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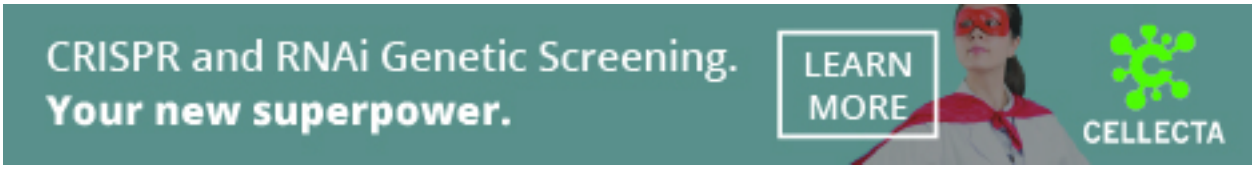

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RESEARCH

Bovine Natural Resistance Associated Macrophage Protein 1 (*Nramp1*) Gene

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The *Bcg/Ity/Lsh* locus is a major gene controlling early phases of infection with intracellular parasites in mice. *Natural resistance associated macrophage protein 1* (*Nramp1*) has been shown to be the *Bcg* gene in mice. Analysis of a bovine cDNA homolog of murine *Nramp1*, designated as bovine *NRAMP1*, predicted a 548-amino-acid protein with hydrophobic domains, an amino-terminal SH3-binding domain, and a conserved consensus transport motif. Northern blotting indicated that bovine *NRAMP1* was expressed primarily in macrophages and tissues of the recticuloendothelial system. Bovine *NRAMP1* was mapped to BTA 2 within syntenic loci conserved on HSA 2q and MMU 1.

Intracellular bacterial zoonotic diseases like brucellosis and tuberculosis cause significant losses in worldwide livestock industries despite widespread application of antimicrobials, vaccination, quarantine, test, and/or slaughter. Despite spending billions of dollars since the inception of the bovine tuberculosis eradication program in 1917 and the subsequent development of the bovine brucellosis eradication program in 1939, these diseases still plague the North American livestock industries and are a threat to public health (Dietrich et al. 1986a; Dietrich et al. 1986b; Essey and Koller 1994; Martin et al. 1994). The lack of success in eradicating infectious diseases with these approaches indicates the need for new strategies such as genetically based natural disease resistance (Templeton et al. 1988). Genetic studies in mice have demonstrated that innate susceptibility to *Mycobacterium bovis* (BCG), *Leishmania donovani*, *Salmonella typhimurim* and several atypical mycobacteriae is controlled by a single gene on *Mus musculus* autosome (MMU) 1, called *Bcg*, *Lsh*, or *Ity* (Plant et al. 1982; Skamene et al. 1984; Goto et al. 1989; Frelier et al. 1990; Mock et al. 1990; Schurr et al. 1991; de Chastellier et al. 1993). *Bcg* mediates antimicrobial activity of macrophages against intracellular parasites early during infection (Gros et al. 1983; Blackwell et al. 1991; Roach et al. 1991; Roach et al. 1994).

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Cattle that are naturally resistant (R) or susceptible (S) to brucellosis were identified by in vivo *Brucella abortus* challenge experiments (Harmon et al. 1985). In vitro studies demonstrated that macrophages from R cattle were better able to control intracellular replication of *B. abortus* (Harmon et al. 1989; Price et al. 1990; Campbell and Adams 1992). These observations were comparable to the differences in macrophage function between mice R or S to *M. bovis*-BCG, *S. typhimurim*, and *L. donovani* controlled by the *Bcg/Lsh/Ity* gene(s) (Gros et al. 1983; Blackwell et al. 1991; Radzioch et al. 1991; Roach et al. 1991; Blackwell et al. 1994; Kramnik et al. 1994).

In mice, a 30-cM segment on MMU 1 (Skow et al. 1987; Mock et al. 1990; Malo et al. 1993) that includes *Bcg* was reported to be conserved on *Homo sapiens* autosome (HSA) 2q (Cellier et al. 1994; White et al. 1994) and *Bos taurus* autosome (BTA) 2 (Womack and Moll 1986; Adkison et al. 1988; Fries et al. 1993; Beever et al. 1994). Vidal et al. (1993) isolated a murine *Bcg* candidate gene, designated *natural resistance associated macrophage protein* (*Nramp*), that apparently encodes a polytopic integral membrane protein that has structural features similar to prokaryotic and eukaryotic transporters. Recently, studies using knockout mice have shown that *Nramp1* is the *Bcg/Lsh/Ity* gene (Vidal et al. 1995). The authors suggest that the murine Nramp protein might function in the phagolysosomal membrane as a concentrator of oxidation products of nitric ox-

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ide, mediating cytocidal or cytostatic activity against the ingested parasites of infected macrophages (Vidal et al. 1993; Malo and Skamene 1994; Cellier et al. 1994; Malo et al. 1994). A study was undertaken to determine if a bovine homolog of the murine *Nramp1* gene was expressed in bovine macrophages and involved in susceptibility of cattle to *B. abortus*.

In this report, we identify a bovine homolog of murine *Nramp1*. The bovine *NRAMP1* was mapped to BTA 2, expressed primarily in macrophages, spleen, and lung and is predicted to encode a protein that has 12 transmembrane segments with one hydrophilic amino-terminal region containing a SH3-binding motif located at the cytoplasmic surface. The gene is designated as bovine *NRAMP1*, because of conserved chromosomal location, tissue expression, and amino acid sequence homology with murine *Nramp1*, and we have evidence of the existence of at least one other bovine *NRAMP* (J. Feng, Y. Li, and J. Templeton, unpubl.) as reported in the mouse (Dosik et al. 1994; Gruenheid et al. 1995).

RESULTS

Isolation of Bovine *NRAMP1* cDNA

Based on the genomic sequence of murine *Nramp1*, oligonucleotide primers designated 1F and 1R were used to amplify a 164-bp segment in bovine genomic DNA. Using a bovine-specific forward primer designated PE2 from this 164-bp bovine sequence and a reverse primer MUT2 from the murine *Nramp1* cDNA sequence (Vidal et al. 1993), a 222-bp product was amplified from reverse transcribed bovine macrophage total RNA. This 222-bp probe was used to screen a bovine splenic cDNA library. Twenty potentially full-length *NRAMP1* clones (~2.3 kb) were obtained, eight of which were sequenced and used to construct the complete sequence. The in-frame initiator codon ATG was located at nucleotide position (pst) 73 from the 5′ end, and was followed by a segment of 1644 nucleotides, forming a single open reading frame (ORF) encoding a protein of 548 residues with a calculated molecular weight of 59.6 KDa. A TGA termination codon located immediately downstream from glycine 548 (nucleotide pst 1717) was followed by an intact AATAAA polyadenylation signal (pst 2257) (Fig. 1A).

Analysis of the Predicted Bovine *NRAMP1* Structure

The first 64 amino-terminal amino acids of bovine *NRAMP1* are rich in proline (11/64), glycine (10/64), serine (8/64), and charged amino acids (10/64), and include two putative protein kinase C (PKC) phosphorylation sites at amino acid pst 37 and 51. Because *Src* Homology (SH3) domains interact specifically with proline-rich peptides, we compared the proline-rich coding fragment PPSPEP (pst 20–26) with several identified SH3-binding sequences (Table 1) (Lim and Richards 1994). The analysis revealed that the PXXP binding motif (Musacchio et al. 1994; Yu et al. 1994) is conserved in bovine *NRAMP1*, which indicates that bovine *NRAMP1* contains an amino-terminal SH3-binding domain. Kyte-Doolittle hydrophilicity analysis disclosed that the surface probability of peptide PPSPEP is 50.3–67.6%, which indicates that the bovine *NRAMP1* SH3-binding motif is most likely located at the inner membrane surface.

Analysis of the remaining bovine *NRAMP1* cDNA sequence indicates the predicted protein to be highly hydrophobic with 12 putative transmembrane domains (Fig. 1B) in agreement with the putative murine and human Nramp structure (Vidal et al. 1993; Barton et al. 1994; Cellier et al. 1994). The bovine *NRAMP1* product contains one potential amino-linked glycosylation site at pst 335, within a highly hydrophilic region between predicted transmembrane (TM) 7 and 8, and four PKC phosphorylation sites on serine (pst 37, 51, 114, and 269, respectively). A 20-amino-acid transport motif is located between the predicted TM domains 8 and 9 and conserved in murine and human Nramp (Fig. 1A,C,D). This conserved motif is known as the binding-protein-dependent transport system inner membrane component signature (Vidal et al. 1993; Malo and Skamene 1994; Cellier et al. 1994; Malo et al. 1994). Based on the hydropathic analysis and conserved transport motif, we propose the membrane-associated topography of bovine NRAMP1 (Fig. 1C) as follows: the amino terminus is located in the cytoplasm, and the following 12 TM domains result in 5 consecutive transmembrane loops. This arrangement would place the SH3-binding motif on the cytoplasmic membrane surface; SH3-binding domain with two potential phosphorylation sites on TM loops 2 and 3 and the TM 6 and TM 7 loops containing one phosphorylation site each, all projecting into the cytoplasm; the TM 7 and TM 8 loop containing one predicted amino-linked extracellular glycosylation site; and the carboxyl terminus in the cytoplasm.

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Homology among Human, Murine, and Bovine NRAMP1 Proteins

Comparison of human, murine, and bovine predicted NRAMP1 protein sequences (Fig. 1D) indicates a remarkable degree of homology (86.9% identical residues between murine and bovine; 88.6% identical residues between human and bovine). The predicted TM segments 1–8 are highly conserved hydrophobic membrane associated domains in the three species, with 99% and 96% identical amino acid sequence between human and bovine, murine and bovine, respectively. The most conserved consecutive region was from TM 8–9 with 100% identity from pst 346 to 456 between human and bovine; 98.2% identical between murine and bovine. Within the TM 8–9 segment, the bovine binding-protein-dependent transport system inner membrane component signature was identical with murine and human NRAMP with one exception (substitution of lysine to arginine at pst 392 in the human; Fig. 1D). Also among these three species, one predicted amino-linked glycosylation site was conserved within the fourth putative extracellular loop between TM 7 and 8; and one consensus PKC phosphorylation site was conserved in the predicted intracytoplasmic loop between TM 6 and 7 at pst 37 (Fig. 1A,C,D) (Vidal et al. 1993; Cellier et al. 1994; Gruenheid et al. 1995).

Amino acid substitutions were not randomly distributed along the sequence of the protein but were significantly clustered within certain regions. The most striking differences were located at extreme ends of the proteins, amino terminus (57.4% identity of pst 1–47 between murine and bovine; 66% identity of pst 1–50 between human and bovine) and carboxyl terminus (57.6% iden-

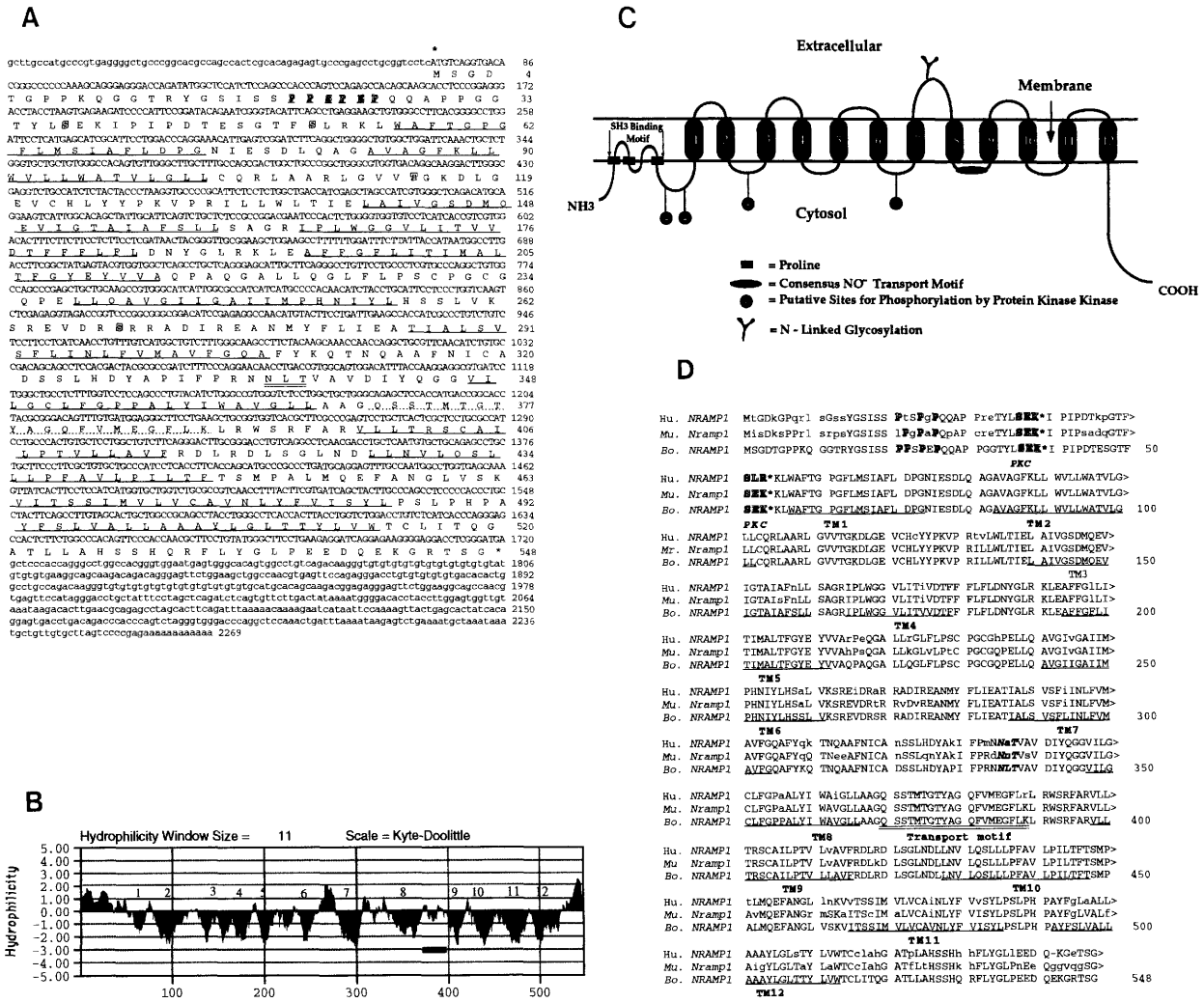

Figure 1 *(See facing page for legend.)*

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Table 1. Sequence Alignment of SH3-binding Motifs

SH3-binding motif	P-5	P-4	P-3	P-2	P-1	P0	P1	P2	P3	P4	P5
Bovine *NRAMP1* (16-26)	G	S	I	S	S	**P**	p	S	**P**	E	p
Human *NRAMP1* (19-29)[a]	S	S	P	T	S	**P**	T	S	**P**	G	p
Src library consensus[b]			R	X	L	**P**	p	L	**P**	R	#
Murine 3BP1 (267-277)[b]	P	T	M	P	P	**P**	L	P	**P**	V	p
Murine 3BP2-40 (2-12)[b]	P	A	Y	P	P	**P**	p	V	**P**	V	p
Murine 3BP2-40 (2-12)[b]	P	A	Y	P	P	**P**	p	V	**P**	V	p
SH3-binding site consensus[b]			X	**p**	#	**P**	**p**	X	**P**		

Capital letters represent amino acid residues in the SH3 binding motifs. X represents nonconserved residues. # represents hydrophobic residues and p represents residues that tend to be proline. The bold capital letter P indicates the completely conserved proline residues.
[a]Cellier et al. 1994.
[b]Yu et al. 1994.

tity of pst 516–548 between murine and bovine; 69.6% identity between human and bovine). The predicted third and fourth extracellular loops at pst 215–237 and pst 307–346 were less conserved in amino acid sequences than the TM domains. Identity was 78.2% between murine and bovine and 82.0% between human and bovine for the predicted third extracellular loop, respectively, and 75.0% identity between murine and bovine and 85% identity between the human and bovine, respectively, for the predicted fourth extracellular loop.

Figure 1 (*A*) Nucleotide sequence (GenBank accession no. U12862) and deduced amino acid sequence of the cDNA clone encoding *NRAMP1* transcript and protein. Nucleotides are numbered positively in the 5′ to 3′ orientation to the right of each lane, starting with the first nucleotide of the 5′ untranslated region and ending with the last nucleotide. The deduced amino acid sequence is shown below the nucleotide sequence. Both the ATG start codon and TGA stop codon are indicated with *. The putative SH3-binding motif PXXP is indicated by outline type font at amino acid pst 21–24 and the bovine homologous sequence is PPSPEP. One predicted amino-linked glycosylation site is indicated by a double line corresponding to the sequence N-X-S/T (amino acid pst 235–237), and four predicted phosphorylation sites for protein kinase C (S/T-X-R/K) are bold with shadow (amino acid pst 37, 51, 114, and 269, respectively). Hydrophobic segments corresponding to putative membrane-spanning (TM) domains are underlined. The binding-protein-dependent transport system inner membrane component signature located between predicted TM8 and TM9 is indicated by a dotted underline (amino acid pst 370–389). (*B*) Hydrophilicity profile of the predicted amino acid sequence of the bovine *NRAMP1* product. The hydrophilicity plot was obtained by standard Macvector 4.1 program analysis, using the algorithm and hydropathy values of Kyte-Doolittle for a window of 11 amino acids. The positions of highly hydrophobic segments forming putative TM domains are indicated in each sequence, together with the position of the conserved membrane transport signature (thick bar) located between predicted TM 8 and 9 domains. (C) Schematic representation of the putative structure of the bovine NRAMP1 protein. The prolines in the SH3-binding motif are shown by black squares on the intracellular membrane surface. The potential PKC phosphorylation sites are illustrated with minus signs, and the putative amino-linked glycosylation site within the TM7 and 8 predicted extracellular domains is identified by a small Y structure. The positions of the 12 putative TM domains are shown, with the predicted polarity of the membrane domains following cytoplasmic and extracellular spaces. The consensus transport motif shared by prokaryotic and eukaryotic transporters is presented by a thick closed black segment located between TM 8 and 9 domains. (*D*) Comparison of the amino acid sequences encoded by *Nramp1* of bovine (*Bo. NRAMP1*), human (*Hu. NRAMP1*) and mouse (*Mu. Nramp1*). Numbering of the amino acid sequences begins with the initiator codons for *Nramp1*. The PPXP proline-rich motif is indicated with outline type at amino acid pst 21–26. The conservation of two PKC sites are shown with outline type followed by an asterisk (*) at amino acid pst 37–39 and amino acid pst 51–53; and one glycosylation site is conserved at amino acid pst 345–347, indicated by bold italic font. The binding-protein-dependent transport system inner membrane component signature located between predicted TM8 and TM9 of the bovine NRAMP1 and conserved in humans and murines is indicated by a double underline (amino acid pst 370–389).

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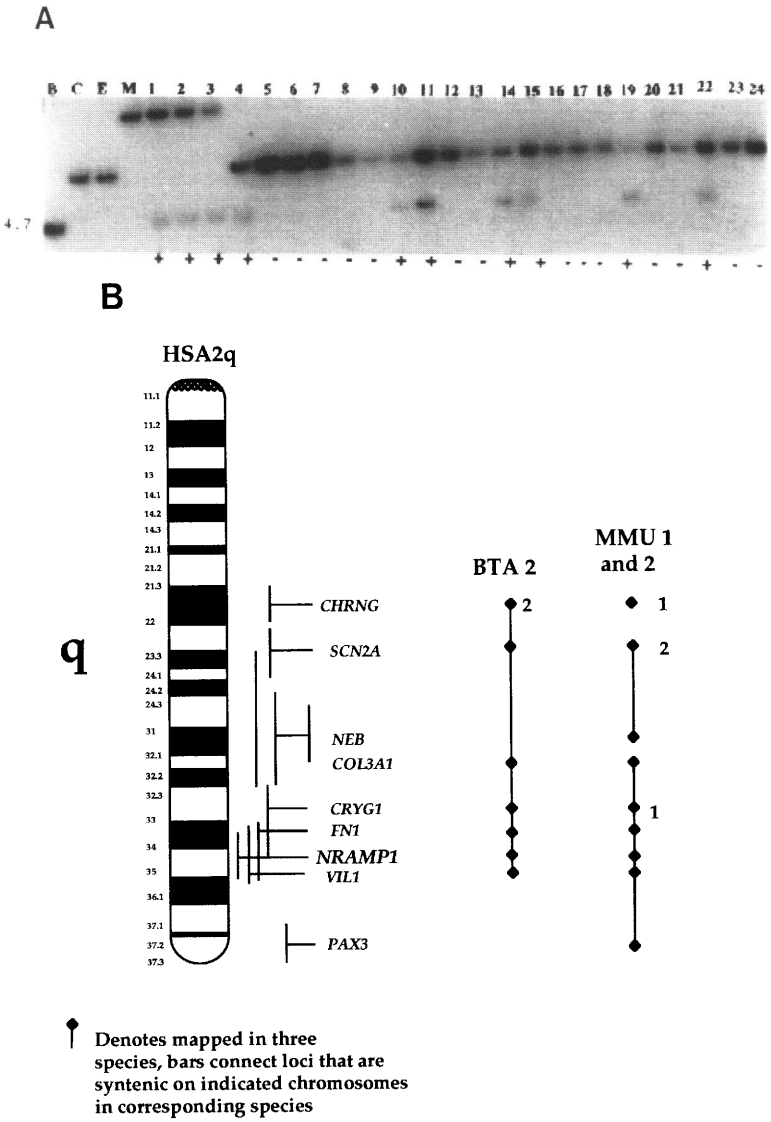

Figure 2 (*A*) Genetic mapping of bovine *NRAMP1* on BTA 2. Autoradiogram of Southern blot of *Hin*dIII-digested DNA from bovine rodent somatic cell hybrids and bovine (B), hamster (C-CHO and E-E36 cell lines), and thymidine kinase-deficient mouse cells (M) controls. It was probed with ^{32}P-labeled bovine 1F/1R probe. Single bovine-specific fragments are identified at 4.7 kb. (*B*) A representation of conserved chromosomal segments among three species around *NRAMP1* locus on HSA 2q, BTA 2, and proximal MMU1.

Genetic Mapping of Bovine *NRAMP1*

The syntenic arrangement of bovine *NRAMP1* was determined by somatic cell hybrid segregation analysis (Womack and Moll 1986; White et al. 1994). DNA from 87 bovine x rodent somatic hybrid cells was digested with *Hin*dIII and hybridized to a PCR-generated probe using 1F/1R primers (Fig. 2A). The bovine-specific *Hin*dIII restriction fragment of 4.7 kb was easily discriminated from fragments representing the hamster and mouse homologs, permitting detection of bovine-specific fragments in each cell line. A pairwise concordancy analysis indicated that bovine *NRAMP1* segregated 100% concordantly with γ-*crystallin*, which has been assigned to BTA 2 (Fig. 2B). The results of analyzing 87 somatic hybrids were 28 positive for γ-*crystallin* and bovine *NRAMP1*, and 59 were negative for γ-*crystallin* and bovine *NRAMP1*. A group of bovine syntenic loci including *villin*, γ-*crystallin* (Adkison et al. 1988; Beever et al. 1994), and *Interleukin-8 receptor* (J. Feng, Y. Li, and J. Templeton, unpubl.) has been mapped to a region of BTA 2 and are conserved on HSA 2q (White et al. 1994) and proximal MMU 1 (Cerretti et al. 1993), which were closely linked to the *Bcg/Ity/Lsh* locus in the mouse (Fig. 2B). These results support further the homology among human, bovine, and murine *NRAMP*.

Cell-Specific Expression of Bovine *NRAMP1* mRNA

To test whether bovine *NRAMP1* was expressed primarily in macrophage populations, we analyzed total RNA prepared from 14 different bovine tissues (peripheral blood lymphocytes, liver lymph node, spleen, tonsil, lung, kidney, thymus, heart, skeletal muscle, jejunum, colon, ovary, uterus, brain, and cultured macrophages) by Northern blot analysis using a 1F/1R PCR generated bovine DNA probe (Fig. 3). A band of ~2.3 kb was detected in macrophage, spleen, and lung RNA, but was absent in the RNA analyzed from the other tissues. These results indicate that bovine *NRAMP1* is expressed principally in the macrophage and the reticuloendothelial (RE) system.

DISCUSSION

The bovine homolog of human and murine *Nramp* is highly conserved. The bovine *NRAMP1* cDNA would encode a protein with an overall

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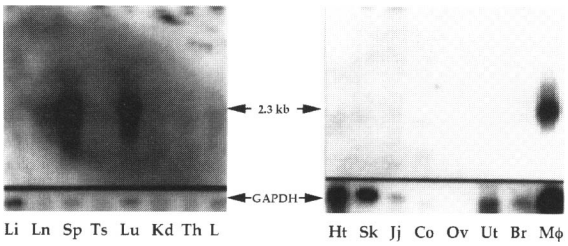

Figure 3 Northern blot of RNA from bovine tissues including liver (Li), lymph node (Ln), spleen (Sp), tonsil (Ts), lung (Lu), kidney (Kd), thymus (Th), heart (Ht), skeletal muscle (Sk), jejunum (Jj), colon (Co), ovary (Ov), uterus (Ut), brain (Br), blood monocyte-derived macrophage (Mϕ), and lymphocyte (L). The blots were hybridized with α-[^{32}P] dCTP labeled bovine 1F/1R probe (top) or GAPDH (bottom). Bovine *NRAMP1* was detected in spleen, lung, and macrophages as a 2.3-kb fragments.

predicted amino acid sequence homology of 86.9% and 88.6% to the human and murine *Nramp* product, respectively. Northern blot analysis indicated that bovine *NRAMP1* is expressed principally in the RE organs and macrophages as in the human and murine *NRAMP* (Vidal et al. 1993; Cellier et al. 1994; Gruenheid et al. 1995). The bovine *NRAMP1* has been mapped to BTA 2 within a group of syntenic loci conserved on HSA 2q and murine chromosome 1 overlapping the *Bcg/Ity/Lsh* locus (Adkison et al. 1988; Beever et al. 1994; Cellier et al. 1994; White et al. 1994). Additionally, we have shown *interleukin-8 receptor* to be linked to bovine *NRAMP1* (J. Feng, unpubl.). Our data further extend the large conserved synteny of bovine, human, and murine genes on these chromosomes.

The potential mechanisms for NRAMP controlling natural resistance has been recently reviewed by others (Vidal et al. 1993; Blackwell et al. 1994; Cellier et al. 1994; Ivanyi 1994; Blackwell et al. 1995; Nathan 1995; Vidal et al. 1995). Recently, Medina et al. (1996) reported that Nramp1 does not have the same potency in regulating natural resistance to virulent *M. tuberculosis* challenge infection in mice as Nramp1 does in regulating natural resistance to *M. bovis*-BCG, *S. typhimurium,* or *L. donovani.* However, given the conservation of the *NRAMP1* genes in at least three species, it is likely that the fundamental function of NRAMP1 is conserved against the different intracellular pathogens, i.e., Mycobacteriae, Brucellae, Salmonellae, and Leishmania, but more genes may be active in controlling infections caused by wild-type virulent pathogens. This would be consistent with previous reports by Blackwell et al. (1980), Roberts et al. (1989), Brett et al. (1992), and Apt et al. (1993). In a preliminary study of 22 unrelated cattle phenotyped as naturally resistant or susceptible to an in vivo challenge of virulent *B. abortus* as described in Harmon et al. (1989) and Price et al. (1990), there was a significant association ($P = 0.0089$) of *NRAMP1* with these two different phenotypes based on single stranded conformation analysis (data not shown). The significant association of bovine *NRAMP1* with natural resistance to virulent *B. abortus* offers the possibility for selecting and breeding domestic and freeranging ungulates that are naturally resistant to these important diseases and could play a key role in a new strategy to control these worldwide zoonotic pathogens.

METHODS

Animals

The cattle used in these experiments for cloning of *NRAMP1* were a purebred Angus (*Bos taurus*) bull and crossbred cows produced by a three-way cross—F1 [Jersey (*B. taurus*) × Hereford (*Bos taurus*)] × American Brahman (*Bos indicus*) (Harmon et al. 1985). All experimental protocols are university reviewed and approved.

Isolation and Sequencing of cDNA Clones

The fragment of bovine *NRAMP1* was amplified with PCR primers 1F/1R from a murine *Nramp1* genomic sequence. Sequence analysis showed that this PCR product contained 90% nucleotide identity with the third exon (nucleotide pst 338–458) of murine *Nramp1*. RT–PCR was performed on bovine macrophage mRNA using PE2 (5′-CGTGGTGAC-AGGCAAGGACT-3′, bovine *NRAMP1* cDNA nucleotide pst 402–425) and MUT2 (5′-CCAAGA-AGAGGAAGAAGAAGG-TGTC-3′, murine *Nramp* cDNA nucleotide pst 600–624). A 222-bp product was generated to screen a λgt11 cDNA library made from bovine spleen (Clontech). 1×10^6 clones were screened by in situ plaque hybridization radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmole) (DuPont, NEN Research Products) by hexamer priming (1×10^9 to 2×10^9 cpm/μg). Filters were washed under conditions of increasing stringency up to 1× SSC, 0.1% SDS at 65°C for 30 min. Positive clones were verified using PCR with primers PE2/MUT-2 and subsequently PCR amplified to obtain a 2.3-kb insert with λgt11 insert screening amplimers. This PCR product was gel purified and ligated into pT7BlueT-Vector (Novagen). Both strands of plasmid DNA were sequenced by the dideoxy method of Sanger et al. (1977) using modified T7 DNA polymerase (USB) and [α-^{35}S]dATP (3000 Ci/mmole) (NEN Research Products, Boston, MA). All sequence data were compiled and analyzed using MacVector 4.1 software (Eastman Kodak, New Haven, CT).

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Genetic Mapping

Bovine–hamster hybrid somatic cell panel blots (kindly supplied by Dr. James E. Womack, Texas A&M University) (Womack and Moll 1986; Womack 1994; Adkison et al. 1988) were hybridized with the 1F/1R PCR-generated probe (4×10^8 to 8×10^8 cpm/μg) (Feinberg and Vogelstein 1983). Hybridization was performed at 42°C for 18 hr in 20 ml of 50% formamide, 5× SSC, 1× Denhardt's solution, and 20 mM $NaPO_4$ (pH = 6.8), followed by washing once in 2× SSC, 0.5% SDS at room temperature for 15 min, two successive washes in 1× SSC, 0.1 % SDS at 65°C for 30 min (Adkison et al. 1988). All gene probes were labeled with the random primed DNA labeling method with α-[^{32}P] dCTP (3000 Ci/mmole) (NEN Research Products, Boston, MA). Synteny was ascertained by analysis of concordancy of the probe with known marker genes as described (Womack and Moll 1986; Adkison et al. 1988; Womack 1994).

Northern Blot Analysis

Monocyte-derived macrophages were harvested and cultured as described (Campbell and Adams 1992). Total RNA was isolated from peripheral blood lymphocytes, liver, lymph node, spleen, tonsil, lung, kidney, thymus, heart, skeletal muscle, jejunum, colon, ovary, uterus, brain, and cultured macrophages using standard techniques (Chirgwin et al. 1979). Of the total RNA from these tissues, 10 μg were separated on 1% formaldehyde agarose gels, transferred to Nytran plus membranes (Schleicher & Schuell). Blots were prehybridized in 20 ml of 50% formamide, 10% dextran sulfate, 4.7× SSPE (1× SSPE is 10 mM sodium phosphate, 1 mM EDTA, 150 mM NaCl), 0.47× Denhardt's solution, 0.1% SDS, 0.18 mg/ml heat-denatured salmon sperm DNA, and 0.34% fat-free milk for 4 hr at 42°C. Hybridization at 42°C for 18 hr was done in the same solution containing 2×10^8 cpm/gml [^{32}P]-radiolabeled probe 1F/1R fragment. Final wash conditions were 0.2× SSC, 0.1% SDS at 68°C for 30 min. GAPD was used as a positive control.

RT–PCR

Total RNA (0.5 μg) was transcribed in 25 μl reaction at 37°C for 60 min with MMLV reverse transcriptase (GIBCO–BRL). cDNA amplification was performed at 95°C (5 min) followed by 32 cycles of 94°C (1 min), 58°C (1 min), and 72°C (1 min) with 1 mM $MgCl_2$, 2 μl 10× PCR buffer, 1 Unit *Taq* polymerase (Perkin-Elmer) and 4 μl RT template in a final volume of 25 μl.

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