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**mRNA expression of the acute phase protein haptoglobin in blood
and milk somatic cells and cellular localisation within the mammary
gland of dairy cows**

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Meinen Eltern

Abstract

mRNA expression of the acute phase protein haptoglobin in blood and milk somatic cells and cellular localisation within the mammary gland of dairy cows

The haptoglobin (Hp) concentration in blood and milk is increasingly discussed as a diagnostic marker for bovine mastitis. This acute phase protein is mainly synthesised in the liver, however, Hp mRNA was also discovered in tissue homogenates of the bovine mammary gland. The aim of this study was to advance this finding by allocating Hp mRNA expression within the bovine mammary gland at the cellular level. Possible candidates are, firstly, cells of the mammary gland itself and, secondly, circulating leukocytes infiltrating the udder. The presence of the Hp mRNA in the latter cells was investigated by RT-PCR carried out with RNA extracted from bovine blood leukocytes as well as from milk somatic cells. In order to localise Hp mRNA expression at the cellular level within the mammary gland a protocol for in situ hybridisation was developed. Subsequently, this was applied to bovine mammary tissues infected with *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* (*Staph. aureus*) by intracisternal inoculation 0, 6, 12 or 24 h pre-slaughter. In addition, blood and milk samples from these cows were analysed for the concentration of Hp protein collected during the 24 h infection and immediately pre-slaughter, respectively. In comparison to cattle, human leukocytes and mammary gland tissue were evaluated for Hp mRNA by RT-PCR, because of existing, however contradicting evidence on Hp expression in man.

The results demonstrate that Hp mRNA is present in bovine leukocytes as well as milk somatic cells. Within the bovine mammary gland Hp mRNA expression could generally be assigned to the alveolar epithelial cells. In addition, *E. coli* pathogens caused a marked upregulation of Hp mRNA expression within hours after start of infection, whereas Hp mRNA expression in response to *Staph. aureus* infection appeared unchanged during the initial 24 h post-infection. These differences in mRNA presence between the pathogen types were matched by Hp protein concentrations measured in blood and milk. In humans, Hp mRNA was also evident in the mammary gland and in 50% of the leukocytes samples tested. In conclusion, this study identified novel cellular sites of Hp mRNA expression in dairy cows and, thus, possible sources of the Hp protein found in milk.

Zusammenfassung

mRNA Expression des Akute Phase Proteins Haptoglobin in Blut- und Milchezellen sowie zelluläre Lokalisation im Euter von Milchkühen

Die Konzentration von Haptoglobin (Hp) in Blut und Milch wird als diagnostischer Marker für die Mastitis des Rindes zunehmend diskutiert. Dieses Akute Phase Protein wird hauptsächlich in der Leber synthetisiert, jedoch konnte Hp mRNA auch in Gewebehomogenaten der bovinen Milchdrüse nachgewiesen werden. Auf diesem Ergebnis aufbauend war das Ziel dieser Studie, die Hp mRNA Expression innerhalb der bovinen Milchdrüse auf zellulärer Ebene zuzuweisen. Dafür in Frage kommen einerseits Zellen der Milchdrüse selbst und andererseits Leukozyten, die aus dem Blut in das Euter einwandern. Um die Präsenz von Hp mRNA in den letztgenannten Zellen zu untersuchen, wurde mit RNA aus bovinen Blutleukozyten und aus Milchezellen eine RT-PCR durchgeführt. Zur zellulären Lokalisierung der Hp mRNA Expression in der Milchdrüse wurde ein Protokoll für eine in situ Hybridisierung entwickelt. Mit diesem Protokoll wurde anschließend Milchdrüsengewebe von Kühen untersucht, welches 0, 6, 12 oder 24 h vor der Schlachtung durch intrazisternale Applikation mit *Escherichia coli* (*E. coli*) oder *Staphylococcus aureus* (*Staph. aureus*) infiziert wurde. Zusätzlich wurde die Hp-Proteinkonzentration in Blut- und Milchproben gemessen, die diesen Kühen während der 24-stündigen Infektion bzw. unmittelbar vor der Schlachtung genommen worden waren. Im Vergleich zum Rind wurden wegen bereits bestehender, aber widersprüchlicher Angaben zur Hp Expression beim Menschen zudem Leukozyten und Milchdrüsengewebe des Menschen mittels RT-PCR auf Hp mRNA getestet.

Die Ergebnisse zeigen, dass Hp mRNA in bovinen Leukozyten und Milchezellen vorhanden ist. Im Euter konnte die Hp mRNA Expression den Zellen des Alveolarepithels zugeordnet werden. Zusätzlich zeigte sich, dass in diesen Epithelzellen *E. coli* Pathogene innerhalb von Stunden nach Beginn der Infektion einen deutlichen Anstieg der Hp mRNA Expression verursachten, wogegen diese Expression als Antwort auf die *Staph. aureus* Infektion in den ersten 24 h unverändert blieb. Ähnliche Unterschiede zwischen den Pathogentypen ergaben sich für die Hp Konzentrationen in Blut und Milch. Beim Menschen wurde Hp mRNA in der Brustdrüse und in 50 % der untersuchten Leukozytenproben nachgewiesen. Mit dem erstmaligen Nachweis von Hp mRNA Expression in den beschriebenen Zellen konnten somit mögliche Quellen von Hp Protein in der Milch identifiziert werden.

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Abbreviations

ACTH	Adrenocorticotrophic hormone
AP	Alkaline phosphatase
APP(s)	Acute phase protein(s)
APR	Acute phase reaction
as	Antisense
ATP	Adenosine triphosphate
bHp	Bovine haptoglobin
BCIP	5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BLAST	Basic local alignment search tool
CD	Cluster of differentiation
cDNA	Complementary DNA
cfu	Colony forming unit
C/EBP β	CCAAT/enhancer binding protein beta
CRP	C-reactive protein
CTP	Cytidine triphosphate
Da	Dalton
dATP	Desoxy-adenosine triphosphate
dCTP	Desoxy-cytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Desoxy-guanosine triphosphate
DIG	Digoxigenin
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
DNP	Dinitrophenol
dNTP	Desoxynucleosides triphosphates
dTTP	Desoxy-thymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme linked immunosorbent assay
Fab	Fragment antigen binding
Fig	Figure
<i>g</i>	Earth's gravity (9.8 m/sec ²)
GTC	Guanidiniumthiocyanate
GTP	Guanosine triphosphate
Hb	Haemoglobin
hHp	Human haptoglobin
Hp	Haptoglobin
IL	Interleukin
ISH	In situ hybridisation
IU	International unit
LPS	Lipopolysaccharide
MA	Maleic acid
MNC	Mononuclear cells
MOPS	3-Morpholinopropanesulfonic acid
mRNA	Messenger RNA
NBT	Nitro blue tetrazolium
nt	Nucleotide
NTP	Nucleosides triphosphates
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Pig-Map	Pig major acute phase protein
PNC	Polymorphonuclear cells
POD	Peroxidase, commonly horseradish peroxidase
PVA	Polyvinyl alcohol
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Reverse transcription

s	Sense
SAA	Serum amyloid A
sec	Seconds, in contrast to the SI unit "s" that is used herein for sense probe
sem	Standard error of the mean
SSC	Sodium chloride – sodium citrate buffer
<i>Staph. aureus</i>	<i>Staphylococcus aureus</i>
STAT	Signal transducers and activators of transcription
Tab	Table
TNF	Tumour necrosis factor
TTP	Thymidine triphosphate
TEA	Triethanolamine
Tris	Tris-hydroxymethyl aminomethane
tRNA	Transfer RNA
TSA	Tyramide signal amplification
U	Unit
u	Uracil
UTP	Uridine triphosphate
UV	Ultra violet

1 Introduction

The role of the acute phase protein haptoglobin (Hp) during infection of the mammary gland in cattle has been investigated in recent years with increasing interest. The chapters of the introduction below provide a brief overview of the current knowledge of Hp in general and more specifically its involvement during bovine mastitis. Finally, the aims of this study are defined.

1.1 Haptoglobin (Hp)

1.1.1 Occurrence and sites of haptoglobin synthesis

Hp is mainly found in plasma, but is also present in many other body fluids in mammals such as milk, urine, cord serum, cerebrospinal fluid, amniotic fluid and saliva (Katnik and Dobryszczycka, 1990; Hiss et al., 2003; Hiss et al., 2004).

Liver is the primary site of Hp synthesis (Miller et al., 1951). In addition, Hp expression has been reported in a variety of extrahepatic tissues. Hp mRNA could be detected in spleen, thymus, heart, lung and kidney of the rat after lipopolysaccharide (LPS) challenge (Kalmovarin et al., 1991). Similarly, Hp mRNA was also found in murine adipocytes at a basal level and at elevated levels after LPS challenge (Friedrichs et al., 1995). These researchers estimated the basal level of Hp mRNA in adipose tissue to be 10-15% of the levels in liver. Moreover, murine lung epithelial cells express Hp mRNA (Yang et al., 2000). There is also evidence of Hp mRNA in the reproductive tract. Hp mRNA expression was shown in rabbit oviductal tissue from 6 h post-conception to day 3 and in the uterus on days 5 and 6 post-conception (Herrler et al., 2004), in human endometrium (Sharpe-Timms et al., 2000) and in bovine oviduct (Lavery et al., 2004). In addition, macrophages and eosinophils as well as epidermal keratinocytes express Hp in humans (Yang et al., 2000; Li et al., 2005). Finally, Hp mRNA was identified in the mammary gland of cows (Hiss et al., 2004). Summarising for cattle, liver, oviduct and mammary gland are the only sites currently recognised of Hp mRNA expression.

1.1.2 Structure and phenotypes

Hp is a α_2 -glycoprotein and is synthesised as a single chain polypeptide, which is cleaved into an amino-terminal α -chain and a carboxy-terminal β -chain (Bowman and Kurosky, 1982). There are two major α chains, a shorter and a longer variation, designated as α_1 and α_2 chains, respectively. Sequence studies showed that the Hp α_2 allele arose from a partial gene duplication of the Hp α_1 allele (Smithies et al., 1962). The basic Hp isoform, Hp1-1, is a monomer in which two $\alpha_1\beta$ Hp molecules are linked by a single disulfide bond through their respective α_1 -chains (Smithies et al., 1962). This phenotype is found in most mammals except the *Bovidae* and *Cervidae* families. They exhibit the Hp2-2 type, a polymer of the $\alpha_2\beta$ Hp molecule (Travis and Sanders, 1972; Busby and Travis, 1978). Humans are the only species known to display phenotypic heterogeneity. Besides the homozygous Hp1-1 and Hp2-2 type, they can be heterozygous for the α chain locus giving rise to the Hp2-1 type (Smithies and Walker, 1956). Hp2-1 consists of two $\alpha_1\beta$ subunits flanking a $\alpha_2\beta$ polymer. Eckersall and Conner (1990) assessed the molecular mass of the bovine Hp molecule and the according α_2 and β chains as > 1000 kDa, 16 kDa and 40 kDa, respectively, thus confirming a similarity in molecular size of the two subunits to the human Hp2-2 types (Travis and Sanders, 1972).

1.1.3 Physiological functions of Hp

The main physiological tasks assigned to Hp are transport and immunomodulatory properties. The best recognised function of haptoglobin is to bind free haemoglobin (Hb) and to transport Hb to the liver. More specifically, after the release of Hb into plasma, a physiological phenomenon associated with haemolysis or apoptosis of erythrocytes, Hp can attach to Hb by non-covalent binding at a ratio of 1:1 (Fraser and Smith, 1971). This Hp-Hb complex cannot pass the glomerular filtration in the kidney due to its large molecular size, thereby preventing renal losses of the small Hb molecule (Fagoonee et al., 2005). Instead, the Hp-Hb complex is metabolised by CD163-positive monocytes/macrophages making the Hb-iron available for new Hb synthesis (Kristiansen et al., 2001). Besides the recycling of Hb-iron, the formation of the Hp-Hb complex possesses two additional benefits. On the one hand, Hp has a bacteriostatic effect by hampering the iron requiring process of bacterial replication as shown in rats inoculated with pathogenic *Escherichia coli* (*E. coli*) (Eaton et al., 1982). On the other hand, Hp was assigned an anti-oxidative role by inhibiting Hb-driven free radical oxidative tissue damage (Miller et al., 1997).

Another important property associated with Hp is the modulation of the inflammatory response by acting on different immune cells. Hp suppresses the production of pro-inflammatory, but not anti-inflammatory cytokines in human monocytes and inhibits the respiratory burst activity of human neutrophils (Oh et al., 1990; Arredouani et al., 2005). In addition, lymphocyte proliferation normally occurring after stimulation with concanavalin A or LPS in rabbits was reduced in the presence of Hp (Baseler and Burrell, 1983). In addition, Hp appears to act as a systemic regulator of dendritic cell function by preventing functional maturation of epidermal Langerhans cells, i.e. their transformation to cells capable of presenting antigens to T-cells (Xie et al., 2000).

Finally, there is evidence that Hp stimulates angiogenesis, thus supporting tissue repair under inflammatory conditions (Cid et al., 1993).

1.2 Haptoglobin – an acute phase protein

1.2.1 The acute phase reaction and acute phase proteins

Hp belongs to the group of Acute Phase Proteins (APPs) which come into play during the Acute Phase Reaction (APR). The APR was defined for the first time in 1941 by Abernethy and Avery (1941) and describes the organism's response to injury, infection or trauma of a tissue as well as to immunological disorders e.g. rheumatoid arthritis. It comprises a complex cascade of reactions to prevent further tissue damage, eliminate any infective organisms and enhance the healing process in order to restore homeostasis. It is initiated by macrophages of the affected tissue or by blood monocytes which release a wide range of mediators including cytokines. These cytokines act on fibroblasts and endothelial cells in the near vicinity causing a second release of cytokines. Only this second wave of cytokines triggers the actual cascade of complex reactions as part of the APR occurring locally and systemically. Locally, cytokines mediate leukocyte recruitment, in particular neutrophils and mononuclear cells, to the sites of inflammation. Systemically, they act on the immune system, bone marrow, brain and liver, and the reaction comprises the generation of a febrile response, an increase in adrenocorticotrophic hormone (ACTH) secretion, leukocytosis and alteration of the hepatic APP gene expression. This change of hepatic APP expression leads to increases as well as decreases of APP plasma concentrations dividing them into positive and negative APPs,

respectively (summarised according to the reviews of Heinrich et al. (1990) and Baumann and Gauldie (1994)). Since Hp is produced at elevated levels during the APR, it is categorised as a positive APP (Skinner et al., 1991; Dobryszczycka, 1997).

1.2.2 Cytokine control of APP synthesis

The APP synthesis is controlled by cytokines as mentioned above. They act directly upon specific receptors of hepatocytes prompting APP production (Peters et al., 1997). APPs can be divided into two major categories according to their regulators: type 1 APP production is induced by interleukin (IL)-1 and tumour necrosis factor- α (TNF- α), whereas type 2 APP synthesis is elicited by IL-6 (Baumann and Gauldie, 1994). IL-6 is believed to be the primary stimulator of most APP genes, however, there is evidence that IL-1 and TNF- α can amplify the effects of IL-6 (Heinrich et al., 1990). In cattle, IL-6 could be established as the principal regulator of Hp production in hepatocytes (Yoshioka et al., 2002); hence, it can be classified as type 2 APP in this species. Similarly, Hp is ranked as type 2 APP in man, however, as type 1 in the rat (Baumann and Gauldie, 1994).

Induced by IL-6, the actual Hp gene transcription within a cell is mediated by signal transducers and activators of transcription proteins (STAT) of which STAT3 has been described as the main signalling protein in mice hepatocytes in vitro (Kim and Baumann, 1997). After binding of IL-6 to its receptor, STAT3 is activated at the cytoplasmic side of the IL-6 receptor by phosphorylation. Once activated it translocates to the nucleus. In mice, the three main regulatory elements of the Hp gene promoter are two recognition sites for the transcription factor CCAAT/enhancer binding protein beta (C/EBP β) flanking a STAT interaction site. Binding of STAT3 to this interaction site has been identified as the key upregulator of murine Hp gene transcription induced by IL-6, whereas binding of other STAT proteins, e.g. STAT5, exerts inhibitory effects (Kim and Baumann, 1997; Wang et al., 2001).

Besides their direct regulatory effect, cytokines can also act via the pituitary-adrenal axis on APP production. They mediate an increased release of glucocorticoids by causing a higher secretion of ACTH during the APR. Glucocorticoids, in turn, enhance the APP production in hepatocytes on the one hand, and reduce the release of cytokines from monocytes and macrophages on the other hand (Heinrich et al., 1990). However, the effect on Hp expression in hepatocytes via this route appears low compared to the direct route (Marinkovic and Baumann, 1990). The signalling pathway of ACTH on the Hp promoter has yet to be fully

characterised; a direct glucocorticoid receptor binding site in the murine Hp promotor is suspected (Pajovic et al., 1994).

1.2.3 Species specific APP response during APR

Several plasma proteins are known as APPs, however, depending on the species the protein pattern of each single APP during the APR is highly varying. In cattle, Hp and serum amyloid A (SAA) are considered as the most prominent APPs, whereas C-reactive protein (CRP) is normally present in circulation and its concentration remains unchanged during an acute phase (Eckersall and Conner, 1988; Gronlund et al., 2003; Pedersen et al., 2003). In contrast, CRP is recognised as a major reactant in the pig together with pig-Map (pig major acute phase protein) and also Hp (Lampreave et al., 1994; Gonzalez-Ramon et al., 2000). In man, CRP besides SAA shows the highest increases during an APR, whereas Hp increases only moderately (Heinrich et al., 1990). Similarly in the dog, CRP is classified as a major APP, whereas in the rat, α_2 -macroglobulin and α_1 -acid glycoprotein are the APPs with the greatest increase of concentration during the APR (Eckersall and Conner, 1988; Heinrich et al., 1990).

1.3 Haptoglobin as an indicator of mastitis and other disorders in cattle

In healthy cattle, Hp blood concentrations have been described as low or even not detectable (Morimatsu et al., 1992; Eckersall et al., 2001), whereas numerous studies have reported pathological increases during the APR in response to mastitis and other disorders. In cases of mastitis, evaluating Hp not only in blood, but also in milk has recently been demonstrated to be beneficial. Hp concentration in milk appears to react with higher fold increases and earlier to intramammary infection than Hp concentration in blood. Hiss et al. (2004) found a 10-fold increase in blood Hp concentration and even a 150-fold increase in milk levels after intramammary inoculation with LPS compared to pre-challenge values; the Hp levels in milk from the control quarter remained unchanged. Likewise, Pedersen et al. (2003) identified an 8-fold rise in milk Hp concentration as a result of an experimental inoculation into mammary gland quarters with *Streptococcus uberis* compared to pre-challenge and control quarter levels, but no change in serum levels. Another major mastitis pathogen is *Staphylococcus*

aureus (*Staph. aureus*). Elevations of Hp blood and milk concentrations were observed in cows with experimental *Staph. aureus* mastitis, however, the increase was 20-fold in both fluids (Gronlund et al., 2003). During the subsequent chronic phase of the infection the elevation of the concentration of SAA in blood was more pronounced than Hp. This is in contrast to a later investigation by the same research group where Hp was detectable more often than SAA in serum samples from cows with natural chronic subclinical mastitis (Gronlund et al., 2005).

Besides mastitis, the bovine Hp response during an APR induced by other disrupting incidents has also been investigated intensively. For example, prolonged transport of calves over two days significantly affected Hp blood levels (Murata and Miyamoto, 1993), and cows undergoing invasive surgery showed a 50-fold increase in their blood Hp concentrations (Morimatsu et al., 1992). Also experimental challenges with LPS or turpentine applied to cattle intravenously or subcutaneously elicited higher serum concentrations than pre-challenge values (Conner et al., 1988; Jacobsen et al., 2004).

All these cases demonstrate the substantial involvement of Hp during the bovine APR. More importantly, though, some researchers have made an attempt at establishing the diagnostic value of Hp by correlating the level of Hp concentration to the severity of the disease. Indeed, Godson et al. (1996) demonstrated in an experimental infection model for bovine respiratory disease that Hp could be detected in infected animals before the onset of clinical signs and that its concentration could be used as an indicator of disease severity. In contrast, other authors found that in the case of a respiratory disease Hp concentration is not correlated with severity (Wittum et al., 1996). Again in two other experimental studies already mentioned above, a dose-dependency of Hp serum levels was recognised after LPS or turpentine injection (Conner et al., 1988; Jacobsen et al., 2004).

Particularly in the case of mastitis, considering it is the second major reason for dairy cows leaving the herd (Arbeitsgemeinschaft Deutscher Rinderzüchter, 2005), an objective and rapidly assessable indicator of the disease is desirable that allows the effective discrimination between healthy and diseased animals, preferably even quarters. In the event of naturally occurring clinical mastitis, serum Hp levels provided sensitivities and specificities for differentiating between healthy cows and cows affected by mastitis of 82% and 94%, respectively; for milk Hp the corresponding values were assessed at 86% and 100% (Eckersall et al., 2001). Analyses of Hp in milk from cows with naturally occurring subclinical mastitis yielded a sensitivity of 85% and a specificity of 94% (Hiss et al., 2005).

Gronlund et al. (2005) concluded from their above mentioned work with cows suffering of natural chronic subclinical mastitis that the absence of any detectable Hp as well as SAA in milk was a good indicator of healthy quarters.

In summary, although some potential could be demonstrated, further refinements are clearly needed before Hp fulfils the requirements of a reliable indicator of disease, especially of inflammation in the bovine mammary gland. A more detailed knowledge of the physiological processes in healthy and infected mammary glands regarding the major bovine APP Hp might aid in advancing the diagnostic value of this APP for mastitis.

Recently, the presence of Hp mRNA was verified in homogenates of bovine mammary glands (Hiss et al., 2004). Even an increase in the abundance of local Hp mRNA was triggered by LPS challenge into the quarter. This suggests that Hp protein is produced locally within the mammary gland to encounter bacterial invasion. The exact cell type expressing Hp could not be identified by the above method based on RNA from homogenates. Possible candidates are cells of the gland itself as well as immune cells known to infiltrate this organ in the healthy but particularly during the diseased state (Kehrli and Shuster, 1994). The elucidation of these two possibilities would further specify the results by Hiss et al. (2004). Human studies on Hp mRNA expression in leukocytes provide contradicting evidence: Wagner et al. (1996) found no Hp mRNA expression in the entire population of human peripheral blood leukocytes, whereas Yang et al. (2000) reported Hp mRNA in peripheral eosinophilic granulocytes. No information on Hp mRNA expression in mammary gland in any species other than cattle could be retrieved from the current literature.

1.4 In situ hybridisation

The localisation of Hp mRNA at the cellular level can be achieved by in situ hybridisation (ISH) applied to tissue sections. The general principle of this method is described here since it represented an integral part of this study.

ISH is a technique that allows specific nucleic acid sequences to be directly localised in morphologically preserved chromosomes, cells or tissue. For this purpose, a labelled nucleic acid (probe) is generated which is specific for, but complementary to the target nucleic acid. This allows annealing of the two nucleic acids to form a double stranded complex (hybrid) which can be visualised by detection of the label. RNA-RNA hybrids are considered to be the

most stable hybrids, followed by RNA-DNA, and DNA-DNA hybrids being the least stable (Morel and Cavalier, 2000).

There are several options for labels. Besides labelling with radioisotopes (^3H , ^{35}S , ^{32}P and ^{33}P) as immediate constituents of the nucleotides which are visualised by autoradiography, nonradioactive molecules can be coupled to the probes which in turn can be detected by two different methods. First, in the direct method the label molecule (fluorescein) is bound to the nucleic acid probe and can be detected directly under the fluorescence microscope. Second, the indirect procedures require the probe to contain a reporter molecule with antigenic properties (biotin, digoxigenin (DIG)). Subsequently, the reporter molecules is visualised by an immunologic pathway, i.e. via an antibody either coupled to fluorescein or to an enzyme finally activating chromogenic substrates. These signals can be detected by fluorescence or brightfield microscopy, respectively. Moreover, unique to the immunological pathway is the possibility of extending the immunologic detection system by the use of several antibodies in succession to further enhance the ISH signal. This is not possible with a radioactive system. The advantages of nonradioactive probes are denoted as safety, stability, rapid results and single cell resolution, while generally a higher sensitivity is credited to radioactive probes (summarised according to Wilkinson (1998) and Morel and Cavalier (2000)). However, Femino et al. (1998) were capable of visualising a single RNA transcript in single cells by fluorescence ISH.

In the present study, the target nucleic acid was Hp mRNA to be detected by the indirect method via DIG.

1.5 Aim of this study

Hp mRNA expression has recently been validated in homogenates of the bovine mammary gland, but the exact cell types responsible for Hp mRNA synthesis have not yet been specified. The aim of this study was to advance this finding by allocating Hp gene expression within the bovine mammary gland at the cellular level. This was to be addressed by investigating immune cells of the blood for the presence of Hp mRNA, since these cells can infiltrate the udder. In support of this, milk somatic cells were to undergo the same examination since they comprise mainly blood derived immune cells. The main task was to localise in situ Hp mRNA within the bovine mammary gland. For this purpose, an

experimental infection model was to be applied by inoculating cows intramammarily with *E. coli* or *Staph. aureus* pathogens. Besides determining sites of Hp mRNA expression in general, this also allowed assessing in situ possible differences specific for these two mastitis pathogens regarding site and abundance of Hp mRNA within the mammary gland. Finally, human blood cells and human mammary gland were to be tested in comparison.

2 Materials and Methods

2.1 General laboratory practice

Particular care was taken to work ribonuclease (RNase) free. Generally, working benches were cleaned with 80% ethanol. Before the start of ISH, benches and pipettes were additionally wiped with 0.1 M HCl. The inside of the cryostat was cleaned with chloroform. Glass and metal ware was baked at 180°C for at least 6 h. Thermostable plastic ware was autoclaved at 121°C for 20 min.

Chemicals, composition of buffers and solutions, instruments and consumables used in this study are listed in the appendix.

2.2 Sample origin and collection

2.2.1 Dairy cattle

Animals from three different sources were available for this study depending on the type of samples required.

Firstly, for Hp mRNA detection by polymerase chain reaction (PCR) within cells of blood and milk the appropriate samples were taken from nine cows appearing healthy and selected at random out of 25 German Holsteins (Deutsche Schwarzbunte) of the dairy herd at the research station Frankenforst in Königswinter. This research station belongs to the University of Bonn, Germany.

Secondly, mammary gland tissue for in situ hybridisation was derived from six German Holstein dairy cows in the middle of their first or second lactation after experimental intramammary infection. The milk of these animals had been tested free of mastitis pathogens by weekly bacteriological tests three times pre-trial and, additionally, to have somatic cell

counts < 100,000 cells/ml in all three samples. The six animals were split into two groups of three each and experimentally infected intracisternally with either *E. coli* (500 cfu) or *Staph. aureus* (10^4 cfu). These pathogens contained in 2 ml saline were administered into the front right, hind right and hind left quarter at 0 h (T0), 12 h (T12) and 18 h (T18) after the start of the trial, respectively. The front left quarter received 2 ml pathogen-free saline at T0. The cows were milked at T0, T12 and at 24 h after start of trial (T24) and were finally slaughtered at T24. This experiment was conducted by Dr. Holm Zerbe and his staff at the University of Veterinary Medicine, Hanover, Germany, who provided the trial animals and pathogens and enabled the sampling of the tissues required for this study. The pathogens applied in this trial originated from milk of clinical mastitis cases tested positively for exactly one of the two desired pathogen types. The isolated bacteria had been grown in pure culture and stored in glycerol at -20°C.

Thirdly, liver samples for control purposes were collected from cows at the abattoir in Euskirchen, Germany

Cows from these three sources are in the following referred to as Frankenforst cows, Hanover cows and Euskirchen cows, respectively.

2.2.1.1 Blood

For leukocyte preparation, blood from six Frankenforst cows was taken by venipuncture from the jugular vein into 50 ml disposable polypropylene tubes preloaded with 10 µl heparin (i.e. 5000 IU heparin/l blood). Heparin concentration had been optimised in pilot studies to reduce adherence of leukocytes for their subsequent harvest: 5000 IU/l produced superior results compared to 10,000 or 15,000 IU/l blood. Immediately after sampling the tubes were gently inverted to mix the heparin with the blood and then transported in ice water to the laboratory for the immediate isolation of leukocytes.

Serum samples from blood withdrawn from the six Hanover cows at T0, T12, T18 and T24 were prepared by the cooperating group in Hanover and made available for the current study. Samples were stored at -20°C until Hp protein assay.

2.2.1.2 Milk

For the preparation of somatic cells, milk was collected from another three Frankenforst cows during morning milking. The quarter for sampling was chosen by ease of access to that quarter, which was first brushed to remove any loose particles of dirt, skin or hair. Next, the

bottom part of the quarter and the entire teat were disinfected with 80% ethanol. After the first few squirts were discarded, 200 ml of milk were hand-stripped into an autoclaved glass jar. The milk was kept in ice water during transportation to the laboratory and then processed immediately for cell preparation.

Milk samples of all four quarters of the six Hanover cows were taken just prior to slaughter (T24) in a similar manner as described above. At T24 the four individual quarters had been exposed to the pathogens for a total of 0 h, 6 h, 12 h and 24 h, respectively. Milk was skimmed after centrifugation (2000 g, 15 min, 4°C) and stored at -20°C until Hp protein analysis.

2.2.1.3 Mammary gland and liver tissue

Tissue samples of the mammary gland were taken immediately after slaughter from the Hanover cows for in situ hybridisation. The main focus of the evaluation for Hp mRNA was on secretory tissue and not teat, cistern and duct system; hence, the samples were excised from the gland accordingly. Cubes (approximately 1 cm³) were removed with scalpel, forceps and scissors, rinsed in 0.9% NaCl and transferred to a 7 ml sample vial. Cubes of the same tissue were either snap frozen in liquid nitrogen directly, or first embedded in Tissue Tek[®] OCT[™] Compound within the vial and then snap frozen. The samples were transported on dry ice to the laboratory and stored at -80°C. Liver samples of these cows were taken likewise.

In addition, liver tissue samples were sourced from Euskirchen cows in a similar manner. Total RNA was extracted from these samples and served as PCR control.

2.2.2 Humans

Four human volunteers (three females, one male) aged between 21 and 34 years and appearing healthy were available for blood collection.

2.2.2.1 Blood

For leukocytes preparation, blood of the four candidates was taken from a vein in the inner elbow region into four 12 ml syringes preloaded with 60 µl of a heparin solution. This heparin solution was prepared by diluting the purchased heparin 1:25 in sterile saline. This extra step yielded improved results in cell preparation from human whole blood samples compared to using undiluted heparin directly. Final heparin concentration was 5000 IU/l blood.

Immediately after sampling, the tubes were gently inverted to mix the heparin with the blood, and then transported in ice water to the laboratory for immediate cell isolation.

2.2.2.2 Liver tissue

No liver tissue samples were processed for RNA extraction. Instead, human liver Poly (A)⁺ RNA was kindly donated by Dr. Jens Gruber, RWTH Aachen University, Germany.

2.2.2.3 Mammary gland

Total RNA of human mammary gland was purchased from BD Biosciences, Heidelberg, Germany. Hence, no tissue samples were required for RNA extraction.

2.3 Principal molecular biology methods used

2.3.1 RNA preparation

2.3.1.1 Total RNA extraction

Total RNA was prepared from the isolated cells or tissue samples according to the method by Chomczynski and Sacchi (1987) with some modifications (Chomczynski and Mackey, 1995). All solutions used were prechilled to 4°C. Tissue samples (approximately 200 to 400 mg) were homogenised in a protein-denaturing GTC-working solution (1 ml/100 mg tissue) with a dispersing tool (Ultra Turrax), whereas blood or milk cell pellets were dissolved in GTC-working solution by simply pipetting up and down. After addition of water saturated phenol (1 vol/vol GTC-solution) and 1-bromine-3-chlorine propane (0.2 vol/vol GTC-solution) and subsequent thorough mixing, the solution obtained had to be of milky appearance. If this was not the case, further 1-bromine-3-chlorine propane was added up to 0.8 vol/vol GTC-solution until the required colour was achieved. This solution was blended with 2 M Na-acetate (0.2 vol/vol GTC solution), mixed thoroughly for 1 min, incubated on ice for 15 min and centrifuged (1.5 ml tube: 10,000 g, 4°C for 20 min; 15 ml tube: 5000 g, 4°C for 30 min). The RNA, present in the aqueous supernatant, was precipitated by an equal volume of isopropanol during 5 min at -80°C and subsequent centrifugation as mentioned above. After two washing

steps with 1 ml 70% ethanol, including 15 min incubation at room temperature during the second wash, and centrifugation at 10,000 g, 4°C for 5 min, the RNA-pellet was dissolved in DEPC-treated water and stored at -80°C.

For liver tissue an additional protein denaturing and RNA precipitation step was included following the first precipitation by isopropanol to further remove connective tissue components and, thus, improve RNA purity. This step, though part of the original protocol, had previously been tested to be optional when extracting RNA from blood and milk somatic cells; in fact, its omission led to a higher RNA yield from these cells. The step comprised: GTC-working solution (100 µl/100 mg tissue) and 2 M Na-acetate (0.1 vol/vol GTC-solution) were added in which the pellet was to be dissolved during 20 min incubation at room temperature. Again the RNA was precipitated in an equal volume of isopropanol as described above.

2.3.1.2 Quantification of RNA by optical density

The total RNA dissolved in the DEPC-treated water was quantified by measuring the absorption of nucleic acids at 260 nm in a spectrophotometer. One unit of optical density at 260 nm (OD₂₆₀) corresponds to an RNA quantity of 40 µg/ml. This leads to the following equation to estimate the RNA concentration:

$$\text{RNA } (\mu\text{g/ml}) = \text{OD}_{260} * 40 \mu\text{g/ml} * \text{dilution factor.}$$

For this work the solution was diluted 1:100.

As a measure of protein contamination the ratio of absorbance at 260 nm (wave length of high absorption by nucleic acids) to the absorbance of 280 nm (wave length of high absorption by proteins) was established. Only samples with a ratio > 1.6 were used for further analyses.

2.3.1.3 DNase digest

The RNA samples underwent a digest of DNA to prevent false positive results produced by any residual genomic DNA in the subsequent DNA amplification reactions. Depending on the amount and the concentration of the original RNA as well as the desired amount and concentration of the RNA required for the subsequent reverse transcription, the desoxyribonuclease (DNase) digest was carried out in a total reaction volume of 15 or 25 µl. For each assay, a master mix was prepared with DNase I (0.5 µl/sample) and the appropriate volume of 10x reaction buffer (1.5 or 2.5 µl). This mix was aliquoted to the 1.5 ml tubes

prefilled with the amount of RNA solution to be subjected to the digest. The final volume was adjusted with DEPC-treated water. The digest was performed during 15 min incubation at 37°C and the reaction stopped at 75°C for 5 min. The RNA concentration of the solutions obtained was quantified again by photometry as described above. If this RNA concentration was $> 0.5 \mu\text{g}/\mu\text{l}$, an aliquot was diluted to a working concentration of $0.3 \mu\text{g}/\mu\text{l}$.

2.3.1.4 Quality control of the RNA extracted

The integrity of the RNA extracted was verified by denaturing gel electrophoresis. For this purpose $3 \mu\text{g}$ of total RNA were mixed with $3 \mu\text{l}$ 5x RNA loading buffer and an appropriate volume of DEPC-treated water to achieve a total volume of $15 \mu\text{l}$. This mixture was denatured at 65°C for 10 min. Immediately afterwards the samples were cooled on ice to preserve the denatured structure of the RNA. The gel contained 1.2% agarose which was initially dissolved in ultra pure water by boiling. After cooling down to 60°C, 10x MOPS buffer and deionised formaldehyde were added resulting in a final concentration of 1x MOPS and 12.5 M formaldehyde. The gel was immersed in 1x MOPS buffer and run at 3.5 V/cm for 1.5 h after pipetting the samples into the gel slots. The RNA bands were visualised under UV light (FluorImager SI).

A high quality of extracted total RNA is indicated by the presence of two distinct bands representing the ribosomal RNA (rRNA) subunits 28S and 18S after agarose gel electrophoresis. Ideally, the intensity of the 28S band should be twice the intensity of the 18S band. In contrast, these bands appear smeared or are even completely lacking when the RNA is partially degraded (Ivell, 1998). In the first place, these bands are a measure of the quality of rRNA only, but they are also considered to reflect the quality of *total* RNA including mRNA that was of interest for this study.

2.3.2 Reverse transcription (RT)

The RT serves to synthesise a first strand complementary DNA (cDNA) from an RNA template (Gerard and Grandgenett, 1975). The enzyme reverse transcriptase catalyses this reaction in the presence of primers. For this work the method of random priming with randomised hexanucleotide sequences was chosen allowing complete transcription of total RNA into cDNA (Veres et al., 1987).

The protocol had previously been validated for the transcription of 1 µg total RNA in a reaction volume of 20 µl. The RNA was denatured at 65°C for 5 min and immediately cooled down to 4°C to preserve its linearised structure. The random hexamer primers annealed to this denatured RNA during 10 min at 27°C, followed by the reverse transcriptase activity for 1 h at 42°C. The reaction was stopped at 99°C for 1 min, and the preparation cooled to 4°C. See Table 2.1 for reagents used and their final amounts.

Tab. 2.1: RT reagents used and their final amount in 20 µl reaction volume.

RT reagents	Final amount in 20 µl
Ultra-pure H ₂ O	Ad 20 µl
Random hexamer	50 pmol
5x Reaction buffer	1x
dNTP (dATP, dCTP, dGTP and dTTP)	10 nmol of each
Ribonuclease inhibitor	20 U
Reverse transcriptase	200 U

2.3.3 Polymerase chain reaction (PCR)

The PCR is a technique to generate a large number of copies of DNA segments. Similar to the RT, the key reagents are the primers and an enzyme, the DNA polymerase. However, contrary to the RT, the PCR primers for this study were chosen to act not at random, but specifically for a DNA region and, hence, for the gene of interest. The principle of the PCR is the 20 to 40-fold cyclic repetition of three steps: firstly, denaturing of double stranded DNA or a DNA-RNA complex to single strands, secondly, annealing of the primers and, thirdly, extending of the primer sequence to a complementary copy of the whole template (Mullis and Faloona, 1987).

2.3.3.1 Selection of primers for the genes of interest

The main gene of interest was Hp. Species specific primers were applied to samples of bovine and human origin, since the use of bovine Hp primers did not yield any PCR products from

human liver RNA. Also two different primer pairs (bHp1 and bHp2) for the bovine Hp gene were applied yielding PCR products of different lengths. The longer PCR fragment (bHp2) became relevant only during establishing the in situ hybridisation protocol and was not used in standard PCR investigations which were carried out with bHp1 primers. For convenience, in the following bHp1 and bHp2 may denote not only the respective PCR fragments but also the corresponding primer pairs.

Besides the Hp gene, primers for bovine β -actin as internal control gene were selected and applied to samples of both species. Specificity of the PCR products for both species was established by sequencing (Value Read, MWG Biotech AG, Ebersberg, Germany).

Table 2.2 provides details on NIH gene bank accession number, the literature source of the primer sequences, the PCR fragment size, and the primer sequences used. The cDNA sequences of bHp and hHp currently known are given in the appendix.

Tab. 2.2: NIH gene bank accession number, literature source of PCR primer sequences, the PCR fragment size and the forward (F) and reverse (R) primer sequences for the genes bHp, hHp, and β -actin.

PCR frag- ment	NIH gene bank accession	Literature source	Frag- ment size (bp)	Primer sequences
bHp1	AJ271156	Hiss et al. (2004)	174	F = 5'-GTCTCCCAGCATAACCTCATCTC-3' R = 5'-AACCACCTTCTCCACCTCTACAA-3'
bHp2	AJ271156	Lavery et al. (2004)	302	F = 5'-CCAAAGGCAGCTTTCCTTGG-3' R = 5'-GGAAGGTAGGCAGATGGGCAT-3'
hHp	NM_005143	Chen et al. (1998)	338	F = 5'-CCTGAATGTGAAGCAGTATGT-3' R = 5'-TTCTGTTTGAGTTTGATGAGC-3'
β -actin	AY141970	Fitzpatrick et al. (2002)	226	F = 5'-CGTGGGCCGCCCTAGGCACCA-3' R = 5'-GGGGGCCTCGGTCAGCAGCAC-3'

2.3.3.2 Implementation of the PCR

A protocol optimised in the laboratory beforehand was used to implement the PCR. A Master Mix was prepared containing 10x *Taq* buffer with $(\text{NH}_4)_2\text{SO}_4$, MgCl_2 , dNTPs, *Taq* DNA polymerase and the forward and reverse primers (Table 2.3). This mixture (47 μl) was added to 3 μl of cDNA to achieve a total PCR reaction volume of 50 μl . The temperature and time conditions for the complete PCR are shown in Table 2.4.

Tab. 2.3: PCR reagents used and their final concentration in 50 μl .

PCR reagents	Final concentration in 50 μl
Ultra-pure H_2O	Ad 50 μl
10x <i>Taq</i> buffer with $(\text{NH}_4)_2\text{SO}_4$	1x
MgCl_2	2 mM
dNTPs (dATP, dCTP, dGTP and dTTP)	100 μM each
Primer forward	0.4 μM
Primer reverse	0.4 μM
<i>Taq</i> DNA polymerase	1.0 U

Tab. 2.4: Temperature and time conditions of the PCR steps.

	Fragment			
	bHp1	bHp2	hHp	β -actin
Hot start	72°C	72°C	72°C	72°C
Denaturation	95°C 1 min	95°C 1 min	95°C 1 min	95°C 1 min
Cycle	Denaturation	94°C 40 sec	94°C 40 sec	94°C 30 sec
	Primer annealing	55°C 20 sec	60°C 30 sec	55°C 30 sec
	Extension	72°C 20 sec	72°C 40 sec	72°C 1 min
Number of cycles	37	34	34	30
Final extension	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5 min
Cooling	4°C ∞	4°C ∞	4°C ∞	4°C ∞

2.3.3.3 Visualisation of PCR products by DNA gel electrophoresis

A successful PCR is characterised by the formation of numerous PCR products of the correct fragment length determined by the PCR primers. These PCR products were checked by DNA gel electrophoresis. PCR products (10 µl) with 5x DNA loading buffer were separated in a 2% agarose gel containing 0.3 µg ethidium bromide/ml in 1x TBE buffer at 4 V/cm for 60 to 75 min. After the electrophoresis the DNA bands were visualised by UV-laser scanning system (FluorImager SI) and analyzed by image analysis software (Peak Finder, ImageQuaNT™). The fragment size was assessed by comparison to a DNA molecular weight marker (200 ng, ΦX174 DNA/*Bsu*RI (*Hae*III)).

2.3.3.4 Reamplification

Negative results from the standard PCR method were to be confirmed by PCR reamplification. For this purpose, 1 µl of first course PCR products plus 2 µl of ultra-pure H₂O, instead of 3 µl cDNA, were reamplified under the PCR conditions mentioned above. Reamplification of PCR products is a tool widely used as part of the differential display method identifying and isolating genes differentially expressed (Wang et al., 1998).

2.3.4 Controls for RT-PCR

Liver RNA was used as positive RT-PCR control. One µg total RNA from bovine liver and 0.075 µg Poly (A)⁺ mRNA from human liver were subjected to RT-PCR, taking into account that total RNA comprises approximately 5 to 10% of Poly (A)⁺ mRNA (Alberts et al., 1997). Water was used as negative RT-PCR control. In order to control any residual genomic DNA, each RNA sample was also subjected to RT-PCR without the addition of reverse transcriptase. β-actin served as a suitable internal control gene to check the integrity of the cDNA synthesis and PCR. For β-actin primers and PCR condition refer to Tables 2.2 and 2.4.

2.4 Detection of Hp mRNA in bovine blood and milk somatic cells as well as in human blood cells and mammary gland by RT-PCR

Cells were to be extracted from the collected blood and milk for further investigation of Hp mRNA within these cells. Before processing the required buffers (1x PBS, 2x PBS) and ultra-pure H₂O were chilled to 4°C unless stated otherwise. It was important that the PBS was free of the divalent cations Mg²⁺ and Ca²⁺ to prevent coagulation of the blood cells. During processing the samples were kept in ice water. The centrifugation was carried out in a swing-out rotor, without brakes and at 4°C unless stated otherwise.

2.4.1 Blood processing for cell separation

2.4.1.1 Isolation of leukocytes

The isolation of leukocytes was based on their lower density compared to erythrocytes. After centrifugation of heparinised whole blood the leukocytes aggregate at the plasma-erythrocyte interphase and form the so called "buffy coat". Lysis of undesirable erythrocytes was carried out following the protocol of Krüger (2001).

Upon arrival at the laboratory 48 ml of whole blood of each individual were transferred in equal portions to four 15 ml tubes and centrifuged for 20 min at 1100 g (bovine blood) and 700 g (human blood). After discarding the plasma, the buffy coats of the four tubes were collected with a sterile Pasteur pipette and combined in one 15 ml tube. Contaminating erythrocytes were removed by hypotonic lysis: 5 ml ultra-pure H₂O were added and mixed by inversion for 20 sec; thereafter isotonicity was restored with 5 ml 2x PBS, followed by centrifugation at 100 g for 8 min. For complete elimination of erythrocytes the lysis was repeated. It appeared helpful to resuspend the cell pellet in 1 ml 1x PBS before the second lysis to allow an optimal mixing of the water with all cells during the short incubation time. After the lyses the pellet was again resuspended and the cells washed in 10 ml 1x PBS; of this a 100 µl subsample was removed for cell differentiation by a smear and cell count in a counting chamber (see below). Finally, the cell suspension was centrifuged as above, the supernatant discarded and the pellet stored at -80°C until further processing.

2.4.1.2 Isolation of mononuclear and polymorphonuclear cells

The isolation of mononuclear (lymphocytes and monocytes) and polymorphonuclear leukocytes (neutrophilic, basophilic and eosinophilic granulocytes) is based on the principle of density gradient centrifugation. Blood is applied to a separation medium which after centrifugation and due to different sedimentation rates accumulates the mononuclear cells at the plasma-medium interphase, and the polymorphonuclear cells and erythrocytes as a pellet below the medium.

This procedure was applied only to bovine blood and according to the protocol of Krüger (2001) with some modifications. Fifty ml of heparinised blood were diluted 1:2 with 1x PBS. Ninety ml of this mixture were split into three equal portions of 30 ml each and the remaining 10 ml discarded. Each portion was then carefully layered on 15 ml Histopaque[®]-1077 in a 50 ml vial and centrifuged (1100 g, 30 min, room temperature). Both PBS and Histopaque[®]-1077 were employed at room temperature.

For the harvest of mononuclear cells (MNC), the upper plasma layer was aspirated to within 0.5 cm of the opaque interphase and discarded. The interphases, containing the MNC, of the three tubes were then transferred by a Pasteur pipette to a new 50 ml tube and pooled. These cells were washed with 25 ml 1x PBS and centrifuged at 430 g for 10 min. If an unexpected erythrocyte contamination was observed in the pellet obtained, a lysis was introduced as described above with 20 ml ultra-pure H₂O, 20 sec incubation time, 20 ml 2x PBS and centrifugation at 430 g for 10 min. This pellet was washed again with 10 ml PBS. After a 100 µl subsample had been taken for a smear and cell count, the suspension was centrifuged at 250 g for 10 min. The supernatant was discarded and the pellet stored at -80°C.

Polymorphonuclear cells (PNC) were prepared as follows: In the original tube after the removal of the mononuclear cells, the Histopaque[®]-1077 phase was aspirated to within 0.5 cm of the red pellet and discarded. The contaminating erythrocytes in the pellet were lysed hypotonically similarly to above by 20 ml ultra-pure H₂O, 20 sec incubation period, 20 ml 2x PBS, and the suspension centrifuged at 100 g for 8 min. After resuspension of the pellet in 1 ml 1x PBS, the solution was transferred to a 15 ml tube and the lysis repeated with a volume of 5 ml ultra-pure H₂O and 5 ml 2x PBS. Finally, the pellets of the three repeats were pooled, washed in 10 ml 1x PBS. After a 100 µl subsample had been taken, the suspension was centrifuged at 100 g for 8 min. The supernatant was discarded and the pellet stored at -80°C.

2.4.1.3 Leukocyte differentiation by blood smears

Firstly, from all whole blood samples a white blood cell differential was established to check for any irregularities in each individual. Secondly, a differential was required of the extracted cells to determine their composition and, in particular, purity of the isolated cell fractions.

For this purpose one drop of whole blood or one drop of purified cells diluted 1:2 in bovine plasma beforehand was spread onto a SuperFrost[®] Plus microscope slide by a glass cover slip. Mixing the isolated cells with plasma enhanced the attachment of these cells to the slide. The cell-free plasma used herein was prepared from the blood of one trial animal and kept at -20°C. After drying overnight at 37°C the slides were stained according to the method of Pappenheim (Romeis, 1989) and 100 cells were differentiated microscopically with 660x magnification.

2.4.1.4 Cell count and cell viability

An estimate of the number of cells isolated and their viability after the purification process was obtained by the use of a counting chamber and Trypan Blue staining, respectively. The Trypan Blue penetrates into non-vital cells only, whereas vital cells exclude the stain. The presence of contaminating erythrocytes was also assessed in the counting chamber and differentiated from the leukocytes by their smaller size.

For this purpose, 25 µl of the 100 µl subsample taken before (see above) were mixed with 25 µl of Trypan Blue solution (Trypan Blue stain diluted 1:4 in ultra-pure H₂O), applied to the Neubauer counting chamber according to the manufacturer's protocol and the cells evaluated at 260x enlargement within 30 min thereafter. Cells were counted in eight small squares of the counting chamber instead of the 64 recommended by the manufacturer, since only an estimate of the isolated cells was needed.

2.4.2 Milk processing for cell separation

For cell preparation from cow's milk the protocol by Boutinaud et al. (2002) preparing somatic cells from goat's milk was adapted. Somatic cells were isolated from 200 ml milk. The milk was diluted 1:2 with 1x PBS and centrifuged in two 200 ml beakers at 1000 g for 15 min. The fat layer was removed with a spatula and a Pasteur pipette, and the skimmed milk was discarded. After each cell pellet had been resuspended in 1 ml 1x PBS the two suspensions were combined in one 50 ml tube, washed with 48 ml 1x PBS and centrifuged at

200 g for 10 min. Again, the pellet was dissolved in 1 ml 1x PBS and subsequently transferred to a 1.5 ml tube. After a further centrifugation step the pellet was frozen at -80°C.

An attempt was made to analyze the extracted cells for type or count similarly to blood cells. However, fat globules within the milk interfered with the accuracy of differentiating cells in a smear or counting cells in the counting chamber, both leading to unsatisfying results. Hence, these analyses were abandoned.

2.4.3 RT-PCR with RNA from separated cells

The cells separated from blood and milk were processed for total RNA extraction and RT-PCR with bHp1 and hHp primers for cows and humans, respectively, as described above. Since standard RT-PCR for the hHp gene revealed no positive results with RNA from human blood cells, these PCR products underwent reamplification.

2.4.4 RT-PCR with human mammary gland RNA

Human mammary gland total RNA, as described above in "Sample origin", had been purchased and, hence, required no extraction from tissue sample. However, prior to RT-PCR, this RNA was subjected to a DNase digest to ensure removal of possible contaminations with genomic DNA.

2.5 Cellular localisation of Hp mRNA in the mammary gland by in situ hybridisation (ISH)

Hp mRNA was to be localised in tissue sections of the bovine mammary gland by ISH. As mentioned in the introduction a non-radioactive probe with the reporter molecule DIG to be detected by the indirect method was applied for this purpose. It involved the following six steps:

1. Generation of a nucleic acid probe
2. Tissue preparation including sectioning and fixation
3. Pretreatment to increase accessibility of target RNA
4. Hybridisation of probe to the target RNA
5. Posthybridisation washes to remove unbound or unspecifically bound probe
6. Immunological detection of hybrids and visualisation.

2.5.1 Generation of RNA probe

The RNA probes used for ISH were generated and simultaneously labelled by in vitro transcription from a DNA template (Morel and Cavalier, 2000), and the purified product subsequently quantified in a dot blot. Two homologous probes and one heterologous (human) probe were tested on bovine tissue. They are referred to as bHp1, bHp2 and hHp probes in the following corresponding to the PCR primers employed in the template amplification. Hence, the probe lengths were 174, 302 and 338 nt, respectively. These three probes were tested in the following chronological order: bHp1, hHp and bHp2. An ISH protocol could be established with bHp1 probe for liver with moderate staining, whereas no staining was achieved in the mammary gland using the same conditions. Similar unsatisfying results were obtained with the longer, but heterologous hHp probe in both tissues. Finally, best results were achieved with the bHp2 probe, the corresponding primer sequence of which was only published while establishing this ISH protocol (Lavery et al., 2004).

2.5.1.1 DNA template synthesis and purification

The DNA template required for the probe synthesis consisted of purified RT-PCR products from bovine or human liver RNA. Two different templates were required allowing the separate synthesis of antisense (as) and sense (s) probes. This was achieved by extending one of the two Hp PCR primers at its 5'-end by the sequence of the T7-RNA-Polymerase promotor plus some "filling" sequences to allow optimal attachment of the enzyme. This filling sequence had been successfully tested by Hakemann (2002). One primer sequence is schematically shown in Figure 2.1.

The PCR with the Hp (forward) and T7+Hp (reverse) primers led to the template for the antisense probe and with T7+Hp (forward) and Hp (reverse) primers to the template for the sense probe. The sequence of all three standard Hp primer pairs and the appropriate conditions for PCR amplification can be found above (Tables 2.2 and 2.4). The PCR products were assessed on a 2% agarose gel for quality and quantity, using image analysis software (Volume Report, ImageQuaNT™) for quantification.

The subsequent purification of the obtained PCR products provided optimal conditions for the transcription reaction by removing contaminations of primers, buffers and enzymes, and was carried out using the QIAquick PCR-purification kit according to the manufacturer's protocol.

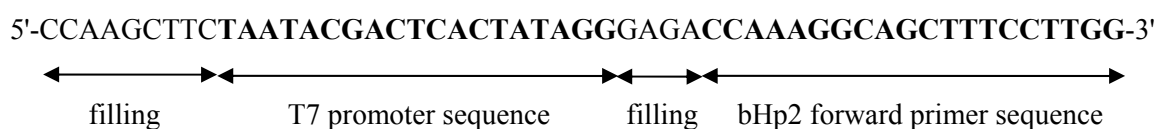


Fig. 2.1: bHp2 forward primer extended by the T7 promoter sequence and filling sequences. This primer together with the standard bHp2 reverse primer was used to generate a DNA template required for ISH sense probe synthesis.

2.5.1.2 Probe synthesis by in vitro transcription

The in vitro transcription of the DNA template to single-stranded RNA probe in the presence of Dig labelled UTPs by the T7 RNA polymerase was carried out according to the manufacturer's instructions of the DIG RNA labelling kit (Roche) with some modifications (personal communication with PD Dr. Stephan Baader, Institute of Anatomy, Anatomy and Cell Biology Group, University of Bonn, Germany) as described below. The Dig labelled UTPs as part of the NTP labelling mixture leads to a probe with a Dig labelled nucleotide

incorporated every 20th to 25th nucleotide. 200 ng DNA template were added to DEPC-treated H₂O to a final volume of 13 µl and left to stay at room temperature for the Master Mix to be added. This Master Mix was prepared at room temperature with

- 2 µl 10x Dig NTP labelling mixture
- 2 µl 10x transcription buffer
- 1 µl RNase inhibitor
- 2 µl T7 RNA polymerase

and added to the template. It was important that the transcription buffer was brought to room temperature before mixing to avoid DNA precipitation by the spermidin contained in the buffer. Consequently, the complete Master Mix was prepared and left to stay at room temperature. However, care was taken to work as time efficiently as possible. After gentle mixing and brief centrifugation the preparation was incubated at 37°C for 2 h. After this incubation, a second Master Mix containing

- 1 µl 10x transcription buffer
- 1 µl 10x Dig NTP labelling mixture
- 7 µl DEPC-treated H₂O
- 1 µl T7 RNA polymerase

was added and incubated at 37°C for one additional hour to increase the yield of the reaction. The reaction was stopped by adding 3 µl 0.2 M EDTA (pH 8.0). In the case of several reactions per probe the content of tubes containing the same probe was pooled at this stage.

Next, the yielded probe was purified to remove undesirable components of the transcription reaction, particularly salts. First, the RNA was precipitated in 0.1 vol 4 M LiCl and 2.5 vol 100% ice cold ethanol at -20°C for a minimum of 1 h, preferably overnight. After centrifugation (13,000 g, 15 min, 4°C) the pellet was washed in 200 µl 70% ice cold ethanol and centrifuged again for 10 min. The pellet obtained was air dried, dissolved in 100 µl DEPC-treated H₂O and stored at -20°C.

2.5.1.3 Probe quantification by dot blot

As the final step of probe generation, the yield of the probe was quantified by a direct detection method (dot blot). A series of dilutions of Dig labelled RNA probe was applied to a positively charged nylon membrane. In parallel, the nylon membrane was loaded with a dilution series of Dig labelled RNA of known concentration ranging from 10 ng/µl to 30 pg/µl serving as standard. The nylon membrane was subjected to immunological detection and the

probe concentration determined by eye assessment of the staining intensities of the probe compared to the standard. NBT and BCIP were used in combination as substrate. BCIP is oxidised to indigo while NBT in a simultaneous coupled reaction is reduced to diformazan, both of which lead to the formation of blue insoluble precipitates. The colour of the precipitates might be recognised not only as blue but also as brownish or purplish depending on the type of membrane used (Roche, 2002).

First, the Dig labelled control RNA stock (100 ng/μl) provided with the above mentioned kit was diluted 1:10 in RNA dilution buffer to a working concentration of 10 ng/μl. Next, dilution series of this working solution as well as of the undiluted probe were created: 1:3.3, 1:10, 1:33, 1:100, and 1:330, 1 μl each of which was loaded onto the nylon membrane placed in a plastic petri dish. Next the membrane underwent the following immunological detection:

- UV light to fix the RNA to the membrane, 5 min
- 10 ml MA buffer to equilibrate, 1 min
- 9 ml MA buffer + 1 ml 10% Blocking Reagent, 30 min, shaking (100 rpm, Shaker 3005)
- brief wash with MA buffer
- 15 ml MA buffer + 3 μl Anti-Dig-AP (Fab fragments, Roche; 1:5000 dilution), 30 min, shaking (100 rpm)
- substrate buffer 1 to equilibrate, 2 min
- 10 ml substrate buffer + 45 μl NBT + 35 μl BCIP, 10 – 30 min, incubate protected from light until suitable staining had developed
- TE buffer to stop the reaction, 10 min
- air dry membrane.

Finally, the probe concentration was determined by eye assessment. A stock subsample was diluted in DEPC-treated H₂O to a working concentration of 10 ng/μl, aliquoted and stored at -20°C. Labelled probes are stable for 1 year when stored at -20°C according to Roche.

It was important to ensure that the nylon membrane never dried out during processing until the staining was completed.

2.5.2 Preparation and fixation of sections

The preparation of cryostat sections comprises three steps: warming of the tissue sample, cutting the sections and fixing the sections.

First, the vials containing the tissue samples stored at -80°C were placed into the cryomicrotome (Leica) for a minimum of 3 h to allow the sample to warm to the chamber temperature in the cryostat (-21°C).

Second, sections of a thickness of $12\text{ }\mu\text{m}$ (liver, mammary gland) were prepared at a specimen temperature of -18°C and a cutting angle of $2-3^{\circ}$, and were deposited on room temperature warm SuperFrost® Plus microscope slides. These slides were immediately placed on the quick freeze shelf (-45°C) for the section to refreeze and then kept in a precooled Hellendahl glass jar within the cryomicrotome until fixation. The temperatures mentioned for cryochamber and specimen had experimentally been optimised for liver and mammary gland tissue. An increasing section thickness to $18\text{ }\mu\text{m}$ for mammary gland tissue was also tested leaving some cells not cut but intact. Hence, their RNA was retained within the cell until permeabilisation and, consequently, might produce better staining results. However, this could not be confirmed and, thus, the idea was abandoned. In addition, deposition of the sections onto slides having room temperature rather than being precooled to cryochamber temperature considerably improved adherence, particularly with mammary gland tissue.

Third, the sections were fixed in 4% paraformaldehyde (PFA) at room temperature. The aim of the fixation process is to inhibit cellular metabolism, to deactivate lytic enzymes and endogenous RNases, but also to conserve cellular morphology and nucleic acid integrity. PFA achieves this by cross-linking proteins and nucleic acids (summarised according to Morel and Cavalier (2000)). Three fixation times were tested: 5, 15 or 30 min. Considering that PFA penetrates tissue at a rate of 4 mm/h (Romeis, 1989), fixing $12\text{ }\mu\text{m}$ sections for 5 min would be sufficient. However, initial trials with 5 min fixation yielded no positive staining results, possibly caused by too short a fixation time. Hence, the fixation period was extended to 15 min and even 30 min as suggested by Lavery et al. (2004) of which the former turned out to be satisfying for the purposes of the present study. Fixation was followed by two washing steps in 1x PBS for 5 min each.

Similarly as for the dot blot, once the sections were immersed in the fixation solution it was important to ensure that the sections never dried out during any subsequent steps until the staining was completed.

2.5.3 Pretreatment of sections

The main aim of the pretreatments is to make the target nucleic acid accessible to a probe. This may include modifying or destroying membrane and histone proteins, improving

signal/background ratio as well as denaturing the target nucleic acid and the probe (Morel and Cavalier, 2000). This chapter describes a selection of single steps which are currently reported in the literature to achieve this aim and which were also tested separately or in combination for this work. The selection was based on the necessity of the steps determined by factors such as fixation method or tissue type.

One key issue turned out to be the deproteinisation step which seems to be a common application to paraffin embedded sections (Molenaar et al., 1992; Mayer et al., 2002; Sayed-Ahmed et al., 2003), but on which the literature reports are highly varying for frozen tissues. While some researchers (Knabel et al., 1998; Sinowatz et al., 2000) are applying a combination of Proteinase K and HCl, others work successfully by omitting this step completely (Jung et al., 1994; Lavery et al., 2004). Hence, some effort was spent to find the optimal treatment of liver and mammary gland tissue which turned out to be the entire exclusion of the deproteinisation step.

Similarly, an attempt was made to determine a treatment of the sections just prior to the hybridisation that provides optimal conditions for a successful hybridisation afterwards (prehybridisation step). Three options were compared: first, equilibration with 2x SSC only; second, equilibration with 2x SSC followed by a mixture of equal volumes of 2x SSC and hybridisation buffer (without probe); third, a dehydrating alcohol series. Since the latter two options did not reveal any additional benefit over the former, option one was preferred because it involved the least work, time and chemicals.

The other measures applied are briefly summarised as follows: acetylation of sections was always carried out, whereas the post-fixation alcohol series was optionally tested and the quenching of endogenous peroxidase applied only when required by the chosen hybrid revelation system. The paragraphs below describe in more detail the implementation of the different pretreatment steps, a selection of which was assessed each round. It is refrained from mentioning all combinations assessed as this presentation would be too comprehensive. The steps are listed in chronological order as applied in this study, however other orders are possible (Morel and Cavalier, 2000; Plenz et al., 2003). They were carried out in Hellendahl glass jars at room temperature unless noted otherwise.

2.5.3.1 Alcohol series

After the fixation a graded alcohol series can be incorporated into the protocol (Jung et al., 1994). Ethanol not only has fixating properties, but also extracts lipids from tissue (Romeis,

1989). This latter function might be important in mammary gland tissue with epithelial cells containing fat vesicles. The sections underwent an ascending alcohol series (50%, 70%, 90% and 100% ethanol (denatured), freshly diluted in DEPC-treated H₂O, 5 min each), followed by a descending alcohol series (90%, 70%, 50% ethanol, re-using the solutions from the ascending series, 5 min each) and a final washing step in 1x PBS for 5 min. Inclusion of these two alcohol series was compared to the omission of both.

2.5.3.2 Inactivation of endogenous peroxidase

This step was included only when applying the Tyramide Signal Amplification (TSA) system (see below "revelation of hybrid"). It required the inactivation of endogenous peroxidase since one of the antibodies used for the immunological detection was coupled to horseradish peroxidase (POD). This was assured by incubating the sections in 0.3% H₂O₂ (1:100 dilution of 30% H₂O₂ in 1x PBS) for 15 min and washing twice in 1x PBS for 5 min.

2.5.3.3 Deproteinisation

As suggested by the literature mentioned above, two deproteinisation treatments (enzymatically with Proteinase K and chemically with HCl) were tested in combination compared to the entire exclusion of their application. Proteinase K is considered to be a selective protease for proteins associated with nucleic acids. After equilibration in 1x PBS for 5 min, different concentrations (1, 10 and 20 µg/ml 1x PBS) of Proteinase K as well as different incubation periods (10 and 30 min) were evaluated in a 2x3 Latin square design. The selection of PBS as dissolvent and treatment at room temperature, in contrast to 37°C often seen in the literature (Molenaar et al., 1992; Knabel et al., 1998), was suggested by PD Dr. Stephan Baader because of ease of handling (personal communication). Alternatively to PBS, effects of dissolving Proteinase K in 0.2 M Tris, 2 mM CaCl₂ were also tested (Molenaar et al., 1992; Morel and Cavalier, 2000). No differences were observed, hence, for subsequent Proteinase K treatment the ready-to-use buffer 1x PBS was used. The Proteinase K incubation was succeeded by two washes in 1x PBS for 5 min. Subsequently, sections were subjected to HCl at a concentration of 0.2 M for 10 min, conditions commonly reported in the literature for this treatment (Molenaar et al., 1992; Knabel et al., 1998; Morel and Cavalier, 2000)

2.5.3.4 Acetylation

The acetylation reaction is effective in reducing background by transforming the reactive amine group (NH_3^+) of proteins into a substituted amine group ($-\text{NH}-\text{CO}-\text{CH}_3$, neutral) by reacting the sections with acetic anhydride ($\text{CH}_3-\text{CO}-\text{CH}_3$) in a triethanolamine buffer. For this purpose a 0.25% acetic anhydride solution in 0.1 M TEA buffer was prepared only just prior to usage because of the instability of acetic anhydride. The sections were incubated in this solution for 10 min.

2.5.3.5 Prehybridisation

Three options were compared as prehybridisation treatment:

Option 1: Sections were equilibrated in 2x SSC for 10 min.

Option 2: Initially, the sections were equilibrated in 2x SSC for 10 min and then pre-hybridised with 90 μl of a mixture of equal volumes of 2x SSC and hybridisation buffer for at least 30 min while the microscope slide was placed horizontally in a humid chamber (stainless steel grid in a stainless steel box containing DEPC-treated H_2O at the bottom). Prior to the application of the mixture the slide was dried with paper tissue omitting the section generously to minimise dilution effects.

One aim of the prehybridisation is to saturate nonspecific sites that might adsorb probe molecules. This saturation is achieved by subjecting the sections to macromolecules such as Denhardt's solution, RNA or DNA contained in the hybridisation buffer. However, no probe is added.

Option 3: The sections were subjected to a dehydrating alcohol series (50%, 70%, 90%, and 100% ethanol) for 5 min each and then air dried. This eliminates any dilution of the subsequently applied hybridisation buffer with probe.

2.5.4 Hybridisation

In situ hybridisation is the step during which the target nucleic acid, having been rendered accessible by pretreatments to the probe, will be capable of hybridising with this probe in given conditions. For this purpose, the probe has to be complimentary, thus anti-sense, to the target nucleic acid. A successful hybridisation is characterised by the formation of stable and specific hybrids. Crucial for a successful hybridisation is the choice of hybridisation buffer, hybridisation temperature, Na^+ concentration, type of probe and length of probe. The

composition of the hybridisation buffer was based on the protocol of Molenaar et al. (1992) with some minor adjustments for non-radioactive probes and is shown in Table 2.5. The salinity of this buffer was 0.54 M and the hybridisation temperature was 52°C. As a reminder, three DIG labelled RNA probes were tested: bHp1 (174 nt), bHp2 (302 nt), and hHp (338 nt). In addition, different total amounts of probe were tested: 10 to 60 ng per section for the standard ISH and 3 to 30 ng per section for ISH with the TSA (see below).

The probe was applied to the section as follows: After thawing the probe on ice, the appropriate amount was added to the hybridisation buffer to yield a total volume of 50 µl. This solution was mixed gently, but thoroughly because of the viscosity of the buffer. Next, this mixture was exposed to 80°C for 5 min to denature the RNA probe and then immediately placed in ice to preserve the denatured structure. Following the final step of pretreatment, the slide was dried with paper tissue omitting the actual section. Then the section was carefully overlaid with 50 µl of the hybridisation mixture and covered with a Hybri-Slip (40 mm x 24 mm) cut in half. Finally, the slide was placed horizontally in a humid chamber filled with DEPC-treated H₂O and incubated at 52°C overnight (16 to 18 h).

Tab. 2.5: Reagents of hybridisation buffer.

Reagent	Concentration	Purpose
Dextran sulphate	10%	A nonionic polymer, it increases the effectiveness of hybridisation by accelerating the reaction through concentrating the solute through the volume that it occupies in the solution. It also has considerable blocking activity.
Formamide, deionised	50%	It reduces the hybridisation temperature necessary for the hybridisation to take place and, thus, preserves better tissue morphology.
NaCl	150 mM	Hybrids form in the presence of Na ⁺ ions. Their concentration has a direct effect on their stability.
SSC	2x	As for NaCl
Yeast tRNA	0.2 mg/ml	Macromolecules that produce saturation of nonspecific bonds by competition, hence its high concentration.
Fish sperm DNA	1 mg/ml	As for yeast tRNA
Denhardt's solution	1x	Macromolecules also saturating nonspecific bonds by competition

2.5.5 Posthybridisation washes

The main aim of the posthybridisation washes is to increase the specificity of the hybridisation signal or, in other words, to reduce background by removing probe that has bound unspecifically or not at all. This can be achieved by controlling temperature and salinity of washing buffer as well as including RNase treatment.

After the hybridisation step the Hybri-Slips were rinsed off by carefully flushing 2x SSC with a pipette between slip and slide and the slides were placed in a Hellendahl glass jar filled with 2x SSC. The sections were washed twice in 2x SSC at 45°C for 10 min each, twice in 50% formamide/50% 2x SSC at 45°C for 10 and 20 min, respectively, and, finally, twice in 0.2x SSC on the shaker (100 rpm) at room temperature for 10 min each.

Next, sections were subjected to an RNase digest. Two different types of enzymes (RNase A, RNase T1), their combination and different enzyme concentrations were tested in 2x SSC buffer during 30 min at 37°C, based on the literature (Yang et al., 1999; Goldammer et al., 2004; Lavery et al., 2004):

1. 10 µg RNase A/ml
2. 10 µg RNase A + 100 U RNase T1/ml
3. 5 µg RNase A + 50 U RNase T1/ml
4. 5 µg RNase A + 25 U RNase T1/ml
5. without RNase.

Option 3 (5 µg RNase A + 50 U RNase T1/ml) returned results optimal for the purpose of this study. The digest was followed by three washes in 2x SSC on the shaker (100 rpm) for 10 min each. Extra care was taken during the handling of RNase to avoid any undesirable contaminations in the laboratory.

2.5.6 Revelation of hybrid

2.5.6.1 Immunological detection of hybrid without signal amplification

Generally, the choice of the revelation method depends on the type of hybrid label. The DIG labelled probe used in this study was detected immunologically by an anti-DIG antibody coupled with alkaline phosphatase which in turn reacts with the substrate NBT/BCIP. Phosphatase hydrolysis of BCIP is coupled to the reduction of NBT, yielding a formazan and

an indigo dye that together form a black-purple coloured precipitate at the site of reaction (see Figure 2.2).

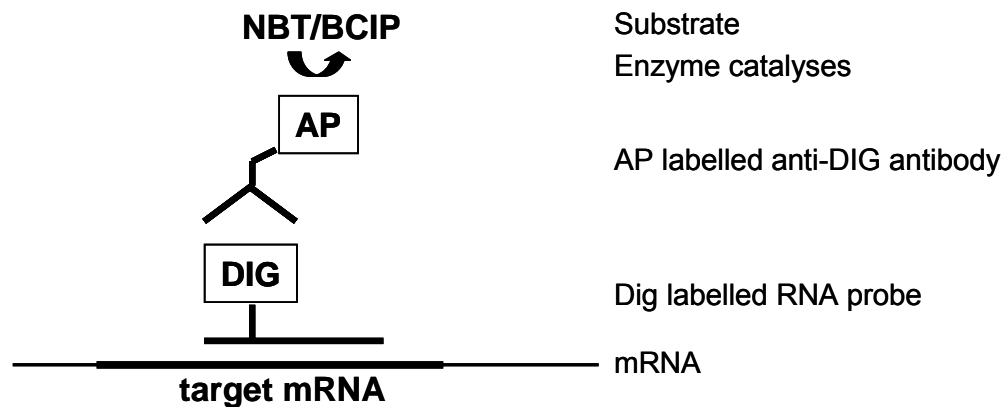


Fig. 2.2: Diagram of hybrid detection system.

The effects of two kinds of substrate buffer were compared (substrate buffer 1 and 2). Substrate buffer 2 contained 50 mM MgCl_2 in addition to substrate buffer 1. This was recommended in older protocols and explained with the need for Mg of the enzyme AP for optimal activity. However, meanwhile it is suggested that endogenous Mg present in the section is sufficient (personal communication with technical support at Roche) and was confirmed herein. Furthermore, the effect of adding 1 mM or 5 mM levamisole to the staining solution was considered (Roche, 2002; Goldammer et al., 2004), which is supposed to inactivate endogenous phosphatase. Since no significant background staining was obtained when exposing the sections to all steps without the addition of probe and without the addition of levamisole, this option was abandoned.

Similarly to the probe quantification by dot blot, the combination of NBT and BCIP were used as substrates. Staining of bluish, brownish or purplish colour was considered as specific ISH staining.

The following steps were conducted:

- MA buffer, 10 min (equilibration)
- 1% blocking solution (10% blocking reagent diluted 1:10 in MA buffer), 30 min with agitation (100 rpm)
- Slides were dried with a tissue adjacent to the sections, the sections encircled with a special pen (PAP Pen Super) creating a water repellent barrier

- 100 µl Anti-DIG-AP antibody (Fab fragment, Roche), diluted 1:500 in 1% blocking solution, in a humid chamber in the dark for 2 h at room temperature
- MA buffer containing 0.1% Tween 20, 15 min, with agitation (100 rpm) (wash off excessive antibody)
- MA buffer, three times 15 min, with agitation (100 rpm) (washes)
- Substrate buffer 1 or 2, 10 min (equilibration)
- Substrate incubation (final concentration: 337.5 µg NBT/ml, 175 µg BCIP/ml in substrate buffer 1 or 2; i.e. 225 µl NBT + 175 µl BCIP in 50 ml substrate buffer), protected from light, 2 h or more, mainly overnight, until optimal staining achieved; addition of 1 mM or 5 mM levamisole was tested
- TE buffer, 10 min (termination of substrate reaction)
- Mounting with Kaiser's glycerol gelatine

2.5.6.2 Immunological detection of hybrid with signal amplification

Strong specific staining was observed for Hp mRNA in liver tissue with the above described protocol, whereas this could not be achieved in mammary gland sections. This pointed to low levels of Hp mRNA expression in that tissue, but also emphasised the need for signal enhancement integrated into the ISH protocol. In this study two options were investigated: polyvinyl alcohol (PVA) as supplement to the staining solution as described above, and the inclusion of the tyramide signal amplification (TSA) system as an additional step during the revelation process.

Polyvinyl alcohol

As part of the staining solution, PVA can reduce the diffusion of reaction intermediates by enhancing the formazan formation during the phosphatase reaction and, thus, is leading to an increase in signal sensitivity up to 20-fold without increasing background (De Block and Debrouwer, 1993).

Following equilibration in standard substrate buffer, the sections incubated in PVA-substrate buffer plus substrates (337.5 µg NBT/ml, 175 µg BCIP/ml) at 30°C. When colour development was optimal, slides were washed thrice for 5 min in ultra-pure H₂O, the sections then dehydrated once in 70% ethanol and twice in 100% ethanol 15 sec each and finally mounted with Kaiser's glycerol gelatine.

Tyramide signal amplification

Tyramide is a phenolic compound that precipitates after oxidation. It can be labelled and its label, in this study dinitrophenol (DNP), can be detected immunologically in a similar manner as described above. The principle of TSA-DNP technology is that several DNP molecules are coupled to each single tyramide molecule. Therefore, after oxidation of TSA by the enzyme peroxidase, numerous DNP labels are deposited immediately adjacent to the site of the enzyme and, consequently, a higher number of substrate molecules can be precipitated at the site of detectable mRNA in comparison to the standard immunological detection (see Figure 2.3).

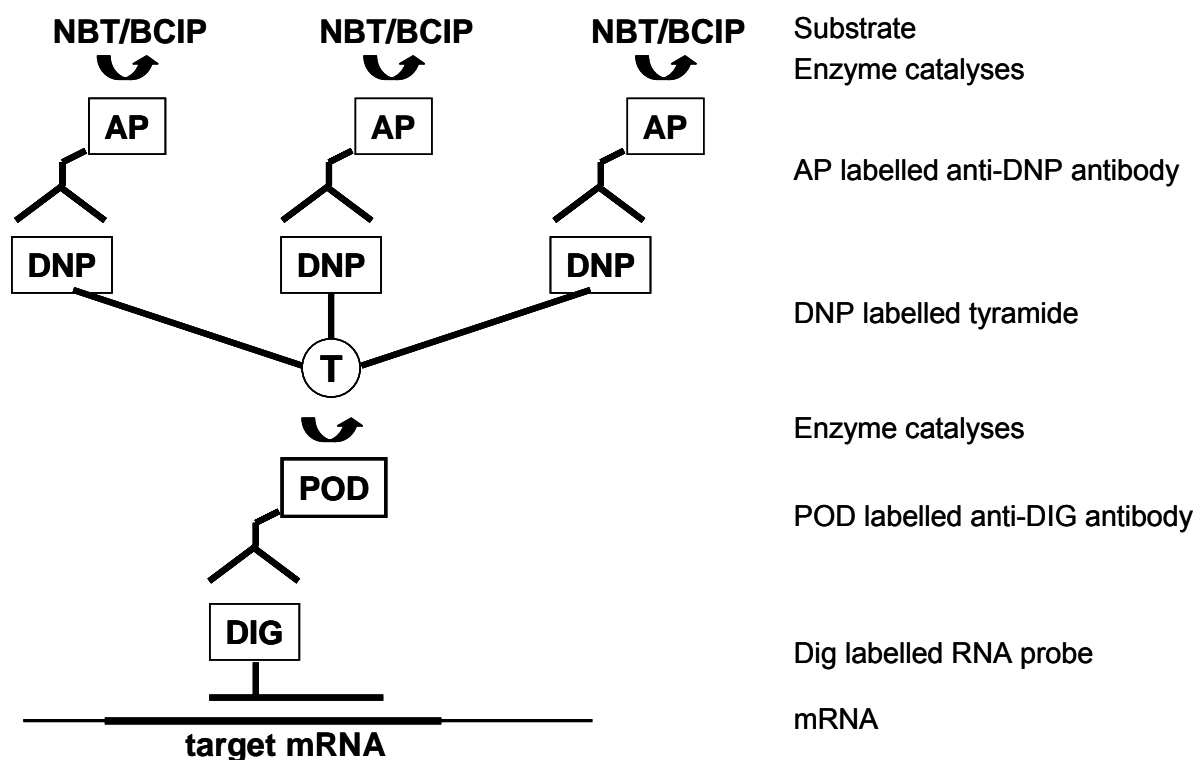


Fig. 2.3: Diagram of the hybrid detection system including tyramide signal amplification.

The TSA Plus DNP AP system was integrated after the posthybridisation washes and carried out according to the manufacturer's instruction (PerkinElmer LAS). As also recommended by the manufacturer and suggested by Yang et al. (1999), different incubation times and reagent dilutions had to be adapted for this study:

- TN buffer, 5 min
- Encircle section with PAP Pen Super
- 100 µl TNB blocking buffer, 30 min

- 90 µl Anti-DIG-POD antibody (Fab fragment, Roche) diluted 1:100 or 1:500 in TNB blocking buffer, protected from light, 30-60 min
- TNT wash buffer, three times 5 min with agitation (100 rpm)
- 90 µl TSA Plus DNP working solution (DNP amplification reagent stock solution diluted 1:50 in 1x Plus Amplification diluent (TSA kit)), 3 or 10 min
- TNT wash buffer, three times 5 min with agitation (100 rpm)
- 90 µl Anti-DNP-AP (TSA kit) diluted 1:100 in TNB blocking buffer, protected from light, 30-60 min
- TNT wash buffer, three times 5 min with agitation (100 rpm)
- Substrate buffer 1, 10 min
- Substrate (337.5 µg NBT/ml, 175 µg BCIP/ml substrate buffer 1), protected from light, 10-50 min
- TE buffer, 10 min
- Mounting with Kaiser's glycerol gelatine.

Optimal results were produced by the 1:100 dilution of the Anti-DIG-POD antibody, longer incubation times of both antibodies (60 min) as well as the TSA Plus DNP working solution (10 min) and substrate incubation for 50 min.

2.5.7 Counterstaining

Two types of counterstaining were tested. First, sections were exposed to 0.1% Nuclear Fast Red in 5% aluminium sulphate for 1 min, washed twice in tap water for 10 min. Alternatively, sections were placed in Mayer's hemalum solution for 3-5 sec, followed by 1% potassium aluminium sulphate for 10 sec to stop the staining reaction and washed briefly in ultra-pure H₂O. These steps were applied after the substrate reaction was completed, but before sections were mounted with Kaiser's glycerol gelatine. The reactions lead to a red or blue staining of the nucleus, respectively. Both procedures were sourced from Roche (Roche (2002) and personal communication with their technical support, respectively).

2.5.8 Evaluation and scoring of the ISH staining signals

The staining of the sections was assessed by light microscopy (Microscope DM LB, Leica). In addition, the staining of each section was scored evaluating one section per tissue: A reference grid (1 x 1 cm; 10 x 10 squares) placed in the microscope ocular was superimposed

over the section, and stained cells of 24 squares (randomly picked for the grid, but fixed for all sections) were counted at a total magnification of 260.

Instruments for photographing (Routine microscope, Axioskop 2 MOT, Zeiss; Polaroid DMC Ie camera) were kindly made available by PD Dr. Stephan Baader.

2.5.9 Controls

2.5.9.1 Negative controls

Hybridisation without probe

In order to control background staining, the complete ISH reaction was carried out without the addition of probe to the hybridisation buffer. This tool represents a negative control and is of particular use when working with signal amplification systems such as TSA.

Application of sense probe

The sense probe serves as another negative control. Since its sequence is identical to the target mRNA, it does not attach to the target mRNA and, thus, is not leading to staining. For all treatments tested, a section hybridised with sense probe was always prepared in parallel to each section hybridised with antisense probe. The "sense section" was semi-adjacent to the "antisense section".

2.5.9.2 Positive controls

Casein probe

Casein is a protein that is widely synthesised in the alveolar epithelium of the lactating mammary gland (Molenaar et al., 1992). In situ hybridisation with an RNA probe for this protein applied to cryosections of bovine mammary gland served as positive control in this study to be able to demonstrate the functioning of ISH in this organ. Both as and s probes (750 and 850 nt, respectively) originated from amplifications of plasmid inserts which had been kindly donated by Prof. Dr. Hans-Martin Seyfert, Research Institute for the Biology of Farm Animals, Dummerstorf, Germany. Prior to this study, these probes had been tested successfully in the same laboratory by Claudia Hakemann during her PhD studies in an ISH

applied to paraffin embedded sections (Hakemann, 2002). Her protocol was adapted for the current study with cryosections.

Liver tissue

ISH with bovine liver cryosections served as positive control for all three Hp probes produced since this organ is the main site of Hp synthesis.

2.6 Measurement of Hp protein in blood and milk

The Hp protein concentration was determined in the serum and milk samples of the Hanover cows by enzyme linked immunosorbent assay (ELISA). The assay (Hiss et al., 2004) was slightly modified such that serum Hp was applied for coating and standard curve instead of Hp purified from bovine serum. The standard used herein had been calibrated against a standard obtained from European Union Concerted Action on the standardisation of animal APP (QLKS-T-1999-0153).

2.7 Statistical analysis

The data from both the ISH signal scoring and the Hp protein analysis were analysed by a nonparametric paired samples test (Friedman's test) in SPSS 12.0. A p-value < 0.05 was considered significant.

3 Results

3.1 Detection of Hp mRNA in blood and milk somatic cells of cattle and man as well as in human mammary gland by RT-PCR

3.1.1 Blood and milk somatic cell preparation

When isolating leukocytes initially with a heparin concentration of 10,000 IU/l blood, the first centrifugation step produced a buffy coat with cells adhering too strongly to allow proper subsequent isolation. Similar difficulties were observed when increasing heparin concentration to 15,000 IU/l. Finally, best results were achieved with 5000 IU heparin/l blood. No difficulties occurred when preparing milk somatic cells.

The process from blood or milk sampling until freezing of the final cell pellet to extract RNA from required 4 h maximally.

3.1.2 Blood cells – differential count, number and viability

The differential cell count of the whole blood samples from the six Frankenforst (F) cows and the four humans are presented in Table 3.1, all being close to the normal range for their species. The number of isolated leukocytes varied between 8 to 152 x 10⁶ cells from 48 ml whole blood, and the number of isolated cells per MNC and PNC fraction varied between 36 to 62 x 10⁶ cells originating from 45 ml whole blood (Table 3.2). The extracted cells showed a viability above 85%. The purity of five MNC and PNC fractions was greater than 94%, while the PNC fraction of cow F6 contained 85% polymorphonuclear and 15% mononuclear cells (Table 3.2). The contamination of isolated white blood cells with erythrocytes was established in the counting chamber and was less than 2% for all samples. This contamination was not taken into account when assessing composition of isolated fractions (Table 3.1).

Tab. 3.1: White blood cell differential of six cows and four humans.

Candidate	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)
Cow F1	70	7	18	4	1
Cow F2	58	2	26	14	0
Cow F3	67	2	29	2	0
Cow F4	58	0	36	6	0
Cow F5	60	2	30	8	0
Cow F6	71	2	27	0	0
Normal range for cattle ⁽¹⁾	45-65	2-6	25-45	1-10	0-2
Volunteer 1	34	0	64	2	0
Volunteer 2	41	1	57	1	0
Volunteer 3	29	0	70	1	0
Volunteer 4	25	0	72	3	0
Normal values for man ⁽²⁾	31	6	60	2	<1

⁽¹⁾ Kraft and Dürr (1999); ⁽²⁾ Klinker and Silbernagel (1994)

Tab. 3.2: Number and viability of isolated blood cell fractions and where applicable their composition.

Candidate	Isolated blood cell fraction	Number of separated cells per fraction (x 10 ⁶)	Cell viability (%)	Fraction composition	
				MNC (%)	PNC (%)
Cow F1	leukocytes	48	95	-	-
Cow F2	leukocytes	152	91	-	-
Cow F3	leukocytes	136	99	-	-
Cow F4	MNC	52	85	98	2
Cow F4	PNC	46	99	4	96
Cow F5	MNC	49	90	95	5
Cow F5	PNC	49	93	4	96
Cow F6	MNC	62	89	94	6
Cow F6	PNC	36	93	15	85
Volunteer 1	leukocytes	25	94	-	-
Volunteer 2	leukocytes	11 ⁽¹⁾	96	-	-
Volunteer 3	leukocytes	8	100	-	-
Volunteer 4	leukocytes	12	97	-	-

⁽¹⁾ Only 24 ml whole blood were available from volunteer 2.

3.1.3 Milk somatic cells

Since there was no cell count and smear carried out with the extracted milk cells, there are no results available for the parameters cell count, viability and differential in milk.

3.1.4 Quality of extracted total RNA

Figure 3.1 illustrates the gel electrophoresis results from a representative selection of blood and milk somatic cell RNA samples. Two bands were visible in all samples representing the 28S and 18S rRNA subunits. The fluorescence intensity of the 28S band was twice the intensity of the 18S band in three samples (lane 2, 3 and 5) whereas of approximately identical intensity in the other three samples (lane 1, 4 and 6).

It must be noted that the total amount of extracted RNA from PNC (2.3-4.6 μg) was around ten-fold lower compared to MNC (39.5-60.0 μg). Considering that per sample 1 μg RNA was required for each cDNA synthesis (two preparations: with and without the transcriptase enzyme), the amount of PNC RNA was insufficient to also allow RNA gel electrophoresis. Priority was given to cDNA synthesis.

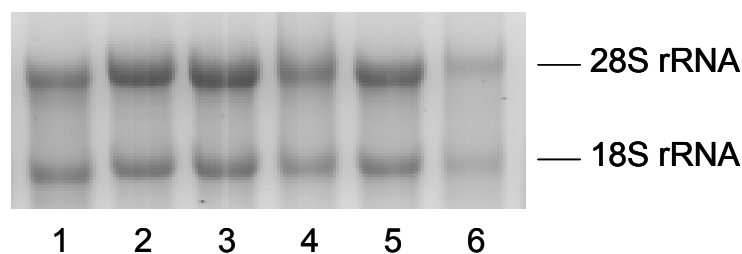


Fig. 3.1: Denaturing gel electrophoresis of RNA extracted from blood cells (lane 1 to 3) and milk somatic cells (lane 4 to 6).

3.1.5 Qualitative RT-PCR results

3.1.5.1 Bovine leukocytes

The leukocytes of all three cows examined showed a distinct RT-PCR signal for bHp (174 bp), while the same samples subjected to RT-PCR without reverse transcriptase did not yield any signal (Figure 3.2). The controls (liver and water with bHp primers as positive and negative control, respectively, as well as all samples with β -actin primers as internal control) worked as required.

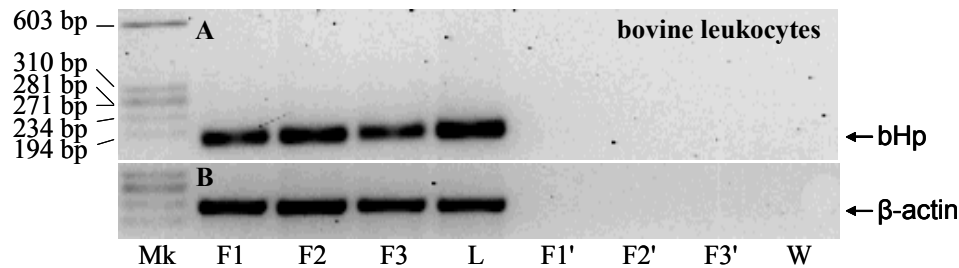


Fig. 3.2: Qualitative RT-PCR detection of bovine Hp mRNA (174 bp) in bovine blood leukocytes (A) parallel to the detection of β -actin mRNA (226 bp) as internal control in the same cells (B). Lanes F1-F3 contain PCR products of RNA from three individual Frankenforst cows. The same RNA samples underwent RT-PCR without the addition of reverse transcriptase (lanes F1'-F3'). Mk = marker, L = bovine liver (positive control), W = water control.

3.1.5.2 Bovine mononuclear and polymorphonuclear blood cells

Figure 3.3 shows the RT-PCR results of bHp mRNA in bovine MNC and PNC. In both leukocytes subpopulations a clear signal was found for the Hp gene, though the signals of cow F6 were of weaker intensity. The RT-PCR controls as described above for blood leukocytes worked successfully.

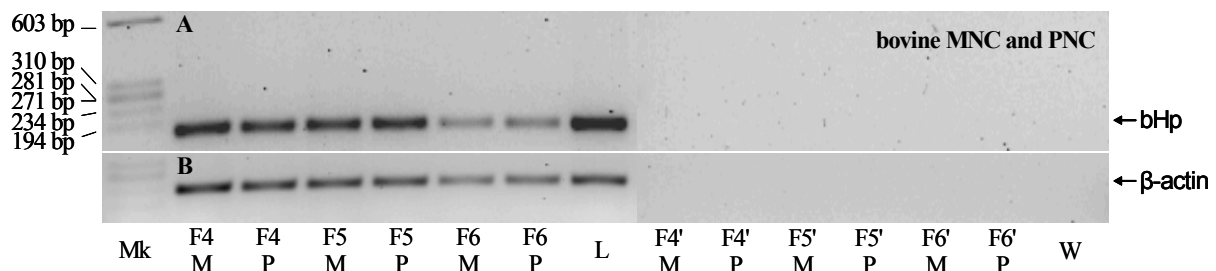


Fig. 3.3: Qualitative RT-PCR detection of bovine Hp mRNA (174 bp) in bovine blood MNC and PNC (A) parallel to the detection of β -actin mRNA (226 bp) as internal control in the same cells (B). Lanes F4-F6 contain PCR products of RNA from three individual Frankenforst cows each of whose leukocytes were divided into mononuclear cells (M) and polymorphonuclear cells (P). The same RNA samples underwent RT-PCR without the addition of reverse transcriptase (lanes F4'-F6'). Mk = marker, L = bovine liver (positive control), W = water control.

3.1.5.3 Bovine milk somatic cells

In bovine milk somatic cells, distinct RT-PCR fragments for the bHp gene were found, though the bHp signal for cow F7 was of weaker intensity compared to the other two cows (Figure 3.4). Similarly for cow F7, the internal control gene β -actin showed a slightly weaker signal. This suggests that RNA extracted from this animal's milk might generally have been of minor quality, which would also explain the results for the bHp gene. The RT-PCR controls worked as desired.

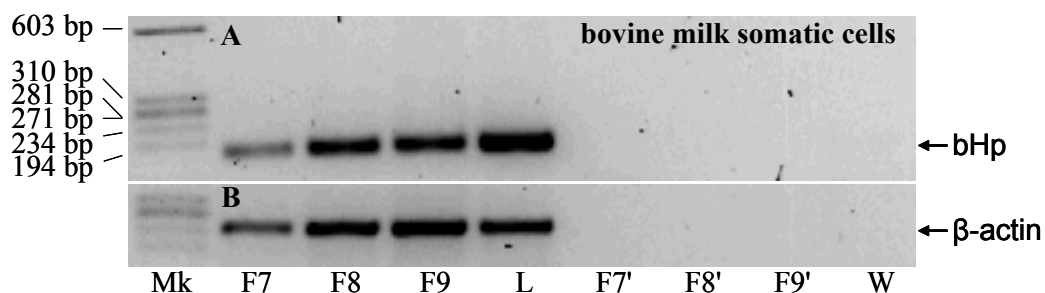


Fig. 3.4: Qualitative RT-PCR detection of bovine Hp mRNA (174 bp) in bovine milk somatic cells (A) parallel to the detection of β -actin mRNA (226 bp) as internal control in the same cells (B). Lanes F7-F9 contain PCR products of RNA from three individual Frankenforst cows. The same RNA samples underwent RT-PCR without the addition of reverse transcriptase (lanes F7'-F9'). Mk = marker, L = bovine liver (positive control), W = water control.

3.1.5.4 Human leukocytes

The leukocytes of all four human candidates did not present a hHp signal after applying the standard RT-PCR method (Figure 3.5, A). However, hHp specific transcripts (338 bp) were found after reamplification of the first course PCR products, but only in two out of four individuals (Figure 3.5, B). Standard RT-PCR detected β -actin transcripts in samples of all four donors (Figure 3.5, C). RT-PCR and reamplification without the addition of reverse transcriptase did not produce any signal for the Hp as well as the β -actin gene in all samples. This emphasizes that no contaminating genomic DNA had been present in the RNA samples used, which in turn suggests that the positive results seen after reamplification originate from Hp mRNA present in the samples. Also RT-PCR and reamplification of the positive and negative PCR controls, liver and water respectively, presented results as required.

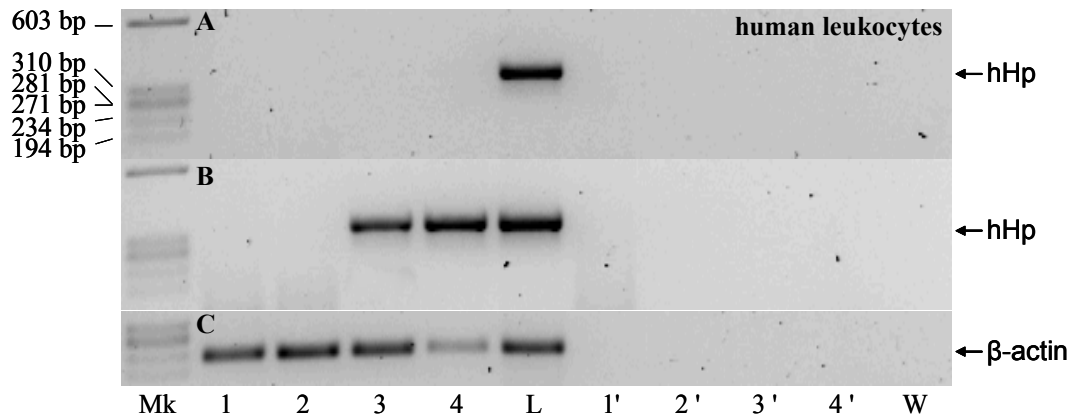


Fig. 3.5: Qualitative detection of human Hp mRNA (338 bp) in human blood leukocytes after RT-PCR (A) and reamplification of 1 μ l of these first course RT-PCR products (B), parallel to the standard RT-PCR detection of β -actin mRNA (226 bp) as internal control in the same cells (C). Lanes 1-4 contain PCR products of RNA from four individual test persons. The same RNA samples underwent RT-PCR and reamplification without the addition of reverse transcriptase (lanes 1'-4'). Mk = marker, L = human liver (positive control), W = water control.

3.1.5.5 Human mammary gland

Figure 3.6 illustrates RT-PCR products of Hp mRNA in human mammary gland alongside human liver as control organ. The other RT-PCR controls (water with hHp primers as well as all samples with β -actin primers) worked as required.

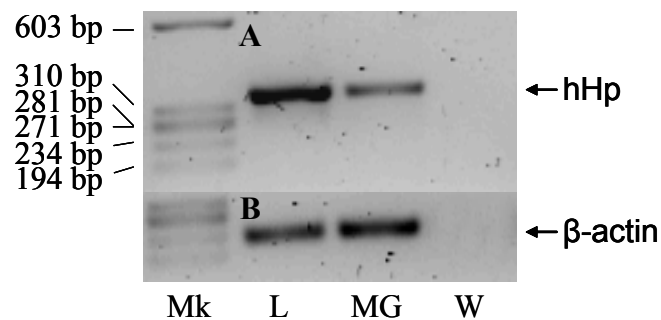


Fig. 3.6: Qualitative RT-PCR detection of human Hp mRNA (338 bp) in human mammary gland (MG) (A) parallel to the detection of β -actin mRNA (226 bp) as internal control in the same cells (B). Mk = marker, L = human liver (positive control), W = water control.

3.2 Cellular localisation of Hp mRNA in infected bovine mammary glands by ISH

3.2.1 Establishment of suitable ISH protocols

3.2.1.1 ISH controls

Casein mRNA was detected in bovine mammary gland tissue by a ISH protocol which was based on the study by Hakemann (2002). However, modifications were necessary since the current research was carried out with cryosections instead of paraffin embedded sections. Also minor changes were made to the order of the pretreatments and the composition of some buffers to fit in with the ISH procedures with Hp probes. The detailed protocol is presented in the appendix.

Hp mRNA was detected in bovine liver tissue by in situ hybridisation with all three probes, however best results were achieved applying bHp2 probe by the protocol also given in the appendix.

3.2.1.2 Hp mRNA in the bovine mammary gland

The finally established protocol for best detection of Hp mRNA in the bovine mammary gland is presented in the appendix. The three key elements of this protocol were:

1. Omission of deproteinisation
2. Introduction of the longer homologous bHp2 probe
3. Introduction of TSA

Overall, implementing the longer bHp2 probe into the ISH protocol applied to bovine liver (omitting deproteinisation) had generated hybridisation signals noticeably stronger than with the bHp1 probe. Likewise, with a previous protocol, still containing a deproteinisation pretreatment, the heterologous hHp probe had returned similarly weak staining results as the bHp1 probe in the bovine liver, therefore the homologous probes were preferred. In summary, bHp2 was the probe of choice.

Most importantly for the detection of Hp mRNA in the bovine mammary gland was the introduction of signal amplification by the TSA system to an existing ISH protocol with which Hp mRNA had already been established for bovine liver with no deproteinisation and employment of the bHp2 probe. It caused a marked increase in signal intensity, whereas the addition of PVA to the substrate buffer produced no noticeable signal enhancement. These staining signals produced by the TSA appeared to be even more distinct, i.e. with less background, when including alcohol series after fixation. Moreover, when initially establishing the ISH protocol with the TSA system, the amount of 3 ng probe/section had generated equally good staining as the amount of 30 ng probe/section, which is why the lesser amount was applied subsequently to save probe.

Finally, neither haemalaun nor Nuclear Fast Red treatments produced counterstains of satisfying contrast to the NBT/BCIP reaction products. Therefore, it was refrained from this option. No attempt had been undertaken to optimise the protocols further for the current purposes.

3.2.2 Demonstration of ISH results

3.2.2.1 ISH controls

With the established ISH protocols the following results were achieved for the ISH controls:

Distinct staining for casein mRNA was detected in the alveolar epithelium and no signal in the stroma of the mammary gland. The section hybridised with the sense probe remained unstained (Figure 3.7; A, A').

Strong ISH signals were found in hepatocytes but no staining in stroma, bile duct and blood vessels. Also no staining was present in liver sections hybridised with the sense probe (Figure 3.7; B, B').

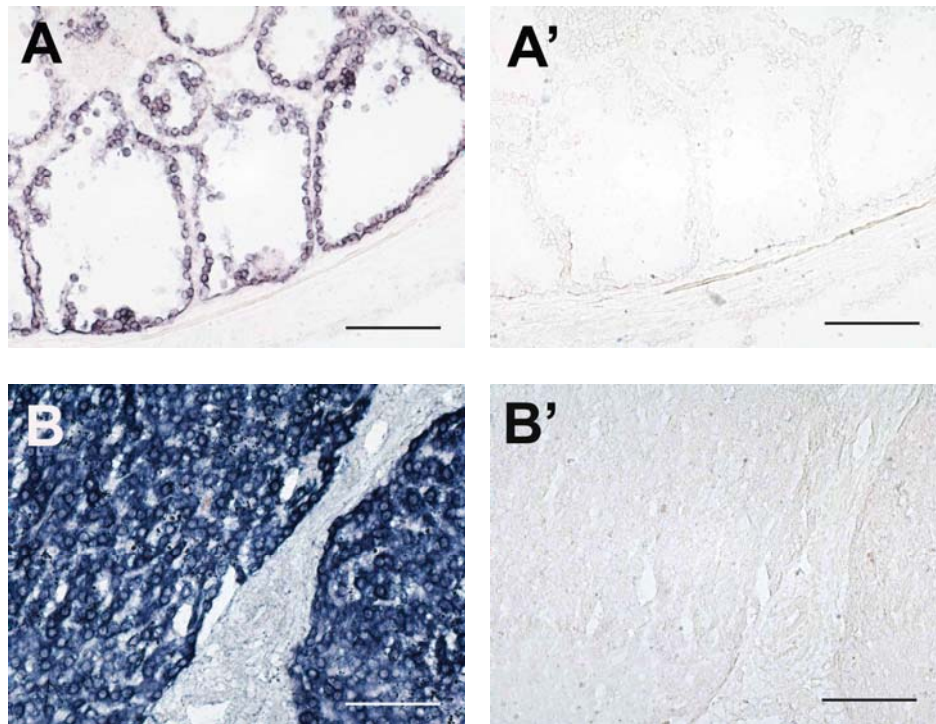


Fig. 3.7: Controls for ISH: Detection of casein mRNA in bovine mammary epithelium (A) and of Hp mRNA in bovine liver hepatocytes (B). Tissues were hybridised with antisense (A, B) and sense probe (A', B'). Bars represent 100 μ m.

3.2.2.2 Hp mRNA in infected bovine mammary glands

All control sections, i.e. sections of the bovine mammary gland incubated with bHp2 sense probe (see Figure 3.8 (A' to D'), Figure 3.9 (A' to D') and Figure 3.10 (A')) or without any probe (data not shown), were devoid of any specific hybridisation signal.

Hp mRNA was visualised in the alveolar epithelium cells of bovine mammary glands by ISH with bHp2 antisense probe. Specific signals for Hp mRNA were found in udders previously infected with *E. coli* as well as glands infected with *Staph. aureus*; however, the two treatment groups exhibited markedly different staining patterns. Figures 3.8 and 3.9 present the results of one cow per treatment group.

In *E. coli* infected cows, the number of cells stained per mammary alveolus was low in the control quarter (Figure 3.8; A) and increased with increasing incubation time of the pathogen in the other quarters (Figure 3.8; B to D). In detail, while Hp mRNA was demonstrated only in single cells scattered across the section of the control quarter, the stained cells appeared in patches of isolated areas of adjacent alveoli after 6 h incubation time. The size of these areas expanded further in quarters infected for 12 h, until the staining was uniformly distributed in

the section of the quarter exposed to *E. coli* for 24 h. This pattern of Hp expression was repeated in the samples of the other *E. coli* cows.

Mammary glands infected with *Staph. aureus*, in contrast to *E. coli* treated udders, showed a minimal level of Hp mRNA expression detected by ISH with slight variations between the three cows in this treatment group. Sections of quarters of the first cow infected at T0 and at T18 with *Staph. aureus* or having received only saline were completely devoid of any positive Hp mRNA signal (Figure 3.9; A, B, D), while a small number of stained epithelium cells was present in the T12 quarter (Figure 3.9; C). In fact, the hybridisation signals in the sections of this quarter were confined only to those cells depicted in this photo. Similarly for one other cow of the same treatment group, ISH signal was restricted to a patch of few stained cells in sections from also one single quarter only, namely the quarter treated with saline. The other three quarters of that cow remained without a positive ISH signal (data not shown). Moreover, the sections of the third cow were even completely devoid of any ISH signal (data not shown).

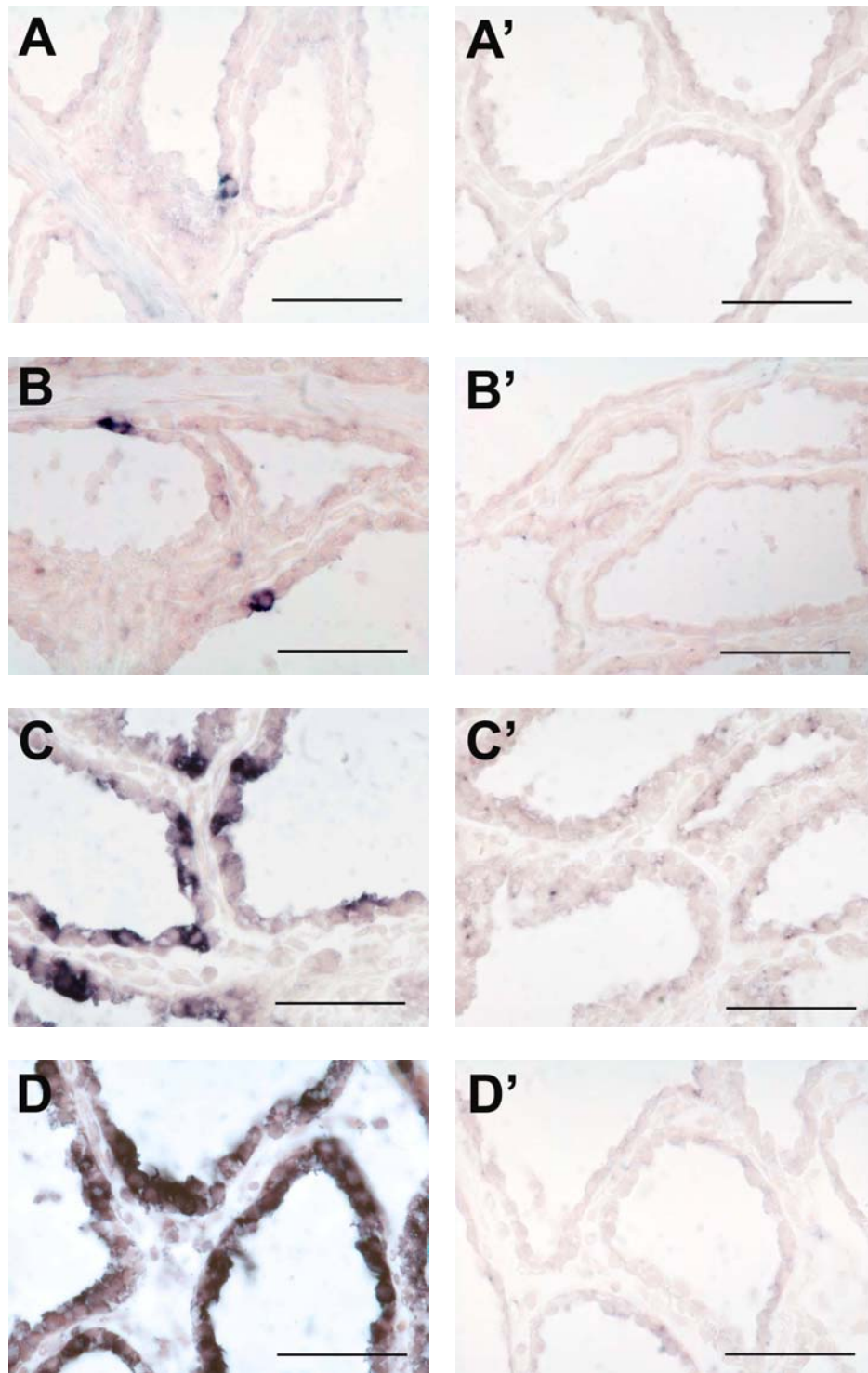


Fig. 3.8: Localisation of Hp mRNA expression in bovine mammary gland epithelium by in situ hybridisation (purple stained cells). Three udder quarters had been infected intracisternally with *E. coli* at 6 (B, B'), 12 (C, C'), and 24 h (D, D') pre-slaughter, the fourth quarter had received saline as control 24 h pre-slaughter (A, A'). Tissue was hybridised with antisense (A to D) and sense probe (A' to D'). Bars represent 50 µm.

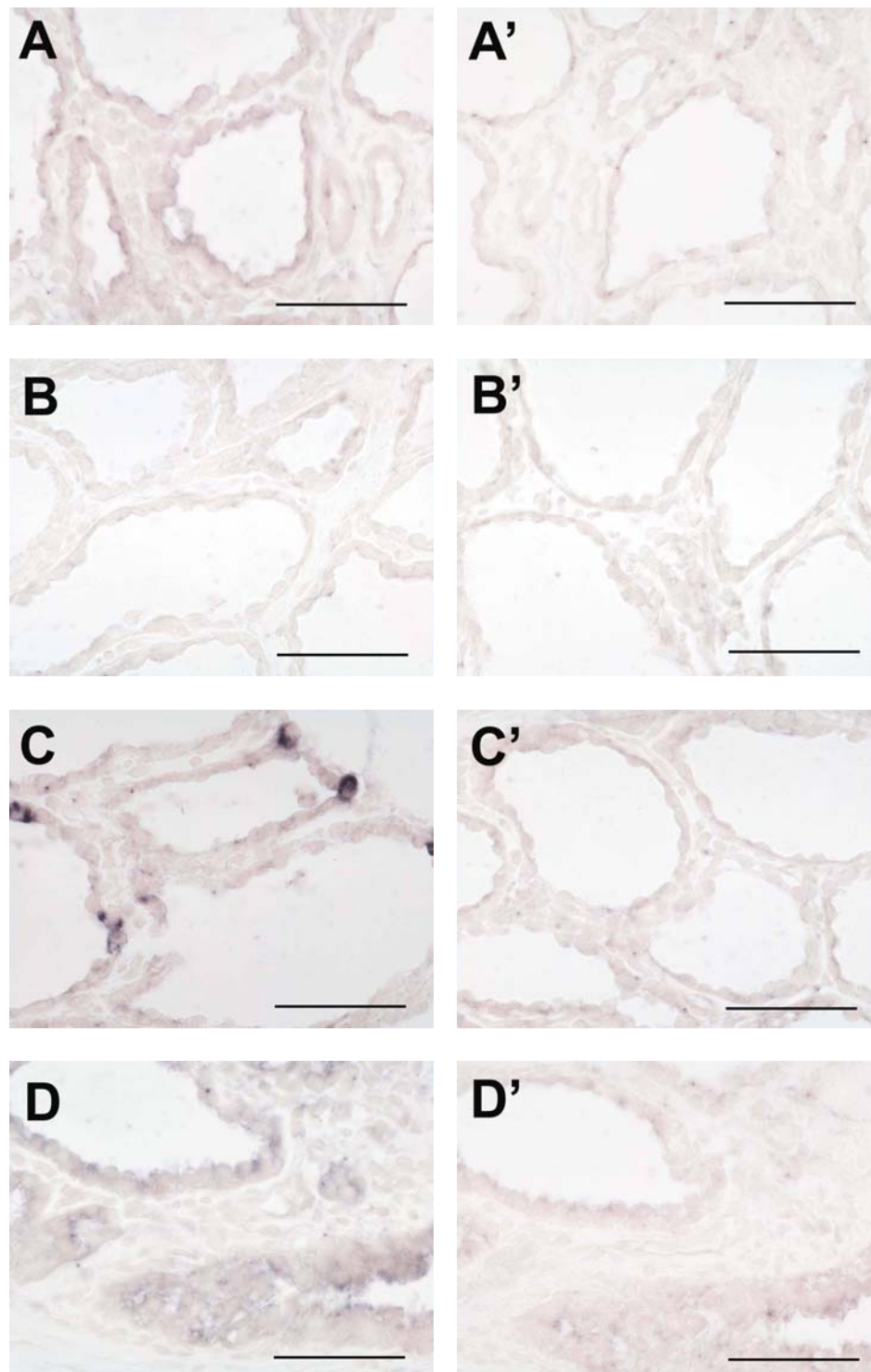


Fig. 3.9: Localisation of *Hp* mRNA expression in bovine mammary gland epithelium by in situ hybridisation (purple stained cells). Three udder quarters had been infected intracisternally with *Staph. aureus* at 6 (B, B'), 12 (C, C'), and 24 h (D, D') pre-slaughter, the fourth quarter had received saline as control 24 h pre-slaughter (A, A'). Tissue was hybridised with antisense (A to D) and sense probe (A' to D'). Bars represent 50 µm.

The differences in mammary Hp mRNA expression between the two treatment groups assessed by quantifying stained cells with the aid of a reference grid is summarised in Table 3.3. The number of stained cells per section differed significantly between the four quarters infected with *E. coli* for varying lengths of time: the quarter infected for 24 h showed considerably more stained cells than the quarters treated with saline or infected for 6 h and even 12 h. In contrast, all of the corresponding figures for the *Staph. aureus* treated cows were extremely low or even zero and remained unchanged over the experimental period.

Tab. 3.3: Abundance of Hp mRNA in the bovine mammary gland visualised by ISH was quantified by counting ISH staining signals via a reference grid (stained cells/square). Cows were infected intramammarily by *E. coli* (n = 3; EC1 to EC3) or *Staph. aureus* (n = 3; SA1 to SA3), the four quarters of each were exposed to the pathogens for varying lengths of time pre-slaughter (0, 6, 12 and 24 h, respectively). Mean values (\pm sem) per treatment group are also given.

Cow	Duration of infection per quarter				p-value
	0 h	6 h	12 h	24 h	
EC1	0.2	0.3	0.4	26.3	
EC2	0	0	0	18.3	
EC3	0.8	1.1	4.7	6.8	
Mean \pm sem	0.3 \pm 0.2	0.5 \pm 0.3	1.7 \pm 1.5	17.1 \pm 5.7	< 0.05
SA1	0.04	0	0	0	
SA2	0	0.13	0	0	
SA3	0	0	0	0	
Mean \pm sem	0.01	0.04	0	0	> 0.05

In addition to the alveolar epithelium, Hp mRNA was also expressed in the multilayered milk duct epithelium. This evaluation was possible because milk ducts were found in some sections prepared from the secretory tissue. Figure 3.10 (A, A') shows results from a quarter infected with *E. coli* for 12 h; single cells of the epithelium with ISH staining were also observed in a quarter infected for 24 h (data not shown). In contrast, no staining occurred in a quarter treated with *Staph. aureus* for 24 h (Figure 3.9; D). No attempt was undertaken to assess the expression in milk ducts of other quarters.

Next to parenchymal cells, also some cells within the stroma presented ISH specific staining for Hp mRNA (Figure 3.10; B1, B2). Their presence was limited to the sections derived from *E. coli* cows only and therein to no more than two cells per section. Identification of those cells was not possible since counterstaining could not be optimised.

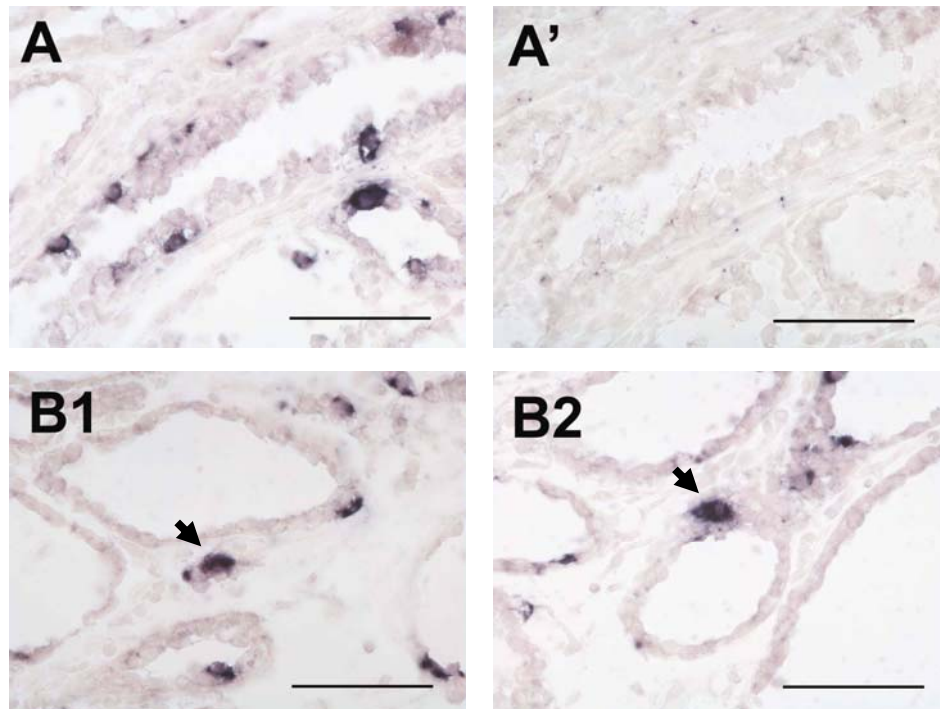


Fig. 3.10: Localisation of Hp mRNA expression in milk duct (A) and stroma (B1, B2; arrows) of the bovine mammary gland by in situ hybridisation. Quarter had been infected with *E. coli* 12 h pre-slaughter. Tissue was hybridised with anti-sense (A, B1, B2) and sense probe (A'). Bars represent 50 µm.

3.3 Hp protein concentration in blood and milk of dairy cows affected by mastitis

Hp concentration in serum differed significantly between samples collected from *E. coli* cows at four time points during the 24 h infection period, which is in contrast to samples from *Staph. aureus* cows (Table 3.4). Pre-stimulus values were numerically similar in both treatment groups; however, blood Hp concentration rose 55-fold but only 2-fold during 24 h of infection by *E. coli* and *Staph. aureus*, respectively. Likewise, milk samples collected from the four quarters of the *E. coli* cows immediately prior to slaughter showed a significant difference in their Hp concentration, but no significant changes of Hp concentrations were observed in milk from quarters affected by *Staph. aureus* (Table 3.5). The Hp concentration in milk from quarters exposed to *E. coli* for 24 h was 165-fold higher than in the control quarter.

Tab. 3.4: Hp concentration ($\mu\text{g/ml}$) in serum from six cows before and during the course of experimental intramammary infection with *E. coli* (EC1 to EC3) or *Staph. aureus* (SA1 to SA3). Three quarters of each cows were inoculated at 0, 12 and 18 h, respectively. Cows were slaughtered after 24 h. Mean values (\pm sem) per treatment group are also given.

Cow	Time since initial intramammary infection				p-value
	0 h	12 h	18 h	24 h	
EC1	27.8	35.8	221.0	1040.0	
EC2	18.2	22.6	76.8	785.0	
EC3	12.0	50.0	55.0	1410.0	
Mean \pm sem	19.3 \pm 4.6	36.1 \pm 7.9	117.6 \pm 52.1	1078.3 \pm 181.4	< 0.05
SA1	22.7	23.7	35.1	19.9	
SA2	23.1	35.5	43.4	135.0	
SA3	24.0	31.0	25.0	10.0	
Mean \pm sem	23.3 \pm 0.4	30.1 \pm 3.4	34.5 \pm 5.3	55.0 \pm 40.1	> 0.05

Tab. 3.5: Hp concentration ($\mu\text{g/ml}$) in quarter milk from six cows at slaughter previously infected intramammarily with *E. coli* (EC1 to EC 3) or *Staph. aureus* (SA 1 to SA3). The four quarters were exposed to the pathogens for varying lengths of time pre-slaughter (0, 6, 12 and 24 h, respectively). Mean values (\pm sem) per treatment group are also given.

Cow	Duration of infection per quarter				p-value
	0 h	6 h	12 h	24 h	
EC1	2.0	2.5	8.1	294.0	
EC2	4.1	2.7	45.4	433.0	
EC3	2.0	5.0	114.0	642.0	
Mean \pm sem	2.7 \pm 0.7	3.4 \pm 0.8	56.5 \pm 30.9	456.3 \pm 101.1	< 0.05
SA1	0	0	0	0	
SA2	2.9	5.1	2.8	1.8	
SA3	5.0	1.0	2.0	4.0	
Mean \pm sem	2.6 \pm 1.4	2.0 \pm 1.6	1.6 \pm 0.8	1.9 \pm 1.2	> 0.05

4 Discussion

4.1 Discussion of the methods applied

4.1.1 Isolation of blood and milk somatic cells and subsequent RNA extraction for RT-PCR

Effect of blood and milk processing on Hp mRNA expression

The cell isolation process, i.e. taking blood or milk samples until freezing the final cell pellet for RNA extraction, required up to 4 h. This delay might have had an enhancing effect on Hp mRNA expression: Duvigneau et al. (2003) showed with porcine peripheral immune cells a significant increase in mRNA expression of both cytokines IL-1 and IL-6 in these cells 6 h after sampling compared to processing immediately or after 1 or 2 h. Assuming a similar time course in cattle and man and knowing that Hp synthesis in both species can be primed by IL-6 (Baumann and Gauldie, 1994; Yoshioka et al., 2002) as well as that the effects of IL-6 can even be enhanced by IL-1 (Heinrich et al., 1990), the amount of Hp mRNA in the isolated cell populations might have been affected by the processing time in both species. On the other hand, it might be speculated that bovine leukocytes are more strongly affected by the length of processing time with regards to Hp mRNA expression than human leukocytes. Since Hp is considered as a major APP in cattle, but not in man, the Hp transcription might be upregulated more quickly in bovine immune cells during the stress situation of cell isolation than in human immune cells. Such a triggered additional Hp mRNA expression would have to be preceded by transcription and translation of IL-6. However, as the duration of IL-6 translation and subsequent Hp transcription is not known in both species, the proportion of Hp mRNA expression caused by the processing delay cannot be precisely determined.

Whether the processing time, besides affecting cytokine production, might also have a direct effect on Hp mRNA expression itself in blood or milk somatic cells was not discussed by Duvigneau et al. (2003). However, such an option cannot be excluded, although the

underlying mechanism is not known. Similarly, the effects of other processing parameters (e.g. sampling procedure, centrifugation speed, centrifugation temperature, laboratory temperature) on Hp mRNA expression in these cells could not be addressed in the current study.

Leukocyte isolation

The adherence of leukocytes by forming a firm buffy coat after initial centrifugation caused difficulties when using a heparin concentration of 10,000 IU/l or more. An increase in heparin concentration could not eliminate this problem as might be expected; instead best results were achieved with 5000 IU/l. Some researchers worked successfully with 10,000 IU/l (Duvigneau et al., 2003), while others managed with 2500 IU/l for leukocyte isolation (Dixit et al., 2003).

Purity of isolated bovine MNC and PNC fractions

Precise allocation of Hp mRNA to a single subpopulation isolated from bovine leukocytes is difficult due to the cross-contamination by cells of the other subpopulation of up to 5%, and in one sample even up to 15%. The use of fluorescence-activated cell sorting or magnetic bead sorting techniques might have produced cleaner cell populations, although low purity rates of only 78% have also been reported (Schubbert et al., 1997).

It might be speculated that despite the cross-contamination the observed Hp mRNA expression in each fraction originates indeed from cells which were correctly allocated to their fraction. Bovine Hp mRNA production in the liver is known to be triggered by the presence of the cytokines IL-1, TNF- α or IL-6 (Yoshioka et al., 2002). Since these cytokines are reported to be generated in human monocytes (Hartel et al., 2004), bovine B-cells (Amills et al., 2004) and human polymorphonuclear granulocytes (Palma et al., 1992), they might also initiate Hp mRNA expression directly in all these leukocyte cell type in bovines. On the other hand, Hp mRNA expression in blood cells might occur without prior cytokine stimulus considering that these animals appeared healthy and not undergoing an acute phase. Likewise the option must be considered that a minority of cells contributes overproportionally to the amount of total Hp mRNA present. Both these latter possibilities impede a precise allocation of Hp mRNA expression to one cell type or subpopulation.

RNA extraction: quality and total amount

The quality of the RNA extracted from blood and milk somatic cells varied as assessed by gel electrophoresis. Only three out of the six presented samples showed strong 28S and 18S

rRNA bands; also only in the same three samples the intensity of the 28S band was twice that of the 18S band as is ideally the case (Ivell, 1998). This reflects that the quality of the extracted RNA in some samples was impaired. However, RNA, which showed both 28S and 18S bands of identical intensity after gel electrophoresis, was also described to be of high quality (Chomczynski and Sacchi, 1987). Also, the gel electrophoresis visualises only the large rRNA subunits (28S ca. 5000 bp; 18S ca. 2000 bp; Alberts et al. (1997)). Considering that degradation of larger molecules is more likely than degradation of smaller molecules, smaller RNA units might still be intact in partially degraded total RNA. The Hp fragments to be detected ranged between 174 bp and 338 bp, and the fragment size of the control gene β -actin was 226 bp, all of which are considerably smaller than the rRNA subunits. In all samples β -actin fragments were present after RT-PCR, suggesting that indeed small RNA molecules were still intact in all samples used; yet, it must be noted that in the cells investigated the abundance of mRNA of the control gene β -actin was expected to be markedly higher than of the Hp gene. On the other hand, it has been demonstrated that gene expression profiles obtained from RNA samples, which were experimentally partially degraded to different extents, displayed a high degree of similarity to gene expression profiles from intact RNA samples (Schoor et al., 2003). These researchers concluded that RNA samples of suboptimal quality might still lead to meaningful results. In summary, it was assumed that the quality of the total RNA extracted from blood and milk somatic cells was adequate for the investigation of Hp mRNA presence by RT-PCR in the current study.

The amount of RNA extracted from bovine MNC was approximately 10-fold that from PNC. This might be explained by the difference between the functions associated with these two cells fractions. The MNC fraction is dominated by lymphocytes whose main immune function is to produce antibodies, thus requiring a certain level of expression. In fact, memory cells, a kind of lymphocytes, can be active for years. In contrast, PNC consist mainly of polymorphonuclear granulocytes which have been characterised as terminally differentiated cells with insignificant RNA or protein synthesis, consistent with their limited quantity of ribosomes and endoplasmic reticulum (Cline, 1966; Bainton et al., 1971) and whose life span covers only a few days. In cattle, they circulate in the blood stream around 9 h and function as phagocytes in the mammary gland for 1 to 2 d (Van Oostveldt et al., 2001).

RT-PCR

The method of RT-PCR worked successfully as demonstrated by the results of the PCR control samples. Possible methodical causes of the differences observed between the RT-PCR results from human and from bovine leukocytes are discussed below in chapter 4.2.

4.1.2 ISH to detect Hp mRNA

Tissue preparation and section fixation

Tissue preparation is critical for the integrity of mRNA. For this work, tissue cubes were immediately excised after slaughter and snap frozen to prepare cryosections. Alternatively, paraffin embedded tissue can be used. The most important advantage of frozen tissue over fixed and paraffin embedded tissue is the higher preservation of intact mRNA which is essential when aiming at detecting low abundant target transcripts. A loss of up to 30% of RNA has been reported to occur during the process of tissue fixing and paraffin embedding (Jin and Lloyd, 1997; Roche, 2002). Moreover, a high level of RNase activity has been reported in rat mammary tissue (Liu et al., 1979), also emphasizing the need for suppressing RNase activity as rapidly as possible after tissue excision, in particular from mammary tissue. Therefore, since the number of Hp mRNA molecules was considered to be low in the bovine mammary gland, it was opted for preparing cryosections from snap frozen mammary tissue. However, common disadvantages of cryosections are, firstly, that more care has to be taken when handling frozen, thus not yet fixed tissue cubes and sections to minimise effects of possible active ribonucleases, secondly, the quality of morphology may considerably suffer during the preparation process and, finally, a section thickness of 10 to 20 µm may also interfere with optimal cellular localisation (Liu et al., 1998; Wilkinson, 1998). Sections from paraffin embedded tissue are usually obtained at a smaller thickness of 5 to 10 µm (Wilkinson, 1998).

In addition, cryosections were always prepared on the day of use to avoid RNA losses during storage. While storage of cryosections is possible, e.g. dehydrated in an airtight box containing a drying agent (Wilkinson, 1998; Morel and Cavalier, 2000), signal intensity was reported to be inferior in desiccated sections stored at -80°C for two days (Yang et al., 1999). On the other hand, other researchers observed no signal losses when storing dehydrated cryosections for 5 to 7 d which had already been fixed in 4% PFA (Udovic, 1993; Köhler, 2000). However, this option was not tested in the current study in order to minimise the

number of possible variables interfering with the ISH outcome. Instead, freshly prepared frozen sections underwent a fixation process immediately after cutting to stabilise the mRNA and to prevent RNase activity and were employed in the ISH immediately afterwards.

Tissue pretreatment

The main aim of treating the sections prior to hybridisation is to make the tissue permeable such that the target nucleic acid is rendered accessible to the probe. As already mentioned in the chapter "Materials and Methods" the literature reports a large variety of partly controversial options available to achieve this aim. One key finding when developing the present ISH protocol to detect Hp mRNA in mammary gland was that best staining results were yielded when omitting the entire deproteinisation step. This feature is consistent with protocols described by Jung et al. (1994) and Lavery et al. (2004), but contrary to Knabel et al. (1998) and Sinowatz et al. (2000) who applied a combination of Proteinase K and HCl to their cryosections. Obviously, no general recommendation can be made for cryosections with regard to deproteinisation. In fact, the importance of optimising the pretreatment for each single tissue type has been pointed out by other researchers (Singer et al., 1986; Yang et al., 1999). The reason as to why strongest staining signals were achieved without deproteinising the sections cannot be completely clarified. Subjecting cryosections to 4% PFA for 15 min as used herein can be considered as a mild fixation with modest cross-linking between proteins and nucleic acids (Singer et al., 1986), thus leaving the target mRNA possibly still accessible to the probe. Increased cross-linking lowers the accessibility of the target sequence (Wilkinson, 1998).

Size and homology of probe

Clearly, longer probes can give stronger signals because more labelled nucleotides can be incorporated into them. In addition, the stability of the hybrid increases with the size of the probe (Wilkinson, 1998). However, the penetration of the tissue sections might diminish with increasing probe length. A probe length of 200 to 400 nt has been recommended for optimal penetration and signalling, while longer probes can be cleaved into shorter fragments by partial alkaline hydrolysis to simplify penetration (Wilkinson, 1998). However, Yang et al. (1999) found that RNA probes (up to 2600 nt) not only readily permeated cryosections, but also yielded stronger signals than hydrolysed probes of equivalent starting length.

The introduction of a third probe, the longer homologous probe bHp2 with 302 nt, was crucial to the success of the present ISH method to localise Hp mRNA in bovine mammary gland.

Already in hepatocytes with their high abundance of Hp mRNA it was observed that neither the use of the shorter homologous bHp1 probe (174 nt), nor the heterologous, 338 nt long hHp2 probe achieved results as satisfying as with the bHp2 probe. The bHp1 probe, which was tested first, might have simply been too short to provide a sufficient number of DIG molecules for a strong ISH signal. According to Roche, the manufacturer of the DIG RNA labelling kit, every 20th to 25th nucleotide of the synthesised probe is DIG-UTP, i.e. bHp1 contained only seven to nine DIG-UTP. For that reason, the longer, though heterologous probe hHp2 was tested next. However, no improvement in the signal intensity could be recognised, which might be explained by the alignment identities of 82% between the two known cDNA sequences of bHp and hHp and 81% between the known bHp sequence and the actual hHp probe sequence, respectively. The appendix illustrates the exact alignment of these bovine and human sequences. A total of 35 mismatches becomes obvious between the bHp cDNA sequence and the hHp probe sequence. Most of these mismatches, namely 31, are confined to 1 bp only, while the other gaps are of greater size. The longest gap segment stretches even over 8 bp (bHp cDNA sequence bp 119 to 126). It is worth noting that the initial 79 nt of the human Hp probe could not be aligned to the bovine Hp cDNA sequence since of the latter a length of only 302 bp is currently known (see Appendix for details). As a result of these mismatches, less stable hybrids are likely to have been produced between the human probe and the bovine Hp mRNA sequence in situ. Therefore, this might have caused the probe to become detached subsequently by stringent washes and RNase digest yielding inferior staining signals. Finally, the third probe, bHp2, was introduced yielding optimal staining results. This probe was not only homologous but also with 302 nt almost twice as long as bHp1 and even shorter than hHp. The specificity of this bHp2 probe was not tested in this study, however, homologous hybridisation of this probe to the bHp gene had previously been demonstrated with Northern analysis (Lavery et al., 2004).

It must also be mentioned at this stage that another vital part of the established ISH protocol was the TSA system. The TSA system was added to the ISH protocol that already used the bHp2 probe; whether equally meaningful results would have been achieved in the bovine mammary gland by combining the TSA and the shorter bHp1 probe together in one protocol was not investigated.

Posthybridisation washes

A key component of the posthybridisation washes was the RNase digest, the aim of which is to increase signal specificity by reducing background noise. A number of concentrations and

combinations of two RNases (RNase A and RNase T1) were tested of which the application of 5 µg RNase A + 50 U RNase T1/ml for 30 min at 37°C was chosen. This was only half the concentrations used by Goldammer et al. (2004), but more than used by Yang et al. (1999). In fact, the latter researchers declared an RNase digest even as unnecessary, because they observed not only a considerably stronger hybridisation signal in sections without RNase digest, but most importantly non-specific background was not significantly reduced by RNase digest. While developing the current protocol for in situ localisation of Hp mRNA in the mammary gland, a loss of signal intensity was also frequently recognised when introducing an RNase digest as seen by Yang et al. (1999). Nevertheless, it was decided to maintain the RNase digest to ensure a high specificity of ISH signal.

Amplification of hybridisation signal

Two options of signal enhancers were tested: PVA as part of the substrate buffer and the tyramide signal amplification system. The alkaline phosphatase reaction with NBT/BCIP is relatively slow; reaction intermediates can diffuse into the medium and might not be available for the final precipitation of formazan. Hence, reduction of the signal can be the consequence (Van Noorden and Jonges, 1987). The addition of 10% PVA to the substrate buffer successfully enhanced this enzyme reaction resulting in a 20-fold increase in sensitivity (De Block and Debrouwer, 1993). However, no signal enhancement with 10% PVA in the substrate buffer could be demonstrated in this study which is why this option was not further pursued.

In contrast, the addition of the signal amplification system TSA to the immunodetection process, the principle of which was introduced in chapter Materials and Methods, was crucial to the outcome of this experiment and finally produced the desired strong hybridisation signals for Hp mRNA in the mammary gland. A number of studies have applied this kind of signal enhancement for detection of low abundance mRNAs (Van de Corput et al., 1998; Yang et al., 1999; Evans et al., 2003; Karatsaidis et al., 2003), of which only the former study used tyramide conjugated to DNP instead of biotin. For this work also tyramide conjugated to DNP was purchased. The advantage of DNP over biotin labelling is that DNP is not an endogenous substance in mammalian tissue which could interfere with the specificity, whereas endogenous biotin of animal tissue has to be inactivated prior to detection to avoid such interference. A negative aspect of increased sensitivity by TSA is also a possible increase in background noise. In order to overcome this side effect the manufacturer suggested decreasing probe concentration. Indeed, Yang et al. (1999) found that high non-

specific staining in lingual tissue with sense and antisense probe alike could be counteracted by reducing the probe concentration 10-fold. Although no major background signals were discovered after the introduction of the TSA system, a 10-fold lower probe concentration was also found to be sufficient in the current study. Incubation time of both antibodies, Anti-DIG-POD and Anti-DNP-AP, was prolonged from 30 min recommended by the manufacturer to 1 h as established by Yang et al. (1999) with the idea to further amplify hybridisation signal, in particular in mammary sections from *Staph. aureus* cows.

EnVision+, a two-layer dextran polymer visualisation system, was also considered when choosing a means of signal amplification. Wiedorn et al. (2001) reported that this method, although superior to conventional ISH, did not achieve the same sensitivity as the TSA-biotin system. Therefore, the TSA based signal enhancement was preferred.

Sensitivity of the ISH method applied

While Hp mRNA in epithelium cells could clearly be detected by the chosen ISH method, this was surprisingly not the case for leukocytes in the mammary gland. These cells typically infiltrate healthy and, in markedly higher numbers, infected udders (Frost et al., 1982; Gudding et al., 1984). The two cells stained in the stroma of the *E. coli* cow (Figure 3.10; B1, B2) are too few to represent leukocytes since a higher number of them can be expected within an infected gland. This lack of ISH signal is in contrast to the results of the first investigation in this current study identifying Hp mRNA in isolated bovine peripheral blood leukocytes and milk somatic cells and might be explained by an insufficient sensitivity of the ISH method applied. Lavery et al. (2004) failed to detect Hp mRNA in bovine oviduct by in situ hybridisation using radioactive probes and even the same probe sequence, yet revealed a positive signal by Northern blotting. Jin and Lloyd (1997) pointed out that a detection limit of non-radioactive ISH was 20 transcripts/cell, though Femino et al. (1998) managed to visualise even a single RNA transcript by fluorescence ISH. It is not known how many Hp mRNA transcripts are commonly present in leukocytes, in particular in polymorphonuclear granulocytes. Real-time RT-PCR analysis of these cells would be a tool to quantify that further.

On the other hand, sensitivity could be improved by increasing probe length. However, the limiting factor for increasing the length of the bHp probe is the current knowledge of the bovine partial Hp cDNA sequence of only 302 bp equalling the probe length used. Complete sequences of cDNA could be yielded by rapid amplification of cDNA ends (RACE). This is a PCR-based technique which was developed to facilitate the cloning of full-length cDNA 5'-

and 3'-ends after a partial cDNA sequence has been obtained by other methods (Schaefer, 1995).

ISH controls

Both controls worked successfully: Hp mRNA in bovine liver as well as casein mRNA in bovine mammary gland could be localised by ISH. Thus, it could be documented that on the one hand Hp mRNA could principally be visualised by chosen ISH conditions and, on the other hand, in situ localisation of an mRNA in the mammary gland was possible. Both protocols, in contrast to Hp mRNA detection in the mammary gland, could be applied without the introduction of signal amplification reflecting the high abundance of each kind of mRNA in the respective organ. However, one important difference between the two protocols is the additional deproteinisation with Proteinase K and HCl in the ISH for casein mRNA. While the exclusion of deproteinisation was crucial for the Hp mRNA analysis as mentioned before, this omission was tested only after the protocol for casein mRNA had been validated. Therefore, the actual importance of deproteinisation of mammary gland sections to detect casein mRNA cannot be resolved. Whether the greater length of the casein antisense probe (750 nt) compared to the Hp probes played a role in requiring this extra permeabilisation step was not in the interest of this study.

4.2 Discussion of the results obtained

Hp mRNA expression in bovine liver

Hp mRNA expression was detected by ISH in bovine hepatocytes, supporting the general view that the liver is the main site of Hp synthesis (Miller et al., 1951). Moreover, it confirms the findings by Lavery et al. (2004) who similarly applied the ISH method to detect Hp mRNA in bovine liver, although they used autoradiographic instead of chromogenic ISH. The staining observed in the present study was uniform throughout the liver lobules. While this is in contrast to the predominant expression of Hp mRNA in the periportal region as seen in mice (Peters et al., 1997), it might reflect a species difference between cattle and mouse. Since the specificity of the bHp2 probe used was tested by Northern analysis previously (Lavery et al., 2004), it can be excluded that the staining of hepatocytes was unspecific. Further "fine tuning" of the ISH method with reduced amount of probe, hybridisation time or

substrate incubation time might clarify the existence of a possible gradient of Hp mRNA expression also throughout the bovine liver.

Hp mRNA expression in blood leukocytes, milk somatic cells and mammary gland

This study allocates Hp mRNA expression to circulating leukocytes, milk somatic cells and mammary epithelial cells in cattle which specifies novel sites of Hp gene expression in this species not previously reported in the literature. However, Hp mRNA expression outside the liver is not unknown, but has been verified in a variety of tissues in bovines and other mammals as outlined in the introduction. In particular, the presence of Hp mRNA was demonstrated in human epidermal keratinocytes and endometrium (Sharpe-Timms et al., 2000; Li et al., 2005), representing different kinds of epithelial cells. This underlines that epithelial cells are capable of Hp gene expression and supports the current finding of Hp mRNA in the epithelium of the bovine mammary gland. Moreover, it has been established that mammary epithelial cells can transcribe IL-6 in vitro (Okada et al., 1997; Pareek et al., 2005), the main cytokine known to be in charge of inducing Hp gene expression in bovine hepatocytes (Yoshioka et al., 2002). In fact, the study by Pareek et al. (2005) demonstrated even a 10-fold increase of IL-6 mRNA abundance 6 h after LPS challenge. The precise regulation of Hp gene expression in the bovine mammary gland is not known, however, if it also required a cytokine stimulus as in liver, these latter studies would further emphasize the specificity of the ISH signals for Hp mRNA in the mammary epithelium obtained in the present experiment, particularly seen in sections from cows with *E. coli* infection.

Besides Hp mRNA expression, also the expression of other APPs is well recognised in the bovine mammary gland epithelium, e.g. lactoferrin, mammary-associated-SAA 3 and mammary SAA 3 homologue (Molenaar et al., 1996; McDonald et al., 2001; Molenaar et al., 2002).

Hp mRNA expression detected in bovine leukocytes is in agreement with one human study reporting on Hp mRNA expression in eosinophils (Yang et al., 2000). Similarly, lymphocytes, monocytes and plasma cells express mRNA of another APP, namely SAA, though this again was proven only in humans or human cell lines (Ray and Ray, 1997; Urieli-Shoval et al., 1998). The Hp mRNA expression discovered in bovine milk somatic cells confirms the present results obtained for the blood cells as well as mammary epithelial cells of this species, since milk somatic cells generally comprise more than 90% blood-derived cells next to epithelial cells exfoliated from mammary epithelium (Harmon, 1994; Boutinaud and Jammes, 2002).

Most importantly, this cellular localisation of Hp gene expression advances the recent finding of Hp mRNA in homogenates of the bovine udder (Hiss et al., 2004). Their method did not allow to distinguish between different cell types expressing Hp mRNA. The current study clearly specified possible sites of Hp mRNA synthesis in the mammary gland as epithelium cells of the gland itself as well as immune cells of the blood.

The effects of intramammary infection and type of pathogen on the transcript level of Hp detected in the mammary gland by ISH will be discussed below.

In humans, Hp mRNA was detected in isolated leukocytes. These latter results varied between individuals. Moreover, Hp specific bands were seen only after reamplification of first course PCR products. The lack of Hp mRNA in human leukocytes by our standard RT-PCR method, though in contrast to bovines, is in agreement with results from the less sensitive Northern Blot analysis of human blood cells by Wagner et al. (1996). However, the presence of Hp specific transcripts after reamplification of the first course PCR products in two out of four individuals might reflect a weak Hp gene expression in human leukocytes. Even in the cells of the fourth subject a distinct signal of Hp specific PCR products was observed after reamplification despite a reduced intensity of the band of the housekeeping gene β -actin. Yang et al. (2000) detected Hp mRNA in circulating eosinophils of healthy donors by ISH, but not in other blood cells. Eosinophils typically comprise only around 2% of total leukocytes in healthy individuals (Klinke and Silbernagel, 1994). The differential cell count of the four test persons in this current work revealed 1-3% eosinophils of total leukocytes, thus all being close to the normal values. Therefore, the fact that only a minority of leukocytes appear to exhibit Hp mRNA expression, might explain the extremely low levels observed in the present samples.

The current study identified Hp mRNA not only in extracts of the human mammary gland but is also the first study to report on Hp mRNA expression in this organ. This finding is in accordance with Hiss et al. (2004) who showed the equivalent for cows. Clearly, standard RT-PCR results cannot be compared in absolute terms. However, given the low abundance of Hp mRNA in human leukocytes as determined by PCR in contrast to the strong PCR signal in the human mammary gland, this might be interpreted as an indication of Hp mRNA synthesis locally within this gland. As shown within this study for cows, alveolar epithelium cells are possible candidates. Gene expression of another acute phase protein, namely SAA, in normal human breast epithelium could already be established (Urieli-Shoval et al., 1998).

Nevertheless, here still remains the task to identify the exact cell type of Hp mRNA expression which is possible via ISH.

Hp mRNA expression: bovine vs. human leukocytes

The difference observed between cattle and man in the abundance of Hp transcripts in leukocytes, as determined by PCR amplification and reamplification, might be explained by two main factors: methodical or species differences. On the one hand, species specific PCR primers were applied to the samples of bovine and human origin. While primers have an effect on the PCR amplification rate (Cuypers et al., 1992), nothing is known about the efficiency of the two primer pairs bHp1 and hHp applied in this current leukocyte analysis. Hence, their PCR results cannot be compared quantitatively in a precise manner. On the other hand, a strong band for the human liver control was visualised by gel electrophoretic resolution, the intensity of which was similar to the signal intensity of the bovine liver and even bovine blood cell samples. From this might be speculated that the entire absence of first course PCR products and the presence of reamplification products in only 50% of the leukocyte samples of the examined individuals might indeed reflect a lower abundance of Hp mRNA in these human cells and, thus, a species difference between man and cattle regarding Hp mRNA presence in circulation leukocytes. This difference of Hp mRNA abundance could be determined by a generally lower expression rate, but similar degradation rate of Hp mRNA or, alternatively, by a similar expression rate, but higher degradation rate in humans compared to bovines. Quantitative real-time RT-PCR might elucidate this possible difference further, although not identical primer pairs applied to bovine and human samples would again be a limiting factor for proper comparison. Despite these uncertainties, a well known difference between cattle and man exists concerning the acute phase protein Hp. In cattle, Hp is considered to be one of the most prominent indicators of inflammation (Eckersall and Conner, 1988), whereas in man the Hp blood concentration reacts only moderately to inflammation (Heinrich et al., 1990). Instead, CRP is the most sensitive APP in man (Heinrich et al., 1990).

Effects of intramammary infection on local Hp mRNA expression as well as on Hp protein levels in blood and milk

ISH with sections from intramammarily infected bovine udders revealed that the transcript level of Hp detected in each mammary gland quarter was remarkably influenced by type as well as duration of infection. The substantial rise in the abundance of Hp mRNA in mammary quarters during the progress of *E. coli* infection indicates a strong immune response by the

cow. This also confirms the changes of Hp mRNA expression found in mammary gland biopsies after *E. coli*-LPS intramammary challenge (Hiss et al., 2004). In that study, a first elevation of Hp mRNA transcripts was detected already 3 h post challenge, increasing further thereafter. In contrast, in *Staph. aureus* treated cows, no marked changes in Hp mRNA expression in the mammary gland could be recognised during the initial 24 h of infection, which could be interpreted in the way that these cows maintained their basal level of expression. No literature reporting on Hp mRNA in response to *Staph. aureus* intramammary infection could be sourced for comparison. However, mammary epithelium cells exposed in vitro 24 h to LPS responded with a significant 10-fold increase in SAA mRNA expression, in contrast to in vitro infection with *Staph. aureus* which caused no significant changes in SAA mRNA (Wellnitz and Kerr, 2004). The low expression of Hp mRNA observed herein in the control quarters of both treatment groups indicates a general basal level of Hp expression within the bovine mammary gland. Although an increase in Hp mRNA expression was noticed also in control quarters in the above mentioned study by Hiss et al. (2004), this elevation was attributed to a local immune response to biopsy sampling from those quarters.

Similarly to the mRNA levels, *E. coli* and *Staph. aureus* exerted different effects also on Hp protein levels in both blood and milk. *E. coli* treatment led to substantially elevated mean Hp concentrations in both blood (55-fold) and particularly milk (165-fold), whereas *Staph. aureus* treatment caused only minimal changes (5-fold increase in blood Hp and no increase in milk Hp in one cow; negligible changes in blood and milk of the two other cows). The latter findings contradict results from another study involving dairy cows also experimentally infected with *Staph. aureus* into the mammary gland (Gronlund et al., 2003), which exhibited 20-fold increases in blood as well as milk Hp levels. However, these researchers measured the first above normal values in blood and in milk only 24 h post-infection, and the peak values were reached after 72 h and 54 h, respectively. This suggests that in the present experiment the time period of the first 24 h post-inoculation might have been insufficiently long to comprise the entire immune response to *Staph. aureus* and, thus, might account for the lack of noticeable changes in Hp mRNA and protein levels. On the other hand, the early and significant immune response in terms of Hp protein concentration discovered in both blood and milk to *E. coli* intramammary infection is in accordance with the findings by Salonen et al. (1996) and Hiss et al. (2004) obtained from similar experimental intramammary challenges. Salonen et al. (1996), studying Hp levels only in blood, documented an elevation above normal values after 12 h and reaching the peak value 72 h post-challenge equalling to a 52-fold total increase. More interestingly, Hiss et al. (2004) detected within hours not only a

significant change to basal values of blood concentration, but also a significant rise in milk Hp concentration following intramammary infection with *E. coli*-LPS. In fact, while in their study basal concentrations of blood and milk Hp were numerically similar to those in the current study, the respective values rose 10 and 150-fold after 12 h, but only less than 2 and 20-fold in this current investigation after the same time. This stronger response might be due to their use of the endotoxin directly rather than the original bacterium *E. coli* or due to the use of a different *E. coli* strain for endotoxin extraction.

These observed divergent effects of *E. coli* and *Staph. aureus* on Hp transcript and on protein levels in the mammary gland and in blood and milk, respectively, are consistent with general differences reported on the immune response to these two pathogens. Intramammary infection by *E. coli* is acute in nature, eliminated spontaneously by the cow and, thus, cured within a few days (Smith and Hogan, 1993; Hoebe et al., 2000). In contrast, while an infection with *Staph. aureus* can also start with an acute phase, it is mostly less severe and often becomes chronic and subclinical (Sutra and Poutrel, 1994). It was speculated that the latter effect might be due to an insufficient immune response to this pathogen (Sutra and Poutrel, 1994). A key component of the immune response is the upregulation of cytokine production in macrophages and endothelial cells (Dinarello, 2000; Arredouani et al., 2005). Subsequently, cytokines initiate not only the recruitment of leukocytes to inflammatory sites but also the enhanced synthesis of acute phase proteins as part of the acute phase response (Cybulsky et al., 1988; Ohtsuka et al., 2001; Yoshioka et al., 2002). Besides TNF- α , IL-1 and IL-6 already mentioned in the introduction, IL-8 is another important cytokine produced at higher rates during the immune response and mediates the transendothelial neutrophil migration (Huber et al., 1991). Therefore it is not surprising that studies applying intramammary infection models with *E. coli* and *Staph. aureus* demonstrated indeed differences in the level of blood and even milk cytokines. For instance, after intramammary infection with *E. coli* a strong and rapid concentration increase was detected of Hp protein in blood as well as of inflammatory cytokines in whey, such as TNF- α , IL-1 β and IL-8, compared to only a moderate increase in Hp blood concentrations and even absence of detectable cytokines in milk in *Staph. aureus* infected animals (Riollet et al., 2001). Similarly, both *E. coli* and *Staph. aureus* intramammary infection induced increases in milk IL-1 β levels, although with a delay in the *Staph. aureus* cows, but only *E. coli* elicited the production of TNF- α and IL-8 (Bannerman et al., 2004). In summary, these prominent differences in cytokine concentrations exerted by *E. coli* and *Staph. aureus* as seen in the previous two studies might account for the different

outcomes in Hp mRNA and protein levels analysed in the present study applying the same kinds of mastitis pathogens.

Other elements involved in the immune response are Toll-like receptors (TLR) immediately at the beginning of signalling cascade. TLR are recognised as cell surface receptors located on phagocytes. After recognition of pathogens they initiate a signalling pathway to counteract these pathogens at the end of which stands the expression of certain genes, in particular of cytokines (Takeda and Akira, 2005). Different kinds of TLR come into play depending on the pathogen present. For example, TLR 2 is generally described as the essential receptors for components of Gram-positive bacteria (e.g. *Staph. aureus*) and TLR4 as the essential receptor of LPS as a major constituent of Gram-negative bacteria (e.g. *E. coli*) (Takeda and Akira, 2005). However, Goldammer et al. (2004) reported that TLR 2 as well as TLR 4 increased simultaneously in bovine mammary glands during mastitis, but interestingly even markedly higher in glands infected with *Staph. aureus* compared to *E. coli*. These results are unexpected and do not fit in with the general findings on the immune response to *E. coli* and *Staph. aureus* mastitis.

A weakness of this study is the limited knowledge of the actual virulence of the specific strains used in this trial and how representative they are for strains causing mastitis. Since they were isolated and cultured from clinical mastitis cases, it can be assumed that they cause clinical mastitis again after experimental reapplication into the mammary gland. Obviously, the severity of the disease is also dose dependant, but no general recommendation can be made. The current study used a dose of 500 and 10,000 cfu/quarter of *E. coli* and *Staph. aureus*, respectively, whereas Bannerman et al. (2004) achieved clinical mastitis with as little as 72 to 74 cfu/quarter of either bacteria type and yet observed significant variations in the immune response. Again other researchers applied 1500 cfu and 100,000 cfu/quarter of *E. coli* and *Staph. aureus*, respectively (Salonen et al., 1996; Gronlund et al., 2003). Hence, it is difficult to judge how representative the infection models used in this study are for *E. coli* and *Staph. aureus* mastitis. However, evaluating the Hp response during the progress of mastitis by applying the bacteria into the quarters of one mammary gland at different time points relative to slaughter is a valid model, as has been verified by other researchers (Frost et al., 1982; Frost et al., 1984). Furthermore, the fact that the local immune response within the mammary gland remains confined to the affected quarter has been well established (Frost et al., 1984; Mehrzad et al., 2004).

Potential physiological benefits of local Hp expression in immune and mammary gland cells

The physiological benefit of local Hp expression in immune and mammary epithelial cells and, in particular, the rapid availability of the protein in the mammary gland during infection might be manifold. One important characteristic of Hp is its down-regulation of the host immune response by acting on the circulating immune cells: Hp suppresses production of pro-inflammatory, but not anti-inflammatory cytokines in monocytes, inhibits the respiratory burst activity in neutrophils and reduces lymphocyte proliferation as was shown in vitro (Baseler and Burrell, 1983; Oh et al., 1990; Arredouani et al., 2005). All these three functions of Hp might come into play also in the mammary gland, especially during *E. coli* infection where the rapid rise of Hp mRNA was observed. For instance, the presence of Hp in epithelial cells might allow the former effect to occur likewise in these cells capable of producing also the pro-inflammatory cytokines TNF- α and IL-1 (Okada et al., 1997). In fact, by producing simultaneously IL-6, the main inducer of Hp protein synthesis, these cells might exert an overall negative feedback on pro-inflammatory cytokine production within themselves. Furthermore, since the influx of neutrophils occurs already within a few hours after onset of intramammary infection, particularly *E. coli* infection, the almost immediate enhancement of local Hp production to encounter possible tissue damage caused by neutrophil respiratory burst activity might be advantageous over the supply delay resulting from systemic up-regulation of Hp production in the liver.

Another well known ability of Hp is to bind haemoglobin and thereby not only hampering the iron requiring process of bacterial replication as was shown for *E. coli* (Eaton et al., 1982), but also inhibiting oxidative damage caused by free haemoglobin (Miller et al., 1997). A bacteriostatic effect of Hp on *Staph. aureus* has yet to be defined. Red blood cells can appear in the milk of the inflamed quarters as a result of the dramatic alteration in mammary blood flow and microvascular integrity during mastitis (Vandeputte-Van Messom et al., 1993).

Finally, Hp is able to promote tissue repair through stimulating angiogenesis as has been reported under chronic inflammatory conditions (Cid et al., 1993). Tissue damage is a feature often accompanying mastitis, in particular *E. coli* mastitis (Burvenich et al., 2003).

5 Conclusions

The current study clearly identified novel sites of mRNA expression of the acute phase protein haptoglobin in cattle, namely circulating immune cells, milk somatic cells and epithelial cells of the mammary gland. Hp mRNA presence was verified even in both subpopulations of the peripheral blood cells examined: the mononuclear cells and the polymorphonuclear cells. In addition, besides generally assigning Hp mRNA expression in the mammary gland to the alveolar epithelium, it can be concluded from the experimental infection model that some mastitis pathogens are able to alter level of Hp mRNA expression in these cells. *E. coli* caused a marked upregulation of Hp mRNA expression within hours after start of infection, whereas Hp mRNA expression in response to *Staph. aureus* infections appeared unchanged over 24 h. These differences in mRNA presence were matched by Hp protein concentrations measured in blood and milk. Finally, Hp mRNA expression became evident in human leukocytes and human mammary gland. In conclusion, this study identified novel cellular sites of Hp mRNA expression in dairy cows and, thus, possible sources of the Hp protein found in milk. It also confirmed that Hp plays a role in the encounter of bacterial infections in the bovine mammary gland. However, the explicit significance of this local production and immediate availability of the protein have to be determined by future studies.

6 Literature

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7 Appendix

7.1 Chemicals, solutions, consumables, instruments and software

Chemicals

Applichem, Darmstadt, Germany

Agarose, boric acid, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), 1-bromine-3-chlorine propane, 50x Denhardt's solution, diethylpyrocarbonate (DEPC), ethylenediaminetetraacetic acid disodium salt (EDTA), formamide deionised, isoamyl alcohol, 3-morpholinopropanesulfonic acid (MOPS), nitro blue tetrazolium (NBT), potassium chloride (KCl), Proteinase K, sodium acetate anhydrous, sodium chloride (NaCl), tri-sodium citrate dihydrate ($3 \text{ Na-citrate} * 2\text{H}_2\text{O}$)

BD Biosciences, Heidelberg, Germany

Human mammary gland total RNA

Carl-Roth GmbH & Co, Karlsruhe, Germany

37% Formaldehyde, glacial acetic acid, glycerine, guanidiniumthiocyanate (GTC), hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), isopropanol, lithium chloride (LiCl), magnesium chloride hexahydrate ($\text{MgCl}_2 * 6 \text{ H}_2\text{O}$), 2-mercaptoethanol, phenol, potassium aluminium sulphate, sodium hydroxide (NaOH) pellets, Tris-hydroxymethyl aminomethane (Tris-Base), Tween[®]20

Chroma-Gesellschaft, Münster, Germany

Orange G

Eurobio, Les Ulis Cedex B, France

Ethidium bromide (10 mg/ml)

Fermentas, St. Leon-Rot, Germany

Desoxynucleotides triphosphates (dNTP), DNA marker Φ X174 DNA/*Bsu*RI (*Hae*III), magnesium chloride (MgCl_2), RevertAid™ M-MuLV reverse transcriptase and 5x reaction buffer, RiboLock™ Ribonuclease Inhibitor, RNase A, RNase T1, *Taq* DNA polymerase and 10x *Taq* buffer with ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$)

Invitrogen, Karlsruhe, Germany

Agarose, primer, random hexamers

KMF Laborchemie Handels GmbH, Lohmar, Germany

Ethanol denatured with 1% methyl ethyl ketones

Merck Eurolab GmbH, Darmstadt, Germany

Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$), Mayer's hemalum solution, di-methyl sulfoxide (DMSO), Kaiser's glycerol gelatine, di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$), potassium dihydrogen phosphate anhydrous (KH_2PO_4)

Oxoid GmbH, Wesel, Germany

Kochsalz-Tabletten für Bakteriologie

PerkinElmer, LAS, Rodgau Jügesheim, Germany

TSA Plus DNP AP kit

Qiagen, Hilden, Germany

QIAquick PCR-purification kit

Roche Diagnostics GmbH, Mannheim, Germany

Anti-Dig-AP Fab fragments, blocking reagent, DNase I and 10x reaction buffer, fish sperm DNA (10 mg/ml), DIG RNA labelling kit

Ratiopharm GmbH, Ulm, Germany

Heparin-Natrium-5000-ratiopharm (5000 IU/0.2 ml ampoule)

Serva, Electrophoresis GmbH, Heidelberg, Germany

Trypan Blue stain

Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

Acetic anhydride, bromophenol blue solution saturated, dextran sulphate, Histopaque®-1077, di-methylformamide (DMF), lauroylsarcosine sodium salt, levamisole, maleic acid, 0.1%

Nuclear Fast Red in 5% aluminium sulphate, paraformaldehyde (PFA), polyvinyl alcohol 70-100 kDa (PVA), primer, triethanolamine (TEA), yeast tRNA

Vogel Medizinische Technik und Elektronik, Gießen, Germany

Tissue Tek[®] O.C.T.[™] Compound (Sakura)

Buffers and solutions – general

DEPC-treated H₂O

1000 ml ultra-pure H₂O

0.1% = 1 ml DEPC

It was mixed thoroughly several times at room temperature, incubated under safety hood overnight and autoclaved.

0.5 M EDTA

0.5 M = 46.53 g EDTA

ad 250 ml ultra-pure H₂O

pH was adjusted to 8.0. Autoclaved.

0.9% NaCl

0.9 % = 2 tablets NaCl

ad 1000 ml ultra-pure H₂O

Autoclaved.

10x PBS

1.37 M = 80 g NaCl

0.027 M = 2 g KCl

0.015 M = 2 g KH₂PO₄ anhydrous

0.096 M = 15 g Na₂HPO₄ * 2 H₂O

ad 1000 ml ultra-pure H₂O

pH was adjusted with NaOH to 7.4. Buffer was treated with 0.1% (1 ml) DEPC as above and autoclaved.

1x PBS

100 ml	10x PBS
900 ml	ultra-pure H ₂ O

For in situ hybridisation buffer was treated with 0.1% (1 ml) DEPC as above and autoclaved.

2x PBS

200 ml	10x PBS
800 ml	ultra-pure H ₂ O

80% Ethanol for cleaning

800 ml	100% Ethanol, denatured
200 ml	ultra-pure H ₂ O

Ultra-pure H₂O

Water, pre-purified by reverse osmosis, was further purified by ion exchange resins to a resistivity of 18.2 MΩ*cm, photo-oxidised (185 nm) and ultra-filtered (0.2 µm) (Elga Labwater USF PureLab Plus UV/UF). Finally, this water was autoclaved.

Buffers and solutions for RNA extraction, RT-PCR and gel electrophoreses**1-Bromine-3-chlorine propane**

48 ml	1-Bromine-3-chlorine propane
1 ml	Isoamyl alcohol

Stored at 4°C.

5x DNA loading buffer

50 mg	Orange G
2 ml	Glycerine
400 ml	0.5 M EDTA, pH 8.0
ad 10 ml	ultra-pure H ₂ O

Aliquots were stored at -20°C and 4°C long term and short term, respectively.

Ethidium bromide, 1 mg/ml

Ethidium bromide (10 mg/ml) was diluted 1:10 in DEPC-treated H₂O.

GTC stock solution

4 M = 47.26 g GTC

Some DEPC-treated H₂O was added.

25 mM = 3.33 ml 0.75 M Na-citrate, pH 7.0

0.5% = 5 ml 10% (w/v) Lauroylsarcosine sodium salt

75 ml DEPC-treated H₂O

The reagents were solved, if necessary by heating to 50°C.

ad 100 ml DEPC-treated H₂O

Stored at 4°C for up to three months.

GTC working solution

100 ml GTC stock solution

780 µl 2-Mercaptoethanol

Stored at 4°C up to one month.

10% (w/v) Lauroylsarcosine sodium salt

10% = 5g Lauroylsarcosine sodium salt

ad 50 ml ultra-pure H₂O

10x MOPS buffer

0.2 M = 41.8 g MOPS

800 ml ultra-pure H₂O

pH was adjusted to 7.0.

50 mM = 16.6 ml 3 M Na-acetate

10 mM = 20 ml 0.5 M EDTA, pH 8.0

ad 1000 ml ultra-pure H₂O

Autoclaved and stored at room temperature protected from light.

2 M Na-acetate

2 M = 16.4 g Sodium acetate, anhydrous

40 ml Glacial acetic acid

Heated to 40°C and pH adjusted to 4.0.

ad 100 ml DEPC-treated H₂O

3 M Na-acetate

3 M = 24.6 g Sodium acetate, anhydrous
 ad 100 ml ultra-pure H₂O
 No pH adjustment.

0.75 M Na-citrate

0.75 M = 22.06 g 3-Na-citrate * 2 H₂O
 ad 100 ml DEPC-treated H₂O
 pH was adjusted to 7.0.

5x RNA loading buffer

226 µl Bromophenol blue solution saturated
 20 µl 0.5 M EDTA, pH 8.0
 25 µl Ethidium bromide (10 mg/ml)
 180 µl 37% Formaldehyde
 500 µl 100% Glycerine
 771 µl Formamide, deionised
 1000 µl 10x MOPS buffer
 Aliquots were stored at 4°C for up to three months.

10x TBE buffer

890 mM = 108 g Tris-Base
 890 mM = 55 g Boric acid
 20 mM = 40 ml 0.5 M EDTA, pH 8.0
 ad 1000 ml ultra-pure H₂O
 Autoclaved and stored at room temperature.

Buffers and solutions for in situ hybridisation**BCIP (50 mg BCIP/ml DMSO)**

300 mg BCIP
 ad 6 ml DMSO

Aliquots were stored at -20°C and 4°C long term and short term, respectively, protected from light. Working aliquot was brought to room temperature prior to usage, since DMSO freezes at 4°C.

10% Blocking Reagent

10% = 20 g Blocking reagent

150 ml MA buffer

Mixture was brought to boil thrice in microwave and stirred gently in between. After cooling down to room temperature:

ad 200 ml MA buffer

Solution was autoclaved and aliquots were stored at -20°C.

1 M CaCl₂

1 M = 14.7 g CaCl₂*2H₂O

ad 100 ml DEPC-treated H₂O

Autoclaved.

50% Dextran sulphate

50% = 2 g Dextran sulphate

2.5 ml DEPC-treated H₂O

This was left to dissolve overnight.

ad 4 ml DEPC-treated H₂O

This was always prepared fresh in a 25 ml measuring cylinder one day prior to preparation of hybridisation buffer.

DNP amplification reagent stock solution

DNP amplification reagent (TSA Plus DNP AP kit) reconstituted in 0.3 or 0.15 ml DMSO, depending on kit size. Stored at 4°C.

0.2 M HCl

16.56 ml 37% HCl

ad 1000 ml DEPC-treated H₂O

Not autoclaved.

Hybridisation buffer, 20 ml

To

10% = 4 ml 50% Dextran sulphate

was added the following:

150 mM = 1200 μ l 2.5 M NaCl

50% = 10 ml Formamide, deionised

2x = 2 ml 20x SSC

0.2 mg/ml = 400 μ l 10 mg/ml yeast tRNA

1x = 400 μ l 50x Denhardt's solution

1 mg/ml = 2 ml 10 mg/ml Fish sperm DNA

This was mixed thoroughly and left to stay until air bubbles had disappeared. Aliquots (1 ml) were stored at -20°C.

4 M LiCl

4 M = 16.96 g LiCl

ad 100 ml ultra-pure H₂O

Solution was treated with 0.1% (100 μ l) DEPC as above and autoclaved.

MA buffer

100 mM = 11.61 g MA

150 mM = 8.77 g NaCl

pH was adjusted to 7.5 with NaOH pellets and 5 M NaOH.

ad 1000 ml ultra-pure H₂O

Autoclaved.

2.5 M NaCl

2.5 M = 14.61 g NaCl

ad 100 ml ultra-pure H₂O

Solution was treated with 0.1% (100 μ l) DEPC as above and autoclaved.

NBT (75 mg NBT/ml 70% DMF)

300 mg NBT

ad 4 ml 70% DMF

Aliquots were stored at -20°C and 4°C long term and short term, respectively, protected from light.

4% (w/v) PFA

10 g	PFA
220 ml	1x PBS

This was brought to 65°C under ventilation hood and then 5 µl 5 M NaOH was added for the solution to become clear. If necessary, the solution was left at 65°C for 30 to 60 min until PFA was completely dissolved. After cooling to room temperature, pH was adjusted to 7.3.

ad 250 ml	1x PBS
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Stored protected from light and used within two weeks (preferably prepared fresh).

Proteinase K (20 mg/ml)

20 mg	Proteinase K
1 ml	DEPC-treated H ₂ O

Aliquots were stored at -20°C and after thawing once not reused.

PVA substrate buffer

10% = 10 g	PVA
90 ml	Substrate buffer 1

It was dissolved at 90°C and brought again to room temperature:

ad 100 ml	Substrate buffer 1
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RNA dilution buffer

5 ml	DEPC-treated H ₂ O
3 ml	20x SSC
2 ml	37% Formaldehyde

Aliquots were stored at -20°C.

20x SSC

3 M = 87.66 g	NaCl
0.3 M = 44.12 g	3 Na-citrate * 2H ₂ O
ad 500 ml	ultra-pure H ₂ O

pH was adjusted to 7.0. Solution was treated with 1% (500 µl) DEPC as above and then autoclaved.

2x SSC

20x SSC was diluted 1:10 in DEPC-treated H₂O or ultra-pure H₂O for work pre- or post-hybridisation, respectively.

Substrate buffer 1

100 mM = 12.1 g Tris-Base

100 mM = 5.84 g NaCl

pH was adjusted to 9.5 with 5 M HCl.

ad 1000 ml ultra-pure H₂O

Autoclaved.

Substrate buffer 2

100 mM = 12.1 g Tris-Base

100 mM = 5.84 g NaCl

800 ml ultra-pure H₂O

pH was adjusted to 9.5 with 5 M HCl.

50 mM = 10.16 g MgCl₂ * 6 H₂O

ad 1000 ml ultra-pure H₂O

Buffer was filtered sterilize. It was not autoclaved to avoid Mg-precipitates.

0.1 M TEA buffer

0.1 M = 13.2 ml TEA

900 ml DEPC-treated H₂O

pH was adjusted to 8.0 with 37% HCl.

ad 1000 ml DEPC-treated H₂O

Autoclaved and stored at room temperature protected from light.

TE buffer

10 mM = 5 ml 1 M Tris-HCl

1 mM = 1 ml 0.5 M EDTA

pH was adjusted to 8.0.

ad 500 ml ultra-pure H₂O

Autoclaved.

TN buffer

0.1 M = 12.11g Tris-Base
0.15 M = 8.77 g NaCl
pH was adjusted to 7.5 with 37% HCl.
ad 1000 ml ultra-pure H₂O

TNB blocking buffer

0.5% = 0.5 g Blocking reagent (TSA Plus DNP AP kit)
100 ml TN buffer

Blocking reagent was added slowly in small increments to TN buffer while stirring and, if necessary, heated to 60°C to dissolve completely. Aliquots were stored at -20°C.

TNT wash buffer

0.05% = 2.5 ml 10% Tween 20
497.5 ml TN buffer

1 M Tris-HCl

1 M = 60.57 g Tris-Base
pH was adjusted to 8.0 with 37% HCl.
ad 500 ml DEPC-treated H₂O
Autoclaved.

Yeast tRNA (10 mg/ml)

25 mg = 500 units Yeast tRNA
2500 µl DEPC-treated H₂O

tRNA was dissolved by incubating on ice for at least 1 h. Aliquots were stored at -20°C.

Important consumables**Amersham Pharmacia Biotech, Uppsala, Sweden**

Nylon membrane Hybond-N+

Carl-Roth, Karlsruhe, Germany

Hellendahl jars, Rotilabo[®]-Probenröhrchen (7 ml sample vial)

Menzel-Gläser, Braunschweig, Germany

SuperFrost[®] Plus microscope slides

Sigma, Taufenkirchen, Germany

Hybri-Slips (40 mm x 24 mm)

Zytomed, Berlin, Germany

PAP Pen Super

Main instruments

Autoclave	Systec-Table Autoklave, Model 2540 EL, Tuttnauer, Breda, The Netherlands
Camera	Polaroid DMC Ie, Cambridge, MA, USA
Centrifuge	Beckman CL-GPKR (swing out rotor), Munich, Germany
Centrifuge	Biofuge primo R, Heraeus Instruments GmbH, Osterode, Germany
Centrifuge	1K15, Sigma, Taufkirchen, Germany
Cryostat	CM 3050 S, Leica, Nussloch, Germany
Fluor Imager SI	Amersham Pharmacia Biotech, Uppsala, Sweden
HeizThermoMixer	HTM 130 RP, HLC, Haep Labor Consult, Bovenden, Germany
Microscope	DM LB, Leica, Wetzlar, Germany
Microscope	Routine microscope, Axioskop 2 MOT, Zeiss, Jena, Germany
pH Meter	Orion, ORI037002, pH/mV/ISE/Temp Meter, Colora, Lorch, Germany
Shaker	3005, Gesellschaft für Labortechnik (GFL), Burgwedel, Germany
Spectrophotometer	U-2000/1 Hitachi UV/VIS-Computer-Double-Beam-Spectrophotometer, Colora, Lorch, Germany
Thermocycler	MJ Research, Biozym Diagnostik, Oldendorf, Germany
Thermocycler	Trio-Thermocycler V2.23 [©] Biotron 190, Biometra, Göttingen, Germany
Ultra Turrax	T25, IKA Labortechnik, Staufen, Germany
UV lamp	TCP-20.M Vilbert Lourmat, Marne-la-Vallée Cedex 1, France

Water purifier	Elga Labwater, PureLab Plus™ UV/UF, Celle, Germany
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Software

BLAST programme	http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1990)
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Peak Finder	ImageQuaNT™, Version 4.1, Molecular Dynamics, Amersham Pharmacia Biotech, Uppsala, Sweden
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Statistics programme	SPSS 12.0, Chicago, Illinois, USA
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Volume Report	ImageQuaNT™, Version 4.1, Molecular Dynamics, Amersham Pharmacia Biotech, Uppsala, Sweden
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7.2 Hp cDNA sequences and alignment

Partial bovine Hp cDNA sequence

Source: PubMed, Nucleotide, accession number: AJ 271156

bHp1 primers in bold embracing 174 nt (33-206 nt):

```

1  ccaaaggcag ctttccttgg caggccaaga tggttctcca gcataacctc atctcgggag
61 ccacgctcat caatgaacga tggctcctca ccacagctaa aaatctctac ctgggtcaca
121 gtagtgacaa aaaagcaaag gacatcactc ctactttaag actctatgtg gggaagaacc
181 agcttgtaga ggtggagaag gtgggttctcc accctgacca ctccaaggta gacattgggc
241 tcatcaaact cagacagaag gtacctgtca atgacaaagt aatgcccatac tgcctacctt
301 cc

```

bHp2 primers in bold embracing 302 nt (1-302 nt):

```

1  ccaaaggcag ctttccttgg caggccaaga tggttctcca gcataacctc atctcgggag
61 ccacgctcat caatgaacga tggctcctca ccacagctaa aaatctctac ctgggtcaca
121 gtagtgacaa aaaagcaaag gacatcactc ctactttaag actctatgtg gggaagaacc
181 agcttgtaga ggtggagaag gtgggttctcc accctgacca ctccaaggta gacattgggc
241 tcatcaaact cagacagaag gtacctgtca atgacaaagt aatgcccatac tgcctacctt
301 cc

```

Partial human Hp cDNA sequence

Source: PubMed, Nucleotide, accession number: NM_005143

hHp primers in bold embracing 338 nt (471-808 nt):

```

1  agatgccccca cagcactgct cttccagagg caagaccaac caagatgagt gccctgggag
61  ctgtcattgc cctcctgctc tggggacagc tttttgcagt ggactcaggc aatgatgtca
121 cggatatcgc agatgacggc tgcccgaagc cccccgagat tgcacatggc tatgtggagc
181 actcggttcg ctaccagtgt aagaactact acaaactgcg cacagaagga gatggagtat
241 acaccttaaa tgataagaag cagtggataa ataaggctgt tggagataaa cttcctgaat
301 gtgaagcaga tgacggctgc ccgaagcccc ccgagattgc acatggctat gtggagcact
361 cggttcgcta ccagtgtaa aactactaca aactgcgcac agaaggagat ggagtgtaca
421 ccttaaacaa tgagaagcag tggataaata aggctgttgg agataaactt cctgaatgtg
481 aagcagtatg tgggaagccc aagaatccgg caaaccacag gcagcggatc ctgggtggac
541 acctggatgc caaaggcagc tttccctggc aggctaagat ggtttcccac cataatctca
601 ccacaggatgc cacgctgatc aatgaacaat ggctgctgac cacggctaaa aatctcttcc
661 tgaaccattc agaaaatgca acagcgaaaag acattgcccc tactttaaca ctctatgtgg
721 ggaaaaagca gcttgtagag attgagaagg ttgttctaca ccctaactac tcccaggtag
781 atattggggt catcaaactc aaacagaagg tgtctgttaa tgagagagtg atgcccatct
841 gcctaccttc aaaggattat gcagaagtag ggcgtgtggg ttatgtttct ggctgggggc
901 gaaatgcaa ttttaaattt actgaccatc tgaagtatgt catgctgcct gtggctgacc
961 aagaccaatg cataaggcat tatgaaggca gcacagtccc cgaaaagaag acaccgaaga
1021 gccctgtagg ggtgcagccc atactgaatg aacacacctt ctgtgctggc atgtctaagt
1081 accaagaaga cacctgctat ggcgatgcgg gcagtgcctt tgccgttcac gacctggagg
1141 aggacacctg gtatgcgact gggatcttaa gctttgataa gagctgtgct gtggctgagt
1201 atggtgtgta tgtgaagggt acttccatcc aggactgggt tcagaagacc atagctgaga
1261 actaatgcaa ggctggccgg aagcccttgc ctgaaagcaa gatttcagcc tggaaagagg
1321 caaagtggac gggagtggac aggagtggat gcgataagat gtggtttgaa gctgatgggt
1381 gccagccctg cattgctgag tcaatcaata aagagctttc ttttgaccca ttt

```

Alignment of bovine and human Hp cDNA sequences

Source: PubMed, Basic local alignment search tool (BLAST) (Altschul et al., 1990)

Identities = 248/301 (82%)

The fragment contained in the hHp probe is set in bold, i.e. 259 nt of the hHp2 probe (base 550 to 808) can be aligned to the currently known bovine cDNA sequence. Identities = 212/259 (81%). The starting sequence (79 nt) of the hHp2 probe (base 470 to 549) cannot be aligned to the bHp cDNA sequence due to the limited current knowledge of the latter.

```
bHp: 1  ccaaaggcagcttttccttggcaggccaagatgggtctcccagcataacctcatctcgggag 60
      |||
hHp: 550 ccaaaggcagcttttccttggcaggctaagatgggttcccaccataatctcaccacaggtg 609
```

```
bHp: 61  ccacgctcatcaatgaacgatgggtcctcaccacagctaaaaatctctacctgggtcaca 120
      |||
hHp: 610 ccacgctgatcaatgaacaatgggtgctgaccacggctaaaaatctcttcctgaaccatt 669
```

```
bHp: 121 gtagtgacaaaaaagcaaaggacatcactcctactttaagactctatgtggggaagaacc 180
      |
hHp: 670 cagaaaatgcaacagcgaaagacattgcccctactttaacactctatgtggggaaaaaagc 729
```

```
bHp: 181 agcttgtagaggtggagaagggtggttctccaccctgaccactccaaggtagacattgggc 240
      |||
hHp: 730 agcttgtagagattgagaagggtgttctacaccctaactactcccaggtagatattgggc 789
```

```
bHp: 241 tcatcaaactcagacagaagggtacctgtcaatgacaaagtaatgcccatctgcctacctt 300
      |||
hHp: 790 tcatcaaactcaaacagaagggtgtctgttaatgagagagtgatgcccatctgcctacctt 849
```

```
bHp: 301 cc 302
      |
hHp: 850 ca 851
```

7.3 ISH protocols

All steps were carried out in Hellendahl glass jars and at room temperature, unless stated otherwise.

Detection of casein mRNA in bovine mammary gland

Fixation and pretreatment

- 12 µm cryosections
- 4% PFA, 15 min
- 1x PBS, 2 x 5 min
- 50% ethanol, 5 min
- 70% ethanol, 5 min
- 90% ethanol, 5 min
- 100% ethanol, 5 min
- 90% ethanol (reusing the alcohol from above), 5 min
- 70% ethanol (reusing the alcohol from above), 5 min
- 50% ethanol (reusing the alcohol from above), 5 min
- 1x PBS, 2 x 5 min
- 10 µg Proteinase K/ml in 1x PBS, 10 min
- 1x PBS, 2 x 5 min
- 0.2 M HCl, 10 min
- 0.1 M TEA + 0.25% acetic anhydride, 10 min
- 2x SSC, 10 min

Hybridisation

15 ng of casein probe (working solution 10 ng probe/ μ l DEPC-treated H₂O) were mixed with hybridisation buffer up to a final volume of 50 μ l, denatured at 80°C for 5 min and applied to each section. Hybridisation occurred in a humid chamber at 52°C overnight (16-18 h).

Posthybridisation washes

- 2x SSC, 2 x 10 min, 45°C
- 50% formamide/50% 2x SSC, 10 min, 45°C
- 50% formamide/50% 2x SSC, 20 min, 45°C
- 0.2x SSC, 2 x 10 min, agitation (100 rpm)
- 5 μ g RNase A/ml + 50 U RNase T1/ml, 30 min, 37°C
- 2x SSC, 3 x 10 min, agitation (100 rpm)

Immunological detection

- MA buffer, 10 min
- 1% blocking solution in MA buffer, 30 min, agitation (100 rpm)
- 100 μ l Anti-Dig-AP antibody/section (not in glass jar), 1:500 diluted in MA buffer, 2 h in humid chamber
- 0.1% Tween in MA buffer, 15 min, agitation (100 rpm)
- MA buffer, 3 x 15 min, agitation (100 rpm)
- Substrate buffer 1, 10 min
- Substrate (0.3375 mg NBT/ml + 0.175 mg BCIP/ml substrate buffer 1), incubated overnight protected from light
- TE buffer, 10 min
- Kaiser's glycerol gelatine for mounting

Detection of Hp mRNA in bovine liver*Fixation and pretreatment*

- 12 μ m cryosections

- 4% PFA, 15 min
- 1x PBS, 2 x 5 min
- 0.1 M TEA + 0.25% acetic anhydride, 10 min
- 2x SSC, 10 min

Hybridisation

30 ng of bHp2 probe (working solution 10 ng probe/ μ l DEPC-treated H₂O) were mixed with hybridisation buffer up to a final volume of 50 μ l, denatured at 80°C for 5 min and applied to each section. Hybridisation occurred in a humid chamber at 52°C overnight (16-18 h).

Posthybridisation washes

- 2x SSC, 2 x 10 min, 45°C
- 50% formamide/50% 2x SSC, 10 min, 45°C
- 50% formamide/50% 2x SSC, 20 min, 45°C
- 0.2x SSC, 2 x 10 min, agitation (100 rpm)
- 5 μ g RNase A/ml + 50 U RNase T1/ml, 30 min, 37°C
- 2x SSC, 3 x 10 min, agitation (100 rpm)

Immunological detection

- MA buffer, 10 min
- 1% blocking solution in MA buffer, 30 min, agitation (100 rpm)
- 100 μ l Anti-Dig-AP antibody/section (not in glass jar), 1:500 diluted in MA buffer, 2 h in humid chamber
- 0.1% Tween in MA buffer, 15 min, agitation (100 rpm)
- MA buffer, 3 x 15 min, agitation (100 rpm)
- Substrate buffer 1, 10 min
- Substrate (0.3375 mg NBT/ml + 0.175 mg BCIP/ml substrate buffer 1), incubated overnight protected from light
- TE buffer, 10 min
- Kaiser's glycerol gelatine for mounting

Detection of Hp mRNA in bovine mammary gland

Fixation and pretreatment

- 12 µm cryosections
- 4% PFA, 15 min
- 1x PBS, 2 x 5 min
- 50% ethanol, 5 min
- 70% ethanol, 5 min
- 90% ethanol, 5 min
- 100% ethanol, 5 min
- 90% ethanol (reusing the alcohol from above), 5 min
- 70% ethanol (reusing the alcohol from above), 5 min
- 50% ethanol (reusing the alcohol from above), 5 min
- 1x PBS, 2 x 5 min
- 0.1 M TEA + 0.25% acetic anhydride, 10 min
- 2x SSC, 10 min.

Hybridisation

3 ng of bHp2 probe (working solution 10 ng probe/µl DEPC-treated H₂O) were mixed with hybridisation buffer up to a final volume of 50 µl, denatured at 80°C for 5 min and applied to each section. Hybridisation occurred in a humid chamber at 52°C overnight (16-18 h).

Posthybridisation washes

- 2x SSC, 2 x 10 min, 45°C
- 50% formamide/50% 2x SSC, 10 min, 45°C
- 50% formamide/50% 2x SSC, 20 min, 45°C
- 0.2x SSC, 2 x 10 min, agitation (100 rpm)

- 5 µg RNase A/ml + 50 U RNase T1/ml, 30 min, 37°C
- 2x SSC, 3 x 10 min, agitation (100 rpm).

Immunological detection with TSA

- TN buffer, 5 min
- 100 µl TNB blocking buffer/section, 30 min
- 90 µl Anti-DIG-POD antibody/section, diluted 1:100 in TNB buffer, 1 h in humid chamber protected from light
- TNT wash buffer, 3 x 5 min, agitation (100 rpm)
- 90 µl TSA Plus DNP working solution (DNP amplification reagent stock solution diluted 1:50 in 1x Plus Amplification diluent (TSA kit)), 10 min
- TNT wash buffer, 3 x 5 min, agitation (100 rpm)
- 90 µl Anti-DNP-AP/section, diluted 1:100 in TNB blocking buffer, 1 h in humid chamber protected from light
- TNT wash buffer, 3 x 5 min, agitation (100 rpm)
- Substrate buffer 1, 10 min
- Substrate (0.3375 mg NBT/ml + 0.175 mg BCIP/ml substrate buffer 1), protected from light, 50 min
- TE buffer, 10 min
- Kaiser's glycerol gelatine for mounting.

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