Specific interaction of the nonstructural protein NS1 of minute virus of mice (MVM) with [ACCA]$_2$ motifs in the centre of the right-end MVM DNA palindrome induces hairpin-primed viral DNA replication

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Minute virus of mice (MVM) is an autonomous member of the genus Parvovirus of the family Paroviridae (Siegl et al., 1985). MVM virions contain a linear single-stranded DNA genome of 5149 nt (Astell et al., 1985). Characteristically, short palindromic sequences at both DNA termini fold into stable hairpin structures and are indispensable for viral DNA replication (Astell et al., 1985, 1986; Berns, 1990; Bourguignon et al., 1976; Tattersall & Ward, 1976). The genomic left-end hairpin serves as a primer for complementary (c)-strand synthesis, giving rise to double-stranded (ds) replicative form (RF) DNA, which is subsequently closed at its right terminus through the activity of cellular ligases (Baldauf et al., 1997; Cotmore et al., 1999). This covalently closed DNA species, designated cRF DNA (Baldauf et al., 1997), serves as a substrate for sequence- and strand-specific nicking executed by the parvovirus replication protein NS1 (Cotmore & Tattersall, 1998; Cotmore et al., 2000; Willwand et al., 1997). NS1 nicks cRF DNA within the c-strand close to the right-end hairpin stem. The 3’-OH primer created is thus extended, leading to the unfolding of the hairpin in front of the replication machinery and concomitant copying of the right-end palindromic sequence (Berns, 1990). The resulting extended DNA molecule was designated 5’eRF DNA by Baldauf et al. (1997), referring to the fact that the genomic 5’ end is at the right terminus of the RF DNA. The further fate of 5’eRF DNA relies on a structural transition converting the right-end duplex palindrome into two hairpins, thus recreating a primer for strand-displacement synthesis (Berns, 1990; Cossons et al., 1996a, b; Tattersall & Ward, 1976; Willwand et al., 1998).

Unidirectional extension of this primer leads to the copying of the c-strand, while the original DNA, the v-strand, is displaced. Due to the covalent coupling of the original c- and v-strands at the left terminus, the displaced v-strand then serves as a template for the continuation of DNA synthesis, resulting in the formation of a left-to-left end dimer both in vivo (Tullis et al., 1994) and in vitro (Baldauf et al., 1997). In the further course of replication, the above-mentioned events, including NS1-mediated opening of the viral DNA at the right-end hairpin stem, formation of the palindromic duplex end, structural transition of the duplex palindrome into hairpins and extension of the hairpin primer, are thought to recur (Berns, 1990). This gives rise to higher order concatemers that are subsequently resolved into monomeric DNA molecules through the activity of NS1 in conjunction with cellular factors (Christensen et al., 1997; Cotmore et al., 1992, 1993).

Although the structural transition of the right-end palindrome from linear duplex to hairpin can be driven by cellular factors to a limited extent in vitro (Cossons et al., 1996a, b; Willwand et al., 1998), efficient processing requires the viral NS1 protein (Willwand et al., 1998). We recently proposed a molecular scheme for the structural transition process, involving the interaction of NS1 with [ACCA]$_2$ motifs in the DNA replication, as measured in a reconstituted in vitro replication system. Thus, [ACCA]$_2$ sequence motifs are essential as NS1-binding elements in the context of the structural transition of the right-end MVM palindrome.
right-end MVM DNA palindrome (Willwand et al., 1998). [ACCA]$_2$ motifs constitute specific NS1-binding elements (Cotmore et al., 1995) and might permit access of NS1 to the viral DNA terminus, thereby facilitating the local unwinding of the two DNA strands and their refolding into hairpins. Binding of NS1 to [ACCA]$_2$ motifs within the MVM right-end palindrome, including those depicted in Fig. 1(A), was reported recently (Cotmore et al., 2000). As part of an investigation into the importance of these motifs for the NS1-induced structural change in the MVM right-end palindrome, their binding affinities for NS1 were assessed further. Site-specific binding of NS1 to DNA was monitored according to Cotmore et al. (1995). Synthetic dsDNA oligodeoxynucleotides, in which the central region of the right-end palindrome was present in its wild-type (wt) form (wtAC) or harboured mutated [ACCA]$_2$ motifs (ΔAC2 or ΔAC1/2) (Fig. 1A), were 5’ end-labelled.
using the Klenow fragment of *Escherichia coli* polymerase I, 
$[^{32}P]dCTP$, gel-purified and used as binding substrates. The degree of end labelling was comparable for all three oligodeoxynucleotides, as measured by autoradiography after polyacrylamide gel electrophoresis (Fig. 1B, lanes 7–9). To analyse NS1-binding, the DNA substrates were incubated with recombinant NS1 followed by the addition of NS1-specific antibodies and immunoprecipitation of bound DNA (Cotmore *et al*., 1995). As apparent from Fig. 1(B), wtAC and AAC2 DNA were both efficiently immunoprecipitated in the presence of NS1 (Fig. 1B, lanes 3 and 5) but not in its absence (Fig. 1B, lanes 4 and 6). In contrast, only a small portion of the ΔAC1/2 mutant, devoid of [ACCA]$_2$ motifs, was recovered in the presence of NS1 (Fig. 1B, lane 1). Residual ΔAC1/2 precipitation is likely to result from the reported nonspecific DNA-binding capacity of NS1 (Cotmore *et al*., 1995). Altogether, these results are thus consistent with the specific binding of NS1 to wtAC and AAC2 via the [ACCA]$_2$ motif(s).

Assuming that the [ACCA]$_2$ motif mediates the specific binding of NS1 to the duplex right-end palindromic, one would predict that ΔAC2 DNA, which contains a single such element, interacts with a lower efficiency with NS1 as compared with wtAC DNA, which comprises three of these motifs. This could not be assessed from the above-mentioned binding experiments, which were performed with an excess amount of NS1. Therefore, competition-binding experiments were carried out to compare the capacities of unlabelled wtAC and AAC2 DNA for inhibiting the specific interaction of NS1 with radiolabelled probes. As illustrated in Fig. 1(C), binding of NS1 to wtAC DNA (Fig. 1C, lane 1) was efficiently inhibited by a 30-fold molar excess of unlabelled wtAC (Fig. 1C, lane 2), while it was only reduced in part by the same amount of ΔAC2 (Fig. 1C, lane 3). Similarly, binding of NS1 to radiolabelled AAC2 DNA (Fig. 1C, lane 4) was more efficiently competed by wtAC (Fig. 1C, lane 5) than by ΔAC2 DNA (Fig. 1C, lane 6). Altogether, these binding and competition studies confirm the specificity of NS1 interaction with [ACCA]$_2$ motifs in the central part of the right-end palindrome.

As depicted in Fig. 2(A), the duplex-to-hairpin transition occurring at the MVM right-end palindromic has been assumed to be facilitated as a result of NS1-binding to [ACCA]$_2$ motifs on both sides of the axis of symmetry of the extended duplex palindromic, followed by unwinding of the c-strand DNA, cruciformation, branch migration and hairpin building (Willwand *et al*., 1998). This model predicts that elimination of [ACCA]$_2$ motifs from the right-end palindromic should interfere with the generation of hairpin primers by preventing NS1 from specifically binding to the palindrome. To test this hypothesis, the mutant MVM 5′ telomeric clones, pAAC2 and pAAC1/2, were produced from plasmid p98 (Antonietti *et al*., 1988) by substituting ΔAC2 or AAC1/2 DNA for the corresponding wt 87 bp region within the right-end palindromic. Since the unrelated elements with which the [ACCA]$_2$ motifs II and II′ were replaced in the mutant clones were inverted repeats, the 5′ telomere of these clones retained the potential of the wt constructs for forming hairpins, at least as far as DNA sequence was concerned. The structural transition of the extended 5′ telomere was determined using the wt and mutant DNA templates described above, linearized by Sall cleavage, in conjunction with a reconstituted *in vitro* system (Willwand *et al*., 1998). This system allows NS1-induced hairpin formation at linear duplex termini and elongation of the hairpin 3′ end by the Klenow fragment of *E. coli* DNA polymerase I. While hairpin-primed DNA replication is undetectable in this system in the presence of Klenow polymerase alone, the addition of NS1 suffices for its induction (Willwand *et al*., 1998). Analysis of the *in vitro*-generated DNA involves PshAI digestion, yielding short right-end DNA fragments that can be readily separated based on their electrophoretic characteristics into refolded and elongated (product DNA) or outstretched fragments (input DNA) (Baldauf *et al*., 1997). As shown in Fig. 2(B, lanes 1, 3 and 5), *in vitro* replication reactions carried out in the absence of NS1 gave rise to one labelled PshAI product in amounts that were similar for wt and mutant DNA templates. This DNA product, marked ‘d’, had been identified in previous studies as the duplex (open) DNA right-end terminus, presumably labelled through filling of the Sall-restricted end of the linearized template DNA. An additional band, migrating much faster and more heavily labelled, was detected when replication was carried out in conjunction with NS1 and Klenow polymerase (Fig. 2B, lane 2). This species, marked ‘h’, was previously assigned to the processed terminal hairpin fragment (Baldauf *et al*., 1997). Importantly, the formation of this turn-around species was greatly reduced when either pAAC2 or pAAC1/2 was used as template instead of the wt form in the replication assay (Fig. 2B, lanes 4 and 6). Inhibition of the right-end structural transition due to the alteration of both outward [ACCA]$_2$ motifs (AAC2) was not reinforced by further mutating the inward motif (AAC1/2). This leads us to conclude that the former two elements (II and II′) constitute codeterminants of NS1-induced remodelling of the duplex right-end telomere, although we did not formally exclude that motif I may cooperate with motifs II and II′ in driving this reaction. It should be stated that, in the presence of NS1, the ‘d’ band was reduced to a similar extent, irrespective of whether replication was carried out with wt or mutant input DNA (Fig. 2B, lanes 4 and 6). This failure of ‘d’ DNA induction may be assigned to the fact that, at least in the first place, only a very small proportion of template DNA gets remodelled and replicated *in vitro* (Baldauf *et al*., 1997) and, in the second place, NS1 is encumbered with unspecific DNA-binding and nuclease activities that are liable to interfere with the end-labelling reaction (Cotmore *et al*., 1995; data not shown). The residual amount of hairpin-primed replication from the terminus of ΔAC1/2 template DNA is likely to be due to the nonspecific DNA binding and helicase activities of NS1 (Cotmore *et al*., 1995; Wilson *et al*., 1991). Altogether, these results argue for a role of the [ACCA]$_2$ motifs, present on both sides of the
Fig. 2. For legend see facing page.
palindrome’s right-end axis of symmetry, as regulatory cis-elements mediating the NS1-induced structural transition of the MVM right-end terminus.

Our results substantiate the structural transition mechanism hypothesized in our previous report (Willwand et al., 1998) and depicted in Fig. 2(A), in which unwinding of the cDNA strands in the palindrome leads to cruciformation, followed by branch migration and hairpin folding (Scheffler et al., 1988). According to this mechanism, the increase in free energy associated with the breakage of intermolecular base pairing within the duplex palindrome would be compensated by the simultaneous intramolecular reannealing. Unwinding of the right-end of the MVM DNA palindrome in a sequence-specific fashion is not without precedent. Replication of poxvirus DNA, which also contains palindromic telomeres, involves a duplex-to-hairpin transition step that proved to require inverted copies of a core target sequence within the terminal palindromes (DeLange et al., 1987). From the present study, we would predict that the structural transition of poxvirus palindromes relies on the interaction of this core target sequence with viral or cellular factor(s), the nature of which remains to be identified.

Unwinding of dsDNA by helicases is usually independent of the DNA nucleotide sequence, as was shown for NS1 (Wilson et al., 1991). However, some viral helicases, such as the large T antigen of simian virus 40 (SV40), display both unspecific helicase activity and specific unwinding activity, the latter of which relies on the binding of the large T antigen to SV40 origin DNA and serves as the initiation of SV40 DNA replication (Dean et al., 1987). The present study shows that the MVM NS1 protein, in addition to its role as a classical unspecific helicase, is also able to unwind the MVM right-end palindrome in a sequence-specific manner, participating in this way in the initiation of MVM RF DNA replication.

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References


Fig. 2. (A) Schematic representation of the proposed molecular events leading to NS1-induced formation of a hairpin primer at the MVM DNA right-hand terminus. Boxed sequences constitute NS1-binding [ACCA]2 motifs. NS1 is depicted as an ellipse. (B) Hairpin-primed initiation of DNA replication at the MVM right-end palindrome. Wt (pWTAC) and mutant (pΔAC2 and pΔAC1/2) forms of linearized MVM p98re DNA (as depicted in Fig. 1A) were incubated in the presence of the large Klenow fragment of *E. coli* polymerase I in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of NS1. Reaction products were digested with PstI and analysed by 5% polyacrylamide gel electrophoresis. d, Duplex form; h, hairpin form.


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