

Binding of Endonuclease VII to Cruciform DNA

VISUALIZATION IN THE ELECTRON MICROSCOPE*

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The binding of Holliday structure resolving endonuclease VII to cruciform DNA was studied in the electron microscope. The protein was found to bind either to the junction or to one of the arms or an end of one of the arms of the construct. The amount of bound protein was determined by measuring the size of the complexes. On average, one complex containing three dimers was found per one molecule of cruciform DNA.

Endonuclease VII (endo-VII)¹ is the product of gene 49 of phage T4 (T4). It is a versatile endonuclease with a broad substrate profile. Besides resolving four-way and three-way junctions, the nuclease also recognizes mismatches, heteroduplex loops, single strand overhangs, flap substrates, nicks, gaps, bent DNA, bulky adducts (all reviewed in Ref. 1) and, as recently shown, apyrimidinic sites (AP-sites) (2). The enzyme introduces staggered nicks flanking the targets within a distance of 2–6 nucleotides. In the case of Holliday structures, this results in precise resolution of the junction and formation of patch and splice recombinants. The enzyme is essential for phage growth *in vivo* and is responsible for resolving branches in the newly synthesized DNA that otherwise block the translocation of the molecule during DNA packaging (3–5).

Endo-VII contains 157 amino acids and has a molecular mass of 18.2 kDa (6, 7). The protein forms dimers in solution that easily exchange subunits among each other (8–10). Formation of dimers activates the nuclease (11). Each dimer contains two DNA binding sites composed of one N- and one C-terminal portion of each subunit and two cleavage sites (12). In theory, one dimer would be sufficient to cleave two strands flanking the junction of a Holliday structure or its substitute, a cruciform DNA. Interestingly, electromobility shift assays (EMSA) indicated that the synthetic cruciform DNA CF01 binds in total three dimers of endo-VII (12). To further study

the DNA binding of endo-VII, we have visualized the DNA-protein complexes formed between a cruciform DNA and the protein in the electron microscope and have calculated the amount of protein bound by visual inspection. Again we find that three dimers of endo-VII bind to the junction of the cruciform DNA.

EXPERIMENTAL PROCEDURES

Chemicals and Radiochemicals—Acrylamide/bisacrylamide (37.5:1) for native- or SDS-polyacrylamide gel electrophoresis was purchased from Bio-Rad (München, Germany). [γ -³²P]dATP (specific activity > 5000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Braunschweig, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Oligonucleotides and DNA—Synthetic oligonucleotides were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). The sequences of the four oligonucleotides and the protocol of their assembly giving cruciform DNA CF01 were described earlier (13). Synthesis of cruciform DNA J12, kindly provided by Jack Griffith, was described earlier (14).

Electron Microscopy—The preparation of samples for the electron microscope was done essentially as described earlier (14). Briefly, a 50-fold molar excess of endo-VII dimer was incubated for 15 min at room temperature with 100 ng of J12 DNA in 50 μ l of 10 mM HEPES (pH 7.5) and 100 mM NaCl. After fixation at room temperature for 5 min with 0.8% (v/v) glutaraldehyde, the solution containing protein-DNA complexes was filtered through a 2-ml Bio-Gel A5 m-column (Bio-Rad) to remove excess unbound protein. The samples were then mixed with spermidine buffer giving a final concentration of 0.1 mM spermidine, 0.1 mM MgCl₂, 7.5 mM NaCl, and 5 mM KCl and then transferred to carbon foils. After a rotary showcast at 10^{−7} torr with tungsten, the samples were examined in a Phillips CM12 electron microscope.

Protein Purifications—Proteins endo-VII^{WT} and cleavage-deficient mutant protein [Asp]-EVII(1–156) were used for the EM studies. Proteins endo-VII^{WT} and His-tagged covalently linked endo-VII dimer CD321 were used for EMSA. Expression and purifications of the proteins and removal of the 6xHis tag (where applicable) were performed as described before (10–13).

Electrophoretic Mobility Shift Assay—DNA-binding reactions were performed in 10 μ l of binding buffer (50 mM Tris HCl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol). Varying amounts of protein were incubated with 1 fmol (100 pM final concentration) of radioactively labeled cruciform DNA CF01 at 16 °C. After 15 min, 2.5 μ l of loading buffer (40 mM Tris-HCl, pH 7.5, 4 mM EDTA, 25% glycerol, 0.05% bromophenol blue, and 400 μ g/ml bovine serum albumin) were added to each sample. The mixtures were immediately loaded onto an 8% native PAA gel and run at 4 °C for 2.5 h at 7 V/cm in electrophoresis buffer (6.7 mM Tris HCl pH 8.1, 3.3 mM sodium acetate, and 2 mM EDTA). The DNA was visualized by autoradiography of the dried gels and quantitated by phosphorimaging on a Fuji BAS1000.

RESULTS AND DISCUSSION

Endo-VII has a general tendency to bind to single-stranded DNA as well as double-stranded DNA (15, 16). The protein also binds with high affinity to cruciform DNA and mismatch-containing oligonucleotides (10, 11, 17). When synthetic cruciform DNA CF01 was exposed to increasing amounts of endo-VII, three distinct shift positions were revealed by band shift analyses suggesting one, two, and three units of protein binding in succession to the DNA (Fig. 1a, lanes 1 through 7). The size of the smallest binding unit of endo-VII was determined as a dimer because a synthetic covalently linked endo-VII dimer shifted the DNA to the same positions in the gel as the endo-VII wild-type protein (11).

To further analyze the modalities of endo-VII-binding to cruciform DNA, we loaded the protein on cruciform DNA and inspected the samples after fixation with glutaraldehyde in the

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¹ The abbreviations used are: endonuclease VII, endo-VII; EMSA, electrophoretic mobility shift assays; EM, electron microscope.

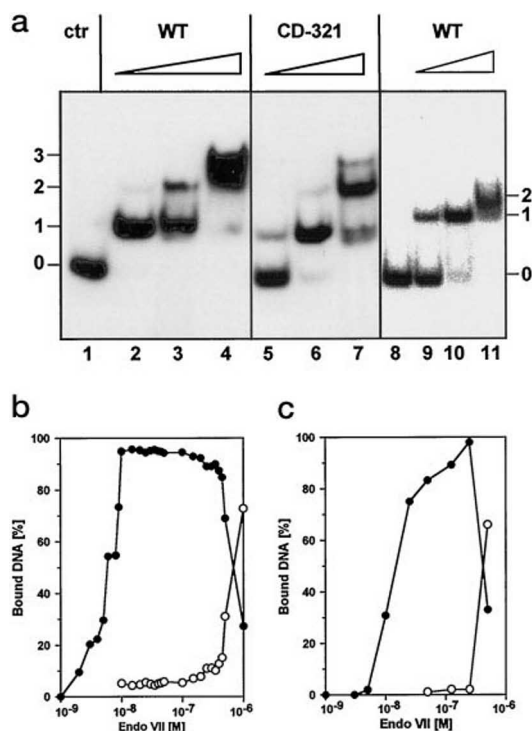


FIG. 1. **Binding of endo-VII proteins to cruciform DNA CF01.** *a*, EMSA of cruciform DNA CF01 or mismatch-DNA Bea46MM^M. Samples containing 1 fmol of radioactively labeled cruciform DNA CF01 or Bea46MM^M were incubated with 20, 100, or 500 nmol of endo-VII^{WT} (lanes 2–4 and 9–11) or the covalent dimer CD-321 (lanes 5–7). The reaction products were separated on native 8% PAA gel as described under “Experimental Procedures.” Bands were visualized by autoradiography of dried gels. Lane 1 and 8 contain mock-treated substrate DNA. Positions containing free DNA CF01 or DNA complexed with one, two, and three dimers are marked with 0–3 to the left of the figure. Positions containing free DNA Bea46MM^M or DNA complexed with one and two dimers are marked with 0–2 to the right of the figure. Measurements of the dissociation constants of endo-VII binding to CF01 (*b*) or Bea46MM^M (*c*). The fraction of bound DNA was plotted against the logarithm of the molarity of endo-VII dimers. Symbols: ●, DNA at the first shift position; ○, DNA at the second shift position.

electron microscope. The same procedure and the same DNA were recently used successfully to demonstrate the binding of tumor suppressor protein p53 to cruciform DNA (14).

Two forms of endo-VII were used in these studies. These were the enzymatically active wild-type protein (pEVII^{WT}) and the enzymatically inactive mutant protein ([Asp]-EVII-(1–156)) which lacks the ultimate C-terminal amino acid 157 and carries a point mutation in codon 62 (EVII-N62D). This mutant has recently been described (10, 18).

Typical photographs from the analyses are shown in Fig. 2. A list summarizing and classifying all samples investigated is given in Table I. Among 151 inspected intact cruciform DNA molecules (Fig. 2*a*), 132 were found associated with distinct protein complexes after the reaction with EVII^{WT}. The complexes were either located at the junction (43 complexes) (Fig. 2*b*), somewhere along the arms (47 complexes) (Fig. 2*c*) or at the end of one of the arms (42 complexes) (Fig. 2*d*). A similar result was obtained when EVII^{WT} was replaced by mutant protein [Asp]-EVII-(1–156). In this experiment, a total of 138 molecules were inspected, and 51 of them had protein complexes bound to the junction, 30 carried complexes somewhere along one of the arms, and 48 had the protein attached to the end of one arm. In both experiments, the majority of DNA-protein complexes contained only one protein complex per DNA. A molar ratio of endo-VII dimers to cruciform DNA molecules of 50:1 was found to give the best results in terms of

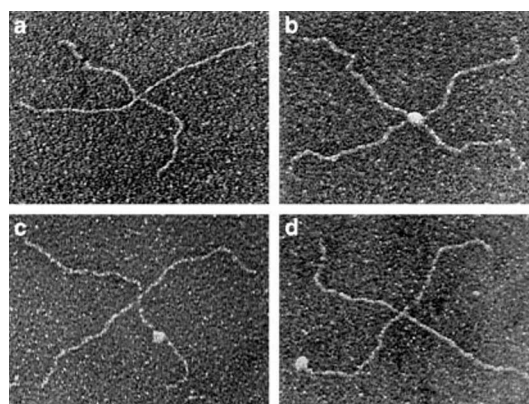


FIG. 2. **Binding of endo-VII proteins to cruciform DNA J12 visualized by electron microscopy.** 100 ng of cruciform DNA J12 was incubated with a 50-fold molar excess of endo-VII^{WT} dimers and visualized in the EM as described under “Experimental Procedures.” *a*, free DNA J12; *b*, endo-VII^{WT} bound to the junction of cruciform DNA J12; *c*, endo-VII^{WT} bound to the center of one arm of J12; *d*, endo-VII^{WT} bound to the end of one arm of J12.

number and size of protein-DNA complexes. When a 10-fold higher concentration of protein was used, large amorphous aggregates of protein were found along with “regular” sized complexes. The aggregates were considered unspecific and left out of this study. Lower amounts of endo-VII^{WT} resulted in fewer countable complexes, again of regular size.

When the mutant protein was used in the presence of 10 mM MgCl₂ (which is possible because the mutant protein is enzymatically inactive and does not degrade the substrate), the number of molecules with protein complexes bound to the junction was significantly higher (73 complexes) when compared with those with protein bound along one arm (32 complexes) or at the end of one arm (34 complexes). This suggests that Mg²⁺ may increase the affinity of endo-VII to the junction. Mg²⁺-induced changes in the secondary structure of cruciform DNA have recently been reported (19). They may eventually make the structure more apt to bind endo-VII protein.

How much endo-VII binds to the junction of a cruciform DNA? We approached the problem by measuring the diameters of protein complexes on enlarged photos and compared these numbers to measures of the length of one arm of the cruciform DNA on the same photo. One arm contains 565 base pairs with a predicted length of 1,921 Å. The relative diameter of the protein complex was then calculated to be 171 ± 43 Å as averaged from ten samples. Remarkably, this number was nearly the same as the diameter of p53^{dimer} protein complexes (169 Å) formed under the same experimental conditions using the same cruciform DNA (14). The mass of DNA bound p53^{dimer} complexes was originally estimated from comparisons to the mass of T7 DNA polymerase, used as an internal standard, times the ratio of the projected areas to the 3/2 power (14). The calculated mass of endo-VII complexes was about 108 kDa,² suggesting that on the average three dimers of endo-VII bind to cruciform DNA. This is consistent with the results obtained by EMSA (Fig. 1, lanes 1 through 7). However, we want to emphasize that the molar ratio of endo-VII protein to cruciform DNA required to shift the DNA to the third position in the gel was about 50-fold higher here than the ratio used in the EM analyses. We suspect that the binding equilibrium between protein and DNA is shifted under the EM conditions such that the back reaction is prevented, possibly by the fixation with glutaraldehyde. Unfortunately, this hypothesis could not be

² Mass EVII^{complex} = mass p53 ((diameter EVII^{complex})/(diameter p53^{dimer}))^{3/2} = 106 kDa (171 Å/169 Å)^{3/2} = 107.8 kDa.

TABLE I
Compilation of endo-VII-cruciform DNA complexes as analyzed by electron microscopy

DNA J12 was visualized in the EM after incubation of 100 ng of the DNA with a 50-fold molar excess of dimers of endo-VII^{WT} or mutant protein [Asp]-EVII-(1–156) in the absence or presence of 10 mM MgCl₂.

	MgCl ₂	Total	Complexes bound to			Free DNA
			Junction	Arm	End	
		%		%		%
WT endo VII	–	151 (100)	43 (28)	47 (31)	42 (28)	19 (13)
[Asp]-EVII-(1–156)	–	138 (100)	51 (37)	30 (22)	48 (35)	9 (6)
[Asp]-EVII-(1–156)	+	148 (100)	73 (49)	32 (22)	34 (23)	9 (6)

tested with cruciform DNA CF01 and EMSA using EM cross-linking conditions because the substrate was not stable (results not shown).

Interestingly, when the same protein concentration was used for shift experiments with linear double-stranded DNA containing a C/C mismatch, two shift positions were obtained (Fig. 1a, lanes 8–11). From the band shift experiment shown in Fig. 1a, a dissociation constant (K_d) of 5 nM (5×10^{-9} M) was calculated for endo-VII^{WT} and cruciform DNA CF01 (Fig. 1b) and 14 nM (1.4×10^{-8} M) for mismatch containing substrate Bea46MM¹¹ (Fig. 1c). A consistent failure to obtain footprints with endo-VII and any of its substrates including cruciform DNA and mismatch-containing DNA (results not shown) indicate that the affinity of the protein to DNA is too low. Only when extremely high protein to DNA ratios ($\gg 1000$ -fold molar excess of endo-VII) are chosen is footprinting possible (20).

Resolution of Holliday structures requires a minimum of two staggered nicks placed across the junction, which could in principle be done by one protein dimer. The three-dimensional structure of a cruciform DNA in solution was predicted by model building as an X-shaped scissors like arrangement of the arms in space with a small angle of 60° between two pairs of arms and a compensatory wide angle of 120° between the other two pairs of arms (19, 21). Binding of endo-VII to cruciform DNA was reported to cause a structural change from an X-shaped molecule into a planar + -shaped molecule (22). Based on the two-fold symmetry of the cruciform DNA, one can easily envisage how binding of two arms by one endo-VII dimer (comprising two DNA-binding sites) would be sufficient to resolve the structure. By the same structural argument, one can also envisage how a second endo-VII dimer binds to the same DNA molecule without interfering with the first dimer. Binding of three dimers, however, is not understood. We therefore believe that binding of the third dimer is unspecific, possibly reflecting protein-protein interactions *via* differently charged surfaces of the protein. The recently obtained crystal structure of endo-VII revealed that the dimer has two differently charged surfaces indeed.³ Dwelling upon this argument further, the two dimers

binding to a mismatch oligonucleotide would then reflect one dimer binding specifically to the two arms flanking the mismatch and one dimer adhered nonspecifically to the first.

It should be noted, that the amount of endo-VII protein required to shift DNA CF01 to the first, second, and third position in the gel is about 1,000-, 20,000-, and 300,000-fold higher, respectively, than is required for cleavage of 50% of the same DNA in the nuclease reaction. We therefore assume that the three-dimer complexes reflect the special situation caused by high protein concentrations that may or may not be relevant *in vivo*.

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REFERENCES

- Kemper, B. (1997) in *Biochemistry, Genetics and Cell Biology* (Nickoloff, J. A., and Hoekstra, M., eds) pp. 179–204, Humana Press, Totowa, NJ
- Greger, B., and Kemper, B. (1998) *Nucleic Acids Res.* **26**, 4432–4438
- Flemming, M., Deumling, B., and Kemper, B. (1993) *Virology* **196**, 910–913
- Kemper, B., and Brown, D. T. (1976) *J. Virol.* **18**, 1000–1015
- Frankel, F. R., Batcheler, M. L., and Clark, C. F. (1971) *J. Mol. Biol.* **62**, 439–463
- Tomaschewski, J., and Rueger, W. (1987) *Nucleic Acids Res.* **15**, 3632–3633
- Barth, K. A., Powell, D., Trupin, M., and Mosig, G. (1988) *Genetics* **120**, 329–343
- Kosak, H. G., and Kemper, B. (1990) *Eur. J. Biochem.* **194**, 779–784
- Giraud-Panis, M. J. E., and Lilley, D. M. J. (1997) *EMBO J.* **16**, 2528–2534
- Golz, S., Christoph, A., Birkenkamp-Demtroeder, K., and Kemper, B. (1997) *Eur. J. Biochem.* **245**, 573–580
- Birkenbihl, R. P., and Kemper, B. (1998) *EMBO J.* **17**, 4527–4534
- Birkenbihl, R. P., and Kemper, B. (1998) *J. Mol. Biol.* **280**, 73–83
- Golz, S., Birkenbihl, R., and Kemper, B. (1995) *DNA Res.* **2**, 277–284
- Lee, S., Cavallo, L., and Griffith, J. (1997) *J. Biol. Chem.* **272**, 7532–7539
- Kemper, B., and Garabett, M. (1981) *Eur. J. Biochem.* **115**, 123–131
- Kemper, B., Garabett, M., and Courage, U. (1981) *Eur. J. Biochem.* **115**, 133–141
- Golz, S., Birkenkamp-Demtroeder, K., and Kemper, B. (1998) *Nucleic Acids Res.* **26**, 1132–1133
- Christoph, A., Heesberg, G., and Kemper, B. (1998) *J. Mol. Biol.* **277**, 529–540
- Bhattacharyya, A., Murchie, A. I. H., von Kitzing, E., Diekmann, S., Kemper, B., and Lilley, D. M. J. (1991) *J. Mol. Biol.* **221**, 1191–1207
- Parsons, C. A., Kemper, B., and West, S. C. (1990) *J. Biol. Chem.* **265**, 9285–9289
- von Kitzing, E., Lilley, D. M., and Diekmann, S. (1990) *Nucleic Acids Res.* **18**, 2671–2683
- Pöhler, J. R. G., Giraud-Panis, M. J., and Lilley, D. M. (1996) *J. Mol. Biol.* **260**, 678–696

³ D. Suck, personal communication.