The Formation of a Flexible DNA-binding Protein Chain Is Required for Efficient DNA Unwinding and Adenovirus DNA Chain Elongation

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The adenovirus DNA-binding protein (DBP) binds cooperatively to single-stranded DNA (ssDNA) and stimulates both initiation and elongation of DNA replication. DBP consists of a globular core domain and a C-terminal arm that hooks onto a neighboring DBP molecule to form a stable protein chain with the DNA bound to the internal surface of the chain. This multimerization is the driving force for ATP-independent DNA unwinding by DBP during elongation. As shown by x-ray diffraction of different crystal forms of the C-terminal domain, the C-terminal arm can adopt different conformations, leading to flexibility in the protein chain. This flexibility is a function of the hinge region, the part of the protein joining the C-terminal arm to the protein core. To investigate the function of the flexibility, proline residues were introduced in the hinge region, and the proteins were purified to homogeneity after baculovirus expression. The mutant proteins were still able to bind ss- and double-stranded DNA with approximately the same affinity as wild type, and the binding to ssDNA was found to be cooperative. All mutant proteins were able to stimulate the initiation of DNA replication to near wild type levels. However, the proline mutants could not support elongation of DNA replication efficiently. Even the elongation up to 26 nucleotides was severely impaired. This defect was also seen when DNA unwinding was studied. Binding studies of DBP to homo-oligonucleotides showed an inability of the proline mutants to bind to poly(dA)₄₀, indicating an inability to adapt to specific DNA conformations. Our data suggest that the flexibility of the protein chain formed by DBP is important in binding and unwinding of DNA during adenovirus DNA replication. A model explaining the need for flexibility of the C-terminal arm is proposed.

Adenovirus DNA replication can be reconstituted in vitro, using three viral proteins, adenovirus DNA polymerase (pol), precursor terminal protein (pTP), and the DNA-binding protein (DBP). Optimal replication efficiency is obtained when two cellular transcription factors are added, nuclear factor I (NFI) and Oct 1 (for reviews see Refs. 1–3).

The adenoviral dsDNA genome contains two terminal proteins (TP) covalently linked to the 5′-ends. The inverted terminal repeats contain the origins of replication. pTP and pol are tightly associated in solution. During initiation of replication pTP functions as a primer to which the first nucleotide, dCTP, is covalently coupled. Both NFI and Oct 1 stimulate the initiation by recruiting the pTP-pol complex to the origin of replication (4–7). Initiation starts opposite position 4 of the template strand. After formation of a pTP-trinucleotide (pTP-CAT) (8), the complex jumps back and CAT becomes paired with template residues 1–3. Shortly after jumping back, the polymerase dissociates from pTP and elongation proceeds via strand displacement (9).

DBP has several functions during the adenovirus life cycle. Besides DNA replication, the protein is involved in transcriptional control and mRNA stability (10, 11), transformation (12), virion assembly (13), and determination of the host range (14, 15).

DBP performs several functions in DNA replication. During initiation, it stimulates directly the formation of a pTP-CAT intermediate by lowering the Kₘ value of the reaction (16), possibly via a direct interaction with the pTP-pol complex. Indirectly, DBP stimulates initiation by increasing the binding of NFI to the origin (17–19). The stimulation of initiation by DBP is most pronounced at low pTP-pol concentrations, suggesting a role of DBP in recruitment of the pTP-pol complex to the origin. Furthermore, DBP plays an essential role during the elongation phase of DNA replication, where it helps to unwind the parental strand (20) and enhances the processivity of the polymerase (17). This is achieved by cooperative binding to the displaced strand during replication, thereby protecting it from nuclease digestion and facilitating strand displacement. Strand displacement is ATP-independent and requires only the helix DNA unwinding activity of DBP, unlike helicase activity, which does require ATP (21–23). Finally, DBP enhances the renaturation of complementary displaced strands (20).

The crystal structure of the C-terminal domain of DBP (amino acids 174–529, ΔN-DBP) has been solved (24). This region contains the DNA binding domain and is functional in in vitro assays (25–27). The protein is mainly globular except for the last 17 amino acids, which form a protruding arm. This C-terminal arm binds to a hydrophobic cleft of another DBP molecule, allowing protein chain formation which is essential for the function of DBP in elongation and cooperative binding on ssDNA as shown by deletion studies (28) (Fig. 1B). A second crystal structure of ΔN-DBP has been described, which super-
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Here we have studied the role of the flexibility of the C-terminal arm of ssDNA binding and DNA replication using DBP with mutations in the hinge region that are expected to impose except for the C-terminal arm (Fig. 1) (29). Comparison of the structure of the last 17 amino acids of the crystal forms 1 and 2 demonstrates a difference in the arrangement of residues Asn512–Leu515, called the hinge region. Further investigations have revealed amino acids Asn512 and Ser514 to be responsible mainly for the conformational changes. The ability of the C-terminal arm to adopt several orientations suggests that the DBP protein chain is flexible and can adopt different arrangements. This effect could explain different estimations of the length of the binding sites found for different homopolymer nucleotides (29, 30).

Here we have studied the role of the flexibility of the C-terminal arm in ssDNA binding and DNA replication using DBP with mutations in the hinge region that are expected to lead to reduced flexibility. We find that the proline mutations severely impair the capability of DBP to sustain elongation and to unwind DNA, although protein chain formation is still possible. This indicates that flexibility of the protein chain is essential for its function, possibly by enabling adaptation to different DNA conformations.

EXPERIMENTAL PROCEDURES

Construction of Mutants—The hinge mutants were prepared by PCR in two steps from the adenovirus 5 ΔN-DBP (amino acids 174–529) gene (31) in the pVL1392 baculovirus vector. In the first step the 5′ primer was TTATGATCTGTTAATTCAATGCGCCATGGTCTCTTCAC and the 3′ primers were CCGCCGATCCATGGCCCACATGCAGGACACGGGGGCACT, CCGCCGATCCATGGCCCACATGCAGGAGCCAGGTGTTGCA, and CCGCCGATCCATGGCCCACATGCAGGAGGATTGGCCGCACT for the N512P, V513L, and N5V512–514PP mutants, respectively. In the second PCR step the generated PCR product was, in each case, annealed with the 3′ end of a common 3′ primer GGCAGCAATTTCTCAAATATGGGAAAAGGTTGTCGCGGCACTGATATGGCCAC, and the DNA was amplified using the same 5′ primer as above.

In each case the constructs were prepared by isolating, purifying, and annealing three DNA fragments as follows: (a) the pVL1392-5N-DBP vector processed with EagI (cleavage site at position 126) and opened 5′ with EcoRI; (b) this fragment further cut with BanI (cleavage site at position 897) to yield the EagI-BanI piece; and (c) the second PCR product digested with BanI and EcoRI. The third fragment (containing the mutations) was sequenced to check the correctness of the construct. ΔN-DBP (aa 174–511) was constructed and purified as described by Dekker et al. (28).

Purification of ΔN-DBP and Mutant DBP from Baculovirus-infected Cells—Monolayers of SF9 cells were infected with recombinant baculoviruses at 28 °C for 72 h. The titer giving optimal protein expression levels was determined beforehand in pilot experiments. The purification of wild-type and mutant DBP was as follows. Cells were harvested, washed twice with PBS, and resuspended in 50 mM Tris-Cl (pH 8.0), 5 mM MgCl2, 1 mM EDTA, 10 mM KCl, 250 mM NaCl, followed by homogenizing using a Dounce homogenizer. The solution was clarified by centrifugation at 60,000 × g for 30 min at 4 °C. The supernatant was diluted in DEAE buffer, 25 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.1 mM PMFS, 1 mM EDTA, 20% glycerol, to lower the salt concentration to 100 mM. The diluted supernatant was loaded on a DEAE-FF-Sepharose column equilibrated with 200 mM NaCl. The flow-through was applied to an ssDNA-cellulose column equilibrated in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.1 mM PMFS, 20% glycerol, and 50 mM NaCl (ssDNA buffer). The column was washed in buffer containing 500 mM NaCl, and the protein was eluted with buffer containing 2 mM NaCl. The protein was dialyzed to 100 mM against 25 mM Hepes-KOH (pH 8.0), 100 mM NaCl. To concentrate the protein, the solution was loaded on a MonoS FPLC column equilibrated with 25 mM Hepes-KOH (pH 8.0), 20% glycerol, and 80 mM NaCl and developed with a linear gradient of 80–600 mM NaCl. The proteins eluted around 200 mM and were shown to be at least 95% homogeneous as judged by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

DNA Binding Assays—For the ssDNA electrophoretic mobility shift assay, a 114-bp EcoRI/XhoI fragment from pHR1 was Klenow end-labeled in the presence of [α-32P]dCTP and denatured by boiling. The dsDNA assays were performed with a 50-nm dsDNA oligonucleotide (TD50), containing the first 50 base pairs of the Ad5 origin.

Binding assays were performed in a final volume of 20 μl of buffer containing 20 mM Hepes-KOH (pH 8.0), 100 mM NaCl, 4 mM MgCl2, 0.4 mM EDTA, 4% Ficoll, 1 μg of bovine serum albumin, 0.05 ng of denatured DNA or dsDNA, and the indicated amounts of ΔN-DBP or mutants. Bound and free DNAs were separated on a 10% polyacrylamide gel at room temperature. The running buffer contained 0.5 TBE and 0.01% Nonidet P-40. Gels were dried and quantified using a PhosphorImager Storm 820 from Molecular Dynamics with ImageQuant 4.2a, Build 13 software. The concentration at which 50% of the ssDNA is complexed with DBP is used as a measure of the ssDNA binding affinity. A more accurate calculation as described by Verrijzer et al. (32) was not possible since binding of DBP to ssDNA is cooperative and does not fit a normal Scatchard plot.

DNA Unwinding—DNA unwinding assays were performed using a partially double-stranded oligonucleotide consisting of the last 50 bases of the template strand of the adenoviral origin of replication, hybridized with an oligonucleotide containing the complementary bases 15–50 from the displaced strand, thereby creating a dsDNA oligonucleotide with a 3′ (template) 15-base pair single-stranded overhang. Both strands were 5′-labeled prior to hybridization. DNA (0.5 ng) and indicated amounts of DBP or mutant forms of DBP were incubated for 1 h at 30 °C in a total volume of 25 μl in a buffer containing 25 mM Hepes-KOH (pH 8.0), 1 mM EDTA, 0.1 mM PMFS, 20% glycerol, 500 mM NaCl, 0.2% Nonidet P-40, 0.5 mM EDTA, 1 μg of bovine serum albumin, and 100 mM NaCl. Reactions were stopped by addition of 5 μl of 40% sucrose, 1.2% SDS, and 0.1% bromphenol blue and 0.1% xylene cyanol. Products were analyzed on a 12.5% SDS-polyacrylamide gel using a running buffer containing 1 TBE and 0.2% SDS. Gels were dried and quantified using a PhosphorImager.

DNA Replication on TP-DNA—The pTP-pol complex was purified as described (8). Terminal protein containing Ad5 DNA (TP-DNA) was obtained as described (33). Adenovirus DNA replication was performed in a final reaction volume of 25 μl in the presence of 25 μl of buffer containing (pH 7.5), 50 mM NaCl, 1.5 mM MgCl2, 1 mM DTT, 500 nM [α-32P]dCTP, and 40 μM dATP, dGTP, and dTTP, and 30 ng of TP-DNA cut with XhoI. 140 ng of pTP-pol was added to the reaction. The amounts of ΔN-DBP and mutants are indicated in the legends. After incubation for 45 min at 37 °C reactions were stopped by addition of 2.8 μl of stop mix (40% sucrose, 1% SDS, 0.1% bromphenol blue, 0.1% xylene cyanol). Reaction products were analyzed on a 1.5% agarose gel. Gels were dried followed by autoradiography. Replication products were quantified by densitometric analysis using a PhosphorImager. PhosphorImager software. The concentration at which 50% of the ssDNA is complexed with DBP is used as a measure of the ssDNA binding affinity. A more accurate calculation as described by Verrijzer et al. (32) was not possible since binding of DBP to ssDNA is cooperative and does not fit a normal Scatchard plot.
mutants are indicated in the legends. Reactions were performed at 37 °C for TP-DNA and at 30 °C when a synthetic oligonucleotide was used. After 45 min the reactions were stopped by addition of 80 mM EDTA. The samples were precipitated with 20% trichloroacetic acid for 30 min on ice. Precipitates were washed with 5% trichloroacetic acid, resolved in sample buffer, and analyzed on an SDS-7.5% polyacrylamide gel and autoradiographed. Initiation products were quantified by densitometric analysis using a PhosphorImager.

**RESULTS**

The Hinge Mutants Bind ss- and dsDNA with Approximately Wild Type-like Affinity—ΔN-DBP (wild type) and the mutants N512P (P-DBP), V513L (L-DBP), and NVS512–514PPP (PPP-DBP) were purified and assayed for their ability to bind to ss- and dsDNA as shown in Fig. 2. All proteins bound cooperatively to ssDNA resulting in fully saturated protein-DNA complexes without intermediate complexes (Fig. 2A). Multiple intermediate complexes can be seen when ΔC-DBP was used in this assay (Fig. 2B). ΔC-DBP lacks the C-terminal arm and is therefore not able to form multimers on ssDNA, resulting in the loss of cooperative binding (28). To estimate the binding affinity, the concentration required to shift 50% of the probe was determined after quantification (Table I). All proteins bound to ssDNA with approximately wild type affinity. The slight differences in binding affinity for the different mutants are not significant in view of the scattering of the data around the 50% shift point.

The binding of the mutants to a 50-base pair dsDNA probe is shown in Fig. 2C. All three mutants were able to bind to dsDNA with wild type affinity. Binding to dsDNA is not cooperative for DBP, and therefore intermediate complexes can be found in this assay. We assume that the band with the slowest migration is fully saturated, whereas the fastest migrating band contains only 1 monomer. However, we have not studied the stoichiometry of the bands in detail. The lack of cooperativity also leads to a lower binding affinity that is reflected by the higher concentration of proteins needed to obtain a shifted complex (35).

The Hinge Mutants, in Particular PPP-DBP, Are Defective in Stimulating DNA Replication—We tested the hinge mutants for their activity to support DNA replication in an in vitro assay, see Fig. 3A. Ad5 DNA isolated from virus particles and containing the terminal protein was digested with XhoI and used as template. The reaction was carried out in the presence of pTP-pol, NFI, Oct 1, radiolabeled dNTPs, and increasing amounts of wild type or mutant DBP. Analysis of the products on agarose gels shows specific labeling of two restriction fragments, B and C, containing the origin of replication. In addition two labeled fragments migrating with higher mobility are observed which contain ssDNA. These originate from second rounds and further of displacement synthesis, indicative of effective template usage (Fig. 3B). Quantitation of the total DNA replication activity, the 1st as well as further rounds together, was performed and compared for the 500 (lanes 3, 7, 11, and 14) and 1000 ng (4, 8, 12, and 15) lanes. P-DBP had a slightly lowered replication activity (4.2- to 1.1-fold), whereas PPP-DBP had a 100- to 50-fold decrease in activity and could

![Image](image-url)

**FIG. 2** The hinge mutants have a wild type-like ss- and dsDNA binding affinity. A, gel retardation assay on a 114-nt ssDNA probe. The highest amount of DBP and mutants was 5 ng. Proteins were diluted by successive 2-fold steps. The percentage of complexed DNA was quantified, and the calculated values of the 50% shift concentration are shown in Table I. B, ΔC-DBP on a 114-nt ssDNA probe. The highest concentration of protein is 1000 ng and is diluted by successive 2-fold steps. C, gel retardation assay on a 50-bp dsDNA probe. The highest amounts of DBP and mutants were 1000 ng. Proteins were diluted by successive 2-fold steps.

### Table I

| Protein   | Protein concentration at which 50% of DNA has shifted | σ  |
|-----------|-------------------------------------------------------|----|
| ΔN-DBP    | 0.15                                                  | 0.07 (n = 7) |
| L-DBP     | 0.17                                                  | 0.05 (n = 5) |
| P-DBP     | 0.23                                                  | 0.06 (n = 5) |
| PPP-DBP   | 0.25                                                  | 0.3 (n = 5)  |
hardly support any replication. L-DBP behaved wild type-like with a slight decrease in activity (1.4-fold) at 500 ng. The differences in activity are most prominent at low protein concentrations.

To test whether the loss of activity for PPP-DBP is due to the presence of inhibitors in the protein solution, a control was performed by mixing in PPP-DBP in a D N-DBP replication (lane 18). Inhibition of DNA replication was not detected. Lane 17, the negative control (no DBP), contains some background bands, caused by aspecific labeling of all XhoI TP-DNA fragments not related to protein-primed replication.

The Hinge Mutants Stimulate Initiation but Are Defective in Early Elongation—To test whether the observed decrease in replication efficiency is due to reduced initiation levels, an initiation assay was performed. The first step in initiation is the covalent coupling of dCTP to the pTP of the pTP-pol complex. This reaction is enhanced considerably by DBP when the pTP-pol concentration is low (28).

A low amount of pTP-pol (50 ng) was incubated together with TP-DNA and dCTP. The stimulation of pTP-C formation was determined for two DBP concentrations (250 and 500 ng) (Fig. 4A). Without DBP only a low level of initiation was observed (1%). L-DBP and P-DBP stimulated the initiation like wild type with activities ranging from 86 to 116%. For a quantitation see Fig. 4A. The stimulation by PPP-DBP was slightly lower (25–36%) but was still considerable.

To distinguish between defects during the initiation and elongation phase, we performed a partial elongation. This leads to the formation of a pTP-CAT initiation intermediates (8) as
was used.

The winding was quantified and presented in a graph. The percentage of the unwinding was quantified and presented in a graph. The "boiled lane" was used to determine 0% of unwinding and the boiled lane was used to determine the 100% unwinding value.

Well as a pTP-26 nucleotide product indicative of early elongation (Fig. 4B). The elongation to initiation ratio was calculated after quantitation (Fig. 4B). This ratio is indicative of the ability to stimulate the elongation by DBP. Whereas ΔN-DBP as well as L-DBP and P-DBP were all able to stimulate elongation efficiently, PPP-DBP was deficient indicating that stimulation of elongation is already inhibited at an early stage of elongation.

The pTP-CAT formation is slightly stimulated by all mutant DBPs, but the absolute rate of stimulation is lower than that in Fig. 4A, due to the higher pTP-pol (100 ng) concentration, and for PPP-DBP the increase is minimal as this mutant is already less efficient stimulating initiation (Fig. 4A).

For ΔN-DBP the pTP-CAT formation decreases at the highest amount of protein (1000 ng), due to efficient elongation of this intermediate. For L- and P-DBP, however, no decrease in pTP-CAT formation was observed, presumably since higher protein concentrations are required for optimal stimulation of elongation by these mutants.

A small band below the pTP-C and pTP-26n products was also observed, presumably due to degradation of pTP.

Unwinding of DNA Correlates with the Reduction in DNA Replication Activity—During elongation, DBP destabilizes dsDNA and facilitates elongation of the DNA polymerase. The unwinding of dsDNA is ATP-independent and only DBP is required, cooperative binding of the DBP monomers to the displaced strand being the driving force (21–23). Defects in unwinding will therefore result in diminished, or loss of, replication activity. An unwinding assay was performed with a partial duplex DNA (TD15) to investigate the unwinding activity of DBP and the hinge mutants (Fig. 5A). The percentages of unwinding were calculated and presented in Fig. 5B. All proteins were able to unwind TD15, but the activity of the hinge mutants was diminished as higher protein concentrations were required. The differences in unwinding activity were calculated from the slope of the curves. Most prominent was the decrease of unwinding activity for PPP-DBP (125-fold), whereas the reductions for ΔN- and P-DBP were 125- and 23-fold, respectively (Fig. 5B). The decrease in unwinding activity for PPP-DBP can account for the large decrease found in the DNA replication activity during the elongation phase.

Binding to Poly(dA)₄₀ Is Diminished for P- and PPP-DBP—

Both the unwinding and replication assays show large differences in activity for the hinge mutants, with L-DBP behaving like wild type, P-DBP having an intermediate effect, and PPP-DBP being most severely impaired. An explanation for these results could be that the flexibility of the C-terminal arm is gradually reduced. This could lead to a change in the multiprotein chain making adaptation to rigid or irregularly shaped DNA more difficult. To investigate this, we tested the proteins for binding to homo-oligonucleotides containing either 40 A, T, C, or G residues. Binding to poly(dT)₄₀, (dC)₄₀, and (dG)₄₀ showed only small differences and are not shown. In contrast, P- and PPP-DBP are unable to bind to poly(dA)₄₀ efficiently (Fig. 6). The highest concentration used in Fig. 6 was 40 ng for P- and PPP-DBP. No binding of P- and PPP-DBP could be found up to 1000 ng (data not shown). This suggests that reduction of the flexibility of the C-terminal arm can result in loss of the ability to bind particular sequences, possibly caused by the presence of aberrant secondary or tertiary structures in these homopolymers.

**DISCUSSION**

Reduction of the Flexibility of the C-terminal Arm Does Not Result in Loss of ss- and dsDNA Binding—The ss- and dsDNA binding capabilities of the hinge mutants were not significantly reduced. This is in contrast with deletion of the C-terminal arm (ΔC-DBP), which leads to a 100-fold reduction in ssDNA binding, whereas no change in dsDNA binding is detectable (28, 34). This has been explained by the lack of cooperativity in ΔC-DBP. Since the cooperative binding is not lost in the hinge mutants, this strongly suggests that the hinge mutants are still able to form multimers on DNA. Direct assays to test this (electron microscopy with negative staining and dynamic light scattering) were inconclusive perhaps due to aggregation problems.

A small decrease in ssDNA binding was found for P- and PPP-DBP. A low resolution crystal structure of DBP complexed with ssDNA shows that although Asn512 approaches the DNA, it is unlikely to make any interaction with the phosphate backbone (31). Rather than a loss of direct contacts, the slight decrease in binding affinity for ssDNA could be due to the reduced flexibility of the C-terminal arm. Possibly, the mutant protein chain is not able to adapt to certain secondary struc-
The proteins were diluted by 2-fold successive steps.

atures or conformations in the ssDNA strand as indicated by the inability to bind poly(dA) efficiently.

Elongation of Replication Is Dependent on the Flexibility of the C-terminal Arm—P-DBP and, in particular, PPP-DBP have a reduced replication activity. No strong differences in stimulation of initiation were detected, and direct assays show that the main defect lies in elongation. This is in agreement with the reduced unwinding. A similar effect was observed upon deletion of the C-terminal arm (28). Like PPP-DBP, the ΔC-DBP mutant was still able to stimulate initiation with a slight reduction in efficiency while being unable to support replication or DNA unwinding. From this we have concluded earlier that oligomerization of DBP is the driving force of ATP-independent DNA unwinding during the elongation phase. Although this may be true, multimerization apparently is not the only requirement for DBP to function effectively in elongation. Previously we showed the need for an intact flexible loop located between aa Lys236 and aa Ser332 that ensures high affinity binding to ssDNA (34). Here we suggest yet another requirement, i.e. the need for flexibility in the protein chain even in the presence of an intact flexible loop and multimerization.

Model—What could be the function of the flexibility of the C-terminal arm during elongation and replication fork destabilization? We propose (Fig. 7) that reduction of the flexibility of the C-terminal arm leads to an inability to hook into a neighboring DBP molecule when bound to the replication fork. In particular we assume that a conformational change is required to accommodate the transition of DBP bound to the double-stranded parental strands to that when bound to the displaced single strand, which is situated at the ssDNA part of the replication fork. The high off rate of DBP on dsDNA coupled to an impairment of the transition will prevent unwinding at the replication fork. Why is multimerization possible when bound on single-stranded DNA and not in the replication fork? Presumably less mobility exists in the replication fork, compared with single-stranded DNA.

As an addition to the model, binding of a less flexible DBP protein to the displaced strand could also lead to difficulties when AT tracts, hairpins, or other secondary DNA structures are encountered. This might be reflected by the problems in binding poly(dA) which has an aberrant structure and a different binding site for DBP (30, 35).

The model might explain the lack of unwinding by hinge region mutants during elongation. This situation might also apply to the early stages of elongation. For stimulation of initiation DBP monomers suffice (28), but even in elongation multimerization is required. Although the conformation of the preinitiation complex and the changes occurring during transition of initiation to early elongation are unknown, we assume that the same flexibility of DBP is needed at this stage. Alternatively, we could envisage the dsDNA breathing and the irreversible steps that occur when DBP binds cooperatively to the part unwound by breathing to be slowed down with the mutants. However, we consider this to be less likely because this effect would mainly influence unwinding of long stretches of DNA, and we observe already a block in unwinding with a 35-bp probe.

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REFERENCES

1. Hay, R. T., Freeman, A., Leith, I., Monaghan, A., and Webster, A. (1995) Curr. Top. Microbiol. Immunol. 199, 31–48
2. van der Vliet, P. C. (1995) Curr. Top. Microbiol. Immunol. 199, 1–30
3. de Jong, R. N., and van der Vliet, P. C. (1999) Gene (Amst.) 236, 1–12
4. Bosher, J., Robinson, E. C., and Hay, R. T. (1990) New Biol. 2, 1083–1090
5. Chen, M., Mermod, N., and Horwitz, M. S. (1990) J. Biol. Chem. 265, 18634–18642
6. Coenjaerts, F., and van der Vliet, P. C. (1994) Nucleic Acids Res. 22, 5235–5240
7. Dekker, J., van Oosterhout, J. A., and van der Vliet, P. C. (1996) Mol. Cell. Biol. 16, 4073–4080
8. King, A. J., and van der Vliet, P. C. (1994) EMBO J. 13, 5786–5792
9. King, A. J., Teeterstra, W. R., and van der Vliet, P. C. (1997) J. Biol. Chem. 272, 24617–24623
10. Zijderveld, D. C., d’Adda, D. F., Giacono, G., Timmers, H. T., and van der Vliet, P. C. (1994) J. Virol. 68, 8288–8295
11. Clegghon, V., Voelkerding, K., Morin, N., Delsert, C., and Klessig, D. F. (1989) J. Virol. 63, 2289–2299
12. Rubenstein, F. E., and Ginsberg, H. S. (1974) Internat. J. of Cancer 13, 170–174
13. Nicolas, J. C., Sarnow, P., Girard, M., and Levine, A. J. (1985) Virology 126, 228–239
14. Anderson, C. W., Hardy, M. M., Dunn, J. J., and Klessig, D. F. (1983) J. Virol. 48, 31–39
15. Harfst, E., and Leppard, K. N. (1999) Virus Genes 18, 97–106
16. Mul, Y. M., and van der Vliet, P. C. (1993) Nucleic Acids Res. 21, 641–647
17. Lindenhoum, J. O., Field, J., and Horwitz, J. (1986) J. Biol. Chem. 261, 10218–10227
18. Cleat, P. H., and Hay, R. T. (1989) EMBO J. 8, 1841–1848
19. Stuiver, M. H., and van der Vliet, P. C. (1990) J. Virol. 64, 379–386
20. Zijderveld, D. C., Stiwer, M. H., and van der Vliet, P. C. (1993) Nucleic Acids Res. 21, 2591–2598
21. Pronek, R., Van Driel, W., and van der Vliet, P. C. (1994) FEBS Lett. 337, 33–38
22. Monaghan, A., Webster, A., and Hay, R. T. (1994) Nucleic Acids Res. 22, 3379–3386
23. de Jong, R. N., and van der Vliet, P. C. (1999) EMBO J. 18, 10218–10227
24. Cleat, P. H., and Hay, R. T. (1989) EMBO J. 8, 1841–1848
25. Stiwer, M. H., and van der Vliet, P. C. (1990) J. Virol. 64, 379–386
26. Zijderveld, D. C., Stiwer, M. H., and van der Vliet, P. C. (1993) Nucleic Acids Res. 21, 2591–2598
27. Pronek, R., Van Driel, W., and van der Vliet, P. C. (1994) FEBS Lett. 337, 33–38
28. Monaghan, A., Webster, A., and Hay, R. T. (1994) Nucleic Acids Res. 22, 3379–3386
29. de Jong, R. N., and van der Vliet, P. C. (1999) EMBO J. 18, 1841–1848
30. Stiwer, M. H., and van der Vliet, P. C. (1990) J. Virol. 64, 379–386
31. Pronek, R., Van Driel, W., and van der Vliet, P. C. (1994) FEBS Lett. 337, 33–38
32. Monaghan, A., Webster, A., and Hay, R. T. (1994) Nucleic Acids Res. 22, 3379–3386
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23. Zijderveld, D. C., and van der Vliet, P. C. (1994) *J. Virol.* 68, 1158–1164
24. Tucker, P. A., Tsernoglou, D., Tucker, A. D., Coenjaerts, F. E., Leenders, H., and van der Vliet, P. C. (1994) *EMBO J.* 13, 2994–3002
25. Ariga, H., Klein, H., Levine, A. J., and Horwitz, M. S. (1980) *Virology* 101, 307–310
26. Friedfeld, B. R., Krevolin, M. D., and Horwitz, M. S. (1983) *Virology* 124, 380–389
27. Tsernoglou, D., Tsugita, A., Tucker, A. D., and van der Vliet, P. C. (1985) *FEBS Lett.* 188, 248–252
28. Dekker, J., Kanellopoulos, P. N., Loonstra, A. K., van Oosterhout, J. A., Leonard, K., Tucker, P. A., and van der Vliet, P. C. (1997) *EMBO J.* 16, 1455–1463
29. Kanellopoulos, P. N., Tsernoglou, D., van der Vliet, P. C., and Tucker, P. A. (1996) *J. Mol. Biol.* 257, 1–8
30. van Amerongen, H., van Grondelle, R., and van der Vliet, P. C. (1987) *Biochemistry* 26, 4646–4652
31. Kanellopoulos, P. N., van der Zandt, H., Tsernoglou, D., van der Vliet, P.C., and Tucker, P. A. (1995) *J. Struct. Biol.* 115, 113–116
32. Verrijzer, C. P., Kal, A. J., and van der Vliet, P. C. (1990) *Genes Dev.* 4, 1964–1974
33. Dekker, J., Coenjaerts, F. E., and van der Vliet, P. C. (1996) in *Viral Genome Methods* (K. W. Adoph, ed.) pp. 269–282, CRC Press, Boca Raton, FL
34. Dekker, J., Kanellopoulos, P. N., van Oosterhout, J. A., Stier, G., Tucker, P. A., and van der Vliet, P. C. (1998) *J. Mol. Biol.* 277, 825–838
35. Kuil, M. E., van Amerongen, H., van der Vliet, P. C., and van Grondelle, R. (1989) *Biochemistry* 28, 9795–9800