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The minute virus of mice (MVM) nonstructural protein NS1 induces nicking of MVM DNA at a unique site of the right-end telomere in both hairpin and duplex conformations *in vitro*

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The right-end telomere of replicative form (RF) DNA of the autonomous parvovirus minute virus of mice (MVM) consists of a sequence that is self-complementary except for a three nucleotide loop around the axis of symmetry and an interior bulge of three unpaired nucleotides on one strand (designated the right-end 'bubble'). This right-end inverted repeat can exist in the form of a folded-back strand (hairpin conformation) or in an extended form, base-paired to a copy strand (duplex conformation). We recently reported that the right-end telomere is processed in an A9 cell extract supplemented with the MVM nonstructural protein NS1. This processing is shown here to result from the NS1-dependent nicking of the complementary strand at a unique position 21 nt inboard of the folded-back genomic 5′ end. DNA species terminating in duplex or hairpin configurations, or in a mutated structure that has lost the right-end bulge, are all cleaved in the presence of NS1, indicating that features distinguishing these structures are not prerequisites for nicking under the *in vitro* conditions tested. Cleavage of the hairpin structure is followed by strand-displacement synthesis, generating the right-end duplex conformation, while processing of the duplex structure leads to the release of free right-end telomeres. In the majority of molecules, displacement synthesis at the right terminus stops a few nucleotides before reaching the end of the template strand, possibly due to NS1 which is covalently bound to this end. A fraction of the right-end duplex product undergoes melting and re-folding into hairpin structures (formation of a 'rabbit-ear' structure).

Introduction

Minute virus of mice (MVM) is a member of the genus *Parvovirus* of the family *Parvoviridae* (Siegl *et al.*, 1985). The MVM genome contained in purified virions (Cotmore & Tattersall, 1989) is linear single-stranded DNA, 5149 nt in length (Astell *et al.*, 1986), terminating in short self-complementary sequences that fold into stable hairpin structures (Bourguignon *et al.*, 1976). Early in infection, the incoming genome is converted into a double-stranded replicative form (RF) by extension of the 3′ (left-end) hairpin until the growing strand reaches the folded-back 5′ terminus at the genomic right end (Tattersall *et al.*, 1973). A subsequent ligation step was proposed resulting in a covalently closed DNA (cRF) (Cotmore & Tattersall, 1987), a form which has been detected in parvovirus-infected cells (Cotmore *et al.*, 1989; Löchelt *et al.*, 1989) and which was recently generated *in vitro* (Baldauf *et al.*, 1997). RF amplification is assumed to involve opening of cRF at its right end by the major MVM nonstructural protein, NS1, followed by displacement and copying of the right-end telomere (Astell *et al.*, 1985). Melting of the extended duplex terminus thus created and re-formation of hairpin structures are thought to provide a primer for strand-displacement synthesis leading to the formation of dimer RF DNA (Cotmore & Tattersall, 1987; Baldauf *et al.*, 1997; Cossons *et al.*, 1996). In agreement with this model, dimer-length as well as higher order concatemeric molecules have been isolated from parvovirus-infected cells (Ward & Dadachanji, 1978).

We have recently developed an *in vitro* DNA replication system that is based on mouse A9 cell extract and supports nicking of the right-end hairpin of MVM cRF DNA when supplemented with recombinant baculovirus-produced MVM NS1. Nicking is followed by the generation of a molecule

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extended at its right end and designated 5′eRF, given that the RF right end corresponds to the genomic 5′ terminus. We noted that the 5′eRF species is a target for further NS1-induced nicking at the right-end inverted repeat, indicating that this reaction does not require the substrate to have a covalently closed hairpin structure. However, the efficiency of NS1-mediated nicking of the MVM right end may still depend on its secondary structure. This possibility is raised by recent *in vivo* data obtained with a mutant MVM virus, designated MVMx, in which the three nucleotides normally unpaired within the right-end hairpin are fully base-paired, eliminating the bulge from this position (Costello *et al.*, 1995). During infection of A9 cells, the ratio of duplex to hairpin RF right ends was found to be significantly lower for the MVMx virus as compared to MVMwt. This may indicate that the NS1-mediated processing of the right-end hairpin into the duplex form is somewhat impaired in the absence of the bulge, implying that a hairpin, complete with bulge, is a more favourable substrate for NS1 than the extended duplex structure, since the latter contains no bulge. A preference for the hairpin structure may minimize the reported nicking of the extended right end of 5′eRF molecules (Baldauf *et al.*, 1997), a process whose usefulness is not evident and which leads to the release of free right-end telomeres.

In the present study, we tested the influence of the bulge on NS1-induced *in vitro* processing of the right-end telomere of MVM RF DNA. Nicking and extension of this telomere were found to occur with a similar efficiency, irrespective of the presence of the bulge. This result and our previous analysis of *in vitro* replication of cRF and 5′eRF molecules (Baldauf *et al.*, 1997) indicate that neither the covalently closed structure nor the bulge are prerequisites for the NS1-mediated resolution of the right end of replicative forms.

Methods

■ **Cultivation of cells, preparation of extracts and production of recombinant NS1.** Mouse A9 cells were grown in suspension and processed for the preparation of cytosolic extracts as previously described (Baldauf *et al.*, 1997). Nuclear extracts were prepared according to Dignam *et al.* (1983). NS1 was produced in Sf9 insect cells infected with recombinant baculovirus (kindly provided by D. Pintel) and purified by affinity chromatography as previously reported (Baldauf *et al.*, 1997).

■ **DNA templates.** A9 cells were infected with the prototype strain of minute virus of mice (MVMp) or the mutant form, MVMx, and RF DNA was extracted according to the Hirt method (Hirt, 1967) modified as previously described (Baldauf *et al.*, 1997). RF DNA templates were purified on neutral and alkaline sucrose gradients (Straus *et al.*, 1976).

■ ***In vitro* replication and analysis of product DNA.** *In vitro* replication was carried out as reported (Baldauf *et al.*, 1997) except for the use of a mixture of cytosolic and nuclear extract (60 μg and 30 μg respectively of total protein per reaction) instead of pure cytosolic extract. Purified baculovirus-expressed NS1 was added where indicated. Replication products were analysed either directly, or after restriction digestion, on 5% polyacrylamide gels. Analysis under denaturing conditions was performed in 6% polyacrylamide–7 M urea gels which were run at 72 °C to ensure complete denaturation of hairpin segments. Size markers consisted of a yeast DNA ladder obtained according to the method of Sanger *et al.* (1977) or Maxam–Gilbert sequencing products (Maxam & Gilbert, 1980) of a right-end MVM DNA fragment (nt 4916–5068).

Results

Copying and segregation of the MVM right-end hairpin

We recently presented an *in vitro* replication assay that measures specific nicking of the right-end hairpin of MVM cRF DNA by recombinant NS1 followed by DNA extension at the nick site (Baldauf *et al.*, 1997). These events, which are schematically depicted in Fig. 1 (centre portion), were demonstrated by cleavage of the *in vitro*-labelled replication product with *Psh*AI at nt 4916 followed by fractionation of the DNA fragments on a polyacrylamide gel (Baldauf *et al.*, 1997). A representative experiment of this type is illustrated in Fig. 2(A), lanes 1 and 2. MVM cRF DNA used as a template gave rise to a major labelled DNA species, designated E, and two minor species, designated (T + H)1 and (T + H)2.

(i) Species E migrated at the position expected for the right-end *Psh*AI fragment of extended duplex RF DNA. This was confirmed by further digestion of gel-purified band E with the restriction enzyme *Afl*III (Fig. 2B, lanes 1 and 2). *Afl*III cuts MVM DNA at nt 4984 and 5104, generating three segments from the right-end extended *Psh*AI fragment (marked a, b and c in Fig. 1, centre portion). *Afl*III subfragments were found at the anticipated positions of left-hand (70 bp) and internal (120 bp) segments b and a, respectively, while the third subfragment migrated around the 68 bp position and thus was slightly retarded in comparison with the 61/64 bp long right-end segment c expected from the reported size of *in vivo*-synthesized extended RF DNA (Astell *et al.*, 1985; Cotmore & Tattersall, 1989). This retardation may be due to proteinase K-resistant residues of the NS1 protein which is known to become covalently attached to the 5′ ends of *in vivo* (Cotmore & Tattersall, 1988) and *in vitro* (Baldauf *et al.*, 1997) processed RF molecules.

(ii) The identity of the faster migrating species (T + H)1 (Fig. 2A, lane 2) was also investigated by gel purification and digestion with *Afl*III. For reasons to be explained below, this DNA species is actually made up of two components that could not be resolved in the gel shown in Fig. 2(A), hence the designation (T + H). As illustrated in Fig. 2(B), lanes 3 and 4, *Afl*III cleaved (T + H)1 DNA into two major fragments, one of which comigrated with the E segment b (70 bp) while the other one ran ahead of it. This is in line with the assumption that the main component of species (T + H)1 consists of the right-end *Psh*AI fragment in the turn-around configuration, since *Afl*III digestion of this fragment should generate segment b and a smaller turn-around segment, marked a′ in Fig. 1, right-hand part. The identification of species T1 with a turn-around right-end *Psh*AI fragment was confirmed by the denaturing gel

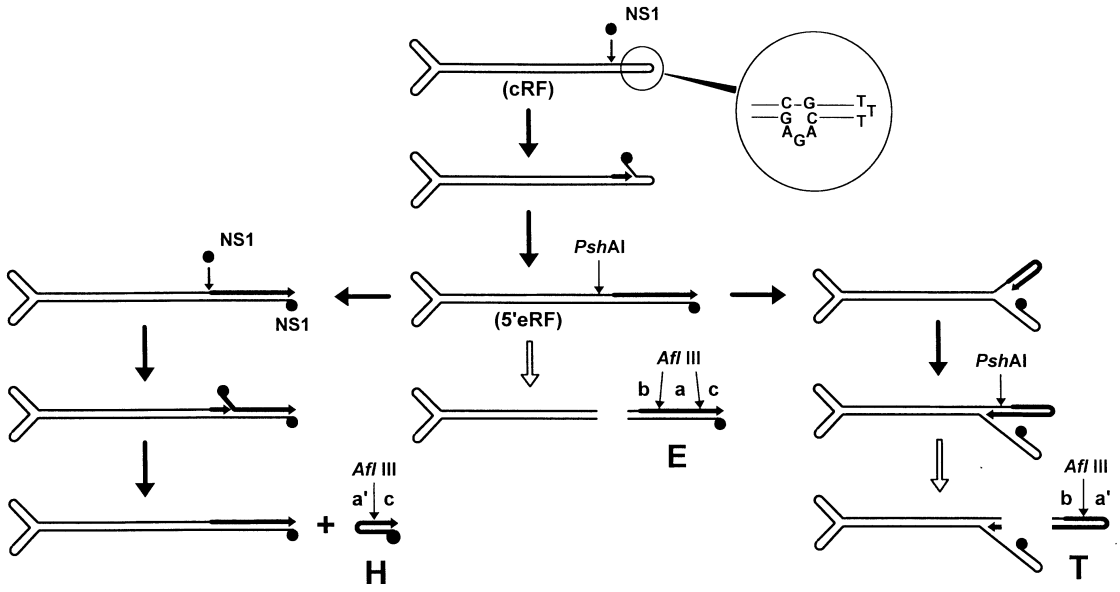

Fig. 1. Proposed scheme for replication reactions occurring at the right terminus of MVM RF DNA. The 3′-hydroxyl primer is symbolized by a small arrow. NS1 is depicted as a solid circle. Thin and heavy lines represent parental and newly synthesized DNA, respectively. H denotes segregated right-end hairpin DNA, while E and T designate *Psh*AI-generated right-end extended and turn-around fragments. *Afl*III-derived DNA segments were named a, b, c and a′. Recognition sites for *Psh*AI and *Afl*III are shown. The *Psh*AI digestion involved in T production occurs after primer elongation up to the restriction site. See text for details.

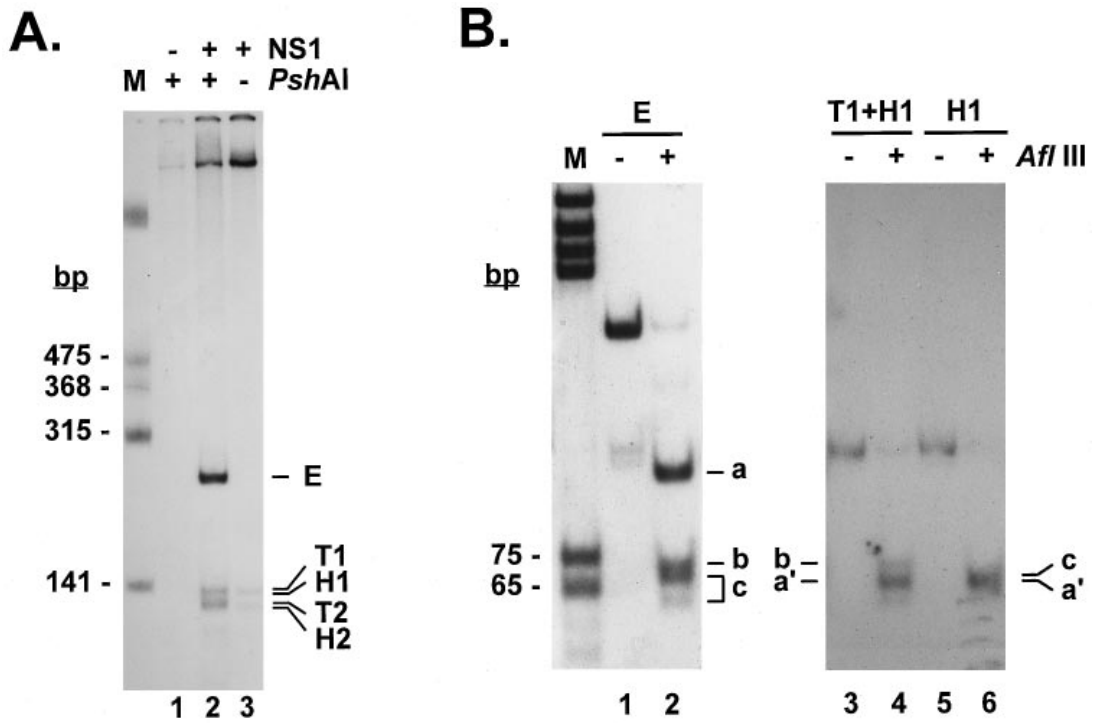

Fig. 2. Resolution of cRF DNA at its right end. (A) cRF DNA was incubated in A9 extract in the absence (lane 1) or presence (lanes 2 and 3) of NS1. Replication products were analysed directly (lane 3) or after digestion with *Psh*AI (lanes 1 and 2) on a neutral 5% polyacrylamide gel. Lane M shows the migration of 3′ end-labelled *Taq*I restriction fragments of pBR322 DNA used as size markers. (B) Gel-purified E (T+H)1 and H1 DNA (see Fig. 1) were fractionated as in panel (A) with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) previous digestion with *Afl*III. A *Hin*fI digest of pGEM-5 DNA was run under M. The size of marker fragments in base-pairs is indicated.

analysis illustrated below. Labelling of the T1 species was unlikely to result from repair synthesis taking place on the input cRF template, since detection of this fragment was dependent on NS1 (Fig. 2A, compare lanes 1 and 2). Furthermore, the level of unspecific labelling in the assay was low. Therefore, these data were interpreted in terms of the mechanism depicted in Fig. 1 (right-hand part), i.e. a fraction of copied right-end telomeres was folded back into the 'rabbit-eared' structure and was extended at least past the *Psh*AI site.

(iii) Labelled DNA products were also detected at the (T+H)1 position in the absence of *Psh*AI digestion, although to a lesser extent (Fig. 2A, lane 3). Digestion of this DNA with *Afl*III gave rise to one broad band that comigrated with segments c and a′ (Fig. 2B, lanes 5 and 6). As depicted in Fig. 1 (left-hand part), this was the pattern expected from free right-end telomeres that would fold back into a hairpin structure after being displaced from RF DNA through secondary rounds of NS1-induced nicking and extension reactions (Baldauf *et al.*, 1997). Accordingly, this DNA species was designated H1 for hairpin. The H1-comigrating band from the *Psh*AI-treated sample (Fig. 2B, lane 3) must also contain H1 besides T1, hence its above designation (T+H)1. At the gel resolution level, the H1 and T1 species could not be separated, in keeping with the fact that the NS1 and *Psh*AI cleavage sites defining their respective free ends are only a few nucleotides apart.

(iv) In addition to T1/H1 DNA, faster-migrating species were detected at the T2/H2 position (Fig. 2A, lanes 2 and 3). The analysis of the (T+H)2 and H2 species gave essentially the same results as with (T+H)1 and H1 DNA, respectively, except for a small difference in the migration of the turn-around *Afl*III fragment a′ (data not shown). This difference could be ascribed to a structural modification due to the repair of the right-end bulge on the hairpin stem (see below). Therefore, the T1/T2 and H1/H2 species are referred to collectively as T and H in Fig. 1, respectively.

Structural requirements for right-end telomere nicking and extension

The conclusion that *in vitro*-produced duplex right termini may be re-nicked and re-extended in the presence of NS1 (as illustrated in Fig. 1, left-hand part) implies that the closed right-end hairpin structure of cRF is not a prerequisite for these events. This was ascertained by showing that 5′eRF DNA indeed served as a substrate for NS1-induced strand-displacement synthesis at the right-end telomere, as measured by the appearance of labelled E and H fragments during analysis of *Psh*AI-digested or undigested product DNA (Fig. 3, lanes 2 and 5). Given that re-nicking of the extended product of *in vitro*-processed cRF DNA is a rare event (compare the relative intensities of bands H and E in Fig. 2A, lanes 3 and 2, respectively), the efficiencies of cRF versus 5′eRF cleavage and extension could be assessed from the labelling of E and H species with both types of substrates. As illustrated in Fig. 3, the duplex right terminus and the closed hairpin telomere were

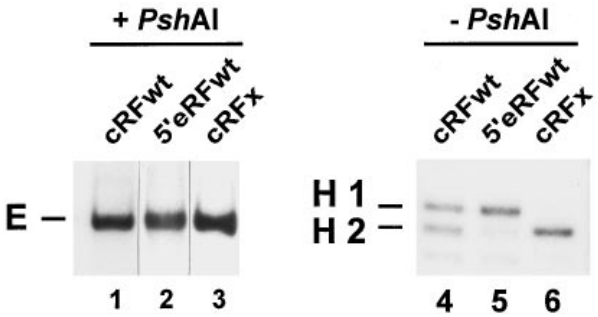

Fig. 3. Influence of the right-end structure on the initiation of MVM RF DNA replication. The right-end duplex intermediate (5′eRF) and bubble-containing (cRFwt) or bubble-less (cRFx) right-end hairpin molecules were compared for their ability to serve as substrates in *in vitro* strand-displacement synthesis initiated by NS-mediated nicking at the right-end telomere. Products were analysed on neutral 5% polyacrylamide gels as in Fig. 2 either directly (lanes 4–6) or after digestion with *Psh*AI (lanes 1–3). Only the E and H regions of the gels are shown.

processed with similar efficiency, producing comparable amounts of the *in vitro*-extended *Psh*AI fragment (lane 2 versus 1) and displaced hairpin DNA (lane 5 versus 4). Therefore, the hairpin structure appeared to have little influence on the capacity of the right-end telomere to undergo NS1-dependent nicking and extension under the *in vitro* conditions tested. The variable observance of hairpin products (H1 or H2) for different templates is discussed in detail below.

The mutant virus MVMx was produced by insertion of three complementary nucleotides opposite the unpaired nt 5024–5026 of MVMp, eliminating the bulge within the right-end hairpin (Costello *et al.*, 1995). During infection of A9 cells, replication of the bulge-less MVMx DNA was impaired as compared to the replication of wt MVMp DNA (Costello *et al.*, 1995), raising the possibility that the bulge controls in *cis* the processing of the right-end telomere. To directly test this possibility we compared cRF templates from MVMwt- and MVMx-infected cells for their competence to be nicked and extended in the presence of NS1. As is apparent in Fig. 3 from the labelling of either E (lanes 1 and 3) or H (lanes 4 and 6) DNA, no impairment of right-terminal processing was observed in the absence of the bulge. Therefore, the *in vitro* assay used revealed no evidence for a functional role of the bulge in nicking and extension of the right-end telomere.

Structural analysis of replication products

As stated above, the hairpin products released from *in vitro*-processed cRFwt DNA migrated as two discrete species denoted H1 and H2 (Fig. 3, lane 4). In contrast, similarly assayed 5′eRFwt and cRFx substrates each yielded a single major low-molecular-mass product that comigrated with H1 or H2, respectively (Fig. 3, lanes 5 and 6). The various H DNA products were further characterized to understand the reason for the heterogeneity. Gel-purified band H1 (from cRFwt and 5′eRFwt templates) and H2 (from cRFwt and cRFx templates) were analysed under denaturing conditions on a

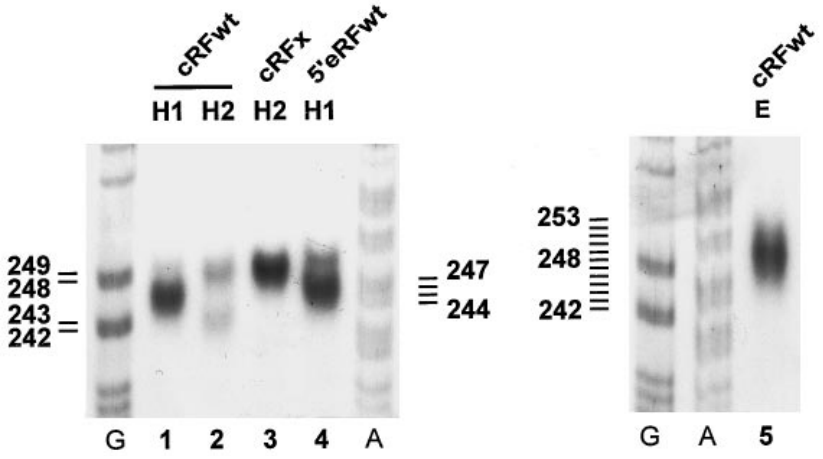

Fig. 4. Determination of the size of *in vitro*-processed right-end telomeres. H1 and H2 designate right-end telomeres released during *in vitro* strand-displacement synthesis from cRF or 5′eRF templates isolated from cells infected with wild-type or mutant (x) MVM (lanes 1–4). E corresponds to the extended right-end *Psh*AI fragment of duplex DNA generated *in vitro* from cRFwt template (lane 5). DNA species were purified on neutral 5% polyacrylamide gels as in Fig. 2, recovered by electroelution and further analysed on a 6% polyacrylamide–urea sequencing gel. G and A tracts of yeast DNA sequencing reactions were run as size markers (lanes G and A respectively). The sizes in nucleotides are indicated.

polyacrylamide–urea gel. The H1 species released during the processing of both cRFwt and 5′eRF wt migrated as a broad spot corresponding to a size of 244–249 nt (Fig. 4, lanes 1 and 4). Notwithstanding the heterogeneity, this length agreed with the identity of H1 as a displaced right-end telomere (see Fig. 1, left-hand part). The H2 product of cRFwt DNA replication was distinguished by the fact that it was resolved into two spots bracketing the H1 species and corresponding to an average length difference of six nucleotides (Fig. 4, lane 2). This raised the possibility that H2 DNA resulted from an *in vitro* mismatch-correction process (Umar *et al.*, 1994) which eliminated the bubble by inserting (upper spot) or deleting (lower spot) three nucleotides. Consistent with this hypothesis, the H2 upper spot comigrated, under denaturing conditions, with the cRFx segregation product, in which the bulge was removed by a three nucleotide insertion (Fig. 4, lanes 2 and 3). Furthermore, the slower migration of the H1 species in comparison with H2 molecules originating from both cRFwt and cRFx under neutral conditions (Fig. 3, lanes 4 and 6) fits with the known electrophoretic retardation of mismatch-containing versus fully base-paired double-stranded DNA fragments (Costello *et al.*, 1995; Lilley, 1995). It is also worth noting that most hairpins released from 5′eRF templates were of the H1 type (Fig. 3, lane 5; Fig. 4, lane 4), in agreement with the fact that the bulge is not present and thus cannot be eliminated in the extended telomere. The right-end E fragment resulting from *Psh*AI digestion of *in vitro*-extended cRFwt templates was also gel-purified and analysed under denaturing conditions (Fig. 4, lane 5). Labelled E strands showed the same length heterogeneity as H1 products (see below) but were of an average longer size, consistent with the position of the *Psh*AI restriction site a few nucleotides inboard of the right-end telomere (see Fig. 1).

Mapping of the NS1 nick site

The heterogeneity of the E and H species observed by gel electrophoresis under denaturing conditions may result from NS1-mediated nicking of the right-end telomere at different positions, thus giving rise to terminal extensions of various lengths. Alternatively, NS1 may nick the right-end telomere at a single site, but extension of the complementary strand fails to go to completeness. This premature arrest of extension may result from steric hindrance due to the NS1 protein which is covalently linked to the 5′ ends of RF molecules produced *in vivo* (Cotmore & Tattersall, 1988) and *in vitro* (Baldauf *et al.*, 1997). This possibility was tested by determining whether gel-purified native E and H1 DNA produced from cRF template contained recessed 3′ ends which could be filled-in by means of the Klenow fragment of *E. coli* DNA polymerase I, knowing that bound NS1 was mostly removed during the proteinase K digestion step of DNA isolation. As a control, a 220 bp long *Ava*II-generated pGEM DNA fragment having 3′-recessed ends was 5′-end labelled, gel-purified and treated with Klenow polymerase in the same way. Untreated and Klenow enzyme-treated samples were analysed in a sequencing gel along with yeast DNA ladders. As shown in Fig. 5(A), lanes 1 and 2, incubation of the control pGEM DNA fragment with Klenow polymerase resulted in a size shift expected for the filling of the *Ava*II-generated recessed ends. Similarly, the Klenow enzyme treatment converted the heterogeneous E and H1 patterns into single bands migrating on the high-molecular-mass edge of the respective original smears (Fig. 5A, lanes 3–6). We conclude that the NS1-induced cleavage of the right-end telomere is site-specific, but that the ensuing extension reaction is incomplete except for a small (yet detectable) minority of molecules (Fig. 5A, lanes 3 and 5).

In order to map the NS1 nick site, the gel-purified H1 species produced from a 5′eRF template was digested with *Afl*III and subsequently treated with Klenow polymerase. This procedure was expected to yield a DNA fragment extending from the NS1 to the *Afl*III cleavage sites in which any recessed ends, either caused by incomplete extension or asymmetric *Afl*III digestion, were filled-in by the Klenow enzyme. This sample was analysed by electrophoresis through a

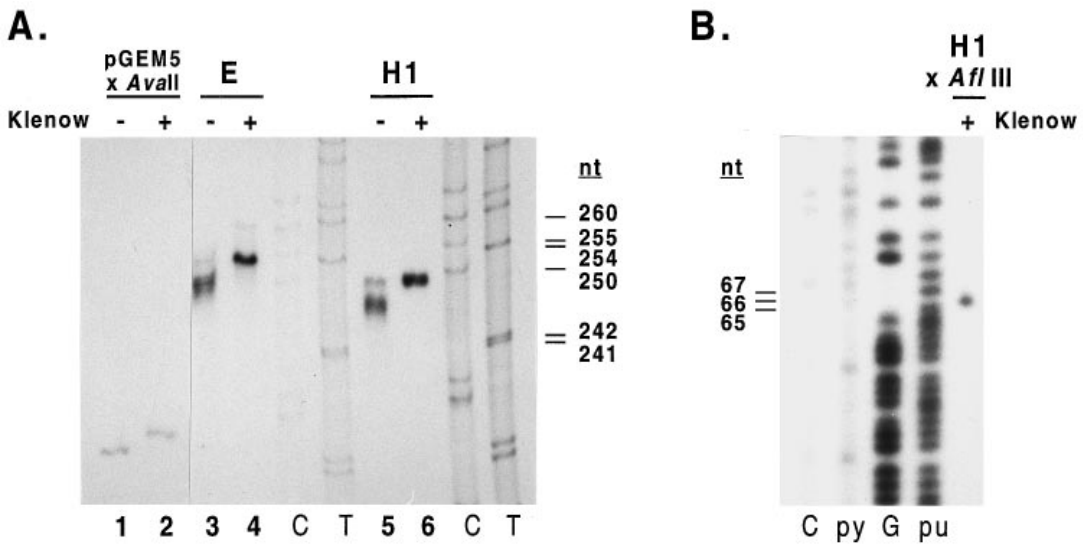

Fig. 5. Completion of the extension of *in vitro*-processed right-end telomeres. (A) Duplex RF right-end *Psh*AI restriction fragment (E, lanes 3 and 4) and displaced right-end hairpin DNA (H1, lanes 5 and 6) were obtained *in vitro* from cRFwt template, purified on a neutral 5% polyacrylamide gel as in Fig. 2 and recovered by electroelution. DNA species were treated (+) or not (−) with Klenow polymerase and analysed on a 6% polyacrylamide–urea sequencing gel. As a control, the *Ava*II digestion product of pGEM-5 DNA, which has 3′-recessed termini, was treated in the same way (lanes 1 and 2). C and T tracts of yeast DNA sequencing reactions were run as size markers, with the lengths (in nucleotides) indicated on the right. (B) Purified H1 DNA was digested with *Afl*III, treated with Klenow polymerase and analysed in a sequencing gel as described for panel (A). Maxam and Gilbert sequencing products of the MVM p98 *Psh*AI–*Sal*I fragment (nt 4916–5068) were used as size markers.

Fig. 6. Schematic representation of functional elements within the MVM DNA right-end telomere. The position of the NS1 nick site, as deduced from the sizes of DNA species analysed in Fig. 5, is indicated relative to the MVM 5′-terminal genomic hairpin. Nucleotide numbering is according to Astell *et al*. (1985). Possible NS1 binding sites (open boxes; Cotmore *et al*., 1995) and the NS1 nick site consensus (shaded box; Cotmore & Tattersall, 1994) are shown.

polyacrylamide–urea gel along with Maxam–Gilbert sequencing products from a *Psh*AI–*Sal*I fragment (nt 4916–5068) of the MVM infectious DNA clone p98 (Antonietti *et al*., 1988). According to these size markers, the NS1-*Afl*III H1 fragment was 66 nt in length (Fig. 5B), corresponding to an NS1 nick site located 21 nt inboard of the folded-back genomic 5′ end. A 66 nt long oligodeoxynucleotide synthesized according to the MVM sequence from this position to the filled-in *Afl*III site (nt 4922–4988) was found to comigrate with the fragment above, confirming the determination of the nick site (data not shown). A nick site at this position fits with the length of 254 nt determined for the full-length *Psh*AI-produced E fragment analysed in Fig. 5(A) and predicts a size of 248 nt for the full-length H1 strand. Actually, the H1 DNA species was slightly retarded and migrated at the 249 nt position (Fig. 5A), which is likely to be due to the residual amino acids of the NS1 protein covalently bound to the 5′ end and not totally removed by the proteinase K treatment (see Fig. 1 and Baldauf *et al*., 1997). Similarly, a faint band could be detected at the 67 nt position after overexposure of Fig. 5(B) (data not shown), and may correspond to the amino acid-bound strand of the NS1-*Afl*III H1 fragment. This is in line with the fact that only the 66 nt band was detected in *Afl*III-digested E DNA produced from the cRF template (data not shown), in keeping with the lack of association of the labelled E DNA strand with NS1 (see Fig. 1). Thus, the data of Fig. 5(A, B) both indicate NS1-mediated nicking of MVM DNA at a unique position 21 nt inboard of

the folded-back genomic 5′ end, i.e. between T and A opposite nt 4922 and 4923, as depicted in Fig. 6.

Discussion

We recently reported that the MVM double-stranded DNA intermediate that is covalently closed at both ends (cRF) is specifically resolved at the right terminus when incubated in mouse A9 cell extract supplemented with the MVM non-structural protein NS1. This resolution involves the NS1-induced nicking of the right-end hairpin and subsequent elongation of the primer thus created, leading to the formation of a right-hand extended DNA molecule (5′eRF). Furthermore, previous data (Baldauf *et al*., 1997) substantiated in the present work indicate that 5′eRF DNA can be re-nicked by NS1 and undergoes (an) additional round(s) of strand-displacement synthesis resulting in the release of free right-end telomeres which fold back into the hairpin configuration (H DNA). This shedding of H DNA from RF molecules seems futile for the virus and is possibly minimized under *in vivo* conditions as a result of the structural transformation of the extended right-end telomere into a 'rabbit-eared' configuration allowing further strand elongation and production of multimeric intermediates (Cotmore & Tattersall, 1987; Ward & Dadachanji, 1978). The 5′eRF DNA restriction pattern presented in this work also provides evidence for 'rabbit-eared' formation at a low frequency *in vitro*.

The investigation of the role of the hairpin in the NS1-dependent processing of the right-end telomere is complicated by the fact that (i) re-nicking events might mask original differences in the affinity of NS1 towards distinct DNA structures and (ii) 'rabbit-ear' formation within a portion of terminally extended duplex molecules recreates a hairpin substrate for NS1-induced events. However, a measurement of the relative levels of precursor incorporation into released H DNA versus extended right-end telomeres (*Psh*AI fragment E) indicated that secondary rounds of NS1-mediated nicking and extension were infrequent. Furthermore, only a minor fraction of 5′eRF was found to become rearranged into the 'rabbit-eared' structure as apparent from the relative amounts of *Psh*AI right-end turn-around (T) versus extended (E) fragments in Fig. 2. Therefore, it seemed justified to draw a conclusion from the comparison of cRF and 5′eRF substrates as to the influence of the DNA right-end structure on NS1-induced processing.

Similar amounts of radiolabelled right-end extension products were detected irrespective of whether cRF or 5′eRF was used as a template, arguing against a major effect of the hairpin on the sensitivity of the right terminus to NS1-dependent nicking and extension. This was additionally supported by the fact that cRF DNA used as template, and therefore present in high excess over the small amount of 5′eRF product formed during the reaction (see above), did not prevent the occurrence of secondary rounds of cleavage and displacement synthesis from extended product molecules. Strand-displacement synthesis at the right terminus as monitored in the present assay requires DNA nicking followed by primer extension. Therefore, it cannot be ruled out that a difference between hairpin and extended substrates in the first step still exists but is masked by the limiting availability of (a) cellular factor(s) involved in the second step. This possibility is unlikely, however, since the extent of right-end telomere processing was found to increase in proportion to the amount of both template DNA and NS1 for a given extract concentration (data not shown). It should also be mentioned that the reactivity of DNA substrates present in the *in vitro* assay might be modulated by their binding to cellular extract proteins. A dependence of protein binding upon DNA conformation might therefore result in structure-dependent variations in replication efficiencies. However, varying the concentration of cellular proteins in the *in vitro* assay was found to influence the reactivity of both kinds of substrates in a comparable way. This argues against a major effect of the DNA conformation upon protein-mediated modulation of substrate reactivity (data not shown).

Altogether, these results support the conclusion that NS1 drives the resolution of MVM hairpin and extended right-end telomeres with similar efficiency. This contrasts with recent reports concerning the resolution activity of the equivalent nonstructural proteins Rep 68/78 encoded by adeno-associated virus (AAV), a helper-dependent genus of the *Parvoviridae*. Indeed, the hairpin structure was found to be a preferred target, in comparison with the extended form, for Rep binding to the AAV inverted terminal repeat (Ashktorab & Srivasta, 1989; Im & Muzyczka, 1989; Ryan *et al*., 1996) and subsequent site-specific nicking (Snyder *et al*., 1993). It is worth noting in this respect that the cRF DNA of autonomous parvoviruses like MVM terminates in closed hairpins at both ends but that only the right-end hairpin is resolved in the presence of NS1, as recently demonstrated *in vitro* (Baldauf *et al*., 1997). This feature accounts for the fact that the right terminus of MVM DNA exists in two possible orientations (designated flip and flop), due to the transfer of the imperfect hairpin from one strand to the other (Rhode & Klaassen, 1982). In contrast, the left terminus (comprising the genomic 3′ end) is found in one orientation only (Astell *et al*., 1985), because its resolution by NS1 does not take place in monomeric RF but at the level of the (3′–3′) bridge of head-to-head multimeric intermediates (Cotmore *et al*., 1993; Cotmore & Tattersall, 1994; Liu *et al*., 1994). The involvement of NS1 in both types of reactions may be related to its ability to act with similar efficiencies on hairpin and extended termini. Multimeric RF molecules are also detected in AAV-infected cells, yet their resolution may not involve the bridge region (Berns, 1990). This is consistent with the occurrence of sequence inversion at both ends of the AAV genome (Lusby *et al*., 1980).

The right-end hairpin of MVM DNA contains a three nucleotide bulge loop (the so-called 'bubble'). Bulge loops have been described as important functional elements within

DNA and RNA molecules (for a review see Lilley, 1995). The conservation of this right-terminal mismatch among a number of autonomous parvoviruses suggests that it constitutes a recognition element of a process involving the hairpin, given that the mismatch is present in the stem of the hairpin but not the right-end duplex configuration. The finding that bulge-less and natural right-end hairpins are resolved with similar efficiency in the presence of NS1 argues against an involvement of the bulge in the initiation of RF replication. Thus, the reduced amount of extended versus turn-around RF right termini, as observed in cells infected with the bubble-less mutant MVMx (Costello *et al*., 1995), may result from a role of the bulge in DNA replication step(s) subsequent to the initiation event analysed here, e.g. in gene expression or genome encapsidation. Encapsidation of the MVM viral strand is thought to be coupled with its release from monomeric RF DNA by strand displacement synthesis initiated at the right terminus (Müller & Siegl, 1983). The bulge loop on the right-end hairpin might therefore be involved in recognition of the displaced genome by preformed capsids. Experiments demonstrating MVM capsid binding to the genomic left end failed to reveal any interaction with the right-end hairpin (Willwand & Hirt, 1991). However, this result does not rule out that the bulge plays a role in encapsidation by inducing a structural change in the right-end telomere. It has been noted that inverted repeated sequences encompassing the bulge region may generate a cruciform structure within the right-end hairpin (Cotmore & Tattersall, 1987). Cruciform extrusion should be facilitated by the bulge which can be calculated to reduce the energy for the structural transition from 129·7 to 81·2 kJ/mol (Jaeger *et al*., 1989, 1990; Zuker, 1989). The cruciform configuration at the DNA right end may conceivably contribute to encapsidation, given its resemblance to the genomic left-end hairpin structure that was shown to interact with capsids (Willwand & Hirt, 1991).

The *in vitro*-resolved right terminus was analysed in order to map the NS1 nick site. This study revealed an unexpected size heterogeneity of *in vitro*-resolved right-end telomeres, ascribed to the fact that the displacement synthesis reaction does not always go to completion. This premature arrest of elongation is hypothesized to result from steric hindrance by the NS1 protein, which becomes covalently bound to the 5′ end of nicked DNA (Cotmore & Tattersall, 1988; Baldauf *et al*., 1997). In agreement with this possibility, the extension reaction could be completed in the presence of Klenow DNA polymerase on proteinase K-treated gel-purified product DNA. Occurrence *in vivo* of this premature arrest of right-end strand-displacement synthesis (see below) would not be detrimental, since the sequences within the inverted terminal repeat that are located 5′ to the axis of symmetry are repaired after hairpin refolding and further elongation. Taking this into consideration, the nick site was mapped 21 nt inboard of the folded-back genomic 5′ end. The position of the NS1 cleavage site has been inferred before from the analysis of extended RF molecules isolated from MVM-infected cells. According to the sequencing data of Astell *et al*. (1985), nicking was suggested to take place 18 nt inboard of the genomic hairpin. Yet, the *in vivo*-produced RF DNA showed size heterogeneity at the right end, in agreement with our *in vitro* results. The assignment of this heterogeneity to incomplete extension may account for the present location of the NS1 nick site three nucleotides further away from the right-end hairpin, after experimental completion of strand elongation. Furthermore, primer extension studies of RF DNA extracted from MVM-infected cells led Cotmore & Tattersall (1989) to localize the NS1-mediated nick site 21 nt from the folded-back genomic 5′ end, in line with the present determination.

As stated above, NS1 induces the nicking and resolution not only of the right-end (5′) telomere but also of the left-end (3′) inverted repeat duplicated in the form of a 3′–3′ bridge inside multimeric intermediates (Cotmore *et al*., 1993; Cotmore & Tattersall, 1994; Liu *et al*., 1994). The CTWWTCA sequence (where W designates A or T) was previously noticed in the vicinity of both the left-end and right-end inverted repeats of MVM DNA (Cotmore & Tattersall, 1994). The present work mapped the NS1-mediated cleavage of the right-end telomere between the second and third nucleotide of this sequence (depicted in Fig. 6), which is equivalent to the position determined by Cotmore & Tattersall (1994) for the major *in vitro* nick site of the 3′–3′ bridge. Cotmore *et al*. (1995) recently showed that NS1 interacts with ACCA repeats that are present in particular in the vicinity of the 3′–3′ nick site. Another NS1-binding element of somewhat lower affinity is the CAACCAA sequence (Cotmore *et al*., 1995). This motif is present both 3′ and 5′ to the NS1 nick site (Fig. 6). NS1 was shown to bind to given ACCA elements in an asymmetrical fashion, projecting more to the 5′ side than to the 3′ side of the binding motif (Cotmore *et al*., 1995). This would favour the usage of the CAACCAA element located 3′ to the nick site (Fig. 6) in the NS1-mediated cleavage reaction. We are currently conducting a mutational analysis of the right-end region of MVM DNA in order to assess the respective roles played by these elements in the NS1-directed resolution of right-end telomeres.

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References

Antonietti, J.-P., Sahli, R., Beard, P. & Hirt, B. (1988). Characterization of the cell type-specific determinant in the genome of minute virus of mice. *Journal of Virology* **62**, 552–557.

Ashktorab, H. & Srivasta, A. (1989). Identification of nuclear proteins that specifically interact with adeno-associated virus type 2 inverted terminal repeat hairpin DNA. *Journal of Virology* **63**, 3034–3039.

Astell, C. R., Chow, M. B. & Ward, D. C. (1985). Sequence analysis of the termini of virion and replicative forms of minute virus of mice DNA suggests a modified rolling hairpin model for autonomous parvovirus DNA replication. *Journal of Virology* **54**, 171–177.

Astell, C. R., Gardiner, E. M. & Tattersall, P. (1986). DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. *Journal of Virology* **57**, 656–669.

Baldauf, A., Willwand, K., Mumtsidu, E., Nüesch, J. & Rommelaere, J. (1997). Specific initiation of replication at the right-end telomere of the closed species of minute virus of mice replicative form DNA. *Journal of Virology* **71**, 971–980.

Berns, K. I. (1990). Parvovirus replication. *Microbiological Reviews* **54**, 316–329.

Bourguignon, G. J., Tattersall, P. J. & Ward, D. C. (1976). DNA of minute virus of mice: self-priming, nonpermuted, single-stranded genome with a 5′-terminal hairpin duplex. *Journal of Virology* **20**, 290–306.

Cossons, N., Faust, E. A. & Zannis-Hadjopoulos, M. (1996). DNA polymerase-dependent formation of a hairpin structure at the 5′ terminal palindrome of the minute virus of mice genome. *Virology* **216**, 258–264.

Costello, E., Sahli, R., Hirt, B. & Beard, P. (1995). The mismatched nucleotides in the 5′-terminal hairpin of minute virus of mice are required for efficient viral DNA replication. *Journal of Virology* **69**, 7489–7496.

Cotmore, S. F. & Tattersall, P. (1987). The autonomously replicating parvoviruses of vertebrates. *Advances in Virus Research* **33**, 91–173.

Cotmore, S. F. & Tattersall, P. (1988). The NS-1 polypeptide of minute virus of mice is covalently attached to the 5′ termini of duplex replicative-form DNA and progeny single strands. *Journal of Virology* **62**, 851–860.

Cotmore, S. F. & Tattersall, P. (1989). A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. *Journal of Virology* **63**, 3902–3911.

Cotmore, S. F. & Tattersall, P. (1994). An asymmetric nucleotide in the parvoviral 3′ hairpin directs segregation of a single active origin of DNA replication. *EMBO Journal* **13**, 4145–4152.

Cotmore, S. F., Gunther, M. & Tattersall, P. (1989). Evidence for a ligation step in the DNA replication of the autonomous parvovirus minute virus of mice. *Journal of Virology* **63**, 1002–1006.

Cotmore, S. F., Nüesch, J. P. F. & Tattersall, P. (1993). Asymmetric resolution of a parvovirus palindrome *in vitro*. *Journal of Virology* **67**, 1579–1589.

Cotmore, S. F., Christensen, J., Nüesch, J. P. & Tattersall, P. (1995). The NS1 polypeptide of the murine parvovirus minute virus of mice binds to DNA sequences containing the motif [ACCA]2-3. *Journal of Virology* **69**, 1652–1660.

Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Research* **11**, 1475–1489.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *Journal of Molecular Biology* **26**, 365–369.

Im, D.-S. & Muzyczka, N. (1989). Factors that bind to adeno-associated virus terminal repeats. *Journal of Virology* **63**, 3095–3104.

Jaeger, J. A., Turner, D. H. & Zuker, M. (1989). Improved predictions of secondary structures for RNA. *Proceedings of the National Academy of Sciences, USA* **86**, 7706–7710.

Jaeger, J. A., Turner, D. H. & Zuker, M. (1990). Predicting optimal and suboptimal secondary structure for RNA. *Methods in Enzymology* **183**, 281–306.

Lilley, D. M. J. (1995). Kinking of DNA and RNA by base bulges. *Proceedings of the National Academy of Sciences, USA* **92**, 7140–7142.

Liu, Q., Yong, C. B. & Astell, C. R. (1994). *In vitro* resolution of the dimer bridge of the minute virus of mice (MVM) genome supports the modified rolling hairpin model for MVM replication. *Virology* **201**, 251–262.

Löchelt, M., Delius, H. & Kaaden, O.-R. (1989). A novel replicative form DNA of Aleutian disease virus: the covalently closed linear DNA of the parvoviruses. *Journal of General Virology* **70**, 1105–1116.

Lusby, E., Fife, K. H. & Berns, K. I. (1980). Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *Journal of Virology* **34**, 402–409.

Maxam, A. M. & Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods in Enzymology* **65**, 499–560.

Müller, D.-E. & Siegl, G. (1983). Maturation of parvovirus LuIII in a subcellular system. I. Optimal conditions for *in vitro* synthesis and encapsidation of viral DNA. *Journal of General Virology* **64**, 1043–1054.

Rhode, S. L. & Klaassen, B. (1982). DNA sequence of the 5′-terminus containing the replication origin of parvovirus replicative form DNA. *Journal of Virology* **41**, 990–999.

Ryan, J. H., Zolotukhin, S. & Muzyczka, N. (1996). Sequence requirements for binding of Rep68 to the adeno-associated virus terminal repeats. *Journal of Virology* **70**, 1542–1553.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.

Straus, S. E., Sebring, E. D. & Rose, J. A. (1976). Concatemers of alternating plus and minus strands are intermediates in adeno-associated virus DNA synthesis. *Proceedings of the National Academy of Sciences, USA* **73**, 742–746.

Siegl, G., Bates, R. C., Berns, K. I., Carter, B. J., Kelly, D. C., Kurstak, E. & Tattersall, P. (1985). Characteristics and taxonomy of Parvoviridae. *Intervirology* **23**, 61–73.

Snyder, R. O., Im, D.-S., Ni, T., Xiao, X., Samulski, R. J. & Muzyczka, N. (1993). Features of the adeno-associated virus origin involved in substrate recognition by the viral Rep protein. *Journal of Virology* **67**, 6096–6104.

Tattersall, P., Crawford, L. V. & Shatkin, A. J. (1973). Replication of the parvovirus MVM. II. Isolation and characterization of intermediates in the replication of the viral deoxyribonucleic acid. *Journal of Virology* **12**, 1446–1456.

Umar, A., Boyer, J. C. & Kunkel, T. A. (1994). DNA loop repair by human cell extract. *Science* **266**, 814–816.

Ward, D. C. & Dadachanji, D. K. (1978). Replication of minute virus of mice DNA. In *Replication of Mammalian Parvoviruses*, pp. 297–313. Edited by D. C. Ward & P. Tattersall. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Willwand, K. & Hirt, B. (1991). The minute virus of mice capsid specifically recognizes the 3′ hairpin structure of the viral replicative-form DNA: mapping of the binding site by hydroxyl radical footprinting. *Journal of Virology* **65**, 4629–4635.

Zuker, M. (1989). On finding all suboptimal foldings of an RNA molecule. *Science* **244**, 48–52.

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