A STUDY OF TWO CANDIDATE GENES FOR RESISTANCE TO *SALMONELLA* INFECTION IN CHICKENS: *NRAMP1* AND *TNC*

by

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Abstract

Salmonellosis is an important foodborne disease with world-wide economic and public health significance. Domestic poultry constitute one of the largest reservoirs of *Salmonella* in animal populations. Susceptibility to this infection varies in different chicken lines suggesting the involvement of host genetic factors. The gene, *Nramp1*, and the locus, *Lps*, are well-characterized in the mouse and are known to be involved in the early, innate responses to *Salmonella* infection. In our laboratory, we have used a comparative gene approach to examine whether NRAMP1 and LPS are important to chicken salmonellosis.

This thesis has continued our comparative examination through three objectives. The first objective was to determine the frequency of the *NRAMP1* $Arg^{223} \rightarrow Gln^{223}$ variant, originally found in *Salmonella*-susceptible chicken line C, in outbred chicken and pedigreed stock. Our second objective was to genetically map chicken *TNC*, a closely linked marker to *LPS*. A chicken backcross reference population was used to map this gene: the Compton mapping panel. We mapped *TNC* to microchromosome E41W17 in a region of conserved synteny with mouse chromosome 4 and human chromosome 9q. The third objective of this thesis was to develop chicken NRAMP1 antibodies in order to eventually characterize the protein's function and expression. The antisera raised in New Zealand White rabbits identified a 90-100 kDa species on a Western blot that most likely corresponds to the glycosylated chicken NRAMP1.

Résumé

La salmonellose est une cause importante d'intoxication alimentaire ayant un impact significatif sur l'économie mondiale et la santé publique. L'élevage de volailles constitue un réservoir important de *Salmonella* au sein de la population animale. La susceptibilité à cette infection varie selon les différentes lignées de poulet, suggérant l'implication d'une composante génétique chez l'hôte. Le gène *Nramp1* et le locus *Lps* sont bien caractérisés chez la souris et leur rôle est reconnu dans la réponse immunitaire précoce et innée à l'infection à *Salmonella*. Dans notre laboratoire nous avons utilisé une approche de génétique comparative afin d'étudier si les gènes NRAMP1 et LPS sont importants au niveau de la salmonellose chez le poulet.

Ce projet de maîtrise nous a permis de continuer notre étude comparative selon trois objectifs. Le premier objectif était de déterminer la fréquence de la mutation $Arg^{223} \rightarrow Gln^{223}$, originellement retrouvée chez la lignée de poulets C sensibles à *Salmonella*, dans des poulets non-consanguins. Notre second objectif était de cartographier génétiquement le gène *TNC* de poulet, un marqueur étroitement lié à LPS. Une population de référence de poulets issus d'un rétrocroisement est été utilisées pour cartographier ce gène: le catalogue Compton. Nous avons cartographié *TNC* sur le microchromosome E41W17, dans une région conservée avec le chromosome 4 de la souris et le chromosome 9q humain. Le troisième objectif de ce projet de maîtrise était de générer des anticorps de façon à éventuellement caractériser la fonction et l'expression de la protéine. Les antisera générés dans les lapins New Zealand White ont permis d'identifier une bande de 90-100 kDa par immunobuvardage, ce qui correspondrait à la forme glycosylée du gène NRAMP1.

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AIDS	autoimmune deficiency syndrome
Ala	alanine
AMBP	alpha-1-microglobulin bikunin
Amp^r	ampicillin resistance
Arg	arginine
aro A	aromatic gene
BAC	bacterial artificial chromosome
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane receptor
CHO	Chinese hamster ovary
chr	chromosome
cM	centiMorgans
cpm	counts per minute
crp	cyclic AMP receptor protein
cya	adenylate cyclase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FISH	fluorescence *in situ* hybridization
galE	UDP-galactose epimerase
Gln	glutamine
GST	glutathione-S-transferase
GST-N	GST-fusion protein
HIV	human immunodeficiency virus

IL-1	interleukin 1
IL-1 R	interleukin 1 receptor
Ile	isoleucine
INFγ	interferon gamma
inv	invasion genes
IPTG	isopropyl-beta-D- thiogalactoside
Ity^r	*Salmonella*-resistant allele
Ity^s	*Salmonella*-susceptible allele
JF	Red Jungle Fowl
kDa	kiloDaltons
LB	Luria-Bertani
LBP	LPS binding protein
LPS	lipopolysaccharide
Lps^d	LPS defective allele
Lps^n	LPS normal allele
MCS	multiple cloning site
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB
Nramp1	natural resistance-associated macrophage protein 1
nu	nude
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ptac	tac promotor
PVDF	polyvinylidene fluoride
RES	reticuloendothelial system
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
RT PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
spa	secretory proteins
SSC	salt-sodium-citrate
SSCP	single strand conformation polymorphism
SSLP	single strand length polymorphism
TBE	Tris-borate-EDTA
TBST	Tris buffered saline-Tween
TE	Tris-EDTA
Thr	threonine
Tlr4	toll-like receptor 4
Tnc	tenascin C
TNFα	tumour necrosis factor alpha
TM	transmembrane
Val	valine
WL	White Leghorn
xid	X-linked immunodeficiency
YAC	yeast artifical chromosome

Introduction

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Introduction

(1) *Salmonella*: The Pathogen

Classification and Habitat:

The genus, *Salmonella*, belongs to the family Enterobacteriaceae. *Salmonellae* are commonly isolated from the intestines of humans and animals but can also survive for long periods of time in the environment (Bopp et. al., 1999). Over 2200 serotypes of this bacteria are known to exist (Lillehoj, 1991). *Salmonella* is a facultative, intracellular parasite that is classified as a Gram negative bacteria due to the lipopolysaccharide (LPS) component in its outer membrane (Neidhardt et. al., 1990). Variations in the structure of the LPS molecule are used to identify specific *Salmonella* serotypes (Falkow and Malkanos, 1990). Serotypes can also be classified based on differences in their capsular and flagellar antigens (Ekperigin and Nagaraja, 1998). Pathogenically, *Salmonellae* can be divided into two main groups: the small number of serotypes that produce severe systemic disease in a restricted number of host species and those serotypes (about 2000 in number) that are not restricted to a particular host species, most often existing in the carrier state within healthy animals (Cooper, 1994; Barrow, 1996). Examples of the first group include *Salmonella typhi* which causes typhoid fever in humans and *S. gallinarum* which produces fowl typhoid. *S.* Typhimurium and *S. enteritidis* are typical members of the second group of *Salmonella* serotypes: their epidemiology is more complex, they infect a broad range of animal hosts and commonly cause food-poisoning in humans (Falkow and Malkanos, 1990).

Pathogenesis:

After oral ingestion, *Salmonella* spp. must first survive the low pH of the stomach. The bacteria that survive the low pH have developed an adaptive acid tolerance response, enabling them to enter the intestine (Gorden and Small, 1993). *Salmonella* infect the host by penetrating the intestinal mucosa through the epithelial cells (Jones and Falkow, 1996). The first step of the invasion process involves adherence of the bacteria to the host cell surface. Adhesins are cell surface molecules which facilitate the localization of *Salmonellae* to the appropriate target tissues and permit binding to tissue cells (Medzhitov et. al., 1997). The invasion of non-phagocytic cells has been called translocation or macropinocytosis and is bacterial-induced (Finlay and Cossart, 1997). Translocation refers to the method by which *Salmonella* invade the host cell, by moving through the epithelium rather than by disrupting the intercellular junctions (Neidhardt et. al., 1990). Macropinocytosis, primarily, describes the mechanism by which *Salmonellae* enter non-phagocytic epithelial cells. The bacteria send signals to the host cell inducing membrane ruffling and cytoskeletal rearrangements culminating in bacterial uptake through the formation of a membrane-bound vacuole (Finlay and Cossart, 1997). This invasion process depends largely on ligand-receptor mechanisms which are mostly unknown (Aderem and Underhill, 1999). However, recent data demonstrate that upon binding to epithelial cells, *S.* Typhimurium utilize a type III secretion system specialized to deliver virulence factors directly to host cells (Mecas and Strauss, 1999). Once inside the vacuole of the epithelial cell, the bacteria multiply and eventually exit the epithelium to infect the reticuloendothelial system (RES) (Foster and Spector, 1995). *Salmonellae* become quickly associated with their principal target of infection, the macrophage. Sequestered within the macrophage, the bacteria can multiply while evading the host's humoral defenses. The abduction of the macrophage is either induced by the bacteria, as in macropinocytosis, or may be instigated unwittingly by the macrophage itself via phagocytosis (Finlay and Cossart, 1997). Once inside the macrophage, the bacteria take up

residence within the intracellular phagosomes (Cellier et. al., 1996). Phagosomes undergo successive fusion events which lead to their strong acidification, the delivery of hydrolytic enzymes, the generation of toxic oxygen radicals and, finally, microbicidal action (Abbas et. al., 1994). *Salmonella* have evolved strategies to interfere with the phagosomal maturation process facilitating their survival within an otherwise hostile milieu (Skamene et. al., 1998). There is an alternative theory that *Salmonella* do not inhibit the phagosomal maturation process but are simply resistant to degradation within the macrophage (Gulig, 1995). *Salmonella* bacteria are not always localized to the gastrointestinal tract and RES, some highly virulent strains are capable of spreading to the bloodstream, causing bacteremia, and affecting several organ systems (Ekperigin and Nagaraja, 1998).

The virulence factors of *Salmonella* are controlled genetically as are the responses from the host; whether the disease progresses or is stifled depends on the interplay of the host immune system and the virulence of the bacteria (Jones and Falkow, 1996). In combat with the host are the *Salmonella* virulence genes which are involved at every step of infection. The numerous acid-tolerance genes are important for bacterial survival in the harsh conditions of the stomach and phagosomes (Foster and Spector, 1995). Several different invasion genes (*inv*), along with other secretory proteins (*spa*), participate in the macropinocytosis process, such as, the *inv A, E, G* and *C* loci and the *spa S* and *L* (Finlay and Cossart, 1997). Mutants defective in these loci cannot induce membrane ruffling and will not invade. These mutants are also unable to induce apoptosis, or programmed cell death, in macrophages. Apoptosis of the macrophage is thought to control the number of cells capable of attacking the bacteria (Finlay and Cossart, 1997). *Salmonellae* are subject to oxidative damage within the phagosomes of the macrophage. *Rec A* mutants, defective in DNA repair, have increased susceptibility to oxidative damage (Gulig, 1995). Any one of the aforementioned virulence genes influence the outcome of a *Salmonella* infection.

(2) Human Salmonellosis

Non-Typhoid Salmonellosis:

Non-typhoid salmonellosis refers to infections caused by *Salmonella* serotypes other than *S. typhi*. The incidence of non-typhoid salmonellosis has increased dramatically over the past twenty years in North America and Europe due to changes in animal farming, the mechanization of food processing and the mass distribution of food (Falkow and Malkanos, 1990). In underdeveloped countries, acute gastroenteritis caused by non-typhoid *Salmonella* is widespread among malnourished children and is the principal cause of death in this age group (Kariuki et. al., 1996).

Food animals that do not develop salmonellosis, and those that recover from the disease, become carriers and serve as reservoirs of disease. In the majority of cases, humans acquire non-typhoidal *Salmonella* spp. by the ingestion of contaminated food, most commonly poultry and poultry products (primarily eggs) (Rubino, 1997). However, salmonellosis can also be transmitted by direct contact with animals, by non-animal foods, by water and, occasionally, by human contact (Bopp et. al., 1999).

Non-typhoidal *Salmonella* strains usually cause an intestinal infection, namely gastroenteritis, lasting one to two weeks which is accompanied by diarrhea, fever, abdominal cramps and vomiting (Bopp et. al., 1999). The spectrum of disease ranges from healthy carrier to severe dysentery-like syndromes, the more severe symptoms usually occurring in infants and adults over fifty years old (Falkow and Malkanos, 1990). *S. enteritidis* and *S.* Typhimurium are among the most common serotypes and are encountered world-wide (Cowden, 1996).

Widespread outbreaks of these and other *Salmonella* strains have occurred due to the international and national distribution of contaminated food: *S. agena* first spread around the world as a consequence of the use of contaminated chicken feed and 250 000

cases of *S. enteritidis* infection, across the United States, were caused by the co-transportation of raw eggs and ice cream (Tauxe and Hughes, 1996).

Non-typhoidal *Salmonella* strains are becoming increasingly resistant to a variety of antimicrobial drugs. This phenomenon is largely due to the widespread use of antibiotics in the animal reservoir and due to an increase in the use of several major antibiotics by clinicians in both developed and developing countries (Tauxe, 1999; Cowden, 1996). Multi-drug resistant *Salmonella* bacteria are potentially lethal pathogens especially in patients with bacteremia or a compromised immune system.

Typhoid:

Typhoid fever, caused by the bacterium, *S. typhi*, was extremely common at the beginning of the twentieth century but is now largely controlled in the industrialized countries through public health measures, such as disinfection of drinking water, sewage treatment and milk pasteurization (Tauxe, 1999). In 1900, the incidence of typhoid fever was approximately 100 per 100 000 population; by 1920, it had decreased to 33.8 and by 1950, to 1.7 (Anon., 1999). However, in the developing world, typhoid is still a major cause of disease (Griffin, 1999). The seriousness of typhoid is compounded by the fact that, as with non-typhoidal *Salmonella*, many strains of *S. typhi* are becoming resistant to a variety of antimicrobial agents, causing epidemics in parts of central and southeast Asia (Murdoch et. al., 1998). Carriers may be of importance as reservoirs of infection; about 3% of infected persons continue to excrete *Salmonella* one year after symptoms have disappeared (Falkow and Malkanos, 1990).

Often, the first signs of infection are enterocolitis with diarrhea, frequently accompanied by several nonspecific symptoms such as headache, dizziness and/or abdominal cramping (Hornick et. al., 1970). Fever and other symptoms of bacteremia accompany the spread of bacilli from the infected cells of the RES into the bloodstream

(Falkow and Malkanos, 1990). The course of illness is usually severe and prolonged, lasting several weeks if untreated or untreatable.

It was recently found that *S. typhi*, but not *S.* Typhimurium, uses the cystic fibrosis transmembrane receptor (CFTR) to gain entry into epithelial cells (Pier et. al., 1998) allowing the bacteria to penetrate the intestinal epithelium barrier. The study by Pier et. al. (1998) suggests that these two closely related *Salmonella* serotypes invade cells through different pathways which could partially explain their pathological differences.

Salmonella and Human Immunodeficiency Virus (HIV):

In the early to mid-1980s it was already clear that non-typhoidal *Salmonella* was an opportunistic pathogen associated with HIV and the acquired immunodeficiency syndrome (AIDS) (Glaser et. al., 1985; Fischl et. al., 1986). Data has suggested that risk for non-typhoidal *Salmonella* infection is increased 20 to 100 fold among AIDS patients (Morris and Potter, 1997). It has also been found that, due to the immunosuppression caused by AIDS, *Salmonella* infections are more likely to progress to septicemia. In fact, bacteremia has been used as an indicator, since the early 1980s, as the first manifestation of AIDS if associated with HIV seropositivity (Bottone et. al., 1984). Multi-drug resistant *Salmonella* presents a very serious problem for AIDS patients due to the fact that they are more likely to develop bacteremia, a condition that can be ultimately fatal. *S.* typhimurium and *S. enteritidis* are the serotypes most commonly isolated from AIDS patients and are the serotypes most associated with bacteremia in these patients (Sperber et. al., 1987). Studies outside of North America suggest that AIDS patients have a similar increase in risk for infection with *S. typhi* as well as an increase in the severity of infection, in areas endemic to typhoid fever (Morris and Potter, 1997).

(3) Genetic Control of Resistance to Salmonella in Mouse

Phases of Infection:

Investigations into the genetics of *Salmonella* infection have, most frequently, been carried out using a mouse model. This model allows researchers to set up appropriate informative crosses in a prospective, directed fashion within controlled experimental conditions (Sudbery, 1998). In this way, the infection can be dissected into its component parts allowing the possibility of identifying individual genes in a multigenic sequence that control an entire complex trait. Susceptibility to infection is one such complex trait that involves the action of many genes within the host organism. In order to study the progression of *S.* Typhimurium infection under laboratory conditions, Mastroeni et. al. (1994) translated susceptibility into a simple phenotype, that of, bacterial replication within the mouse. They found that sublethal intravenous infection with *S.* Typhimurium in inbred mouse strains has four distinct phases: (1) an early inactivation of the inoculum, (2) exponential growth in the RES (also known as the "early immune phase"), (3) suppression of bacterial growth or plateau phase and (4) the clearance of organisms from tissues (also known as the "late immune phase").

Phase one takes place within twenty four hours of inoculation. *Salmonellae* are cleared from the blood by activated complement factors. This response from the host reduces the number of bacteria that reach the RES.

Phase two is unaffected by antibody response, which may delay but not suppress exponential growth. However, this phase is strongly influenced by bacterial virulence and genetically defined resistance in the mouse (Maskell et. al., 1987). There are three classified genes in the mouse that are believed to be key to survival during this early phase of *S.* Typhimurium infection. *Nramp1* (natural resistance-associated macrophage protein 1) is a gene on mouse chromosome one (chr 1) which controls for the differential growth rate of *S.*

Typhimurium in resistant and susceptible inbred mice (Gros et. al., 1981). Another gene important to the outcome of phase two in resistant and susceptible mice is *Lps* mapped to a single, autosomal locus on mouse chr 4 (Qureshi et. al., 1999b). The gene that corresponds to this locus is the recently cloned, toll-like receptor 4 (*Tlr4*). *Tlr4* codes for a transmembrane receptor expressed on the mouse macrophage which is believed to be part of the complex machinery that recognizes the bacterial LPS (Wright, 1999). *Nf-Il6* is also involved with the early, innate immune phase of *Salmonella* infection in mice. This gene is a transcription factor whose production is triggered by bacterial LPS and is able to induce several important cytokines in activated macrophages (Tanaka et. al., 1995).

Phase three is the plateau phase which begins toward the end of the first week of infection (Mastroeni et. al., 1994). This phase is characterized by an influx of monocytes, predominantly macrophages, which control bacterial growth. TNF α and INF γ are important mediators throughout this phase.

Phase four, considered the late immune phase is regulated by genes inside and outside the major histocompatibility complex (MHC), such as, *nu* (nude) (Nauciel et. al., 1990), *xid* (X-linked immunodeficiency) (O'Brien et. al., 1981) and certain H2 (MHC) haplotypes (Nauciel et. al., 1988). Clearance of bacteria from the RES occurs during this phase and requires the work of T cells (Mastroeni et. al., 1994).

Innate vs. Acquired Immunity:

The general consensus is that in many bacterial infections, particularly those caused by facultative, intracellular pathogens, both arms of the immune system are involved (Barrow, 1996). Innate (or natural) immunity uses germline genes to evaluate an antigen, deploying a rapid, generalized barrier against microorganisms deemed dangerous (Fearon, 1997). One important mechanism of natural immunity against intracellular microbes is phagocytosis via cells, such as, the macrophage (Abbas et. al., 1994). Macrophage receptors

are capable of identifying components of bacterial cell walls, such as LPS, and subsequently engulfing and degrading these pathogens (Janeway et. al., 1997). The macrophage is capable of secreting cytokines that enhance antibacterial responses and presenting antigen to cells involved in the acquired (or adaptive) immune response (Barrow, 1996). Acquired immunity is the system of B and T lymphocytes, each of which express a specific antigen receptor that has arisen by somatic gene rearrangement (Fearon, 1997). Once B and T lymphocytes have been activated, via the antigen receptors, they mature into cells that defend the host by secreting antibodies, activating macrophages and killing infected cells (Abbas et. al., 1994). Innate immunity is involved with controlling the immediate threat of pathogen invasion, whereas, the acquired defenses, because of their specificity, take up to two weeks to mount an effective response. In effect, the acquired immune system compresses evolution into two weeks, allowing the selection of specific antigen receptors to keep up with variant microorganisms (Fearon, 1997).

Salmonella is a facultative, intracellular pathogen that must primarily evade the innate immune system of the host in order to effectively battle, or completely avoid, the more targeted acquired immune response. *Salmonella* survive within the host successfully by resisting elimination by phagocytes (Abbas et. al., 1994). These bacteria hijack the most important phagocyte involved with innate immunity by living within the phagosome of the macrophage. It is believed that the *Salmonellae* are capable of preventing the acidification of the phagosome (an important step in bacterial killing) by preventing its fusion with the acid-containing lysosome (Finlay et. al., 1992; Gulig, 1995; Skamene et. al., 1998). *Salmonellae* must also be able to evade the B and T lymphocyte response which is invariably induced in the infected host. *S.* Typhimurium uses antigenic variation, via DNA rearrangements, to elude the specific immune response of the host (Janeway et. al., 1997). *S.* Typhimurium is capable of inverting a segment of its DNA that codes for a flagellin gene, enabling it to turn off expression of one gene and turn on the expression of a second, which codes for an antigenically distinct protein.

These techniques of evasion account for the success of the *Salmonella* bacteria. The entire repertoire of mechanisms used by *Salmonella* against the host immune system is as yet unknown. The interaction of innate and acquired immunity of the host with the virulence factors of the pathogen is probably even more complex than we presently realize.

Nramp1:

Salmonellosis is a multigenically controlled disease evidenced by the fact that different strains of mice show different levels of susceptibility to infection. However, the *Ity* locus (for "immunity to typhimurium") has been shown to have a major effect on susceptibility to *S.* Typhimurium (Plant and Glynn, 1976; 1979). Mice possess either *Salmonella* sensitive, Ity^s allele, or carry the dominant resistant Ity^r allele. Mice homozygous for the susceptibility allele succumb to overwhelming sepsis following injection of only a few organisms (Jones and Falkow, 1996). Whereas, homozygous or heterozygous Ity^r mice have almost complete inhibition of bacterial growth with a moderate, non-specific immune response. The strain distribution pattern of Ity^{r+s} correspond exactly with two independently discovered loci known as *Bcg* and *Lsh*, which together control for natural resistance to infection with other antigenically and taxonomically unrelated, intracellular parasites, namely, *Mycobacterium bovis* and *Leishmania donovani* (Cellier et. al., 1996).

The three loci, *Ity/Bcg/Lsh*, were located on mouse chr 1 by previous linkage studies (Skamene et. al., 1982). A positional cloning approach was used in order to isolate the gene which was eventually named *Nramp1*, natural resistance-associated macrophage protein 1 (Vidal et. al., 1993). Sequence analysis of the deduced Nramp1 primary amino acid sequence identified a 60 kDa polypeptide with characteristics of an integral membrane protein, including 12 transmembrane domains (Vidal et. al., 1993). Nramp1 was found to contain a sequence motif also found in several bacterial transport proteins which suggested that Nramp1 could be involved in the membrane transport processes of the macrophage,

perhaps as an ion transporter or channel (Cellier et. al., 1996; Skamene et. al., 1998). Computer analysis revealed Nramp1 homologues identified in the fly *Drosophila* (Cellier et. al., 1995), in plants (Belouchi et. al., 1995), in birds (Hu et. al., 1995), in yeast (West et. al., 1992), in many species of *Mycobacteria* (Skamene et. al., 1998) and in many mammals, including humans (Cellier et. al., 1995). Nramp has been found to define a family of proteins extremely conserved in evolution from bacteria to man, suggesting a fundamental role in all living organisms (Cellier et. al., 1995).

Sequencing of the *Nramp1* gene in resistant and susceptible inbred mouse strains revealed that susceptibility to *Mycobacterium* infection was associated with a nonconservative substitution from glycine to aspartic acid at position 169 within the fourth predicted transmembrane domain of the protein (Malo et. al., 1994). The next step was to create a mouse mutant completely lacking an *Nramp1* gene on a resistant background. Although the *Nramp1* null mutant mice were viable, they lost their natural resistance to *Salmonella*, *Mycobacterium* and *Leishmania* (Vidal et. al., 1995). Secondly, it was shown that C57BL/6J susceptible mice gained resistance to infection with *Salmonella*, *Mycobacterium* and *Leishmania* after transgenesis with the *Nramp1* resistant allele (Govoni et. al., 1996). These experiments are considered evidence that *Nramp1* is a gene responsible for differential resistance and susceptibility to these diseases in the mouse.

In order to initiate biochemical classification of *Nramp1*, Nramp1 antisera were raised in rabbits (Vidal et. al., 1996). These antisera were used to determine the molecular basis of susceptibility in mice with the defective *Nramp1* allele. The study by Vidal et. al. (1996) detected the Nramp1 protein only in macrophage membranes derived from resistant mice. The macrophages from susceptible mice had no trace of the Nramp1 protein. This implies that the mutation in the *Nramp1* gene could affect the insertion or folding of Nramp1 within the macrophage membrane. This could lead to the failure of the protein to mature, causing its rapid degradation.

A common step in the intracellular behaviour of *Salmonella*, *Mycobacteria* and *Leishmania* is the transit of these organisms through the phagosome (Skamene et. al., 1998). This information was used in order to determine the subcellular location of Nramp1. Phagosomes containing *S.* Typhimurium have been shown to have greatly decreased fusion with lysosomes and other late endocytic compartments and to exhibit delayed and attenuated acidification (Gruenheid et. al., 1997). The Nramp1 protein is thought to be involved with the enhancement of the fusion process, over-riding the effect of the bacteria, and allowing acidification of the phagosome (Hackam et. al., 1998). This same group found that the Nramp1 protein was recruited to the membrane of the phagosome upon phagocytosis and remained during the organelle's maturation to the phagolysosome. It is also thought that the Nramp1 ion transporter may compete with *Salmonella* bacteria for a particular substrate, thus sabatoging bacterial replication within the phagosome (Skamene et. al., 1998).

At present, the exact biochemical function of *Nramp1* has not been determined. It is hypothesized that the gene encodes an ion transporter that effects the intracellular milieu of the phagolysosome (Cellier et. al., 1995, 1996). The ion or ions that are transported by Nramp1 remain unknown, however, many possibilities have been suggested (Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, among other divalent cations) based on the functional studies of Nramp protein homologues in various species (Supek et. al., 1996; Gunshin et. al., 1997).

Lps/Tlr4:

LPS, or endotoxin, is an abundant outer cell membrane glycolipid and common structural constituent of Gram negative bacteria. Recognition of LPS by host cells, such as the macrophage, which is an important cell in endotoxin-mediated effects, causes dramatic transcriptional up regulation of a wide range of proinflammatory genes, including tumour necrosis factor alpha (TNFα), interleukin 1 (IL-1), IL-6 and IL-8 (Gulig, 1995). LPS-dependent activation of these genes has been shown to be controlled by the transcription

factors, nuclear factor (NF)-κB (Wright, 1999). This response can lead to a toxic sepsis, however, a rapid response to LPS by the host allows an effective clearance of bacteria before wide dissemination occurs (Poltorak et. al., 1998b). While B and T lymphocytes are also cells that respond to LPS and are important to bacterial clearance, the phenotype of LPS-tolerant mice corresponds precisely with that expected of a defective innate immunity (Wright, 1999). These animals are extremely susceptible to Gram negative infection but respond normally to Gram positive challenge and do not have other health defects.

The study of the *Lps* locus was made possible through the use of the mouse strain, C3H/HeJ, that shows hyporesponsiveness to bacterial LPS and is susceptible to Gram negative bacterial infection (Qureshi et. al., 1996). In 1977, the endotoxin hyporesponsive state was ascribed to a single, co-dominant locus (Lps^d) which was mapped to mouse chr 4 (Watson et. al., 1977). Macrophages of Lps^d mice fail to respond to endotoxin and therefore do not induce the cellular and inflammatory responses which would normally protect against infection (Gulig, 1995). The death of susceptible mice following infection with *S.* Typhimurium appears to result from uncontrolled multiplication of bacteria in the RES and reduced recruitment of inflammatory cells to the RES (Qureshi et. al., 1999b).

Tlr4 was mapped and cloned by two groups, Poltorak et. al. (1998a, b) and by Qureshi et. al. (1999a). Both groups performed genetic mapping techniques to narrow the position of the *Lps* locus on mouse chr 4, aligned the target region on YACs and BACs (yeast and bacterial artificial chromosomes) and searched for candidate genes using exon trapping, cDNA selection, comparative mapping and DNA sequencing. Qureshi et. al. (1999a) identified three transcription units within the *Lps* candidate region, including *Tlr4*. Poltorak et. al. (1998b) found only two genes in the entire target region, one being a metallo-proteinase not expressed in macrophages, and *Tlr4*. Thus, *Tlr4* was cloned because of its mapping position and because it was found to be part of the TLR family whose members are involved with innate immune recognition of infectious pathogens.

A single missense mutation was observed in the mutant C3H/HeJ inbred strain, involving a highly conserved proline within a portion of *Tlr4* that exhibits the strongest degree of evolutionary conservation (Qureshi et. al., 1999a). The C57BL/10 ScNcr *Lps* mutant mouse strain was found to have no *Tlr4* expression at all (Poltorak et. al., 1998b). This null allele behaves as a recessive mutation, the presence of a single wild type *Tlr4* allele is sufficient to permit normal LPS signal transduction.

The TLR protein family has homologues in mammals, insects and plants implying an ancient evolutionary origin (Qureshi et. al., 1999b). TLR4 is a transmembrane protein with structural and functional similarities to IL-1R (inter-leukin 1 receptor), a trans-membrane signalling protein that is part of the toll receptor family (reviewed by: Qureshi et. al., 1999a, b). Recent observations indicate that human TLR4 can be an effective signaling molecule in mammalian cells and drives NF-κB activation (Wright, 1999). As mentionned earlier, NF-κB is a transcription factor intimately involved with the LPS response.

The signalling pathway of LPS has been partially deduced based on many years of experimental investigations. Virulent Gram negative bacteria induce inflammation by shedding LPS from their outer membrane; the LPS monomers then aggregate in the host's vasculature (Qureshi et. al., 1999b). It has been shown that LPS is concentrated from the plasma by a LPS binding protein (LBP) (Poltorak et. al., 1998b). CD14, recognized as a major receptor for LPS, exists in both a soluble form (sCD14) and as a surface molecule on mononuclear cells (mCD14), is known to play a role in LPS signaling (Wright, 1999). What happens to LPS signaling after this point is conjecture. As CD14 has no transmembrane domain, it is believed that another co-receptor is involved in LPS signal transduction (Potorak et. al., 1998a) Recent studies have determined two distinct routes of LPS trafficking with two distinct biological outcomes. These studies state that monomeric LPS is delivered to the golgi apparatus independently of mCD14 , a process associated with cell stimulation, whereas, LPS aggregates are delivered to the lysosome in association with mCD14, a process associated with detoxification and clearing (Thieblemont and Wright,

1999; Vasselon et. al., 1999). Additionally, macrophages from *Lps*^{d} mice are defective in vesicular transport of LPS (Vasselon et. al., 1999). All of this evidence indicates that TLR4 could be a CD14 co-receptor which is somehow involved with intracellular signaling and/or trafficking, however, the role of TLR4 is still unknown.

(4) Salmonellosis in the Chicken

Importance:

Salmonella is an important foodborne disease affecting populations globally (Finlay et. al., 1992). Domestic poultry constitute the largest single reservoir of *Salmonella* in animal populations (Lillehoj, 1991). Present animal husbandry practices are condusive to horizontal transmission of *Salmonella* due to high stock density farms (Cooper, 1994). However, due to logistical and economic reasons these farming practices persist. For the same reasons, present processing technologies in poultry slaughtering plants cannot guarantee a *Salmonella*-free final product (Cooper, 1994). Although there is a general complaint about the paucity of information on the chicken model, several studies have shed some light on the pathogenesis and genetics of *Salmonella* infection in chickens. These studies increase our knowledge of salmonellosis which facilitates efficient control of the pathogen, improved treatments for the disease and improved breeding of genetically resistant lines of chicken.

Contamination:

Salmonellae are usually associated with food poisoning by virtue of their ability to colonize the alimentary tracts of livestock, particularly poultry (Lillehoj, 1991). This results in considerable contamination of carcasses at slaughter, with entry of *Salmonellae* into

human food. A few sick birds can cause a rapid spread of a particular strain through a flock as the housing, water and feeding systems become contaminated (Turner et. al., 1998). Infection of newly hatched chicks, which have a relatively simple gut flora, can result in rapid multiplication and extensive excretion which also has the potential to cause a chicken epidemic (Bumstead and Barrow, 1993). Certain *Salmonella* serotypes are capable of becoming localized in the ovary or oviduct, resulting in contamination of the egg contents, or contaminating the egg surface as it passes through the cloaca or after laying (Gast and Beard, 1990). In either case, it can result in contaminated hatching of infected progeny. Present control methods are either inadequate or create additional problems. The reduction of *Salmonella* contamination, via hygenic measures, is expensive requiring the change of animal husbandry practices, reducing cross-contamination along the slaughter line, among other measures (Rubino, 1997).

Infection:

A) Pathogenesis:

Several lines of evidence indicate that the overall sequence of events following infection with *Salmonella* is similar in the mouse and chicken (Cooper, 1994; Barrow, 1996). The severity of disease varies according to the serotype of *Salmonella* and strain of chicken but generally there is always some invasion of the intestinal epithelium and RES (Henderson et. al., 1999). The mechanism whereby *Salmonella* colonize the alimentary tract, migrate to the RES and where they are ingested by phagocytic cells of the immune system is unknown in chickens (Turner et. al., 1998). However, the bacteria do reach the blood stream, probably intracellularly, and reside in the spleen, liver, bone marrow (Zhang-Barber et. al., 1999), as well as, thymus and lung (Hu et. al., 1996). *Salmonella* infection usually occurs as an acute systemic form in young chickens but in adults it is most often localized

and chronic (Lillehoj, 1991). Newly hatched chicks can succumb to massive multiplication of *Salmonella* with large numbers being excreted in the faeces for several weeks (Barrow, 1996). Young chicks then acquire a more complex gut flora which is able to control the *Salmonellae* so that infection results in excretion of fewer bacteria, for a shorter period.

B) Immune Responses to Salmonella *Infection in the Chicken:*

Although infection of chickens with *S.* Typhimurium has been studied in some detail, the relative roles of the acquired and innate immune response in clearance of the bacteria from chickens is still unclear. It seems likely that acquired immunity, rather than innate immunity, is responsible for tissue clearance in the chicken (Zhang-Barber et. al., 1999). A significant innate response to *S.* Typhimurium infection, resulting in anti-LPS antibodies, has been reported in chickens within five days of infection (Cooper, 1994). Light and electron microscopic examinations of intestines taken from chickens after infection with various species of *Salmonella* show an influx of heterophils and macrophages to the surface of the intestine (Barrow et. al., 1987). The heterophil is considered the equivalent to the mammalian neutrophil, a phagocyte, and is an important cell in the containment of bacterial growth (Kogut, 1994). *Salmonella* has been shown to survive and replicate within macrophages from many hosts, including chickens (Henderson et. al., 1999).

C) Resistance to Salmonella *in the Chicken:*

Bumstead and Barrow (1993) reported that different breeds of chicken show variation in their response to infection with *S.* Typhimurium. Chicken lines are either resistant (lines W1,61, N) or susceptible (lines C, 72, 15I). Susceptible chickens have growth in the RES during the first week of infection and die within 10-12 days (Bumstead and Barrow, 1988). Further studies determined that the lines previously shown to be

resistant to *S.* Typhimurium were also found to be resistant to *S. gallinarum*, *S. pullorum* and *S. enteritidis* and lines susceptible to *S.* Typhimurium were also more susceptible to the other serotypes (Bumstead and Barrow, 1993). These results suggest that there may be a general mechanism of resistance that may apply to all *Salmonella* serotypes in chicken. The differences in susceptibility to *Salmonella* infection has been shown to be correlated with the bacterial load in liver and spleen (Barrow et. al., 1987). The many experiments carried out by Bumstead and Barrow (1987, 1988, 1993) suggest that resistance to salmonellosis can be determined by the action of phagocytic cells during the initial phase of infection in chickens. Genetic variation in the number and activity of macrophages in chickens has been described (Qureshi et. al., 1986).

D) Prophylaxis:

The ideal vaccine would provide strong protection against intestinal and systemic infection and needs to be avirulent for humans. Several vaccines have been developed to control *Salmonella* infection in chickens with varying success. Vaccination with live attenuated strains of host specific *Salmonella* serotypes induces a strong protective immunity and has been successfully used over the years (Zhang-Barber et. al., 1999). The 9R fowl typhoid vaccine confers strong protection against systemic disease in adult chickens although it retains some virulence and can be passed to eggs (Barrow, 1996). Other vaccines that have been developed against host-specific serotypes have not been as effective as the 9R vaccine.

Vaccination against non-host specific serotypes of *Salmonella* have not been as clearly successful as the host-specific live attenuated vaccines. The reason for this has been explained by a lack of information on the immunological interactions between *Salmonella* organisms and the chicken host. Thus, poultry vaccination is still at an antidiluvian level of development. A strain of wild type *S.* Typhimurium was mutated to produce a new strain

that was defective in the enzyme, UDP-galactose epimerase (gal E), which blocks LPS synthesis (Janeway et. al., 1997). After it was administered to one day old chicks, reduced feacal shedding was noted, as well as, reduced carrier status. However, the innate immune response in these chicks was low meaning that the immunity probably would not last. Chickens have been vaccinated orally with a *S.* Typhimurium strain with mutations in the genes encoding adenylate cyclase (*cya*) and cyclic AMP receptor protein (*crp*) (Cooper, 1994). Two successive doses prevented colonization of the caeca by a homologous serotype. Barrow et. al. (1990) found that a strain of *S. enteritidis* with a deletion in the *aro A* (aromatic) gene, given by oral and intramuscular routes, did not protect the gut wall of laying hens and did not protect the ovary. It was found that this mutant did not survive well in the gut of chickens. All of these vaccines are considered live vaccines, killed vaccines have not been as effective in eliciting immune responses in poulty.

Antibiotics have been used in chickens for more than four decades in order to prevent infectious diseases such as *Salmonella*, to decrease the amount of feed needed and to increase the rate of weight gain (DuPont and Steele, 1987). About 22 microbial drugs have been used on poultry farms and the most frequently used antibiotics are tetracycline, sulfa, penicillin and neomycin (DuPont and Steele, 1987). However, antibiotic therapy in food production animals is increasingly coming under close scrutiny, largely because of the fear of increased levels of resistance in food-borne human pathogens, such as, *Salmonella* (Barrow, 1996).

Competitive exclusion involves the administration of broad-spectrum gut flora from healthy adult chickens to newly-hatched birds (McMullin, 1999). Administration of competitive exclusion products can produce a degree of protection against salmonellosis in the early life of the young chick (Zhang-Barber et. al., 1999). This method of control of *Salmonella* has become more popular as antibiotics have lost their charm.

(5) Experimental Infection of *S.* Typhimurium in Chicken

Historically, *S.* Typhimurium has been the strain used when investigating this disease in the laboratory. *S.* Typhimurium is a gram negative bacteria which causes severe outbreaks of salmonellosis in many kinds of animal species and is frequently the cause of sporadic outbreaks of gastroenteritis in humans (Lillehoj, 1991). Although *S.* Typhimurium infection is of less economic significance than *S. gallinarum* or *S. pullorum*, which are avian specific serotypes, it can nevertheless be useful in the study of chicken salmonellosis. However, one must keep in mind that some lines of evidence suggest that different *Salmonella* serotypes have alternative mechanisms for host colonization (Turner et. al., 1998; Henderson et. al., 1999).

The similar early phenotypic response to infection with *S.* Typhimurium in chicken and mouse inspired the hypothesis that the genetic factors in this process may also be similar. The gene, *Nramp1*, and the locus, *Lps*, are well-characterized in the mouse and are known to be involved in early, innate responses to *S.* Typhimurium infection. Hu et. al. (1997) used a comparative gene approach to determine whether these genes were also important in chicken salmonellosis. This type of study facilitates the transfer of information from "map rich" (eg. mouse) to "map poor" species (eg. chicken) thereby speeding progress toward disease gene identification (Nadeau and Sankoff, 1997). The fact that both of these genes are each part of separate gene families that can trace homologies with species spanning millions of years of evolution (Cellier et. al., 1996; Qureshi et. al., 1999b) also lent support to the idea that they could be important in the chicken. The chicken *NRAMP1* homolog was cloned and mapped to chr 7q13 revealing a segment highly conserved in evolution between mammals (mouse chr1 and human chr 2q) and birds (Hu et. al., 1996). Further analysis revealed structural similarities between chicken, mouse and human *NRAMP1*, the presence of similar regulatory elements within the promoter regions of the gene and similar tissue expression (RES) (Hu et. al., 1996). The degree of conservation

between species lends support to the hypothesis that the *Nramp1* gene is important to survival and the similarity of the physical aspects of chicken, human and mouse NRAMP1 suggests a similar protein function. The next step was to investigate the role of *Nramp1* in differential resistance and susceptibility to *S.* Typhimurium infection in chicken. Two approaches were used: linkage and sequence analysis. Eleven polymorphisms were found in *Salmonella*-resistant and *Salmonella*-susceptible chicken lines, ten of which were silent or conservative mutations and only one was a non-conservative mutation found in susceptible line C. A positively charged amino acid residue Arg^{223} was replaced by a polar residue, Gln^{223}, located at the extracellular junction between predicted transmembrane 5 (TM5) and TM5-6 interval (Hu et. al., 1997). While this mutation does not cause an obvious loss of function, it could possibly alter the physical properties of the proposed transmembrane domain which could lead to protein malfunction. The role of the *Lps* locus in chickens was also examined. This locus could only be tested indirectly because it had not yet been cloned at the time these experiments were carried out. Instead, a DNA marker closely linked to *Lps* in the mouse (*Tnc*) was used in chickens to determine linkage with disease phenotype (Hu et. al., 1997).

The linkage analysis, for both loci, was carried out using a chicken DNA panel containing 425 progeny specifically using susceptible line C (Gln^{223}) and resistant line W1 (Arg^{223}) bred at the Institute for Animal Health (IAH), Compton, England. The panel was created using a backcross between two birds derived from inbred White Leghorn lines that differed in their disease resistance (Burt et. al., 1995). Once the progression of *Salmonella* infection was divided into two phases, an early phase (0-1 week) and a late phase (1-2 weeks), survival analysis revealed that both *NRAMP1* and *TNC* were involved in early phase resistance (Hu et. al., 1997). Together these genes were responsible for 33% differential resistance and susceptibility of parental lines W1 and C to *S.* Typhimurium infection. The candidate gene approach was successful in identifying two chromosomal regions important to *Salmonella* disease progression, and which influence survival, in the chicken. The

statistical analysis also revealed that, though these genes are significant, other unknown genes influence resistance and susceptibility to salmonellosis. Guillot et. al. (1996) found that susceptible lines of chicken vary in survival time which also suggest that different genes and mechanisms of resistance could be involved with infection.

(6) Objectives

The first objective of this thesis was to gain insight into the frequency and importance of the $Arg^{223} \rightarrow Gln^{223}$ NRAMP1 variation previously found in *Salmonella*-susceptible line C chickens (Hu et. al., 1997). Several outbred lines and pedigreed stock were tested for the presence of this non-conservative substitution. This type of information could be useful to determine whether this mutation is associated with disease in other lines of chickens. Ultimately, chicken breeders could test their stock for susceptiblity genes in order to control *Salmonella* in their chicken populations.

The second objective of this thesis was to genetically map chicken *TNC*. The chicken genome is about one third the size of the mammalian genome (1.2×10^9 bases) consisting of 8 pairs of macrochromosomes, 30 pairs of microchromosomes and the sex chromosomes, Z and W (the female being the heterogametic sex) (Burt et. al., 1995). In the chicken, three different linkage maps, the East Lansing, Compton and Wageningen/Euribrid, have been created using three different mapping populations.The three maps were used to create an improved single consensus map that has gradually been developed for linkage analysis (Groenen et. al., 2000). The Wageningen/Euribrid panel was developed by the Animal Breeding and Genetics group of Wageningen University, The Netherlands, and was based on 456 F_2 chickens from a cross between two broiler dam lines originating from the white Plymouth Rock breed. The East Lansing panel was created at the USDA Avian Disease and Oncology Laboratory in the United States by mating a partially inbred Red Jungle Fowl with a highly inbred White Leghorn and the Compton panel was established at

the Institute for Animal Health in the United Kingdom through the mating of two White Leghorn lines that differ in their susceptibility to disease. We used the Compton panel to map *TNC*. This panel was originally created in order to locate genes responsible for resistance to *Salmonella* infection (Bumstead and Palyga, 1992). All three panels can be used in evolutionary studies through comparative genome mapping of chicken with other phylogenetically distant species (Jones et. al., 1997; Nanda et. al., 1999) or they can be used to determine important loci in the chicken that are associated with economically important traits (Burt et. al., 1995; Smith et. al., 1997). The association of *TNC* with the phenotype of *Salmonella* resistance may be due to the gene itself or to a closely linked gene in the chicken (Hu et. al., 1996). In the mouse and human genomes, *Tnc* is known to be closely linked to $Tlr4^{Lps}$ (Qureshi et. al., 1996), however, it is, as yet, unknown whether this same linkage group exists in the chicken. Once *TLR4* is mapped in the chicken, we will be able to determine the respective importance of these two genes in the progression of *Salmonella* infection.

The third objective of this project was to develop chicken NRAMP1 antibodies. Previous studies carried out in our laboratory have focused primarily on the *NRAMP1* gene itself and not on its protein. Northern blot studies revealed that chicken NRAMP1 was expressed predominantly in spleen, liver, thymus and lung which is similar to the tissue expression in mice and humans (Hu et. al., 1996). Hu et. al. (1996) also found that mRNA transcript levels were similar in both resistant and susceptible chickens suggesting that differential resistance of chickens to *S.* Typhimurium was not due to altered NRAMP1 mRNA expression. Several sequence polymorphisms were detected by sequence analysis of C and W1 NRAMP1 mRNA samples, however, they did not represent an obvious loss of function. Therefore, in order to characterize the role of the NRAMP1 protein in susceptible and resistant chicken lines, we developed anti-chicken NRAMP1 antibodies in the rabbit. Once these particular antibodies are raised, it is possible to determine the type of cell expressing chicken NRAMP1, the subcellular location of chicken NRAMP1, and the

amount of protein produced in susceptible chicken lines. Our project used as a model NRAMP1 antibody studies originally carried out in the mouse (Vidal et. al., 1996) in an attempt to raise antibodies specific to chicken NRAMP1.

Materials and Methods

Materials and Methods:

(1) Chickens:

Inbred White Leghorn chicken lines W1, C, N, 6_1, 7_2 and 15I were maintained at the Institute for Animal Health (Compton, UK) in specific pathogen-free facilities. Lines N, 15I, 6_1 and 7_2 were derived from the flocks held at the Avian Diseases and Oncology Laboratory (East Lansing, USA), line C was originally developed at the Northern Poultry Breeding Station (Reaseheath, UK) and line W1 was acquired from Dr. J. Ivanyi (Wellcome Laboratories, Beckenham, UK). Inbred lines 6_1, W1 and N are resistant to *S.* Typhimurium, *S. gallinarum*, *S. pullorum* and *S. enteriditis*, whereas, inbred lines 15I, 7_2 and C are relatively susceptible. The partially inbred Red Jungle Fowl and inbred White Leghorn were maintained at the Avian Diseases and Oncology Laboratory (East Lansing, USA).

The outbred chicken lines used in the genotyping experiments were obtained from the Institut National de la Recherche Agronomique (INRA: Nouzilly, France). Line Y11 is an experimental meat-type line, line L2 is an egg-type line, B13 is a White Leghorn inbred line homozygous for the MHC of the B13 haplotype and line PA12 is a White Leghorn line (Protais et. al., 1996). Samples from pure line pedigreed stock consisting of six commercial egg-type and 6 commercial meat-type chicken lines obtained from Shaver Poultry Breeding Farms (Cambridge, ON, Canada) were also used in the genotyping experiments.

Chicken line FC (Hyline International, Dallas Centre, USA) was used for the splenocyte membrane and cytosolic preparations.

(2) Chicken *NRAMP1* Genotyping:

Preparation of DNA from Blood:

Blood samples were collected from 120 pure pedigreed chickens (60 meat-type and 60 egg-type) at Shaver Poultry Breeding Farms (Cambridge, ON). In our laboratory, cell lysis buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 1% Triton 100) was added to the blood in order to break down the cell membrane. The nuclei were centrifuged out of the solution for 15 minutes at 4°C at 1300g. The pelleted nuclei were then washed two additional times with cell lysis buffer and centrifuged under the same conditions. The pellet was resuspended in Tris/EDTA (TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 1% SDS in order to break open the nuclei. A Proteinase K (10 mg/ml) digestion was carried out overnight at 50°C. The DNA was extracted using a phenol and chloroform mixture. The DNA was subsequently precipitated with ice cold ethanol overnight at -20°C. The resultant DNA was spooled out of solution and dissolved in TE at 65°C. Concentrations were determined based on the binding of Hoechst 33258 fluorescent dye to thymidine and adenine bases and measured using a fluorometer (Hoefer Scientific Instruments, San Francisco, CA) so that each DNA sample was diluted to 10 µg/ml for PCR amplification.

PCR amplification and Restriction Enzyme Digest:

PCR amplifications of genomic DNA were carried out in 50 µl reaction volumes containing 10 ng of template DNA. Cycling conditions were as follows: incubation for 2 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 52°C, 1 minute at 72°C and a final extension step of 7 minutes at 72°C. The DNA used was isolated from 60 meat-type and 60 egg-type chickens (previously described) and 28 chicken DNA samples

(from lines Y11, L2, B13, PA12) kindly provided by Dr. F. Lanthier (INRA, France). The primers used to amplify the region of *NRAMP1* showing an altered *Eag*1 restriction site between *Salmonella*-susceptible line C and *Salmonella*-susceptible line W were (5')CAAGCTGGAGGCCTTCTTCGGCTT(3') (position 622-645) and (5')GAGGAGTGCAGGAAGATGTTATGG(3') (position 805-828) (Hu et. al., 1997). The PCR products, after subjection to restrition enzyme digest, were detected by ethidium bromide staining on 2% agarose gels. Chickens carrying the $Arg^{223} \rightarrow Gln^{223}$ mutation have one band (approximately 200 bp) on the gel while wild type chickens have two bands (approximately 70 bp and 130 bp) indicating successful *Eag*1 digestion.

(3) Production of Anti-chicken NRAMP1 Antibodies:

Immunogens:

Oligopeptides used for immunization were selected within the most hydrophobic regions of the chicken NRAMP1 protein. Nucleotide and predicted amino acid positions for NRAMP1 cDNA, DNA and protein are as described by Hu et. al. (1996). A DNA fragment encoding the amino-terminal domain (residues 3-62) of the NRAMP1 protein was generated via polymerase chain reaction (PCR) using oligonucleotides GST1 of sequence (5')**TCTGGATCCGGCCCTGCTATGGCA**(3') (*NRAMP1* nucleotides 35 to 56 are in bold and the *Bam*H1 site is underlined) and GST2 of sequence (5')ACAGAATTCCTGAAGCTGAAGCCG(3') (*NRAMP1* nucleotides 211 to 196 are in bold and the introduced *Eco*R1 site is underlined). The amplification reaction was carried out for 20 cycles (1 min 94°C, 1 min 55°C and 1 min 72°C) using 1ng of cDNA template under experimental conditions suggested by the supplier of *Taq* polymerase (Amersham/Pharmacia). A similar strategy was used to produce a DNA fragment encoding a carboxy-terminal domain (residues 523-555) of the NRAMP1 protein using primers

GST3 (5')ATGGATCC**ACGTGCAGCATTGCTCAC**(3') (*NRAMP1* nucleotides 1592-1609 are in bold and the introduced *Bam*H1 site is underlined) and GST4 (5')ATACTTAAG**TGCGGCCCTGCCAG**(3') (*NRAMP1* nucleotides 1693-1679 are in bold and the introduced *Eco*R1 site is underlined). The same PCR conditions were used to amplify the carboxy-terminal using 1ng of chicken genomic DNA. We could not use a cDNA template to amplify the carboxy-terminal because there is no cDNA clone isolated for this region of chicken NRAMP1.

Preparation of Immunogens and Plasmid for Cloning:

The amplified fragments representing the amino- and carboxy-terminal domains were subjected to restriction endonuclease digestion with *Bam*H1/*Eco*R1 (NEB, Beverly, MA) in preparation for cloning into the bacterial expression vector, PGEX-2T (Amersham/Pharmacia). The digestion of the DNA fragment was carried out using bovine serum albumin (BSA) (100x) (Sigma, St. Louis, Missouri), 10 units each of *Bam*H1 and *Eco*RI with corresponding enzyme buffer (10x) and 3μg of the amino- or carboxy-terminal domain PCR products, at 37°C for four hours. The plasmid, PGEX-2T, was isolated from a single colony of *Escherichia coli* (provided by the laboratory of Dr. S. Vidal, Department of Biochemistry, University of Ottawa) grown up in 500 ml antibiotic-containing Luria-Bertani medium (LB). This large preparation of *E. coli* was harvested by centrifugation and lysed, first by adding 0.01M EDTA in a solution of 0.05M Tris (pH 8.0) and 0.05M dextrose, secondly by exposing the cells to detergent and alkali (0.2M NaOH, 1% SDS), and thirdly by treatment with 3M KAcetate and 10% acetic acid. The plasmid DNA was subsequently purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient. PGEX-2T was then digested with the same restriction enzymes as the amino terminal DNA fragment, using the same principals. Both the fragment and the plasmid were electrophoresed in agarose gels (2% and 1%, respectively) and isolated from the gels with GeneClean II Kit (Bio/Can

Scientific; Mississauga, ON) under experimental conditions suggested by the supplier. The DNA fragments were thus ready to be cloned into the corresponding site of PGEX-2T.

Cloning and Transformation:

The cloning was performed via ligation with subsequent transformation of the plasmid constructs into heat shocked *E. coli* XL-1-blue MRF' (Stratagene, La Jolla, CA) competent cells. Ligation was carried out at 16°C overnight using 1 unit of bacteriophage T4 DNA ligase (Gibco, Rockville, MD) in the appropriate buffer (10x) following the protocol in Maniatis et. al. (1989). The amount of insert DNA used in the ligation reaction was calculated using the ratio of the amount of vector (ng) multiplied by the insert size (bp) to the size of the vector (bp). The XL-1-blue cells were made competent by exposure to a combination of divalent cations followed by treatment with dimethyl sulfoxide (DMSO; Sigma), reducing agents and hexamminecobalt chloride (Maniatis et. al., 1989). These cells were then ultimately prepared for the acceptance of plasmid DNA by heat shocking the XL-1-blue cells. The circularized recombinant with the amino-terminal fragment thus transforms *E. coli* to ampicillin resistance and this allowed selection of successful transformants. These transformed *E. coli* were grown on ampicillin-containing petri plates. As negative control XL-1-blue without plasmid and XL-1-blue transformed with linearized vector were grown on ampicillin plates and as positive control XL-1-blue transformed with intact PGEX-2T were grown on ampicillin plates.

In order to verify the integrity of the cloned DNA fragment, a PCR reaction was carried out with the primers GST1 and GST2 using a DNA preparation (Wizard Plus Miniprep DNA Purification System; Promega, Madison, Wisconsin) from the transformed bacteria. Direct sequencing was also carried out on the cloned plasmid DNA using primers GST1 and GST2. The cycle-sequencing method was used via the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham/Pharmacia). The sequencing

reactions were cycled 30 times (30 secs 95°C, 30 secs 55°C, 1 min 72°C) using 300 ng of DNA template, 20 ng of either primer GST1 or GST2. The products were run on a glycerol-tolerant gel (7M urea, 8% polyacrylamide, 5x Tris/taurine/EDTA).

Purification of GST Fusion Protein:

In order to purify the GST-fusion proteins, transformed *E. coli* XL-1-blue MRF' bacterial cultures, were induced with 0.1mM isopropyl β-D-thiogalactoside (IPTG) for six hours. These bacteria were then lysed by sonication in phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, pH 7.4). The GST chimera was recovered by affinity chromatography using Glutathione Sepharose 4B beads (Amersham/Pharmacia) and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were measured using a detergent-modified Lowry assay (Markwell et. al., 1981). The GST-fusion protein samples (20-50 μg) were denatured at 100°C for three minutes in SDS gel-loading buffer (1x: 50mM Tris-Cl pH 6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) before loading on the gel. The fusion protein band was excised from the gel after staining with Coomassie blue.

The GST protein was also purified using the same method above except that the *E. coli* bacteria employed to produce this protein had been tranformed using the PGEX-2T vector without an insert.

Production of Antibodies:

Polyclonal antibodies against the GST-fusion protein were raised in New Zealand White male rabbits. Fusion protein GST-N excised from the SDS gel was fragmented and emulsified in an equal volume of complete Freund's adjuvant (Gibco) and dispensed as

four 250 µl sub-cutaneous injections. This was followed by three booster injections of electroeluted fusion proteins emulsified in incomplete Freund's adjuvant at three week intervals. Preimmune and immune serum were taken from rabbits by arterial puncture. Immune sera were taken one week after the second, third and fourth immunizations. These sera were then tested for the presence of serum antibodies.

Preparation of Chicken Splenocyte Membrane and Cytosolic Fractions:

Chicken spleens, taken from the chicken line FC, were donated by the laboratory of Dr. M. Ratcliffe, Department Microbiology and Immunology, McGill University. Splenocytes were extracted, homogenized and suspended in RPMI (Gibco). Using a hematocytometer, the cell suspension concentration was adjusted to $2x10^7$ cells per ml. Lympholyte M (Cedar Lane Laboratories Ltd., Hornby, ON) was layered under the cell suspension forming an interface between the two solutions. The layered solutions were then centrifuged for 20 minutes at 1000g at room temperature. A splenocyte layer was formed above the dense Lympholyte-M and below the RPMI in a density gradient. Splenocytes were carefully removed using a Pasteur pipette, diluted with RPMI and re-centrifuged at 800g for 10 minutes. Splenocyte crude membrane fractions were prepared according to a previously described procedure (Canonne-Hergaux et. al., 1999). The splenocytes were ground to a powder using a sterilized ceramic mortar and pestle precooled on dry ice and "Dounce" homogenized in ice cold extraction buffer (0.25M sucrose, 0.03M L-histidine, pH 7.2, protein inhibitors). The homogenate was then centrifuged at 6000g for 15 minutes. The homogenate supernatant was ultracentrifuged (Beckman SW41 for 1 hour at 4°C at 80 000g) and the resultant supernatant provided the cytosolic fraction of the chicken splenocytes. The pellet from the ultracentrifuged cells, corresponding to the membrane fraction, was resuspended in 150 µl of fresh, ice cold sucrose/histidine extraction buffer.

The protein concentration of the cytosolic and membrane fractions were estimated by a detergent-modified Lowry protein assay (Markwell et. al., 1981).

Immunoblotting Analysis:

Chicken splenocyte membranes (20-50 μg) and cytosolic fractions (20-50 μg) were boiled for three minutes in SDS-loading sample buffer (1x) and run on a 10% SDS-polyacrylamide gel along with 5-10 μg of GST protein and 5-10 μg of GST-N. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) at 150 mA for 40 minutes using a semi-dry system. Membranes were blocked overnight at 4°C in a 10% milk/Tris buffered saline-Tween solution (TBST: 10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween-20) and then incubated with primary antibody in 10% milk and TBST (1:1000) for one hour at room temperature. The blot was then incubated with the secondary antibody, horseradish peroxidase conjugated goat anti-rabbit IgG antiserum (Sigma), in 10% milk and TBST (1:10 000) for one hour at room temperature. The Super Signal West Pico chemoluminescent substrate (Pierce, Rockford, IL) was used to reveal the immune complexes. The Western blot was incubated for 5 minutes in a one-to-one ratio of each chemoluminescent substrate (luminol + an enhancer and hydrogen peroxide). The light emission created by the chemical reaction of horseradish peroxidase (secondary antibody) and the chemoluminescent substrates was recorded using Kodak Biomax MR film. The film was exposed to the light emission for 30 seconds to 2 minutes and then developed.

(4) Mapping of Chicken Tenascin C (*TNC*)

Chicken Mapping Panels:

The Compton reference mapping panel was used in the mapping experiments. This mapping panel, developed at the Institute for Animal Health (Compton, UK), was derived from the White Leghorn lines N (*Salmonella*-resistant) and 15I (*Salmonella*-susceptible). Approximately 190 RFLPs were used to define 36 autosomal linkage groups from a panel of 56 progeny. Approximately 400 markers have been mapped within this reference population. The breeding and maintenance of (Nx15I)F1x15I segregating backcross chickens have been described previously (Bumstead and Palyga, 1992).

Genomic DNA Preparation and Southern Blot:

High molecular weight chicken genomic DNA samples obtained from N (resistant) and 15I (susceptible) chickens were digested to completion with numerous restriction enzymes (100x BSA, 10 units restriction enzyme, 10x enzyme buffer, 5µg DNA). The digested DNA, in gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Amersham/Pharmacia)) was separated by gel electrophoresis through a 1% agarose gel cast in 1x Tris-borate/EDTA elecrophoresis buffer (TBE) containing ethidium bromide (0.5µg/ml). The DNA was transferred via Southern blot onto nylon Hybond membrane (Amersham/Pharmacia).

Hybridization Probe:

Human tenascin C cDNA partial clone was kindly provided by Dr. Luciano Zardi from the Laboratory of Cell Biology, Genoa, Italy. The clone contained 2126 bp of human

TNC from 791 bp to 2917 bp of the sequence and was amplified by PCR (1 min 94°C, 1 min 60°C, 2 min 72°C; for 35 cycles) using λgt11 primers (Clonetech Laboratories Inc., Palo Alto, CA). The PCR product was migrated on a 1% agarose gel and the band corresponding to human *TNC* was excised, treated with beta-agarase (NEB), and subjected to phenol-chloroform extraction. The product was labeled with ^{32}P (3000 Ci/mmol) using Klenow enzyme (1000 U/ml) then purified using Sephadex G-50 (Amersham/Pharmacia) pre-equilibrated in 1x salt-Tris/EDTA (STE: 0.1M NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0). This labeled probe was then used, on the previously described Southern blot membrane, to test for the presence of restriction fragment length polymorphisms (RFLPs). Hybridization was performed in 50% formamide, 10% dextran sulfate, 1x Denhardt's solution, 0.1% SDS, 5x salt-sodium-citrate (SSC) and 10μg/ml denatured salmon sperm DNA at 65°C overnight (probe counts = 10^6cpm/ml hybridization solution).

DNA Markers used for genotyping:

DNA markers for the gene, α-1-microglobulin-bikunin (*AMBP*), and the microsatellite markers, *ADL199*, *ADL202*, *MCW151* and *HUJ002*, all mapping to chicken microchromosome E41W17, were tested for polymorphisms in the parental strains of the Compton reference population. All primers used to amplify the microsatellite markers were obtained from Drs. H. Chang and J. Dodgson of the Avian Disease and Oncology Laboratory, East Lansing, USA. The sequences for the *AMBP* primers were obtained from GenBank, accession number, X54818. These oligonucleotides were radio-labelled with ^{33}P and used in PCR reactions (1 min 94°C, 1 min 52°C, 1 min 72°C; for 35 cycles) to make probes. All PCR reactions were run on an 8% polyacrylamide sequencing gel under denaturing conditions.

Identification of TNC *Polymorphism for the East Lansing Panel:*

An approximately 2000 bp intron segment of chicken *TNC* was amplified using primer TNC-U of sequence (5') GCCCCTCACTGCTTTTGTC (3') (position 525-533) and primer TNC-L (5') GCCCCCACTGGAAAACCTA (3') (position 559-577). The primers were kindly provided by Dr. F. Lanthier (INRA, France). The amplification reaction was carried out using 40 ng of DNA template from the East Lansing backcross reference population parental strains, (JFxWL)F1 and WL, as well as the JF founder male, and 0.5 μM of each primer for 35 cycles (1 min 94°C, 1 min 59°C, 2 mins 72°C). The resultant DNA fragments were identified on a 1% agarose gel by ethidium bromide staining and were gel purified using GeneClean II Kit. These DNA were then sequenced with the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit, as previously described, using primer TNC-U. New primers were used in sequencing reactions to search for a point mutation within the *TNC* intron. A series of four point mutations were found between the JF founder male and the WL backcross parental strain approximately 800 bp into the *TNC* intron using primer TNC-U3 (5') ACCTATACATCCCTTGTACAT (3').

Linkage Analysis:

Single-stranded length polymorphisms (SSLP) were found between (Nx15I)F1 and 15I Compton backcross parental strains with microsatellite markers *MCW151*, *HUJ002* and *ADL202*, all mapping to chicken microchromosome E41W17. These polymorphisms were typed, in concurrence with the *TNC* RFLP, in the Compton backcross mapping panel.

Linkage analysis was carried out using the computer program MapManager version 2.6 (Manly, 1993). Gene order was determined by minimizing the number of double crossover events required to explain haplotype distributions.

Screening of Chicken Cosmid Library:

An attempt was made to identify a cosmid clone corresponding to chicken *TNC* in order to verify its chromosomal position via fluorescence *in-situ* hybridization (FISH). The human *TNC* probe, previously described, was used to screen a chicken genomic library ($1.4x10^6$ independent clones) constructed in the *Bam*H1 site of cosmid vector pWE15 (Stratagene). The chicken library was plated on nitrocellulose filters (Amersham/Pharmacia) on LB plates containing ampicillin. Replicas of the colony forming units (cfu) on the LB plates were made using Hybond nylon filters (Amersham/Pharmacia). The replicas were incubated with the ^{32}P radio-labelled human *TNC* probe in hybridization solution (35-50% formamide) at 60°C overnight. The replica filters were washed in 2x SSC for 20 minutes at room temperature, 0.5x SSC for 20 minutes at 50°C and 0.5-0.2x SSC for 30 minutes at 60°C. The hybridized library was then exposed to a Kodak X-OMAT X-ray film overnight at -70°C using an intensifying screen. The second screenings were carried out under conditions of high stringency (see conditions for chicken TNC probe).

A chicken probe was also created via reverse transcriptase (RT) PCR (1 min 94°C, 1 min 65°C, 2 min 72°C; for 35 cycles) using chicken RNA previously isolated by Hu et. al. (1996). Primers for the chicken probe were designed from the chicken *TNC* sequence, exon 4190-5661 bp, amplifying a 1431 bp segment. The hybridization and washing conditions used for the probe were of high stringency. The replicas were incubated with the chicken probe in hybridization solution containing 50% formamide at 65°C overnight. The final wash was in 0.1x SSC for 30 minutes at 65°C and the hybridized library was exposed to X-ray film for 4 hours or overnight at -70°C using two intensifying screens.

Results

Results:

(1) *NRAMP1* Genotyping:

Dr. Malo's laboratory has previously shown that resistance to infection with *S.* Typhimurium in chickens is inherited as a complex trait and that *NRAMP1* accounts for 18% of the early differential resistance to infection. In addition, we have reported the sequence analysis of *NRAMP1* transcripts from three susceptible chicken lines (7_2, 15I, C) and three resistant chicken lines (N, 6_1, W1). Nucleotide sequence analysis of the coding portion of *NRAMP1* in these chicken lines revealed 11 sequence variants, 8 of which were silent mutations, whereas, 3 were nonsilent mutations: $Thr^{55} \rightarrow Ala^{55}$, $Arg^{223} \rightarrow Gln^{223}$, $Val^{307} \rightarrow Ile^{307}$. $Thr^{55} \rightarrow Ala^{55}$ and $Val^{307} \rightarrow Ile^{307}$ are two conservative mutations located in the amino-terminal portion of *NRAMP1* and between predicted TM 7 and TM 8, respectively. These two conservative changes were not associated with susceptibility to infection. However, the $Arg^{223} \rightarrow Gln^{223}$ substitution within the predicted TM 5-6 interval is specific to susceptible line C and was not present in any of the resistant lines tested (Hu et. al., 1997).

In order to gain some insight into the frequency and importance of the $Arg^{223} \rightarrow Gln^{223}$ variant in different chicken populations, we tested the genotypes of 28 outbred chicken DNAs (kindly provided by Dr. F. Lanthier, INRA, France) and 120 samples from 12 pure line pedigreed stock (kindly provided by the Shaver Poultry Breeding Farms Ltd., Cambridge, ON). The samples from INRA were obtained from four chicken lines: line Y11, an experimental meat-type line; line L2, an egg-type line; B13, a White Leghorn inbred line homozygous for the MHC of the B13 haplotype; and line PA12, a White Leghorn line (Protais et. al., 1996). These chicken lines have been tested for their susceptibility to infection with *S. enteritidis*. The commercial egg-type line, L2, was found to be the most susceptible to this pathogen. The samples from Shaver Poultry Breeding Farms were obtained from 6 commercial egg-type and 6 commercial meat-type chicken lines.

These chicken lines have been developed for markets in the USA, Japan and Northern Europe for high egg mass, shell quality with early maturity or for competitive growth rate and high breast yield with high fertility. The *Salmonella*-susceptibility status of these chicken lines is not known.

We tested the genotype frequencies of *NRAMP1* in the chicken lines from the INRA and the Shaver Breeding Farms using a previously described *NRAMP1* polymorphism (Hu et. al., 1997). This polymorphism relied on a disruption of an *Eag*1 restriction site at the $Arg^{223} \rightarrow Gln^{223}$ substitution. It was originally found that the resistant W1 line retains the restriction enzyme site while the *Salmonella*-susceptible line C DNA does not. Our results showed, however, that none of the chickens tested bear the $Arg^{223} \rightarrow Gln^{223}$ mutation, all of the chickens presented the resistant W1 genotype. This suggests that the $Arg^{223} \rightarrow Gln^{223}$ variant is rare and unique to *Salmonella*-susceptible line C.

(2) Production and characterization of anti-NRAMP1 specific antisera:

We generated anti-chicken NRAMP1 antisera to study the localization and regulation of NRAMP1 in normal chicken tissues. These antibodies will be further used to determine the cell type expressing NRAMP1, the protein's subcellular location and the amount of protein produced. Oligopeptides derived from chicken NRAMP1 were synthesized in order to create fusion proteins which were used as immunogens in rabbits. The oligopeptides were chosen from the extreme amino-terminal and carboxy-terminal ends of chicken NRAMP1. Their sequences have previously been found to be unique to chicken NRAMP1 and to be unlike other NRAMP-related proteins. These factors increase the likelihood that antibodies will be raised to the immunogen and not to rabbit NRAMP1 or to other NRAMP membrane proteins. The DNA fragment (position 38-211) encoding the amino-terminal (position 3-62) was successfully amplified via PCR using primers GST1 and GST2 and was subsequently purified. However, the amplification of the DNA fragment

(position 1592-1693) encoding the carboxy-terminal of NRAMP1 (position 523-555) using primers GST3 and GST4, was comparatively weak. Despite this fact, both oligonucleotides were used in subcloning experiments with the PGEX-2T vector.

The 177 bp amino-terminal oligonucleotide was subcloned into the multiple cloning site (MCS) of the PGEX-2T plasmid (Figure 1). The PGEX-2T vector has, among other attributes, an introduced GST gene from *Schistosoma japonicum*, a tac promotor, as well as, ampicillin resistance gene. The antibiotic resistance was used to select bacteria that had successfully incorporated the circularized PGEX-2T. In order to verify the integrity of the amino-terminal insert, primers GST1 and GST2 were used to sequence the purified plasmids. These sequencing reactions revealed that the plasmid did indeed contain the insert at the MCS (Figure 2). After several attempts, we did not succeed in the cloning of the PCR product representing the carboxy-terminal of NRAMP1.

The MCS is located at the end of the GST gene of the PGEX-2T plasmid which allows expression of the cloned NRAMP1 amino-terminal insert as a fusion protein to GST (GST-N). The GST gene is directed by the tac promotor, which is induced by IPTG, enabling controlled production of GST-N. The GST segment of the fusion protein provides an affinity tail for purification on Glutathione 4B Sepharose beads. In our case, PGEX-2T plasmids, with and without inserts, were induced with IPTG and both products were purified by affinity chromatography. The resultant GST protein and the chicken NRAMP1 amino-terminal GST-fusion protein were run on a 12% SDS-polyacrylamide gel (Figure 3). This gel revealed a band corresponding to the 27 500 Da GST protein (lane 4) and another band corresponding to the 34 000 Da GST-fusion protein (lanes 1-3). The size of the fusion protein on the gel corresponded to its predicted size. The fusion protein size was predicted by first converting the number of base pairs in the insert (177) to amino acids (59), this number was then multiplied by the average molecular weight of one amino acid (110 Da x 59 = 6490 Da) and the fusion protein size was determined simply by adding the size of the GST protein (27 500Da) to the predicted size of the insert (6490 Da). The smaller bands in

FIGURE 1: Schematic representation of PGEX-2T expression vector with NRAMP1 amino- and carboxy-terminal inserts:

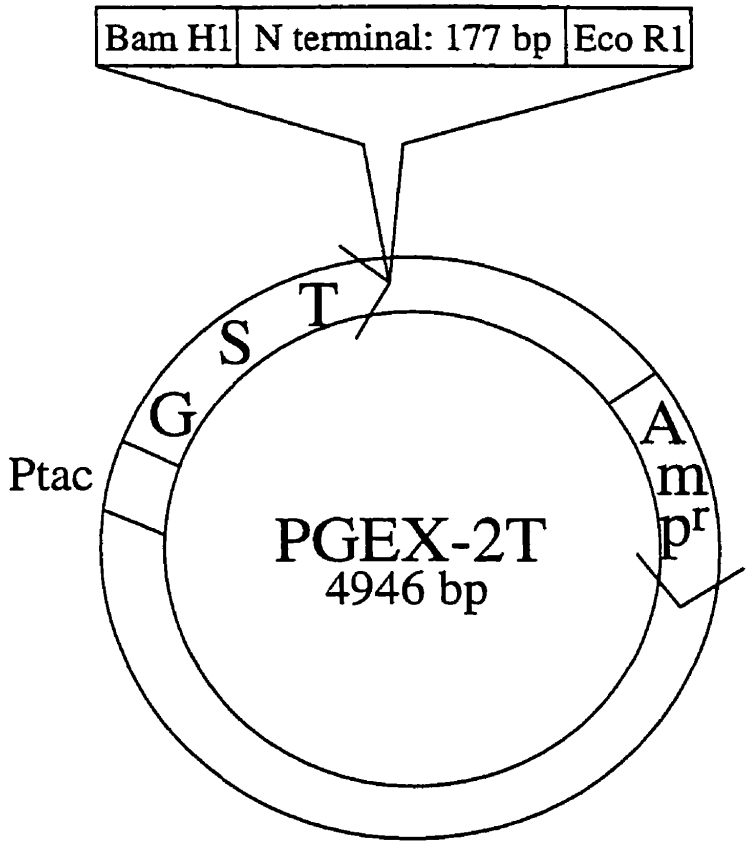
(A) The 177 bp chicken NRAMP1 fragment (position 38-211) from highly sequence divergent amino-terminal region showing *Bam*H1 and *Eco*R1 restriction enzyme sites, positioned at the PGEX-2T MCS

(B) The 111 bp NRAMP1 fragment (position 1592-1693) from highly sequence divergent carboxy-terminal region with *Bam*H1 and *Eco*R1 sites, positioned at the MCS of PGEX-2T

Amp^r = ampicillin resistance

Ptac = inducible tac promotor

A


Bam H1
N terminal: 177 bp
Eco R1
T
S
G
Ptac
A
m
p^r
PGEX-2T
4946 bp

B

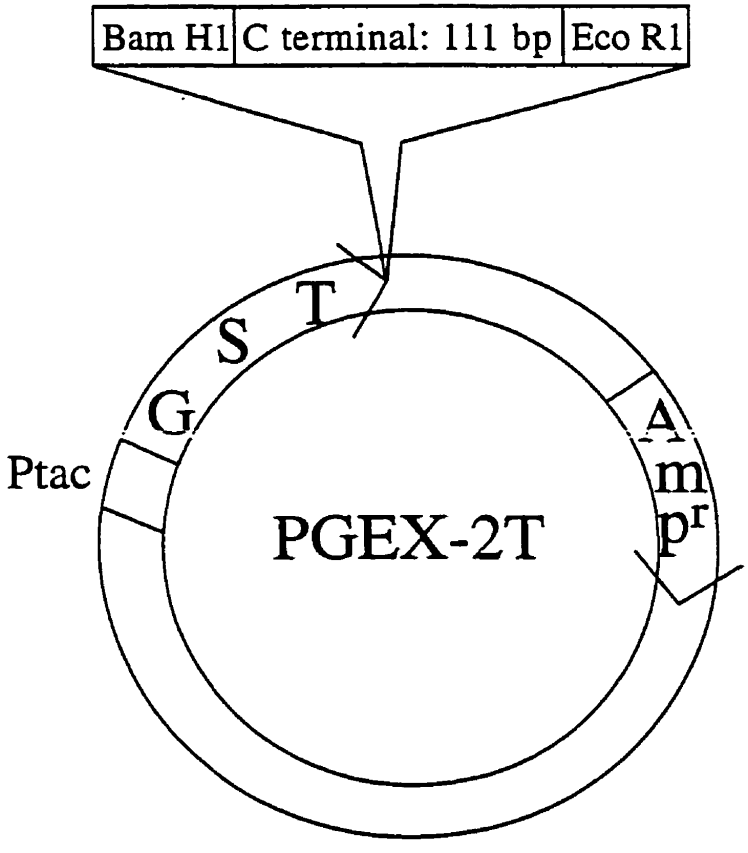

Bam H1
C terminal: 111 bp
Eco R1
T
S
G
Ptac
A
m
p^r
PGEX-2T

FIGURE 2: Sequence of NRAMP1 amino-terminal fragment cloned into MCS of PGEX-2T:

The NRAMP1 amino-terminal fragment (position 38-211) with *Bam*H1 and *Eco*R1 restriction sites underlined and PGEX-2T sequence in bold. The oligopeptide sequence (residues 3-62) is shown in uppercase letters.

5′ **aaaatcggatctggttccgcgt**ggatccggccctgctatggcatcattggagccaggcctcgctggg
G S G P A M A S L E P G L A G

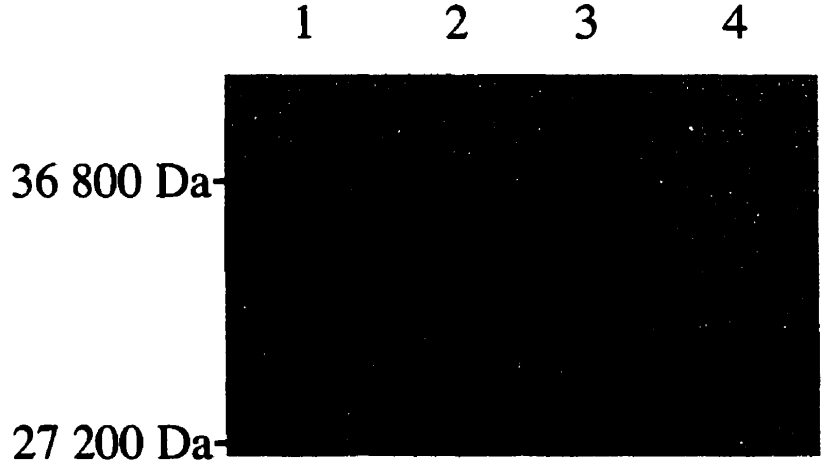
tccctgaaccgaggccaaactgacgccagcaatgtccctgttcccccacatcaccccgtcccccatgct
S L N R G Q T D A S N V P V P P H H P V P H A

cagacctacctggatgagctcatcagcatccccaaaggcagcacgcccggcttcagcttcagg**aattca**
Q T Y L D E L I S I P K G S T P G F S F R

tcgtgactgactgacgatctg 3′

FIGURE 3: SDS-polyacrylamide preparative gel:

The GST fusion protein (≅ 34 kDa) from purified cell lysate of PGEX-2T (+ amino-terminal insert) transformants after induction with IPTG is represented in lanes 1 to 3. Unstable fusion proteins are seen in the same lanes at smaller molecular weights. The GST protein (≅ 27.5 kDa) from IPTG induced PGEX-2T transformants (- insert) is represented in lane 4. The positions of molecular size standards in kiloDaltons (kDa) are indicated it the left of the gel.


1
2
3
4
36 800 Da
27 200 Da

lanes 1 to 3 of Figure 3 probably represent unstable fusion proteins that have broken down and released the 27 500 Da GST moiety, as well as, other partial proteolytic fragments.

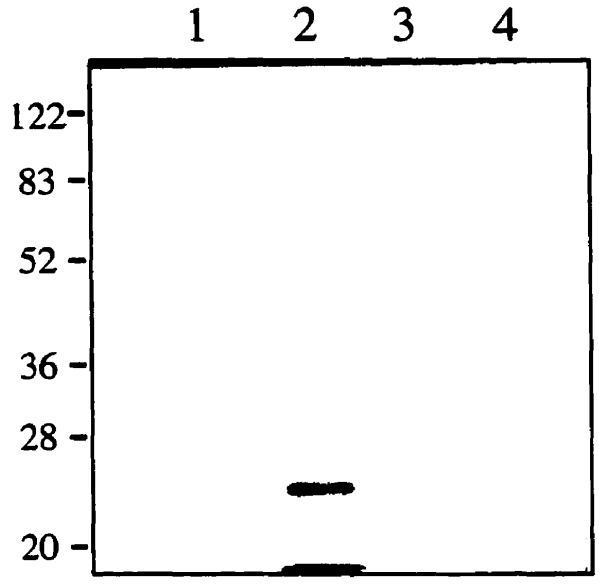
The GST-N fusion protein band was cut from the preparative gel, fragmented and then emulsified in an equal volume of complete Freund's adjuvant. The mixture was administered as four sub-cutaneous injections to two New Zealand White male rabbits. This was followed by three booster shots of electroeluted fusion protein emulsified in incomplete Freund's adjuvant at three week intervals. Immune sera were taken one week after the second, third and fourth immunizations. These sera were then tested for the presence of specific antibodies by Western blot analysis against the membrane and cytosolic fractions of chicken splenocytes, the GST-fusion protein and the GST protein. Once these proteins were separated by size on a 10% SDS-polyacrylamide gel they were transferred onto a PVDF membranes. The membranes were blocked with 10% milk in TBST solution, presented with the immune sera raised against the GST-N fusion protein and subsequently incubated with a secondary antibody conjugated with horseradish peroxidase. The immune complexes were identified by activating the membrane-bound horseradish peroxidase through the addition of a chemoluminescent substrate. The light emitting signals were exposed on Kodak Biomax MR film and developed for 30 seconds to 2 minutes.

Hyperimmune antisera were generated from two rabbits and the results from one of the rabbits is shown in Figure 4. The membrane fraction of chicken splenocytes is represented in lane 1, lane 2, represents the cytosolic fraction, lane 3, the GST protein and lane 4, the GST-N fusion protein. The anti-chicken NRAMP1 antibody specifically detected a major protein species of 90-100 kDa in chicken splenocytes (cytosolic and membrane preparations). This immunoreactive band was more abundant in the cytosolic fraction and was absent in the membrane and cytosolic extracts incubated with preimmune sera. Additional bands were detected at approximately 19 kDa, 27 kDa and 35 kDa. The 19 kDa and 27 kDa bands were also detected with the preimmune sera, whereas the 35 kDa band was present predominantly in the splenocyte membrane fraction exposed to the immune

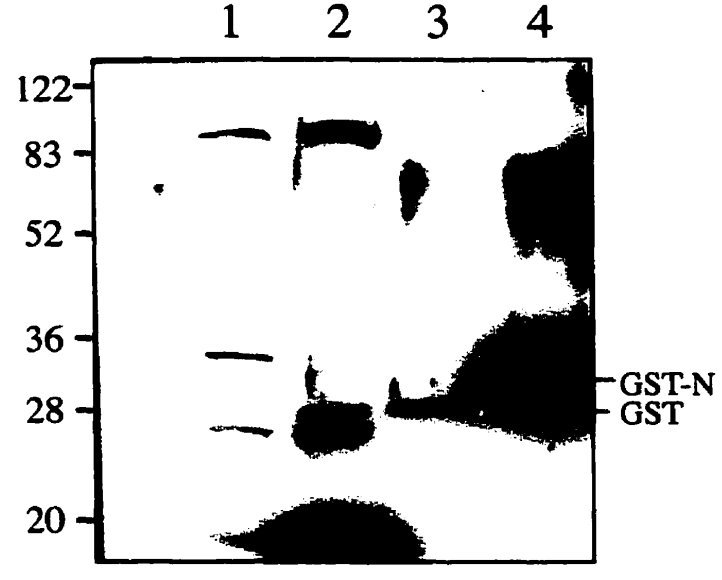
FIGURE 4: Identification of anti-NRAMP1 antibodies by immunoblotting:

The membrane fraction of chicken splenocytes (lane 1), cytosolic fraction (lane 2), GST protein (lane 3) and GST-N fusion protein (lane 4) were introduced to preimmune and immune sera raised against the GST-N fusion protein via Western blot. Immune complexes were detected by horse radish peroxidase reaction on film exposed for 30 seconds. The positions of molecular size standards in kDa are indicated to the left of the gel.

Preimmune



Immune



sera. This immunoreactive band may represent a degradation product of the native NRAMP1 protein.

(3) Tenascin Linkage Analysis:

We have shown that *TNC* (a closely-linked marker to *Tlr4*^{Lps}), is linked to resistance to salmonellosis in chickens (Hu et. al., 1997). Together *NRAMP1* and *TNC* explain 33% of the genetic variance observed between *Salmonella*-susceptible line C and resistant line W1. *NRAMP1* has been mapped to chicken chr 7 by our group (Hu et. al., 1995) and we report here the mapping of *TNC*.

High molecular-weight genomic DNA samples obtained from N (resistant) and 15I (susceptible) chickens were digested to completion with numerous restriction enzymes, electrophoresed, blotted and hybridized to the human cDNA *TNC* probe (Figure 5). Several RFLPs were detected with restriction enzymes *Bgl*II, *Kpn*1, *Stu*1, *Hinc*II, *Hae*III, *Dde*1 and *Nde*1. The segregation of the *Bgl*II, *Hae*III and *Nde*1 RFLPs identified with the TNC probe were then followed in a panel of 56 backcross progeny from (Nx15I)F1x15I mating. The *TNC* inheritance pattern was then compared with the segregation of 800 DNA markers previously assigned to this particular backcross panel (Bumstead and Palyga, 1992; web page: http://poultry.mph.msu.edu/). These results suggested that the likely location for *TNC* is on chicken chr 4 (LOD score=3.0, p=0.01), however, many double cross-overs were found between *TNC* and its nearest linked microsatellite markers, *LEI0085*, 24.1 cM proximal and *LEI0063*, 25 cM distal (Figure 6).

Subsequently, a search to find a cosmid clone corresponding to chicken *TNC* was carried out using a chicken genomic library. This cosmid clone could have been used in fluorescence *in situ* hybridization (FISH) in order to confirm the map location of chicken *TNC*. A human *TNC* probe as well as a synthesized chicken *TNC* probe were used to screen the genomic library. The chicken probe was created by RT PCR using RNA previously

FIGURE 5: Autoradiogram of a Southern blot:

Southern blot analysis of genomic DNA from (Nx15I)F1 and 15I parents of the (Nx15I)F1x15I backcross progeny. DNA samples were digested with *Nde*1, *Hinc*II, *Stu*1, *Sal*1, *Sac*1, *Pst*1, *Bgl*II and hybridized to a partial human *TNC* probe. *Bgl*II and *Nde*1 polymorphisms were used to follow the segregation pattern of *TNC* in backcross chickens.

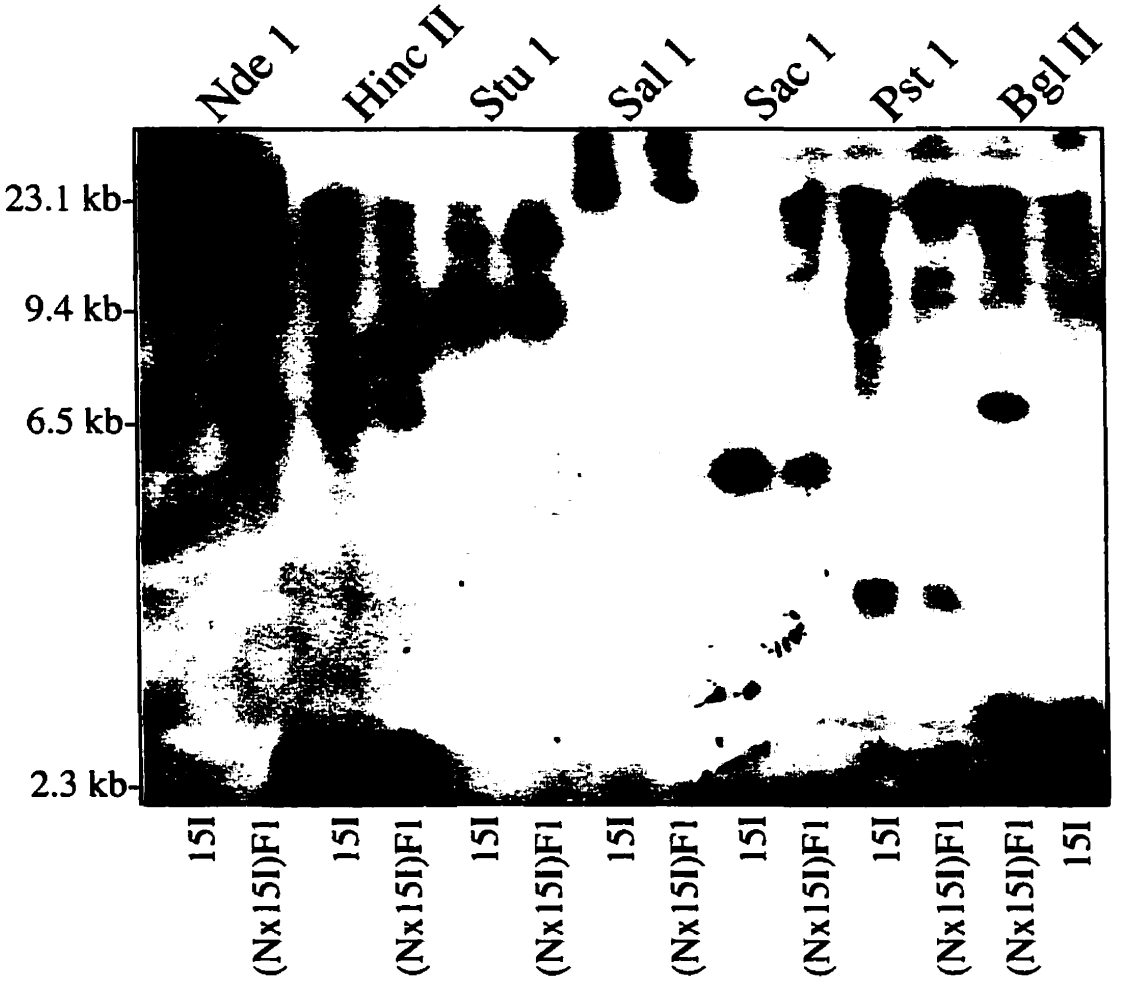

Nde 1
Hinc II
Stu 1
Sal 1
Sac 1
Pst 1
Bgl II
23.1 kb
9.4 kb
6.5 kb
2.3 kb
15I
(Nx15I)F1
15I
(Nx15I)F1
15I
(Nx15I)F1
15I
(Nx15I)F1
15I
(Nx15I)F1
15I
(Nx15I)F1
(Nx15I)F1
15I

FIGURE 6: RFLP analysis of 56 (Nx15I)F1x15I chickens:

Distribution of the F1 alleles (N) and 15I allele (I) in the Compton (Nx15I)F1x15I backcross reference panel for *TNC* and its two nearest linked markers, *LEI0085* (24.1 cM proximal) and *LEI0063* (25 cM distal) on chr 4. Double cross-overs are indicated in diagram with "X".

? = unknown

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
LEI0085	N	N	N	N	N	N	I	I	I	N	?	I	I	N	N	I	N	N	N	N	N	N	I	N	I	N	N	I	N
		x					x		x		?			x	x							x		x					x
TNC	N	I	N	N	N	N	N	I	N	N	I	I	I	I	I	I	N	N	N	N	N	I	I	I	I	N	N	I	I
		x					x		x		x			x	x							x		x					x
LEI0063	N	N	N	N	N	N	I	I	I	N	N	I	I	N	N	I	N	N	N	N	N	N	I	N	I	N	N	I	N

	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
	N	N	N	I	I	I	?	I	N	N	N	N	N	N	I	N	N	N	N	N	I	I	I	I	N	I	N
	x		x								x			x													x
	I	N	I	I	I	I	N	I	N	N	I	N	N	I	I	N	N	N	N	N	I	I	I	I	N	I	I
	x		x								x			x													x
	N	N	N	I	I	I	N	I	N	N	N	N	N	N	I	N	N	N	N	N	I	I	I	I	N	I	N

isolated in our laboratory and primers designed from a chicken *TNC* sequence obtained from GenBank, accession number, M23121. Unfortunately, both probes yielded only false positives, therefore, no cosmid clones corresponding to chicken *TNC* were isolated from the chicken library.

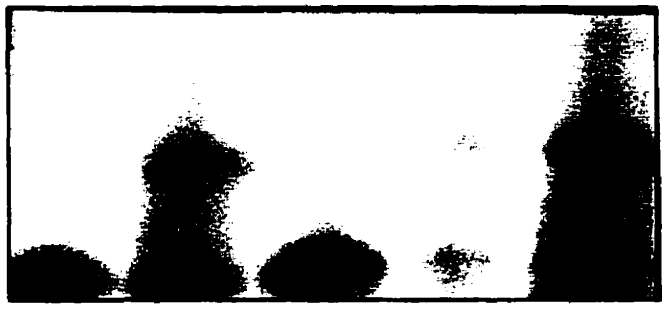
Because of the worrying number of double cross-overs between chicken *TNC* and its nearest linked markers and due to the inability to isolate a chicken *TNC* cosmid clone, we decided to investigate other linkage possibilities for this gene. An article by Nanda et. al. (1999), based on evolutionary studies between human and chicken, uncovered similarities between human chr 9 and chicken microchromosome E41W17. This portion of human chr 9 is also orthologous to mouse chr 4 where human and mouse *TNC* resides. Three markers from the desired region of chicken microchromosome E41W17 were found to show polymorphisms between the parental lines used to generate the Compton (Figure 7) backcross mapping panel. These polymorphisms were subsequently typed in this reference population. The Compton panel is composed of a backcross between two birds from partially inbred White Leghorn lines that differ in their disease resistance. The LOD score between *TNC* and its nearest marker on microchromosome E41W17 in the Compton mapping panel was slightly higher (LOD score=3.6; p=0.01) than previously indicated in linkage to chicken chr 4 (Figure 8).

Experiments have been initiated to map *TNC* in the East Lansing backcross panel. This panel was created from a cross between a single, partially inbred Red Jungle Fowl (JF) male and a highly inbred White Leghorn (WL) female; two F1 males were each mated to 8 or 10 White Leghorn females to produce the backcross progeny (Crittenden et. al., 1993). A polymorphism of 4 point mutations was found between the DNA sequence of the founder JF male and the WL female backcross parental strain (Figure 9). The polymorphism will be followed in the East Lansing reference population, (JFxWL)F1xWL, in concurrence with polymorphic markers mapping to the E41W17 microchromosome that we have already typed in this panel.

FIGURE 7: SSLP for Compton panel:

8% polyacrylamide denaturing gel showing SSLPs for markers *HUJ002* (A), *MCW151* (B), *ADL202* (C). Reported approximate size of amplified fragment for (A) is 125 bp, (B) 255-269 bp and (C) 200 bp.

A



B

C

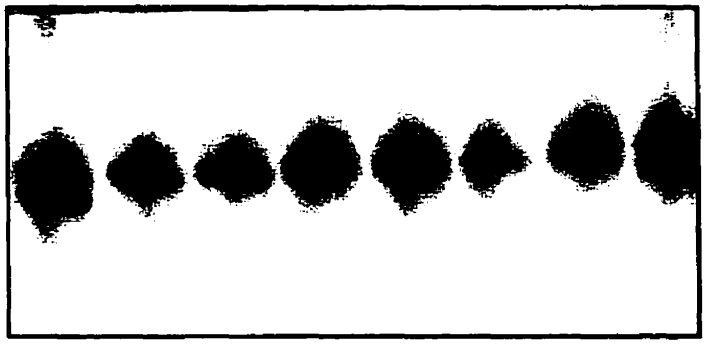
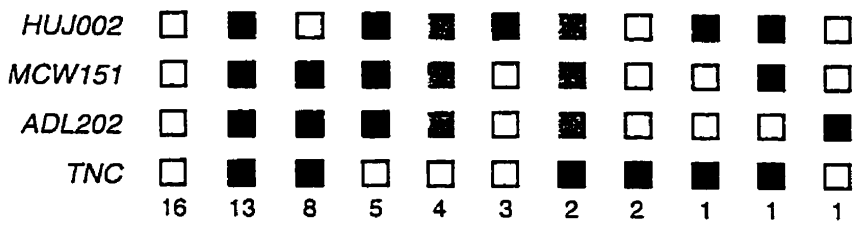


FIGURE 8: Segregation analysis of *TNC* and flanking markers in 56 (Nx15I)F1x15I backcross chicken progeny:

(A) Each column represents a chromosomal haplotype identified in the backcross progeny. Each locus is listed on the left. 15I alleles (open boxes), F1 alleles (black boxes) and unknown (grey boxes) are shown. The number of backcross chicken inheriting each type of chromosome is listed at the bottom of each column

(B) Partial genetic map of the linkage group carrying *TNC* in chicken. Recombination distances between loci (in centiMorgans = cM) are shown to the left of the chromosome.

A


HUJ002
MCW151
ADL202
TNC
16 13 8 5 4 3 2 2 1 1 1

B

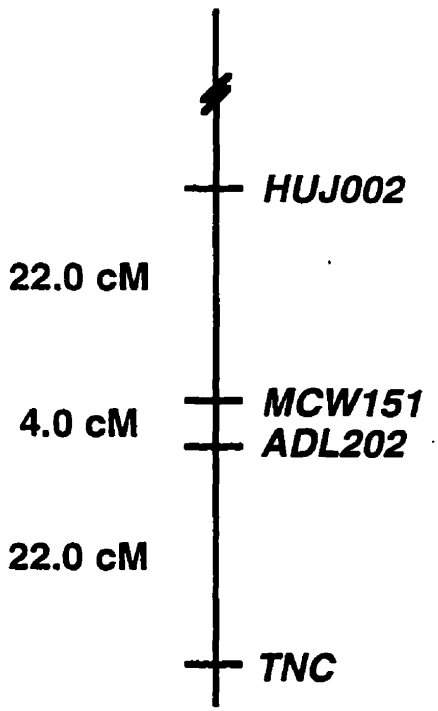

HUJ002
22.0 cM
MCW151
4.0 cM
ADL202
22.0 cM
TNC

FIGURE 9: *TNC* sequence polymorphism for East Lansing backcross parental strains:

The polymorphism found between founder JF male (A) and WL female backcross parental strain (B) are shown as four point mutations in bold type. The primers that will be used to amplify a 120 bp fragment encompassing the polymorphism are shown in uppercase letters. The resultant DNA fragments will be used to find an SSCP.

A

5′
ACCTATACATCCCTTGTACATccctacacctatacatccctggaggcattcaaggccaagctggatg

tggctctgggcagcctggtctggtggttggtaaGGGTGCACATAGCAGGAGTT
3′

B

5′
ACCTATACATCCCTTGTACATccctacacctatacattcctggaggcatccaaagccaagctggatg

tggctctgggcagcctggtctggtggttggtaaGGGTGCACATAGCAGGAGTT
3′

We originally mapped chicken *TNC* using a low density marker chicken reference population. This linkage analysis was carried out using a human *TNC* probe that readily detected polymorphisms in the chicken *TNC* gene. Whereas, the use of human DNA on a chicken reference panel proved successful, the sparcity of markers in the panel itself proved problematic. The gene was mapped to an area of the chicken genome with very few markers and there were many cross-over events between it and its nearest linked markers. In this case, it was more advantageous to use a comparative mapping approach through the identification of a candidate gene region. Genes already defined on human chr 9 were used to map a conserved syntenic region on chicken microchromosome E41W17 (Nanda et. al., 1999). *AMBP* is one of the genes in this conserved syntenic group and is known to be linked to *TNC* and *TLR4* in humans and mice (chr 4) (Qureshi et. al., 1996). Previous to the article by Nanda et. al. (1999), linkage conservation has been reported between chicken and mammals (Burt et. al., 1995; Hu et. al., 1995). With this information we were able to test for linkage of *TNC* to ths chicken microchromosome. This deductive mapping method was shown to be more accurate than the previous linkage analysis. Once the chicken genetic map is more established in all regions of the genome, traditional linkage analysis in the chicken will be more accurate and reliable.

Discussion

Discussion:

In most cases, multiple genes as well as environmental factors affect host-pathogen interactions. The genetic approach to the study of disease can allow the identification of genes and proteins implicated in key physiological events and can provide an understanding of the mechanism by which alterations in these genes cause disease (Vidal and Gros, 1994). Often, these genes have been well-conserved throughout evolution. It is possible, once a disease gene has been identified in one species, to use orthologous genes as candidates for linkage to the corresponding disease in another species.

In our laboratory, we have used the genetic approach to study salmonellosis in chickens. *Salmonella* spp. are intracellular facultative pathogens causing localized or systemic infections, in addition to a chronic, asymptomatic carrier state, in a wide range of hosts (Zhang-Barber et. al., 1999). The consequences of infection by these bacteria are of world-wide economic and public health significance. The incidence of salmonellosis has increased dramatically over the past 20 years in North America and Europe (Falkow and Malkanos, 1990). In developing countries, *Salmonella* is a major killer especially among malnourished children (Kariuki, 1996). *Salmonella* is also known to be an opportunistic pathogen associated with HIV and AIDS (Morris and Potter, 1997). The infection in these patients can be of increased severity and could be ultimately fatal. A compounded problem is that *Salmonella* bacteria have become increasingly resistant to a variety of antimicrobial drugs which complicates treatment for all individuals (Rowe et. al., 1999; Cowden, 1996).

Dr. Malo's laboratory has focused on two genes, *Nramp1* (the gene encoding for the *Ity* locus, Vidal et. al., 1993) and *Tlr4* (the gene encoding for the *Lps* mutation, Poltorak et. al., 1998; Qureshi et. al., 1999), identified first in the mouse and known to be involved in *Salmonella* infection. These genes are known to be involved in the early non-immune phase of host response, more precisely, in controlling macrophage function (Cellier et. al., 1995; reviewed in Qureshi et. al., 1996). It has previously been demonstrated that host resistance

to infection with *Salmonella* in chicken is complex and controlled by several genes acting at different steps of the host immunological response (Bumstead and Barrow, 1988; Hu et. al., 1995). Our group used the knowledge previously developed in the mouse model to examine the candidacy of *Nramp1* and *Tlr4* as influencing factors involved in resistance to *Salmonella* infection in the chicken. Hu et. al. (1995 and 1996) cloned and mapped the *NRAMP1* gene in chicken, examined its genetic structure and its mRNA expression. It was found that, based on the predicted amino acid sequence, the chicken NRAMP1 is 68% identical to mouse and human NRAMP1 sequences. The overall genetic structure and regulatory elements in the promotor region were also found to be similar between mouse, human and chicken. Northern blot analysis of total cellular RNA isolated from a variety of chicken tissues demonstrated that the highest expression of chicken NRAMP1 is in the spleen and thymus, with much lower expression in the liver and lung (Hu et. al., 1996). These results are similar to studies in mouse and humans where *NRAMP1* mRNA levels were lower in liver than in spleen (Vidal et. al., 1993; Cellier et. al., 1994). The high tissue expression of NRAMP1 in the thymus is unique to chicken. The cell type expressing NRAMP1 in the thymus is unknown, however, it is possible that the gene could be expressed in T cells, macrophages or dendritic cells (Hu et. al., 1996).

This information supports the hypothesis that NRAMP1 may play a similar role in mouse, human and chicken. Using this information, Hu et. al. (1997) set out to determine whether resistance to *S.* Typhimurium is linked to *NRAMP1* and *LPS* in chickens. At that time, *Lps* was not cloned, therefore, a locus tightly linked to *Lps* in the mouse genome (*Tnc*), was tested in the chicken. *NRAMP1* could be tested directly due to a nonconservative $Arg^{223} \rightarrow Gln^{223}$ substitution within the predicted TM 5-6 interval which is specific to *Salmonella*-susceptible line C and not found in any resistant line (Hu et. al., 1997). The analysis of 425 backcross chicken progeny (W1xC)F1xC derived from parental lines, C (susceptible) and W1 (resistant), revealed that *NRAMP1* and *TNC* account for 33% of early differential resistance to infection with *S.* Typhimurium in chickens. This research carried

out in our laboratory has brought new insights to the genetics of chicken salmonellosis. Using the candidate gene approach, Hu et. al. (1995, 1996 and 1997) demonstrated that species separated by millions of years of evolution could be compared genetically in order to elucidate important loci involved with disease. The orthologues, *NRAMP1* and *TNC*, were ultimately shown to be linked to *S.* Typhimurium resistance in the chicken.

Once *NRAMP1* and *TNC* were established as important loci involved with chicken salmonellosis, we initiated studies to elucidate the function of the NRAMP1 protein in the chicken and to map chicken *TNC*. At the beginning of this thesis, we studied the frequency of the $Arg^{223} \rightarrow Gln^{223}$ polymorphism in outbred chicken lines. The polymorphism could be followed directly in these chickens through a disruption of an *Eag*1 site based on the $Agr^{223} \rightarrow Gln^{223}$ substitution found previously in line C. The isolated chicken DNA from the Shaver Poultry Breeding Farms and the DNA from the INRA were tested for disruption of this restriction enzyme site. These chickens did not bear the $Arg^{223} \rightarrow Gln^{223}$ mutation, indicating that this mutation is rare and unique to *Salmonella*-susceptible line C.

Secondly, we raised antibodies against chicken NRAMP1 in order to study the expression pattern of the protein in resistant and susceptible chickens. These protein studies would enable us to better understand the role of NRAMP1 in chicken salmonellosis and the mechanism of susceptibility. This information could improve treatment of *Salmonella* infection in chickens or influence breeding practices which could eventually lead to better control of salmonellosis in the human population.

Finally, we examined *TNC* as a marker for *LPS* in chicken, a locus responsible for differential resistance to *S.* Typhimurium infection noted by Hu et. al. (1997). *Tnc* has been studied extensively in the mouse and is believed to be part of a family of extracellular matrix proteins (Chiquet-Ehrismann et. al., 1995). The protein is highly regulated during embryogenesis and is re-expressed in adulthood in connection with tumours and many other pathological conditions. Its actual function is not yet known, however, among its many hypothesized functions is the modulation of the recruitment of macrophages (Talts et. al.,

1999). The *TNC* gene has been studied in the chicken and could have similar functions as those reported in the mouse (Spring et. al., 1989). It is possible that this gene could play a role in *Salmonella* infection in chicken and the mapping of *TNC* could be useful for this reason. It would also be interesting to determine whether *TNC* is part of a conserved linkage group with *TLR4*, as it is with humans (chr 9) and mice (chr 4).

Chicken NRAMP1 Antisera Characterization

In order to elucidate the mechanism of NRAMP1 function in the chicken, we initiated the biochemical characterization of the NRAMP1 protein. Oligopeptides were synthesized from the extreme amino- and carboxy-terminals of NRAMP1 which are regions known to be unique to chicken (Hu et. al., 1996). The use of these unique oligopeptides decreases the likelihood of making antibodies to rabbit NRAMP1 or to other NRAMP related members. The amino-terminal oligonucleotide was readily amplified, using primers GST1 and GST2, from a chicken cDNA template previously isolated in our laboratory. In comparison, the carboxy-terminal oligonucleotide was only weakly amplified from chicken genomic DNA. The differences in amplification intensities can be explained by the use of different templates (cDNA is more readily accessed by the PCR machinery than genomic DNA). We could not use a cDNA template to amplify the carboxy-terminal because there is no cDNA clone isolated for this region of chicken NRAMP1. The amino-terminal oligonucleotide was then purified and successfully ligated into the MCS of the PGEX-2T plasmid (Figure 1 A). Direct sequencing reactions using primers GST1 and GST2 verified the integrity the amino-terminal fragment (Figure 2). The carboxy-terminal was not as successfully cloned into the plasmid. When direct sequencing was attempted using primers GST3 and GST4, the reactions revealed that the PCR amplified DNA fragment had not been ligated at all (no sequencing product). The low yield of carboxy-

terminal PCR DNA produced and the small size of the fragment (111 bp) could explain the unsuccessful ligation reactions.

The PGEX-2T plasmid was designed so that foreign oligopeptides could be expressed, in transformed *E. coli* bacteria, as GST-fusion proteins. There is a high probability of creating specific antibodies to the introduced oligopeptide because the GST moiety should not cause immunological complications (Smith and Corcoran, 1990). In our case, we induced large preparations of *E. coli* transformants with 100 mM of IPTG for 6 hours producing GST-N fusion proteins (PGEX-2T + insert) and GST proteins (PGEX-2T - insert). Transformants that expressed the GST protein were identified, by size, on a 12% SDS-polyacrylamide gel migrating at 27.5 kDa (Figure 3, lane 4). Transformants expressing the desired GST-N fusion protein were identified on the same gel by the absence of the 27.5 kDa GST molecule and the presence of a novel, larger species corresponding to the predicted 34 kDa size (Figure 3, lanes 1-3). Unstable fusion proteins were detected on the gel as smaller bands below the larger fusion proteins. After the preparative gel was destained the strong GST-N band could be visualized clearly. This allowed the band to be excised precisely from the gel in order to prepare the immunogen, with little chance of contamination by the smaller unstable fusion proteins.

The GST-N band, cut from the preparative gel and homogenized with Freund's adjuvant, was used to immunize two male New Zealand rabbits. Anti-chicken NRAMP1 immune sera were collected at pre-determined intervals and tested by Western blot analysis. Both rabbits had very strong immune responses evidenced by strong background on the Western blots (data not shown). The percentage of milk in the blocking solution was increased from 5% to 10% in order to help control the high background. The optimal primary antibody concentration was found to be 1 in 1000 and the concentration of the secondary antibody at 1 in 10 000. These changes only alleviated the immune sera background problem for one of the samples. The immune sera from this rabbit detected the GST-N protein (Figure 4, lane 4) and the GST protein (Figure 4, lane 3) and neither of

these bands were detected by the preimmune sera (Figure 4, lanes 4 and 3). The recognition of the GST-N fusion protein by the immune sera is to be expected. The strength of the GST-N band and the lighter shaded signal above it are probably due to overloading the sample on the original gel. The recognition of the GST protein is a potential problem, however, according to Smith and Corcoran (1990) the GST carrier is less immunogenic than other carriers and should not interfere with specific antibody-antigen interactions. Therefore, if the antisera is specific to chicken NRAMP1 as well as to GST, this should not complicate the reading of the Western blot. The signal against the GST fusion protein is much stronger than that against GST indicating that more antibodies could have been raised to the chicken NRAMP1 oligopeptide. The only antibodies that could be cross-reactive are the ones that were against GST alone. Although there is no known chicken GST protein, there may be a related protein in the chicken that could be recognized by our antisera.

The first two lanes of the preimmune and immune Western blots represent the membrane and cytosolic fractions of isolated chicken splenocytes. Our immune sera recognized four bands in the cytosolic fraction (Figure 4, lane 2) and the preimmune sera revealed two bands (Figure 4, lane 2). The cytosolic fraction contained some splenocyte debris, running at a low molecular weight, that was not completely removed by centrifugation. The rabbit already had made antibodies to a protein in this "debris" evidenced by the signals in the preimmune sera. The stimulation of the rabbit's immune system by our injections of adjuvant and antigen caused the increased production of these preimmune antibodies, as witnessed by the stronger bands in the immune Western blot. The immune sera revealed a band of molecular weight 90-100 kDa, in both the membrane (lane 1) and cytosolic fractions. This 90-100 kDa protein most likely represents chicken NRAMP1. We detected NRAMP1 both in the membrane and cytosolic fractions of chicken splenocytes. This result is in contrast to the finding of Vidal et. al. (1996) who detected mouse Nramp1 in the splenocyte membranes only. It is probable that chicken NRAMP1 is a membrane protein (Hu et. al., 1996), however, very little else is known about chicken

NRAMP1 and its role in macrophage physiology. Our results could, therefore, indicate that NRAMP1 is expressed in the membrane and cytosol of chicken splenocytes or could simply demonstrate that our isolated cytosolic fraction is not pure. The predicted chicken NRAMP1 protein size based on its primary amino acid sequence is estimated to be 60 kDa. The analysis of the chicken NRAMP1 polypeptide predicted two N-linked glycosylation sites (Hu et. al., 1996). Glycosylization could explain why the putative NRAMP1 protein runs at 90-100 kDa instead of 60 kDa. In a similar investigation into the characteristics of mouse Nramp1, Vidal et. al. (1996) reported a 90-100 kDa band that represented the Nramp1 protein in murine macrophages. It is believed that heavy glycosylation accounts for more than 50% of the mature mouse Nramp1 protein's weight. All other bands in lanes 1 and 2 of the immune Western blot are of unknown origin, they may represent degradation products from NRAMP1 or may be cross-reactive species.

The quality of NRAMP1 antisera could be improved by their purification. The specificity of the antisera needs to be further tested using transfected CHO cells that overexpress chicken NRAMP1 that carries an epitope tag (*cmyc* or HA) fused in-frame at its carboxy-terminus. An anti-tag monoclonal antibody can then be used in immunoblotting experiments to verify the specificity of the signal. Non-specific antibodies can be removed by using an acetone extract of a cell line or tissue that is known not to express chicken NRAMP1. Additionally, a concentration of the GST protein could be added to the milk:TBST:primary antibody solution so that the protein may compete for rogue antibodies causing GST-influenced bands to potentially disappear from the Western blots. Purification of the IgG molecules in our antisera can be carried out using Protein A beads. This may increase the precision of the horseradish peroxidase secondary antibodies used to generate the chemoluminescent signal. Lastly, immunoprecipitation could be used to detect chicken NRAMP1 in splenocytes because of its high sensitivity and specificity.

TNC Mapping Experiments

The current chicken genetic linkage map has been constructed from three different reference mapping populations: Compton, UK, East Lansing, USA, and Wageningen/Euribrid (Animal Breeding and Genetics Group of the Wageningen University, the Netherlands) with at least 1889 loci and approximately 235 representing known genes (Groenen et. al., 2000). The East Lansing panel was created from a cross between a partially inbred Red Jungle Fowl male and a highly inbred White Leghorn female (Crittenden et. al., 1993). Two F1 males were each mated to 8 or 10 White Leghorn females to produce the backcross progeny. The Wageningen/Euribrid population consisted of 456 F_2 birds from a cross between two broiler dam lines originating from the white Plymouth Rock breed. The Compton panel used a backcross between two birds derived from inbred White Leghorn lines that differed in their disease resistance (Bumstead and Palyga, 1992). Specifically, these lines differed in their resistance (N) or susceptibility (15I) to salmonellosis. We used a mapping panel from this population consisting of 56 backcross progeny from a mating between an F1 hen (produced by a 15IxN cross) and a 15I cockerel to map chicken *TNC*.

Our first attempt to genetically map chicken *TNC* consisted of following the segregation of restriction enzyme RFLPs, identified with a human *TNC* probe, in the Compton backcross mapping panel (Figure 5). The inheritance pattern was then compared to the segregation pattern of 800 loci defining 39 autosomal linkage groups. This analysis revealed that *TNC* was located within a linkage group on chicken chr 4 (Figure 6). The nearest DNA markers, *LEI0085* (proximal) and *LEI0063* (distal), mapped to positions about 25 cM from *TNC*. Additionally, this region of the chicken genome contains very few known genes. Although, *TNC* was linked to all surrounding loci in this region with a LOD score of 3, there were many double recombinants between *TNC* and its nearest linked markers (Figure 6). As the distance between two loci increases, so does the probability that

multiple recombination events will occur between them (Silver, 1995). The double crossovers between *TNC* and *LEI0085* or *LEI0063* could either mean that *TNC* is genetically linked but truly distant from these DNA markers or that *TNC* is not linked to this region of chicken chr 4. These data were the best linkage results we obtained using a low density marker chicken genetic map.

In order to prove that chicken *TNC* was on chr 4, we decided to carry out a FISH analysis. Once a cosmid clone corresponding to chicken *TNC* is identified from a chicken cDNA library, the clone can be fluorescently labelled and used as a probe on metaphase chromosomes from chicken. The fluorescent label can reliably indicate whether the isolated clone maps to a specific chromosome or not. Unfortunately, we were unable to isolate a *TNC* cosmid clone from our library. We used both a human and then a chicken *TNC* probe obtaining some positive signals on the first screen, however, with subsequent screening all signals were revealed to be false positives. It is possible that *TNC* is under-represented in this chicken genomic library.

Because of the number of double crossovers between chicken *TNC* and its nearest linked DNA markers and due to the inability to isolate a chicken *TNC* cosmid clone, another type of approach to map this gene was initiated. While examining the evolution of avian and mammalian sex chromosomes through comparative gene mapping, Nanda et. al. (1999) found 11 orthologous genes between chicken Z and human chr 9. This group also found that the distal long arm of human chr 9 showed conserved synteny with the chicken linkage group E41W17, which corresponds to a microchromosome. This portion of human chr 9 contains the alpha microglobulin (*AMBP*) gene which is tightly linked to *TNC* and *TLR4* and are all part of a syntenic group conserved on mouse chr 4 (Qureshi et. al., 1996). Based on this information, we decided to follow the segregation of three microsatellite markers mapping to the *AMBP* region on microchromosome E41W17 with *TNC* polymorphisms found in the Compton chicken mapping panel. This was carried out in order to determine whether *TNC* and *AMBP* are part of a conserved linkage group in chicken, as they are in

mouse and human. We found that *TNC* most likely maps to the E41W17 microchromosome. The LOD score for the original mapping of *TNC* to chr 4 with the Compton panel was 3.0 and our mapping to E41W17, using the same panel, gave a LOD score of 3.6 which is significantly improved.

We have initiated experiments to map *TNC* in the East Lansing backcross mapping population in order to firmly establish our mapping results from the Compton panel. We found, by sequencing an intron within the chicken *TNC* gene, a series of four point mutations between the JF founder male and the WL backcross parental strain approximately 800 bp into the intron (Figure 9). After amplification of a region (≅200 bp) encompassing the point mutations, the polymorphism could potentially be followed on a 6% non-denaturing polyacrylamide gel in order to reveal an SSCP in the East Lansing panel. This technique is capable of detecting changes in migration patterns between DNA samples differing in a single point mutation. The SSCP will subsequently be followed in concurrance with SSLPs we have previously found in the East Lansing population using microsatellite markers mapped to E41W17.

The presence of conserved synteny between species, such as human, mouse and chicken, reflects the common phylogenetic origin of these species and probably also the ancestral genomic organization (Nanda et. al., 1999). Comparative mapping data based on the chicken consensus map shows considerable chromosomal conservation between humans and chicken during evolution (Groenen et. al., 2000). This regional conservation was not found, to the same degree, between the mouse and chicken. Chromosomal rearrangement events within conserved regions are believed to provide a mechanism for creating species diversity (Lundin, 1993 in Hu et. al., 1995). However, as many as 87 common chromosomal regions have been identified between human and chicken, making the chicken an excellent evolutionary model organism (Groenen et. al., 2000). The chicken michrochromsomes appear to be more gene dense than the machrochromosomes (Burt et.

al., 1995). Based on this information there is speculation that these microchromosomes most likely represent, in almost their entirety, large fragments of specific human chromosomes (Groenen et. al., 2000). This appears to be the case with the microchromosome, E41W17, on which we believe *TNC* resides. All of this information supports the possibility of a conserved *AMBP-TNC-TLR4* linkage group on this microchromosome.

Although these evolutionarily distant species retain many conserved genes and gene regions, it is probable that their regulation, expression and function are different, as witnessed by the vast apparent dissimilarities between humans, mice and chickens. Our study of chicken NRAMP1 presented results contrary to those found in mouse and humans. It is possible that this protein has different properties than that previously found in other species. The similarities, at the genetic level, of such divergent groups as birds, mice and humans are very useful for gene mapping purposes and very interesting from an evolutionary standpoint. However, the differences, at the level of gene expression, are just as important to the understanding of the fine mechanisms of our own and other species' biology, immunology, pathology, among others.

The work described here was essential in order to continue the research project initiated in our laboratory on the functional characterization of NRAMP1 and TNC in chicken resistance to salmonellosis. Future work will involve improving the specificity of our antibodies by removing cross-reactive species and purifying the IgG fraction. We would like to determine the cell type expressing NRAMP1 in chicken since this gene is expressed in the thymus, a finding contrary to the detected expression in mammals. It is possible that chicken NRAMP1 may be associated with a wider variety of cells than seen in mammals. The expression pattern of NRAMP1 in *Salmonella*-susceptible chicken line C needs to be determined in order to conclude whether the $Arg^{223} \rightarrow Gln^{223}$ variant is associated with disease and, if so, how it is associated with disease. The subcellular localization of NRAMP1 in chickens will be of particular interest since we have shown that

NRAMP1 is present in the cytosolic and membrane fractions of chicken splenocytes which is different to that shown in mammals.

With the recent cloning of *Lps* in the mouse, it is now possible to map and clone this locus in the chicken. This is a project that is currently in progress in our laboratory. The information will allow us to directly test whether this gene is important in chicken salmonellosis. This does not, however, make the study of chicken *TNC* obsolete. Once $TLR4^{LPS}$ is mapped in the chicken we will be able to see if *TLR4* and *TNC* are retained as part of a conserved linkage group as they are in mice and humans. Linkage conservation has been reported between chicken and mammalian chromosomes (reviewed in Burt et. al., 1995 and Groenen et. al., 2000). It is possible that regional chromosomal organization is critical for the activity of genes in a particular region, therefore, it would be interesting to uncover this linkage group in chicken. Hu et. al. (1997), as mentioned earlier, developed a chicken experimental model in order to study *Salmonella* infection in susceptible (C) and resistant (W1) chicken lines. They found that it was clear that many genetic and nongenetic factors affect both the early and late phases of *S.* Typhimurium infection. This is another reason to continue to study the chicken *TNC* gene. If it is found that *TNC* is not linked to *LPS* in chicken, we will have to explain this locus' influence on differential resistance and susceptibility to *S.* Typhimurium. It is possible that *TNC* could be one of the many disease genes involved with *Salmonella* infection. A large scale chicken genome scan is also being carried out in our laboratory. The genome scan will help identify more regions of the chicken genome that control resistance to infection with *Salmonella* and may help verify previous work done.

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