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13. ABSTRACT *(Maximum 200 words)*

The Rel/NF-κB family of ubiquitous, inducible transcription factors has been implicated in such diverse cellular processes as proliferation, differentiation, and apoptosis. Given that these processes occur during mammary gland morphogeneis and that NF-κB activity is upregulated in breast cancer derived cell lines and primary ductal cell carcinomas, the expression and activity of NF-κB factors in the murine mammary gland during pregnancy, lactation, and involution was examined. Northern, western, and immunohistochemical analyses reveal dynamic expression patterns for RelA, p105/p50, p100/p52, and IκBα in mammary epithelial cells. NF-κB activity in the mammary gland, as assessed by electrophoretic mobility shift assay (EMSA) and by the expression of a luciferase reporter driven by the NF-κB responsive human immunodeficiency virus long terminal repeat (HIV-LTR) in transgenic mice, is highest during pregnancy. KappaB-activity levels decrease dramatically during lactation, and rise during involution. RelA and p50 containing complexes are present in nuclear extracts from pregnant and involuting glands. These data suggest that NF-κB may play a role in mammary gland proliferation and differentiation during pregnancy, as well as apoptosis during involution.

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Dana M. Bradley 4/29/98

PI - Signature Date

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Introduction

The Rel/Nuclear Factor-Kappa B (NF-κB) family of ubiquitous transcription factors is composed of multiprotein complexes that rapidly respond to a wide variety of growth factor, cytokine, and pathogenic stimuli (Verma et al., 1995). Authentic NF-κB is a heterodimer consisting of a 50 kD polypeptide (Ghosh et al., 1990) and a 65 kD polypeptide, RelA (Nolan et al., 1991). These two proteins are highly homologous in their amino-termini, sharing a 300 amino acid Rel homology domain (RHD). All members of NF-κB share this RHD, which contains DNA binding, dimerization, and basal transcription factor binding domains, as well as a specific inhibitor binding domain and a nuclear localization signal (Verma et al., 1995). NF-κB complexes are normally sequestered in the cytoplasm by association with one member of a family of specific inhibitory proteins, termed Inhibitors of NF-κB (IκBs). The two most well characterized IκBs, IκBα and IκBβ, are serine phosphorylated by one of two known IκB kinases (IKKs), IKK-α or IKK-β, upon growth factor, cytokine, or pathogenic stimulation (DiDonato et al., 1997; Mercurio et al., 1997; Régnier et al., 1997; Woronicz et al., 1997; Zhandi et al., 1997). Phosphorylation signals ubiquitination and degradation of IκBα or IκBβ, subsequently allowing NF-κB to translocate to the nucleus and modulate gene expression. Recent data suggests that while both IKK-α and IKK-β are activated by NF-κB-inducing kinase (NIK) through the tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1) signaling pathways, IKK-β may also be responsive to mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1) activation (Ling et al., 1998; Nakano et al., 1998; Yin et al., 1998). These data begin to explain how such a wide array of stimuli can converge to activate NF-κB.

NF-κB activity has been implicated in the balance between cellular proliferation, differentiation, and apoptosis in several cell culture models and *in vivo*. It has also been demonstrated that variant forms of NF-κB factors possess oncogenic potential. For example, mice that are nullizygous for the proto-oncogene c-*rel* show diminished B-cell and T-cell proliferation (Köntegen et al., 1995). Recent data demonstrat that c-Rel containing complexes are capable of transcriptionally downregulating *bcl* -2 expression in FL5.12 progenitor B-cells, and constitutive inhibition of NF-κB activity in these cells delays entry into apotosis following cytokine withdrawl (Sohur et al., submitted). The oncogenic variant v-Rel is responsible for avian reticuloendotheliosis virus (REV-T) induced lymphoma (Theilen et al., 1966; Chen and Temin, 1982; Chen and Temin, 1986), and translocations within the c-*rel* gene have been associated with human follicular and diffuse large cell lymphoma (Lu et al., 1991). The genes encoding two other NF-κB family members, Bcl-3 and p100/p52 (lyt-10), have been identified in chromosomal breakpoint junctions associated with B-cell chronic lymphocytic leukemia and B-cell non-Hodgkin lymphoma, respectively (Ohno et al., 1990; Kerr et al., 1992; Neri et al., 1991). Targeted disruption of RelA is lethal at embryonic day 15-16 as a result of massive liver apoptosis (Beg et al., 1995). Several cell culture models suggest that RelA is anti-apoptotic (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). Mice nullizygous for RelB demonstrate dual proliferative defects, as demonstrated by myeloid hyperplasia and abnormal hematopoiesis, as well as a decrease in the thymic dendritic cell population (Weih et al., 1995). Mice lacking p50 through targeted disruption also demonstrate a defect in B-cell proliferation in response to bacterial lipopolysaccharide (Sha et al., 1995). Collectively, these data demonstrate that several NF-κB family members participate in proliferation and apoptosis *in vivo*, as well as contributing to carcinogenesis.

Interruption of NF-κB activity *in vivo* through the use of an N-terminal deletion mutant of IκBα (IκBα-ΔN), which acts as a transdominant inhibitor by interrupting the signal transduction cascade responsible for the degradation of IκBα, lends credence to the argument that NF-κB activity modulates proliferation, differentiation, and/or apoptosis. Transgenic mice expressing IκBα-ΔN in B-cells and T-cells show both decreased proliferation and increased apoptosis in the respective cell types. In addition, a reduction in the levels of mature B- and T-cells in these mice

suggests a defect in differentiation (Boothby et al., 1997; Yull et al., submitted). Introduction of an avian retrovirus or adenovirus harboring IκBα-ΔN into the limb field of a developing chick embryo results in abberant limb morphogenesis and disruption of the apical ectodermal ridge (Bushdid et al., 1998; Kanegae et al., 1998). The infected limbs exhibit severe truncation and the loss of distal structures, as well as an apical ectodermal ridge defect characterized by the loss of normal pseudostratified columnar epithelial morphology. These data suggest a role for NF-κB in patterning and epithelial-mesenchymal interactions, as c-Rel is highly expressed in the mesenchyme underlying the apical ectodermal ridge during limb morphogenesis (Bushdid et al., 1998). In addition, expression of IκBα-ΔN in chick embryo fibroblasts prolongs the G_2 to M transition of the cell cycle (Bushdid, unpublished observations). These data demonstrate that inhibition of NF-κB activity alters that capacity of various cell types to proliferate, differentiate, and die in an appropriate manner.

Epithelial cell proliferation and differentiation during pregnancy, as well as involution following cessation of nursing, occur during normal postnatal mammary gland development. In addition, the mammary gland is a major target organ for carcinogenesis, representing the second highest cause of cancer mortality in women within the United States at 17% and the highest rate of new cancer cases in women at 31% (Parker et al., 1996). Given (I) the role for NF-κB in proliferation, differentiation, and apoptosis, (ii) the upregulation of p100/p52 levels in several breast cancer cell lines and in primary human ductal cell carcinomas (Dejardin et al., 1995; Sovak et al., 1997), (iii) and the upregulation of NF-κB activity associated with estrogen receptor negative cell lines and primary breast tumors, which are highly invasive, metatstatic, and refractory to hormone and chemotherapies (Nakshartri et al., 1997), the expression and activity of NF-κB in the developing murine mammary gland was investigated. Northern, western, and immunohistochemical analyses for the expression of RelA, p50, and IκBα in mammary glands were performed from virgin, pregnant, lactating, and post-wean female mice. These analyses reveal dynamic expression patterns, with elevated levels of nuclear RelA in mammary epithelial cells during pregnancy. Active RelA and p50 containing complexes were identified in nuclear extracts from the mammary glands of pregnant and post-wean animals via supershifts in electrophoretic mobility shift assays (EMSAs). In addition, RelA disappears from specific binding complexes in lactating mice, while p50 remains. NF-κB activity in murine mammary glands *in vivo* was assessed and quantified by the activity of a luciferase reporter gene driven by an NF-κB responsive enhancer element, the human immunodeficiency virus long terminal repeat (HIV-LTR), in transgenic mice (termed HLLs; Yull et al., submitted). The pattern of reporter activity complements the pattern of EMSA binding activity oabserved in that luciferase activity is highest during pregnancy, decreases to the level of non-transgenic control mice during lactation, and increases during the involution phase of mammary gland development. These data demonstrate that NF-κB is active during pregnancy and involution in the murine mammary gland, and suggest that NF-κB may play an integral role in these processes.

Body

Experimental Methods

Mouse stocks and maintenence: B6D2 mice (Harlan-Sprawgue-Dawley) were used for all expression and EMSA analyses. HLL mice from line 20 and 27 on a B6D2 genetic background were used for *in vivo* activity assays. Presence of the luciferase transgene was assessed by PCR and/or Southern blot, and confirmed by constitutive luciferase activity in the brain (Yull et al., submitted). All mice were maintained in microisolators and given mouse chow (Purina) and water *ad libitum*.

Northern and western analyses: Total cellular RNA was taken from the entire #4 mammary glands of virgin mice, after 10.5 and 16.5 days of pregnancy, after 10.5 days of lactation, and after 1, 3,

and 5 days post weaning of pups at 21 days of lactation using the Tri-Reagent (Molecular Research Center, Inc.). 10 μg of RNA were loaded on a 6.29% formaldehyde, 1% agarose gel in 1X MOPS buffer and electrophoresed. The RNA was transferred to a nylon membrane (Hybond-N). Probes were generated by random priming (Stratagene Prime-it II Kit) a 325 bp Xba/XhoI fragment from the 3' end of the murine IκBα cDNA and a 589 bp NcoI/XbaI fragment purified from the 3' end of human p100/p52. The specific activity for each probe was on the order of 10^9 cpm/μg. Membranes were prehybridized in 5X SSC, 5X Denhart's solution, 0.5% SDS, 0.08% Salmon sperm DNA, and hybrized in the same solution plus probe overnight. Membranes were washed at hybridization temperature 2 times in 0.5X SSC, 0.1% SDS, for 15 minutes and two times in 40 mM sodium phosphate (pH 7.0), 0.05% SDS. Following washes, membranes were exposed to a phosphoimager plate overnight for quantification and to X-ray film at -80°C to visualize specific RNAs. IκBα RNA expression panel shows 4 day exposure for IκBα and overnight exposure for p100/p52.

Total cellular protein was isolated from mammary glands over the time course listed for RNA using the Tri-Reagent extraction protocol. Protein (35 μg) was loaded on a 12% SDS-polyacrylamide gel and elecrophoresed. Proteins were transferred to a PVDF (Immobilon) membrane. The membrane was blocked overnight at 4°C in PBS plus 0.01% Tween-20 (PBST) and 5% milk, washed briefly at room temperature in PBST, and incubated for 1 hour at room temperature with primary antibody (rabbit anti-IκBα, Santa Cruz Biotechnology, 1:1000 dilution). The membrane was washed overnight in PBST at 4°C. Following wash, the membrane was incubated with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Southern Biotechnology Associates, 1:7000) for 1 hour at RT, washed, and developed using chemilluminescence protocol (NEN). IκBα protein expression panel shows 20 minute exposure.

Immunohistochemistry: Mammary glands from B6D2 female mice were collected, fixed in 4% paraformaldehyde in PBS at 4°C for 2 days, dehydrated, embedded in paraffin, and 5 to 7 μm sections prepared. For immunostaining, slides were prewarmed to 56°C for 1 hour prior to deparaffinization, and rehydration. Antigen retrieval was accomplished by microwaving (4 times 5 minutes at high power) in citrate buffer. Specimens were rinsed in PBS prior to blocking of non-specific interactions with 5% BSA in PBS for 1 hour at ambient temperature. The slides were exposed to anti-p100/p52 (Santa Cruz Biotechnology, 1:1000), anti-IκBα (Santa Cruz Biotechnology, 1:500), or anti-RelA (Santa Cruz Biotechnology, 1:50) antisera in PBS for 1 hour at room temperature in a humidified chamber. Slides were washed extensively in PBS prior to incubation with a FITC-labeled secondary goat anti-rabbit sera (Southern Biotechnology, 1:50) or Cy3-labeled secondary goat anti-rabbit sera (Jackson ImmunoResearch, 1:2500) for 1 hour in the dark. After extensive washings, the samples were stained in DAPI (1:10,000 dilution of 1 mg/ml stock) in water for 15 minutes at room temperature. The slides were again washed extensively prior to mounting of coverslips in Prolong Anti-Fade (Molecular BioProbes). Visualization of fluorescent chromophores was performed on an Olympus BX60 at 60X magnification.

Electrophoretic Mobility Shift Assays: Nuclear and cytoplasmic extracts from entire #4 mammary glands over the time course were collected. Samples were collected, frozen, and stored in liquid nitrogen until processed. The samples were dounce homogenized in PBS, the cells pelleted by centrifugation at 12,000 rpm for 15s at 4°C , the pellet resuspended in 400 μl of lysis buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF), and the samples placed on ice. Following incubation for 15 minutes, 25 μl of 10% Nonidet-P40 were added and the samples immediately vortexed for 10s. The suspension was centrifuged at 12,000 rpm for 30s at 4°C, and the supernatant (cytoplasmic extract) was removed, aliquotted, frozen on dry ice, and stored at -80°C. The remaining pellet was resuspended in lysis buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF), and incubated on ice, shaking, for 15 minutes. The suspension was centrifuged at 12,000 rpm for 5 minutes at 4°C, and the supernatant (nuclear extract) aliquotted, frozen on dry ice, and stored at -80°C. Protein concentrations were determined by Bio-Rad Protein Assay.

MCF-7 cytoplasmic extracts prepared as above were used as positive controls for binding and supershifts, as they harbor RelA and p50 (Dejardin et al., 1995). Cells were maintained in DMEM (Gibco-BRL) plus 10% fetal bovine serum, (Gibco-BRL) 1X penicillin/streptomycin, and 6 ng/mL bovine insulin (Sigma) in 5% CO_2. MCF-7 cells at approximately 80% confluency were harvested for extract preparation.

EMSA conditions for NF-κB have been outlined elsewhere (Baldwin et al., 1991). Probe for the HIV-LTR was (+ strand only) 5' AAA GGT ACC GAA TTC CTA GAG GGG ACT TTC GCG TCT CGA GAA AG. Briefly, 10 μg of mammary nuclear extract or 1 μg MCF-7 cytoplasmic extract were incubated on ice for 45 minutes in binding buffer containing 1 μg poly-dIdC (Boeringher-Mannheim) and, where appropriate, 2 μg specific antiserum (anti-RelA, SC-109X, anti-p50, SC-114X, anti-cRel, SC-70X, anti-p52, K-27X, Santa Cruz Biotechnology). Following incubation, the samples were incubated with 20,000 cpm of end-labeled probe for 25 minutes at ambient temperature. Prior to addition of the probe, MCF-7 cytoplasmic extracts were activated with 0.05% deoxycholate, then neutralized with 0.05% Nonidet-P40 to dissociate IκB from the NF-κB complex (Baeuerle and Baltimore, 1988). The samples were then loaded on a 4% polyacrylamide gel and electrophoresed at 150V for 2 hours. The gel was dried at 80°C under vacuum for 2 hours (Bio-Rad Model 583 gel dryer) and exposed to X-ray film for 2-4 days at-80°C.

Luciferase Assay: Cell extracts from entire #4 mammary glands from HLL line 20 mice over the time course were used for luciferase assay. Samples were collected and frozen on liquid nitrogen until processed. The samples were dounce homogenized in PBS, the cells pelleted by centrifugation at 12,000 rpm for 15s at 4°C, processed into cell lysates, and analyzed for luciferase activity according to manufacturer's instructions (Analytical Bioluminescence). Protein concentrations in the extracts were determined by BioRad Protein Assay, and the values were used to normalize relative light unit (RLU) data.

Results and Discussion

RelA, p100/p52, and IκBα are differentially expressed in the developing mammary gland during pregnancy, lactaion, and involution

To begin to elucidate the role of NF-κB during normal mammary gland proliferation, differentiation, and involution, the expression patterns of p100/p52 and IκBα were examined in virgin, pregnant, lactating, and post-wean female B6D2 mice. Northern analysis reveals that p100/p52 and IκBα have dynamic expression patterns throughout the course of post-natal mammary gland morphogenesis. RNA levels for p100/p52 are high during pregnancy and lactation, while expression decreases during involution (Figure 1). The expression of IκBα varies even more dramatically, increasing during pregnancy, decreasing to near absence during lactation, and rising again during involution. The protein levels for IκBα vary as well, with high levels in the virgin gland that wane during pregnancy (Figure 1). The lower band appearing during lactation likely represents a degradation product. Like RNA levels, protein levels increase during involution. The apparant discrepancy between expression at the RNA level and the protein level during pregnancy may be explained by the regulation, in part, of the IκBα promoter by NF-κB (Verma et al., 1995; our unpublished data). Active NF-κB complexes present in the mammary gland during pregnancy may upregulate the transcription of IκBα in pregnant and involuting glands. C-*rel* mRNA expression has not been detected by northern analysis (data not shown), and the expression studies for other family members, including RelA and p105/p50, are in progress.

To determine the cells types expressing RelA, p100/p52 and IκBα, as well as the subcellular localization of p100/p52 and RelA, immunohistochemistry was performed on sections taken from murine mammary glands at various stages of pregnancy, lactation, and involution post-weaning. At 16.5 dpc (days post coitem), both p100/p52 and IκBα are localized to the cytoplasm

of epithelial cells (Figure 2). This pattern of expression is consistent throughout all stages of development investigated thus far, including virgin, earlier stages of pregnancy, lactation, and involution. At both 12.5 and 16.5 dpc, RelA is localized to both the cytoplasm and nuclei of epithelial cells (Figure 3). These data suggest that specific NF-κB family members are expressed in mammary epithelial cells, and the presence of RelA in epithelial cell nuclei suggests that complexes capable of transactivation exist in the mammary gland during pregnancy. Expression and subcellular localization studies for p105/p50 and for RelA at all stages of mammary development are ongoing. In addition, the Cy3 fluorochrome is currently being used to reduce the background fluorescence, as endogenous proteins within the mammary gland emit flourescence at the same wavelengthas the FITC-conjugated secondary antibody used in initial studies. Nuclear localization of RelA and p105/p50 will be confirmed by confocal microscopy.

Active Nuclear RelA and p50 Containing Complexes are Most Abundant During Pregnancy and Involution

Given the increase in IκBα mRNA levels during pregnancy and involution, as well as the presence of nuclear RelA in mammary epithelial cells during these same stages (Figures 1 and 3), electrophoretic mobility shift assay (EMSA) were performed with mammary gland nuclear extracts to assess the pattern of NF-κB DNA binding activity over the course of pregnancy, lactation, and involution. As seen in Figure 4, several nuclear protein complexes from mammary nuclear extracts are capable of binding to an oligonucleotide probe bearing the κB enhancer element from the human immunodeficiency virus long terminal repeat (HIV-LTR). This κB enhancer binds a broad range of NF-κB homodimeric and heterodimeric complexes (Nabel and Baltimore, 1987; Kawakami et al., 1988; unpublished observations). Specificity of the protein:DNA complexes is demonstrated by the addition of excess unlabeled competitor oligonucleotides (lanes 4, 12, 19, and 24), demonstrating specificity. DNA binding activity is highest during pregnancy (lanes 1-9), decreases substantially through lactation (lanes 10-17), and then increases following forced cessation of nursing (lanes 18-23). In order to identify specific NF-κB components in these complexes, supershifts were performed using antibodies recognizing specific family members. Complexes containing p50 and RelA were identified in samples taken from pregnant and involuting glands (lanes 6 and 7, 21 and 22). The specificity of the supershifting antisera was confirmed by using MCF-7 cytoplasmic extracts, which are known to harbor RelA and p50, as positive controls (Dejardin et al., 1995). Although p50 containing complexes were also detected in nuclear extracts from lactation time points (lane 14), RelA was not detected (lane 15). As RelA is capable of transactivation, these data suggest that transcription mediated by NF-κB is decreased in the lactating gland. Neither c-Rel nor p52 containing complexes were detected in these extracts at any time point (data not shown). These data demonstrate that nuclear RelA and p50 containing complexes are able to bind a κB element exist in the mammary gland and are most abundant during pregnancy and involution.

NF-κB is Active in the Mammary Gland *In Vivo* During Pregnancy and Involution

NF-κB complexes that are capable of DNA binding *in vitro* are abundant in the mammary gland during pregnancy and involution (Figure 4). In order to assess the transcriptional activity of NF-κB *in vivo*, the production of an NF-κB responsive reporter was monitored in the mammary glands of transgenic mice over the time course indicated in Figure 5. Transgenic mice bearing the HIV-LTR enhancer driving the *Photinus* luciferase cDNA, termed HLLs, have been used previously to detect and quantify both constitutive and induced NF-κB activity (Yull et al., submitted; Blackwell et al., unpublished observations). Mammary glands from 2 independent HLL lines were collected, cell lysates processed, and assayed for luciferase activity. The data,

measured in relative light units (RLUs), were normalized based on protein concentrations within each sample. Luciferase activity rises in the mammary gland from virgin to mid-pregnancy (12.5 dpc to 14.5 dpc), and then begins to decrease in late pregnancy (18.5 dpc, Figure 5). KappaB-activity levels decreased dramatically during lactation, matching the levels measured in non-transgenic control glands. Following removal of the pups after 10.5 days of lactation, κB activity begins to increase once more during involution. These results demonstrate that NF-κB is transcriptionally active during pregnancy and involution, but not during lactation. Future studies will include immunohistochemical and/or *in situ* hybridization detection of luciferase in transgenic mammary gland sections to assess cell-type specificity for kappaB activity and possible colocalization with proliferating and/or apoptosing epithelial cells.

Conclusions

The data presented in this report demonstrate (1) Rel/NF-κB and IκB family members, including p100/p52, p105/p50, RelA, and IκBα are expressed in the epithelial component of the murine mammary gland; (2) RelA and p50 containing complexes that are capable of binding DNA exist in the nuclei of pregnant and involuting glands, whereas complexes containing p50 and lacking RelA exist in lactating glands; (3) NF-κB mediated transcriptional activity *in vivo* is highest in the pregnant and involuting mammary gland. The temporal activity patterns of NF-κB in the mammary gland suggest that NF-κB may function in epithelial cell proliferation during pregnancy and/or apoptosis during involution. As perturbations in proliferation and apoptosis can contribute to neoplastic transformation, understanding the involvement of NF-κB in these processes will likely contribute to the understanding of normal post-natal mammary gland morphogenesis and the pathways that lead to neoplasia.

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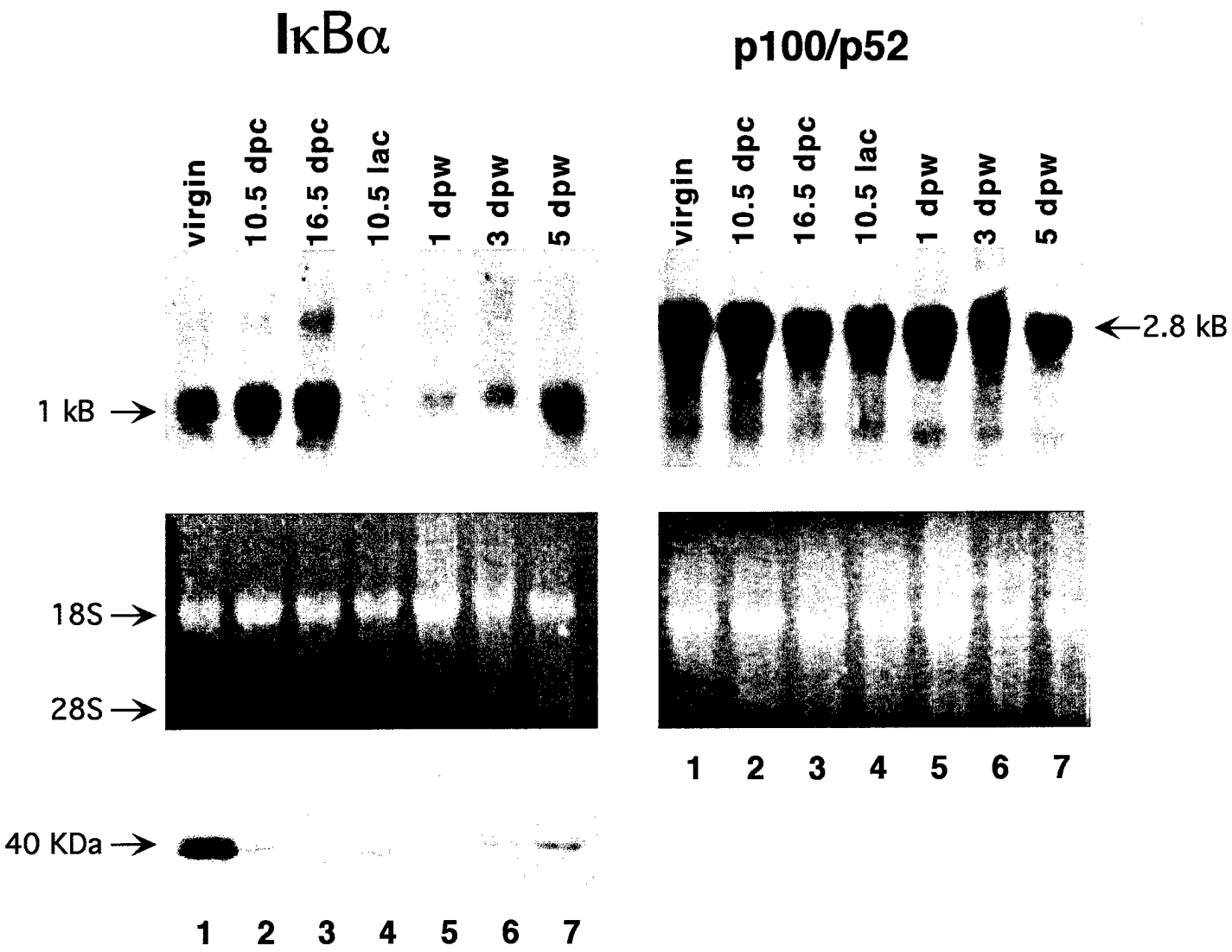

Figure 1: IκBα and p100/p52 are expressed in the mammary gland over the course of post-natal morphogenesis. The expression of IκBα RNA (top left) and protein (bottom left) is dynamic, with an increase during pregnancy, a dramatic decrease during lactation, and a rise involution post weaning. The expression of p100/p52 is more consistent over the time course, with a slight decrease during involution. RNA loading was consistent, as demonstrated by ethidium bromide stained gels (middle left and bottom right).

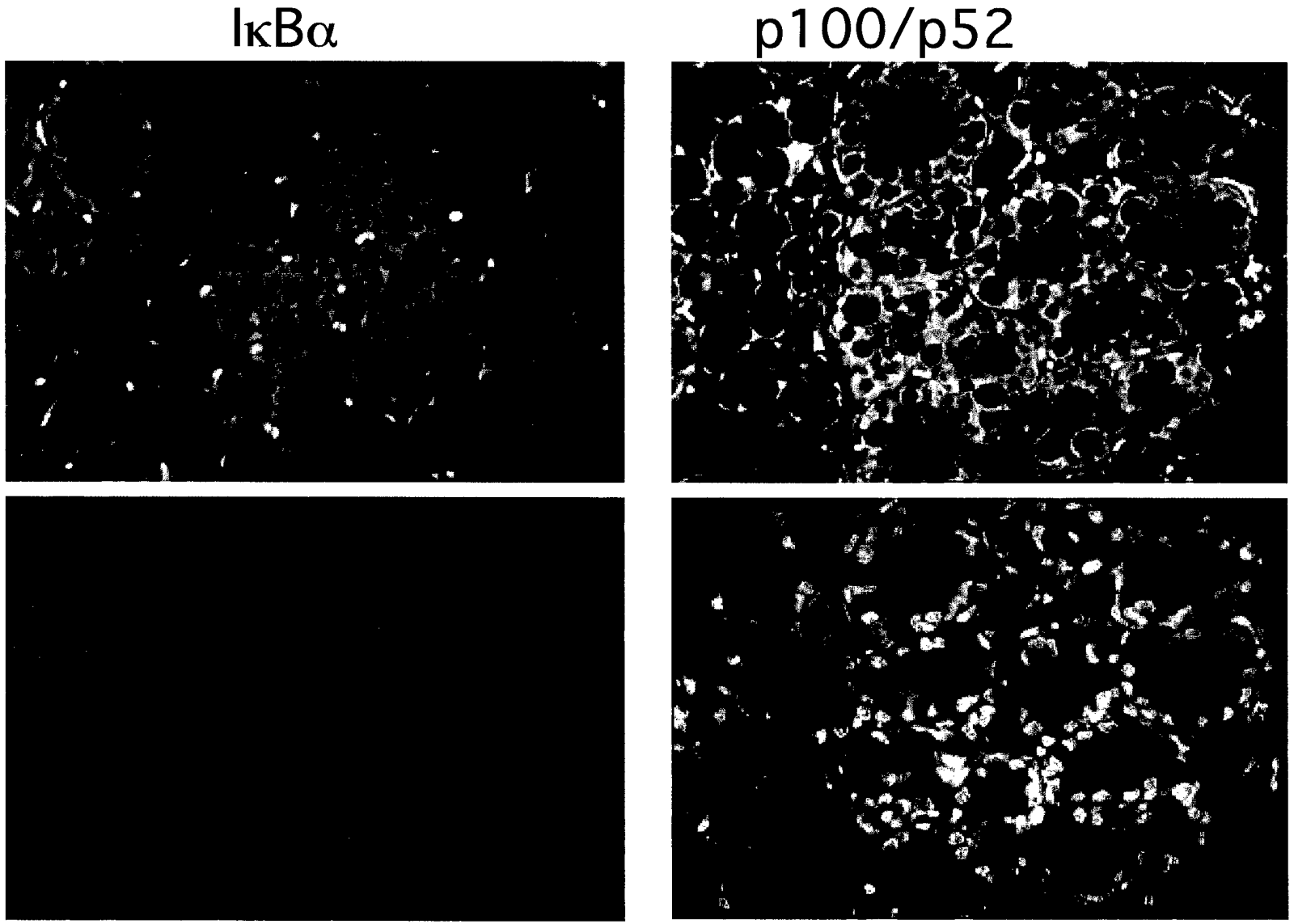

Figure 2: IκBα and p100/p52 are localized to the cytoplasm of mammary epithelial cells during post-natal mammary gland morphogenesis. Panels show the expression of IκBα (top left) and p100/p52 (top right) at 16.5 dpc as detected by immunohistochemistry using an FITC-conjugated secondary antibody. Expression is limited to the cytoplasm, as seen by complementary DAPI nuclear staining in the same section (bottom panels). This expression pattern is consistent throughout pregnancy, lactation, and involution (data not shown).

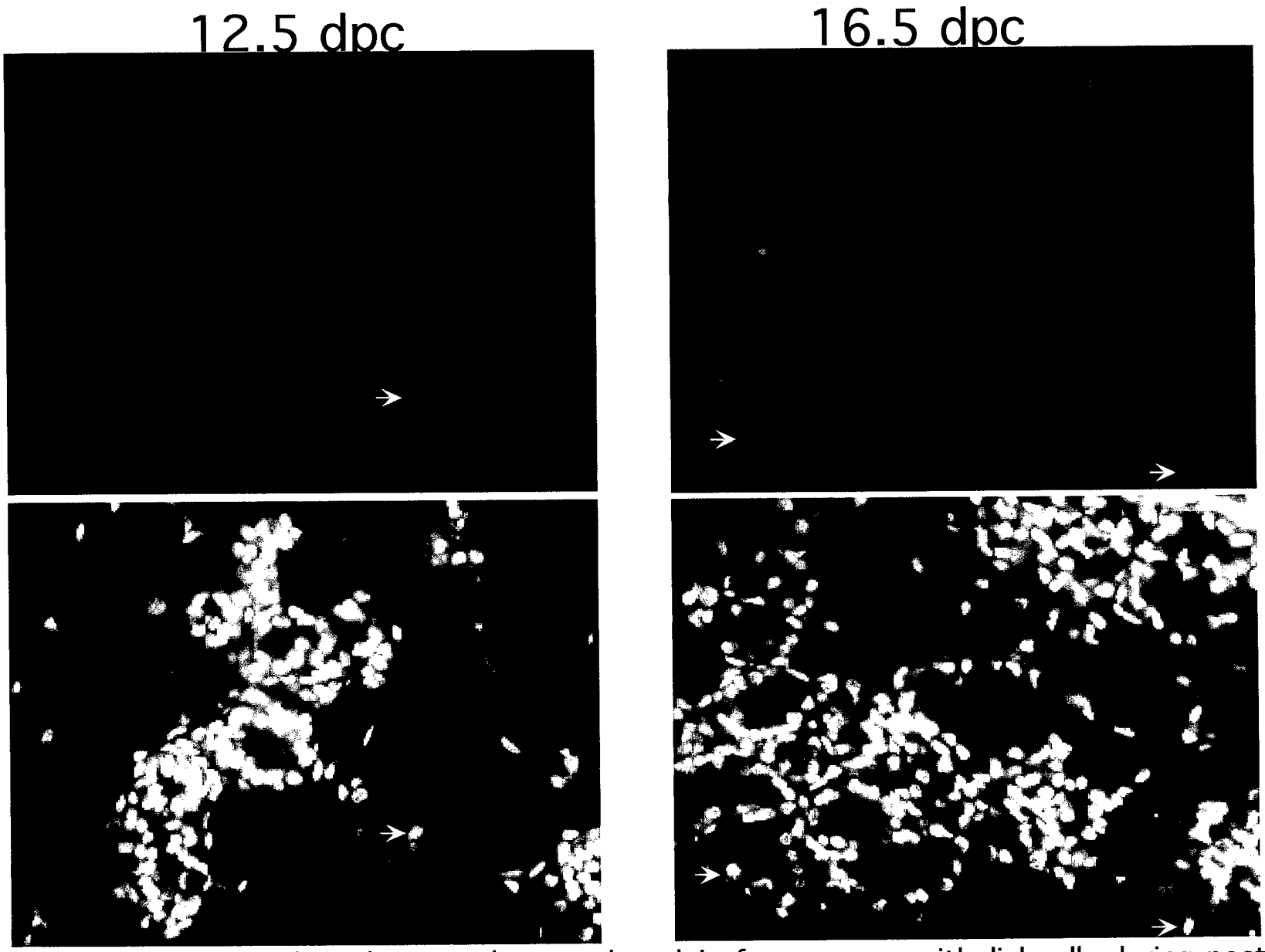

Figure 3. RelA is localized to the cytoplasm and nuclei of mammary epithelial cells during post-natal mammary gland morphogenesis. Panels show the expression of RelA at 12.5 dpc (top left) and at 16.5 dpc (top right) as detected by immunohistochemistry using a Cy3-conjugated secondary antibody. Expression is both nuclear (arrows) and cytoplasmic, as seen by complementary DAPI nuclear staining in the same section (bottom panels). Staining is specific, as the fluorescence intensity is much greater than with normal rabbit serum or with secondary antibody alone (data not shown).

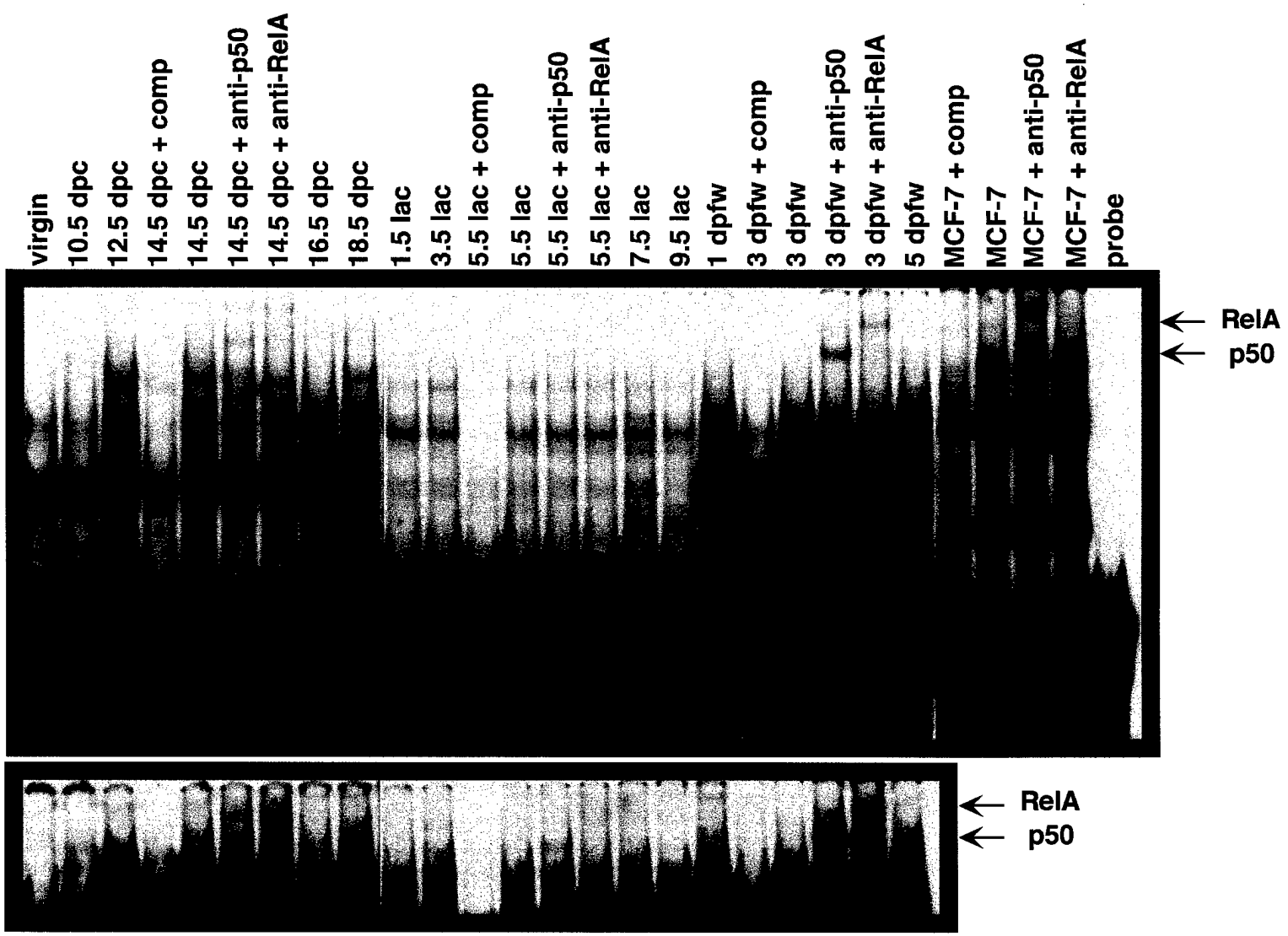

Figure 4: KappaB binding activity changes throughout post-natal mammary gland development. Nuclear extracts were collected and flash frozen at the indicated times. Protein concentrations were determined by Bradford Reagent and 10 μg protein was combined with 20,000 cpm of radiolabelled HIV-LTR κB1 double stranded oligonucleotide as probe. Where indicated, a 50X excess of unlabeled oligonucleotide was added at the time of probe addition to demonstrate specificity. Where indicated, 2 μg anti-p50 or anti-RelA sera was added to protein extract to demonstrate NF-κB subunit specificity.

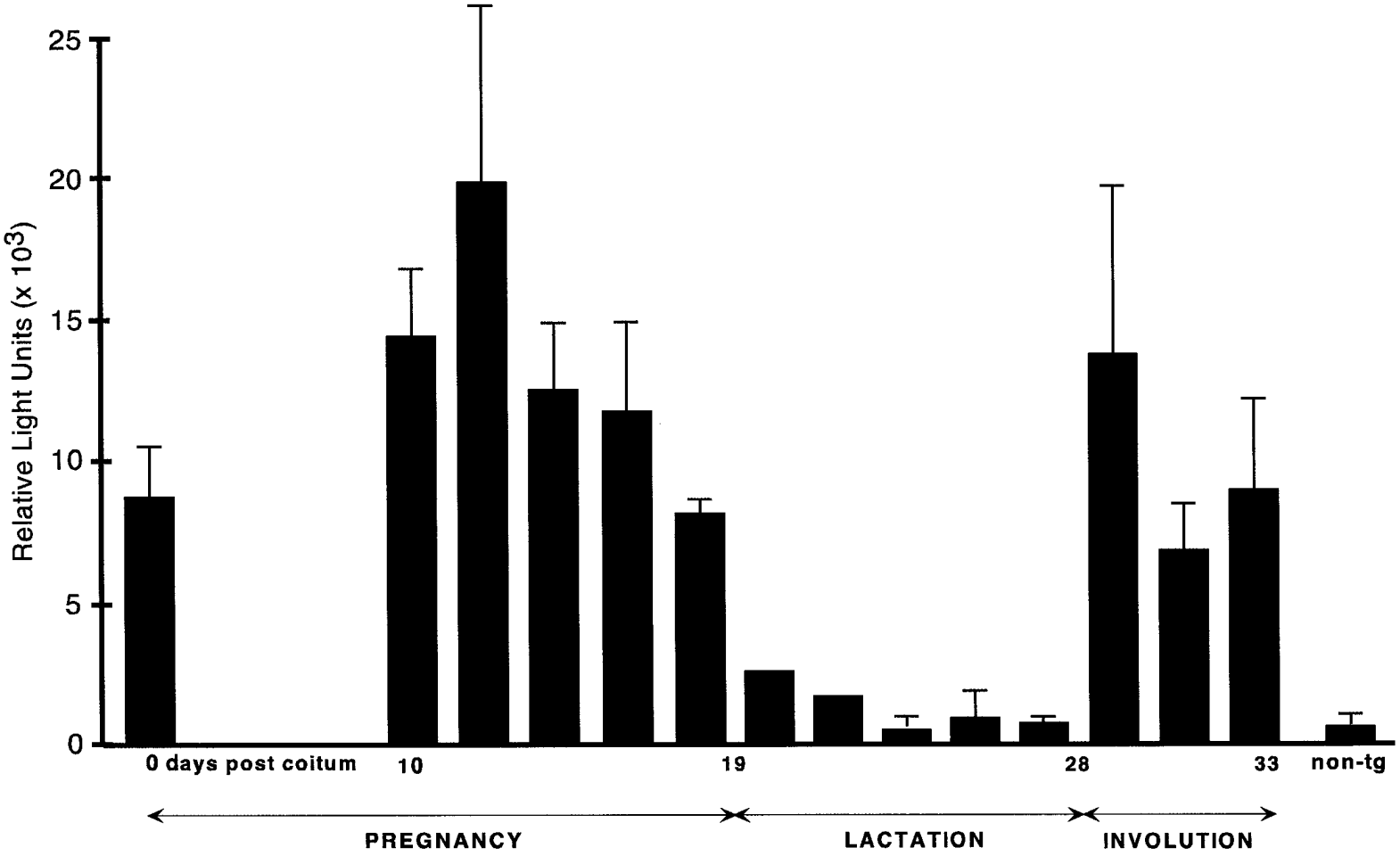

Figure 5: KappaB activity is regulated throughout post-natal mammary gland morphogensis. At the indicated time points, female HLL (lines 20 and 27) were euthanized and the #4 mammary gland was excised, flash frozen, and subsequently thawed and homogenized in luciferase buffer. 20 μls protein lysate was analyzed via luminometer according to the manufacturer's recommendations. Data shown were normalized by protein concentration and are the average of at least two and up to four independent mice with the standard error of the mean. Non-transgenic (non-tg) control lysates were taken from B6D2 female mice at 10.5 dpc.