The role of NF-κB and C/EBP factors during pathogen-mediated activation of bovine interleukin 8 and beta-defensin in mammary epithelial cells

Inaugural dissertation for the academic degree

Doctor rerum naturalium

of

Mathematisch-Naturwissenschaftlichen Fakultät

Universität Rostock

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Rostock

2009

URN: urn:nbn:de:gbv:28-diss2009-0185-5

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Date of defense: October 19th, 2009

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1. INTRODUCTION

1.1 Mastitis as a challenge in general immunology

Mastitis is an inflammation of the mammary gland. According to the clinical symptoms, mastitis is classified into two categories, acute and chronic mastitis. The apparent clinical symptoms, such as swelling, heat, redness and pains, usual appear in the acute cases. Subclinical, chronic mastitis is always a moderate and persistent inflammation and makes a great impact on dairy industry. Acute mastitis is caused commonly by *Escherichia coli* (*E. coli*), but also *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* sp., Fungi and yeasts. In contrast, Gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis*, are the most prevalent pathogens causing chronic mastitis.

Mastitis remains a highly prevalent disease, although people take efforts to control it. It is also a costly disease in dairy industry (Seegers et al., 2003). It is estimated that financial losses resulting from mastitis amount to US$ 2 billion in the USA alone (Nash et al., 2000), and worldwide losses are estimated at US$ 25 billion annually. The major economic losses are from reduction of milk yield and quality, usage of massive antibiotic, extended calving intervals and high culling rate. Hence, prevention and treatment of mastitis becomes a serious burden to producers and are of primary concern to the dairy industry. Subclinical mastitis remains at the forefront of interest in the international scale.

The establishment and persistence of an infection depend on both intrinsic virulence factors of the pathogen (Hornef et al., 2002) and capacity of the host's immune response to the pathogen (Burvenich et al., 2003). Unlike the adaptive immune system, which is long-lasting and specific for microorganism, the innate immune response is ubiquitous, non-specific and short acting. It mediates the predominant defence at early stages of an infection. Moreover, the innate immunity represents the first line of active defence against invading pathogens after their penetrating the physical barriers. Hence, it is crucial to comprehensively understand the immune mechanism, especially innate immune mechanism of bovine mammary gland, for developing strategies to reduce incidence of udder infections.

1.2 Innate immunity of the bovine mammary gland

The bovine mammary gland is a secretory organ with an organ-specific immune competence. Although mammary gland is always exposed to copious microorganisms, individual pathogen rarely causes mammary gland infection. The main reasons are from the physical barrier function of the epithelia of mammary gland and the presence of antimicrobial substances. However, once the barriers are breached, the host initiates immune defence against the invading pathogens.

Innate immunity mechanisms contribute to humoral immunity and cellular immunity. The former includes activation and/or release of various humoral mediators, such as complement cascade, cytokines and antimicrobial substances. The latter involves the activation of immune cells and/or their recruitment to sites of infection and inflammation, for example, neutrophils, macrophages, natural killer cells (NK) and dendritic cells (DC).

The contribution of the complement system to the defence of the bovine mammary gland has been reviewed (Rainard, 2003). Complement is present in milk of the healthy mammary gland at the low but significant concentration. The chemotactic complement fragment C5a is reported to induce the migration of neutrophils through the mammary epithelium *in vitro* and *in vivo* (Persson et al., 1993; Smits et al., 1996). It is also a potent stimulator of neutrophils (Rainard et al., 2000), which is important for the efficient phagocytosis in milk during infection. But the role of C5a in the initiation of the inflammatory response of the mammary gland remains questionable.

Lactoferrin (Lf) is firstly known for its iron-chelating property which is thought to establish its bacteriostatic function to protect against oxygen radical catalyzed by free iron (Legrand et al., 2004). Bovine Lf may suppress the LPS-induced IL-6 response of inflammatory cells (MattsbyBaltzer et al., 1996), but the contribution of Lf in inflammation control during *E. coli* mastitis remains to be specified. Lf may cooperate with other defence components, such as complement or lysozyme. For example, bovine Lf has been shown to modulate complement activation. The Lf binding to *S. agalactiae* activates the classical pathway of complement system, resulting in the opsonization of the bacteria (Rainard, 1993). Interestingly, activation of the alternative pathway by bovine Lf results in an increase of C3 deposition on *S. aureus* (Kai et al., 2002).

Neutrophils and macrophages are the predominant recruited cells into the milk or the mammary gland, although their abundances vary at the different physiological stages. For example, Neutrophils are the major cell type in colostrum, while the macrophage dominates in dry standing and lactating cows (Lee et al., 1980; Sordillo et al., 1991). Although milk

macrophages are less efficient than milk neutrophils in phagocytosis, they can ingest the common mastitis pathogens (Mullan et al., 1985). Moreover, they are potential antigen-presenting cells, and are implicated in the detection of invading pathogens and the initiation of the inflammatory response. Interestingly, the function of macrophages in mammary gland markedly decrease during the periparturient period, and this alteration has been linked to the increased mastitis incidence (Sordillo and Streicher, 2002; Waller, 2000).

Mammary epithelial cells (MEC) are another crucial cell type in mammary gland. It has been demonstrated that bovine mammary epithelial cells (bMEC) are able to produce a variety of inflammatory mediators or effectors in response to invading pathogens. They are composed of cytokines (TNFα etc.) and/or chemokines (interleukin 8, CXCL5, CXCL6 etc.) (Pareek et al., 2005; Strandberg et al., 2005; Wellnitz and Kerr, 2004; Yang et al., 2008), antimicrobial peptides (BNBD, LAP, etc) (Goldammer et al., 2004; Swanson et al., 2004). Hence, bMEC provides a relevant model for the research of innate immunity of bovine mammary gland. So far, the established two bMEC cell lines include MAC-T (Huynh et al., 1991) and BME-UV (Zavizion et al., 1996). Then, how do these cells recognize the extracellular pathogen?

1.3 Toll-like receptors (TLRs): main receptors perceiving the pathogen presence

Toll-like receptors (TLRs) have been identified as major receptors recognizing conserved bacterial structures known as pathogen-associated molecular patterns (PAMPs) (Kopp and Medzhitov, 2003). Mammalian TLRs family comprises 13 transmembrane receptors (TLR1 to TLR13). These TLRs were reported to specifically recognize different PAMPs. For example, TLR2 is the receptor for peptidoglycan and lipoteichoic acid (LTA) of Gram-positive bacteria. TLR4 recognizes lipopolysaccharides (LPS) of Gram-negative bacteria, TLR5 binds bacterial flagellin, and TLR9 recognizes bacterial CpG DNA (Van Amersfoort et al., 2003). Upon recognition of PAMPs by specific TLR or multiple TLRs, the signal transduction pathway is initiated. This features activation of downstream adaptors, such as Myeloid differentiation marker 88 (MyD88) or the TIR-domain-containing adaptor protein (TRIF). As a result, nuclear factor κB (NF-κB) is activated and transcription of the immune-related genes, such as chemokines and β-defensins, is enhanced (Takeda et al., 2003). Given the important functions of chemokines and β-defensins in innate immunity system, it is valuable to elucidate the molecular mechanisms regulating their expression in response to pathogen stimulation. In the following sections, I focus on the mechanisms regulating expression of the chemokine interleukin 8 and β-defensins.

1.4 Interleukin 8 (IL-8): a chemokine

The chemokine family consists of functionally diverse proteins mediating inflammatory responses. They are small proteins (8-10 kD) with similar 3-dimensional structures. Four subfamilies are classified according to the presence of a cysteine containing signature motif near their N-terminus (CXC, CC, C and CX3C) (Mackay, 2001; Rossi and Zlotnik, 2000). To date, 18 bovine chemokines are known.

Interleukin-8 (IL-8), a member of the CXC chemokine family, is an important activator of neutrophils and a chemoattractant for neutrophils, T-cells and basophil cells. Hence, it plays a crucial role in inflammatory disease (Harada et al., 1994; Matsushima and Oppenheim, 1989). It is not constitutively but inducibly produced by pathogen signal in many cell types, including macrophages, epithelial cells, monocytes, dermal fibroblasts, endothelial cells, keratinocytes, mesangial cells and several human tumour cell lines (Roger et al., 1998).

1.4.1 Inducible expression of IL-8

It has been reported that IL-8 is induced by LPS (Yoshimura et al., 1987), *S. aureus* entertoxin A (Schmid and Weissmann, 1987), cytokines (IL-1 and TNF-α) (Matsushima et al., 1988) and phorbol myristate acetate (PMA) (Mukaida et al., 1989). In mammary epithelial cells, IL-8 mRNA expression is up-regulated by LPS and LTA (Strandberg et al., 2005). An increase in IL-8 was also reported in milk from quarters stimulated by LPS (Bannerman et al., 2003).

IL-8 is also inductively produced by whole bacteria. In milk cells from mammary glands infected with Gram-negative *E. coli* bacteria, IL-8 mRNA transcription is increased (Lee et al., 2006). Primary bovine MEC cells markedly increase IL-8 transcription upon induction with heat-inactivated *E. coli* (Yang et al., 2008). In contrast to Gram-negative bacteria, intramammary infections with Gram-positive or wall-less bacteria are shown to induce a delayed and/or diminished IL-8 response. In two studies using different *S. uberis* strains, milk IL-8 is not increased until 30 h and 66 h after infection (Bannerman et al., 2004a; Rambeaud et al., 2003). Another *S. aureus* strain does not increase milk IL-8 following experimental intramammary infection (Bannerman et al., 2004b). However, the differential transcription of IL-8 mRNA has also been confirmed in experimental mammary tissue infected with other strains of *E. coli* and *S. aureus* (Yang et al., 2008). Hence, the IL-8 gene expression is cell-type and stimulus specific (Roebuck, 1999).

1.4.2 Molecular regulation of the human IL-8 expression

How can cells respond dynamically to a variety of stimuli? One view is that the ubiquitous, stimulus- and cell type-specific activators can be assembled into a nucleoprotein complex called enhanceosome (Carey, 1998). In the enhanceosome model, the binding of different activators to their recognition sites generates a unique network. Each enhanceosome provides a specific activation surface, which is chemically and spatially complementary to the target surfaces of coactivators and to the basal transcriptional machinery. In this way, they can be recruited to DNA to generate synergistic transcription.

IL-8 regulation is concentrated on the transcriptional level. Since stimulus- and cell-type specific activators are involved in the course of IL-8 transcription, the regulation pattern is altered owing to different cell types and stimuli. The regulation of the human IL-8 gene has been well documented. Functional studies show that the human IL-8 transcriptional responses to inflammatory stimuli are rapid and require approximate 150 nucleotides upstream of the TATA box. The region possesses DNA binding sites of Activator protein 1 (AP1), CCAAT/enhancer binding protein (C/EBP) and NF-κB. Among them, NF-κB is an important factor to activate the human IL-8 promoter in most cases. AP-1 might be involved in inflammatory response in epithelial cells. It is demonstrated that AP-1 and NF-κB are essential transcription factors for IL-1β-induced the IL-8 gene expression in human vascular smooth muscle cells (Jung et al., 2002). In human airway smooth muscle cells, transcriptional regulation of IL-8 by bradykinin involves AP1, C/EBP and NF-κB (Zhu et al., 2003). Transcription factors C/EBPβ and NF-κB synergistically activate the human IL-8 transcription in the cited examples (Kunsch et al., 1994; Mahe et al., 1991; Matsusaka et al., 1993). In contrast, cyclic mechanical stretch does not increase the NF-κB activity, but AP1 and C/EBP are required for IL-8 activation in the same cell type (Kumar et al., 2003). Hence, the three transcription factors may play an important role in the modulation of the human IL-8 gene. The types of factor(s) implicated in the IL-8 activation appear to be dependent on the cell types and stimuli types.

The modulation of the bovine IL-8 gene is poorly understood. Therefore, it is valuable for rational control mastitis to investigate the regulatory mechanism of the bovine IL-8 gene under pathogen stimulation.

1.5 Antimicrobial peptides: β-defensins

1.5.1 Classification and structures of defensins

In contrast to IL-8, defensins are small, cysteine-rich, cationic antimicrobial peptides widely expressed in nearly all higher eukaryotes, from insects and plants to amphibians and mammals**.** Defensins constitute a large, highly conserved multigene family. On the basis of the numbers and patterns of disulfide bridges, defensins are divided into three groups, α-, β- and θ-defensins (Ganz, 2003).

α-defensins are abundant in neutrophils (Ayabe et al., 2000). They are small polypepetides with 29-35 residues and a six-cysteine motif that forms three intramolecular disulphide bonds (Cys1-Cys6, Cys2-Cys4, Cys3- Cys5) (Lehrer and Ganz, 2002).

β-defensins differ from α-defensins in size (> 30 amino acid residues) and cysteine pairing (Cys1-Cys5, Cys2-Cys4, Cys3- Cys6). They are expressed in several organs, such as tongue, airways, skeletal muscles, esophagus, intestine and skin. However, α- and β-defensins have similar tertiary structures, featuring triple-stranded antiparallel β sheets (Pardi et al., 1992; Zimmermann et al., 1995).

θ-defensins are identified only in rhesus monkeys so far. They are circular mini-defensins (18 amino acids) generated by two α-defensin mRNA precursors, which acquired a stop codon between the third and the fourth codon for cysteine (Lehrer and Ganz, 2002).

1.5.2 β-defensin in mammal

In mammal, copious β-defensins have been identified. The first mammalian β-defensin designated TAP (tracheal antimicrobial peptide) was isolated from cow trachea (Diamond et al., 1991). Lingual antimicrobial peptide (LAP) was subsequently purified from cow tongue and shown to be expressed in various epithelia (Schonwetter et al., 1995). Apart from TAP and LAP, 13 different bovine β-defensins (BNBD1-13) are identified from neutrophils (Selsted et al., 1993), and EBD from enteric epithelium cells (Tarver et al., 1998). In a recent report, six novel β-defensin members (named DEFB401-DEFB405 and LAP-like) in bovine are characterized (Roosen et al., 2004). In mouse, four β-defensins are expressed in keratinocytes or various epithelial cells (Bals et al., 1999; Jia et al., 2000; Morrison et al., 1999). Six human β-defensins (hBD1-6) have been characterized (Bals et al., 1998; Bensch et al., 1995; Garcia et al., 2001a; Harder et al., 1997; Kao et al., 2003; Yamaguchi et al., 2002). Among them, hBD-1, 2 and 3 are produced by keratinocytes and various epithelial cells (Diamond et al., 1996; Harder et al., 1997; Liu et al., 1998; Russell et al., 1996; Zhao et al.,

1996). hBD-4 is strongly expressed in the testis and gastric antrum (Garcia et al., 2001b), whereas hBD-5 and hBD-6 are specifically expressed in the epididymis (Yamaguchi et al., 2002).

1.5.3 Inducible expression of β-defensins

Unlike α- and θ-defensins, β-defensins transcription is inducible. hBD-2 expression is induced in monocytes by bacteria, LPS and interferon-γ (IFN-γ) (Duits et al., 2002; Fang et al., 2003). IL-1β markedly stimulates hBD-2 mRNA increase in tracheal epithelial cells (Hiratsuka et al., 2003). hBD-3 mRNA concentration is strongly induced by IFN-γ in keratinocytes (Harder et al., 2004). hBD-4 mRNA in respiratory epithelial cells is upregulated by bacterial exposure or PMA (Garcia et al., 2001b). It was also reported that both TAP and LAP transcription are enhanced in bovine tracheal epithelial cells (TEC) challenged with LPS or TNFα (Russell et al., 1996). A lot of β-defensins are expressed in mammary gland or mammary epithelial cells in response to mastitis, including LAP, β-defensin 5 (BNBD5), TAP, DEFB1, BNBD3, BNBD9, BNBD12 and DEFB401 (Goldammer et al., 2004; Roosen et al., 2004; Strandberg et al., 2005). The data indicate that β-defensins may play a significant role in host immune response (Fellermann and Stange, 2001).

1.5.4 Roles of β-defensins in mammalian immunity

β-defensins can kill or inactivate bacteria, fungi or some enveloped viruses *in vitro* and are generally considered to be direct effectors of innate antimicrobial immunity (Ganz and Lehrer, 1998; Lehrer and Ganz, 1999). For example, hBD-2 exhibits a potent antimicrobial activity against Gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa*, but only a bacteriostatic activity against Gram-positive *S. aureus* (Schibli et al., 2002). Although the antimicrobial mechanism of β-defensins remains poorly understood, it is commonly thought that their antimicrobial activities result from their spatially separated charged and hydrophobic regions. These enable the peptides to insert into the cell membrane to impair membrane integrity and functions, which ultimately leads to the lysis of microorganism cells (Sahl et al., 2005). In addition to potent antimicrobial activity in innate immunity, β-defensins also seemingly play roles in adaptive immunity, which provides a link between innate and adaptive immunity (Yang et al., 1999). For example, hBD-1 is chemotactic for immature, but not mature DCs (Yang et al., 1999), hBD-2 for $CD45R0^+$ memory T cells (Yang et al., 1999). Murine β-defensins 2 and 3 (mBD2 and mBD3) can act on murine immature but not mature DCs (Biragyn et al., 2001).

1.5.5 Regulation of β-defensins expression

The regulation of the human hBD genes has been well documented. Different from the hBD-3 gene, the hBD-2 promoter contains NF-κB response element (Becker et al., 2000; Harder et al., 1997). Moreover, NF-κB binding to the pκB1 attachment site (-205 to -186) was found to be crucial for the transcriptional regulation of the hBD-2 gene in response to IL-1β and TNF-α in A549 cells (Tsutsumi-Ishii and Nagaoka, 2003).

In bovine primary TEC cells, the induction of TAP is regulated at the transcriptional level in response to LPS and the mutant strain (WD3) of *B. bronchiseptica*. NF-κB p50 and p65 were found to be involved in the regulation, but C/EBPβ has no alteration after stimulation in EMSA assay (Diamond et al., 2000; Legarda et al., 2005). A more recent report shows that NF-κB and NF-IL6 (C/EBPβ) are indispensable for expression of the bovine BNBD5 gene under different stimuli (Yang et al., 2006).

1.6 Nuclear factors (NF-κB)

Nuclear factors (NF-κB) or Rel proteins are a family of structurally-related eukaryotic transcription factors, which are implicated in the regulation of inflammatory and immune responses, developmental processes, cellular growth and apoptosis. In mammals, the NF-κB family is composed of five transcription factors: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Ghosh et al., 1998). All of them possess a highly conserved ca. 300 amino acids DNA-binding/dimerization domain called the Rel homology domain (RHD) (Fig. 1). The NF-κB members can form homodimers and heterodimers via RHD and bind to NF-κB binding sites to modulate gene expression. The consensus sequence of the common NF-κB site is GGGPuNNPyPyCC, where Pu stands for purine base, Py is pyrimidine and N symbolizes any nucleotide (Ivanov et al., 1995; Weih et al., 2001). Three members p65, c-Rel and RelB contain transactivation domains (TADs) in C-terminal, which enable them to activate target gene expression. In contrast, p50 and p52 have no TAD (Fig. 1). Homodimers or heterodimers of p50 and p52 were reported to repress NF-κB-site-dependent transcription *in vivo* (Lernbecher et al., 1993), possibly by competing for DNA binding with other transcriptionally active dimers (*e.g.* p50/p65). p50 or p52 also often forms heterodimers with the other NF-κB members to activate transcription. Alternatively, they might interact with the other proteins possessing a TAD, such as Bcl-3 to enhance target gene transcription. Surprisingly, p50/p50 homodimer has been demonstrated to activate NF-κB-site-dependent transcription *in vitro* (Lin et al., 1995).

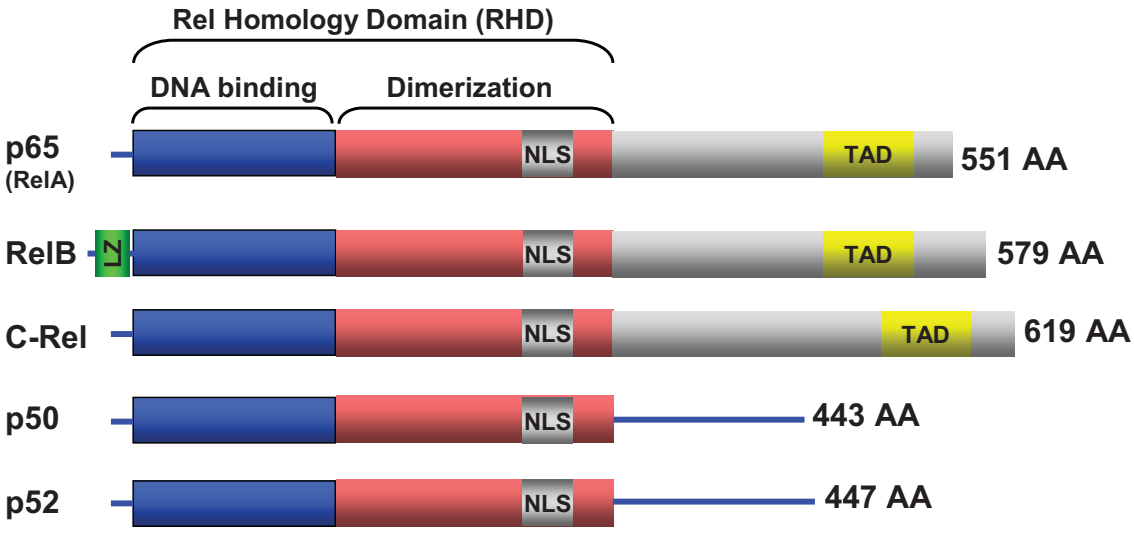


Figure 1. Members of the NF-κB (or Rel) family

All members encompass a well-conserved RHD, which comprises DNA-binding and dimerization domains, and a nuclear localization signal (NLS). RelB has a unique N-terminal leucine zipper (LZ) domain. TAD: transactivation domain only in p65, RelB and c-Rel, The above image is adapted with minor modification (Xiao, 2004).

1.6.1 Inhibition of NF-κB

NF-κB factors are generally been thought to reside in the cytoplasm in an inactive form bound by its inhibitory proteins, the members of the IκB family (Finco et al., 1994; Verma et al., 1995). However, serial findings have demonstrated that NF-κB may shuttle between the nucleus and cytoplasm (Carlotti et al., 2000; Huang et al., 2000; Tam et al., 2001; Tam and Sen, 2001). Hence, the majority of NF-κB-IκB complexes are present in the cytoplasm, since potent nuclear export signals (NES) in the IκB proteins overcome the NLS of the NF-κB subunits (Tam et al., 2000). The crystal structures of IκBα and IκBβ revealed that the IκB proteins mask only NLS of p65, whereas the NLS of p50 remains accessible (Chen et al., 1998; Croy et al., 2004; Huxford et al., 1998).

1.6.2 Activation of NF-κB

NF-κB can be activated by various extracellular stimuli, including inflammatory cytokines, bacterial components (such as LPS), viral infection, physiological, physical or oxidative stresses, a variety of mitogens among others (Ghosh et al., 1998; Pahl, 1999). In general, two events are considered in NF-κB activation. One is the release of NF-κB from the NF-κB-IκB complex and its translocation into the nucleus. During the event, the activated IκB kinase (IKK) complex phosphorylates IκB to make the latter degraded by proteasome. As a result,

NF-κB is released from the NF-κB-IκB complex. Two pathways may be involved in the course, the classical pathway or the alternative one. It was reported that the former is involved in innate immunity and the latter maybe in adaptive immunity. Since the alternative pathway of NF-κB activation is required for B cell maturation and formation of secondary lymphoid organs (Senftleben et al., 2001).The post-translational modification of NF-κB is another activation event. I will explain the type in details in the following part.

1.6.3 Phosphorylation and acetylation of NF-κB

Phosphorylation and acetylation of NF-κB p65 are main molecular events in post-translational modification of NF-κB factor, which controlling the strength and duration of the NF-κB transcriptional response. It has been documented that NF-κB p65 is phosphorylated at least at 4 different serine sites, including Ser^{276} and Ser^{311} in the RHD and Ser^{529} and Ser^{536} in the TAD (Duran et al., 2003; Hu et al., 2004; Sakurai et al., 2003; Zhong et al., 1998). Mitogen and stress-activated protein kinase (MSK1 and MSK2) and protein kinase A (PKA) are involved in phosphorylation at Ser^{276} (Chen and Greene, 2004; Vermeulen et al., 2003). Phosphorylation of p65 at Ser^{276} enhances the interaction of p65 with the coactivator CREB-binding protein (CBP) and its structural homolog p300 (Zhong et al., 1998). PKCζ phosphorylates p65 at Ser^{311}. However, in PKCζ –deficient cells, p65 fails to interact with CBP in response to TNFα (Duran et al., 2003). Ser^{529} and Ser^{536} are catalyzed by IKK and casein kinase II (CK2) respectively (Chen and Greene, 2004; Perkins, 2006). Similar to Ser^{276}, phosphorylation at Ser^{536} increases assembly of p65 and CBP/p300. However, the mutation of Ser^{536} into Ala abrogates this interaction (Chen et al., 2005).

Similarly, NF-κB acetylation has been demonstrated to be important for NF-κB function (Chen et al., 2001; Kiernan et al., 2003). The acetylation of NF-κB p50 at lys^{431}, lys^{440} and lys^{441} seems to enhance the transcriptional activity and DNA binding activity of modified heterodimers (Deng et al., 2003; Deng and Wu, 2003; Furia et al., 2002). Three acetylation sites (lys^{218}, lys^{221} and lys^{310}) have been identified in NF-κB p65, and they play distinct roles in the transcriptional activities of NF-κB p65 (Chen et al., 2002). For example, acetylation at lys^{310} is required for the transciptional activity of p65, but has no effect on its DNA binding or interaction with IkBα. The acetylation of p65 at lys^{218} and lys^{221} enhances its DNA-binding activity and impair the assembly with IκBα, thereby extending the duration of the NF-κB response in the nucleus. Deacetylation of p65 at lys^{218} and lys^{221} by HDAC3 greatly enhances the binding to IkBα. The reversible acetylation of p65 serves as an intranuclear molecular

switch regulating the strength and duration of the NF-κB response (Chen et al., 2001). A recent study provides a link between acetylation and phosphorylation of p65. It demonstrates that phosphorylations at Ser^{276} and Ser^{536} increase the p65 acetylation at lys^{310} (Chen et al., 2005). Taken together, phosphorylation and acetylation play key roles in NF-κB activation.

1.6.4 Transcriptional coactivators and interactive proteins of NF-κB

Although NF-κB is an essential transcription factor involved in the expression regulation of many genes, it does not function alone. It is just one component of the complicated regulation complex required for gene transcription. Some important transcriptional coactivators are also implicated in the complex. It was reported that NF-κB p65 binds to the CBP and p300 (Gerritsen et al., 1997; Perkins et al., 1997; Zhong et al., 1998). Additionally, CBP can also associate with the CBP associated factor (p/CAF) (Yang et al., 1996) and with the RNA polymerase II holoenzyme (Nakajima et al., 1997). other components of the transcriptional complex may associate with CBP to form a large regulatory complex by facilitating the sequence-specific activators to the basal transcriptional machinery. These alter the chromatin structure in two ways, since both CBP (Bannister and Kouzarides, 1996) and p/CAF (Yang et al., 1996) contain histone acetyltransferase (HAT) domains and have strong HAT activities. Hyperacetylated histones have been identified in transcriptionally active chromatin, whereas hypoacetylated histones exist in the heterochromatin (Turner, 1998).

Many transcription factors have been reported to interact with NF-κB, including C/EBP (Ruocco et al., 1996; Stein et al., 1993b), AP-1 (Stein et al., 1993a) , Sp-1 (Perkins et al., 1993; Perkins et al., 1994), signal transducer and activator of transcription 6 (STAT6) (Shen and Stavnezer, 1998) among others. Since NF-κB in genes regulation is frequently accompanied with C/EBP factors, I would like to introduce C/EBP as follows.

1.7 CCAAT-enhancer binding proteins (C/EBP)

The C/EBP is a family of transcription factors involved in the regulation of cellular differentiation in multiple tissues. Six different members (C/EBPα to ζ) have been isolated and characterized (Akira et al., 1990; Cao et al., 1991; Descombes et al., 1990; Poli et al., 1990; Ron and Habener, 1992; Williams et al., 1991). All members contain a basic DNA-binding domain and a leucine zipper motif (bZIP) in the C-terminal 55-65 amino acid residues (Akira et al., 1990; Landschulz et al., 1988a; Poli et al., 1990; Ron and Habener, 1992). Due to the high identity in the bZIP domain, the members of C/EBP can form homo- or

heterodimers (Chumakov et al., 1997; Williams et al., 1991) binding to a consensus C/EBP binding site (Landschulz et al., 1988a; Landschulz et al., 1988b).

Conversely, the N-terminal in the C/EBP factors are divergent (<20% sequence identity).The C/EBP-α, -β, -δ and -γ genes are intronless, whereas C/EBP-ε and - ζ contain two and four exons respectively. Importantly, most members of the C/EBP family can be translated into different isoforms from the same mRNA molecules. C/EBPα may be translated into two protein isoforms, 42 kD and 30 kD, with the latter having a lower transcriptional activity (Lin et al., 1993; Ossipow et al., 1993). C/EBPβ mRNA produces at least three different protein isoforms: the liver-enriched transcriptional activating proteins (LAP1, 39 kD), LAP2 (36 kD) and liver inhibitory protein (LIP, 20 kD). LIP always acts as a dominant-negative (DN) transcriptional repressor due to lack of most of transactivation domain. LAP2 and LIP are the major forms (Descombes and Schibler, 1991). At least four isoforms can be produced (32 kD, 30 kD, 27 kD and 14 kD) by the same C/EBPε mRNA molecule. The activation potential of the 30 kD form is lower than that of the 32 kD form, and the 14 kD form lacks an intact transcriptional activation domain (Lekstrom-Himes, 2001; Yamanaka et al., 1997b).

1.7.1 Role of C/EBP in inflammatory and immune response

C/EBP-α, -β and -δ are differentially regulated by inflammatory agents, including LPS and cytokines. It was shown that C/EBPβ and C/EBPδ mRNA concentrations are increased by inflammatory stimuli in a number of cell types, such as hepatocytes, macrophages, renal mesangial cells and astroglial cells, whereas C/EBPα transcription is decreased (Akira et al., 1990; Alam et al., 1992; Cardinaux et al., 2000; Granger et al., 2000; Kinoshita et al., 1992; Poli et al., 1990; Poli, 1998; Ramji et al., 1993; Tengku-Muhammad et al., 2000). C/EBPβ-deficient mice show that expression of serum amyloid A (SAA), the stress associated protein (SAP), α1-acid glycoprotein, complement C3 and TNFα was impaired (Poli, 1998; Screpanti et al., 1995; Tanaka et al., 1995). Delayed migration of neutrophils along with an impaired bactericidal response was observed in the C/EBPε-deficient mice (Lekstrom-Himes and Xanthopoulos, 1999). Additionally, a number of inflammatory/immune-related genes are down-regulated in the C/EBPε-deficient mice (Tavor et al., 2002; Yamanaka et al., 1997a). These findings clearly demonstrate that C/EBP factors play a crucial role in inflammatory and immune response.

1.7.2 Autoregulation and phosphorylation of the C/EBP family factors

The autoregulation of C/EBPα gene expression is known in a species-specific manner. The murine C/EBPα gene expression is autoregulated by C/EBPα or C/EBPβ via the C/EBP binding site in its proximal promoter region (Christy et al., 1991; Legraverend et al., 1993). The human C/EBPα promoter lacks a C/EBP binding site, however, its expression can still be autoregulated by C/EBPα indirectly via stimulating the DNA-binding activity of upstream stimulatory factor (USF) to its binding site in the proximal promoter region (Timchenko et al., 1995). Likewise, the promoters of the C/EBPβ genes in mouse, rat and chicken are also autoregulated (Chang et al., 1995; Mink et al., 1999; Niehof et al., 2001).

Phosphorylation modification is also involved in the regulation of C/EBPβ function. C/EBPβ is normally repressive since negative regulatory regions mask its transactivation domains (Kowenzleutz et al., 1994; Williams et al., 1995). However, phosphorylation of the negative regulatory regions can abolish the repression. A typical example relevant to inflammation/immune is that the transactivation potential of C/EBPβ is induced by phosphorylation at Thr^{235} through the Ras/mitogen-activated protein kinase (MAPK) pathway (Nakajima et al., 1993; Zhu et al., 2002).

1.7.3 Interaction of C/EBP with NF-κB

A number of transcription factors have been reported to physically and functionally interact with C/EBP factors. Here I only introduce the interaction of C/EBP with NF-κB factors. It has been demonstrated that the C/EBP and NF-κB factors synergistically enhanced expression of the inflammatory/immune-related genes encoding SAA1, A2, A3, and α1-acid glycoprotein, as well as the cytokines IL-6, IL-8, and IL-12 and Granulocyte-Colony Stimulating Factor (G-CSF) (Betts et al., 1993; Dunn et al., 1994; Lee et al., 1996; Li and Liao, 1992; Matsusaka et al., 1993; Plevy et al., 1997; Ray et al., 1995; Vietor et al., 1996). The interaction is also the main event in the modulation of the human immunodeficiency virus (HIV) long terminal repeat (LTR) (Ruocco et al., 1996). Apart from the cooperative activation, C/EBP and NF-κB factors have also showed antagonistic effects (Brasier et al., 1990; Stein et al., 1993b). It is general thought that promoter structure and cell-type specificity are likely to play a major role. Although the mechanisms for cooperative effects have not yet been entirely clarified, an efficient interaction requires the integrity of both the NF-κB RHD and the C/EBP leucine zipper domain (Leclair et al., 1992).

The conformation of chromatin is also known to contribute to gene regulation and is best studied in the endogenous context. Therefore, in the following parts, I will focus on the roles of chromatin remodeling and the related DNA methylation status in transcription.

1.8 Chromatin remodeling

1.8.1 Overview of chromatin remodeling

In eukaryotes, DNA and protein complex are assembled into higher-order structures designated as chromatin, which maintains the majority of genes in an inactive state. The main reason is that the cell-type specific transcription factors and RNA polymerases are restrained from access to the respective binding sites. However, under some situations, the chromatin encompassing that gene and its regulatory regions must be remodeled to make them accessible to cell-specific transcription activators, which also permits the complicated transcription machinery containing RNA polymerases to initiate transcription. There are two broad types of enzymes involved in chromatin remodeling. They are ATP-dependent remodeling enzymes and HAT. Although the mechanisms of chromatin remodeling are unclear, the enzymes are believed to involve in alteration of the chromatin structure and in acetylation of histones or non-histones that somehow increase accessibility to transcription factors. Now, I will focus on the link between acetylation and genes transcription.

1.8.2 The link between acetylation and transcription

The core histones, particularly H3 and H4, can be acetylated by HAT at the ε-amino groups of lysine residues in the N-terminal tails. The charge neutralization may cause an affinity reduction of DNA for histone but an increase for transcription factors to activate genes. Given are positive examples, but not universal, correlation between the extent of core histone acetylation and inducible gene activity. For example, the transcriptional activation of the IFN-β gene is associated with the localized hyperacetylation of histones H3 and H4 (Parekh and Maniatis, 1999). Similarly, histone acetyltransferase activity of yeast Gcn5p is required for the activation of targets genes *in vivo* (Kuo et al., 1998). However, a detailed acetylation analysis of histone H4 in *Drosophila* polytene chromosomes reveals that acetylation of H4 at lys^5 or lys^8 is distributed throughout euchromatin, whereas acetylation at lys^{12} is preferentially associated with heterochromatin, which is generally thought to be transcriptionally repressed (Turner et al., 1992). Hence, the correlation between histone acetylation and transcriptional activity does not appear to be simple and general.

Apart from the histone acetylation, it is reported that there is a link between nonhistone acetylation and transcription. For example, the factor p300 and its homolog CBP, p/CAF contain HAT activity. However, they not only acetylate histones, but also acetylate general transcription factors (GTFs) (e.g. TFIIE), activator/repressors (e.g. p53), and architectural proteins (e.g. high mobility group protein [HMG] I (Y)). p300 and CBP interact with the activation domains of numerous transcription factors and the nuclear receptors, and acetylate the transcription factors to enhance genes transcription (Torchia et al., 1998). These interactions provide a direct means of targeting the acetylation to a surrounding regulatory region.

1.9 DNA Methylation

1.9.1 Methods for DNA methylation analysis

In mammalian DNA, approximate 80% of all CpG dinucleotides are methylated. DNA methylation seems to be part of a system affecting chromatin structure and transcriptional regulation. The methylation reaction is common catalyzed by DNA methyltransferase (Dnmt) by adding a methyl group to carbon 5 of the cytosine ring. Methods for DNA methylation analysis can be divided roughly into two types: global and gene-specific methylation analysis. Chromatographic methods and methyl accepting capacity assay are two methods for measuring the overall level of methyl cytosines in genome. In order to analyze gene-specific methylation, a large number of techniques have been developed. Most early studies used methylation-sensitive restriction enzymes (e.g. *Hpa*II) to digest DNA followed by southern detection or PCR amplification. Recently, bisulfite reaction-based methods have become very popular such as methylation specific PCR (MSP) (Herman et al., 1996) and bisulfite genomic sequencing PCR (BSP) (Clark et al., 1994; Frommer et al., 1992). In the study, I have adopted BSP (Fig. 2). This technique begins with the treatment of genomic DNA with bisulfite. Under appropriate reaction conditions, bisulfite converts cytosine into uracil, but 5-methylcytosine remains nonreactive. After bisulfite treatment, the DNA region of interest is amplified by PCR and the PCR products are sequenced (either directly or after cloning into a plasmid vector). By comparison with the known DNA sequence, the cytosines that were methylated within the genomic DNA can be identified because they will be the only cytosines that were not converted into uracil.

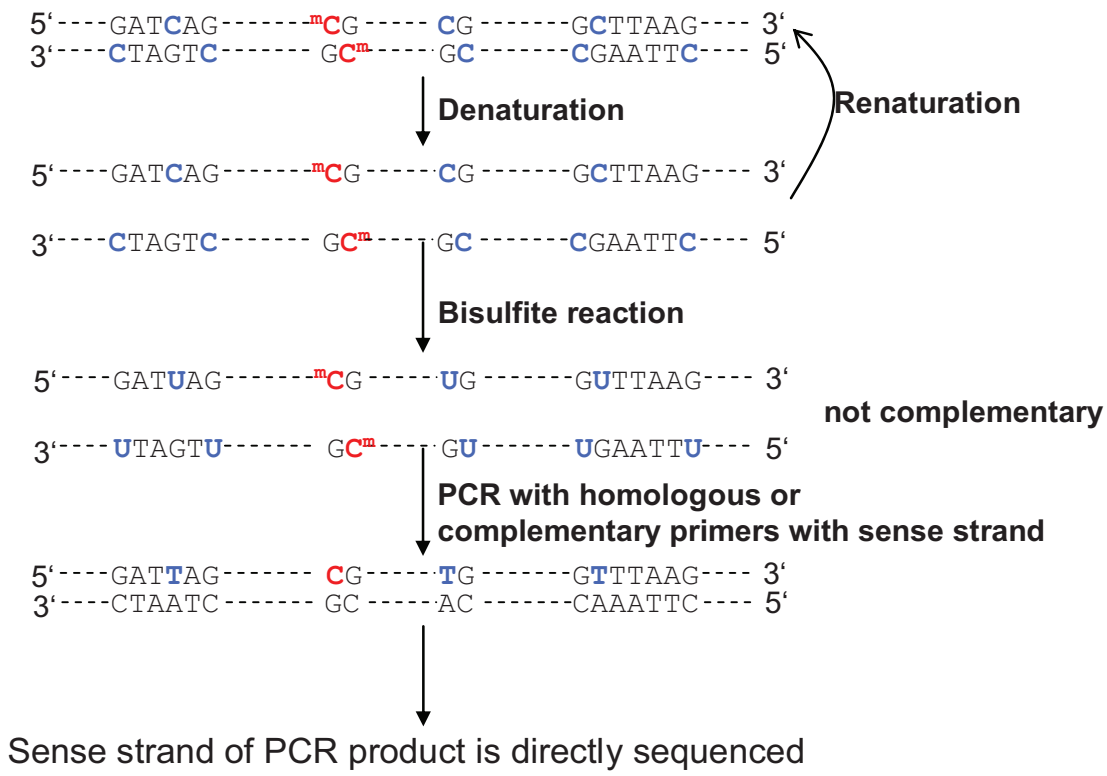


Figure 2. Bisulfite genomic sequencing procedure

The originally complementary genomic double-stranded DNA was shown firstly. The methylated cytosine residues are shown in red. The unmethylated cytosine and their corresponding uracil and thymine conversion products are shown in blue. The image is adapted with minor modification (Clark et al., 1994).

1.9.2 Roles of DNA methylation

Gene knock-out studies indicate that CpG methylation in mammals is an indispensable process. Targeted deletion of Dnmt1 results in a marked reduction in the level of DNA methylation in embryonic stem cells (ES) (25–30% of wild-type levels) and is lethal early in embryogenesis (Li et al., 1992). However, that methylation somehow orchestrates changes in chromatin structure during normal development is not well-supported by experimental evidence. Importantly, with the exception of the few imprinted genes and those genes located on the inactive X chromosome in females, the CpG islands located in the promoter regions of genes are usually unmethylated irrespective of the expression status of the gene (Bird, 1992). Hence, DNA methylation in the promoter region does not appear to be a general regulatory mechanism in the expression of most CpG island-containing genes, even when they exhibit tissue-specific expression patterns. However, some have argued that hypomethylation frequently follows transcriptional activation in the tissue-specific expression of genes. Therefore, the link between methylation and gene quiescence may not be causal (Li et al., 1992).

1.10 Goals:

In order to find an alternative strategy for controlling mastitis, we hope to investigate the earlier events associated with pathogen-specific activation of the immune defence in the udder, which will be helpful to decrease incidence of mastitis by regulating the target molecules. This requires us firstly analyzing the tissue-specific activation of model genes. I selected the LAP gene and the IL-8 gene as the paradigms for an immune relevant effector gene and a "sentinel" gene in MEC, respectively. Subsequently, we need to analyze the pathogen-specific modulation. To achieve the two long-term goals, my goals are:

- To validate LAP as a paradigm of high relevance to innate immune response;
- To analyze molecular mechanism of pathogen-mediated activation of the LAP gene both at the transcriptional level and at the chromatin level;
- To compare and contrast the regulatory mechanism between LAP and IL-8 as an alternative, even earlier regulated paradigm.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Name	Manufacturer
Amp PCR system 9600	Perkin Elmer
Bioscan QC2000	BioScan Inc
Branson Sonifier 250	Branson
Geltrockner Slab Gel Dryer SGD4050	Savant
Homogenizator Ultra-Turrax® T25 basic	IKA®-Werke
Image Eraser	Amersham Biosciences
LI-COR model 4000L infrared fluorescence DNA sequencer	MWG-Biotech
Lightcycler	Roche Applied Science
Luminometer Lumat LB9501	EG&G Berthold
Mini Trans-Blot System	Bio-Rad laboratories Inc.
Phosphor Screen	Amersham Biosciences
Spectrophotometer NanoDrop ND-1000 UV/Vis	NanoDrop Technologies
Storm 840 Phosphor Image System	Molecular Dynamics

2.1.2 Reagents

Name	Manufacturer
2 % N,N'-methylene-bis-acrylamide	Merck
5′- α- ^{32}P-dCTP (10 µCi / µl)	GE Healthcare
37% Formaldehyde	Roth
40 % Acrylamide	Merck
AHT (anhydrotetracycline)	IBA
Bio-RAD protein assay	Bio-Rad laboratories Inc.
Dimethyl sulfoxide (DMSO)	Roche
DSG (Disuccinimidyl glutarate)	Pierce
FuGENE 6 Transfection Reagent	Roche
G418 sulfate	Serva
BenchMark™ Pre-Stained Protein Ladder	Invitrogen
Lipofectamine™ 2000 Reagent	Invitrogen
Long-Ranger™ gel solution	AT Biochem, USA
Nonidet P-40	Sigma
Phenylmethylsulfonyl fluoride (PMSF)	Fluka
Poly (dIdC)	Roche
Protease Inhibitor Cocktails	Sigma
Roti®-Block	Roth
Strep-tag Protein Ladder (lyophilized)	IBA
The IRD800 labelled sequencing primers	MWG BIOTECH
TRIzol	Invitrogen
UltraPure™ Glycogen (20mg/ml)	GIBCOBRL

2.1.3 Kits

Name	Manufacturer
Chromatin Immunoprecipitation (ChIP) Assay Kit	Upstate
Dual Luciferase ®Reporter Assay System	Promega
EndoFree® Plasmid Maxi Kit (10)	Qiagen
EpiTect Bisulfite Kit (48)	Qiagen
Expand High FidelityPLUS PCR System	Roche
FastStart DNA Master^{PLUS} SYBR Green I Kit	Roche
FastStart Taq DNA Polymerase	Roche
GeneRacer™ Core Kit	Invitrogen
GeneRacer Superscript™ II RT Module	Invitrogen
High Pure PCR Product Purification Kit	Roche
HotStarTaq Plus Master Mix Kit (250)	Qiagen
mini Quick Spin Columns	Roche
pGEM®-T-Easy-Vector-System I	Promega
SuperScript™ II Reverse Transcriptase	Invitrogen
Thermo Sequenase fluorescent labelled primer cycle sequencing kit	Amersham Pharmacia

2.1.4 Strains and plasmids

Strain or plasmids	Manufacturer
E. coli 1303	As described (Petzl et al., 2008)
E. coli XL1-Blue	Stratagene
pASK-IBA3plus	IBA
pBR328	Collection of our lab
pCDNA 3.1+	Invitrogen
pGEM®-T Easy	Promega
pGL3 Basic	Promega
phRL-TK	Promega

2.1.5 Enzymes

Name	Manufacturer
DNA polymerase large (Klenow) fragment	Promega
FastStart Taq DNA Polymerase	Roche
Proteinase K	Serva
Restriction enzymes	MBI Fermentas
RNase A	Serva
RNase T1	Sigma-Aldrich
Superscript II Reverse Transcriptase	Invitrogen
T4 DNA ligase	Promega

2.1.6 Antibodies

Name	Cata. Num.		Origin	Company
anti-NF-κB p65 (H-286)	sc-7151x	Primary	Rabbit	Santa Cruz
anti-NF-κB p50 (NLS)	sc-114x	Primary	Rabbit	Santa Cruz
anti-C/EBPβ (Δ 198)	sc-746x	Primary	Rabbit	Santa Cruz
anti-C/EBPε (S-16)	sc-31931x	Primary	Goat	Santa Cruz
anti-RNA Pol II (H-224)	sc-9001x	Primary	Rabbit	Santa Cruz
anti-NF-AT C1 (k-18)	sc-1149x	Primary	Goat	Santa Cruz
anti-Rabbit IgG AP	A-3687	Secondary	Goat	Sigma
Strep-Tactin AP conjugate	2-1503-001	Secondary		IBA

2.1.7 Tissue samples

All udder samples were collected in the abattoir of the FBN, Dummerstorf. Tissues were shock frozen in liquid nitrogen and stored at -80°C for extended periods. The preparation of experimental infected udder has been described (Vanselow et al., 2006).

2.1.8 Cells and cell lines

Cells	Full name	Source
HC11	The murine mammary epithelial cell line	Donated by Dr. Wolfgang Doppler, Austria
pbMEC	Primary bovine mammary epithelial cell	Prepared by our lab
HEK 293	Human embryonic kidney cell line	Purchased from ECACC
MAC-T	bovine mammary alveolar cell line	Donated by Dr. Ynte Schukken, USA

Reagents	Manufacturer
Antibiotic antimycotic solution (100 ×)	GIBCO
Dulbecco's Modification of Eagles Medium (DMEM)	Biochrom AG
Epidermal growth factor (EGF)	Biochrom AG
Fetal calf serum (FCS)	Pan
Gentamycin	GIBCO
Insulin	Biochrom AG
Kanamycin	GIBCO
L-Glutamine	Biochrom AG
Pen-strep	GIBCO
RPMI 1640 medium	Biochrom AG
Sodium pyruvate	Biochrom AG
Trypsin/EDTA solution	Biochrom AG

2.2 Molecular cloning methods

2.2.1 Preparation of plasmid DNA

2.2.1.1 Mini-or midi-preparation of plasmid DNA

The preparation for plasmid DNA in the study is based on alkaline lysis either in minipreparation or in midipreparation.

Preparation of SI, SII and SIII as follows

Solution I	50mM Glucose, 10mM EDTA, 25mM Tris-HCl (pH8.0)
Solution II	0.2 N NaOH, 1% SDS, prepare freshly
Solution III	3M KAc (pH4.8), 11% CH3COOH

Preparation of plasmid DNA in different scales

	Minipreparation	**Midipreparation**
Culture (ml)	1.4	50
Centrifugation	14000 rpm, 1-5min	3750 rpm, 10-20min
SI (ml)	0.1	2
SII (ml)	0.2	4
SIII (ml)	0.15	3
TE (µl)	40	1400
RNase A (10mg/ml) (µl)	1	5
RNase T1 (100 U/µl) (µl)	0.1	0.3
Proteinase K (20mg/ml) (µl)	0.2	2.5
2% SDS (µl)	0.75	2.5µl of 20% SDS
TE (µl)	40	200

2.2.1.2 Preparation of endotoxin free plasmid DNA by Qiagen kit

EndoFree® Plasmid Maxi Kit from Qiagen is used to prepare endotoxin free plasmid DNA used in transfection. The patented endotoxin free plasmid procedure from Qiagen integrates endotoxin removal into the standard plasmid purification procedure. The neutralized bacterial lysate is filtered and incubated with a specific endotoxin removal buffer. The endotoxin removal buffer prevents LPS molecules from binding to the resin in the QIAGEN-tips. The resulting plasmid DNA per µg contains less than 0.1U endotoxin. The procedures were done as recommended by manufacturer.

2.2.2 RNA techniques

2.2.2.1 RNA extraction from cell culture and frozen tissue

1) Aspirate the medium and lyse cells directly by adding 1 ml TRIzol reagent to the target well, and pipet the cell lysate up and down several times and transfer into a RNase-free tube.

   If RNA is extracted from frozen tissue, add 1 ml TRIzol reagent to 100 mg tissue and homogenize for 10 s with Homogenizator Ultra-Turrax T25.
2) Incubate the homogenized samples for 5 min at room temperature (RT).
3) Add 0.2 ml of chloroform per 1 ml of TRIzol reagent. Shake tubes vigorously for 15 s and incubate for 2-3 min at RT.
4) Centrifuge at 11500 rpm (Eppendorf 5417R) for 15 min at 4 °C.
5) Transfer the aqueous phase to a fresh tube. Precipitate the RNA by mixing with 1 volume of isopropanol.
6) Incubate for 10 min at RT, and centrifuge at 11500 rpm for 10 min at 4°C.
7) Discard the supernatant and wash the RNA pellet once with 1 ml of 70% ethanol per 1 ml of TRIzol reagent used for the initial homogenization.
8) Centrifuge at 11500 rpm for 5 min at 4 °C.
9) Briefly air dry the pellet and dissolve RNA in DEPC-treated water.
10) Store at -80 °C.

2.2.2.2 RNA electrophoresis

1) In order to prepare RNA gel, melt 0.72 g of agarose in 43.27 ml of DEPC-treated water.
2) Cool to 65°C in a water bath and add 10.73 ml of 37% formaldehyde (FA) and 6 ml 10× MOPS buffer. Mix thoroughly and pour onto the gel supporter.
3) Mix 4 volumes of RNA sample (1µg) and 1 volume of 5×RNA treatment buffer. Incubate for 3-5 min at 65°C and chill on ice.
4) Prior to running the gel, equilibrate RNA gel in 1×MOPS buffer. Then load the RNA samples onto the equilibrated gel.
5) Run the gel at 5-7 v/cm in 1 × MOPS buffer.

10 × MOPS buffer	400 mM MOPS, 100 mM NaAc, 10 mM EDTA
RNA loading buffer	15% Glycerin, 0.4% Bromophenol blue, 0.4% Xylencyanol, 1mMEDTA
5×RNA treatment buffer	10 µl Formamid, 3.5 µl Formaldehyde, 1 µl 10×MOPS buffer, 2 µl RNA loading buffer, 0.1µl Ethidium bromide

2.2.3 Polymerase Chain Reaction (PCR)

2.2.3.1 Standard PCR and high fidelity PCR

In the construction of expression vectors, in order to reduce the error rate of amplification and produce amplification fragments with better fidelity, Expand high fidelity^{plus} PCR system from Roche was applied in high fidelity PCR.

Standard PCR		High fidelity PCR	
Template	10 -50 ng	Template (µl)	variable
10× buffer with Mg^{2+} (µl)	2.5	5 × buffer with Mg^{2+} (µl)	5
10 mM dNTP mix (µl)	0.5	10 mM dNTP mix (µl)	0.5
25 µM forward primer (µl)	0.25	25 µM forward primer (µl)	0.25
25 µM reverse primer (µl)	0.25	25 µM reverse primer (µl)	0.25
FastStart Taq (5U/µl) (µl)	0.1-0.2	Enzyme Blend (5 U/µl) (µl)	0.25
H_2O (µl)	variable	H_2O (µl)	variable
Total volume (µl)	25	Total volume (µl)	25

Step	PCR programs	Classic PCR program		Touch-down PCR program	
1	Denaturation	95 °C	5 min	95 °C	5 min
2	Denaturation	95 °C	30 sec	95 °C	30 sec
3	Annealing	60 °C	30 sec	65 °C	30 sec, -1 °C/Cycle
4	Extension	72 °C	1 kb/ min	72 °C	1 kb/ min
5		Go to step 2, 35-40 cycles		Go to step 2, 5 cycles	
6	Denaturation			95 °C	30 sec
7	Annealing			60 °C	30 sec
8	Extension			72 °C	1 kb/ min
9				Go to step 6, 28 cycles	
10	Final extension	72 °C	7min	72 °C	7min
11		6 °C	Pause	6 °C	Pause

2.2.3.2 Reverse transcription PCR (RT-PCR)

Reverse transcription PCR is done as the manual of SuperScript™ II Reverse Transcriptase kit recommended by the supplier (Invitrogen). A brief description of the First-Strand cDNA synthesis using SuperScriptase II for RT-PCR is given below.

Step1: add the following components to a nuclease-free microcentrifuge tube:

1-2 µg total RNA

1 µl 50 µM oligo $(dT)_{20}$, or 2 pmol of gene-specific primer

1 µl 10 mM dNTP mix

Add sterile, distilled H_2O to 12 µl

Incubate the mixture for 5 min at 65 °C, incubate for at least 1 min on ice.

Step2: collect the mixture by brief centrifugation and add

4 µl 5×First-Strand Buffer

2 µl 0.1 M DTT

1 µl H_2O

42 °C, 2 min. Add 1 µl of Superscript^{TM} II RT (200 U/ µl)

Incubate for 50 min at 42 °C, inactivate the reaction for 15 min at 70 °C.

Step3: purify the cDNA with the purification kit from Roche.

2.2.3.3 Real-time PCR

Real-time PCR is used to quantify the target template. It monitors the amount of fluorescent group incorporated during PCR amplifications as an indicator of the amount of PCR product during each PCR cycle (i.e. in real time). This signal increases in direct proportion to the amount of PCR product in a reaction. As the PCR product accumulates, fluorescence increases. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct value is inversely proportional to the amount of target nucleic acid in the sample. i.e. Ct reflects the amount of template DNA. According to the principle, a standard curve is prepared and used to quantify the copies of template DNA. In the study, template copies are measured by SYBR Green method in Lightcycler (Roche). The dye SYBR green nonspecifically binds to the minor groove of the double-stranded DNA but not to single strand DNA. For each reaction, 5 µl of a containing template solution (40-75 ng cDNA or other DNA) and 5 µl of master mixture (2.6 µl H_2O, 0.2 µl of 25 µM forward primer, 0.2 µl of 25 µM reverse primer and 2 µl master mix from kit) are combined.

2.2.3.3.1 Preparation of standard curve

The standard curve is established from serial dilutions of a plasmid DNA harboring the target DNA region. The plasmid DNA is linearized with a restriction enzyme, purified and measured the DNA concentration. According to DNA molecular weight and concentration, the copy number is calculated in a definite volume. Serial dilutions are done to contain the copy numbers of 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 respectively in 5 µl volumes. The Ct values of resulting templates are measured in real-time PCR by the same reaction condition as the unknown concentration samples. The standard curve is plotted with the Ct values and log copies of the serially diluted DNA concentrations. The copy numbers of samples are calculated according to the established standard curve.

2.2.3.3.2 Real-time PCR programs

Program	Pre-incubation (1 Cycle)		
Segment Number	Temperature Target (°C)	Hold Time (Sec)	Slope (C°/sec)
1	95	600	20
Program	Amplication (40 Cycles)		
1	95	15	20
2	60	5	20
3	72	20	20
4	Depend on melting curve	5	20
Program	Melting Curve		
1	95	0	20
2	65	15	20
3	95	0	0.1
Program	Cooling		
1	40	60	20

2.2.4 Plasmid construction techniques

2.2.4.1 DNA electrophoresis

DNA fragments are resolved in 0.8-2 % agarose gel containing 0.5 µg/ml of ethidium bromide (EB) in 1×TBE buffer (90 mM Tris-base, 90 mM Boric acid, 2 mM EDTA pH 8.0). The loaded gel with DNA samples is run at constant voltage (5V/cm) until the target band is separated from others.

2.2.4.2 Purification of DNA from agarose gel

After DNA fragment is isolated from adjacent bands by DNA electrophoresis, the target band is retrieved with razor blade without DNA contamination on the UV tray. Excised DNA slice is weighted and add 300 µl binding buffer per 100 mg of DNA slice in the same tube, the subsequent procedures are conducted as recommendation of the High Pure Product Purification Kit from Roche.

2.2.4.3 Restriction digestion of DNA fragment

In order to prepare DNA fragment with two protruding termini, the resulting DNA fragment from PCR is digested with appropriate restriction enzymes (RE) which share the same restriction buffer or sequentially digestion with interest REs in the corresponding buffer. In general, 5-10 Units of RE is applied to digest 5 µg DNA in 50 µl reaction volume. The digestion reaction is incubated at the recommended temperature overnight. Purified interest fragment is ready for ligation.

To generate one protruding end and one blunted end, the DNA fragment is blunted with Klenow firstly, followed with a RE digestion to generate a protruding terminus. It is common that the digestion reaction with Klenow is performed as follows.

DNA fragment	x µl
dNTP	1 µl
10× Klenow buffer	5 µl
Klenow (5U/µl)	0.5 µl
H_2O	y µl
Total volume	50 µl
React for 30 min at 37 °C, inactivate Klenow for 15 min at 75 °C.	

2.2.4.4 Preparation of vector for cloning

For the vector and fragment used in one ligation reaction, they are common digested with the same REs to produce the same target ends, the principle and order of digestion is similar to preparation of DNA fragment. In order to possess the same end, isocaudomer is alternately used. The digestion reaction is generally performed that 10-20 Units of RE is applied to digest 10 µg of vector DNA in 100 µl reaction volume overnight. The digestion efficiency is checked in 0.8 % agarose gel. If desirable, the target band is retrieved for ligation reaction.

2.2.4.5 Ligation

In the study, the ligation reaction is performed at 16 °C for 1-2 h or 4°C overnight. In general, the amount of DNA fragment is 3-fold molar excess of vector amount.

DNA fragment	vector	2× rapid ligation buffer	T4 ligation enzyme	H_2O	Total volume
x	y	5 µl	0.5 µl	10-x-y	10 µl

2.2.5 Transformation

2.2.5.1 Preparation of competent cells

1) Pick a single colony of *E. coli* XL1-Blue from a freshly streaked tetracycline (Tet) plate (15µg Tet /ml LB medium) into 5 ml of SOB–medium, incubate overnight at 37 °C with vigorous agitation.
2) Inoculate 5 ml culture into 50 ml PSI medium in 1 l flask and continue to incubate until OD_{600}=0.45-0.55.
3) Transfer the culture into 50 ml Falcon tube, keep it on ice for 15 min.
4) Harvest bacterial cells by centrifugation at 3000 rpm for 15 min at 4 °C, remove supernatant.
5) Resuspend carefully in 15 ml of ice cold RF1 medium, incubate on ice for 10 min.
6) Centrifuge at 3000 rpm for 15 min, remove supernatant.
7) Resuspend the bacterial pellet in 4 ml ice cold RF2 medium, incubate on ice for 15 min.
8) Snap freeze 40-80 µl aliquots in liquid nitrogen.
9) Store at -80 °C.

LB medium	10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl, adjust pH to 7.0 and autoclave to sterile
SOB-Medium	2 % trypton, 0.5 % yeast extract, 20 mM NaCl, 2.5 mM KCl, after being autoclaved and add the steriled 10 mM $MgSO_4$ and 10 mM $MgCl_2$
PSI-Medium	2 % trypton, 0.5 % yeast extract, 10 mM NaCl, 5 mM KCl, after being autoclaved and add the steriled 20 mM $MgSO_4$
RF1-Medium	100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, pH7.5, 15 % Glycerol, adjust pH to 5.8 , sterile by filtration
RF2-Medium	100 mM MOPS (pH 6.8), 10 mM RbCl, 75 mM $CaCl_2$, 15 % Glycerol, adjust pH to 6.8, sterile by filtration

2.2.5.2 Transformation procedure

1) Add 5µl of the ligated DNA constructs (ca.20 ng) into the tube containing 40-80µl competent cell, mix gently and incubate on ice for 30min.
2) Incubate the tube at 42 °C waterbath for 90 sec exactly and chill on ice for 1-2 min.
3) Add 0.4 ml LB medium without antibiotics and incubate for 45min at 37 °C with gentle agitation (<100 rpm).
4) Spread appropriate volume of transformed cells onto LB plate. If necessary, supplement 40 µl of 2 % X-gal and 7 µl of 20 % IPTG.
5) Incubate at 37 °C overnight.

2.2.6 PCR mediated mutagenesis and deletion

PCR mediated mutagenesis and deletion is used to generate mutation and deletion of *cis*-elements. The principle of PCR-mediated deletion and mutagenesis is based on regular PCR technique. However, the sites which are intended to be deleted or mutated are introduced by the specific overlapping internal primers. PCR-mediated deletion is selected as an example to be described (Fig. 3). The two primary PCRs are performed with primer set P1 (BASEND_f: 5′-GTACTAACATACGCTCTCCATC-3′) and dP3 (specific deletion primer3), dP4 (specific deletion primer4) and P2 (Luc_r: 5′-GCCTTATGCAGTTGCTCTCCA-3′), respectively. Two PCR products (product1 and product2) are achieved. Equal molar amount of product1 and product2 are mixed to run overlap extension PCR. After being denatured and annealed, the strands with complementary areas in internal primers are bound together and filled into full length PCR product3 by polymerase. After 5-10 cycles, outer primers P1 and P2 are supplemented into the PCR reaction to amplify the product3. The resulting DNA fragment has been deleted target area (Fig. 3 fragment d). The target DNA fragment and vector are digested with the appropriate RE, respectively. The subsequent procedures are similar to the classic cloning procedure.

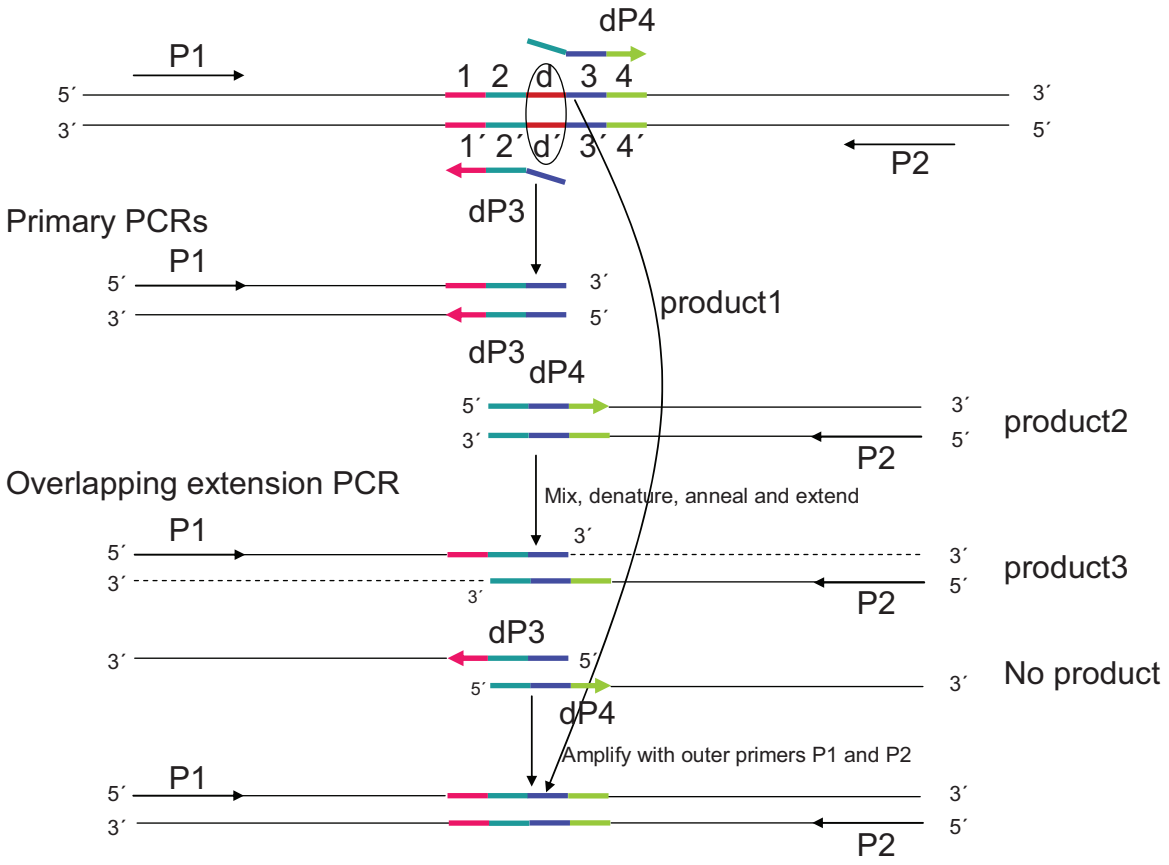


Figure 3. Schematic diagram of PCR-mediated deletion method

In order to delete the target area named “d” and “d′” in red, two primary PCRs and one overlap extension PCR are required to be done. The adjacent areas are designated as 1, 2, 3 and 4, respectively and their corresponding complementary strand as 1′, 2′, 3′ and 4′. Four primers are P1, P2, dP3 and dP4. Dashed lines are fill-in DNA sequences.

2.2.7 Rapid Amplification of 5′ cDNA Ends (5′ RACE)

5′ RACE is a method to identify 5′ ends of cDNA using known cDNA sequence. Since transcription start site (+1) locates at 5′ end of cDNA and promoter commonly resides in the vicinity of transcription start site. Thus, 5′ RACE is a valuable tool to further define promoter. 5′ RACE is conducted according to the manual of manufacturer (Invitrogen). This technique comprises the following steps. Firstly, calf intestinal phosphatase (CIP) selectively removes the 5′ phosphates of truncated mRNA and non-mRNA. Secondly, tobacco acid pyrophosphatase (TAP) removes the 5′ cap structure from intact, full-length mRNA. Thirdly, RNA Oligo from supplier is ligated to the 5′ end of the decapped mRNA using T4 RNA ligase. Fourthly, mRNA is reverse transcribed with gene specific primer or oligo(dT) primer. The resulting cDNA is amplified, cloned and sequenced. Transcription start site is determined by comparing with genomic sequence.

2.3 Protein techniques

2.3.1 Determination of total protein concentration (Bradford assay)

Bio-Rad's protein assay (from Bio-RAD laboratories inc.) is employed in the study. Bio-Rad's protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. In a reaction, 200 µl Bio-RAD protein assay is diluted with 800 µl H_2O, added the target protein solution or the standard protein, mixed well and incubated for at least 5 min at room temperature. The absorption at 595 nm (A595) is measured by spectrometer. The standard curve is plotted with A595 and the amount of standard protein bovine serum albumin (BSA). The total protein concentration of the unknown concentration sample is calculated according to the standard curve.

2.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a technique resolving proteins according to different molecular weights. It can be used to estimate relative molecular weight, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. In the present study, Laemmli method is used. 0.1 % Coomassie® Brilliant BlueR-250 acts as a staining reagent. The technique comprises a couple of steps: gel preparation, samples treatment, running gel, staining gel and destaining gel. All recipes are listed below.

Reagents	**10 % Separating gel**	**5 % Stacking gel**
H_2O	3.5 ml	2.2 ml
40 % Acrylamide	2.5 ml	0.5 ml
2 % N,N'-methylene-bis-acrylamide	1.4 ml	0.27 ml
1.5 M Tris-HCl buffer	2.5 ml (pH8.8)	1 ml (pH 6.8)
20 % SDS	50 µl	20 µl
20 % APS (ammonium persulfate)	50 µl	20 µl
TEMED (N,N,N',N'-Tetramethylethylene diamine)	8 µl	4 µl
Total volume	10 ml	4 ml

2 x Loading buffer	100 mM Tris-HCl pH6.8, 20 % glycerol, 4 % SDS, 0.2 % bromophenol blue, 5 % 2-mercaptoethanol
1x Running buffer	25 mM Tris base, 250 mM Glycine, 0.1% SDS
Staining buffer	40 % Methanol, 10 % Glacial acetic acid, 0.1 % Coomassie® Brilliant Blue R-250
Destaining buffer	10 % Methanol, 10 % Glacial acetic acid

2.3.3 Western blot

Western blot is used to detect specific proteins in a given protein sample. Protein molecules are resolved in SDS-PAGE gel according to different molecular weights and are subsequently blotted onto a membrane, such as nitrocellulose or polyvinylidene difluoride (PVDF). The protein on membrane is identified by diagnostic primary antibody. The latter is recognized by secondary antibody conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP). Colorless substrates are converted into the colored product to visualize the interest protein. In our lab, we adopt two transfer membrane devices: sandwich devices by capillary action and by eletrophoretic transfer Mini Trans-Blot System from Bio-Rad laboratories, Inc..

1) After SDS-PAGE, immerse the gel, membrane, filter papers and support pads in Transfer buffer to ensure that they are thoroughly soaked.
2) Assemble the transfer sandwich to make the membrane directly contact the gel, avoid air bubbles trapped between the gel and the membrane.
3) Put the ready sandwich device into the electrophoretic tank containing sufficient Transfer buffer and ensure the membrane closest to the anode. The electrophoretic transfer starts with an appropriate and constant current. The velocity of transfer depends on the current and molecular weight of interest protein.
4) Place the marked membrane in Blocking buffer in a sealed bag and incubate at room temperature for 2 h with agitation or leave the blot overnight at 4°C.
5) Wash the membrane three times for 5 min each in Washing buffer.
6) Immerse the membrane in the diluted primary antibody solution by Washing buffer. Incubate at room temperature for 1 h with agitation.
7) To remove unbound antibody, repeat step 5) and immerse the membrane in the diluted secondary antibody conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) and incubate 1 h at room temperature with agitation. In my study, AP is preferable.
8) To remove unbound conjugate, wash the blot as step 5).
9) Equilibrate the membrane in Detection III solution
10) Incubate the membrane in the fresh AP substrate solution contain the substrate bromo-chloro-indolyl-phosphate (BCIP) and a dye, Nitro Blue Tetrazolium (NBT). Since it is light sensitive, color development should be done in dark.
11) After the desired bands are visible, the reaction is stopped by rinsing the membrane in TE buffer.

Transfer buffer	50 mM Tris-HCl, pH 8.5, 20 % methanol, 15 g/l Glycine
10 × PBS (pH 8.2)	1.37 M NaCl, 78.10 mM Na_2PO_4, 26.80 mM KCl, 14.70 mM KH_2PO_4
Blocking buffer	1× Roti-Block and 1× PBS pH 8.2
Washing buffer	1 × Roti-Block, 0.2 % Triton X-100, 0.1 % SDS
Detection III	100 mM Tris-HCl, pH 9.5, 50mM $MgCl_2$, 100 mM NaCl
Color solution	175µg/ml BCIP, 337.5 µg/ml NBT in Detection III
TE buffer	10 mM Tris-HCl, pH8.0, 1mM EDTA

2.4 Mammalian cell culture and reporter gene assay

2.4.1 Cell types and media

Cells	Media
HC11	RPMI supplemented with 10% FCS, 2mM L-Glutamine, 100 µg/ml Gentamycin, 5 µg/ml Insulin, 10 ng/ml EGF
pbMEC	DMEM supplemented with 10% FCS, 4mM L-Glutamine, 50 µg/ml Gentamycin, 1mM Sodium pyruvate
HEK 293	DMEM supplemented with 10% FCS, 4mM L-Glutamine, 50 µg/ml Gentamycin,
MAC-T	DMEM supplemented with 10% FCS, 4mM L-Glutamine, 100 µg/ml Pen-strep

2.4.2 Cell culture and cryopreservation

2.4.2.1 Cryopreservation of cells

Continuous cell culture can lead to the accumulation of unwanted karyotype alterations and also increases the possibility of contamination by bacteria or other unwanted organisms. The only insurance against loss of the cell line is to ensure that adequate numbers of vials are cryopreserved for future use.

1) View the cultures under a phase-contrast inverted microscope to assess cell density and to confirm without contamination. Cells for cryopreservation should be in log growth phase with greater than 90% viability.
2) Centrifuge the cells at 1500 rpm for 5 min and aspirate the supernatant from the tube.
3) Suspend the cells into 1ml 100 % FCS, and add dropwise equal amount of the premixture containing 20 % DMSO and 80 % FCS.
4) Quickly aliquot 1 ml [(1–2) × 10^6 cells] into each of the prepared cryovials.
5) Place cryovials containing these cells inside a passive freezing container and stored at –80°C overnight.
6) Transfer to liquid nitrogen for the long-term storage.

2.4.2.2 Thaw of the cryopreserved cells

1) Prepare one tube containing 9 ml of growth medium prewarmed to 37°C.
2) Remove one vial of cells from liquid nitrogen.
3) Transfer the vial of cells to a 37°C water bath until the suspension is just thawed.

4) In the cell culture hood, transfer the contents of the vial slowly into the tube containing the growth medium.
5) Centrifuge the cells at 1500 rpm for 5 min to obtain a pellet.
6) Aspirate the supernatant containing DMSO and suspend the cell pellet in 10 ml complete growth medium.
7) Transfer the cells to a tissue culture dish (100 mm) and incubate at 37°C, 5% CO_2.
8) Examine cultures daily using a phase-contrast inverted microscope to ensure that the culture was not contaminated during the freeze–thaw process and that the cells are growing well.

2.4.2.3 Subculture of cells

Maintenance of healthy, viable cells requires routine medium exchanges to ensure that the nutrients in the medium do not become depleted and that pH of the medium does not become acidic (i.e., turn yellow).

In the study, cells are subcultured when they reach 70–80% confluence.

1) Aspirate the medium and wash cells three times with prewarmed PBS to remove any residual growth medium.
2) Add 1 ml of 0.25 % trypsin and incubate the dish for 3-5 min at 37 °C in a 5 % CO_2 incubator. Rock the dish, supplement adequate medium containing FCS to inhibit trypsin activity and centrifuge to collect cells.
3) Discard the medium and add fresh complete growth medium. Transfer a small aliquot of the well-mixed cell suspension into a fresh dish containing prewarmed complete growth medium, ensuring that the resulting cell density is in the optimal range.
4) Repeat this subculture step every 2–3 d to maintain cells in an exponential growth phase.

2.4.3 Transient transfection

Transfection is a process of introducing nucleic acids into eukaryotic cells by nonviral methods. General transfection reagents, like calcium phosphate, DEAE-dextran or cationic lipid-based reagents, can coat DNA, neutralize or even create an overall positive charge to the molecule to make it easier for the DNA: transfection reagent complex to cross the negatively charged membrane. In the present study, lipofectamine™ 2000 Reagent is adopted as the transfection reagent to analyze transient expression of the transfected genes. Several reporter systems have been employed, such as luciferase (firefly and renilla), β-galactosidase, alkaline phosphatase and green fluorescent protein (GFP). In the study, the target promoter sequence is inserted in the promoterless pGL3 Basic vector, which expresses firefly luciferase activity.

The luciferase activity reflects the extensity of the target promoter activity.

2.4.3.1 Transient transfection mediated by lipofectamine™ 2000 Reagent

Normally, transient transfection is done in 6-well plate containing the corresponding medium without antibiotics.

1) Plate ca. 3×10^5 cells in 6-well plate prior to one day before transfection so that the cell density will be 70 %-80 % at the time of transfection.
2) Dilute DNA (less than 4 μg) in 100 μl of medium without serum, mix gently.
3) Dilute the appropriate amount of lipofectamine™ 2000 in 100μl of serum-free medium [lipofectamine™2000(μl)/DNA(μg) =2:1]. Incubate for 5 min at room temperature.
4) Combine the diluted DNA with diluted lipofectamine™ 2000, mix gently and incubate for additional 20 min at room temperature.
5) Add the 200 μl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth. Incubate cells at 37 °C in a 5 % CO_2 incubator.
6) Twenty four hours after transfection, cells are washed, trypsinized and distributed into 6 wells of 24-well plate evenly. Three wells are kept untreated as control, the other three wells will be induced for the expected times with 1×10^7 heat-inactivated *E. coli* 1303/ml. In the study, the LAP reporter gene is induced for 24 h and the IL-8 for 6 h.
7) Forty eight hours posttransfection, cells are harvested and assayed reporter gene activity according to the following protocol.

2.4.3.2 Assay of reporter gene activity

In the assay, Dual Luciferase® Reporter Assay System kit from Promega is used. The procedures are done according to the instruction of supplier. The activity measurement is conducted manually in Luminometer Lumat LB9501 of EG&G Berthold company.

2.5 Electrophoretic Mobility Shift Assay (EMSA)

EMSA provides a simple and rapid method for identifying DNA-binding proteins. The assay is based on the observation that DNA-protein complexes migrate more slowly than free DNA fragments or double-stranded oligonucleotides in a nondenaturing polyacrylamide gel. The assay is composed of 3 main steps: preparation of nuclear extract (NE); labeling of probe; preparation of EMSA reaction, electrophoresis and radioautography.

2.5.1 Preparation of nuclear extract from cultured cells

1) Wash cells 3 times with ice-cold PBS.
2) Trypsinize the cells and transfer cell suspension into a 1.5ml tube.
3) Spin down for 5 min at 2400 rpm (Eppendorf 5417R) at 4ºC. Wash the pellet with ice cold PBS.
4) Remove the supernatant, and resuspend cell pellet in 400 µl of buffer A. Incubate the tube for 15 min on ice.
5) Add 25 µl of 10% Nonidet P-40 (NP-40), and shake for 1 min at 4ºC by hand.
6) Pellet nuclei at 10000 rpm for 1 min at 4ºC.
7) Remove the supernatant carefully and completely.
8) Add 50 µl of buffer B to the tube, and shake vigorously for 20 min at 4ºC to lyse nuclei and release chromatin-associated proteins.
9) Centrifuge at 14000 rpm for 5 min at 4ºC.
10) Transfer the supernatant containing nuclear extract to a new tube.
11) Determine protein content of the extract by Brandford method and store nuclear extracts at -80ºC. The extracts are ready for EMSA.

Buffer A	Buffer B
10 mM HEPES (pH 7.9, adjusted with KOH)	20 mM HEPES (as left)
10 mM KCl	0.4 M NaCl
0.1 mM EDTA	1 mM EDTA
	1 mM EGTA
0.1 mM EGTA	1 mM DTT (add freshly)
1 mM DTT (add freshly)	1 mM PMSF
1 mM PMSF	20 mM NaF
20 mM NaF	1 mM Sodium Orthovanadate (Na_3VO_4)
1 mM Sodium Orthovanadate (Na_3VO_4)	1/200 proteinase inhibitor cocktail (Sigma)
1/10 Proteinase inhibitor cocktail (Sigma)	

2.5.2 Labeling and purification of probe

1) Mix 5 µl of the longer primer (50µM) and 10 µl of the shorter primer (50µM), supplement with 30µl of H_2O, boil for 5 min, chill on ice immediately.
2) Prepare cold and hot probe reaction on ice according to the following table.

Cold probe reaction	Volume (µl)	Hot probe reaction	Volume (µl)
Original primer mix	36	Original primer mix	0.72
10× Klenow buffer	5	10× Klenow buffer	2
dNTPs (10 mM each)	1	dNTPs (dATP,dGTP,dTTP,10mM each)	0.5
		5′- α-^{32}P dCTP (10 µCi/µl)	2
Klenow	1	Klenow	1
H_2O	7	H_2O	13.78
Total volume	50	Total volume	20

The prepared cold probe reaction and hot probe reaction are incubated for 1h at 25°C, thermal inactivate Klenow for 10min at 75°C.

3) Purify hot probe by mini quick spin column (Roche), dilute 5 times and measure its activity (cpm/µl) by Bioscan QC2000 (BioScan Inc. Washington DC, USA).

2.5.3 Preparation of EMSA reaction, electrophoresis and radioautography

EMSA reaction should be prepared on ice.

1) Prepare 2 × binding buffer for NF-κB and C/EBP

2 × C/EBP binding buffer	2 × NF-κB binding buffer
10 mM $MgCl_2$	20mM Tris-HCl (pH 8.0)
0.2 mM EDTA	20% Glycerol
2 mM DTT *	2 mM EDTA
100 mM NaCl	100 mM NaCl
20% Glycerol	0.4 mM PMSF
20 mM HEPES (pH7.9)	2 mM DTT *
0.4 mM PMSF *	

*, PMSF and DTT should be added freshly.

2) Prepare EMSA reactions according to the below table

Standard binding reaction		Competition binding reaction		Supershift binding reaction	
X µl	dd H_2O	X µl	dd H_2O	X µl	dd H_2O
1 µl	PolydI/dC (1 µg/µl)	1 µl	PolydI/dC (1 µg/µl)	1 µl	PolydI/dC (1 µg/µl)
10µl	2 × binding buffer	10µl	2 × binding buffer	10µl	2 × binding buffer
Y µl	Nuclear extract (2-20µg)	Y µl	Nuclear extract (2-20 µg)	Y µl	Nuclear extract (2-20µg)
1 µl	Hot probe(40fmol/µl)	1 µl	Hot probe (40 fmol/µl)	1 µl	Hot probe (40 fmol/µl)
		1 µl	Cold or mutant probe (4 pmol/µl)	0.5-1µl	Antibody (2 µg/µl)
20µl	Total volume	20µl	Total volume	20µl	Total volume

10 min prior to the addition of hot probe, antibody is added into the supershift reaction and is incubated for 10 min on ice. Afterward, the reaction continues to be incubated for additional 20 min at room temperature. After 6% non-denaturing PAGE gel is run for 1h in advance, load the samples onto it.

3) Run the big gel at 260 V for about 1.45 h in 0.5 x TBE running buffer.
4) Dry the gel for 30 min-1 h at 75°C and expose it overnight.
5) Scan the dried gel with Storm 840 Phosphor Image System from Molecular Dynamics. If necessary, quantify the target bands by Image Quant 5.2 software from the supplier.

2.6 Chromatin Immuno Precipitation (ChIP)

The chromatin structure plays important role in gene regulation by controlling promoter access for inducible transcription factors. DNase I footprinting assay is a common technique to identify the DNA region bound by a transcription factor by assessing nucleotides resistance to nuclease attack. Although it is able to provide the protein binding pattern to its natural chromatin, the technique can not efficiently identify the weak or partial DNA binding and is also powerless to precisely identify the prevented complex. In the study, in order to avoid

some drawbacks of DNase I footprinting assay, ChIP assay is used to investigate binding of transcription factors within natural chromatin context, and temporally change of the DNA-protein complex at various conditions. In this assay, intact cells are fixed with the crosslinker formaldehyde to link protein to DNA. The cells are then sonicated to fragment chromatin. The crosslinked DNA-protein complex is enriched by immunoprecipitation. The retrieved complex is analyzed by real-time PCR to quantify the specific DNA targets. Chromatin Immunoprecipitation (ChIP) Assay Kit from Upstate is used and the protocol recommended by Upstate is modified and provided as follows.

2.6.1 Preparation of cell lysate

1) All cell dishes (3 ×100mm dishes) should be treated with 1×10^7 heat killed *E. coli* 1303/ml medium for the expected times. Wash cells three times with room temperature PBS and add 10ml of PBS containing 1 mM $MgCl_2$, pH8.0 into each dish exactly.
2) Add 2 mM Disuccinimidyl glutarate (DSG) into cell dish directly while agitation. Cells are fixed with DSG for 45min at room temperature with gentle agitation.
3) Cells are washed with room temperature PBS twice.
4) Add 10 ml PBS/Mg^{2+}, pH 8.0, containing 1 % formaldehyde and incubate for 10 min at room temperature with agitation.
5) Quench formaldehyde by adding 125 mM glycine into the above dishes, whirl well and keep them at room temperature for 5 min, then transfer the dishes on the ice.
6) Wash cells for three times with ice cold PBS.
7) Add 1 ml ice cold PBS containing 0.5 % Protease Inhibitor Cocktail II and 1mM PMSF into dishes.
8) Scrape cells into conical tubes.
9) Spin at 1000 rpm for 5 min at 4°C to pellet cells.
10) Remove supernatant. Resuspend cell pellet in 3×900 µl of L1 buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% NP-40, 10 % glycerol) supplemented with proteases inhibitors and incubate for 15 min on ice to remove free cytoplasmic proteins. Precipitate nuclei by centrifugation at 1000 rpm for 5 min.
11) Resuspend nuclei in 0.9 ml of SDS lysis buffer (Upstate) supplemented with 0.5 % Protease Inhibitor Cocktail II and 1mM PMSF, keep them on ice for 10 min.

2.6.2 Sonication to shear chromatin

12) Shear chromatin with Branson sonifier (setting 2, duty 40 %) to 200-1000 bp.
13) In order to analyze the size of the sheared DNA, 40 µl aliquot is transferred into new tubes and supplemented with 1.6 µl of 5M NaCl, 1 µl of 20% SDS and 0.4 µl of 20mg/ml proteinase K. Reverse the crosslink reaction for 1h at 65°C. Analyze the purified DNA in 2% agarose gel.
14) If desired, spin the original sheared chromatin at 14000 rpm for 10 min at 4°C to remove insoluble materials and store the sheared crosslinked chromatin at -80°C.

2.6.3 Immunoprecipitation (IP) of crosslinked protein/DNA

15) Aliquot the sheared crosslinked chromatin for the number of desired immunoprecipitations and put on ice.
16) Per 100 µl of the above chromatin is added with 900 µl of Dilution Buffer with 0.5 % Protease Inhibitor Cocktail II and 1mM PMSF.
17) Preclear the diluted chromatin with Protein A agarose. In the study, add 80 µl of Protein A agarose for 320 µl soluble chromatin, incubate for 2 h at 4°C with agitation.
18) Supplement 10 µg normal rabbit IgG and continue to incubate for additional 1 h.
19) Pellet agarose by brief centrifugation at 3000-5000 g for 1 min at 4°C.
20) Collect the supernatant and transfer 10 % (100µl) of the supernatant as Input and save at 4°C until step 26.
21) Aliquot 1 ml of supernatant fraction into fresh microfuge tubes and add 4 µg of the interest antibody or normal rabbit IgG, incubate overnight at 4°C with agitation.
22) Add 80 µl of Protein A agarose for 3 h at 4°C with agitation.
23) Pellet Protein A agarose by brief centrifugation at 3000-5000 g for 1 min and remove the supernatant fraction.
24) Wash the Protein A agarose-antibody/chromatin complex by resuspending the beads in 1 ml of the cold buffers in the order listed below and incubate for 3-5 min on a rotating platform followed by brief centrifugation at 3000-5000 g for 1 min and careful removal of the supernatant fraction:
    a. Low Salt Immune Complex Wash Buffer, one wash
    b. High Salt Immune Complex Wash Buffer, one wash
    c. LiCl Immune Complex Wash Buffer, one wash
    d. TE Buffer , two washes

2.6.4 Elution of protein/DNA complexes

25) Prepare Elution Buffer (1% SDS and 0.1 M $NaHCO_3$) for all IP tubes as well as all Input tubes (see step 20).
26) For Input tubes (see step 20), add 200 µl of Elution Buffer and set aside at room temperature until reverse crosslink.
27) Add 200 µl of Elution Buffer to each tube containing the antibody/agarose complex. Mix by flicking tube gently.
28) Incubate for 15 min at room temperature.
29) Pellet agarose by brief centrifugation at 3000-5000 g for 1 min and collect supernatant into new microfuge tubes.
30) Add additional 100 µl of Elution Buffer to each tube, repeat steps 28-29 and combine eluates (total volume = 300 µl).

2.6.5 Reverse crosslink of protein/DNA complexes to free DNA

To all tubes (IPs and Inputs) with 300 µl eluates, add 12 µl of 5 M NaCl (0.2 M fc), 15 µl of 1 M Tris-HCl, pH 6.5, 6 µl of 0.5 M EDTA, and 3 µl of 20 mg/ml Proteinase K, incubate for 4h at 65°C to reverse crosslink the DNA/protein complex.

2.6.6 Purification of DNA

DNA is purified with phenol/chloroform extraction and dissolved into 36 µl H_2O. During the step, 0.2µg/µl glycogen is used as a carrier to make DNA pellet visible. Purification kit is not recommended until test.

2.6.7 Quantification of the template by real-time PCR

1-5 µl of template DNA is used in PCR reaction. PCR is run with gene-specific primers for 40 cycles at 60 °C annealing temperature. The relative copies are calculated according to the formula (% input = 100 × copies of IPed DNA/ copies of Inputed DNA).

2.7 Chromatin Accessibility by Real-Time PCR (CHART-PCR)

It has been well documents that chromatin remodeling plays a role in the regulation of the inducible transcription in eukaryotes (Almer et al., 1986). CHART-PCR provides a sensitive, quantitative method to evaluate the chromatin remodeling events *in vivo* (Rao et al., 2001). Its basis is that the condensed chromatin restricts the accessibility of restriction enzyme whereas the loosed chromatin DNA is digested with restriction enzyme. Real-time PCR was used to measure the digestion extent of the target chromatin DNA, which reflects the alteration of

chromatin structure indirectly. The method comprises 4 steps: Nuclei preparation; Digestion of nuclei with restriction enzyme; DNA purification; Quantification of undigested DNA and analysis of data. In the present study, I originally applied two-enzyme digestion strategy to decrease the viscosity of genomic DNA and to increase the reproducibility of the method. The selection criterion of the second restriction enzyme is that no recognition site of the enzyme lies within the intended amplification region.

1) 50-100 mg tissue is ground into powder under liquid nitrogen and suspended in 5 ml of ice-cold LS buffer with NP-40.
2) Homogenize with Ultra-Turrax T25 for the optimal time by checking the nuclei under microscope. Add 4 ml of ice-cold LS buffer with NP-40 and mix.
3) Filter the homogenate through siliconed glass wool.
4) Spin down the nuclei at 1500 rpm for 2 min at 4°C.
5) Wash the nuclear pellet with 2 ml of ice-cold 1× restriction buffer.
6) Spin down at 2000 rpm for 2 min at 4°C.
7) Resuspend the nuclei in 100 µl of ice cold 1× restriction buffer.
8) Estimate DNA concentration by diluting 1 µl of aliquot with 9 µl of 5% SDS solution and vortex.
9) Restriction digestion is performed with ca. 2 µg chromatin and 20 Units restriction enzyme (*Hind*III for LAP and BNBD5) for 60 min at 37°C in 200 µl reaction volume.
10) Thermal inactivate the first restriction enzyme (*Hind*III) at 65 °C for 20 min, followed by adding 5 Units of the second RE (*Eco*RV) and incubate at 37 °C overnight.
11) Add 3 µl of RNase A, 0.3 µl of RNase T1 and 6.7 µl of TE buffer per tube and incubate for 30min at 37°C. Add equal volume of proteinase K buffer with 3 µl proteinase K (20 mg/ml), and incubate for 4 h at 56°C.
12) Purify DNA with kit and dissolve into 50 µl water.
13) Measure DNA concentration exactly by Nanodrop.
14) Run real-time PCR with 25-50 ng DNA in 10 µl reaction volume.

LS buffer	10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 % NP-40, 0.5% Protease Inhibitor Cocktail
Proteinase K buffer+PK	50 mM Tris-HCl (pH 8.0), 150 mM EDTA, 100 mM NaCl, supplemented freshly and orderly with 6 mM DTT, 0.27 µg/µl Proteinase K and 1 % SDS

2.8 DNA methylation analysis

The methylation state of CpG dinucleotides site is thought to be linked to the structure of chromatin and to the regulation of promoter function by modulating DNA-protein interactions. The bisulfite sequencing PCR is applied to analyze changes of methylation status. In the bisulfite reaction, non-methylated cytosine residues in DNA are converted into uracil, while 5-methylcytosines remain unaltered. The converted DNA is amplified with gene-specific primers and sequenced. All cytosine residues remaining in the sequence represent previously methylated cytosines in the genome. The procedures consisted of genomic DNA preparation, bisulfite conversion, amplification and sequencing.

2.8.1 Preparation of genomic DNA from tissue and pbMEC cells

1) 100 mg-1g tissue is ground into powder under liquid nitrogen.
2) Add 250 mg tissue powder into 3 ml proteinase K buffer with 0.5 mg/ml proteinase K. (Harvest cells from 6-well plate and suspend cell pellet into proteinase K buffer.)
3) Mix gently and incubate at 56 °C overnight.
4) Cool down to room temperature. Add an equal volume of phenol/chloroform. Mix gently by inverting the tube for 10 min.
5) Remove the upper aqueous phase to a new tube. Add 1/10 volume of 3 M NaAc (pH 5.2), mix. Add 0.6-1 volume of isopropanol and mix gently but thoroughly.
6) Pellet the stringy DNA by centrifugation.
7) Wash the DNA pellet with 70 % ethanol.
8) Air-dry the pellet for 15-20 min at RT and dissolve in 200 µl of TE (pH 8.0).

Proteinase K buffer	50 mM Tris-HCl (pH 8.0), 150 mM EDTA, 100 mM NaCl, supplemented freshly and orderly with 6 mM DTT, 0.5 mg/ml Proteinase K and 1 % SDS
TE (pH 8.0)	10 mM Tris-HCl, 1 mM EDTA (pH 8.0)

2.8.2 Bisulfite conversion reaction

Sodium bisulfite conversion reaction is done according to the manual of manufacturer (Qiagen). Briefly,

1) Prepare bisulfite reactions according to the following table

Component	Volume (µl)/reaction
DNA solution (1 ng – 2 µg)	< 20
RNase-free H_2O	Variable
Bisulfite Mix (dissolved)	85
DNA Protect Buffer	35
Total volume	140

2) Run the following bisulfite conversion program in thermal cycler.

Steps	Temperature (°C)	Time (min)
Denaturation	99	5
Incubation	60	25
Denaturation	99	5
Incubation	60	85
Denaturation	99	5
Incubation	60	175
Hold	20	Indefinite

3) Cleanup of bisulfite converted DNA according to the manual of manufacturer.

2.8.3 Bisulfite Sequencing PCR (BSP)

2.8.3.1 Designation of methylation specific primers

The primer design is critical and different from regular PCR. Generally, the double DNA strands are analyzed separately since they are no longer complementary. The PCR is initiated with the forward primer with G-rich and the reverse primer that should be C-rich. (The forward primer is homologous, but the reverse is complementary to the sequenced strand).

2.8.3.2 Primary PCR , nested PCR and PCR program

Primary PCR		Nested PCR	
Component	Volume (µl) /reaction	Component	Volume (µl) /reaction
Modified DNA	0.5	Purified primary PCR product	1.25
10× buffer	2.5	10× buffer	2.5
dNTP (10mM each)	0.5	dNTP (10mM each)	0.5
Forward primer	0.25	Forward nested primer	0.25
Reverse primer	0.25	Reverse nested primer	0.25
Taq from Roche	0.1	Taq from Roche	0.1
H_2O	20.9	H_2O	20.9
Total volume	25	Total volume	25

Touchdown PCR Steps		Temperature	Time
1	Denaturation	94°C	5 min
2	Denaturation	94°C	30 sec
3	Annealing	62°C -1°C/Cycle	30 sec
4	Extension	72°C	2 min
Go to step 2, 10 cycles			
5	Denaturation	94°C	30 sec
6	Annealing	52°C	30 sec
7	Extension	72°C	2 min
Go to step 5, 28 cycles			
8	Final extension	72°C	7 min

2.8.4 Direct sequencing

2.8.4.1 Direct sequencing by ABI

The sequencing by ABI was done by Mrs. Fuchs who directs sequencing in our unit.

2.8.4.2 Direct sequencing by Li-COR

1) Preparation of 8 % polyacrylamide sequencing gel according to the following table

Component	66cm sequencing gel
Urea	21.0 g
Long Ranger™ 50% solution	4.3 ml
10 × TBE	5.0 ml
H_2O	32 ml
DMSO	500 µl
10 % APS	350 µl
Degas for 15min and store at least for 15min at 4°C, , add TEMED immediately prior to pour gel	
TEMED	50 µl

2) PCR reaction

H_2O	7.6 µl
DMSO	0.4 µl
The IRD800 labelled Sequencing primer(2 pmol/µl)	1.0 µl
PCR product (300ng/1000bp)	X µl
Mix 2 µl of the above mixture and 2 µl from the Thermo Sequenase fluorescent labeled primer cycle sequencing kit, add 5 µl liquid wax separately. Run PCR in Amp PCR system 9600 (Perkin Elmer)	

3) PCR program

Steps		Time (sec)	Temperature (°C)
1	Denaturation	300	95
2	Denaturation	30	95
3	Annealing	30	59
4	Extension	60	72
Go to step 2, 30 cycles			

After PCR, mix 4µl loading buffer (formamide dye), store the mixture in –20 °C.

4) Electrophoresis

Run the sequencing gel without any samples on Li-COR for 30 min to 1 h in 0.8×TBE buffer. Thaw the sequencing reaction samples for 2 min at 70 °C and immediately load 1µl to corresponding lane. Each sequencing reaction is loaded into four lanes A, C, G and T in adjacent orders. Run gel at a constant power of 50 watts, 2200 V, 37 mA, 45°C overnight.

2.8.5 Data analysis

In order to quantify the C/T polymorphic sites, the sequence gel files are further processed and evaluated with the PHRED software, version 0.020425 as described (Vanselow et al., 2005). The percentage of methylation is calculated as the portion of the C peak area [% methylation= peak area of 100 ×C / (peak area of C + peak area of T). In the study, to decrease the error of sequencing method, the percentage of methylation from different animals is regressed according to a regression equation. The similar equation was designed as described (Vanselow et al., 2005). The regression equation is established as follows. The clone with 100% C in target CpG site is combined with the one with 100% T by various proportions (two clones are donated by Dr. Jens Vanselow). The mixtures are used as templates to run PCR and PCR products are purified, sequenced and calculated by the similar method to the target samples. A plot is done by the theoretical percentage of methylation versus the actual one from the sequenced C/T peaks.

3. RESULTS

3.1 Concepts and tools of the study

To dissect the kinetics of NF-κB-dependent gene expression and to determine which distinct mechanisms regulate the expression of different immune genes during mastitis, I selected the LAP and the IL-8 genes as the paradigms for an immune relevant "effector" gene and a "sentinel" gene in MEC, respectively. Heat-inactivated *E. coli* 1303, which causes a stronger effect than *S. aureus*, served as an inducer. The detailed study for regulatory mechanism of the LAP and the IL-8 genes will provide basic knowledge which in later steps may be scrutinized to eventually unravel pathogen-specific mechanisms of immune activation in the udder.

For most genes, important regulatory events generally occur at the promoter regions. Therefore, I focused the regulatory events of the two target genes on the promoter regions, which mainly comprise the regulatory analyses at the transcriptional level and at the chromatin level.

3.1.1 Construction of reporter genes with serial deletions and mutations of the LAP promoter

Construction of reporter genes is the first step to determine the relevant *cis*-elements to gene expression and induction. To generate the LAP truncated promoter fragments with diverse 5′ ends and the same 3′ end, PCR amplifications were run using the wild-type LAP promoter reporter construct as a template with the different forward primers and the same reverse primer (the different forward primers: GCAGATTCAAAGCCCTAAGCGA, TTTTCTGGGGATTTCCACAGCCTCATTAGCATA or GTGACTGACCCTGCTTTGTGCT. The same reverse primer: GCCTTATGCAGTTGCTCTCCA). PCR products were firstly blunted with DNA polymerase large (Klenow) fragment (Promega Corp.), afterward, digested with *Xho*I and inserted into the *Kpn*I/Klenow/*Xho*I sites of the wild-type LAP promoter reporter. The validated clones were applied into reporter gene assay (Fig. 9).

Serial mutation constructions (Fig. 10) were generated by PCR-mediated mutagenesis. PCRs were run similar to the PCR-mediated deletion technique as described (Fig. 3). Primer1 and primer2 (P1 and P2) were BASEND_f: 5′-GTACTAACATACGCTCTCCATC-3′ and Luc_r: 5′-GCCTTATGCAGTTGCTCTCCA-3′. The mutated primers (mP3 and mP4) in PCR were listed in Table 1. All fragments obtained by overlapping extension PCR were digested with

restriction enzymes *Kpn*I and *Xho*I, and inserted into the *Kpn*I/*Xho*I sites of promoterless pGL3 Basic vector. All constructs were verified by restriction enzyme analysis and sequencing.

Table 1. Primers for serial mutations of C/EBP and NF-κB binding sites in the LAP promoter

Clones	mP3* (reverse)	mP4* (forward)
CE1m	5′-AGACTT**TCCAT**TG**TT**CCTTCGCTTAGGGCTTTGAA-3′	5′-CGAAGG**AA**CA**ATGGA**AAGTCTGTGCCCCTGCCAG-3′
CE2m	5′-GGCAGGGG**GACTAGTC**GGGGCACAGACTTGCCTG-3′	5′-GCCCC**GACTAGTC**CCCCTGCCCATCAGCTGA-3′
CE3m	5′-ATGAGGC**GACTT**AAATCCCCAGAAAAAGCTTTC-3′	5′-GGGGATTT**AAGTC**GCCTCATTAGCATACGAGTG-3′
κBmG	5′-CTGTGGAAATCC**G**CAGAAAAAGCTTTCCTGCTG-3′	5′-TTTTCTG**C**GGATTTCCACAGCCTCATTAGCATA-3′
κBmTTCC	5′-GAGGCTGT**ACTG**ATCCCCAGAAAAAGCTTTCCT-3′	5′-TGGGGAT**CAGT**ACAGCCTCATTAGCATACGAG-3′

*: The mutated sequences are shown as the underlined bold letters.

3.1.2 Construction of reporter genes with wild-type or serial deletions of the IL-8 promoter

The wild-type IL-8 promoter construct (-1294) was retrieved from bovine genomic DNA by primary PCR (forward primer: GCAAGGAGGTCCAACCAGTC; reverse primer: TTGGAAGTCATATTTGAACAAGAG) and nested PCR (forward primer: CCTGAGGCTGGGAGGGATTG; reverse primer: TTGGAAGTCATATTTGAACAAGAG). PCR product was blunted with DNA polymerase large (Klenow) fragment and inserted into the *Sma*I site of promoterless pGL3 Basic vector. The right-orientated clone was screened (Fig. 29 clone -1294).

Clone -755 (Fig. 29) was from the wild-type construct -1294, which was digested with *Kpn*I and followed by self ligation. The wild-type construct -1294 was digested with *Kpn*I and *Pvu*II, respectively, blunted with DNA polymerase large (Klenow) fragment and followed by self ligation. The resulting clone is the shorter promoter construct -345 (Fig. 29). The two deletion constructs -121 and -56 (Fig. 29) were generated by PCRs using the wild-type IL-8 construct -1294 as a template. Forward primers in PCR are respective ATGCGGTACCGTGCTCTCAAAGGGCGGATG or ATGCGGTACCTGCACTTGTTCCCTCTTCTTAC. The same reverse primer (GCCTTATGCAGTTGCTCTCCA) is used in the two PCR reactions. Both resulting PCR products were digested with *Kpn*I and *Xho*I and inserted into the *Kpn*I/*Xho*I sites of pGL3 Basic vector. To construct ΔC/κB (Fig. 29), PCR-mediated deletion technique was applied as described (Fig. 3). The PCR fragments from two primer sets

(CCTGAGGCTGGGAGGGATTG and GCACCATCATGTATGCAACCATCCGCCCTTTG; ATGGTTGCATACATGATGGTGCACTTGTTCCC and GCCTTATGCAGTTGCTCTCCA) were amplified by primary PCRs. The target fragment was obtained by overlapping extension PCR with the two primary PCR fragments. The resulting interest fragment was blunted with Klenow, digested with *Bgl*II and inserted into the *Sma*I/*Bgl*II sites of pGL3 Basic vector. Restriction enzyme mapping and sequencing confirmed all constructs.

3.1.3 The cloning of NF-κB p65 and p50 eukaryotic expression vectors

In order to analyze the function of different NF-κB factors for LAP and IL-8 expression, I cloned NF-κB p65 and p50 as eukaryotic expression vectors. Total RNA from the infected udder for 24h was reversely transcribed into cDNA by the specific primers of NF-κB p65 and p50. To retrieve the p65 fragment, the resulting cDNA was used as a template to run primary PCR with primers GGCGAATGGCTCGACAGTAG and TGGAGCCGAACATCAGGATA. The following nested PCR was performed with forward primer CAGTGGTACCATGGACGACCTCTTCCCCC and reverse primer AGTCCTCGAGGGAGCTGATCTGACTCAGAA containing *Kpn*I and *Xho*I recognition sites, respectively. The nested PCR product was digested with *Kpn*I and *Xho*I and cloned into the *Kpn*I/*Xho*I sites of pASK-IBA43plus. Similarly, to retrieve the p50 fragment, primary PCR was carried out with primary primers TCAAACTCCAGAATGGCAGA and CACTGTCCCCGTTCTCATC, and nested PCR was performed using primer set CAGTGAATTCATGGCAGAAGACGACCCGT and AGTCGTCGACAGGGTCATTTTTAGATGGGGT. *Eco*RI and *Sal*I recognition sites were introduced into the nested forward and reverse primers respectively. The nested PCR product was digested with *Eco*RI and *Sal*I, and was cloned into the *Eco*RI/*Sal*I sites of pASK-IBA43plus. cDNA segments featuring with sequences of a 5′ his-tag and a 3′ strep-tag were subsequently subcloned into pcDNA3.1+ (Invitrogen) respectively. A schematic diagram of recombinant NF-κB p65 and p50 proteins is shown (Fig. 4). The clones were verified by sequencing. Their expressions were validated by western blot (Fig. 5) with specific antibodies, α-p65 and α-p50 (Santa cruz). As shown, HEK293 cells express multiple endogenous p50 molecules (Fig. 5 right lower). The recombinant p50 has greater molecular mass than the endogenous p50 due to those dual tags.

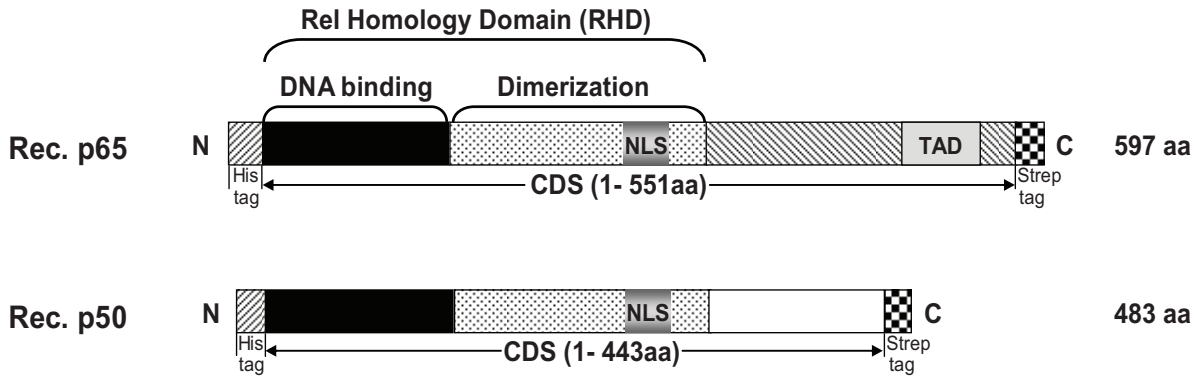


Figure 4. Schematic diagram of recombinant NF-κB p65 and p50 proteins

NF-κB p65 and p50 recombinant proteins are constructed as dual-tagged proteins in the eukaryotes systems. Both proteins contain polyhistidine-linked amino acids (25 aa for p65, 21 aa for p50) at their N-termini and a Strep-tag (21 aa for p65 and 19 aa for p50) at C-termini. They are illustrated as left hatched boxes and right checkered boxes, respectively. The other boxes represent distinct protein domains. Black boxes, DNA binding domains; Stippled boxes, Dimerization domains; NLS, the nuclear localization signal; TAD, transactivation domain, only in p65. Shown are amounts of amino acids in bovine recombinant p65 and p50 proteins on the right margin.

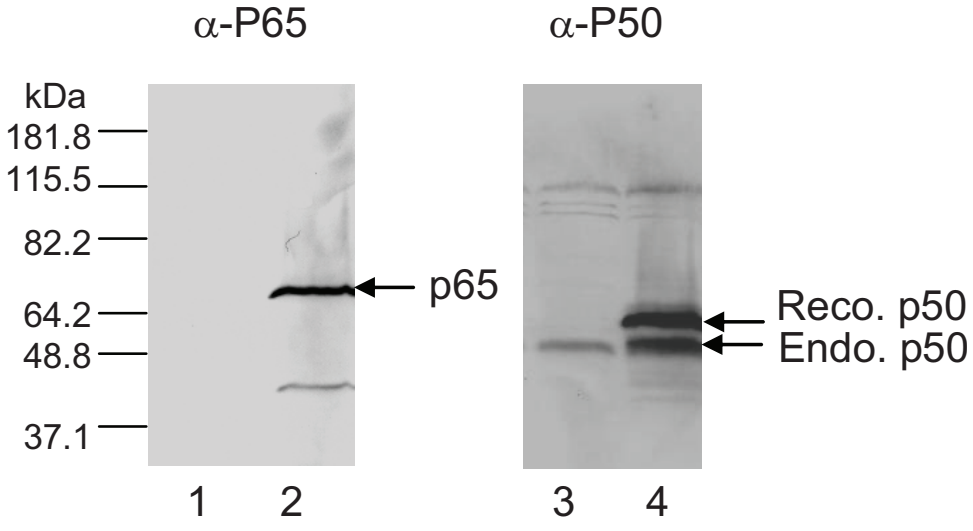


Figure 5. The western blot of NF-κB p50 and p65 in HEK293 cells

HEK293 cells were transfected with the expression constructs of NF-κB p50 and p65 and harvested to prepare the whole cell extract. Lane 1 and lane 3 are from the transfected cells with empty vectors, lane 2 and lane 4 are extracts from whole cells transfected with expression vectors of p65 or p50. Equal amounts of total protein were loaded into SDS-PAGE, specific rabbit antibody against p65 or p50 was used to detect the expression of p65 or p50. Goat anti-rabbit IgG conjugated with AP was used as the secondary antibody. The molecular mass standard is shown on the left lane. The desired bands are shown by arrows. Reco. p50 stands for recombinant p50 and Endo. p50 for endogenous p50.

3.2 Time course of pathogen-induced expression of LAP and IL-8 differs

To understand the time course of LAP and IL-8 in response to pathogen during mastitis, mRNA copy numbers of LAP and IL-8 were measured by real-time PCR at the different induction times with pathogen *E. coli* strain 1303. Maximal mRNA concentration of LAP was reached at 24h after stimulation within the tested induction times (Fig. 6). 105-fold induction over the untreated level was obtained in pbMEC. IL-8 mRNA concentration, in contrast, peaked (190-fold induction) already at 1 h after challenge with the same pathogen in pbMEC cells and was reduced thereafter to stable levels, but at much higher levels than found in the unstimulated control (Fig. 6). Obviously, the target genes LAP and IL-8 exhibit a markedly different temporal pattern of gene expression during the response of the cells to the extracellular pathogen. Hence, I classify LAP as a late-immune-gene and IL-8 as an early-immune-gene according to their response to the pathogen *E. coli*. What is the difference in regulation between the late-immune-gene LAP and the early-immune-gene IL-8? In the present study, I have probed the regulatory mechanisms of the LAP and the IL-8 genes.

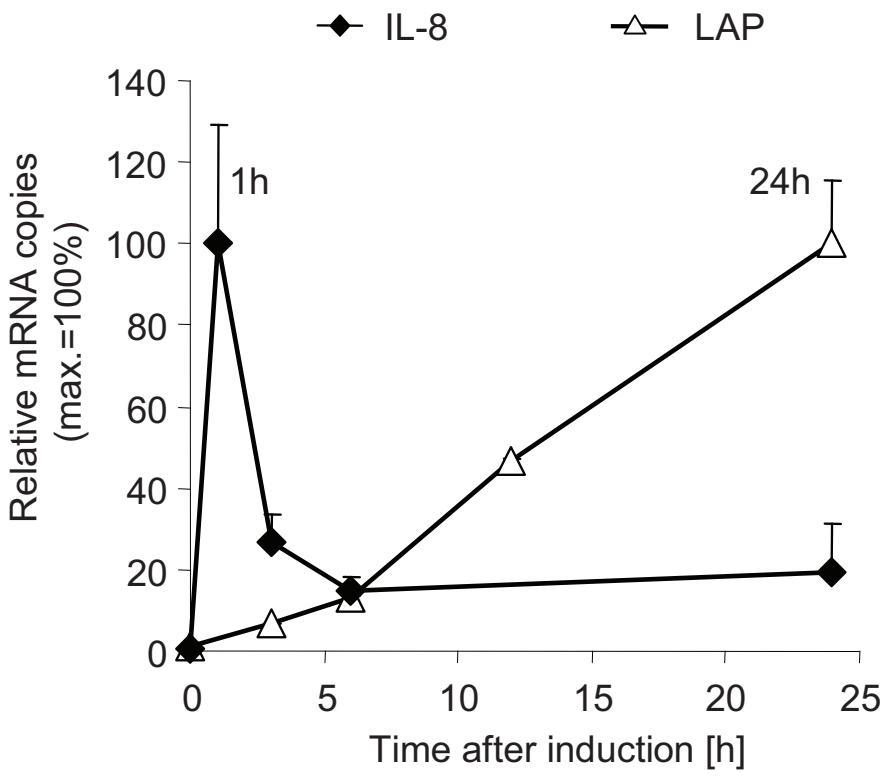


Figure 6. The transcription analysis of LAP and IL-8 in pbMEC by real-time PCR

pbMEC from healthy cows were induced for the indicated times with 1×10^7 heat-inactivated *E. coli* 1303/ml medium. RNA were prepared, reversely transcribed and measured by real-time PCR. Throughout the study, equal concentration of the same inducer was applied unless otherwise noted.

3.3 Transcriptional regulation of late-immune-gene LAP

3.3.1 Definition of the LAP promoter

3.3.1.1 The determination of the transcription start site (+1) of the LAP gene

The primary step to analyze the transcriptional regulation of a gene requires identifying its promoter. The promoter commonly includes the DNA sequence in the vicinity of the transcription start site (+1) that directs activation or repression of transcription. On the basis of this definition, the location of the promoter region can be identified simply by mapping the transcription start site.

Twenty three nucleotides upstream of the ATG of the published LAP mRNA sequence (Sequence # S76279) (Schonwetter et al., 1995) could not be found in contig of *Bos taurus* chromosome 27 genome (Sequence # NW_001502105.1), and no 5′ UTR (untranslated region) of the LAP gene was reported. Therefore, it was necessary to define the transcription start site of the LAP gene for further analyses. The transcription start site for a gene may be determined by identifying the 5′ end of its mRNA. In the study, I adopted the GeneRacer™ method to conduct a 5′ RACE amplification. The major transcription start site (+1, tss_1) identified from the most prominent 5′ amplicon was found to reside 125 nucleotides upstream from the start codon of translation (Fig. 7). The target sequence shows 100% identity with the genomic contig (Sequence # NW_001502105.1). The minor transcription start sites (tss_2 and tss_3) are located at +16 and +25 downstream of tss1, respectively. mRNA transcripts which are initiated from the three transcription start sites (tss1-tss3) include the start codon ATG at +125/+128. Their deduced translation products are 64-aa full length LAP peptides. The canonical TATAAA sequence resides downstream of all resulting transcription start sites. Taken together, LAP transcription is initiated by multiple transcription start sites, and these transcripts are initiated 5′ to the TATA box (Fig. 7).

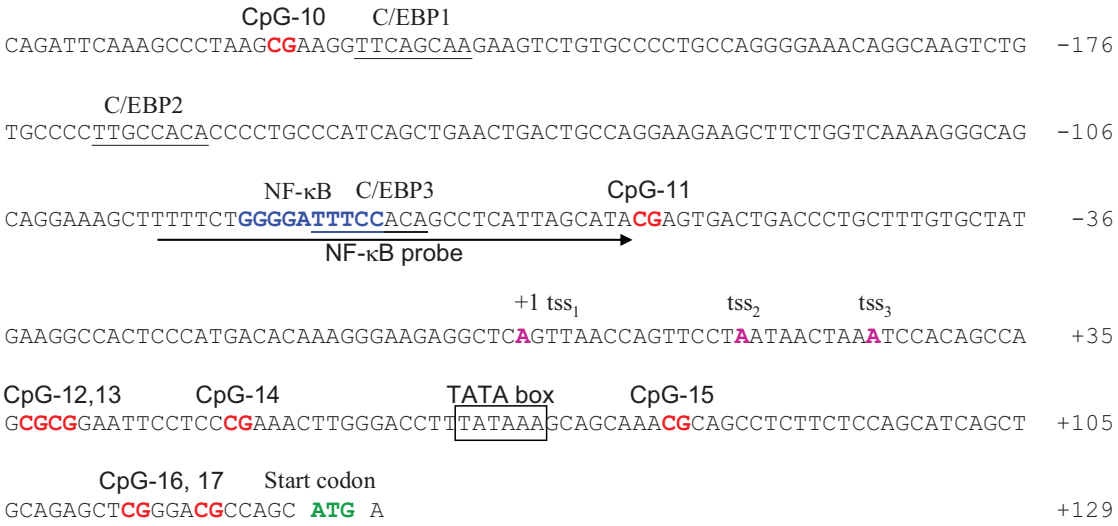


Figure 7. The sequence of the proximal LAP promoter

tss, transcription start site in purple letters; CpG sites for methylation analyses are shown in red letters; C/EBP binding site, underlined letters; NF-κB binding site, blue letters; The NF-κB probe used in EMSA is shown as arrow; Start codon of translation ATG, green letters; TATA box, rectangle box. Position numbers are indicated relative to the most 5´-located tss1 (+1).

3.3.1.2 *In silico* identification of putative transcription factors binding sites of the LAP promoter

The computational analysis of the LAP promoter sequence with the MatInspector software shows one putative NF-κB site and 3 C/EBP sites, which are termed as C/EBP site1, C/EBP site2 and C/EBP site3 from the distal to the proximal location, respectively (Fig. 8). The NF-κB binding site overlaps with the C/EBP site3 and a NFAT (nuclear factor of activated T cells) binding site. Within the 13 bp-sequence (GGGGATTTCCACA) at -89/-77, the ATTTCC motif is shared by NF-κB and C/EBP site3 (Fig. 8). Detailed sequence information on the putative transcription factors binding sites is shown in Fig. 7.

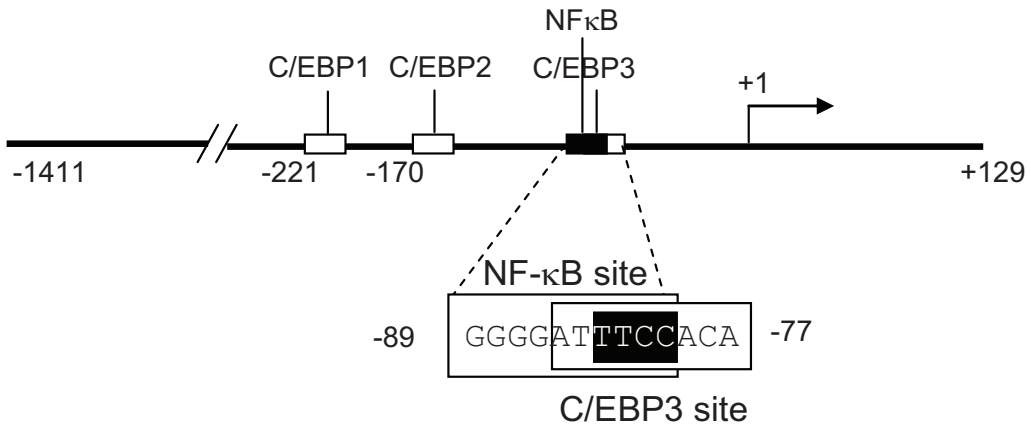


Figure 8. Schematic representation of putative transcription factors bound to the LAP promoter

The binding sites of putative transcription factors were analyzed with the software MatInspector (at http://www.genomatix.de). The core sequences of these binding sites are shown in Fig. 7. The transcription start site (+1) was determined by 5′ RACE. TTCC with black background was discussed in section 3.3.5.1.

3.3.2 Experimental identification of *cis*-elements crucially relevant to LAP expression and induction

3.3.2.1 The mapping of relevant elements in the LAP promoter using promoter deletions in reporter gene assay

To delineate the relevant region for LAP expression and induction, reporter plasmids harboring a series of 5′ deletion promoters were transiently transfected into HC11 cells. More than 25-fold basal activities over the promoterless pGL3 Basic vector were achieved for the LAP promoter. Heat-inactivated *E. coli* strikingly induced LAP expression more than 10-fold (Fig. 9). The data demonstrate that the basal activity of the LAP promoter is very strong, and that the HC11 cell line is a suitable cell model for regulation analysis of the LAP promoter. Both basal activity and induction were abolished after deleting the region containing the putative NF-κB and C/EBP site3 at -95/-58 (Fig. 9). More upstream sequences are only of minor relevance. Hence, the region at -95/-58 is highly relevant to the basal activity and induction of the LAP promoter.

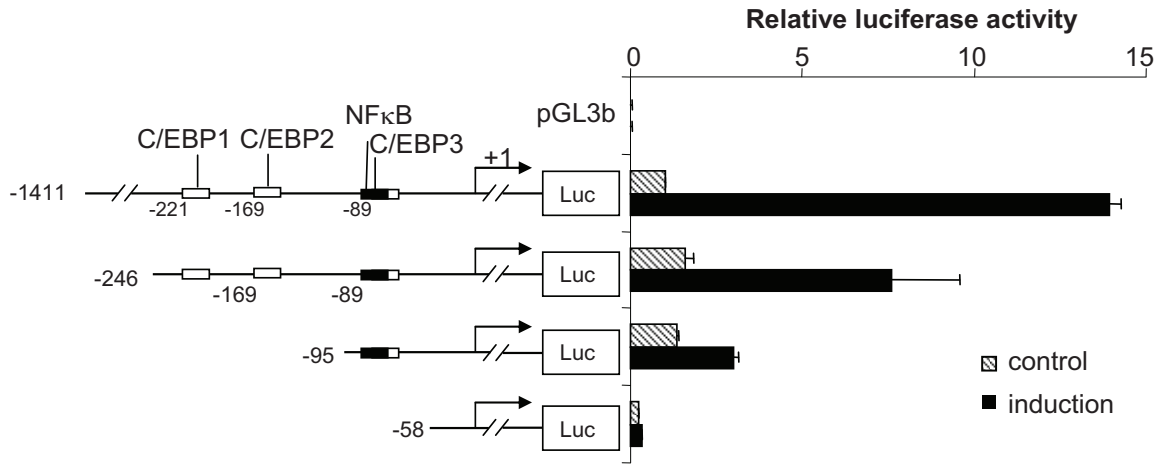


Figure 9. Identification of the key region to LAP expression and induction

Serially 5′ truncated promoter segments of the bovine LAP promoter were inserted into the promoterless pGL3 Basic vector, upstream of the firefly luciferase encoding gene and transiently transfected into HC11 cells. After 24h, 1×10^7/ml heat-inactivated *E. coli* 1303 were added into the induction group. The basal activity of the longest promoter -1411 is set as 1. The luciferase activities are shown as the mean ± S.E.M. from the representative of three independent experiments.

3.3.2.2 Identification of NF-κB and C/EBP3 binding sites as the highly relevant cis-elements to LAP expression and induction

To fine map the *cis*-elements crucial for LAP induction and expression, reporter genes were constructed either featuring a single point mutation or multiple-base substitutions in the putative transcription factor binding sites of the LAP promoter. Reporter gene assays were conducted in HC11 cells. The mutated sequences of mutants are shown in Fig. 10 left. Only a single base substitution of the NF-κB site (κBmG) dramatically reduced induction to 8% and basal expression to 50% (Fig. 10b). This shows that the NF-κB site is indispensable for maintaining LAP induction and basal expression. Similarly, multiple-base substitutions of the NF-κB site (κBmTTCC) and mutation of C/EBP site3 (CE3m) decreased LAP basal activity and induction (Fig. 10f and e), adding supportive evidence that the putative NF-κB/CEBP3 site is crucial to LAP induction and expression. In contrast, mutation of the C/EBP site2 (CE2m) did not influence both LAP basal activity and the extent of induced activity (Fig. 10d). Mutating the distal C/EBP site1 (CE1m) significantly enhanced both LAP basal activity and induction (Fig. 10c). Thus, C/EBP site1 is a repressive site. Taken together, the findings firmly establish that the NF-κB/CEBP3 overlapping site is crucial for LAP basal activity and induction.

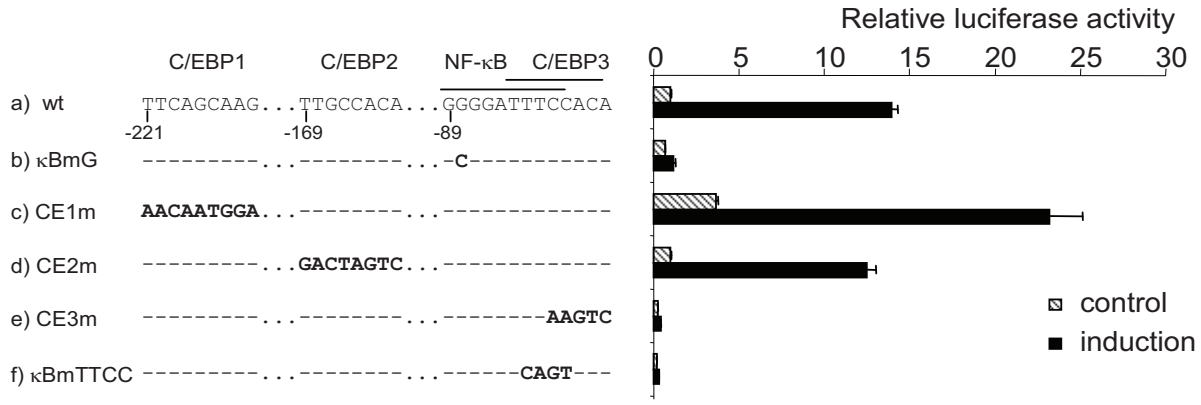


Figure 10. Mutation analysis of putative NF-κB and C/EBP binding sites of the LAP promoter in HC11 cells

Alterations of the LAP promoter sequence are listed on the left. The positions of the core sequences of the individual binding motifs are indicated below the wild-type sequence. The mutated sequences in mutants are shown in bold letters. The unaltered sequences are indicated as hyphens. The stipples indicate not shown bases. The fragment with single point mutation or multiple-base substitution mutations of putative NF-κB or C/EBP binding sites were inserted into the promoterless pGL3 Basic vectors. These clones were transfected into HC11 cells. Cells were kept untreated (control) or induced for 24h with heat-inactivated *E. coli* 1303 before harvest. The control activity of the wild-type is set as 1. The data are shown mean ± S.E.M from the representative in triplicate, of three independent experiments.

3.3.3 Effect of NF-κB factors on LAP expression

3.3.3.1 NF-κB p65 and p50 factors bind to the NF-κB binding site in the LAP promoter *in vitro*

The mapping and mutational analyses have clearly shown that the NF-κB binding site is pivotal for the function of the LAP promoter. I wanted to know which factors of the NF-κB family matter. To address the question, EMSA assays were performed using nuclear extracts from control or 2h induced HC11 cells. The 33bp-oligonucleotide probe derived from the LAP promoter containing the NF-κB binding site and C/EBP site3 (Fig. 7). A representative gel is shown (Fig. 11A). Three specific complexes (C1-C3) were eventually formed (Fig. 11 double-head arrows) and could be competed away with a 100-fold molar excess of the unlabeled wild-type competitor (Fig. 11A lane5 and lane7), but not by another unlabeled competitor featuring the mutation of the NF-κB core sequence (κBmTTCC) (Fig. 11A lane4 and lane8). Thus, the bands are the specific NF-κB-DNA complexes. To identify which member(s) (p65 and/or p50) of the NF-κB factor family binds indeed to the LAP promoter, antibody against NF-κB p65 or p50 was added into the EMSA reaction. Antibodies against NF-κB p65 shifted away the complexes C1 and C2 forming “so called” supershifted bands (Fig. 11A lane2 vs. lane3 and lane10 vs. lane9). Anti-p50, however, shifted away all three

complexes, C1, C2 and C3 (Fig. 11A lane1 vs. lane3 and lane 11 vs. lane9). This apparently shows that NF-κB p65 is contained in the complexes C1 and C2, and complexes C1, C2 and C3 possess NF-κB p50. In conclusion, complex C1 contains p65 and p50. C2 also comprises p65 and p50. But the molecular weight of C1 is heavier than that of C2, therefore, C1 may contain unknown factor (s) in addition to p65 and p50.

I wanted to know whether different mutants in Fig. 10 left influence the binding of NF-κB factor, competition assays were done with nuclear extracts from 18h induction HC11 cells. The same wild-type probe was labeled. Three specific complexes (C1-C3) for NF-κB were shown (Fig. 11B, lane 2, 3 and 4) similar to the left panel. Comparison of lane 2 (competitor κBGm) with lane 4 (shift reaction) (Fig. 11B) showed that compositions of the bands were the same in two lanes except that the signal magnitude by κBGm competitor was slightly lower. Hence, single-base mutation (G→C) of NF-κB binding site has effectively abolished the binding of NF-κB factors. Additionally, the composition pattern of the complexes in lane 3 was the same as that of shift reaction in lane 4 (Fig. 11B). It implies that the binding of NF-κB factors is completely disrupted by the mutation of κBmTTCC. Taken together, both mutations (κBGm, κBmTTCC) block the binding of NF-κB factors to the LAP promoter. Combined with data from Fig. 10, NF-κB factor is crucial for LAP basal expression and induction.

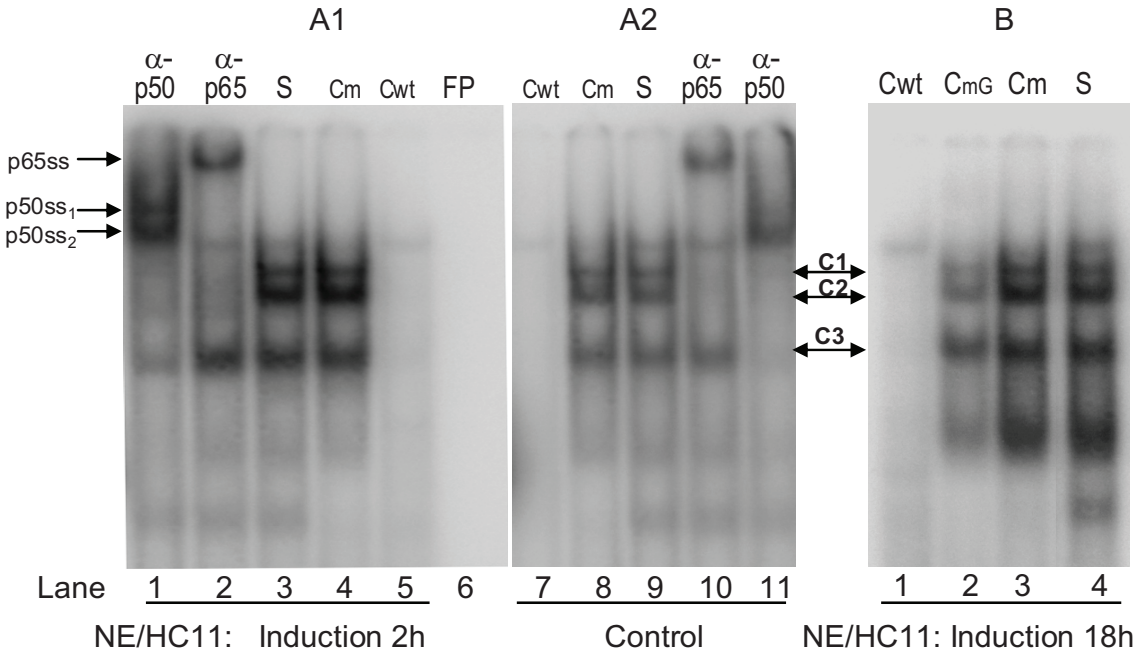


Figure 11. Binding analyses of NF-κB p65 and p50 to the LAP promoter by EMSA

EMSAs were performed using a labeled probe with NF-κB binding site of the LAP promoter. Double-head arrows between panel A and B refer to NF-κB specific complexes (C1-C3) and p65 or p50 supershift bands (ss) are shown on the left by arrows. Lanes are listed at the bottom of each image. A) Equal amounts of nuclear extracts (NE) were used in panel A1 and A2 taken from 2h induced and uninduced (control) HC11 cells. The assays (lane1-11) were resolved in the identical gel. Cwt and Cm indicate the wild-type and the mutated overlapping site competitors (κBmTTCC). Their mutated sequences are the same as those of panel B. B) Competition assays, 10 μg of the nuclear extracts from HC11 cells induced for 18h were used in the reactions except for FP (free probe). Unlabeled competitors (the wild-type and the mutated probes) were applied in 100-fold molar excess. Cwt, CmG and Cm represent the wild-type, the mutated κBmG and κBmTTCC competitors, respectively. The mutated sequences are the same as the corresponding mutants (listed in Fig.10 left).

3.3.3.2 Induction makes many more cells recruit NF-κB p65/p65 homodimers to the LAP promoter *in vivo*

To examine the recruitment of NF-κB factor to the native chromatin, chromatin immunoprecipitation (ChIP) assays were carried out using pbMEC cells. The chromatins from pbMEC cells induced for different times were incubated with antibody against NF-κB p65 (sc-7151x), p50 (sc-114x) or normal rabbit IgG (sc-2027x), respectively. Twenty four hours of induction made many more cells open their LAP promoters for p65 binding [183-fold increase relative to the resting cells] (Fig. 12). Neither NF-κB factor was detected on the LAP promoter in the resting cells (Fig. 12). Very few cells are accessible for NF-κB p50 to the promoter after induction for 3h. In contrast, no DNA was detected when chromatin was incubated with control IgG (Fig. 12 open columns). Taken together, it is apparent that NF-κB

p65 homodimers are recruited to the LAP promoter at 24 h after induction, and hence may be the key factor to LAP expression and induction.

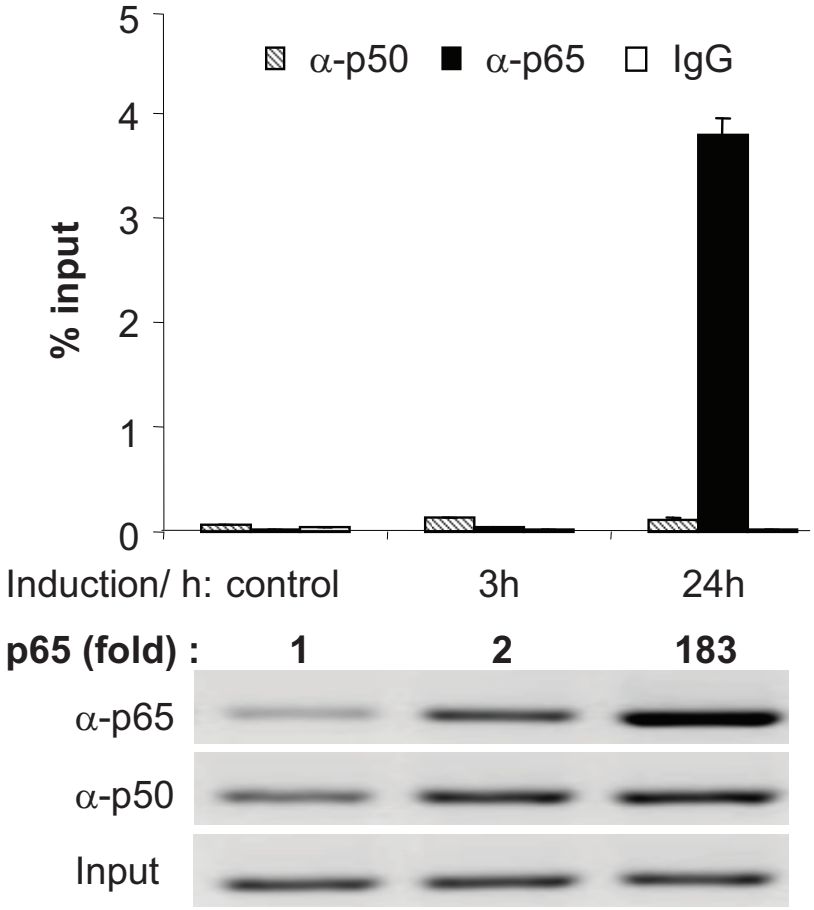


Figure 12. ChIP analysis of recruitment of NF-κB p65 and p50 to the LAP promoter

Shown are the yields of DNA associated with p65 and p50 before and after induction. pbMEC were untreated (control) or induced for the indicated times. Four μg of specific antibodies or normal rabbit IgG was used in immunoprecipitations. The immune complexes were captured by protein A agarose. The purified DNA was used as template in real-time PCR. Details are given in Materials and Methods section. The yield (% input) of uninduced p65 is set as 1. After induction with heat-inactivated *E. coli*1303, the increase (fold) of cells bound by p65 is shown. To validate the specificity of the PCR, products were run on agarose gels after the real-time PCR amplification. Specific PCR products were confirmed by ethidium bromide staining.

3.3.3.3 NF-κB p65 activates, but p50 inhibits the LAP promoter activity in a dose-dependent manner

To investigate the function of NF-κB p65 and p50 in the transcriptional regulation of the LAP gene, their respective expression vectors were transiently transfected into HC11 cells together with the wild-type LAP reporter. In HC11 cells, p65 activates, but p50 down-regulates the LAP promoter activity (Fig. 13). To know the function specificity of p65 and p50, increasing amounts (10, 30, 100, 300 and 1000 ng) of p65 or p50 expression vector were cotransfected into HC11 cells with the wild-type LAP reporter. As the amount of p65 expression construct was increased, luciferase activity was enhanced gradually. High amount (1μg) of p65 also displayed 1.8-fold increase of luciferase activity relative to the mock group. In contrast, p50 expression vector decreased luciferase activity with the increasing of p50 amount. Thus NF-κB p65 up-regulates, but p50 down-regulates the LAP promoter activity in a dose-dependent manner (Fig. 13A and 13B). The finding is consistent with the ChIP data that only NF-κB p65 significantly binds to the active LAP promoter. Taken together, NF-κB p65 drives LAP expression and induction.

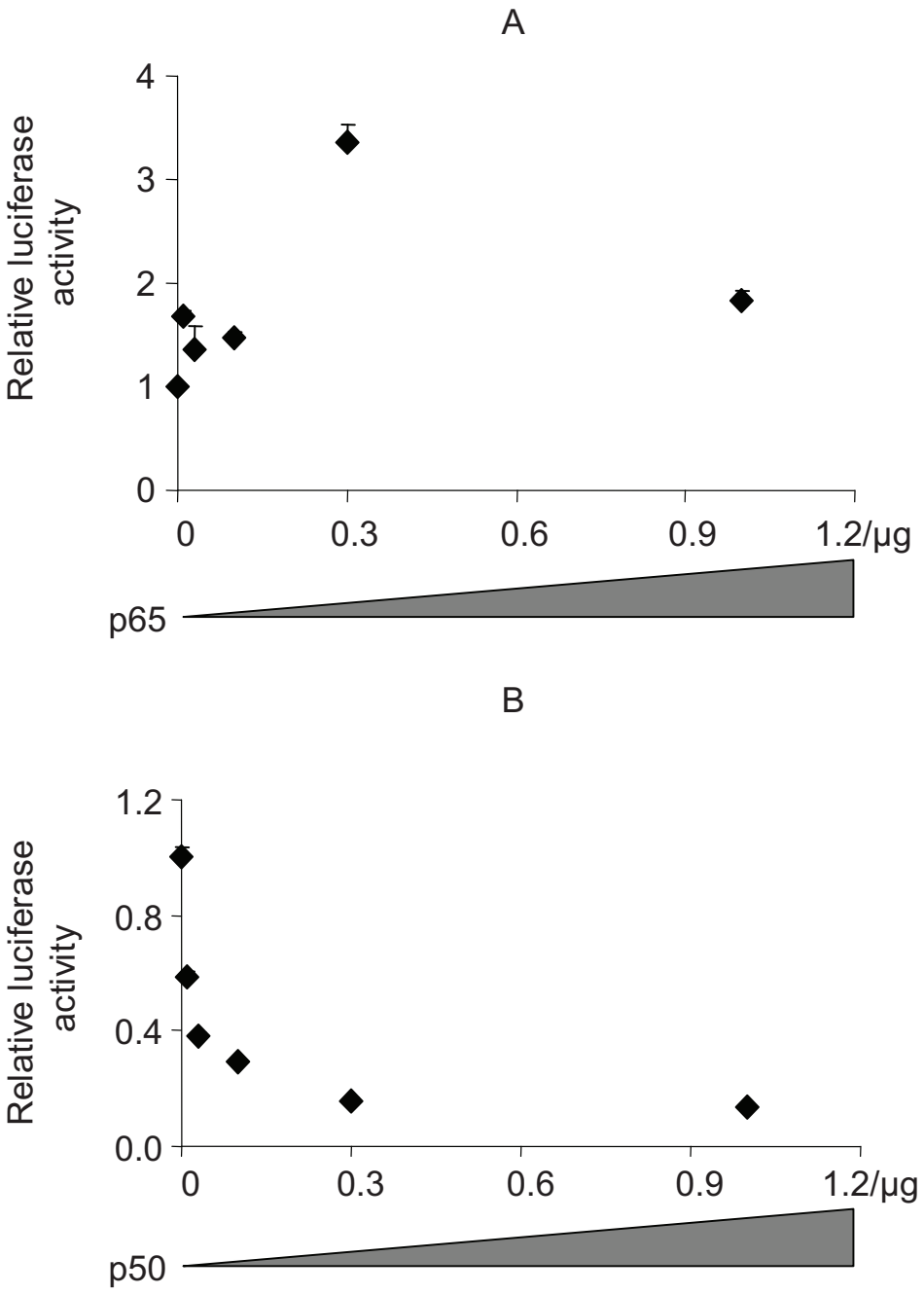


Figure 13. Functional analysis of NF-κB factors in the LAP promoter

HC11 cells were transiently transfected with 1000 ng of the wild-type LAP reporter construct together with increasing amounts of p65 (A) or p50 expression vector (B) (10 ng to 1000 ng). Total amount of transfected DNA was kept constant by adjusting with the empty vector. The luciferase activities were given relative to that of LAP reporter.

3.3.4 Effect of C/EBP factors on LAP expression

3.3.4.1 EMSA shows that C/EBP-β and -ε may function by direct binding to C/EBP site3

The mutation analysis shows that the NF-κB/CEBP overlapping site is crucial to LAP expression and induction. I wondered how C/EBP factors influence LAP expression and induction. To analyze the binding of different C/EBP factors to the C/EBP site1, site2 and site3 in the LAP promoter, EMSA was performed with equal amounts of nuclear extracts (NE) from HC11 cells transfected with empty vector (mock) or with expression constructs of dominant negative (DN)-C/EBP-α, -β, -δ or -ε (Fig. 14 and appendix Fig. 37). In order to avoid the disturbance of the endogenous C/EBP factors to effectively discriminate the specific binding of different C/EBP factors, the truncated C/EBP factors (DN-C/EBPs) in N-terminus, which possess the lighter molecular weight, were employed in EMSA analysis. Furthermore, to effectively recognize the specific binding bands of the C/EBP factors to C/EBP site3 and to make this recognition not be influenced by the binding of NF-κB to the overlapping site, the mutated probe for NF-κB (LAP_κBmG) was used in EMSA (Fig. 14). Expectedly, no specific complexes for NF-κB bound to the mutated probe (Fig. 14 lane2). This provides further evidence that κBmG mutant completely disrupts the binding of NF-κB factors. Respective contrast lane3, lane4, lane5 or lane6 with lane2 in Fig. 14 and contrast lane3, lane4, lane5 or lane6 with lane2 in Appendix Fig. 37, the specific C/EBP-α, -β, -δ and -ε bands were identified and indicated by arrows (Fig. 14 and Appendix Fig. 37). No C/EBP factors bound to putative C/EBP site2. C/EBP site 1 was weakly occupied with C/EBP-α, -β, -δ and -ε factors (Appendix Fig. 37). Only C/EBP-β and -ε strongly bound to C/EBP site3 (Fig. 14). Moreover, the affinity of the C/EBP site3 for C/EBP factors was higher than that of the C/EBP site1. Combined with the mutation analysis, C/EBP-β and -ε may influence LAP expression and induction by direct binding.

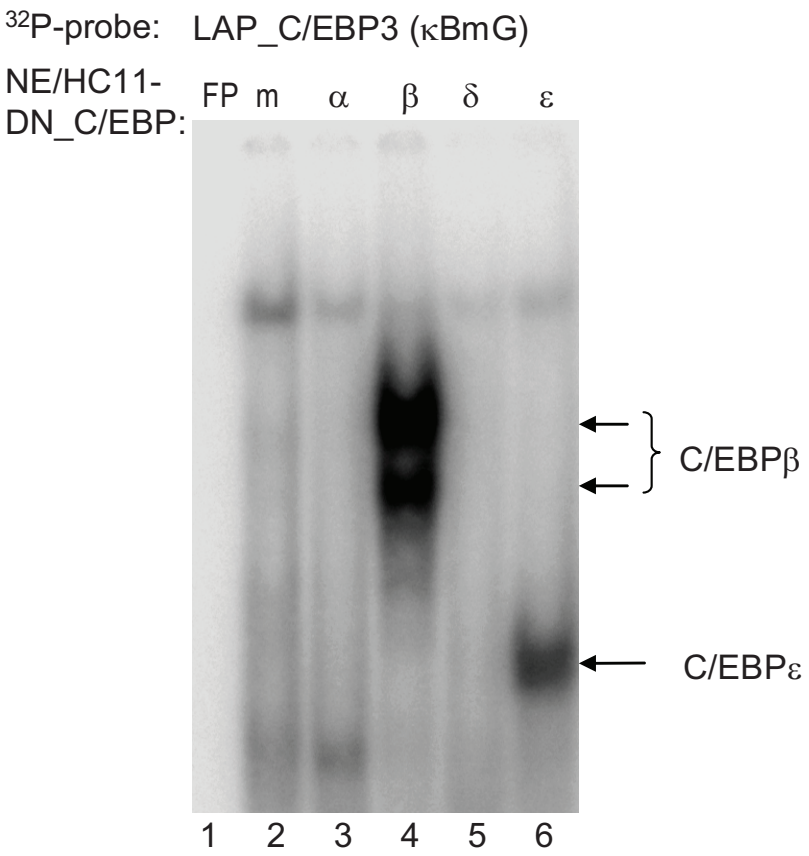


Figure 14. Binding analysis of the different C/EBP factors in the LAP promoter by EMSA

Nuclear extracts (NE) were from HC11 cells transfected with different expression constructs of DN-C/EBP-α, -β, -δ or -ε. Equal amounts of NE (2 μg) were incubated with the labeled probes as indicated. FP, free probe, without nuclear extract. Radiolabeled probes derived from NF-κB/CEBP3 overlapping site of the LAP promoter, but the binding of NF-κB was blocked (LAP_κBmG). The C/EBP specific complexes are indicated by arrows. Lanes are given at the bottom.

3.3.4.2 Functional analysis of C/EBP factors in the LAP promoter

3.3.4.2.1 All C/EBP factors down-regulate the LAP promoter activity

As described in the previous part, C/EBP factors only bind weakly to the distal C/EBP site1 but only C/EBP-β and -ε strongly bind to the proximal C/EBP site3. To clarify which member of the different C/EBP factors matters in the regulation of the LAP promoter activity, equal amounts of C/EBP factors were cotransfected into HC11 cells together with the wild-type LAP reporter. The result showed that all C/EBP factors down-regulate the LAP promoter activity to different extents (Fig. 15). Taken the above data together, the LAP promoter activity may be down-regulated by C/EBP-β and -ε via direct binding and by C/EBP-α and -δ via indirect binding.

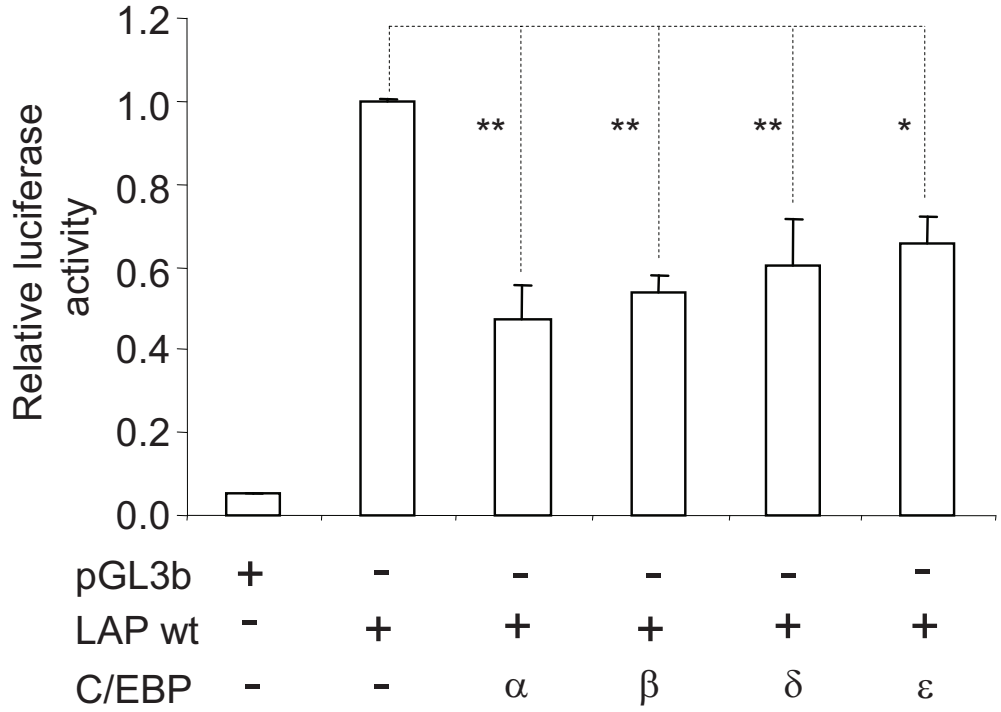


Figure 15. Functional analysis of C/EBP factors in the LAP promoter

C/EBP-α, -β, -δ or -ε expression vector was cotransfected into HC11 cultures, along with equal amounts of the reporter vector. The luciferase activity of the wild-type LAP promoter is set as 1. pGL3 Basic vector is served as a reference to evaluate the activity of the LAP reporter. The relative luciferase activities are shown as the mean ± S.E.M from three independent experiments. **, $p < 0.01$. *, $p < 0.05$.

3.3.4.2.2 C/EBPβ reduces basal activity but not inducibility, whereas C/EBPε blocks LAP promoter completely

The EMSA assay shows that C/EBP-β and –ε directly and strongly bind to the key NF-κB/CEBP3 overlapping site of the LAP promoter. Hence, C/EBP-β and –ε may play a more important role in regulating LAP promoter activity. To further determine the effect of C/EBP factors on the LAP promoter activity, increasing amounts of C/EBP-β or C/EBP-ε were cotransfected into HC11 cells, together with constant amount of the wild-type LAP reporter plasmid. The result showed that less than 0.1 μg of C/EBPβ did not influence basal activity of the LAP promoter (Fig. 16A). Increasing amounts of C/EBPβ gradually reduced the basal activity of the LAP promoter. However, the fold induction was barely changed (Fig. 16A). That is to say, high amounts of C/EBPβ reduced only the basal activity but not the induction extent of the LAP promoter. In contrast, 0.1 μg of C/EBPε significantly down-regulated only the basal activity of the LAP promoter (Fig. 16B). However, increasing amounts of this factor reduced both basal and induced activity. More than 0.9 μg of C/EBPε (equal amount to the

LAP wild-type reporter) abolished both basal and inducibility of the LAP promoter in HC11 cells (Fig. 16B). Hence, C/EBPε reduces both basal activity and induction of the LAP promoter in a dose-dependent manner. Taken together, C/EBPβ decreases only basal activity, and C/EBPε down-regulates not only basal activity but also induction of the LAP promoter in a dose-dependent manner, although the reduction is much lower than the non-titration experiments.

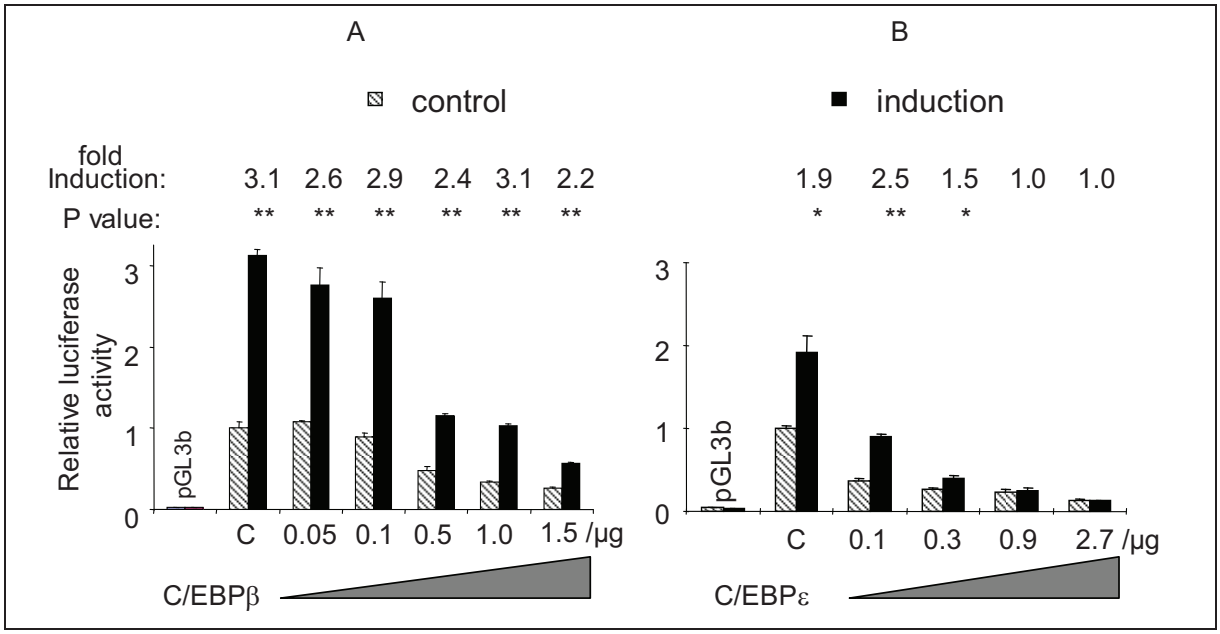


Figure 16. Dose-dependent analysis of C/EBPβ and C/EBPε for the LAP promoter in HC11 cells

The constant amount of the wild-type LAP reporter (1.5 µg for panel A, 0.9 µg for panel B) and the increasing amounts of C/EBPβ or C/EBPε were transiently transfected into HC11 cells. The heat-inactivated *E. coli* 1303 was incubated into the cell culture for 24h. The luciferase activity of the respective uninduced LAP reporter is set as 1. The total amount of DNA in each well is maintained constant by adding empty clone, **, $p < 0.01$. *, $p < 0.05$.

3.3.5 The combined effect of C/EBP and NF-κB factors on LAP expression

3.3.5.1 C/EBPε represses the LAP promoter by TTCC motif, independent of the NF-κB binding

The previous results firmly established that NF-κB factors and their site are essential for function of the LAP promoter, and the crucial C/EBP binding site3 overlaps with the NF-κB binding site. Hence, I intended to analyze whether the repressive effect of C/EBP factors on the LAP promoter activity is exerted via interference with NF-κB binding, or rather solely conveyed by C/EBP factors. Both C/EBP-β and –ε factors avidly bind to this site and C/EBPε was chosen to address this question. The repressive effects were compared after cotransfection of high amounts of this factor together with either the wild-type LAP reporter,

or the reporter genes harboring different mutations in the NF-κB binding motif. The mutants comprise i) the mutant κBmG, in which the single-base substitution blocks NF-κB binding, and ii) the mutant κBmTTCC, in which NF-κB and possibly C/EBP binding are abolished. It was found that C/EBPε repressed strongly the activity of that construct (κBmG) abolishing NF-κB binding only (Fig. 17). Hence, C/EBP factors diminish the LAP promoter activity by themselves, independent of the binding of NF-κB. However, after the TTCC motif (Fig. 8) was mutated into CAGT (κBmTTCC), the capability of C/EBPε to further reduce the activity of the LAP promoter was abolished. Thus the TTCC motif is the repressive site of C/EBPε. Taken together, C/EBPε represses the LAP promoter through binding to the TTCC motif, independent of the NF-κB binding.

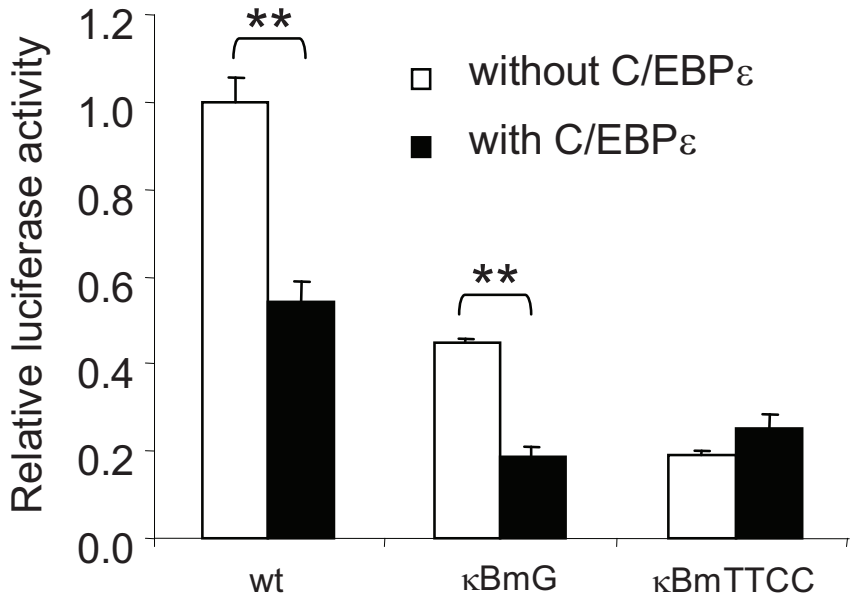


Figure 17. Analysis of C/EBPε repressive effect and acting site in the LAP promoter

HC11 cells were transfected with 900 ng of the wild-type LAP reporter (wt) or equal amount of the mutated LAP reporter with NF-κB single-base substitution (κBmG) or multiple-base substitution κBmTTCC, with/without 2700 ng of C/EBPε expression vector. Luciferase activity was shown relative to the wt LAP reporter. **, $p < 0.01$.

3.3.5.2 C/EBP–β and –ε also repress the LAP promoter by diminishing NF-κB p65-mediated activation

As we have shown, NF-κB p65 activates but C/EBP-β, -ε repress the LAP promoter activity independent of the binding of NF-κB. To further investigate how C/EBP-β and -ε play roles in the presence of NF-κB p65, C/EBP-β or -ε expression vector was cotransfected into HC11 cells together with the wild-type LAP reporter and NF-κB p65 expression construct. As described in the previous experiments, NF-κB p65 was found to activate the LAP promoter. After addition of the expression vectors of C/EBPβ or C/EBPε into the transfection system, the NF-κB p65-mediated activations were diminished (Fig. 18). Thus, C/EBP-β and –ε play the repressive roles in the LAP regulation by diminishing NF-κB p65-mediated activation.

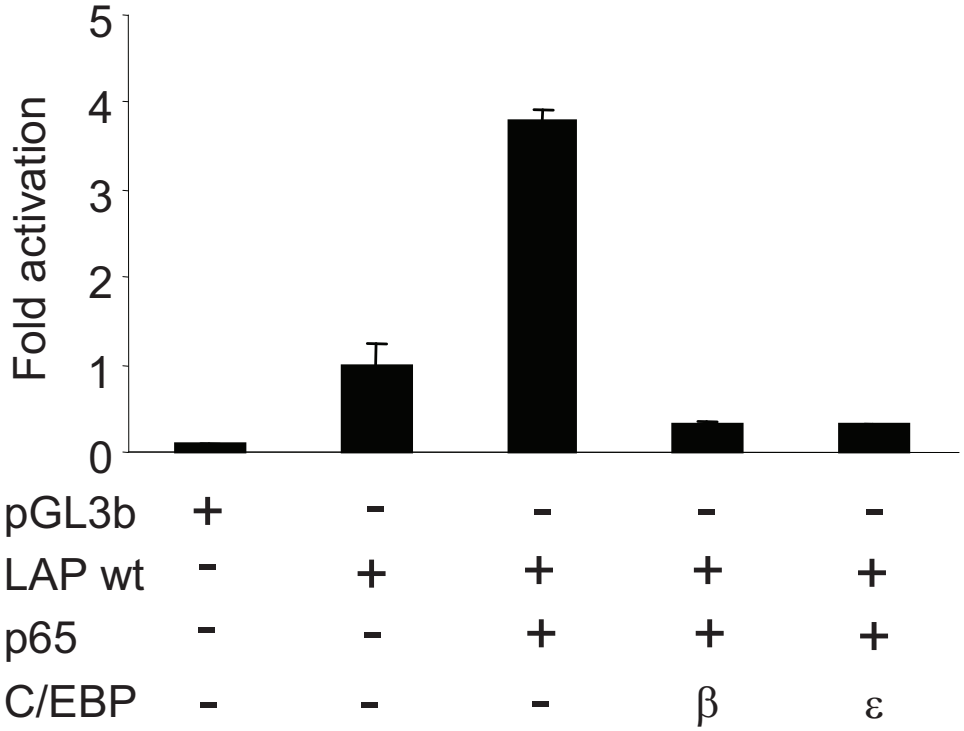


Figure 18. Functional analysis of C/EBP–β or –ε in the LAP promoter, in the presence of NF-κB p65

HC11 cells were cotransfected with 1 µg of NF-κB p65 expression vector, together with or without C/EBP-β or –ε expression vector. 1 µg of LAP reporter gene and the respective C/EBP expression vector were used. Luciferase activity of the wt LAP reporter plasmid is set as 1. Fold activations of transcription factors are shown as the mean ± S.E.M. from the representative in triplicate of two independent experiments.

3.3.5.3 C/EBPε and NF-κB factors may compete for the overlapping site in the LAP promoter

It was shown, so far, that C/EBP-β and -ε or NF-κB p65 and p50 can individually bind to the overlapping site of the LAP proximal promoter and that only NF-κB p65 activates the LAP activity. Both C/EBP-β and –ε repress the LAP promoter activity by diminishing NF-κB p65-mediated activation. In order to find the cause that C/EBP attenuates NF-κB p65-mediated activation, I carried out EMSA with nuclear extracts from the untreated HC11 cells (Fig. 19A). Also, the untreated HC11 which expressed DN-C/EBPε (Fig. 19B) or the induced HC11 expressing DN-C/EBPε (Fig. 19C). The result showed that the amount of NF-κB binding to the probe was not changed, regardless of the presence or absence of overexpressed DN- C/EBPε (Fig. 19A vs. 19B). Induction made more NF-κB bind to the probe as previous, despite DN-C/EBPε was added into the reaction (Fig. 19B vs. 19C). i.e. high amounts of DN-C/EBPε can not diminish the binding of NF-κB factors to the probe. It shows that the probe has higher affinity for NF-κB than for C/EBPε, and that C/EBPε and NF-κB bind to the different probe molecules *in vitro*. One factor (e.g., NF-κB) maybe precludes the binding of another factor (e.g., C/EBP). Thus, the proportion of endogenous NF-κB to C/EBP factors appears to be a main regulatory event for LAP expression.

In conclusion, the molecular mechanism of the LAP promoter at the transcriptional level has been clarified. NF-κB p65 triggers LAP expression and induction. Moreover, C/EBP factors play the repressive role by diminishing the activation of NF-κB p65 to LAP expression and are independent of NF-κB. C/EBP factors and NF-κB competitively bind to the NF-κB/CEBP3 overlapping site in the LAP promoter. Hence, the proportion of endogenous NF-κB to C/EBP factors appears to be a main regulatory event for LAP expression.

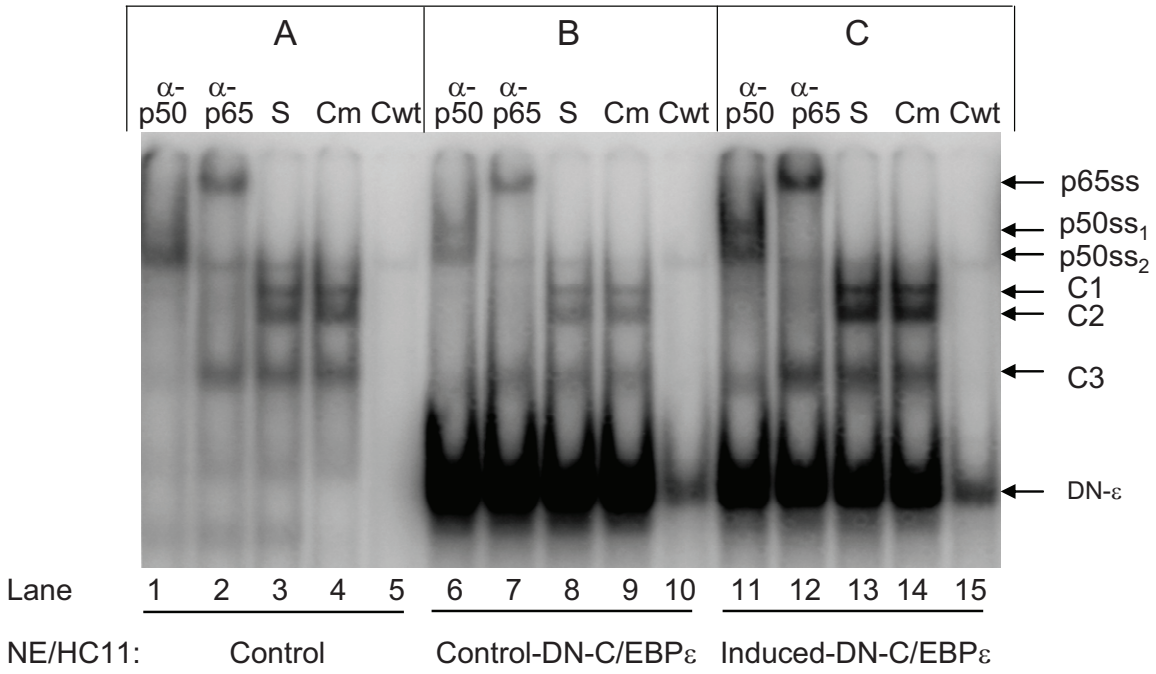


Figure 19. EMSA analysis of NF-κB p65 and C/EBPε binding to the LAP promoter

Oligonucleotide containing the NF-κB/CEBP3 overlapping site in the LAP promoter was labeled. The labeled probes were incubated with 10μg nuclear extract from control HC11 cells transfected with empty vector (A), control cells transfected with DN-C/EBPε (B) and 2h induced HC11 cells transfected with DN-C/EBPε (C). The unlabeled wild-type (Cwt) or mutated probe (Cm: κBmTTCC) was used to compete the specific binding at 100-fold molar excess. The supershift binding reactions (α-p65 or α-p50) were incubated with specific antibodies for 10 min at room temperature prior to the addition of labeled probe. The complexes were resolved in 6 % non-denaturing polyacrylamide gel. The dried gel was exposed overnight and scanned by Storm 840. The supershift bands, NF-κB specific complexes (C1-C3) and DN-ε band are indicated on the right. S: shift reaction.

3.4 Role of chromatin remodeling in induction of β-defensin encoding gene

3.4.1 Time course and induction extent of LAP and NF-κB are uncoupled

The above evidences show that transcription factor NF-κB p65 drives LAP expression and induction. Hence, its alteration directly affects LAP expression and induction. It is necessary to know the alteration of NF-κB at the different induction times.

3.4.1.1 NF-κB rapidly increases only two-four fold after induction in HC11 cells

The NF-κB motif of the LAP promoter was used as a probe in EMSA to monitor NF-κB alteration. Nuclear extracts were from HC11 cell cultures induced for the indicated times (Fig. 20A). Image Quant 5.2 software from Molecular dynamics was used to quantify the supershifted bands of NF-κB p50 (Fig. 20B filled squares) and p65 (Fig. 20B filled triangles), respectively. The data showed that the change patterns of the two factors were similar. Both the amount of NF-κB p50 and p65 were increased to 2-4 fold after induction for 2h. Afterwards, it decreased slowly. Differently, the previous results showed that LAP expression was activated by only NF-κB p65 and repressed by p50. I wanted to further check alteration of active NF-κB at the different induction times.

To the end, another technique---reporter gene assay was applied. HC11 cells were transfected with ELAM reporter harboring five tandem NF-κB attachment sites. Cells were harvested after induction for the indicated times and renilla activities were assayed. The result demonstrated that NF-κB activity peaked by 3-fold increase at 6h after induction and afterward decreased (Fig. 20B open circles). Although the time point with the maximal NF-κB activity was shifted from 2h (in EMSA) to 6h (in reporter gene assay), the profile *in vivo* was similar to the data from EMSA *in vitro*. Taken together, 3-fold more active NF-κB is increased in cells at 6h after induction.

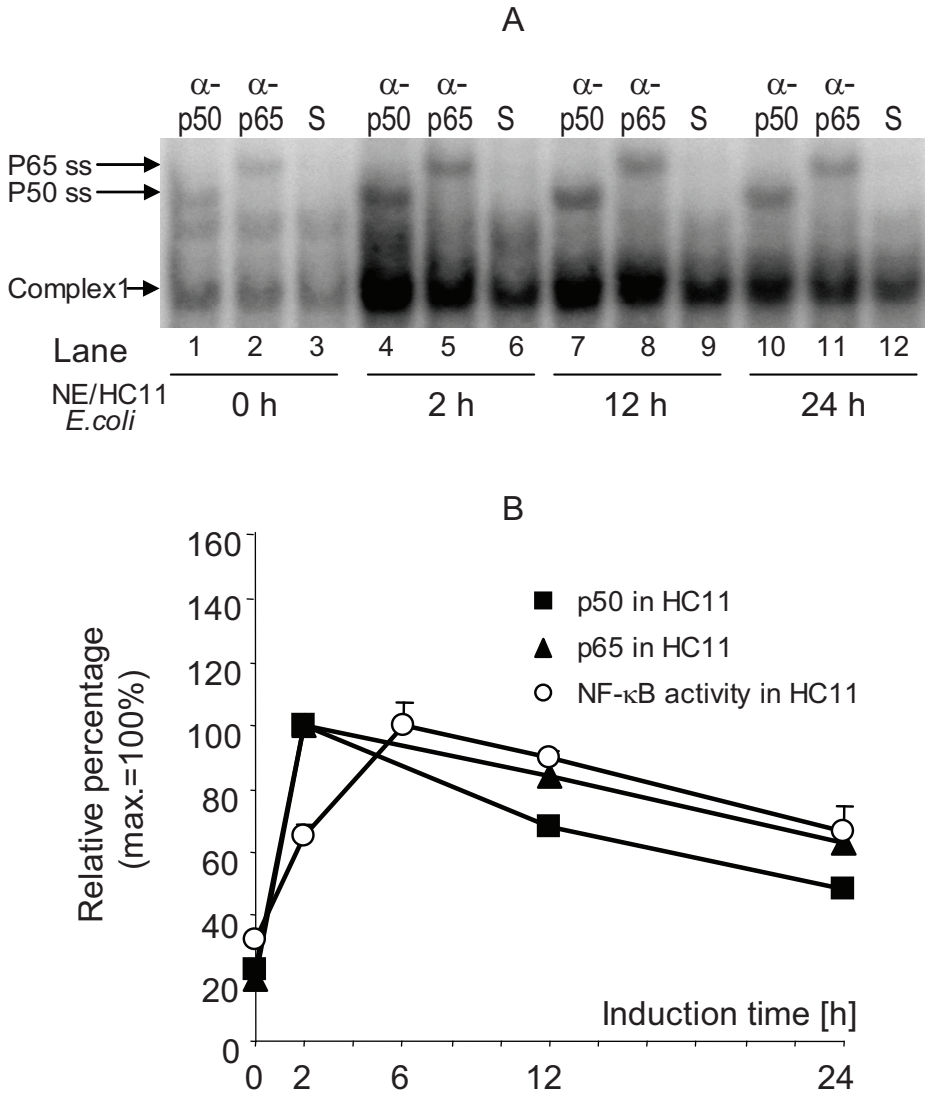


Figure 20. Alteration of NF-κB in HC11 cells

A) EMSA was performed with nuclear extracts (NE) from HC11 cells without inducer or incubated with 1×10^7 /ml heat-inactivated *E. coli* strain 1303 for the indicated times (0, 2h, 12h and 24h). 10 µg NE was incubated with labeled 33-oligonucleotides probe as previous. The complexes were resolved in 6 % non-denaturing PAGE, and dried gel was exposed overnight, scanned with Storm480 and analyzed by Image Quant 5.2 software of Molecular dynamics. B) Alterations of NF-κB p50 (filled squares) and p65 (filled triangles) in HC11 derived from the quantified image on the left. NF-κB activities in HC11 (open circles) were from transfection assay. 20 ng ELAM reporter construct harboring five tandem NF-κB attachment sites was transfected into HC11 cells. Inducer was added for the indicated times prior to the harvest. Renilla activities were assayed. The maximal values in three graphs were set as 100 %.

3.4.1.2 LAP mRNA accumulation is much stronger and later than NF-κB activation. The resting LAP promoter is insulated against NF-κB factor

The above reporter gene assay shows that active NF-κB is increased 3-fold at 6h after induction in HC11 (murine MEC cells). Previous results show that NF-κB p65 drives LAP expression and induction. I wanted to detect whether similar change happened in LAP promoter activity in primary bovine MEC (pbMEC). To address the question, the wild-type LAP promoter reporter was transiently transfected into pbMEC cells. The cells were induced for the indicated times prior to the harvest. The result showed that the extent of the LAP promoter reporter activity was increased gradually with the prolongation of the induction time and reached the highest at 12h. No apparent change between 12h and 24h time point. Consistent with increase (3-fold) of active NF-κB in murine MEC (Fig. 20B open circles), only 2.6-fold of LAP promoter reporter activity was achieved relative to the uninduced level in bovine MEC (Fig. 21 open circles). However, LAP mRNA concentration in pbMEC (Fig. 21 filled triangles) was increased to more than 100-fold 24h after induction, which was much stronger than increase of LAP promoter activity (2.6-fold) and active NF-κB (3-fold). More importantly, in udder, much more (700-fold) increase of LAP mRNA concentration was reached (Fig. 21 filled squares). Obviously, peak of LAP mRNA (24h) is much later than that of NF-κB (2h) (Fig. 20). Taken together, LAP mRNA accumulation and NF-κB activation are uncoupled and the former is much stronger and later than the latter.

Comparison of the minimal with the maximal activities, LAP promoter activity increased from 38 % up to 100 % maximal activity 12 h after induction. However, LAP mRNA copies of the resting cultures reached only 1% of the maximal level (100 %) 24h after induction in pbMEC. The difference in LAP mRNA concentration between resting (unstimulated) (0.14 %) and 24h after full pathogen induction (100 %) was even stronger in udder. Contrast the levels at the resting stage at the three lays, 38 % of maximal LAP promoter activity was higher than 1 % or 0.14 % of maximal LAP mRNA concentration in pbMEC or in udder. The data clearly shows that the LAP promoter exhibits different levels of insulation against NF-κB in the resting stage, the order of insulation magnitude is udder > pbMEC >> reporter.

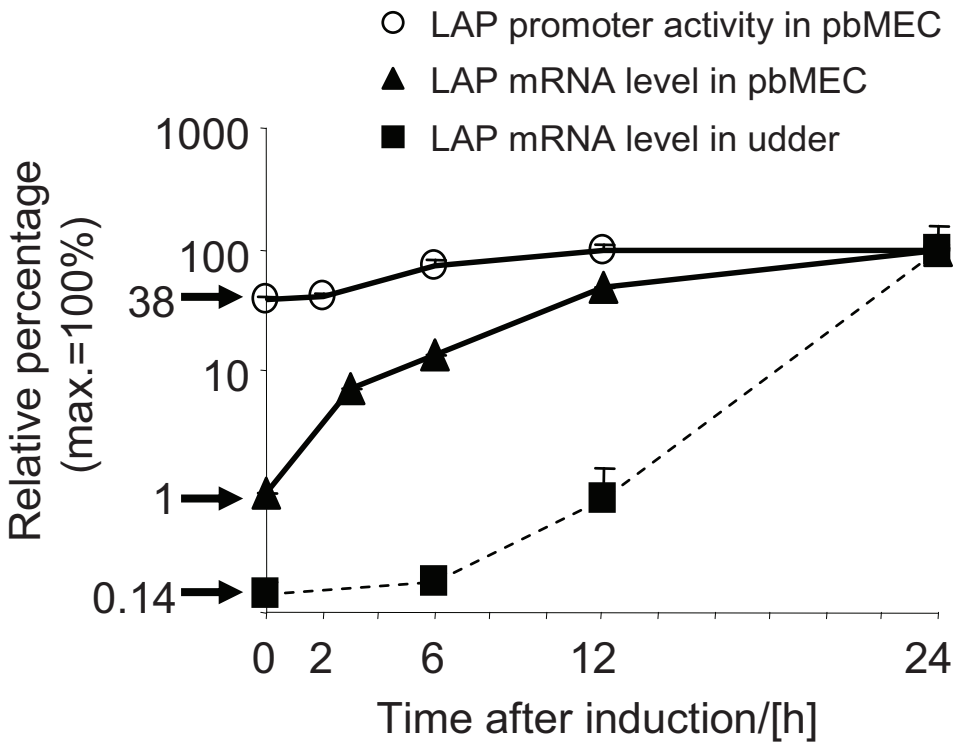


Figure 21. Alteration of LAP promoter activity and mRNA concentration in pbMEC and udder

pbMEC cells from healthy cows were transient transfected with the wild-type LAP reporter, harboring the LAP promoter and expressing firefly activity, and induced for the indicated times. The firefly activity reflects the LAP promoter activity (open circles). LAP mRNA level in pbMEC (filled triangles) is adapted from Fig. 6. In the two experiments, 1×10^7/ml heat-inactivated *E. coli* strain 1303 induced pbMEC cells for the indicated times before harvest. In order to obtain udder samples from 4 healthy and lactating cows, four different udder quarters of each cow was infected for the indicated times with 500 CFU *E. coli* strain 1303 before culling. The same measurement technique of LAP mRNA copies for udder samples was applied as Fig. 6. Ordinate is shown as logarithmic scale. The maximal values in three graphs are set as 100 %.

3.4.2 β-defensin induction is associated with decompaction of chromatin in udder

In the resting cells of udder, LAP promoter insulates against NF-κB to a greater extent. I wanted to investigate whether chromatin structure in udder is remodeled after induction to be accessible to NF-κB p65. CHART-PCR (Chromatin accessibility by real-time PCR) was performed to detect alteration of chromatin structure. CHART-PCR is based on the fact that the condensed chromatin restricts transcription factors, as well as restriction enzyme, access to DNA, whereas the loosed chromatin DNA is accessible for them, hereby can be digested with restriction enzyme. Real-time PCR is used to detect the digestion degree reflecting the degree of chromatin compaction. Chromatin from control and infected udder samples was digested with restriction enzyme *Hind*III for 1h. The active transcriptional regions of the LAP

and the BNBD5 (β-defensin gene 5) genes were chosen as target regions in which encompass the NF-κB/CEBP overlapping site. Two *Hind*III sites lie in the region of the LAP promoter and one *Hind*III site in the BNBD5 target region. The result showed that protection percentage of LAP chromatin was high significantly reduced from 95.1% (Fig. 22A open diamonds, ordinate) down to 59.7% (Fig. 22A filled diamonds, ordinate) after induction for 24h ($p<0.01$). Similarly, protection percentage of BNBD5 chromatin was significantly reduced from 100.8 % down to 82.5 % (not shown) after induction for 24h ($p<0.05$). The data show that induction decompacts the chromatin around crucial sites of β-defensin promoters. Combined with the mRNA copies (Fig. 22A abscissa), it shows that β-defensin induction is associated with decompaction of chromatin in udder. In contrast, the control αs1-casein gene data (Fig. 22B) from the same animals show that αs1-casein induction is associated with compaction of chromatin in udder.

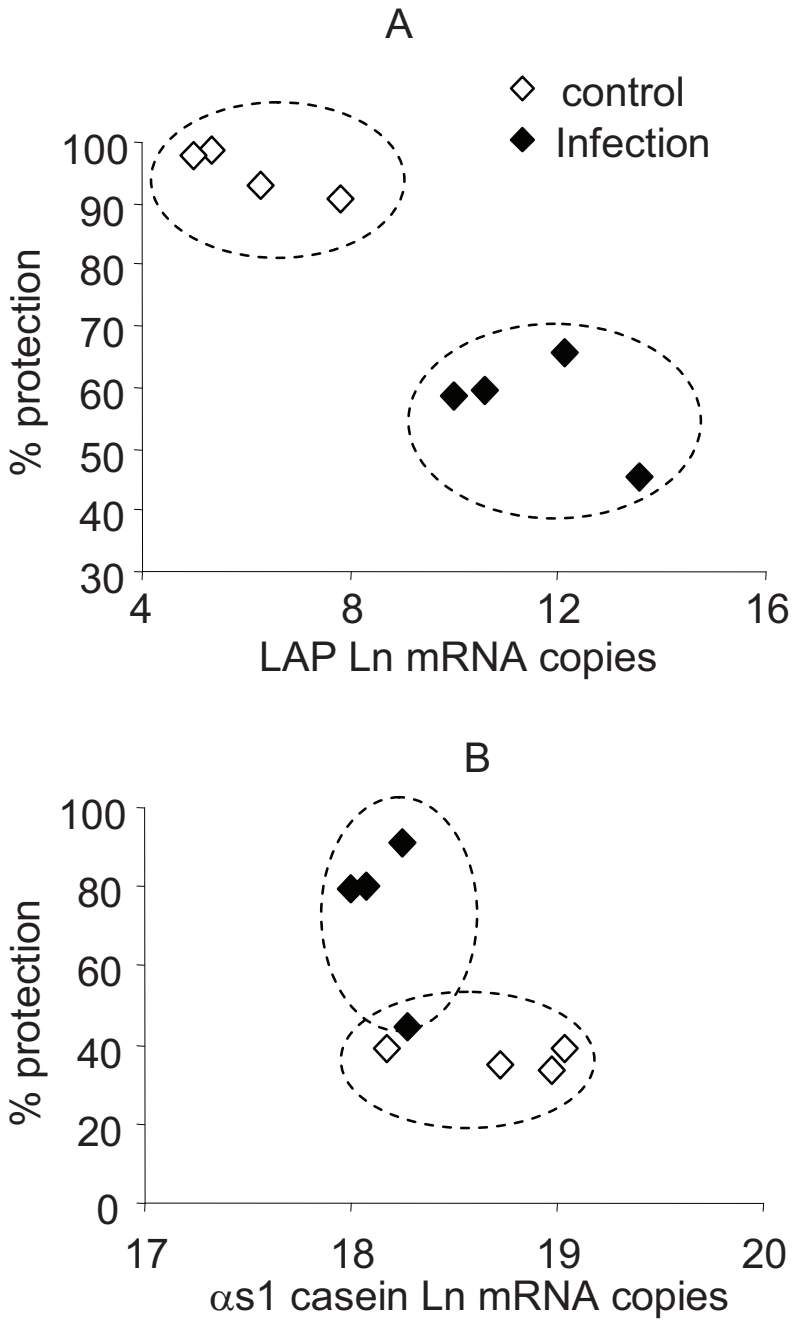
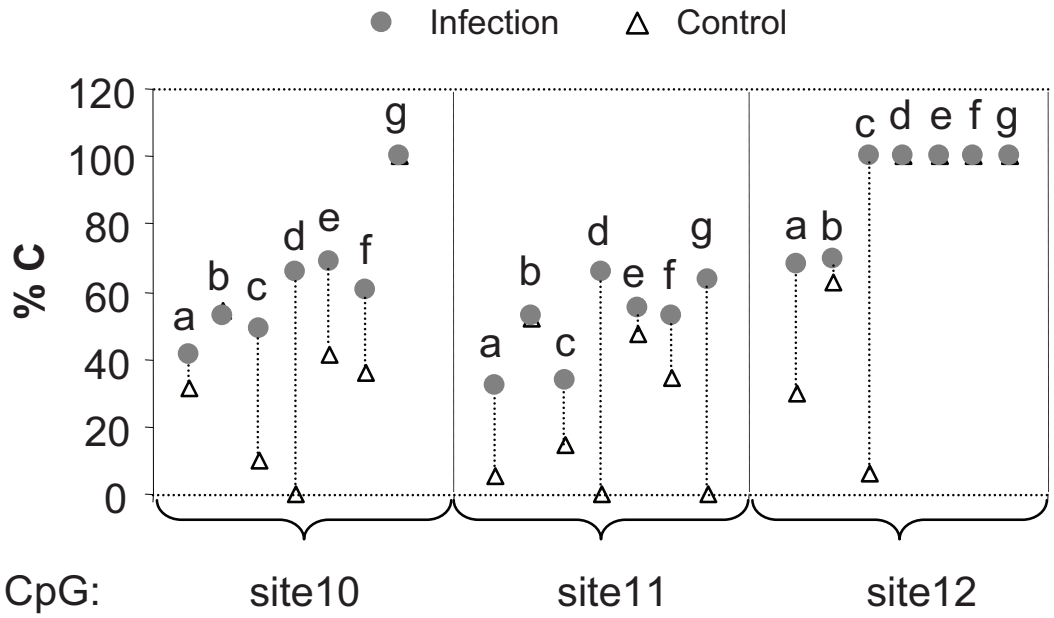


Figure 22. Alterations of chromatin compaction and mRNA copies for the LAP gene and αs1-casein gene

The ln mRNA copies (abscissa) of control (open diamonds) or 24 h infected (filled diamonds) udder samples (n=4 animals) are plotted against the degree of chromatin protection from *Hind*III for LAP or from *Dde*I for αs1-casein (ordinate, from CHART-PCR). Shown are mean values from two independent experiments. Chart PCR data of αs1-casein has been described (Vanselow et al., 2006). *, $p < 0.05$, **, $p < 0.01$.

3.4.3 Chromatin decompaction of the LAP promoter during induction is associated with hypermethylation

The previous results demonstrated that the LAP chromatin compaction is loosed in the active transcription region (Fig. 22) possibly modulating the accessibility for transcription factors to their target binding sites. I wanted to know whether DNA methylation correlates with the chromatin remodeling. To address the question, I analyzed and quantified the degree of the DNA methylation of LAP promoter region with the bisulfite sequencing technique. Udder tissues were derived from 7 healthy Holstein cows. After milking at the fourth month of their first lactation, one quarter was challenged with 500 CFU of *E. coli* strain 1303 for 24h before culling and one quarter was left untreated as control. All 17 CpG sites in the 1.5-kb LAP promoter were analyzed. No methylation alteration happened in CpG 1-9 (located at -1411/-730) and CpG 16-17 (located at +81/+130) before and after infection (not shown). However, the methylation patterns of CpG sites 10-15 at -220 to +81 are differential in different animal individuals. Surprisingly, in all individuals, no demethylation was found in CpG sites 10-15 after infection. Hypermethylation was displayed in the some individuals except for the animal individuals not showing alteration of methylation (not shown). The hypermethylation region covers ~140 bp upstream and downstream of the NF-κB binding site. The student t-test show that only CpG site 11 display hypermethylation (p <0.05) in all 7 tested animal individuals in a statistically significant fashion (Fig. 23). CpG site10 and site11 are encompassed respectively in the forward and reverse primers used in CHART-PCR. i.e., CpG in the loosed chromatin is hypermethylated but not hypomethylated. However, DNA methylation is known to be relevant to silencing genes in most cases (Jaenisch and Bird, 2003; Li, 2002).



CpG	Quarters	Site 10	Site 11	Site 12
Mean value (% C)	Infection	62.7	50.7*	91.0
	Control	38.8	22.0	71.3

Figure 23. Methylation analysis of the LAP promoter in udder

Genomic DNAs were prepared from the uninfected and 24h infected udder quarters of same animals. Total 7 different animals are indicated with lower case letters (a-g). EpiTect Bisulfite Kit from Qiagen was served to carry out the bisulfite conversion reaction. Purified DNA with converted U or C was used as a template to amplify the target fragment with specific methylation primers in primary PCR and secondary PCR. PCR products were sequenced directly by ABI sequencing system. The symbols from the same animal (open triangles: control quarter, grey dots: infected quarter) are connected with dashed lines. All symbols for the same CpG site are shown by compartments. Mean values from 7 animals are listed in table.

Our lab has reported that NF-κB is essential for pathogen-mediated activation of the β-defensin gene 5 (BNBD5) (Yang et al., 2006), which is another pivotal member of bovine β-defensins. I wanted to know whether NF-κB plays a different role in LAP and in BNBD5 regulation.

3.5 Lower affinity for NF-κB of the β-defensin gene 5 (BNBD5) promoter is a key cause of lower expression compared with the LAP promoter

3.5.1 Both basal and induced activities of the BNBD5 promoter are much lower than those of the LAP promoter

To our knowledge, LAP expression explains 65 %-80 % defensin messages among five bovine β-defensins (LAP, BNBD5, TAP, EBD and BNBD4) (Wei Yang's thesis). LAP is the predominant member of the five peptides in the infected pbMEC and udder. To investigate the cause of the differential transcription, equal amounts of the wild-type LAP reporter and the BNBD5 reporter were transfected into HC11 cells respectively. The result apparently showed that more than 50-fold activities for LAP basal activity and only 2.4-fold for BNBD5 were found over the promoterless pGL3 Basic. After induction, the LAP basal activity was enhanced to 5.9-fold relative to the unstimulated level. Only 2.3-fold induction was achieved in the BNBD5 case (Table 2). This shows that both basal activity and induction of the LAP promoter are significantly higher than those of the BNBD5 gene. This result is consistent with the finding that LAP is the most abundant β-defensin encoding gene in MEC among the tested five members of that gene family.

Table 2. Contrast basal activity and inducibility between the LAP promoter and the BNBD5 promoter

Promoters	Fold of pGL3 Basic		
	Control	Induction	Fold induction
BNBD5	2.4±0.3	5.4±1.4	2.3
LAP	50.1±10.9**	296.8±58.6**	5.9

HC11 cells were transiently transfected with 1.5 μg of the wild-type LAP promoter or the BNBD5 promoter, and either left uninduced or induced with 1×10^7 / ml heat-inactivated *E. coli* 1303 for 24h. Shown were the luciferase activities relative to the uninduced promoterless pGL3 Basic. Results represent the mean ± S.E.M. of two independent experiments. **, $p < 0.01$.

3.5.2 The function of NF-κB p65 or p50 in BNBD5 regulation is equivalent to that in LAP

Since NF-κB is essential for pathogen-mediated induction of the BNBD5 encoding gene in mammary epithelial cells (Yang et al., 2006), I wanted to know what function of the NF-κB factors exert in regulating the BNBD5 expression. HC11 cells were transiently transfected with p65 or p50 expression vector and the BNBD5 reporter. The result showed that NF-κB p65 transactivated the BNBD5 promoter to 40-fold in HC11 cells relative to mock group (Fig. 24), whereas, NF-κB p50 significantly repressed BNBD5 basal activity from 1 to 0.5 in the same cell line. In conclusion, the patterns of p65 activation and p50 repression to the BNBD5 promoter are comparable to those of the LAP promoter as described in the previous part (cf. Fig. 13).

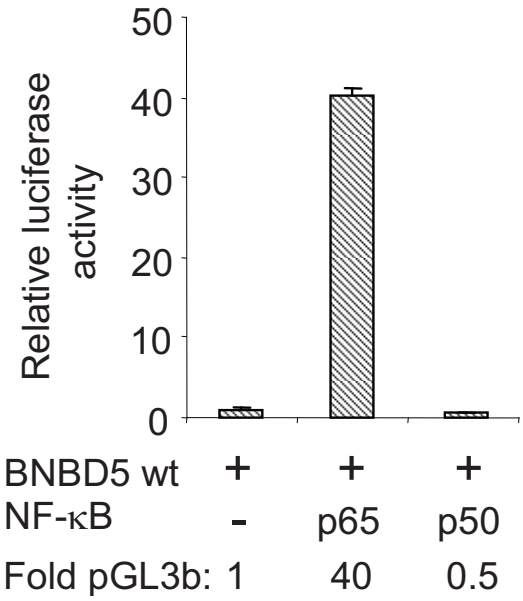


Figure 24. Functional analysis of NF-κB in the BNBD5 promoter

HC11 cells were transiently transfected with the wild-type BNBD5 reporter and equal amounts of NF-κB p65 or p50 expression vector. Luciferase activities from whole cell lysate were assayed at 48h after transfection and were shown as the mean ± S.E.M relative to the wild-type BNBD5 promoter.

3.5.3 Both the NF-κB-like site and the C/EBP-like site3 in the BNBD5 promoter differ from the canonical sites in the LAP promoter

To find the reason for the differential levels of expressions between the BNBD5 promoter and the LAP promoter, the attachment sites of NF-κB and C/EBP transcription factors in the two promoters were aligned. The data show that both C/EBP site1 and site2 are well conserved in the two promoters despite the locations are distinct (not shown). Combination with the data

from the LAP promoter, I focused on comparison of the crucial NF-κB/CEBP3 overlapping site (Table 3). A canonical NF-κB site (GGGG**A**TTTCC) is encompassed in the LAP promoter, but only a NF-κB-like site (GGGGTTTCC) in the BNBD5 promoter according to the consensus sequence of NF-κB (GGGPuNNPyPyCC, Pu: purine, N: any nucleotide, Py: pyrimidine) (Ivanov et al., 1995; Weih et al., 2001). Comparison of the NF-κB binding sites between the LAP- and the BNBD5 promoters indicates that the fifth base A is missing within NF-κB binding site of the BNBD5 promoter (Table 3). Likewise, Comparison the C/EBP site3 sequence (TTTGGAAA**C)** in minus strand of the BNBD5 promoter with C/EBP consensus sequence T(T/G)NNGNAA(**T/G**) (Akira et al., 1990), the ninth conserved T/G within consensus sequence is changed into C in minus strand of the BNBD5 promoter. Similarly, this alteration results from the absence of the same base A in the NF-κB/CEBP3 overlapping site. Hence, the BNBD5 promoter contains two noncanonical sites, the NF-κB-like site and the C/EBP-like site3.

Combining with the cotransfection data, the absence of single-base A has no effect on the function of NF-κB in the BNBD5 promoter. I wanted to investigate whether the absence of base A influences the binding ability of NF-κB to the BNBD5 promoter.

Table 3. Sequence comparison of the NF-κB/CEBP3 overlapping site between the BNBD5 promoter and the LAP promoter

Promoter	NF-κB/CEBP3 overlapping site(+ strand)
LAP	GGGG**A**TTTCC**ACA** (-89/-77)
BNBD5	GGG**G** TTTCC**AAA** (-191/-181)

Note: the positions in the LAP promoter were determined relative to the major transcription start site (in the study). For the BNBD5 promoter, the transcription start site s1 was used as a reference (Yang et al., 2006). NF-κB binding sites are indicated in underlined letters. The core sequences of C/EBP site3 in plus strand are shown in reverse arrows.

3.5.4 NF-κB factors bind with lower affinity to the BNBD5 promoter than to the LAP promoter

To explain the above question, EMSA assays were conducted with nuclear extract from the uninduced HC11 cells and 18 h induced cells (Fig. 25). The labeled probes derived from the BNBD5 promoter and the LAP promoter, which contained respective NF-κB binding sites. All complexes in Fig. 25A or Fig. 25B were resolved in the identical gels and nearly equal activity (cpm) of labeled probes was added in the BNBD5 and LAP reactions. In the BNBD5

case, p50 supershift bands were visible and induction increased the binding amount of NF-κB to the BNBD5 promoter (Fig. 25A lane4 vs. lane6). This pattern was comparable with that of the LAP promoter (Fig. 25A right panel). However, it was obvious that stronger NF-κB p50 binding bands (3-fold) were found in the labeled LAP probe than the BNBD5 probe. Hence, the binding capability of NF-κB p50 to the LAP promoter is stronger than that to the BNBD5 promoter. In order to contrast the binding fashion of NF-κB p65 to the two promoters, another EMSA (Fig. 25B) was conducted with equal amount of nuclear extract of the induced HC11 cells for 2h, shorter than panel A (Fig. 25A, 18h). Similarly, more NF-κB p65 bound to the LAP promoter than to the BNBD5 promoter (5.8-fold). Hence, the LAP promoter possesses much stronger binding ability for NF-κB p65 than the BNBD5 promoter.

Taken together, the absence of single-base A within NF-κB binding site of the BNBD5 promoter obviously attenuates the NF-κB binding. The low affinity of NF-κB *cis*-element for NF-κB factors may be an important cause of lower basal activity and induction of the BNBD5 promoter.

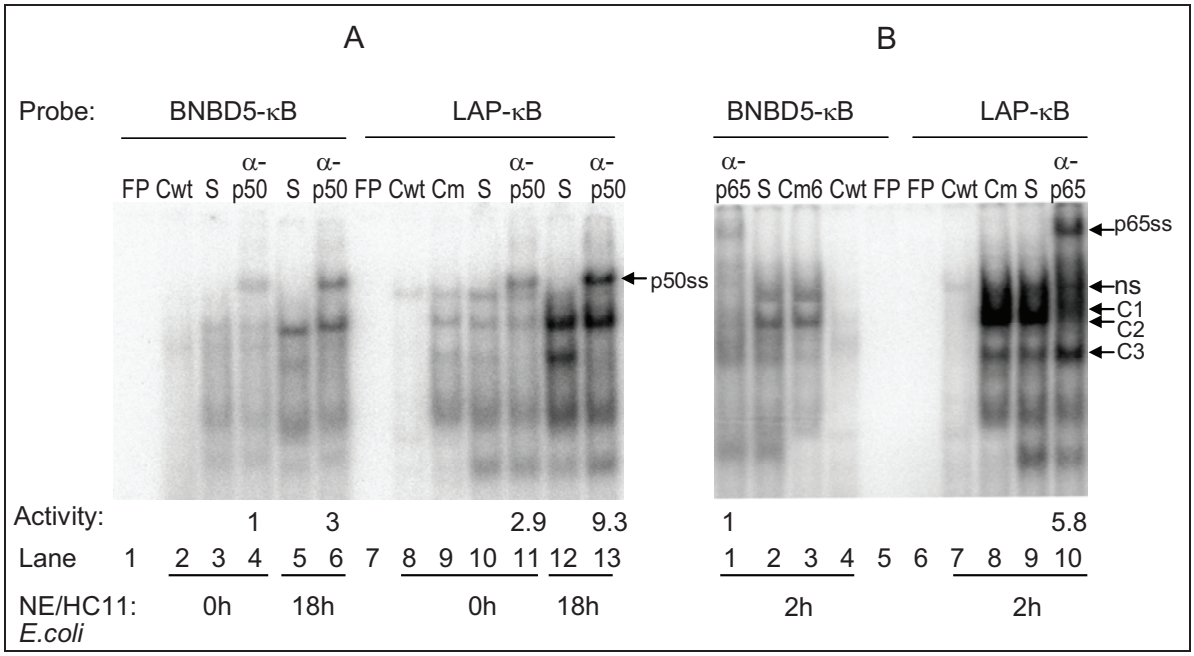


Figure 25. Comparison of binding ability of NF-κB to the BNBD5 promoter and the LAP promoter by EMSA

NEs were prepared from the uninduced HC11 cells and the induced cells for the indicated times. The labeled probes contained NF-κB binding site from the BNBD5 promoter or from the LAP promoter, respectively. For the supershift reactions, antibodies were added prior to the labeled probes. FP: free probe, without NE; Cwt: the wild-type competitor; Cm6: the mutated competitor of NF-κB site in the BNBD5 promoter as described (Yang et al., 2006) Cm: the mutated competitor (κBmTTCC) of NF-κB site in the LAP promoter (in the study); S: shift reaction; α-p65, α-p50: antibodies against p65 and p50; p65 ss and p50 ss: supershift bands indicated as arrows; C1, C2 and C3: NF-κB specific complexes; ns: non-specific band.

Activity was analyzed by Image Quant 5.2 software of Molecular Dynamics and normalized to activity per cpm. A) 5 μg NEs were subjected into the reactions. 1 μg of anti-p50 is from rabbit origin. The activity of p50 ss from control NE to the BNBD5 probe is set as 1. B) 10 μg of NEs were explored except for FP. anti-p65 was added in the supershift reactions. The activity of p65 ss from 2h NE to the BNBD5 probe is set as 1.

In conclusion, for the late immune β-defensin genes, two main regulatory events happen *in vivo*. Firstly, induction decompacts chromatin which hereby becomes accessible for transcription factors. Secondly, NF-κB p65 is selectively recruited to the native chromatin and activates expression of the late β-defensin genes. During the recruitment, affinity of promoters for NF-κB p65 limits the expression of β-defensin genes, and C/EBP factors play the repressive role by diminishing the activation of NF-κB p65.

3.6 Transcriptional regulation of the early-immune-gene IL-8

Considering the differential responses of proinflammatory genes and late immune genes to extracellular pathogen stimulus, I investigated the regulatory mechanism of the early-immune-gene IL-8.

3.6.1 Definition of the IL-8 promoter

3.6.1.1 Time course of the IL-8 induction

As described in section 3.1.2, I constructed the wild-type IL-8 promoter reporter gene, to confirm the inducibility of the construct and to select an optimal time span of induction, I analyzed the relative IL-8 promoter activity at the different induction times (10min, 20min, 30min, 1h, 3h and 24h) in HC11 cells. The result showed that the IL-8 promoter activity did not increase until 1h after induction (Fig. 26 insert), and subsequently increased rapidly and reached maximal activity at 6h after induction. The continuous prolongation of the induction time reduced the IL-8 promoter activity and 69 % of the maximal activity was kept at 24h after induction (Fig. 26). Hence, I adopted 6h as time span for treatment with the inducer throughout the study. Furthermore, the data also indicate that the time course of the IL-8 promoter activity in reporter gene assay in HC11 cells differs from that of the transcription of IL-8 in pbMEC cells (Fig. 6). Maybe, the transfected and "naked" promoter conceivably recruits a complement of TFs differing from that of the promoter of the residing endogenous gene.

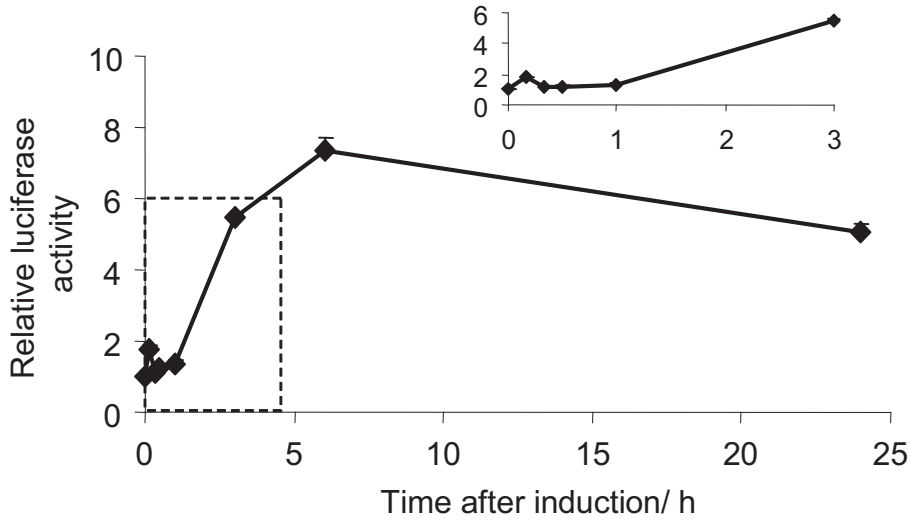


Figure 26. Induction of the IL-8 promoter

HC11 cells were transiently transfected with the wild-type IL-8 promoter and induced at the different time points. The duration of pathogen in cells ranged from 10min to 24h. The cells were lysed with 100 µl passive lysis buffer (Promega) for 20 min at room temperature. The supernatant of lysate was measured firefly luciferase activity. The uninduced luciferase

activity is set as 1. The relative luciferase activity is given in y-axis. The experiment was duplicated independently and a representative experiment is shown. The error bar (S.E.M.) is from 3 parallel assays. The alteration of luciferase within 3h after induction is shown by insert.

3.6.1.2 Determination of the transcription start site (+1) of the IL-8 gene

A similar strategy as applied for analysis of the regulation of the late-immune-gene was used to analyze the transcriptional regulation of the IL-8 gene. A 5′ RACE amplification experiment was applied to determine the transcription start site of the IL-8 gene. The 5′ UTR identified here conforms by 100% with the *Bos taurus* chromosome 6 genomic contig (Sequence # NW_001495205.2) as deposited in the EMBL database. The TATA box lies in -29/-24 upstream the transcription start site (+1). The start codon of translation ATG resides at position +85 (Fig. 27).

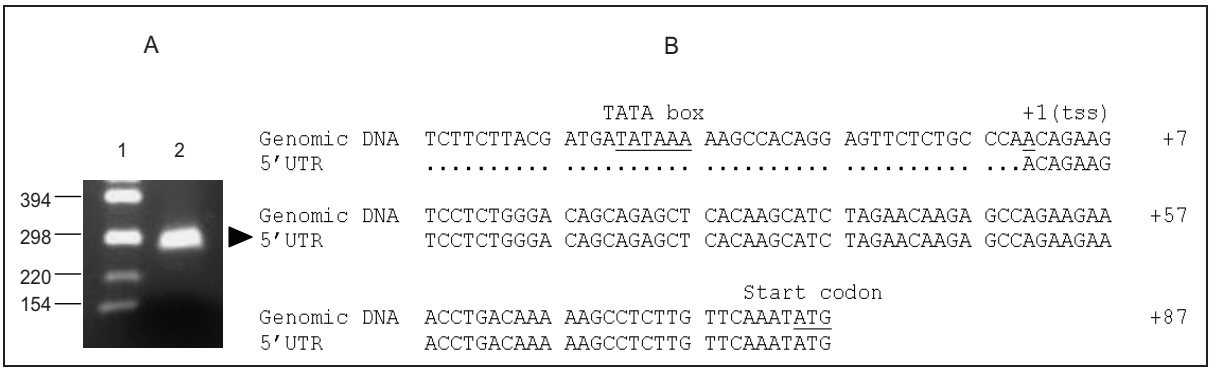


Figure 27. The determination of the transcription start site (+1) of the IL-8 gene

Total RNA, which derived from pbMEC induced for 1h with 1×10^7/ml heat-inactivated *E. coli* strain 1303, was dephosphorylated, decapped sequentially. The RNA Oligo was ligated to decapped mRNA and reversely transcribed into cDNA. The resulting cDNA end was amplified by primary and nested PCR. The strongest fragment was purified and ligated into pGEM®-T Easy. Five clones were sequenced and the sequences were blasted with bovine genomic DNA. A) 2% agarose gel stained by ethidium bromide, lane 1: DNA marker, lane2: the nested PCR products amplified with the GeneRacer™ 5′ nested primer and the IL-8 specific reverse primer (s1112, IL8_R3). B) Alignment of 5′UTR and genomic DNA, the numbers indicate the position of the last base in each line.

3.6.1.3 *In silico* identification of putative transcription factor binding sites in the IL-8 promoter

Analysis of the promoter sequence with the MatInspector software reveals four putative AP1 binding sites, two NFAT binding sites, three C/EBP binding sites and one NF-κB binding site in the retrieved 1389 bp-IL-8 promoter. The C/EBP binding site closest to the transcription start site (+1) is designated C/EBP site1. Among three potential C/EBP sites, C/EBP site2 is overlapping with a NF-κB binding site in the proximal IL-8 promoter (Fig. 28).

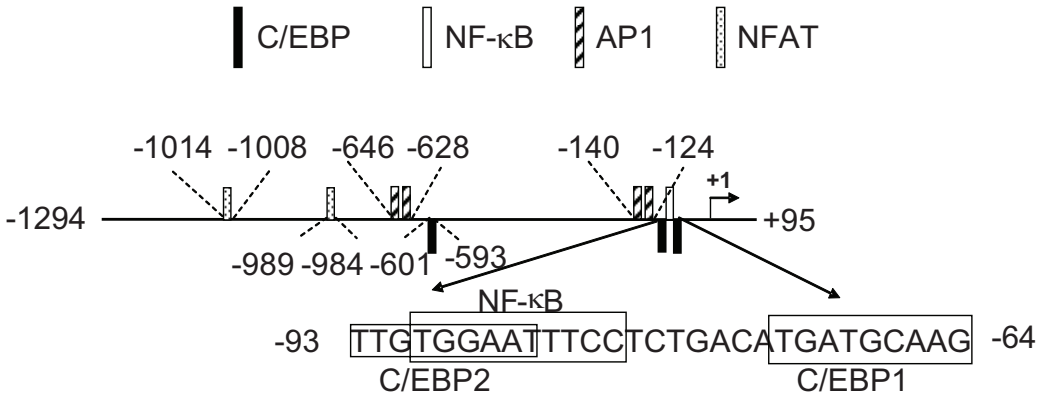


Figure 28. Analysis of putative transcription factors bind to the IL-8 promoter

The binding sites of putative transcription factors were analyzed with software MatInspector in http://www.genomatix.de. The transcription start site (+1) was determined by 5′ RACE.

3.6.2 Experimental identification of *cis*-elements crucially relevant to IL-8 expression and induction

To define the elements relevant for IL-8 expression and induction, a series of deletion plasmids were constructed on the basis of computational analysis of putative transcription factors and were transiently transfected into HC11 cells. After induction for 6h with heat-inactivated *E. coli*, the IL-8 reporter gene activity was found to be 5.3-fold higher than that of the uninduced control (Fig. 29). Serially shortening the promoter from -1294 down to -345 bp altered neither the basal activity nor the extent of activation. However, deleting the area between -121 and -56 bp effectively diminished both basal and induced promoter activity. The importance of this short element was verified by selectively removing the short segment of 29 bp out from the wild-type reporter plasmid (-1294). This short deletion (ΔC/κB) completely abolished the promoter activity. One putative NF-κB site and two proximal C/EBP sites are included within the short segment. Hence, the 29 bp segment (-93/-64) with putative NF-κB and two C/EBP binding sites is pivotal for IL-8 basal activity and induction (Fig. 29).

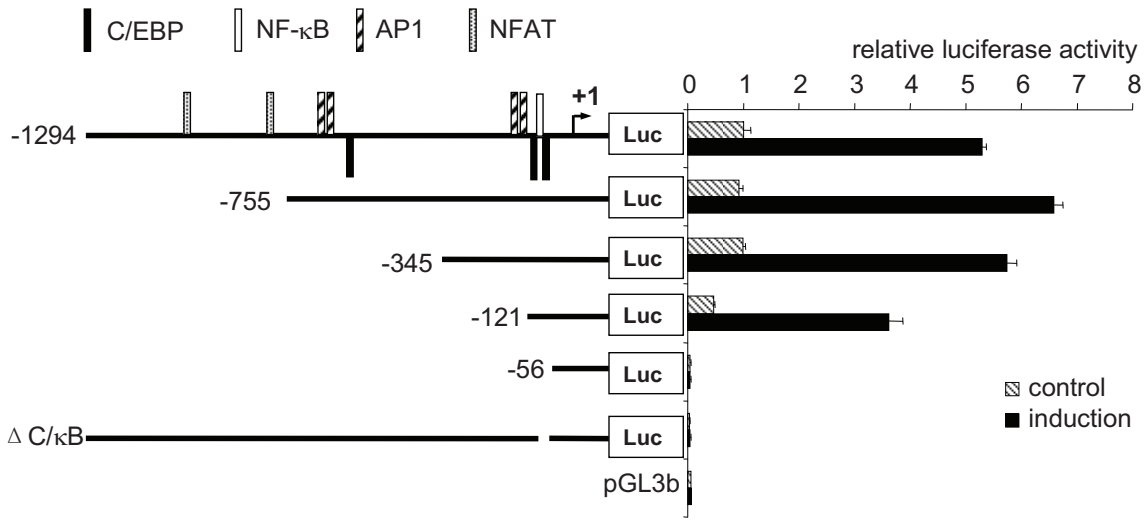


Figure 29. Identification of elements relevant to IL-8 expression and induction in HC11 cells

Schematic representative of 5′ serial deletions and the deletion (ΔC/κB) of the proximal region of the IL-8 promoter is shown on the left. The names of clones are indicated on the left. The relative positions of various transcription factors are shown by vertical rectangle with different fills on the wild-type IL-8 promoter (-1294). Except that clone ΔC/κB deletes 29 bp of the proximal region, the other IL-8 promoter luciferase reporter constructs contain different 5′ ends and the same 3′ end at + 95 bp relative to the transcription start site. HC11 cells were transiently transfected with 1 μg of the wild-type IL-8 luciferase reporter constructs or serial deletion constructs. 42h after transfected, cells were induced for 6h with 1×10^7 /ml heat-inactivated *E. coli* 1303. The luciferase activity of the wild-type IL-8 promoter is set as 1.

3.6.3 Induction quickly converts NF-κB p65/p65 homodimers to p65/p50 heterodimers i*n vivo*

The transcription of the IL-8 gene is already increased strongly at 1h after induction by *E. coli* (Fig. 6). I wanted to know whether the promoters of more cells are occupied by RNA polymerase II molecules at the same time point *in vivo*. To this end, I analyzed the recruitment of RNA polymerase II to the IL-8 promoter with the ChIP assay. The data showed that induction led to more cells (4-fold) opening their promoters for RNA Polymerase II relative to the uninduced cells (not shown). Hence, the assay provides convincing evidence that an increase of cells having recruited RNA polymerase II is the cause of stronger transcription at 1h after induction.

Furthermore, I analyzed the recruitment of NF-κB p65, p50 and C/EBPβ to native IL-8 chromatin. I precipitated the chromatin with specific antibodies (anti NF-κB p65, p50 and C/EBPβ) and non-specific control IgG. ChIP assay showed that NF-κB p65, but not p50 occupied the inactive IL-8 promoter. However, induction quickly recruited p50 to the active

promoter. After induction for 24h, no NF-κB bound to the IL-8 promoter, and the IL-8 promoter went back to basal activity (Fig. 30A). Therefore, induction alters the composition of NF-κB from p65/p65 homodimers on the quiescent promoter to p65/p50 heterodimers on the maximally stimulated IL-8 promoter. Surprisingly, cells having recruited C/EBPβ were not significantly increased after 1h stimulation with heat-inactivated *E. coli* (Fig. 30B).

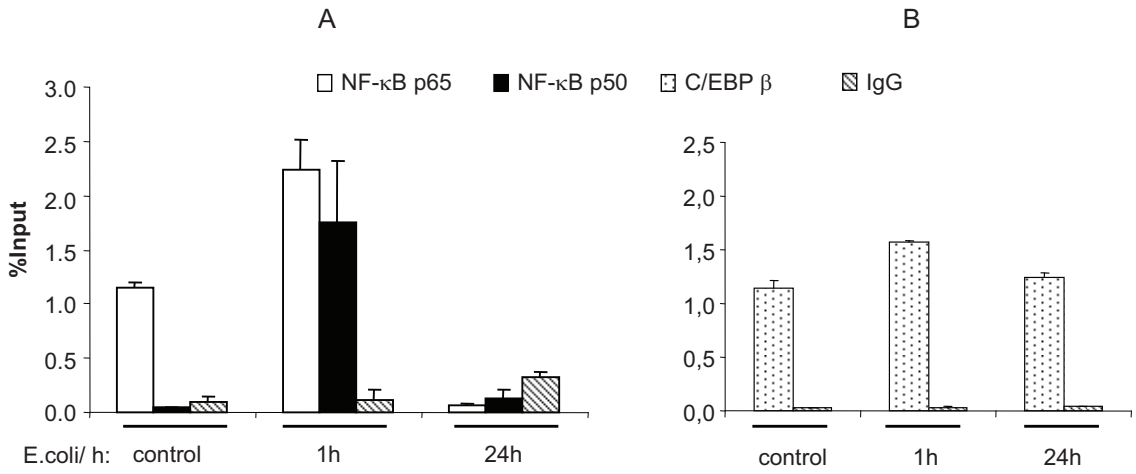


Figure 30. ChIP analysis of recruitment for NF-κB and C/EBP to the IL-8 promoter in pbMEC

ChIP assays were carried out with chromatin from control and the induced pbMEC cells as the indicated times with heat-inactivated *E. coli* 1303. Antibody against NF-κB p65, NF-κB p50, C/EBPβ or control IgG is indicated. Immunoprecipitated DNA was amplified by quantitative real-time PCR with specific primers for the IL-8 promoter.

3.6.4 Functional analysis of NF-κB or C/EBP in the IL-8 promoter

3.6.4.1 NF-κB regulates the IL-8 promoter activity in a cell-type specific fashion

To examine the function of NF-κB in regulation of the IL-8 gene, equal or increasing amounts of the expression vectors for NF-κB p65 or p50 were cotransfected into pbMEC or HEK 293 cells, together with the reporter gene driven by the wild-type IL-8 promoter. In the human embryonic kidney cell line, HEK293, NF-κB p65 strongly activated the IL-8 promoter to 3.5-fold over the untreated level, and p50 did not affect the promoter activity (Table 4). This result could be expected, since NF-κB p65 is known to have a transactivation domain, which is deficient in NF-κB p50.

Table 4. The effect of NF-κB factors on the IL-8 promoter in HEK293 cells

	NF-κB		
	-	p65	p50
IL-8 reporter activity	1.00±0.05	3.54±0.08	1.04±0.01

1 μg of NF-κB p65 or p50 expression construct was cotransfected in human embryonic kidney cell (HEK293) together with 1μg of the wt IL-8 reporter. The amount of total DNA was kept constant by supplement with empty vector pCDNA 3.1+.

Surprisingly, NF-κB factors diminished the IL-8 promoter activity in pbMEC (primary bovine MEC) cells. With the increasing amounts of transfected p65 or p50 from 0.1, 0.5 to 1.5 μg, the basal activity and extent of induced expression of IL-8 were reduced in a dose-dependent manner. The effect of p65 was quicker than that of equal amounts of p50 (Fig. 31). Taken together, both NF-κB p65 and p50 regulate the IL-8 promoter activity in a cell-type specific manner.

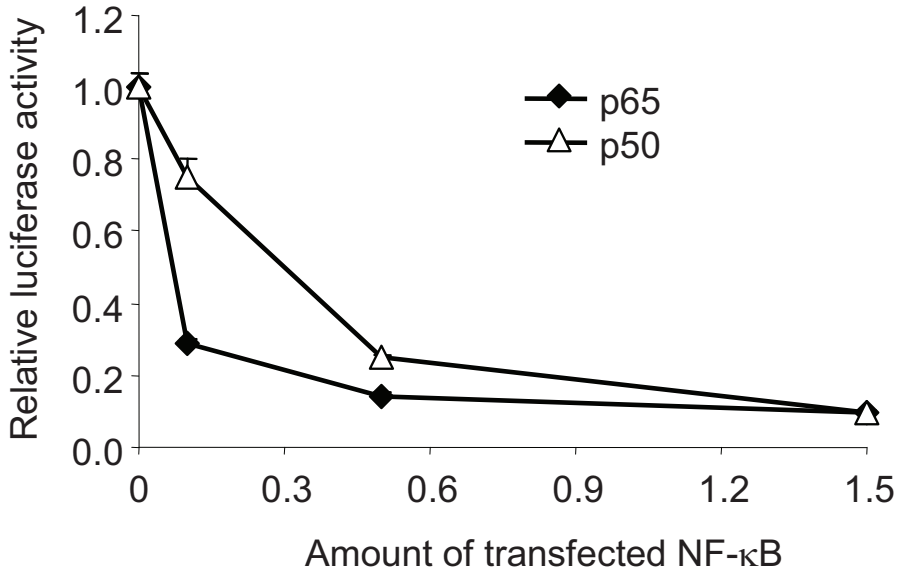


Figure 31. NF-κB regulates the IL-8 promoter activity in a cell-type specific manner

Constant amount of the wt IL-8 reporter and increasing amounts of NF-κB p65 or p50 expression construct (0.1, 0.5 and 1.5μg) were cotransfected into primary bovine MEC (pbMEC). The activity of the wt IL-8 promoter is always set as 1. The other details were described in Materials and Methods.

To validate the observation, I preincubated pbMEC cells with the inhibitor PDTC (pyrrolidine dithiocarbamate), known to block NF-κB activation, and evaluated the alteration of IL-8 transcription by real-time PCR. The data showed that IL-8 was de-repressed and mRNA copy

numbers were increased to 6 fold after preincubation with PDTC (Fig. 32). This provides further evidence that NF-κB represses IL-8 basal expression in MEC cells.

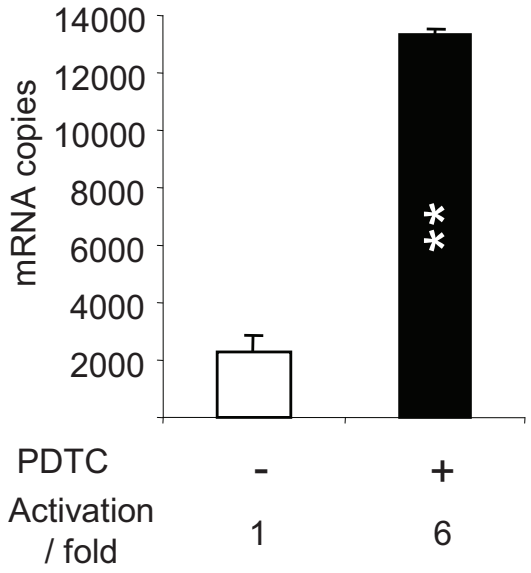


Figure 32. Effect of NF-κB inhibitor PDTC on IL-8 transcription in pbMEC

pbMEC cells were pretreated with 50 μM PDTC for 1h. Total RNA was prepared with TRizol reagent, and reversely transcribed into cDNA. mRNA copy numbers were measured by real-time PCR as described in Materials and Methods.

To determine the relevant site of the transfected NF-κB effect in IL-8 regulation, clone Δ C/κB lacking NF-κB binding site was cotransfected into HC11 cells together with the expression construct of p65 or p50. Compared with the wild-type IL-8 promoter, the mutant blocked the cell-type specific effect of NF-κB completely (not shown). The data show that the region from -93 to -64 is the acting site of NF-κB factor.

Hence, NF-κB regulates IL-8 expression via motif -93/-64 in a cell-type specific fashion.

3.6.4.2 C/EBPs activate IL-8 expression mainly through the proximal C/EBP sites

To inspect the function of C/EBP members in the IL-8 expression, HC11 cells were transiently transfected with equal amounts of full length C/EBPs expression vectors (α, β, δ and ε) or equal amounts of DN-C/EBPs (Dominant Negative-C/EBPs) expression vectors, together with the wild-type IL-8 reporter (clone -1294). The data showed the overexpressed C/EBP members (α, β, δ and ε) markedly activated the IL-8 promoter with the different efficiencies (Fig. 33A) in HC11 cells. The activation folds of the respective factors (the luciferase activity of the untreated is set as 1) were 20-, 10-, 7-, 2-fold for C/EBP-α, -ε, -β,

and -δ, respectively. Hence, the amount of endogenous C/EBP is a rate-limiting factor to the IL-8 promoter. The similar activation (2-fold activation) is obtained in pbMEC cells cotransfected with C/EBPβ (not shown). In contrast, DN-C/EBP members diminished IL-8 activity due to the absence of transactivation domains (not shown). Hence, the transactivation domains of C/EBPs are indispensable to activate IL-8 expression in MEC.

Furthermore, to decide the main acting site of C/EBP factors, the mutant ΔC/κB was cotransfected together with C/EBP factors into HC11 cells. C/EBP-mediated activation to the IL-8 promoter was decreased dramatically relative to those of the wild-type promoter (Fig. 33B). However, C/EBPα still activated the mutant ΔC/κB to 5.2-fold relative to the activity of the wild-type IL-8 promoter, even if the two proximal C/EBP sites were deleted. This shows that C/EBPα activates the IL-8 promoter not only by the proximal sites but also by the distal site (-601/-593, Fig. 28). Taken together, the two proximal C/EBP sites from -93 to -64 are the main acting sites of C/EBPs.

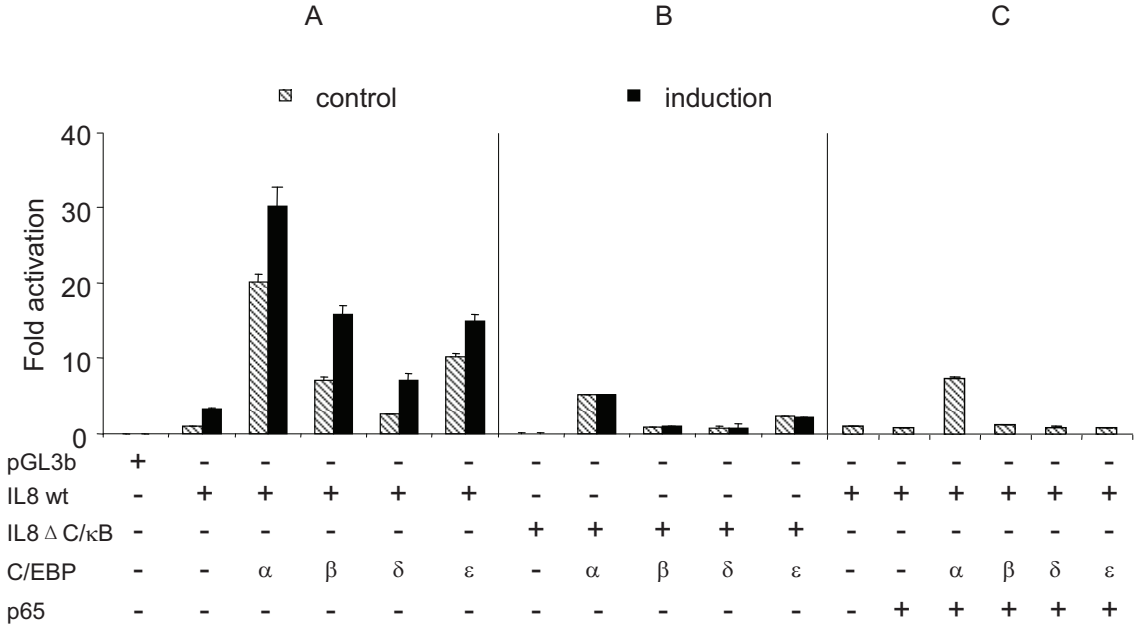


Figure 33. Effect of C/EBPs alone or combined with NF-κB p65 on the IL-8 promoter in HC11 cells

HC11 cells were transiently transfected with 1 µg of the wild-type IL-8 reporter (clone -1294) (A or C) or the deletion-construct, IL-8 ΔC/κB (B) and 1µg of C/EBPs expression vectors and/or equal amounts of NF-κB p65. Cells were induced for 6h with heat-inactivated *E. coli* 1303 before harvest (black column) or without treatment (hatched column). Fold activation was presented relative to the luciferase activity of the IL-8 promoter. Total amount of transfected DNA was kept constant by adding empty vector. After transfection for 48h, cells were lysed and assayed luciferase activity according to the manufacturer's manual.

3.6.5 NF-κB p65 attenuates the C/EBP-mediated activations of the IL-8 promoter

To analyze synergistic effects of C/EBPs (α, β, δ and ε) and NF-κB p65 on the IL-8 promoter, the wild-type IL-8 reporter gene was cotransfected with or without NF-κB p65 and C/EBPs expression vectors into HC11 cells. Compared Fig. 33C with Fig. 33A, NF-κB p65 reduced the activation of C/EBP factors on the IL-8 promoter. Only C/EBPα kept its partial potential to activate the IL-8 promoter in the presence of p65. Other C/EBP-β, –δ and -ε had no obvious effect on the IL-8 promoter activity in HC11 cells at the same condition (Fig. 33C).

The data together show that overexpression of NF-κB p50 quenches the IL-8 promoter activity in pbMEC cells, also in HC11 cells. However, NF-κB p65 has different effects in pbMEC cells compared with in HC11 cells on the IL-8 promoter activity. In pbMEC cells, p65 always and strongly reduces the IL-8 promoter activity. In HC11 cells, NF-κB p65 does not alter the IL-8 promoter activity (not shown). Furthermore, it disrupts the activating effect of the C/EBP-β, –δ and –ε. Taken ChIP data that NF-κB p65 homodimers reside on the resting IL-8 promoter together, NF-κB p65 homodimers insulate the IL-8 promoter against active C/EBP factors by the proximal site.

4. DISCUSSION

4.1 The concepts in inflammatory/immune response and mastitis

TLR receptors (Fig. 34) recognize pathogens and activate downstream adaptor molecules such as MyD88 or TRIF (Akira and Takeda, 2004). As a result, latent cytoplasmic NF-κB factors are activated and translocate into the nucleus. NF-κB heterodimers or homodimers recognize the cognate binding site (Leung et al., 2004) and induce expression of NF-κB-dependent genes. These include inflammatory mediator genes, such as IL-8, TNFα, and also effector genes of innate immunity, such as β-defensin encoding genes. IL-8, as a chemoattractant, recruits neutrophils to the site of inflammation (Fig. 34). Afterwards, neutrophils release their toxic contents. Since the toxic contents do not discriminate between the extracellular pathogen and the host itself, the collateral damage to host is unavoidable (Nathan, 2002). Hence, the inflammatory response of the host must be tightly regulated. However, on the other hand, the expression of antimicrobial peptide encoding genes, such as LAP and BNBD5, protects the host from infection. Hence, it is valuable to investigate the precise modulation of the innate immune response and inflammation for selective control.

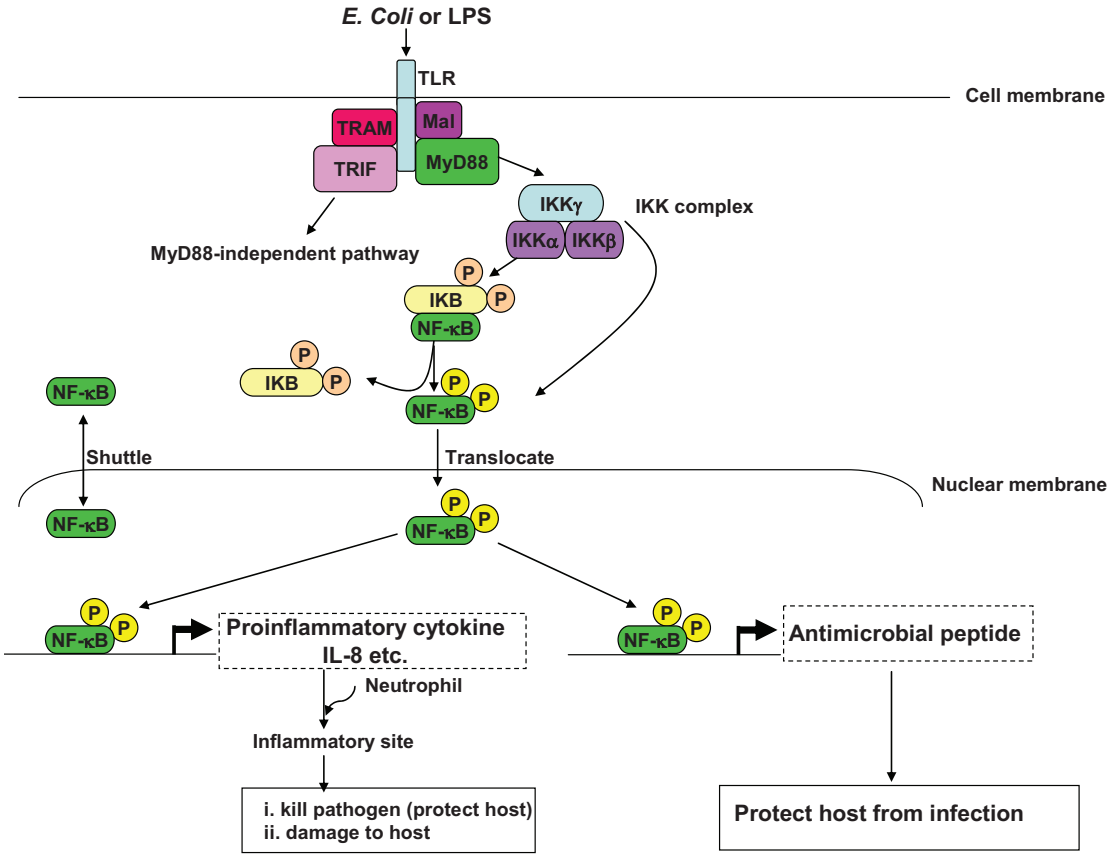


Figure 34. The activated TLR signalling pathway by *E. coli* or LPS

Moreover, chronic mastitis, as a prevalent inflammation in dairy cows, also results from the interplay between host and pathogen. In order to find alternative strategies to decrease the incidence of chronic mastitis, one needs to understand the key regulatory event of inflammatory and innate immune response in the mammary gland. To date, little is known about regulation of inflammation and immune defence in this organ.

It has been known that mammary epithelial cell (MEC) is the dominant cell type in parenchyma of mammary gland. It is a front line which meets pathogens invading the mammary gland. Its relevance for inflammation surveillance and also for immune defence has only recently been well appreciated (Gunther et al., 2009; Strandberg et al., 2005). Hence, MEC is suitable cell model for exploring the immune and inflammatory response mechanism of mammary gland.

Proinflammatory cytokine and antimicrobial peptide are typical samples of NF-κB-dependent target genes and play completely different roles in host defence system. In bovine MEC, *E. coli* strongly induces the expression of proinflammatory cytokine IL-8 (Yang et al., 2008), and LAP is the dominant antimicrobial peptide in the identical cells (Wei Yang's dissertation). Therefore, in the study, I chose the IL-8 and the LAP gene as paradigms for "sentinel" and "effector" respectively to identify at the molecular level the key regulators for mammary immune defence and inflammation.

4.2 NF-κB and C/EBP factors are crucial for inflammation and innate immune response

In the present study, four lines evidences indicate that both NF-κB and C/EBP factors are crucial for inflammatory and immune response genes (IL-8 and LAP) expression.

Firstly, serial deletion and mutation analyses indicate that both NF-κB and C/EBP binding sites are essential for basal activity and induction of early (IL-8) and late (LAP) immune responsive genes in bovine (Fig. 9, 10 and 29).

Secondly, NF-κB and C/EBP can bind to the LAP probe in EMSA assay (Fig. 11 and 14). Thirdly, ChIP data show that only p65 is recruited to the activated LAP promoter (Fig. 12). Differently, the active IL-8 promoter is occupied with p65/p50 and C/EBP factors (Fig. 30). Finally, cotransfections of NF-κB or C/EBP expression vectors demonstrate that they play important roles in both IL-8 and LAP activation (Fig. 13, 15, 31 and 33A).

In agreement with the current findings, NF-κB is long known to induce multiple inflammatory genes expression, for example, pro-inflammatory cytokines IL-8 (refers to the following section), IL-6 (Libermann and Baltimore, 1990), GM-CSF (Cakouros et al., 2001), adhesion molecules [ICAM-1] (Rajan et al., 2008). As stated in the introduction section,

C/EBP is also known as key trans-acting factors in inflammation and immune response. C/EBPβ-deficient mice impair expression of proinflammatory cytokine IL-12, TNFα, G-CSF etc. (Screpanti et al., 1995; Tanaka et al., 1995). C/EBPβ and NF-κB p50 are also implicated in cytokine-induced regulation of the CRP encoding gene (Singh et al., 2007).

4.3 Roles of AP1, NF-κB and C/EBP factors in IL-8 regulation

Extensive studies were focused to elucidate the roles of transcription factors in the regulation of human IL-8 expression. However, little is known about their roles in regulating the expression of the bovine IL-8 encoding gene, especially in the MEC. Alignment of the bovine and the human IL-8 promoters reveals that the binding sites of AP1 and NF-κB share 100 % identity between the two species. In the bovine proximal IL-8 promoter, there are two C/EBP binding sites (C/EBP site1 and C/EBP site2). C/EBP site2 overlaps with NF-κB site at -93/-81, and C/EBP1 stands alone. In contrast, the human IL-8 promoter only features one C/EBP site residing upstream of the NF-κB binding site, not overlapping with the latter one (Roebuck, 1999). This indicates that the two transcription factors may play different roles in the two species in regarding the regulation of the IL-8 promoters.

Several previous studies show that NF-κB, C/EBP and AP1 display distinct roles in the human IL-8 regulation, dependent on different cell-types and various stimuli (Roebuck, 1999). For example, induction of the human IL-8, by IL-1β, phorbol myristate acetate (PMA), *Pseudomonas aeruginosa* or TNFα, requires synergistic activation of NF-κB and C/EBPβ (Kunsch et al., 1994; Mukaida et al., 1990; Stein and Baldwin, Jr., 1993; Venza et al., 2009). AP1 is involved in the human IL-8 activation in response to an IL-1β or a bradykinin stimulus in human vascular or airway smooth muscle cells (Jung et al., 2002; Zhu et al., 2003). In contrast to these reports, the current result shows that the deletion encompassing two proximal AP1 binding sites does not obviously influence the bovine IL-8 induction in MEC, and only slightly decreases basal activity (Fig. 29). It seems that AP1 is not essential for the bovine IL-8 regulation after stimulation of MEC by heat-inactivated *E. coli*. However, it was reported that both AP1 and NF-κB binding sites are responsible for activating the human IL-8 gene expression in other epithelial cell lines (Okamoto et al., 1994; Yasumoto et al., 1992). This discrepancy may result from i) different *cis*-elements of the IL-8 promoter in the two species; ii) cell-type specificity; iii) stimulus specificity; or iv) all these differences together.

4.3.1 Role of NF-κB factors in bovine IL-8 regulation

My results show that NF-κB p65/p65 homodimers block IL-8 basal expression. In support of this, ChIP experiments conducted *in vivo* in bovine MEC cells indicate that NF-κB factors p65 homodimers, but not p65/p50 heterodimers reside on the resting IL-8 promoter. Consistent with the observation, functional analyses show that NF-κB p65 represses the IL-8 promoter in pbMEC cells. PDTC, extensively used as an inhibitor of NF-κB activation (Brennan and O'Neill, 1996), allowed to further evaluate the effect of NF-κB on the IL-8 promoter activity. The data indicates that after NF-κB activation is blocked by inhibitor PDTC, the IL-8 promoter is derepressed and the mRNA expression is increased to 6-fold (Fig. 32). All these results show that p65/p65 homodimers block IL-8 basal expression. This is in contrast to the previous notion that NF-κB complexes are absent in nuclei and solely sequestered in cytosol in the unstimulated cells. However, as shown in Fig. 20, NF-κB is present in substantial amounts in nuclei of the resting cells amounting to 20-30% of that at 2h after induction. In agreement with the observation, recent findings show that NF-κB dynamically shuttles between cytosol and nucleus (Birbach et al., 2002; Huang et al., 2000; Tam et al., 2000). Therefore, a steady state and low level of NF-κB p65 is always present in the nuclei irrespective of the presence or absence of stimulus. Furthermore, Wada et al. reported that p65/p65 homodimers have DNA binding activity (Wada et al., 2001). Hence, it is reasonable to find that NF-κB p65 resides on the resting IL-8 promoter.

However, p65 is known to contain transactivation domain necessary to activate genes (Schmitz and Baeuerle, 1991). The key issue is which mechanism makes NF-κB p65 repress but not activate the basal transcription of IL-8. It is possible that NF-κB p65 interacts with transcriptional corepressor factors to block the IL-8 gene activity. Lee et al reported that the cotransfection of SMRT corepressor (the Silencing Mediator of Retinoic acid and Thyroid hormone receptors) together with p65 decreases p65-mediated activation (Lee et al., 2000). Another supportive report is that HDAC1, known to have deacetylation activity, is involved in NF-κB p65-mediated repression to the death gene Bnip3 (Shaw et al., 2006). Additionally, HDAC1 interplays with NF-κB p65 and the HDAC inhibitor trichostatin A (TSA) causes an increase of both basal and induced expression of the hIL-8 promoter (Ashburner et al., 2001). More evidences confirm that deacetylation of p65 represses genes expression (Yeung et al., 2004).

More importantly, the occupancy of p65/p65 homodimers in the IL-8 promoter also provides a clue that the chromatin of the IL-8 promoter in the resting pbMEC cells appears to be accessible to transcription factors.

In contrast to NF-κB p65/p65 homodimers binding to the resting promoter, only stimulus-induced NF-κB p65/p50 heterodimers are necessary for bovine IL-8 activation. Since ChIP data indicate that induction rapidly alters NF-κB p65/p65 homodimers into NF-κB p65/p50 heterodimers (Fig. 30A). NF-κB p65/p50 heterodimer is reported to be the most abundant form of NF-κB and is known to act as a strong transactivator (Chen and Ghosh, 1999). I surmise that the repressive NF-κB p65 in resting cells seems to be different from the p65 molecule in the active state. NF-κB p65 binding to the active promoter has possibly been phosphorylated. Four phosphorylation sites (Ser^{276} and Ser^{311} in RHD and Ser^{529} and Ser^{536} in TAD) were found to be crucial for the complete activation of p65 (Duran et al., 2003; Perkins, 2006; Wang et al., 2000; Zhong et al., 1998). The previous report also indicates that LPS stimulation causes the nuclear translocation of the NF-κB complexes containing phosphorylated p65 by PKA to activate transcription (Zhong et al., 2002).

4.3.2 Role of C/EBP factors in bovine IL-8 regulation

A well documented event is that C/EBP factors are involved in inflammatory response and immunity (Cloutier et al., 2009; Singh et al., 2007). In agreement with these findings, my data demonstrate that C/EBP activates the proinflammatory gene IL-8 expression following a pathogen stimulus. Transient transfection assays show that different C/EBP factors activate the bovine IL-8 promoter at different extents. The activation potentials for the factor C/EBP-α, –ε, –β and –δ in descending order are 20-, 10-, 7- and 2-fold, respectively (Fig. 33A).

Surprisingly, ChIP data show that C/EBP factors seemingly unchangedly occupy the IL-8 promoter at all times irrespective of induction (Fig. 30B). As my study has been completed, a recent observation showed that the C/EBPβ factor is constitutively bound to the human IL-8 promoter, both in resting and LPS-activated neutrophils (Cloutier et al., 2009). This observation perfectly matches my finding. I surmise that C/EBP seems to be activated by phosphorylation. Interestingly, C/EBPβ is induced by phosphorylation of Thr^{235} by a Ras/ MAPK pathway (Nakajima et al., 1993) and the inductive expression of IL-8 is regulated by a mitogen-activated protein kinase pathway (MAPK) (Jung et al., 2002; Smith et al., 2001).

Alternatively, it is also known that C/EBP factors have multiple isoforms from different AUG initiation codons with the same open reading frame (Welm et al., 1999). Among them, C/EBPβ has three dominant isoforms and various isoforms display different transcription

activation potentials (Ossipow et al., 1993). Conceivably, selective binding of different C/EBPβ isoforms leads to the different activities of the IL-8 promoter before and after stimulation. A supportive evidence is that synthesis of the different C/EBPβ isoforms is regulated by LPS in liver (An et al., 1996). Combination of different isoforms is dependent on cell type and culture conditions (Descombes and Schibler, 1991). The results suggest that a complicated mechanism regulates synthesis of C/EBP isoforms in a given cell line. More experiments are required to elucidate the issue.

In conclusion, C/EBP but not NF-κB factors activate IL8 activity. NF-κB p65 homodimers insulate the IL-8 promoter against C/EBP factors.

4.4 Molecular mechanism of LAP

Regarding molecular mechanism controlling β-defensin expression, extensive researches have previously seen to understand the regulation of the human hBD-2 and the bovine TAP, BNBD5. Very little is known about regulation of the predominant member of bovine β-defensin in bovine MEC, which is LAP. My study provides a detailed analyses regarding molecular control mechanism of that important late immune responsive gene both at the chromatin level and at the transcriptional level.

4.4.1 Roles of chromatin remodeling and DNA methylation in LAP regulation

A number of genes have been shown to be regulated by chromatin remodeling (Albrecht et al., 2004; Holloway et al., 2003; Rao et al., 2001). Moreover, only delayed accessible genes, but not fast accessible genes, require stimulus-dependent chromatin remodeling to make the NF-κB sites accessible (Saccani et al., 2001). Consistent with the reports, the present study demonstrates that the chromatin structure within the binding region of NF-κB and C/EBP factors is significantly remodeled in the LAP promoter after challenging the udder with live pathogens for 24h. The tightly condensed LAP promoter is opened (Fig. 22). However, a different pattern appears in the cell model pbMEC. The CHART result indicates that the LAP promoter chromatin in pbMEC is not significantly remodeled after induction. The degree of protection of the chromatin at the different conditions (unstimulated, 6h and 21h after induction) is unchanged and maintains at about 85% (data not shown). It seems that in the MEC model cell, chromatin is always open for transcription factors. Thus, the cell model pbMEC can not completely reflect all those physiological mechanism operating in the udder tissue. This assumption is supported by a recent report (Gunther et al., 2009).

It is generally assumed that DNA methylation within the promoter region represses the gene transcription (Nan et al., 1997; Newell-Price et al., 2000). As stated above, the LAP chromatin is decompacted and transcription is more active in response to pathogen-stimulus. However, pathogen invasion causes unusual hypermethylation of CpG site11 (located at -50) contiguous to NF-κB binding site (-89/-80) at the identical time point. That is, in this case, the hypermethylation is associated with chromatin decompaction. This is unexpected. Interestingly, it is well-documented that methylation of the promoter for an immediate-early "frog virus3" encoding gene and adenovirus promoter do not inhibit transcription. Trans-acting protein of the host or the virus-induced can override the inhibitory effect of methylation to facilitate the transcription (Thompson et al., 1986; Thompson et al., 1988). Furthermore, it is also possible that Coactivator-associated Arginine Methyltransferase (CARM1), which is responsible for histone H3 methylation of NF-κB-dependent genes, enhances NF-κB p65 recruitment to the LAP promoter by interaction. The supportive evidence is that CARM1 enhances NF-κB p65 recruitment to the target promoters (Covic et al., 2005). Although many possibilities are given for the unusual case here and someone also argues that the association between DNA methylation and gene silence may not be causal (Li et al., 1992), it remains open for further analyses if and how the observed hypermethylation at the LAP promoter influences the transcription of the LAP gene.

4.4.2 Role of transcription factors in LAP regulation

4.4.2.1 NF-κB p65 drives the late-gene LAP expression

NF-κB factors are crucial in β-defensin regulation. LPS activates the NF-κB p65/p50 heterodimer binding to the NF-κB binding site in the proximal hBD-2 promoter (O'Neil et al., 1999; Tsutsumi-Ishii and Nagaoka, 2002), resulting in upregulation of hBD-2 transcription. Serial deletion of the TAP promoter and EMSA show that NF-κB p50 and p65 are involved in the TAP regulation (Diamond et al., 2000). Another evidence stems also from NF-κB SN50, a peptide inhibiting translocation of the p50-containing NF-κB active complex to the nucleus, it reduces TAP mRNA expression in bovine tracheal epithelial cells (Legarda et al., 2005). A more recent report shows that the bovine BNBD5 induction depends on binding of NF-κB (Yang et al., 2006). In the present study, deletion and mutation analysis of the LAP promoter clearly shows that the NF-κB binding site is crucial for the basal activity of LAP and also for its induction (Fig. 9 and 10). NF-κB factors both p65 and p50 can bind to the site as evidenced in EMSA assays (Fig. 11A). Cotransfection experiments demonstrate that only p65

strongly activates the LAP promoter (Fig. 13). This conclusion is augmented by ChIP analysis. Twenty four hours after induction, the percentage of cells having recruited p65 to the active LAP promoter is dramatically increased. Thus, NF-κB p65 is the major activator for LAP expression. This is convincing, since NF-κB p65 possesses transactivation domain (TAD). This coincides with a previous observation that the fusion construct of full-length p65 sequences to DNA binding domain of the yeast GAL4 transcription factor strongly increased a CAT reporter activity (Schmitz and Baeuerle, 1991). The mutation of Ser^{529} in the TAD of p65 into Ala blocks the ability of p65 to activate transcription in response to TNFα (Wang and Baldwin, 1998).

4.4.2.2 C/EBPs repress the late-gene LAP expression

In contrast to NF-κB, C/EBPs repress p65-mediated activation of LAP expression. In accord with my observations, a previous report has shown that C/EBPα suppresses the NF-κB-mediated transactivation of the HCMV IE1/2 promoter (Human Cytomegalovirus IE1/2 promoter). EMSA analysis has demonstrated that NF-κB and C/EBP synergistically bind to the same oligonucleotide probe and form a heavier complex (Prosch et al., 2001). In contrast, my EMSA assay shows that NF-κB and C/EBP can not bind to the same oligonucleotide molecule (Fig. 19). They may competitively bind to the NF-κB/CEBP overlapping site. Probably spatial hindrance makes the overlapping site inaccessible for simultaneous binding of both factors. This conclusion is supported by another report that the NF-κB-like Bpi protein (a cytokine inducible protein) competes with the constitutive C/EBP-like BPcs protein (constitutive proteins) for the NF-κB/CEBP overlapping sites on the APRE (Acute-Phase Response Element) promoter (Brasier et al., 1990). My EMSA also shows that the LAP promoter has higher affinity for NF-κB factor than for C/EBP factor. It seems that the proportion of endogenous NF-κB over C/EBP factors is another regulatory mechanism in LAP regulation.

4.5 Both the IL-8 and the LAP genes are regulated in a cell-type specific manner

As stated previously, whether a specified transcription factor is involved in the target gene regulation appears to depend on cell types. Now, even if the same factor is involved in the regulation of a given gene, it displays distinguishable functions in different cell types. We chose the predominant factors of NF-κB family, p65 and p50 to address the question. I contrasted the activation extent of the two factors for the target paradigm genes, the IL-8 gene

and the LAP genes, in MEC and in HEK293 cells. The MEC model is the mouse MEC (HC11 cell line). HEK293 is an embryonic kidney cell line. The results are summarized in Table 5. The data shows that, in the case of IL-8, i) p65 displays an opposite pattern in MEC versus in HEK293. p65 significantly decreases the activity of the IL-8 luciferase reporter gene in MEC. Especially in pbMEC, a stronger decrease is observed. Contrarily, in HEK293, p65 obviously increases the activity of the same reporter gene. ii) p50 reduces the IL-8 luciferase reporter gene activity in MEC but has no effect in HEK293. Taken the above data together, the bovine IL-8 gene is regulated in a cell-type specific manner, which is comparable with the human IL-8 regulation (Roebuck, 1999).

Regarding the LAP encoding gene, p65 activates the LAP promoter-driven luciferase activity both in MEC and HEK293, stronger in HEK293 than in MEC. However, p50 regulates the activity in opposite directions contrasted in MEK with in HEK293. Hence, the LAP gene is also regulated in a cell-type specific manner.

The cell-type specific regulation possibly results from the cell-type specific complement of auxiliary transcription factors present in different cell types. These may be mandatory to relay the effect of those key transcription factors (e.g. NF-κB, C/EBP) onto the promoter of the respective target genes.

Table 5. Summary for cell-type specific regulation of the IL-8 and the LAP genes

	MEC		HEK293	
	IL-8	LAP	IL-8	LAP
p65	**Blocked**	**Activated**	**Activated**	**Activated**
p50	**Blocked**	**Blocked**	Neutral	**Activated**

4.6 Two-level tight regulation results in expression in temporal order of different effector genes in inflammatory and immune response

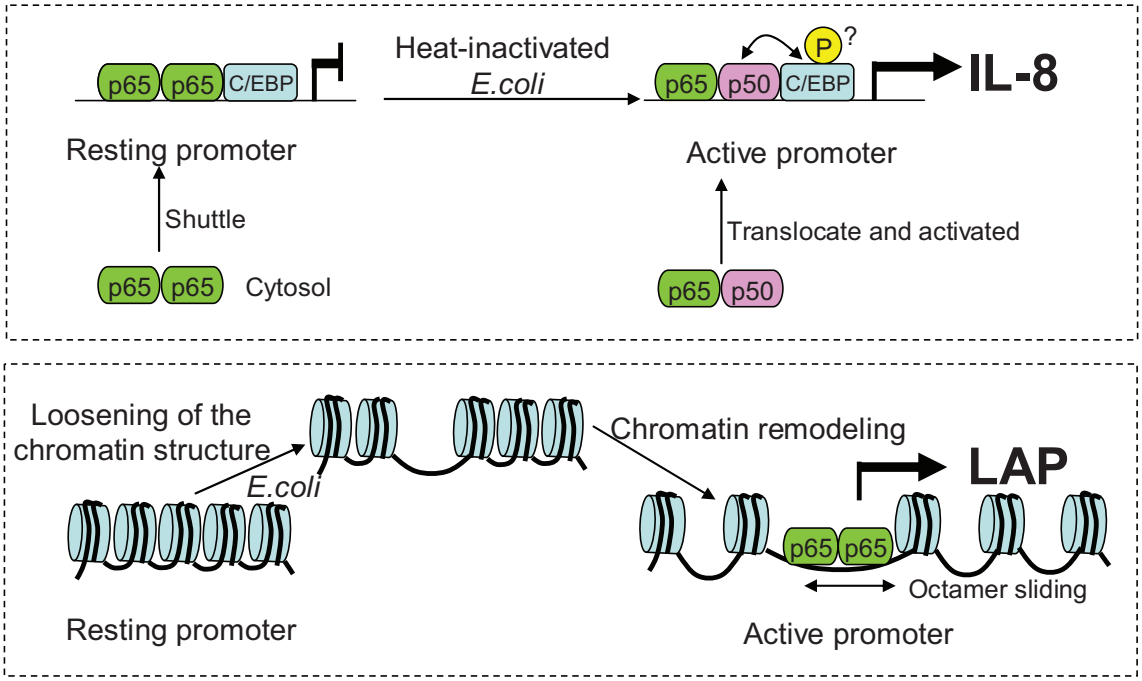


Figure 35. Model for transcriptional regulation of IL-8 and LAP in response to heat-inactivated *E. coli*

On the basis of the present findings, a model is proposed (Fig. 35). As a paradigm for "sentinel" gene, early IL-8 gene initiates rapidly transcription (refer to fig. 6) in response to heat-inactivated *E. coli* stimulation. Once being stimulated, the IL-8 promoter replaces immediately the p65/p65 homodimers from shuttling with the translocated and the activated NF-κB p65/p50 heterodimers. Interestingly, NF-κB p50 is known to physically interacts with C/EBPβ to induce CRP expression (Cha-Molstad et al., 2000). Hence, p65/p50 heterodimers probably interact with the active C/EBP factors to activate pathogen-mediated IL-8 expression. The early gene may enhance its inducible expression by fast post-translation modification of transcription factors. Regulation of the gene expression is rapid and tight.

In contrast, continuous expression of the late induced LAP gene is beneficial for protecting the host from infection. As the present data indicate, the late LAP gene is regulated at two levels. The first one is regulation at the chromatin level. The chromatin is strictly condensed in the resting state, and transcription is almost blocked and the basal expression is very low. Twenty-four hours after challenged with live *E. coli* pathogens, the LAP promoter chromatin is opened (Fig. 22), and NF-κB p65 is recruited (Fig. 12) to activate the LAP promoter activity (Fig. 13). However, chromatin remodeling mechanism cannot be studied in detail, since the most adequate cell model, the pbMEC, apparently does not display these changes in

chromatin compaction at the LAP promoter. Nevertheless, the ChIP data clearly show that induction greatly increases the percentage of cells having recruited p65 onto the LAP promoter. This occurs independently from chromatin remodeling. Unexpectedly, amount of cells having recruited p65 is not obviously increased within 3h after induction (Fig. 12). It is not consistent with the fact that NF-κB molecules in nuclei are nearly the most at the same time point (Fig. 20). Hence, factors independent from chromatin structure are also highly relevant for NF-κB p65 recruitment onto the LAP promoter.

Extensive evidences support the current findings that the two groups of NF-κB-dependent genes (inflammatory mediator and antimicrobial peptide) exhibit differential expression and modulation (Fig. 35). Recent studies suggest that TLR-induced genes in an *in vitro* system of LPS tolerance are categorized into two classes, class T and class NT. The former includes pro-inflammatory mediators. The latter includes antimicrobial gene. They display distinct patterns of histone modifications (Foster et al., 2007). Interestingly, another report shows that NF-κB binding occurs in two distinct "waves" in LPS-stimulated macrophages, a fast recruitment to constitutively and immediately accessible promoters and a late recruitment to inductively and lately accessible promoters. The latter promoters require stimulus-dependent modifications in chromatin structure to make NF-κB site accessible (Saccani et al., 2001). A validated microarray data analysis and hierarchical clustering have also shown distinct expression profiles of NF-κB-dependent genes in response to TNFα (Tian et al., 2005). These observations strongly match findings in the study. Thus, the two types of pathogen-induced responses are differentially regulated and can be dissociated. The present data highlight a remarkable role of C/EBP factors for pathogen-induced IL-8 expression. Hence, C/EBP factors might be a novel therapeutic target for selective control of inflammation and innate immunity. This is quite a novel perspective.

5. SUMMARY

Mammals perceive pathogens by TLR-receptors which are known to subsequently activate the NF-κB family of transcription factors in the cytoplasm. This study analyzed at the molecular level how this common stimulus differentially activates in Mammary Epithelial Cell (MEC) the expression of the genes encoding IL-8 and LAP, serving as paradigms for either quickly activated proinflammatory cytokines or slowly responding effectors of immune response, respectively.

- The kinetics of pathogen-induced expression of both genes is very different. The IL-8 mRNA concentration peaks quickly (1h) after pathogen contact, while LAP mRNA accumulates steadily with induction time after a time lag of 1 h in response to pathogen contact.

- NF-κB and C/EBP factors play opposite roles in pathogen-induced expression of both genes. The quiescent IL-8 promoter is occupied by NF-κB p65 homodimers. Activation recruits NF-κB p50 which may cooperate with the activated C/EBP factors to induce the IL-8 promoter activity. On the contrary, chromatin in the quiescent LAP promoter is tightly compacted prohibiting NF-κB or C/EBP factor binding. The pathogen stimulus results in de-compaction of the chromatin and subsequently NF-κB p65 homodimers greatly enhance the promoter activity. C/EBP factors conversely attenuate the LAP promoter activity.

- Core data were obtained with Chromatin Immunoprecipitation techniques and validated further by establishing and using vectors capable to over-express all those relevant bovine transcription factors in reporter gene assays. Application of those vectors also highlighted that the roles of NF-κB factors in regulating IL-8 and LAP expression are cell type specific.

The data show that induction of inflammation and mounting the β-defensin protection in the udder are controlled by different mechanisms. This might offer novel strategies to selectively control inflammation and separately activate effectors of innate immunity in the udder.

6. ACKNOWLEDGEMENTS

This work would not have been possible without the support and encouragement of my colleagues and friends.

In the first place, I am deeply indebted to my supervisor Prof. Dr. Hans-Martin Seyfert. It is he who provided me the precious opportunity of Ph.D study and of our family union here. No matter when and what difficulties I encounter, he is always enthusiastic, patient to help me. Upon my arrival here, I deeply felt his considerate care. He bought numerous daily necessities for us, and many toys and other necessities of his house were moved by him into my flat. His generous, selfless help makes our life become easier. Throughout my experimental work and writing of thesis period, he provided encouragements, sound advices, critical comments and lots of good ideas. His meticulous work attitude and excellent professional ethics have benefited me and will continue in my future work. My gratitude to him much surpasses these I listed.

It is a pleasure to express my gratitude wholeheartedly to our lab colleagues Angelika Deike, Bärbel Pletz, Anne Berndt, Birgit Wego and Martina Kunze. Their diligent, particular work makes the whole lab be run smoothly which greatly facilitates our experimental work. Especially, I would like to express my thanks to Mrs. Deike for her willingness to share her experiences with me, which is very fruitful for shaping up my ideas. I am also grateful to Mrs. Deike and Mrs. Berndt for their excellent techniques for pbMEC cells. I would like to thank Mrs. Pletz for generously providing uncountable tableware to meet our daily demand. Every time I touch those things, I always remember Mrs. Pletz. It is my pleasure to thank Mrs. Wego for providing toys and kindergarten information.

I want to express my gratitude to Kathrin Esch, Dr. Alexander Rebl and Dr. Juliane Günther for their enthusiastic help in my study and life. Whenever I needed, they always try their best to help me.

Sincerely thank is given to Dr. Jens Vanselow for sharing DNA methylation protocol and experiences, and Dr. Rainer Fürbaß for sharing CHART PCR protocol and experiences. With their help, I rapidly mastered the two key techniques.

I would like to thank other members of molecular biology research unit for their encouragement and help, our head Prof. Dr. Klaus Wimmers and teachers Dr. Ronald M. Brunner, Dr. Tom Goldammer and Dr. Christa Kühn.

I am grateful to Maren Anders for patiently instructing me methylation sequencing by LI-COR. I want to express my cordial gratitude to Dr. Fuchs for her kind help in methylation sequencing and daily sequencing.

I am indebted to my many colleagues for providing a stimulating and fun environment for learning and growing, especially thanks to Annett Eberlein, Sylvio Tetzlaff, Bodo Brand, Sabine Schütze and Philipp Westphal for daily help.

I want to appreciate our unit secretary Mrs. Masermann, the personnel Mrs. Saß and Mrs. Künzel for assisting me in many different ways.

My special thanks to my best friend Wei Yang in my graduate study and his wife Yongjun Wei. From our departure to arrival in Germany, they provided every journey details. Their hospitable treatment and guidance in life let us feel no strangeness here. Even if in the United States, they always give me timely and friendly help.

I wish to thank my parents for their raising, supporting, culturing and loving me. I dedicate this thesis to them. I offer my heartfelt thanks to my sisters and brothers for their support, encouragement and patient care of our parents.

Especially, I would like to give my special thanks to my husband Xuanming Shi and my son Weitao shi for the unlimited happiness they brought me. Their lasting love enabled me to smoothly complete this work. Specially thank is given to Xuanming for his valuable advices in the designation of experiments and analyses of results. Without his support, it is unimaginable for me to complete this work.

I am very grateful to DFG For585 (grant se326/14-2) for supporting my whole work.

Finally, I would like to express my gratitude to all those that ever gave me help during my three-year study and life in FBN, as well as expressing my apology for those that I did not mention here.

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APPENDIX

I. Supplementary data

I.I Sonication to fragment the chromatin in ChIP

In ChIP assay, sonication was used to fragment the chromatin into pieces comprising 200 bp to 1000 bp. The shorter the fragments are, the higher the resolution is but the lower the yield is. The effect is shown in Fig. 36A, B and C. Comparison of panel A with panel B reveals that the smaller chromatin fragments were increased and the bigger molecules were decreased accordingly. Reversal of cross-linking for two nucleosomes generated about 400 bp naked DNA. This indicates that sonication efficiently fragments chromatin.

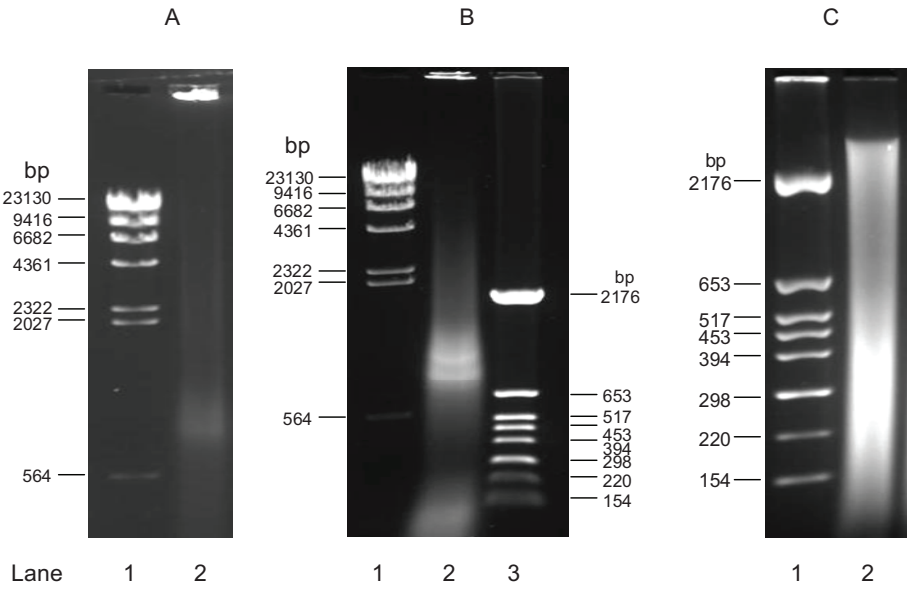

Figure 36. Analysis for pbMEC chromatin after sonication

A) The sheared chromatin of pbMEC by sonication was resolved on 0.8 % agarose gel and stained by ethidium bromide. Lane 1: λ DNA/HindIII marker, lane 2: the sheared chromatin for 20 sec. B) The sheared chromatin of pbMEC for 9×20 sec, Lane 1: λ DNA/*Hind*III marker, lane 2: two nucleosomes, lane3: pBR328/*Hinf*I marker. C) Naked DNA after chromatin reversal of cross-linking from B.

I.II Binding analysis of C/EBP factors to C/EBP site1 and site 2 in the LAP promoter

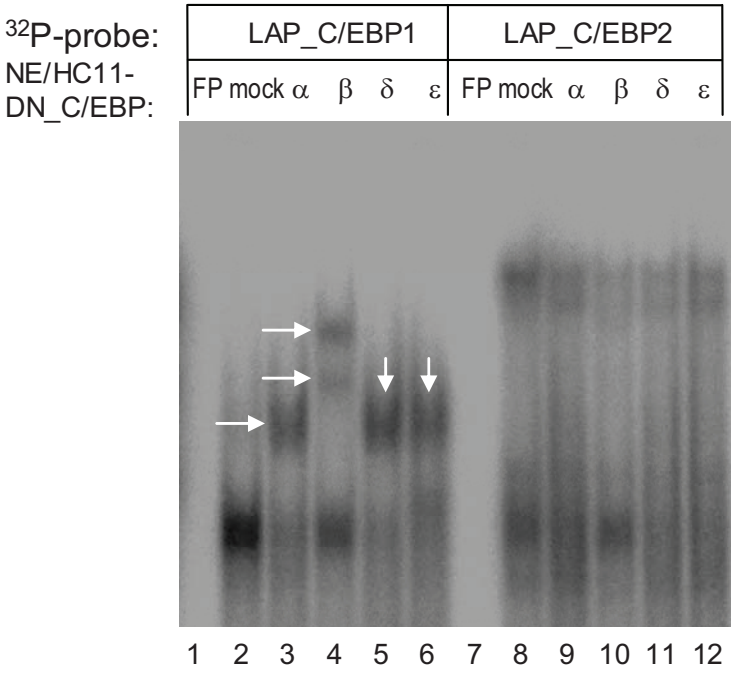

Figure 37. Affinity analysis of the C/EBP binding motifs for different C/EBP factors in the LAP promoter by EMSA

Nuclear extracts (NE) were from HC11 cells transfected with different expression constructs of DN-C/EBP-α, -β, -δ or -ε. Equal amounts of NE (2 μg) were incubated with the labeled probes as indicated. FP, free probe, without nuclear extract. Radiolabeled probes derived from C/EBP site1 (LAP_C/EBP site1), C/EBP site2 (LAP_C/EBP site2). The C/EBP specific complexes are indicated by arrows. Lanes are given at the bottom.

I.III Distribution of CpG sites and hypermethylated site 11 of the LAP promoter

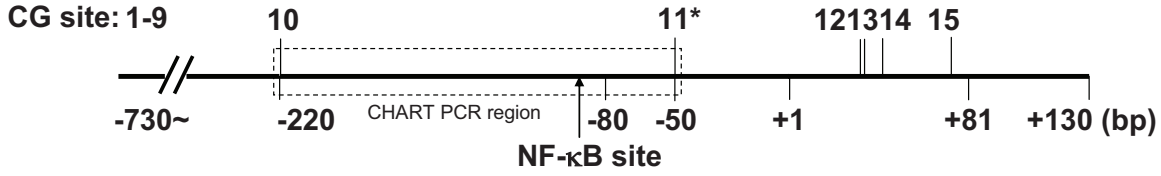

Figure 38. Map of CpG sites and hypermethylated site 11 of the LAP promoter

The vertical lines refer to CpG sites. Distributions of CpG sites in the LAP promoter are listed by the actual scale. The actual positions are given at the bottom. The transcription start site (+1) was determined by 5′ RACE. The core sequence of NF-κB binding site is located at -89/-80. Hypermethylated region was defined by bisulfite genomic sequencing. CHART PCR region is indicated by a dashed rectangle. *, $p < 0.05$.

II. List of primers

II.I List of primers for EMSA

Series No.	Name	Sequence (5′-3′)
336	Nf_κBf	AGCTTTTTCTGGGGTTTCCAAAGCCTCATT
337	Nf_κBr	AATGAGGCTT
1333	LAP_κB_Efm	TTTTCTGGGGATCAGTACAGCCTCATTAGCATA
1334	LAP_κB_Er	TATGCTAATG
1335	LAP_κB_Ef	TTTTCTGGGGATTTCCACAGCCTCATTAGCATA
1336	LAP_CE_Efm	AAGCGAAGGAACAATGGAAAGTCTGTGC
1337	LAP_CE_Er	GCACAGACTT
1338	LAP_CE_Ef	AAGCGAAGGTTCAGCAAGAAGTCTGTGC
1339	LAP_CE_mr	AGACTTTCCATTGTTCCTTCGCTTAGGGCTTTGAA
1340	LAP_CE_mf	CGAAGGAACAATGGAAAGTCTGTGCCCCTGCCAG
1388	LAP_CE2_Er	CAGGGGTGTGGCAAGGGGCACAGACTTG
1389	LAP_CE2_Erm	CAGGGGGACTAGTCGGGGCACAGACTTG
1390	LAP_CE2_Ef	CAAGTCTGTGC
1473	LAP_κB_Efm3	TTTTCTGCGGATTTAAGCAGCCTCATTAGCATA
1474	LAP_κB_Efm2	TTTTCTGGGGATTTAAGCAGCCTCATTAGCATA
1475	NFAT_r	TATGAAACCAA
1476	NFAT_f	CGCCCAAAGAGGAAAATTGGTTTCATA
1486	CEBP_con_r	TGCAGATTG
1487	CEBP_con_f	TGCAGATTGCGCAATCTGCA
1488	CEBP_con_rm	TGCAGAGAC
1489	CEBP_con_fm	TGCAGAGACTAGTCTCTGCA
1512	NFATcon_f	CGCCCAAAGAGGAAAATTTGTTTCATA
1513	NFATcon_r	TATGAAACAAA
1533	LAP_κB_Efm4	TTTTCTGCGGATTTCCACAGCCTCATTAGCATA
1674	LAP_CE3_Ef	TTTTCTGGGGATTTAAGTCGCCTCATTAGCATA

II.II List of primers for measuring mRNA copies

Series No.	Name	Sequence (5′-3′)
689	uDef_f	AGGCTCCATCACCTGCTCCT
690	uDef_r	GAAACAGGTGCCAATCTGTCTC
792	Oligo(dT)20	TTTTTTTTTTTTTTTTTTTN
951	cas_427f	AACAATCCATGACCATCCTGAC
981	BT_TNFa_f1	CTTCTGCCTGCTGCACTTCG
982	BT_TNFa_r2	GAGTTGATGTCGGCTACAACG
983	BT_TNFa_r1	CTGTGAGTAGATGAGGTAAAGC
1112	IL8_R3	GGCCCACTCTCAATAACTCTC
1159	LAP_sr	CCTGCAGCATTTTACTTGGGCT
1160	LAP_sf	AGGCTCCATCACCTGCTCCTT

II.III List of primers for cloning

Series No.	Name	Sequence (5′-3′)
72	5_Lucr	GCCTTATGCAGTTGCTCTCCA
92	BASENDf	GTACTAACATACGCTCTCCATC
587	730_mu_NFκB	GCAGGAAAGCTTTTTCTGGAGATCTCAAAGCCTCATTAGC
715	LAP_r	AGCATTTTACTTGGGCTCCGAG
718	LAP_Kpn_f	TAGGTACCGTCTTTCTCCACTTTAACTGTTCC
719	LAP_Xho_r	ATCTCGAGGCTGGCGTCCCGAGCTCTGC
1330	LAP_Pcf	GTGACTGACCCTGCTTTGTGCT
1331	LAP_κB_mr	GAGGCTGTACTGATCCCCAGAAAAAGCTTTCCT
1332	LAP_κB_mf	TGGGGATCAGTACAGCCTCATTAGCATACGAG
1391	LAP_CE2_mr	GGCAGGGGGACTAGTCGGGGCACAGACTTGCCTG
1392	LAP_CE2_mf	GCCCCGACTAGTCCCCCTGCCCATCAGCTGA
1462	IBA_Hind_Xho_f	ATGCAAGCTTCTCGAGATGGCTAGCAGAGGATCGC
1463	IBA_Strep_BamHI_r	ATGCGGATCCTTATTATTTTTCGAACTGCGG
1535	bBD5_κBm2_r	TTTGGAAACCGCAGAAAAAGCTTTCCTGCTGC
1536	bBD5_κBm2_f	GCTTTTTCTGCGGTTTCCAAAGCCTCATTAGC
1537	bBD5_κBm_r	GGCTTTGAGATCTCCAGAAAAAGCTTTCCTGCTG
1538	bBD5_κBm_f	TTTCTGGAGATCTCAAAGCCTCATTAGCATACAAG
1539	bκB_p50SalI_r	AGTCGTCGACAGGGTCATTTTTAGATGGGGT
1540	bκB_p50EcoRI_f	CAGTGAATTCATGGCAGAAGACGACCCGT
1541	bκB_p50_r2	CACTGTCCCCGTTCTCATC
1542	bκB_p50_r1	GGCGTTGGCGTGCTGCTT
1543	bκB_p65XhoI_r	AGTCCTCGAGTTAGGAGCTGATCTGACTC
1544	bκB_p65KpnI_f	CAGTGGTACCATGGACGACCTCTTCCCCC
1545	bκB_p65_r2	TGGAGCCGAACATCAGGATA
1546	bκB_p65_r1	TCTGCCTAAGCACCTCCAAA
1557	pcDNA3,1_f	ACCCACTGCTTACTGGCTTA
1558	pcDNA3,1_r	GGGGGAGGGGCAAACAAC
1596	pEGFP_f	CCAAAATGTCGTAACAACTCCG
1597	pEGFP_r	CTCCTCGCCCTTGCTCAC
1600	LAP_CE3_mr	ATGAGGCGACTTAAATCCCCAGAAAAAGCTTTC
1601	LAP_CE3_mf	GGGGATTTAAGTCGCCTCATTAGCATACGAGTG
1602	LAP_κBG_mr	CTGTGGAAATCCGCAGAAAAAGCTTTCCTGCTG
1649	bκB_p50_f1	TCAAACTCCAGAATGGCAGA
1650	bκB_p65XhoI_r2	AGTCCTCGAGGGAGCTGATCTGACTCAGAA
1651	bκB_p65_f1	GGCGAATGGCTCGACAGTAG
1673	bκB_p50SalI_r	AGTCGTCGACTCAAGGGTCATTTTTAGATGGGGT
1681	CGFP_r	CTTTGATTCCATTCTTACCGC
1682	CGFP_f	TCTACTGTTTCTCCATACCCG
1683	NGFP_r	CTAGTTATTGCTCAGCGGTG
1684	NGFP_f	ACGTTCCCATCATGGCAGACA
1685	bκB_p50SmaI_r	GCATCCCGGGAGGGTCATTTTTAGATGGGGT
1686	bκB_p65SmaI_r3	GCATCCCGGGGGAGCTGATCTGACTCAGAA
1689	bIL8__luc_r	TTGGAAGTCATATTTGAACAAGAG
1690	bIL8_luc_f	GCAAGGAGGTCCAACCAGTC
1698	bIL8_luc_f2	CCTGAGGCTGGGAGGGATTG
1720	bIL8_luc_r2	GCACCATCATGTATGCAACCATCCGCCCTTTG
1721	bIL8_luc_f5	ATGGTTGCATACATGATGGTGCACTTGTTCCC
1722	bIL8_luc_f4	ATGCGGTACCGTGCTCTCAAAGGGCGGATG
1723	bIL8_luc_f3	ATGCGGTACCTGCACTTGTTCCCTCTTCTTAC

II.IV List of primers for CHART-PCR and ChIP

Series No.	Name	Sequence (5′-3′)
1042	BN5_CHf	CTGGAAGGGTGAGGGTGGGT
1039	LAP_PCHr	CAAAGCAGGGTCAGTCACTCG
1040	LAP_PCHf	GCAGATTCAAAGCCCTAAGCGA
1041	BN5_CHr	CCTGGAGGAATTCCATGCAGA
1161	TNFα_Pf	TTGAACCCTTCTGAAAAAGACAC
1162	TNFα_Pf2	GGTGGAGAAAGAGGTGTCTTC
1163	TNFα_Ir	TCTGCTTACTCATTCGTCCAAC
1409	CAS_CHr	CACAAAATACCACTGAGAAATACCT
1410	CAS_CHf	TAGGGTTGGATAAGGAAATGCT
1647	bTNFα_κB_f	CCTAAAGGAACGGAAACAGTG
1648	bTNFα_κB_r	TCTCATTCAACCAGCGGAAAA
1652	BN5_CHf3	CACAGGAAAACAGTGGGCAGT
1653	BN5_CHf2	AGGTGCCATTTCCTTCTGCAC
1664	bTNFα_κB_r2	TTGGTGGAGAAACCCATGAG
1665	BT_IL8_CHr	CGTAAGAAGAGGGAACAAGTG
1666	BT_IL8_CHf	ACTACCATTGACAACAATGTATC
1687	bTNFα_κB_r3	CATTCAACCAGCGGAAAACT
1688	bTNFα_κB_f2	CCCTAAAGGAACGGAAACAGT
1696	BN5_CHf3	GTCTGGGGAGCGGGTAAGCA
1697	BN5_CHf4	TGGAAGGGTGAGGGTGGGT
1765	BT_IL8_CHf2	ACAAAGCTTGGGTCACACAG
1766	bTNFα_κB_f3	CCGAGCGGGCGGAGTGTA
1769	LAP_PCHf2	CCAGGAAGAAGCTTCTGGTC
1807	bIL8_Ch_PolIIf	CACTTGTTCCCTCTTCTTACG
1808	bTNFα_Ch_PolIIr	GCTGCTTGTCTCTCTCCTG
1809	bTNFα_Ch_PolIIf	GTTTTCCGCTGGTTGAATGAG
1810	LAP_Ch_PolIIr	TGATGGAGCCTCATGCTGG
1811	LAP_Ch_PolIIf	AGAGGCTCAGTTAACCAGTT

II.V List of primers for methylation

Series No.	Name	Sequence (5′-3′)
1136	LAP_P_ID	GGGGAGATAGGTTTTTTGAGGT
1135	LAP_bi_f	GATTGTGAGTTTTGATTGTATGG
1134	LAP_bi_r	AACATAACCAAACATACACATACAC
1440	LAP_P_ID3	GGAGATAGGTTTTTTGAGGTAGG
1441	LAP_P_ID2	TTTTGATGGAGGGGAGATAGG
1499	LAP_bi_r2	AAACACAAACTTCTTACTAAACCT
1500	LAP_bi_r1	AAACTTCTTCCTAACAATCAAT
1501	LAP_bi_f1	TAGGAAGAAAGTTATTAGGAAA
1534	LAP_bi_f2	TGTTTAGATTTAGTGGGTAAAG
1563	LAP_bi_r3	CTAACTTCTCAAAAACATCAAA
1564	LAP_bi_r4	CACCTAACCCAACAAACAAAAC
1565	LAP_bi_f3	TTTTTGTAAAAGGAATTATAGATA
1566	LAP_bi_r5	AATTAACTAAACCTCTTCCCTTT
1567	LAP_bi_f4	AAAGGGAAGAGGTTTAGTTAATT
Dr. Vanselow	GE843f	TTGGTTAGGGAATTGGATTTTATATGTT
Dr. Vanselow	GE845r	ATTCCAACAATTCACAACTATATTCCT
Dr. Vanselow	GE919*f	AAGTGTTATAAGTAAGATTTGGTATAGT

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V. Abbreviation list

A	
Ab	Antibody
AP-1	Activator protein-1
AP	Alkaline phosphatase
APRE	Acute-phase response element
APS	Ammonium persulfate
B	
BCIP	Bromo-chloro-indolyl-phosphate
BNBD	β-defensin gene 5
bp	Base pair
BSP	Bisulfite genomic sequencing PCR
BSA	Bbovine serum albumin
bZIP	basic leucine zipper motif
C	
CARM1	Coactivator-associated arginine methyltransferase
CBP	Cyclic AMP response element binding protein (CREB)-binding protein
C/EBP	CCAAT/enhancer binding proteins
CFU	Colony form unit
CHART-PCR	Chromatin Accessibility by Real-Time PCR
ChIP	Chromatin Immuno Precipitation
CIP	Calf intestinal phosphatase
CRP	C-reactive protein
D	
d	Day
DC	Dendritic cells
DMEM	Dulbecco's Modification of Eagles Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
Dnmt1	DNA methyltransferase 1
DSG	Disuccinimidyl glutarate
DTT	Dithiothreitol
E	
EB	Ethidium bromide
EBD	Enteric β defensin
E.coli	*Escherichia coli*
EDTA	sodium ethlenediaminetetraacetate
EGF	Epidermal growth factor
ELAM	Endothelial leukocyte adhesion molecule
EMSA	Electrophoretic Mobility Shift Assay
ES	Embryonic stem cells
F	
FA	Formaldehyde
fc	final concentration
FCS	Fetal calf serum
G	
g	gram
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GTFs	General transcription factors
H	
h	hour
HAT	Histone acetyltransferase
hBD	human beta defensin

HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HMG	High mobility group protein
HRP	Horseradish peroxidase
I	
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IκB	Inhibitory κB
IKK	IκB kinase
IL	Interleukin
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside
K	
kg	kilogram
L	
l	liter
LAP	Lingual antimicrobial peptide
LAP1 ,LAP2	Liver-enriched transcriptional activating proteins
Lf	Lactoferrin
LIP	Liver inhibitory protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTR	Long terminal repeat
M	
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cells
MG	Mammary gland
min	minute
µl	microliter
ml	milliliter
mRNA	messenger RNA
MSP	Methylation specific PCR
MyD88	Myeloid differentiation marker 88
N	
NBT	Nitro Blue Tetrazolium
NE	Nuclear extracts
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B chain
ng	nanogram
NLS	Nuclear localization sequence
NP-40	Nonidet P-40
P	
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
pbMEC	primary bovine mammary epithelial cell
p/CAF	the CBP associated factor
PCR	Polymerase chain reaction
PDTC	Pyrrolidine dithiocarbamate
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonylfluorid
Pu	Purine
PVDF	Polyvinylidene difluoride
Py	Pyrimidine

R	
5′ RACE	Rapid Amplification of 5′ CDNA Ends
RE	Restriction enzyme
RHD	Rel-homology domain
RT	Room temperature
RT-PCR	Reverse transcription PCR
S	
S. aureus	*staphylococcus aureus*
S. agalactiae	*staphylococcus agalactiae*
SAA	Serum amyloid A
SAP	Stress associated protein
SDS	Sodium dodecyl sulfate
S.E.M.	the standard error of the mean
STAT 6	Signal transducer and activator of transcription 6
T	
TAD	Transactivation domain
TAP	Tracheal antimicrobial peptide
TAP	Tobacco acid pyrophosphatase
TBE	Tris-borate- EDTA
TEMED	N,N,N',N'-Tetramethylethylene diamine
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRIF	TIR-domain-containing adaptor protein
Tss	Transcription start site
U	
UTR	Untranslated region
W	
Wt	Wild-type
V	
V	Voltage
X	
X-gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside

VI. Amino Acid Abbreviations

Full name	One-letter code	Three-letter code
Alanine	A	Ala
Cysteine	C	Cys
Aspartate	D	Asp
Glutamate	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Proline	P	Pro
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr

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Declaration on oath

I hereby declare that I myself prepared the work presented in this dissertation and that I did not use any other resources than indicated.

Shuzhen Liu 2009, Rostock