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Aggregation of RecA-Derived Peptides on Single-Stranded Oligonucleotides Triggered by Schiff Base-Mediated Crosslinking

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Abstract—We here show that single-stranded oligonucleotides containing 5-formyl-2′-deoxyuridine (fdU) can crosslink the peptides derived from the DNA binding site of RecA protein through a Schiff base formation. The ability of crosslinking of fdU-containing oligonucleotides was investigated using a series of peptides whose amino acid residues spanning the center of the RecA-derived peptide were sequentially replaced with lysine. Circular dichroism (CD) spectroscopy, gel mobility shift assay and sedimentation experiment demonstrated that crosslinking reaction proceeded efficiently only when the peptides bound to the oligonucleotides.

5-Formyl-2′-deoxyuridine (fdU) (Fig. 1) is one of oxidative thymine lesions in DNA formed by ionizing radiation[1–3] and photochemical sensitization.[4,5] fdU has been known to be mutagenic and cytotoxic. Mutagenic effect of fdU is largely ascribed to mispairing during DNA replication[6–8,12] and cytotoxicity is likely to result from inhibition of thymidylate synthetase and thymidine kinase.[9–12] Moreover, it has been suggested that the formyl group of fdU may react with proteins to form crosslinks, which would be of importance in mutagenesis and cytotoxicity.[2,6] In organic solvents, fdU and its sugar-protected derivatives can form a Schiff base with various amines.[13,14] However, until today direct observation of crosslinking via Schiff base formation between oligonucleotides containing fdU and proteins has not been reported yet.

To assess the crosslinking capability of fdU, we chose a RecA derived peptide as its target. *Escherichia coli* RecA protein promotes the strand exchange between two homologous DNA molecules.[15] RecA first polymerizes onto the ssDNA, producing a nucleoprotein filament. This complex then captures dsDNA to search the homologous region to the resident ssDNA. A 20 amino acid residue peptide spanning the RecA loop L2 region (FECO peptide in Fig. 2a) has been shown not only bind to both ss- and dsDNA's but also catalyze the pairing reaction.[16] Determination of the structure of FECO–DNA complex should provide useful information on the mechanism of strand exchange reaction catalyzed by RecA protein. However, FECO–DNA complex formed by polymerization of FECO onto DNA is a heterogeneitic aggregate. Owing to the heterogeneity, structural characterization of the complex has been a challenge to X-ray crystallography and NMR spectroscopy. This peptide has one unique lysine, and hence seems to be a suitable molecule to test the crosslinking activity of fdU in the oligonucleotide. Here we report the results of crosslinking experiments of the oligonucleotide containing fdU with the RecA-derived peptides.

We synthesized a series of peptides (Fig. 2a) whose amino acid residues spanning the center of the loop L2 of RecA were sequentially replaced with lysine to find out the appropriate position for Schiff base formation with fdU. Based on the results of mutation study of whole RecA,[17] the positions to be replaced with lysine were selected so that DNA binding activity of mutants would be retained. Further, peptides were designed to

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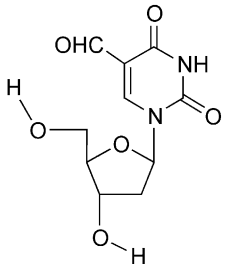

Figure 1. Structure of 5-formyl-2′-deoxyuridine.

A peptides

FECO	NQIRMKIGVMFGNPETTTGG
K6R	NQIRMRIGVMFGNPETTTGG
G8K	NQIRMRIKVMFGNPETTTGG
G8^{d}K	NQIRMRI^{d}KVMFGNPETTTGG
V9K	NQIRMRIGKMFGNPETTTGG
M10K	NQIRMRIGVKFGNPETTTGG
F11K	NQIRMRIGVMKGNPETTTGG
G12K	NQIRMRIGVMFKNPETTTGG

B oligonucleotides

I* : 5′-TTXTTXTTXTTXTTXTTXTTXTTU^{DIG}-3′

II* :5′-^{DIG}CATTATACTTAXTTTTATGTTCT-3′

III*:5′-^{DIG}CATTATACTTATTTTTATGTTCT-3′

Figure 2. Sequences of the peptides and oligonucleotides used in this study. X denotes the 5-formyl-2′-deoxyuridine unit.

have a single lysine by replacing the congenital lysine with arginine to avoid the complexity arising from multiple reaction points. All peptides were acetylated at the N-terminus, amidated at the C-terminus. Peptides were prepared by solid-phase synthesis using Fmoc chemistry, and then purified by HPLC. The identity and the purity of each purified peptide were assessed by HPLC and TOF mass spectrometry.[18] Concentration of each stock solution was estimated using the UV absorption at 205 nm (concentration (mg/mL) = 31 A units).[19]

First, we tested the binding of RecA peptides to ssDNA. As the binding of the peptides to the natural DNA was too weak to obtain gel shifts, it was assessed by CD spectroscopy. On binding to ssDNA, RecA peptides change the conformation from a random coil to a β-structure, which can be easily monitored by CD in the far-UV region where the spectroscopic signals primarily arise from the peptide bonds.[16,19] In our experiments, the CD from DNA was negligible. The β-structure is characterized by a maximum at ~190 nm and a minimum at ~220 nm. The conformation of K6R changed from a random coil to a β-structure by adding ssDNA $(dT)_{21}$ (Fig. 3a), showing that the replacement of lysine with arginine does not impair the ssDNA binding activity of the RecA peptide. Thus, K6R is suitable for the starting reference. Effects of ssDNA on the CD spectra of six mutant peptides studied here are compared in Figure 3b. The order of their propensity to form the β-structure was found to be FECO > G8K > M10K, G12K > V9K > F11K, reflecting their affinity towards ssDNA.

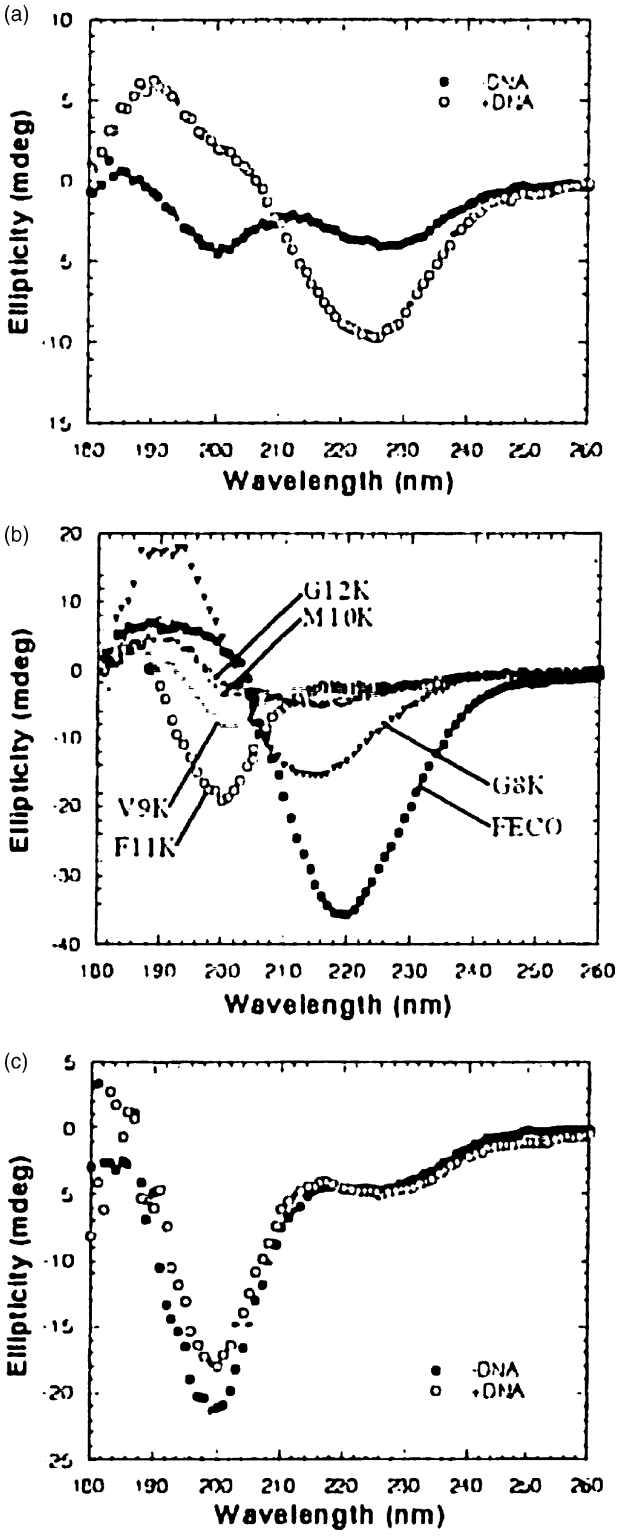

Figure 3. CD spectra of RecA-derived peptides (300 microM) in the presence and absence of $(dT)_{21}$ (15 microM): (a) CD of K6R in the presence (open circle) and absence (filled circle) of ssDNA; (b) CD of FECO and its mutant peptides in the presence of ssDNA; (c) CD of G8^{d}K in the presence (open circle) and absence (filled circle) of ssDNA. CD spectra were measured in 10 mM sodium phosphate buffer pH 7.4.

Interestingly, Gly8 is totally conserved among 64 eubacterial RecAs.[15,20] Since Gly has no side chain, we also became interested in the effect of stereochemistry of amino acid at the 8th position on ssDNA-binding. Thus, we synthesized the peptide G8^{d}K in which L-Lys of G8K was replaced with D-Lys. Figure 3c shows that the addition of ssDNA did not induce any conformational change in G8^{d}K, indicating the complete loss of DNA binding ability. The stereochemistry of Lys is rigorously limited to L-configuration.

Oligonucleotides containing 5-formyl uracils were prepared as described previously.[13,21,22] Oligonucleotides were non-radioactively labeled with DIG (digoxigenin) according to the manufacture's manual (Roche Diagnostics). **I** was labeled at the 3′-end. Figure 4a shows the results of crosslinking experiment for FECO and its lysine-scanned mutants. DIG-labeled DNA **I***, (Fig. 2b), which has seven units of fdU, was incubated with 160 μM of peptide in PBS binding buffer containing 1 mM $MgCl_2$ (1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, 1 mM $MgCl_2$, 0.1% IGEPAL CA-630 and 10% glycerol, pH 7.4). The resultant Schiff bases were reduced by $NaBH_3CN$ to fix the crosslink and analyzed on 10% polyacrylamide gel electrophoresis. DNA was electroblotted from the gel to the nylon membrane. DIG-labeled DNA on the membrane was visualized by chemical luminescence. The reaction with FECO resulted in the complete disappearance of **I*** on the gel, showing that the crosslinking reaction proceeded efficiently. G8K also crosslinked with **I***, affording the high molecular weight molecules. The other four peptides did not give any detectable crosslinked product. These results are consistent with those of CD, indicating that crosslinking through Schiff base formation proceeded efficiently only when the peptides bound to ssDNA. The failure of attempts to obtain gel shifts with the natural ssDNA and RecA derived peptides (data not shown) also supports the Schiff base formation between fdU and lysine.

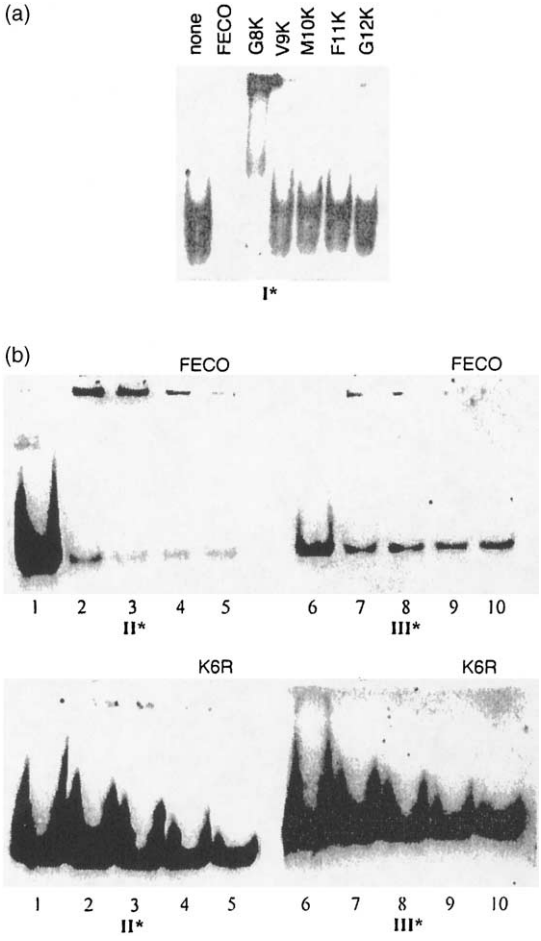

Figure 4. Electrophoretic mobility shift assays of crosslinking reactions. (a) 0.1 microM of DIG-labeled ssDNA I* was incubated with 160 microM of each peptide for 30 min at room temperature and reduced with $NaBH_3CN$ for 30 min. Each sample was resolved on a 10% polyacrylamide gel and visualized by chemical luminescence. (b) 0.1 microM of DIG-labeled ssDNAs (**II*** and **III***) were reacted with serially diluted FECO or K6R. Lanes 1–5, **II***; lanes 6–10, **III***. Lanes 1 and 6, free probe; lanes 2–5 and lanes 7–10 contain 10, 20, 40, 80 microM of peptides, respectively.

Although FECO reacted with **I***, reaction product could not be visualized. We speculated that the crosslinking reaction between FECO and **I*** may have caused formation of insoluble large aggregate in the reaction mixture. To further understand the reaction mechanism between RecA derived peptides and oligonucleotides containing fdU, we conducted the second crosslinking experiments. In order to reduce the extent of aggregation, we synthesized 23 mer oligonucleotide **II** that has only one unit of fdU. The oligonucleotide **III** is a natural type, whose sequence is the same as that of **II**. They were labeled with DIG at the 5′-end (Fig. 2b). The position of DIG, whether 3′ or 5′-end, does not affect the results in this study. Figure 4b shows the results of the crosslinking experiment with **II*** and **III***. We examined four peptide-DNA combinations: FECO–**II***, FECO–**III***, K6R–**II***, and K6R–**III***. The DIG-labeled DNA was incubated with serially diluted peptides (10–80 μM) in PBS buffer containing 10 mM $MgCl_2$ and reduced by $NaBH_3CN$. The reaction mixture was separated on a 12% polyacrylamide gel and analyzed as described above. Although CD experiments have demonstrated that both FECO and K6R can bind to ssDNA, only the combination of FECO–**II***, in which lysine and fdU coexisted, gave new clear bands. Since the new bands were detected in the position of wells and there were no bands between free DNA and the well, the products were likely to be aggregates. Interestingly, the reaction produts exhibited more intense bands at the lower peptide concentrations compared with the higher ones. This is probably due to the formation of large aggregates that cannot get into the gel at higher peptide concentrations. This reaction requires not only DNA binding of peptides but also coexistence of lysine and fdU in the complex. We therefore conclude that this reaction process includes Schiff base formation. In the other peptide–DNA combinations, the band intensity of free DNA slightly decreased as the peptide concentrations increased, suggesting the adhesion of peptide–DNA complex to the tube.

To find out if the peptide–DNA complex detected on the PAGE is an aggregate, we assayed for aggregation[23] under the same condition of the second crosslinking

using the same oligonucleotides (**II*** and **III***, Fig. 2b) and 10 μM of peptides (FECO, K6R, G8K). When FECO or G8K was mixed with ssDNA **II***, most of the DNA was found in aggregates that sedimented to the bottom of a tube after centrifugation (Fig. 5). Neither FECO nor G8K caused the aggregation of ssDNA **III*** which lacks fdU. K6R did not cause the aggregation of ssDNA with and without fdU. The aggregation required both lysine from peptide and fdU from ssDNA, supporting the formation of Schiff base. Thus, aggregation seems to be triggered by Schiff base formation between lysine residue and fdU in FECO–**II*** and G8K–**II*** complex. As anticipated from the results of PAGE, we confirmed that the final reaction product between peptides (FECO or G8K) and **II*** was an aggregate.

The formed Schiff base slows down the dissociation rate of a bound RecA peptide from DNA and facilitates the polymerization of the peptides on the DNA by promoting interpeptide interactions, which would result in aggregates. Since the Schiff base is probably stabilized in the aggregates, the ordinarily unfavorable equilibrium for the formation of Schiff bases in aqueous solution can shift to the product side. In the crosslinking reaction using fdU, the background arising from nonspecific reaction would be very low by virtue of the low reactivity of formyl group of fdU in aqueous solution. Thus, oligonucleotides containing fdU will be a novel probe that can specifically detect the lysines in the close proximity of fdU. This crosslinking is especially advantageous to entrap kinetically unstable complexes which cannot survive during gel electrophoresis such as RecA peptide.

We demonstrated the usefulness of fdU as a crosslinking reagent using RecA peptide. Photo-crosslinking studies have mapped DNA-binding sites of RecA protein to loop L1 and L2.[24,25] Since only L2 has a lysine in the two loops, it is intriguing to consider direct application of this technique to whole RecA protein. RecA is thought to have two kinds of DNA binding sites, site I and site II[26–28] and it may be possible to discriminate these two sites using fdU-containing oligonucleotides for differential proximity probing of RecA protein.[25] As all mutant RecA proteins that have the same replacement with lysine as in the RecA peptides used in this study are functional in vivo,[17] if combined with fdU-mediated crosslinking, lysine scanning study for the whole RecA protein may be more informative than for RecA peptides.

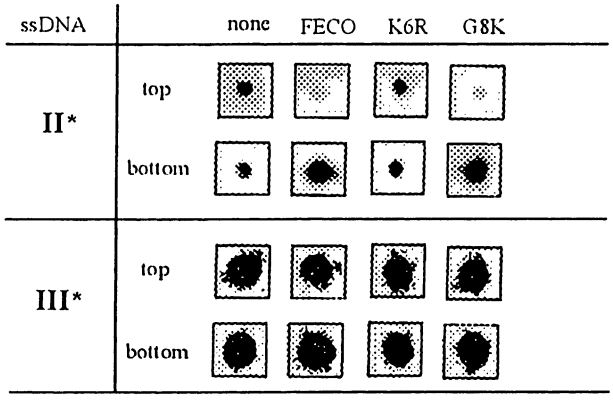

Figure 5. Assays for aggregation of ssDNA. 0.1 μM of DIG-labeled ssDNAs (**II*** and **III***) were reacted with 10 μM of FECO or K6R for 30 min at room temperature and reduced with $NaBH_3CN$ for 30 min. After centrifugation, aliquots of 2 μL were taken from the top of the supernatant and from the bottom of the tube and were spotted onto a positively charged nylon membrane to detect DIG-labeled ssDNA by chemical luminescence.

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[MH^+] 2219.5, found 2220.7; F11K calcd for [MH^+] 2203.5, found 2203.4; G12K calcd for [MH^+] 2293.7, found 2294.0.
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