Epitope Mapping of Anti-recA Protein IgGs by Region Specified Polymerase Chain Reaction Mutagenesis*

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Monoclonal IgGs were shown to be useful for the specific inhibition of a set of activities of the recA protein, a key protein in homologous genetic recombination. The mapping of the epitopes for these IgGs and site-directed mutagenesis based on the mapping will facilitate location of the functionally active sites on the tertiary structure of the protein, which is being solved by means of physicochemical techniques. We developed a novel technique for region-specific mutagenesis and applied the technique to epitope mapping. Using the polymerase chain reaction in the presence of deoxyribonuclease triphosphate, we introduced random base substitutions specifically into a region of the recA gene defined by a pair of primers. RecA mutants exhibiting altered antigenicity were selected, in plaque-immunoblotting experiments, from libraries of mutagenized recA genes constructed on the λgt11 expression vector. Mutant recA genes were obtained at the frequency of about 10⁻² among the plaques expressing fused recA genes and then each one was expressed as a whole protein, which was characterized by enzyme-linked immunosorbsorbent assay. Analyzing the DNA sequences of the mutant recA genes, we located at the amino acid sequence level the epitopes for two anti-recA IgGs which could not be located in previous studies. One of the antibodies was shown to prevent self-assembly of the recA protein and the other was suggested to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space around or near the relevant epitope.

The recA protein, and its prokaryotic and virus (T4 phage) analogues promote “homologous pairing” and “strand exchange” between homologous double-stranded and single-stranded DNAs through ATP (or dATP-) dependent reactions in vitro, and were shown to play an essential role in homologous genetic recombination in vivo. Homologous pairing is the formation of an intermolecular duplex (“heteroduplex”) between a couple of homologous single-stranded and double-stranded DNAs, and strand exchange is the processing of the heteroduplex, such as its elongation. Each of these reactions consist of a number of substeps and the recA protein or its analogues appear to have various active sites that promote each of these substeps, such as an ATP-binding site, ATPase catalytic center, binding site for single-stranded DNA, binding site for double-stranded DNA, and sites for self-polymerization. The localization of these active sites on the tertiary structure of the recA protein is essential for understanding the mechanisms of the underlying biochemical functions of the protein. However, only sites related to ATPase have been partly located at the amino acid sequence level. The mapping of mutation sites as well as x-ray crystallographic analysis of the protein are the main means to this end. A series of our studies involving the use of anti-recA protein monoclonal IgGs is also an approach to the same goal (see Shibata et al., 1991, for review).

We have isolated clones of mouse hybridomas which produce anti-recA protein IgGs (Makino et al., 1985). Two (ARM193 and ARM191) of these anti-recA protein IgGs each inhibit a set of activities of the recA protein without affecting the others; i.e. ARM193 severely inhibits the unwinding of the double helix and strand exchange, but allows homologous pairing and single-stranded DNA-dependent ATP hydrolysis (Ikawa et al., 1989; Makino et al., 1985, 1987). On the other hand, ARM191 inhibits the homologous pairing and unwinding of the double helix, but only affects the single-stranded DNA-dependent ATP hydrolysis a little (Makino et al., 1985). ARM193 was suggested to affect the site for the interaction between recA polypeptides (Ikawa et al., 1989) and ARM191 to affect the site on the recA polypeptide for the binding to double-stranded DNA (Makino et al., 1985). We preliminarily located the epitopes for both ARM193 and ARM191 in a C-terminal 88 amino acid region (Phe²⁶⁰-Glu³⁴⁷) of the recA polypeptide by examining the cross-reaction of proteolytic fragments. However, we failed to map them more precisely, since none of the subfragments of the 88-amino acid region exhibited significant cross-reaction with either of the IgGs (Ikeda et al., 1990). Therefore, it was necessary to introduce another technique to overcome the problem. Here, we describe a novel technique for region-specified mutagenesis and, as an application of this technique, the mapping of the epitopes of ARM193 and ARM191 at the amino acid sequence level in distinct but slightly overlapped subregions in the C-terminal 88-amino acid region.

MATERIALS AND METHODS

recA Protein—The purified recA protein was fraction V prepared as described (Shibata et al. 1981).

Oligonucleotides—Oligonucleotides were synthesized with a DNA synthesizer (Du Pont-New England Nuclear CODEX300) and purified with NENSORB PREP (Du Pont-New England Nuclear).

Techniques for Cloning of DNA—Treatment with restriction endonucleases and DNA ligase, and the isolation and cloning of the DNA fragments onto vectors were carried out as described (Berger and Kimmel, 1987; Maniatis et al., 1982).

Antibodies and Immunochemical Techniques—The anti-recA pro-

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Epitope Mapping on the recA Protein by PCR Mutagenesis

Isolation of RcoRI-fragments
DNA-library formation on λgt11-vector
Transfer onto membranes

FIG. 1. Region-specified PCR mutagenesis. DNA encoding the E. coli recA gene flanked by primers 1 and 2 was amplified by PCR in the presence of dITP with the use of Taq DNA polymerase. EcoRI fragments of the amplified DNA which encoded the C-terminal region of the recA protein were cloned on a λgt11 expression vector to construct DNA libraries of the mutagenized recA genes. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the recA gene was connected to the lacZ gene in-frame. Proteins expressed in the plaques obtained from the libraries were transferred to a pair of membranes and then the cross-reaction with either anti-recA protein IgG ARM191 or ARM193 was tested. The plaques showing cross-reaction with only one of the IgGs (indicated by arrows) were picked up and subjected to further cross-reaction tests. The closed circles in the big circles at the bottom of the figure denote plaques which showed cross-reaction with the indicated IgG.

DNA encoding the N-terminal region of the recA polypeptide was put under the control of the Tac promoter on a multicopy plasmid (pKK223-3). EcoRI fragments of the mutagenized recA gene were inserted at the EcoRI site at the codons for Glu75-Phe76.

FIG. 2. Structure of pMI996 for expression of mutated recA genes. DNA encoding the N-terminal region of the recA polypeptide was put under the control of the Tac promoter on a multicopy plasmid (pKK223-3). EcoRI fragments of the mutagenized recA gene were inserted at the EcoRI site at the codons for Glu75-Phe76.

The enzyme-linked immunosorbent assay (ELISA) was carried out as described previously. We used affinity purified preparations of these IgGs. An anti-recA protein monoclonal IgG, MAb156, was isolated by Karu and Allen (Karu and Allen, 1982), and a purified preparation of this IgG was provided by Dr. Alexander Karu (University of California, Berkeley) and Dr. A. John Clark (University of California, Berkeley).

Thus, the amounts of ARM191 and ARM193 bound to the recA protein were specifically measured by ELISA with the use of an appropriate subclass-specific antibody. A: □, the binding of ARM191 in the absence of ARM193; ■, the binding of ARM191 in the presence of 5 μg of ARM193/ml. B: ○, the binding of ARM193 in the absence of ARM191; ●, the binding of ARM193 in the presence of 5 μg of ARM191/ml.

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The procedure was described in detail in the legend to Fig. 1. Arrowheads indicate mutants which showed altered cross-reaction; mutant D (the top ones in A1 and A2) shows no cross-reaction with ARM191, and mutant 47 (B1 and B2) none with ARM193.

Testing of the Competition between ARM191 and ARM193—The IgGs, ARM191 and ARM193, belong to subclasses 1 and 2b, respectively (Ikeda et al., 1990), and thus each could be assayed by use of anti-mouse IgG1 and anti-mouse IgG2b antibodies, respectively. A solution of the purified recA protein (0.2 μg/ml) was put into the wells of a microtiter plate to coat the walls of the wells. Each solution of an indicated amount (no dilution: 1.5 μg/ml) of a tested anti-recA protein IgG (50 μl) contained 0 or 5 μg of the other anti-recA protein IgG/ml. The amounts of the tested anti-recA protein IgG that bound to the wells were measured by ELISA with anti-mouse IgG1 or anti-mouse IgG2b antibodies (Zymed Laboratories, Inc.).

Table I

<table>
<thead>
<tr>
<th>dITP</th>
<th>Total plaques</th>
<th>Number of plaques picked up on the first selection</th>
<th>Total number of mutants identified</th>
<th>Number of species of mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1800</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.2</td>
<td>1100</td>
<td>21</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>2200</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>200</td>
<td>2000</td>
<td>21</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>

TABLE I

Isolation of mutants by region-specified PCR mutagenesis

Testing of the Competition between ARM191 and ARM193—The preliminary mapping of the epitopes of ARM191 and ARM193 indicated that both epitopes were located between Phe260 and Gin285 (Ikeda et al., 1990). First, we examined whether or not these anti-recA protein-IgGs showed competition in the cross-reaction with the recA protein. Since ARM191 and ARM193 belong to different IgG subclasses, each can be discriminated through the use of subclass-specific antibodies on ELISA. Fig. 3 indicates that the presence of one of these IgGs did not affect the binding of the other IgG to the recA protein. We conclude that the epitope for ARM191 and ARM193 are different. Thus, we tried to locate the epitopes of these anti-recA protein IgGs more precisely.

Development of Region-specified PCR Mutagenesis and Isolation of Mutants—Since we were not able to locate the epitopes of ARM191 and ARM193 by examinations of the cross-reaction of subfragments of the recA polypeptide, we developed a novel technique for region-specified mutagenesis which could be applied for mapping of the epitopes. The whole process for the isolation of mutants causing altered cross-reaction consists of three stages (Fig. 1): the introduction of region-specified random base substitutions by means of PCR, construction of libraries of the mutagenized DNA with the use of the AgtT expression vector, and in situ testing for cross-reaction of the mutated polypeptides expressed in the plaques obtained from the libraries. In PCR (Saiki et al., 1985), Taq DNA polymerase causes the misincorporation of nucleotides (Eckert and Kunkel, 1990). We added deoxynucleosine 5'-triphosphate (dITP), at 200 μM, to the reaction mixture for PCR to enhance the misincorporation (Martin and Castro, 1985), and specifically amplified a DNA region defined by a pair of oligonucleotides (primers 1 and 2). Primer 1 includes the initiation codon of the recA polypeptide and examined by means of immunoblotting experiments, as described previously (Ikeda et al., 1990; Morishima et al., 1990). The plaques showing cross-reaction with only one of the IgGs (indicated by arrows in Fig. 1; examples are shown in Fig. 4) were picked up, and the phages were obtained after repeated single plaque isolation and testing by means of immunoblotting experiments.

DNA Sequence Analysis—The tested DNAs were cloned on pUC119 and then their sequences were analyzed by the dideoxynucleotide chain termination method (Sanger et al., 1977); the labeling reaction was carried out by use of the double-stranded template according to a manual for Sequenase (United States Biochemical Co., Cleveland, OH), and the products were analyzed with the use of an automated DNA sequence analyzer (Du Pont GENESIS2000). We analyzed both stands in most of the cases.
Epitope Mapping on the recA Protein by PCR Mutagenesis

**Table II**

<table>
<thead>
<tr>
<th>Mutation site on recA polypeptide</th>
<th>Name of mutant</th>
<th>Change in DNA sequence</th>
<th>Amino acid replaced</th>
<th>Cross-reactivity on immunoblotting*</th>
<th>Half-maximum values on ELISA µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>ARMI91</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>283</td>
<td>CTG → CGG</td>
<td>Leu → Pro</td>
<td>+</td>
<td>0.017</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>291</td>
<td>TAC → TGC</td>
<td>Tyr → Cys</td>
<td>+</td>
<td>0.069</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>296</td>
<td>GAG → GGG</td>
<td>Glu → Gly</td>
<td>+ &gt;30</td>
<td>0.27</td>
</tr>
<tr>
<td>Average of &quot;plus&quot; recA polypeptide (S.D.)</td>
<td>305</td>
<td>GCG → GGG</td>
<td>Ala → Gly</td>
<td>+</td>
<td>0.53</td>
</tr>
<tr>
<td>308</td>
<td>308</td>
<td>TG G → TG C</td>
<td>Trp → Cys</td>
<td>+ &gt;30</td>
<td>0.021</td>
</tr>
<tr>
<td>309</td>
<td>309</td>
<td>GTG → CCG</td>
<td>Leu → Pro</td>
<td>+ &gt;7.5</td>
<td>0.023</td>
</tr>
<tr>
<td>312</td>
<td>312</td>
<td>AAC → AAA</td>
<td>Asn → Lys</td>
<td>+ &gt;9.5</td>
<td>(0.024)</td>
</tr>
<tr>
<td>315</td>
<td>315</td>
<td>ACC → AT C</td>
<td>Thr → Ile</td>
<td>+</td>
<td>0.013</td>
</tr>
<tr>
<td>332</td>
<td>332</td>
<td>AAC → GAC</td>
<td>Asn → Asp</td>
<td>+</td>
<td>0.012</td>
</tr>
<tr>
<td>333</td>
<td>333</td>
<td>TCA → CCA</td>
<td>Ser → Pro</td>
<td>+</td>
<td>0.015</td>
</tr>
<tr>
<td>337</td>
<td>337</td>
<td>TTC → TCC</td>
<td>Phe → Ser</td>
<td>+ NT*</td>
<td>NT</td>
</tr>
<tr>
<td>338</td>
<td>338</td>
<td>TCT → TGT</td>
<td>Ser → Phe</td>
<td>+</td>
<td>0.0085 &gt;30</td>
</tr>
<tr>
<td>339</td>
<td>339</td>
<td>AAA → AGA</td>
<td>Lys → Arg</td>
<td>+</td>
<td>0.013</td>
</tr>
<tr>
<td>340</td>
<td>340</td>
<td>AAC → GAC</td>
<td>Asn → Asp</td>
<td>+</td>
<td>0.012</td>
</tr>
<tr>
<td>341</td>
<td>341</td>
<td>AT C → ACC</td>
<td>Ile → Thr</td>
<td>+ &gt;30</td>
<td>0.085</td>
</tr>
<tr>
<td>342</td>
<td>342</td>
<td>G TA → GCA</td>
<td>Val → Ala</td>
<td>-</td>
<td>0.023</td>
</tr>
<tr>
<td>267</td>
<td>267</td>
<td>32.3, 32.3, 36.3' GTG → GCC</td>
<td>No replacement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>321</td>
<td>321</td>
<td>30.3, 30.3, 30.3' AA G → AAA</td>
<td>No replacement</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*† Proteins showing the plus phenotype with respect to cross-reaction with the relevant IgG.
† Shadow letters indicate bases substituted.
† All mutants having the same substitution were isolated from the same PCR preparation, except mutants 23 and 34.
† Mutants 30, 33, 36, and 41 have more than two base substitutions, each of which is indicated, e.g. 30.1 and 30.2.
* NT, not tested.

primer 2 includes a complementary sequence about 80 bases downstream of its stop codon (Fig. 1). Primer 2 was designed so as to have an EcoRI cutting site. At more than 200 µM, dITP severely inhibited the amplification of DNA by PCR (data not shown). After 25 cycles of PCR, the amplified DNA was treated with EcoRI and the EcoRI fragment encoding the region of the recA polypeptide extending from Phe320 to the C-terminal Phe was isolated. The fragment was inserted into the EcoRI site of the λgt11 expression vector (Young and Davis, 1983). The proteins in the plaques were examined as to their in situ cross-reaction with anti-recA protein IgGs ARM191 and ARM193. As shown in Fig. 4, most of the plaques expressing the fused recA polypeptide showed cross-reaction to both IgGs. We could pick up about 1% of these plaques as candidates of mutants which showed cross-reaction to only one of the IgGs (indicated by arrowheads in Fig. 4; Table I).

From the selected plaques, phages were recovered and the inserted EcoRI fragments were reisolated. The DNA fragments were recloned on the pUC119 vector (Vieira and Messing, 1987) and their DNA sequences were analyzed by the dideoxyribonucleotide chain termination method (Sanger et al., 1977). We analyzed both strands in most of the cases by use of double-stranded form and an appropriate primer. The triplets which were changed by mutations are shown in Table II. During the development of the new technique for mutagenesis, we obtained 19 mutants exhibiting altered cross-reaction to either ARM191 or ARM193 from among the 52 plaques tested (Table I). So far as defined, all mutations were the substitution of amino acid(s) caused by base substitution(s) (Table II). Some of the mutants have a second (third) base substitution which does not affect the amino acid sequence. Of the base substitutions defined, 83% are transitions and 17% transversions.

On the first screening, the addition of dITP appeared to have only a little effect on the PCR mutagenesis (Table I). However, when we characterized the mutants isolated, we found that the addition of dITP is essential for this mutagenesis (Table I). Without dITP or with 0.2 µM dITP, we picked up 5 and 21 plaques, respectively, on the first screening, but obtained only 3 kinds of mutants. When dITP was added at 200 µM, we picked up 21 plaques from among about 2000 plaques expressing the fused recA polypeptide, and from these groups we finally isolated 11 mutants which were categorized into 10 kinds. Three of them (mutants 30, 33, and 36) had base substitutions at two or three sites (see Table II). These findings indicate the significant enhancement of the yield and variation on mutagenesis with dITP, and the very high yield of base substitutions under an appropriate set of conditions.

Some mutants have base substitutions at the same sites. Except mutants 23 and 34 (substitution of Ser320), such overlapped mutants were isolated from the same PCR preparations. Thus, these overlapped mutations seem to be created at an early cycle in PCR and amplified during the procedure.
Epitope Mapping on the recA Protein by PCR Mutagenesis

Fig. 5. Variation of the antigen concentration on ELISA does not change the amount of IgG giving a half-maximum signal. The wells of a microtiter plate were coated with the purified recA protein at the indicated concentrations. On the other hand, the concentration of the anti-recA protein IgG (ARM191 in A; ARM193 in B) was adjusted to 30 μg/ml ("nondilution"), and then a series of dilutions of the IgG was put into individual wells. The bound IgG was measured by ELISA. The concentrations of the recA protein were as follows: □, 2 μg/ml; ○, 0.2 μg/ml; △, 0.1 μg/ml; △, 0.05 μg/ml; ■, 0.02 μg/ml; ●, without recA protein.

Except for in these cases, base substitutions appeared to be introduced at random in the amplified DNA.

Cross-reaction of Mutant recA Proteins—We constructed a plasmid (pMI996; Fig. 2) in which the wild type recA gene was under the control of the Tac promoter on a multicopy plasmid (a derivative of pKK223-3 (Brosius and Holy, 1984). We replaced the EcoRI-EcoRI region encoding the C-terminal 93 amino acids of the wild type recA polypeptide with the EcoRI fragments on which we found mutation(s) (Fig. 2). Then, a mutant of E. coli in which the whole recA gene was deleted was transformed with these plasmids. The expression of the mutant recA genes was induced and cell-free extracts were prepared, followed by quantitative assaying (ELISA) for cross-reaction with ARM191 and ARM193. For comparison of the extent of cross-reaction of a tested IgG with the recA protein, we determined the amount of the IgG giving a half-maximum signal on ELISA. As shown in Fig. 5, variations in the amount of recA protein did not significantly change the amount of the IgG which gave the half-maximum signal on ELISA. We calculated the amounts of ARM191 and ARM193 giving a half-maximum signal to be 0.0277 (σ = 0.017) and 0.29 (σ = 0.10) μg/ml, respectively, from the data in this figure.
in a region defined by a pair of primers. From among the plaque expressing the fused recA gene, we obtained mutants of the recA gene at the frequency of 0.5% under the optimized conditions. Using this mutagenesis, we identified regions of the recA polypeptide in which amino acid substitutions prevent the cross-reaction with ARM191, ARM193, and/or MAb156. The region for ARM191 and that for ARM193 are different, but slightly overlap each other; i.e. that for the former IgG comprises positions 283 through about 320, and that for the latter positions through 315–338. Since ARM191 and ARM193 showed no competition, Thr^{215} and Ile^{219} and/or Val^{223} would be located on different sides of a local structure or the whole molecule of the recA polypeptide.

The substitution of an amino acid might interfere with the cross-reaction with an IgG directly or through a very local change in the tertiary structure, or indirectly through extensive alteration of the tertiary structure of the recA protein. The Leu^{283} to Pro substitution in mutant 38 could cause gross alteration of the tertiary structure of the recA protein. The clustering of other amino acid substitutions in a particular region is favorable for the first two possibilities rather than the last one. ARM193 was shown to prevent the self-assembly of the recA protein and ARM191 to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space at or around the relevant epitope. We are testing these possibilities by examining the effects of the mutant recA proteins isolated in this study and those constructed by another round of region-specific PCR mutagenesis. Determination of the epitope loci and the inhibitory effects of these IgGs, together with the tertiary structure of the recA protein, will facilitate the understanding of the function of the protein in relation to its structure.

The technique of region-specific random base substitutions involving the use of PCR employed in this study is very useful not only for epitope mapping, as described in this paper, but is also widely useful for studies on the function of a gene and an enzyme or protein, because of the flexibility as to specifying a target region, and the high yield of the random base substitutions.

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REFERENCES


**DISCUSSION**

Region-specific PCR mutagenesis is an efficient tool for introducing random base substitution mutations specifically

A J. Clark and A. Karu, personal communication.