the surface of APCs, (ii) are structurally related to the Ig supergene family with an Ig V– and Ig C–like domain, and (iii) costimulate T cells to produce IL-2 and proliferate. However, B7-1 and B7-2 differ in that B7-2 mRNA is constitutively expressed in unstimulated B cells, whereas B7-1 mRNA does not appear until 4 hours and cell surface protein is not detected until 24 hours (*8*, *9*). Unstimulated human B cells do not express CTLA-4 counter-receptors on the cell surface and do not costimulate T cell proliferation (*7*). Therefore, expression of B7-2 mRNA in unstimulated B cells would allow rapid expression of B7-2 protein on the cell surface after activation, presumably from stored mRNA or protein. Expression of B7-1 and B7-2 in cell lines and human B cell neoplasms differ (*11*), suggesting they may have biologically distinct functions.

The functional necessity for multiple CD28 and CTLA-4 counter-receptors is unknown. Indeed, whereas CD28 can clearly transduce a costimulatory signal, the signaling function of CTLA-4 is currently unknown. A T cell, which has received a first signal from the TCR, must commit within 12 to 24 hours to either activation or anergy (*12–15*). Although B7-1 (*12*, *13*) or cross-linking by CD28 mAb (*15*) can provide a costimulatory signal that prevents the induction of anergy in vitro, the late appearance (between 24 and 48 hours) of B7-1 on B cells (*9*) and macrophages (*16*) after activation suggests that B7-1 is not the critical costimulatory molecule that regulates whether a T cell becomes activated or anergic. We postulate that the earlier appearance of B7-2 provides this critical costimulatory signal (*17*, *18*). If confirmed, then B7-1 costimulation is more likely to provide a signal that results in T cell clonal expansion. Identification and characterization of B7-2 will enable further manipulation of the CD28 pathway, potentially leading to therapeutic strategies for the control of autoimmunity, transplantation tolerance, and the stimulation of viral and tumor immunity.

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Gene Replacement in *Toxoplasma gondii* with Chloramphenicol Acetyltransferase as Selectable Marker

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A system for stable transformation of *Toxoplasma gondii* tachyzoites was developed that exploited the susceptibility of *Toxoplasma* to chloramphenicol. Introduction of the chloramphenicol acetyltransferase (CAT) gene fused to *Toxoplasma* flanking sequences followed by chloramphenicol selection resulted in parasites stably expressing CAT. A construct incorporating the tandemly repeated gene, *B1*, targeted efficiently to its homologous chromosomal locus. Knockout of the single-copy gene, *ROP1*, was also successful. Stable transformation should permit the identification and analysis of *Toxoplasma* genes important in the interaction of this opportunistic parasite with its host.

Toxoplasma gondii is an obligate intracellular parasite that can infect most warm-blooded vertebrates. In humans, infection can result in severe congenital defects and life-threatening infection in immunocompromised hosts. Recently, the parasite has received increased attention as an opportunistic pathogen affecting up to 25% of patients with acquired immunodeficiency syndrome (AIDS) (*1*). Detailed study of the basic biology of the host-parasite interaction has been hampered by the absence of a methodology to introduce and stably express or knockout genes in this organism. Despite intensive efforts, transfection of intracellular eukaryotic parasites was unsuccessful until recently when transient CAT expression driven by the upstream regions of three *Toxoplasma* genes was achieved (*2*).

Efforts to stably transform *Toxoplasma* were complicated by the inability of these parasites to replicate outside host cells. Neomycin and hygromycin, drugs commonly used for selection of stable transformants in systems such as protozoan parasites from the kinetoplastida order (*3–5*), kill host cells as efficiently as they kill parasites. We have developed an alternative system in which CAT is used as an efficient and easily detectable selectable marker in *Toxoplasma*.

Chloramphenicol and other antibiotics that inhibit prokaryotic translation have potent effects against *Plasmodium* and are currently used for malaria chemotherapy. It has been thought that *Toxoplasma* tachyzoites are not susceptible to chloramphenicol in vitro. We have found, however, that chloramphenicol, like the mechanistically similar antibiotic clindamycin (*6*), has a potent parasiticidal effect, but one that is delayed in onset: Parasites complete two to three cycles of host cell lysis (approximately 7 days or 20 to 25 divisions) before any effect of drug is evident. Daily visual inspection of cultures indicated that 10 μM chloramphenicol killed more than 90% of parasites, but had no obvious effects on the host monolayer. These results indicate that *Toxoplasma gondii* has a highly specific susceptibility to drugs inhibiting prokaryotic translation. This may reflect a unique target for chemotherapy in these coccidian parasites, for example, targets in mitochondria or other organelles as suggested for *Plasmodium* (*7*, *8*).

Our initial constructs (Fig. 1A) utilized the upstream and downstream regions of a gene, *SAG1*, that encodes the major surface antigen p30. These sequences have been previously shown to drive efficient CAT expression (*2*). Stable transfection, if by means of homologous recombination with replacement of *SAG1*, seemed likely to be a deleterious event given that *Toxoplasma* tachyzoites are haploid and *SAG1* is single-copy. [Indeed, Sag1$^-$ mutants are impaired in virulence and intracellular growth (*9*, *10*).] We therefore added to the construct a segment of *B1*, a gene of unknown function that is tandemly reiterated approximately 35 times within the genome (*11*). In transient transfection, this *B1*-containing construct induced CAT activity similar to that induced by a construct lacking *B1* (*12*).

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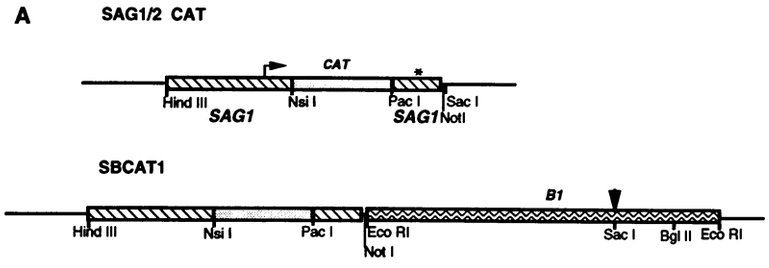


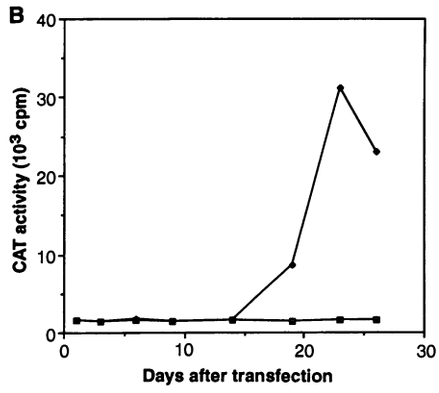


Fig. 1. Stable transformation of *Toxoplasma* with CAT. (**A**) Constructs. The starting plasmid, SAG1/2 CAT, was Bluescript SK+ (thin line) containing *SAG1* upstream and downstream regions (hatched boxes) fused to *CAT* (stippled box) (*2*). The plasmid contains ~800 bp upstream of the AUG start codon of *SAG1* and 300 bp downstream of the stop codon. The transcription start site (bent arrow) and polyadenylation site (*) are indicated. An Eco RI genomic DNA fragment containing a single *B1* repeat (wavy box) was ligated to Sac I–cut, phosphatased SAG1/2 CAT. Both insert and plasmid were treated with T4 DNA polymerase before ligation, destroying the Sac I cloning site in the vector. The resulting plasmid, SBCAT1, was linearized for use in transformation at the remaining Sac I site within the *B1* repeat (arrow). (**B**) Chloramphenicol selection of transfected parasites. A solvent phase-partition assay (*16*) was used for CAT assays on 10^7 parasites transfected with 10 μg of linearized SBCAT1 and maintained in the absence (open squares) or presence (solid squares) of 20 μM chloramphenicol. Chloramphenicol selection was begun 3 days after transfection. Data are presented with all assays in the linear range of detection. Days after transfection are indicated (*17*).

Linearized and circular plasmid were electroporated into freshly lysed tachyzoites under conditions previously established for transient transfection (*2*). Parasites were inoculated onto confluent monolayers of human foreskin fibroblasts (HFF) and allowed to infect and pass through one lytic cycle in host cells before passage and selection (*13*). We found that selection with 20 μM chloramphenicol was optimal. [This represented the minimal concentration able to kill 100% of wild-type parasites. Although effective for selection, 10 μM chloramphenicol sometimes allowed growth of parasites that were not stably transformed with CAT, whereas selection with 100 μM chloramphenicol did not yield any stable transformants (*12*).] Use of 20 μM chloramphenicol resulted in the emergence of chloramphenicol-resistant presumptive transformants in transfected cultures after approximately 10 days of selection. High CAT activity was detected in parasite populations transfected with both 10 and 40 μg of linearized SBCAT1 plasmid after approximately 2 to 3 weeks of selection (Fig. 1B). No CAT activity was detectable in parasites transfected and passaged in parallel without drug selection. Stable transformants transfected with 10 μg of plasmid were cloned and analyzed further.

The minimum amount of plasmid required to yield stable recombinants has varied depending on the construct used, but 10 to 50 μg usually yields stable transformants. We have also found that selection can be initiated as soon as 16 hours after transformation. Parasites used in these experiments were cloned after 4 to 6 weeks of selection, but stably transformed parasites can be cloned as early as 10 days after transformation (when wild-type cells are dying and a chloramphenicol-resistant population is beginning to emerge).

Although no difference in CAT activity was seen in transient assays, circular plasmids were a less efficient source of stably transforming DNA than linear plasmids (*12*). Exact quantitation of parasite survival in transfected populations proved difficult owing to the delayed effects of the drug and the necessity to pass parasites onto new monolayers during selection.

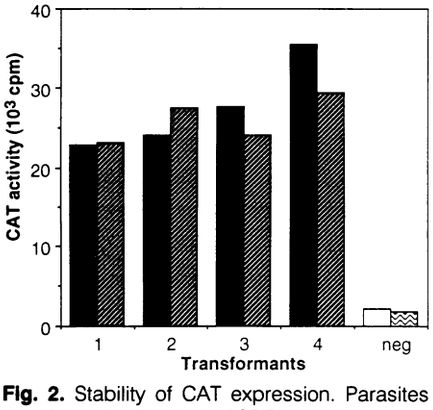


Fig. 2. Stability of CAT expression. Parasites transformed with 10 μg of SBCAT1 were cloned by limiting dilution in the presence of 10 μM chloramphenicol after 6 weeks of selection. Only wells containing a single plaque were used. Assays were done on parasites grown in chloramphenicol (hatched bars) or grown without drug (solid bars) for 2 weeks. Each number represents a separate individual clone. Negative controls include parasites transformed with an irrelevant plasmid 16 hours earlier (open bar) and an assay done with no lysate (wavy bar). CAT assays were done on 10^7 parasites.

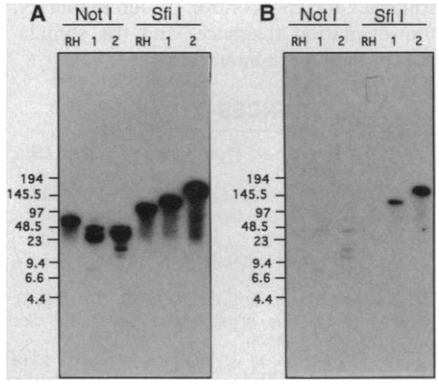


Fig. 3. Southern blot analysis of SBCAT1 recombinants. Chromosomal plugs were prepared as described (*18*) from wild-type RH strain and cloned stable transformants obtained after transfection of SBCAT1. Plugs were digested in situ with 100 U of Not I or Sfi I (New England Biolabs) overnight as suggested by the manufacturer. Pulsed-field gel electrophoresis was done with a Geneline I TAFE (Beckman) apparatus using a two-step program (150 V, 30-s switch for 10 hours followed by 150 V, 15-s switch for 10 hours). DNA from plugs were separated in 1% LE agarose (FMC Corp.) in 0.25× TAE (1× = 0.04 M tris-acetate, 0.001 M EDTA). The gel was stained with ethidium bromide and ultraviolet cross-linked with 60 mJ before transfer to Nytran by alkaline capillary transfer. The membrane was probed with the 2.2-kb *B1* fragment (**A**) and a 600-bp *CAT* fragment (**B**). (Both were labeled by random priming with ^{32}P-labeled deoxycytidine 5′-triphosphate (dCTP). The membrane was stripped with NaOH before reprobing.) The membrane was washed at a final stringency of 65°C with 0.1× standard saline citrate–0.25% Sarkosyl for 1 hour and exposed at −70°C. Sizes of λ DNA ladder and λ phage DNA cut with Hind III are shown for comparison. RH, wild-type parasites; 1, a representative clone from experiment 1; and 2, a representative clone from experiment 2. Molecular size standards are indicated at left (in kilobases).

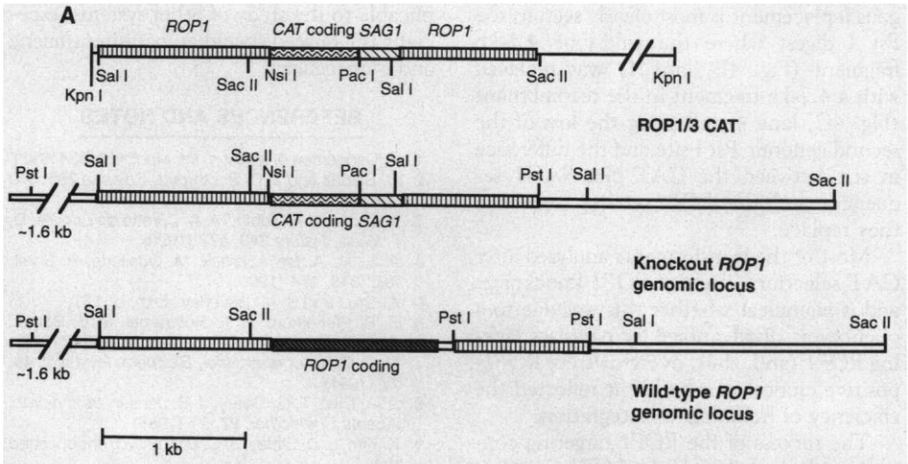
Once selected, transformants did not require growth in drug for maintenance of CAT expression (Fig. 2). We have maintained some clones for 250 generations (12 weeks) in the absence of drug without any loss of CAT activity.

Given this stability, it appeared unlikely that the gene was carried as an extrachromosomal element. This was confirmed by Southern (DNA) blot analyses, with a *CAT* probe, of clones obtained from three separate experiments and transfected with 10 μg of the linearized SBCAT1 construct. Hybridization of uncut DNA did not indicate the presence of plasmid-sized extrachromosomal DNA. Instead, the intensity and size of bands obtained with a variety of restriction enzymes suggested that multiple copies of the plasmid integrated into the genome. Homologous recombination with replacement of *SAG1* had not occurred, because all clones continued to express p30 (*12*).

As a test of whether integration had occurred in the *B1* repeat locus, chromosomal plugs of representative clones from separate experiments were digested with Not I, an enzyme that cuts the plasmid vector but not the *B1* repeat, and Sfi I, which cuts in neither *B1* nor the vector. Digests were then analyzed by pulsed-field gel electrophoresis and Southern blotting. The blot was hybridized with a *B1* probe (Fig. 3A) and a *CAT* probe (Fig. 3B).

Sfi I digestion of DNA from both clones 1 and 2, as compared with digestion of wild-type DNA, revealed a single, larger Sfi I fragment that hybridized to the *B1* probe (Fig. 3A). The *CAT* probe hybridized to a fragment of identical size as the *B1* fragment in both transformants, but did not hybridize to wild-type DNA (Fig. 3B). This indicated that targeting to the *B1* locus had occurred.

To confirm these results, we analyzed Not I digests. The first recombinant had multiple copies of SBCAT1 integrated tandemly into the *B1* tandem repeat, as evidenced by the appearance of a plasmid-sized Not I fragment and two larger fragments (~35 and 45 kb) and the disappearance of the original fragment (~75 kb) when the blot was probed with *B1*. The second recombinant had multiple copies of SBCAT1, which had integrated at separate sites within the *B1* repeat locus as reflected by the presence of multiple *B1*-hybridizing fragments smaller than the *B1* Not I fragment present in wild-type DNA. As expected, the Not I digests probed with *CAT* had one less hybridizing band in each of the recombinants (35 kb in clone 1 and 40 kb in clone 2), which would correspond to the segment of the *B1* tandem repeat downstream of *CAT* and the Not I site (Fig. 1A). In all clones analyzed, SBCAT1 had integrated within the *B1* locus, presumably by homologous recombination. Although our linearized construct was designed to facilitate gene replacement, we were unable to ascertain whether this had occurred, owing to the many endogenous copies of *B1*.



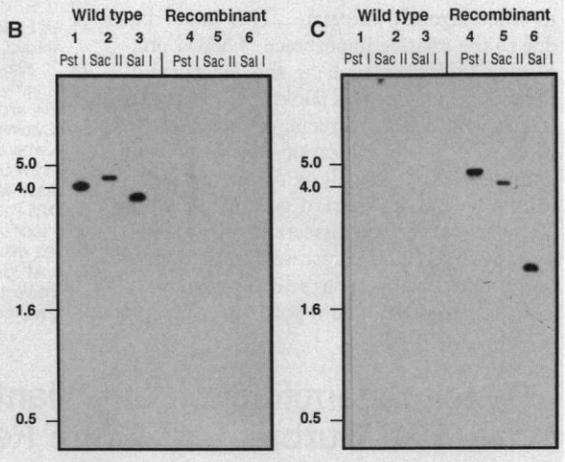


Fig. 4. Knockout of *ROP1* with ROP1/3 CAT. (**A**) ROP1/3 CAT construct, knockout and wild-type *ROP1* genomic loci. ROP1/3 CAT was cloned by insertion of a 950-bp Pst I fragment from the downstream region of *ROP1* into Bam HI–cut phosphatased ROP1/2 CAT (*2*). Insert and plasmid were treated with T4 DNA polymerase before ligation, thus destroying the Bam HI cloning site on the vector and the Pst I sites on the insert. The ROP1/3 CAT contains ~1.3 kb of *ROP1* upstream sequence, including the promoter and 5′ untranslated region (from the genomic Sal I site to the second AUG), 600 bp of *CAT* coding region (embraced by Nsi I and Pac I sites, which contain the start and stop codons, respectively), 300 bp of *SAG1* 3′ region (identical to that in constructs in Fig. 1 and containing polyadenylation and presumptive termination signals), and the 950-bp Pst I fragment from the *ROP1* 3′ untranslated and downstream region. The thin line indicates Bluescript II SK+ vector sequence. Vertical lines indicate *ROP1* regions in the construct; clear boxes indicate *ROP1* genomic sequences not present in the plasmid. *ROP1* coding region, *CAT* coding region, and *SAG1* sequences are indicated. ROP1/3 CAT was linearized for transfection at the Kpn I site in the polylinker. (**B** and **C**) Southern blot analysis. Genomic DNA from wild-type RH parasites (lanes 1 to 3) and the *ROP1* knockout recombinant (lanes 4 to 6) were digested with Pst I (lanes 1 and 4), Sac II (lanes 2 and 5), and Sal I (lanes 3 and 6) in duplicate. After transfer, membranes were probed in parallel with a 1.2-kb fragment encompassing the exact *ROP1* coding region (B) or a 600-bp *CAT* fragment (C) (both labeled by random priming with [^{32}P]dCTP). The blots were washed at a final stringency of 0.2× SSC–0.1% SDS at 65°C.

We wished to determine whether targeting to two single-copy loci, *SAG1* and *ROP1*, occurred as efficiently as to the *B1* locus. Transfection with the SAG1/2 CAT plasmid did not result in either *SAG1* knockout (as determined by protein immunoblot of individual clones and cytofluorometric analysis of the stably transformed population) or homologous recombination without replacement (as determined by Southern analysis of randomly isolated clones) (*12*).

However, knockout of *ROP1* was successful (Fig. 4). A plasmid, ROP1/3 CAT (Fig. 4A), was constructed that has 5′ and 3′ regions from *ROP1* flanking *CAT*. After transfection and selection for chloramphenicol-resistant clones (*14*), recombinants were screened by protein immunoblot for the presence or absence of ROP1 protein. Two ROP1$^-$ recombinants were thus identified (*15*). Southern blot analysis of a knockout recombinant (the other was not characterized) indicated that the coding region of *ROP1* was no longer present (Fig. 4B): a single copy of ROP1/3 CAT had integrated into the *ROP1* locus by homologous recombination (Fig. 4C). Homolo-

gous replacement is most clearly seen in the Pst I digest where the wild-type 4.2-kb fragment (Fig. 4B, lane 1) was replaced with a 4.7-kb fragment in the recombinant (Fig. 4C, lane 4) reflecting the loss of the second genomic Pst I site and the difference in size between the *CAT* plus *SAG1* sequences and the *ROP1* coding sequence they replace.

Most of the transformants analyzed after CAT selection were not *ROP1* knockouts, and it is unclear whether this was due to a phenotypic disadvantage for parasites lacking *ROP1* (and, thus, overgrowth of *ROP1*-positive clones) or whether it reflected the efficiency of homologous integration.

The success of the *ROP1* targeting contrasts with the failure of the *SAG1* construct to homologously integrate. This could be due to the relative importance of *SAG1* and *ROP1*, to factors such as the length and nature of the sequences flanking *CAT* in the relevant plasmid constructs, or both.

Stable transformation of *Toxoplasma*, in conjunction with analysis of mutants and utilization of transmission genetics, should allow the molecular dissection of the biology of intracellular parasitism. In addition, the use of *CAT* as a selectable marker rather than as a simple reporter may be applicable to the study of other systems, especially the related coccidian parasites *Eimeria* and *Plasmodium*.

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14. Transfection was done with 20 μg of ROP1/3 CAT plasmid linearized with Kpn I. Selection was begun 16 hours after transfection with 20 μM chloramphenicol. Parasites were cloned by limiting dilution in drug 10 days after transfection.
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Genetic Identification of a Hantavirus Associated with an Outbreak of Acute Respiratory Illness

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A mysterious respiratory illness with high mortality was recently reported in the southwestern United States. Serologic studies implicated the hantaviruses, rodent-borne RNA viruses usually associated elsewhere in the world with hemorrhagic fever with renal syndrome. A genetic detection assay amplified hantavirus-specific DNA fragments from RNA extracted from the tissues of patients and deer mice (*Peromyscus maniculatus*) caught at or near patient residences. Nucleotide sequence analysis revealed the associated virus to be a new hantavirus and provided a direct genetic link between infection in patients and rodents.

An outbreak of a respiratory illness with high mortality was recently reported in the shared border region (Four Corners) of New Mexico, Arizona, and Colorado in the southwestern United States (*1*). Patients were defined as having unexplained adult respiratory distress syndrome (ARDS) or acute bilateral pulmonary interstitial infiltrates in the presence or absence of prodromal symptoms (*2*). Mortality in confirmed patients has been in excess of 75%, frequently in previously healthy adults between 20 and 40 years of age. Serologic surveys of patients failed to detect evidence of agents normally associated with severe respiratory illness but did detect immune cross-reactivity with previously characterized hantavirus antigens (*1*). This finding was unexpected because hantaviruses had not previously been associated with outbreaks of acute human disease in North America nor had hantaviruses found elsewhere in the world been associated with a severe, predominantly respiratory illness.

Hantaviruses are rodent-borne viruses belonging to the family Bunyaviridae. They possess a negative sense, single-stranded RNA genome consisting of three segments, designated large (L), medium (M), and small (S), which encode the virus polymerase protein (L), the glycoproteins G1 and G2, and the nucleocapsid (N) protein, respectively (*3–7*). At least four distinct virus serotypes have been clearly defined that differ in their overall geographic distribution, rodent host, and degree of pathogenicity for humans (*8*). The Hantaan (HTN) serotype viruses, associated with field mice (*Apodemus agrarius*) and found predominantly throughout Korea, China, and far eastern Russia, cause severe hemorrhagic fever with renal syndrome (HFRS). The Seoul (SEO) serotype viruses are probably found worldwide, which reflects the range of their primary host, *Rattus norvegicus*. The SEO viruses have been associated with a generally more moderate form of HFRS, particularly in Korea and China. Recently, three cases of mild HFRS disease associated with SEO-related virus were described in the United States, although the SEO virus was probably introduced into the United States by colonization of its Eurasian rat host (*9*). The Puumala (PUU) serotype viruses, found throughout Scandinavia and Europe west of the Ural Mountains, are associated with a relatively mild form of HFRS. The primary

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