

**Szent István University**  
**Postgraduate School of Veterinary Science**

**The transcriptional regulation of the bovine neonatal Fc receptor**

**Ph.D. thesis**

written by

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## Abbreviations

293	human embryonic kidney epithelial cells	IL-2	interleukin-2
AP1	activating protein 1	IRF	interferon regulatory factor
BAEC	bovine aortic endothelial cells	IRF-E	interferon regulatory factor element
bFcRn	bovine neonatal Fc receptor	ISGF3	interferon stimulated gene factor 3
bp	base pair	ISRE	IFN stimulated response element
BSA	bovine serum albumin	I $\kappa$ B	inhibitory $\kappa$ B
$\beta$ 2m	$\beta$ <sub>2</sub> -microglobulin	Jak	Janus kinase
cAMP	adenosine 3',5'-cyclic monophosphate	MAC-T	bovine mammary epithelial cells
CBP	CREB binding protein	MEM	Minimum essential medium Eagle
ChIP	chromatin immunoprecipitation	MHC	major histocompatibility complex
CREB	cAMP response element binding protein	ML	maximum likelihood
DEAE	diethylaminoethyl	NEMO	NF $\kappa$ B essential modulator
DLR	Dual-Luciferase Reporter	NF1	nuclear factor 1
DMEM	Dulbecco's modified Eagle's medium	NF $\kappa$ B	nuclear factor $\kappa$ B
DMSO	dimethylsulphoxide	NJ	neighbour-joining
DNA	deoxyribonucleic acid	NLS	nuclear localization signal
dNTP	2'-deoxynucleotide 5'-triphosphate	PCR	polymerase chain reaction
DTT	1,4-dimercapto-2,3-butanediol	PEI	polyethylenimine
EDTA	ethylenediamine-tetraacetic acid	qPCR	quantitative polymerase chain reaction
Fc $\gamma$ rt	Fc gamma receptor	RHD	Rel homology domain
FcRn	neonatal Fc receptor	STAT	signal transducer and activator of transcription
FCS	fetal calf serum	TAD	transactivation domain
GAS	INF- $\gamma$ activation site	TESS	Transcription Element Search System
GFP	green fluorescent protein	TF	transcription factor
GM-CSF	granulocyte-macrophage colony-stimulating factor	TNF	tumour necrosis factor
HC11	mouse mammary epithelial cells	TSS	transcription start site
HDAC	histone deacetylase	Tyk	tyrosine kinase
HeLa	human cervical adenocarcinoma cells	VNTR	variable number of tandem repeats
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	$\beta$ 2m	$\beta$ <sub>2</sub> -microglobulin
hFcRn	human neonatal Fc receptor	$\beta$ -TRCP	$\beta$ -transducin repeat-containing protein
IgA	immunoglobulin A		
IgG	immunoglobulin G		
IKK	I $\kappa$ B kinase complex		

# 1. Summary

The neonatal Fc receptor (FcRn), like other MHC class I molecules, is composed of an  $\alpha$ -chain and a  $\beta_2$ -microglobulin ( $\beta_2m$ ). This receptor has been detected in the bovine mammary gland, small intestine, lower respiratory system, and endothelial cells. While epithelial FcRn is involved in IgG transport through these barriers, the FcRn expressed in capillary endothelial cells is responsible for regulating the IgG catabolism. Due to these crucial immunological functions, the gene regulation of the bovine FcRn (bFcRn) may contribute to the immune homeostasis. Although gene expression is controlled at multiple levels, one of the most important is transcriptional regulation. Accordingly the sequences of the human and mouse FcRn  $\alpha$ -chain *cis*-regulatory region have been published and their preliminary examination has been achieved, but their transcriptional regulation has not been adequately unravelled.

In order to reveal the regulation of the bFcRn transcription, the 5'-flanking sequence of the bFcRn  $\alpha$ -chain gene was isolated, cloned and functionally examined. The bFcRn  $\alpha$ -chain *cis*-regulatory region was induced by NF $\kappa$ B in the luciferase reporter gene assays of human and bovine cell models. Three functional  $\kappa$ B binding sites were identified in the *cis*-regulatory region using site-directed mutagenesis accompanied by luciferase reporter gene assays, and it was verified that these  $\kappa$ B sites were responsible for the complete NF $\kappa$ B responsiveness of the bFcRn *cis*-regulatory region. The  $\kappa$ B binding sites were also tested in gel retardation assay verifying their binding ability to NF $\kappa$ B complex with p65 content. These *in vitro* findings indicated, in accordance with the present *in vivo* data, that the bFcRn was under the control of an important transcriptional pathway activated during infection and inflammation.

The  $\beta_2m$  is a chaperone of FcRn and other MHC class I (-like) proteins ensuring the appropriate function of these molecules. To fulfil this function, it is expressed ubiquitously under constitutive and cytokine-induced transcriptional controls. Transcriptional elements of the  $\beta_2m$  *cis*-regulatory region have been experimentally well characterized in human, and it has been found that a  $\kappa$ B and an ISRE sites were responsible for the cytokine-induced regulation.

The 5'-flanking sequence of the bovine  $\beta_2m$  (b $\beta_2m$ ) has been isolated and cloned in order to assess its cytokine-induced gene expression in relation to FcRn. Although the ISRE site was conserved in the cattle, there was a deletion in the b $\beta_2m$   $\kappa$ B site compared to the human orthologue, and there was no NF $\kappa$ B responsiveness of the b $\beta_2m$  *cis*-regulatory region in the luciferase reporter gene assays of human and bovine cell models. To the contrary, the b $\beta_2m$   $\kappa$ B site did bind the NF $\kappa$ B complex with p65 content in gel retardation assay rendering these

*in vitro* results controversial. *In vivo* data about the mRNA level of the b $\beta$ 2m upon LPS induction are also contradictory, therefore the NF $\kappa$ B inducibility of the b $\beta$ 2m *cis*-regulatory region cannot be deduced from the present data. The functionality of the b $\beta$ 2m ISRE site was confirmed *in vitro* by gel retardation and luciferase reporter gene assays, thus, the b $\beta$ 2m ISRE site mediated the IFN- $\gamma$  induction similarly to its human orthologue, and there were no differences in the ISRE-mediated transcriptional regulation of this gene in cattle.

In order to establish a species-specific system that can be used to analyze gene regulation in bovine, the full length coding sequence of the bovine p65 (bp65) subunit of NF $\kappa$ B was isolated and cloned. The cloned bp65 was expressed in mammalian cells, and it induced the NF $\kappa$ B-specific luciferase reporter gene expression. Using gel retardation assay, it was demonstrated that the cloned bp65 bound to the consensus  $\kappa$ B sequence. The comparison of the bp65 with its human and mouse orthologues at amino acid level showed high homology in both the DNA-binding domain, known as Rel homology domain (RHD) and the transactivation domain (TAD). The phylogenetic analysis at DNA level provided a new insight into the evolution of the NF $\kappa$ B family, and it was able to resolve the topology of the mammalian p65 molecules. Although, the RHD was conserved in vertebrates, the TAD sequences deviated from each other, and showed faster molecular evolution than RHD sequences.

## 2. Introduction

### 2.1. The function and expression of the neonatal Fc receptor

With the discovery of immunoglobulins, immunoglobulin G (IgG) was recognized to possess two unique properties: selective pre- or postnatal transepithelial transport across the placenta in humans and the intestinal epithelium in rodents, and a prolonged half-life relative to other serum proteins, suggesting protection from catabolism in adults. These observations were noted by Francis Brambell, who further predicted the presence of a saturable receptor responsible for both biological functions (BRAMBELL, 1966). Two decades after these predictions, biochemical and ultimately molecular biological evidence was obtained in the late 1980s for the presence of a receptor that was physiologically active in neonatal rodent epithelium. However, not until recently with the generation of X-ray crystallographic structures, subsequent structure–function analyses and the creation of a knock-out animal, has it become clear that the so-called neonatal Fc receptor for IgG (FcRn) is responsible for both of the aforementioned functional attributes of IgG physiology: its transport across the neonatal epithelium of rodents and the avoidance of catabolism (QIAO *et al.*, 2007).

The FcRn molecule is a heterodimer composed of a 50-kDa transmembrane  $\alpha$ -chain subunit that is noncovalently associated with the 12-kDa  $\beta_2$ -microglobulin ( $\beta_2m$ ) subunit (Figure 1). The  $\alpha$ -chain, that bears the most of FcRn specific attributes, is related to the major histocompatibility complex (MHC) class I molecules. The gene for the  $\alpha$ -chain, termed *Fcgrt* (Fc gamma receptor), has been independently cloned first from rat (SIMISTER and MOSTOV, 1989), then, from human (STORY *et al.*, 1994), mouse (KANDIL *et al.*, 1995), cattle (KACSKOVICS *et al.*, 2000), possum (ADAMSKI *et al.*, 2000), sheep (MAYER *et al.*, 2002), swine (SCHNULLE and HURLEY, 2003, ZHAO *et al.*, 2003) and dromedary (KACSKOVICS *et al.*, 2006b). The crystal structure of the FcRn has been solved and it has been confirmed that the extracellular domains of FcRn were structurally similar to MHC class I molecules (BURMEISTER *et al.*, 1994). FcRn binds immunoglobulin G (IgG, Figure 1) and albumin, and the interaction sites have been localized by a combination of site-directed mutagenesis and X-ray crystallography (ANDERSEN *et al.*, 2006, GHETIE and WARD, 2000).

IgG, the main immunoglobulin that is primarily found in serum and in extracellular space, is produced by B lymphocytes in the peripheral lymph nodes and the spleen. Maternal IgG endows the fetus with protection against congenital infection and also provides adequate

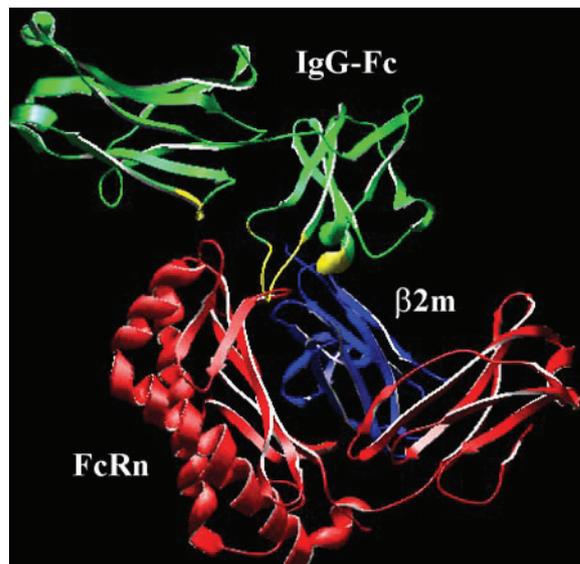


Figure 1  
The 3D model of the bovine FcRn and the bound Fc region of IgG (MAYER *et al.*, 2004b).

immunity for the first weeks of independent life, since at birth the offspring is exposed to a similar antigenic environment as its mother. FcRn as an IgG receptor plays a major role in transmission of passive immunity to the fetus and young (JUNGHANS, 1997).

Although, there is some prenatal transfer of IgG through the yolk sac, rodents acquire most of their IgG from colostrum and milk through the small intestine during lactation. FcRn molecules in the intestinal epithelium of suckling rodents bind to IgG deriving from maternal colostrum and milk and transport it by endocytosis across the cell in an apical to basolateral direction, passing it into the digestive circulation of the newborn (RODEWALD, 1973, SIMISTER and REES, 1985). At the time of weaning (approximately 14 days of age), FcRn expression is down-regulated approximately 1000-fold within the rodents epithelium at the time of epithelial closure and simultaneously with the cessation of IgG transport (JENKINS *et al.*, 2003). FcRn binds IgG in a strictly pH dependent manner, thus, IgG binds to the luminal surface of the intestinal epithelial cells via FcRn at pH 6.0, and IgG dissociates from FcRn on the basolateral surface at pH 7.4 after transcytosing across the cells (ISRAEL *et al.*, 1995, RODEWALD, 1976). In addition, IgG is endocytosed through fluid-phase endocytosis in the rat yolk sac, where the pH is neutral, and the binding of IgG to FcRn occurs in the acidic environment of the apical endosome (Figure 2 a)(ROBERTS *et al.*, 1990). In human, most of the IgGs are transmitted to the fetus from the maternal vascular system of the placenta. Besides the presumable participation of other Fc receptors, the IgG transmission occurs via FcRn located in the syncytiotrophoblast layer of the placenta, which binds maternal

circulatory IgG and transports it into the bloodstream of the fetus (LEACH *et al.*, 1996, SIMISTER, 2003).

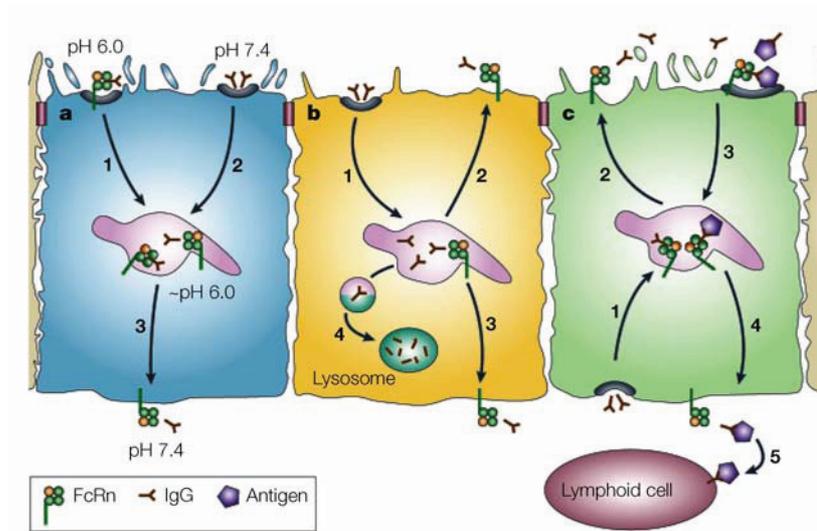


Figure 2

The function of FcRn. a. Transport of IgG. In the small intestine of suckling rats, where the extracellular pH is mildly acidic, IgG binds to FcRn located on enterocytes, where it is endocytosed (step 1). In cells, such as those that line the yolk sac, where the pH of the extracellular fluid is neutral, IgG is internalized by fluid-phase endocytosis (step 2). In this case, binding to FcRn occurs in the acidic environment of the early endosome. FcRn-IgG complexes are transported to the basolateral cell surface (step 3), where the neutral pH at the serosal side of the tissue promotes ligand dissociation and secretion. b. Regulation of IgG catabolism. In endothelial cells, IgG is taken up by fluid-phase endocytosis and delivered to endosomes (step 1), where it interacts with FcRn. Ligand, bound to receptor, is either recycled back to the apical plasma membrane where it is returned to blood (step 2), or transported to, and released at, the basolateral pole of the cell (step 3). When IgG concentrations are high and binding to FcRn becomes limiting, unbound IgG is delivered along with bulk fluid to lysosomes where it is degraded (step 4) c. FcRn-IgG complex in immune activation and tolerance. IgG is endocytosed at the basolateral pole of the cell (step 1) and transported by FcRn to the apical pole of the cell, where it is released into secretions (step 2). Following binding to antigen, the IgG-antigen complexes, internalized by fluid-phase endocytosis or through their interactions with FcRn (step 3), are transcytosed in the opposite direction (step 4), delivering immune complexes to the lamina propria for subsequent induction of immune activation or tolerance (step 5) (ROJAS and APODACA, 2002).

In ruminants, protective IgGs are transferred from the maternal mammary gland to the neonate via the colostrum to mediate passive immunity (BRAMBELL, 1969). Upon ingestion of the colostrum, IgGs are transported across the intestinal barrier of the neonate into its blood (KACSKOVICS, 2004). Whereas this intestinal passage appears to be somewhat non-specific for types of IgG (NEWBY and BOURNE, 1976a), there is a high selectivity in the passage of these proteins from the maternal plasma across the mammary barrier into colostrum, and only IgG1 is transferred in large amounts (BUTLER, 1999, SASAKI *et al.*, 1976). There is a rapid drop in the concentration of all lacteal IgGs immediately postpartum (BUTLER, 1983), and the selectivity of this transfer has led to the speculation that a specific transport mechanism across the mammary epithelial cell barrier is involved. Accumulating evidence supports that

FcRn contributes to this transport in mammary glands of ruminants. The sheep FcRn is expressed exclusively in the epithelial cells of the acini in the mammary gland, and there is a remarkable cellular redistribution of this receptor around parturition with a downward expressional trend postpartum (MAYER *et al.*, 2002). Re-analysing this issue in cattle has confirmed the data derived from sheep (KACSKOVICS *et al.*, 2000, MAYER, 2005, MAYER *et al.*, 2005). Moreover, it has been found that there is a correlation between haplotypes of the bovine FcRn  $\alpha$ -chain and  $\beta$ 2m and the IgG concentration in neonatal calves (CLAWSON *et al.*, 2004, LAEGREID *et al.*, 2002).

Regarding mucosal protection, where immunoglobulins are transported onto mucosal surfaces, it is important to note that secretory immunoglobulin A (IgA) is the major immunoglobulin in the external secretion of non-ruminants. However, FcRn-mediated mucosal protection might be important in the lower respiratory and female genital tracts of humans in which the concentration of IgG is greater than IgA (ROJAS and APODACA, 2002). In ruminants IgG1 dominates at many mucosal surfaces (BUTLER, 1983), which can be explained by the fact that ruminant IgG1, similarly IgA, is resistant to proteolysis (NEWBY and BOURNE, 1976b). Since ruminant FcRn has been detected from multiple mucosal tissues, more recently in the lower respiratory tract of bovine (MAYER *et al.*, 2004a), one may argue that IgG1 secretion is an FcRn dependent process in these tissues (KACSKOVICS, 2004).

In addition to mediating the transfer of IgG, FcRn is also important in regulating the amount of IgG in blood. The half-life of IgG is longer compared to the half-life of other Ig classes in mammals, and the rate of IgG turnover increases as the amount of IgG in blood rises (BRAMBELL *et al.*, 1964). Turnover occurs in endothelial cells, and involves a saturable process, and it has been hypothesized that the process is receptor mediated. Several results verify that the FcRn takes part in the IgG catabolism, as it is localized at the endothelial cells of small arterioles and capillaries in muscle and liver (BORVAK *et al.*, 1998), and the serum half-life of IgG in  $\beta$ 2m-deficient mice is abnormally low in comparison to control animals (GHETIE *et al.*, 1996). Moreover, the interaction sites of IgG and Fc-fragment for FcRn and the IgG parts, which are responsible for its half-life in serum, tightly overlap in mouse (KIM *et al.*, 1994, MEDESAN *et al.*, 1997). Bovine FcRn (bFcRn) is also expressed in endothelial cells, and the results from the interaction of human IgG on bFcRn imply that it is involved in IgG homeostasis in cattle (KACSKOVICS *et al.*, 2006a). In the model, describing the FcRn-mediated IgG homeostasis, IgG is taken up by fluid-phase endocytosis and delivered to endosomes of endothelial cells (Figure 2 b) (GHETIE and WARD, 2000). The fate of endocytosed IgG varies depending on the concentration of internalized IgG, which is directly

proportional to the concentration in blood. At modest levels of IgG, most of the ligand binds to FcRn and it is either recycled or delivered by transcytosis to the basolateral surface. When the IgG level is high, FcRn is saturated, and the non-receptor-bound IgG is delivered along with other fluid-phase cargo to the lysosomes, where it is then degraded. Recently, this model has been supported by *in vitro* experimental data (WARD *et al.*, 2003).

It has been also hypothesized that FcRn that is functionally expressed by monocytes, macrophages and dendritic cells protects IgG from catabolism to prolong the IgG half-life in extracellular or intracellular environment, which may impact the antigen presentation functions of these cells (ZHU *et al.*, 2001). Furthermore, FcRn fulfils a major role in IgG-mediated phagocytosis in human neutrophils (VIDARSSON *et al.*, 2006). In addition to the role of FcRn in IgG catabolism, FcRn has been recently implicated in prolonging the half-life of serum albumin by a similar mechanism (CHAUDHURY *et al.*, 2006, CHAUDHURY *et al.*, 2003).

FcRn is expressed in adult human intestinal epithelial cells, where it transcytoses IgG in both directions, and has led to the proposal of a new function for FcRn (DICKINSON *et al.*, 1999, ISRAEL *et al.*, 1997). The model of this function entails the fluid-phase internalization and transport of IgG from the interstitial space to the intestinal lumen, where it is released into secretions (Figure 2 c). After binding with its cognate antigen in the lumen, the IgG-antigen complexes are transcytosed in the opposite direction, delivering immune complexes to the lamina propria for subsequent induction of immune activation or tolerance (YOSHIDA *et al.*, 2006). In the respiratory epithelium, the luminal-to-serosal transport of Fc fragment, derived from IgG, has been verified further supporting this model (SPIEKERMANN *et al.*, 2002).

## **2.2. Transcriptional regulation of the neonatal Fc receptor**

### **2.2.1 Transcription and transcription factors**

The process of transcription, whereby an RNA product is produced from the DNA, is an essential element in gene expression, and an attractive control point for regulating the expression of genes in a particular cell type or in response to a particular signal. The regulation of transcription is achieved by the coordinated cooperation of specific DNA sequence elements and specific proteins. These proteins can directly or indirectly bind to the short specific DNA sequence elements, which are located upstream and downstream from transcription start site (TSS). The DNA sequence elements are known as *cis*-acting or *cis*-regulatory elements and the DNA region that bears them as *cis*-regulatory region, while the proteins are referred to as *trans*-acting or *trans*-regulatory elements. The most essential *cis*-

acting element is the TATA-box, which is found in very many but not all genes, and plays fundamental role in binding basal transcriptional complex including RNA polymerase II itself, which is the enzyme responsible for transcribing protein coding genes. Although the TATA-box is found in most eukaryotic genes, it is absent in some, where a specific sequence known as initiator elements replaces it (WEIS and REINBERG, 1992). It is worth mentioning that the initiator sequences have not been characterized completely, thus the binding requirement of the basal transcriptional complex is unknown in several *cis*-regulatory regions. The region in the close proximity of TSS, which binds the basal transcription complex, is defined as the promoter or core promoter. The promoter maintains the constitutive transcription, that means the basal level of transcription of a particular gene in a particular cell type. In addition, numerous *cis*-acting elements are located outside the promoter, which are often termed enhancers. They act by influencing the basal activity of the promoter through transcription factors (TF), although they lack promoter activity themselves (LATCHMAN, 1998).

The name of transcription factor indicates all *trans*-acting elements, which can bind to DNA in sequence-specific manner and can directly or indirectly influence the function of basal transcription complex. It is noteworthy that their indirect influence occurs via chromatin remodelling which can alter the recruitment of basal transcriptional complex (FRY and PETERSON, 2001). Some transcription factors such as Sp1 (LANIA *et al.*, 1997) or some members of the NF1 family (GRONOSTAJSKI, 2000) contribute to the constitutive transcription in order to strengthen the activity of the basal transcriptional complex that binds the promoter with low affinity. Apart from *cis*-acting elements and the corresponding transcription factors, which are involved in the constitutive transcription itself, the TF binding sites are found only in genes transcribed in a particular cell type or in response to a specific stimulus. TF binding sites in the *cis*-regulatory regions of cell type specific genes play a critical role in producing their cell type specific pattern of expression by binding transcription factors which are present in an active form only in a particular cell type where the gene will be activated. For example, the *cis*-regulatory regions of the immunoglobulin heavy- and light-chain genes contain a TF binding site known as the octamer motif, which can confer B cell specific expression on these genes through binding corresponding transcription factor, called Oct-2, expressed only in B cells (WIRTH *et al.*, 1987). Genes that are activated or inhibited at the transcriptional level in response to a specific stimulus share specific TF binding sites in their *cis*-regulatory regions. In turn, such TF binding sites act by binding the corresponding and specific transcription factor that becomes activated in response to the stimulus. Once activated, this factor interacts directly or indirectly with the basal transcriptional complex

resulting in increased or decreased transcription of the genes. For example, the *cis*-regulatory regions of genes that harbour cAMP response element can be activated by cAMP through the binding of cAMP response element binding protein (CREB) (MONTMINY, 1997), or genes contain a TF binding site for the activating protein-1 (AP1), which produces induction of gene expression in response to phorbol ester treatment (KARIN *et al.*, 1997). It is worth mentioning, that the transcription factors often share a common DNA-binding domain, which ensures sequence-specific binding to the common TF binding site, but they may radically deviate in transactivation potential or cell-specific expression. These transcription factors based on common DNA-binding domain are classified in a transcription factor family, for instance, the Ets TF family includes transcription factors that contain Ets-domain, and bind to Ets binding site, but is linked with diverse biological processes (SHARROCKS, 2001).

The mechanism of transcription and transcriptional regulation in specific genes of immune system do not differ from those of other genes, but the transcription factors and TF families are at least partly immune-specific in the same way as the receptors on the cell surface and signal transduction pathways. Therefore, immunological stimuli act via immune-specific signal transduction pathways, and often end at the members of mostly immune-specific TF family such as NF $\kappa$ B, interferon regulatory factor (IRF), AP1, and signal transducer and activator of transcription (STAT) families (FOLETTA *et al.*, 1998, HORVATH, 2000, LEVY and DARNELL, 2002). In the present study, NF $\kappa$ B and IRF families have a special importance, thus their features are further expounded.

### 2.2.2 Nuclear factor $\kappa$ B

The NF $\kappa$ B/Rel superfamily comprises a variety of transcription factors that share a DNA-binding domain of common origin, known as the Rel homology domain (RHD), but they have diverse functions and mechanisms of action (GRAEF *et al.*, 2001). Three groups of proteins belong to the NF $\kappa$ B/Rel superfamily; nuclear factor of activated T cell (NFAT) proteins, tonicity enhancer-binding protein (TonEBP) and NF $\kappa$ B proteins, of which the family of NF $\kappa$ B proteins is one of the best-studied transcription factors in biology (DIXIT and MAK, 2002, GHOSH and KARIN, 2002). The members of the NF $\kappa$ B family play an indispensable role in controlling both innate and adaptive immunity (LI and VERMA, 2002), among other crucial regulatory functions. Moreover, the most significant common features of innate immunity throughout the animal kingdom, are the central positions of Toll-like receptor signaling pathways and the NF $\kappa$ B family (KARIN and BEN-NERIAH, 2000, ZHANG and GHOSH, 2001), including the basic mechanism of NF $\kappa$ B activation in mammals and insects (SILVERMAN and MANIATIS, 2001). In the quiescent state of NF $\kappa$ B system, NF $\kappa$ B

proteins are retained in the cytoplasm by the members of the inhibitory  $\kappa$ B (I $\kappa$ B) proteins. Appropriate signaling pathways, activated by an astonishing number of extracellular signals, terminally lead to the degradation of I $\kappa$ Bs by the  $\beta$ -transducin repeat-containing protein ( $\beta$ -TRCP) ubiquitin proteasome in mammals and the Slimb proteasome in insects (Figure 3). The degradation permits the nuclear translocation of NF $\kappa$ B, where it stimulates the transcription of various immune-related genes such as the mammalian interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)  $\alpha$  (PAHL, 1999) through a family of specific DNA-binding sites known as  $\kappa$ B sites. Among other genes, NF $\kappa$ B activates the transcription of I $\kappa$ B, thus the increased amount of I $\kappa$ B prevents the nuclear translocation of NF $\kappa$ B, and an autoregulative feedback loop is triggered (GHOSH *et al.*, 1998). The above mentioned common characteristics of NF $\kappa$ B activation

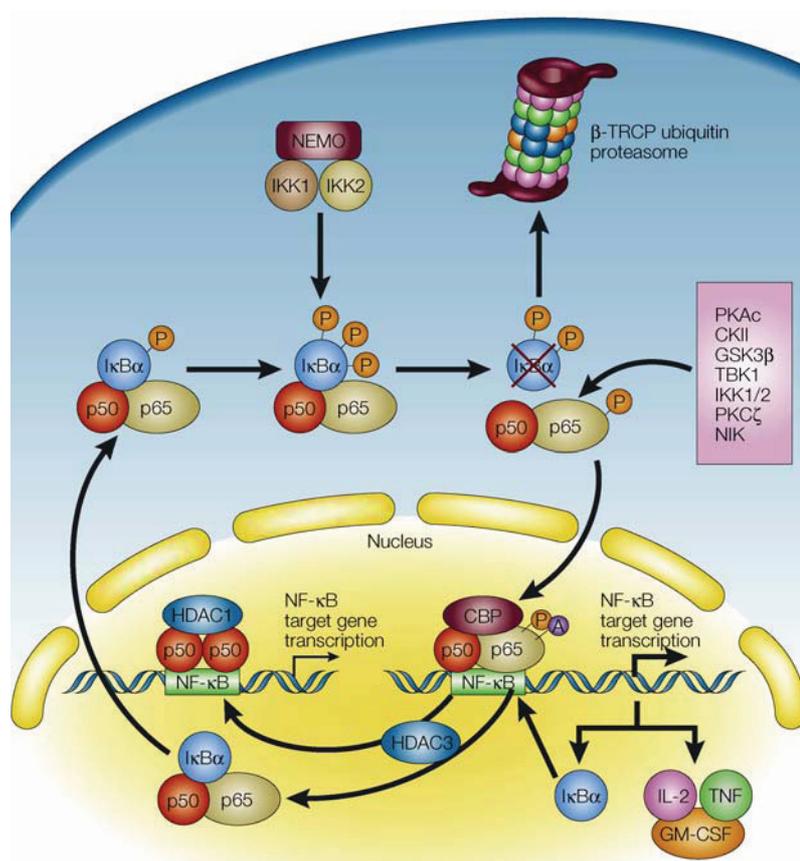


Figure 3

The model of NF $\kappa$ B regulation at multiple level. After induction, IKK complex, which consists of IKK1, IKK2 and NF $\kappa$ B essential modulator (NEMO), phosphorylate I $\kappa$ B, which leads to its degradation by the  $\beta$ -TRCP ubiquitin proteasome and allows NF $\kappa$ B dimers to enter the nucleus. During or after I $\kappa$ B degradation, p50 is phosphorylated, which is essential for its binding of CBP and replacing the p50-p50-histone deacetylase (HDAC) 1 complex as well as activating the transcription of target genes, such as I $\kappa$ B $\alpha$ , IL-2, GM-CSF and TNF. p50-p50-HDAC1 complexes repress transcription, whereas p50-p65-CBP complexes activate transcription via chromatin remodelling. Furthermore, HDAC3 might help to switch off NF $\kappa$ B activity by deacetylating p65 and enhancing the binding affinity between p50-p65 and I $\kappa$ B $\alpha$ . Various kinases (in purple box) might be involved in p65 phosphorylation (LI and VERMA, 2002).

have been recently confirmed in the horseshoe crab, *Carcinoscorpius rotundicauda*, which is the most ancient arthropod (WANG *et al.*, 2006). Beside the arthropod NFκB molecules, invertebrate and deuterostome NFκB homologues have been also described in ascidians (KAWAI *et al.*, 2005, SHIMADA *et al.*, 2001), and their contribution to the function of the immune system has been evaluated in sea urchin as well (PANCER *et al.*, 1999). On the contrary, however, NFκB proteins are absent in the worm *Caenorhabditis elegans* (PUJOL *et al.*, 2001), thus the ancient origin of NFκB has not been unravelled.

In addition to the ancient functions in innate immunity, NFκB proteins contribute to the development and the function of T and B lymphocytes (BAEUERLE and HENKEL, 1994). To perform these divergent immunological functions, paralogous NFκB genes evolved by duplication of a unique ancestral gene (HUGUET *et al.*, 1997). In mammals, the NFκB family of transcription factors contains five members, p65 (RelA), c-Rel, RelB, NFκB1 (p50, p105) and NFκB2 (p52, p100), which constitute homo- and heterodimers of different composition. Heterologous transactivation domains (TAD) are found in p65, c-Rel, and RelB, therefore dimers that contain any of them can activate transcription. In contrast, active NFκB1 (p50) and NFκB2 (p52) produced from precursor proteins (p105 and p100) lack TAD, so their homodimers cannot activate transcription (BEINKE and LEY, 2004). Moreover, NFκB1 homodimer can be associated with histone deacetylase (HDAC) 1 and can actively repress transcription through the chromatin structure near to the target genes (Figure 3). The balance between different NFκB homo- and heterodimers will determine which dimers are bound to specific κB sites and thereby regulate the level of transcriptional activity (CAAMANO and HUNTER, 2002). Besides, the classical signaling pathways of NFκB, which are triggered by well-known signal molecules such as LPS and TNF-α (GOETZ *et al.*, 2004), and are merged in IκB kinase complex (IKK) to phosphorylate and degrade IκBs (Figure 3), and alternative signaling pathways (HAYDEN and GHOSH, 2004) activate NFκB dimers of different composition (BONIZZI and KARIN, 2004). Consequently, different NFκB dimers in the same cell can influence the transcription in a signal-dependent manner. It is worth mentioning that NFκB proteins are expressed in a cell- and tissue-specific pattern, which provides an additional level of regulation. RelB, c-Rel and NFκB2 are expressed specifically in lymphoid cells and tissues, whereas p65 and NFκB1 are ubiquitously expressed, and the p65/NFκB1 heterodimers constitute the most common, inducible NFκB binding activity (CAAMANO and HUNTER, 2002).

The p65 is the only ubiquitously expressed mammalian NFκB protein which contains TAD, and its vital importance is confirmed by experiments showing that lack of p65 subunit is lethal to such embryos. By contrast mice that lack each of the other four members are merely

immunodeficient without lethality (LI and VERMA, 2002). The p65 comprises two specific domains, the N-terminal RHD and the C-terminal TAD, which incorporate two typical features of transcription factors, the sequence-specific DNA-binding and the transcription influential abilities. Apart from DNA-binding, RHD is responsible for dimerization and interaction with I $\kappa$ B family members, such as I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  (GHOSH and KARIN, 2002). The three dimensional crystal structures of p65/NF $\kappa$ B1 heterodimer RHD regions complexed to DNA, and the N-terminal regions of p65/NF $\kappa$ B1 heterodimer bound to I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$  have been solved (BERKOWITZ *et al.*, 2002, CHEN *et al.*, 1998, HUXFORD *et al.*, 1998, JACOBS and HARRISON, 1998, MALEK *et al.*, 2003). Therefore the specific amino acid residues of RHD contributing to DNA-binding, dimerization and I $\kappa$ B interaction are well-defined. A linker region is localized between RHD and TAD, which bears a nuclear localization signal (NLS) in close proximity to RHD. The cytoplasmic localization of the NF $\kappa$ B/I $\kappa$ B complex is due to masking of the NLS by the I $\kappa$ B proteins. Thus I $\kappa$ B degradation would simply lead to the unmasking of the NLS allowing free NF $\kappa$ B dimers to enter into the nucleus (GHOSH and KARIN, 2002). The squelching, deletion and mutational analyses of the p65 C-terminal region have demonstrated the strong transactivation potential of TAD, which was divided into two functional parts, the TAD1 and the TAD2 (SCHMITZ and BAEUERLE, 1991, SCHMITZ *et al.*, 1994, SCHMITZ *et al.*, 1995). However, the crystal structure of p65 TAD has not been determined yet. In addition to the regulation of NF $\kappa$ B activity, such as I $\kappa$ B degradation, I $\kappa$ B autoregulative feedback loop and the balance of different NF $\kappa$ B dimers, the posttranslational modifications of p65 represent a further level in its regulation (Figure 3). The posttranslational modifications of p65 extends from the phosphorylation of both the RHD and TAD to the acetylation of an undefined region (CHEN *et al.*, 2001), and influences mainly the transactivation potential, as well as the ability of DNA-binding and dimerization (VIATOUR *et al.*, 2005). These effects are often attained through transcription coactivators like the CREB binding protein (CBP), which exerts chromatin remodelling (LI and VERMA, 2002, NATOLI *et al.*, 2005).

### 2.2.3 The interferon regulatory factor family

Interferons (IFNs) are a family of multi-functional cytokines, which mediate cellular resistance against viral infection and play diverse roles in the immune response to pathogens, immunomodulation and hematopoietic development. IFN- $\alpha$  and IFN- $\beta$  are produced by virus-infected cells and constitute the primary response against virus infection, whereas IFN- $\gamma$ , produced by activated T cells and natural killer cells, is crucial in eliciting the proper immune response and pathogen clearance (MAMANE *et al.*, 1999). To exert an influence on the

transcription of their target genes, IFNs bind to the type I IFN receptors (IFNAR1 and IFNAR2) and type II IFN receptor (IFNGR1), and activate Janus family protein tyrosine kinases such as Janus kinases (Jak1, Jak2) and tyrosine kinase 2 (Tyk2), then, the activation of these Janus kinases causes site-specific tyrosine phosphorylation of STAT1 and STAT2 (Figure 4). Although phosphorylated STATs can activate transcription themselves through a specific *cis*-acting element, known as INF- $\gamma$  activation site (GAS), they also induce the transcription of some IRF family members such as IRF1. Therefore several target genes of IFNs are directly regulated by the IRF family members (STARK *et al.*, 1998). In addition, phosphorylated STATs, in combination with IRF9, form a heterotrimeric TF complex, termed

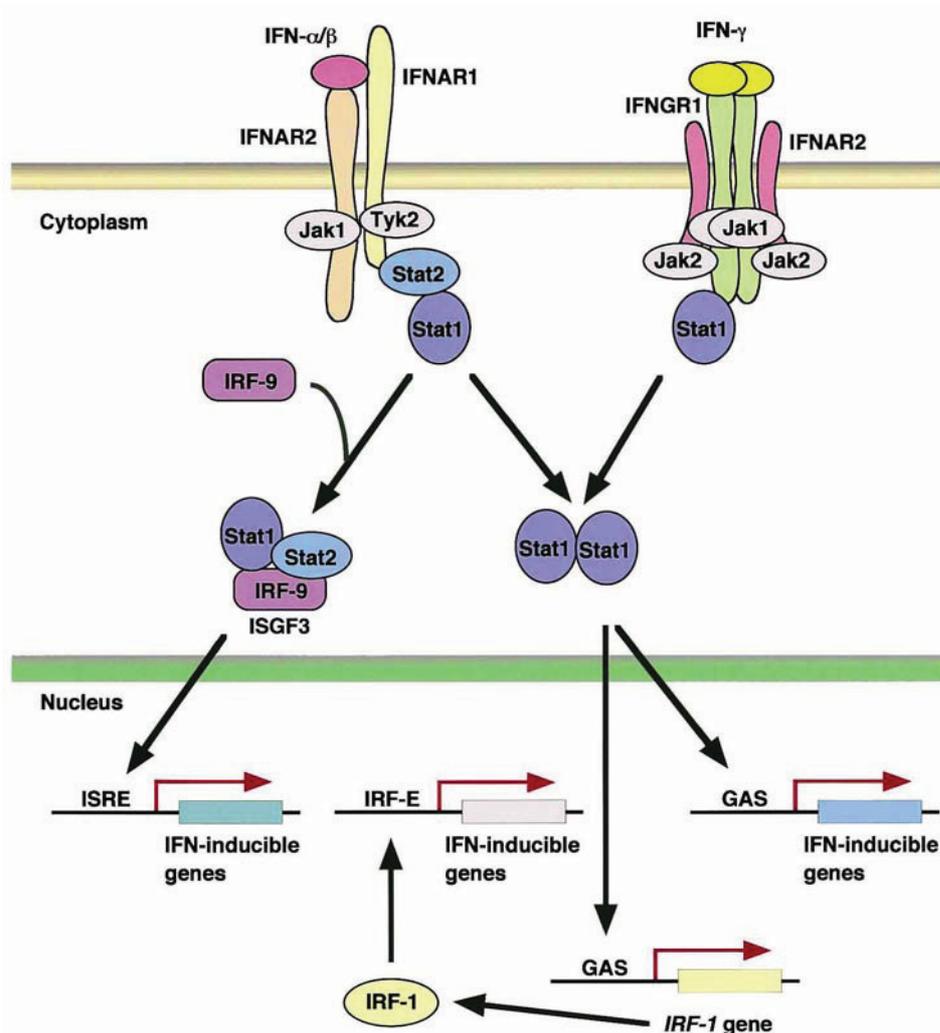


Figure 4

Signal transduction and transcriptional regulation in IFN system. IFNs can activate the transcription of their target genes via the Jak-STAT pathway including IFN receptors (IFNAR1, IFNAR2, IFNGR1) and Janus kinases (Jak1, Jak2, Tyk2). The *cis*-regulatory regions of IFN-inducible genes are activated directly by STATs binding to GAS site or indirectly by IRF family members binding to ISRE and IRF-E sites. In addition, ISRE site can bind heterotrimeric TF complex, ISGF3, which consists of STAT1, STAT2 and IRF9 to activate the transcription of some INF target genes (SATO *et al.*, 2001).

interferon stimulated gene factor (ISGF) 3, which can also activate the transcription of some IFN target genes (Figure 4).

The best characterized members of the IRF family are IRF1 and IRF2, but the family has recently expanded to include seven additional members: IRF3, IRF4, IRF5, IRF6, IRF7, IRF8, IRF9. All members of the family share homology in their N-terminus encompassing the DNA-binding domain that contains a characteristic repeat of five tryptophan residues. Through this DNA-binding domain, IRF family members bind to similar TF binding sites, termed IFN stimulated response element (ISRE), which is almost undistinguishable from the IRF element (IRF-E, Figure 4). The members are ubiquitously expressed, except for IRF4 and IRF8, whose expression is restricted to hematopoietic cells. The mRNA level of IRF1 is dramatically upregulated upon viral infection or IFN stimulation via STATs, therefore it takes part in the establishment of antiviral response, but also contributes to the nitric oxide synthesis against bacterial infection, immunomodulation or the development of T cells and natural killer cells (SATO *et al.*, 2001). IRF2, IRF3, IRF7 and IRF9 are also responsible for the tight regulation of antiviral response, whereas IRF4 and IRF8 are presumably required for the development and function of hematopoietic cells. Information about the other IRF family members has been scarce regarding their functions (TANIGUCHI *et al.*, 2001).

#### 2.2.4 Previous studies on the transcriptional regulation of the FcRn $\alpha$ -chain

Insufficient information is currently available regarding the transcriptional regulation of FcRn. Whereas the sequences of the human and mouse FcRn  $\alpha$ -chain genes including 5'-flanking sequences and the *cis*-regulatory region of possum and rat have been reported (JIANG *et al.*, 2004, KANDIL *et al.*, 1995, MIKULSKA *et al.*, 2000, WESTERN *et al.*, 2003), the elements that are critical in the promoter activity of FcRn  $\alpha$ -chain in these species have been only partially identified.

The *cis*-regulatory region of FcRn  $\alpha$ -chain is not associated with typical TATA or initiator sequences in human and rodents (KANDIL *et al.*, 1995, MIKULSKA *et al.*, 2000). A preliminary study of human FcRn (hFcRn) *cis*-regulatory region has verified the promoter activity of the segment, which spans from -660 bp to +300 bp compared to TSS, by reporter gene assay, and has predicted Sp1-like, AP1 or CREB, and Ets family binding sites in the proximal promoter region by the electrophoretic mobility shift assay (EMSA) of relatively large promoter fragments (MIKULSKA and SIMISTER, 2000). A variable number of tandem repeats (VNTR) region within the hFcRn 5'-untranslated region that can influence the promoter activity has been observed in another study (SACHS *et al.*, 2006). In reporter gene assay, the FcRn *cis*-regulatory regions containing the two most common VNTR alleles in

Caucasians can activate the transcription in different degrees, and quantitative polymerase chain reaction (qPCR) on monocytes has shown the same relation. In addition, the elevated FcRn transcription in monocytes is accompanied by the elevated IgG binding, but these VNTR alleles have not been functionally connected with any *trans*-acting elements. Furthermore, the location of rat core promoter has been defined from -157 bp to +135 bp by reporter gene assay, and two clusters of five Sp1-like sites that contribute to constitutive activity of core promoter have been identified by EMSA, DNase I footprinting and the reporter gene analysis of mutant core promoter constructs. The two Sp1-like sites of the proximal cluster have been also confirmed by supershift assay of Sp1, Sp2 and Sp3 transcription factors (JIANG *et al.*, 2004). A study of mouse *cis*-regulatory region (TIWARI and JUNGHANS, 2005) has been started from the fact, that FcRn is expressed in a developmentally regulated manner in rodent intestine. In suckling mice, high level of FcRn expression occurs in their enterocytes until day 14 following birth, afterwards the intestinal expression drops dramatically and is nearly undetectable in adults. Two upstream regions that have repressor and activator functions have been identified from -372 bp to -141 bp and from -105 bp to -1 bp by reporter gene assay. The proximal region bears an Ets site, a Sp1-like site and a NF1 site according to EMSA and the reporter gene analysis of constructs harbouring mutant site has shown that the Ets site has repressor and the Sp1-like site has activator functions, but the NF1 has failed to influence the reporter gene activity. None of these sites bind protein in EMSA using nuclear extract from neonatal intestine, therefore the high expression level of mouse FcRn in neonatal intestine is not adequately supported by experimental analyses, and the developmental regulation of *cis*-regulatory region has not been clarified.

In summary, Sp1-like sites that may contribute to the constitutive transcription and an Ets site that may repress the transcription (SHARROCKS, 2001) have been successfully identified, but the transcriptional regulation has failed to correlate with the well characterized immunological function of FcRn, and transcription factors that possess relevant immunological functions has not been implicated in the transcriptional regulation of FcRn  $\alpha$ -chain.

### 2.2.5 The $\beta_2$ -microglobulin and previous data about its transcriptional regulation

Besides the crucial role of  $\beta_2m$  in IgG transport and metabolism as a component of FcRn molecule (GHETIE *et al.*, 1996, ISRAEL *et al.*, 1995),  $\beta_2m$  is best known for its association with the MHC class I heavy chain. Classical MHC class I molecules and  $\beta_2m$  are ubiquitously

expressed in most adult tissues to present antigen-derived peptides to cytotoxic T lymphocytes and important in protection against natural killer cell mediated cytotoxicity (HUDSON and PLOEGH, 2002).  $\beta$ 2m is also associated with MHC class Ib or class I-like molecules, which have more restricted tissue distribution, and have more specialized functions in antigen presentation and iron metabolism (BRAUD *et al.*, 1999, WAHEED *et al.*, 2002).

In contrast to FcRn, the constitutive and immune-regulated transcription of  $\beta$ 2m has been well characterized in human. The constitutive regulation is similar to that of MHC class I genes (VAN DEN ELSEN *et al.*, 2004), their *cis*-regulatory region possess an SXY module in close proximity of TSS (Figure 14) which binds a multi-protein complex, and which is regulated through an MHC-specific enhanceosome (GOBIN *et al.*, 1998, GOBIN *et al.*, 2001). In addition, an E-box is only located in  $\beta$ 2m *cis*-regulatory region contributing to the constitutive transcription (GOBIN *et al.*, 2003). A  $\kappa$ B and an ISRE site located upstream from SXY module are mainly responsible for the cytokine-induced regulation, although they also contribute to the constitutive expression. The ISRE site mediates strong INF- $\gamma$  inducibility and weaker INF- $\alpha$  and INF- $\beta$  activation, whereas the degree of TNF- $\alpha$  induction is the same as those of INF- $\alpha$  and INF- $\beta$ , and the mutation of  $\kappa$ B and ISRE sites delete cytokine-inducibility. Both p50 and p65 subunits of NF $\kappa$ B bind to the  $\kappa$ B site according to supershift assay, and the activity of the *cis*-regulatory region can be induced by p65 overexpression. IRF1 and IRF3 overexpression can activate the transcription, while IRF2, IRF4 and IRF8 overexpression fail to induce it in reporter gene assay, although supershift assay has verified the presence of IRF1 and IRF2 in the IFN- $\gamma$ -specific complex of ISRE site. Thus the basal level of  $\beta$ 2m transcription is enhanced by cytokines to meet local requirements for an adequate antigen presentation, IgG transport, and possibly also to fulfill any of its other function (GOBIN *et al.*, 2003).

### 3. Aims of the present study

The role of the IgG-Fc receptor FcRn in transporting IgGs through epithelial cells and regulating IgG homeostasis has been recently shown in selected species. However, insufficient information is currently available regarding the regulation of the FcRn expression. Whereas the sequences of the human and murine FcRn genes and the promoter sequence of the possum have been reported, the elements that are critical in directing FcRn transcriptional activity in these species have been only partially identified.

The primary aim of this study was to investigate the transcriptional regulation of the bovine FcRn  $\alpha$ -chain, because it was presumed that the expression regulation of the FcRn molecule was achieved through the  $\alpha$ -chain, which was mainly responsible for the specific features of the FcRn molecule. Based on the initial *in silico* promoter analysis, putative binding sites for transcription factors within 5'-flanking sequence of the bFcRn were identified, and some of these putative binding sites reflected the possible responsiveness of this gene in inflammatory reactions. As FcRn is a heterodimer and composed of the  $\alpha$ -chain and the  $\beta_2$ -microglobulin, thus the transcriptional co-regulation of the bovine  $\beta_2m$  and FcRn  $\alpha$ -chain was thought an interesting issue, and both of these genes were analyzed with regard to their transcriptional regulation.

To perform these aims, the methods for the investigation of the *cis*-regulatory region, such as luciferase reporter gene assay, gel retardation assay and site-directed mutagenesis had to be set up in our laboratory. Naturally, the set up of these methods did not only serve the present study, but it supported the further aims of our laboratory. Having these techniques established, the effect of the NF $\kappa$ B and IRF transcription factors was analyzed in the regulation of these genes. During the experimental work of the present study, the demand arose to generate a fully bovine-specific cell model of the NF $\kappa$ B gene regulation, therefore the cloning and characterization of the bovine p65 was added to the original aims.

## 4. Materials and Methods

### 4.1. *In silico* transcription factor binding site analysis

Transcription of genes is controlled primarily by transcription factors recognizing and binding to DNA sequence motifs, termed transcription factor binding sites, in *cis*-regulatory region of the genes, leading to activation or repression of their transcription. TF binding sites comprising 5-25 base pairs are specific for a TF or a TF family, but also exhibit sequence variability in different degrees. Position weight matrix based on a multiple alignment of the known binding site sequences of a particular TF is used for the quantitative description of TF binding site (WASSERMAN and SANDELIN, 2004). Attempting to find the putative binding sites of a particular *cis*-regulatory region *in silico* is essentially performed with a database of position weight matrices representing different TF binding sites and a computer program developed to scan a DNA sequence against that database. A quantitative score for each potential TF binding site is produced by the program, which characterizes the similarity between the potential binding site and the corresponding position weight matrix. If the score is high enough, above a particular threshold value determined experimentally or statistically by the user, then the potential binding site is identified as a putative TF binding site (PRESTRIDGE, 2000). While the matrix-based search is considered to be sensitive, the major drawback of using position weight matrix in identifying TF binding sites is that only a small fraction of the predicted putative binding sites is functionally significant (QIU, 2003). These kind of false positive matches can be reduced by increasing the threshold value, but the higher threshold value results in more false negative matches, functionally significant binding sites that are not predicted as putative binding sites (PICKERT *et al.*, 1998). However, even optimized threshold values will most likely yield lots of false positive and false negative matches, thus the putative TF binding sites must be experimentally investigated before being accepted as functional sites.

In order to search putative binding sites in the *cis*-regulatory region of the bovine FcRn, the unpublished 5'-flanking sequence of the bFcRn  $\alpha$ -chain was analysed by Transcription Element Search System (TESS) software (SCHUG and OVERTON, 1997), which is accessible on-line and free of charge. The searches were performed by matrix-based search using mammalian position weight matrices (WINGENDER *et al.*, 2001) and relatively high thresholds (minimum lg likelihood ratio: 6, maximum lg likelihood deficit: 6-10). When the result of the initial search justified the necessity, the initial threshold was reduced, and the

putative binding sites were confirmed by other computer programs, such as TFSEARCH (HEINEMEYER *et al.*, 1998).

#### **4.2. Cloning, mutagenesis, and the generation of different constructs used in the present study**

Different types of constructs were generated, most of them were produced by integrating the *cis*-regulatory region of target gene upstream of the reporter gene. However, cDNA segments were inserted into cloning or mammalian expression vectors in several cases. The *cis*-regulatory regions were cloned into pGL3-basic (Promega) firefly luciferase reporter gene vector (GROSKREUTZ *et al.*, 1995). The original 5'-flanking sequences of the bovine FcRn and  $\beta$ 2m (Appendix) were isolated and cloned by Yaofeng Zhao and Lennart Hammarström (Clinical Immunology, Karolinska University Hospital, Stockholm, Sweden) with whom our laboratory collaborated. Likewise they generated the bovine FcRn and  $\beta$ 2m luciferase reporter gene constructs, namely pGL3-bFcRn-1787+92, pGL3-bFcRn-1112+92, pGL3-bFcRn-525+92 and pGL3-b $\beta$ 2m-1934+49. The human FcRn luciferase reporter gene constructs, termed pGL3-hFcRn-1970+218 and pGL3-hFcRn-611+116, were produced by Imre Kacs Kovics. The constructs were labelled as follows: the first tag indicated the vector, while the second tag specified the gene or the *cis*-regulatory region of the gene including its positions of the 5'- and 3'-ends relative to TSS. If the constructs contained mutated *cis*-regulatory regions, the third tag designated the mutated TF binding site including its position of the 3'-end.

The basis of molecular cloning was developed by the late 1980s, thus the basic techniques belonging to molecular cloning such as polymerase chain reaction (PCR) performed with *Taq* polymerase, restriction endonuclease digestion, ligation and transformation were performed in accordance with the two most popular, comprehensive manuals (AUSUBEL, 1987, SAMBROOK *et al.*, 1988). Briefly, the purification of DNA fragments from gel or solution was carried out by QIAquick Gel Extraction kit (Qiagen), which was always applied before ligation by T4 ligase (New England Biolabs). DH5 $\alpha$  *E. coli* strain was used for cloning, and cloned constructs were purified by Qiagen Plasmid kits (Qiagen). PCR using *Taq* polymerase (Promega) was performed for the screening of the constructs. The generated constructs were always verified by sequencing and restriction endonuclease digestions.

#### 4.2.1 Mutagenesis of the bovine FcRn and $\beta$ 2m luciferase reporter gene constructs

Site-directed mutagenesis is a powerful method for analyzing the individual TF binding sites within a *cis*-regulatory region. By replacing discrete segments of DNA with heterologous segments of the same length, the topological and spatial organization of the DNA helix is maintained. This allows the contribution of the individual TF binding sites to be determined by a reporter gene system in the context of the native DNA helix configuration (GUSTIN and BURK, 2000). To generate mutated TF binding sites in a *cis*-regulatory region is to synthesize enzymatically a new DNA, while in the meantime incorporating the desired mutations into their newly synthesized DNA. Other than attempting to introduce mutations into the product, the methods or protocols for DNA mutagenesis are essentially the same as those for DNA synthesis or for other molecular biological manipulations, such as cloning, sequencing and probe labelling. PCR provides a plausible approach to the site-directed mutagenesis because of its simplicity over other time-consuming and labour-intensive techniques. On the other hand, the main drawback of using PCR for DNA mutagenesis is the relatively high rate of sequence errors in PCR products, often creating undesired mutations in addition to intended ones. Taq, the most widely used thermostable polymerase, lacks the 3'-5' exonuclease activity that proofreads any errors caused by 5'-3' DNA polymerase during DNA synthesis. Amplification through many cycles therefore accumulates errors. This applies oddly to the mutagenesis techniques that produce the entire mutant *cis*-regulatory region, and replace the wild-type *cis*-regulatory region in a vector. Other polymerases, such as Pfu and Vent, which carry 3'-5' exonuclease activity, provide 6-15 times the sequence fidelity of Taq, thus polymerases with 3'-5' exonuclease activity are preferred for PCR-based site-directed mutagenesis (LING and ROBINSON, 1997).

Two kind of these PCR-based, site-directed mutagenesis methods have been used in this study, the ligation method of two PCR products carrying mutations and the megaprimer method (Figure 5). The first method involves two primer pairs, one pair spans the *cis*-regulatory region from the 5'-end to the TF binding site that is needed to mutate and the other pair from the TF binding site to the 3'-end. The two middle mutagenic primers that cover the TF binding site overlap each other, and contain a common restriction digestion site. For producing the entire mutant *cis*-regulatory region, three PCRs are needed to perform in all. Two of them amplify the two halves of the *cis*-regulatory region, then the resulting two PCR products carrying mutation are digested and ligated followed by amplifying the entire mutant *cis*-regulatory region from the ligated PCR products. Megaprimer mutagenesis reduces the number of primers to three and the number of PCRs to two for each mutation. Two of the

primers are located at the ends of the *cis*-regulatory region, the third mutagenic primer in the middle of the *cis*-regulatory region. The first PCR, using one primer in the end position and the middle mutagenic primer, amplifies a double-stranded megaprimer containing mutations introduced by the mutagenic primer. The second PCR, using the megaprimer and the other primer in the end position produces the entire mutant *cis*-regulatory region (HARLOW *et al.*, 1996, LING and ROBINSON, 1997).

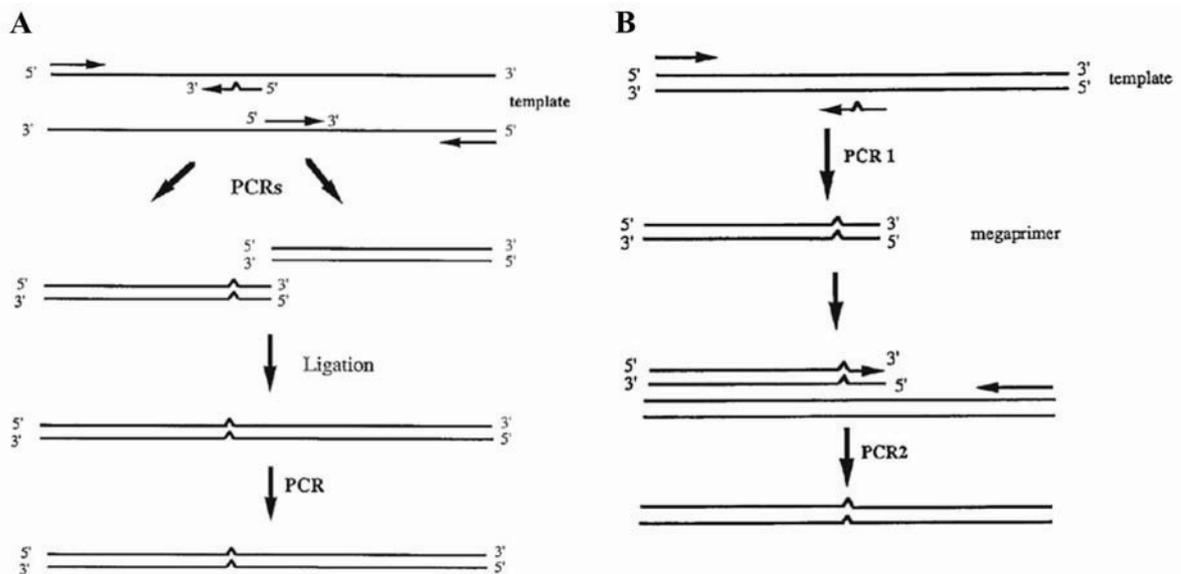


Figure 5

The schematic picture of site-directed mutagenesis methods based on PCR used in this study. A. Ligation method using two middle mutagenic primers. B. Megaprimer method using one middle mutagenic primer (LING and ROBINSON, 1997).

For generating the luciferase reporter gene constructs containing the mutant  $\kappa$ B site in the bFcRn *cis*-regulatory region by ligation method (GUSTIN and BURK, 2000, GUSTIN and BURK, 1993), the flanking primers of the bovine FcRn *cis*-regulatory region from -1112 bp to +92 bp (Table 1) and two middle mutagenic primers for each mutation were used (Table 2). The primers were designed based on the unpublished 5'-flanking sequences of the bFcRn  $\alpha$ -chain (Appendix). The introduced mutant base pairs were designed on the basis of the position weight matrix of  $\kappa$ B site from TRANSFAC v6.0 database (MATYS *et al.*, 2003). Each mutagenic primer pair harboured a unique restriction digestion site that was not present in the pGL3-basic vector to facilitate the screening of mutant constructs. PCRs that generated the two halves of the *cis*-regulatory region were performed using 1.25 U of Deep Vent proofreading DNA polymerase (New England Biolabs) and 1 ng of pGL3-bFcRn-1112+92 construct as template per reaction. The total reaction volume was 50  $\mu$ l, and the final concentrations of the dNTP and each primer were 200  $\mu$ M and 1 pmol/ $\mu$ l, respectively. The

PCR temperature profile used was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at the temperature depending on the melting temperature of primers (Table 2) for 30 sec, extension at 72 °C for the time

Table 1

The flanking primers of the bFcRn and b $\beta$ 2m *cis*-regulatory region. The restriction digestion sites are underlined, s and as mean sense and antisense, and bp are corresponding to the sequences in Appendix.

The gene of <i>cis</i> -regulatory region	Sequence	Direction and binding positions (bp)	Restriction digestion site	The temperature of annealing
bovine FcRn	5'-CCG <u>ACGCGT</u> GACACGACTGAAGGGGTTA-3'	s, -1112 - -1093	<i>MluI</i>	61 °C
	5'-TTT <u>AAGCTT</u> GCCGCGATCCCTTCCCTCTG-3'	as, +92 +73	<i>HindIII</i>	
bovine $\beta$ 2m	5'-ATAGGT <u>ACCCA</u> AGGTATTGGAGTTTCAGCTT-3'	s, -1934 - -1913	<i>KpnI</i>	58 °C
	5'-CGAGCT <u>AGCCC</u> ACGAAGCGAGCCATC-3'	as, +30 - +50	<i>NheI</i>	

depending on the length of the desired PCR products (1 min was reckoned per the 1000 bp of length) and final extension at 72 °C for 10 min. After the amplifications, mutant PCR products were purified, digested by the unique restriction endonuclease and ligated. The entire mutant *cis*-regulatory region produced by ligation was amplified by PCR using the flanking primers of the bovine FcRn *cis*-regulatory region and the above mentioned PCR conditions. The resultant products that harboured different mutant  $\kappa$ B binding sites were purified, digested with the endonuclease of the flanking primers (Table 1) and cloned into the pGL3-basic vector. The constructs were named for pGL3-bFcRn-m $\kappa$ B-612, pGL3-bFcRn-m $\kappa$ B-758, and pGL3-bFcRn-m $\kappa$ B-840.

Table 2

The mutagenic primers were used in this study. The restriction digestion sites that were built into the primers are underlined, the mutated bases are indicated by bold letters, s and as mean sense and antisense, and bp are corresponding to the sequences in Appendix..

The name of the resultant construct	Sequence	Direction and binding positions (bp)	Restriction digestion site	The temperature of annealing
pGL3-bFcRn-m $\kappa$ B-612	5'-CCCAAG <u><b>GACGTCC</b></u> ATCAGACACATTAAGT-3'	as, --633 - -616	<i>AatII</i>	61 °C
	5'-GGGACCACTTAATGTGTCTGGGG <u><b>GACGTC</b></u> TTG-3'	s, -612 - -585		57 °C
pGL3-bFcRn-m $\kappa$ B-758	5'-GGTGGCGGTGGGGT <u><b>ACTAGT</b></u> TTTTTT-3'	as, -749 - -774	<i>SpeI</i>	57 °C
	5'-CCC <u><b>ACTAGT</b></u> ACTGAACCGTACACTAAATGAAAAG-3'	s, -768 - 739		58 °C
pGL3-bFcRn-m $\kappa$ B-840	5'-TGGTTTGCC <u><b>GAATTC</b></u> TTTAACAGCATGCCG-3'	as, -831 - -859	<i>EcoRI</i>	59 °C
	5'-AG <u><b>GAATTC</b></u> CGGCAAACAGTGGTTAAGACTCTGCC-3'	s, -840 - -814		60 °C
pGL3-b $\beta$ 2m-mISRE-122	5'-CTAGAAAATGAGCCTGAGGACGGGGAAGCC-3'	s, -141 - -111	-	61 °C

The *cis*-regulatory region carrying all three different mutated binding sites was generated by the sequential repeating of the ligation mutagenesis protocol. First, the pGL3-bFcRn-m $\kappa$ B-

840 was used as template for the mutagenesis of the  $\kappa$ B -758 site, which resulted in a construct containing two mutant binding sites, the pGL3-bFcRn-m $\kappa$ B-758-840. Secondly, the pGL3-bFcRn-m $\kappa$ B-758-840 was applied as template for the mutagenesis of the  $\kappa$ B -612 site, which resulted in the pGL3-bFcRn-m $\kappa$ B-612-758-840 construct.

In order to generate the luciferase reporter gene vector containing the mutant ISRE site in bovine  $\beta$ 2m *cis*-regulatory regions, megaprimer mutagenesis method was achieved (DATTA, 1995). Two PCRs were used for the mutagenesis using the flanking primers of the bovine  $\beta$ 2m *cis*-regulatory region from -1934 bp to +49 bp (Table 1) and a middle mutagenic primer (Table 2). The primers were designed based on the unpublished 5'-flanking sequences of the  $\beta$ 2m (Appendix). The introduced mutant base pairs were designed on the basis of the position weight matrix of ISRE site from TRANSFAC v6.0 database (MATYS *et al.*, 2003). The first PCR, which produced the megaprimer, was performed using 2.5 U of Deep Vent polymerase, 10 ng of pGL3-b $\beta$ 2m-1934+49 construct as template, 250 pmol of the antisense flanking primer and mutagenic primer, and 10 mmol of dNTP in 50  $\mu$ l of total reaction volume. The PCR temperature profile used was as follows: initial denaturation at 94 °C for 3 min, followed by 20 cycles of denaturation at 94 °C for 30 sec, annealing at the 61 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 5 min. After the PCR, the reaction mixture was run on 1% agarose gel to remove antisense flanking primer, then megaprimer was cut from gel and purified. The purified megaprimer was used in the second PCR. To maximize the yield of the final product, second PCR was carried out for 5 cycles using only the megaprimer, so the reaction mixture consisted of 2.5 U of Deep Vent polymerase, 10 ng of pGL3-b $\beta$ 2m-1934+49 construct as template, 50 ng of megaprimer, and 10 mmol of dNTP in 50  $\mu$ l of total reaction volume, and the following two-step PCR profile was fulfilled: initial denaturation at 94 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 1 min, annealing and extension at 72 °C for 3 min. After the 5 cycles, 2  $\mu$ l of 50 pmol/ $\mu$ l sense flanking primer was added, and the reaction was continued following the temperature profile: 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 3 min, and final extension at 72 °C for 5 min. The resultant product harbouring the mutant ISRE binding sites was purified, digested with the endonuclease of the flanking primers (Table 1) and cloned into the pGL3-basic vector. The construct was named for pGL3-b $\beta$ 2m-mISRE-122.

#### 4.2.2 Generation of the pGL3-bFcRn-838+92 and pGL3-bFcRn-1112-845/-525+92 luciferase reporter gene constructs

In order to estimate the transactivation potential of the bovine FcRn *cis*-regulatory region segments that were located between -1112 and -526 bp, and upstream and downstream from the  $\kappa$ B -840 site, the variants of the pGL3-bFcRn- $\kappa$ B-840 luciferase reporter gene construct were generated utilizing the *EcoRI* restriction site of the mutated TF binding site. The segment ranging from the 5'-end of the *cis*-regulatory region to the restriction site of the  $\kappa$ B -840 site was cut out from the pGL3-bFcRn- $\kappa$ B-840 construct by *MluI* and *EcoRI* restriction endonucleases. After this, blunt-ends were generated from the 5'-overhang ends of the digested restriction sites by Klenow fragment (Promega) followed by the blunt-end ligation and cloning of the opened construct. The resultant construct was termed for pGL3-bFcRn-838+92.

To produce a *cis*-regulatory region containing the upstream *cis*-regulatory segment from the  $\kappa$ B -840 site and the downstream segment from -525 bp, two constructs, pGL3-bFcRn- $\kappa$ B-840 and pGL3-bFcRn-525+92, were applied. First, the *EcoRI* site of the pGL3-bFcRn- $\kappa$ B-840 was digested, and blunt-ends were generated from the ends of the digested site. Second, the boundary of the 3'-end of the *cis*-regulatory region and multiple cloning site was cut by *HindIII*, consequently the *cis*-regulatory region from -846 bp was removed, and the resultant, opened construct comprised the upstream *cis*-regulatory segment from the  $\kappa$ B -840 site with a 3'-blunt-end, and a *HindIII* compatible 5'-end at the other side of the multiple cloning site. In parallel with the digestions of pGL3-bFcRn- $\kappa$ B-840, the pGL3-bFcRn-525+92 construct was digested at the 5'-end of the *cis*-regulatory region by *MluI*, and blunt-ends were generated from the ends of the digested site. Then the construct was digested at the 3'-end of the *cis*-regulatory region by *HindIII*, thus the *cis*-regulatory region from -525 bp to +92 bp with 5'-blunt-end and *HindIII* compatible 3'-end was cut out from the construct. This *cis*-regulatory segment was directionally ligated with the opened construct derived from pGL3-bFcRn- $\kappa$ B-840 at the blunt-ends and *HindIII* compatible ends. The resultant construct was cloned and named for pGL3-bFcRn-1112-845/-525+92.

#### 4.2.3 Confirmation of the deletion in the $\kappa$ B site of bovine $\beta$ 2m

To confirm that the  $\kappa$ B deletion of  $\beta$ 2m was present in several animals and different breeds apart from the original source derived from an Angus individual, the 5'-flanking sequences of  $\beta$ 2m from two individual belonging to Angus breed and three individual belonging to Holstein-Friesian breed were amplified by PCR, cloned and sequenced. The primers were designed based on the unpublished 5'-flanking sequences of the  $\beta$ 2m (Appendix). The PCR

was performed using 1 U of Deep Vent DNA polymerase, 500 ng of bovine genomic DNA, 50 pmol of the primers corresponding to the bases -245 - -230 (5'-GGG TAC GCG TTG TGC GCC GCA AGC TT-3') and +43 - +60 (5'-GCA GCT CGA GAA GGA CCA AGG CCA CGA A-3'), and 10 mmol of dNTP in 50 µl of total reaction volume. The PCR temperature profile used was as follows: initial denaturation at 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C for 1 sec, annealing at 64 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 10 min. The resultant products were purified, digested at the endonuclease sites of the primers, *MluI* and *XhoI*, directionally cloned into the pGL3-basic vectors, and sequenced.

#### 4.2.4 Generation of a *Renilla* internal control plasmid

To monitor transfection efficiency and to avoid the down-regulation of the expression of the internal control vector driven by virus promoters (HUSZAR *et al.*, 2001, IBRAHIM *et al.*, 2000), a segment of the human  $\beta$ -actin *cis*-regulatory region was cloned into *Renilla* internal control vector. As the selected segment was composed of very high (74 %) G + C content, a two-step PCR protocol with merged annealing and extension was carried out instead of the standard three-step PCR protocol (DUTTON *et al.*, 1993). The PCR was performed using 3 U of Deep Vent DNA polymerase, 650 ng of human genomic DNA, 50 pmol of the primers corresponding to the bases of the human  $\beta$ -actin gene (Genbank AY582799) -213 - -195 (5'-CCC AAG CTT CTC CTC TTC CTC ATT CTC G-3') and +914 - +932 (5'-CCT GAA TTC GTG AGC TGC GAG AAT AGC C-3'), and 10 mmol of dNTP in 50 µl of total reaction volume. The PCR temperature profile used was as follows: initial denaturation at 98 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 1 min, annealing and extension at 66 °C for 5 min, and final extension at 72 °C for 10 min. The resultant product was purified, digested at the endonuclease sites of the primers, *HindIII* and *EcoRI*, and directionally cloned into the phRL-null vector (Promega). The *Renilla* construct was termed for phRL-h $\beta$ actin-213+932.

#### 4.2.5 Cloning of the bovine p65 subunit of NF $\kappa$ B cDNA

The bovine transcription factor p65 (bp65) was cloned in order to estimate its potential species-specific influence for the bovine FcRn and  $\beta$ 2m *cis*-regulatory region. For this purpose, total RNA was isolated by TRIzol Reagent (Invitrogen) from primary bovine aortic endothelial cells (BAEC) as it expresses the p65 subunit of NF $\kappa$ B (ANRATHER *et al.*, 1997). One µg of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega) with the (dT)17-adaptor primer (5'-GAC TCG AGT

CGA CAT CGA(T)17-3') and cDNA quality was verified by PCR of the bovine  $\beta$ -actin (5'-ACC ATC GGC AAT GAG C-3', 5'-CGT GTT GGC GTA GAG GTC-3'). A cDNA segment which contained the complete coding sequence of the bp65 was amplified with 1 U of Deep Vent DNA polymerase using primers locating in the boundary of the 5'-untranslated region (UTR) and the start of the coding region (5'-CGG GGT ACC GGC CAT GGA CGA CTT CTT C-3') and in the 3'-UTR (5'-GGC CCC GGG CTC CCA GAA TCC ATC AGT GTG-3') of bp65 mRNA, which were designed based on bovine EST sequences (Genbank AW464277, BF889526, BI774155, CN790494) and the human p65 mRNA (Genbank M62399). The total reaction volume was 50  $\mu$ l, and the final concentrations of the dNTP and each primer were 200  $\mu$ M and 1 pmol/ $\mu$ l, respectively. The following PCR profile was fulfilled: initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 96 °C for 45 sec, annealing at 69 °C for 30 sec, extension at 72 °C for 3 min, and final extension at 72 °C for 10 min. The resultant products from three independent amplifications were modified with A-overhangs using Qiagen A-Addition Kit (Qiagen) and cloned into pGEM-T TA vectors (Promega). The correct insert was selected by sequencing and comparing the inserts, cut out with *KpnI*, *NotI* endonucleases from pGEM-T and cloned into pcDNA3.1/Hygro(+) (Invitrogen) mammalian expression vector. The vector was named for pcDNA3.1/Hygro(+)-bp65.

### 4.3. Cells, cell culture and treatments

The basic techniques of cell culture, such as subculturing and cryopreservation were achieved according to one of the most widespread manuals (FRESHNEY, 2000). Briefly, cells were rinsed with Dulbecco's phosphate buffered saline (Sigma), incubated with trypsin-EDTA (Sigma) for cell harvesting, and cryopreserved in cell culture freezing medium-DMSO (Invitrogen).

HeLa S3 (human cervical adenocarcinoma cells, a kind gift from Dr. P. Gálfi, Szent István University, Budapest, Hungary) and 293 (human embryonic kidney epithelial cells, a kind gift from Dr. B. Gereben, Institute of Experimental Medicine, Budapest, Hungary) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen), penicillin (50 U/ml, Sigma), streptomycin (50  $\mu$ g/ml, Sigma) and sodium pyruvate (1 mM, Sigma). Primary bovine aortic endothelial cells (BAEC, Cambrex Bio Science) were propagated in DMEM containing 10% FCS, penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml), human epidermal growth factor (10 ng/ml, Sigma) and hydrocortisone (1  $\mu$ g/ml, Sigma). HC11 cell line (mouse mammary epithelial

cells) was regularly cultured in RPMI-1640 (Invitrogen) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), mouse epidermal growth factor (10 ng/ml, Sigma), and insulin (5 µg/ml, Sigma). After transfection for luciferase reporter gene assay, HC11 cells were maintained in medium with FCS reduced to 2% and without mouse epidermal growth factor (GEYMAYER and DOPPLER, 2000). MAC-T cell line (bovine mammary epithelial cells, a kind gift from Dr. X. Zhao, McGill University, Quebec, Canada) was grown in DMEM supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), insulin (5 µg/ml) and hydrocortisone (1 µg/ml). All cells were cultured in 5% CO<sub>2</sub> at 37 °C. For gel retardation assay, BAEC cells were incubated with 100 ng/ml LPS (*E. coli* 026:B6 serotype, Sigma) for 1 hour, with 100 ng/ml bovine INF-γ for 24 hours, and HeLa S3 cells were treated with 20 ng/ml human TNF-α (Sigma) for 1 hour.

#### **4.4. Transfection**

The direct transfer of genetic material into eukaryotic cells is a crucial technique for the investigation of both gene function and gene therapy. Viral particles transfer nucleic acid naturally to cells by the process of infection, and the process of nucleic acid transfer to cells by artificial, non-viral methods is referred to as transfection. The understanding of eukaryotic transcriptional regulation has benefited greatly by the co-development of *in vitro* transfection techniques together with genetic reporter genes, since transcription activity can be studied through the transfection of reporter genes linked operationally to *cis*-regulatory regions. Many techniques have been developed for the purpose of transfecting plasmid DNA vectors and other nucleic acids into cultured cells. The following characteristics describe the desirable attributes for the successful DNA transfection techniques: highly efficient delivery of DNA to nucleus, low cellular toxicity, minimal interference with normal cellular physiology, ease of use and reproducibility. Some chemical reagents such as cationic polymers, DEAE-dextran, calcium phosphate and cationic liposomes match these attributes, and are often utilized for *in vitro* DNA transfection. One common feature of these chemical reagents is their cationic nature. On the one hand, the positively charged molecules associate with the negatively charged phosphates of the DNA backbone. On the other hand, an overall net positive charge of the DNA-reagent complexes is typically effective, because it allows closer apposition of these DNA complexes with the outer cell membrane having negative surface charges (SCHENBORN, 2000).

Two kinds of techniques based on the above-mentioned chemical reagents have been used in this study: one is a cationic polymer technique founded on polyethylenimine (PEI), and the

other is the calcium phosphate co-precipitation method. PEI is an organic macromolecule with the highest cationic-charge-density potential. PEI compacts DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. The high cationic-charge-density potential may enable to buffer the endosomal pH and protects DNA from degradation. It may also cause a continuous proton influx that induces endosome osmotic swelling and rupture providing an escape mechanism for DNA particles to the cytoplasm and finally to the nucleus (BEHR, 1997). Thus PEI due to its advantageous chemical features ensures an effective and reproducible transfection with low toxicity (BOUSSIF *et al.*, 1995). The calcium phosphate co-precipitation method is one of the first and most widely used *in vitro* transfection methods. The calcium phosphate co-precipitation method consists of mixing DNA with calcium chloride and a phosphate buffer, forming a fine precipitate and distributing over cultured cells (SCHENBORN and GOIFFON, 2000). The mechanism for transfection of DNA with calcium phosphate may involve endocytosis and some protection of DNA from nucleases in a similar way as the mechanism of PEI (LOYTER *et al.*, 1982). Continued popularity of this method is based on the readily available and inexpensive components, however, the precise conditions for precipitate formation are difficult to control and reproduce among the experiments (JORDAN *et al.*, 1996).

MAC-T, 293 and HC11 cell lines and BAEC cells were transfected for luciferase reporter gene assay by the polyethylenimine method (BOUSSIF *et al.*, 1995). For each cell type, it is critical to optimize the conditions of the particular method (SCHENBORN, 2000), thus the PEI transfection was optimized for several parameters. The optimization resulted in the parameters outlined in the next. One day before transfection, cells were seeded into 24-well plates (Corning) to become subconfluent by the next day. Before transfection, the cells were rinsed twice with FCS and antibiotics free minimum essential medium Eagle (MEM, Sigma) except for 293 cells, because they weakly attached to the surface of cell culture plate and the repeated rinses would have removed the cells. After this, 400  $\mu$ l FCS and antibiotics free MEM was added to cells. PEI (Aldrich) solution was used for the transfection of 293, HC11, MAC-T and jetPEI (Qbiogene) for the transfection of BAEC cells. Different cell types were transfected using different amounts of DNA and PEI solutions (Table 3). PEI or jetPEI solutions were mixed with DNA solutions, added to the cells and incubated on the cells for 3 hours at 37 °C in 5% CO<sub>2</sub>. After incubation, the cells were rinsed once with the media of the particular cell types except for 293 cells, and 1 ml of the particular media were added to all cell types. Cells were harvested two or three days after transfection (Table 3).

For gel retardation assays, MAC-T cells were transfected using the calcium phosphate co-precipitation method as described (JORDAN *et al.*, 1996). Briefly, the transfection was carried out in 100-mm (diameter) dish (Corning) at 70% confluence. One hour before the precipitate was added, the medium was replaced with 9 ml of fresh medium. 500  $\mu$ l of 250 mM calcium chloride solution containing 4  $\mu$ g of pcDNA3.1/Hygro(+)-bp65 or enhanced green fluorescent protein (GFP) expression vector, pEGFP-N3 (BD Biosciences, Clontech) and 4  $\mu$ g of pUC19 carrier DNA and 500  $\mu$ l of HEPES buffered saline containing 1.5 mM sodium phosphate were mixed and incubated for 5 min on ice. The mixed solutions were added to the cells, and were incubated for 5 hours at 37 °C in 5% CO<sub>2</sub>. After incubation, a glycerol shock was applied, when the cells were exposed to 20% glycerol in DPBS. After 1 min the glycerol was removed, the cells were rinsed twice with DPBS, and 10 ml of medium was added to the cells. The cells were then incubated for 2 days before they were harvested.

Table 3

The parameters of transfection for luciferase reporter gene assay. <sup>a</sup> The molar ratio of PEI amine nitrogen and DNA phosphate; 1  $\mu$ l of 0,45 mg/ml PEI solution contains 10 nmol of amine nitrogen, 1  $\mu$ g of DNA contains 3 nmol of phosphate (BOUSSIF *et al.*, 1995).

Cells or cell line	Seeded cell number one day before transfection	N <sub>PEI</sub> /P <sub>DNA</sub> <sup>a</sup>	total DNA (ng/well)	Harvesting time after transfection (hour)
293	2.5x10 <sup>5</sup>	35	1400	48
BAEC	1.5x10 <sup>5</sup>	5	1600	48
HC11	1.2x10 <sup>5</sup>	16	1400	72
MAC-T	1.0x10 <sup>5</sup>	16	1400	48

#### 4.5. Reporter gene assays

Genetic reporter genes have contributed greatly to the *in vitro* and *in vivo* studies from the eukaryotic gene expression to the boarder ensemble of molecular events that define phenotype expression. The term reporter gene is used to define a gene with a readily measurable phenotype, such as the amount of reporter mRNA and reporter protein, or the enzymatic activity of the reporter protein, which can be distinguished easily over a background of endogenous proteins. Therefore a defined nucleotide sequence, which, when introduced into a biological system, yields a measurable phenotype upon expression. Reporter genes are used to investigate signal transduction pathways, *cis*-regulatory regions, transcription factors, mRNA processing, and translation. In addition, reporters are also used to monitor transfection efficiencies, protein-protein interactions, protein subcellular localization and recombinant events. Several reporter genes available today impart a range of performance criteria to

choose from, including convenience, sensitivity, reproducibility, linearity and dynamics (NAYLOR, 1999, SCHENBORN and GROSKREUTZ, 1999, WOOD, 1995).

Analysis of *cis*-regulatory regions is the most common application for the reporter genes. For these types of studies, native or modified *cis*-regulatory regions are cloned normally upstream of reporter gene to generate reporter gene vector containing chimeric gene. Reporter gene vectors allow characterizing the *trans*-activity ability related to *cis*-regulatory regions, because the expression or activity of a reporter gene is correlated to changes in the transcriptional activity of the *cis*-regulatory regions. In addition, the *cis*-acting transcriptional elements, such as TF binding sites can be deleted by site-directed mutagenesis and tested in the reporter gene assay to identify them. For the investigation of a TF binding site, whose transcription factor is not present in quiescent nucleus, the endogenous transcription factor is activated through signal transduction cascade, or the transcription factor is overexpressed by the co-transfer of the transcription factor expression vector besides the reporter gene vector. Similarly, uncharacterized transcription factor can be assayed by the co-transfer of the particular transcription factor expression vector and a reporter gene vector harbouring the corresponding TF binding site. The vectors used for reporter gene assay are usually introduced into cultured cells by standard transfection methods (HIMES and SHANNON, 2000, SCHENBORN and GROSKREUTZ, 1999).

When comparing the ability of two *cis*-regulatory regions to regulate the expression of a reporter gene, following transfection of cells in culture, it is necessary to control for the potential difference in transfection efficiency (FARR and ROMAN, 1992). This is usually done by adding a second reporter gene vector, known as internal control vector, encoding a different reporter gene, so two individual reporter genes are simultaneously expressed within a single system. Typically, the experimental reporter coupled to the investigated *cis*-regulatory region is correlated with the effect of specific experimental condition, while the activity of the constitutively expressed control reporter provides an internal control that serves as the baseline response. Normalizing the activity of the experimental reporter to the activity of the internal control reporter minimizes experimental variability caused by differences in transfection efficiency. In addition, other sources of variability, such as differences in pipetting volumes or cell viability, can be effectively eliminated (SHERF *et al.*, 1996).

For the detection and optimization of transfection efficiency, enhanced GFP was used in some experiments. GFP from sea pansy, *Aequorea victoria*, is unique among light-emitting proteins in that it is autofluorescent and therefore does not require the presence of any cofactors or substrates for the light generation. Moreover, several GFP mutants are now available, which exhibit improved fluorescence properties over wild type GFP. The greatest advantage of GFP

and its mutant variants is that in the absence of cell lyses, noninvasive monitoring of gene expression in living cells is possible. On the contrary, the prevalence of other fluorescent molecules limits the sensitivity of GFP, thus GFP is less suitable for the quantitative measurement of transcription (NAYLOR, 1999, WOOD, 1995).

In this study, Dual-Luciferase Reporter (DLR) Assay System (Promega) has been utilized for determining the *trans*-activity ability of the *cis*-regulatory regions and bovine p65 subunit of NFκB and identifying the functional TF binding sites. DLR assay combines two luciferase reporter genes, those of the firefly (*Photinus pyralis*) and the sea pansy (*Renilla reniformis*), which provide the superior assay characteristics of bioluminescent reporters (BRONSTEIN *et al.*, 1994). Firefly and *Renilla* luciferase possess distinct evolutionary origins and therefore have dissimilar enzyme structures and substrate requirements, thus activities from each reporter can be measured sequentially in the same cell lysate. Firefly luciferase, which is a popular reporter gene due to its convenience and sensitivity, is utilized as experimental reporter in DLR assay. It is a monomer that requires no post-translational modification, thus the matured enzyme is produced directly upon translation and its catalytic competence is attained immediately after release from the ribosome (WOOD, 1998). In addition, no endogenous firefly luciferase activity exists in mammalian cells, so the background level of assay is low. The linear range of assay extends over 8 order of magnitude of enzyme concentration, providing detection of 1 femtomole, approximately  $10^{-20}$  mole of enzyme. To respond rapidly to change in gene expression, the genetic reporter must have a short half-life in biological system. Firefly luciferase also fulfils this criterion, its half-life is 3 hours in mammalian cells (WOOD, 1995). Beside its advantageous characteristics, firefly luciferase assay is sensitive for some conditions, for example the assay is optimized at 20-25 °C (BRONSTEIN *et al.*, 1994). *Renilla* luciferase provides many of the same benefits as firefly luciferase, although it is not generally preferred over firefly luciferase because its assay chemistry is somewhat more limited, and therefore it is utilized as control reporter in DLR (WOOD, 1998).

In order to optimize transfection on MAC-T, 293 and HC11 cell lines and BAEC cells, GFP and firefly luciferase reporters were applied by transfecting pEGFP-N3 and pGL3-control (Promega) vectors into the cells. In the case of GFP reporter, two independent parallels were observed with Axiovert 135 fluorescence microscope (Zeiss), and the means of the parallels were indicated without standard deviation. For measuring firefly luciferase reporter, Steady-Glo or Bright-Glo luciferase assay system (Promega) were used. Cells were harvested according to the manufacturer's instruction of DLR Assay System, the luciferase activities of

three independent parallels were determined using Luminoskan Ascent luminometer (Thermo Labsystem).

For the investigation of bovine FcRn and  $\beta$ 2m *cis*-regulatory regions, the following pGL3 luciferase reporter gene constructs were used: pGL3-basic, -bFcRn-1787+92, -bFcRn-1112+92, -bFcRn-525+92, -bFcRn-838+92, -bFcRn-1112-845/-525+92, -bFcRn-m $\kappa$ B-612, -bFcRn-m $\kappa$ B-758, -bFcRn-m $\kappa$ B-840, -bFcRn-m $\kappa$ B-612-758-840, -hFcRn-1970+218, pGL3-hFcRn-611+116, - $\beta$ 2m-1934+49 and - $\beta$ 2m-mISRE-122. To assess the specific inducing ability of bovine p65, pNF $\kappa$ B-Luc (Clontech, a kind gift from Prof. Dr. E. Duda, Biological Research Center, Szeged, Hungary) containing four tandem consensus  $\kappa$ B sites (5'-GGGAATTTCC-3') was applied. In addition, two luciferase reporter gene constructs harbouring AP1 or GAS binding sites, namely pAP1-Luc (Clontech, a kind gift from Prof. Dr. E. Duda, Biological Research Center, Szeged, Hungary) and m67-Luc (BESSER *et al.*, 1999), were used as positive control for the experiments of c-Jun and STAT3 overexpressions. The pGL3 constructs, pNF $\kappa$ B-Luc, pAP1-Luc or m67-Luc were transfected with the presence or absence of transcription factor expression vectors, which expressed human p50, human p65, human IRF1, mouse c-Jun, mouse STAT3, or bovine p65, namely pETp50, pETp65 (NAUMANN *et al.*, 1993), pRc/RSV-IRF1 (GOBIN *et al.*, 2003) (a kind gift from Prof. Dr. P. J. van den Elsen, Leiden University Medical Center, Leiden, Netherlands), pcDNA3-c-Jun (SHEN and STAVNEZER, 2001), pBABE-STAT3 (BROMBERG *et al.*, 1999) and

Table 4

The transfected luciferase reporter gene, internal control and transcription factor expression vectors for luciferase reporter gene assay in cell types were used.

Cells or cell line	pGL3 constructs (ng/well)	pNF $\kappa$ B-Luc (ng/well)	pET-p65 (ng/well)	pRc/RSV-IRF1 pcDNA3-c-Jun pBABE-STAT3 (ng/well)	pcDNA3.1/Hygro(+)-bp65 (ng/well)	pRL-TK (ng/well)	phRL-h $\beta$ actin -213+932 (ng/well)	total DNA (ng/well)
293	50	10	100	200	10	100	0.1	1400
BAEC	400	100	-	-	10	-	1	1600
HC11	200	50	100	200	5	200	1	1400
MAC-T	200	50	-	-	1 or 5	-	1	1400

pcDNA3.1/Hygro(+)-bp65. An internal control vector, pRL-TK (Promega) or phRL-h $\beta$ actin-213+932, was also co-transfected, and the necessary vectors for reporter gene assay were made up with pUC19 carrier DNA to maximize the transfection efficiency (FEKETE *et al.*, 2004). In different cell types different amounts of total DNA, luciferase reporter gene vectors, internal control vectors and transcription factor expression vectors were transfected (Table 4). Cells were harvested according to the manufacturer's instructions of the DLR Assay System (Promega), and the luciferase activities of three independent transfections were determined in

triplicate using a Luminoskan Ascent luminometer. The backgrounds of both firefly and *Renilla* luciferase activities were measured in non-transfected cells, and were subtracted from the measured values of luciferase reporter genes. Luciferase activity was normalized to *Renilla* activity, and the results expressed relative to normalized activity derived from pGL3-basic.

#### **4.6. Gel retardation assays**

The definition of gel retardation assays collects highly similar techniques that derive from EMSA and are founded on a common basic principle. Gel retardation assays rely on the obvious principle that a fragment of DNA to which a protein has bound move more slowly in gel electrophoresis than the same DNA fragment without bound protein. The assays are carried out by radioactively labelling the specific DNA sequence, whose protein-binding properties are being investigated. The labelled DNA is then incubated with a nuclear extract prepared in such a way as to contain the DNA-binding proteins. In this way DNA-protein complexes are allowed to form in the binding reaction. The complexes are then electrophoresed on a non-denaturing polyacrylamide gel and the position of the radioactive DNA is visualized by autoradiography. If no protein has bound to the DNA, all the radioactive labelled DNA will be at the bottom of the gel, whereas if a stable protein-DNA complex has formed, radioactive DNA to which the protein has bound will migrate more slowly and hence will be significantly retarded. This basic technique can be used therefore to identify the proteins which can bind to a particular DNA sequence *in vitro*. Some modifications of the basic technique further extend the area of utilization. The DNA sequence specificity of the protein within the complex can be tested by competing for binding with non-radiolabelled DNA fragments. If a molar excess of a DNA fragment capable of binding the same protein is introduced into binding reaction, much of the protein will bind to the unlabelled DNA, leaving less protein available for binding to the labelled DNA. This will lead to a reduction in, or elimination of the band corresponding to the complex formed by that protein. In contrast, if the non-radiolabelled DNA cannot bind the same sequence as the labelled DNA, the complex with the labelled DNA will form and the corresponding band will be visualized as before. The identification of proteins within the complex may be made by antibodies against known proteins in the binding reaction. Sufficient antibody can bind to the complex, causing further electrophoretic retardation of the complex. This kind of modified gel retardation assay is known as supershift assay (DENT *et al.*, 1999, LATCHMAN, 1998).

BAEC treated with LPS for 1 hour and with bovine IFN- $\gamma$  for 24 hours, and HeLa S3 cells treated with TNF- $\alpha$  for 1 hour were harvested for the preparation of nuclear extracts immediately after the treatment, while MAC-T cells transfected with bp65 were harvested 2 days after the transfection. All nuclear extracts (BAEC, LPS-treated BAEC, IFN- $\gamma$ -treated BAEC, HeLa, TNF- $\alpha$ -treated HeLa, MAC-T, bp65-transfected MAC-T) were prepared using CellLytic Nuclear Extraction kit (Sigma) followed by the determination of the total amount of protein using Micro BCA Protein Assay Reagent kit (Pierce) according to the manufacturer's instructions. The single-strand oligonucleotides were labelled with T4 polynucleotide kinase (New England Biolabs) and 1  $\mu$ l  $\gamma$ -[32]-ATP ( $\sim$ 5  $\mu$ Ci, Institute of Isotopes Co., Budapest, Hungary), annealed to form double-stranded oligonucleotides harbouring  $\kappa$ B, ISRE, IRF-E, and Sp1 sites (Table 5). The purification of labelled, double-strand oligomer was carried out with Sephadex mini Quick Spin Column (Roche). The binding reaction was performed in 10  $\mu$ l total volume for 20 min at room temperature using 1  $\mu$ l labelled oligomer, nuclear extracts containing 6  $\mu$ g of total protein, and, in some cases, 1  $\mu$ g of rabbit anti-p65 antibody (epitope corresponding to amino acids 1-286, H-286, Santa Cruz Biotechnology). The final composition of the binding buffer contained 4 mM HEPES, pH=7.9, 20 mM KCl, 0.4 mM DTT, 0.2 mM EDTA, 0.5 mg/ml BSA, 50  $\mu$ g/ml poly(dI-dC) (Sigma), 0.1 % IGEPAL (Sigma), and 4 V/V% glycerol (ZEOLD *et al.*, 2006). The samples were run on 5% nondenaturing polyacrylamide gel in 0.25x TBE buffer at 200 V for 2 hours. The gels were fixed with 10 V/V% acetic acid solution, dried onto Whatmann 3M paper and exposed to Hyperfilm MP (Amersham Biosciences).

Table 5

The following oligonucleotides were used for gel retardation assay. The TF binding sites are underlined.

The name of the oligonucleotide	Sequence
consensus IRF-E	5'-GGAAGCGAAAATGAAATGACT-3'
consensus $\kappa$ B	5'-AGTTGAGGGGACTTCCAGGC-3'
consensus Sp1	5'-ATTCGATCGGGGCGGGCGAGC-3'
bFcRn $\kappa$ B-612	5'-AATGTGTCTGGGATCTCCTTGGGAT-3'
bFcRn $\kappa$ B-758	5'-CAAAAAAAGAAAAACCCACCGTACA-3'
bFcRn $\kappa$ B-840	5'-CGGCATGCTGGGAAATCCCTGGCAAA-3'
b $\beta$ 2m ISRE-122	5'-TAACTAGAAAATGAAACTGAAAACGGGG-3'
b $\beta$ 2m $\kappa$ B-110	5'-TGAAAACGGGAAGCCTGTTTCTA-3'

#### 4.7. Phylogenetic analysis

Phylogenetic analysis of DNA or amino acid sequences has become an important tool for studying the evolutionary history of organisms. Besides investigating the evolutionary classification of organisms, it is indispensable for clarifying the evolutionary pattern of multigene families. For this purpose, phylogenetics concerns with coding regions at the level of both amino acid and DNA. First, a multiple alignment of the particular sequences is generated generally by using computer programs, and then phylogenetic tree is reconstructed by a statistical method. In practice, sequences often include insertions and deletions resulting in gaps, when the sequences are aligned, but statistical methods for reconstructing phylogenetic trees cannot take these gaps into account. Therefore a set of sequence data comprises only nucleotide identities and nucleotide mutations for other nucleotides, known as substitutions. In addition, the initiation and termination codons should be excluded in the analysis, because they remain unchanged in most cases (NEI and KUMAR, 2000).

There are many statistical methods that can be used for reconstructing phylogenetic trees from molecular data. Two of them have been used in this study, a distance method, namely the neighbour-joining (NJ) method (SAITOU and NEI, 1987), and the maximum likelihood (ML) method (FELSENSTEIN, 1981). NJ method is based on a distance such as p and Poisson correction distances at amino acid level or Jukes and Cantor's and Kimura's two-parameter distances at DNA level, similarly to other distance methods. In this method, the sum of all branch length estimates is computed for topologies, and the topology with the smallest sum of all branch length is chosen as the best tree, but all possible topologies are not examined as in minimum evolution method. NJ method, therefore, is regarded as a simplified version of the minimum evolution method. In ML method, the likelihood of observing a particular set of sequence data is maximized for each topology, and the topology that gives the highest maximum likelihood is chosen as the final tree (NEI and KUMAR, 2000).

Comparison of the rates of synonymous substitutions, which are silent and do not change amino acids, and non-synonymous substitutions, which alter amino acids encoded by codons, provides an important means for studying the mechanisms of DNA sequence evolution. Synonymous substitutions are apparently free from natural selection, and the rate of synonymous substitution is similar for many genes, indeed. By contrast, the rate of non-synonymous substitution is generally much lower than that of synonymous substitution and varies extensively from gene to gene. This is considered to be due to purifying selection, the extent of which varies from gene to gene. The rates of synonymous and non-synonymous substitution are defined as the number of synonymous substitutions per synonymous site ( $d_s$ )

and the number of non-synonymous substitutions per non-synonymous site ( $d_N$ ) for a pair of sequences (NEI and KUMAR, 2000).

There are several methods for estimating  $d_S$  and  $d_N$ . In this study, one of the evolutionary pathway methods, the Nei-Gojobori method (NEI and GOJOBORI, 1986) has been used, in which all possible evolutionary pathways between each pair of homologous codons of two DNA sequences are considered for estimating  $d_S$  and  $d_N$ .

The sequences of the NF $\kappa$ B/Rel superfamily (Table 6) were downloaded from the DDBJ/EMBL/Genbank databases. The sequence editing and comparisons were accomplished

Table 6

Sequences of the NF $\kappa$ B/Rel superfamily used for the reconstruction of phylogenetic trees. <sup>a</sup> labels the protein names of the NF $\kappa$ B/Rel superfamily used in this study, whereas their alternative names are in parentheses. <sup>b</sup> indicates the Genbank accession numbers of the DNA sequences that were used in this study. (pr) designates sequences in Genbank predicted by automated computational analysis and derived from annotated genomic sequences.

Species	Protein <sup>a</sup>	Accession no. <sup>b</sup>	Species	Protein	Accession no.
<i>Bos taurus</i> (cattle)	p65 (RelA)	DQ355511	<i>Xenopus laevis</i> (clawed frog)	p65 (Xrel1)	M60785
<i>Canis familiaris</i> (dog)	p65 (RelA)	XM_540850 (pr)		c-Rel (Xrel2)	Z49252
<i>Homo sapiens</i> (human)	p65 (RelA)	M62399		RelB	D63332
	c-Rel	X75042	<i>Danio rerio</i> (zebra fish)	p65	AY163839
	RelB (Irel)	M83221		c-Rel	AY163837
	NF $\kappa$ B1 (p50)	M55643		NF $\kappa$ B2 (p52)	AY163838
	NF $\kappa$ B2 (p52)	S76638	<i>Ciona intestinalis</i> (sea squirt)	Rel (Ci-rel1)	AY692136
	NFAT1	U43341		NF $\kappa$ B	AB210574
<i>Pan troglodytes</i> (chimpanzee)	p65 (RelA)	XM_522064 (pr)	<i>Halocynthia roretzi</i> (sea squirt)	Rel (As-rel1)	AB051857
<i>Mus musculus</i> (mouse)	p65 (RelA)	M61909	<i>Strongylocentrotus purpuratus</i> (sea urchin)	NF $\kappa$ B (SpNF $\kappa$ B)	AF064258
	c-Rel	X15842			
	RelB	M83380	<i>Drosophila melanogaster</i> (fruitfly)	Dorsal	M23702
	NF $\kappa$ B1 (p50)	M57999		Dif	L29015
	NF $\kappa$ B2 (p52)	AF155372		Relish	U62005
<i>Rattus norvegicus</i> (rat)	p65 (RelA)	BC079457	<i>Aedes aegypti</i> (yellow fever mosquito)	Dorsal (Rel1)	AY748242
<i>Gallus gallus</i> (chicken)	p65 (RelA)	D13721		Relish	AF498105
	c-Rel	X52193	<i>Carcinoscorpius rotundicauda</i> (horseshoe crab)	NF $\kappa$ B	DQ090482
	NF $\kappa$ B1 (p50)	M86930		Relish	DQ345784
	NF $\kappa$ B2 (p52)	U00111			

using GeneDoc v2.6.002 program (NICHOLAS and NICHOLAS, 1997), amino acid and DNA sequences were aligned using the ClustalX v1.83 program (THOMPSON *et al.*, 1997), and the final alignments were done by hand. The final alignments that were used in the construction of the phylogenetic trees contained 226 sites in the case of amino acid sequence for the Rel homology domain, and 675 sites and 332 sites in the cases of DNA sequences of RHD and transactivation domain, respectively. The phylogenetic trees were reconstructed by the NJ method with the use of MEGA v3.1 program (KUMAR *et al.*, 2004), and the ML

method using PHYLIP v3.65 program package. The distances for NJ phylogenetic trees were calculated with amino acid Poisson correction and Kimura's two-parameter distances. Human NFAT1 and *Xenopus* p65 sequences were used as outgroup for the phylogenetic trees of RHD and TAD, respectively. To assess the reliability of the inferred trees, bootstrap tests (FELSENSTEIN, 1985) were performed with 1000 bootstrap replications in the case of NJ phylogenetic trees and 100 bootstrap replications in the case of ML phylogenetic trees. The rates of synonymous ( $d_S$ ) and non-synonymous ( $d_N$ ) mutations were calculated by the Nei-Gojobori method based on the mammalian DNA sequences of 273 codons from RHD and 123 codons from TAD using the MEGA v3.1 program.

## 5. Results

### 5.1. The role of the NF $\kappa$ B in the transcriptional regulation of the bovine FcRn $\alpha$ -chain

#### 5.1.1 Investigation by luciferase reporter gene assay combined with *in silico* promoter analysis

There was no information about the quantitative change of mRNA level or transcriptional regulation of bFcRn  $\alpha$ -chain until the recent past, despite the fact, that the immunohistochemical investigation of bFcRn in mammary gland biopsies implied some expression changes around parturition (MAYER *et al.*, 2005). Although gene expression is controlled at multiple levels and the above mentioned bFcRn expression changes were detected at the protein level, it was hypothesized that gene expression of the bFcRn was also regulated at the transcription level.

Therefore the 5'-flanking sequence of the bFcRn was isolated, its promoter activity was demonstrated and compared to 5'-flanking sequence of the hFcRn (DOLESCHALL *et al.*, 2005). The comparison of about 2 kb 5'-flanking sequences revealed considerable divergence in a similar way like the comparison of human and mouse upstream regions (TIWARI and JUNGHANS, 2005). Although the FcRn is related to MHC class I molecules, the 5'-flanking sequence of the bFcRn differed from those of MHC class I molecules, as it did not contain typical TATA or other initiator sequences similarly to the human and rodent FcRn sequences. The bFcRn  $\alpha$ -chain gene has acquired several repetitive sequences in its 5'-flanking region including multiple short and long interspersed nuclear elements (DOLESCHALL *et al.*, 2005). It is worth mentioning that the two functional Sp1-like sites from the cluster that is located proximal in rat FcRn core promoter (JIANG *et al.*, 2004) were well conserved in both bovine and human, moreover, one of these rat Sp1-like sites was identical to the functional Sp1-like site in the mouse FcRn *cis*-regulatory region. Therefore these consensus Sp1-like sites in vicinity of TSS may contribute to the constitutive transcription of FcRn in mammals in accordance with the fact that Sp1 is important in enhancing the transcription of some TATA-less genes (EMAMI *et al.*, 1998).

An *in silico* promoter analysis was performed to localize putative binding sites for transcription factors within the 5'-flanking sequence using TESS and TFSEARCH programs (DOLESCHALL *et al.*, 2005). Among several other potential candidate sequences, a highly consensus  $\kappa$ B binding site was detected (Figure 6). The luciferase reporter construct

```

001 GACACGACTG AAGTGGTTTA GCAGCAGCAG CAGCACATGC AGGGAAAAAA 050
051 AATGTA AAAA AACACAACA ACCTTGCAAG AAAATATTTG CAGCAGCATT 100
101 AGTCATAATA GCCAAAAAAG TGAAAAACAAC CAAATGTCCA TCAGCTGCTA 150
151 AAGGGATGAA CCAAGTGTGG TCAATGCATA CGACGGAGTA TCATCCAGCC 200
201 GTGAAGAGGA ACGAAGTCCT GCCGCATGCT ACCATGTGGC TAAATCTTGA 250
251 AAACGGCATG CTGGGAAATT CCCTGGCAAA CCAGTGGTTA AGACTCTGCA 300
----- (16.27) NF-kappaB, NF-kappaB1, NF-kappaB2 M00208
===== == (14.71) NF-kappaB M00054
===== == (16.51) NF-kappaB M00054
----- == (11.11) NF-kappaB M00054
----- == (12.27) NF-kappaB1 M00051
301 TTTTCATTGT GATCTTCTGC AAGTCCTGCG GGGAGGCCAA AAAAAGAAAA 350
351 ACCCCACCGT ACACTAAATG AAAGATGCCA GATATAAAAT GCCACATTAA 400
401 TTTGAAATGT CCTGAATTGG CAAATCCATG GTGACAGAAA GTAAATTTGT 450
451 GGTTCAGGG GCAGGGGGGA GAAGGGACCA CTTAATGTGT CTGGGATCTC 500
501 CTTTGGGATG ATGAAAAAGT TCTGGAAC TA GATCGTGGTG ATGGTTGCAC 550
551 AACATTGTGA GTGCACCAA TGTCAC TAAT AGTAA 585

```

Figure 6

The *in silico* promoter analysis of the bFcRn *cis*-regulatory region. The analysis was performed by TESS software using the value 6 of the minimum lg likelihood ratio and 6 of maximum lg likelihood deficit. The search using strict thresholds resulted in a κB binding site that was highly similar to the consensus κB site. The *cis*-regulatory region is depicted from -1112 bp to -526 bp, and only NFκB binding sites are illustrated. The scores of TF binding sites are in the parentheses, the higher score represents the better similarity to the particular position weight matrix. The names of position weight matrices are written in light blue.

harbouring the longest segment of the bFcRn *cis*-regulatory region, pGL3-bFcRn-1787+92, was tested using overexpression of human p65 in 293 and HC11 cell lines (Figure 7). The

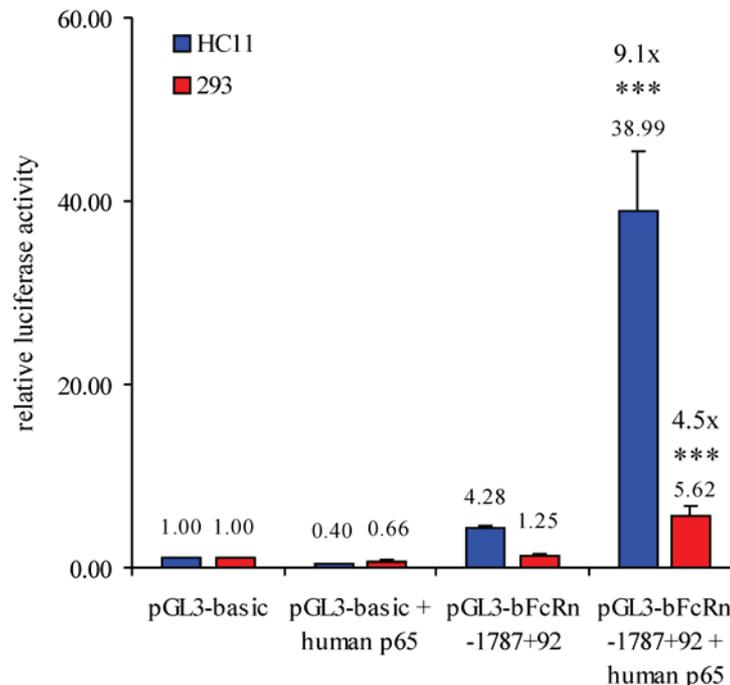


Figure 7

The induction of the bFcRn *cis*-regulatory region by human p65 overexpression in luciferase reporter gene assay. The luciferase activity of pGL3-bFcRn-1787+92 was significantly induced according to Student's test at the level 0.001 (\*\*\*) in HC11 and 293 cell lines, whereas the luciferase activity of negative control, pGL3-basic, failed to be affected. The means of normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments.

luciferase activity of pGL3-bFcRn-1787+92 induced by human p65 showed 4.5 and 9.1 fold induction in 293 and HC11 cells, respectively. These inductions were significant according to the Student's test at the 0.001 level, whereas the pGL3-basic vector could not be induced. The NFκB responsiveness of pGL3-bFcRn-1787+92 was also tested by human p50 and p65 co-expression, but this experiment did not result in any difference compared to the induction, which was performed with purely the p65 overexpression (data not shown).

For supporting the p65 responsiveness of the bFcRn *cis*-regulatory region and generalizing NFκB-mediated gene regulation in other mammals, an about 2200 bp and an about 700 bp segment of the hFcRn were investigated in luciferase reporter gene assay (Figure 8). Both segments could be significantly activated according to the Student's test at the 0.001 level by human p65 overexpression, whereas pGL3-basic failed to be induced in 293 cells. Further studies using site-directed mutagenesis accompanied by luciferase reporter gene assay and gel retardation assay demonstrated a functional κB site, which was partially responsible for p65 responsiveness of hFcRn and could bind NFκB complex containing p65 (data not shown) (SZALAI, 2005). The inducibility of the bFcRn *cis*-regulatory region by other immunologically relevant transcription factors was also tested to map the possible relation

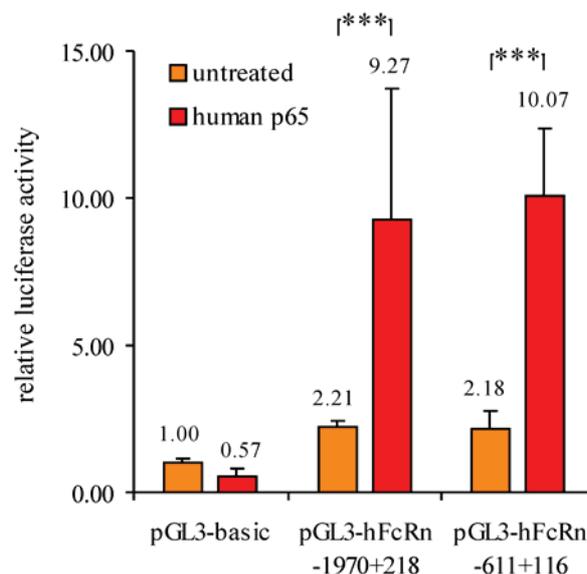


Figure 8

The activation of the hFcRn *cis*-regulatory region by human p65 overexpression in luciferase reporter gene assay. The luciferase activities of pGL3-hFcRn-1970+218 and pGL3-hFcRn-611+116 were significantly induced according to Student's test at the level 0.001 (\*\*\*) in 293 cell lines, whereas the luciferase activity of negative control, pGL3-basic, failed to be affected. The means of normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments.

among the bFcRn transcription and the other immune-related gene regulations. The pGL3-bFcRn-1787+92 was not activated by IRF1, c-Jun or STAT3 overexpression in 293 and HC11

cell line, but the positive control vectors of these transcription factors could be induced in both cell lines indicating that cell models of these transcriptional regulations function properly (data not shown).

The responsiveness of the bFcRn *cis*-regulatory region was higher in HC11, a cell line that has been widely used for NFκB-mediated transcriptional regulation (GEYMAYER and DOPPLER, 2000), therefore the further luciferase reporter experiments were achieved in these cells. The 3'-end of the κB binding site was located at -840 bp, and the segments of the bFcRn *cis*-regulatory region in different lengths also implied the importance of this binding site in luciferase reporter gene assay. Two luciferase reporter constructs containing the κB-840 binding site, pGL3-bFcRn-1787+92 and pGL3-bFcRn-1112+92, could be induced by human p65, but the luciferase reporter construct harbouring the shortest segment of bFcRn *cis*-regulatory region, pGL3-bFcRn-525+92, failed to be induced by human p65 (Figure 9). As the responsiveness of pGL3-bFcRn-1787+92 and pGL3-bFcRn-1112+92 was practically the

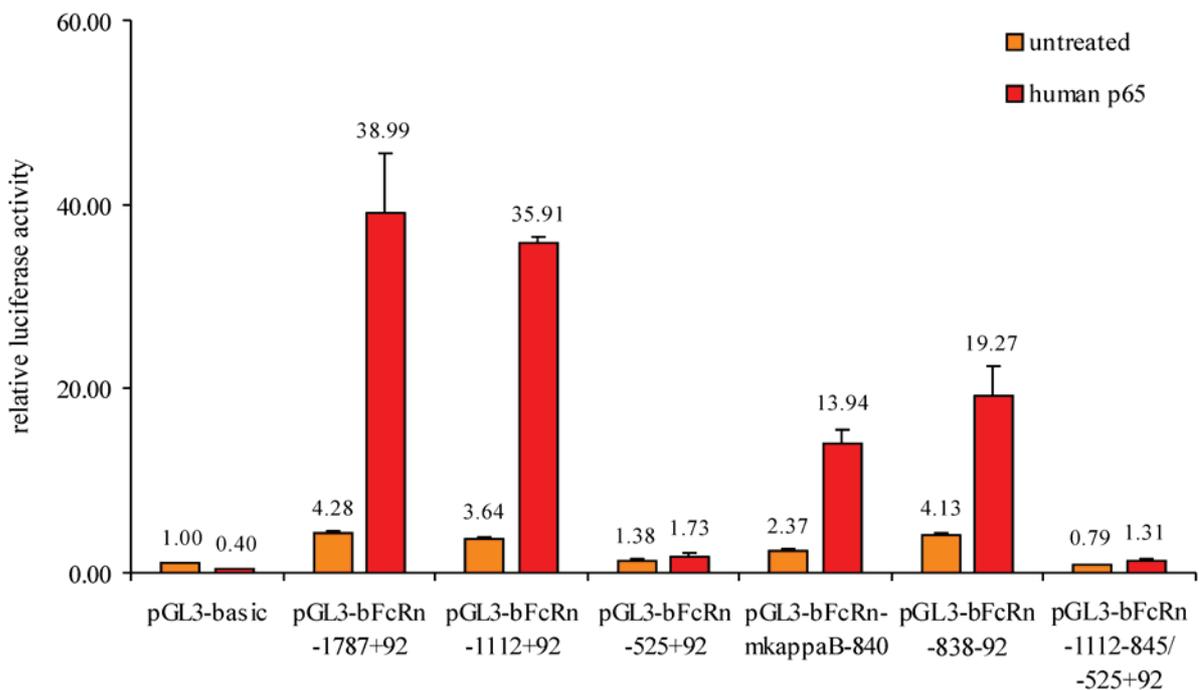


Figure 9

The induction of the bFcRn *cis*-regulatory segments in different length and pGL3-bFcRn-mκB-840 by human p65 overexpression in luciferase reporter gene assay of HC11 cells. The luciferase activity of the pGL3-bFcRn-1787+92 and pGL3-bFcRn-1112+92 were induced, but pGL3-bFcRn-525+92 failed to be induced by human p65 implying that the *cis*-regulatory region from -1112 bp to -526 bp harboured the p65 responsiveness. The responsiveness of pGL3-bFcRn-mκB-840 was reduced compared to the responsiveness of pGL3-bFcRn-1112+92, but it was not deleted completely indicating that the κB-840 binding site contributed to the p65 inducibility. The luciferase activity of the pGL3-bFcRn-838+92 was partially induced in similar rate as pGL3-bFcRn-mκB-840, whereas the luciferase activity of pGL3-bFcRn-1112-845/-525+92 failed to be induced implying that the region from -838 bp to -526 bp harboured at least one κB binding site. The means of

normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments. same, and pGL3-bFcRn-525+92 could not be induced, it was concluded that the *cis*-regulatory region from -1112 bp to -526 bp carried the p65 inducibility, for which the  $\kappa$ B-840 site was presumably responsible.

To reveal the contribution of the  $\kappa$ B-840 site to the p65 inducibility of the bFcRn *cis*-regulatory region, a luciferase reporter construct harbouring mutated  $\kappa$ B-840 site, pGL3-bFcRn-m $\kappa$ B-840, was generated and investigated in luciferase reporter gene assay (Figure 10). The responsiveness of pGL3-bFcRn-m $\kappa$ B-840 was significantly reduced according to the

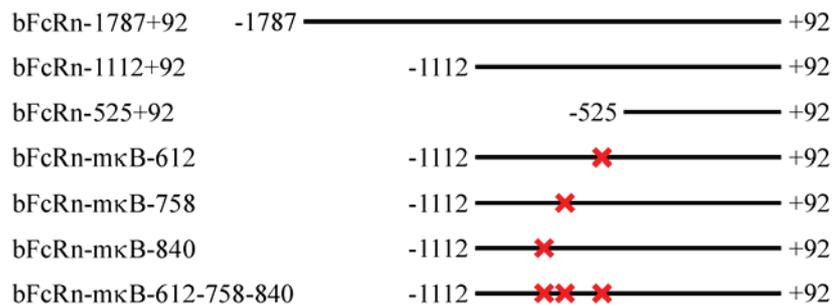


Figure 10

The positions of the mutated bFcRn  $\kappa$ B binding sites (red x) proportionately depicted on the bFcRn *cis*-regulatory segments for luciferase reporter gene assay.

Student's test at the 0.001 level compared to the inducibility of pGL3-bFcRn-1112+92 that contained a bFcRn *cis*-regulatory region in the same length, but the responsiveness was not

001	GACACGACTG	AAGTGGTTTA	GCAGCAGCAG	CAGCACATGC	AGGGAAAAAA	050
051	AATGTAAAAA	AACAACAACA	ACCTTGCAAG	AAAATATTTG	CAGCAGCATT	100
101	AGTCATAATA	GCCAAAAAAG	TGAAAAACAAC	CAAATGTCCA	TCAGCTGCTA	150
151	AAGGGATGAA	CCAAGTGTGG	TCAATGCATA	CGACGGAGTA	TCATCCAGCC	200
201	GTGAAGAGGA	ACGAAGTCCT	GCCGCATGCT	ACCATGTGGC	TAAATCTTGA	250
251	AAACGGCATG	CTGGGAAATT	CCCTGGCAAA	CCAGTGGTTA	AGACTCTGCA	300
				-----	(10.91) NF-kappaB, NF-kappaB1, NF-kappaB2	M00208
				-----	(7.72) NF-kappaB, NF-kappaB1, NF-kappaB2	M00208
				-----	(16.27) NF-kappaB, NF-kappaB1, NF-kappaB2	M00208
				-----	(9.04) NF-kappaB	M00054
				=====	(14.71) NF-kappaB	M00054
				=====	(16.51) NF-kappaB	M00054
				-----	(11.11) NF-kappaB	M00054
301	TTTTCATTGT	GATCTTCTGC	AAGTCTTGCG	GGGAGGCCAA	AAAAAGAAAA	350
351	ACCCACCGT	ACACTAAATG	AAAGATGCCA	GATATAAAAT	GCCACATTAA	400
				-----	(8.26) NF-kappaB	M00054
401	TTTGAAATGT	CCTGAATTGG	CAAATCCATG	GTGACAGAAA	GTAAATTTGT	450
451	GGTTGCAGGG	GCAGGGGGGA	GAAGGGACCA	CTTAATGTGT	CTGGGATCTC	500
501	CTTTGGGATG	ATGAAAAAGT	TCTGGAAC TA	GATCGTGGTG	ATGGTTGCAC	550
				--	(5.29) NF-kappaB	M00054
551	AACATTGTGA	GTGCACCAAA	TGTCAC TAAT	AGTAA		585

Figure 11

The repeated *in silico* promoter analysis of the bFcRn *cis*-regulatory region. The analysis restricted to the TF binding sites of the NF $\kappa$ B family was performed by TESS software using the value 6 of the minimum lg likelihood ratio and 10 of the maximum lg likelihood deficit. The search using relatively low threshold values revealed two other  $\kappa$ B binding sites that showed low similarity to the consensus  $\kappa$ B site. The *cis*-regulatory region is depicted from -1112 bp to -526 bp. The scores of the TF binding sites are in the parentheses, the higher

score represents the better similarity to the particular position weight matrix. The names of the position weight matrices are written with light blue.

deleted completely (Figure 9). From this result, it was inferred that the *cis*-regulatory region from -1112 bp to -526 bp has another  $\kappa$ B binding site or sites beside the  $\kappa$ B-840 site. In order to find these possible  $\kappa$ B sites, a new *in silico* promoter analysis was performed using lower thresholds with mere use of the position weight matrices of the NF $\kappa$ B family. The promoter analysis revealed two new  $\kappa$ B binding sites, which were located downstream from the  $\kappa$ B-840 site (Figure 11), and the sites were termed for  $\kappa$ B -612 and  $\kappa$ B-758 sites. It was technically simpler to confirm the p65 responsiveness of the *cis*-regulatory regions were located upstream and downstream from  $\kappa$ B-840 site than the mutagenesis of the new  $\kappa$ B sites, thus two luciferase reporter constructs, pGL3-bFcRn-838+92 and pGL3-bFcRn-1112-845/-525+92 were first generated using the restriction digestion site of the mutated  $\kappa$ B-840 site. There was no considerable difference between the responsiveness of the pGL3-bFcRn-838+92 and the pGL3-bFcRn-m $\kappa$ B-840, while the elimination of the *cis*-regulatory regions from -844 to -526 completely deleted the p65 inducibility in the same way as it was deleted in pGL3-bFcRn-525+92 (Figure 9). This result supported the presumption that at least one of the two new  $\kappa$ B sites contributed to the p65 responsiveness.

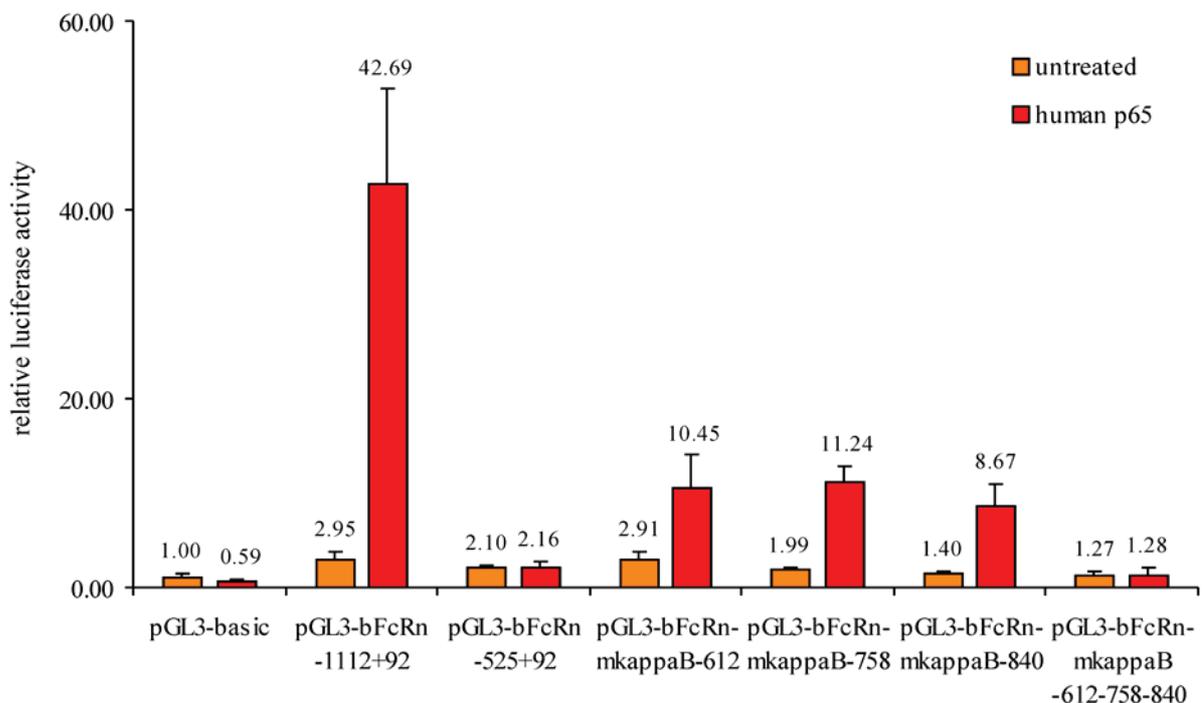


Figure 12

The induction of the mutated bFcRn luciferase reporter constructs by human p65 overexpression in HC11 cells. The responsiveness of the single mutated constructs, pGL3-bFcRn-m $\kappa$ B-612, pGL3-bFcRn-m $\kappa$ B-758 and pGL3-bFcRn-m $\kappa$ B-840, was reduced compared to the responsiveness of pGL3-bFcRn-1112+92, but it was not deleted completely indicating that all of these sites contributed to p65 inducibility. The luciferase activity of the pGL3-bFcRn-m $\kappa$ B-612-758-840 failed to be induced indicating that these three  $\kappa$ B sites were responsible for p65 inducibility of bFcRn *cis*-regulatory region. The means of normalized luciferase activities are above the

columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments.

To test the  $\kappa$ B -612 and  $\kappa$ B-758 sites, two new mutated luciferase reporter constructs were made, pGL3-bFcRn- $\kappa$ B-612 and pGL3-bFcRn- $\kappa$ B-758 (Figure 10). The responsiveness of the pGL3-bFcRn- $\kappa$ B-612 and the pGL3-bFcRn- $\kappa$ B-758 were significantly reduced according to the Student's test at the 0.001 level compared to the inducibility of pGL3-bFcRn-1112+92, but the responsiveness of them are not deleted completely in similar manner for pGL3-bFcRn- $\kappa$ B-840 (Figure 12). It was indicated that all of these  $\kappa$ B sites were responsible for the p65-mediated induction. The mutagenesis of all  $\kappa$ B sites, the name of the luciferase reporter construct that harboured them was pGL3-bFcRn- $\kappa$ B-612-758-840, resulted in the complete deletion of p65 inducibility to verify that the cumulative effect of the  $\kappa$ B-612,  $\kappa$ B-758 and  $\kappa$ B-840 sites caused the p65 responsiveness of the bFcRn *cis*-regulatory region spanning from -1787 bp to +92 bp (Figure 12).

Taking into account the results of luciferase reporter gene assay in respect to the bFcRn *cis*-regulatory region, the responsiveness to human p65 overexpression was verified in 293 and HC11 cell lines, and three  $\kappa$ B sites were identified in HC11, which were responsible for the entire p65 inducibility.

### 5.1.2 The gel retardation assay of $\kappa$ B binding sites

For investigating the binding ability of bFcRn  $\kappa$ B sites *in vitro*, EMSA and supershift assay were carried out using radiolabelled oligonucleotide representing the  $\kappa$ B-612,  $\kappa$ B-758,  $\kappa$ B-840

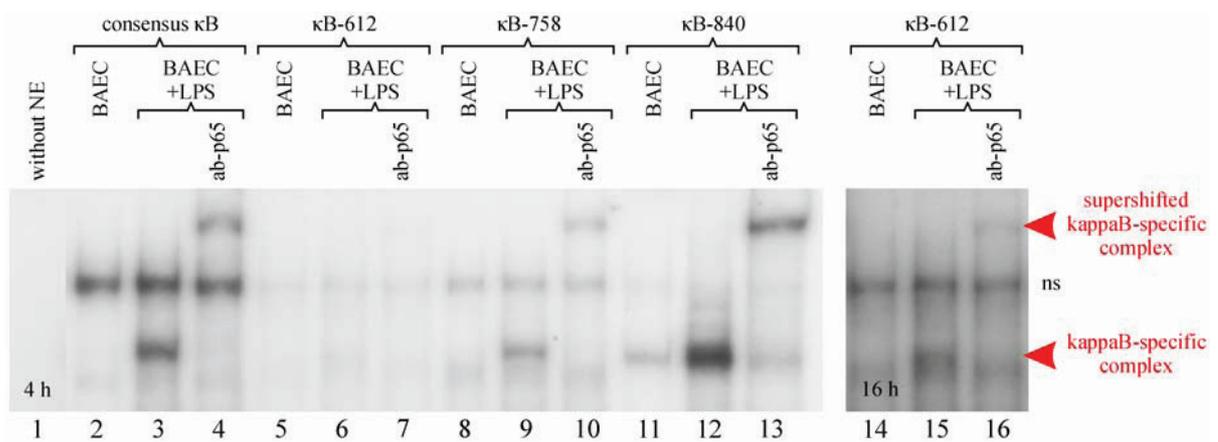


Figure 13

Gel retardation assay for the investigation of the bFcRn  $\kappa$ B binding sites. Nuclear extracts (NE) prepared from untreated BAEC (BAEC) and LPS-treated BAEC (BAEC +LPS). Nuclear extracts were incubated and run with labelled oligonucleotides representing the  $\kappa$ B-612,  $\kappa$ B-758,  $\kappa$ B-840 and consensus  $\kappa$ B sites in absence or presence of p65 antibody (ab-p65). Distinct  $\kappa$ B-specific protein-DNA complexes (red arrow, kappaB-specific complex) were detected using nuclear extracts from LPS-treated BAEC cells (lane 3, 9, 12), and these complexes could be further shifted by p65 antibody (red arrow, supershifted kappaB-specific complex, lane 4, 10, 13). In the case of  $\kappa$ B-612 oligonucleotide, the NF $\kappa$ B-specific complex and its supershifted version appeared only with

16 hours (16 h) film exposition time (lane 15, 16). The  $\kappa$ B-specific complexes could not be observed using nuclear extract from BAEC cells (lane 2, 8, 11, 14). "ns" means non-specific bands. and consensus  $\kappa$ B sites, and nuclear extracts were prepared from BAEC and LPS-treated BAEC cells. Distinct protein-DNA complexes were detected by bFcRn  $\kappa$ B and consensus  $\kappa$ B oligonucleotides using the nuclear extracts from LPS-treated BAEC cells, although this complex could be observed at the  $\kappa$ B-612 oligonucleotide using 16 hours film exposition (Figure 13, red arrow,  $\kappa$ B-specific complex, lane 3, 9, 12, 15). The bands of these complexes could not be observed at the  $\kappa$ B-612,  $\kappa$ B-758 and consensus  $\kappa$ B oligonucleotides, when the nuclear extracts from untreated BAEC cells were used (lane 2, 8, 14) indicating that there was no NF $\kappa$ B in the nucleus of untreated BAEC cells as it was expected. On the other hand, an identically migrating band was found at  $\kappa$ B-840 oligonucleotide (lane 11) using the nuclear extracts from untreated BAEC cells. In the case of the  $\kappa$ B-612,  $\kappa$ B-758 and consensus  $\kappa$ B oligonucleotides, the disappearance of the complexes and the simultaneous emergence of the supershifted bands were detected in the presence of p65 antibody (red arrow, supershifted  $\kappa$ B-specific complex, lane 4, 10, 16), while the disappearance could not be observed completely in the case of the  $\kappa$ B-840 oligonucleotide (lane 13). The presence of supershifted bands verified unambiguously that the complexes were  $\kappa$ B-specific and contained p65. The faint band of the  $\kappa$ B-840 oligonucleotide that migrated identically with the  $\kappa$ B-specific complexes represented a non-specific complex in all likelihood, because it was present with similar intensity using the nuclear extract from untreated BAEC and beside the supershifted  $\kappa$ B-specific complex. However, this gel retardation assay was not quantitative, the apparent differences in the intensity of the  $\kappa$ B-612,  $\kappa$ B-758 and  $\kappa$ B-840 complexes were obvious, moreover the differences were well reproducible in the parallel experiments. In summary, the oligonucleotides representing the bFcRn  $\kappa$ B sites could bind to the bovine NF $\kappa$ B *in vitro* supporting the results of the luciferase reporter gene assay.

## **5.2. The role of the NF $\kappa$ B and IRF1 transcription factors in the transcriptional regulation of the bovine $\beta$ 2m**

### **5.2.1 Comparative analysis of the bovine $\beta$ 2m *cis*-regulatory region**

In contrast to the immune regulation of the FcRn transcription, the transcriptional regulation of the human  $\beta$ 2m *cis*-regulatory region has been experimentally well characterized, thus the investigation of the bovine  $\beta$ 2m transcription was built on the previous human studies (GOBIN *et al.*, 2003, GOBIN *et al.*, 1998, GOBIN *et al.*, 2001).

As a first step, the 5'-flanking sequence of  $\beta 2m$  was isolated and compared with its human counterpart (Figure 14). In human, there is an SXY module and an E-box that ensure high

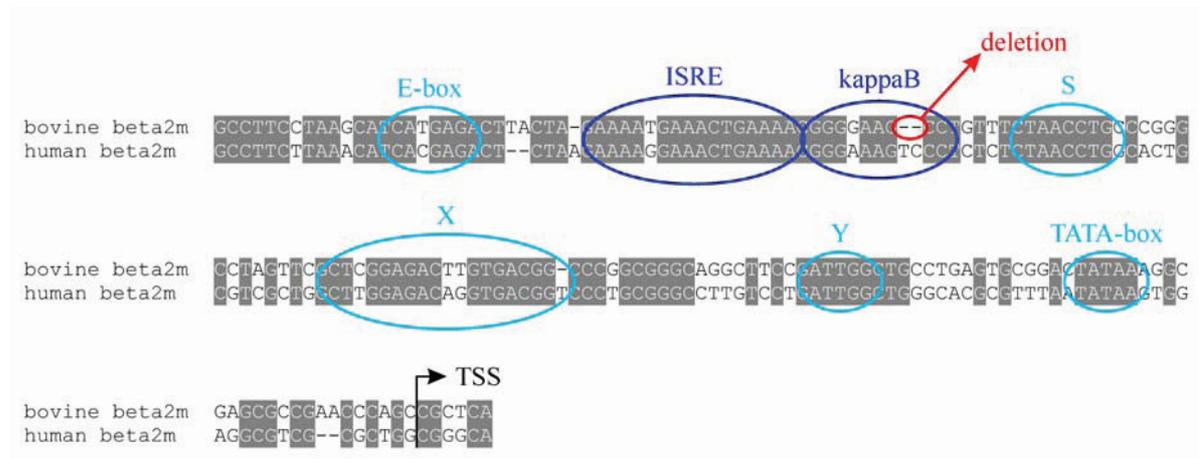


Figure 14

The comparison of the human and bovine  $\beta 2m$  cis-regulatory regions. Although the constitutive binding site (SXY module, E-box, TATA-box, light blue) near to the transcription start site (TSS) and ISRE binding site (blue) are well conserved, the bovine  $\kappa B$  site shows a two-nucleotide deletion compared with the human orthologue. The  $\kappa B$  site has been confirmed by sequencing the promoter in several animals belonging to Angus and Holstein-Friesian breeds.

constitutive expression (GOBIN *et al.*, 1998, GOBIN *et al.*, 2001), and these constitutive TF binding sites were well conserved, as well as the TATA-box in the  $\beta 2m$  cis-regulatory region. Moreover, the complete elimination of the segment harbouring SXY module and an E-box from  $\beta 2m$  cis-regulatory region resulted in a strong reduction of the constitutive expression according to the evidence of luciferase reporter gene assay (data not shown). A  $\kappa B$  and an ISRE site what are mainly responsible for the cytokine-induced regulation in human, although they also contribute to the constitutive expression (GOBIN *et al.*, 2003). The ISRE site was also well conserved in bovine, but the  $\kappa B$  site showed two-nucleotide deletion compared with its human orthologue (Figure 14, red arrow). The existence of the  $\kappa B$  deletion was confirmed by sequencing the 5'-flanking sequence of  $\beta 2m$  in several animals belonging to Angus and Holstein-Friesian breeds, hence the possibility of random mutation, that was characteristic of one individual, was excluded. This finding was supported by the fact that the mRNA level of  $\beta 2m$  induced bovine TNF- $\alpha$  and LPS has not changed in bovine aortic endothelial cells according to a previous study (VAN KAMPEN and MALLARD, 2001).

### 5.2.2 The investigation of the $\kappa B$ binding site

The luciferase reporter construct harbouring a long segment of the  $\beta 2m$  cis-regulatory region, pGL3- $\beta 2m$ -1934+49, was examined using the overexpression of the human p65 in

293 and HC11 cell lines (Figure 15). The luciferase activities of pGL3-b $\beta$ 2m-1934+49 failed to be induced by human p65, whereas the reporter gene vector with consensus  $\kappa$ B binding

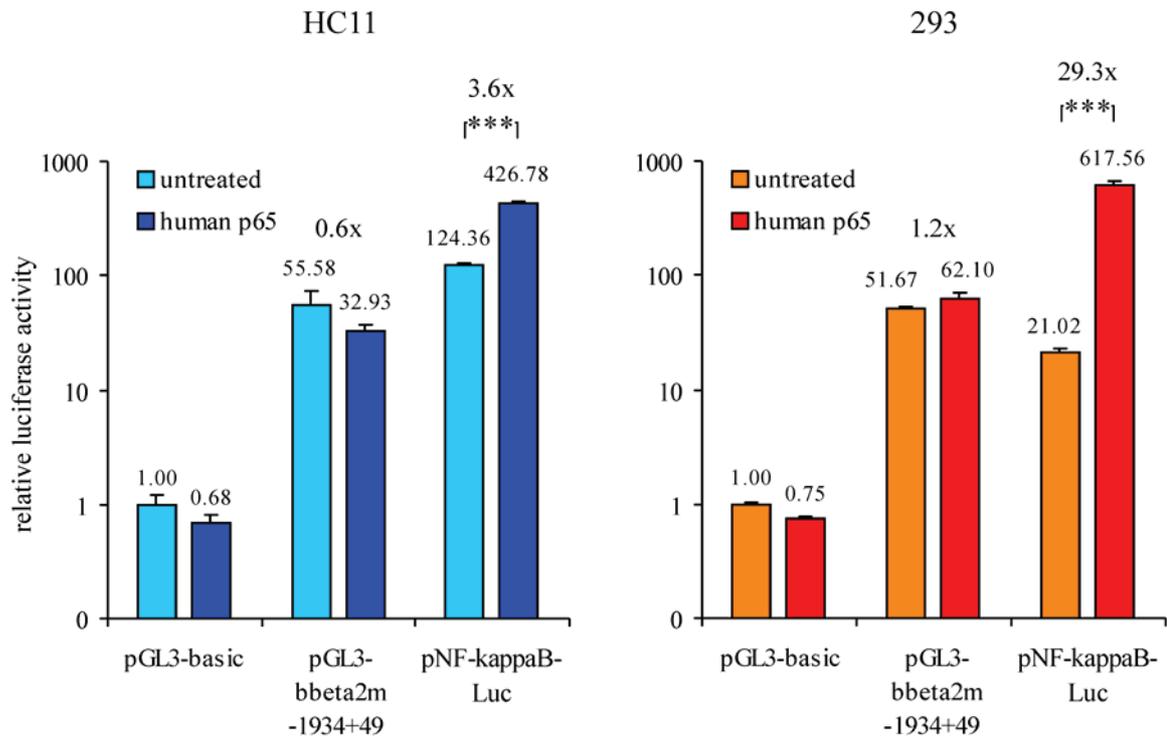


Figure 15

The induction of the  $\beta$ 2m *cis*-regulatory region by human p65 overexpression in luciferase reporter gene assays of HC11 and 293 cell lines. The luciferase activities of pGL3-b $\beta$ 2m-1934+49 failed to be induced by human p65, whereas the positive control, pNF $\kappa$ B-Luc, was induced according to Student's test at the level 0.001 (\*\*\*) in both cell lines. These results predicted that the bovine beta2m could not be induced by NF $\kappa$ B presumably due to the deletion in the  $\kappa$ B-110 site. The means of normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments.

sites, pNF $\kappa$ B-Luc, was significantly induced according to the Student's test at the 0.001 level in both cell lines. These results suggested that the  $\beta$ 2m could not be induced by NF $\kappa$ B presumably due to the deletion in the  $\kappa$ B-110 site.

To confirm the results of the luciferase reporter gene assay, EMSA and supershift assay were performed using radiolabelled oligonucleotide representing the  $\beta$ 2m  $\kappa$ B-110 and consensus site  $\kappa$ B, and nuclear extracts were prepared from BAEC and LPS-treated BAEC cells. Distinct  $\kappa$ B-specific protein-DNA complex (Figure 16, red arrow, kappaB-specific complex, lane 3) could be detected using nuclear extracts from LPS-treated BAEC cells with the consensus  $\kappa$ B oligonucleotide, which was shifted with the p65 antibody (red arrow, supershifted kappaB-specific complex, lane 4). The  $\kappa$ B-specific complex could not be observed using nuclear extracts from non-stimulated BAEC cells (lane 2, 5, 8). A weak  $\kappa$ B-specific and supershifted complexes, which were observed well using 16 hours film exposition, were generated with the

$\kappa$ B-110 oligonucleotide (lane 9, 10). This indicated that the  $\kappa$ B-110 site could bind to NF $\kappa$ B complex containing p65 in spite of non-conserved composition, hence the results of luciferase reporter gene and gel retardation assays were contradictory.

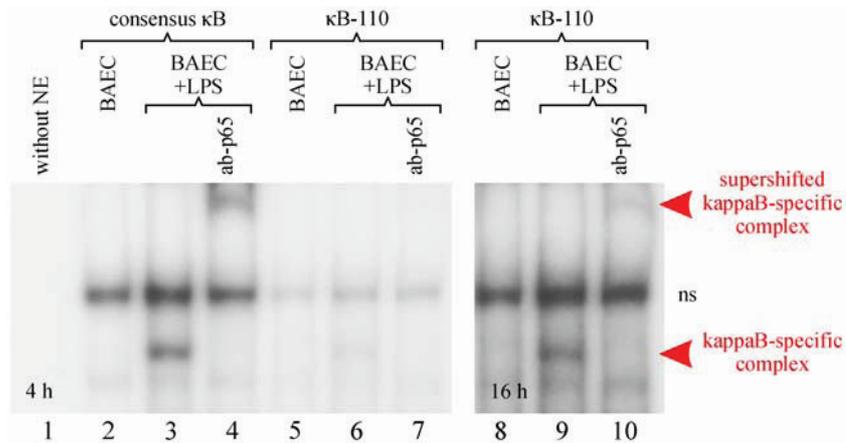


Figure 16

Gel retardation assay for the investigation of the  $\beta$ 2m  $\kappa$ B binding site. Nuclear extracts (NE) were prepared from untreated and LPS-treated BAEC cells (BAEC and BAEC +LPS). Nuclear extracts were incubated and run with the labelled oligonucleotides representing the  $\kappa$ B-110 and consensus  $\kappa$ B sites in absence or presence of p65 antibody (ab-p65). Distinct  $\kappa$ B-specific protein-DNA complex (red arrow, kappaB-specific complex) was detected using nuclear extract from LPS-treated BAEC cells with the consensus  $\kappa$ B site (lane 3), and could be supershifted with p65 antibody (red arrow, supershifted kappaB-specific complex, lane 4). A  $\kappa$ B-specific and supershifted complexes, which could be observed well using 16 hours film exposition (16 h), were generated with the  $\kappa$ B-110 site (lane 15, 16). This result indicated that *in vitro* the  $\beta$ 2m  $\kappa$ B site could bind to NF $\kappa$ B complex containing p65 in spite of non-conserved composition. The  $\kappa$ B-specific complexes could not be observed using nuclear extract from BAEC cells (lane 2, 8). "ns" means non-specific bands.

### 5.2.3 The investigation of the ISRE binding site

The luciferase reporter construct, pGL3- $\beta$ 2m-1934+49, was also used to study the effect of the human IRF1 overexpression in 293 and HC11 cell lines in order to examine the INF- $\gamma$  responsiveness of the  $\beta$ 2m *cis*-regulatory region. In both cell lines, the luciferase activity of pGL3- $\beta$ 2m-1934+49 was significantly induced by human IRF1 according to the Student's test at the 0.001 level, whereas the pGL3-basic vector failed to be induced (Figure 17). To reveal the contribution of ISRE-122 binding site for the IRF1 responsiveness of the  $\beta$ 2m *cis*-regulatory region, a luciferase reporter construct containing mutant ISRE-122 site, pGL3- $\beta$ 2m-mISRE-122, was generated. The IRF1 inducibility of the pGL3- $\beta$ 2m-mISRE-122 was abolished in HC11 cells, and its basic luciferase activities was significantly reduced according to the Student's test at the 0.001 level compared to the activities of the pGL3- $\beta$ 2m-1934+49 construct (Figure 17). This finding confirmed the functional similarity between the human and bovine ISRE sites, thus it could be concluded that the ISRE-122 site contributed to the INF- $\gamma$  mediated and constitutive transcription of the  $\beta$ 2m *cis*-regulatory region.

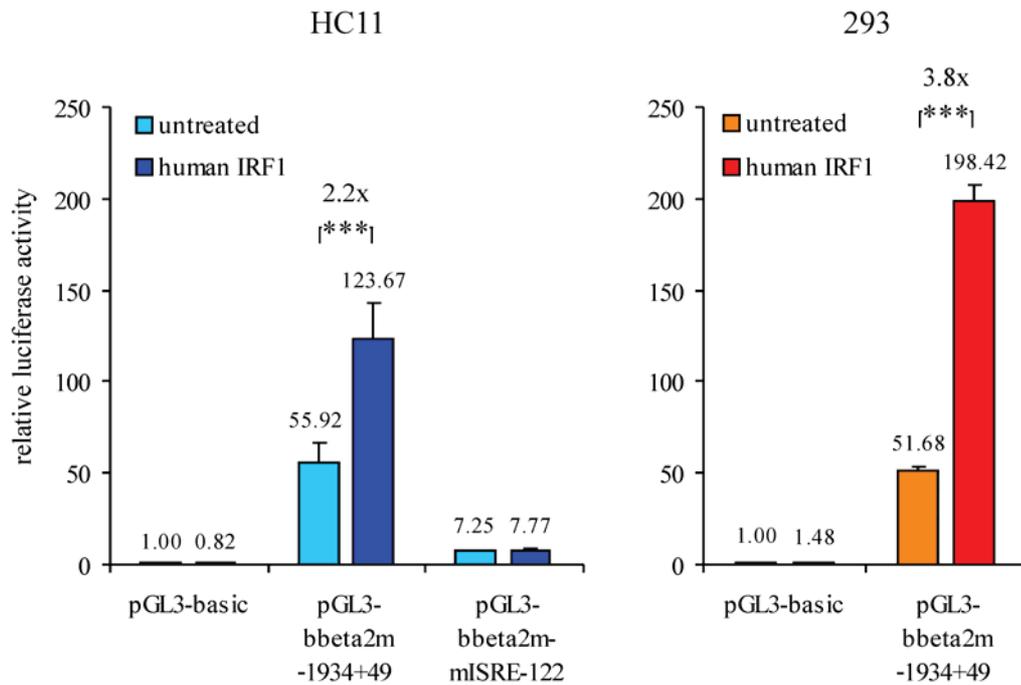


Figure 17

The induction of the  $\beta 2m$  *cis*-regulatory region by human IRF1 overexpression in luciferase reporter gene assays of HC11 and 293 cell lines. The luciferase activity of the negative control, pGL3-basic, failed to be induced by IRF1, whereas the luciferase activity of pGL3- $\beta 2m$ -1934+49 was induced according to Student's test at the level 0.001 (\*\*\*) in both cell lines. The IRF1 induction of pGL3- $\beta 2m$ -mISRE-122 was abolished in HC11 cells. The means of normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments.

For examining the binding ability of the  $\beta 2m$  ISRE binding site *in vitro*, EMSA and supershift assay were performed using radiolabelled oligonucleotide representing the ISRE-122 site and nuclear extracts were prepared from BAEC and bovine INF- $\gamma$  -treated BAEC cells. Distinct ISRE-specific protein-DNA complex was detected using nuclear extracts from bovine INF- $\gamma$ -treated BAEC cells (Figure 18, red arrow, ISRE-specific complex, lane 5), whereas the ISRE-specific complex was not observed using nuclear extracts from non-stimulated BAEC cells (lane 2, 3, 4). The addition of unlabelled specific competitor oligonucleotide containing IRF-E binding site (IRF-E comp.) resulted in the disappearance of the ISRE-specific complex (lane 6), while the unlabelled unspecific competitor oligonucleotide containing Sp1 binding site (Sp1 uncomp.) did not (lane 7). These findings suggested that the members of interferon regulatory factors constituted the ISRE-specific complex, as it was expected.

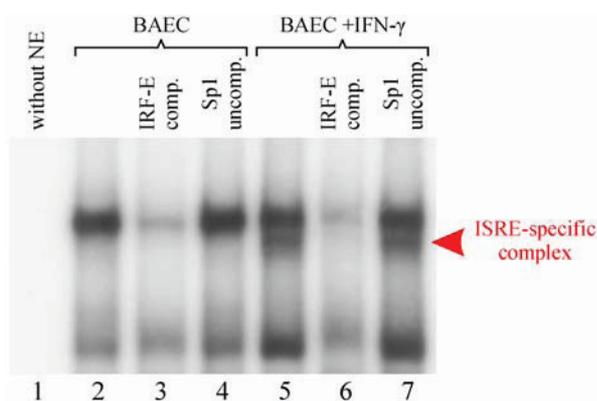


Figure 18

Gel retardation assay for the investigation of b $\beta$ 2m ISRE binding site. Nuclear extracts (NE) was prepared from untreated and bovine IFN- $\gamma$ -treated BAEC cells (BAEC and BAEC +IFN- $\gamma$ ). Nuclear extracts were incubated and run with the labelled oligonucleotides representing ISRE-122 binding site. Distinct ISRE-specific protein-DNA complex (red arrow, ISRE-specific complex) was detected using nuclear extract from bovine IFN- $\gamma$ -treated BAEC cells (lane 5), whereas the ISRE-specific complex could not be observed using nuclear extracts from BAEC cells (lane 2-4). The addition of unlabelled specific competitor oligonucleotide containing IRF-E binding site (IRF-E comp.) resulted in the disappearance of the ISRE-specific complex (lane 6), while the unlabelled unspecific competitor oligonucleotide containing Sp1 binding site (Sp1 uncomp.) did not (lane 7). These findings suggested that the members of interferon regulatory factors constituted the ISRE-specific complex.

### 5.3. Cloning and characterization of the bovine p65 subunit of NF $\kappa$ B

#### 5.3.1 Isolation of the bovine p65 subunit of NF $\kappa$ B cDNA

Although transcription factors are highly conserved molecules, and their use in different species models is common, it has been thought that tiny differences in the transcription factor might influence binding and transcriptional activity. Thus, in order to establish a bovine specific system, the coding sequence of bp65 was isolated by PCRs. The amplifications were performed with high fidelity polymerase to reduce the occurrence of random mutations, and yielded DNA fragments of about 1700 bp. To exclude the possibility of random mutagenesis, the three DNA fragments derived from independent amplifications were independently cloned, and then, the independent clones were completely sequenced and compared with each other. The sequence data derived from the clones resulted in a cDNA sequence of 1746 bp, encompassing part of the 5'-UTR with the consensus Kozak translational initiating sequence (KOZAK, 1989), and the entire coding sequence including the 3'-UTR of the bp65 (Figure 19). The sequence data have been submitted to Genbank under the accession number DQ355511. The data were compared to the cloned and characterized vertebrate p65 sequences, and exhibited a high identity to the coding region of human (RUBEN *et al.*, 1991) and mouse (NOLAN *et al.*, 1991) p65 (88.9% and 84.7%, respectively) and a moderate identity to chicken (IKEDA *et al.*, 1993), clawed frog (KAO and HOPWOOD, 1991) and

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1   CCGGCCATGGACGACCTCTTCCCCCTCATCTTCCCTGCAGAGCCGGCGCAGGCCTCTGGCCCCATGTGGAGATCATCGA
    M D D L F P L I F P A E P A Q A S G P Y V E I I E 25
81  GCAGCCCAAGCAGCGGGGCATGCGCTTCCGCTACAAGTGAGGGCCGCTCAGCTGGCAGCATCCCTGGAGAGAGGAGCA
    Q P K Q R G M R F R Y K C E G R S A G S I P G E R S T 52
161 CGGACACCAACCAAGACCCACCCCACTATCAAGATCAATGGCTACACCGGGCCAGGGACAGTCCGCATCTCCCTGGTCAC
    D T T K T H P T I K I N G Y T G P G T V R I S L V T 78
241 AAGGACCCCCCTCACCGGCCTCACCTCACGAGCTAGTGGGAAAGACTGCCGGATGGCTTCTATGAGGCTGAGCTCTG
    K D P P H R P H P H E L V G K D C R D G F Y E A E L C 105
321 CCCGGACCGCTGCATCCACAGCTTTCAGAACCTGGGGATCCAGTGTGTAAGAAGCGGGACCTGGAGCAGGCCATCAGCC
    P D R C I H S F Q N L G I Q C V K K R D L E Q A I S Q 132
401 AGCGCATCCAGACCAACAACACCCCTTCCAAGTTCCCATAGAAAGAGCAGCGCGGGACTACGACCTGAATGCCGTCCGG
    R I Q T N N N P F Q V P I E E Q R G D Y D L N A V R 158
481 CTCTGCTTCCAGGTGACAGTGCGGGACCCGGCAGGCAGGCCCTCCGCCCTGGCGCTGTCTCTCACCCATCTTTGAG
    L C F Q V T V R D P A G R P L R L A P V L S H P I F D 185
561 CAACCGCGCCCAACACCGCCGAGCTCAAGATCTGCCGGTGAATCGGAACCTCTGGGAGCTGCCTCGGCGGGGATGAGA
    N R A P N T A E L K I C R V N R N S G S C L G G D E I 212
641 TCTTCTGCTGTGTGACAAGGTGCAGAAAGAGGACATCGAGGTGATTTTACGGGACCAAGCTGGGAGGCCCCGAGGCTCT
    F L L C D K V Q K E D I E V Y F T G P G W E A R G S 238
721 TTTTACAAGCTGACGTGCACCGCAAGTGGCCATCGTGTTCGGGACGCGCCCTACCGGACCCCGGCTGCAGGCCCC
    F S Q A D V H R Q V A I V F R T P P Y A D P G L Q A P 265
801 TGTGCGCGTCTCCATGCAGCTGCGGGCCCTTCCGATCGGGAGCTCAGTGAGCCATGGAAATCCAGTACTTGCCAGACA
    V R V S M Q L R R P S D R E L S E P M E F Q Y L P D T 292
881 CAGACGATCGTCACCGGATTGAGGAGAAGCGCAAAAGGACGTACGAGACCTTCAAGAGCATCATGAAAAAGAGCCCTTTC
    D D R H R I E E K R K R T Y E T F K S I M K K S P F 318
961 AATGGACCCACCGACCCCGGCTCCAACCCGGCGCATCGTGTGCCTAACCGGGCTCAGCCTCCATCCCAAGCCAGC
    N G P T D P R P P T R R I A V P N R G S A S I P K P A 345
1041 TCCCCAGCCCTATTCTTTACGCCATCTCTCAGCACCATCAACTTTGAGGAGTTTTCCCCTCATGGTCTTTCTTCTGGGC
    P Q P Y S F T P S L S T I N F E E F S P M V F P S G Q 372
1121 AGATCCCAAGCCAGACCTCGGCCTTGGCAACAGCCCCAACCCTGCTGACCCAGACCAGTCTGGCCCCAGCCCCG
    I P S Q T S A L A P A P T P V L T Q T Q V L A P A P 398
1201 GCCCCAGCCCGAGGCATGGCATCAACCTGGCCAGGCCCTAGCCCCAGCCCTGGCTCAGGCTGTGACCCTCGCCTGCCCC
    A P A P G M A S T L A Q A L A P G L A Q A V T P P A P 425
1281 CAGGACCAACCCAGACCGGGGAAGGGACACTGCAGAGGCCCTGCTGCAGCTGCAGTTTGATACCGATGAAGACCTGGGG
    R T N Q T G E G T L T E A L L Q L Q F D T D E D L G A 452
1361 CTCTGCTTGGCAACAACACTGACCCTGCCGTGTTACAGACCTGGCATCTGTCGACAACCTCTGAGTTTCAGCAGTTGCTG
    L L G N N T D P A V F T D L A S V D N S E F Q Q L L 478
1441 AACCAGGGTGTACCCATGGGCCCCACACAGCTGAGCCCATGCTGATGGAGTACCCTGAGGCTATAACTCGCCTGGTGAC
    N Q G V P M G P H T A E P M L M E Y P E A I T R L V T 505
1521 AGGGTCCCAAGGCCCCCTGACCCAGCTCCCACTCCCTGGGGCCCCCTGGGCTACCAATGGCCTCTCTCGGGGATG
    G S Q R P P D P A P T P L G P P G L T N G L L S G D E 532
1601 AAGACTTCTCTCCATCGCGGATGTGGACTTCTCAGCCCTTCTGAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCA
    D F S S I A D V D F S A L L S Q I S S * 551
1681 GCCCAGCGCAGTGGGTTAGCAAGGGATTGAGCCCTCCCAAAGCACACACTGATGGATTCTGGGAG

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Figure 19

The nucleotide sequence and deduced amino acid sequence of the bovine p65 subunit of NFκB. The consensus Kozak translational initiating sequence is underlined, while black arrows mark the exon-intron boundaries based on the bovine gene predicted to be similar to the transcription factor of p65 (Genbank LOC508233).

zebra fish (CORREA *et al.*, 2004) (57.4%, 52.2% and 50.6%). The resulting bp65 sequence was completely identical with the recently deposited complete bovine mRNA sequence (Genbank XM\_584983 and the corresponding gene under accession number LOC508233), which was predicted by automated computational analysis to be similar to the transcription factor of p65. This record was generated from an annotated genomic sequence using gene

prediction method supported by EST evidences. It is worth mentioning that at the time, when this study was initiated, some EST fragments that partially covered bp65 were used to design the cloning primers, however the predicted bp65 had been deposited before the functional studies were completed and thus we could compare the sequences. In addition, based on the genomic structure of the predicted bp65, the exon-intron boundaries of bp65 gene were analyzed (Figure 19) and found to be identical to their human and mouse orthologues. A DNA fragment of the bp65 correspondent with the submitted sequence was recloned from a pGEM-T vector into pcDNA3.1/Hygro(+) mammalian expression vector, and its integrity was verified by sequencing.

### 5.3.2 Comparison of the bovine and vertebrate p65 subunits of the NF $\kappa$ B protein sequence

The comparison of the deduced amino acid sequence from bp65 to its human, mouse, chicken, clawed frog and zebra fish orthologues exhibit apparently different degrees of homology from various regions (Figure 20). The comparison identified the expected domains of bp65 as Rel homology domain, transactivation domain 1 and 2. RHD has 97.1%-98.5% identity in mammals and 66.7%-78.9% among vertebrate classes (Table 7), while the amino acid residues that contribute to the DNA base specific contacts, DNA backbone contacts and dimer interface according to the crystal structure of mouse p65/NF $\kappa$ B1 heterodimer and  $\kappa$ B site (BERKOWITZ *et al.*, 2002), are conserved except for a group of dimerization residues (Leu-202, Pro-203, Gly-204, Asp-205) in chicken. RHD residues that contact I $\kappa$ B proteins (data not shown) based on the crystal structure of the p65/NF $\kappa$ B1 heterodimer and I $\kappa$ B proteins complexes (HUXFORD *et al.*, 1998), are identical in mammals, but 46.2% of them are variable in vertebrates. The nuclear localization signal is identical in vertebrates, thus the I $\kappa$ B contacts to NLS, which contributes to NF $\kappa$ B masking by the I $\kappa$ B proteins (MALEK *et al.*, 2003), are also conserved. In addition, the serine residues of RHD (Ser-205, Ser-276, Ser-281) which take part in the phosphorylation-dependent, *cis*-acting element-specific transactivation (ANRATHER *et al.*, 2005) are entirely conserved. The amino acid sequences outside RHD have shown great variability due to the frequent frame shift mutations and the



the lowest identity among regions of the p65, 62.7%-68.8% in the case of mammals and 9.8%-27.3% among vertebrate classes. Frame shift mutations and insertions-deletions contribute to this extremely low identity of the linker regions in vertebrates, while they also have an effect on the homology of TADs. The most pronounced sequence diversity between TADs is found in zebra fish p65, in that it lacks the C-terminal 53 amino acids using bp65 TAD as a point of reference, hence zebra fish TAD is not regarded as a completely homologous sequence to other vertebrate TADs. Apart from the zebra fish TAD, there is 87.6%-90.1% identity in mammals and 22.4%-32.2% identity in vertebrate TADs, which are located close to the values of RHD in mammals and considerably far away from the values of RHD in vertebrates. TAD1 regions exhibit higher identity (90.0%-96.7% and 32.4%-42.4%) than entire TADs. The most characterized serine residue of TAD1, Ser-536, which modulates the transcriptional activity of the p65 by phosphorylation (VIATOUR *et al.*, 2005), is well conserved, whereas other functional serine residues of TAD (Ser-468, Ser-529) are conserved only in mammals. These results indicated that the functionally known relevant amino acid residues of p65 were highly conserved in mammals, while similar levels of high homology could be observed in the cases of NLS, and the residues of RHD that contribute to the DNA contacts and the dimerization in vertebrates. In contrast, the level of homology was moderate in the TAD sequences of vertebrate species.

Table 7

The domain-by-domain comparison of the amino acid identity in Rel homology domain (RHD), linker region and transactivation domains (entire TAD, TAD1) of the cloned and characterized vertebrate p65 sequences.

	Bos	Homo	Mus	Gallus	Xenopus	
Homo	98.5					RHD
Mus	97.4	97.1				
Gallus	71.8	72.2	71.8			
Xenopus	78.5	78.2	78.9	70.2		
Danio	72.1	72.1	72.8	66.7	71.4	
Homo	68.0					linker region
Mus	62.7	68.8				
Gallus	25.2	27.3	26.0			
Xenopus	13.8	13.7	13.1	19.1		
Danio	13.0	12.2	10.0	14.2	9.8	
Homo	90.1					entire TAD
Mus	88.4	87.6				
Gallus	32.2	32.2	31.4			
Xenopus	30.1	30.1	30.9	22.4		
Homo	93.3					
Mus	90.0	96.7				
Gallus	39.4	42.4	42.4			
Xenopus	39.4	39.4	39.4	32.4		

### 5.3.3 Expression and characterization of the bovine p65 subunit of NFκB

To assess the transactivation ability of the cloned bp65 and to indirectly investigate its sequence-specific DNA binding ability, mammalian expression vector harbouring the coding sequence of bp65 (pcDNA3.1/Hygro(+)-bp65) and luciferase reporter gene constructions were cotransfected in mammalian cell lines and primary cells. The overexpressed bp65 significantly induced the luciferase activity of the reporter gene vector containing consensus κB sites, called pNFκB-Luc, in 293, BAEC, HC11 and MAC-T cells (Figure 21). However, it failed to affect the luciferase activity of the reporter gene vector without consensus κB site, the pGL3-basic, in all cell types. The fold inductions of the pNFκB-Luc were different in these cell lines and primary cells (29.4x in 293, 4.0x in HC11, 179.4x in MAC-T and 7.3x in BAEC cells), but NFκB-specific induction occurred similarly in human and mouse cell lines as well as in bovine cells, suggesting that there was no species-specific difference of p65 induction in mammals.

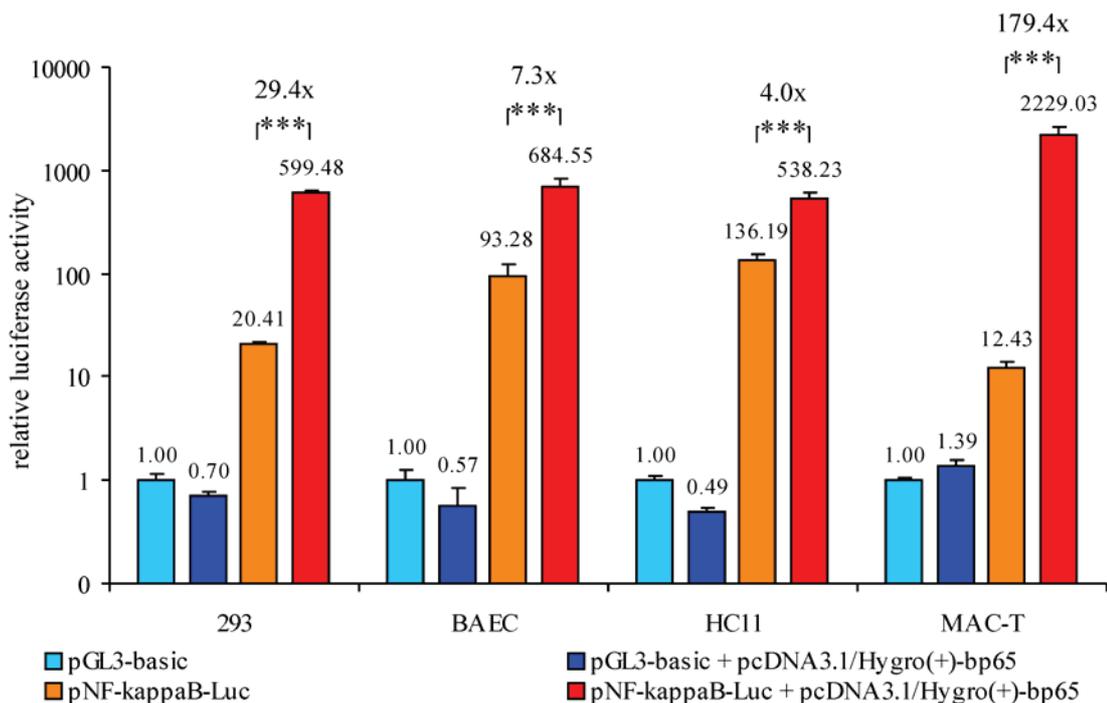


Figure 21

The induction of luciferase activities by bp65 overexpression. The luciferase activities of the pNFκB-Luc were significantly induced in 293, BAEC, HC11 and MAC-T cells, whereas the luciferase activities of the negative control, the pGL3-basic, failed to be affected. The means of normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments. The fold inductions of pNFκB-Luc are above columns, and \*\*\* indicates the significant difference according to Student's test at the level 0.001.

Nuclear extracts were prepared from BAEC, LPS-treated BAEC, MAC-T and bp65-transfected MAC-T cells. Nuclear extracts were also collected from HeLa S3 cells before and after treatment with TNF- $\alpha$ . These extracts were then used to investigate the *in vitro* sequence-specific DNA binding ability of the cloned bp65 by gel retardation techniques using labelled consensus  $\kappa$ B oligonucleotide. Distinct endogenous protein-DNA complexes were detected using the nuclear extracts from TNF- $\alpha$ -treated HeLa S3 and LPS-treated BAEC cells (Figure 22, red arrow, kappaB-specific complex, lane 3, 6). There was no difference between the migration of human and bovine molecular complexes, suggesting that the molecular weight and the conformation of these molecules were similar. These identically migrating complexes were not observed when the nuclear extracts from untreated HeLa S3 and BAEC cells were used (lane 2, 5). The disappearance of the complexes and the simultaneous emergence of the band supershifts were also found identical in the presence of p65 antibody (red arrow, supershifted kappaB-specific complex, lane 4, 7). The nuclear extracts from bp65 transfected MAC-T cells contained cloned bp65 molecules in all likelihood, since the transactivation effect in luciferase reporter gene assay could not occur in another way. The appearance of the  $\kappa$ B-specific complex containing the cloned bp65 resembled the endogenous bovine complex (lane 8, 9), however, it could not be perfectly sequestered from the lower non-specific band. The supershifted complex of the cloned bp65 proved to be identical to the

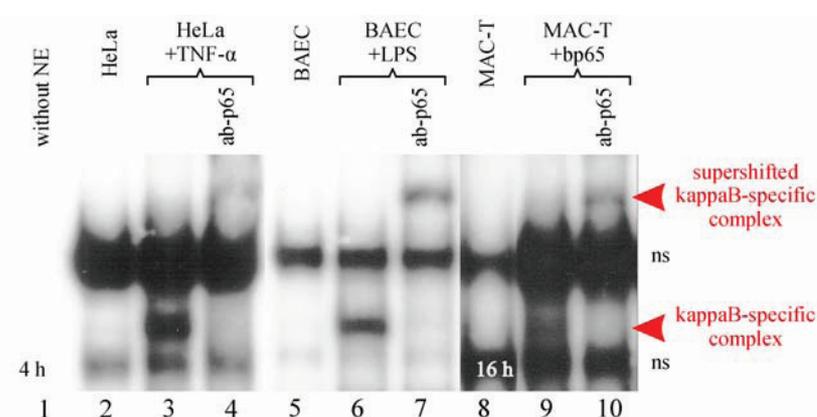


Figure 22

Gel retardation assay for the detection of  $\kappa$ B-specific complex containing p65. Nuclear extracts (NE) prepared from untreated HeLa S3 (HeLa), TNF- $\alpha$ -treated HeLa S3 (HeLa +TNF- $\alpha$ ), untreated BAEC (BAEC), LPS-treated BAEC (BAEC +LPS), untransfected MAC-T (MAC-T) and bp65 transfected MAC-T (MAC-T +bp65). Nuclear extracts were incubated and run with labelled oligonucleotide representing consensus  $\kappa$ B site in absence or presence of p65 antibody (ab-p65). Distinct  $\kappa$ B-specific protein-DNA complexes (red arrow, kappaB-specific complex) were detected using nuclear extracts from TNF- $\alpha$ -treated HeLa S3 and LPS-treated BAEC cells (lane 3, 6). These identically migrated complexes could not be observed using nuclear extracts from untreated HeLa S3 and BAEC cells (lane 2, 5), and could further shift with p65 antibody (red arrow, supershifted kappaB-specific complex, lane 4, 7). The appearance of  $\kappa$ B-specific complex and its supershifted version containing cloned bp65 from transfected MAC-T cells ranged with endogenous bovine complex (lane 8, 9). "ns" indicates non-specific bands.

endogenous bovine complex (lane 10). In addition to the  $\kappa$ B-specific and the supershifted band of the cloned bp65, other specific bands did not appear. Taking into account the results of the gel retardation experiments, it was concluded that cloned bp65 could constitute complex with endogenously expressed bovine NF $\kappa$ B proteins, and was capable of binding the consensus  $\kappa$ B sequence.

#### 5.3.4 Phylogenetic and genetic analysis

The NJ trees of NF $\kappa$ B proteins (Table 6, Figure 23 A) based on the amino acid and DNA sequences of the Rel homology domain (Figure 23 B, C, Appendix) were constructed in order to better understand the evolutionary relationship of the NF $\kappa$ B family and the position of the bp65 in the evolutionary history of the NF $\kappa$ B family. The clustering of the main groups from the different vertebrate genes and the groups of the invertebrate protostome and deuterostome genes were essentially the same in the amino acid and DNA NJ trees (NJ RHD amino acid, NJ RHD DNA) except for the position of *Ciona* and sea urchin NF $\kappa$ B. Besides, there was a multifurcated branch among the cluster of the vertebrates paralogous genes containing transactivation domain (p65, c-Rel and RelB) and the cluster of the ascidian Rel genes in both trees. The NJ RHD amino acid tree could not reliably resolve the branching order of the mammalian p65 proteins (Figure 23 B), and the position of *Drosophila* Dif and *Carcinoscorpius* NF $\kappa$ B was inverted (Supplement 1) compared to the NJ RHD DNA tree. The branching order of *Gallus* and *Xenopus* p65 supported by 59% bootstrap value did not reflect the accepted species evolution (HEDGES, 2002) in the NJ RHD amino acid tree. This result corresponded with the NJ tree based on the amino acid sequence of RHDs in a previous study (HUGUET *et al.*, 1997) in the same way as the topology of the common taxa of the two amino acid RHD trees. The NJ RHD DNA tree of mammalian p65 also comprised a discrepancy of species evolution, since the position of *Danio* and *Xenopus* p65 was inverted compared to the expected relationship. To verify the topology of the NJ RHD DNA tree, a phylogenetic tree was constructed by maximum likelihood method using the same dataset (ML RHD DNA, Appendix). The ML RHD DNA tree largely confirmed the topology of NJ RHD DNA tree, however, the position of the mammalian p65 did not correlate in the two RHD DNA trees. In addition, the ML RHD DNA tree could resolve the position of the ascidian Rel genes, and contained the *Danio* and *Xenopus* p65 with the expected evolutionary relationship, but the topology of Relish genes slightly deviated in two RHD DNA trees. It is worth mentioning that the bootstrap values of the ML RHD DNA tree were relatively low compared to the values of NJ RHD DNA tree.

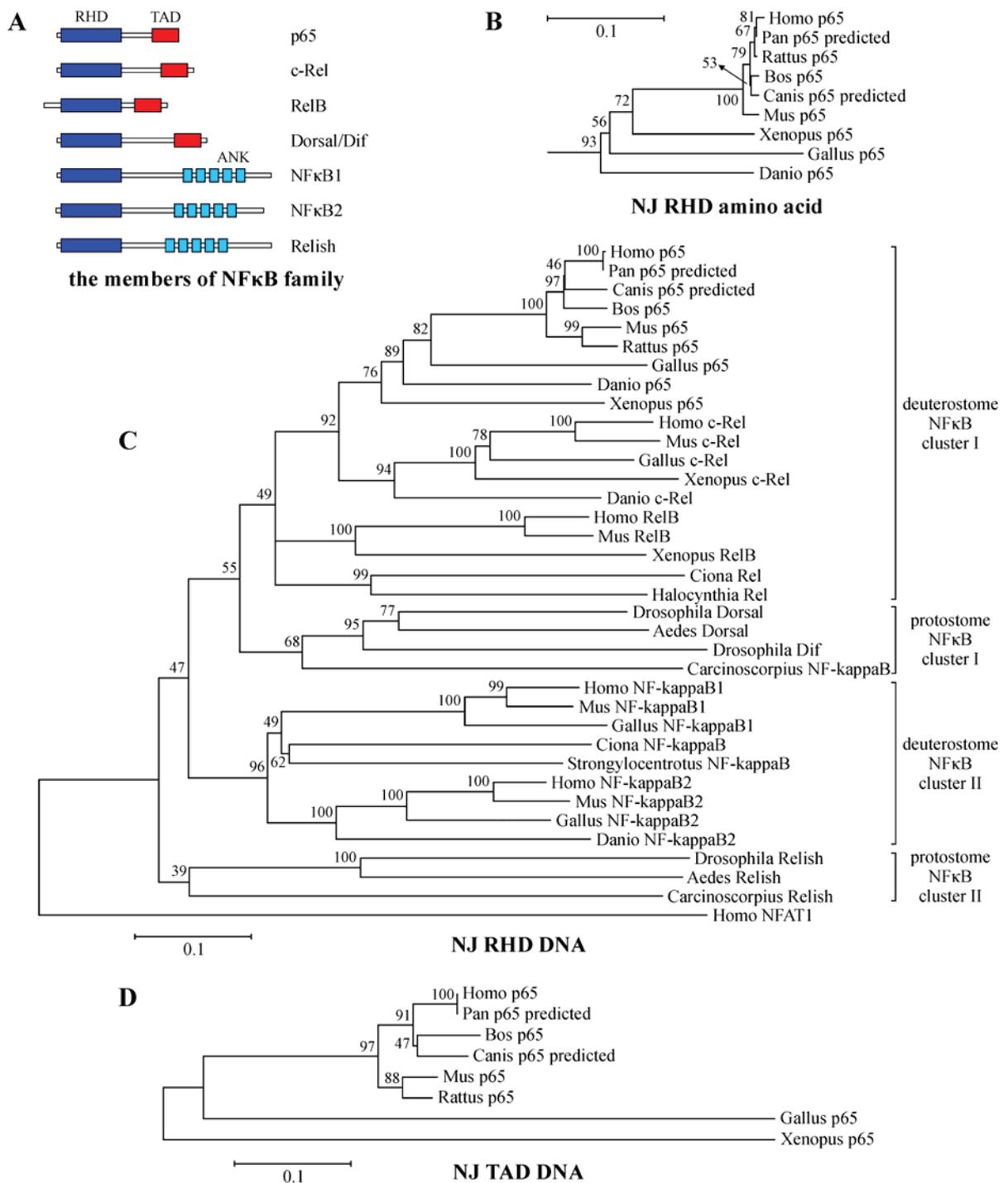


Figure 23

Rooted phylogenetic trees of the NFκB family. (A) All members of the NFκB family have a structurally conserved Rel homology domain (RHD). RelB, p65 and c-Rel comprise transactivation domain (TAD) in vertebrates, while Dorsal and Dif in invertebrate. NFκB1 and NFκB2 in vertebrates and Relish in invertebrates do not contain TAD, but they share repeated structural elements, the ankyrin repeat (ANK). (B) Rooted phylogenetic tree of vertebrate p65 based on amino acid sequence of Rel homology domain (RHD) by NJ method. (C) Rooted phylogenetic tree of NFκB family based on DNA sequence of the Rel homology domain by neighbor-joining method using human NFAT1 as the outgroup. The clusters of the NFκB family are indicated on the right side of the tree. (D) Rooted phylogenetic tree of vertebrate p65 based on DNA sequence of the transactivation domain (TAD) by NJ method using *Xenopus* p65 as the outgroup. Bootstrap values are next to the corresponding nodes. The scale bar indicates the genetic distance.

Nonetheless, the bp65 reliably clustered to other p65 proteins in all phylogenetic trees, thus this result validated its homology to the p65 molecules. Four clusters of the NFκB family could be defined based on the RHD trees, which follow the function and evolution of the NFκB proteins. The first cluster was labelled deuterostome NFκB cluster I, which included three paralogous genes of vertebrates containing TAD, p65, c-Rel and RelB, and the ascidian Rel genes. The second cluster was the protostome NFκB cluster I comprising of the protostome genes with TAD. It is clear that the genes of the protostome NFκB cluster I may be considered as orthologues of p65, c-Rel and RelB (HUGUET *et al.*, 1997). Similar relationships characterized the third and fourth clusters of the genes that do not contain TAD. The deuterostome NFκB cluster II included two paralogous vertebrate genes, NFκB1 and NFκB2, and the invertebrate deuterostome NFκB genes, while Relish genes, the orthologues of NFκB1 and NFκB2, were involved in the protostome NFκB cluster II cluster.

To clarify the branch order of mammalian p65 proteins, a phylogenetic tree of p65 genes was reconstructed by NJ and ML method based on the DNA sequences of TADs (NJ TAD DNA, ML TAD DNA, Figure 23 D, Appendix), assuming that the lower amino acid identity of TAD compared to that of RHD is the consequence of faster molecular evolution. The two TAD DNA trees corresponded with each other, the branch order of trees resolved the topology of the mammals, however, the bootstrap value of *Bos* and *Canis* node was moderate in both NJ and ML TAD DNA tree (47% and 46%, respectively).

To estimate the dynamic of molecular evolution in RHD and TAD sequences, the ratios of the rate of non-synonymous (amino acid-altering) versus the rate of synonymous (silent) mutations ( $d_N/d_S$ ) were determined by the Nei-Gojobori method in mammalian DNA sequences. The average rates of synonymous mutations did not show significant difference according to the Student's test at the 0.01 level in the two domains ( $0.331 \pm 0.072$  in RHD and  $0.272 \pm 0.047$  in TAD), therefore the silent nucleotide changes occurred in similar scale. The value of average  $d_N/d_S$  were  $0.023 \pm 0.011$  in RHD and  $0.174 \pm 0.038$  in TAD, the difference was significant according to the Student's test at the 0.01 level. This result indicated that the selective pressure acting on RHD is larger than that acting on TAD, and the assumption for the faster molecular evolution of TAD based on lower amino acid identity was correct.

#### 5.4. The investigation of NFκB-mediated induction of bovine FcRn and β2m transcription in bovine specific model

The NFκB responsiveness of the bFcRn and bβ2m *cis*-regulatory region was examined with luciferase reporter gene assay using bp65 overexpression in bovine cell line, MAC-T. The luciferase activities of pGL3-bβ2m-1934+49 failed to be induced by pcDNA3.1/Hygro(+)-bp65, whereas pGL3-bFcRn-1787+92 and pNFκB-Luc were significantly induced according to the Student's test at the 0.01 level (Figure 24). Taking into account the findings of the luciferase reporter gene assay in 293, HC11 and MAC-T, there was no qualitative differences among the results in the different cell lines, and the contradiction between the results of the luciferase reporter gene assay and gel retardation assay could not be resolved.

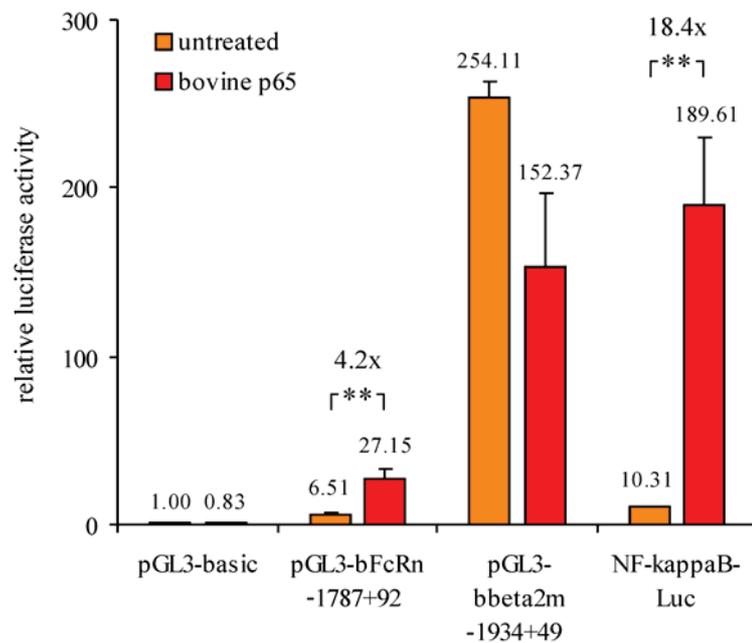


Figure 24

The induction of the bFcRn and bβ2m *cis*-regulatory region by bovine p65 overexpression in luciferase reporter gene assays of MAC-T cell line. The luciferase activities of pGL3-bβ2m-1934+49 failed to be induced by bovine p65, whereas the luciferase activities of the pGL3-bFcRn-1787+92 and pNFκB-Luc were induced according to Student's test at the level 0.01 (\*\*) in MAC-T. The means of normalized luciferase activities are above the columns, which are expressed relative to the activity driven from the pGL3-basic vector, and the error bars indicate the standard deviation from three independent experiments.

## 6. Discussion

The expression profile of FcRn reflects its functional pattern, therefore high levels are expressed in neonatal rodent enterocytes, rabbit fetal yolk sac, human placental syncytiotrophoblast and mammalian mammary glands, where FcRn functions as IgG transport receptor. Besides, in mammalian endothelial cells, it functions as protection receptor to maintain high plasma IgG throughout life (GHETIE and WARD, 2000). Recently, FcRn has also been implicated in prolonging the half-life of serum albumin by a similar mechanism (CHAUDHURY *et al.*, 2006, CHAUDHURY *et al.*, 2003). In addition, FcRn expression has been reported at low levels in other cell types, tissues and organs, such as kidney, pancreas, liver and human small intestine, where it is also implicated in IgG protection and recycling. FcRn is developmentally down-regulated at the time of weaning in the rodent intestine (JENKINS *et al.*, 2003), however, it has been recently appreciated that FcRn continues to be expressed in adult life in humans, pigs, cattle, sheep, monkeys and even rodents. This high degree of recently appreciated complexity in FcRn expression and consequently function focuses significant attention on the manner in which FcRn expression is regulated. However, very little is known about how FcRn is transcriptionally regulated. Characterization of human, mouse and rat FcRn promoter regions has revealed several putative promoter binding sites, and some of these have been shown to be functional binding sites for Sp1-like factors (JIANG *et al.*, 2004, MIKULSKA and SIMISTER, 2000, TIWARI and JUNGHANS, 2005). Although the crucial regulatory element that drives FcRn gene expression remains unknown in these species and thus it is unknown how FcRn is transcriptionally regulated (QIAO *et al.*, 2007), nevertheless, the cytokine-mediated transcriptional regulation of FcRn has been also raised by others (GOBIN *et al.*, 2003).

The pattern of FcRn expression is well characterized in ruminant mammary gland, and the obvious change in the subcellular localization of the receptor in the mammary epithelial cells around the time of parturition in ewes, its presence in the crypt epithelial cells of the neonatal lamb as well as in the lower respiratory tract led to the hypothesis, that this receptor is involved in IgG1 transport across these barriers (KACSKOVICS, 2004). This hypothesis is further supported by the fact that allotypic variants of both bFcRn  $\alpha$ -chain and b $\beta$ 2m influence serum IgG concentration in newborn calves (CLAWSON *et al.*, 2004, LAEGREID *et al.*, 2002). In addition, bFcRn is also expressed in endothelial cells and can bind both bovine and human IgGs *in vitro* suggesting that it also takes part in IgG homeostasis (KACSKOVICS *et al.*, 2006a). Despite these important functions of this receptor in an economically important species, which is already a target to involve in human therapy

(KUROIWA *et al.*, 2002), the expression changes have not been available from bovine until the very recent past (RABOT *et al.*, 2006).

## 6.1. Comments for the methods

For analyzing gene regulation, the methods that were intended to use were established. These techniques had to be introduced at first in the laboratory, thus some of these issues call for an explanation to better illuminate the applied methods and the results of study.

At first the site-directed mutagenesis of TF binding sites was achieved by ligation method, and then by megaprimer method. There is no difference in the outcome of the two methods, but undesired mutations are generated by failure of DNA polymerase more frequently in the ligation method. The rate of undesired mutations with a particular DNA polymerase depends on the number of cycles in PCR, therefore the amplification cycles should be kept at a minimum to avoid any random mutations (DATTA, 1995). The cycle number that was carried out in the megaprimer mutagenesis was 50 instead of 60, which was used for the ligation method. In addition, the ligation method consists of more steps than the mutagenesis method, which results in more possibility for the errors, and renders it more time-consuming. One variant of megaprimer method offers a mutagenesis that can be performed with only 30 amplification cycles in less than 5 hours (PICARD *et al.*, 1994), but quite often it did not work in our hand, because the mutagenic primers presumably were not suitable. However, the general rule should be followed that good primers have to be designed to ensure successful DNA mutagenesis (LING and ROBINSON, 1997), and the sequence around TF binding site, which is needed to mutate, often limits the quality of possible primers.

The pivotal part of the reporter gene assay is the DNA transfer into the cells, and as a reporter gene assay cannot be carried out without effective DNA transfer, it is highly important to optimize the DNA transfer (SCHENBORN, 2000). The optimization of PEI transfection was achieved in each cell line and cell type that was used, but the process of the optimization remained essentially the same. The preliminary optimization was fulfilled by GFP due to its simplicity and low cost. On the other hand, the results of transfection efficiency from GFP reporter experiments did not correlate exactly with the results from firefly luciferase reporter experiments, thus GFP results were considered as tendencies, and were always verified by firefly luciferase reporter assay. The main optimized parameter was the  $N_{\text{PEI}}/P_{\text{DNA}}$  molar ratio, which was calculated on the basis of PEI amine nitrogen per DNA phosphate (BOUSSIF *et al.*, 1995). In addition, the amount of total DNA, the confluence of cells at the time of transfection, the harvesting time of cells, the number of rinses before transfection and the

number of rinses after transfection were also optimized. Prudent optimization was tried to achieve for all cells types, but it did not make sense to spend very long time with this kind of complex optimization, thus the refinement of transfection protocols were performed during the large number of experiments for shedding light on transcriptional regulation of bFcRn and b $\beta$ 2m.

The reporter gene assay accompanied by the site-directed mutagenesis represents the single technique, which can reveal the contribution of the individual *cis*-acting elements to the transactivation of a transcription factor interacting with the investigated *cis*-regulatory region, thus it is almost indispensable for unravelling the *in vivo* transcriptional regulation of a particular gene in response to a particular stimulus (NAYLOR, 1999). Although the transcription of transiently transferred reporter gene occurs inside the eukaryotic nucleus, the reporter gene assay cannot be regarded as a clear *in vivo* model, because the investigated *cis*-regulatory region is not organized into chromatin structure (NARLIKAR *et al.*, 2002). In eukaryotic cells, one of the essential features is the chromatin structure of DNA (KHORASANIZADEH, 2004), and transcription factors often exert their transactivation abilities through remodelling the chromatin structure (FRY and PETERSON, 2001). For this reason, the responsiveness of a particular *cis*-regulatory region linked to a reporter gene in response to a particular transcription factor is seldom in proportion to the responsiveness of the particular *cis*-regulatory region integrated in genome, and it is suitable for drawing qualitative rather than quantitative conclusions in respect of *in vivo* transcription. The site-directed mutagenesis of the corresponding TF binding site can confirm the responsiveness of the *cis*-regulatory region in response to a transcription factor, because the responsiveness will be moderated or completely eliminated if the transcription factor cannot bind to its binding site, therefore the manipulated *cis*-regulatory region can behave in accordance with the wild type sequences. Naturally, the examination of *in vivo* mRNA level in response to a stimulus related to a particular transcription factor by qPCR (BUSTIN *et al.*, 2005) or Northern-blot provides an obvious solution to validate the result of reporter gene assay, but some other methods such as gel retardation and chromatin immunoprecipitation (ChIP) assays (DAS *et al.*, 2004) are also capable of validating it. It is more difficult to verify a negative result from reporter gene assay, when there is no responsiveness to a particular transcription factor, and it should be decided whether the *in vivo* transcription activity of the investigated gene does not change indeed or the reporter gene assay fails to reflect the reality. For instance, pNF $\kappa$ B-Luc can be induced by bovine or human p65 overexpression in several PEI-transfected cell types (Figure 15, 21), but it fails to be induced by human p65 overexpression in calcium phosphate-transfected 293 cells, whereas other reporter constructs containing wild type *cis*-regulatory

region can be activated at the same conditions (data not shown). It is senseless to perform site-directed mutagenesis for confirming the negative result of reporter gene assay, while the outcome of gel retardation and ChIP assays becomes more doubtful in the absence of proper information from the localization of the particular TF binding sites (WELLS and FARNHAM, 2002). In many cases, the single rational possibility is provided by the examination of *in vivo* mRNA level. Taking everything into account, the responsiveness or unresponsiveness of the investigated *cis*-regulatory region in reporter gene assay alone does not unambiguously prove that *in vivo* transcription regulation occurs similarly, instead it should be considered as a reliable prediction. This approach also concerns partly other *in vitro* methods, such as gel retardation assay and especially their negative results. Nevertheless, it would be careless to conclude that methods that *in vivo* investigate the transcriptional regulation are generally better than those *in vitro*. For example, the investigation of *in vivo* binding ability in a *cis*-regulatory region by ChIP assay is usually in need of previous information from the localization of TF binding sites, which are revealed mostly by *in vitro* techniques, furthermore, it is quite complicated or very costly to reach some target cells for qPCR (PINZANI *et al.*, 2006), which can limit their use in some cases. Therefore there is no ultimate method that has proved its superiority over other methods up to the present, and the features of the investigated gene should especially determine the applied methods and experimental strategy (URNOV, 2003).

## **6.2. The immune regulation of the bovine FcRn $\alpha$ -chain transcription**

The regulation of FcRn  $\alpha$ -chain gene expression occurs at least partially at the level of transcription (JIANG *et al.*, 2004, TIWARI and JUNGHANS, 2005), but the connection between the immunological functions of the transcriptional regulation of FcRn has not been established. Therefore an about 1900 bp of the bFcRn  $\alpha$ -chain *cis*-regulatory region was investigated by luciferase reporter gene assay with regards to the immune-specific transcriptional regulation. The *cis*-regulatory region could be induced by human p65 in both human and mouse cell lines, then the NF $\kappa$ B responsiveness was validated in bovine-specific model using bp65 overexpression and bovine mammary gland. Besides NF $\kappa$ B, other immune-related transcription factors were also tested in luciferase reporter gene assay. Based on our experiments, IRF1, c-Jun, and STAT3 failed to induce the bFcRn *cis*-regulatory region, whereas their positive controls were activated. These negative results were not confirmed by other methods, hence their ineffectiveness for the transcription of the bFcRn can be only predicted. Three functional  $\kappa$ B binding sites, the  $\kappa$ B-612,  $\kappa$ B-758 and  $\kappa$ B-840, have been

identified in the *cis*-regulatory region using site-directed mutagenesis accompanied by luciferase reporter gene assay. One by one, the mutant  $\kappa$ B sites could reduce the p65 responsiveness, whereas the mutagenesis of all three  $\kappa$ B sites completely deleted the inducibility of the bFcRn *cis*-regulatory region indicating that these  $\kappa$ B sites have been responsible for the p65 inducibility of the bFcRn. The sequence-specific binding ability of the bFcRn  $\kappa$ B sites were verified by EMSA and supershift assay proving that these sites can bind NF $\kappa$ B complex that comprises p65. The NF $\kappa$ B responsiveness of the bFcRn transcription has confirmed that human FcRn can be also induced in response to p65 overexpression in luciferase reporter gene assay. A functional  $\kappa$ B binding site in the hFcRn *cis*-regulatory region has been identified using site-directed mutagenesis and gel retardation assays (SZALAI, 2005). It is noteworthy, that the hFcRn  $\kappa$ B site is located about 200 bp upstream from TSS, thus it is not regarded as a homologous sequence of the bFcRn  $\kappa$ B sites. Moreover, the bFcRn  $\kappa$ B sites are located in a bovine-specific, short interspersed nuclear element (DOLESCHALL *et al.*, 2005), thus the bovine and human  $\kappa$ B sites of FcRn reflect a functional relation rather than common evolutionary origin.

Although the above mentioned results clearly support the NF $\kappa$ B inducibility of bFcRn transcription, the cell type, tissue, or organ specificity of this NF $\kappa$ B action has not been investigated until the very recent past. Nowadays the increase of the bFcRn mRNA level caused by LPS has been found in bovine mammary gland (RABOT *et al.*, 2006) indicating that the NF $\kappa$ B-mediated increase of bFcRn level may play some role in mastitis (HANSEN *et al.*, 2004). Around parturition, IgG level changes in direct proportion to cytokine levels such as TNF- $\alpha$  in bovine mammary gland (BUTLER, 1983, HAGIWARA *et al.*, 2000) raised the possibility that NF $\kappa$ B-mediated increase of bFcRn level contributes to the temporal change of IgG level in colostrum and milk. In addition, the bFcRn mRNA level is also increased by LPS induction in the spleen and liver of mouse FcRn knockout-bFcRn transgenic mice (Balázs Bender, Zsuzsanna Bősze and Imre Kacs Kovics's personal communication). Although LPS can influence the transcription via ISRE site in some cases (PALSSON-MCDERMOTT and O'NEILL, 2004), the negative result from the luciferase reporter gene assay with IRF1 overexpression further supports that the described  $\kappa$ B sites are responsible for LPS-induced mRNA increase of the bFcRn.

The p50 and p65 subunits of NF $\kappa$ B are ubiquitously expressed (GHOSH *et al.*, 1998), thus they coexist with FcRn in all cell types, tissues and organs where FcRn exerts its influence. Therefore NF $\kappa$ B can potentially act on the FcRn-mediated IgG functions in every place where FcRn are expressed. It may be especially important for the immune functions classically linked to NF $\kappa$ B, such as infection or inflammation (CAAMANO and HUNTER, 2002). One

may speculate, when LPS induces NF $\kappa$ B in a bacterial infection, then FcRn expression will be upregulated and that may raise the level of IgG transport to bovine lower respiratory tract (MAYER *et al.*, 2004a).

In summary, NF $\kappa$ B responsiveness of bFcRn has been verified using luciferase reporter gene assay accompanied by site-directed mutagenesis and gel retardation assay in accordance with the present *in vivo* data about the LPS-induced alteration of mRNA. NF $\kappa$ B is the first described immune-related transcription factor which can bind to the bFcRn *cis*-regulatory region and activate bFcRn transcription modulating presumably the bFcRn expression and function. The NF $\kappa$ B-mediated alteration of the bFcRn function may be supposed at all presumed immune processes that belong to the bFcRn, but the expression changes and function of bFcRn are inadequately clarified *in vivo* providing very narrow base for these hypotheses. Therefore the significance of the bFcRn transcriptional regulation mediated by NF $\kappa$ B cannot assess exactly at the present time and leaves room for *in vivo* analysing these hypotheses in the bovine transgenic mice, which have been created recently (BENDER *et al.*, 2005) or in cattle by using proteomics or other systems biological approaches.

### **6.3. The immune regulation of the bovine $\beta$ 2m transcription**

As the  $\beta$ 2m is a chaperone of MHC class I (-like) proteins, its function is related to these molecules, and it has key positions in antigen presentation, IgG transport and homeostasis, as well as iron metabolism (TYSOE-CALNON *et al.*, 1991). The association of  $\beta$ 2m with FcRn  $\alpha$ -chain is also well documented in human (GHETIE *et al.*, 1996, GHETIE and WARD, 2000, ISRAEL *et al.*, 1995), and the bovine  $\beta$ 2m is equivalent with its human orthologue in its major features (SHIELDS *et al.*, 1998). The transcriptional regulation of the human  $\beta$ 2m is experimentally well characterized, thus the investigation of immune regulation of the b $\beta$ 2m transcription was built on previous human studies (GOBIN *et al.*, 2003, GOBIN *et al.*, 1998, GOBIN *et al.*, 2001).

The  $\kappa$ B and ISRE sites that are responsible for the cytokine-induced regulation in human were only partially conserved in bovine as it is shown by the sequence comparison. The  $\kappa$ B site showed a two-nucleotide deletion in bovine, which was confirmed by sequencing this 5'-flanking segment of several individuals. There was no p65 responsiveness for an about 2000 bp b $\beta$ 2m *cis*-regulatory region using luciferase reporter gene assay neither in human, nor in bovine cell models, which is in keeping with the deletion in the  $\kappa$ B site. In spite of the two-nucleotide deletion in the  $\kappa$ B site and the negative results of the luciferase reporter gene assay, the b $\beta$ 2m  $\kappa$ B site could bind the NF $\kappa$ B complex containing p65 subunit in gel retardation

assay rendering data from *in vitro* experiments controversial. In addition, the available *in vivo* data are also contradictory, since the b $\beta$ 2m mRNA level in BAEC increases upon LPS induction investigated by qPCR (preliminary data from Balázs Mayer, Zoltán Doleschall and Imre Kacs Kovics), whereas other qPCR data exhibit no increase in the same model (VAN KAMPEN and MALLARD, 2001). Therefore the functionality of the b $\beta$ 2m  $\kappa$ B site cannot be established on the basis of the present data, nor can its significance be assessed at the present time.

The b $\beta$ 2m ISRE binding site, ISRE-122, was well conserved based on the sequence comparison between bovine and human. The about 2000 bp b $\beta$ 2m *cis*-regulatory region was induced by human IRF1 overexpression, and the contribution of the ISRE-122 to the IRF1 responsiveness was identified by site-directed mutagenesis accompanied by luciferase reporter gene assay. Thus the ISRE-122 site is responsible for the IRF1 inducibility and also takes part in the constitutive transcription in the same way as the orthologue ISRE site in human (GOBIN *et al.*, 2003). The ISRE-122 site could bind the INF- $\gamma$ -specific complex in gel retardation assay further supporting its functionality and the results from luciferase reporter gene assay. In addition, the b $\beta$ 2m mRNA level in BAEC increases upon bovine INF- $\gamma$  induction investigated by qPCR (preliminary data from Balázs Mayer, Zoltán Doleschall and Imre Kacs Kovics) providing *in vivo* confirmation that the b $\beta$ 2m ISRE site functions in similar way as its human orthologue.

Taking all things into account, the role of b $\beta$ 2m  $\kappa$ B analyzed by *in vitro* methods were controversial, and need further studies to clarify it. On the other hand, based on the results of luciferase reporter gene assay, gel retardation assay and qPCR, the ISRE site mediates the IFN- $\gamma$  induction similarly to its human orthologue (GOBIN *et al.*, 2003), thus there are no differences in the ISRE-mediated transcriptional regulation of this gene in cattle. Although the IFN-mediated function of b $\beta$ 2m has not been adequately investigated in bovine, the other function of b $\beta$ 2m highly correspond to that of human (KIEVITS and IVANYI, 1987, SHIELDS *et al.*, 1998), thus its IFN-mediated function presumably resembles this  $\beta$ 2m function in human (PINE, 2002, SERVANT *et al.*, 2002).

#### **6.4. Cloning and characterization of the bovine p65 subunit of NF $\kappa$ B**

In order to establish a species-specific system that can be used to analyze gene regulation in bovine, the bovine p65 subunit of the NF $\kappa$ B family, which plays a central role in the transcriptional regulation of the immune system (LI and VERMA, 2002), has been isolated and verified its homology to the known p65 molecules. The comparisons on the basis of

amino acid and DNA alignments of the vertebrate p65 proteins have identified the expected regions of the bp65 as the Rel homology domain, the nuclear localization signal and the transactivation domain 1 and 2 (GHOSH and KARIN, 2002). The homology of RHD and TAD was found to be very high in the cloned and characterized mammalian p65 molecules. The amino acid residues with known functions were identical in bovine, human and mouse, suggesting no species-specific difference in the function among the mammalian p65 molecules. In accordance with the sequence comparisons, the first experimental analyses of bp65 functions have not revealed any discrepancies compared to the p65 functions that have been characterized in mammals so far (LI and VERMA, 2002), however, they could not exclude the existence of species-specific differences. The band patterns of the  $\kappa$ B-specific protein-DNA complexes examined by gel retardation techniques were identical in human and bovine. These  $\kappa$ B-specific human and bovine complexes containing p65 represent p65/NF $\kappa$ B1 heterodimers in TNF- $\alpha$ -treated HeLa cells (LU *et al.*, 2002) and LPS-treated BAEC (ANRATHER *et al.*, 1997), which is expected, because only the p65 and NF $\kappa$ B1 are expressed outside mammalian lymphoid cells and tissues (CAAMANO and HUNTER, 2002). NF $\kappa$ B transactivation normally occurred in our experiments under the effect of the bp65 overexpression in bovine cells likewise in human (293) and mouse (HC11) cell lines. Therefore the bp65 is able to form interactions with human and mouse transcription apparatuses.

Some members of the invertebrate NF $\kappa$ B system have been recently isolated (KAWAI *et al.*, 2005, SHIN *et al.*, 2005, WANG *et al.*, 2006), and stimulated the revision of the former comprehensive phylogenetic analysis of the NF $\kappa$ B family (HUGUET *et al.*, 1997). Our results based on the comprehensive phylogenetic analyses of the RHD amino acid and DNA sequences confirmed the fundamental conclusions of the above mentioned study, however, the clusters of the NF $\kappa$ B family were defined disparately as a result of recent data. In addition, the phylogenetic analysis of the NF $\kappa$ B family based on the DNA sequences proved to be more reliable than the analysis based on the amino acid sequences in the case of mammals because a few amino acid mutations were generated during this relatively short evolution time. The phylogeny of the major mammalian groups are unresolved, for example, it is not yet clear whether primates (*Homo, Pan*) are closer to rodents (*Mus, Rattus*) or ruminants (*Bos*) because different results have been obtained with different gene analyses (HEDGES and KUMAR, 2002). The phylogenetic trees of mammalian TAD sequences using neighbour-joining and maximum likelihood methods indicated that primates and ruminants were the closer relatives, whereas rodents were the most divergent of the three groups. The doubtfulness of mammalian phylogeny is better understood taking into account that the major groups of mammals, such as

primates, rodents and ruminants diverged during about the first 10 million years from the 92 million years of mammalian evolution (HEDGES, 2002). Therefore DNA mutations that are capable of distinguishing these groups originate from a short period of the mammalian evolution. Interestingly, the ascidian Rel genes appear to be the ancient form of deuterostome NF $\kappa$ B genes containing transactivation domain, and may represent the state before the duplications of the unique ancestral gene. Although the gene duplication event that resulted in p65 and c-Rel genes occurred after the genesis of vertebrates in all likelihood, the relation of the p65 and c-Rel cluster, RelB genes and the ascidian Rel genes is ambiguous in the NJ RHD amino acid and DNA trees. Beside low bootstrap values, the invertebrate and deuterostome NF $\kappa$ B genes and the NF $\kappa$ B1 genes are clustered together in RHD DNA trees, but not in RHD amino acid tree, thus the phylogenetic position of the invertebrate and deuterostome NF $\kappa$ B genes is more ambiguous.

The RHD of p65 proteins is well-conserved among vertebrate species, whereas the vertebrate TAD sequences exhibit only a moderate homology at the level of both amino acid and DNA. Beside nucleotide substitutions, frequent frame shift mutations and insertions-deletions occurred in vertebrate TAD sequences, so often that about 50 amino acids of the C-terminus of *Danio* p65 TAD became deleted (CORREA *et al.*, 2004). Similarly, the C-terminus of the C-terminal TAD of the signal transducer and activator of transcription 1 (STAT1), which also regulates the transcription of immune-related genes, is deleted in grass carp (*Carassius auratus*) (ZHANG and GUI, 2004), although this kind of C-terminal deletion among the TAD sequences of STAT family members derived from different species does not consequently accompany the loss of interaction with transcription coactivator (PAULSON *et al.*, 1999). The average values of  $d_N/d_S$  in mammalian RHD and TAD (0.023 and 0.174, respectively) are lower than the average values of mammalian genes (0.231) published in a study based on 47 mammalian genes (ZHANG, 2000), however the value of these genes refers to the entire coding regions, and not merely to the functional domains. The  $d_N/d_S$  value of mammalian RHDs is extremely low, for example lower than the value of DNA-binding domain (0.053) in mammalian STAT5 transcription factors (SEYFERT *et al.*, 2000), and indicates a high selection pressure for the sequence preservation. The TAD of p65 proteins is less conserved than RHD, which follows from the faster molecular evolution of TAD. In general, the DNA-binding regions of transcription factors are much more conserved than the transactivation regions (KACZYNSKI *et al.*, 2003, LAUDET *et al.*, 1992), and the variability of the protein sequences in transactivation region can change the coactivator interaction and the function of transcription factor (HSIA and MCGINNIS, 2003). Taking all things into account, the high diversity of vertebrate TAD sequences may result in the alteration of the transcription factor

functions of p65, and may indirectly modify the immune functions of NF $\kappa$ B, whereas the alteration in the functions of vertebrate RHDs is less probable.

## 7. New scientific results

It has been proven using luciferase reporter gene assay that the transcription of the bovine FcRn  $\alpha$ -chain is under the control of p65 in mammalian- and bovine-specific cell models. Three  $\kappa$ B binding sites have been identified in the *cis*-regulator region of the bFcRn  $\alpha$ -chain using site-directed mutagenesis accompanied by luciferase reporter gene assay and gel retardation assay, which are completely responsible for the p65 responsiveness of the bFcRn  $\alpha$ -chain, and can bind NF $\kappa$ B complex with p65 content.

It has been confirmed by luciferase reporter gene assay that the bovine  $\beta$ 2m ISRE binding site mediates the IRF1 induction in mammalian cell models similarly to its human orthologue. In addition, the b $\beta$ 2m ISRE site can bind IFN- $\gamma$  specific complex in EMSA.

The bovine p65 cDNA sequence has been cloned and its basic transcription factor specific features have been characterized by luciferase reporter gene and gel retardation assays.

The phylogenetic analysis has provided a new insight into the evolution of the NF $\kappa$ B family, and it has resolved the topology of the mammalian p65 molecules. Genetic analysis has proven that the mammalian TAD sequences have undergone faster molecular evolution than RHD sequences.

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## 9. The candidate's publications

### 9.1. Articles related to the present study in foreign journals

Doleschall M., Zhao Y., Mayer B., Hammarstrom L. and Kacskovics I. (2005) Isolation of the gene encoding the bovine neonatal Fc receptor. *Vet Immunol Immunopathol* 108, 145-150

Mayer B., Doleschall M., Bender B., Bartyik J., Bosze Z., Frenyo L. V. and Kacskovics I. (2005) Expression of the neonatal Fc receptor (FcRn) in the bovine mammary gland. *J Dairy Res* 72, 107-112

Doleschall M., Mayer B., Cervenak J., Cervenak L. and Kacskovics I. (2007) Cloning, expression and characterization of the bovine p65 subunit of NFκB. *Dev Comp Immunol* 31, 945-961

### 9.2. Articles related to the present study in Hungarian journals

Kis Zs., Mayer B., Juhász V., Doleschall M., Frenyo L. V. and Kacskovics I. (2004) A szarvasmarha neonatalis Fc receptor (bFcRn) tőgybeli expressziója és IgG kötő képessége. [Expression in the udder and IgG binding capacity of bovine neonatal Fc receptor (bFcRn).] *Magyar Állatorvosok Lapja* 10, 598-605

Kacskovics I., Mayer B., Kis Zs., Doleschall M., Bősze Zs. and Bender B. (2005) Az IgG transzportját és katabolizmusát szabályozó FcRn génregulációs elemzések szarvasmarhában. [Investigation on the contribution of the FcRn gene regulation for the IgG transport and catabolism in cattle.] *Magyar Tudomány* 6, 714-23

Doleschall M. and Kacskovics I. (2007) Az NFκB és transzkripciós faktor tulajdonságainak vizsgálata [The NFκB and the methods for the investigation of its transcription factor features.] *Magyar Immunológia* (accepted)

### 9.3. Publications related to the present study in conference materials

Kis, Zs., Mayer B., Doleschall M., Jancsik V., Frenyó L. V. and Kacskovics I. (2003) In vitro rendszerek létrehozása az FcRn-receptor által közvetített IgG-kötés és transzport elemzésére. [In vitro systems to study the bovine FcRn mediated IgG binding and transcytosis.] *Magyar Immunológia* 2003/3, 30-31

Doleschall M., Zhao Y., Mayer B., Kis Zs., Frenyó L. V., Hammarström L. and Kacs Kovics I. (2004) A szarvasmarha FcRn nehéz és könnyű lánc promoterének jellemzése. [Characterization of the bovine FcRn heavy and light chain promoters.] Committee on Veterinary Science of Hungarian Academy of Sciences, Academic Report

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Doleschall M., Zhao Y., Mayer B., Hammarström L. and Kacs Kovics I. (2005) A szarvasmarha FcRn nehéz lánc transzkripció szabályozásának elemzése. [Analysis of the transcriptional regulation of the bovine FcRn heavy chain.] Committee on Veterinary Science of Hungarian Academy of Sciences, Academic Report

Doleschall M., Mayer B. and Kacs Kovics I. (2006) A szarvasmarha NFκB p65 alegységének molekuláris klónozása és funkcionális vizsgálata. [Molecular cloning and functional characterization of the bovine p65 subunit of NFκB.] Committee on Veterinary Science of Hungarian Academy of Sciences, Academic Report

Mayer B., Doleschall M., Benkő S., Cervenak J., Ludányi K., Doleschall Z., Cervenak L., Rajnavölgyi É. and Kacs Kovics I. (2006) Human FcRn α-chain is differentially expressed in monocytes, macrophages and dendritic cells. 16<sup>th</sup> European Immunology Congress, Paris, France

Doleschall M., Mayer B., Cervenak J., Zhao Y., Doleschall Z., Cervenak L., Hammarström L. and Kacs Kovics I. (2006) NFκB does not induce bovine β<sub>2</sub>-microglobulin expression due to a deletion in its promoter. 16<sup>th</sup> European Immunology Congress, Paris, France

Kacs Kovics I., Mayer B., Ari K., Doleschall M., Cervenák J., Bajcsy Cs., Mádl I. and Szenci O. (2006) Újszülött borjak szérum IgG szintjének és katabolizmusának elemzése. [Analysis of serum IgG level and catabolism in neonate calves.] XVII. Hungarian Buiatric Congress, Gyula, Hungary

#### **9.4. Publications unrelated to the present study**

Fekete C., Gereben B., Doleschall M., Harney J. W., Dora J. M., Bianco A. C., Sarkar S., Liposits Z., Rand W., Emerson C., Kacskovics I., Larsen P. R. and Lechan R. M. (2004) Lipopolysaccharide induces type 2 iodothyronine deiodinase in the mediobasal hypothalamus: implications for the nonthyroidal illness syndrome. *Endocrinology* 145, 1649-1655

Zeöld A., Doleschall M., Kacskovics I., Fekete Cs. and Gereben B. (2005) Characterization of the NF- $\kappa$ B responsiveness of the human *dio2* gene. *Thyroid* 15 Suppl. 1, S24-25

Zeold A., Doleschall M., Haffner M. C., Capelo L. P., Menyhart J., Liposits Z., da Silva W. S., Bianco A. C., Kacskovics I., Fekete C. and Gereben B. (2006) Characterization of the nuclear factor- $\kappa$ B responsiveness of the human *dio2* gene. *Endocrinology* 147, 4419-4429

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# 11. Appendix

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-1934                                     CAAGGTATTGGAGT -1921
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-560 CTCCTTCATGCCAGATGGTTCTCTGAGCTGGCTCCATGAGGCTCGAGGTTCTTGGAAACGTTTCTGCCAGAGAATCC -481
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-160 CTAAGCATCATGAGACTTACTAGAAAATGAAACTGAAAACGGGGAAGCCTGTTTCTAACCTGCCCGGGCTAGTTCGCTC -81
-80 GGAGACTTGTGACGGCCCGGGGCGAGGCTTCCGATTGGCTGCTGAGTGCGGACTATAAAGGCGAGCGCCGCAACCAGC -1
+1 CGTCACTCCGCCACCAGCGCAGTGTGCAAGGATGGCTCGCTTCTGGCTTGGTCTTCTCGGGCTGTGTGCTGT +79

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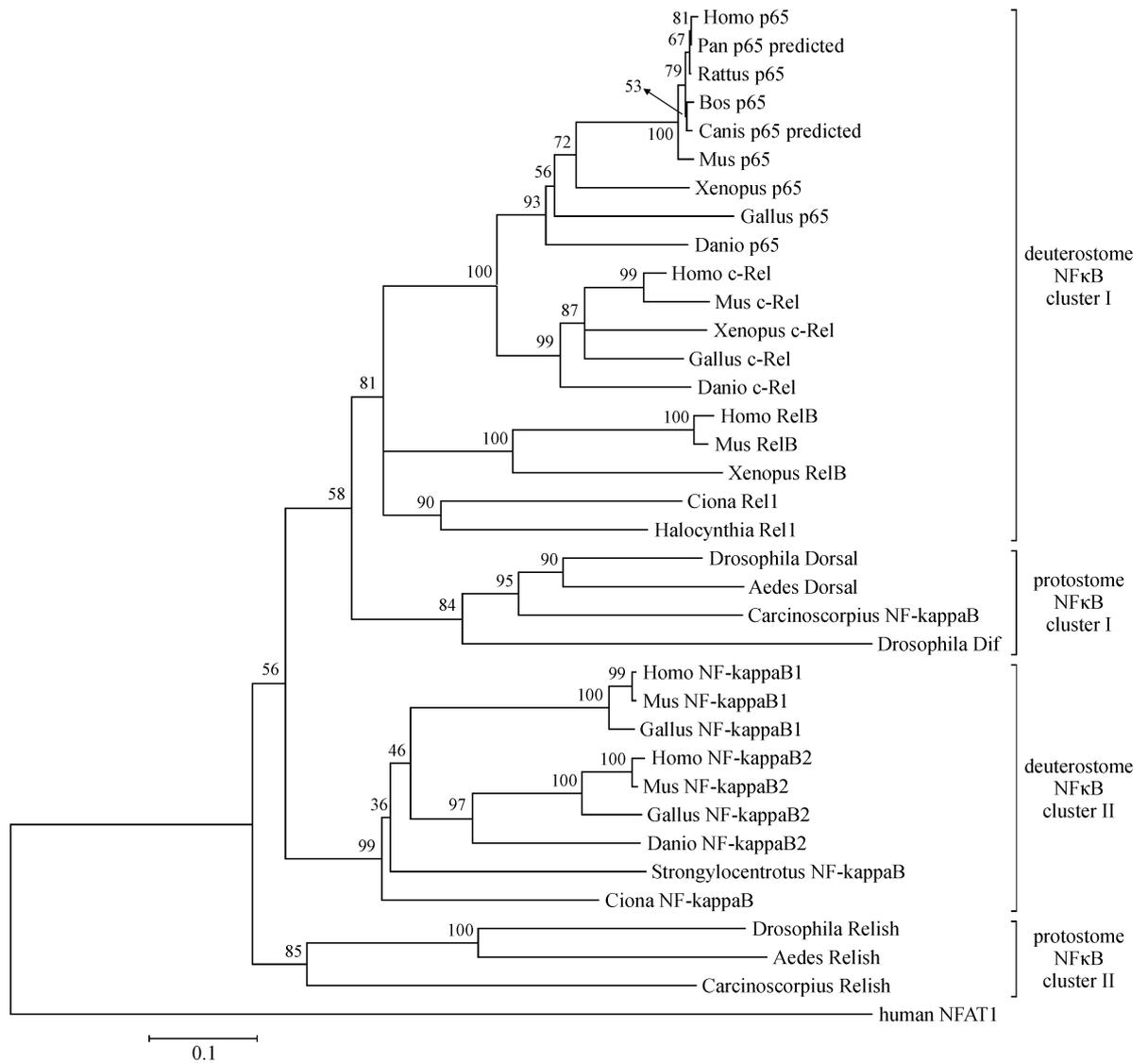
The 5'-flanking sequences of the bovine  $\beta 2m$ . The positions of bases are compared to the TSS.

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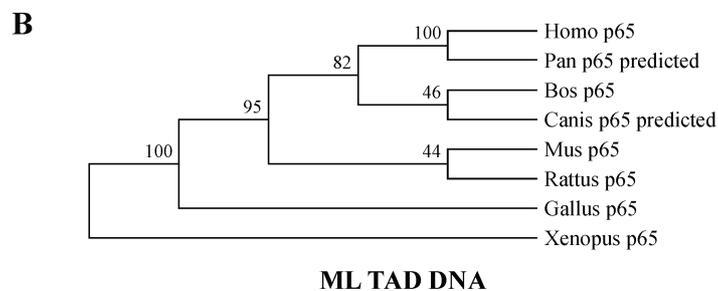
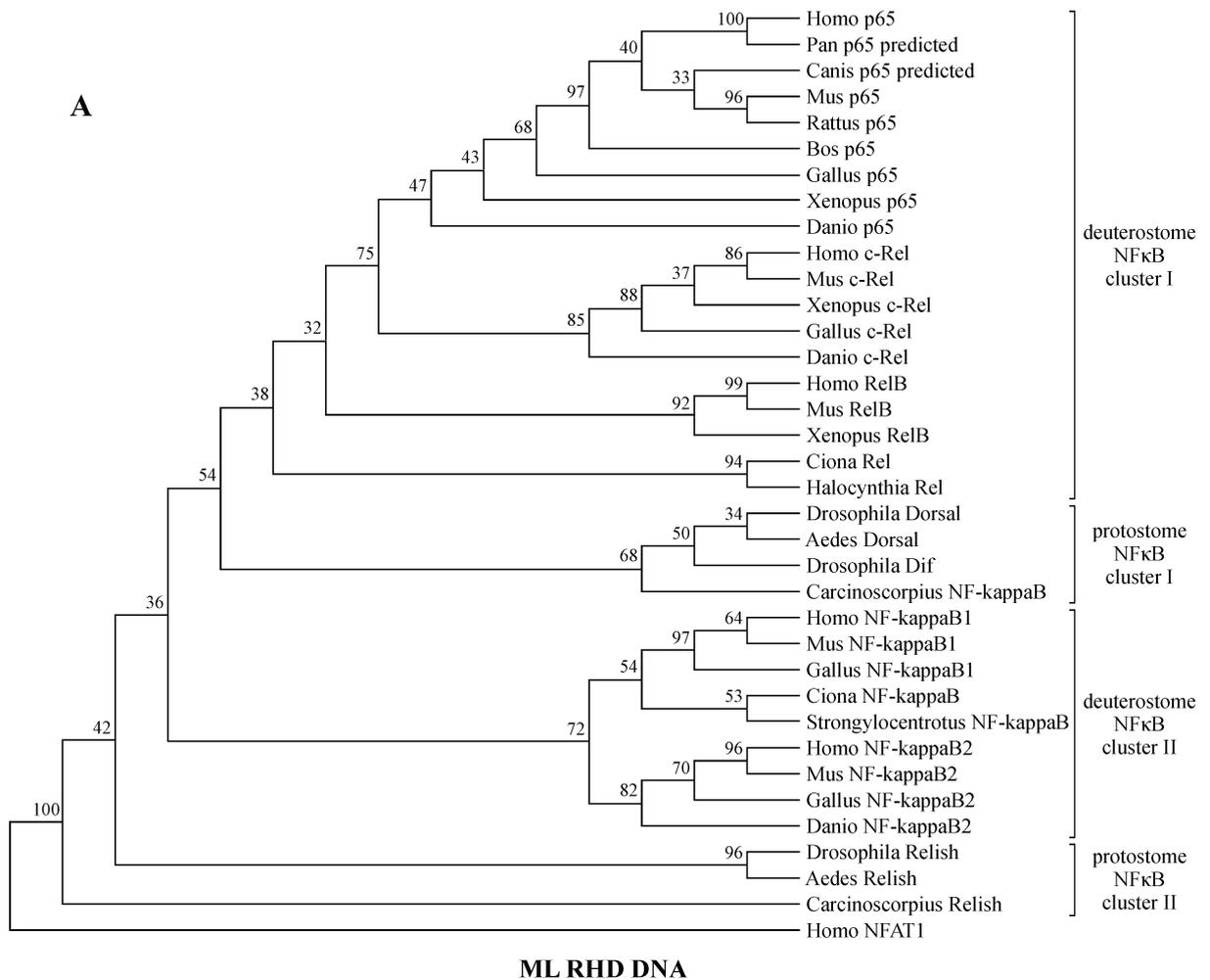
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-1760 ATTCTGGATGTTTCTGTGTGGGTGTTTGGGGGTGAAATTACACTGAAATCAGTGGACTCTGAGTAGAGCCGACCACCC -1681
-1680 CACCATAATGTGGGTGGGCTCATCCAATCAGTGAAGGTGGAAGACTGACCTTCCCTCAAGCAAAGGGGAACCCCTGCCA -1601
-1600 GCGGACTGCGTTTAGATTTTCTGCAAGATGAGCTTCTGGGTTTTCCAGATAGCCTGCCTTTGGACTTGAAGTGTAA -1521
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+81 AGGGATCGCGGCTGCCAGTGAAGTCCAGAGCTGGGGCCCTAACCAAGGAGAAGGGCCGGGCGCAGAACTCCCGGGT +160

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The 5'-flanking sequences of the bovine FcRn. The positions of bases are compared to the TSS.



Rooted phylogenetic tree of the NFκB family based on amino acid sequence of Rel homology domain (RHD) by NJ method using human NFAT1 as the outgroup. Bootstrap values are next to the corresponding nodes. The scale bar indicates the genetic distance.



Rooted phylogenetic tree of the NFκB family based on DNA sequence of Rel homology domain by ML method using human NFAT1 as the outgroup (ML RHD DNA). The clusters of the NFκB family are indicated on the right side of the tree. Rooted phylogenetic tree of vertebrate p65 based on DNA sequence of the transactivation domain (TAD) by ML method using *Xenopus* p65 as the outgroup (ML TAD DNA). Bootstrap values are next to the corresponding nodes.