Winter 1-1-2006

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Published online 8 September 2006

Nucleic Acids Research, 2006, Vol. 34, No. 16 ***e111***
doi:10.1093/nar/gkl642

Site-specific labeling of supercoiled DNA

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Received July 7, 2006; Revised and Accepted August 16, 2006

ABSTRACT

Visualization of site-specific labels in long linear or circular DNA allows unambiguous identification of various local DNA structures. Here we describe a novel and efficient approach to site-specific DNA labeling. The restriction enzyme SfiI binds to DNA but leaves it intact in the presence of calcium and therefore may serve as a protein label of 13 bp recognition sites. Since SfiI requires simultaneous interaction with two DNA recognition sites for stable binding, this requirement is satisfied by providing an isolated recognition site in the DNA target and an additional short DNA duplex also containing the recognition site. The SfiI/DNA complexes were visualized with AFM and the specificity of the labeling was confirmed by the length measurements. Using this approach, two sites in plasmid DNA were labeled in the presence of a large excess of the helper duplex to compete with the formation of looped structures of the intramolecular synaptic complex. We show that the labeling procedure does not interfere with the superhelical tension-driven formation of alternative DNA structures such as cruciforms. The complex is relatively stable at low and high pH (pH 5 and 9) making the developed approach attractive for use at conditions requiring the pH change.

INTRODUCTION

Visualization of site-specific labels in long linear or circular DNA allows unambiguous identification of various local DNA structures, such as bent DNA (1–3), as well as the protein binding sites (4–6) at predicted distances from the labels. The formation and stability of local structures, as well as protein functions, depend on the level of superhelical tension in DNA, therefore there is a great demand for a simple labeling procedure that does not change the DNA topology. Different approaches have been proposed for the sequence-specific DNA labeling. Some of them utilize the site-specific binding of oligonucleotides (7–9) or PNA fragments (10–12). The major drawbacks of these approaches are the complexity of their designs and the involvement of multiple experimental steps, and/or their dependence on DNA supercoiling. Restriction enzymes and methylases rendered inactive by mutations have been suggested for direct DNA labeling (13), however moderate specific affinities of 10^{-7} to 10^{-9} M limit their use for routine labeling. We have recently used AFM to study very stable complexes of the restriction enzyme SfiI with DNA which was kept undigested by replacing Mg^{2+} cations in the buffer with Ca^{2+} (14). SfiI binds as a tetramer (total molecular weight of 124 kDa), which is easily identified in the AFM images. Thus, SfiI appears to be an excellent candidate label of specific sites in circular DNA. However, the requirement of two DNA recognition sites for the stable complex formation complicates the use of SfiI for site-specific labeling: (i) SfiI poorly binds to an isolated recognition site; (ii) SfiI binding to DNA molecules with more than one recognition site results in DNA looping that changes the overall DNA topology and therefore limits the propensity of DNA molecules for intrinsic and protein-induced structural rearrangements. Here we show that these complications can be overcome by inducing stable SfiI binding to its target site in DNA with the help of a short DNA duplex that provides the second SfiI recognition site. Using the oligonucleotide duplex in excess to target DNA preferentially drives the formation of an intermolecular (*trans*) synaptic complexes which contain SfiI, long DNA molecule and the oligonucleotide duplex, rather than the *trans* complex formed by SfiI-mediated bridging of two recognition sites in two long DNA fragments or an intramolecular or *cis* complex formed by SfiI binding to the two recognition sites in the same circular molecule. This labeling procedure does not interfere with cruciform formation and has very low dependence on the DNA supercoiling. In addition, the complex dissociates slowly in moderately acidic (pH 5) or alkaline (pH 9) media.

MATERIALS AND METHODS

Materials

All enzymes and BSA were from New England Biolabs (Beverly, MA). Concentrations of commercial SfiI stocks

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were determined by comparing the densitometric traces of a Coomassie Blue-stained SDS–PAGE gel of SfiI and of reference quantities of BSA (15). The pUC8-derivative plasmids pEO200, pEOF200 and pEOF250 were prepared as described previously (14). Each plasmid contains two SfiI recognition sites (SfiI recognition sequence is underlined): 5′-GGCC-ACCCCGGCC-3′ and 5′-GGCCTCGAGGGCC-3′ (pEO200 and pEOF200) and 5′-GGCCTTGTGGGCC-3′ and 5′-GGCC-TCGAGGGCC-3′ (pEOF250). The two sites are separated by 200 bp of random sequence in plasmid pEO200 and by 300 bp in plasmid pEOF200. The latter plasmid contains a 106 bp inverted repeat F14C (16), centrally located between the recognition sites. Plasmid pEOF250 also contains the F14C inverted repeat asymmetrically located between the SfiI sites that are 350 bp apart. Open circular DNA was obtained by digestion of supercoiled plasmids with a nicking enzyme Nt.BstNBI as recommended by the supplier. After phenol–chloroform extraction, DNA was purified by ethanol precipitation.

The 362 bp DNA fragment with one SfiI recognition site was obtained by a double digestion of plasmid pEOF250 with NspI and HindIII restriction endonucleases. The fragment was purified from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA), ethanol precipitated and dissolved in HE buffer (10 mM HEPES and 1 mM EDTA, pH 7.5). Its concentration was determined from DNA absorption at 260 nm using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The 17 bp duplex was prepared from the oligonucleotide 5′-GGGGCCTCGAGGGCCAT-3′ and its complement (Integrated DNA Technologies, Coralville, IA) by mixing in TNM buffer (10 mM Tris–HCl, 50 mM NaCl and 10 mM $MgCl_2$), heating to 95°C and slow cooling to room temperature. The duplex formation was monitored with a non-denaturing PAGE.

Labeling procedure

A typical labeling reaction mixture contained a 2:1 molar ratio of the SfiI tetramer per DNA recognition site, e.g. 100 fmol of SfiI tetramer and 25 fmol of plasmid DNA (two recognition sites in each molecule), in 10 µl of reaction buffer A (10 mM HEPES, 50 mM NaCl, 2 mM $CaCl_2$, 0.1 mM EDTA and 1 mM DTT, pH 7.5). The mixture was incubated

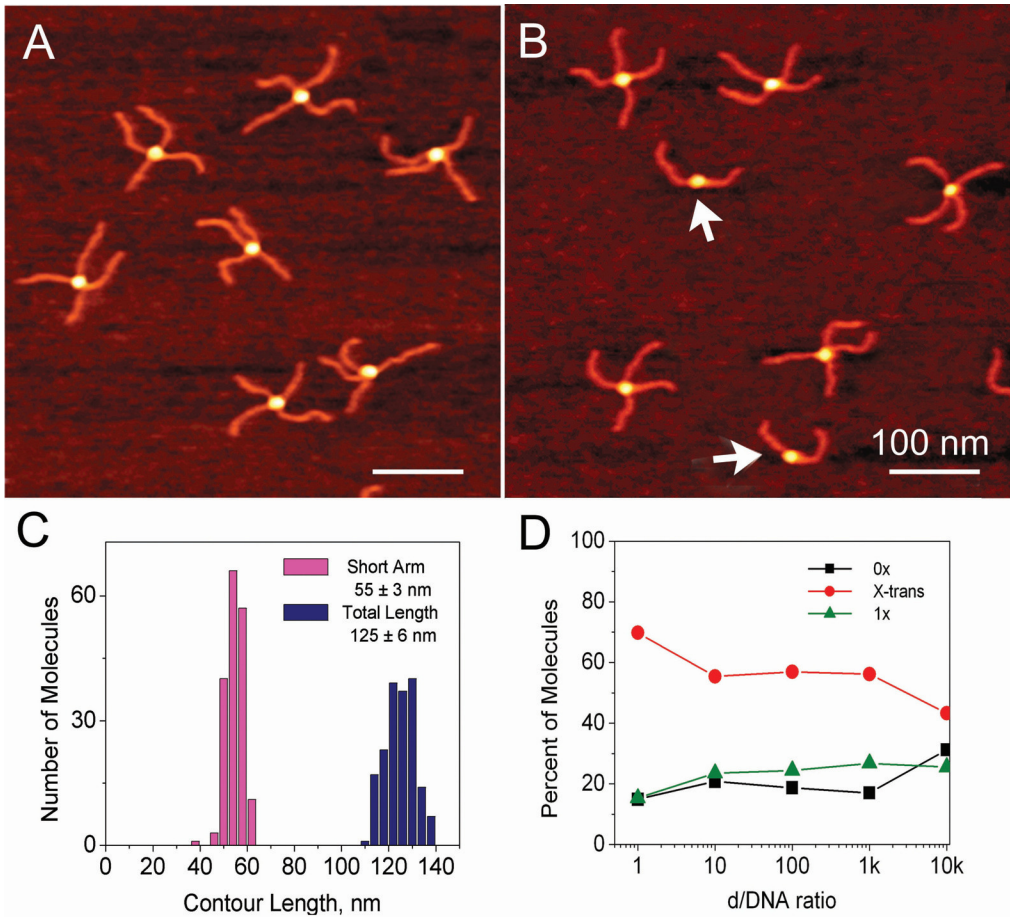

Figure 1. SfiI binding to the 362 bp DNA fragment containing one enzyme recognition site. (**A**) AFM images of the SfiI/DNA fragment complexes. 82% of DNA fragments are involved in a *trans* X-shaped synaptic complex containing the SfiI tetramer and two fragment molecules. (**B**) AFM images of the SfiI/DNA fragment complexes prepared in the presence of the 17 bp duplex at a 1000-fold excess over the fragment. DNA fragments labeled with SfiI are marked with arrows. (**C**) The contour length measurements for DNA fragment with the protein bound (blue) and the distance of the SfiI position from the end of the fragment (pink). (**D**) The yields of unlabeled DNA (×0) and different type of the SfiI/DNA complexes: complex containing two 362 bp fragments (X-*trans*) and complex containing one 362 bp fragment and one 17 bp duplex (×1), at different duplex-to-fragment ratios in the reaction mixture.

for 15 min at room temperature followed by addition of the DNA duplex and subsequent incubation for 15 min. The complex was purified by filtration through a Millipore UFC7 column, and the protein-bound DNA was eluted with 20 µl of reaction buffer. To test the stability of labeled DNA at different pH, the buffer was changed after the first round of filtration and additional two rounds of filtration were done with another buffer on the same column. Low pH acetate buffer (100 mM sodium acetate, 2 mM $CaCl_2$ and 1 mM DTT, pH 5.0) and high pH bicarbonate buffer (100 mM sodium bicarbonate, $CaCl_2$ and 1 mM DTT, pH 9.0) were used for this procedure.

AFM imaging procedure

Atomic force microscopy (AFM) procedure has been described previously (14,17). Briefly, freshly cleaved mica was treated with 167 µM water solution of aminopropylsilatrane (17) for 30 min. DNA samples (3–4 µl) were placed onto APS-mica for 2 min; then the sample was rinsed with deionized water (Labconco Co., Kansas City, MO) and dried in argon flow. Images were acquired in air with MultiMode SPM NanoScope IV system (Veeco/Digital Instruments, Santa Barbara, CA) and TESP probes (Tapping Mode Etched Silicon Probes, spring constant ~42 N/m and resonant frequency ~320 kHz). Image processing was performed with the Femtoscan software (Advanced Technologies Center, Moscow, Russia). Statistical analysis of the yield of different DNA structures was performed with the sets of 200–400 molecules for each sample.

RESULTS AND DISCUSSION

The possibility to use SfiI for the site-specific DNA labeling was tested with the 362 bp DNA fragment with a well-defined position of the SfiI binding site. In the presence of SfiI two fragment molecules form the X-shaped synaptic structure with the protein positioned at a cross point (Figure 1A). Ca^{2+} was used to stabilize the synaptic complex. In the presence of calcium SfiI can normally bind to DNA, however its cleavage activity is inhibited (18). At DNA concentration in the nanomolar range, the yield of synaptic complexes was 65–70%. The yield of the two-fragment synaptic complex decreased to 45–55% when the 17 bp DNA duplex was added to the reaction mixture even at the 1:1 molar ratio. At the same time separate DNA fragments carrying clearly identified SfiI molecules were observed. These were the synaptic complexes involving the 362 bp fragment and the 17 bp duplex. The latter was not visible due to its small size. Images of the SfiI/DNA complexes prepared at a 1000-fold excess of the 17 bp duplex over the 362 bp fragment are shown

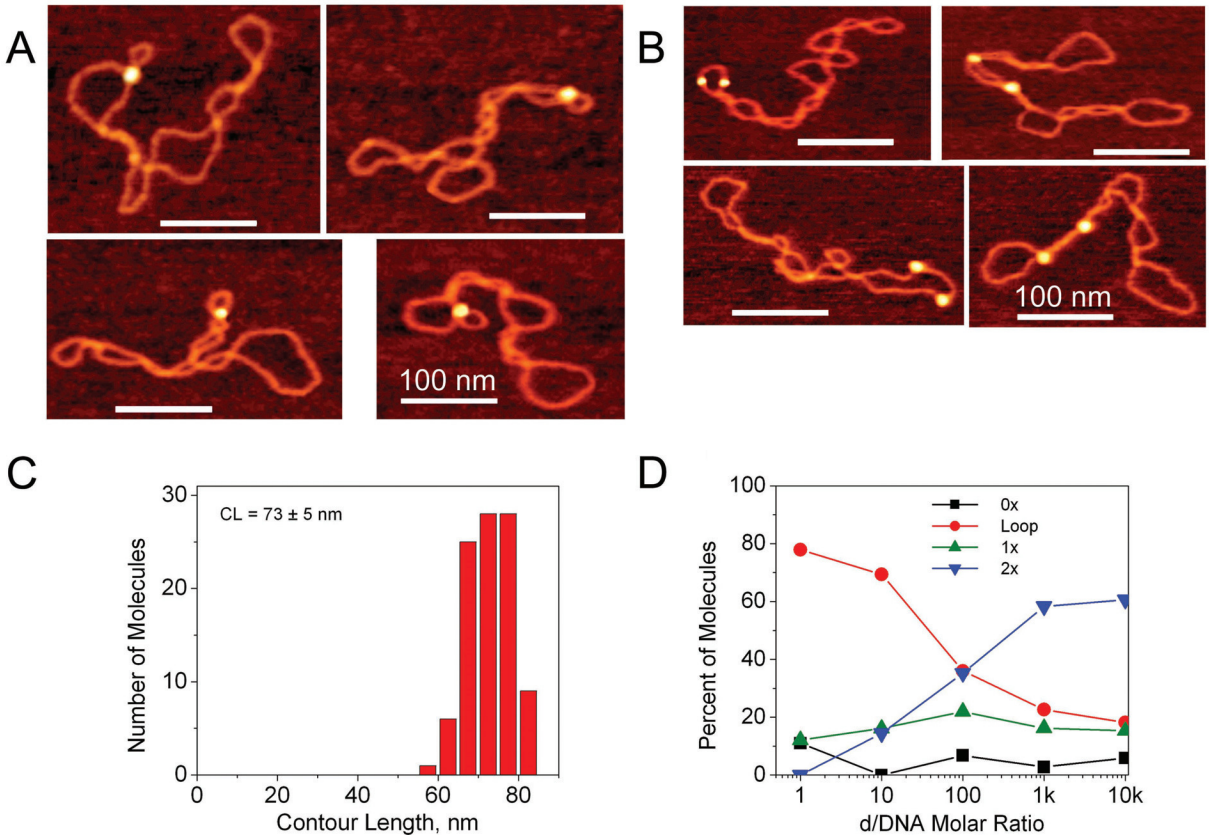

Figure 2. SfiI binding to supercoiled plasmid pEO200 containing two enzyme recognition sites. (**A**) Images of the plasmid in complex with SfiI. The intramolecular synaptic complex appears as a loop closed by the SfiI tetramer. (**B**) AFM images of the double-labeled DNA prepared in the presence of the 17 bp duplex at a 1000-fold excess over the plasmid. (**C**) The contour length (CL) measurements between two sites in double-labeled molecules. (**D**) The yields of unlabeled DNA (×0) and different type of the SfiI/DNA complexes: the SfiI-closed loop (loop) and complexes containing the 17 bp duplex at one (×1) or both SfiI recognition sites (×2), at different duplex-to-plasmid ratios in the reaction mixture.

in Figure 1B. The labeled individual DNA molecules are indicated with arrows. Note that no such complexes were observed in the absence of the duplex. The length measurements data are shown in Figure 1C. The short arm length of 53 ± 2 nm and the total fragment length of 125 ± 5 nm correspond to the 169 bp distance between the recognition site and one fragment end, and the total fragment length of 362 bp. The contour length measurements show that the protein position on the 362 bp fragment is slightly asymmetric and perfectly coincided with the expected position of the SfiI binding site. A minor number of unspecific complexes could be found with protein sitting at the end of the fragment. Only 3 of 1716 complexes analyzed had the position of the protein exceeding 3 SD values. The standard deviation for the measurements of the SfiI position taken over >300 complexes was 7.3% of the mean value compared with 5.5% for the contour length measurements on bare DNA. This means that the accuracy for the SfiI labeling procedure is only 20% less than contour length measurements regardless of the inevitable loss of accuracy due to the large protein size. The dependencies of the yields of different types of complexes on the duplex-to-fragment ratio are shown in Figure 1D. This graph shows that even at the equimolar duplex-to-fragment ratio, 15% of protein–DNA complexes involved labeled 362 bp fragments. The yield of the protein-labeled DNA fragments increased with increasing duplex concentration and reached 30%. Overall, the data obtained support the feasibility of using the SfiI based approach for site-specific labeling.

To extend the proposed approach to labeling supercoiled DNA we used plasmid pEO200 containing two recognition sites at a 200 bp distance. Enzyme binding to two sites should either result in DNA looping if the SfiI tetramer brings together two recognition sites in the same plasmid, or in a short duplex-assisted binding of two SfiI tetramers to both recognition sites in the plasmid (double-labeled circular DNA). AFM images of the complex formed by SfiI with plasmid pEO200 in the absence of the 17 bp duplex are shown in Figure 2A where DNA loops closed by the SfiI tetramer are seen. The loop size of 72 ± 5 nm is very close to the expected value of 68 nm for the 200 bp distance between the two SfiI binding sites. In the presence of the 17 bp duplex, double-labeled plasmids are observed in addition to those containing the SfiI-closed loops. At a 1000-fold molar excess of the 17 bp duplex over the plasmid, the plasmid molecules labeled at both recognition sites (Figure 2B) are the major type of the complexes (~60%). The measured distances between the labels have a narrow distribution around the mean value of 73 ± 5 nm (Figure 2C), which is the same as the size of the SfiI-closed loops and which agrees well with the expected distance of 68 nm or 200 bp between the two recognition sites. We also tested plasmid labeling in a broad range of the 17 bp duplex concentrations and determined the yields of various products (Figure 2D). At increasing duplex concentrations, the yield of double-labeled plasmids increased at the expense of plasmids with the SfiI-closed loops. The percentage of double-labeled molecules leveled off at ~55–60% when the molar duplex-to-plasmid ratio was >1000:1. Thus, the data obtained show unambiguously that the proposed methodology is capable of labeling two sites within the plasmid.

The experiments described above were performed with a natively supercoiled DNA sample (superhelical density, $\sigma = -0.05$). It levels off at ~100:1 for this sample. Also, since plasmid superhelicity may vary in a broad range, we tested whether the labeling efficiency was affected by DNA supercoiling. To evaluate the effect of DNA supercoiling, we performed experiments with an open circular DNA obtained by the treatment of plasmid pEO200 with the site-specific nicking enzyme Nt.BstNBI (Figure 3). SfiI binding to the plasmid in the absence of the 17 bp duplex resulted in the formation of SfiI-closed DNA loops (Figure 3A). The yield of looped structures was ~80%. Adding of the competing duplex changes the morphology of SfiI-DNA complexes. Figure 3B shows the images of selected double-labeled molecules (ca. 30% of total different complexes) taken for the sample prepared at the 1000-fold molar excess of the 17 bp duplex over relaxed plasmid. Similarly to the previous data for supercoiled DNA (Figure 2D), the experiments with

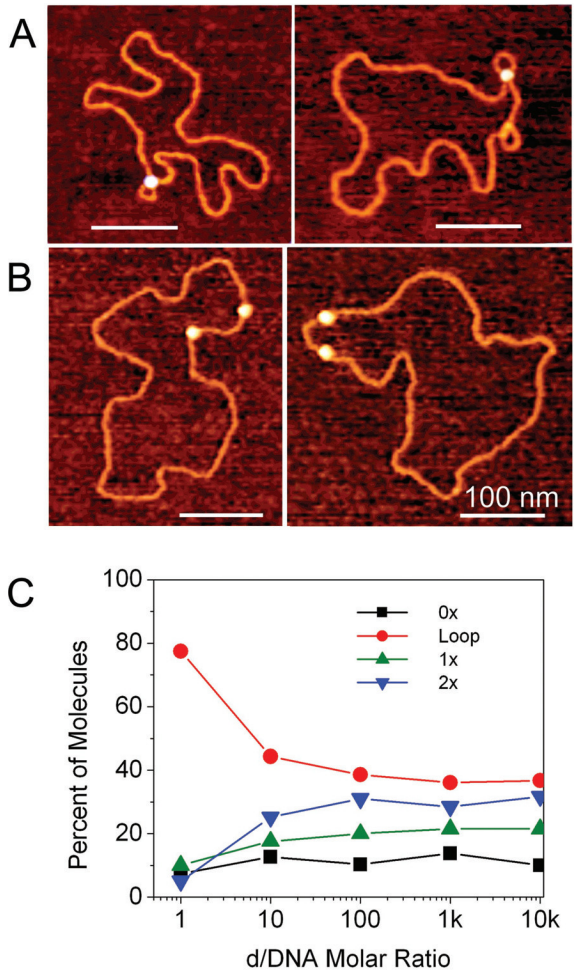

Figure 3. SfiI binding to open circular plasmid pEO200. (**A**) AFM images of the molecules with SfiI-closed loops obtained in binding reactions without 17 bp duplex. (**B**) Images of the double-labeled molecules prepared in the presence of the 17 bp duplex at a 1000-fold excess over the plasmid. (**C**) The yields of unlabeled DNA (×0) and different type of the SfiI/DNA complexes: the SfiI-closed loop (loop) and complexes containing the 17 bp duplex at one (×1) or both SfiI recognition sites (×2), at different duplex-to-plasmid ratios in the reaction mixture.

different duplex:plasmid ratios were performed and various types of the SfiI complexes were counted. The yields of the complexes with open circular DNA leveled off when the duplex/plasmid ratio reached 1000:1 (Figure 3C). Interestingly, the double labeling of relaxed DNA was two times less efficient compared with the supercoiled DNA. Given the higher probability of the site juxtaposition in supercoiled DNA compared with an open circular DNA, one should anticipate an opposite trend—higher efficiency of the short duplex-assisted SfiI binding to separate sites in relaxed DNA. However, the obtained results suggest that other factors, such as the relative orientation of protein binding sites (phasing) and local DNA winding angles, both of which depend on DNA supercoiling, can contribute to the stability of the *cis* synaptic complex in supercoiled DNA.

We also tested for the possible interference of this labeling procedure with the formation of alternative DNA structures stabilized by negative DNA supercoiling. We used plasmid pEOF200 which differs from plasmid pEO200 by insertion of the inverted repeat F14C, capable of cruciform formation at the supercoiled density below $\sigma = -0.03$ (2,19), into the 200 bp segment between the two SfiI binding sites. If the cruciform extruded, the distance between the recognition sites would be 200 bp. Selected images of the plasmid pEOF200 complexed with SfiI in the absence and presence of the 17 bp duplex are shown in Figure 4A and B, respectively. In the absence of the 17 bp duplex, 86% of plasmid molecules had SfiI-closed loops (Figure 4A) with the cruciform positioned at the loop apex (the cruciforms are indicated with arrows). The number of looped complexes decreased two times (to ~40%) when the 1000-fold molar excess of the 17 bp duplex was added to the reaction mixture. This decrease was accompanied primarily by the formation of the double-labeled complexes shown in Figure 4B. The contour length measurements show that the labels are positioned at the distance of 35 ± 2 nm from the center of the cruciform (Figure 4C), which correlates well with the expected 100 bp distance between the recognition sites and the cruciform. Statistical analysis performed over more then 300 molecules showed that the standard deviation for the SfiI position measured relative to the cruciform position is 6.3%. This value is slightly exceeding the accuracy for the length measurements (5.5%) suggesting that the accuracy of the labeling is 15% less than the accuracy for the contour length measurements.

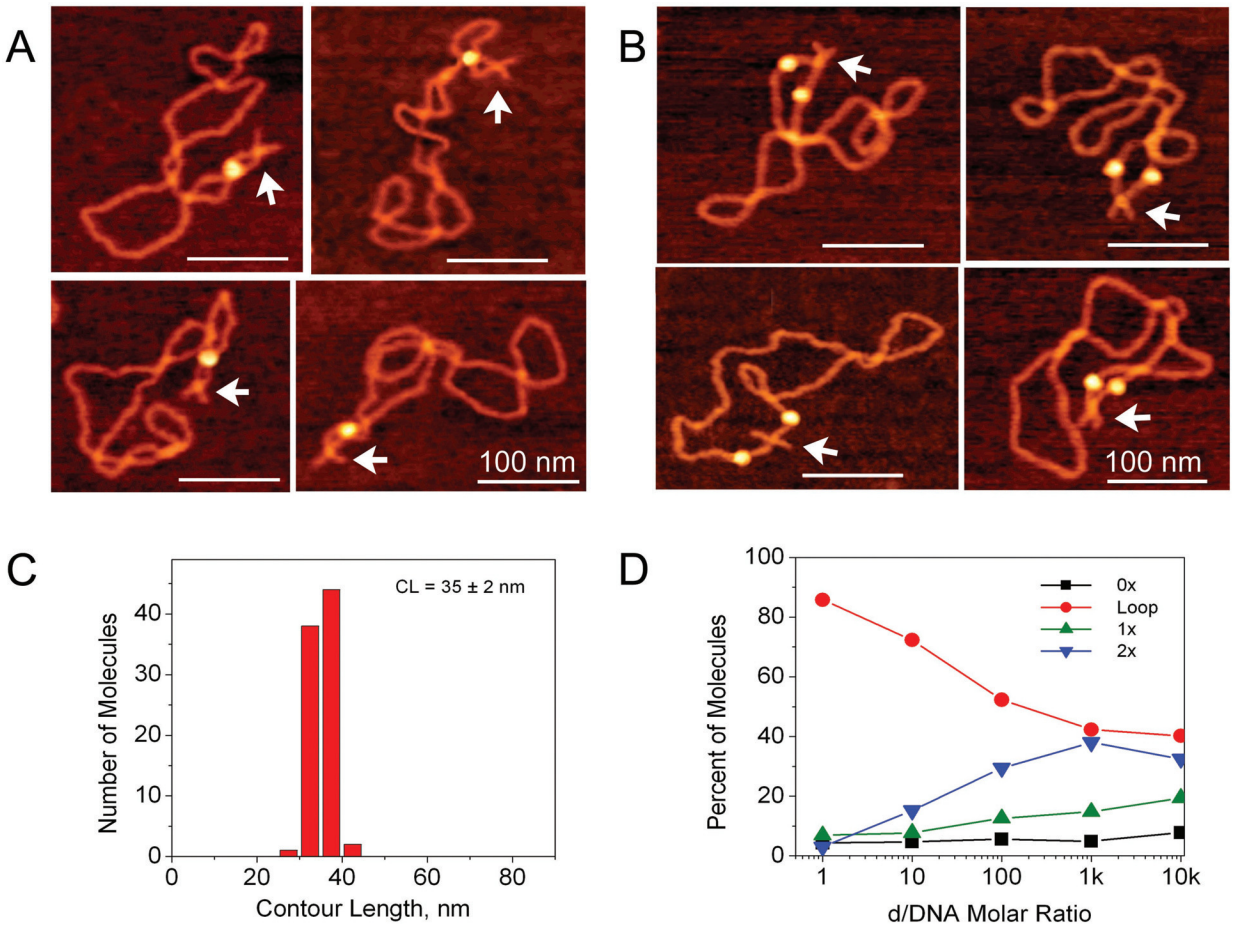

Figure 4. SfiI binding to supercoiled plasmid pEOF200 containing the cruciform. (**A**) AFM images for the SfiI/DNA complexes prepared without the duplex; 86% of molecules have the SfiI-closed loop and the cruciform. The cruciforms are indicated with arrows. (**B**) Selected AFM images of the double-labeled molecules in the sample obtained in the presence of the 17 bp duplex at a 1000-fold excess over the plasmid. (**C**) Measurements of the contour length (CL) between the cruciform and either of the labeled positions. (**D**) The yields of unlabeled DNA (×0) and different type of the SfiI/DNA complexes: the SfiI-closed loop (loop) and complexes containing the 17 bp duplex at one (×1) or both SfiI recognition sites (×2), at different duplex-to-plasmid ratios in the reaction mixture.

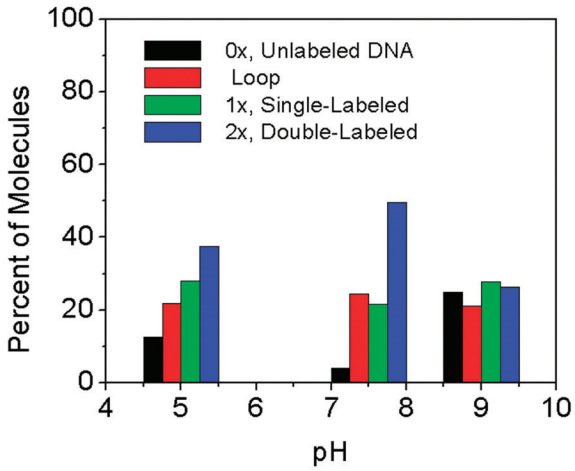

Figure 5. The pH effects on the yield of various types of SfiI complexes with plasmid pEO200. Protein binding to DNA was accomplished in the presence of the 17 bp duplex at a 1000-fold excess over the plasmid. Percentages of unlabeled DNA (×0, black bars) and different type of the SfiI/DNA complexes: the SfiI-closed loop (loop, red bars) and complexes containing the 17 bp duplex at one (×1, green bars) or both SfiI recognition sites (×2, blue bars).

The titration data obtained in a broad range of duplex concentrations (Figure 4D) show that the percentage of the SfiI-closed loops decreased with the increase of the duplex concentration and reached a plateau at ~40% when the duplex-to-plasmid ratio was above 1000:1. At the same time, the number of double-labeled molecules increased up to ~35%, whereas the number of molecules labeled at the single position increased slowly to ~20%.

If SfiI/DNA complexes are to be used for identification of pH-dependent local DNA structures, such as H-DNA (16), it is instructive to evaluate the stability of complexes at different pH. For this experiment, SfiI was bound to plasmid pEO200 at the duplex-to-plasmid ratio of 1000:1 to obtain a high yield of the double-labeled complexes. The sample, prepared at pH 7.5, was divided into three aliquots and the pH was adjusted to 5.0 and 9.0 in two of them. Figure 5 shows that the percentage of the double-labeled molecules decreased from 50% at pH 7.5 to 38 and 26% at pH 5.0 and pH 9.0, respectively. This decrease in double-labeled molecules was accompanied by an increase in the amount of unlabeled DNA (from 3 to 4% at pH 7.5 to 12% at pH 5.0 and to 25% at pH 9.0). It is quite surprising that the complex remains stable under conditions quite far from the most optimal ones. Such a relatively high complex stability is a useful feature for the labeling procedure extending the range of the conditions for its use.

CONCLUSION

The data obtained show that the restriction enzyme SfiI can be used as a site-specific label for circular DNA molecules if a short DNA duplex is used as a helper to stabilize the formation of the site-specific synaptic complex. SfiI binds as tetramer of the total molecular weight ca 124 kDa which is easily distinguished on the DNA molecule by AFM (and EM). The protocol is simple and requires an inexpensive commercially available protein. Importantly, the weak dependence of labeling efficiency on DNA supercoiling, significantly broadens the range of the labeling application protocol. The fact that labeling does not interfere with the formation of cruciforms is another important feature of the developed method in applications where alternative structures are involved. The tolerance of the complex to the pH change is another important feature of the SfiI based protocol extending the application from pH 5 to 9. Although the major focus of the paper is the labeling of circular DNA, the procedure can be applied to labeling of linear molecules. The labeling of linear molecules can be useful if the molecules are long and the position of the SfiI site is in relatively close proximity to the areas of interest. The proposed approach utilizes SfiI enzyme that requires relatively long site for the recognition that may not present in the plasmid of interest. The procedure can be extended to other type II enzymes recognizing shorter DNA sequences the enzymatic activity of which is blocked by replacing Mg^{2+} with Ca^{2+} (e.g. NgoMIV). Our recent experiments showed that EcoRII restriction enzyme belonging to the same type II family (20) can be another potential candidate. This enzyme forms the synaptic complexes in the dimeric form compared to the tetrameric form for SfiI. It also does not cut in the absence of Mg^{2+} cations and is capable of binding DNA in the absence of divalent cations. However these properties in terms of the accuracy of the labeling need to be investigated thoroughly. This work is in progress.

ACKNOWLEDGEMENTS

The authors thank E. Oussatcheva for plasmid preparation, and M. Karymov, A. Krasnoslobodtsev, L. Shlyakhtenko and other members of the laboratory for useful and stimulating discussions. The work was supported by the NIH grant GM 062235 to Y.L.L. Funding to pay the Open Access publication charges for this article was provided by the National Institutes of Health (NIH).

Conflict of interest statement. None declared.

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