The multifunctional replication protein of autonomous parvoviruses, NS1, is vital for viral genome replication and for the control of viral protein production. Two DNA-interacting domains of NS1, the N-terminal and helicase domains, are necessary for these functions. In addition, the N and C termini of NS1 are required for activation of viral promotor P38. By comparison with the structural and biochemical data from other parvoviruses, we identified potential DNA-interacting amino acid residues from canine parvovirus NS1. The role of the identified amino acids in NS1 binding dynamics was studied by mutagenesis, fluorescence recovery after photobleaching, and computer simulations. Mutations in the predicted DNA-interacting amino acids of the N-terminal and helicase domains increased the intranuclear binding dynamics of NS1 dramatically. A substantial increase in binding dynamics was also observed for NS1 mutants that targeted the metal ion coordination site in the N terminus. Interestingly, contrary to other mutants, deletion of the C terminus resulted in slower binding dynamics of NS1. P38 transactivation was severely reduced in both N-terminal DNA recognition and in C-terminal deletion mutants. These data suggest that the intranuclear dynamics of NS1 are largely characterized by its sequence-specific and -nonspecific binding to double-stranded DNA. Moreover, binding of NS1 is equally dependent on the N-terminal domain and conserved β-loop of the helicase domain.

Parvoviruses are small viruses with a T=1 capsid composed of VP1 (~10%) and VP2 (~90%) proteins. Their ~5.3-kb single-stranded DNA (ssDNA) genome contains two transcription units. Canine parvovirus (CPV) capsid proteins (VP1 and VP2) are produced from the right-hand-side transcription unit, and nonstructural proteins (NS1 and NS2) are produced from the left-hand-side transcription unit (1). NS1 is the only essential nonstructural protein of CPV (2).

The parvovirus genome is exposed from the capsid in the nucleus of the host cell. In S-phase cells, the host replication machinery starts the virus genome replication from the inverted terminal repeats. First, the ssDNA virus genome is complemented to a covalently closed single-stranded DNA (dsDNA) replicative form. Next, the NS1 protein is produced and starts to control genome replication. NS1, a 76.7-kDa multifunctional nuclear phosphoprotein, has multiple essential functions in the virus life cycle. Parvoviral NS1 proteins belong to the superfamily 3 (SF3) helicases (3, 4). The helicase domain, including a conserved ATP binding pocket, is located in the middle of the polypeptide chain (5). Flanking the helicase domain N and C terminals are the origin of replication (ori) binding and promoter transactivation domains, respectively (6, 7). Many functions of NS1 are dependent on its interactions with DNA. Viral replication induces formation of two ori recognition complexes. These are named OriL and OriR, according to their positioning in the left- and right-hand sides of the genome, respectively. In both complexes, NS1 binds to the dsDNA in a sequence-specific manner. It forms a ternary complex with endogenous proteins: the glucocorticoid modulatory element binding proteins in OriL (8) and high-mobility group proteins in OriR (9). Both recognitions lead to nicking of the dsDNA genome in a strand- and site-specific manner and covalent linking of NS1 to the emerging 5′ end (10, 11).

The NS1 helicase activity is mandatory for viral genome replication (12). The NS1-DNA interactions in the helicase mode are sequence independent, but they require an ssDNA overhang for initiation, as indicated in in vitro experiments (8, 13). Ori recognition, nicking, and helicase activity are dependent on ATP. However, a requirement for energy input is evident only for the helicase function, and ATP binding is likely to promote NS1 oligomerization for the other activities (8, 9, 12, 14, 15).

The N-terminal domain of NS1 recognizes the viral ori sequence and nicks the dsDNA genome in a site- and strand-specific manner (10, 11). Ori recognition has been studied with related Rep proteins of adeno-associated viruses (AAVs). Structural studies with the ori binding domain of AAV serotype 5 (AAV5) Rep proteins have revealed multiple DNA recognition modes (16). In Rep, the interactions with ori are maintained with two loops that reside at one of the facets of this domain. The amino acids critical for nicking are located at the catalytic interface, which is on a different facet. Catalytic amino acids include two histidines (the HUH motif), a glutamate amino acid residue of the metal ion coordination site, and a nearby tyrosine that forms a covalent bond with the nicked DNA (16). Parvoviral genome replication is dependent on helicase activity of NS1. Structural information and biochemical studies of SF3 helicases have shown that the β-hairpin of the conserved B′ motif is critical for ssDNA binding and for helicase activity (17–20). Parvoviral NS1 proteins are transactivators of the viral capsid protein promoter. The transactivation do-
main is mapped to the C-terminal 67 amino acid residues in the minute virus of mice (MVM) NS1 (6, 21).

Fluorescence recovery after photobleaching (FRAP) is a widely used microscopy method that allows measurement of protein dynamics in living cells. Various mathematical models and computer simulations can be used to extract diffusion and binding properties of molecules from the FRAP data (22). Quantitative analysis of the FRAP experimental data is challenging in a multi-component system like the nucleus, which consists of a plethora of freely diffusing and interacting molecules in unknown concentrations (23). However, reliable information of protein dynamics can be obtained and used to predict a protein’s function in the cell (24, 25).

Our aim was to determine which regions in CPV NS1 mediate its interactions with DNA and how these interactions contribute to the binding of the protein in living cells. To this end, we used the DNA-bound structure of the AAV5 Rep protein and available biochemical data to pinpoint prominent amino acids in the CPV NS1 sequence. We mutated identified amino acids, expressed the mutants as fluorescent fusion proteins in NLFK cells, and followed their dynamics by using the FRAP technique. Computer simulations of the FRAP data suggest that NS1 has two independent binding modes toward an immobile binding partner. These results indicate that the N terminal and the helicase domain both strongly affect the DNA binding properties of NS1.

MATERIALS AND METHODS

Sequences and modeling. Parvovirus NS1 protein sequences were retrieved from UniprotKB (26) by using BLAST (27) with the CPV-N NS1 sequence as a reference (Swiss-Prot ID P12929). Alignment was produced from sequences of Parvovirus genus NS1 proteins (with Swiss-Prot ID numbers in parentheses): feline panleukopenia virus (FPV; P24842), mouse parvovirus 1 (MPV-1; Q83429), the immunosuppressive variant of MVM (MVMi; P07300), Killham rat virus (KRV; P88899), H1 parvovirus (H1; P03133), LuIII virus (P36311), and porcine parvovirus (PPV) NADL-2 (P18547). Alignments were done in the Bodil modeling environment (28) using the Malign algorithm. Alignment figures were generated with ClustalX (29). Isoelectric points for proteins were analyzed with the Sequence Manipulation suite (30).

The comparative model of the CPV NS1 helicase domain has been described before (15). In brief, the monomeric model was built with Modeller (31), and it is based on alignment of amino acid residues 277 to 490 of the AAV2 Rep40 protein (PDB accession numbers 1SHH and 1U0J) (18, 32) and residues 338 to 556 of the CPV NS1 protein. The hexameric model was built by superimposing modeled CPV NS1 subunits to the hexameric structure of a simian virus 40 L tag protein (PDB accession number 1N25 [33]) by using the Bodil program. Protein structure images were produced by using the PyMol program (34).

Constructs. All mutations were made to NS1-deYFP, which contains NS1 cloned to the multiple cloning site of the pEYFP-N3 plasmid (Clontech), conserved mutations at the P38 promoter area of the NS1 gene, and an ATG-to-ACG mutation at the start codon of the enhanced yellow fluorescent protein (EYFP) gene. This construct has been characterized (15). Mutations were made with PCR-based site-directed mutagenesis or the two-step PCR method. Primer sequences are available on request. The correctness of all constructs was confirmed by sequencing.

Fixed-cell studies. In infection studies, an NLFK cell line stably expressing H2B-ECFP (37) was used. Cells were fixed at 24 h or 48 h posttransfection and postinfection (p.t./p.i.) with 4% paraformaldehyde (PFA; 20 min at room temperature [RT]). CPV capsids were detected with a capsid-specific mouse antibody (a gift from Colin Parrish, Cornell University, Ithaca, NY) followed by goat anti-mouse Alexa 633 secondary antibody (Molecular Probes, Eugene, OR). Imaging was done using an Olympus FV-1000 confocal microscope with the UPLSAPO 60× oil immersion objective, numerical aperture (NA) of 1.35 (Olympus, Tokyo, Japan). The percentage of infected NS1-deYFP-expressing cells was calculated by using nuclear capsid antibody labeling and H2B-enhanced cyan fluorescent protein (ECFP) marginalization as markers for infection. Image analysis was done with ImageJ (38).

Replication efficacy. The effect of DNA-interacting mutations on viral replicative efficacy was examined in secondary infection studies. NLFK cells were plated on 3-cm-diameter culture dishes and transfected on the following day with pIC constructs. Medium from transfected cells (2 ml) was collected at 2, 3, or 4 days p.t., and concentrated to 50 μl in 100-kDa filter tubes (Amicon Ultra-4; Millipore). Twenty-five microliters of concentrated medium was applied to NLFK cells on a coverslip and the cells were incubated at 37°C for 15 min, 2 ml of fresh culture medium was added, and cells were moved back to the incubator. At 48 h postinoculation, cells were fixed with 4% PFA, labeled with anti-NS1 monoclonal antibody (Alexa 488-conjugated anti-mouse secondary antibody), and embedded with ProLong antifade reagent containing DAPI (4’,6-diamidino-2-phenylindole; Molecular Probes). Cells were imaged with an Olympus FV-1000 microscope and a 20× objective (NA, 0.75; 2× zoom). Six to eight randomly selected fields of cells from two independent experiments with each pIC construct and each time point were imaged. The infectivity percentage was calculated by dividing the number of NS1-positive nuclei by the total number of DAPI-stained nuclei (over 2,000 cells in each sample). NS1- and DAPI-positive nuclei were segmented using the k-means clustering algorithm in ImageJ.

FRAP. In FRAP experiments, NLFK cells were cultivated on glass-bottom dishes (MatTek Cultureware, Ashland, MA). Cells were transfected with plasmids using TransIT-LT1 transfection reagent according to the manufacturer’s instructions (Mirus Bio, Madison, WI). In infected samples, infection was done immediately after transfection. Confocal imaging was performed with an LSM 510 inverted laser scanning microscope (Carl Zeiss AG, Jena, Germany) with a Plan-Neofluor 63× (NA, 1.25) oil immersion objective. The sample holder and objective were heated to 37°C. The image size was 256 by 256 pixels with a resolution of 100 nm/pixel, and the pinhole was adjusted to 2 air units. A 514-nm argon laser line was used to excite the EYFP construct. In FRAP experiments, a high laser intensity (100% of 25 mW) was used to bleach 1-μm-wide (10 pixels) rectangular regions in seven iterations from the nucleus, avoiding the nucleolus. Images were acquired after bleaching with a low laser intensity (0.5 to 2%) with the time interval depending on the recovery speed of each construct (0.5 to 5 frames per second [fps]). Appropriate imaging rates were determined in preliminary studies. In noninfected cells, the rates were as follows: 4 fps for K406M, AU-K470/2A, AU-K470/2A-dC67, K470/2A-dC67, K2A-K470/2A, K2A-dC67, and K2A-K470/2A-dC67; 2 fps for E121A, AU, K2A, K470/2A, AU-K2A, and AU-dC67; 1 fps for NS1 and Y212F; ½ fps for dC67. For infected cells, imaging rates were as follows: 2 fps for K406M and K2A-K470/2A-dC67; 1 fps for E121A, AU, AU-K470/2A, AU-K470/2A-dC67, AU-K2A, AU-dC67, K470/2A-dC67, K2A-K470/2A, and K2A-dC67; ½ fps for NS1, Y212F, K2A, K470/2A, and dC67. ImageJ and spreadsheet software were used to analyze recovery data. FRAP data were normalized as follows (24): \( F_{\text{rel}} = \frac{B_t}{B_0} \left( \frac{N_t}{N_0} \right) \); relative fluorescence at time point \( t \) \( (F_{\text{rel}}) \) was calculated.
from the fluorescence of the bleach area at time point \( t (B) \), the average fluorescence of the bleach area before the bleach \( (B_0) \), the total fluorescence of the nucleus at time point \( t (N) \), and the average fluorescence of the nucleus before the bleach \( (N_0) \). The background value was measured from the outside of the cell and subtracted from all measured values. A minimum of 20 cells from at least 2 individual experiments were analyzed for infected samples and noninfected single-site mutants, while a minimum of 10 cells was imaged for noninfected double and triple mutants. Half-recovery was defined as the median value between the first image after the bleaching and full recovery. Student’s \( t \) test (two-tailed, unequal variance) was used to evaluate statistical significance in the change of the recovery time point values.

**Virtual cell simulations.** Virtual Cell software was used to simulate line FRAP data (39). The nucleus was simulated as an ellipsoid, with a width of 10 \( \mu \)m and a height of 15 \( \mu \)m. The time step of the simulation was set to 10 ms, and images were collected with the same frame rate as in the FRAP studies. The bleaching pulse was modeled as a laser light-induced general reaction. The bleaching region of interest was set to correspond to a 1-\( \mu \)m strip, as in the FRAP experiments. The length of the bleach pulse was adjusted to 50 ms, and the first recovery image was collected 50 ms after the bleach phase to simulate the image acquisition of the FRAP experiments. In addition, bleaching due to the imaging laser was modeled as a second continuous bleaching reaction in the nucleus. The binding reactions were modeled as a mass action reaction, in which the free ligand (freely diffusing NS1) reacted with the substrate (immobile binding partner) and formed a complex (immobile NS1). The concentrations of free binding sites and NS1 were set to 1 \( \mu \)M. In this situation, the pseudo-on-rate \( (k_{on}) \) is a product of the binding site concentration and the real on-rate of the reaction. The diffusion coefficient for monomeric NS1-deYFP in simulations was 18.8 \( \mu \)m/s. This estimate was based on mass scaling from the diffusion coefficient of freely diffusing EYFP (15, 35). The binding reaction \( k_{on} \) and \( k_{off} \) rates were changed in the modeling process until the recovery in the simulation fit the measured data. The reaction map for simulations is shown below in Fig. 4.

**In vitro FRAP.** In vitro FRAP (iFRAP) experiments were conducted essentially as described earlier (15, 40). Cells were grown on live-cell-imaging dishes and transfected 24 h before the experiments. When indicated, the infection was done immediately after the transfection. Before imaging, cells were washed with permeabilization buffer (20 mM HEPES, 110 mM K-acetate, 2 mM Mg-acetate; pH 7.5), and permeabilized with 100 \( \mu \)g/ml digitonin in the same buffer (6 min, RT). Imaging was done in a pH 6.8 permeabilization buffer. The buffer was supplemented with 1 mM ATP or 1 mM ADP (Sigma) from freshly prepared stock solutions when indicated. In salt concentration studies, imaging was done at different salt concentrations (20 mM HEPES, 2 mM Mg-acetate, 50 mM or 200 mM NaCl; pH 6.8). In all iFRAP experiments, the bleach area was a 2-\( \mu \)m-wide rectangle, the image acquisition rate was 5 fps, and data were collected for 20 s after the bleaching. Other imaging parameters were the same as in the live-cell FRAP experiments. Normalization was done as described for the FRAP experiments, except that for the ATP measurements, the recoveries were further normalized to between 0 and 1 (from the minimum value after bleaching to full recovery) to emphasize differences in the recovery phase.

**RESULTS**

**Identification of DNA-interacting amino acids in CPV NS1.** The N-terminal \( \sim 260 \) amino acid residues of NS1 from representative members of the *Parvovirus* genus were aligned with the N-terminal 193 amino acid residues of the AAV5 Rep sequence (41). Overall sequence identity between Rep and NS1 sequences was very low in this region (<17%), prohibiting reliable modeling of the CPV N-terminal domain. However, closer inspection showed that many critical amino acids were conserved between Rep and NS1 proteins. In Rep proteins, the N-terminal domain functions in the ORI and Rep binding site recognition as well as in catalytic site- and strand-specific nicking. Structures of this Rep protein domain have revealed that dsDNA recognition and nicking activities reside at the different facets (41).

The catalytic DNA-nicking activity of parvoviral replication proteins is associated with the divalent metal ion coordination site, which contains a conserved HUH motif (two histidines flanking a hydrophobic amino acid) (42) and a linking tyrosine (11). In the structure of the N-terminal domain from AAV5 Rep, the amino acids H89 and H91 form the HUH motif and together with E82 coordinate a Zn\(^{2+}\) ion (Fig. 1A) (41). According to the protein alignment, the corresponding amino acids in the CPV NS1 are H129, H131, and E121, and they are conserved in the *Parvovirus* genus (Fig. 1A and C). According to the alignment and previous results obtained with MVM NS1 (11), the tyrosine that covalently links NS1 to the 5’ end of the nicked DNA is Y212 in CPV NS1 (Fig. 1C).

dsDNA recognition in AAV5 Rep is principally mediated by the two surface loops of the N-terminal domain (amino acids 101 to 118 and 135 to 144) (16). Overall conservation of this region is poor between NS1 and Rep proteins. However, two lysines, K196 and K197 in CPV NS1 (K137 and K138 in AAV5 Rep) (Fig. 1A) are conserved in the *Parvovirus* genus members. In our hexameric model, K470 and K472 are aligned in the central cavity and are freely accessible to the solvent (Fig. 1B) (15).

**Mutations and fluorescent constructs.** According to the above analysis, we mutated the regions that are expected to target different DNA binding functions of the CPV NS1 (Fig. 1D). Sequence-specific dsDNA recognition was targeted with a K2A mutant (mutated amino acids K196A and K197A), nicking activity was targeted with the Y212F mutation, metal ion coordination was targeted with E121A and AAU (H129A and H131A) mutants, and helicase activity was targeted with mutations at one, or both, of the lysines in the B’ motif (K470A, K472A, or K470/2A). In addition to the DNA binding mutations, the transactivation activity of the CPV NS1 was targeted with a C-terminal deletion mutant, in which amino acids corresponding to the C-terminal 67 amino acids in the MVM NS1 were removed (dc67). To characterize the role of the modifications to the function of the CPV NS1, we cloned them as fluorescent EYFP fusions under a cytomegalovirus promoter (EYFP constructs). To inhibit the P38 promotor-driven expression of the free EYFP, we introduced conserved mutations to the TATA box area of the P38 promoter and mutated the start codon of EYFP (deYFP constructs in Fig. 1D) (35). Moreover, constructs that combined two or three of the above mutations in NS1 were made. To confirm the integrity of the fusion proteins, cells were transfected with deYFP constructs, and the whole-cell lysates were analyzed with Western blotting. All constructs, excluding the dc67 deletion, showed a major band of \( \sim 120 \) kDa that was recognizable with both anti-GFP (Fig. 2A) and anti-NS1 (results not shown) antibodies. All constructs with the dc67 deletion migrated as an \( \sim 100 \)-kDa band that was recogniz-
able with anti-GFP antibody. This deletion mutant could not be recognized with anti-NS1 antibody, since its epitope is located at the C terminus of the protein. The apparent size of all proteins was larger than their theoretical size of 104 kDa (97 kDa for dC67-deYFP). This was in good agreement with Western blot results obtained for wild-type (wt) CPV NS1 and for previously characterized NS1 fluorescent fusion proteins, which were detected 20-kDa larger than their theoretical size (15, 35). Fluorescence microscopy analysis of transfected cells showed that all NS1-deYFP fusion proteins were predominantly nuclear in both infected and noninfected cells (results not shown).

**Effects on transactivation and infectivity.** We first characterized whether induced mutations had changed NS1 P38 promoter transactivation properties. We transfected NLFK cells with EYFP constructs, which had a functional P38 promoter, and followed expression of free EYFP. Equal amounts of total cellular lysates from transfected cells were analyzed by Western blotting using an anti-GFP antibody. An EYFP-sized band was only detectable in

![FIG 1](http://jvi.asm.org/)

**FIG 1** Single-site DNA binding mutants. (A) Cartoon presentation of the structure of the AAV5 Rep N-terminal domain (PDB accession number 1M55) (41). Amino acids in the catalytic domain and the ORI binding loop are shown as stick models, and bound Zn ion is shown as a sphere. Amino acids are numbered according to Rep, and alignment with CPV NS1 is shown in parentheses. (B) Surface presentation of the hexameric model from the CPV NS1 helicase domain (amino acids 338 to 556). Two lysines in the β-hairpin of the B′ motif are shown in red. (C) Alignment of the N termini of AAV5 Rep and parvoviral NS1 proteins. Numbering below the alignments is according to that for CPV NS1. (D) Schematic presentation of fluorescent NS1 mutant constructs.
NS1, E121A, AUA, Y212F, and K470/2A samples (Fig. 2B, anti-GFP). Next, we transfected cells with infectious clones (pIC) harboring the same mutations and blotted for capsid proteins (Fig. 2B, anti-VP). Remarkably, the same constructs as in the EYFP experiment showed prominent capsid protein production. These experiments strongly suggest that mutations in the predicted dsDNA binding facet of the N-terminal domain (K2A) and the deletion of the C terminus abolish the CPV NS1s transactivation ability.

To characterize how the induced mutations affected the replication efficacy of the virus, we monitored the effects of mutations on virion production. Medium from mutated and nonmutated infectious clone-transfected cells was collected 24, 48, or 72 h p.t., concentrated, and used to inoculate new cells. Immunofluorescence analysis demonstrated that only the nonmutated pIC was able to produce detectable amounts of infectious viruses in the culture medium. The infectivity percentage of the pIC medium collected at 24, 48, and 72 h p.t. was 1.2% ± 0.7%, 5.6% ± 3.8%, and 9.1% ± 4.8% (mean ± standard deviation), respectively (Fig. 2C). All mutated infectious clones showed only a trace amounts of infected cells. The highest infectivity rate (0.25% ± 0.4%) was observed for medium collected from pIC-E121A at 24 h p.t. The infectivity of other mutants was below 0.2% at all time points. This suggests that all mutations inhibit either virion production or cellular release of virions to the surrounding medium.

The effect of NS1 mutations on progression of the infection was studied in histone H2B-ECFP-expressing cells. Cells were fixed at 24 h or 48 h posttransfection and postinfection, and antibody labeled for viral capsids was added to the cultures. Nuclear accumulation of the virus capsids and marginalization of the host genome labeled with H2B-ECFP were used as markers for infection (15, 35). Studies showed that expression of mutated NS1-deYFP constructs inhibited CPV infection. At 24 h p.t./p.i., 82% of NS1-deYFP-expressing cells were infected, and the amount of infected cells increased to 91% at 48 h p.t./p.i. (Fig. 2D). At 24 h p.t./p.i., the highest infectivity of mutant NS1-expressing cells was seen for AUA-deYFP (~42%), and similar infection percentages were observed for cells expressing E121A-deYFP (~41%), K2A-deYFP (~37%), and Y212F-deYFP (~38%). Infectivity at 24 h p.t./p.i. was markedly reduced in cells expressing dC67-deYFP.
Distribution analysis of viral capsid protein and chromatin showed variations between mutants. Of infected NS1-deYFP-expressing cells, ~7% had a phenotype where capsid proteins accumulated to the nuclei (Fig. 2D, black bar), ~56% had the host chromatin marginalized toward the nuclear envelope but no nuclear capsid labeling (Fig. 2D, white bar), and ~36% showed that both the marginalized genome and nuclear virus capsid accumulation (gray bar). Similarly, most mutant NS1-expressing cells showed all three phenotypes observed in NS1-deYFP, although at different levels. Interestingly, infected K470/2A-deYFP-expressing cells did not contain any cells showing host chromatin marginalization at 24 h.p.t./p.i., and a proportion of this phenotype was the smallest of all mutants at 48 h.p.t./p.i. (~53%). The proportion of the cells with nuclear capsid accumulation was smallest in infected dC67-deYFP transfectants (~17% and ~15% at 24 h.p.t./p.i. and 48 h.p.t./p.i., respectively).

To conclude, all of the mutations inhibited virion production in the secondary infection assay. In addition, infectivity was clearly reduced in cells that expressed mutated NS1 proteins, compared to those expressing nonmutated NS1. Moreover, two of the mutants had distinct distribution-of-infection phenotypes: the helicase mutant K470/2A deYFP transfectants were least often associated with marginalization of the host genome, and the C-terminal deletion dC67 mutant was least often associated with nuclear capsid accumulation.

**FRAP analysis of NS1 mutants.** The binding properties of the fluorescent NS1 mutants were studied using the FRAP technique. Cells were transfected with deYFP constructs (Fig. 1D) and analyzed at 24 h.p.t./p.i. (Fig. 3A) or at 24 h.p.t. (Fig. 3B). To gain further insight into the recovery properties of the mutants, we also included in the analysis the previously characterized K406M, an NS1 mutant that does not bind ATP (15). In FRAP studies, a 1-μm-wide strip from the nucleus and outside the nucleolus was bleached with high laser intensity, and redistribution of the fluorescence was monitored until it reached equilibrium. First, the effect of the diffusion on recovery was monitored by following the changes in the shape of the fluorescence gradient between images taken after bleaching and at the half-recovery time point (results not shown). The fluorescence gradient was smoothed in all mutants, indicating that they all underwent diffusion-dependent recovery (43).

Next, we compared the half-recovery time points (τ/2) of FRAP recoveries for the mutants expressed in infected and in noninfected cells (Fig. 3A and B). In noninfected cells, the τ/2 of NS1-deYFP was 10.65 ± 3.74 s (n = 20 cells; average ± standard deviation), and the fastest recovery was seen with the helicase mutant K470/2A-deYFP (τ/2, 1.08 ± 0.4 s; n = 25) and slowest for the dC67-deYFP mutant (τ/2, 20.96 ± 4.47 s; n = 23). The τ/2 of the Y212F-deYFP mutant (τ/2, 7.82 ± 2.46 s; n = 22) was closest to that for NS1-deYFP. Constructs with mutations in the N-terminal domain had τ/2 values that were very similar to each other and to that for K470/2A-deYFP but considerably faster than for NS1-deYFP. The τ/2 values for the two metal ion coordination mutants, E121A-deYFP (1.2 ± 0.47 s; n = 20) and AUA-deYFP (1.55 ± 0.63 s; n = 20) were not statistically different from each other (P > 0.053), while K2A-deYFP (τ/2, 1.1 ± 0.30 s; n = 23) was significantly slower than AUA-deYFP (P < 0.0079). Interestingly, the τ/2 for the K406M-deYFP mutant was significantly faster (P < 0.001) than for K470/2A-deYFP (τ/2, 0.48 ± 0.07 s; n = 24).

All constructs, excluding dC67-deYFP, showed drastically slower τ/2 values after infection (Fig. 3A). The dC67-deYFP mutant had similar τ/2 values regardless of infection (P > 0.9). The recovery τ/2 in nonmutated NS1-deYFP was ~2.5 times slower in infected than in noninfected cells, and a similar change was seen for Y212F-deYFP. Considerable larger changes in recovery τ/2 values, ranging from ~7.4- to ~12.5-fold slower values in infected than in noninfected cells, were observed for other mutants and for K406M-deYFP. Compared to NS1-deYFP (τ/2, 27 ± 8.91 s; n = 20), all mutants had significantly faster recovery in infected cells (P < 0.05). The fastest recovery was observed for the helicase mutant K470/2A-deYFP (τ/2, 11 ± 4.61 s; n = 22), and the slowest was observed with dC67-deYFP (21.22 ± 9.68 s; n = 23). Two metal ion coordination mutants, E121A-deYFP (τ/2, 11.1 ± 3.48 s; n = 22) and AUA-deYFP (τ/2, 11.5 ± 4.30 s; n = 23) had similar values of τ/2, while the K2A τ/2 (13.9 ± 4.84 s; n = 21) was significantly slower than that of E121A-deYFP (P < 0.0036). In infected cells, the Y212F-deYFP mutant (τ/2, 18.5 ± 10.1; n = 23) had a τ/2 comparable to those for the dC67-deYFP and K2A-deYFP mutants. Finally, the τ/2 values for all mutants were significantly faster than for K406M-deYFP (4.0 ± 1.7 s; n = 23; P < 0.001).

**Virtual cell simulations of NS1 mutants.** FRAP experiments of the single-site mutants in infected and noninfected cells were reproduced in the virtual cell simulation environment to gain insight into the binding properties of the constructs (Fig. 4). According to previous results, a diffusion coefficient of 18.8 μm²/s was used for the NS1-deYFP fusion (15, 35). Attempts to simulate FRAP recoveries with only free diffusing proteins or a single binding site did not correctly reproduce the experimental data (results not shown). Addition of a second binding site to the simulations yielded good fits to the experimental data in noninfected cells (Fig. 3B), but an immobile fraction was needed to simulate the FRAP values in infected cells (Fig. 3A). Binding pseudo-on (k_{on}) and off (k_{off}) rate constants for both reactions were sampled until the experimental FRAP was correctly reproduced (Fig. 3C).

In infected cells, the immobile fraction was 4% for all other constructs but 2% for Y212F (Fig. 3C). The first binding reaction (B1) had higher k_{on} and k_{off} rates in all cases and hence faster turnover than for the second binding reaction (B2). In infected cells, ~30.5% of the nonmutated NS1 was bound to the B1 fraction, with a binding time of 20 s (1/k_{1,off}, ~35%) was bound to the B2 fraction with a binding time of ~143 s (1/k_{2,off}), and ~30.5% was freely diffusing for 17.2 [1/(k_{1,off} + k_{2,off})] (Fig. 3C). Binding times of Y212F and dC67 mutants were identical to NS1. However, their free diffusion times were a bit longer than NS1, and hence the distribution of the proteins at equilibrium was slightly different. In Y212F, the fraction of B2 had decreased, while both B1 and freely diffusing populations were increased compared to nonmutated NS1. Similar changes were observed in dC67 simulations. Recoveries of the two metal ion coordination mutants, E121A and AUA, were reproduced with the same simulation parameters. Both were bound to B1 for 12.5 s (~37.6%), B2 for 55.56 s (~20.9%), and diffusing freely for 11.11 s (~37.6%). Interestingly, the FRAP results for K470/2A-deYFP and K2A were highly similar. Slightly longer binding times were observed in simul-
FIG 3 FRAP and virtual cell simulations. (A and B) FRAP results for the single-site NS1 mutants in infected (A) and noninfected (B) cells (n = 20). Normalized recovery curves are shown in blue, and virtual cell simulations are in red for each construct. Error bars are standard deviations. Half-recovery times (t₁/₂), measured in seconds, are shown as means ± the standard deviations. (C) Simulation parameters for infected and noninfected recoveries. Residence times (in seconds) and proteins fractions (percentage) for both binding reactions and free diffusion are shown. The immobile fraction (as a percentage) was used to simulate recovery in infected cells. Corresponding pseudo-on-rate (kₑₒᵣₙ) and off-rate (kₒᵣₙ) constants for both binding reactions are shown (per second).
tions for the helicase mutant K470/2A, which was bound to the B1 for \( \sim 14.3 \text{ s (37.1\%)} \), to B2 for \( 66.7 \text{ s (21.8\%)} \), and diffusing freely for \( 12.5 \text{ s (37.1\%)} \). The K2A binding time to B1 was \( 16.67 \text{ s (36\%)} \), \( 66.67 \text{ s (24\%)} \) for B2, and it was diffusing freely for \( 14.3 \text{ s (37.1\%)} \). The shortest binding times were seen for the K406M mutant. It was bound to B1 for \( 4 \text{ s (40\%)} \), B2 for \( 50 \text{ s (16\%)} \), and freely diffusing for \( 3.9 \text{ s (40\%)} \).

In noninfected cells, the recovery of NS1-deYFP was considerably faster than in infected cells. In equilibrium, \( \sim 31\% \) of NS1-deYFP was bound to B1, with a binding time of \( 6.3 \text{ s (36\%)} \), \( 66.67 \text{ s (24\%)} \) for B2, and it was diffusing freely for \( \sim 14.3 \text{ s (36\%)} \). The shortest binding times were seen for the K406M mutant. It was bound to B1 for \( 4 \text{ s (40\%)} \), B2 for \( 50 \text{ s (16\%)} \), and freely diffusing for \( 3.9 \text{ s (40\%)} \).

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In infected cells, constructs could be grouped roughly into two categories: NS1-like binding (NS1, Y212F, and dC67) and faster-turnover binding (E121A, AUA, K2A, and K470/2A). Mutants in the NS1-like category differed only in the pseudo-on rate of B2, which was slightly slower than in NS1 in all mutants. Mutants in the faster-turnover category had increased turnover (higher pseudo-on and off rates) in B1 and over 2-times-higher off rates in B2 compared to NS1. In noninfected cells, single-site mutants could be grouped into three categories: NS1-like binding (NS1 and Y212F), slower turnover (dC67), and faster turnover (E121A, AUA, K2A, and K470/2A). dC67 had clearly lower pseudo-on and off rates in both B1 and B2 reactions than NS1. The faster-turnover group was more heterogeneous than in infected cells. Compared to NS1, all had faster turnover in the B1 reaction, but the off rate in B2 was slower for E121A.

**FIG 4** Virtual cell simulation reaction map. (A) NS1 binding was modeled with two binding reactions to immobile targets (bound 1 and bound 2). Reactions were defined with individual on and off rates (\( k_{1\text{on}} \) and \( k_{1\text{off}} \), and \( k_{2\text{on}} \) and \( k_{2\text{off}} \)). (B) Schematic of the virtual cell simulation reactions. Green circles represent reactants, and yellow circles represent reactions. The unbound population of NS1 (free) can undergo two reversible binding reactions (K1 and K2) with immobile substrate (DNA1 and DNA2), leading to complex formation (bound 1 and bound 2). For every step, there is a concentration-dependent probability for switching to one of the irreversibly bleached populations [bound 1 or 2 (bleached); free (bleached)]. This is caused by imaging (imaging laser) or bleach pulse (bleach laser). Bleached molecules are still able to compete for binding sites. In case of infection, some proteins were immobile [immobile and immobile (bleached)] and did not compete for binding sites.
turnover mutants was an increased freely diffusing population and decreased B2 population at equilibrium. Finally, the ATP binding mutant K406M was clearly different from all others. It had a highly increased turnover in B1 and a slightly increased turnover in B2 than NS1.

In vitro FRAP. The dynamics and function of NS1 are dependent on binding and hydrolysis of ATP molecules (15, 44, 45). We tested if NS1 mutants retained the ATP dependency of their binding dynamics. To this end, we used ivFRAP method, where the plasma membrane is permeabilized with digitonin and FRAP imaging is conducted in a buffer solution (40). This treatment effectively removes small soluble molecules, e.g., ATP, from the cytoplasm and nucleus (46). By changing the constituents of the imaging buffer, we monitored the ATP dependency of the binding dynamics.

Binding of all single-site mutants and NS1 was enhanced in noninfected cells upon addition of ATP (Fig. 5). The fast recovery of NS1-deYFP without ATP was markedly slowed in the presence of ATP, and a very similar behavior was seen for mutants Y212F-deYFP, K2A-deYFP, and AUA-deYFP (Fig. 5) and also to a lesser extent for K470/2A-deYFP. Without ATP, the recovery of the dC67-deYFP mutant was more complete than for NS1-deYFP during the imaging period (Fig. 5). This could indicate a smaller freely diffusing population for dC67-deYFP compared to NS1-deYFP. Upon addition of ATP, the recoveries of the dC67-deYFP and NS1-deYFP constructs were very similar.

FRAP analysis of combined mutations. The above FRAP results implied that the N-terminal and helicase domains affect both the fast (B1) and the slow (B2) turnover binding reactions. To assess the possible cooperative role on dynamics of NS1, we combined N-terminal (AUA and K2A), helicase (K470/2A), and C-terminal (dC67) mutations as fluorescent constructs and studied their dynamics with the FRAP technique by comparing recovery $t_{1/2}$ values for the mutants.

First, we combined N-terminal metal ion coordination mutant AUA with other mutations. In noninfected cells, $t_{1/2}$ was faster in AUA-K2A and AUA-K470/2A mutants (Fig. 6, CPV-), while $t_{1/2}$ was markedly slower in the AUA-dC67 mutant than the AUA mutant. Again, the triple-mutant AUA-K470/2A-dC67 had a fast $t_{1/2}$ that was comparable to that for AUA-K470/2A. In infected cells, AUA-K2A, AUA-K470/2A, and AUA-K470/2A-dC67 had faster $t_{1/2}$ values than the AUA mutant, and the recovery for AUA-dC67 was again slower (Fig. 6, CPV+).

Results with K2A combined mutants were similar to those of AUA mutants. Compared to K2A, a faster recovery was seen for mutants K2A-AUA, K2A-K470/2A, and K2A-K470/2A-dC67 in both infected and noninfected cells.

The helicase mutant K470/2A-deYFP had the fastest $t_{1/2}$ of all single mutants, and combining it with other mutations had only a marginal effect in noninfected cells. Interestingly, this was also
true for the C-terminal deletion mutant (K470/2A-dC67), which was seen to slow the recovery of AUA and K2A mutants. In infected cells, K470/2A-AUA and K470/2A-AUA-dC67 had faster recoveries than K470/2A, while the recoveries of K470/2A-AUA and K470/2A-AUA-dC67 were even faster.

C-terminal deletion mutant dC67-deYFP had the slowest recovery of all single mutations in noninfected cells. Increasingly faster recoveries were observed in noninfected cells upon addition of AUA, K2A, K470/2A, AUA-K470/2A, and K2A-K470/2A mutations. A slower overall recovery but a very similar trend upon addition of combined mutations was seen in infected cells.

Overall, deletion of the C terminus slowed recovery of both AUA and K2A mutants. A slower recovery associated with the dC67 mutation was not seen in any construct with a helicase mutation (K470/2A-dC67, K2A-K470/2A-dC67, or AUA-K470/2A-dC67). This suggests that deletion of the NS1 C terminus enables NS1 to bind more tightly, and this binding is dependent on its helicase domain.

DISCUSSION
DNA binding is essential for many functions of the parvoviral replication protein NS1. The structure of NS1 is modular, and many of its functions are mapped to a specific domain. The functions of these domains in CPV NS1 have not been characterized previously, and only a minority of the information on NS1s of other parvoviruses has been obtained from living cells, within the context of their natural cellular environment. By using comparative modeling, sequence comparison with homologous proteins, and prior biochemical knowledge obtained from the NS1 proteins of other parvoviruses, we searched for amino acids that are critical for DNA binding in the CPV NS1. Our data showed that certain amino acids are conserved in NS1 proteins of autonomous parvoviruses. Mutation in any of the identified amino acids stops virus production from a CPV infectious clone, further supporting the vital role of these amino acids. ATP binding is known to be crucial for the function of the CPV NS1 (15). All proposed DNA binding mutants did, however, retain their ATP binding ability, and hence this property does not explain our results. In addition, CPV’s ability to infect cells that expressed any of the mutated NS1 proteins was reduced compared to cells expressing nonmutated NS1. These data strongly suggest that we have identified amino acids that are critical for NS1 function and virus replication and that mutated forms are able to inhibit NS1 function in a dominant negative manner.

During replication, NS1 recognizes and binds to tetrancleotide repeat ACCA-containing sequences at the viral ORI regions (7, 47). NS1 is also a transcriptional regulator and, most importantly, transactivator of the capsid protein promoter P38 (48). The transactivation region in the P38 promoter and ORI region share the same ACCA sequence, suggesting that the same domain of the NS1 protein is responsible for DNA binding in both (47). Both of these sequence-specific interactions are dependent on the N-terminal domain of NS1, but the exact amino acids that mediate this interaction are not known (7, 48). In the related AAV Rep protein, DNA recognition has been more thoroughly characterized. In analogy to NS1, the Rep protein recognizes tetrancleotide repeats of the Rep binding site (RBS) in the AAV genome. In the structure of the AAV5 Rep RBS, two surface loops of the N-terminal domain maintain the majority of the contacts with the DNA (16). We compared the sequences of CPV NS1 and AAV5 Rep in order to find the conserved amino acids in the RBS binding region. The N-terminal domains of paroviral NS1 proteins are 40 to 50 amino acids longer than in Rep, and the sequences are quite dissimilar. The binding sequences in the DNA targets of the proteins are quite different, which might explain the differences in these proteins (7, 16). However, two consecutive lysines in AAV5 Rep, K137 and K138, that are located in the DNA recognition loop are conserved in autonomous parvoviruses and correspond to K196 and K197 in CPV NS1. The same loop is important for dsDNA binding in the AAV2 Rep protein (49). Mutation of these lysines to alanines in the K2A mutant severely reduced its P38 transactivation activity. Compared to NS1, K2A had faster binding kinetics in both infected and noninfected cells. In infection, the nucleus of an infected cell is filled with virus-specific DNA that offers sequence-specific targets for NS1, while nonspecific interactions with host chromatin dominate in noninfected cells. Observed differences in the binding kinetics suggest that the K2A mutant is defective in its nonspecific DNA binding as well in virus-specific DNA recognition.

Besides the N-terminal domain, the transactivation activity of NS1 is dependent on the C terminus (21). Transactivation activity is retained in a fusion protein where the C-terminal 126 amino acids of MVM NS1 are fused with the DNA binding domain of LexA, a dimeric bacterial repressor protein (6). This supports the idea that the C terminus of NS1 does not directly bind to the DNA but is able to function as a transcriptional activator when it is guided to the promoter. A deletion of 67 amino acids from the C terminus of MVM NS1 is enough to remove its capsid promoter transactivation activity (21). Our results showed the same for CPV NS1. Like K2A, a C-terminally truncated NS1, dC67, was severely reduced in a P38 transactivation assay. In addition, the infected dC67 transfectants showed the smallest percentage of cells with nuclear capsid labeling. This suggests that the dC67 mutant inhibits the transactivation activity of wt NS1 in a dominant negative manner. In noninfected cells, dC67 recovery was markedly slower than NS1. This suggests that dC67’s ability to bind nonviral DNA is stronger than that of nonmutated NS1. The C-terminal deletion in dC67 changes the theoretical isoelectric point of NS1 from 7 to 7.3, which could explain the differences in their binding properties. However, the binding kinetics of dC67 and NS1 were very similar in infected cells, where sequence-specific interactions dominate. This argues that dC67 has lost its DNA binding specificity compared to NS1. The C terminus of NS1 could have a regulatory role in controlling NS1’s binding specificity; the strong binding to DNA would only occur in the presence of virus-specific DNA sequence. Removal of this control switch in dC67 would allow NS1 to bind strongly to nonspecific dsDNA. Interestingly, a similar mechanism has been recently proposed for a human T-cell leukemia virus nucleocapsid (NC) protein that is a nucleic acid chaperone (50). The cationic N terminus and anionic C terminus of the NC protein were concluded to form an intramolecular interaction, leading to reduced chaperone function. Removal of the C termini enhanced the NC proteins’ chaperone activities. A similar mechanism where the anionic C terminus of NS1 inhibits the strong nonspecific DNA binding could explain the slower dynamics of the dC67 mutant in noninfected cells. In infection, the nucleus is filled with virus-specific DNA, leading to slower, more dC67-like dynamics for NS1. Alternatively, interactions with the endogenous transcription factors could provide an explanation for the differences in DNA binding properties of the dC67 and...
nonmutated NS1 in noninfected cells. The MVM NS1 interacts with sp1 and presumably with other endogenous transcription factors (51). The sp1 interaction is mediated by the C-terminal domain (6, 52), which is deleted from the dC67 mutant. The binding of the NS1-sp1 complex would be fine-tuned toward virus-specific DNA sequences. This in turn could lead to an increase in overall dynamics in noninfected cells, where specific binding sites are scarce.

Considering the close functional relationship between ORI recognition and nicking in parvoviruses, it is not surprising that the nicking activity is also located in the N-terminal domain (11). In studies with the AAV5 Rep structure have revealed that the amino acids responsible for RBS recognition and those responsible for nicking reside on different facets of the N-terminal domain (16). Some of the amino acids that are important for the nicking reaction have been characterized in autonomous parvovirus NS1 proteins (11). The catalytic site contains a conserved metal ion coordination center characterized by two conserved histidines and an acidic amino acid (42). Mutations at the conserved histidines in this HUH motif have been reported to abolish nicking activity in the MVM NS1 protein (11). According to the sequence comparison, H129, H131, and E121 form the metal ion coordination cluster in the CPV NS1. Moreover, the nicking activity is dependent on a specific tyrosine that links the protein covalently to the 5′ end of the cleaved DNA strand (11). Comparison with MVM NS1 and Rep proteins suggests that the linking tyrosine in CPV NS1 is Y212. The homologous tyrosine in AAV5 Rep, Y153, is in proximity to the HUH motif but does not participate in the binding of the ion (16). In AAV2, the nicking is dependent on two tyrosine residues (53); however, based on results obtained with MVM NS1, just one tyrosine is sufficient for autonomous paroviruses (11). Despite the close spatial proximity of the mutated sites with K2A, both AUA and E121A mutants retained their P38 transactivation activity, suggesting that these mutants have retained their sequence-specific dsDNA binding properties. Similar mutations in MVM NS1 abolish its dsDNA nicking activity, similar to mutation in the linking tyrosine (11). In our FRAP experiments, both E121A and AUA had faster recoveries in both infected and in noninfected cells than did NS1. This suggests a reduction in both nonspecific and sequence-specific DNA binding properties of these mutants. The remaining N-terminal mutant, Y212F, had almost no effect on the binding characteristics of CPV NS1. The only difference, compared to NS1, was a reduction in the immobile fraction. It is possible that the immobile fraction in FRAP experiments represents covalent NS1-DNA complexes that arise from the nicking reaction. The reduced immobile fraction in Y212F would be explained by its expected reduction in nicking activity. However, a similar reduction in the immobile fraction was not seen with other presumably nonnicking mutants, and we cannot confirm that the small reduction seen in Y212F was not due to experimental noise. Differences in binding kinetics of Y212F compared to AUA and E121A suggest that the lack of nicking activity is not enough to explain the faster recovery of AUA and E121A mutants. Since these mutations target a metal ion coordination site, it is possible that these amino acids have a general stabilizing role in the N-terminal domain or participate in correct positioning of the DNA at the catalytic center. Alternatively, the AUA and E121A mutations, but not Y212F, could abolish protein-protein interactions that are important for the DNA binding of NS1.

Paroviral NS1 proteins function as helicases, and the SF3 helicase domain resides in the middle of the protein (5), at residues 338 to 556 in CPV NS1 (15). SF3 helicases are thought to encircle ssDNA as a hexameric ring, and the interactions with the DNA are mediated by the β-hairpin of the B′ motif (17, 54). In the ssDNA-bound structure of bovine papillomavirus (BPV) E1, a lysine and a histidine of the β-hairpin directly interact with the backbone phosphates of consecutive bases in translocated DNA (17). In AAV5 Rep, the β-hairpin has two lysines, the first of which is reported to be critical for DNA binding (18, 20). Both lysines are conserved in the autonomous parvovirus NS1 proteins and correspond to K470 and K472 in CPV NS1. The hexameric model of the CPV NS1 supports the idea that these lysines are important for ssDNA translocation. Both K470 and K472 are at the surface of the protein and point toward the central cavity. The coinfection of K470/2A-transfected cells lead to a low percentage of cells with marginalized chromatin, a phenotype that is linked to virus genome replication in CPV infection (15, 35). In the P38 transactivation assay, the K470/2A mutant showed activity comparable to that of NS1. These results suggest that the K470/2A mutant has retained its sequence-specific dsDNA recognition but is deficient in viral genome replication, presumably due to the absence of helicase activity.

K470/2A and K2A mutants had the fastest binding kinetics of all DNA binding mutants in infected and noninfected cells. Our comparison also included a CPV NS1 mutant, K406M, that does not show ATP-induced DNA binding (15). Recovery of K406M was even faster than K470/2A or K2A regardless of the infection status. This suggested that ATP-dependent changes in NS1 binding are mediated through multiple DNA binding interactions. Interestingly, the mutants K2A and K470/2A, which targeted ORI binding and helicase domains, respectively, had very similar recoveries in both infected and noninfected cells. Both were characterized by similar increases in turnover in both binding reactions in infected cells. In noninfected cells, their turnover was faster than NS1 in the first binding reaction, while binding to the second binding site was reduced. This strikingly similar behavior for the two mutants could indicate that, although the amino acids reside in different domains of the NS1 protein, they both contribute to the same DNA binding function. A similar observation has been recently reported for the BPV E1 protein (55). Even though BPV E1 and CPV NS1 are not closely related at the sequence level, they share analogous roles in replication of the virus genome. Like NS1, E1 is a viral initiator protein. It has an N-terminal ORI binding domain and a central SF3 helicase domain that are connected by a small multimerization region. The function of E1 has been recently investigated, and structures of the N-terminal and helical domains are available (17, 56). E1 binds to the ORI sequence as a head-to-head double trimer and causes local melting of the dsDNA and formation of a head-to-head double hexameric helicase ring (19, 57). E1 is known to bind sequence specifically to the dsDNA with its N-terminal domain (58), while nonspecific dsDNA binding is needed for E1 double-trimer formation (55). Interestingly, the β-hairpin of the conserved B′ motif and two loops in the helicase domain are important for the nonspecific dsDNA binding of E1 (55). Considering the similar binding characteristics of the CPV NS1 mutants, N-terminal K2A and helicase domain K470/2A, in noninfected cells, we suggest that paroviral NS1 proteins share a similar nonspecific dsDNA binding mechanism with the BPV E1 protein. The dynamics for the double mu-
tant were faster than that of either single mutant, indicating further-reduced binding. These data suggest that the K2A and K470/2A mutations target a common and unique DNA binding reaction of NS1.

One of the inherent challenges in interpreting FRAP results is the lack of detailed knowledge of the concentrations of the binding components. During the experiments, infected cells were selected based on the marginalization of the host chromatin, which is a reliable indicator of viral genome replication (35). This approach gives a good sample-to-sample reproducibility for FRAP results, indicating relatively constant concentrations of binding partners. However, expression of NS1 mutants, especially K470/2A, in infected cells might lead to differences in the viral genome concentrations between different mutants. The change in the concentration of NS1 binding sites would directly affect the binding pseudo-on-rate constant. However, the binding off-rate constant, and thus the time during which the NS1 stays bound, would not be affected. These possible differences in the binding site concentration should be taken into consideration when comparing binding properties of different mutants in infected cells. In our recent studies, the dynamics of the CPV NS1 protein in infected and noninfected cells were concluded to have at least two independent binding modes (15,35). In addition, we previously showed that ATP binding has a strong influence on NS1 dynamics, and it is associated with slow turnover for the binding reaction (15). Based on these results, we hypothesized that the fast-turnover binding originates from the interactions of the N-terminal ORI recognition domain, while the slow-turnover binding represents an ATP-dependent helicase activity of NS1. Much to our surprise, the dynamics of the ORI binding mutant, K2A, and the helicase domain mutant, K470/2A, in the present study were very similar. This strongly suggests that our original interpretation on the origin of the two binding modes was oversimplified. Instead, both the slow- and the fast-turnover binding reactions of NS1 are dependent on interactions that are mediated by both the N-terminal ORI binding domain and the helicase domain. Hence, we propose that the two observed binding modes of NS1 originate from the nonspecific and sequence-specific interactions with the dsDNA.

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