. Expression of RelA and AhR yields a novel URE NF-κB element binding complex. **A)** Co-transfection with AhR and RelA expression vectors leads to formation of a novel complex. Confluent plates (100 mm^2 dishes) of Hs578T cells were transfected with either 52 μg *pcDNA3* empty vector, or 50 μg *pcDNA3-AhR* in the absence or presence of 2 μg *pEVRF-p65* expression plasmid using 70 μl FUGENE reagent. After 24 h, nuclear proteins were isolated using the method of Dignam et al. (22), and subjected to EMSA. N indicates position of a new complex; 1, indicates position of previously observed major complex. **B)** Competition EMSA confirms the specificity of the major bands. Nuclear extracts of Hs578T cells co-transfected with *pcDNA3-AhR* and *pEVRF-p65* were pre-incubated with either 4- or 20-fold molar excess unlabelled wildtype (URE) or mutant (mt URE) URE prior to the 30 min incubation reaction with the radiolabelled URE. Two nonspecific bands were identified and marked with an asterix (*). **C)** Nuclear extracts from the AhR and RelA expression vector co-transfected cells, prepared as described above in Fig. 3, were incubated with the URE probe. Following a 30 min binding reaction, antibodies were added where indicated, the reactions incubated for an additional 1 h, and subjected to EMSA. Results of two separate analyses are shown. **D)** lane 1, no antibody; lane 2, 1 μl RelA-specific antibody (#1226, kindly provided by N. Rice), lane 3, 1 μl AhR-specific antibody (BioMol #SA-210); Two nonspecific bands were identified and marked with an asterix (*). A faster migrating, nonspecific band, that appears upon addition of antibody #1226 with the probe alone, is indicated by a double asterisk (**). B) lane 1, no antibody; 1 μl RelA-specific antibody (sc-372X); lane 3, 1 μl p50-specific antibody (sc-114). Specific binding complexes are indicated as band 1 and band N, as above.

Fig. 6. H&E stained section of mammary tumor (adenocarcinoma) from a 9 month old, multiparous, female MMTV-c-Rel transgenic mouse.

Fig. 7. EMSA analysis was performed on nuclear extracts (5 ug) of a mammary tumor or a normal mamary gland from the same MMTV-c-Rel mouse as above, using an NF-κB oligonucleotide as probe +/- c-Rel or p50 antibodies.

Fig. 8. Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 ug *pEVRF p65* or 20 ug *T7-pcDNA2-AhR* DNA alone or in combination with 30 ul FUGENE transfection reagent. Total transfected DNA was maintained at 24 ug by addition of *pcDNA3* plasmid. Alternatively, cells were transfected with *pcDNA3* plasmid alone (Vector). After 48 h, cells were harvested and samples of whole cell extracts (40ug) subjected to immunoblot analysis for c-Myc (786-4) and β-actin (AC-15) proteins.

Appendix

1. List of key research accomplishments:
   - NF-κB is functionally activated in HMECs malignantly transformed by environmental carcinogens
   - In premalignant HMECs immortalized by carcinogen treatment *in vitro,* NF-κB activity was dysregulated in quiescence.
   - Six founder lines of transgenic mice with targeted ectopic expression of the c-Rel subunit in the mammary gland were established, and studies are in progress to directly test the role of NF-κB/Rel in the mammary gland
   - One of the mice has developed mammary tumor, which clearly overexpresses functional c-Rel protein.
   - Aromatic Hydrocarbon Receptor (AhR) and RelA (p65) cooperate to transactivate the c-*myc* promoter in the MCF-10F HMECs, as well as in the Hs578T breast cancer cells, and leads to activation of the endogenous c-*myc* gene in MCF-10F cells.
2. Degrees Obtained
   - Ph.D. defense passed by the Principal Investigator. Formal degree to be awarded on June of 2001 upon completion of M.D. degree at Boston University School of Medicine.
3. Manuscripts/Presentations

   - **D.W. Kim**, M.A. Sovak, G. J. Zanieski, G. Nonet, R. Romieu-Mourez, A. W. Lau, L.J. Haefer, P. Yaswen, M. Stampfer, A.E. Rogers, J. Russo,G.E. Sonenshein. Activation of NF-κB/Rel Occurs Early During Neoplastic Transformation of Mammary Cell. Carcinogenesis, vol 21 no. 5, pp. 871-879, 2000

   - **D. W. Kim,** L. Gazourian, S. A. Quadri, R. Romieu, D. H. Sherr, and G. E. Sonenshein. The Aromatic Hydrocarbon Receptor/Transcription Factor (AhR) and the p65 Nuclear Factor-κB Subunit Cooperate to Transactivate the c-*myc* Promoter. Manuscript submitted to Oncogene, 2000

   - **D. W. Kim, M.A. Sovak, G. Zanieski, A. Lau, L.Gazourian, S. A. Quadri, R. Romieu-Mourez, G. Nonet, L.J. Haefer, P. Yaswen, M. Stampfer, J. Russo, A. E. Rogers, P. Toselli, D. Sherr, and G. E. Sonenshein.** ROLE OF NF-κB/REL, AHR AND C-MYC IN BREAST CANCER. DOD ERA OF HOPE CONFERENCE 20001, ATLANTA GA.

   - **D.W. Kim**, M.A. Sovak, M. Arsura, G. J. Zanieski, K. Kavanagh, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, G.E. Sonenshein. Early Activation of NF-κB/Rel During Neoplastic Transformation of Mammary Cells. Russek Day Presentation, Boston Unviersity School of Medicine (2nd Prize Award).

Figures

Figure 1

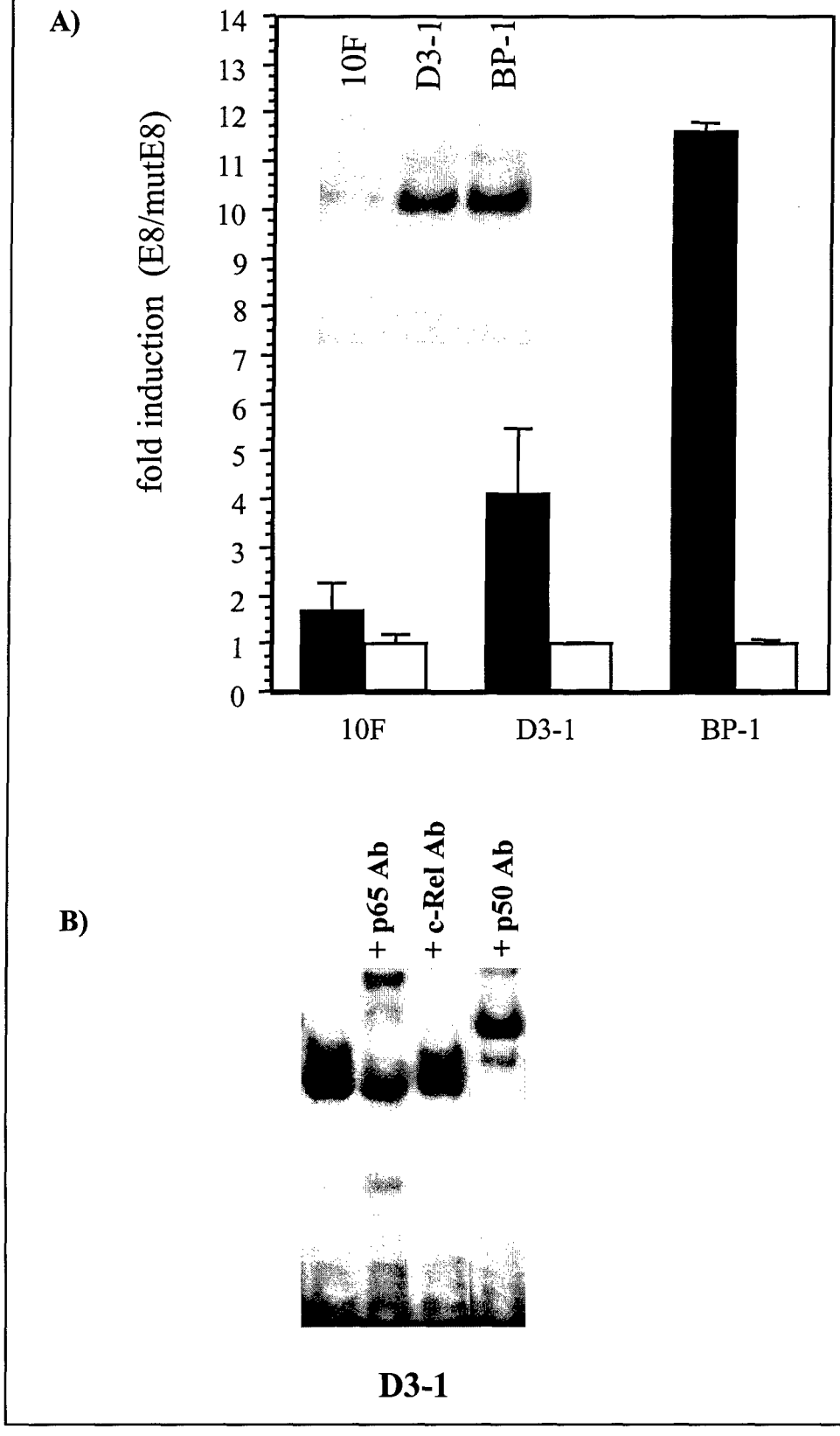
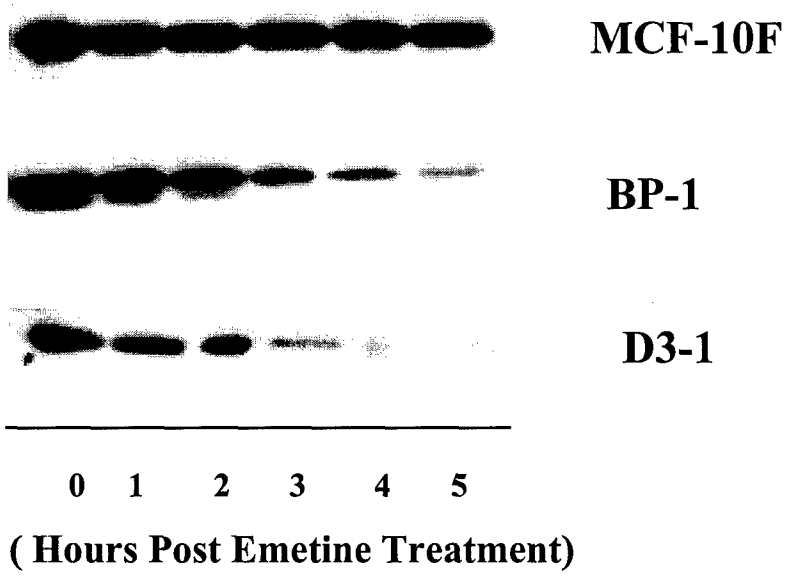


Figure 2

A)


MCF-10F
BP-1
D3-1
0 1 2 3 4 5
(Hours Post Emetine Treatment)

B)

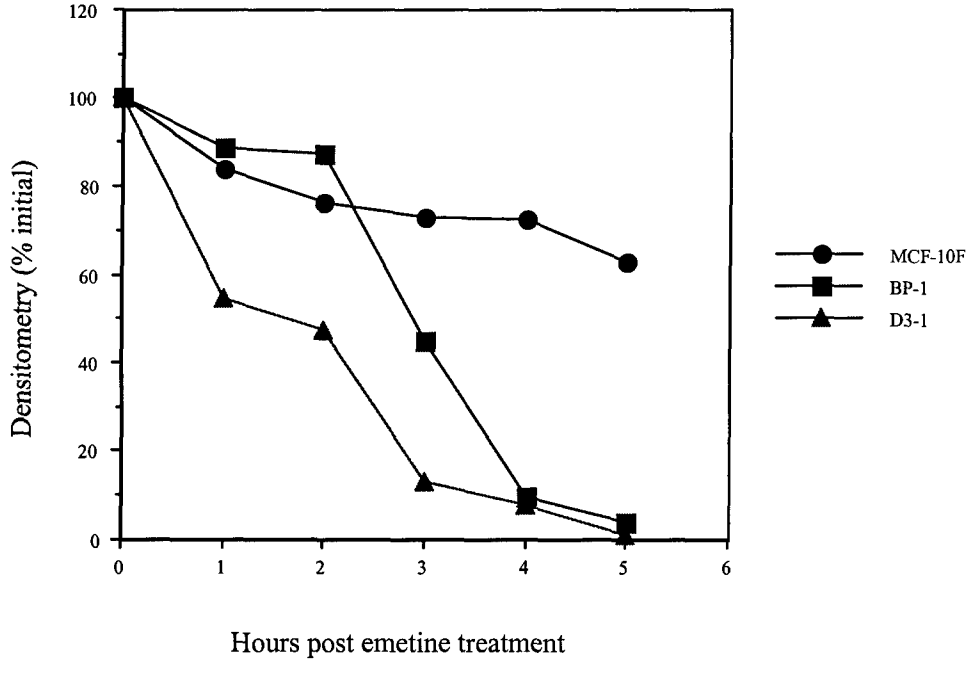
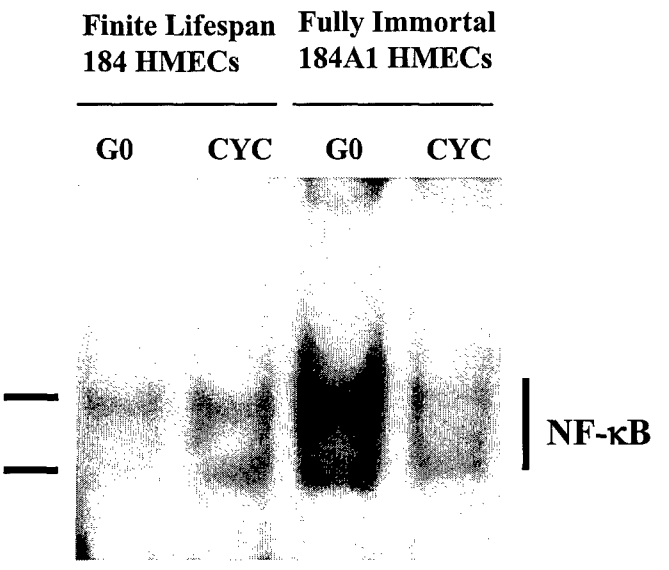

120
100
80
60
40
20
0
Densitometry (% initial)
0 1 2 3 4 5 6
Hours post emetine treatment
MCF-10F
BP-1
D3-1

Figure 3



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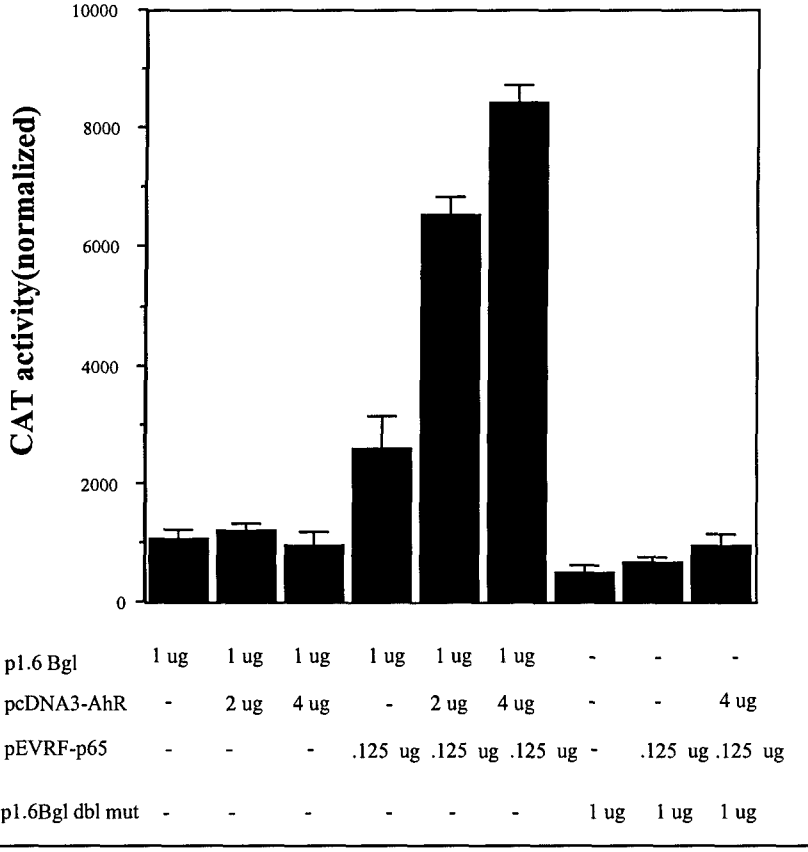

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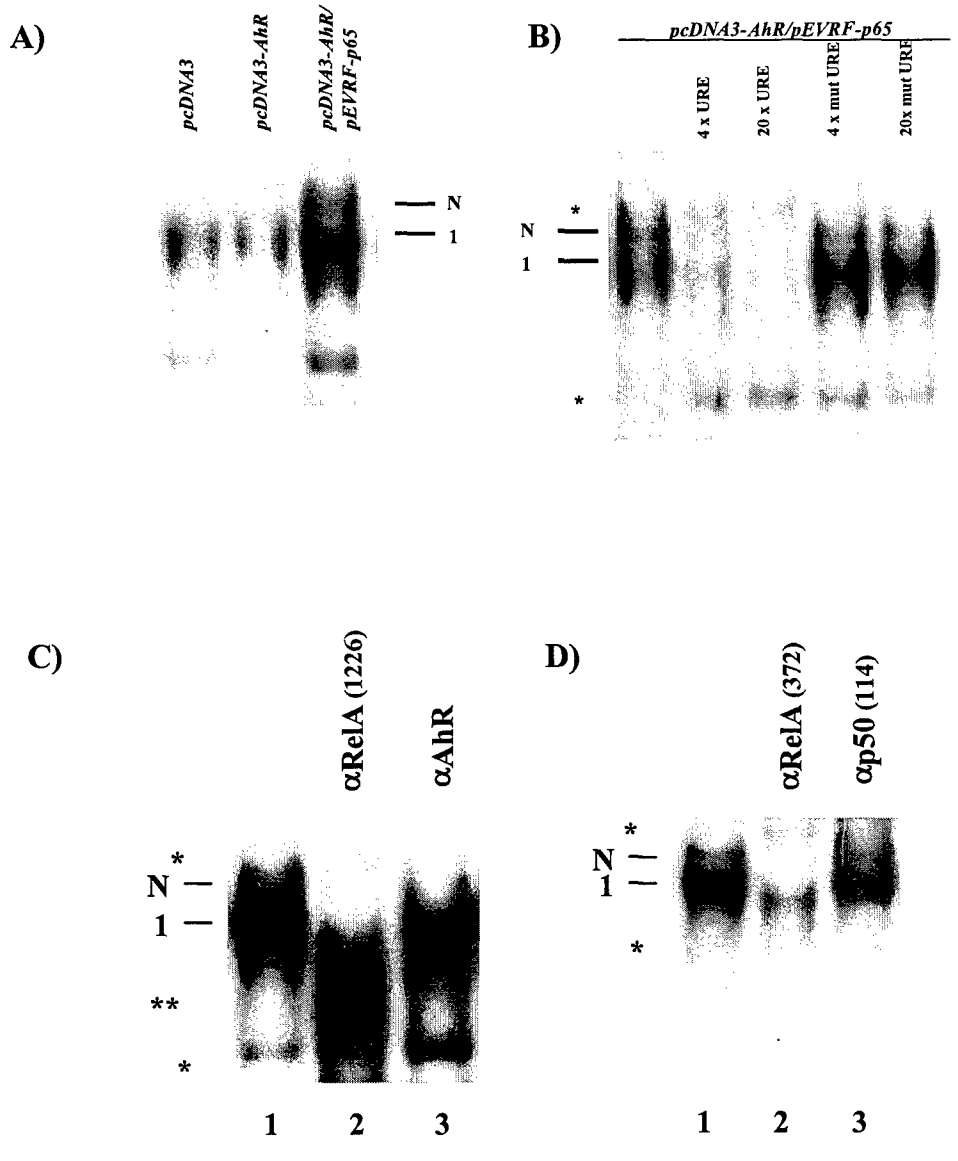
A)
pcDNA3
pcDNA3-AhR
pcDNA3-AhR/ pEVRF-p65
N
1
B)
pcDNA3-AhR/pEVRF-p65
4 x URE
20 x URE
4 x mut URE
20x mut URE
N
1
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*
C)
αRelA (1226)
αAhR
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D)
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Figure 6

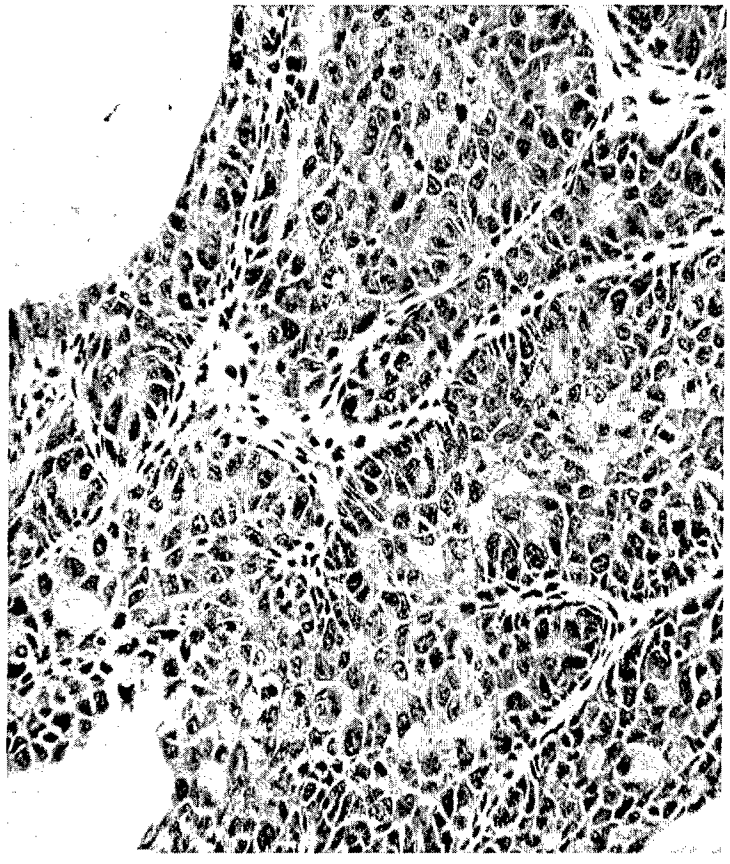
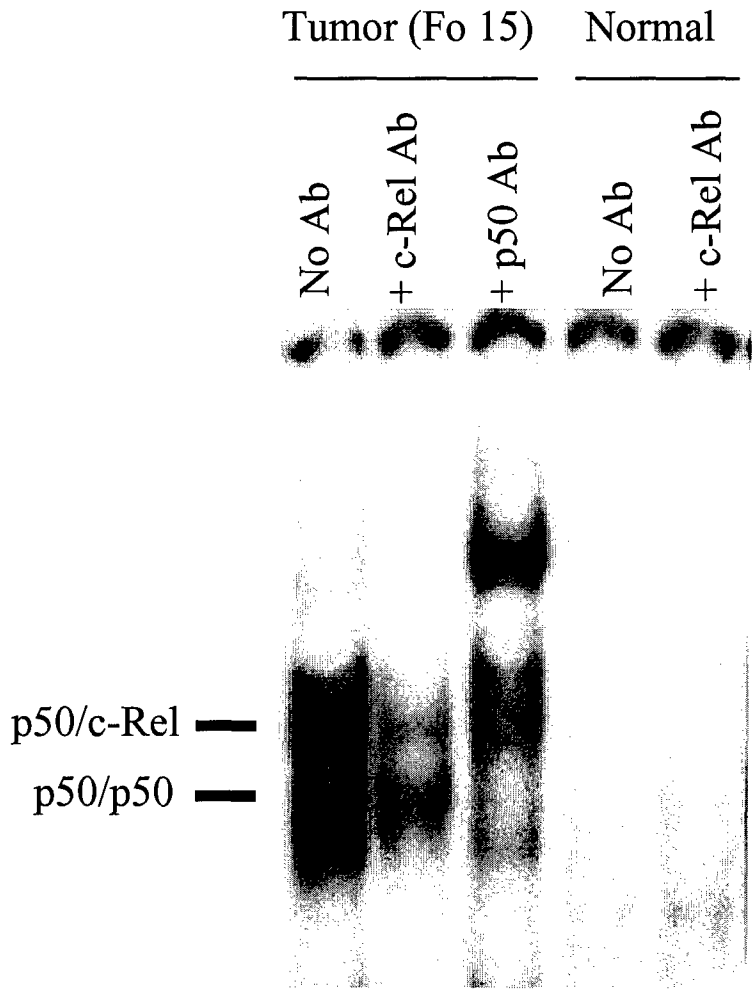


Figure 7


Tumor (Fo 15)
Normal
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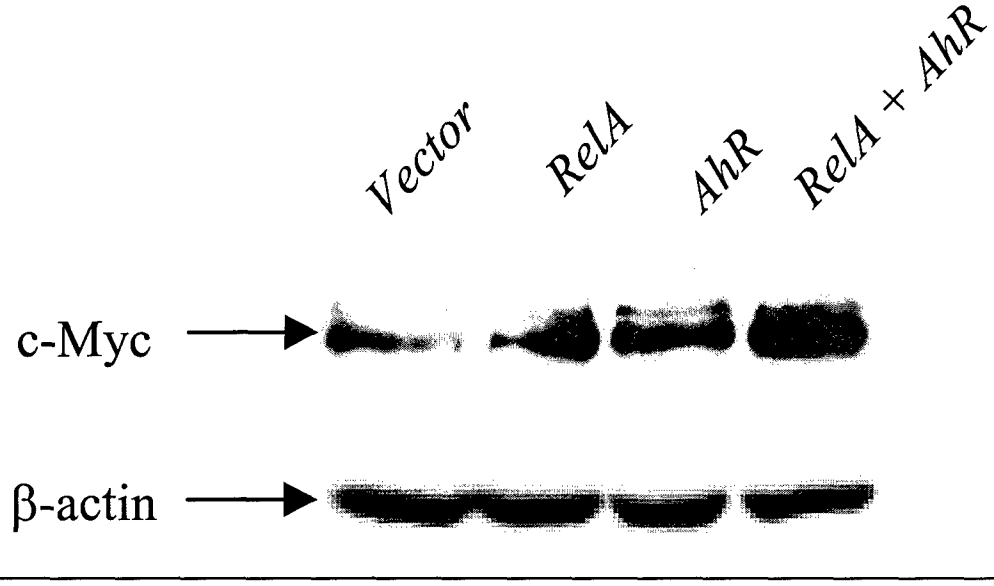
+ c-Rel Ab
+ p50 Ab
No Ab
+ c-Rel Ab
p50/c-Rel
p50/p50

Figure 8



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Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells

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NF-κB/Rel is a family of transcription factors which are expressed in all cells; however, in most non-B cells, they are sequestered in the cytoplasm in inactive complexes with specific inhibitory proteins, termed IκBs. We have recently shown that NF-κB/Rel factors are aberrantly activated in human breast cancer and rodent mammary tumors, and function to promote tumor cell survival and proliferation. Here, we have examined the time-course of induction of NF-κB/Rel factors upon carcinogen treatment of female Sprague–Dawley (S–D) rats *in vivo* and in human mammary epithelial cells (HMECs) in culture. We observed that NF-κB/Rel activation is an early event, occurring prior to malignant transformation. In S–D rats, increased NF-κB/Rel binding was detected in nuclear extracts of mammary glands from 40% of animals 3 weeks post-treatment with 15 mg/kg 7,12-dimethylbenz[*a*]anthracene (DMBA); this is prior to formation of tumors which normally begin to be detected after 7–9 weeks. In non-tumorigenic MCF-10F cells, *in vitro* malignant transformation upon treatment with either DMBA or benzo[*a*]pyrene (B[*a*]P) resulted in a 4- to 12-fold increase in activity of classical NF-κB (p65/p50). NF-κB induction was corrrelated with a decrease in the stability of the NF-κB-specific inhibitory protein IκB-α. Ectopic expression of the transactivating p65 subunit of NF-κB in MCF-10F cells induced the c-*myc* oncogene promoter, which is driven by two NF-κB elements, and endogenous c-Myc levels. Furthermore, reduction mammoplasty-derived HMECs, immortalized following B[*a*]P exposure, showed dysregulated induction of classical NF-κB prior to malignant transformation. Together these findings suggest that activation of NF-κB plays an early, critical role in the carcinogen-driven transformation of mammary glands.

Introduction

NF-κB/Rel is a family of dimeric transcription factors distinguished by the presence of a Rel homology domain of about 300 amino acids in length which determines much of its function. Classical NF-κB is a heterodimer composed of p65 and p50 subunits (1). Other members of the mammalian Rel family include c-Rel, p52 (also called lyt10) and RelB. The p65 and RelB, and c-Rel subunits, have either potent or moderate transactivation potential, respectively, whereas the p50 and p52 subunits bind avidly, but have only modest transactivation abilities (1). In most cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm, bound to one of the specific inhibitory proteins, termed IκBs, of which IκB-α is the paradigm. A variety of agents can induce NF-κB/Rel (2). Activation of NF-κB involves phosphorylation, followed by ubiquitination and proteosome-mediated degradation of IκB, which allows for translocation of active NF-κB complex into the nucleus where it can bind to κB responsive elements (2).

NF-κB/Rel has now been established as a factor promoting survival from apoptosis (3). An anti-apoptotic function for constitutively expressed NF-κB/Rel factors has been demonstrated in several cell types, including B lymphocytes (4,5) and hepatocytes (6,7). Several groups also found that induction of NF-κB/Rel upon treatment with tumor necrosis factor α (TNF-α), radiation and chemotherapeutic agents can protect cells from apoptosis (8–11). More recently, we showed that aberrant activation of NF-κB/Rel occurs in human breast cancer cells and that specific inhibition of this activity leads to the induction of apoptosis (12). There is also increasing evidence that the NF-κB/Rel family is important in control of cell proliferation and oncogenesis, e.g. correlation of factor activation has been reported in various types of cancer (13).

It has been suggested that some of the rise in breast cancer rates reflects increased exposure to and bioaccumulation of lipophilic environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), like benzo[*a*]pyrene (B[*a*]P), and related organochlorines (14). This hypothesis has been drawn, in part, from epidemiologic studies associating elevated breast cancer rates with PAH exposure (15–18) and from studies demonstrating increased levels of aromatic hydrocarbons in breast carcinomas (19,20), and in sera from breast cancer patients (18). Furthermore, many studies have shown that PAHs can cause malignant transformation of rodent models *in vivo* (21) and human mammary epithelial cells (HMECs) *in vitro* (22). For example, in Sprague–Dawley (S–D) rats, a single intragastric dose of DMBA induces mammary tumors within 7–20 weeks (21). While much has been learned about the effects of carcinogen treatment on gene activation and DNA adduct formation (23), the exact molecular mechanism(s) by which this transformation occurs has yet to be elucidated. Recently, we observed that NF-κB/Rel factors are aberrantly activated in 86% of the mammary tumors induced by DMBA-treatment of S–D rats compared with the normal mammary glands of the age-matched, vehicle-treated control animals (12). Here, we have examined the time course of carcinogen-mediated induction of NF-κB/Rel using the S–D rat model and the

Abbreviations: B[*a*]P, benzo[*a*]pyrene; CAT, chloramphenicol acetyltransferase; DMBA, 7,12-dimethylbenz[*a*]anthracene; EMSA, electrophoretic mobility shift analysis; HMECs, human mammary epithelial cells; PAHs, polycyclic aromatic hydrocarbons; S–D, Sprague–Dawley.

in vitro treated HMECs. We show that NF-κB/Rel activation occurs prior to malignant transformation, suggesting it plays an early and potentially important role in the progression of breast epithelial cells towards a malignant phenotype.

Materials and methods

Cell growth and treatment conditions

Hs578T tumor cell line was derived from a carcinosarcoma and is epithelial in origin (24). MCF-10F (HMECs) were established from a patient with fibrocystic disease and do not display characteristics of a malignant phenotype (22). These cells represent a non-tumorigenic, immortally transformed cell line. The D3-1 and BP-1 lines were derived by DMBA- and B[*a*]P-mediated transformation of MCF-10F cells, respectively (22), and were cultured as published previously (22). 184 HMEC strain, derived from reduction mammoplasty tissue, and the 184A1 cell line, which emerged from 184 HMECs after exposure to B[*a*]P, were cultured as described (25). These HMECs were arrested in G_0 by exposure for 48 h to medium lacking EGF and containing the anti-EGF receptor antibody, Mab 225, as described previously (26). For IκB-α protein turnover assays, exponentially growing cells were treated with 20 μg/ml emetine (Sigma Chemical Co., St Louis, MO) and cytoplasmic extracts (50 μg/sample) subjected to immunoblot analysis with the IκB-α antibody (sc# 371; Santa Cruz Biotechnology, Santa Cruz, CA), essentially as described previously (12).

Electrophoretic mobility shift analysis (EMSA)

Nuclear extracts were prepared from breast cancer cells essentially as described previously (12). Briefly, cells were washed twice with ice-cold PBS (Ca^{2+} and Mg^{2+} free) containing protease inhibitors [0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin (LP)]. They were then resuspended in 1 ml of cold hypotonic RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.4) containing 0.5% NP-40 detergent plus protease inhibitors as above. Following a 15 min incubation on ice, the cells were dounce homogenized until cell lysis occurred. Nuclei were resuspended in two packed nuclear volumes of extraction buffer C (420 mM KCl, 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) plus protease inhibitors as above, and incubated on ice for 30 min. Protein concentration was determined using the Bio-Rad protein assay, following the manufacturer's directions (Bio-Rad Laboratories, Hercules, CA). The sequence of the NF-κB-containing oligonucleotide from the *c-myc* gene (27) is as follows: 5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′, where the underlined region indicates the core binding element. The sequences of the PU.1- and TCF-1-containing oligonucleotides are as follows, PU.1: 5′-GATCTACTTCTGCTTTTG-3′; TCF-1: 5′-GGGAGACTGAGAACAAAGC-GCTCTCACAC-3′ (28). For labeling of the double-stranded oligonucleotide, which has 5′ overhangs allowing fill-in with DNA polymerase I, a 150–300 ng sample was incubated for 30 min at 37°C in a solution adjusted to a final concentration of 50 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 μM each of dATP and dTTP, 50 μCi each of [^{32}P]dCTP and [^{32}P]dGTP, and 5 U of Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA). For the binding reaction, ^{32}P-labeled oligonucleotide (20 000–25 000 c.p.m.) was incubated with 5 μg of nuclear extract, 5 μl sample buffer (10 mM HEPES, 4 mM DTT, 0.5% Triton X-100 and 2.5% glycerol), 2.5 μg poly(dI–dC) as non-specific competitor, and the salt concentration adjusted to 100 mM using buffer C. The reaction was carried out at room temperature for 30 min. DNA–protein complexes were subjected to electrophoresis at 11 V/cm and resolved on a 4.5% polyacrylamide gel (using 30% acrylamide/0.8% bisacrylamide) with 0.5× TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0). Supershift analyses were performed by incubating with the appropriate antibodies for an additional 1 h after the binding reaction had taken place. For supershift/blocking analysis, either of two antibodies against the p50 subunit were used; these were either kindly provided by K.LeClair (Procept Inc., Cambridge, MA) or purchased (sc-114; Santa Cruz Biotechnology). Antibodies against the p65 and c-Rel subunits were sc-109 and sc-372 (Santa Cruz Biotechnology) and #1226, kindly provided by N.Rice (NCI, Frederick, MD) and sc-070 (Santa Cruz Biotechnology), respectively.

Transfection and transactivation analysis

Wild-type (E8) and mutant (mutE8) NF-κB element–thymidine kinase (TK) promoter–chloramphenicol acetyltransferase (CAT) reporter vectors were constructed as reported previously (27). Briefly, these consisted of two copies of either the wild-type NF-κB element from upstream of the *c-myc* promoter, sequence given above, or versions with the two internal G residues converted to C residues, which significantly reduces NF-κB binding and transactivation (27). Each cell type displayed very large differences in transfection efficiency, and thus optimization of the specific transfection protocol was performed for each individual line. D3-1 cells were transfected by a modified calcium phosphate protocol, as described previously (12). MCF-10F and BP-1 cells were transfected using Lipofectamine reagent (Gibco BRL, Gaithersburg, MD). Cultures of 184 and 184 A1 cells were transfected using Cytofectin (Glen Research, Sterling, VA). All cells were harvested using reporter lysis buffer (Promega, Madison, WI) and CAT assays and luciferase assays were performed as published previously (12). Alternatively, confluent cultures of MCF-10F cells were transiently transfected with the p1.6 Bgl *c-myc* promoter CAT, containing 1.2 kb of upstream and 0.4 kb exon 1 sequences, including the two NF-κB elements (29), using FUGENE transfection reagent (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's instructions. Vector pEVRF-p65, encoding murine p65 protein (kindly provided by R.Sen, Brandeis University, Waltham, MA), was co-transfected, as indicated. Total DNA transfected was maintained at 4–5 μg. Cells were harvested after 24 h and extracts normalized for protein assayed, as above.

Transfection and immunoblot analysis

Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 μg pEVRF-p65 plus 20 μg pcDNA3 plasmid or with 24 μg pcDNA3 plasmid DNA with 30 μl FUGENE transfection reagent. After 48 h, cells were rinsed with cold PBS, and harvested in lysis buffer (50 mM Tris–HCl pH 8.0, 5 mM EDTA pH 8.0, 150 mM NaCl, 0.5 mM DTT, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mM PMSF, 0.5% NP-40). Whole-cell extracts (WCEs) were obtained by sonication, followed by centrifugation at 14 000 r.p.m. for 30 min. Samples (40 μg) of WCEs were subjected to electrophoresis and immunoblot analysis, as above. Blots were probed with rabbit anti-c-Myc antibody (786-4, a gift from S.Hann, Vanderbilt University, Memphis, TN), and mouse anti-β-actin monoclonal antibody (AC-15; Sigma).

Carcinogen treatment of rats

Virgin female S–D rats, fed AIN-76A diet, were handled according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. For analysis of mammary glands from normal animals, five virgin female S–D rats (Charles River Laboratories, Wilmington, MA), 6 weeks of age, were housed in the AAALAC-approved Laboratory Animal Science Center and fed AIN76 diet for 3 weeks. They were killed with CO_2; all 12 mammary glands were combined for each animal, and nuclear extracts prepared as described previously (12). For the time-course analysis, S–D rats from the same supplier were housed in environmentally controlled animal quarters in the Mallory Institute of Pathology, fed AIN-76A diet, and randomly entered into control or DMBA-treated groups of either four or five animals. At 8 weeks of age they were given either a single intragastric dose of 15 mg/kg DMBA dissolved in sesame oil or given the sesame oil vehicle alone. Rats were predesignated to be killed and necropsied at 6 or 24 h, or 1, 3 or 9 weeks after DMBA or oil administration. Rats were palpated weekly for tumor. At necropsy of each control and DMBA-treated rat, all 12 mammary glands were rapidly removed, combined and frozen in liquid nitrogen for storage. At 9 weeks, two rats were found to have non-palpable tumors in one gland each. These tumors were excluded from the mammary gland samples taken for the time-course study. Nuclear extracts were prepared, as described previously (12), and subjected to EMSA.

Results

Activation of NF-κB/Rel occurs prior to tumor formation in mammary glands of rats treated with DMBA

Recently, we have demonstrated that 86% of the mammary tumors induced by DMBA-treatment of S–D rats displayed aberrant activation of NF-κB/Rel compared with the normal mammary glands of the age-matched, vehicle-treated control animals (12). To determine the nature of the NF-κB binding activity in the normal mammary gland, EMSA was performed on nuclear proteins isolated from the mammary glands of five virgin female 9 week old S–D rats and a radiolabelled NF-κB-containing oligonucleotide, as probe (Figure 1A). A major band (band 1) was seen (Figure 1A), which was better resolved on a light exposure (Figure 1B); many extracts also displayed a minor, slower migrating complex (band 2) seen on a longer exposure (Figure 1A and data not shown). To identify the nature of the subunits within these complexes, antibody supershift/blocking EMSA was performed. Addition of a supershifting antibody against the p50 subunit of NF-κB yielded a new

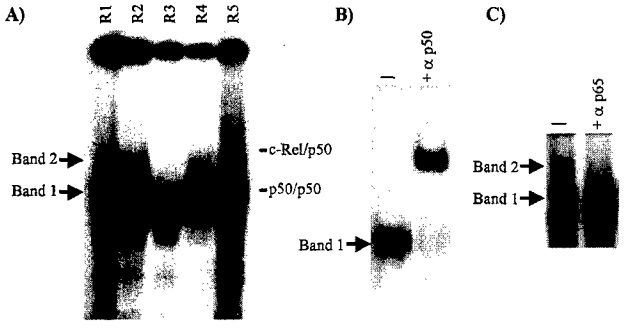

Fig. 1. Nuclear extracts of normal mammary glands of female S–D rats express low levels of NF-κB complexes. (**A**) EMSA was performed using samples (3 μg) of nuclear extracts from mammary glands of five S–D female rats (R1–R5), and an NF-κB oligonucleotide, as probe. Film was exposed for 7 days. The position of the major (band 1) and minor (band 2) complexes are indicated. The positions of the p50 homodimer and p50/c-Rel complexes formed with nuclear extracts of murine WEHI 231 B cells, isolated as described previously (4) and similarly analyzed on the gel, are indicated. (**B**) Samples of Rat 4 nuclear extract (3 μg) were incubated in the absence (–) or presence of 1 μl p50 antibody (sc-114) (α p50) for 60 min and subjected to EMSA using an NF-κB oligonucleotide, as probe. Film was exposed for 6 h. (**C**) Samples of Rat 4 nuclear extract (3 μg) were incubated in the absence (–) or presence of 1 μl p65 antibody sc-372 (α p65) for 60 min and then subjected to EMSA using an NF-κB oligonucleotide, as probe. Film was exposed for 5 days.

band and dramatically reduced formation of the band 1 complex (Figure 1B). Addition of a blocking antibody against p65 ablated formation of band 2 (Figure 1C). Addition of an antibody against c-Rel had no effect on binding (data not shown). From these experiments, we conclude that normal mammary glands express predominantly p50 homodimers with minor amounts of a p65-containing complex. Based on its migration, band 2 likely represents p50/p65 heterodimers.

We performed a time-course experiment to assess the kinetics of activation of NF-κB/Rel in S–D rats as a function of carcinogen treatment (12). S–D rats were administered 15 mg/kg DMBA in sesame oil by intragastric gavage. At this dose, tumors are first detectable after 7 weeks, and numbers increase until ~90% of animals have tumors at 15 weeks (21). Control rats were similarly administered the vehicle alone. Animals were necropsied at 6 and 24 h, and 1, 3 and 9 weeks. For each animal, all 12 mammary glands were excised, combined and nuclear extracts prepared. (At the 9 week time-point, a tumor was observed in one mammary gland of each of two DMBA-treated animals; these were excluded from the remaining glands which were histologically normal.) EMSA analysis was performed using a radiolabelled NF-κB-containing oligonucleotide as probe with extracts from treated or control animals (Figure 2A and B, respectively). A sample of a nuclear extract from an S–D rat mammary tumor, with a typical level of binding, was added to each gel to permit comparison of the relative levels of NF-κB/Rel expression amongst the various samples. As seen previously (12), NF-κB/Rel binding levels were extremely low in mammary gland extracts from control, vehicle-treated rats after a 6 h period (Figure 2B), consistent with data presented in Figure 1A. Similar binding levels were seen with additional control animals, e.g. after various times up to the 9 week period (data not shown). Overall, normal mammary glands exhibited $<10\%$ of the binding levels seen in the tumor sample, as judged by densitometry. After 6 h, 24 h or 1 week following DMBA treatment, no increase in NF-κB/Rel binding was detected. However, at 3 weeks, there was clearly significant activation of formation of all complexes in two of the five animals (nos 46 and 47) that were administered DMBA. The binding levels in these animals approached 39 and 48% of the control tumor sample, as judged by densitometry. At 9 weeks, in two of the five animals (nos 53 and 55) induction of higher constitutive levels of NF-κB/Rel was again detected. Interestingly, these rats were the ones discussed above that had developed one mammary tumor each. The higher intensity of binding in these four samples was not due to unequal loading, as judged by EMSA with an Sp1 oligonucleotide (data not shown). Also, no contamination of B lymphocytes, neutrophils, mast or myeloid cells or of T lymphocytes was detected as judged by EMSA with oligonucleotides containing binding sites for PU.1 and TCF-1 (Figures 2C and D, respectively, and data not shown). Thus, these data indicate that activation of NF-κB/Rel binding in the mammary gland can occur 3 weeks post-DMBA administration, preceding tumor formation which is not detectable prior to 7 weeks of treatment with this dose of carcinogen (21) and was not detected at necropsy until 9 weeks in this experiment.

Transformation of MCF-10F cells by the PAHs DMBA and B[a]P increases NF-κB activity

We next sought to determine whether treatment with PAHs *in vitro* would similarly lead to an induction of NF-κB/Rel activity in HMECs. D3-1 and BP-1 cell lines were derived from the non-tumorigenic MCF-10F cell line by 24 h treatment of either DMBA and B[*a*]P, respectively. Both cell lines exhibit malignant characteristics. BP-1 cells exhibit increased anchorage independent growth, increased chemotaxis and chemoinvasiveness. D3-1 cells exhibit increased chemotactic and chemoinvasive capabilities, but to a lesser extent than BP-1 cells (22). Nuclear extracts from D3-1 and BP-1 cells displayed significantly increased NF-κB binding activity compared with the parental MCF-10F cells (inset Figure 3A). Equal loading was confirmed in EMSA using an Oct-1 probe (data not shown). Two bands were seen with the extracts from the D3-1 and BP-1, which co-migrated with bands seen with nuclear extracts from the MCF-10F cells. (The upper band with MCF-10F extract was better seen on a longer exposure.)

To confirm that the binding activity was functional, transient transfection analysis was performed using E8 and mutE8 multimerized NF-κB element-driven TK promoter–CAT reporter constructs. Since transfection efficiency of these lines varied, the activity of wild-type κB construct was normalized to that of the mutE8 activity, which is reflective of basal reporter activity without any contribution from NF-κB. The parental MCF-10F cells showed a minimal induction of E8 activity over the mutE8 of (~1.7 ± 0.6)-fold (Figure 3A). The D3-1 and BP-1 cells showed a significantly higher level of NF-κB activity of (4.1 ± 1.4)-fold and (11.6 ± 0.2)-fold, respectively. Thus, the relative levels of binding and activity correlate directly.

To confirm that the activity of the E8 vector seen in the transformed cell lines was due to NF-κB/Rel binding, co-transfection analysis was performed with an increasing dose of a vector expressing the NF-κB/Rel specific inhibitory protein IκB-α. The activity of the E8 vector in the D3-1 cells was specifically repressed in a dose-dependent manner by IκB-α (Figure 3B). Similarly, co-transfection with 1.4 μg of vector expressing IκB-α reduced NF-κB-specific activity (3.6 ± 0.1)-fold in the BP-1 cells. Thus, the transformed D3-1 and BP-1 cell lines display increased levels of functional NF-κB/Rel than seen in the parental MCF-10F cells.

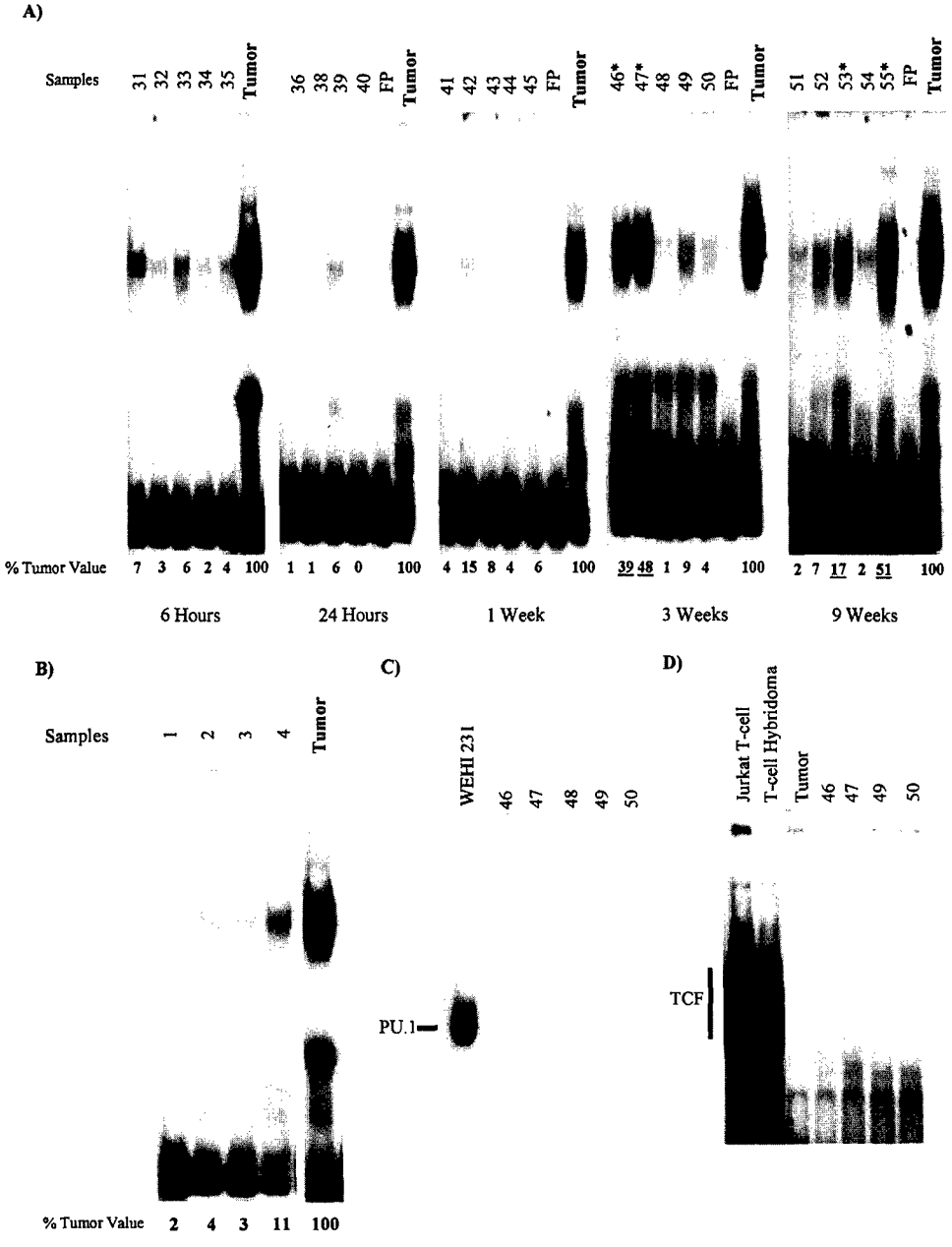

Fig. 2. Nuclear extracts of mammary glands from DMBA-treated S–D rats display high NF-κB/Rel binding activity prior to tumor formation. (A and B) NF-κB EMSA. EMSA was performed using samples (5 μg) of nuclear extracts from mammary glands of rats treated with DMBA (**A**) or representative control 6 h control rats (**B**), and an NF-κB oligonucleotide, as probe. In each panel, a sample (5 μg) of nuclear extract from a DMBA-treated rat mammary tumor (Tumor) was run to allow for comparison between gels, and to serve as a normalization for intensity of binding observed in the various samples. Densitometry analysis was performed to compare the relative level of NF-κB/Rel binding in nuclear extracts from mammary glands from the control or DMBA-treated animals, and intensity values are given as percent of tumor binding intensity (where the tumor intensity was set at 100%). *, animals whose binding intensity were at least one standard deviation above the average intensity of the control animals. (**C**) PU.1 EMSA. EMSA was performed using samples (5 μg) of nuclear extracts from mammary glands of the 3 week DMBA-treated rats (46–50) with the PU.1 oligonucleotide, as probe. As a positive control for binding, nuclear extracts from WEHI 231 B cells were analyzed on the same gel. (**D**) TCF-1 EMSA. Samples (5 μg) of nuclear extracts from mammary glands of the indicated 3 week DMBA-treated rats and a DMBA-induced mammary tumor were subjected to EMSA with the TCF-1 oligonucleotide, as probe (28). As a positive control for binding, nuclear extracts (5 μg) from Jurkat T cells and from an activated T-cell hybridoma were analyzed on the same gel.

Antibody supershift/blocking analysis was performed to identify the NF-κB subunit composition in the various extracts. Addition of an antibody against the p50 subunit to nuclear extracts from MCF-10F cells resulted in a supershift of the both bands, while addition of a blocking antibody against the p65 subunit deleted the upper complex selectively (Figure 4A). Therefore, the upper complex was identified as a p50/p65 complex, also known as classical NF-κB, a potent transactivator (1). Addition of an antibody to the p52 subunit had no effect on binding (data not shown). Thus, based on the faster migration of the bottom band, this complex likely consists of p50/p50 homodimers, which have only minimal transactivation potential. Similar analysis was performed with the D3-1 and BP-1 nuclear extracts (Figure 4B and data not shown). The upper band was eliminated upon addition of the p65 antibody with extracts from both cells, whereas the p50 antibody completely shifted the lower band, and cleared most of the upper band as well (Figure 4B and data not shown).

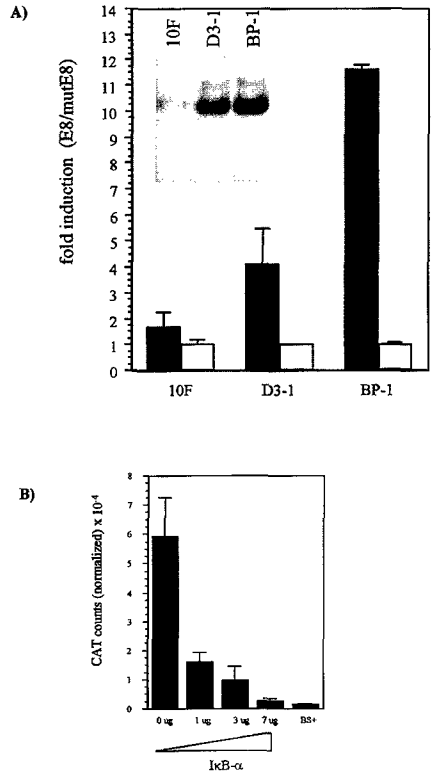

Fig. 3. Carcinogen-transformed D3-1 and BP-1 cells display higher constitutive levels of functional NF-κB than the parental MCF-10F cells. (**A**) Comparison of lines. The MCF-10F cells (10F) and BP-1 cells were transiently transfected by lipofection, in triplicate or duplicate, respectively, with 2 μg E8 or mutE8 reporter construct. Alternatively, D3-1 cells were transfected, in duplicate, using 20 μg of either E8 or mutE8 by the calcium phosphate method. After 24 h (for lipofectamine) or 72 h (for calcium phosphate), extracts were prepared, normalized for protein and assayed for CAT activity. The values for E8 CAT activity are represented as fold induction over mutE8 CAT activity which was set at 1.0 for each cell line. Shown is the representative data from a minimum of two experiments. (Inset) Equal amounts (5 μg) of nuclear extracts from exponentially growing parental MCF-10F cells or transformed D3-1 or BP-1 cells were subjected to EMSA with a radiolabeled oligonucleotide NF-κB element as probe. A representative experiment of two independent assays is shown. (**B**) Activity is inhibited with IκB-α expression. D3-1 cells were transiently transfected, in duplicate, with 15 μg of E8 reporter construct and 0, 1, 3 or 7 μg of PMT2T IκB-α expression vector, in the presence of 2.5 μg TK-luciferase construct for normalization of transfection efficiency. The total amount of plasmid DNA transfected in each sample was adjusted to 25 μg by addition of pBlueScript+ plasmid DNA. Alternatively, 25 μg of pBlueScript DNA was used alone. Lysates were prepared after 72 h and analyzed as described previously (12).

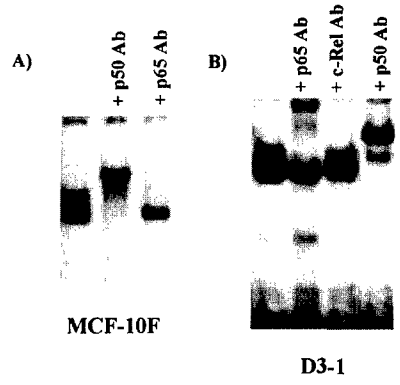

Fig. 4. Supershift analysis reveals classical NF-κB subunits in the binding complex. (**A**) Supershift analysis of MCF10F cells. Following a 30 min incubation of nuclear extracts (5 μg) with the probe, 1 μl of antibody against either the p50 (kindly provided by K.LeClair) or p65 (sc-109) was added as indicated, and the reaction incubated for an additional 1 h and subjected to EMSA as above. (**B**) Supershift analysis of D3-1 cells. Following a 30 min incubation of nuclear extracts (5 μg) with the probe, 1 μl of antibody against the p50 (sc-114), p65 (#1226, kindly provided by N.Rice) or c-Rel (sc-070) was added as indicated, incubated and subjected to EMSA.

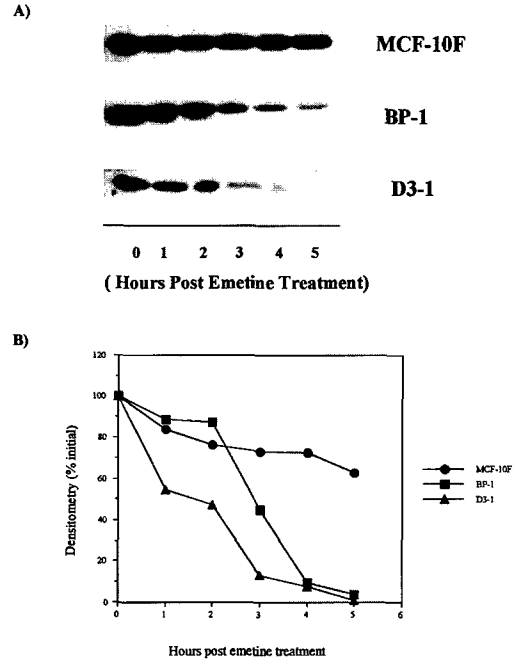

Fig. 5. IκB-α protein in D3-1 and BP-1 cells has a shorter half life than in parental MCF-10F cells. (**A**) MCF-10F, D3-1 and BP-1 cells were incubated in the absence or presence of 20 μg/ml emetine for the indicated periods of time. Cytoplasmic extracts (50 μg protein/lane) were separated by electrophoresis in a 10% SDS–polyacrylamide gel, and subjected to immunoblot analysis for IκB-α protein using SC-371 antibody. These blots are representative of two experiments. (**B**) The immunoblot for IκB-α protein in (A) was quantitated by densitometry, and the data plotted as percent of the original protein value in untreated control cells (0 h). The decay curves were extrapolated by using an exponential best fit analysis.

Addition of an antibody against c-Rel did not affect the pattern of migration with either cell extract. Thus, for the D3-1 and BP-1 cells, the upper band consists of the p50/p65 heterodimers, and the lower band consists of the p50/p50 homodimers, respectively. Thus, carcinogen transformation yields a functional binding complex of classical NF-κB at much higher intensity but of similar subunit composition to the parental MCF-10F cells, consistent with the identical patterns of migration.

IκB-α has a shorter half-life in D3-1 and BP-1 than in MCF-10F cells

In B cells, it has been shown that faster turnover of IκB-α proteins is, at least in part, responsible for the higher levels of functional NF-κB in the nucleus (30). Therefore, we next sought to determine if the increase in NF-κB levels in the transformed D3-1 and BP-1 cells could be correlated with changes in stability of the IκB-α protein. Exponentially growing cells were treated with emetine, a specific inhibitor of elongation of polypeptide chain synthesis, for 0–5 h, and cytoplasmic extracts prepared and subjected to immunoblot analysis for IκB-α protein (Figure 5A). The decay of the IκB-α protein in the BP-1 and D3-1 cells appeared more rapid than that observed in the MCF-10F cells. Equal loading of the

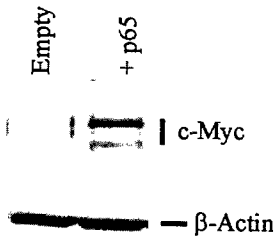

Fig. 6. Ectopic expression of p65 in MCF-10F cells increases c-Myc oncoprotein levels. Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 μg *pEVRF-p65* plus 20 μg *pcDNA3* plasmid or with 24 μg *pcDNA3* plasmid DNA with 30 μl FUGENE transfection reagent. After 48 h, cells were rinsed with cold PBS, and WCEs prepared. Samples (40 μg) were subjected to electrophoresis and immunoblot analysis for c-Myc (786-4, anti-c-Myc antibody), and β-actin (AC-15; Sigma).

lanes was confirmed by probing the same membranes with an antibody to the p65 subunit; this protein appeared to be quite stable even at 5 h post-treatment in all three cell types (data not shown). The resulting autoradiograms were quantified by densitometry and the data plotted in Figure 5B. The half-life of decay of IκB-α protein was determined in the BP-1 and D3-1 cells to be between 2 and 2.5 h, and 1 and 2 h, respectively. In contrast, in the parental MCF-10F cells, a half-life of ~7.8 h was determined. Thus, the chemical transformation of these cells dramatically affects the stability of the IκB-α protein correlating with the higher levels of constitutively active NF-κB associated with the malignant phenotype.

Ectopic p65 expression in MCF-10F cells induces the c-myc oncogene

To begin to assess the potential functional role of the elevated levels of NF-κB activity following transformation of the MCF-10F, we performed transient transfection and measured the effects of increased p65 levels on expression of the c-*myc* oncogene, which contains two NF-κB elements (29). MCF-10F cells, in exponential growth, were transfected in duplicate with 0, 0.5 or 1 μg *pEVRF-p65* vector DNA expressing p65 protein and 1 μg p1.6 Bgl CAT c-*myc* promoter construct. An increase in c-*myc* promoter activity of (18.9 ± 2.3)-fold and (21.3 ± 5.4)-fold was detected with 0.5 and 1.0 μg p65 expression vector, respectively. To verify that p65 expression enhances the endogenous c-*myc* gene, similar transfections were performed. MCF-10F cells were transfected with *pEVRF-p65* vector DNA or with *pcDNA3* plasmid DNA. After 48 h, whole-cell lysates were isolated and subjected to immunoblot analysis for levels of c-Myc and β-actin, as control for equal loading (Figure 6). A significant increase in c-Myc expression was detected upon transfection of the *pEVRF-p65* vector DNA compared with vector DNA alone. In this and two duplicate experiments, a (3.4 ± 0.7)-fold increase was determined by densitometry. In contrast, levels of β-actin remained unchanged (Figure 6). Thus, ectopic expression of the p65 subunit in MCF-10F cells leads to increased c-Myc levels.

HMECs transformed by B[a]P exhibit high levels of NF-κB binding activity even in the premalignant state

To monitor the expression of NF-κB earlier during the transformation process, we compared the normal finite life-span 184 HMEC cell strain with its immortally transformed derivative cell line, 184A1. The 184 HMEC cell strain, derived from reduction mammoplasty tissue, senesces after approximately 80 population doublings. The immortal but non-tumorigenic 184A1 line was established following B[*a*]P treatment of

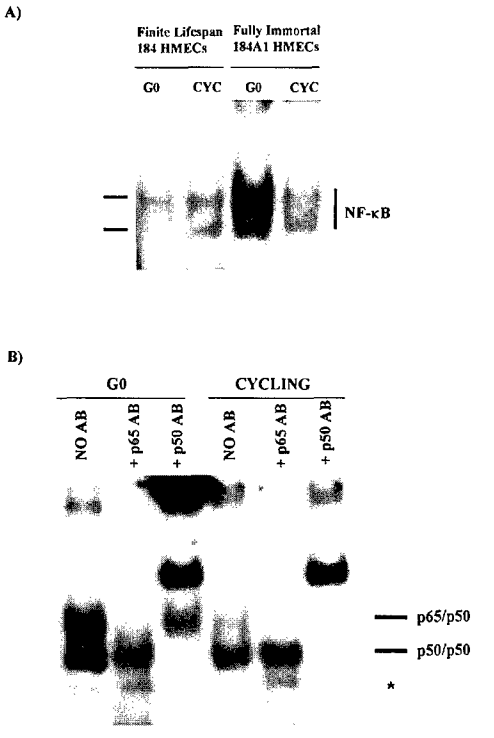

Fig. 7. Dysregulated NF-κB/Rel expression in 184A1 immortalized HMECs. (**A**) 184A1 cells display increased NF-κB binding in quiescence. EMSA was performed with nuclear extracts (5 μg) from finite life-span 184 and fully immortal 184A1 cells following G_0 synchronization upon blockage of EGF receptor signal transduction for 48 h (G_0) or during exponential growth (CYC). Two distinct NF-κB binding complexes were detected. (**B**) Supershift EMSA reveals classical NF-κB in the binding complex of 184A1 cells. Supershift analysis was performed on nuclear extracts (5 μg) harvested from fully immortal 184A1 HMECs in exponential growth, or made quiescent following blockage of EGF receptor signal transduction for 48 h. Following a 30 min incubation of nuclear extracts with the probe, 1 μl of antibody against p50 (sc-114) or p65 (#1226) was added as indicated, and the reaction incubated for an additional 1 h and subjected to EMSA, as above. The upper binding complex is composed of p65/p50 subunits, and lower binding complex is a p50/p50 homodimer. Position of a nonspecific band is indicated by an asterisk (*).

primary cultures of the 184 HMEC strain (25,31). NF-κB binding activity in the finite life-span 184 cells, and the late passage immortal 184A1 cell line was compared. Extracts from both cells gave a similar pattern with two bands detectable (Figure 7A), which co-migrated with the complexes observed in MCF-10F cells (data not shown). The levels of these complexes in the two populations of cycling cells were similar (Figure 7A). The half-life of decay of the IκB-α proteins in these cycling cell populations were also identical; a half-life of ~3.5 h was determined (data not shown). Previously, we had noted that B[*a*]P exposure led to dysregulated gene expression in quiescence (32). Thus, finite life-span and fully immortal cells were made quiescent upon blockage of EGF receptor signal transduction for 48 h, and nuclear extracts used in EMSA (Figure 7A). The levels of binding of finite life-span cells remained unchanged. In contrast, an increase in NF-κB binding activity was evident in the quiescent G_0 immortally transformed 184A1 cells (Figure 7A). In fact, the level was essentially comparable with that found in the D3-1 and BP-1 lines; a commensurate decrease in half-life of decay of IκB-α was determined in these G_0 cells to ~2.5 h (data not shown). Supershift EMSA was performed to identify the subunit components of the complexes in the cycling and quiescent 184A1 HMECs (Figure 7B). In nuclear extracts from 184A1

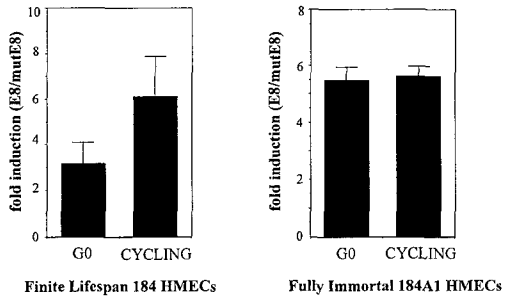

Fig. 8. Transient transfection analysis was performed in both the G_0 and exponential cycling states. Twenty-four hours after plating (110 000 cells/sample), finite life-span 184 and fully immortal 184 A1 cells were transfected, in duplicate, with equivalent amounts of either the E8 or mutE8 plasmid using Cytofectin reagent. Total amount of DNA was normalized to be either 1 or 1.5 μg (equivalent within each experiment) using pBlueScript. Cells were washed in PBS after 5 h and fresh media was added. After 24 h, cells were either given fresh normal medium (CYCLING) or medium minus EGF plus MAb 225 (G_0). Cells were harvested 24 h later in reporter lysis buffer, and assayed for CAT activity. The values for E8 CAT activity are represented as fold induction over mutE8 CAT activity which was set at 1.0 and were normalized for protein levels. Shown is the representative data from a minimum of two experiments.

cells in either growth state, the upper complex was depleted upon addition of an anti-p65 antibody. Addition of an anti-p50 antibody abrogated formation of the bottom complex and reduced the upper complex. Thus, in both cycling and arrested cells, the upper complex is classical NF-κB, composed of a p65 and p50 subunit, while the bottom complex is likely composed of a p50 homodimer. Similar complexes were identified with the 184 cell extracts (data not shown). These findings indicate that NF-κB is dysregulated in the growth-arrested late passage fully immortal 184A1 cells.

To assess the functionality of the increase in NF-κB binding in quiescence, transient transfection analysis was performed (Figure 8). Cells were transfected with E8 and mutE8, as above, and then incubated either in normal medium or medium deprived of EGF receptor signal transduction to make them quiescent. As expected, when the finite life-span 184 HMECs cells were quiescent, the level of E8/mutE8 induction decreased from a 6.1- to a 3.1-fold induction. The fact that the activity decreases most likely reflects the effects that quiescence has on reducing the overall rate of protein synthesis within these cells (26). When the immortally transformed 184A1 HMECs were similarly analyzed, an ~5-fold induction of E8/mutE8 activity was seen in cycling cells, and this induction was maintained even when the cells were made quiescent (Figure 8). This maintenance of E8 activity is most likely due to the increased induction of NF-κB/Rel in the G_0 state, and correlates with the increased binding displayed by EMSA (Figure 7A). Thus, even when growth arrested, NF-κB appears to maintain functionality in the fully immortal cells indicating a dysregulated expression of NF-κB in quiescence. Thus, dysregulation of expression of classical NF-κB can occur significantly prior to malignant transformation of the HMEC in culture.

Discussion

Here we demonstrate that the aberrant activation of NF-κB/Rel, which typifies human and rodent breast cancer, occurs early during the malignant transformation process, and involves de-stabilization of the specific inhibitory protein IκB-α. In S–D rats, activation of NF-κB/Rel occurred in the mammary glands of 40% of the animals 3 weeks after DMBA-treatment, a time-point at which tumor formation was not observed. If not every mammary gland from a DMBA-treated animal has responded, this value potentially represents an underestimation due to the pooling of all 12 mammary glands from individual animals that was performed to facilitate the time course analysis. Furthermore, the observation of NF-κB/Rel activation *in vivo* with this rodent model was extended to cultured HMECs. Carcinogen induced transformation was found to result in elevated levels of NF-κB in the malignantly transformed D3-1 and BP-1 cells compared with their parental, non-malignant MCF-10F cells. This activation of classical NF-κB in the MCF-10F-derived lines correlated with de-stabilization of IκB-α. Furthermore, enhanced expression of p65 in MCF-10F cells led to induction of the c-*myc* oncogene. Lastly, dysregulated expression of NF-κB was seen in the immortal but non-malignant 184A1 cells compared with the finite life-span 184 strain. Given the involvement of NF-κB/Rel factors in control of genes signaling proliferation, cell survival and neoplastic transformation, the early and persistent functional activation of these factors in carcinogen-induced rodent and human mammary systems suggests a potential critical role in the malignant transformation process.

There is mounting evidence for NF-κB/Rel factors mediating signals that induce cell proliferation. Firstly, these factors have been found to transactivate critical genes controlling growth either directly, e.g. c-*myc* (27,29,33), or indirectly, e.g. various interleukins or growth factors (1). Furthermore, constitutive NF-κB/Rel activity has been shown to be essential for proliferation of several cell types, e.g. smooth muscle cells (34), and hepatocytes during liver regeneration after partial hepatectomy or toxic damage (35). In the T lymphoma cell line HuT 78, constitutive NF-κB activation, apparently due to autocrine TNF-α production, was shown to accompany enhanced cellular proliferation (36). Furthermore, we have recently found that ectopic expression of c-Rel subunit permits Hs578T breast cancer cells to overcome TGF-β1-induced growth inhibition (37). Consistent with this role of NF-κB/Rel, the D3-1 and BP-1 cells both proliferate at a faster rate than the parental MCF-10F cells (22), correlating well with the higher NF-κB activity in these cells. Likewise, the late passage 184A1 cells are able to proliferate indefinitely even in the presence of TGF-β1, compared with the parental 184 cells which are TGF-β1-sensitive.

Constitutive or induced activation of NF-κB has been implicated in promoting cell survival by protecting cells from undergoing apoptosis (3). Studies of mice null for the *RelA* gene, encoding the p65 protein, provided the first suggestive evidence (38). Deletion of the p65 subunit is embryonal lethal with liver degeneration due to extensive apoptosis of the hepatocytes (38). Direct evidence for the anti-apoptotic role of NF-κB/Rel was demonstrated in breast cancer cells (12), B-lymphoma cells (3) and in hepatocytes (6): inhibition of constitutive NF-κB/Rel led to cell death by apoptosis. Furthermore, maintenance of NF-κB/Rel activity conferred protection from apoptosis induced by TNF-α or irradiation (8–11), TGF-β1 (7), or B cell receptor signals (4,39). In light of such evidence, the early activation NF-κB/Rel may play a similar role in the mammary gland following DMBA treatment leading to dysregulation of normal control of proliferation and protecting the epithelial cells from apoptosis.

Here we provide evidence that one of the genes regulated by NF-κB/Rel, that may promote epithelial cell proliferation and survival, is the c-*myc* proto-oncogene (40). A drop in

c-*myc* has been found to induce apoptosis of many cell types including breast cancer cell lines (41), while conversely, c-*myc* overexpression is able to promote survival of several cell types, including B cells (40). Furthermore, overexpression of c-*myc* has been implicated in the etiology of breast cancer (42). The regulation of the c-*myc* promoter by NF-κB/Rel was first demonstrated in human breast cancer cells (12). Here, we extend this observation to MCF-10F cells showing that both the c-*myc* promoter and endogenous c-*myc* gene respond to ectopic expression of p65. Interestingly, BP-1 cells overexpress the c-*myc* oncogene (43), and tumors from DMBA treated S–D rats have increased c-*myc* mRNA expression (D.W.Kim and G.E.Sonenshein, unpublished observations). Studies are in progress to determine whether inhibition of NF-κB/Rel activation leads to decreased c-*myc* gene expression and ablates the carcinogen transformation process.

Lastly, aberrant activation of nuclear NF-κB/Rel has been found to correlate with oncogenesis in several different systems, including breast cancer, thyroid carcinoma (44), non-small cell lung carcinoma, colon carcinoma, ovarian carcinoma, prostate cancer (45), Hodgkin's disease (46,47) and various types of lymphomas (13). The results presented here extend this association to carcinogen-induced *in vitro* malignant transformation of HMECs. Furthermore, our findings indicate that the activation of NF-κB/Rel in mammary glands upon carcinogen treatment of rodents is an early event. It was also interesting to note that in our studies with DMBA-treated S–D rats, the two animals studied at 9 weeks which displayed elevated NF-κB/Rel binding activity in the grossly normal mammary glands had non-palpable tumors. Taken together, our results suggest a novel mechanism for carcinogen induction of tumors of the mammary glands, i.e. via the activation of NF-κB/Rel factors. Thus, inhibition of NF-κB may provide a means of intervention at early, as well as later, stages of the transformation process in the mammary gland. Furthermore, the possibility that NF-κB/Rel may serve as a diagnostic marker for pre-malignant mammary glands warrants further investigation. Studies are underway to test directly the role of activation of NF-κB/Rel in neoplastic transformation by enforcing expression of a transactivating subunit of NF-κB/Rel in the mammary glands of transgenic mice.

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