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Mapping and Sequencing of the Canine *NRAMP1* Gene and Identification of Mutations in Leishmaniasis-Susceptible Dogs

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The *NRAMP1* gene (*Slc11a1*) encodes an ion transporter protein involved in the control of intraphagosomal replication of parasites and in macrophage activation. It has been described in mice as the determinant of natural resistance or susceptibility to infection with antigenically unrelated pathogens, including *Leishmania*. Our aims were to sequence and map the canine *Slc11a1* gene and to identify mutations that may be associated with resistance or susceptibility to *Leishmania* infection. The canine *Slc11a1* gene has been mapped to dog chromosome CFA37 and covers 9 kb, including a 700-bp promoter region, 15 exons, and a polymorphic microsatellite in intron 1. It encodes a 547-amino-acid protein that has over 87% identity with the Slc11a1 proteins of different mammalian species. A case-control study with 33 resistant and 84 susceptible dogs showed an association between allele 145 of the microsatellite and susceptible dogs. Sequence variant analysis was performed by direct sequencing of the cDNA and the promoter region of four unrelated beagles experimentally infected with *Leishmania infantum* to search for possible functional mutations. Two of the dogs were classified as susceptible and the other two were classified as resistant based on their immune responses. Two important mutations were found in susceptible dogs: a G-rich region in the promoter that was common to both animals and a complete deletion of exon 11, which encodes the consensus transport motif of the protein, in the unique susceptible dog that needed an additional and prolonged treatment to avoid continuous relapses. A study with a larger dog population would be required to prove the association of these sequence variants with disease susceptibility.

Natural resistance or susceptibility to several antigenically unrelated pathogens, such as *Mycobacterium*, *Salmonella*, and *Leishmania*, has been associated with the locus *Bcg*/*Ity*/*Lsh* in mice (43). The resistant phenotype (Bcg^r) results in macrophage activation producing the destruction of intracellular pathogens early during infection (43). Positional cloning revealed that the *Bcg*/*Ity*/*Lsh* locus corresponds to a gene encoding an ion transporter protein, designated natural resistance-associated macrophage protein (NRAMP1) (52) and referred to here with the new nomenclature, solute carrier family 11 member 1 (Slc11a1).

The Slc11a1 protein is composed of 12 putative transmembrane (TM) domains, an extracellular glycosylated loop, and a transport motif conserved among other transporter proteins in both eukaryotic and prokaryotic organisms (52, 21). A nonconservative amino acid substitution (G169A) in the TM4 domain has been associated with a susceptible phenotype in mice (52, 31). Within macrophages, Slc11a1 has been located in late endocytic compartments and is acquired by the phagosomal membrane after phagocytosis (22). The protein drives divalent metals out of the phagosome, controlling the replication of intracellular pathogens by altering the intravacuolar environment (18). This behavior could also provide an explanation for the observed pleiotropic effects on macrophage function that have been attributed to the *Slc11a1* gene, suggesting a role in infectious and autoimmune disease susceptibility (5).

Slc11a1 has attracted a great deal of interest and has been characterized in different species in a search for polymorphisms involved in disease susceptibility. For instance, a functional repeat polymorphism in the promoter region of the human *Slc11a1* gene was found to drive gene expression, conferring protection against or susceptibility to juvenile rheumatoid arthritis (42) or multiple sclerosis (25). Moreover, a single base change in intron 4 (14) and a 4-bp deletion in the 3′ untranslated region (UTR) (40) were significantly associated with tuberculosis in humans. Recently, a microsatellite in the 3′ UTR also was demonstrated to affect the expression of the bovine *Slc11a1* gene and to control the in vitro replication of *Brucella abortus* (3). Finally, an amino acid substitution (G696A) in chicken Slc11a1 was found only in chicken cell lines susceptible to *Salmonella enterica* serovar Typhimurium (24).

In southern European countries, leishmaniasis is an endemic and zoonotic disease caused by the protozoan parasite *Leishmania infantum*. This parasitic infection in this area is important not only to veterinary medicine, because dogs are considered the main reservoir (4), but also because of its zoonotic aspects. Human leishmaniasis, which traditionally affected young children and infants, is now a common complicating factor in adults infected with human immunodeficiency virus or receiving immunosuppressive drugs (2, 15).

In dogs, leishmaniasis is a systemic disease with a variety of clinical signs, including nonpruritic skin lesions (such as exfo-

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TABLE 1. Mammalian orthologous and dog-specific primers designed to amplify the canine *Slc11a1* gene and cDNA

Primer[a]	Location	Sequence (5′ → 3′)
NRPROM-F	Promoter	CACTTCTGCCTTTCCAAAATGTTTCACA
NRPROM3-F	Promoter	TCTCATAGTTTATCTTACCTGC
NRPROMD-F[b]	Promoter	CCTCTCAGCTAGTCTGAGCC
NRPROM2-R[b]	Promoter	CAGCTGATCTCAGCTGTCCTC
NRPROM3-R[b]	Promoter	AGCTACTCAGCACATGCTTC
NRMICRI1-F[b]	Intron 1	GAGTCTGCTTGAGATTCTCTC
NRMICRI1-R[b]	Intron 1	TATCACCTCCACCCTTCAAAC
NRE2-F	Exon 2	CCTACCTRAGTGAGAAGATCC
NRI2E2-R[b]	Exon 2-intron 2 boundary	GGTTTCCAGCATTCCTACCGGTTC
NRE6I6-F[b]	Exon 6-intron 6 boundary	CCTTGACAACTACGGTGGGT
NRI6-R[b]	Intron 6	CGAAGAAACAGAAGCCCAAG
NRE7-R	Exon 7	CTCATAGCCRAAGGTCAARGCCAT
NRE7-F	Exon 7	GTTGCGGAAGCTGGAAGCCTT
NRE9-F	Exon 9	GAGAAGCCAACATGTACTTCCT
NRE9-R	Exon 9	GCTTGTAGAAGGCCTGCCCAA
NRE10-F	Exon 10	TTCAACATCTGTGCCAACAGCA
NRE10-R	Exon 10	TGCTGTTGGCACAGATGTTGAA
NRE11-R2[b]	Exon 11	CAAACTGTCCCGCGTAGGTGCC
NR3′UTR-R[b]	Exon 15	CACATCAGGAAGCCCATACAG

[a] F, forward; R, reverse.
[b] Dog specific.

liative dermatitis and ulcerations), local or generalized lymphadenopathy, loss of weight, poor appetite, ocular lesions, epistaxis, lameness, renal failure, and diarrhea (45). In this parasitic process, infection is not synonymous with disease. The prevalence of *Leishmania* infection (65%) in an area of endemicity is higher than the seroprevalence (20%) and the prevalence of disease (10%) (11, 47). The immune response in dogs is humoral and cellular, resulting in a wide array of responses: the cellular immune response is protective against *L. infantum*, whereas a strong humoral immune response correlates with disease susceptibility (11, 35, 46).

Based on in vitro and in vivo studies, it is widely accepted that macrophages, where *Slc11a1* is expressed, play a central role in the control of the parasite (1, 48). Moreover, as previously described for *L. donovani* infection, the early outcome of *L. infantum* infection in mice is under the control of the *Slc11a1* gene (28). All of these issues suggest that *Slc11a1* is a strong candidate gene for natural resistance or susceptibility to *Leishmania* infection in dogs. We have addressed this issue by mapping and sequencing the canine *Slc11a1* gene and analyzing sequence variants that could be associated with disease susceptibility in four dogs experimentally infected with *L. infantum*. A microsatellite located in intron 1 of the gene was used in a case-control study to provide evidence of the association with *Leishmania* infection in dogs.

MATERIALS AND METHODS

Study animal. We characterized the canine *Slc11a1* gene in a healthy Rottweiler.

The case-control study was performed with 33 healthy dogs of different breeds that showed a *Leishmania*-specific cellular immune response by means of positive delayed-type hypersensitivity (DTH) to leishmanin (resistant dogs) and 84 dogs of different breeds with patent leishmaniasis diagnosed by a positive *Leishmania*-specific PCR result for bone marrow samples (39) collected from veterinary clinics (susceptible dogs).

Sequence variant analysis was done with samples from four beagles experimentally infected with *L. infantum*. These dogs were inoculated intravenously with 5×10^7 promastigotes of *L. infantum* (MCAN/ES/92/BCN-83/MON-1) diluted in 0.5 ml of saline solution and were kept in the Animalarium at the Facultat de Veterinària, Universitat Autònoma de Barcelona. They were monitored for 5 years, during which time clinical, hematological, biochemical, serological, and parasitological control analyses were performed under the auspices of the Universitat Autònoma de Barcelona animal care committee. Once infection was confirmed and serologic results were positive, dogs were treated when protein and gamma globulin concentrations reached abnormally high values (>7 g/dl). All dogs were subjected to treatment with meglumine antimoniate (20 mg of Sb^{5+}/kg of body weight every 12 h for 20 days) (38, 50) and to a second treatment with liposome-encapsulated meglumine antimoniate (9.8 mg of Sb^{5+}/kg every 24 h for 20 days) (51). One dog was treated again with liposome-encapsulated meglumine antimoniate and allopurinol (10 mg every 12 h for 8 months).

Specific *L. infantum* humoral and cellular immune responses. Specific *L. infantum* humoral and cellular immune responses were assessed as described elsewhere (17) 5 years after experimental infection. Briefly, the humoral response was analyzed by an enzyme-linked immunosorbent assay for detecting specific anti-*Leishmania* immunoglobulin G1 (IgG1) and IgG2 antibodies. The cellular response was analyzed by determining DTH to leishmanin, by a lymphocyte proliferation assay (LPA), and by determining in vitro gamma interferon (IFN-γ) production. The LPA and the determination of IFN-γ production were carried out after incubation of samples with phytohemagglutinin (PHA) or leishmanial soluble antigen (LSA).

Sequencing and mapping of the canine *Slc11a1* gene. Genomic DNA was isolated from peripheral blood as described elsewhere (41). The canine *Slc11a1* gene was PCR amplified from different overlapping fragments corresponding to the promoter (promoter 1), exon 2 to exon 7 (E2-E7), exon 7 to exon 10 (E7-E10), exon 9 to exon 11 (E9-E11), and exon 10 to the 3′ UTR (E10-E15). Mammalian orthologous gene-specific PCR primers are shown in Table 1. PCR conditions and thermocycling profiles are shown in Table 2. Amplification of the 3′ UTR from cDNA was accomplished with an internal primer from the oligo(dT) used in the reverse transcription (RT) reaction (see below). PCR products of the expected sizes were isolated from agarose gels by using a QIAquick gel extraction kit (Qiagen) and were cloned into the PCR 2.1-TOPO vector (TOPO TA cloning kit; Invitrogen). Two independent clones of each fragment were sequenced by using the dideoxy method (BigDye terminator cycle sequencing ready reaction kit, version 2.0; Applied Biosystems) with fluorescent terminators and an automated DNA sequencer (ABI-PRISM; Applied Biosystems).

We used canine *Slc11a1*-specific primers (NRE6I6-F and NRI6-R) to amplify a 179-bp fragment in the RHDF5000 dog whole-genome radiation hybrid panel (53, 54) as previously described (37). PCR conditions and thermocycling profiles are shown in Table 2 (E6-I6). Based on a pairwise analysis with a logarithm of odds ratio score threshold of >8.0, the typing data generated for the *Slc11a1* marker were then incorporated into the integrated radiation hybrid (RH)-linkage map (34) by using the Multimap package (32). Distances (D), calculated as $D = -\ln(1 - \Theta)$, where Θ is the breakage frequency, are expressed in centiRays 5000 (cR5000), where 5000 is the radiation dose used to generate the RH panel.

TABLE 2. PCR conditions and thermocycling profiles used for canine *Slc11a1* sequencing and polymorphism analysis

Region	Primers	$MgCl_2$ concn (mM)	Primer concn (μM)	Conditions for:						No. of cycles	Enzyme(s)[a]
				Denaturation		Annealing		Extension			
				°C	s	°C	s	°C	s		
Promoter 1	NRPROM-F–NRI2E2-R	1.50	0.2	94	30	55	30	72	60	35	A
	NRPROM3-F–NRPROM2-R	1.50	0.2	94	30	54	30	72	60	35	A
Promoter 2	NRPROMD-F–NRPROM3-R	1.50	0.2	94	30	53	30	72	60	35	A
	NRPROMD-F–NRPROM2-R	1.50	0.2	94	30	53	30	72	60	35	A
E2–E7	NRE2-F–NRE7-R	2.25	0.3	92	10	58	30	68	240	10	B
				92	10	58	30	68	240[b]	25	
E2–E9	NRE2-F–NRE9-R	1.50	0.2	94	30	53	30	72	60	35	A
E7–E15	NRE7-F–NRE3′UTR-R	1.50	0.2	94	30	55	30	72	60	35	A
E6–I6	NRE6I6-F–NRI6-R	2.00	0.3	94	30	63[c]	30	72	30	20	D
				94	30	53	30	72	30	10	
E7–E10	NRE7-F–NRE10-R	1.50	0.3	94	15	55	30	68	480	35	C
E9–E11	NRE9-F–NRE11-R2	1.50	0.3	94	15	55	30	68	480	35	C
E10–E15	NRE10-F–NR3′UTR-R	1.50	0.3	94	15	55	30	68	480	35	C
Microsatellite	NRMICRI1-F–NRMICR1-R	1.50	0.2	94	30	55	30	72	30	25	A
Intron 10	NRE10-F–NRE11-R2	1.50	0.2	95	30	55	30	72	45	35	E

[a] Enzymes and commercial kits: A, *Taq* polymerase (Invitrogen); B, Expand long-template PCR system (system 3; *Taq* and *Pwo* polymerases) (Roche); C, Platinum *Pfx* DNA polymerase (with enhancer solution ×1) (Invitrogen); D, AmpliTaq Gold (Applied Biosystems); and E, GC-Rich PCR amplification system (0.5 M GC rich resolution solution and *Taq* and *Tgo* polymerases).
[b] Plus 20 s per cycle.
[c] Minus 0.5°C per cycle.

In this panel, the correspondence between cR5000 and a physical distance has been estimated to be 1 cR5000 for 166 kb. A multipoint analysis was carried out to order the markers belonging to the group in a comprehensive manner, and the intermarker distances were calculated.

Sequence variant analysis. Different tissues were used for RNA isolation. Blood and spleen or liver were extracted postmortem and stored at −80°C until RNA isolation. Peripheral blood mononuclear cells were isolated from heparinized venous blood samples by standard Ficoll-Hypaque (Histopaque 1.077; Sigma, St. Louis, Mo.) density gradient centrifugation as described elsewhere (7). Total RNA was isolated by using Trizol reagent (GibcoBRL-Life Technologies) as specified by the manufacturer. RT-PCR was carried out with a 20-μl final reaction mixture containing 1 μg of total RNA, 1 mM each deoxynucleoside triphosphate, 2 μM oligo(dT) primer, and 200 U of Moloney murine leukemia virus RT (Amersham Life Science). cDNA amplification was accomplished with two overlapping fragments: exon 2 to exon 9 (E2-E9) and exon 7 to exon 15 (E7-E15). PCR conditions and thermocycling profiles are shown in Table 2. PCR products were isolated, cloned, and sequenced as described above.

A seminested PCR was used to amplify the promoter region (promoter 2). A 1-μl sample of the first PCR product (NRPROMD-Fand NRPROM3-R) was used as a template to perform the second PCR (NRPROMD-F and NRPROM2-R). PCR products were isolated, cloned, and sequenced as described above.

The microsatellite located in intron 1 was amplified by using dog-specific primers NRMICRI1-F (fluorescence labeled) and NRMICRI1-R. PCR conditions and thermocycling profiles are shown in Table 2. PCR products were analyzed by capillary electrophoresis with an ABI 3100 genetic analyzer (Applied Biosystems), and labeled PCR products were automatically sized relative to the internal standard (PRISM GeneScan-350 TAMRA; Applied Biosystems) with GeneScan analysis software, version 3.5 (Applied Biosystems). Intron 10 amplification was performed as shown in Table 2 by using a GC-rich PCR amplification system (Roche) and a 50-μl final reaction mixture containing 0.5 M GC-rich resolution solution and 2 U of a *Taq* and *Tgo* DNA polymerase mix, under the manufacturer's conditions.

Computation and statistical analysis. Allele frequencies of the intron 1 microsatellite of the canine *Slc11a1* gene were analyzed by using a two-by-two chi-square test with 1 df.

A neighbor-joining relationship tree for mammalian *Slc11* cDNA sequences, both *Slc11a1* and *Slc11a2*, was created with the assumption that distance is equal to the number of nucleotide differences and with a bootstrap value of 1,000 by using MEGA software, version 2.0 (26).

Nucleotide sequence accession number. The canine *Slc11a1* gene characterized has been deposited under GenBank accession number AF091049.

RESULTS

Sequencing and mapping of the canine *Slc11a1* gene. Mammalian orthologous gene-specific primers were designed to accomplish the complete sequencing of the canine *Slc11a1* gene. The sequences of the primers were designed based on conserved coding regions from all *Slc11a1* sequences from vertebrate species available up to that time: human (6, 12), sheep (9), mouse (21), cow (16, 23), rat (19), pig (49), deer (33), buffalo (M. Hashad et al., unpublished), and bison (J. Feng et al., unpublished). Primer sequences are shown in Table 1. The canine *Slc11a1* gene includes 15 exons and encompasses 9 kb (Fig. 1). Both the sequence and the genomic organization of the coding regions are highly conserved in relation to those of the *Slc11a1* genes from other mammals (Fig. 2). Intron-exon boundaries were determined by comparison of the entire genomic sequence with the beagle cDNA and the mouse gene (21). Intronic regions conform perfectly to the

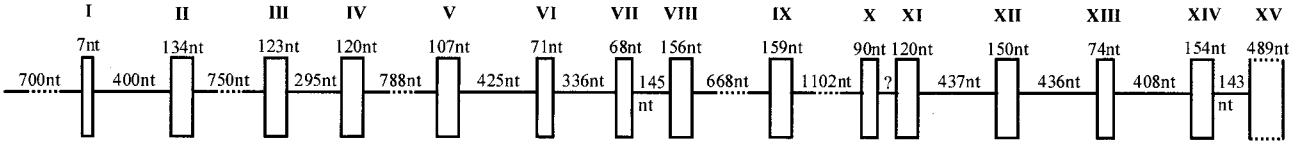

FIG. 1. Genomic structure of the canine *Slc11a1* gene. nt, nucleotides.

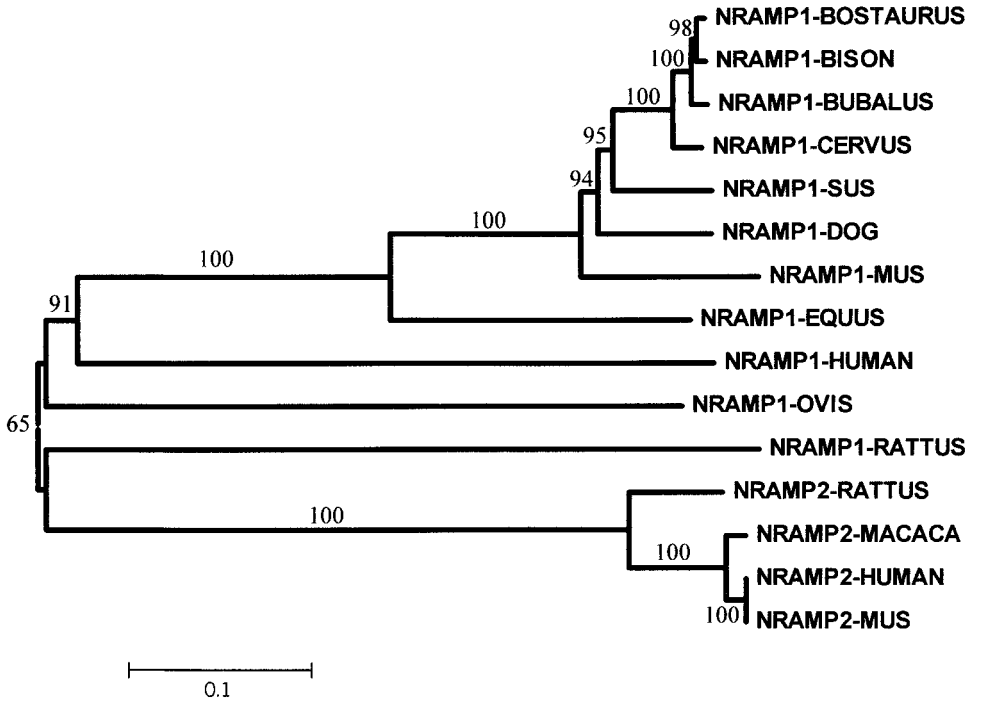

FIG. 2. Neighbor-joining relationship tree of mammalian *Slc11* cDNA sequences. The distance indicates the number of nucleotide differences. Numbers on the nodes are percent bootstrap values from 1,000 replications, and a scale bar for branch lengths is shown.

GT-AG donor-acceptor splice rule and vary in size from 143 to 1,102 bp. In general, they are shorter than those in mice and humans, especially introns 4, 8, and 11. A microsatellite, (TAAA)*n*, was identified in intron 1 (position 1253 of the sequence). Only a partial sequence was obtained for intron 10 due to the presence of GGGGC-CCCCG terminal repeats that confer a complex secondary structure to this region. The canine *Slc11a1* gene encodes a 547-amino-acid protein with amino acid identity of up to 87% with Slc11a1 proteins of different vertebrate species and with the same structure as the other Slc11a1 proteins described up to this time. Slc11a1 is composed of 12 TM domains, three phosphorylation sites for protein kinase C, two N-linked glycosylation sites located in exon 10, and the consensus transport motif located between TM8 and TM9 (exon 11). The G169 residue in TM4 is conserved in the dog Slc11a1 protein, as it is in the human, cow, sheep, pig, buffalo, bison, and deer proteins.

The translational start codon is located at position 689 of the sequence, and the translational stop codon and the 3′ UTR are located in exon 15. The first 680 nucleotides of the sequence includes the 5′ UTR and the promoter of a gene that does not contain a classical TATA box. A sequence motif for a potential initiator element (Inr) which is often found in TATA-less gene promoters was found at position 510. This region also contains several sites for binding of transcription factors involved in tissue-specific transcription and expression or associated with the response to induction by differentiating agents specific to the macrophage (Fig. 3A). Briefly, we have found two potential AP-1 binding sites (TGASTMA), four potential AP-2 binding sites (CCCMNSSS), nine potential IFN-γ binding sites (CWKKANNY), two potential IFN-α/β binding sites (AA RKGA), one potential granulocyte-macrophage colony-stimulating factor binding site (CATTW), three SP-1 binding motifs (GGGCG), a potential binding site for the transforming growth factor β-inducible NF-1 transcription factor (YGG MN_{5-6}GCCAA), and a potential binding site for bacterial lipopolysaccharide (LPS) (NF-IL-6; TKNNGNAAK).

By use of the RHDF5000 dog whole-genome radiation hybrid panel (53, 54) and a pairwise analysis with the Multimap package (32), *Slc11a1* was mapped to RH syntenic group 11 (SRH11) between the microsatellites Ren03C19 and Ren41P15 with Lod scores of 21.7 and 14.8 and distances of 7 cR5000 and 21 cR5000, respectively. RH syntenic group 11 is shown in Fig. 4.

Case-control study. In order to assess the possible role of the *Slc11a1* gene in determining *Leishmania* susceptibility, we performed a case-control study with 33 resistant and 84 susceptible dogs and the intron 1 microsatellite as a marker. The different alleles detected and the allelic distribution observed in the two populations analyzed are shown in Fig. 5. Significant differences between resistant and susceptible animals were observed in the frequency of allele 145 ($P < 0.025$). Furthermore, allele 145 has only been observed in a homozygous state in the susceptible population (0.32), with the exception of one dog in the resistant population (0.03).

Beagle immunoprofiles. In order to search for mutations that could have functional implications, sequence variant analysis was performed with four unrelated beagle dogs experimentally infected with *L. infantum*. Dogs were divided into resistant (R1 and R2) and susceptible (S1 and S2) groups based on their predominant immune response (cellular or humoral, respectively). Resistant dogs did not have clinical relapses after

A.

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TTGGCCTTCTTGGGCATCTAGTTTTAACCTCTCAGCTAGTCTGAGCCCTTCATGTAACAGAAG          63
                                                IFNα/β  AP-1
AAACAAGTTTAGAGAAAGGGCTGGCCTGGTGACAATCTTAAGTGAGTCAGTGGCAGGATCGGGAC       128
                                                AP-1
CAGAATTCCAGCGCCACGTTCTTCTGAGGCTCTCTCTGGCCCGAGTCATCAATGCACGTCCCTGTG      194
IFNγ                                                                IFNγ
GCACTGCCTGACGGTCTTCCCCTCTGAGGCACTCAAGGGGAAACTGAGGCTTGGTAGCCATCAGG      259
        IFNγ            IFNα/β                          SP-1
ACTCCCATTGCCAGGAACTCCAGACTGAAGTGAAGGGCCTGACTGCTTGTGTGGGGCGGATGGGG      324
                IFNγ                            IFNγ                AP-2
GGGGCAGCCATGTGATCTGGACATGAACGCCCAAGTAGCAGGAAATGTCTTGAAATCAGAGCTG       388
                                SP-1  IFNγ / NF-1  GM-CSF       AP-2
GCTTGGGAGACACAGAATATACGGCGCCTGGCAGGAGGCCAAAAGTGTTGTGGGCCCCAGGCCA       452
AP-2                                                        Inr
GGAGGGGAACGAAAGTTCCTGCATTTTGGTCAACTAGACTCTTCCGCCAGAGCTGGCCACTTCTG      517
                IFNγ                    NF-IL6                  SP-1
CCTTTGGAAAGTGTTTCACAATGTATATGAGGACAGCTGAGATCAGCTGAGGATGGCTAGGAGGG      582

CGTGAGCTTGCCCGCTGGCGCGCACCGGCACTCAGAGGGGAAGCATGTGCTGAGTAGCTGCCAGC      647

ATCCCACTCACACGCAGACGGTGCCGGAGCCTGCCGTCCTC  688
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B.

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Rottweiler (allele1)    TTGTGTGGGGCGGGTGAGGGGGGCAG
Rottweiler (allele2)    -------------A--G---------
R1                      ----------------GA--------
R2                      --------A-------G---------
S1                      ----------------G---------
S2                      ----------------G---------
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FIG. 3. Sequence analysis. (A) Promoter and 5′ UTR of the canine *Slc11a1* gene from nucleotide 1 to the translational start codon (position 689). Putative binding sites for transcription factors are indicated with a line above (sense strand) or below (antisense strand) the consensus sequence. (B) G-rich polymorphism of the promoter regions from beagles and a Rottweiler. GM-CSF, granulocyte-macrophage colony-stimulating factor.

the second treatment and were negative for detection of parasite DNA in bone marrow by PCR after 5 years. In susceptible dogs, the disease evolved gradually and became chronic after the second treatment, and a third long treatment was administered to dog S1 to avoid continuous relapses. The results of immunological tests are shown in Table 3.

Sequence variant analysis. *Slc11a1* sequence variants were analyzed at different levels: cDNA, promoter, and microsatellite. cDNA sequences obtained from the experimentally infected beagles were compared with the Rottweiler genomic sequence. Interestingly, two *Slc11a1* cDNAs were coamplified in dog S1: one of the expected size and the other one shorter. The shorter fragment was explained by the complete deletion of exon 11, which includes TM8 and most of the consensus transport motif of the protein. We analyzed this sample at the genomic level (data not shown); after PCR amplification from exon 10 to the 3′ UTR, we found a single band and no evidence

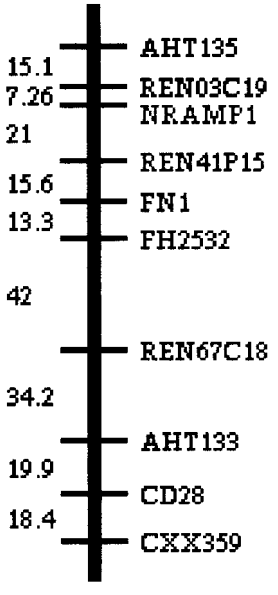

FIG. 4. *Slc11a1* mapped to canine RH syntenic group 11.

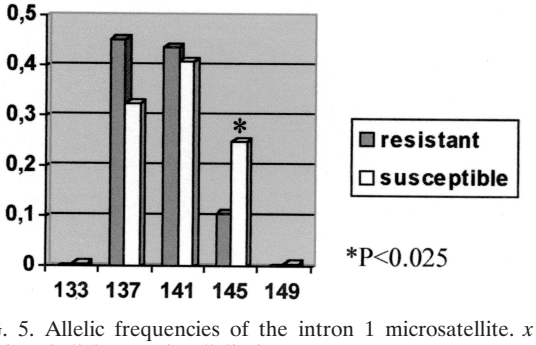

FIG. 5. Allelic frequencies of the intron 1 microsatellite. *x* axis, basepairs of alleles; *y* axis, allelic frequency.

TABLE 3. Assessment of resistance or susceptibility to infection by a range of immunological tests[a]

Dog	OD_{492} for:			DTH response (mm)	OD_{450} in LPA with:			IFN-γ (IU/ml) with:		
	IgG	IgG2	IgG1		UPBMC	PHA	LSA	UPBMC	PHA	LSA
R1	0.929	1.138	0.123	22	0.133	0.916	1.266	80	160	320
R2	0.922	1.294	0.140	15	0.152	1.330	1.600	80	160	640
S1	2.285	3.000	0.513	0	0.100	1.090	0.098	20	40	0
S2	1.560	2.296	0.124	0	0.112	0.512	0.178	0	20	0

[a] OD_{450}, optical density at 450 nm; UPBMC, unstimulated peripheral blood mononuclear cells.

of genomic deletion. Once we sequenced the exon-intron boundaries for intron 10, we found no mutation at the GT-AG donor-acceptor splice sites. However, both bands were also coamplified in two independent RT-PCRs from liver and spleen RNAs from the same animal (Fig. 6). We also found five single-nucleotide polymorphisms that resulted in amino acid substitutions. Four of them were located within the TM domains, and the other one, located in exon 10, produced the loss of an N-glycosylation site. Alignment of the five proteins is shown in Fig. 7.

Differences were also observed between susceptible and resistant animals in the promoter region. There is a G-rich region (positions 310 to 330) in the susceptible animals that is disrupted by G-to-A substitutions in different places in the resistant animals or in the Rottweiler dog sequence (Fig. 3B). The loss of a potential SP-1 binding site in dog R1 and both the gain and the loss of an AP-2 binding site were observed in dog R2 (positions 226 and 328, respectively). The latter dog also had an A-to-G substitution at position 405; this substitution did not modify any transcriptional binding site.

We also analyzed the polymorphism of the $(TAAA)_{9-11}$ microsatellite located in intron 1. Both susceptible animals were homozygous (allele 141), and dogs R1 and R2 were heterozygous (alleles 137/141 and 141/145, respectively).

DISCUSSION

In this study, we sequenced and mapped the canine *Slc11a1* gene as a strong candidate gene for leishmaniasis on the basis of studies with its murine homologue. The canine *Slc11a1* gene has been assigned to linkage group SRH11 located in chromosome CFA37 (8, 37), which includes at least two genes (*FN1* and *CD28*) that have been mapped either to human chromosome 2q35 or mouse chromosome 1. The conserved syntenic group of the fragment delineated by *Slc11a1* and *CD28* can be assumed for dogs, humans, and mice.

In mice, the early outcome of *L. infantum* infection is under the control of the *Slc11a1* gene, which regulates the intraphagosomal replication of parasites inside the macrophage (28). In addition, *Slc11a1* regulates macrophage activation and has multiple pleiotropic effects involved in antigen processing and presentation (27). *Slc11a1* has a relevant function in the modulation of the subsequent immune response that could be predominantly cellular or humoral and that is of great importance in resistance or susceptibility to leishmaniasis (11, 35, 46). To the best of our knowledge, there are no reports describing the genetic factors that could influence *Leishmania* infection in dogs. However, the high degree of conservation of amino acids and structural motifs between the dog and mouse Slc11a1 proteins suggests that they may have a similar role in the progression of the disease. As observed in humans (10), one should not expect an absolute association between *Slc11a1* and *Leishmania* infection, as in the mouse model, but a significant association between canine *Slc11a1* polymorphism and a susceptible phenotype in dogs.

In order to analyze the association between the canine *Slc11a1* gene and leishmaniasis, we performed a case-control study with resistant and susceptible dogs. Significant differences were observed for allele 145 of the intron 1 microsatellite between the two populations. These results suggest an association between *Slc11a1* and leishmaniasis, indicating a role of this gene in disease susceptibility. Sequence variants analyzed in four beagles experimentally infected with *L. infantum* could

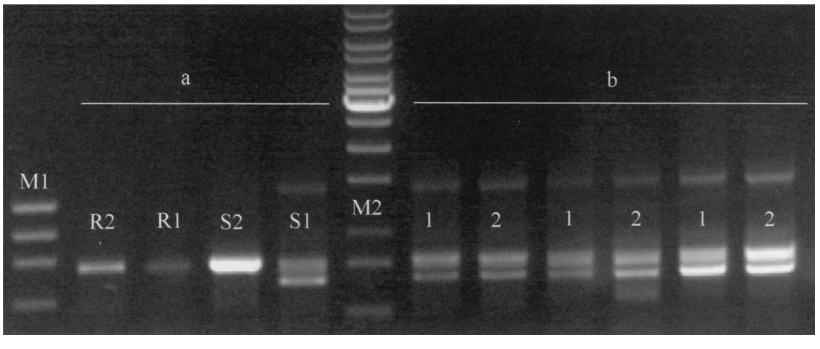

FIG. 6. mRNA alternative splicing in dog S1. (a) PCR amplification from exon 9 to the 3′ UTR of cDNAs from the four beagles experimentally infected with *L. infantum*. R1 and R2 are resistant dogs, and S1 and S2 are susceptible dogs. (b) PCR amplification of the same region of dog S1 from three different RNA extractions. Two independent RT-PCRs of each RNA extraction are shown (lanes 1 and 2). Lane M1, ϕX174 *Hae*III; M2, 1-kb ladder.

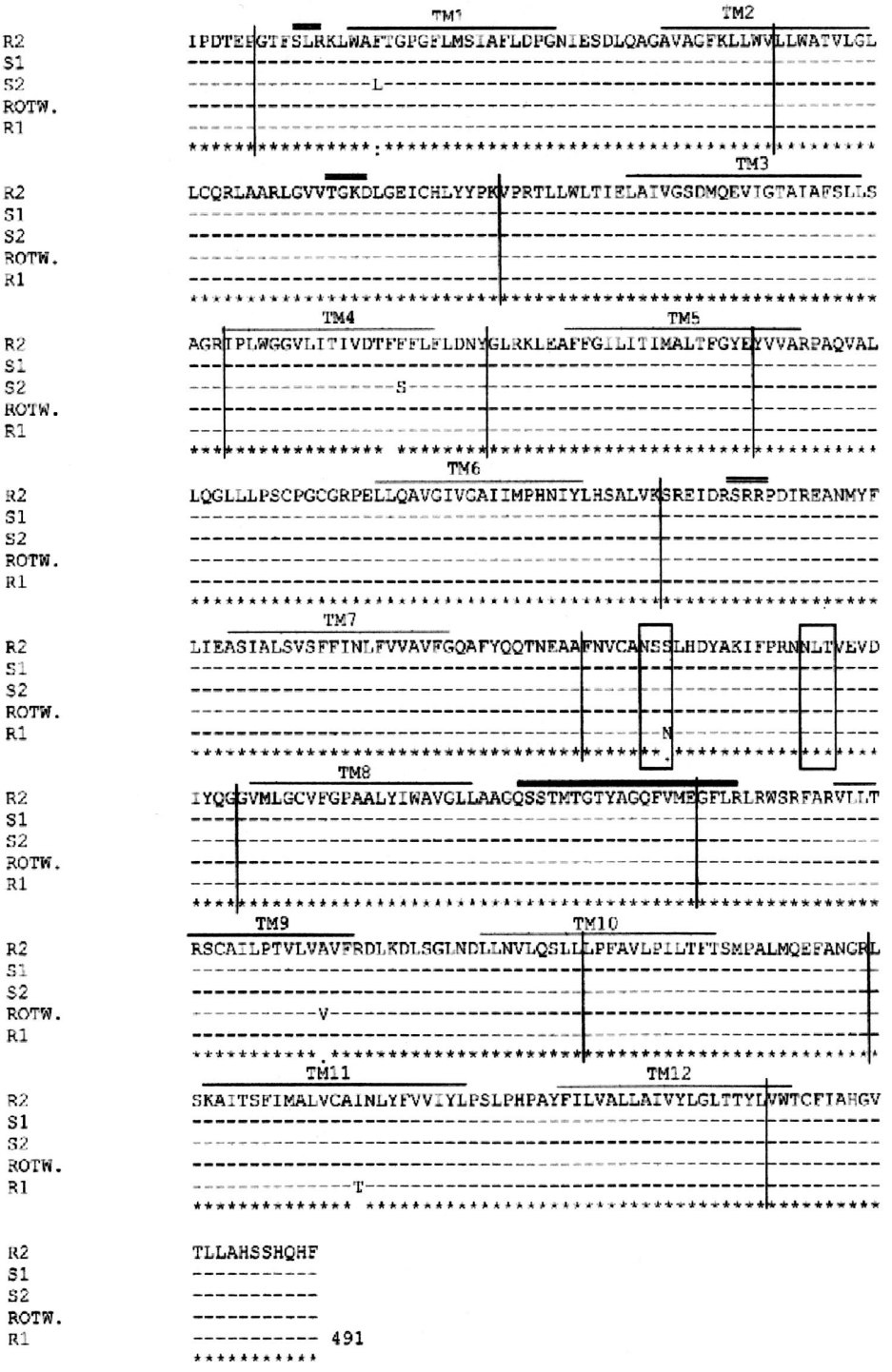

FIG. 7. Multiple alignment of the Slc11a1 proteins from the Rottweiler (ROTW.) and the four beagles experimentally infected with *L. infantum*. For dog S1, the normal allele is shown. TM domains are indicated by thin overlining, and the consensus transport motif of the protein is indicated by thick overlining. Potential N-glycosylation sites are boxed, and potential phosphorylation sites for protein kinase C are indicated by double overlining. Exons are separated by vertical lines.

be associated with possible functional mutations related to their susceptible or protective immune responses. One of the susceptible beagles (S1) had a complete deletion of exon 11 in one of the *Slc11a1* alleles which resulted in the elimination of TM8 and the consensus transport motif of the protein. Since no mutation was observed at the genomic level, including the GT-AG donor-acceptor intron 10 splicing sites, we suggest that the deletion of exon 11 could be caused by alternative mRNA

splicing. Intron 10 has complementary terminal repeats (5′-G GGGCCCCCG-3′) which are canine specific and which could confer a complex secondary structure resulting in alternative mRNA splicing. Alternative splicing has been described for the human *Slc11a1* and *Slc11a2* genes, but in no case was the region of the consensus transport motif of the protein included (29, 30). The loss of the consensus transport motif caused by this deletion could produce a lack of function of the mutated protein, resulting in a higher level of proliferation of the parasite inside the macrophages of susceptible dogs. It is important to note that this dog was the only one that needed a third, sustained treatment to avoid continuous relapses.

We also described five amino acid substitutions, four located in the TM domains of the protein. The most significant mutations were F179S (TM4), I476T (TM11), and S323N (exon 10). The F179S amino acid substitution in TM4 of dog S2 implies a nonconservative change from a hydrophobic to a polar residue. Although this is a region that contains several polar residues (13), this substitution is located in the same TM domain in which the mouse mutation (G169A) was described to be located and resulted in the lack of function of the protein (21, 31). Moreover, this change has never been described for other mammals and has been observed only in Slc11a1 orthologues from plants and prokaryotic organisms. The second amino acid substitution, I476T in TM11 of dog R1, represents a polar change in one of the most hydrophobic regions of the protein (13). Dog R1 also had an S323N substitution in exon 10 that results in the loss of one of the N-glycosylation sites; this substitution has also been described for buffalo and cow Slc11a1 proteins.

The canine *Slc11a1* promoter region has been found to posses nine consensus IFN-γ binding sites and one potential binding site for LPS (NF-IL-6), consistent with the regulation of expression previously described (20). *Slc11a1* gene expression can be regulated during macrophage activation by cytokines and LPS. Furthermore, lymphocytes from resistant *Leishmania*-infected dogs have been found to produce IFN-γ upon parasite-specific stimulation and to lyse infected macrophages in a major histocompatibility complex-restricted manner (36), leading to a cellular protective immune response. Although no polymorphism has been found at the IFN-γ and NF-IL-6 binding sites, different immune responses have been described for resistant and susceptible animals analyzed. Resistant dogs demonstrated *Leishmania*-specific cellular immunity due to their ability to produce IFN-γ, lymphocyte proliferation, and positive DTH. The only polymorphism common to the susceptible animals was the G-rich region in the promoter, which is located close to the region where the human microsatellite has been described to be located. Different alleles in the human dinucleotide microsatellite have been associated with a different ability to drive gene expression and has been correlated with infectious versus autoimmune disease susceptibility (44). A microsatellite located in the 3′ UTR of the bovine *Slc11a1* gene has also been associated with different gene expression controlling the in vitro replication of *B. abortus* (3, 23). All these data suggest that the G-rich region of the *Slc11a1* promoter could also have an effect on gene expression.

In conclusion, the characterization of the canine *Slc11a1* gene, the association between the canine *Slc11a1* gene and disease susceptibility, and the description of new sequence variants will be useful in the elucidation of the genetic factors involved in canine leishmaniasis. However, in order to determine whether each sequence variant described is associated with *Leishmania* susceptibility in dogs, a larger population-based analysis would be required to prove the functional association of *Slc11a1* with the disease.

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