VIROLOGY 209, 122–135 (1995)

Sequence Motifs in the Replicator Protein of Parvovirus MVM Essential for Nicking and Covalent Attachment to the Viral Origin: Identification of the Linking Tyrosine

JÜRG P. F. NÜESCH,*,[1] SUSAN F. COTMORE,* and PETER TATTERSALL*,†,[2]

*Departments of *Laboratory Medicine and †Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510*

Received January 5, 1995; accepted March 1, 1995

Parvoviral DNA replication has many features in common with prokaryotic rolling circle replication (RCR), including the pivotal role of an initiator protein which introduces a site-specific, single strand nick into a duplex origin sequence. In this process, the protein becomes covalently attached to the new 5′ end of the DNA, while making available a 3′ hydroxyl to prime *de novo* synthesis. Sequence comparisons of prokaryotic RCR initiators has revealed a set of three common motifs, two of which, a putative metal coordination site and a downstream active-site tyrosine motif, could be tentatively identified in parvoviral replicator proteins. We have introduced mutations into the NS1 gene of the murine parvovirus minute virus of mice (MVM), *in the putative metal coordination site at H129, and into the three candidate tyrosine motifs at* Y188, Y197, and Y210. Histidine-tagged mutant proteins were expressed in HeLa cells from recombinant vaccinia virus vectors and partially purified. None of the mutant proteins were able to initiate replication of origin-containing plasmids *in vitro*, and each showed impaired site-specific binding to the viral origin, with Y188 and Y197 being most severely defective. If this deficiency was minimized using low salt conditions, however, Y188 and Y197 mutant proteins were able to nick and become *covalently attached to origin DNA, whereas Y210 and H129 mutant proteins were not, suggesting that the latter residues* are part of the catalytic site of the NS1 nickase. Transfer of [^{32}P]phosphate from substrate DNA to NS1, followed by cyanogen bromide cleavage of the complex, gave the single, labeled peptide consistent with Y210 being the linking tyrosine.

INTRODUCTION

Parvoviruses replicate their linear, single-stranded DNA genomes through a series of duplex, concatemeric replication intermediates by a unidirectional, single-strand specific mechanism (Tattersall and Ward, 1976), which in many ways resembles the rolling circle DNA replication (RCR) mechanisms seen in some prokaryotic systems (Baas and Jansz, 1988; Inamoto *et al.*, 1991; Wang *et al.*, 1993). The viruses have short palindromic terminal sequences which are capable of folding into hairpin duplexes in such a way that the nucleotide at each end of the genome is paired with an internal base. Synthesis of the first complementary DNA strand is primed directly from this free 3′ hydroxyl-group (Cotmore and Tattersall, 1987), but at later stages in the infectious cycle replication initiates at site-specific, single-strand nicks introduced by a virally coded initiator protein into origin sequences located at either end of the genome (Cotmore and Tattersall, 1988; Snyder *et al.*, 1990; Cotmore *et al.*, 1993). Parvoviral origins resemble their prokaryotic counterparts (Baas and Jansz, 1988) in that they contain a number of clearly distinguishable domains: a recognition sequence containing the cleavage site (Snyder *et al.*, 1993; Cotmore and Tattersall, 1994), separated by a short spacer region from the specific initiator protein binding sequence (McCarty *et al.*, 1994; Weitzman *et al.*, 1994; Chiorini *et al.*, 1994; Cotmore *et al.*, 1995). The minimal origin sequence at the 3′ end of the minute virus of mice (MVM) genome contains an additional recognition element, a consensus activated transcription factor (ATF) binding site, separated from the initiator binding site by a critical spacer element called the "bubble" (Cotmore and Tattersall, 1994). Given the correct alignment of these domains within the origin, the initiator protein can bind, cleave a specific phosphodiester bond in one DNA strand and, in so doing, transfer itself on to the new 5′ end generated at the cleavage site (Cotmore and Tattersall, 1989; Im and Muzyczka, 1990; Snyder *et al.*, 1990; Cotmore *et al.*, 1993).

The initiator protein of the murine parvovirus MVM diagrammed in Fig. 1A, is a pleiotropic 83 kDa nuclear phosphoprotein called NS1 (Cotmore and Tattersall, 1987, 1988). The covalent linkage that NS1 makes with viral DNA is unaffected by treatment with mild acids or alkali (Astell *et al.*, 1983; Chow *et al.*, 1986) and is therefore presumed to involve a tyrosine phosphodiester bond rather than a serine or threonine linkage. This is consis-

[1] Current address: Angewandte Tumorvirologie, Deutches Krebsforschungzentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany.

[2] To whom correspondence and reprint requests should be addressed. Fax: (203) 785-7340.

0042-6822/95 $6.00

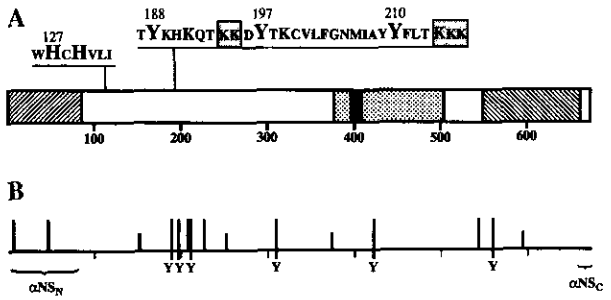

FIG. 1. (A) Diagrammatic structure of the MVM NS1 protein. The positions of functional domains are indicated by boxes within the protein sequence. The stippled region comprises the putative helicase domain homologous with that within the SV40 large T antigen, with the position of the "A" motif of the NTP binding fold shown in black. The cross-hatched region at the N-terminus is the region held in common with the NS2 nonstructural proteins and that at the C-terminus is the transcriptional activation domain (Legendre and Rommelaere, 1994). The two regions of protein sequence within which mutations were constructed for this study are shown above the block diagram. The histidine (H) and tyrosine (Y) residues involved are in bold type and numbered. Boxed and shaded lysine (K) residues are those previously shown to comprise essential elements of the bipartite nuclear localization sequence of NS1 (Nüesch and Tattersall, 1993). (B) Tyrosine residues in NS1 are shown on a bar diagram which is lined up with the NS1 structure shown in A. Tyrosines which are not extensively conserved among the rodent parvoviruses are shown with a short bar, while those which are well conserved are marked with a long bar. Conserved tyrosines shown to be essential for replication (Skiadopoulos and Faust, 1993) are marked with "Y." The protein sequences recognized by the α-$NS1_N$ and α-$NS1_C$ antibodies (Cotmore and Tattersall, 1989) used in the immunoprecipitation and fluorescent antibody staining studies are also indicated.

tent with analyses performed for the analogous parvoviral initiator proteins, Rep 68 and Rep 78, encoded by the helper-dependent virus AAV2 (Snyder *et al.*, 1990) where phosphotyrosine was recovered from acid hydrolysates of Rep:DNA complexes.

Sequence comparisons between the candidate initiator proteins thought or known to mediate rolling circle replication in a wide variety of systems, has revealed three conserved motifs which were suggested to be involved in this type of initiation (Koonin and Ilyina, 1993). Two of these motifs, one encoding a putative metal coordination site for the nickase (HuHuuu, where u is a bulky hydrophobic residue), and the other a consensus active-site tyrosine residue (YxxK, where x is any residue), can be tentatively identified in NS1. The HuHuuu motif has been found in various groups of metalloenzymes in addition to the RCR initiator proteins, suggesting that it may serve as a metal-ion ligand (Koonin and Ilyina, 1993). As such it is thought to function together with the active-site tyrosine, as the catalytic domain for nicking and covalent attachment to the viral DNA. Although candidate sequences can be identified for both of these motifs in MVM NS1, the active site tyrosine motif is particularly hard to pinpoint because its predicted location coincides with a cluster of tyrosine and lysine residues between NS1 amino acid numbers 186 and 215, namely LTYKHKQTKKDYTKCVLFGNMIAYYFLTKK. In this cluster there is a single sequence, involving tyrosine 188, which exactly corresponds to the predicted consensus, but this tyrosine is not conserved throughout the parvoviruses, whereas tyrosine 210, embedded in the sequence YxxxK, is conserved.

One class of rolling circle replicator molecules, typified by the gene A protein of ϕX174, contains a slightly different linking tyrosine motif comprising two closely spaced invariant tyrosine residues ($Yux^K/_RYuxK$), which alternate as the active site tyrosines in successive replication cycles (van Mansfeld *et al.*, 1986). More simply, this motif can be viewed as two adjacent copies of the single motif ($Yxx^K/_R$). Although the NS1 tyrosine cluster does not contain precisely this sequence, some models of MVM DNA replication do postulate a flip-flop cleavage mechanism such as that used by ϕX174 (Astell *et al.*, 1985), and the identified consensus sequences are sufficiently degenerate that precise predictions are not possible.

Previous studies, in which DNA–protein complexes were exposed to limited proteolysis and then immunoprecipitated with antisera directed against different regions of NS1, had shown that the linkage to single-stranded virion DNA was located in the amino-terminal 280 residues of the 672 amino acid protein (Cotmore and Tattersall, 1989). Furthermore, mutation of tyrosine residues at amino acids 6, 42, 209, and 227 in the NS1 gene gave products which were still able to support the replication of defective MVM genomes *in trans* (Skiadopoulos and Faust, 1993). The tyrosine at position 252 is not conserved among different parvoviral NS1 proteins, and substitution of this residue did not affect MVM replication *in vivo*, while mutation of tyrosine 310 did destroy its ability to support replication *in trans* (Skiadopoulos and Faust, 1993), but this residue could be excluded on the basis of the previous immunoprecipitation results (Cotmore and Tattersall, 1989). Thus, the existing experimental evidence pointed to the same cluster of tyrosine residues predicted by the sequence comparisons (Koonin and Ilyina, 1993), but did not distinguish between three candidate tyrosines, at positions 188, 197, and 210 in the NS1 sequence.

To explore the roles of these predicted motifs in NS1, we introduced mutations into critical residues in each consensus. Wild-type and mutant NS1 genes were further modified by addition of a six-histidine amino-terminal tag, to allow rapid and facile isolation of the recombinant proteins, and were expressed in HeLa cells from recombinant vaccinia viruses. We also expressed and isolated control NS1 molecules carrying mutations in a critical lysine residue in the consensus purine triphosphate binding site (Gx_4GKSx_6I) of the putative NS1 helicase

domain (Gorbalenya *et al.*, 1990), which we had previously shown rendered NS1 incapable of supporting excision and replication of the viral telomeres *in vitro* (Nüesch *et al.*, 1992). Nuclear extracts and affinity-purified preparations of wild-type and mutant NS1 molecules were then analyzed for their ability to perform the various steps involved in resolution and replication of MVM termini *in vitro*. Most of the NS1 mutants in the candidate HuHuuu and YxxK motifs showed diminished site-specific DNA binding to the origin sequences, and so were inevitably impaired in all subsequent steps in the replication process. However, using low salt conditions to compensate for this diminished binding affinity, we were able to show that while all mutants showed reduced site specific nicking activity, only substitution of the tyrosine at residue 210 completely inactivated this function, suggesting that amino acid 210 must be the active site tyrosine. To confirm this mutational analysis, we then subjected NS1/DNA complexes generated *in vitro* to cleavage by cyanogen bromide and looked for transfer of [^{32}P]phosphate from the labeled substrate DNA to individual NS1 peptides.

MATERIALS AND METHODS

Viruses and cells

Wild-type vaccinia virus, strain WR, as well as all recombinant vaccinia viruses, were propagated in BSC-40 cells and purified over a sucrose cushion (Mackett *et al.*, 1985). HeLa and BSC-40 cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 5% horse serum; A9ouab'11 and CV-1 cells were grown in DMEM containing 5% fetal calf serum.

Plasmids and mutagenesis

The construction of the plasmid pTM1-NS1$_{wt}$ was described previously (Nüesch *et al.*, 1992). Plasmid pTHis-NS1$_{wt}$ is a modification of pTM1-NS1 that expresses a [His]$_6$-TAG followed by an enterokinase cleavage site at the N-terminus of the MVM NS1 polypeptide. The plasmid was constructed using single step PCR with the primer pair Nu21 5′-GTTGCCATGGCTCACCATCACCACCATCACGGGGACGACGATGACAAG-3′ together with TD1 5′ [2493]-GTGCTCTTTGGCAGC[2497]3′ (MVM nucleotide numbers) on the template pTM*in*N10-NS1 (Nüesch and Tattersall, 1993). The PCR product was digested with *Nco*I and used to replace the *Nco*I fragment in pTM1-NS1ΔRV (Nüesch and Tattersall, 1993). PCR-derived sequences between *Eco*RV (MVM nucleotide 385) and *Bam*H1 (the polylinker) were then replaced with plasmid-derived sequences and the remaining PCR-derived nucleotides were sequenced to verify authenticity. Plasmid pTHisNS1:K405M and pTHisNS1:K405R were constructed by replacing the *Eco*RI to *Bst*EII fragments (MVM nucleotides 1085–1885) in pTHisNS1$_{wt}$ with similar fragments from pTM1-NS1:K405M and pTM1-NS1:K405R (Nüesch *et al.*, 1992), respectively.

Site-directed substitution mutagenesis in the candidate metal coordination site and linkage tyrosine motifs was performed by chimeric PCR as described (Nüesch and Tattersall, 1993) using the outside rightward primer Nu2 5′[261]-ATGGCCGGAAATGCTTACTCT-[281]3′ and the leftward primer TD1 5′[2493]-GTGCTCTTTGGCAGC-[2479]3′. Mutagenesis was performed with individual primer pairs containing a wild-type leftward primer and a mutagenic rightward primer. The first round PCR samples were obtained with Nu2 and the leftward wild-type primer or the mutagenic rightward primer and TD1 separately on the template pDNS5-5. The second round of PCR samples was obtained by combining the overlapping wild-type and the mutant PCR fragments and amplifying them using the outside primers Nu2 and TD1. The resulting hybrid was then digested with *Eco*RV and *Eco*RI and inserted into pTHis-NS1 replacing the wild-type *Eco*RV[384] to *Eco*RI[1085] fragment. Mutants in the HuHuuu motif were constructed in a single PCR reaction using the wild-type primer Nu17 (5′[638]-CCAGCCTTGGTC-[627]3′) and a degenerate primer Nu16 (5′[627]-GACCAAGGCTGGC[**A/G**]CTGCC[**A/G**] TGTACTAAT-[655]3′). Only the single mutant H129R and the double mutant H127;129R were recovered from several screens of the products of this reaction, suggesting that there was a sequence bias in the degenerate oligonucleotide population.

Linking tyrosine motif mutants were also constructed with pairs of wild-type and mutagenic oligonucleotides, Y188F with the wild-type primer Nu19 (5′[821]-AGTAAGTAGAGTAA-[808]3′) and mutagenic primer Nu18 5′-[810]-ACTCTACTTACTTTTAAGCATAAG-[833]3′, Y197F with wild-type primer Nu4 5′[840]-TGGTTTGCTTATGCT-[826]3′ and mutagenic primer Nu22 5′[826]-AGCATAAGCAAACCAAAAAAGACTTTACCAAGTG-[860]3′. Mutants Y209F and Y210F were constructed simultaneously using wild-type primer Nu24 (5′[885]-AAGCAATCATGTTTC-[**A/T**]CT[**A/T**]TTTTTTAAC-[898]3′). All cloned PCR sequences were confirmed by DNA sequencing with Sequenase (USB, Cleveland, OH).

Mutants *sb*MM and *sb*DYKD have been described previously (Nüesch and Tattersall, 1993). In sbMM two methionine codons were substituted for the adjacent lysine residues 194 and 195, whereas in *sb*DYKD, lysines at 214 and 215 were replaced with aspartic acid and tyrosine, respectively, and the isoleucine at position 217 was replaced with aspartic acid.

Production of tk$^-$ recombinant vaccinia viruses containing wild-type and mutant MVM genes

Recombinant vaccinia viruses were prepared as described (Nüesch *et al.*, 1992) by transfecting 10 μg of

each pTHis-NS1-derived plasmid DNA, using Lipofectin (Gibco-BRL, Gaithersburg, MD)-mediated transfection, into CV-1 cells previously infected with wild-type vaccinia virus strain WR. Potentially recombinant vaccinia viruses were screened for NS1 expression in the presence of vTF7-3 by Western transfer using antiserum α-NS_N, directed against the N-terminal 91 amino acids of MVM NS1 (Cotmore and Tattersall, 1986), and by immunofluorescence using antiserum α-$NS1_C$, directed against the C-terminal 16 amino acids of MVM NS1, (Cotmore and Tattersall, 1988), to confirm the production of full-length protein. Virus was purified from stocks grown up after two plaque isolations.

Production and purification of wild-type and mutant His-NS1

NS1 was produced in HeLa cells as previously described (Nuesch *et al.*, 1992; Nuesch and Tattersall, 1993), following double infection with vTF7-3 and the appropriate recombinant vaccinia virus. Cells were harvested 18 hr postinfection and His-NS1 was purified from 10^8 cells using Ni^{2+}-NTA agarose columns. Whole cell extracts were loaded three times on to 0.3- to 0.5-ml Ni^{2+}-NTA agarose (Qiagen, Chatsworth, CA), washed twice each with 5 vol of buffer D (10 m*M* HEPES–KOH, pH 7.8, 50 m*M* NaCl, 7.5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, and 17% glycerol), buffer D containing 4 m*M* imidazole, and finally buffer D containing 10 m*M* imidazole. His-tagged NS1 was then eluted using buffer D containing 80 m*M* imidazole. NS1 preparations were analyzed by discontinuous SDS–PAGE electrophoresis and proteins were detected by Coomassie-blue staining and Western transfer using α-NS_N, as described previously (Nüesch and Tattersall, 1993).

Subcellular localization of NS1

For immunofluorescence studies, A9ouab^{r}11 cells were grown on "spot-slides" (Cel-Line Associates Inc., Newfield, NJ) and infected with vTF7-3. Thirty minutes postinfection cells were transfected with the appropriate pTHis-NS1 plasmid. At 5 hr post-transfection cells were fixed and stained with 1:100 dilution of α-$NS1_C$, as previously described (Nüesch and Tattersall, 1993).

Helicase assays

Helicase assays were performed as described elsewhere (Stahl *et al.*, 1986; Im and Muzyczka, 1990; Wilson *et al.*, 1991). Helicase templates were made by annealing a 17-mer oligonucleotide to single-strand M13 DNA. For M13-26, the annealed primer was extended with Sequenase (USB, Cleveland, OH) in the presence of [α-^{32}P]-dATP and cold dGTP and dTTP but without dCTP, producing a labeled 26-mer oligonucleotide annealed to circular M13 DNA (Im and Muzyczka, 1990). For M13-VAR the primer was extended at room temperature for 5 min in the presence of all dNTPs, supplemented with [α-^{32}P]-dATP, and the elongated fragments terminated at various positions by addition of dideoxy-GTP (Stahl *et al.*, 1986). Between 10 and 300 ng of purified NS1 was incubated with 20 ng of substrate for 15 min at 32° in the presence of 20 m*M* HEPES–KOH, pH 7.8, 25 m*M* NaCl, 5 m*M* KCl, 7.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 8% glycerol, and 3 m*M* ATP. The reaction was terminated by addition of 0.1% SDS, 5 m*M* EDTA, and the products were analyzed on 7% nondenaturing polyacrylamide gels containing 0.1% SDS.

In vitro resolution and replication reactions

In vitro resolution and replication reactions were performed as described previously (Cotmore *et al.*, 1992), using pLEB711 as a substrate for resolution of 3′ concatemeric MVM DNA (Cotmore *et al.*, 1992, 1993) and pTC and pGAA (Cotmore and Tattersall, 1994) as substrates for replication initiation assays. NS1 was supplied either as a whole cell extract from HeLa cells containing NS1 expressed from recombinant vaccinia viruses or as 50 to 200 ng of affinity-purified protein obtained following one-step purification on Ni^{2+}-NTA agarose. The reaction was incubated for 2 hr at 37°, and the [^{32}P]dATP-labeled products were analyzed on 1% agarose gels either directly following deproteinization or after immunoprecipitation using α-NS_N.

Site-specific interaction of NS1 with the origin

Site-specific binding assays using NS1 and the MVM 3′ replication origin were performed as previously described (Cotmore *et al.*, 1995). Plasmid pL1-2TC (Cotmore and Tattersall, 1994) was cut with restriction enzymes *Sau*3A and *Nar*I, giving 30 fragments ranging in size from 9 to 985 basepairs. The 3′ ends of all DNA strands were filled in with Sequenase in the presence of three unlabeled nucleotides and [α-^{32}P]dATP to yield mixtures of labeled, blunt-ended fragments. Binding assays were carried out at 4° in 100 μl of 20 m*M* Tris–HCl, pH 8.0, 10% glycerol, 1% NP-40, 5 m*M* DTT, and 100 m*M* NaCl (buffer B). Site-specific binding assays contained 100–200 ng poly dI:dC, ATP (added to 0.5 m*M* where indicated), and 50 ng of affinity-purified NS1. In standard assays, NS1 was incubated for 10 min on ice with the appropriate mixture of poly dI:dC, EDTA, $MgCl_2$, and NTP as indicated, before the ^{32}P-labeled DNA fragments (10 ng) were added. Samples were incubated for 30 min on ice, after which time 2 μl of α-$NS1_C$ antiserum was added and the incubation continued for another hour. Immune complexes were selected on protein A–Sepharose, deproteinized, and analyzed by electrophoresis on 2.5% agarose gels as described previously (Cotmore *et al.*, 1995).

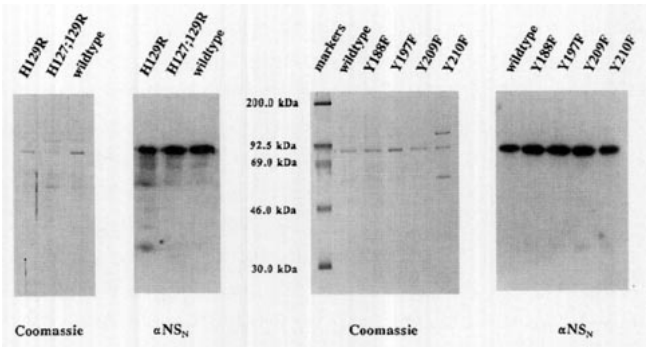

FIG. 2. Expression and purification of wild-type and mutant NS1 proteins. NS1 was expressed in HeLa cells following dual infection with recombinant vaccinia viruses expressing T7 polymerase (vTF7-3) and one of the mutant forms of vHis-NS1. Cells were harvested 18 hr postinfection and His-NS1 purified from crude cell extracts on Ni^{2+}-NTA-agarose. Representative preparations of the wild-type and mutant proteins are shown following SDS–PAGE and staining with Coomassie brilliant blue or analysis by Western-transfer using α-NS_N. Protein concentrations were matched for each assay on the basis of Coomassie blue staining or, in cases where a negative result was obtained, samples were tested at both normal and elevated concentrations.

Nicking of the MVM origin by NS1

The nicking substrate was generated by isolating the 95-basepair *Eco*RI fragment of pL1-2TC containing the "minimal active origin" (Cotmore and Tattersall, 1994), as shown in Fig. 8. The fragment was 3′ end-labeled by fill-in reactions using Sequenase and [α-^{32}P]dATP and [α-^{32}P]dTTP to generate blunt-ends. Between 0.5 and 1.0 ng of substrate was incubated with approximately 200 ng of NS1 in the presence of 20 m*M* HEPES–KOH, pH 7.8, 5 m*M* KCl, 7 m*M* $MgCl_2$, 0.1 m*M* DTT, and 3 m*M* ATP for 1 hr at 37°. The reaction was stopped by the addition of 0.1% SDS, 5 m*M* EDTA, and incubation at 60°. To assess the relative efficiency of the nicking reaction for each different NS1 preparation, reaction products were heat denatured at 95° for 10 min and the covalent NS1–DNA complexes purified by immunoprecipitation using α-NS_N. The covalently attached DNA was then released by proteinase K digestion. Immunoprecipitations were performed in 150 m*M* NaCl, 50 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1% Sodium deoxycholate, 1% Triton X-100, 0.1% SDS, scavenged with formalin-fixed *Staphylococcus* aureus (Boehringer-Mannheim, Mannheim, Germany), deproteinized, and analyzed on denaturing 8% polyacrylamide gels. After proteolysis contaminating input DNA and any full-length complementary strand DNA which had reannealed to the nicked, NS1-associated strands migrated as a discrete band of 95 nucleotides, whereas the specific product derived from NS1 cleavage resulted in a new band of approximately 53 nucleotides.

Cyanogen bromide cleavage of NS1 bound to ^{32}P-labeled origin

The substrates were prepared by separately annealing primers Lori-1 or Lori-2 to denatured pL1-2TC (Fig. 9B). These primers were used previously to delineate the minimal active origin present in the stem of the left end terminal hairpin of MVM (Cotmore and Tattersall, 1994). Each annealed Lori-1 or Lori-2 primer:template was elongated by Sequenase in the presence of either [α-^{32}P]-dATP or [α-^{32}P]dTTP, plus the other three unlabeled dNTPs, to produce double-stranded DNA substrates containing the origin region, in which a single DNA strand was labeled. These substrates were used separately in *in vitro* replication reactions with cell extracts containing wild-type or mutant NS1. Reactions were terminated by digestion of the substrate with DNaseI, which leaves the α-PO_4 group attached to the active site in NS1. The products were then precipitated with TCA and cleaved with cyanogen bromide in 70% HCOOH, as described by Eng *et al.* (1989). The digested products were analyzed on 12–25% gradient SDS–polyacrylamide gels and detected by autoradiography.

RESULTS

Expression and purification of wild-type and mutant NS1 molecules

In order to explore the role of predicted rolling circle replication motifs in NS1, we introduced site-directed mutations into the expression-recombination vector pTHis-$NS1_{wt}$. Two mutations involved the predicted metal coordination site motif [127]-HuHuuu-[132] (NS1 amino-acid numbers), replacing either the second or both of the histidine residues with arginine, to give HcRvLi and RcRvLi, respectively, while in the candidate active-site tyrosine cluster between NS1 residues 186 and 215 ([186]-LTYK-HKQTKKDYTKCVLFGNMIAYYFLTKK-[215]), we mutated the candidate tyrosines Y188, Y197, and Y210 individually to phenylalanines. We also introduced a similar tyrosine to phenylalanine switch at Y209 to serve as a positive control.

Wild-type and mutant NS1 proteins with amino-terminal histidine tags were expressed in HeLa cells, extracted, and purified by passage over Ni^{2+}-NTA-agarose as described under Methods. Although the exact level of purification varied somewhat from preparation to preparation, all mutants were obtained as stable proteins at concentrations suitable for *in vitro* assays as demonstrated by the Coomassie blue staining and Western transfer analyses shown in Fig. 2. The observations were verified by performing individual assays with several different preparations of each mutant.

All candidate RCR mutant proteins translocate to the cell nucleus

Since both the putative metal coordination site and the candidate linkage tyrosine(s) are located close to the nuclear localization signal (Fig. 1A), we analyzed

the intracellular localization of each mutant protein by immunofluorescence. A9 cells were grown on spot-slides, infected with vTF7-3, and super-transfected with the various pTHisNS1-derived plasmid constructs. Cells were fixed 5 hr post-transfection and stained using $\alpha NS1_C$, which recognizes the carboxy-terminal 16 amino acids of NS1. As shown in Fig. 3, each of the RCR mutant NS1 proteins were efficiently translocated to the cell nucleus, indicating that the presence of these mutations did not interfere with recognition of the nuclear localization signal.

NS1 proteins with mutations in candidate RCR motifs are unable to replicate and resolve viral dimer junctions *in vitro*

All mutants were assessed for their ability to support *in vitro* resolution reactions using plasmid pLEB711, which contains the entire MVM dimer junction, as substrate. Samples were tested both as crude extracts or after partial purification on Ni^{2+}-NTA agarose. For the assays shown in Fig. 4, pLEB711 was incubated under *in vitro* replication conditions with various wild-type and mutant forms of NS1, and the reaction products immunoprecipitated with αNS_N in order to separate NS1-initiated products from those synthesized by NS1-independent, repair activities (Cotmore *et al.*, 1992, 1993). After immunoprecipitation, samples were digested with the restriction endonuclease *Sca*I, which cuts these plasmids once in vector sequences, and the immunoadsorbant repelleted, giving separate pellet and supernatant fractions. This process separates those DNA fragments which are themselves covalently attached to NS1 (and thus still associated with the immunoadsorbant after digestion) from the NS1-free fragments which are released into the supernatant (Fig. 4). Resolution of the MVM left-end:left-end concatemer junction is asymmetric (Cotmore *et al.*, 1993) and results in the production of termini in both the NS1-associated "extended" configuration and a covalently closed "turnaround" configuration. If DNA replication is initiated in such reactions, but resolution fails, NS1 is found associated with linear forms of the plasmid. Thus, by analyzing *Sca*I-digested, fractionated reaction products we could expect to identify defects in either initiation of DNA replication or subsequent steps in the resolution process. However, only wild-type and Y209F NS1 preparations were able to support any synthesis at all, and these were both able to carry out the entire resolution reaction. No NS1 dependent replication could be detected for proteins carrying mutations affecting any of the candidate RCR motifs.

Since resolution of the dimer junction is a complicated assay which is relatively insensitive because each initiation event is only associated with the synthesis of a short stretch of labeled DNA, we also explored the ability of mutant NS1 molecules to initiate replication on a more simple, nonpalindromic, substrate carrying a single arm of the dimer junction sequence. The minimal 3′ replication origin of MVM is approximately 50 basepairs in length and is expressed on a single arm of the dimer junction (Cotmore and Tattersall, 1994). The potential origin carried on the other arm of this palindromic structure is inactivated in the virus by substitution of a triplet, GAA, in place the doublet, TC, in the so-called "bubble" sequence of the DNA strand carrying the potential nick site. Thus, by cloning the active and inactive arms of the junction separately, into plasmids pTC and pGAA, respectively, we obtained an almost identical pair of substrates, one with and one without a single, active, NS1-dependent replication origin (Cotmore and Tattersall, 1994). In the presence of standard replication extracts, the active origin present in pTC is recognized by wild-type NS1, allowing the establishment of a unidirectional, single-strand specific replication fork which progresses around the circular plasmid sequences. Since each initiation event results in the synthesis of several kilobases of labeled DNA, this assay is far more sensitive than dimer bridge resolution. Wild-type and mutant NS1 proteins were analyzed in *in vitro* replication assays for their ability to support such "rolling circle" synthesis of active TC-origin plasmids relative to the inactive GAA-plasmids (Fig. 5). Substitution of conserved histidine residues in the putative metal coordination site motif to either HCRVLI or RCRVLI, rendered the mutant NS1 incapable of supporting such synthesis (Fig. 5), indicating the importance of this motif for initiation of the replication fork. Subsequent analyses indicated that the double mutant protein behaved quite abberantly, and further analysis of the importance of this motif was confined to the H129R single mutant.

Molecules carrying substitutions in the candidate active-site tyrosines were also highly impaired for pTC-specific replication, and only the "control" mutant, Y209F, was able to replicate pTC to a substantially higher level than the pGAA control. However, even this mutant seemed to be somewhat defective in its replication efficiency when compared to the wild-type reaction. Qualitative differences, however, were not detected for Y209F, since the proportion of the newly synthesized DNA found covalently attached to both this and wild-type NS1 were equivalent (data not shown). In contrast, NS1 molecules carrying Y188F, Y197F, and Y210F mutations were not able to replicate the pTC construct substantially above control levels, although Y188F and Y197F did appear to support trace levels of TC-specific synthesis (Fig. 5). This suggests that most of the DNA replication supported by these extracts was due to nick-translation rather than NS1-mediated initiation. Although preparations of the mutant Y210F consistently supported higher levels of nonspecific synthesis than the other two candidate mu-

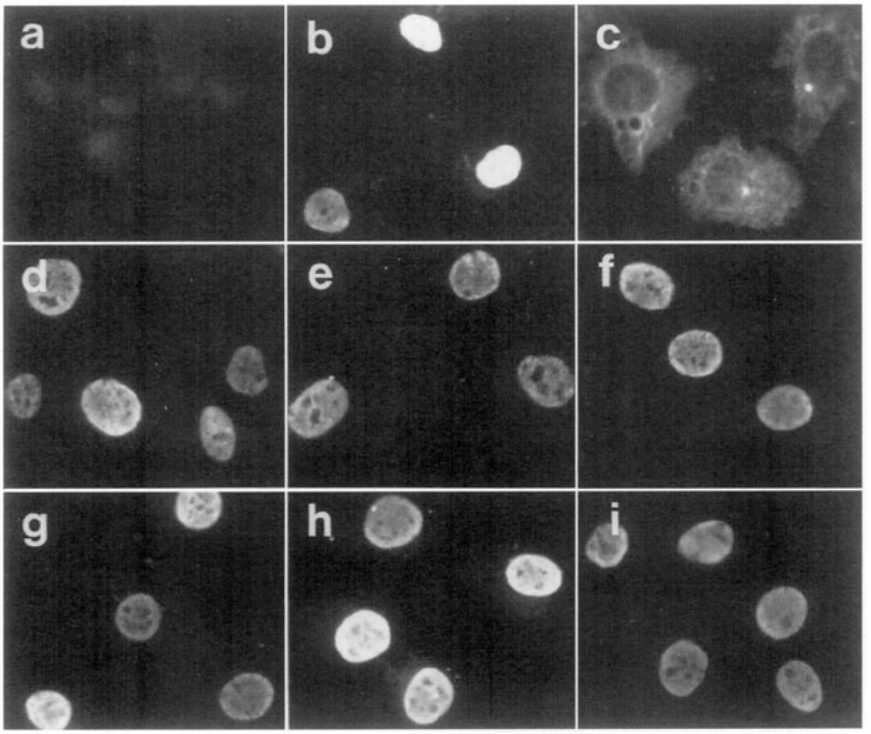

FIG. 3. Immunofluorescence analysis of wild-type and mutant NS1 molecules expressed in A9ouab^{r}11 cells. A9 cells grown on "spot-slides" were infected with 15 PFU/cell vTF7-3 and transfected 30 min later with pTHis-NS1 plasmids. Cells were fixed 5 hr post-transfection and analyzed for the presence of NS1 by immunofluorescent staining using αNS1$_C$. Cells were infected with vTF7-3 alone (a) and after transfection with (b) pTHis-NS1$_{wt}$; (c) pTHis-NS1*sb*DYKD, [which expresses an NS1 molecule with a mutant NLS, thus serving as a cytoplasmic control]; (d) pTHis-NS1:H129R; (e) pTHis-NS1:H127;129R; (f) pTHis-NS1:Y188F; (g) pTHis-NS1:Y197F; (h) pTHis-NS1:Y209F; (i) pTHis-NS1:Y210F.

tants, none of this material could be immunoprecipitated with α-NS$_N$, whereas trace amounts of NS1-associated DNA were observed for the Y188F and Y197F mutants (data not shown).

NS1 proteins with mutations in candidate RCR motifs show impaired binding to the MVM origin

RCR mutant NS1 molecules were assayed for their ability to bind directly to the minimal MVM 3′ replication origin *in vitro*. For these assays the plasmid, pL1-2TC, which contains the minimal origin on a 75-basepair viral insert, was digested with restriction enzymes *Sau*3A and *Nar*I, so that each gave 30 fragments ranging in size from 9 to 985 basepairs. The 3′ ends of all DNA fragments were then labeled with [^{32}P]dGTP, and the resulting mixture incubated with wild-type or mutant NS1 in the presence of cold competitor poly dI:dC. Assays were carried out in the presence of 100 m*M* NaCl, to eliminate nonspecific DNA binding and both with and without 0.5 m*M* ATP (Fig. 6). NS1:DNA complexes were then immunoprecipitated with αNS1$_C$ and the coprecipitated DNA fragments were analyzed on agarose gels. Under the stringent conditions used in these assays all RCR mutants were seen to be impaired in their ability to bind directly to the origin. Wild-type NS1 and the control mutant Y209 showed approximately the same binding efficiency, and in both cases binding was enhanced by the addition of ATP, which is thought to enhance binding by inducing NS1 molecules to multimerize (Cotmore *et al.*, 1995). Of the RCR mutants, H129R showed the best binding (approximately 20% of wild-type levels), followed by Y210F (approximately 5% of wild-type levels), with both Y188F and Y197F giving negligible binding at this salt concentration (Fig. 6). The impaired ability of all our candidate RCR mutants to initiate replication *in vitro* may thus reflect, in part, their inability to establish normal contacts with the origin sequences. However, those mutants which appear most defective in the binding assay, Y188F and Y197F (Fig. 6), were able to establish low level, NS1-specific replication *in vitro* (Fig. 5). This suggests that the remaining mutants, H129R and Y210F, have additional defects.

Wild-type RCR motifs are not essential for helicase activity

The candidate RCR motifs examined in this paper are thought to function together as part of the catalytic domain mediating cleavage and covalent attachment of the NS1 molecule to the viral origin. In order to ask whether mutagenesis of these two motifs affected the intrinsic

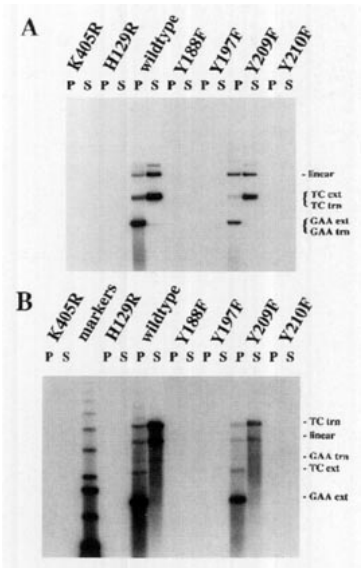

FIG. 4. Agarose gels showing the *in vitro* resolution products of pLEB711 (containing the MVM 3′ concatemer junction sequence), obtained using wild-type and mutant NS1. The newly synthesized, [α-^{32}P]dATP-labeled DNA was immunoprecipitated with α-NS_N, to isolate NS1-initiated complexes, and digested with *Sca*I. NS1-associated restriction fragments were then separated from protein-free fragments by centrifugation to yield pellet fractions (P) which contained all NS1-associated fragments, and NS1-free supernatants (S). Following extensive deproteinization, equivalent samples were electrophoresed on 1% agarose gels under native (A) or denaturing (B) conditions. Predicted resolution products (Cotmore *et al.*, 1993) are indicated as follows: linear, 3396-basepair linear form of the plasmid observed under native and denaturing conditions; GAA, fragment containing telomere derived from GAA arm of the 3′ bridge; TC, fragment with telomere derived from TC arm of the 3′ bridge; species marked "ext" are NS1-associated, extended forms of the telomeres, whereas "trn" denotes covalently closed, turnaround forms of the telomeres. "GAA ext" is 1345 basepairs and "TC ext" is 2170 basepairs under both neutral and alkaline conditions; "GAA trn," 1284 basepairs as a native duplex and 2568 basepairs denatured; likewise, "TC trn" is 2112 basepairs native and 4224 basepairs denatured. "Markers" lane contains "kilobase ladder" molecular weight markers (BRL, Bethesda, MD).

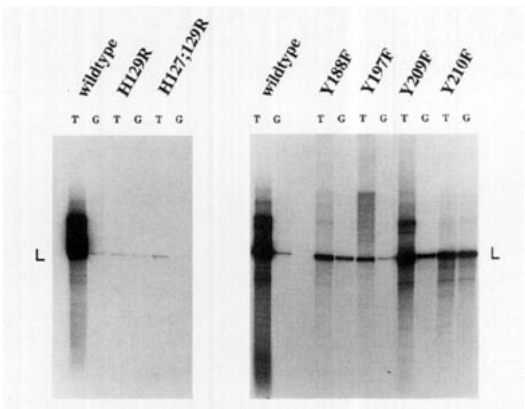

FIG. 5. Agarose gels showing [^{32}P]dATP-labeled replication products generated *in vitro* using substrates pTC, containing the active "TC" arm of the bridge (T) or pGAA containing the inactive "GAA" arm of the bridge (G). DNA was linearized (L) with *Hin*dIII and deproteinized prior to electrophoresis.

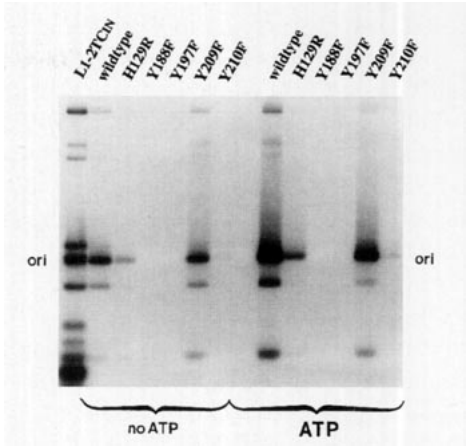

FIG. 6. Site-specific interaction of wild-type and mutant NS1 with the MVM origin. *Sau*3A- and *Nar*I-digested, 3′ end-labeled pL1-2TC plasmid was incubated with NS1 in the presence and absence of ATP. NS1/DNA complexes were then immunoprecipitated using α-$NS1_C$, and the precipitates were analyzed on 2.4% agarose gels. The fragment containing the MVM origin sequences is denoted "ori."

helicase activity of NS1, we analyzed wild-type and mutant proteins for their ability to unwind DNA fragments of various lengths from a circular M13 template. All candidate RCR mutants showed somewhat diminished helicase activity when analyzed relative to wild-type molecules for their ability to unwind the 26-mer template but they were all active, whereas NS1 molecules carrying mutations in the nucleotide binding motif of the helicase domain were completely inactive at all concentrations tested (Fig. 7A).

When wild-type and RCR mutant proteins were tested for their ability to unwind fragments of up to 600 basepair from the M13 template, we obtained essentially similar results (Fig. 7B), with all preparations releasing fragments of the same size spectrum. These observations showed that the candidate RCR mutants did exhibit substantial helicase activity, lending support to the idea that these motifs must be required for an earlier step in the replication reaction. Their somewhat impaired specific helicase activities may simply reflect a diminished ability to bind DNA nonspecifically, just as they were shown to have impaired site-specific binding activity in the previous section.

NS1 molecules with mutations at Y210 or H129 are unable to nick and become covalently attached to the viral origin *in vitro*

We developed an *in vitro* assay to assess the efficiency with which purified forms of NS1 are able to introduce a site-specific, single-strand nick into the viral 3′ origin and in so doing become covalently attached to the new 5′ end of the DNA generated at the nick site. The substrate for these assays was a 95-basepair, linear 3′ end-labeled DNA fragment containing the 75-basepair viral insert

from pL1-2TC, which contains the minimal active MVM left-end origin, plus 20 basepairs of vector sequence. This was incubated with affinity-purified NS1 under low salt conditions and in the presence of Mg-ATP, but in the absence of any additional cellular proteins or competing nonspecific DNA. These conditions allowed NS1 mutants which had an intact nickase function but impaired site-specific binding activity to interact with the substrate and eventually perform the nicking reaction.

Since only a relatively small proportion of the input substrate is nicked by NS1 under these conditions, reaction products were ultimately analyzed for the presence of a covalent linkage between NS1 and the substrate by exposure to conditions designed to denature all noncovalent protein:DNA interactions followed by immunoprecipitation with α-NS_N. This step allowed us to separate specifically nicked DNA from most of the unreacted input substrate prior to analysis, although all precipitates retained some input size DNA whether nicking was achieved or not. Although this contamination only reflects a minor proportion (about 1%) of the input DNA, this constitutes a significant proportion of the DNA found in the precipitates as seen in Fig. 8, but it can be readily identified because it is the same size as the intact substrate. Specifically nicked, NS1-associated DNA strands were identified on sequencing gels after proteolysis as frag-

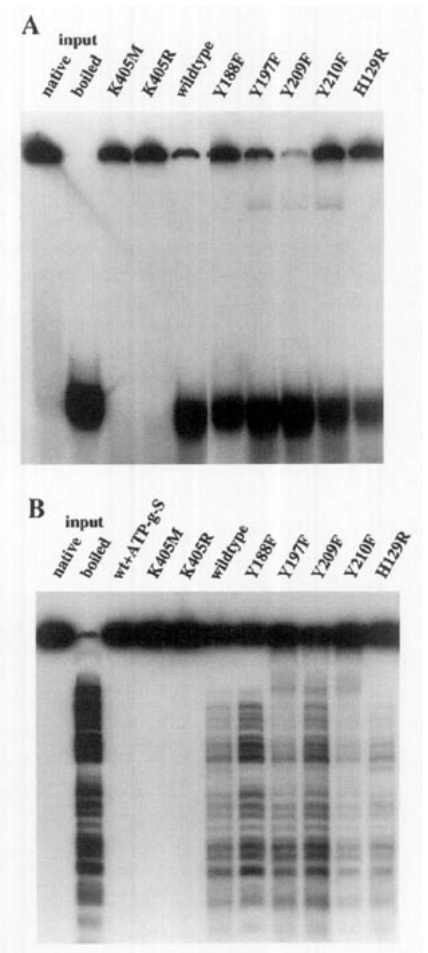

FIG. 7. Helicase-assays of purified wild-type and mutant NS1 proteins. The helicase substrates used to determine general activity of NS1 polypeptides comprised either a ^{32}P-labeled 26-mer annealed to circular M13 template (A) or various length ^{32}P-labeled fragments (30–600 bases) annealed to circular M13 DNA (B). About 20 ng of these substrates was incubated with 10–100 ng of NS1 protein for 15 min at 32°C in the presence of 3 m*M* ATP. The products were analyzed by 7% polyacrylamide electrophoresis containing SDS. The amounts of NS1 used in the assays shown were: K405M, 100 ng; K405R, 100 ng; wild type, 10 ng; Y188F, 10 ng; Y197F, 20 ng; Y209F, 50 ng; Y210F, 50 ng; H129R, 20 ng.

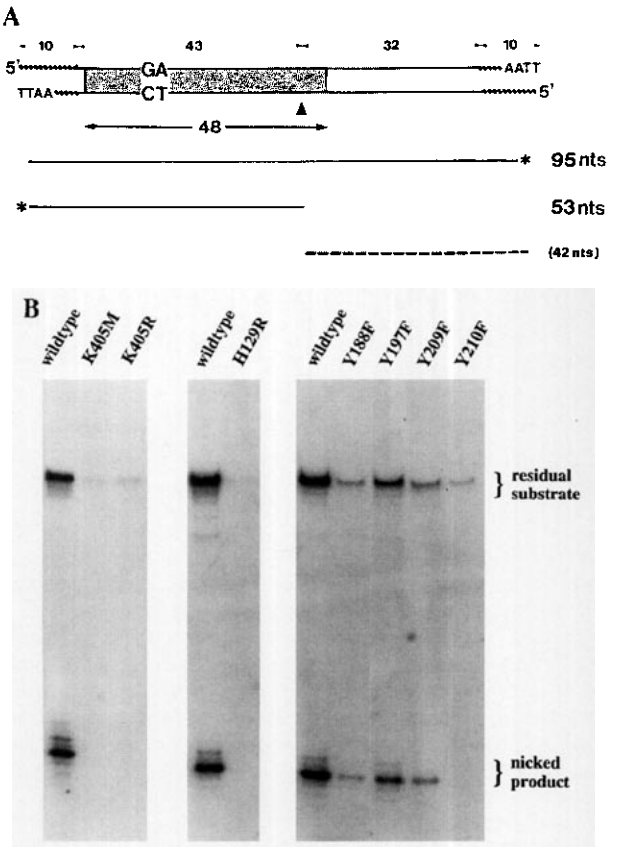

FIG. 8. Nicking of 3′ end-labeled substrate containing the "active" origin isolated from the "TC" arm of pLEB711. (A) Schematic presentation of the substrate. The 95-basepair *Eco*RI fragment of pL1-2TC was 3′ end-labeled with four nucleotides. The stippled segment marks the minimal active origin as determined by Cotmore and Tattersall (1994). The predicted 53- and 42-nucleotide-long nicked products are indicated. (B) For each assay, 3′ end-labeled substrate was incubated with purified NS1 in the presence of ATP. DNA which had become covalently attached to NS1 was then immunoprecipitated using α-NS_N, and the deproteinized precipitates were analyzed on 8% sequencing gels. The sizes of the input substrate and nicked products were determined by running marker sequencing reactions in parallel lanes. Under the conditions used for immunoprecipitation, separated minus and plus strands were able to reanneal (data not shown) and appear in the gel marked "residual substrate." Variations of nicked size DNA are probably due to incomplete removal of residual amino acids attached to the 5′ end.

ments of 53 nucleotides. Substrates labeled at a single 3′ end were used to confirm (data not shown) that this 53 nucleotide product was indeed derived from the predicted DNA strand, as shown in Fig. 8.

Figure 8B shows that no nicking could be detected in this assay with the putative metal coordination site mutant H129R or with NS1 proteins in which the conserved lysine (K405) in the consensus NS1 ATP-binding site was mutated to a methionine or arginine residue. However, the tyrosine mutants Y188F, Y197F, and Y209F were able to nick the origin at the correct site under these conditions, albeit less efficiently than wild-type NS1. In contrast, tyrosine mutant Y210F was completely unable to generate covalent DNA–protein complexes with this substrate (Fig. 8B). Since Y210F consistently failed to nick the origin substrate but was more proficient at binding to this sequence than the other tyrosine substitution mutants (cf., Fig. 6), and because it showed significant helicase activity (Fig. 7), we are reasonably confident that this mutation specifically inactivates the NS1 nickase function.

Two other NS1 substitution mutants were also examined in this assay. Mutant *sb*MM, in which methionine codons were substituted for lysine residues at positions 194 and 195 in the tyrosine cluster, was able to nick origin DNA, while *sb*DYKD, in which the lysine at position 214 in the Y210 YFLTK motif was replaced by aspartic acid (Nüesch and Tattersall, 1993), was unable to form such complexes (data not shown). The latter result supports both our designation of the Y210 residue as the critical active-site tyrosine in the catalytic domain of the NS1 nickase and the prediction made by Koonin and Ilyina (1993) that a basic residue, most likely lysine, downstream of the linking tyrosine would be essential for its function. Our results also show that the conserved HuHuuu element, postulated by these authors to be a metal coordination site, is essential for the nickase activity of NS1.

Labeled origin DNA becomes covalently attached to an NS1-derived peptide which contains Y210, but not Y188 or Y197

In order to support our genetic analysis of candidate active-site tyrosine residues in the catalytic domain of the NS1 nickase, and to assess whether or not Y210 is the only residue capable of mediating a covalent interaction with origin DNA, we monitored $^{32}PO_4$-transfer from labeled substrate DNA to individual NS1 peptides obtained by cyanogen bromide cleavage of the DNA–protein complex. For these reactions we used PCR to label a single strand of the origin fragment derived from pL1-2TC to high specific activity with either [α-^{32}P]dATP or [α-^{32}P]*d*TTP. As a negative control we used a similar substrate which had been labeled on the opposite DNA strand (Fig. 9B). *In vitro* replication assays were performed with these substrates using extracts containing wild-type or mutant NS1 and the reaction products extensively digested with DNaseI, leaving a ^{32}P-labeled α-PO_4 group attached to the linkage tyrosine. To determine the location of this linkage we then cleaved at methionine residues in the labeled protein with cyanogen bromide and analyzed the fragments by SDS–PAGE. As diagrammed in Fig. 9A, tyrosines Y188 and Y197 are located in a cleavage product of predicted molecular weight 14.8 kDa (amino acids 83–206), while Y210 would be present on the adjacent 5.8-kDa peptide (amino acids 207–256). Less likely candidates for the active-site tyrosine, i.e., conserved tyrosines required for replication, Y310, Y422, and Y550, are located on fragments of 1.0 kDa (amino acids 306–313), 18.5 kDa (amino acids 315–483), or 13.5 kDa (amino acids 548–672), respectively, as also indicated in Fig. 9A.

Cyanogen bromide cleavage of reaction products generated in replication reactions employing wild-type NS1 and substrates labeled in the correct DNA strand with either [α-^{32}P]dATP or [α-^{32}P]dTTP (L2-T, L2-A) showed a single ^{32}P-labeled band migrating at 6 kDa (Fig. 9C). No bands could be detected when the incorrect strand was labeled (L1-T) or when either of the mutants K405R or Y210F were used instead of wild-type NS1. These negative results thus confirm that the labeled 6-kDa peptide is derived from reaction products of NS1 with its substrate. Since Y210 is the only candidate tyrosine which resides on a peptide of this size, these results reinforce the genetic data in suggesting that Y210 is the sole active site tyrosine used by NS1 in the relatively simple replication reactions we have been able to reconstitute *in vitro*.

DISCUSSION

We have introduced a series of conservative, single amino acid substitutions into critical positions in candidate sequence motifs thought to be involved in the site-specific nicking activity which initiates rolling-hairpin DNA replication. The mutations were directed at two particular motifs predicted to constitute the catalytic domain of the nickase, but although the first of these motifs was represented by a single sequence in the MVM NS1 protein, the second was ambiguous. Thus, we made mutations in a number of candidate tyrosines and compared the products. However, NS1 is a pleiotropic, multidomain polypeptide involved in many aspects of the viral life cycle (Rhode and Richard, 1987; Tullis *et al.*, 1988; Cotmore and Tattersall, 1989; Brandenburger *et al.*, 1990; Caillet-Fauquet *et al.*, 1990; Doerig *et al.*, 1990; Hanson and Rhode, 1991; Wilson *et al.*, 1991; Cotmore *et al.*, 1992; Legendre and Rommelaere, 1992), and the candidate sequences were all located in the amino-terminal third of the protein, in a complex region already known

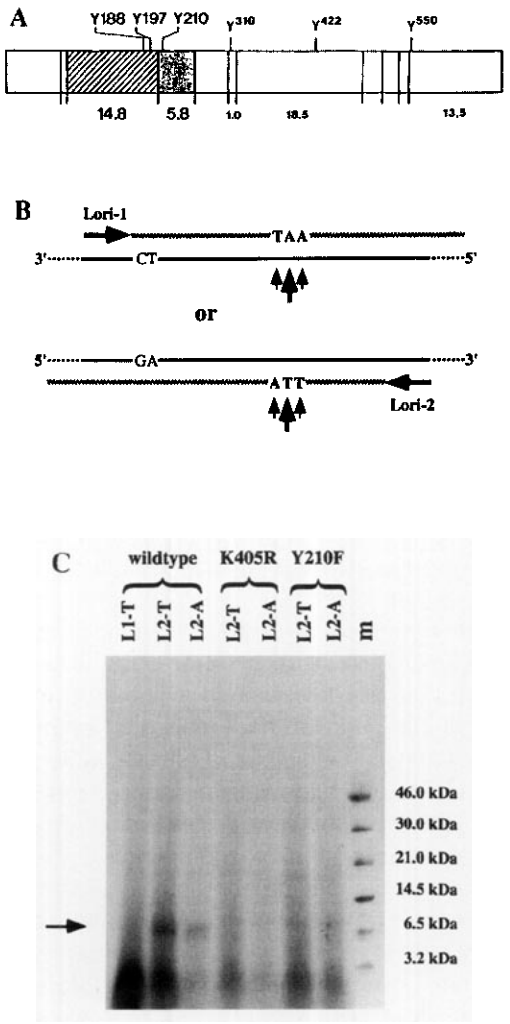

FIG. 9. Localization of the active site for nicking by cleavage of NS1:DNA complexes with cyanogen bromide. (A) Cyanogen bromide cleavage pattern of NS1. Conserved tyrosines, essential for replication activity and the predicted sizes of the corresponding peptides are indicated. The cross-hatched cleavage product contains the candidate linkage tyrosines Y188 and Y197; the stippled peptide contains Y210. (B) Schematic presentation of the substrates used to label the active site. Primers Lori1 or Lori2 were annealed to denatured pL1-2TC in separate reactions. Extension of the primers using [α-^{32}P]dTTP or [α-^{32}P]dATP produced double-strand substrate with either the plus or the minus strand labeled. The αPO_4-group closest to the 5′ end of the nick-site is transferred to the tyrosine during the reaction. (C) Single strand [α-^{32}P]dATP- or [α-^{32}P]dTTP-labeled pTC-plasmids, respectively, were subjected to *in vitro* replication reactions with extracts containing either wild-type, K405R, or Y210F protein. The reaction products were treated with DNaseI leaving proteins which initiated rolling circle synthesis labeled with a terminal $^{32}PO_4$-group at the active site. Labeled proteins were then cleaved at their methionines by cyanogen bromide, and the fragments were analyzed for their size on 12 to 25% SDS–polyacrylamide gradient gels. Lanes contained protein fragments labeled (or not) by reaction with: L1-T, plus strand labeled using [α-^{32}P]dTTP; L2-T, minus strand labeled using [α-^{32}P]dTTP; L2-A, minus strand labeled using [α-^{32}P]dATP; m, low molecular weight ^{14}C-labeled "Rainbow markers" (Amersham, Arlington Heights, IL). The arrow indicates the labeled cleavage products.

to contain the bipartite nuclear localization signal (Nüesch and Tattersall, 1993) and sequences essential for NS1-mediated transactivation of the viral P38-promoter (Skiadopoulos *et al.*, 1992; Skiadopoulos and Faust, 1993) and for its cytotoxicity (Legendre and Rommelaere, 1992). We therefore anticipated that there could be complications in the analyses arising from the overlap of different functional domains. Although, in fact, the nuclear localization signal is not compromised in any of the mutants constructed for this study, to circumvent such potential problems we resorted to a series of *in vitro* assays which we hoped would allow us to explore individual replication functions of the protein.

Crude nuclear extracts and Ni^{2+} ion-affinity purified preparations of all of the mutant proteins in the test group were found to be grossly defective in supporting the *in vitro* replication of plasmids containing MVM origin sequences. However, these are complex reactions dependent upon an ordered sequence of events progressing from the initial recognition of the origin by NS1, through the introduction of a site-specific nick by NS1 and its covalent attachment to the 5′ end of the DNA at the nick site, and leading eventually to the establishment and progression of a viable leading-strand replication fork. In this event, all of the mutants tested proved to be severely defective in the first step of this sequence. They failed to recognize and bind efficiently to the DNA motif, $(ACCA)_2$, which mediates the specific interaction between NS1 and the replication origin (Cotmore *et al.*, 1995). Presumably this defect explains not only why these same mutants are barely able to support initiation and replication on MVM origin-containing plasmid DNA *in vitro,* as reported here, but also why NS1 mutants with changes at similar positions were found to be defective for *trans*-replication when analyzed *in vivo* (Skiadopoulos and Faust, 1993) or for NS1-mediated transactivation of the viral P38-promoter (Skiadopoulos *et al.*, 1992; Skiadopoulos and Faust, 1993) and perhaps even cytotoxicity (Legendre and Rommelaere, 1992). Since we have recently shown that antibodies directed against NS1 amino acids 284 through 459 were able to block specific interactions between NS1 and the origin (Cotmore *et al.*, 1995), the new data presented here indicates that such recognition must either involve a large segment of NS1, extending from at least residue 127 through sequences downstream of residue 284, or that it is multifocal. The catalytic elements of the nickase, identified here as residues 127–132 and Y210, thus appear to be embedded within the specific DNA recognition domain. However, proteins carrying these particular mutations were substantially better at interacting with their cognate site than molecules with mutations at Y188 or Y197, suggesting that the nickase elements are perhaps peripheral to the primary recognition site.

Although the test mutants were compromised in their

ability to recognize the viral origin, the effects of this deficiency can be minimized *in vitro* by using low salt concentrations (Cotmore *et al.,* 1995). Under such conditions all mutants constructed for this study, except H127;129R (not shown), exhibited significant nonspecific helicase activity, and all mutants except those involving the HuHuuu sequence and Y210 showed evidence of being able to nick and become covalently attached to the origin DNA. This latter observation involved the use of a novel site-specific DNA nicking assay in which affinity-purified wild-type and mutant NS1 molecules were incubated, under conditions of low stringency and in the absence of competitor DNA, with a DNA fragment containing the viral left-hand origin labeled with ^{32}P at its 3′ end. Although this assay is inefficient, both wild-type NS1 and the mutants Y188F, Y197F, and T209F were able to nick and become covalently attached to the substrate DNA, and immunoprecipitation of the cleaved and NS1-linked single-strand product with anti-NS1 serum allowed us both to confirm that this was the expected type of cleavage and to purify the product away from most of the unreacted substrate. In fact, this assay only works under conditions of low salt and in the absence of competitor DNA, even for wild-type NS1, and fails to discriminate between the consensus initiation sites present on the replication competent (TC) and inactive (GAA) forms of the viral origin (data not shown). This suggests that the sole substrate requirements for the "nicking" reaction described here are the presence of an NS1-binding sequence and minimal nick site.

In the nicking assay, deleted forms of the MVM 3′ origin, which do not support *in vitro* replication when NS1 is supplied in the form of an unfractionated nuclear extract (Cotmore and Tattersall, 1994), function as well as the full-length origin (data not shown). The minimal sequence required for replication and resolution of the MVM left-hand origin *in vitro* was defined as a 50-base-pair-long region, extending from a point some seven nucleotides beyond the nick site, through an A/T-rich sequence, a cognate NS1-binding site and the so-called "bubble" dinucleotide to a consensus ATF binding site. Within this structure the bubble sequence acts as a critical spacer element, precisely separating the ATF consensus from the NS1-binding sequences in such a way that introduction of a single additional nucleotide at this position completely inactivates the origin (Cotmore and Tattersall, 1994). Since partially purified NS1 is the only protein intentionally added to the nicking assay, it seems probable that an essential factor is missing which would otherwise permit this assay to function under more physiologic conditions. In this situation, low stringency and the lack of competitor DNA presumably permits NS1 to bind maximally to its substrate via both specific and nonspecific interactions, and some site-specific nicking and covalent transfer is able to occur. Thus, although the assay uses relatively high levels of NS1 and low stringency to overcome the blocks to initiation which operate in more complex mixtures, it is useful in the present context since it allows us to explore the essential sequence elements in NS1 required for its nickase activity while excluding other potential endonucleases from the reaction.

This type of genetic analysis showed that the integrity of the HuHuuu cluster and Y210 were absolutely required for site-specific nicking activity. We also approached this question biochemically by determining which cyanogen bromide peptide(s) of NS1 could be labeled by transfer of [^{32}P]phosphate from the substrate DNA during nicking. This approach had the additional advantage of allowing us to look for a second linkage tyrosine, as seen in the gene A protein of ϕX174, which uses two active site tyrosines in alternate rounds of replication (van Mansfeld *et al.,* 1986). However, since the label was transferred exclusively to a single peptide of 5.8 kDa, corresponding to the cleavage product harboring Y210, we feel reasonably confident that this is the only active tyrosine in NS1.

In this study we used previous experimental observations (Cotmore and Tattersall, 1989; Skiadopoulos and Faust, 1993) and computer-assisted predictions derived from comparative data base screening and motif search analysis (Koonin and Ilyina, 1993) to direct our attention to particular NS1 amino acids which were likely to be involved in the catalytic site of this initiator nickase. Since the biochemical data obtained appears to support these predictions, it encourages us to look even more closely at the analogies between parvoviral rolling-hairpin origins and initiators and their many, well-documented, prokaryotic rolling-circle counterparts. Of the three implicated RCR motifs identified by Koonin and Ilyina (1993), parvoviral initiators are unique in retaining only two, arranged so that the putative metal coordination is amino-terminal to the active site tyrosine. This arrangement puts parvoviruses into a subclass of "superfamily II," which contains the geminiviruses of plants and a group of so-called single-strand DNA plasmids belonging to the pUB110 and pMV158 families, which replicate mainly in gram-positive bacteria. The sequence homologies are particularly remarkable because there are at least two other families of prokaryotic replicons, the pT181 plasmid family and the filamentous ssDNA bacteriophages, whose use of RCR replication is well documented, but which do not use the HuHuuu motif. This indicates that RCR initiation can be achieved via alternate mechanisms and suggests that both parvovirus and geminivirus replicons may well have evolved from particular eubacterial ssDNA replicons, with dissemination presumably mediated by recombination. The related prokaryotic systems use a number of principles which have yet to be explored definitively in parvoviral replicons. For example, binding of the initiator protein to a high affinity site in the origin

is used to essentially destabilize the DNA duplex at the nick site and so present single-stranded DNA to the nickase, and most of the replicon initiators catalyze both nicking and joining (termination) reactions, whether or not they use a single active site tyrosine (Baas and Jansz, 1988; Thomas *et al.,* 1990; Pansegrau *et al.,* 1993). In light of the current findings, a closer analysis of such principles would seem to be indicated. However, since parvoviral replicons are small they must rely heavily on cellular proteins to mediate their own preferential replication and have apparently evolved ways to interface the available eukaryotic pathways with the ancient rolling-circle system. Thus, for example, activation of the MVM left-end origin requires binding of both the viral initiator and a host protein, probably a member of the ATF transcription factor family (Cotmore and Tattersall, 1994), while the parvoviral initiator proteins themselves have taken on additional roles in the life cycle. Mounting evidence suggests that NS1 uses the site-specific DNA binding domain of the ancient nickase coupled with a classic acidic transactivator domain, located at its carboxy-terminus, to up-regulate transcription from the viral promoters (J. Christensen, P. Tattersall, and S. F. Cotmore, in preparation; Doerig *et al.,* 1990; Hanson and Rhode, 1991; Christensen *et al.,* 1993; Legendre and Rommelaere, 1994), while the distribution of NS1 DNA binding sites throughout the viral genome suggests that this domain is now also used to serve more structural roles in the viral life cycle (S. F. Cotmore and P. Tattersall, in preparation).

ACKNOWLEDGMENTS

We thank Dr. Eugene Koonin for providing information prior to publication and for stimulating discussions. This work was supported by Public Health Service Grant AI26109 from the National Institutes of Health.

REFERENCES

Astell, C. R., Chow, M. B., and 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. *J. Virol.* **54,** 171–177.

Astell, C. R., Thomson, M., Chow, M. B., and Ward, D. C. (1983). Structure and replication of minute virus of mice DNA. *Cold Spring Harbor Symp. Quant. Biol.* **47,** 751–762.

Baas, P. D., and Jansz, H. S. (1988). Single-stranded DNA phage origins. *Curr. Top. Microbiol. Immunol.* **136,** 31–70.

Brandenburger, A., Legendre, D., Avalosse, B., and Rommelaere, J. (1990). NS-1 and NS-2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. *Virology* **174,** 576–584.

Caillet-Fauquet, P., Perros, M., Brandenburger, A., Spegelaere, P., and Rommelaere, J. (1990). Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. *EMBO J.* **9,** 2989–2995.

Chiorini, J. A., Wiener, S. M., Owens, R. A., Kyostio, S. R. M., Kotin, R. M., and Safer, B. (1994). Sequence requirements for stable binding and function of Rep68 on the AAV type 2 inverted terminal repeat. *J. Virol.* **68,** 7448–7457.

Chow, M., Bodnar, J. W., Polvino, B. M., and Ward, D. C. (1986). Identification and characterization of a protein covalently bound to DNA of minute virus of mice. *J. Virol.* **57,** 1094–1104.

Christensen, J., Storgaard, T., Viuff, B., Aasted, B., and Alexandersen, S. (1993). Comparison of promoter activity in Aleutian mink disease parvovirus, minute virus of mice, and canine parvovirus: Possible role of weak promoters in the pathogenesis of Aleutian mink disease parvovirus infection. *J. Virol.* **67,** 1877–1886.

Cotmore, S. F., Christensen, J., Nüesch, J. P. F., and Tattersall, P. (1995). The NS1 Polypeptide of the murine parvovirus MVM binds to DNA sequences containing the motif [ACCA]2-3. *J. Virol.* **69,** 1652–1660.

Cotmore, S. F., Nüesch, J. P. F., and Tattersall, P. (1992). *In vitro* excision and replication of 5′ telomeres of Minute Virus of Mice DNA from cloned palindromic concatemer junctions. *Virology* **190,** 365–377.

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

Cotmore, S. F., and Tattersall, P. (1986). Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. *J. Virol.* **58,** 724–732.

Cotmore, S. F., and Tattersall, P. (1987). The autonomously replicating parvoviruses of vertebrates. *Adv. Virus Res.* **33,** 91–174.

Cotmore, S. F., and 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. *J. Virol.* **62,** 851–860.

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

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

Doerig, C., Hirt, B., Antonietti, J. P., and Beard, P. (1990). Nonstructural protein of parvoviruses B19 and minute virus of mice controls transcription. *J. Virol.* **64,** 387–396.

Eng, W. K., Pandit, S. D., and Sternglanz, R. (1989). Mapping of the active site tyrosine of eukaryotic DNA topoisomerase I. *J. Biol. Chem.* **264,** 13373–13376.

Gorbalenya, A. E., Koonin, E. V., and Wolf, Y. I. (1990). A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. *FEBS Lett.* **262,** 145–148.

Hanson, N. D., and Rhode, S. L. (1991). Parvovirus NS1 stimulates P4 expression by interaction with the terminal repeats and through DNA amplification. *J. Virol.* **65,** 4325–4333.

Im, D.-S., and Muzyczka, N. (1990). The AAV origin binding protein Rep68 is an ATP-dependent, site-specific endonuclease with DNA helicase activity. *Cell* **61,** 447–457.

Inamoto, S., Yoshioka, Y., and Ohtsubo, E. (1991). Site- and strand-specific nicking in vitro at oriT by the TraY-TraI endonuclease of plasmid R100. *J. Biol. Chem.* **266,** 10086–10092.

Koonin, E. V., and Ilyina, T. V. (1993). Computer-assisted dissection of rolling circle DNA replication. *Biosystems* **30,** 241–268.

Legendre, D., and Rommelaere, J. (1992). Terminal regions of the NS-1 protein of the parvovirus minute virus of mice are involved in cytotoxicity and promoter trans inhibition. *J. Virol.* **66,** 5705–5713.

Legendre, D., and Rommelaere, J. (1994). Targeting of promoters for trans activation by a carboxy-terminal domain of the NS-1 protein of the parvovirus minute virus of mice. *J. Virol.* **68,** 7974–7985.

Mackett, M., Smith, G. L., and Moss, B. (1985). The construction and characterization of vaccinia virus recombinants expressing foreign genes. *In* "DNA Cloning: A Practical Approach" (D. M. Glover, Ed.) IRL Press, Oxford.

McCarty, D. M., Ryan, J. H., Zolotukhin, S., Zhou, X., and Muzyczka, N. (1994). Interaction of the adeno-associated virus Rep protein with a sequence within the A palindrome of the viral terminal repeat. *J. Virol.* **68,** 4998–5006.

Nüesch, J. P., and Tattersall, P. (1993). Nuclear targeting of the parvovi-

ral replicator molecule NS1: Evidence for self-association prior to nuclear transport. *Virology* **196,** 637–651.

Nüesch, J. P. F., Cotmore, S. F., and Tattersall, P. (1992). Expression of functional parvoviral NS1 from recombinant vaccinia virus: Effects of mutations in the nucleotide-binding motif. *Virology* **191,** 406–416.

Pansegrau, W., Schroder, W., and Lanka, E. (1993). Relaxase (Tral) of IncP alpha plasmid RP4 catalyzes a site-specific cleaving-joining reaction of single-stranded DNA. *Proc. Natl. Acad. Sci. USA* **90,** 2925–2929.

Rhode, S. L., and Richard, S. M. (1987). Characterization of the trans-activation-responsive element of the parvovirus H-1 P38 promoter. *J. Virol.* **61,** 2807–2815.

Skiadopoulos, M. H., and Faust, E. A. (1993). Mutational analysis of conserved tyrosines in the NS-1 protein of the parvovirus minute virus of mice. *Virology* **194,** 509–517.

Skiadopoulos, M. H., Salvino, R., Leong, W. L., and Faust, E. A. (1992). Characterization of linker insertion and point mutations in the NS-1 gene of minute virus of mice: Effects on DNA replication and transcriptional activation functions of NS-1. *Virology* **188,** 122–134.

Snyder, R. O., Im, D. S., and Muzyczka, N. (1990). Evidence for covalent attachment of the adeno-associated virus (AAV) rep protein to the ends of the AAV genome. *J. Virol.* **64,** 6204–6213.

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

Snyder, R. O., Samulski, R. J., and Muzyczka, N. (1990). In vitro resolution of covalently joined AAV chromosome ends. *Cell* **60,** 105–113.

Stahl, H., Droge, P., and Knippers, R. (1986). DNA helicase activity of SV40 large tumor antigen. *EMBO J.* **5,** 1939–1944.

Tattersall, P., and Ward, D. C. (1976). Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. *Nature (London)* **263,** 106–109.

Thomas, C. D., Balson, D. F., and Shaw, W. V. (1990). In vitro studies of the initiation of staphylococcal plasmid replication. Specificity of RepD for its origin (oriD) and characterization of the Rep-ori tyrosyl ester intermediate. *J. Biol. Chem.* **265,** 5519–5530.

Tullis, G. E., Labieniec, P. L., Clemens, K. E., and Pintel, D. (1988). Generation and characterization of a temperature-sensitive mutation in the NS-1 gene of the autonomous parvovirus minute virus of mice. *J. Virol.* **62,** 2736–2744.

van Mansfeld, A. D. T. H., Baas, P. D., and Jansz, H. S. (1986). Two juxtaposed tyrosyl-OH groups participate in phi X174 gene A protein catalyzed cleavage and ligation of DNA. *Nucleic Acids Res.* **14,** 4229–4238.

Wang, P-Z., Projan, S. J., Henriquez, V., and Novick, R. P. (1993). Origin recognition specificity in pT181 plasmids is determined by a functionally asymmetric palindromic DNA element. *EMBO J.* **12,** 45–52.

Weitzman, M. D., Kyostio, S. R., Kotin, R. M., and Owens, R. A. (1994). Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc. Natl. Acad. Sci. USA* **91,** 5808–5812.

Wilson, G. M., Jindal, H. K., Yeung, D. E., Chen, W., and Astell, C. R. (1991). Expression of minute virus of mice major nonstructural protein insect cells: Purification and identification of ATPase and helicase activities. *Virology* **185,** 90–98.