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A Novel Parvovirus Isolated from Manchurian Chipmunks

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A novel parvovirus was identified in Manchurian chipmunks inhabiting Korea. Hepatitis B surface antigen (HBsAg) was detected in sera from 4 animals among 62 apparently healthy chipmunks. Electron microscopic examination of the HBsAg-positive sera revealed virus-like spherical particles 20–22 nm in diameter. Extraction of nucleic acid under annealing conditions from the serum samples containing virus-like particles yielded a single species of DNA molecule with the electrophoretic mobility of 5.6-kb double-stranded DNA. Four overlapping clones that encompassed almost the full-length viral genome, except both ends, were obtained. By sequencing these clones, we determined the sequence of 5097 nucleotides of the viral DNA. Two open reading frames were identified, with the left side open reading frame encoding a putative nonstructural protein and the right side open reading frame encoding a putative capsid protein. The nucleotide and amino acid sequences showed significant homology to parvovirus B19 and simian parvovirus, but showed little homology to other mammalian autonomous parvoviruses or adeno-associated viruses. These observations indicate that the virus isolated from Manchurian chipmunks is a novel parvovirus and may be a potentially useful animal model of human B19 infection as a new member of the Erythrovirus genus of the *Parvoviridae*. © 1999 Academic Press

INTRODUCTION

The *Parvoviridae* family comprises a group of single-stranded DNA viruses that are the smallest known animal viruses with a diameter of 18 to 26 nm. Parvovirus B19, the only known human pathogenic parvovirus, was discovered in 1974 during screening of healthy blood donors for hepatitis B (Cossart *et al.,* 1975). In evaluating various tests for hepatitis B surface antigen (HBsAg), a group of anomalous specimens were found, which show positive results in counterimmunoelectrophoresis employing human antisera but negative results in the more sensitive hemagglutination and radioimmunoassay techniques using animal antisera that have been raised by inoculation of purified HBsAg. The new antigen was found to be distinct from HBsAg and consist of parvovirus-like particles. Analysis of the viral particles showed that they contained complementary single strands of DNA of approximately 5.5 kb length with self-priming hairpin termini (Summers *et al.,* 1983; Cotmore and Tattersall, 1984) and that the particles had a buoyant density in cesium chloride of 1.43 g/ml (Clewley, 1984), all characteristics allowing classification of the virus as a member of the *Parvoviridae* family. Although no disease was associated with the new virus at first, human parvovirus B19 is currently known as the etiological agent of erythema infectiosum, arthropathy, aplastic crisis in patients with red cell defects, chronic anemia in immunocompromised patients, and fetal hydrops (Brown *et al.,* 1994; Kerr, 1996).

In an attempt to find a new host of hepadnavirus, we have tested sera from several species of animals for HBsAg reactivity by radioimmunoassay. Four Manchurian chipmunks containing HBsAg and virus-like particles in their sera were identified, suggesting infection of a hepadnavirus in the animals. However, analysis of genetic material from the viral particles showed characteristics allowing classification of the virus as a member of the *Parvoviridae* family. We report here the isolation of a novel parvovirus from chipmunks that shares a significant homology with parvovirus B19 at the DNA and amino acid levels.

RESULTS

Detection of HBsAg and virus-like particles

Of 62 tested animals, HBsAg was detected in the sera of 4 animals. No HBsAg-positive serum produced a more than 10-fold amount of binding to ^{125}I-labeled antibody to HBsAg than that of HBsAg-negative control serum (data not shown). Electron microscopic examination of all the

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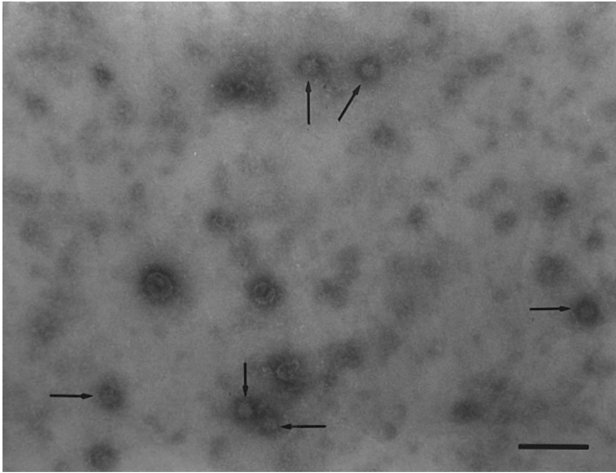

FIG. 1. Electron micrograph of spherical virus-like particles pelleted from HBsAg-positive chipmunk sera (arrows). Bar represents 100 nm.

HBsAg-positive sera revealed numerous spherical virus-like particles with diameters ranging from 20 to 22 nm (Fig. 1). No such particles were found in HBsAg-negative sera.

Isolation and characterization of the viral genome

DNA extracted under annealing conditions from the HBsAg-positive sera containing virus-like particles was analyzed by electrophoresis in neutral agarose gel and alkaline agarose gel. The extracted viral DNA showed the electrophoretic mobility of 5.6-kb double-stranded DNA (Fig. 2A, lane 2) and was resistant to S1 nuclease digestion (Fig. 2A, lane 3). Digestion with restriction endonuclease *Bam*HI cut the molecule apparently at a single site to generate 3.2- and 2.4-kb fragments (data not shown). By analogy with other parvoviruses, these results suggested that the virus produced equimolar amounts of complementary single strands that self-annealed, although we did not prove the presence of single-stranded DNA molecules in the virus particles by extraction under nonannealing conditions.

As the DNA molecules may have complex terminal secondary structures, the strand length of the DNA molecules was analyzed under completely denaturing conditions. The alkali-denatured viral DNA subjected to alkaline agarose gel electrophoresis revealed single-stranded DNA molecules with a size of 5.9 knt (Fig. 2B, lane 2), longer than the size of the DNA molecules before denaturation, strongly suggesting that the DNA molecules have terminal hairpin structures. These findings suggest that the viral genome is a 5.9-knt single-stranded DNA with terminal hairpin structures, consistent with characteristics of *Parvoviridae.*

Cloning and sequencing of viral DNA

As we failed repeatedly to clone restriction fragments of the viral genome, viral DNA was treated with Klenow fragment and then digested with S1 nuclease to produce blunt ends for ligation and then cloned into a TA cloning vector. Four overlapping clones, C2 (nt 1–2371), C27 (nt 793–3309), C7 (nt 3281–3524), and Y2 (nt 3489–5097), that encompassed almost the full-length viral genome were obtained (Fig. 3B). By sequencing these clones, we determined the sequence of 5097 nucleotides of the viral DNA (sequence submitted to GenBank, as an update to Accession No. U86868). Comparison of the full sequence of the chipmunk virus (Fig. 4) with nucleic acid sequences in GenBank confirmed homology with other members of the *Parvoviridae* family. Highest homology was with parvovirus B19 (52.4% identity in 2023 nt of

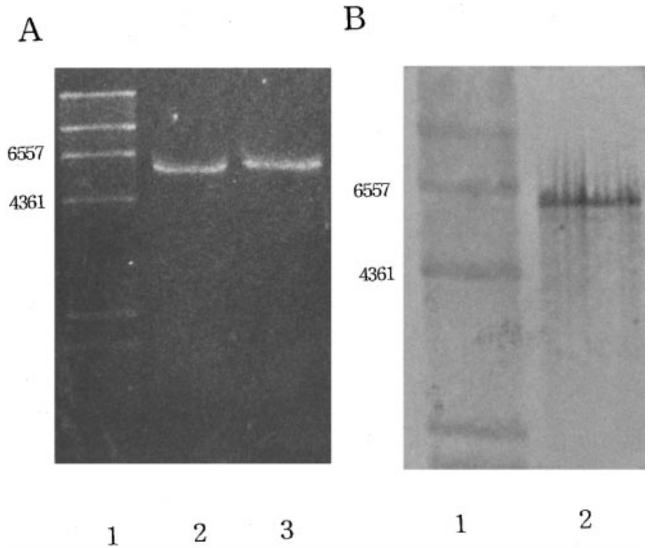

FIG. 2. Analysis of viral DNA extracted under annealing conditions from chipmunk sera containing HBsAg and virus-like particles. (A) Analysis of viral DNA by 1% neutral agarose gel electrophoresis stained with ethidium bromide. (Lane 1) *Hin*dIII digest of phage λ DNA; (lane 2) untreated viral DNA; (lane 3) viral DNA after S1 nuclease digestion. (B) Analysis of alkali-denatured viral DNA by 1.2% alkaline agarose gel electrophoresis and Southern blotting using digoxigenin-labeled viral DNA probe. (Lane 1) Alkali-denatured *Hin*dIII digest of phage λ DNA; (lane 2) alkali-denatured viral DNA.

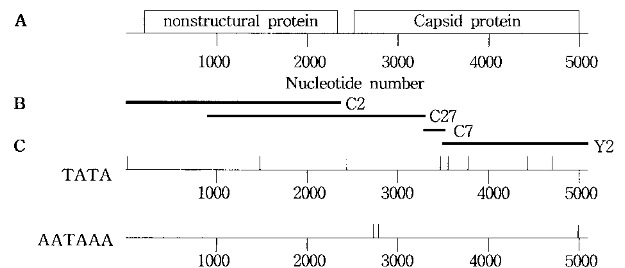

FIG. 3. Genomic organization of chipmunk parvovirus. (A) Open reading frames and (B) clones of the chipmunk parvovirus genome. Four overlapping clones, C2, C27, C7, and Y2, were obtained by direct cloning of chipmunk parvovirus DNA after Klenow fragment treatment followed by S1 nuclease digestion. (C) Possible promoter (TATA) sequences and sites of polyadenylation sequences (AATAAA).

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   1 CCTGGTTGGCTGATAATGGTATATTAGCCCGCGCACTTCCGTGTTCAGATCATTATGCACCGGTGCTTTCGTCTGAGCGGACCTGACGAGGACCCGTCTG

                                                                                                   Non
 101 CTGACTACGAATTCTCCGTCTTGGTGGAAGACCTCCACAGCCAGTTTTGTTCCTGTGTCTCTCCGGGCCTTCATCTACGACCTGTTTTTCTGGTACCATG
                                                                                                     M
     Structural Protein
 201 GCTCAAGCTTGTCTTTCTCTGTCTTGGGCAGATTGCTTTGCCGCTGTCATTAAGTTGCCATGTCCCCTCGAAGAGGTGCTGAGCAACAGCCAGTTTTGGC
     A  Q  A  C  L  S  L  S  W  A  D  C  F  A  A  V  I  K  L  P  C  P  L  E  E  V  L  S  N  S  Q  F  W  Q

 301 AATACTATGTTCTCTGTAAAGATCCGCTTGACTGGCCGGCCTTACAGGTCACTGAGCTGGCTCATGGTTGGGAGGTGGGTGCGTACTGTGCGTTTGCTGA
       Y  Y  V  L  C  K  D  P  L  D  W  P  A  L  Q  V  T  E  L  A  H  G  W  E  V  G  A  Y  C  A  F  A  D

 401 TGCTTTGTATTTGTACCTGGTGGGCAGACTAGCAGACGAGTTTAGTGCGTACTTGCTGTTCTTTCAACTAGAACCAGGTGTGGAAAATCCCCATATTCAT
      A  L  Y  L  Y  L  V  G  R  L  A  D  E  F  S  A  Y  L  L  F  F  Q  L  E  P  G  V  E  N  P  H  I  H

 501 GTTGTGGCACAGGCCACCCAGTTGTCGGCATTTAACTGGCGTCGCATTTTAACTCAGGCATGTCATGACATGGCTCTGGGGTTTTTGAAACCTGACTACT
     V  V  A  Q  A  T  Q  L  S  A  F  N  W  R  R  I  L  T  Q  A  C  H  D  M  A  L  G  F  L  K  P  D  Y  L

 601 TGGGCTGGGCTAAAAATTGTGTGAATATTAAAAAAGACAAGTCTGGACGAATTTTACGGTCAGACTGGCAATTTGTAGAAACTTACCTATTGCCTAAAGT
       G  W  A  K  N  C  V  N  I  K  K  D  K  S  G  R  I  L  R  S  D  W  Q  F  V  E  T  Y  L  L  P  K  V

 701 TCCCCTGAGTAAGGTCTGGTATGCCTGGACTAACAAGCCCGAATTTGAGCCCATAGCTCTCAGTGCCGCTGCGCGGGACAGGCTGATGAGAGGCAACGCA
      P  L  S  K  V  W  Y  A  W  T  N  K  P  E  F  E  P  I  A  L  S  A  A  A  R  D  R  L  M  R  G  N  A

 801 CTTTGTAATCAGCCGGGACCGGGGCCGTCTTTTGGAGACCGGGCAGAAATTCAGGGACCTCCCATTAAAAAGACTAAGGCATCAGATGAGTTTTACACTC
     L  C  N  Q  P  G  P  G  P  S  F  G  D  R  A  E  I  Q  G  P  P  I  K  K  T  K  A  S  D  E  F  Y  T  L

 901 TCTGTCACTGGTTAGCTCAAGAGGGAATATTAACAGAGCCTGCCTGGAGACAGAGAGATTTAGATGGCTATGTGCGTATGCACACCTCTACTCAGGGGAG
       C  H  W  L  A  Q  E  G  I  L  T  E  P  A  W  R  Q  R  D  L  D  G  Y  V  R  M  H  T  S  T  Q  G  R

1001 GCAGCAGGTGGTGTCTGCTCTTGCCATGGCCAAAAACATCATATTGGATAGCATTCCAAACTCTGTGTTTGCCACAAAGGCAGAAGTGGTCACAGAACTC
      Q  Q  V  V  S  A  L  A  M  A  K  N  I  I  L  D  S  I  P  N  S  V  F  A  T  K  A  E  V  V  T  E  L

1101 TGTTTTGAAAGTAACCGCTGTGTGAGGCTCTTGAGAACACAGGGCTATGACCCGGTACAATTTGGCTGTTGGGTGTTACGGTGGCTGGACCGTAAAACGG
     C  F  E  S  N  R  C  V  R  L  L  R  T  Q  G  Y  D  P  V  Q  F  G  C  W  V  L  R  W  L  D  R  K  T  G

1201 GCAAAAAAAATACTATTTGGTTTTATGGGGTCGCTACTACTGGGAAAACTAATCTAGCAAATGCGATTGCCCACTCACTTCCATGTTATGGCTGTGTAAA
       K  K  N  T  I  W  F  Y  G  V  A  T  T  G  K  T  N  L  A  N  A  I  A  H  S  L  P  C  Y  G  C  V  N

1301 CTGGACCAATGAAAACTTCCCCTTTAATGACGCCCCCGACAAATGTGTATTGTTTTGGGACGAGGGTAGAGTCACGGCCAAAATTGTGGAAAGTGTTAAA
       W  T  N  E  N  F  P  F  N  D  A  P  D  K  C  V  L  F  W  D  E  G  R  V  T  A  K  I  V  E  S  V  K

1401 GCTGTGTTGGGAGGCCAAGACATCAGAGTGGATCAGAAGTGTAAGGGGAGCTCTTTCTTAAGGGCTACCCCAGTCATTATAACAAGTAATGGGGACATGA
     A  V  L  G  G  Q  D  I  R  V  D  Q  K  C  K  G  S  S  F  L  R  A  T  P  V  I  I  T  S  N  G  D  M  T

1501 CCGTTGTGCGAGATGGAAATACCACAACCTTCGCCCATCGCCCTGCCTTTAAGGACCGCATGGTCCGCTTAAATTTTGATGTGAGGCTCCCAAATGACTT
       V  V  R  D  G  N  T  T  T  F  A  H  R  P  A  F  K  D  R  M  V  R  L  N  F  D  V  R  L  P  N  D  F

1601 TGGGCTTATCACCCCCACTGAGGTTCGCGAGTGGCTGAGATACTGCAAGGAACAAGGGGACGATTATGAGTTCCCAGACCAGATGTACCAGTTTCCACGA
      G  L  I  T  P  T  E  V  R  E  W  L  R  Y  C  K  E  Q  G  D  D  Y  E  F  P  D  Q  M  Y  Q  F  P  R

1701 GATGTTGTTTCTGTTCCTGCTCCTCCTGCCTTGCCTCAGCCAGGGCCAGTCACAAATGCCCCGGAAGAAGAGATCCTTGATCTCCTTACCCAAACAAACT
     D  V  V  S  V  P  A  P  P  A  L  P  Q  P  G  P  V  T  N  A  P  E  E  E  I  L  D  L  L  T  Q  T  N  F

1801 TCGTCACTCAACCTGGGCTCTCTATTGAGCCGGCCGTTGGACCTGAAGAAGAACCTGATGTCGCAGATCTTGGAGGGTCTCCAGCACCAGCAGTCAGCAG
       V  T  Q  P  G  L  S  I  E  P  A  V  G  P  E  E  E  P  D  V  A  D  L  G  G  S  P  A  P  A  V  S  S

1901 CACCACAGAGTCCAGTGCCGACGAGGACGAGGACGACGACACCTCCTCCTCTGGCGACCACAGAGGAGGAGGAGGAGGGGTCATGGGAGATTTACACGCT
      T  T  E  S  S  A  D  E  D  E  D  D  D  T  S  S  S  G  D  H  R  G  G  G  G  G  V  M  G  D  L  H  A

2001 TCTTCTTCCTCCTTCTTTACTTCCAGTGACTCAGGACTCCCCACTTCCGTCAACACCAGCGACACCCCTTTCTCCTTCAGCCCCGTACCAGTGCACCACC
     S  S  S  S  F  F  T  S  S  D  S  G  L  P  T  S  V  N  T  S  D  T  P  F  S  F  S  P  V  P  V  H  H  H

2101 ACGGACCCCCAACGCTTCTCCCGACCTCACGCCCGACACGCGATCTGGCCCGTGGGCGCCCGTCTTTCCGCCAGTACGAGCCATTGAAAGGCCGGTGTGC
       G  P  P  T  L  L  P  T  S  R  P  T  R  D  L  A  R  G  R  P  S  F  R  Q  Y  E  P  L  K  G  R  C  A

2201 GGACTCGACTACGTTTGGTCGTCCGTCTTGGGCCGCCCCGTGTGCAGTCTACAACACTGCGGAGCTGACTCGTCGTGGAGCAGGTGTCCGAGTTGTGAAG
       D  S  T  T  F  G  R  P  S  W  A  A  P  C  A  V  Y  N  T  A  E  L  T  R  R  G  A  G  V  R  V  V  K
          Non Structural Protein
2301 GGGTCAAGACCAGGTGCGATCTCTGGAAAGTGAGAGACGACCTCGAGGGGCCCAGCGGGGGAGATTTGTTTCGCTGCGCGTATCACACCTTGGAGGACTA
     G  S  R  P  G  A  I  S  G  K  <

2401 TCCATTTGAGGTGCCGACGTCTCCCAGGTGCGCTATACTCTGGAGACTCTCAGAGTTTATGGACGATCACCTTGATTGTGTTGGGACTTTGGACAATTGG
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FIG. 4. Nucleotide sequence of the cloned chipmunk parvovirus genome. Amino acid sequences of the putative nonstructural and capsid proteins are shown below the nucleotide sequence. (Sequence submitted to GenBank, as an update to U86868.)

overlap; B19 nt 2743–4696) and simian parvovirus (51.7% identity in 2034 nt of overlap; SPV nt 2855–4819).

Genomic organization

Analysis of the DNA sequence revealed a genomic organization similar to that of other members of the *Parvoviridae,* with one large open reading frame (nt 198–2331) in the left half of the genome and a second open reading frame (nt 2511–4998) in the right (Fig. 3A). Eight TATA boxes (nt 20, 1478, 2434, 3468, 3552, 3771, 4431, and 4700) and three (AATAAA) polyadenylation signals (nt 2733, 2784, and 4999) were found in the genome (Fig. 3C). The first TATA box (nt 20), located at the analogous region to the P6 promoter of parvovirus B19 (Blundell *et al.,* 1987), appears to be a functional promoter. As with all the *Parvoviridae,* a polyadenylation site was found at the far right site (nt 4999) and polyadenylation sites at nt 2733 or 2784 may represent secondary midgenomic termination sites as in parvovirus B19 (Ozawa *et al.,* 1987).

Viral proteins

By analogy with other parvoviruses, the left open reading frame encoded a putative nonstructural protein of 711 amino acids. The putative protein showed

Capsid Protein

2501 GGGTAGTGGCATGCCCAAGAGAAAGGGCGCCGGGGAGGCTTTTCGTGTGTTGTTGGATGAGTTGTTTGGGGGGATTTTGAGTGTGGGGGGGGATGCCTTT
M P K R K G A G E A F R V L L D E L F G G I L S V G G D A F

2601 GATGACCCTGTATCAGAGCTTGCTGAACACTTAACTTTGAGTGGAATTGGGGATGCTGATACATTTAAAAAATGGCAAGAAAAAGATTTAAGACACATTG
D D P V S E L A E H L T L S G I G D A D T F K K W Q E K D L R H I A

2701 CTCAGCTAGTTGCAGAATTTGAGACGCAGTACAATAAAAAAGAGCTTGATACCCTGGTTGTGGATGAGGTTAAAAAGGTGGCCAATAAAGTGGTTCCCGG
Q L V A E F E T Q Y N K K E L D T L V V D E V K K V A N K V V P G

2801 ATTGGGCGAGACAGGCGCTGCCGTAGCTAACACAGCCAAGCGGTTAAAAACTGACGAGGATCCCTTATCCTTTGGGGCCCCCCCACTAACAGAAAACGCC
L G E T G A A V A N T A K R L K T D E D P L S F G A P P L T E N A

2901 CCGGTTCCCGTTGCGGAGCCAGATGTGGCAATTGTTTCTGAACCAAACAGAGACACTGCTGCAGAACAACTTGAACGAGGCCTGGCAGAGCCTGATCACG
P V P V A E P D V A I V S E P N R D T A A E Q L E R G L A E P D H G

3001 GGGGCATCCACCTACCCGCTGACCGGTATCTTGGCCCTGGCAATCCTCTGGAAAACGGCCCCCCTGTTGACCCGGTTGACGCAGTGGCTCGTATTCACGA
G I H L P A D R Y L G P G N P L E N G P P V D P V D A V A R I H D

3101 CTTTAGGTACGCTGACCTAGAAAAACAGGGCATCAATCCCTACACAACCTACACAATAGCAGATGAGGAGCTCCTTAAAAATCTTGAACATAAAACGGGA
F R Y A D L E K Q G I N P Y T T Y T I A D E E L L K N L E H K T G

3201 GGAAGGGCGGCGATAGCCAGGGCATTTTTTAACTTTAAAAAACTGACATTCCCCCATGCACATCTACAGGGCCCCCTGCCAGCGGTAAAAAGTTGGAAGA
G R A A I A R A F F N F K K L T F P H A H L Q G P L P A V K S W K T

3301 CAGAGCAGCTTGGCCTCGCTGGGATGCAACAAGCTAGCGCGGTGTCCGGTGCGGGGGGAGACCACACCCCTGCTGCGTTGTGGGCTCAGGGTGCTAAGTT
E Q L G L A G M Q Q A S A V S G A G G D H T P A A L W A Q G A K F

3401 TAGTGGGGACTCCGTCACGTGCTTTATGACTCGCAGGTGTTACTTACCGTTTGATGAAGACCCCACTTATAGGGCAATAGCACATAGCGAGAGTGATAGG
S G D S V T C F M T R R C Y L P F D E D P T Y R A I A H S E S D R

3501 AGCAATTTTACTAAAATTATGGTCAATACCGGCACACACACGGTGATGGGATATACCACTCCTTGGCATTATGTAGACTACAACAACATGGCTTTGTTCT
S N F T K I M V N T G T H T V M G Y T T P W H Y V D Y N N M A L F F

3601 TTTCCCCACAAGAATTTCAGTACCTCCTAGAAAACTATGAAGAGATAGCACCCAAGTCACTCACTACGGTCCTGTCTGACCTTGTAGTAAAAGATGTGTC
S P Q E F Q Y L L E N Y E E I A P K S L T T V L S D L V V K D V S

3701 CATCCAAGACCAAAAAACCCAGGTTACAGATAGTGGGACGGGGGGGGTGGCTATCTTTGCTGATGAGTCCTATACCTATCCCTATGTCCTTGGCAACGGT
I Q D Q K T Q V T D S G T G G V A I F A D E S Y T Y P Y V L G N G

3801 CAGCGGACGTTGCCGTCTGACATCCCGATCCAGGTGTACGAACTCCCTAAATATGCGTACCTCACCTGCGGGAAGCGAACAGACGTAGGGATGAAAGGGG
Q R T L P S D I P I Q V Y E L P K Y A Y L T C G K R T D V G M K G G

3901 GCTCTCTCCCTACACATGACAGTGACTTTTTCTTCCTAGAACATGCTATGTTTAAGATTTACAAGACCGGAGACTTTTTTGTCTCCCCCTACTCTTTTCC
S L P T H D S D F F F L E H A M F K I Y K T G D F F V S P Y S F P

4001 TTCCCTAAGACCCCGGAGCCTAATGGGTGCTAGCCAACATTTTTTCATGATGCAAAATCCCTTGTATGACTATGGAATGGACGTCTTGACTGAAATTGGA
S L R P R S L M G A S Q H F F M M Q N P L Y D Y G M D V L T E I G

4101 ACGCACGGGCAATGGTCTTCTCTAGACAAATGGGAGTACCATGGACGCCCGCAAAATTTCTTCCCCGGACCAAAAATTCCCTCTCATGTGGCTGCTGAGG
T H G Q W S S L D K W E Y H G R P Q N F F P G P K I P S H V A A E G

4201 GAGACAGGGGTGGCAAGGCAGAGCTACAAAAGGTAGCTACTGGAACTTCTGTTGGAGATGACTGGTATTCTAGATACACGTTTAGGCCAATGCCCAGTTG
D R G G K A E L Q K V A T G T S V G D D W Y S R Y T F R P M P S C

4301 CCAGGCATACTCCCACGCAGACCCTAAAGATCCAGATTCTGATATTCCTGTTGTGTCCATTGACGCAGTGGCTGCCGGACAACAATCTGAAAAGCCAAAG
Q A Y S H A D P K D P D S D I P V V S I D A V A A G Q Q S E K P K

4401 CCGCCACATGCAAAAGAAAGCAAGTTTCCTTATAAACAGGGAAGACTGCCAAATGATATTGAGATGGCTAAACAGCTTCAAGGGGTCAATGACAAAATGT
P P H A K E S K F P Y K Q G R L P N D I E M A K Q L Q G V N D K M Y

4501 ATCTAGTGCAGACCCTAGCGGGACAAAATACTACCCCCGCACAAATCATTCCACTCATGCCTGGCAGTGTGTGGAATGAACGGGCTCTTCACTACGAGTC
L V Q T L A G Q N T T P A Q I I P L M P G S V W N E R A L H Y E S

4601 TCAAATCTGGACAAAGATTCCTAACCTAGACAAAGGATTTATGACTGACCACCCTGCTCTTGGTGGGTGGGGGATGTCTACCCCTCCACCACAAATATTT
Q I W T K I P N L D K G F M T D H P A L G G W G M S T P P P Q I F

4701 ATAAAAATGATTCCCACACCAGCTCCCTCTGTAGAAGGGGGGGGTACCACAAGCACACTACACCAGTACGCCATATTTAACATGACTGTGAAACTAGAGT
I K M I P T P A P S V E G G G T T S T L H Q Y A I F N M T V K L E F

4801 TTACTCTCAAAAAGCGAGGACTGGCTGGGAGATGGAACCCACAGCCACCTGTAAATCCCCCGTCCGCCGTAGGACACCTGCCTTATGTGCTGTATGACAA
T L K K R G L A G R W N P Q P P V N P P S A V G H L P Y V L Y D N

Capsid Protein

4901 CGGGCAACTAACAGGGGTCTCATCTGATGTGCAATCTCAAAATGGCTATGAAAGATCTGACGAGCTTTGGACTGCAAAGTCACGTGTAAGACACCTTTAA
G Q L T G V S S D V Q S Q N G Y E R S D E L W T A K S R V R H L <

5001 TAAATGATCAATCCTCAAACTGTCTCTTTGTCTTCAATAGCTACAAATCATCAAAGGCAGCTGGTGACGCAATAGCGGCGCCGCCCAGTGGCAAGC

FIG. 4—*Continued*

homology to the nonstructural proteins of other parvoviruses, with highest homology (25.8% overall; 44.3% in a 348-residue overlap; B19 aa 128–474) to the nonstructural protein NS1 of human parvovirus B19 (Figs. 5 and 6, Table 1). As with B19 NS1 protein (Shade *et al.,* 1986), a highly conserved region (aa 326–470) was found in the middle of the open reading frame, which has significant homology with the nonstructural proteins of other parvoviruses, the T antigens of polyoma virus and SV40, and the E1 proteins of papillomavirus (Astell *et al.,* 1987). This region contained several putative protein motifs: a phosphorylation site (aa 336–339; KKNT), an amidation site (aa 334–337; TGKK), and an ATP/GTP binding site (aa 344–351; GVATTGKT). However, the leucine zipper pattern that was found in the nonstructural protein sequence of simian parvovirus (Brown *et al.,* 1995) was not noted.

The right open reading frame encoded a putative capsid protein of 829 amino acids. The putative capsid protein showed 35.9% homology to capsid protein VP1 of parvovirus B19 and 34.1% homology to capsid protein of simian parvovirus, but showed little homology (less than 20%) to the capsid proteins of other members of *Parvoviridae* (Figs. 7 and 8, Table 2).

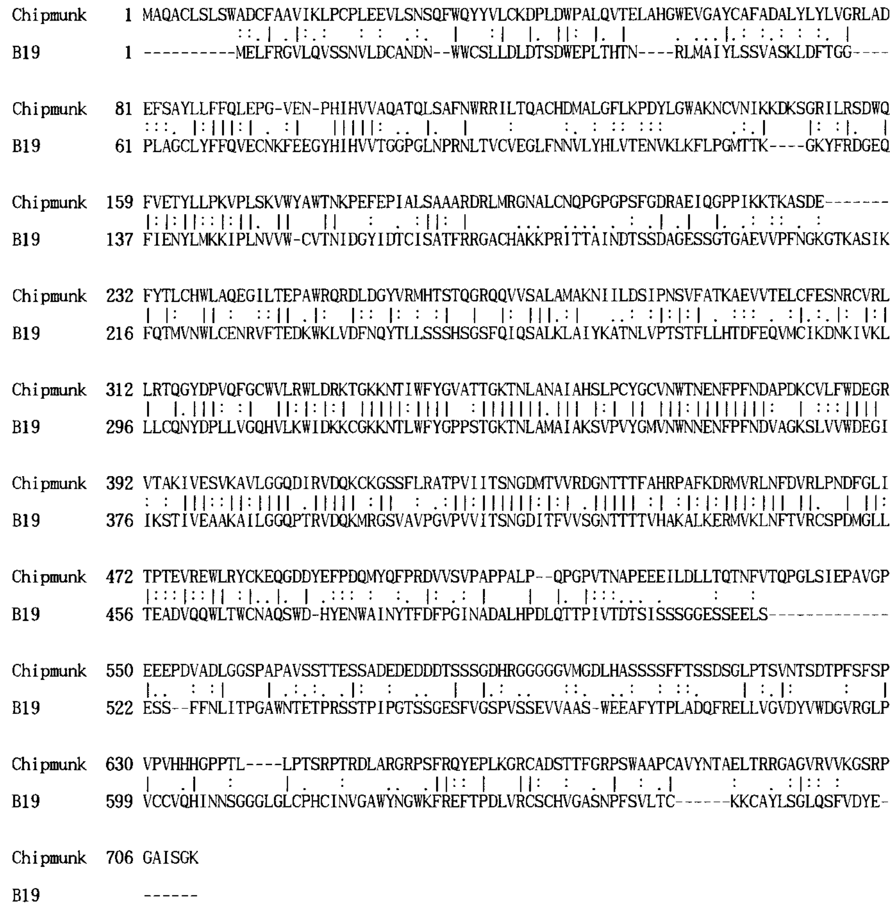

FIG. 5. Amino acid homology between the nonstructural proteins of chipmunk parvovirus and parvovirus B19. Vertical line denotes matching amino acids. A colon denotes amino acids that are positively related using the Standard Probability of Acceptable Mutation (PAM) matrix. A period denotes a neutral relationship, and a blank signifies negatively related amino acids. Dashes indicate gaps inserted in the sequences to increase homology.

DISCUSSION

The *Parvoviridae* family contains two subfamilies: the *Parvovirinae,* which infect vertebrates, and the *Densovirinae,* which infect insects. The *Parvovirinae* are divided into three genera. Most vertebrate disease-causing parvoviruses are autonomous parvoviruses of genus Parvovirus; they replicate in the absence of helper virus. Adeno-associated viruses comprise the second genus, Dependovirus, and require coinfection of target cells with adenovirus or herpesvirus for their replication. The third genus is the newly created Erythrovirus, of which parvovirus B19 is currently the sole member. Parvovirus B19 is autonomous, not requiring the presence of a helper virus, and until recently was classified in the genus Parvovirus. However, parvovirus B19 has some unique features at the molecular level that distinguish it from the other autonomous parvoviruses. The terminal 383 nucleotides at each end of the genome are identical inverted repeats like adeno-associated viruses and considerably longer than the terminal repeats of other autonomous parvoviruses (Deiss *et al.,* 1990). While autonomous paroviruses encapsidate primarily strands of one polarity, B19 encapsidates strands of both polarities with equal frequency (Summers *et al.,* 1983). In contrast to other autonomous paroviruses, B19 has a single P6 promoter (Blundell *et al.,* 1987), from which all transcription, including transcription of the capsid proteins, is initiated with subsequent complex splicing (Ozawa *et al.,* 1987). While other parvoviral transcripts coterminate at a polyadenylation site at the far right side of the genome,

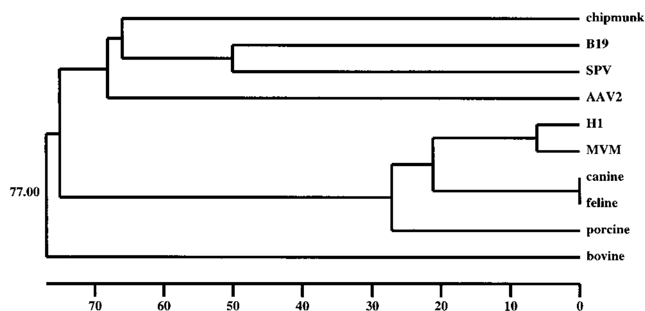

FIG. 6. Phylogenetic tree of the parvoviral nonstructural proteins. Data as used in Table 1. Numbers indicate percentage divergences.

TABLE 1

Percentage Similarities and Divergences of the Amino Acid Sequence of the Nonstructural Proteins of Different Members of *Parvoviridae*

	Similarity										
	1	2	3	4	5	6	7	8	9	10	
Divergence											
1		25.8	23.0	22.1	17.4	17.5	16.9	16.3	16.3	17.1	Chipmunk
2	65.4		41.3	20.9	17.9	18.2	18.2	17.1	17.2	17.2	B19
3	68.0	50.9		22.3	17.4	18.0	17.4	16.6	16.7	15.7	Simian
4	68.0	70.7	67.4		20.6	20.4	20.7	20.4	20.3	15.8	AAV2
5	75.0	76.1	75.7	71.7		91.5	68.2	72.6	72.7	13.9	H1
6	75.2	76.1	75.2	72.2	6.7		68.7	73.1	73.3	14.4	MVM
7	76.5	75.7	76.1	72.6	26.1	25.9		65.8	65.9	13.0	Porcine
8	77.2	77.6	78.0	72.0	21.7	21.5	28.3		99.3	13.7	Canine
9	77.2	77.4	77.8	72.2	21.3	21.1	27.8	0.6		13.6	Feline
10	75.2	75.9	78.0	75.0	78.3	76.7	79.1	77.8	77.8		Bovine

Note. Sequences from PIR database: B19 (671 aa), B24299; AAV2 (621 aa), A03694; H1 (672 aa), A03695; MVM (721 aa), A23008; porcine parvovirus (660 aa), A33302; canine parvovirus (668 aa), A29962; feline parvovirus (668 aa), A36608; bovine parvovirus (726 aa), C26104; simian parvovirus (687 aa), GenBank Accession No. U26342.

several transcripts of B19 terminate in the middle of the genome (Ozawa *et al.,* 1987). Also, in contrast to other mammalian parvoviruses, B19 is difficult to culture. It will not replicate in established cell lines but requires explant cultures of bone marrow (Ozawa *et al.,* 1986; Srivastava and Lu, 1988) or human fetal liver cells (Yaegashi *et al.,* 1989; Brown *et al.,* 1991) for *in vitro* studies. These differences from the other autonomous parvoviruses and its predilection for erythroid cells of bone marrow have led to the classification of B19 as the sole member of the recently created separate genus Erythrovirus (International Committee on Taxonomy of Viruses, 1995). Recently, a simian parvovirus was isolated in cynomolgus monkeys with severe anemia (O'Sullivan *et al.,* 1994), which exhibits a high degree of homology with parvovirus B19 (Brown *et al.,* 1995) and has a similar predilection for erythroid cells (O'Sullivan *et al.,* 1994, 1997). More recently, two more simian parvoviruses have been identified in pig-tailed macaques and rhesus monkeys with anemia, which showed limited sequence homology (70–80%) to both simian parvovirus and parvovirus B19 (Brown and Young, 1997).

We report here the isolation of a novel parvovirus in Manchurian chipmunks. Almost the entire genome, including the whole length of the coding region, has been cloned and sequenced. At the nucleotide and amino acid levels, the chipmunk parvovirus revealed significant homology to the human Erythrovirus, parvovirus B19, and simian parvovirus, but showed little homology to other mammalian autonomous parvoviruses. The high homology to parvovirus B19 and simian parvovirus suggests that the chipmunk parvovirus is closely related to these viruses evolutionarily. In addition, the virus appears to produce equimolar amounts of complementary single strands as with parvovirus B19 and simian parvovirus. It thus could be a potentially useful nonprimate animal system for modeling the molecular biology and pathogenesis of human parvovirus B19 infection. Further studies on the polarity of the genome, nucleotide sequence of both terminal structures, transcription map, and tissue tropism are required to determine whether chipmunk parvovirus can be classified as a new member of the genus Erythrovirus.

At present, we do not know the pathogenetic role of this new parvovirus. As we initially intended to identify a hepadnavirus, only liver tissues were extracted and examined. Histological exam of the liver tissues from chipmunks containing the parvovirus in their sera showed no evidence of liver disease (data not shown). We plan to identify more chipmunks naturally infected with the parvovirus and to examine the viral tissue tropism and pathogenesis in the natural hosts. In addition, experimentally infecting chipmunks with the virus should facilitate understanding of the viral pathogenesis.

Parvovirus B19 was originally discovered by showing positive results in a test for HBsAg, as human antisera employed for the test contained antibody to the new virus B19 (Cossart *et al.,* 1975). It is interesting but difficult to explain that chipmunk parvovirus was also identified by showing positive results in HBsAg radioimmunoassay. The putative proteins of chipmunk parvovirus showed no significant homology to HBsAg. In the AUSRIA II-125 "sandwich" radioimmunoassay used in this study, false-positive results are extremely rare, because tested specimens should bind to both guinea pig antibody to HBsAg (anti-HBs) and human ^{125}I-anti-HBs to show a positive result. Thus, it is quite possible that both human antisera and guinea pig antisera employed for the HBsAg radioimmunoassay contained antibody to the newly identified chipmunk parvovirus. Further seroepidemiological studies are required to determine the host range of the newly identified chipmunk parvovirus and

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Chipmunk   1  MPKRKGAGEAFRVLLDELFGGILSVGGDAFDDPVSELAEHLTLSGIGDADTFKKWQEKDLRHIAQLVAEFETQYNKKELD
B19        1  MSK---KSGKWWESDDKFAKAVYQQFVEFYEKVT---GTDLEL-----IQILKDHYNISLDN-----------------

Chipmunk  81  TLVVDEVKKVANKVVPGLGETGAAVANTAKRLKTDEDPLSFGAPPLTENAPVPVAEPDVAIVSEPNRDTAAEQLERGLAE
B19       52  --PLE------NPSSLFD--LVARIKNNLK---NSPDLYSHHFQSHGQLSDHPHALSSSSSHAEPRGENAVLSSED-LHK

Chipmunk 161  PDHGGIHLPADRYLGPGNPLENGPPVDPVDAVARIHDFRYADLEKQGINPYTTYTIADEELLKNLEHKTGGRAAIARAFF
B19      118  PGQVSVQLPGTNYVGPGNELQAGPPQSAVDSAARIHDFRYSQLAKLGINPYTHWTVADEELLKNIKNETGFQAQVVKDYF

Chipmunk 241  NFKKLTFPHAHLQGPLPAVKSWKTEQLG--LAGMQQASAVSGAG--GDHTPAALWAQGAKFSGDSVTCFMTRRCYLPFDE
B19      198  TLKGAAAPVAHFQGSLPEVPAYNASEKYPSMTSVNSAEASTGAGGGGSNSVKSMWSEGATFSANSVTCTFSRQFLIPYDP

Chipmunk 317  DPTYRAIAHS-ESDRSNFTKIMVNTGTHTVMGYTTPWHYVDYNNMALFFSPQEFQYLLENYEEIAPKSLTTVLSDLVVKD
B19      278  EHHYKVFSPAASSCHNASGKEAKVCTISPIMGYSTPWRYLDFNALNLFFSPLEFQHLIENYGSIAPDALTVTISEIAVKD

Chipmunk 396  VSIQDQK-TQVTDSGTGGVAIFADESYTYPYVLGNGQRTLPSDIPIQVYELPKYAYLTCGKRTDVGMKGGS--LPTHDSD
B19      358  VTDKTGGGVQVTDSTTGRLCMLVDHEYKYPYVLGQGQDTLAPELPIWVYFPPQYAYLTVGDVNTQGISGDSKKLASEESA

Chipmunk 473  FFFLEHAMFKIYKTGDFFVSPYSFPSLRPRSLMGASQHFFMMQNPLYDYGMDVLTEIGTHGQWSSLDKWEYHGRPQNFFP
B19      438  FYVLEHSSFQLLGTGGTASMSYKFPPVPPENLEGCSQHFYEMYNPLYGSRLGVPDTLGGDPKFRSLTHEDHAIQPQNFMP

Chipmunk 553  GPKIPSHVAAEGDRGGKAELQKVATGTSVGDDWYSRYTFRPMPSCQAYSHADPKDPDSDIPVVSIDAVAAGQQSEKPKPP
B19      518  GPLVNSVSTKEGD-SSNTGAGKALTGLSTGTSQNTRISLRPGPVSQPYHHWD-----TDKYVTGINAISHGQTTYGN---

Chipmunk 633  HAKESKFPYKQGRLPNDIEMAKQLQGVNDKMYLVQTLAGQNTTPAQIIPLMPGSVWNERALHYESQIWTKIPNLDKGFMT
B19      589  -AEDKEYQQGVGRFPNEKEQLKQLQGLNMHTYFPNKGT-QQYTDQIERPLMVGSVWNRRALHYESQLWSKIPNLDDSFKT

Chipmunk 713  DHPALGGWGMSTPPPQIFIKMIPTPAPSVEG--GGTTS-TLHQYAIFNMTVKLEFTLKKRGLAGRWNPQPPVNPPSAVGH
B19      667  QFAALGGWGLHQPPPQIFLKILPQSGP--IGGIKSMGITTLVQYAVGIMTVTMTFKLGPRKATGRWNPQPGVYPPHAAGH

Chipmunk 790  LPYVLYDNGQLTGVSSDVQ-SQNGYERSDELWTAKSRVRHL
B19      745  LPYVLYD----PTATDAKQHHRHGYEKPEELWTAKSRVHPL
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FIG. 7. Amino acid homology between the capsid proteins (VP1) of chipmunk parvovirus and parvovirus B19. (Output format as described in the legend to Fig. 5).

to determine whether there are viruses similar to this virus in other hosts.

MATERIALS AND METHODS

Animals

The animals used in this study were apparently healthy Manchurian chipmunks (*Tamias sibiricus asiaticus*) captured in the mountains in the Kyunggido and Kangwondo provinces of Korea. Sera were obtained by cardiac puncture and liver tissues were extracted from the animals.

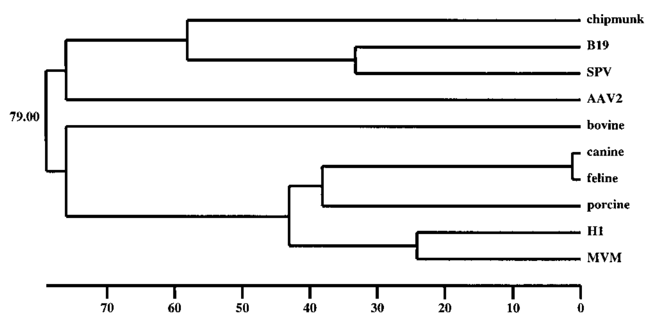

FIG. 8. Phylogenetic tree of the parvoviral capsid proteins (VP1). Data as used in Table 2. Numbers indicate percentage divergences.

Detection of HBsAg

Each serum sample was tested for HBsAg by radioimmunoassay using an AUSRIA II-125 diagnostic kit (Abbott Laboratories, North Chicago, IL).

Electron microscopy

Each serum sample was centrifuged at 10,000 rpm for 10 min; 200 μl of each supernatant was layered over a 11.8-ml gradient of 10–20% (W/VI) sucrose containing 10 mM Tris–HCl (pH 7.4), 0.1 M NaCl, and 5 mM EDTA. After centrifugation for 13 h at 35,000 rpm in a Spinco SW 40.1 rotor at 20°C, the pellet was resuspended in 100 μl of the same buffer, stained with 1% phosphotungstic acid, and examined with an electron microscope.

TABLE 2

Percentage Similarities and Divergences of the Amino Acid Sequence of the Capsid Proteins (VP1) of Different Members of *Parvoviridae*

	Similarity										
	1	2	3	4	5	6	7	8	9	10	
Divergence											
1		35.9	34.1	17.2	14.2	12.8	12.7	13.7	13.5	13.3	Chipmunk
2	56.6		59.9	18.3	15.8	16.0	16.6	16.6	16.7	15.3	B19
3	59.6	33.5		16.5	16.6	15.8	16.7	16.1	16.1	15.4	Simian
4	77.1	75.7	78.1		18.5	18.7	17.7	18.2	18.3	19.1	AAV2
5	81.3	79.2	78.1	75.5		70.2	49.8	51.4	51.9	20.1	H1
6	82.8	79.0	79.3	76.4	24.7		49.7	50.9	51.6	18.9	MVM
7	82.8	78.6	78.8	78.3	43.8	45.7		56.5	57.1	19.3	Porcine
8	82.5	79.0	79.7	78.6	42.0	43.3	38.5		97.3	19.6	Canine
9	82.7	78.8	79.7	78.6	42.2	43.3	38.9	1.0		19.9	Feline
10	81.8	79.5	79.2	76.2	76.2	76.9	77.1	76.5	76.4		Bovine

Note. Sequences from PIR database: B19 (781 aa), A24299; H1 (722 aa), A03699; MVM (718 aa), B23008; porcine parvovirus (723 aa), B33302; canine parvovirus (737 aa), A31163; feline parvovirus (727 aa), B36608; bovine parvovirus (673 aa), A26104; simian parvovirus (818 aa), GenBank U26342; AAV2 (706 aa), sequence as reported by Cassinotti *et al.* (1988).

Isolation and analysis of viral DNA

Virus was concentrated by incubation with human antibody to HBsAg and Protein A–Sepharose followed by pelleting, and DNA was extracted as previously described for cloning of Hepadnaviruses using microquantity serum (Panda *et al.,* 1992). Viral DNA extracted from 300 μl of serum was dissolved in 20 μl of Tris–EDTA buffer (pH 7.5). A portion (2 μl) of the extracted viral DNA was digested at 37°C for 20 min with 10 U S1 nuclease in 50 mM sodium acetate (pH 4.6), 1 mM zinc acetate, 250 mM NaCl, 2.5 μg BSA in a total volume of 10 μl. A sample of untreated viral native DNA and the viral DNA after S1 nuclease digestion were subjected to 1% neutral agarose gel electrophoresis and visualized by ethidium bromide staining (Fig. 2A). The viral native DNA was alkali-denatured and then analyzed by electrophoresis in 1.2% alkaline agarose gel and Southern blotting using digoxigenin-labeled viral DNA probe (Fig. 2B) (Sambrook *et al.,* 1989). The viral DNA probe was labeled by random-primed incorporation of digoxigenin-labeled dUTP (Boehringer Mannheim Biochemica) as instructed by the manufacturer.

Cloning and sequencing of viral DNA

Viral DNA extracted from 100 μl of serum was treated with 5 U of *Escherichia coli* DNA polymerase I Klenow fragment in the presence of 50 μM each dNTP mixture at 30°C for 30 min and then digested with S1 nuclease as previously described. The DNA was then treated with 2.5 U *Taq* DNA polymerase in 1.5 mM $MgCl_2$, 750 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.2 mM each dNTP mixture at 72°C for 30 min in order to add a single deoxyadenosine (A) to the 3′ ends. The product of this reaction was ligated into pCR II vector (Invitrogen, San Diego, CA), transformed into *E. coli* strain TOP 10F′ (Invitrogen), and cultured in the presence of ampicillin (50 μg/ml) with X-gal/IPTG. Ampicillin-resistant white colonies were isolated and their plasmids were screened by Southern hybridization using the digoxigenin-labeled viral DNA probe (Sambrook *et al.,* 1989).

The clones obtained were sequenced by the method of dideoxy chain termination with a *Taq* cycle sequencing kit (Takarashuzo Co.) as instructed by the manufacturer and the DNA sequences were read with an automated sequencer apparatus. Initially "universal" M13 forward and reverse primers were used, and then primers were designed from the previously obtained sequence. Inserts were fully sequenced in both directions.

Nucleotide and amino acid sequence analysis

The sequences were analyzed with Genome Center computer analysis programs at the Korea Research Institute of Bioscience and Biotechnology. DNA sequence homology was analyzed with the lfasta program. Amino acid multiple sequence alignments were determined using the ClustalW program and percentage similarities were calculated from the multiple alignments for all sequence pairs. Phylogenetic trees were drawn by the UPGMA clustering method with divergence values obtained from the ClustalW program. Protein motif analysis was performed with ScanProsite/ProfileScan search programs using the PROSITE data base. DNA motif analysis was performed with TFSITE and TRANSFAC server programs using the TRANSFAC data base.

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