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# DNA sequence comparison between two tissue-specific variants of the autonomous parvovirus, minute virus of mice

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ABSTRACT

We have determined the complete nucleotide sequence of the DNA of the immunosuppressive variant of the parvovirus minute virus of mice (MVMi) and compared it to the published sequence (12) of the fibroblast-specific strain (MVMp). We have found 175 differences between the two viruses, most of which affect single nucleotides. Despite these differences, the genomic organization of MVMp and MVMi is identical. There are 29 amino-acid changes between the putative viral gene products of MVMi and MVMp, 16 of which are conservative. We discuss the possibility that the differential tissue-specificity of the two variants is linked to differences within the non-transcribed region near the 5' end of the viral genomes.

## INTRODUCTION

The autonomous parvoviruses, to which the minute virus of mice (MVM,1) belongs, are non-enveloped icosahedral viruses which infect mammalian cells (2,3). These viruses are dependent for their own replication on factors transiently expressed during the S phase of growing host cells (2-5). In addition, host cells must be in a specific differentiated state to support lytic viral infection (2,6). The genome of the autonomous parvoviruses is a linear single-stranded DNA molecule approximately 5100 bases long. Upon penetration of the virus into the host cell, the virion DNA is uncoated and converted to a double-stranded monomeric replicative form DNA (RF). Multimeric forms of the RF have also been detected (2,3).

In 1976, Bonnard et al. (7) reported that a virus related to MVM was present in the culture fluid of a subline of the murine lymphoma cells EL-4(G-). This virus grew in lymphocytes, was immunosuppressive for allogeneic mixed leukocyte cultures as it inhibited T-cell mediated functions (8), and was therefore called MVMi (for immunosuppressive, 9). The immunosuppressive phenotype of MVMi is linked to its altered host range relative to the prototype strain of MVM (MVMp) which grows in fibroblasts. Both viruses grow in hybrids of the two cell types (10). MVMp and MVMi bind to the same

receptors and are internalized in both fibroblasts and lymphocytes (11). Thus, a factor expressed in permissive cells apparently interacts in an unknown way with a specific determinant of MVMp or MVMi, and allows the lytic growth of the virus. The nature of the determinant, which has to be encoded in the viral DNA, is also unknown.

MVMp and MVMi have been studied mainly in vitro and little is known about their effects in vivo. A comparison of the restriction maps of the intracellular double-stranded RFs of MVMp and MVMi showed that MVMi is clearly different from MVMp but closely related (9). The nucleotide sequence of the genome of MVMp has been published (12), and the genetic organization of the virus has been deduced from the sequence and from the location of viral transcripts (12,13).

In this study, we have determined the complete nucleotide sequence of the genome of MVMi. From the comparison of their nucleotide sequences, we have established that MVMp and MVMi are 96% homologous, and we have confirmed that the genomes of the two viruses are organized in an identical manner. We have also defined regions of sequence variation between the two viruses that probably determine their different tissue specificities.

## MATERIALS AND METHODS

### Materials.

Restriction enzymes purchased from New England Biolabs, Boehringer or Anglian Biotechnologies, were used as suggested by the suppliers. The Klenow fragment of E. coli DNA polymerase I was from either Boehringer or Anglian Biotechnologies. T4 DNA polymerase was from either Bethesda Research Laboratories, or, together with the T4 gene 32 protein, a kind gift of B. Alberts (University of California, San Francisco). NACS 52 resin was from Bethesda Research Laboratories. Terminal transferase and SalI linkers were from P.L. Biochemicals. Low-gelling agarose (type VII) and S1 nuclease were from Sigma. [$\alpha$-$^{32}$P]dATP (400 Ci/mmol) was from the Radiochemical Centre, Amersham, England.

### Virus and cells.

MVMi was grown in the EL-4 lymphoma cell line (14). The original virus was a gift of G. Bonnard (National Institutes of Health, Bethesda, Md.) and was subsequently purified by terminal dilution by P. Tattersall (Yale University, New Haven, Connecticut).

### Bacterial strains, phages and plasmids.

The M13 derivative mWB2344 and the host E. coli strain WB373 (15)

were obtained from W. M. Barnes (Washington University, Saint Louis, MO.), while the plasmid pEMBL9+ and the phage IR1 (16) were obtained through S. Kvist.

The vector mWB2344 can carry inserts as large as 10 kbp in a stable form and is of clear advantage in sequencing DNAs larger than 1 kbp. We derived new vectors from mWB2344: the polylinker of mWB2344 was exchanged with that of M13 mp8 (17) to generate mWB238* (orientation of the polylinker equivalent to M13 mp8). mWB239* was derived from mWB238* by inversion of the polylinker. We then removed the two extra AccI and BamHI sites still present in mWB238* and mWB239* by replacing the short ClaI fragment of these vectors with the corresponding one of M13 mp8. This made them equivalent to M13 mp8 and M13 mp9, and we called them mWB238 and mWB239 respectively.

Cloning procedures.

Most of the recombinant DNA work was done according to standard procedures (18).

Full length MVMi double-stranded replicative form DNA (RF) was cloned after addition of oligo dC tails in pBR322. MVMi RF with oligo dC tails was annealed to pBR322 with oligo dG tails at the PstI site and the resulting hybrid molecules were used to transform E. coli HB101 cells (19). Restriction fragments of the RF were cloned in mWB238* after addition of SalI linkers to each end of the RF.

Addition of SalI linkers and cloning of large fragments of MVMi RF were done as follows: 15 µg of MVMi RF were treated with 100 units of S1 nuclease in a 50 µl reaction containing 50 mM NaOAc pH 4.8, 100 mM NaCl and 1 mM $ZnCl_2$ for 30 min on ice. After phenol-chloroform extraction followed by ethanol precipitation, the DNA was resuspended in 50 µl of T4 DNA polymerase buffer (33 mM Tris-acetate pH 7.9, 66 mM KOAc, 10 mM $MgOAc_2$, 0.5 mM DTT, 200 µM each dNTP and 100 µg/ml BSA) and treated with 2.5 u of T4 DNA polymerase for 15 min at 37°C. The DNA was then extracted and ethanol precipitated and ligated to SalI linkers. After digestion with either XhoI or HindIII, the DNA was ligated to appropriate mWB238* vectors and used to transform WB373 cells.

The terminal MspI fragment at the 5' end of the viral genome was cloned as follows: 5 µg of virion DNA was converted to a double-stranded extended form with 5 u of the Klenow fragment of E. coli DNA polymerase I in a 200 µl reaction containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM $\beta$-mercapto-ethanol, 200 µM each dNTP and 50 µg/ml BSA for 30 min at 15°C. After addition of another 5 u of the Klenow fragment, the reaction

was allowed to proceed for a further 1 hr at 37°C. The DNA was then digested with XbaI and the fragment containing the extended palindrome of the viral 5' end was purified by gel electrophoresis in low-gelling agarose. After digestion with MspI and conversion to a blunt ended form with the Klenow fragment, the DNA molecules were inserted into mWB238.

Plasmid and M13 replicative form DNAs from mini-cultures were purified by a modification of the method of Birnboim and Doly (20). The bacteria from a 1.5 ml culture were pelleted by centrifugation and resuspended in 100 µl of 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA. The cells were then lysed by the addition of 200 µl of 0.2 N NaOH, 1% SDS, 10 mM EDTA and, after gentle mixing, left at room temperature for 5 min. The mixture was then neutralized quickly by the addition of 300-450 µl of 0.3M KOAc pH 4.8, 9 M LiCl and vortexing. The mixture was then kept on ice for 30 min and centrifuged. The pellet containing the denatured DNA was discarded and the renatured plasmid DNA in the supernatant was precipitated with 0.5 vol of isopropanol for 5 min at room temperature, centrifuged at room temperature and washed two times with 70% ethanol. The precipitate was dissolved in 40 µl of 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA and was suitable for analysis with restriction enzymes.

Purification of double-stranded DNA fragments from low-gelling agarose has been described by McMaster et al. (9). When single-stranded DNA was purified, phenol saturated with 10 mM Tris-HCl, 0.1 mM EDTA pH 7.5 was used instead of phenol saturated with 0.5 M NaCl.

DNA sequencing.

The preparation of single-stranded template DNA and the sequencing reactions were done essentially according to Sanger et al. (21,22). To eliminate strong pause sites for the polymerase, we included the T4 gene 32 protein in some of our reactions. The technique was kindly communicated by C. Craik (University of California, San Francisco). This was indispensable for reading the viral strand between position 4915 and 4900 and the copy strand between position 5050 and 5065, which both contain clusters of cytosines. In GC-rich stretches (particularly within the terminal palindromes), reading of the sequencing gels was sometimes very difficult or impossible. Band compressions were resolved by running the sequencing gels at 70°C and reading both strands simultaneously.

Overlapping DNA fragments for sequencing were generated according to Hong (23) with the following modifications. We started with 10-30 µg of supercoiled replicative form DNA of M13 or pEMBL9+ recombinants, and

replaced the selection of high molecular weight DNA with polyethylene glycol by an ionic exchange chromatography using NACS 52 minicolumns.

## RESULTS

### Sequence determination.

We used the monomeric double-stranded replicative form DNA (RF) extracted from infected cells as the main source of material for sequencing. The different clones derived from the RF and others used in the course of the sequence determination are depicted in Fig. 1. The sequence was determined on both strands and overlaps between adjacent fragments were found at least on one strand. The orientation of reference throughout this paper corresponds to that of the viral strand (i.e. the encapsidated DNA strand), unless otherwise specified. The 3' end is on the left and the 5' end is on the right. The RF is the template for RNA synthesis which occurs from left to right. Whenever a nucleotide is mentioned, it refers to a nucleotide in the strand complementary to the virion DNA, unless otherwise specified.

The EcoRI-HindIII fragments from 21 map units (mu) to 52 mu (i2152) and from 52 to 69 mu (i5269, see Fig. 1), and the PstI fragment containing the 5'end, i66100, were derived from MVMi RF cloned in pBR322 after addition of oligo dC tails, and subcloned in M13 mp8 or M13 mp9. i0052, i4198 and i5299 were derived from restriction fragments of MVMi RF cloned in mWB238* or pEMBL 9+ after addition of SalI linkers to both ends of the RF. From these fragments, adjacent overlapping clones were generated using the

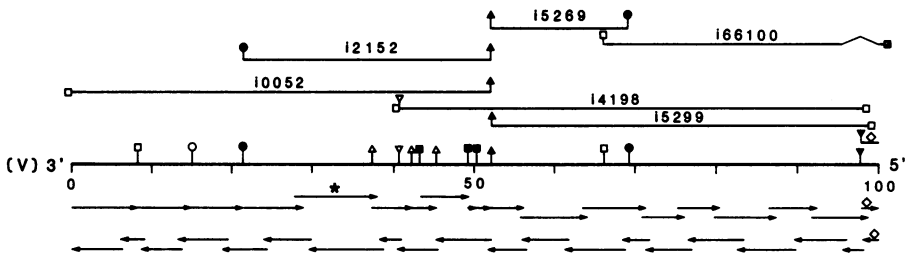


Figure 1. Sequencing approach: the sequences have been determined in the direction of the arrows. The 3' end of the viral strand is on the left. The length of the DNA is expressed in map units (mu), 0 mu corresponds to the 3' end, and 100 mu to the 5' end of the viral DNA. Symbols not explained in the text refer to the following: AluI ; Eco RI ; HindIII ; MspI ; PstI ; PvuII ; SalI linker ; Sau3A1 ; XhoI ; Oligo dG-dC tail .

deletion method of Hong (23), or smaller restriction fragments were subcloned in appropriate M13 vectors. These secondary clones were sequenced by the dideoxy terminator method of Sanger et al. (22). This approach allowed us to determine most of the sequence of the genome of MVMi.

We were not able to obtain the complete terminal sequence of the 5' end in an intact form in cloned RF DNA. The sequences at the 5' and at the 3' end of the viral genome are found in either a hairpin conformation or as extended palindromes (2,3,12,24-26). Only the hairpin configuration is found in the single stranded viral DNA. Therefore, we will refer to the ends of the encapsidated viral DNA as the "hairpins". In the RF, we will only consider the extended palindromic configuration of the ends, and therefore refer to them as the "terminal palindromes". The 5' terminal palindrome or a part of it was always deleted during amplification in E. coli as judged by gel electrophoretic analysis of restriction digests and sequence analysis of cloned MVMi or MVMp DNAs (data not shown). To sequence the nucleotides of the 5' end, we used DNA purified from viral particles. Synthesis of the complementary strand with the Klenow fragment of E. coli DNA polymerase I, using the 3' hairpin as a primer, generated the 5' end with the extended palindromic configuration. The MspI fragment, from position 4984 (the middle of the palindrome) to the 5' terminal nucleotide, i98100 (shown by a diamond in Fig. 1), was cloned in mWB238 and sequenced on both strands.

The portion of the genome between 28 and 37 mu could not be cloned in M13 in the orientation where the viral strand would be propagated in the phage particles, thereby preventing us from deducing the sequence of the viral strand for that area. To overcome this problem, we cloned the SalI-HindIII fragment from 0 to 52 mu in pEMBL9+. Bacteria containing that plasmid produce phage-like particles containing only one of the DNA strands of the plasmid upon superinfection with the filamentous phage IR1 (16). This DNA can then be used for sequence determination. The pEMBL recombinant was subjected to the deletion method of Hong (23). The resulting clones were screened for MVMi inserts starting around position 1500. A clone (shown by an asterisk in Fig. 1) starting at position 1430 was found and sequenced.

We obtained two independent clones containing the 3' terminal palindrome, one derived from the RF with oligo dC tails, the other from the RF to which SalI linkers were added. The nucleotide adjacent either to the oligo dC tail or to the SalI linker was the same in both clones. We assumed

that this nucleotide was the 3' terminal nucleotide and gave it position 1.

We isolated clones consistent with the 5' terminal nucleotide being a "C" (positions 5086 or 5087) or an "A" (5088), suggesting that the size of the encapsidated DNA strand is 5087 ± 1 b. The sequence of the DNA strand complementary to the encapsidated DNA of MVMi is given in the appendix.

The viral strand of MVMp RF contains 18 additional nucleotides at its 5' end relative to encapsidated virion DNA (12,27). In the clone i66100 (Fig. 1), the sequence of the first 17 bases corresponding to the viral strand following the oligo dG tail is identical to the sequence of the copy strand from position 4861 to 4877, as described for MVMp. Following these 17 nucleotides, the sequence is that of the viral strand from position 4858 to 3367. It seems therefore that a deletion occurred during cloning, removing the sequence corresponding to the 5' hairpin and the adjacent nucleotides up to position 4859. This is an example of our inability to amplify in E. coli the 5' terminal palindrome without having it partially or completely deleted by the bacteria.

Three nucleotides are still uncertain: at position 2050 we read "C" in one clone and "A" in another. At positions 3571 and 4285 we read respectively "C" and "G" in two out of three independent clones and "A" in the third clone. These uncertainties are located within the coding part of the MVMi genome and would influence protein sequences in two cases, at positions 2050 and 4285. At the former, "C" to "A" would change Ile to Leu and at the latter "G" to "A" would change Met to Ile.

Sequence comparison.

We have compared the complete nucleotide sequence of the genome of MVMi to the published sequence of MVMp (12). A listing of all the differences found between the two viruses is given in Table 1. For reasons of clarity, the published sequence of MVMp (12) is taken from here on as a reference. To localize inserted nucleotides, the position of the corresponding 5' neighbour in MVMp is given.

There are 175 differences between the two viruses, most of them affecting single nucleotides, while 8% affect two adjacent nucleotides. Therefore, the homology between the two viruses is 96.5%. There are two insertions of one bp, one insertion of three bp and one deletion of one bp in the genome of MVMi relative to MVMp (representing 3% of the differences). Thus, the encapsidated viral DNA of MVMi is approximately four nucleotides longer than that reported for MVMp (12). This size difference is not consistent with a reported deletion of approximately 60 bp in MVMi RF around

Table 1. Location of the nucleotide differences between MVMp and MVMi. The positions are relative to the published sequence of MVMp (12).

| MVMp | Position | MVMi | Amino-acid change | Position affected in the codon |
|---|---|---|---|---|
| G | 53 | T | | |
| | 60 | T | | |
| T | 65 | G | | |
| A | 69 | C | | |
| C | 70 | A | | |
| T | 89 | C | | |
| G | 130 | A | | |
| A | 138 | G | | |
| C | 186 | A | | |
| A | 188 | G | | |
| T | 208 | C | | |
| A | 236 | G | | |
| G | 297 | A | Ala-Thr | 1 |
| A | 355 | C | Asn-Thr | 2 |
| A | 359 | G | | 3 |
| A | 360 | G | Asn-Asp | 1 |
| G | 371 | A | | 3 |
| G | 397 | A | Ser-Asn | 2 |
| A | 407 | G | | 3 |
| T | 458 | C | | 3 |
| A | 489 | T | Thr-Ser | 1 |
| C | 491 | T | " " | 3 |
| T | 529 | A | Phe-Tyr | 2 |
| T | 533 | C | | 3 |
| G | 539 | A | | 3 |
| A | 554 | G | | 3 |
| A | 568 | G | Asn-Ser | 2 |
| G | 575 | A | | 3 |
| T | 582 | G | | 1 |
| T | 583 | C | Phe-Ala | 2 |
| G | 589 | C | Gly-Ala | 2 |
| A | 598 | C | Asn-Thr | 2 |
| A | 611 | G | | 3 |
| A | 623 | G | | 3 |
| A | 662 | C | | 3 |
| G | 686 | A | | 3 |
| A | 704 | G | | 3 |
| A | 752 | G | | 3 |
| A | 799 | G | Asn-Ser | 2 |
| C | 813 | T | | 1 |
| T | 818 | C | | 3 |
| G | 827 | A | | 3 |
| A | 845 | G | | 3 |
| C | 854 | T | | 3 |
| G | 857 | A | | 3 |
| C | 875 | T | | 3 |
| T | 890 | C | | 3 |
| T | 899 | C | | 3 |
| T | 917 | C | | 3 |
| A | 923 | G | | 3 |
| A | 929 | G | | 3 |
| T | 947 | A | | 3 |
| A | 986 | G | | 3 |
| G | 992 | A | | 3 |
| C | 1011 | T | | 1 |
| A | 1103 | G | | 3 |
| T | 1118 | C | | 3 |
| G | 1133 | A | | 3 |
| G | 1160 | A | | 3 |
| A | 1253 | G | | 3 |
| C | 1271 | A | | 3 |
| A | 1289 | G | | 3 |
| G | 1354 | A | Arg-Lys | 2 |
| A | 1355 | G | " " | 3 |
| T | 1413 | C | | 1 |
| T | 1454 | C | | 3 |
| C | 1466 | T | | 3 |
| A | 1502 | G | | 3 |
| C | 1511 | T | | 3 |
| C | 1535 | T | | 3 |
| A | 1541 | G | | 3 |
| C | 1565 | G | | 3 |
| G | 1571 | A | | 3 |
| G | 1685 | A | | 3 |
| A | 1754 | G | | 3 |
| C | 1778 | T | | 3 |
| T | 1793 | C | | 3 |
| C | 1814 | A | | 3 |
| A | 1844 | G | | 3 |
| T | 1913 | C | | 3 |
| A | 1961 | G | | 3 |
| A | 1970 | G | | 3 |
| T | 1984 | C | Leu-Ser | 2 |
| A | 2248 | G | Lys-Arg | 2 |
| C | 2418 | T | | 3 |
| T | 2733 | A | | 3 |
| A | 2821 | G | Ser-Gly | 1 |
| C | 2831 | G | Ala-Gly | 2 |
| A | 2943 | G | | 3 |
| C | 3045 | A | | 3 |
| T | 3057 | C | | 3 |
| A | 3069 | T | | 3 |
| A | 3120 | G | | 3 |
| G | 3132 | A | | 3 |
| A | 3216 | C | | 3 |
| C | 3237 | T | | 3 |
| T | 3272 | C | Leu-Ser | 2 |
| T | 3309 | C | | 3 |
| T | 3349 | C | | 1 |
| C | 3399 | T | | 3 |
| C | 3411 | T | | 3 |
| A | 3420 | G | | 3 |
| T | 3438 | C | | 3 |
| G | 3462 | A | | 3 |
| C | 3468 | T | | 3 |
| G | 3487 | A | Val-Ile | 1 |
| A | 3492 | G | | 3 |
| G | 3501 | A | | 3 |
| | 3521 | G | | |
| | | A | Ile,Pro-Met,Asn,Ser | |
| | | A | | |
| C | 3523 | T | | 1 |
| A | 3573 | T | | 3 |
| G | 3576 | T | | 3 |
| C | 3579 | T | | 3 |
| A | 3582 | G | | 3 |
| C | 3588 | T | | 3 |
| A | 3591 | T | | 3 |
| T | 3594 | A | | 3 |
| T | 3597 | C | | 3 |
| T | 3615 | C | | 3 |
| T | 3616 | C | Ser-Pro | 1 |
| C | 3627 | T | | 3 |
| G | 3636 | A | | 3 |
| C | 3645 | T | | 3 |
| A | 3739 | G | Thr-Ala | 1 |
| A | 3747 | G | | 3 |
| G | 3752 | A | Gly-Glu | 2 |
| C | 3777 | T | | 3 |
| A | 3810 | G | | 3 |
| C | 3855 | T | | 3 |
| A | 3874 | G | Ile-Val | 1 |
| A | 3888 | G | | 3 |
| A | 3893 | G | Lys-Arg | 2 |
| T | 3952 | G | Ser-Ala | 1 |
| T | 3957 | C | | 3 |
| G | 3995 | A | Ser-Asn | 2 |
| T | 4008 | A | | 3 |
| A | 4019 | G | Lys-Arg | 2 |
| A | 4044 | T | | 3 |
| C | 4071 | G | | 3 |
| G | 4098 | A | | 3 |
| T | 4254 | C | | 3 |
| C | 4302 | T | | 3 |
| A | 4314 | G | | 3 |
| T | 4350 | G | | 3 |
| A | 4353 | T | | 3 |
| C | 4356 | T | | 3 |
| C | 4386 | A | | 3 |
| C | 4442 | T | Ala-Val | 2 |
| A | 4473 | T | | 3 |
| T | 4483 | C | | 1 |
| G | 4509 | A | | 3 |
| G | 4515 | A | | 3 |
| C | 4564 | T | | |
| T | 4569 | C | | |
| G | 4616 | A | | |
| G | 4631 | A | | |
| A | 4676 | G | | |
| G | 4707 | A | | |
| C | 4741 | T | | |
| A | 4752 | G | | |
| A | 4753 | G | | |
| A | 4778 | T | | |
| T | 4794 | A | | |
| C | 4824 | | | |
| C | 4837 | A | | |
| | 4861 | T | | |
| T | 4878 | C | | |
| T | 4884 | C | | |
| A | 4904 | G | | |
| A | 4979 | T | | |
| A | 4980 | T | | |
| A | 4981 | T | | |
| T | 5053 | C | | |
| A | 5073 | G | | |
| A | 5079 | G | | |

93 mu (9). Therefore, we cloned the homologous region of MVMp in M13 and sequenced it. The result is shown in Fig. 2: 64 bp, from position 4717 to 4780, are duplicated and separated by a "C" at position 4781. This direct

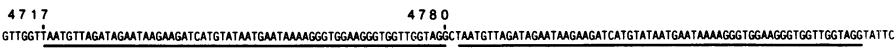


Figure 2. Location of the 64 bp duplicated (underlined) in MVMp. The sequence shown is that of the copy strand, the positions refer to the published sequence of MVMp (12).

repeat in MVMp RF, which had not been detected by Astell et al. (12), accounts for the apparent deletion in MVMi RF.

Among single nucleotide differences, the most frequent correspond to transitions from "A" to "G" (27%), from "T" to "C" (17%), from "G" to "A" (16%) and "C" to "T" (15%). Each possible transversion is represented with frequencies varying from 0.6% (G to C) to 6.3% (A to T). These data are summarized in Table 2.

The distribution of the nucleotide differences along the genome is shown in Fig. 3 as a plot of the local frequency of differences versus the nucleotide position in MVMp RF. Three regions around 11, 17 and 70 mu show more variation than average. One region, between 36 and 60 mu clearly varies less than average. The regions with the highest variation lay within the presumed coding part of the genome, whereas the region with low variation contains the promoter of the second transcription unit (39 mu) and sequences involved in splicing (40-45 mu, see below).

In MVM, only the strand of minus polarity is encapsidated (28, G. K. McMaster unpublished), and the known virus-specific RNAs extracted from infected cells hybridize to the minus strand (13, G. K. McMaster unpublished). The viral RNAs are synthesized from two transcription units

Table 2. Categories of changes corresponding to the differences shown in Table 1. The number of changes in each category is given as well as the percentage relative to the total number of changes (in parentheses).

| TRANSVERSIONS | TRANSITIONS | INSERTIONS | DELETIONS |
|---|---|---|---|
| p ⟶ i | p ⟶ i | bases added in MVMi | base deleted in MVMi |
| A-C: 5 (2.8) | A-G: 48 (27.3) | T: 2 (1.1) | C:1 (0.6) |
| A-T: 11 (6.3) | C-T: 26 (15.8) | GAA:1 (0.6) | |
| C-A: 7 (4.0) | G-A: 28 (15.9) | | |
| C-G: 3 (1.7) | T-C: 30 (17.1) | | |
| G-C: 1 (0.6) | | | |
| G-T: 2 (1.1) | | | |
| T-A: 6 (3.4) | | | |
| T-G: 4 (2.3) | | | |

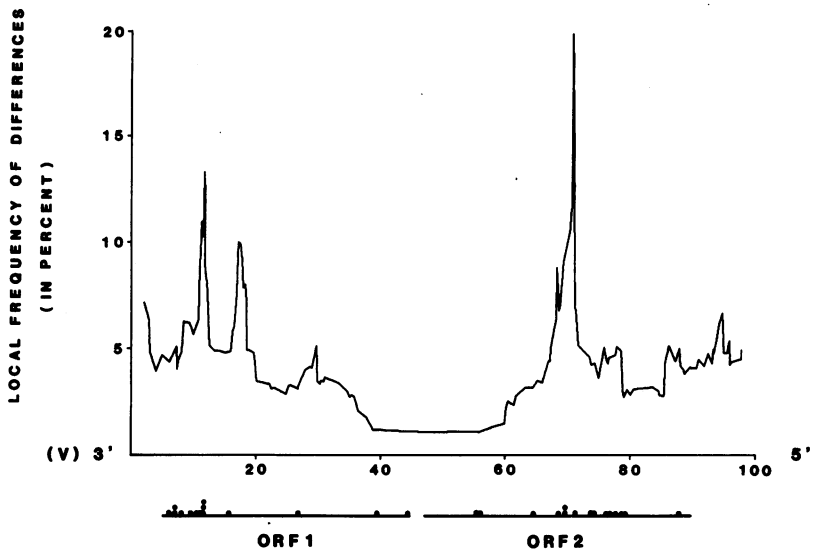


Figure 3. Local frequency of differences between MVMp and MVMi. To determine the local frequency of differences, we have chosen variable length intervals, each containing 11 consecutive differences. The position of the $6^{th}$ difference in each interval is the position of reference for that interval. The upper boundary of the interval is the position of the $5^{th}$ difference upstream of the reference position. The lower boundary of the interval is the position of the $5^{th}$ difference downstream of the reference position. The local frequency was calculated as 11 divided by the length of the interval, multiplied by 100. The position of each nucleotide difference between MVMp and MVMi (see Table 1) is consecutively taken as point of reference. Therefore, the first 5 and the last 5 differences are not represented as individual points in the graph. The other points are represented by the curve connecting them. The published sequence of MVMp (12) is taken as a reference, the orientation 3' to 5' refer to the viral strand, and the positions are expressed in percentage of the genome length (map units). The positions of the corresponding amino-acid changes are shown by dots above the lines representing the two main open-reading frames (ORFs 1 and 2).

(13, G. K. McMaster unpublished) with promoters at positions 180 and 1980 (12,13). At least two spliced mRNAs, 4.8 and 3.3 kb long, are derived from the first transcription unit with a 5' terminal nucleotide at position 201 ± 5 (29). At least one 3 kb spliced mRNA is derived from the second transcription unit. It represents the major mRNA 24 hr post infection, and its 5' end maps at position 2005 ± 5 (29). Polyadenylation signals are found only at three positions near the 5' end of the viral genome (12). The differences between MVMi and MVMp do not affect most of the putative transcription control signals. These include "TATA" boxes, at positions 180

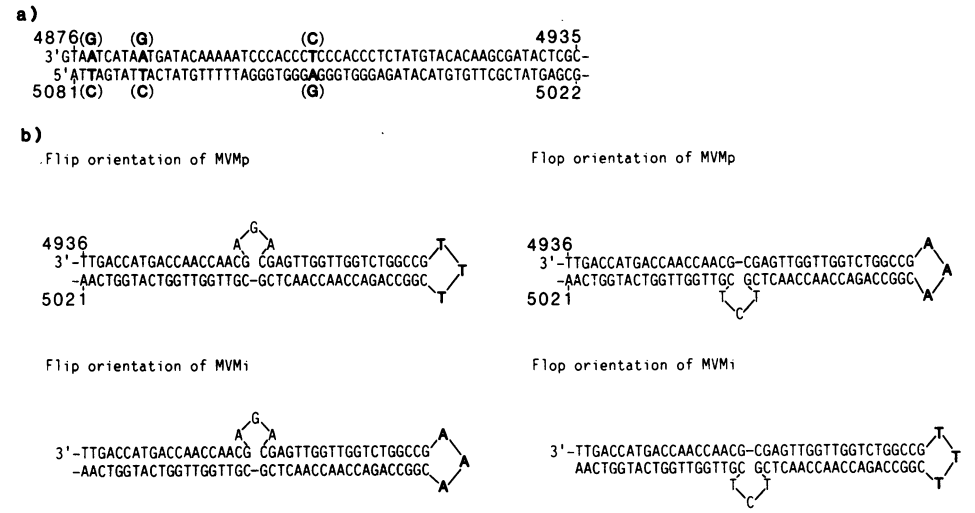


Figure 4. Alternative sequences at the 5' end of the virion DNA, and nucleotide differences between MVMp and MVMi. The differing bases are shown in bold characters (a,b), and those of MVMi in parentheses (a).

and 1980, splice signals around 10 and 42 mu, and two polyadenylation signals (AATAAA, positions 4599 and 4817). The third polyadenylation signal (4752) is lost in MVMi (AATAAA in MVMp corresponds to GGTAAA in MVMi). The corresponding "TATA" boxes and polyadenylation signals in MVMi are underlined in the sequence given in the appendix.

There are two major open reading frames in MVMp (12), one in the left part of the genome (ORF1), the other in the right part (ORF2) (Fig. 3). ORF1 probably codes for non-structural protein(s) while ORF2 codes for the three known capsid proteins (30). 75% of the nucleotide differences found in these two ORFs occur in the third position of the codon, 13% in the second and 12% in the first position (Table 1). The regions with the highest variation (Fig. 3) map within the coding regions of the genome. Although highly different at the nucleotide level, they do not necessarily result in extensive amino-acid changes. In ORF1, from position 261 to 2276, 14 amino-acids (2%) are affected by the nucleotide differences between the two viruses. In ORF2, from position 2343 to 4552, 15 amino-acids (2%) are different and there is an additional amino-acid in MVMi due to the three bp insertion at position 3521. Out of 27 amino-acid changes (the two amino-acid changes at the insertion site are not taken into account), 16 are

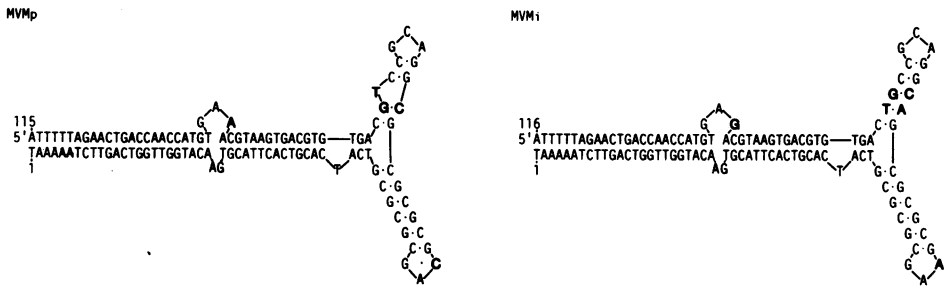


Figure 5. Nucleotide sequence at the 3' end of the encapsidated virion DNA. The nucleotides differing between MVMp and MVMi are shown in bold characters.

conservative. The other changes result in interconversion either between uncharged polar and acidic amino-acids, or between non polar and uncharged polar (Table 1).

In MVMp, the populations of RF and virion DNA are not homogeneous. A sequence heterogeneity is found in the 5' end such that the 5' hairpin (or the corresponding terminal palindrome) in one population is an isomer of the 5' hairpin in the other population (Fig. 4). The two isomers can be differentiated because 6 out of 206 bases do not base-pair in the hairpin, thereby introducing an asymmetry in its structure. This heterogeneity could be explained by an inversion event around the axis of symmetry of the terminal palindrome (flip-flopping) (12,25). Our sequence data for MVMi gave the same result (Fig. 4). In the 5' hairpin (positions 4876-5081), nine nucleotides are changed, six (positions 4878, 4884, 4904, 5053, 5073 and 5079) in the base-paired part of the hairpin and three (4979-4981) in the mismatched region. In fact, MVMi has a structure that is symmetrically related to that of MVMp. For instance, the terminal loop in the "Flip" orientation is "TTT" in MVMp, whereas it is "AAA" in MVMi.

In MVMp, no sequence inversion has been detected in the 3' hairpin (24). The differences in the corresponding palindrome affect nucleotides found in regions where no base-pairing occurs in the encapsidated viral DNA (Fig. 5). Only two unpaired regions (positions 41 and 64-66) are conserved between the two viruses out of the five (41, 51-53, 64-66, 69 and 89-91) that this hairpin possesses.

The 18 additional nucleotides found in the viral strand of the RF (positions 5082-5099) could adopt a hairpin configuration within themselves (Fig. 6). There is one insertion in MVMi, "A" at position 4861 (see legend of Fig. 6), which could make this hairpin more stable in MVMi than in MVMp.

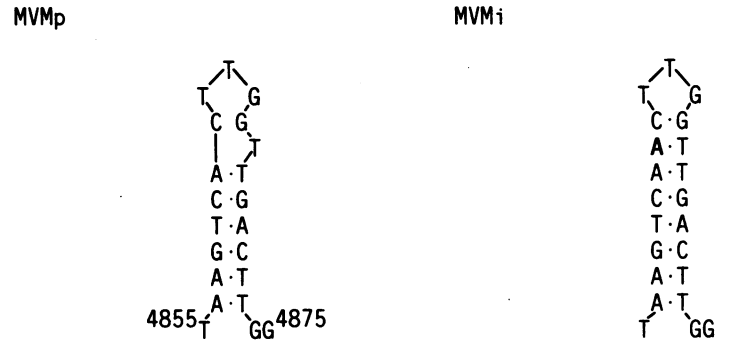


Figure 6. Nucleotide difference within the sequence adjacent to the 5' hairpin in the encapsidated virion DNA. This sequence is identical to the 18 additional nucleotides found in the viral strand of the RF of MVM. The inserted base in MVMi is shown in bold character.

## DISCUSSION

As a part of our effort to understand the distinct biological properties of the prototype of the minute virus of mice (MVMp) and the closely related immunosuppressive variant (MVMi), we have determined the nucleotide sequence of MVMi and compared it with the published sequence of MVMp (12). The genomes of MVMp and MVMi have more than 96% of their sequence in common, which clearly establishes the close relationship between these viruses. The general organization of the genome of MVMi deduced from our sequence determination is also very similar to that of MVMp.

We found 65 additional base pairs in the non-coding part of the 5' end of MVMp (around 93 mu) which are absent from the published sequence. This additional sequence forms a tandem repeat with the adjacent 64 nucleotides in MVMp thus accounting for the apparent deletion in MVMi (9). The reason for this discrepancy is not clear. The strain of MVMp with which we worked might have undergone a duplication of this sequence. Multiple repeats of sequences mapping at a similar position are known to occur in the parvovirus H1 and in defective interfering particles of H1 (25,31), while there are no repeats in the closely related parvovirus H3 (25). The function of these repeated sequence elements is not understood. An unlikely alternative, that we are currently testing, is the hypothesis that the discrepancy results from a sequencing artefact. If the MboII site located within the duplicated 64 bp was used during sequencing analysis of this region (12), the small intervening 65 bp fragment could have been lost.

In addition to the apparent deletion, our data confirm the position of most of the restriction sites found in MVMi RF by McMaster et al. (9). However, our maps for AluI (sites at approximately 22 and 42 map units), HphI (36 mu), HincII (48, 54 and 58 mu), MboI (63 and 85 mu) and EcoRII (33, 34 and 41 mu) differ from those of McMaster et al.

The tissue specificity of MVMp and MVMi depends on an intracellular factor (10,11) which interacts with a strain-specific determinant of MVM. In non-permissive cells, the viral single-stranded DNA is converted to a duplex replicative form (RF) (11), but little or no amplification of the RF takes place in these cells. To distinguish among the possible sequences responsible for tissue-specific growth, we are constructing hybrid viruses between MVMp and MVMi and testing their host range. Preliminary results indicate that the EcoRI fragment corresponding to the genomic 5' end is essential in determining the host range of MVM. We expect therefore that the strain-specific determinant(s) is within this part of the genome of MVM.

Some proteins coded by MVM can be used in trans since MVM provides helper functions for the growth of a deletion mutant of H1 which is defective in both replication and encapsidation (32). Therefore, one would expect that some of these proteins can function in trans in a mixed infection with MVMi and MVMp. Preliminary experiments (unpublished) indicate that MVMi does not help the growth of MVMp in lymphocytes. This would imply that a differential tissue-specific level of expression of an essential viral gene is not involved in the host-range phenotypes exhibited by MVMp and MVMi. Therefore, it seems likely that the recognition of a viral DNA sequence by a host-cell factor, possibly in conjunction with a viral protein, is an obligatory step of the lytic cycle. Two regions of the 5' terminal EcoRI fragment could be responsible for the tissue specificity of the two viruses: the terminal palindrome and the coding region for the COOH-terminal half of the viral capsid proteins. Since the latter alone would be expected to act in trans, we will confine our discussion to the former.

Protein-DNA interaction, for instance during sequence-specific nicking, near or within the terminal palindromes of the viral genome is an important feature of all models of parvoviral DNA replication (2,3,27,33). The structure of these palindromes is highly conserved in the autonomous parvoviruses (24-26), and the 5' terminal palindrome is essential for the replication of the linear genome of MVM (34). A nucleotide change close to or within this palindrome could affect the recognition site of a

hypothetical tissue-specific nuclease of cellular origin (or other host-cell factors), and thereby alter the tissue specificity of replication. Therefore, the insertion of one bp in MVMi within the sequence homologous to the additional 18 bp present in the RF, or the differences found within the 5' terminal palindrome could be involved in the differing host range phenotypes of MVMp and MVMi.

ACKNOWLEDGEMENTS

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APPENDIX

Nucleotide sequence of the genome of MVMi (immunosuppressive variant of MVM). Only the copy-strand is shown, in 5' to 3' orientation. The terminal hairpin regions are indicated in bold characters. "TATA" boxes and polyadenylation signals are underlined.

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   1 ATTTTTAGAA CTGACCAACC ATGTTCACGT AAGTGACGTG ATGACGCGCG CTTCGCGCGC
  61 TGCCTGCGGC AGTCACACGT CACTTACGTC TCACATGGTT GGTCAGTTCT AAAAATGATA
 121 AGCGGTTCAG AGAGTTTAGA CCAAGGCGCG AAAAGGAAGT GGGCGTGGTT TAAAGTATAT
 181 AAGCAAATGC TGAAGTCAGT TACTTATCCT TTCTTTCATT CTGTGAGTCG AGACGCGCAG
 241 AAAGAGAGTA ACCAACTAAC CATGGCTGGA AATGCTTACT CTGATGAAGT TTTGGGAACA
 301 ACCAACTGGT TAAAGGAAAA AAGTAACCAG GAAGTGTTCT CATTTGTTTT TAAAACTGAG
 361 GATGTTCAAC TAAATGGAAA AGATATCGGA TGGAATAATT ACAAAAAGGA GCTGCAGGAG
 421 GACGAGCTGA AATCTTTACA ACGAGGAGCG GAAACTACCT GGGACCAAAG CGAGGACATG
 481 GAATGGGAAT CTACAGTGGA TGAAATGACC AAAAAGCAAG TATTCATTTA TGACTCTTTA
 541 GTTAAAAAAT GTTTGTTTGA AGTGCTTAGC ACAAAAAATA TAGCTCCTGC TGATGTTACT
 601 TGGTTTGTGC AGCATGAATG GGGGAAAGAC CAAGGCTGGC ACTGCCATGT ACTAATTGGA
 661 GGCAAGGACT TTAGTCAAGC TCAAGGAAAA TGGTGGAGAA GGCAGCTAAA TGTTTACTGG
 721 AGCAGATGGT TGGTAACAGC CTGTAATGTG CAGCTAACAC CAGCTGAAAG AATTAAACTA
 781 AGAGAAATAG CAGAAGACAG TGAGTGGGTT ACTTTACTCA CTTATAAACA TAAGCAAACC
 841 AAAAAGGACT ATACTAAATG TGTTCTTTTT GGAAATATGA TTGCTTACTA CTTTTTAACC
 901 AAAAAGAAAA TAAGCACCAG TCCGCCAAGG GACGGAGGCT ATTTTCTAAG CAGTGACTCT
 961 GGCTGGAAAA CTAACTTTTT AAAAGAGGGC GAACGCCATC TAGTGAGCAA ATTATACACT
1021 GATGACATGC GGCCAGAAAC GGTTGAAACC ACAGTAACCA CTGCGCAGGA AACTAAGCGC
1081 GGCAGAATTC AAACTAAAAA AGAGGTTTCT ATTAAAACCA CACTTAAAGA GCTAGTGCAT
1141 AAAAGAGTAA CCTCACCAGA AGACTGGATG ATGATGCAGC CAGACAGTTA CATTGAAATG
1201 ATGGCTCAAC CAGGTGGAGA AAACCTGCTG AAAAATACGC TAGAGATTTG TACGCTAACT
1261 CTAGCCAGAA CAAAAACAGC ATTTGACTTG ATTTTAGAAA AAGCTGAAAC CAGCAAACTA
1321 ACCAACTTTT CACTGCCTGA CACAAGAACC TGCAAGATTT TTGCTTTTCA TGGCTGGAAC
1381 TATGTTAAAG TTTGCCATGC TATTTGCTGT GTTCTAAACA GACAAGGAGG CAAAAGAAAT
1441 ACTGTTTTAT TTCACGGACC AGCCAGTACA GGCAAATCTA TTATTGCACA AGCCATAGCA
1501 CAGGCAGTTG GTAATGTTGG TTGCTATAAT GCAGCTAATG TGAACTTTCC ATTTAATGAC
1561 TGTACGAACA AAAACTTGAT TTGGGTAGAA GAAGCTGGTA ACTTTGGACA GCAAGTAAAC
1621 CAGTTTAAAG CCATTTGCTC TGGTCAAACT ATTCGCATTG ATCAAAAAGG AAAAGGCAGC
1681 AAACAAATTG AACCAACACC AGTCATCATG ACCACAAATG AGAACATTAC AGTGGTCAGA
1741 ATAGGCTGCG AAGAGAGACC AGAACACACT CAACCAATTA GAGACAGAAT GCTCAACATT
1801 CATCTAACAC ATACATTGCC TGGTGACTTT GGTTTGGTTG ACAAGAATGA ATGGCCCATG
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1861 ATTTGTGCTT GGTTGGTAAA GAATGGTTAC CAATCTACCA TGGCAAGCTA CTGCGCTAAA
1921 TGGGGCAAAG TTCCTGATTG GTCAGAAAAC TGGGCGGAGC CGAAGGTGCC GACTCCTATA
1981 AATTCACTAG GTTCGGCACG CTCACCATTC ACGACACCGA AAAGTACGCC TCTCAGCCAG
2041 AACTATGCAC TAACTCCACT TGCATCGGAT CTCGAGGACC TGGCTTTAGA GCCTTGGAGC
2101 ACACCAAATA CTCCTGTTGC GGGCACTGCA GAAACCCAGA ACACTGGGGA AGCTGGTTCC
2161 AAAGCCTGCC AAGATGGTCA ACTGAGCCCA ACTTGGTCAG AGATCGAGGA GGATTTGAGA
2221 GCGTGCTTCG GTGCGGAACC GTTGAAGAGA GACTTCAGCG AGCCGCTGAA CTTGGACTAA
2281 GGTACGATGG CGCCTCCAGC TAAAAGAGCT AAAAGAGGTA AGGGTTTAAG GGATGGTTGG
2341 TTGGTGGGGT ATTAATGTTT AATTACCTGT TTTACAGGCC TGAAATCACT TGGTTTTAGG
2401 TTGGGTGCCT CCTGGCTATA AGTACCTGGG ACCAGGGAAC AGCCTTGACC AAGGAGAACC
2461 AACCAATCCA TCTGACGCCG CTGCCAAAGA GCACGACGAG GCCTATGATC AATACATCAA
2521 ATCTGGAAAA AATCCTTACC TGTACTTCTC TGCTGCTGAT CAACGCTTTA TTGACCAAAC
2581 CAAGGACGCC AAAGACTGGG GAGGCAAGGT TGGTCACTAC TTTTTTAGAA CCAAGCGCGC
2641 TTTTGCACCT AAGCTTGCTA CTGACTCTGA ACCTGGAACT TCTGGTGTAA GCAGAGCTGG
2701 TAAACGCACT AGACCACCTG CTTACATTTT TATAAACCAA GCCAGAGCTA AAAAAAAACT
2761 TACTTCTTCT GCTGCACAGC AAAGCAGTCA AACCATGAGT GATGGCACCA GCCAACCTGA
2821 CGGCGGAAAC GGTGTCCACT CAGCTGCAAG AGTTGAACGA GCAGCTGACG GCCCTGGAGG
2881 CTCTGGGGGT GGGGGCTCTG GCGGGGGTGG GGTTGGTGTT TCTACTGGGT CTTATGATAA
2941 TCAGACGCAT TATAGATTCT TGGGTGACGG CTGGGTAGAA ATTACTGCAC TAGCAACTAG
3001 ACTAGTACAT TTAAACATGC CTAAATCAGA AAACTATTGC AGAATAAGAG TTCACAACAC
3061 AACAGACACT TCAGTCAAAG GCAACATGGC AAAAGATGAT GCTCATGAGC AAATTTGGAC
3121 GCCATGGAGC TTAGTGGATG CTAATGCTTG GGGAGTTTGG CTCCAGCCAA GTGACTGGCA
3181 ATACATTTGC AACACCATGA GCCAGCTTAA CTTGGTCTCA CTTGATCAAG AAATATTTAA
3241 TGTAGTGCTG AAAACTGTTA CAGAGCAAGA CTCAGGAGGT CAAGCTATAA AAATATACAA
3301 CAATGACCTC ACAGCTTGCA TGATGGTTGC AGTAGACTCA AACAACATTC TGCCATACAC
3361 ACCTGCAGCA AACTCAATGG AAACACTTGG TTTCTACCCT TGGAAACCAA CTATAGCATC
3421 GCCATACAGG TACTATTTCT GCGTTGACAG AGATCTTTCA GTAACCTATG AAAATCAAGA
3481 AGGCACAATT GAGCATAATG TAATGGGAAC ACCAAAAGGA ATGAATTCTC AATTTTTTAC
3541 CATTGAGAAC ACACAACAAA TCACATTGCT CAGAACTGGT GATGAGTTTG CTACTGGAAC
3601 CTACTACTTT GACACAAACC CAGTTAAACT TACACACACA TGGCAAACTA ACCGTCAACT
3661 TGGACAGCCT CCACTGCTGT CAACCTTTCC TGAAGCTGAC ACTGATGCAG GTACACTTAC
3721 TGCTCAAGGG AGCAGACATG GAGCAACACA GATGGAGGTT AACTGGGTGA GTGAAGCAAT
3781 TAGAACCAGA CCTGCTCAAG TAGGATTTTG TCAGCCACAC AATGACTTTG AAGCCAGCAG
3841 AGCTGGACCA TTTGCTGCTC CAAAAGTTCC AGCAGATGTT ACTCAAGGAG TGGACAGAGA
3901 AGCCAATGGC AGTGTTAGAT ACAGTTATGG CAAACAGCAT GGTGAAAATT GGGCTGCACA
3961 CGGACCAGCA CCAGAGCGCT ACACATGGGA TGAAACAAAC TTTGGTTCAG GAAGAGACAC
4021 CAGAGATGGT TTTATTCAAT CAGCACCTCT AGTTGTTCCA CCACCACTAA ATGGGATTCT
4081 TACAAATGCA AACCCTATTG GAACTAAAAA TGACATTCAT TTTTCAAATG TTTTTAACAG
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4201 GGACAAAGAA CTAGATCTTG AACACAAACC TAGACTTCAC ATAACTGCTC CATTTGTCTG
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4321 TGATCCAAAC GGAGCCACAC TTTCTAGAAT TGTGACTTAT GGTACATTTT TCTGGAAAGG
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4921 CATGTGTTCG CTATGAGCGA ACTGGTACTG GTTGGTTGCT CTGCTCAACC AACCAGACCG
4981 GCTTTGCCGG TCTGGTTGGT TGAGCGCAAC CAACCAGTAC CAGTTCGCTC ATAGCGAACA
5041 CATGTATCTC CCACCCCCCC ACCCTAAAAA CATAGTGATA CTGATGG

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