Epitopes and Active Sites of the RecA Protein*

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The RecA protein is indispensable for homologous genetic recombination in Escherichia coli. This protein alone promotes the ATP-dependent formation of homologous joint molecules and their processing in vitro. Through the use of a set of anti-RecA protein mouse monoclonal IgGs, we have been attempting to divide the whole process into elementary steps to determine the basic functions of the protein. In order to correlate the basic functions with the active sites on the recA polypeptide, we located the epitopes for the anti-RecA protein-IgGs on the recA polypeptide by means of immunoblotting experiments and an enzyme-linked immunosorbent assay involving isolated proteolytic polypeptides or synthetic ones derived from various regions of the recA polypeptide. The epitopes for anti-RecA protein-IgGs ARM321 and ARM414, both of which are shown to inhibit the DNA-dependent ATP hydrolysis and the formation of homologous joints by the RecA protein, were found to be located between Thr59 and Glu157 and between Glu239 and Lys566, respectively, on the recA polypeptide. IgG ARM193 had been shown to interfere with the protein-protein interaction between two RecA protein molecules, and ARM191 had been suggested to inhibit the binding of double-stranded DNA to the RecA protein. The epitopes for ARM193 and ARM191 were found to be located in a ~90-amino acid region at the C terminus. These results suggest the locations of the active sites and a functional core on the recA polypeptide.

"Homologous pairing," which includes searching for mutually homologous sequences between parental DNA molecules, and the formation of nascent homologous joints through intermolecular base pairing are the most critical and mysterious steps in homologous genetic recombination in vivo. The RecA protein was the first example of a class of proteins which promote homologous pairing of single-stranded and double-stranded DNAs in vitro (McEntee et al., 1978; Shibata et al., 1979), and has been studied extensively to understand the molecular mechanism underlying homologous pairing. Although the RecA protein is a relatively small single polypeptide species (~40 kDa) consisting of 352 amino acid residues (Horii et al., 1980; Sancar et al., 1980), it is involved in not only homologous pairing but also the processing of the nascent homologous joints formed through the former reaction. When double-stranded DNA has an end at or near the joint, the homologous pairing is followed by "strand exchange." Strand exchange is the unidirectional exchange of a parental strand of double-stranded DNA for an incoming strand of single-stranded DNA, and heteroduplex joints formed through homologous pairing are stabilized by this process (Cox et al., 1981; Kahn et al., 1981). When double-stranded DNA is of the closed-circle form and single-stranded DNA is much shorter than the former DNA, the joints are dissociated with the same strand polarity as that in the strand exchange: i.e. 5' to 3' with respect to the displaced strand (Shibata et al., 1982a, 1982b; Wu et al., 1982). The formation and processing of the joint molecules includes various elementary processes which were identified in studies on various DNA substrates and ones involving analogues of substrates or cofactors, mutant RecA proteins and anti-RecA protein monoclonal IgGs. Other than the processes described above, the RecA protein removes the secondary structure of single-stranded DNA through the formation of a filamentous complex in the presence of ATP (Muniyappa et al., 1984; Flory et al., 1984), hydrolyzes ATP in a DNA-dependent manner (Ogawa et al., 1979; Roberts et al., 1979), extensively unwinds the double helix through a progressive reaction ("progressive unwinding": Cunningham et al., 1979a, 1979b; Ohtani et al., 1982), and cleaves SOS-repressor proteins (Roberts et al., 1978).

In general, monoclonal IgGs are supposed to be useful for studying elementary functions involved in a complex reaction process promoted by a protein and for locating the active sites on the protein. We constructed a set of mouse hybridoma clones which produce anti-RecA protein-IgGs (Makino et al., 1985) and have been characterizing them as to their inhibitory effects on the elementary functions of the RecA protein (Makino et al., 1985, 1987). An anti-RecA protein-IgG, ARM193, severely inhibited the RecA protein-promoted progressive unwinding and strand exchange, but affected the homologous pairing by the same protein only a little (Makino et al., 1987). Our previous studies suggested that ARM193 interferes with protein-protein interactions between RecA protein molecules (Makino et al., 1985; Iwabuchi et al., 1983), and cleaves SOS-repressor proteins (Roberts et al., 1978).

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MATERIALS AND METHODS

Proteins and Antibodies

The RecA protein used in this study was Fraction V, which was prepared as described previously (Shibata et al., 1981, 1983). Anti-RecA protein mouse monoclonal IgGs were purified by affinity column chromatography on protein A-Sepharose from mouse ascitic fluid in which hybridoma cells had been grown. The procedure was described in detail previously (Makino et al., 1985; see also Nakagawa et al., 1988). The molecular masses of the recA polypeptide and the IgGs were assumed to be 40 and 160 kDa (Makino et al., 1985), respectively. The amounts of the RecA protein and IgGs were estimated as described previously (Shibata et al., 1981; Makino et al., 1985). The amounts of the synthetic polypeptides and a fragment of the recA polypeptide (Fv 31-8-7) were estimated by their amino acid composition analysis.

Horse heart myoglobin (16.9 kDa) and its fragments (2.5, 6.2, 8.2, and 14.4 kDa), which were used as standards for determination of the molecular masses of the polypeptides, were purchased from RDI Chemicals Ltd. (United Kingdom; molecular weight markers for SDS-polyacrylamide gel electrophoresis).

Endopeptidases

Endoproteinase Arg-C and V8 proteinase (endoproteinase Glu-C) were purchased from Boehringer Mannheim GmbH, Federal Republic of Germany. Lysyl endopeptidase and a-chymotrypsin were purchased from Wako Pure Chemical Industries Ltd. (Japan) and Sigma, respectively.

Buffers

PBS consisted of 50 mM potassium phosphate buffer (pH 7.2) and 150 mM NaCl. PBS-Tween was PBS containing 0.05% Tween 20.

Assaying the Hydrolysis of ATP by the RecA Protein and Its Inhibition by an Anti-RecA Protein-IgG

The reaction mixture (21 μl) for single-stranded DNA-dependent ATP hydrolysis by the RecA protein comprised 31 mM Tris-HCl buffer (pH 7.5), 13 mM NaCl, 1.3 mM [methyl-3H]ATP, 50 μM (in nucleotide residues) circular single-stranded DNA of phage φX174, 2.8 mM diithothreitol, 88 μg of bovine serum albumin (for molecular biology, Boehringer Mannheim) per ml, 2% (v/v) glycerol, and the indicated amount of the RecA protein. After the reaction mixture had been incubated at 37 °C for the indicated time, the reaction was terminated and the amount of ATP hydrolyzed was determined by thin-layer chromatography, as described previously (Shibata et al., 1981).

When the competition as to the binding of the RecA protein to an IgG with a synthetic polypeptide was examined by its ability to prevent the inhibition of the ATPase activity of the RecA protein by the IgG, first the RecA protein (1.0 μM) and the synthesized polypeptide were mixed in the standard reaction buffer lacking an IgG, ATP, and DNA, with the temperature of the reaction mixture equilibrated at 37°C. Then, an anti-RecA protein-IgG (to 1.0 μM) was added, followed by incubation at 37°C for 30 min. A1′ and circular single-stranded DNA were added simultaneously to start the hydrolysis of ATP, followed by incubation for 30 min.

ELISA (Enzyme-Linked Immunosorbent Assay)

Unless otherwise stated, all procedures were carried out at room temperature (25°C). To coat the wells of a microtiter plate with a polypeptide, 50 μl of the polypeptide dissolved in PBS (RecA protein, at 2 μg/ml; synthetic polypeptides Pφ21 and Pφ14, at 10 μg/ml; a fragment of the recA polypeptide, at 4 μg/ml) was put into each well, followed by standing for 4°C for 15 h and then washing three times with ~200 μl of PBS. For blocking, ~100 μl of 1% bovine serum albumin dissolved in PBS was put into each well, followed by standing for 1.5 h and then washing with ~130 μl of PBS. On the other hand, the concentration of anti-RecA protein-IgG was first adjusted to 3 μg/ml in PBS-Tween (no dilution) and then a series of diluted samples in PBS-Tween was prepared. Then, 50 μl of each of the diluted IgG samples was put into a well of the microtiter plate, followed by standing for 1 h. The wells were washed three times with ~130 μl of PBS-Tween. Then, 50 μl of peroxidase-labeled affinity-purified antibody to mouse IgG (500-fold diluent; Catalog No. 14-18-02, Kirkegaard & Perry Laboratories Inc.) was added, followed by standing for 1 h, and then washing of the wells three times with ~130 μl of PBS-Tween and then once with ~200 μl of PBS. To each well, o-phenylenediamine (100 μl; 1 mg/ml dissolved in 0.05 M citric acid, 0.1 M sodium citrate, and 0.15% hydrogen peroxide) was added, followed by standing for 30 min. The coloring reaction was terminated by the addition of 32 μl of 4.5 M sulfuric acid. The absorbance at 490 nm of the samples was measured after the samples had been diluted with 1 M sulfuric acid to adjust the reading to lower than 2.0.

Fragmentation of the RecA Polypeptide with Endopeptidases

Endoproteinase Arg-C and Chymotrypsin—The RecA protein (~0.8 mg/ml) was treated with 11 μg of endoproteinase Arg-C/ml for 22 h or 0.2 μg of chymotrypsin/ml for 4 h in 1.0% ammonium bicarbonate at 37°C.

Lysyl Endopeptidase—The RecA protein (~0.8 mg/ml) was treated with 0.06 μg of lysyl endopeptidase/ml in 31 mM Tris-HCl buffer (pH 7.5) containing 13 mM MeCl. 1.3 mM ATP, and 0.1% SDS at 37°C.

V8 Proteinase (Endoproteinase Glu-C)—The RecA protein (~0.8 mg/ml) was treated with 95 μg of V8 proteinase/ml in 1.0% ammonium bicarbonate containing 0.05% SDS at 37°C for ~20 h.

Purification of the Fragments of the RecA Polypeptide by HPLC

The fragments of the recA polypeptide were injected onto a reverse-phase HPLC column (Cosmosil SC4-300 packed column, 4.6 inner diameter × 250 mm; Nakarai Chemicals Ltd.) that had been equilibrated with solution A (0.1% (v/v) trifluoroacetic acid dissolved in H2O). The sample was eluted with a linear gradient (~2%/min), from 0 to 60%, of solution B (0.1% (v/v) trifluoroacetic acid dissolved in acetonitrile) at 1.0 ml/min at room temperature. In the case of rechromatography, the gradient was reduced to 0.04-0.5%/min. Polypeptide digestion was determined by absorbance at 220 nm.

Polyacrylamide Gel Electrophoresis of the Fragments of the RecA Polypeptide and Immunoblotting Experiments

Unless otherwise stated, the fragments of the recA polypeptide were run through a gel slab (13 cm × 13 cm × 1 mm) containing linear gradients of polyacrylamide (12.5-25%) and urea (1-7 M) in 0.75 M Tris-HCl buffer (pH 8.5), 0.19 M glycine, and 0.1% SDS, at 70 V for ~15 h after the denaturing treatment (Fling and Gregerson, 1986).

Immunoblotting experiments, which included the transfer of the polypeptide from the polyacrylamide gel to a membrane filter, blocking, treatment with a peroxidase-labeled anti-mouse IgG and coloring reactions, were performed as described previously (Nakagawa et al., 1988). Unless otherwise stated, in the present study we used a polystyrene dihydroxy membrane (Immobilon, pore size 0.22 μm; Millipore Ltd.) instead of the nitrocellulose filter used in the previous study. The polypeptide fragments were transferred from the gel to the polyvinylidene difluoride membrane at 36 V/8 cm for ~4 h using 50 mM Tris, 0.19 M glycine, and 20% methanol in a Trans-Blot cell (Bio-Rad).

For estimation of the molecular masses of the polypeptide fragments from their mobilities on gel electrophoresis, the fragments were detected by an immunoblotting experiment and, if a sufficient amount was available, also by staining with Coomassie Brilliant Blue or silver stain (Merril et al., 1980).

Sequence Analysis of the N-terminal Amino Acid Residues of the Fragments of the RecA Polypeptides

A polypeptide fragment was spotted onto a Polybrene-treated glass filter disc (Kennedy et al., 1988), which was then loaded into an automatic protein sequencer (Applied Biosystems, Inc., model 477A) equipped with an on-line phenylthiohydantoin derivative analyzer (Applied Biosystems, Inc., model 120A). Four to five amino acid residues from the N terminus were sequenced for each polypeptide.

Chemical Synthesis of Polypeptides

Polypeptides were synthesized with an automatic chemical synthesizer (Applied Biosystems, Inc., model 430A) equipped with a on-line phenylthiohydantoin derivative analyzer (Applied Biosystems, Inc., model 120A). Four to five amino acid residues from the N terminus were sequenced for each polypeptide.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.
were removed from the products through the use of trifluoromethanesulfonic acid. The polypeptides were further purified by HPLC on a Cosmosil SC-300 column, as described above. The amino acid sequences of the synthesized polypeptides are as follows: P321, TCAF-IAEBHALDFIYARKLVDIDNLCSQPDTCHEQALE, which corresponded to the sequence between residues 89 and 127 of the recA polypeptide, and P414, EGENVVGSETRVKVKNKIAAPFK, which corresponded to the sequence between residues 233 and 256 of the recA polypeptide.

Analysis of the Amino Acid Composition

The protein was hydrolyzed by treatment with 4 N methanesulfonic acid at 104°C for 22 h and the sample was neutralized with NaOH. Then, the amino acid composition was analyzed with an automatic amino acid analyzer (Hitachi model 835).

RESULTS

Fragmentation of the RecA Polypeptide and Immunoblotting Analysis of the Fragments—We examined various proteolytic fragments of the recA polypeptide for their affinity toward anti-RecA protein-IgGs by means of immunoblotting experiments, followed by mapping of the fragments on the recA polypeptide. In this study, we used for anti-RecA protein-IgGs, ARM321, ARM414, ARM191, and ARM193. The binding of these anti-RecA protein monoclonal IgGs to the immobilized RecA protein was compared by ELISA (Fig. 1). Binding of ARM193 to the recA polypeptide was slightly weaker than that of ARM321, ARM414, and ARM191. The latter three anti-RecA protein-IgGs showed almost the same affinity toward the immobilized RecA protein (Fig. 1).

The RecA protein that had been partially digested with an endopeptidase (endoproteinase Arg-C) was electrophoresed through polyacrylamide gel and then subjected to immunoblotting analysis using the anti-RecA protein-IgGs (Fig. 2). The profiles of the bands of the fragments of the RecA polypeptide which reacted with ARM321 and ARM414 were clearly different from each other and from that with ARM193 or ARM191. This indicates that although the inhibitory effects of ARM321 and ARM414 on the in vitro activities of the RecA protein were shown to be identical (Makino et al., 1985), the epitopes for these IgGs are different from each other, and from those recognized by ARM193 and ARM191.

![Fig. 1. ELISA of anti-RecA protein monoclonal IgGs as to their affinities toward the whole recA polypeptide.](http://www.jbc.org/)

The material in each peak of the fragments on the chromatogram was collected and examined as to its reactivity with the IgGs by polyacrylamide gel electrophoresis and immunoblotting analyses. When more than one species of polypeptide was detected on immunoblotting and chemical staining. The purified samples contained a single species of polypeptide which was detected on immunoblotting and chemical staining. Each panel in Fig. 3 shows the profile on immunoblotting of the purified fragments of the recA polypeptide using the anti-RecA protein-IgGs. Each panel shows the proteolytic fragment that migrated at the highest rate in electrophoresis among those which showed cross-reaction with each IgG. Fragments of the RecA protein were prepared and purified by HPLC. Each fragment was electrophoresed through a polyacrylamide gradient gel and then transferred to a membrane. Then the membrane was cut into strips and each strip was treated with the indicated anti-RecA protein-IgG. The bound anti-RecA protein-IgG was detected by a coloring reaction, as described in Fig. 2. Lanes 21, 14, 93, and 91 were treated with anti-RecA protein-IgGs ARM321, ARM414, ARM191, and ARM193, respectively. The fragments tested were as follows: A, Fv 26-6-2; B, Fc223-3; C, Fv 31-8-7.

The profile of the bands of fragments recognized by ARM193 and that in the case of ARM191 appeared to be similar but not identical. Some fragments were detected with one of them but not with the other, and vice versa (Fig. 2).

Fractionation and Mapping of the Fragments of the RecA Polypeptide which Reacted with anti-RecA Protein-IgGs—Then, we examined the binding of the anti-RecA protein-IgGs, ARM321, ARM414, ARM193 and ARM191, to the isolated fragments. The RecA protein was treated with various endopeptidases and the products were fractionated by HPLC. The material in each peak of the fragments on the chromatogram was collected and examined as to its reactivity with the IgGs by polyacrylamide gel electrophoresis and immunoblotting analyses. When more than one species of polypeptide was detected, the samples were repeatedly purified by HPLC until only a single peak was detected in the chromatogram. Fig. 2. Profiles on immunoblotting of the fragments of the recA polypeptide. The RecA protein was partially digested with endoproteinase Arg-C and the products were electrophoresed through a 12.5-25% polyacrylamide gradient gel in the presence of a 1-7 M urea gradient for 13 h under constant voltage (80 V). Then the fragments were transferred to a membrane filter and the filter was cut into strips. Each strip was treated with the indicated anti-RecA protein-IgG. The bound IgG was detected by a coloring reaction catalyzed by an enzyme covalently attached to an anti-mouse IgG antibody. The anti-RecA protein-IgGs used were ARM321 (lane 21), ARM414 (lane 14), ARM193 (lane 93), and ARM191 (lane 91). Lane S, markers for molecular mass determination (from top to bottom: 16.9 kDa, 14.4 kDa, 8.2 kDa, 6.2 kDa, and 2.5 kDa) stained with Coomassie Brilliant Blue (the positions of the bands are indicated by arrows).
smallest in size among the fragments recognized by each of the IgGs tested.

Four or five amino acid residues from the N terminus of each purified fragment were sequenced (Table I, fourth column) and located on the recA polypeptide (Table I, fifth column). The molecular masses of the fragments were determined by polyacrylamide gel electrophoresis under denaturing conditions using marker polypeptides (see “Materials and Methods”; Table I, third column). The C terminus of each fragment was deduced from its size and the specificity of the endopeptidase used for its preparation (Table I, sixth column). Thus, the position of the N terminus of each fragment was precisely determined, but that of the C terminus may not be accurate. The results of immunoblotting analyses, of which three cases are shown in Fig. 3, are summarized in Fig. 4. Considering the overlapping of fragments which did not react with each IgG, we tentatively located the epitope for ARM321 between His97 and Glu130 of the recA polypeptide (residues are numbered from the N terminus of the recA polypeptide), that for ARM414 between Glu283 and Phe285, and those for ARM193 and ARM191 between Phe60 and Glu111. Glu111 was assumed to be the C terminus of Fv 31-8-7, which was found to be the smallest fragment recognized by ARM191 and ARM193 on electrophoresis under denaturing conditions. However, as described later, the true C terminus of this fragment was not Glu111 but Glu247. Antibody ARM321 showed weak cross-reaction with other fragments which did not overlap with the above region, but it showed the strongest cross-reaction with Fl 33-5 (Thr87-Lys245) and Fv 26-5-2 (His79-Glu245) by immunoblotting experiment. As discussed in the following section, this antibody showed slight nonspecific binding to another portion of the recA polypeptide.

Binding of Antibodies ARM321 and ARM414 to Synthetic Polypeptides—Then, we located the epitopes for the anti-RecA protein monoclonal IgGs more precisely. We synthesized polypeptides which consisted of residues 89–127 (39 amino acid residues) from the N terminus (P321) and of residues 233–256 (24 amino acid residues; P414), respectively, and tested them as to their reactivity with all the four anti-RecA protein-IgGs by ELISA. The results shown in Fig. 5 clearly indicate that P321 and P414 were specifically recognized by ARM321 and ARM414, respectively. The affinity of these IgGs toward the synthetic polypeptide appeared to be almost the same as that toward the whole recA polypeptide (compare Fig. 5 with Fig. 1). ARM321 showed weak but significant binding to P414 (Fig. 5B). However, the strength

| Fragments | Endopeptidase used | Determined molecular mass | Amino acid sequence at the N terminus | Region of the recA polypeptide | Candidates | Calculated molecular mass |
|-----------|--------------------|--------------------------|-------------------------------------|-------------------------------|------------|--------------------------|
| Fa 29-6   | Endoproteinase Arg-C | 8.2                      | Gln-Ala-Glu-Phe                     | Glu263                        | Arg248     | 7.6                      |
| Fa 31-7   | Endoproteinase Arg-C | 7.0                      | Lys-Leu-Ala-Gly                     | Lys177                        | Arg460     | 10.7                     |
| Fa 31-11  | Endoproteinase Arg-C | 20                       | Lys-Leu-Ala-Gly                     | Lys177                        | Arg460     | 10.7                     |
| Fa 23-3   | Chymotrypsin        | 2.5                      | Asp-Ile-Arg-Arg                     | Asp236                        | Phe236     | 8.1                      |
| Fa 27-4   | Chymotrypsin        | 6.8                      | X-Glu-X-Ile-Asn                     | X                             | Fv265-2     | 4.8                      |
| Fa 29-8   | Chymotrypsin        | 7.3                      | Gln-Ile-Leu-Tyr                     | Gln286                        | Phe236     | 8.6                      |
| Fa 28-10  | Chymotrypsin        | 8.5                      | Lys-Glu-Ala-Glu                     | Lys256                        | Phe235-2   | 9.2                      |
| Fa 31-3   | Chymotrypsin        | 8.0                      | X-Lys-Leu-Ala                       | X                             | Trp365-6   | 10.8                     |
| Fl 31-5   | Lysyl endopeptidase | 13.8                     | Glu-Gly-Glu-Asn                     | Glu233                        | Phe256     | 8.8                      |
| Fl 31-5-10| Lysyl endopeptidase | 8.5                      | Ile-Ala-Ala-Pro-Phe                 | Ile283                        | Phe256     | 8.8                      |
| Fl 33-5   | Lysyl endopeptidase | 5.1                      | Thr-X-Ala-Phe                       | Thr87                         | Lys172     | 6.7                      |
| Fl 33-6   | Lysyl endopeptidase | 21.5                     | Ala-Glu-Ile-Glu                     | Ala163                        | C          | 22.0                     |
| Fv 25-5-2 | V8 proteinase       | 2.5                      | His-Ala-Leu-Asp                     | His97                         | Glu123     | 2.9                      |
| Fv 278-245-6-2 | V8 proteinase | 3.8                  | Phe-Glu-Ile-Leu                    | Phe260                         | Glu298     | 4.2                      |
| Fv 31-8-7 | V8 proteinase       | 6.0                      | Phe-Glu-Ile-Leu                     | Phe260                         | Glu298     | 4.2                      |

* Molecular mass was estimated from the migration of the peptide on electrophoresis under denaturing conditions.
* The recA polypeptide has Ala and Phe at the N terminus and C terminus, respectively, and consists of 352 amino acid residues. X, the amino acid could not be identified.
* The position of the N terminus of the fragment was determined from the sequence shown in the fourth column. The position of the C terminus was deduced from the size of the fragment and the specificity of the endopeptidase used for its preparation. x- and x+ indicate other candidates. C, C terminus of recA polypeptide (Phe247).
**FIG. 4.** The reactivity of anti-RecA protein-IgGs to fragments of the RecA protein and synthetic polypeptides. The N terminus and C terminus of the fragments were located, as shown in Table I. The arrows at the C termini of the fragments indicate the respective regions of the possible sites of the C termini, considering error in the estimation of the size and the specificity of the protease used for the preparation. The C terminus of fragment Fv 31-8-7 was determined by amino acid composition analysis, as shown in Table II, and analysis of the amino acid residues near the C terminus. The reactivities of the fragments indicated in this figure, except for Fv 31-8-7 and synthetic peptides, were determined from the results of immunoblotting experiments, three cases of which are shown in Fig. 3. The reactivities of Fv 31-8-7 and the synthetic polypeptides were examined by ELISA, as shown in Figs. 7 and 5, respectively. 321, IgG ARM321; 414, IgG ARM414; 191, IgG ARM191; 193, IgG ARM193. **Filled rectangles,** fragments that reacted with the indicated IgGs; **open rectangles,** fragments that did not react with the indicated IgGs; **shadowed rectangles,** fragments that showed faint positive signals for the reactions with the indicated IgGs in immunoblotting experiments. The names (prefixed by F) of the fragments are given on the right side of the panels. P321 and P414 are the synthetic polypeptides. The residues are numbered from the N terminus of the recA polypeptide, which consists of 352 amino acid residues.
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Fig. 4.—continued

Fig. 5. ELISA of anti-RecA protein-IgGs as to their affinities toward the synthetic polypeptides. The wells of a microtiter plate were coated with the indicated synthetic polypeptide at 10 µg/ml (P321 in A and P414 in B). The IgG samples before dilution contained 3 µg of IgG/ml. The anti-RecA protein-IgGs tested were: ARM321 (○), ARM414 (▲), ARM191 (■), and ARM193 (●).

Fig. 6. Competition as to the binding of the RecA protein to anti-RecA protein-IgGs with the synthetic polypeptides. The binding of an IgG to the RecA protein was determined as the extent of inhibition of the single-stranded DNA-dependent ATPase activity of the RecA protein by the IgG. If the test synthetic polypeptide competes with the RecA protein in binding of an IgG, the polypeptide would prevent the inhibition by the IgG of the ATPase activity of the RecA protein. The RecA protein (1.0 µM) was incubated with the indicated amount of a synthetic polypeptide in the reaction buffer (containing 13 mM MgCl₂, but without DNA or ATP) at 37°C for 4 min, and then the indicated anti-RecA protein-IgG (1.0 µM) was added, followed by incubation for 10 min. The hydrolysis of ATP was started by the simultaneous addition of [³²P]ATP and single-stranded DNA (50 µM in nucleotide residues: φX174 phage DNA) and the reaction mixture was incubated at 37°C for 30 min. The amount of hydrolyzed ATP was determined. ○, polypeptide P321 and antibody ARM321; ▲, polypeptide P321 and antibody ARM414; △, polypeptide P414 and antibody ARM414; □, polypeptide P414 and antibody ARM321; ■, both the IgG and the synthetic polypeptide were omitted.

THE SPECIFIC BINDING OF ANTIBODY ARM321 TO SYNTHETIC POLYPEPTIDE P321, AND THAT OF ARM414 TO P414 IN SOLUTION WERE FURTHER TESTED BY MEANS OF COMPETITION EXPERIMENTS. IT HAD BEEN SHOWN THAT BOTH ARM321 AND ARM414 INHIBIT THE SINGLE-STRANDED DNA-DEPENDENT HYDROLYSIS OF ATP BY THE RecA PROTEIN, WHEN THE RecA PROTEIN HAD BEEN PREVIOUSLY INCUBATED WITH EITHER OF THESE ANTIBODIES (MAKINO ET AL. 1985). WE FIRST INCUBATED THE RecA PROTEIN AND EITHER ARM321 OR ARM414 IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF A SYNTHETIC POLYPEPTIDE, AND THEN EXAMINED THE HYDROLYSIS OF ATP. NEITHER SYNTHETIC POLYPEPTIDE P321 OR P414 INHIBITED OR STIMULATED THE SINGLE-STRANDED DNA-DEPENDENT ATPase ACTIVITY OF THE RecA PROTEIN, AND NEITHER OF THEM SHOWED ATPase ACTIVITY BY ITSELF (DATA NOT SHOWN). AS SHOWN IN Fig. 6, P321 AND P414 SPECIFICALLY PREVENTED THE INHIBITION OF ATPase ACTIVITY OF THE RecA PROTEIN BY ARM321 AND ARM414, RESPECTIVELY. IN EITHER CASE, THE INHIBITION BY THE IgG WAS COMPLETELY PREVENTED WHEN THE SYNTHETIC POLYPEPTIDE WAS ADDED AT 4-FOLD OVER THE RecA PROTEIN. THIS INDICATES THAT THE STRENGTH OF THE BINDING OF EITHER IgG TO THE RELEVANT SYNTHETIC POLYPEPTIDE AND THAT TO THE WHOLE recA POLYPEPTIDE ARE OF THE SAME ORDER OF MAGNITUDE.
Based on the specific binding observed on ELISA and the specific competition described above, we conclude that the epitopes for ARM321 and ARM414 reside between Thr<sup>269</sup> and Glu<sup>325</sup>, and between Glu<sup>233</sup> and Lys<sup>256</sup>, respectively (Fig. 8).

**Detailed Localization of the Epitopes for Antibodies ARM193 and ARM191**—Judging from its behavior on gel electrophoresis, fragment Fv 31-8-7 was the smallest proteolytic fragment of the recA polypeptide which reacted with ARM191 or ARM193. The N terminus of Fv 31-8-7 was definitely located on amino acid sequence analysis, but the C terminus of the fragment was tentatively assigned as described in the previous section. This fragment size (66 amino acid residues) was close to the limit of the chemical synthesis in experience. Then, we selected another approach. We obtained an amount of Fv 31-8-7 by partial digestion of the RecA protein with V8 proteinase, followed by purification by HPLC.

Both ARM191 and ARM193 showed cross-reaction with Fv 31-8-7 on ELISA (Fig. 7). The affinities of ARM191 and ARM193 toward Fv 31-8-7 were almost the same as and only slightly weaker than that of these IgGs toward the whole recA polypeptide, respectively (compare Fig. 7 with Fig. 1). As described in the preceding section, neither antibody ARM191 nor ARM193 reacted with synthetic fragments P321 and P414 (Fig. 5). These results indicate that both ARM191 and ARM193 specifically reacted with fragment Fv 31-8-7.

As described above, based on the apparent molecular mass, we tentatively located the C terminus of fragment Fv 31-8-7 at Glu<sup>314</sup> (Table I). We precisely determined the site of the C terminus of fragment Fv 31-8-7 on the recA polypeptide by amino acid composition analysis. Amino acid composition analysis revealed that the above assignment was wrong. If we tentatively located the C terminus of fragment Fv 31-8-7 at Glu<sup>314</sup>, this fragment should have 1 Ser and 1 Pro, but the fragment Fv 31-8-7 was assumed to have G1u<sup>314</sup> at its C terminus. Amino acid composition analysis of fragment Fv 31-8-7 is 1.6 times larger than the molecular mass observed upon gel electrophoresis. Preliminary analysis of the C-terminal amino acid residues by treatment of fragment Fv 31-8-7 with carboxypeptidase W (from wheu; Seikagaku Kogyo Co.) revealed that the C-terminal amino acid residue was Glu, and that Ala, Val, and Gly were released on the treatment. This result is only consistent with Glu<sup>347</sup> as the C terminus of Fv 31-8-7 among candidate sites Glu<sup>269</sup>, Glu<sup>274</sup>, Glu<sup>290</sup>, and Phe<sup>320</sup>. Thus, we conclude that the epitopes for ARM191 and ARM193 reside between Phe<sup>320</sup> and Glu<sup>347</sup> (Fig. 8).

The polypeptide fragment (Fv 31-8-7) was further digested with V8 proteinase, followed by fractionation by HPLC (Cosmosil 5C4-300). The materials in all detected peaks were subjected to immunoblotting after direct blotting of the samples. We found that none of them tested so far gave a positive signal for the reaction with either ARM191 or ARM193. This result may suggest that more than two discontinuous sites of Fv 31-8-7 are required for the binding of ARM191 or ARM193. However, it is also likely that subfragments of Fv 31-8-7 only weakly bound to the filter for the assay and thus were washed out during this assay.

**DISCUSSION**

It is likely that active sites distant from an epitope on the recA polypeptide are also affected by the binding of an IgG, but the localization of the epitopes for anti-recA protein monoclonal IgGs provided some insight as to the loci of the active sites on the recA polypeptide.

The epitopes for anti-recA protein monoclonal IgGs ARM321 and ARM414 were found to be located on the recA polypeptide between Thr<sup>269</sup> and Glu<sup>325</sup> and between Glu<sup>233</sup> and Lys<sup>256</sup>, respectively (Fig. 8). The epitopes for IgGs ARM193 and ARM191 were found to be located in a 90-amino acid region from amino acids 269 and 347 at the limit of the chemical synthesis in experience. Then, we selected another approach. We obtained an amount of Fv 31-8-7 by partial digestion of the RecA protein with V8 proteinase, followed by purification by HPLC.

Both ARM191 and ARM193 showed cross-reaction with Fv 31-8-7 on ELISA (Fig. 7). The affinities of ARM191 and ARM193 toward Fv 31-8-7 were almost the same as and only slightly weaker than that of these IgGs toward the whole recA polypeptide, respectively (compare Fig. 7 with Fig. 1). As described in the preceding section, neither antibody ARM191 nor ARM193 reacted with synthetic fragments P321 and P414 (Fig. 5). These results indicate that both ARM191 and ARM193 specifically reacted with fragment Fv 31-8-7.

As described above, based on the apparent molecular mass, we tentatively located the C terminus of fragment Fv 31-8-7 at Glu<sup>314</sup> (Table I). We precisely determined the site of the C terminus of fragment Fv 31-8-7 on the recA polypeptide by amino acid composition analysis. Amino acid composition analysis revealed that the above assignment was wrong. If fragment Fv 31-8-7 was assumed to have Glu<sup>314</sup> at its C terminus, this fragment should have 1 Ser and 1 Pro, but the expected numbers were both 4 (assuming 8 Gly residues in this region). Other than these 2 residues, the expected numbers of Gly were numbers: 4 (versus 2), Asp plus Asn (12 versus 5), and others did not agree with those observed. Since fragment Fv 31-8-7 was prepared through the use of V8 protease, the C-terminal residue of this fragment is likely to be Glu, and between Glu<sup>314</sup> and the C terminus of the recA polypeptide, residues 318, 320, 325, 343, 347, and 350 are Glu. Among them, Glu<sup>347</sup> can be clearly eliminated as candidates for the C-terminal residue of this fragment, for similar reasons. When the C terminus of the fragment was assumed to be Glu<sup>325</sup>, the expected numbers of amino acid residues (assuming 9 Gly residues) and those observed showed the best agreement among the four candidates, including the C terminus of the recA polypeptide (Table II). The estimated molecular mass of the region from amino acids 269 and 347 is 1.6 times larger than the molecular mass observed upon gel electrophoresis. Preliminary analysis of the C-terminal amino acid residues by treatment of fragment Fv 31-8-7 with carboxypeptidase W (from wheu; Seikagaku Kogyo Co.) revealed that the C-terminal amino acid residue was Glu, and that Ala, Val, and Gly were released on the treatment. This result is only consistent with Glu<sup>347</sup> as the C terminus of Fv 31-8-7 among candidate sites Glu<sup>243</sup>, Glu<sup>274</sup>, Glu<sup>290</sup>, and Phe<sup>320</sup>. Thus, we conclude that the epitopes for ARM191 and ARM193 reside between Phe<sup>320</sup> and Glu<sup>347</sup> (Fig. 8).

The polypeptide fragment (Fv 31-8-7) was further digested with V8 proteinase, followed by fractionation by HPLC (Cosmosil 5C4-300). The materials in all detected peaks were subjected to immunoblotting after direct blotting of the samples. We found that none of them tested so far gave a positive signal for the reaction with either ARM191 or ARM193. This result may suggest that more than two discontinuous sites of Fv 31-8-7 are required for the binding of ARM191 or ARM193. However, it is also likely that subfragments of Fv 31-8-7 only weakly bound to the filter for the assay and thus were washed out during this assay.

**DISCUSSION**

It is likely that active sites distant from an epitope on the recA polypeptide are also affected by the binding of an IgG, but the localization of the epitopes for anti-recA protein monoclonal IgGs provided some insight as to the loci of the active sites on the recA polypeptide.

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| Amino acid | Observed Number of residues<sup>a</sup> | Calculated<sup>b</sup> Number of residues |
|------------|----------------------------------------|------------------------------------------|
| Gly        | 1.42                                  | 9                                        |
| Ala        | 1.08                                  | 6.8                                      |
| Arg        | 0.15                                  | 1                                        |
| Asp + Asn  | 2.13                                  | 13.5                                     |
| Cys        | 0.00                                  | 0                                        |
| Glu + Glu  | 1.94                                  | 12.5                                     |
| His        | 0.00                                  | 0                                        |
| Ile        | 0.73                                  | 4.6                                      |
| Leu        | 1.12                                  | 7.1                                      |
| Lys        | 1.36                                  | 8.6                                      |
| Met        | 0.00                                  | 0                                        |
| Phe        | 0.52                                  | 3.3                                      |
| Pro        | 0.72                                  | 4.6                                      |
| Ser        | 0.70                                  | 4.4                                      |
| Thr        | 0.42                                  | 2.7                                      |
| Tyr        | 0.56                                  | 3.5                                      |
| Val        | 0.79                                  | 4.5                                      |

<sup>a</sup> Assuming that the C terminus is Glu<sup>347</sup>.

<sup>b</sup> The number of residues/molecule was calculated assuming that the number of Gly was 9.
region at the C terminus of the recA polypeptide (Fig. 8). It is unlikely that the epitopes for ARM193 and ARM191 are identical, since (i) the inhibitory effects of these IgGs are different from each other (Makino et al., 1985) and (ii) the profiles on immunoblotting analysis of these IgGs using the partial proteolytic digests of the recA polypeptide were not identical (Fig. 2).

IgG ARM321 was shown to inhibit single- or superhelical double-stranded DNA-dependent ATP hydrolysis and homologous pairing by the RecA protein (Makino et al., 1985). Our preliminary experiment revealed that this IgG inhibits the binding of ATP to the RecA protein in the activation cycle of the RecA protein in the homologous pairing and also the binding of single-stranded DNA to the protein. It was shown that the modification of Cys165 does not affect the binding of ATP to the RecA protein but causes the inhibition of ATP hydrolysis (Kuramitsu et al., 1984). These observations suggest that some residues between amino acids 89 and 129 are closely related to the epitopes for antibody ARM193 and ARM191.

Unlike ARM321 and ARM414, both ARM191 and ARM193 were shown to inhibit the homologous pairing, the processive unwinding and the superhelical double-stranded DNA-dependent ATP hydrolysis by the RecA protein. These results suggest that the region around the epitope for ARM414 is also closely related to the ATPase functions.

It was suggested that the RecA protein has two kinds of DNA-binding site: a primary site, which is essential for all functions of the RecA protein, and a secondary site, which is required only for homologous pairing (Ikawa et al., 1989). Both ARM321 and ARM414 appear to interfere with the binding of single-stranded DNA to the free RecA protein. Unlike ARM321 and ARM414, both ARM191 and ARM193 did not affect the single-stranded DNA-dependent ATP hydrolysis by the RecA protein. These results suggest that the amino acid residues involved in the primary DNA-binding site are located near the epitopes for ARM321 and ARM414. It should also be considered that the binding of these IgGs changes the higher order structure of the RecA protein, preventing the binding of DNA and/or ATP to the protein.

Even in the presence of excess ARM193, the RecA protein promotes single-stranded DNA-dependent ATP hydrolysis and ATP-dependent homologous pairing (Makino et al., 1987). Thus, the binding of ARM193 to the RecA protein seems to interfere little with the binding of single- and double-stranded DNAs to the primary and secondary sites of the RecA protein, the search for homology between two DNA molecules, and the binding and hydrolysis of ATP. On the other hand, ARM193 inhibits the processive unwinding and strand exchange (Shibata et al., 1984; Makino et al., 1987). Our previous study suggested that ARM193 interferes with the protein-protein interaction between RecA protein molecules (Ikawa et al., 1989). The characteristics of ARM193 and the location of its epitope suggest that a site(s) within the C-terminal 90-amino acid region is involved in the protein-protein interaction between RecA protein molecules (see Iwabuchi et al., 1983, and Ikawa et al., 1989). The following observations also suggest that the C-terminal region of the recA polypeptide is the site of species-specific protein-protein interaction: the diversity of the amino acid sequence of this region, especially a 50-amino acid region from the C terminus, was observed among the E. coli RecA, P. aeruginosa RecA, and Serratia marcescens RecA proteins, whereas the amino acid sequences of most other regions are more or less conserved among these three proteins (Sano and Kageyama, 1987; Ball et al., 1990).

As discussed above, this study suggests that the recA polypeptide can be separated functionally into at least two domains: one domain, which includes the epitopes for ARM321 and ARM414, forms a functional core of the RecA protein. This domain is involved in all functions of this protein. The other domain, which includes the epitope for ARM193 in a C-terminal region of the recA polypeptide, seems to be essential to neither ATPase activity of the RecA protein nor homologous pairing, but to protein-protein interactions.

Antibody ARM191 inhibits the homologous pairing, the processive unwinding and the superhelical double-stranded DNA-dependent ATP hydrolysis, but not the single-stranded DNA-dependent ATP hydrolysis by the RecA protein (Makino et al., 1985). The most simple explanation of this mode of inhibition is that ARM191 interferes with the binding of double-stranded DNA to both the primary and secondary DNA-binding sites of the RecA protein, but it does not affect the binding of single-stranded DNA to the primary DNA-binding site, or the binding or hydrolysis of ATP. However, detailed analysis of the effect of the binding of ARM191 to the RecA protein remains to be performed.

Two groups (Krivi et al., 1985; Kobayashi et al., 1987) reported preliminary results as to the location of epitopes for anti-RecA protein monoclonal antibodies in a C-terminal region. The effects of these monoclonal antibodies on the activities of the RecA protein have not been well characterized. That, with two exceptions (ARM321 and ARM414), all anti-RecA protein monoclonal antibodies so far reported recognize the C-terminal region indicates that this region of the recA polypeptide has much stronger antigenicity than the other parts of the polypeptide.

Instead of partial inhibition experiments that elucidate the elementary functions of the RecA protein and the active sites on the recA polypeptide, these anti-RecA protein mono-
clonal IgGs will be useful also for purification of parts of the recA polypeptide, and inactive RecA proteins having deletions, substitutions, or other types of mutation. The preparation of RecA proteins with mutations at known sites will also be very useful for mechanistic studies on the functions of the RecA protein.

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