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Construction of Recombinant Feline Herpesvirus Type 1 Expressing *Toxoplasma gondii* SRS1

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Major factors in transmission of *Toxoplasma gondii* into humans are oocysts in food or water and tissue cysts in meat (Ruiz and Frenkel 1980; Dubey et al. 1990; Buffolano et al. 1996; Baril et al. 1999). Previous studies have demonstrated a positive relation between environmental contamination with oocysts from cat feces and exposure of farm animals to the parasite (Frenkel et al. 1975; Frenkel and Ruiz 1981). Cats shed oocysts after the primary infection, whereas induced immunity prevents cats from oocyst production following subsequent infection (Dubey and Frenkel 1974; Lappin et al. 1989). Thus, vaccination of cats is a significant measure in public health. Live *T. gondii* such as mutant strains or irradiated tachyzoites have been vaccine candidates (Frenkel et al. 1991; Freyre et al. 1993; Omata et al. 1996). However, it is not realistic to immunize both domestic and uncontrolled cats one by one with these vaccines. Further, the live *Toxoplasma* cells carry a risk of accidental infection to humans. To overcome these problems, we constructed a novel candidate for a cat vaccine composed of an immunogenic antigen of *T. gondii* and a viral vector. SRS1 of *T. gondii* is a 46 kDa antigen divergently transcribed by the bi-directional SAG1 promoter (Hehl et al. 1997). Despite the precise role of the antigen has not been specified, vaccination with a recombinant SRS1 produced by *E. coli* modifies host immune responses after infection and partially protects mice from lethal

challenge of *T. gondii* (Mishima et al. in press). Feline herpesvirus type 1 (FHV1) is a world-spread pathogen causing viral rhinotracheitis in cats. Previous studies has suggested that FHV1 can be a useful vector in veterinary field (Yokoyama et al. 1997 reviewed). We used FHV1 as a delivery vector of SRS1 because of two reasons; 1) Host range of FHV1 is strictly restricted to Felidae, and 2) A recombinant FHV1 can automatically spread among cats without inoculating one by one.

The parent FHV1 C7301 strain (Mochizuki et al. 1977) and the modified virus were grown on Crandel feline kidney (CRFK) cells (Crandel et al. 1973) maintained in Dulbecco's modified Eagle's minimum essential medium (Sigma, MO) supplemented with 8% heat inactivated fetal bovine serum and antibiotics. Stock viruses were harvested from culture supernatant of infected CRFK cells. The entire open reading frame (ORF) of SRS1 was amplified from genomic DNA of the RH strain with the polymerase chain reaction (PCR) technique using a primer pair of SRS1-1 (acgaattccGCAAATGGTGAGGA) and SRS1-2 (acgaattcACCTTAGACGG CAC). The amplified SRS1 fragment was inserted between the CAG promoter and a polyadenylic acid (poly-A) in the pCAGGS expression vector (Niwa et al. 1991) kindly provided by Dr. Miyazaki from Osaka University Medical School. The resulting CAG-SRS1-poly-A expression unit was inserted into FHV1 genome as previously described (Yokoyama et al. 1996b).

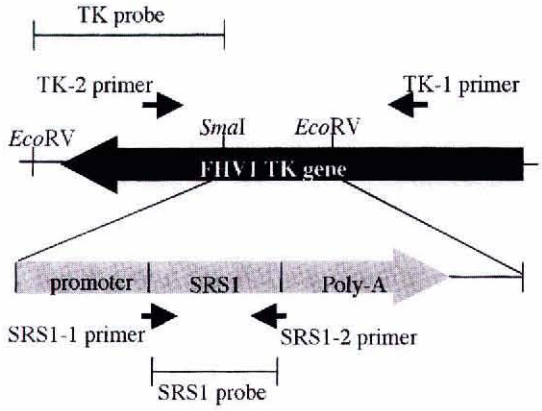

Figure 1. Integration of CAG promoter, SRS1 ORF and polyadenylation signal into the FHV genome. The *Sma*I-*Eco*RV fragment was exchanged. PCR primers and Southern blotting probes are also indicated.

The recombinant virus genome was examined for localization of the inserted expression unit with Southern blotting. FHV/SRS1 genomic DNA was extracted from culture supernatant of infected CRFK cells as previously described (Ishizawa 1991). The DNA was digested with *Eco*RV, electrophoresed and transferred to Hybond N+ membrane (Amersham, England). The membrane was probed with TK and SRS1

probes shown in Fig. 1 by DIG (Roch Diagnostics, Switzerland) system according to the manufacture's specification. TK probe revealed a 1.3 kbp fragment of wild type FHV1 and a 4.5 kbp fragment of FHV/SRS1. The recombination of the virus resulted in an approximately 3 kbp extension of the TK fragment (Fig. 2A). The extension was identical to the expected length for subtraction of TK deletion from the SRS1 insertion after the recombination procedures. Electrophoretic migrations of genomic DNA fragments of SRS1 and TK were also compared in Southern hybridization. The SRS1 probe reacted to a 4.5 kbp fragment of the recombinant genome whereas it revealed no specific reaction to the wild type genome (Fig. 2B). The migration of the SRS1 and TK fragment of the recombinant genome was identical (Fig.2).

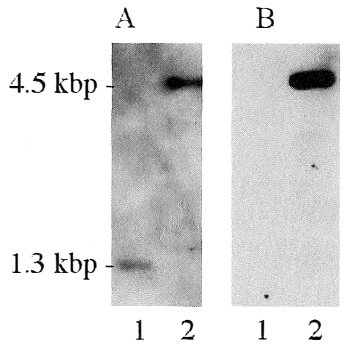

Figure 2. Southern blot analysis on the FHV/SRS1 DNA. Purified DNA from FHV wild type (lane 1) and the recombinant (lane 2) were digested with *Eco*RV and probed for TK (A) and SAG2 (B). The recombinant carried the both sequences in one fragment.

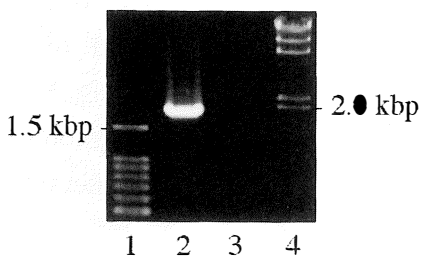

Figure 3. PCR analysis for direction of the inserted SRS1 unit in FHV/SRS1 genome. A pair of TK-1 and SRS1-1 primer increased a corresponding fragment (lane 2) whereas a pair of TK-1 and SRS1-2 did not work (lane 3). Lanes 1 and 4 contained 100 bp ladder marker and l/*hind*III marker, respectively.

The direction of the inserted SRS1 unit was evaluated with PCR assay using two primer pairs of TK-1 and SRS1-1, and TK-1 and SRS1-2. Only a combination of TK-1 and SRS1-1 amplified a DNA fragment (Fig. 3). The amplified fragments has the similar size to the calculated length for a total of the SRS1, poly-A tail and TK sequence between TK-1 primer and *Eco*RV site shown in Fig. 1. These observations evidenced that the SRS1 unit was divergently integrated into the TK region of FHV1 genome as represented in Fig. 1. Infection of FHV1 is often lethal for newborn or

debilitated cats (Povey 1979 reviewed). However, TK deficient FHV1 shows remarkably reduced pathogenicity in cats (Yokoyama et al. 1995 and 1996a). The insertion of the SRS1 expression unit in the present study resulted in a large deletion of TK region. These suggested that FHV/SRS1 is reduced its pathogenicity by this recombination, which is advantageous as a vaccine particularly for kittens.

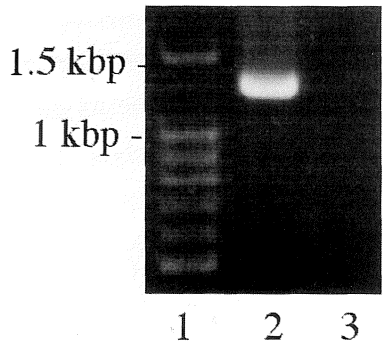

Figure 4. Transcription of SRS1 ORF in FHV/SRS1 infected CRFK cells. Total RNA from the infected cells was examined with RT-PCR (lane 2) and PCR (lane 3). A marker was also indicated in lane 1 (100 bp ladder). RT-PCR amplified a DNA fragment corresponding to the entire ORF of SAG2.

To assess a transcription of SRS1 by the recombinant virus, total RNA from CRFK cells infected with FHV/SRS1 was examined in reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from FHV/SRS1-infected CRFK cells and was treated with DNase. The RNA was tested with RT-PCR using a poly-T reverse-transcription primer and SRS1-1 and SRS1-2 PCR primers shown in Fig. 1. A pair of SRS1-1 and SRS1-2 amplified the entire ORF of SRS1 from the RNA, whereas PCR without reverse transcription did not work (Fig. 4). This result demonstrated that the CAG prompter successfully drove the transcription of the SRS1 gene in the infected cells. The CAG promoter is engineered by connecting the cytomegalovirus immediate early (CMV-IE) enhancer sequence to the modified chicken beta-actin (AG) promoter and both synergisticly enhance transcription of the following sequence in various mammalian cells (Niwa et al. 1991). The AG promoter has a strong ubiquitous activity (Miyazaki et al. 1989). The CMV-IE enhancer/promoter inserted into a recombinant herpes simplex virus type 1 increases transcription of a following sequence by five-fold when CMV is inoculated, and the CMV phosphoprotein pp71 exhibits a stimulation of gene expression (Homer et al. 1999). Although activity of CMV-IE enhancer in FHV/SRS1-infected cells is not evaluated, co-transfection or co-expression of pp77 might enhance the production of the recombinant SRS1 in FHV/SRS1-infected cells.

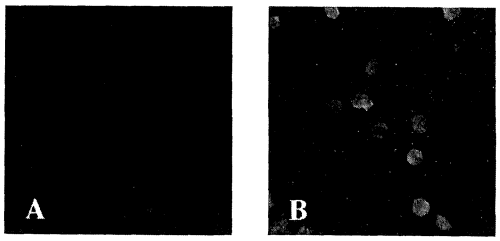

Figure 5. Expression of SRS1 in FHV/SRS1-infected CRFK cells. FHV wild type (A) and FHV/SRS1 (B) infected CRFK cells were examined by IFA using 1:200 diluted mouse serum corrected from mouse chronically infected with *T. gondii*. The assay elicited positive response to FHV/SRS1 infected cells.

Production of recombinant SRS1 in infected cells was evaluated with IFA. CRFK cells infected with FHV/SRS1 were washed and suspended in an appropriate volume of phosphate-buffered saline (PBS). The cell suspension was dropped on glass slides, air-dried and fixed in cold acetone. The slides were incubated for one hour at 37 °C with 1:100 diluted serum collected from a mouse chronically infected with *T. gondii* Beverley strain after absorption with wild FHV1-infected CRFK cells. Then, the slides were washed with PBS, incubated with fluorescein isothiocyanate-conjugated anti-mouse IgM+G+A antibodies (Southern Biotechnology, AL), washed again and observed under fluorescence laser microscopy. An anti-serum from *T. gondii*-infected mouse recognized the FHV/SRS1-infected cells whereas it did not react to wild FHV1-infected cells (Fig. 5). The recombinant FHV/SRS1 product was recognized by the mouse serum induced by *T. gondii* infection. This result suggests that the recombinant SRS1 maintains some immunogenic epitopes of the native antigen. Therefore, immunization with FHV/SRS1 might induce immunity against native SRS1 antigen.

The present study demonstrated that FHV/SRS1 can deliver the SRS1 expression system into cat cells and can express recombinant SRS1 in the infected cells. FHV1 infection is characterized by the feature of latency after primary infection and periodical reactivation. The latency promotes the establishment of endemicity of the virus and the reactivation helps virus spread and re-stimulation of the host immunity. By this viral strategy, a FHV1 base recombinant vaccine might immunize not only domestic cats but also stray or wild cats without inoculating one by one. Therefore, a recombinant FHV1 expressing a *Toxoplasma* immunogenic antigen may be a useful vaccine for cats. Further work is now needed to examine how FHV/SRS1 vaccine influences on protective immunity of cats against *T. gondii* infection.

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