Construction of an Infectious Molecular Clone of the Autonomous Parvovirus Minute Virus of Mice

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The linear single-stranded DNA genome of minute virus of mice, an autonomous parvovirus, was cloned in duplex form into the bacterial plasmid pBR322. The recombinant clones of minute virus of mice were infectious when transfected into monolayers of human 324K cells and produced virus plaques with an efficiency of about 6% that obtained with duplex replicative-form DNA purified from cells infected with minute virus of mice. Southern blot analysis of transfected cells indicated that the cloned minute virus of mice genome requires both termini to be intact for excision and replication as a linear duplex molecule.

Molecular clones of animal viruses capable of propagating as passenger sequences in procaryotic vector-host systems are proving to be immensely valuable tools in the analysis of viral gene function. Here we report the cloning of a double-stranded, infectious copy of the genome of minute virus of mice (MVM), a member of the autonomous parvovirus subgroup (32, 33). The MVM genome is a linear, nonpermuted single strand of DNA five kilobases long, with short palindromic sequences at each end (3; C. R. Astell, M. Thomson, M. B. Chow, and D. C. Ward, Cold Spring Harbor Symp. Quant. Biol., in press). These palindromes form hairpin structures in the single-stranded molecule and appear to contain all the *cis*-acting information necessary for the replication and packaging of the viral genome (12). The hairpin located at the left-hand (3') end of the autonomous parvovirus genome differs in nucleotide sequence and secondary structure from that at the right-hand (5') end. Subgenomic clones of MVM DNA which do not contain these terminal palindromes have been prepared routinely and without difficulty in this laboratory from double-stranded forms of viral DNA. However, we have found it necessary to adopt separate cloning strategies to obtain copies of each MVM terminus and then to recombine two overlapping partial clones in vitro to obtain a full-length genomic clone. This strategy is described in Figure 1.

MVM DNA replicates intracellularly via monomeric and multimeric replicative-form (RF) duplex intermediates (31). Attempts were made to clone terminal sequences of MVM from these forms of RF DNA in both plasmid and bacteriophage vectors. Since RF dimers are left-end-to-

left-end fusions of RF monomers, and RF tetramers are right-end fusions of RF dimers (26, 31), restriction digests of these multimers contain terminal sequences as inverted repeat fragments. Although ligation of either type of fusion fragment into pBR322 or λ dam (11) vectors could be demonstrated by gel analysis, no recombinant molecules containing either viral terminus were obtained after propagation in *Escherichia coli*, paralleling published observations that long inverted repeats are not tolerated in such vector-host systems (8, 9, 17).

The right-hand end was successfully cloned by the addition of synthetic *Bam*HI linkers to MVM duplexes synthesized in vitro from viral DNA. Because MVM packages a unique strand, this approach only attaches linkers to the right-hand end. When the same linker addition strategy was applied to duplex RF molecules obtained from infected cells, we were unable to recover any MVM-containing recombinants. Although it is known that many RF termini exist in the hairpin (turnaround) form, RF populations also contain many molecules which have their ends arranged in the palindromic (extended) configuration (31). However, it appears that the majority of extended form termini are covalently linked through their 5' ends to a protein (Astell et al., in press), and we assume that a residual oligopeptide attached to the 5' ends blocks linker addition to these molecules. Because these extended form termini have 3' hydroxyl groups at their termini (Astell et al., in press), we next attempted to clone them by the addition of 3' homopolymer tails with terminal transferase, as described in Fig. 1. Of the 37 clones isolated, 32 contained sequences from 0 to 8 map units

FIG. 1. Molecular cloning of full-length MVM DNA. Single-stranded viral DNA from the prototype strain MVM(p) was used as a primer-template for the *E. coli* polymerase I Klenow fragment to generate a full-length duplex hairpin molecule with one blunt end at the genomic right-hand terminus (5). This blunt end was ligated (20) to synthetic *Bam*HI linkers and then digested with *Eco*RI (which cuts at 20 and 69 map units) and with *Bam*HI (which does not cut MVM DNA). Digested DNA was ligated into the large *Eco*RI-*Bam*HI fragment of pBR322 and transformed (10) into *E. coli* HB 101 (6), and ampicillin-resistant colonies were analyzed by using minilysate restriction digests (4). pMM610 contains the MVM *Eco*RI partial fragment extending from 20 map units to the *Bam*HI linker on the right-hand end (100 map units). Duplex MVM RF molecules were purified from intracellular viral DNA by chromatography on benzoylated DEAE-cellulose, which removes branched replicative intermediate species and single-stranded DNA (27), and oligo(dC) was then added to available 3' hydroxyl termini by incubation with dCTP and terminal transferase (21). Tailed RF molecules were annealed to DNA from plasmid pAT153 (30) which had been cut at the *Pst*I site and tailed with oligoguanidylate in a similar transferase reaction. After transformation into *E. coli* strain LE392 (29), tetracycline-resistant colonies were screened for MVM terminal sequences by colony hybridization (15), and the inserts were analyzed as described above. pPT206 was the longest insert obtained and is only missing about 4% of the genome from the extreme right-hand end. It contains approximately 20 extra base pairs (presumably deoxyguanylate:deoxycytidylate [poly dG:dC]) at the left-hand end. To provide a selectable marker for a full-length recombinant, the kanamycin-resistance gene from pNG39 (14) was subcloned between the *Bam*HI and *Sal*I sites of pMM610 to give pMM842. The first full-length recombinant, pMM91, was obtained by recombining pMM842 and pPT206 in vitro at the single *Xba*I site (at 85 map units). This was then reconstructed by creating *Pst*I linear molecules, digesting to blunt ends with the 3' to 5' exonuclease of T4 polymerase (7), and ligating on *Bam*HI linkers. After digestion with *Bam*HI, ligation into the *Bam*HI site of pBR322, and transformation into LE392, ampicillin-resistant colonies were analyzed for full-length MVM inserts as described above, resulting in the isolation of pMM984. Care has to be taken during propagation in *E. coli* of all clones containing the complete right-hand end, as they slowly delete a segment of ca. 100 base pairs, centered on map unit 98 and spanning the axis of symmetry of the terminal palindrome, resulting in a loss of plaque-forming ability.

(measured from the left-hand end), but only 5 contained sequences to the right of 85 map units, and none contained a complete right-hand terminus. We attempted to clone the complete viral right-hand end by oligodeoxycytidylate [oli-

go(dC)]-tailing the extended conformer of the terminal *Hae*II fragment (80 to 100 map units) of monomer RF; however, for reasons as yet unclear, all 116 of the recombinants recovered were shorter than the starting material. This

phenomenon was observed even though we could demonstrate that oligo(dC) had been added to each end of the fragment before annealing. Thus, tailing of monomer RF does not appear to be a straightforward method for cloning the viral right-hand terminus, although it is very efficient for obtaining clones of the left-hand end. This conclusion contrasts with the results of Samulski et al. (24), who were able to clone both ends of the DNA of adeno-associated virus type 2, a member of the helper-dependent subgroup of parvoviruses, by using the oligo(dC) procedure. These results are probably due to the presence in adeno-associated virus DNA of an inverted terminal repetition, which gives a hairpin structurally resembling the MVM left-hand terminus, on both ends of the genome (24, 32).

The full-length MVM clone was obtained by recombining the partial clones in vitro, and this recombinant was reconstructed to allow single-step excision of the viral genome with *Bam*HI (Fig. 1).

None of the subgenomic clones were able to form plaques upon transfection into 324K cells, whereas the total genomic clones formed

TABLE 1. Transfection of 324K cells^a

DNA source and manipulation	No. of plaques per μ g of DNA	No. of expts
(a) pBR322, pAT153, pMM610, pMM842, pPT206, pNG39	None detected	4
(b) deproteinized MVM RF	104 $\pm$ 54	7
(c) pMM984	3.3 $\pm$ 1.1	6
(d) pMM984 cut with <i>Bam</i> HI	23 $\pm$ 12	4
(e) MVM insert from (d), double gel-purified	62 $\pm$ 9	5
(f) purified MVM insert from (e), self-ligated	226 $\pm$ 45	4

^a Monolayers of 324K cells (25) were seeded as an even lawn of 5×10^5 cells per 60-mm plastic tissue culture dish and incubated overnight. DNA-calcium phosphate precipitates were prepared by the method of Graham and van der Eb (13) in HEPES-buffered saline (pH 7.05) containing 5 to 10 μ g of A9 cell DNA per ml as the carrier. The medium was removed from each dish, and 0.5 ml of DNA precipitate was added. This solution was incubated for 20 min at room temperature; 5 ml of Autopow M medium plus 5% fetal calf serum was added, and incubation continued at 37°C for 4 h more. The monolayers were then washed with medium and exposed to 1 ml of 25% dimethyl sulfoxide in HEPES-buffered saline. After two rinses with medium, the monolayers were overlaid with 7 to 8 ml of overlay medium, which contained 0.6% agarose (Seakem), 10% fetal calf serum, and 0.2% tryptose phosphate broth and then adjusted to pH 7.8 with 25 mM HEPES-N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid-NaOH buffer. After 6 days at 37°C, plates were stained for 6 to 8 h with 0.02% neutral red in Hanks-buffered salts and read after incubation overnight.

FIG. 2. Analysis of intracellular viral DNA induced by plasmid-derived MVM. Isolated plaques resulting from transfection of 324K cells with pMM91 (as described in footnote a Table 1) were picked at random and inoculated into separate subconfluent cultures of 324K cells; stocks were produced from each by two further passages in similar monolayers. Mouse A9 cells (18) were then infected with each of these stocks at a multiplicity of 10 PFU per cell, and intracellular viral DNA was isolated by the method of Hirt (16) at 21 h after infection. DNA samples were analyzed, before and after digestion with *Eco*RI, by electrophoresis on a 1.4% agarose gel. Lanes 1, 3, 5, 7, and 9 are of undigested samples; lanes 2, 4, 6, 8, and 10 are after *Eco*RI digestion. Lanes 1 and 2 are of DNA induced after infection with authentic MVM virions. Lanes 3 and 4, 5 and 6, 7 and 8, and 9 and 10, are DNA induced by stocks derived from four independent pMM91 plaques. RF and SS denote the positions of MVM duplex RF monomer and single-stranded virion DNA, respectively. Letters denote the *Eco*RI fragments, the map for which is shown below the gel in the stranded convention (2). t denotes the turnaround (hairpin) form of a terminal fragment, and e denotes the extended (palindromic) form (31). The line drawing does not imply that turnaround forms are necessarily on the same molecule or that they are covalently continuous.

plaques at about 6% of the efficiency of gel-purified monomer RF DNA (Table 1). The efficiency of plaque formation was slightly increased by restriction enzyme cleavage within vector sequences, whereas cutting within viral sequences dramatically reduced the number of plaques obtained. Excision of the MVM se-

FIG. 3. Analysis of transfected plasmid DNA. Monolayers of 324K cells were transfected with 10 to 12 μ g of DNA per plate as described in footnote *a* of Table 1. After various times, nuclei were prepared from the cells (22) and total DNA was extracted by sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction and ethanol precipitation. DNA samples were electrophoresed in 1.0% agarose gels, transferred to nitrocellulose (28), and hybridized (1) to pMM984 DNA at 5 ng/ml labeled with 32 P by nick-translation (23). (A) Lane 1 contains 1 ng of intracellular MVM DNA; lanes 2 and 3 contain total DNA from cells transfected with no DNA and carrier DNA, respectively. Lanes 4 through 12 contain total DNA from cells transfected with pMM984 harvested at 0, 2, 3.5, 4, 7, 10, 13, 16, and 52 h after transfection, respectively. The extreme right-hand two lanes are a sevenfold-further exposure of lanes 11 and 12. (B) Intracellular DNA from transfected cells harvested at 7 days after transfection. Lane 1 contains 1 ng of intracellular MVM DNA marker; lane 2 contains an *Eco*RI digest of DNA of lane 1. Lanes 3, 4, and 5 contain total DNA from cells transfected with no DNA, carrier DNA, and pBR322 DNA, respectively. Other lanes contain total DNA from cells transfected with pMM842 DNA (lanes 6 and 7), pPT206 DNA (lanes 8 and 9), pMM91 DNA (lanes 10 and 11), pMM984 DNA (lanes 12 and 13), and MVM RF DNA (lanes 14 and 15). DNAs in lanes 7, 9, 11, 13, and 15 were digested with *Eco*RI before electrophoresis. The extreme right-hand four lanes are an 80-fold-further exposure of lanes 6 through 9. I, II, and III denote supercoiled, relaxed circular, and linear forms, respectively, of pMM984. All other annotations are as in Fig. 2.

quence en bloc with *Bam*HI increased the transfection efficiency 5- to 10-fold, and the gel-purified viral *Bam*HI fragment was nearly as infectious as monomer RF. Interestingly, when this *Bam*HI fragment was ligated at a low DNA concentration to generate a mixture containing about 70% MVM circles, the transfection efficiency went up a further threefold. Viral stocks grown from individual plasmid-derived plaques contained high titers of the hemagglutinin characteristic of MVM virions and induced characteristic MVM-specific nuclear capsid antigens in A9 mouse cells (data not shown). The viral DNA synthesized in A9 cells by four of these independent stocks was analyzed as shown in Fig. 2. The sizes and patterns of both viral RF and its *Eco*RI digestion products, most significantly the RF terminal conformers, are identical for DNA induced by these four plasmid-derived virus stocks and authentic MVM virions. Thus, plaque formation appears to arise by the exact excision of viral DNA sequences from the plasmid, generating infectious virus.

To examine the transition of viral sequences from a colinear insert in the plasmid to a free-replicating linear RF molecule, we examined by Southern blot analysis intracellular plasmid and

viral DNAs at various times after infection. The total amount of plasmid DNA present in transfected cells decreased rapidly over the first 2 days, with a more rapid decrease in form I DNA relative to forms II and III (Fig. 3A). This suggests that the MVM genome does not contain an origin of DNA replication capable of driving extensive plasmid replication or, that if it does, use of this origin is inhibited by a sequence within pBR322, as has been found for simian virus 40-pBR322 chimeras (19). The first significant change in the pattern of intracellular DNA was the appearance (by 52 h), of a band corresponding to monomer RF DNA. Figure 3B shows an analysis of the DNA which had accumulated in transfected cells after 7 days. By this time, monolayers transfected with infectious clones pMM91 and pMM984 or with viral RF monomer DNA contained abundant copies of monomeric and multimeric species of RF DNA, with typical *Eco*RI digestion patterns, whereas little MVM-containing DNA could be detected in monolayers transfected with incomplete clones pMM842 and pPT206. The abundant viral DNA was almost certainly due to secondary infection by the virions produced by the initially transfected cells. After longer exposures, plas-

mid DNA was observed in the pMM610- and pPT206-transfected cells at copy numbers of <1 per cell. When this DNA was digested with *EcoRI*, no bands corresponding to the left- or right-hand monomer RF termini which were excised from pBR322 sequences could be detected in either case, even though pPT206 and pMM842 contain a complete copy of the left-hand and right-hand end, respectively. This finding demonstrates that excision and replication as a linear molecule are dependent on both terminal sequences being present in the same molecule and that, in the absence of either, no other plasmid or viral sequence can substitute. This requirement was best documented by the failure of pPT206 to form replicating linear molecules, because this construct lacks only 180 base pairs of the right-hand terminus, all of which are outside any known coding sequence (Astell et al. in press). The importance of the extreme terminal sequences in linear replication is emphasized by our recent finding (M. J. Merchlinsky and D. C. Ward, unpublished data) that the introduction of a large deletion into the capsid protein-coding sequence of pMM984, although abolishing infectivity, does not adversely affect the ability of the viral insert to be excised and replicated as a linear RF molecule. The mechanism of excision and the roles, if any, played by viral proteins, are the subject of current experiments.

We thank Lynn Enquist for valuable discussion and help with the λ dam cloning attempts and Nigel Grindley for the gift of pNG39 DNA.

This work was supported by Public Health Service grants GM-20124 and CA-16038 to D.C.W. and CA-29303 to P.J.T. from the National Institutes of Health.

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