J. Biochem. **125**, 24-26 (1999)

Crystallization and Preliminary X-Ray Diffraction Studies of a Replication Initiator Protein (RepE54) of the Mini-F Plasmid Complexed with Iteron DNA[1]

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Received November 12, 1998; accepted November 25, 1998

A replication initiator protein (RepE54) complexed with iteron DNA at its binding site was crystallized by the hanging drop vapor diffusion method. The crystals belong to monoclinic space group *C*2 with unit cell dimensions of $a=108.4$ Å, $b=81.9$ Å, $c=73.9$ Å, and $\beta=121.5°$, where one molecule of the protein-DNA complex exists per asymmetric unit. They diffract X-rays up to 2.6 Å resolution with synchrotron radiation.

Key words: crystallization, replication initiator protein, RepE, mini-F plasmid, X-ray diffraction.

The RepE initiator protein of the mini-F plasmid plays an essential role in initiating DNA replication from the origin, *ori2*. RepE exhibits two major functions: initiation of DNA replication from *ori2* (initiator function) and autogenous repression of *repE* transcription (repressor function). The initiation is mediated by the RepE monomers that bind to the *ori2* iterons (direct repeats), whereas the autogenous repression is mediated by the dimer that binds to the *repE* operator, which contains an inverted repeat sequence related to 8-bp of an iteron, as shown schematically in Fig. 1. It is important to elucidate the three-dimensional structure of the RepE-DNA (iteron) complex at the atomic level for a detailed insight into the molecular mechanism by which this protein recognizes an iteron and initiates DNA replication.

A RepE mutant (RepE54) produces hyperactive RepE that cannot form dimers, unlike the wild-type protein that is mostly found as dimers. RepE54 monomers bind to the iterons of *ori2* with very high efficiency but hardly bind to the operator (*1*). While the wild-type protein tends to form aggregates so much that it is difficult to crystallize, RepE54 hardly forms such aggregates. Therefore, RepE54 is thought to be suitable not only for purification and concentration, but also for preparation of the protein-DNA complex.

We first tried to crystallize the RepE54 protein without DNA duplexes by extensive screening, but no crystals were obtained. We have attempted the crystallization of the RepE54 protein complex with various DNA duplexes (synthetic oligomers containing an iteron) to determine the crystal structure of the protein-DNA complex. To facilitate protein purification, the pQE9-*repE54* plasmid was constructed to overexpress RepE54 with a histidine cluster ($6\times$His) at the N-terminal end. The resulting $6\times$His-RepE54 used for crystallization retained both initiator activity *in vivo* and iteron-binding activity *in vitro*, that were essentially identical to those of RepE54 without histidine residues.

Purification of the protein was performed as described previously except for the addition of a Heparin column step (*2*). RepE54 was overexpressed in *Escherichia coli* strain KY1461 (*2*) carrying plasmid pKV7202 (*2*) containing the *repE54* mutation and purified as a His-tagged protein with an Ni^{2+}-nitrilotriacetic acid (NTA) resin (QIAexpress system; QIAGEN) and Heparin columns (Pharmacia). The purified RepE54 gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The final fractions containing the RepE54 protein were dialyzed against a stock buffer [100 mM potassium citrate (pH 6.2), 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol] and then stored at 4°C. The synthesized oligodeoxyribonucleotides used for the complex formation were commercially available. Equimolar amounts of the complementary strands were mixed at a final concentration of 4 mM in an annealing buffer [50 mM Tris-HCl (pH 7.0), 100 mM KCl]. The mixture was heated at 95°C for 10 min and then slowly cooled over a few hours to 20°C for annealing. The DNA duplexes were stored at −20°C.

Each of the DNA duplexes was cocrystallized with RepE54, by the hanging drop vapor diffusion method (*3*). Before crystallization, the protein solution was concentrated with Centricon-10 (Amicon) to about 20 mg/ml. The RepE54 protein was mixed with the iteron DNA in a ratio of 1.2 DNA duplexes (4 mM) to one molecule (0.64 mM). Equal volumes of the protein-DNA and precipitating solutions were mixed on a siliconized coverslip, suspended over a well containing the precipitating solution, and

[1] This work was partly supported by the "Research for the Future" Program (JSPS-RFTF 97L00501) of the Japan Society for the Promotion of Science to K.M.

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allowed to equilibrate at room temperature. The presence of the complex in a component of the co-crystal was confirmed by gel mobility shift analysis of the washed and dissolved crystals. The gel was stained with an ethidium bromide (Et-Br) solution to confirm the presence of DNA and with Coomassie Brilliant Blue to detect the presence of RepE54 in the complex (Fig. 2).

An iteron is a 19 bp direct repeat sequence. In several crystals of protein-DNA complexes, the DNA duplexes are stacked to form a pseudo-continuous helix. Such an arrangement is favored by duplexes containing an integral number of turns. Therefore, 21-22 bp (2 turns) oligonucleotides, which include an iteron with termini that provide blunt-ended or overhanging bases, were surveyed for co-crystallization with the RepE54 protein (*4*). Table I shows the oligonucleotides used for the crystallization (DNA 1-DNA 8) and characterization of the obtained crystals. All the DNA duplexes shown in Table I afforded crystals of the RepE54-DNA complex with polyethylene glycols (PEG-400, 2000, 4000, 6000, and 8000) as precipitants. The crystals gave a slower-moving band corresponding to the formation of a RepE54-DNA complex in the gel shift assay, as shown in Fig. 2. Figure 3 shows the crystals of the protein-DNA complexes successfully obtained for six oligonucleotides. The most suitable crystals for X-ray diffraction studies were grown with a mixture of the protein and a 22 base long DNA duplex (DNA 4), which consists of 5′-CCT-GTGACAAATTGCCCTCAG-3′ and its complement with a T-overhang at each 3′ terminus. Prismatic crystals of the protein-DNA 4 complex grew to approximate dimensions of $0.3\times0.2\times0.2$ mm within 2 weeks from a precipitating solution comprising 100 mM Tris-HCl, pH 8.0, 12% PEG-400, and 200 mM $MgCl_2$.

Diffractions from the crystals extend to 3 Å resolution with a laboratory X-ray source. For characterization of the crystals, oscillation photographs were taken with a DIP-2030 imaging-plate camera system (Mac Science) with Cu-$K\alpha$ radiation generated with a M18XHF^{22}-SRA rotating anode generator operated at 50 kV and 90 mA with a fine focus filament. The crystals belong to monoclinic space group $C2$, with unit cell dimensions of $a=108.4$ Å, $b=81.9$ Å, $c=73.9$ Å, and $\beta=121.5°$. Assuming one complex per asymmetric unit, the Matthews content, V_m, is calculated to be 3.12 Å^3/Da and the solvent content of the crystal to be 60.6% (*5*). Intensity data were collected with 2.5 GeV synchrotron radiation at the BL-18B beam line of the Photon Factory, KEK, Tsukuba. The X-ray beam was monochromatized to 1.00 Å with an Si (111) monochromator and a 0.1 mm aperture collimator was used. Oscillation photographs were taken on a 400×800 mm imaging plate on a multifunction camera for macromolecular crystallography (*6*) equipped with a cylindrical cassette of 430 mm radius. The image data frames were read out on a drum-type imaging plate reader, IPR4080 (*7*). Figure 4 shows the X-ray diffraction pattern in an oscillation photograph of a native crystal. A total of 36 serial oscillation images each with a rotation range of 5.3° (0.3° overlap) and an exposure time of 7 min were collected using one crystal, which covered 180° of the total rotation range. The data were processed with program packages DENZO and SCAL-

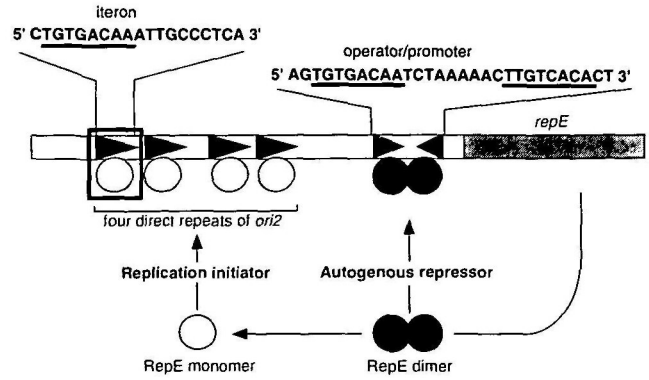

Fig. 1. **The functions of the RepE initiator protein in the mini-F plasmid.** The RepE monomers bind to the four iterons (direct repeats) of *ori2* to initiate replication, whereas the RepE dimers bind to the inverted repeats of the *repE* promoter-operators to repress *repE* transcription. Parts of the repeated sequences are shown at the top of the figure, and portions shared by the direct and inverted repeats are underlined. The box indicates the RepE54-iteron DNA complex crystallized in this work.

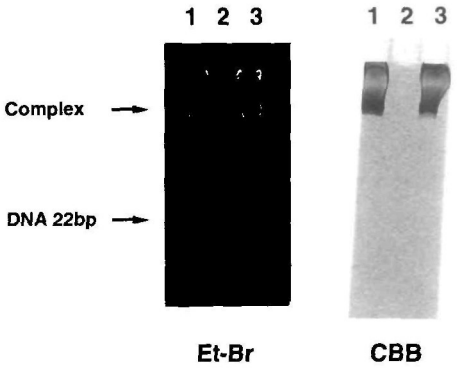

Fig. 2. **Confirmation by gel shift assaying of the crystals of RepE54-DNA complexes.** Samples were electrophoresed on 15% polyacrylamide gels (acrylamide/bis-acrylamide=75:1), and the gels were stained with Coomassie Brilliant Blue (CBB) and ethidium bromide (Et-Br) for the detection of RepE54 and iterons, respectively. Lane 1, mother liquor; lane 2, washing buffer; lane 3, crystals.

TABLE I. **DNA duplexes used in co-crystallization trials.**

DNA	Sequence[a]	Co-crystals obtained
DNA 1	C CTGTGACAAATTGCCCTCA G G GACACTGTTTAACGGGAGT C	Diffracting 5 Å
DNA 2	AC CTGTGACAAATTGCCCTCA G G GACACTGTTTAACGGGAGT CT	Small, cracked
DNA 3	TC CTGTGACAAATTGCCCTCA G G GACACTGTTTAACGGGAGT CT	Small, cracked
DNA 4	C CTGTGACAAATTGCCCTCA GT TG GACACTGTTTAACGGGAGT C	The best crystal diffracting 2.6 Å
DNA 5	CC CTGTGACAAATTGCCCTCA G G GACACTGTTTAACGGGAGT CC	Diffracting 3 Å
DNA 6	T CTGTGACAAATTGCCCTCA T A GACACTGTTTAACGGGAGT A	Very small
DNA 7	TC CTGTGACAAATTGCCCTCA A G GACACTGTTTAACGGGAGT A	Very small
DNA 8	TC CTGTGACAAATTGCCCTCA A G GACACTGTTTAACGGGAGT T	Diffracting 4 Å

[a]The box in each sequence indicates the 19-base pair iteron sequence.

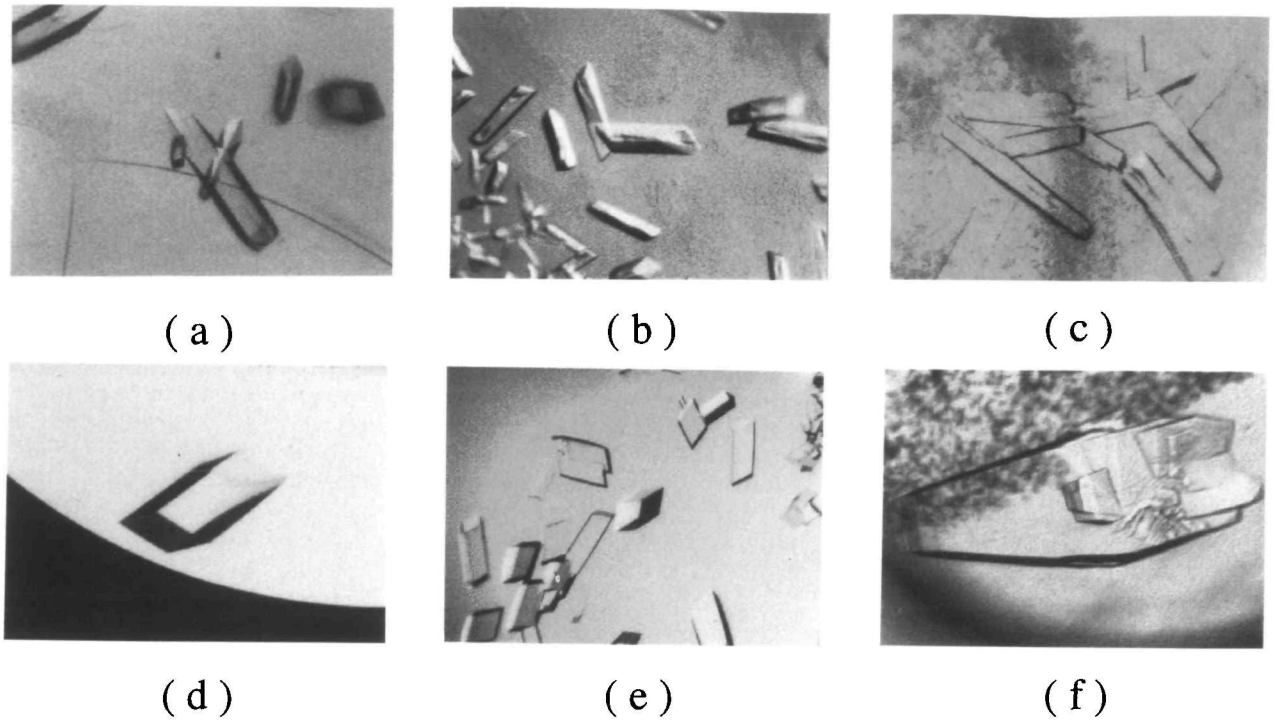

Fig. 3. **Crystals of the RepE54 complexes with DNA duplexes.** Complexes with (a) DNA 1, (b) DNA 2, (c) DNA 3, (d) DNA 4, (e) DNA 5, and (f) DNA 8.

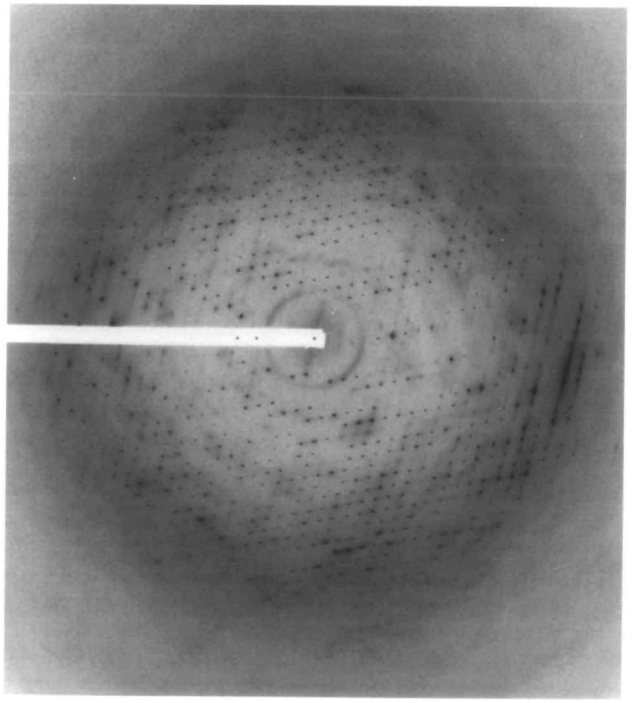

Fig. 4. **Oscillation photograph of a crystal of the RepE54 complex with DNA4 taken with synchrotron radiation.** The oscillation range is 5.3° and the crystal-to-film distance is 430 mm.

EPACK (*8*). Intensity data [$I > \sigma(I)$], consisting of 15,208 unique reflections, which cover 88.7% of the theoretical observations, were obtained with an R_{merge} of 0.035 at 2.6 Å resolution. Structure analysis by means of the multiple isomorphous replacement method is now in progress.

We would like to thank Professor Emeritus N. Sakabe, and Drs. N. Watanabe and M. Suzuki of the Photon Factory, Institute of Material Structure Science, High Energy Accelerator Research Organization, for their kind help in the X-ray diffraction study, which was performed with the approval of the Photon Factory Advisory Committee (Proposal No. 97G095). Thanks are also due to Dr. K. Kamada for the valuable suggestion and to M. Ueda for the technical assistance.

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