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Dissociation of Non-Complementary Second DNA from RecA Filament without ATP Hydrolysis: Mechanism of Search for Homologous DNA[1]

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RecA protein catalyzes the DNA annealing and mimics the DNA strand exchange reaction *in vitro* in the presence of ATP or its non-hydrolyzable analog, adenosine 5′-*O*-3-thiotriphosphate (ATPγS). For these activities RecA coordinates two DNA molecules [Takahashi, M. and Nordén, B. (1994) *Adv. Biophys.* 30, 1-35]. In order to get a better understanding of how RecA performs the search for sequence complementarity or homology between two DNA molecules, the association and dissociation kinetics of a second DNA molecule to and from RecA in the presence of ATPγS have been investigated. The kinetics were monitored by fluorescence measurements of partly etheno-modified poly(dA) assisted by linear dichroism measurements of the flow-oriented complex. The association of the second DNA is fast, regardless of whether the sequence is complementary or not. By contrast, the dissociation kinetics is strongly dependent on sequence complementarity. If the second DNA is complementary to the first, dissociation is extremely slow, whilst that of non-complementary second DNA is fast. In no case does the first DNA leave the RecA fiber. Our findings indicate that the dissociation step is important in the search for homology by RecA.

Key words: RecA protein, homologous recombination, search of homologous DNA, DNA binding, strand exchange.

The RecA protein plays a central role in the genetic (homologous) recombination in *Escherichia coli* by catalyzing the strand exchange reaction and by stimulating the synthesis of proteins involved in the recombination reaction (*1-4*). The protein catalyzes annealing of two complementary DNA strands and mimics the strand exchange reaction *in vitro* in the presence of ATP or its analog (dATP, ATPγS) (*5, 6*). Proteins of similar sequence and with similar functions have been found in various other organisms (*7, 8*). RecA is, therefore, considered as a prototype of proteins which catalyze homologous recombination.

For the strand exchange reaction, RecA first binds to single-stranded DNA with an extremely strong cooperativity (*9, 10*), forming a filamentous complex in which RecA subunits are arranged in a helical manner around the DNA (*9, 11*). In the presence of nucleotide cofactor (ATP or ATPγS), this nucleofilament can bind a second DNA molecule (*12-15*). A second DNA has been found to bind even if it is not homologous or has no complementarity at all with the first DNA strand (*12, 15*). This may seem paradoxical, recalling that the function of RecA involves recognition and preferential binding to homologous (or complementary) parts of a second, entering DNA (*16-18*). Since this recognition does not require hydrolysis of ATP, but also occurs in the presence of a non-hydrolyzable analog, ATPγS (*16-18*), a possible mechanism for the homology recognition step may be, *a priori*, that a complementary DNA sequence associates faster to and/or dissociates more slowly from the RecA filament. To address this question, we have investigated the association and dissociation of the second DNA.

To facilitate the detection of association and dissociation, we have used partly etheno-modified poly(dA) [poly(dA/dεA)] as a second DNA. Poly(dA/dεA) is fluorescent but can base-pair with poly(dT) as can non-modified poly(dA). The association of poly(dA/dεA) to a preformed RecA-ssDNA complex upon mixing could be monitored by following the increase in anisotropy of the fluorescence as the mobility of the nucleobase becomes restricted (*19*). The dissociation was investigated by addition of NaCl, which usually slows down the association and facilitates dissociation measurement (*20*). To extend the conclusion to unmodified and double-stranded DNA, we also investigated the interaction by measuring flow linear dichroism. Flow linear dichroism, which is another form of polarized-light spectroscopy, and which reflects the macroscopic orientation of DNA bases (*21*), has already been used to monitor the binding of two DNA molecules by RecA (*12, 15, 22*).

MATERIALS AND METHODS

Materials—RecA protein was purified as described

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Abbreviations: ATPγS, adenosine 5′-*O*-3-thiotriphosphate; poly(dA/dεA): partly modified poly(dA) containing less than 10% etheno-adenine, LD, linear dichroism; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

previously (*23*). Poly(dA), poly(dC), and poly(dT) were from Pharmacia and their average size was about 250 bases. Oligo(dA), oligo(dC), oligo(dI), and oligo(dT) of 36 bases in length were purchased from Genset. Poly(dA/dεA) was prepared by chemical modification of poly(dA) with chloroacetaldehyde (Merck) as described previously for the preparation of poly(dεA) (*19*), except that the reaction time was reduced to 1 h for partial instead of complete modification. About 7% of bases were modified according to the formula given by Ledneva *et al.* (*24*). ATPγS was from Boehringer Mannheim. The concentration of these reagents was determined from the UV absorption using the following absorption coefficients: $\varepsilon_{278} = 2.15\times10^4\ M^{-1}\cdot cm^{-1}$ for RecA, $\varepsilon_{257} = 8.6\times10^3\ M^{-1}\cdot cm^{-1}$ (in bases) for poly(dA) and oligo(dA), $\varepsilon_{274} = 7.4\times10^3\ M^{-1}\cdot cm^{-1}$ (in bases) for poly(dC) and oligo(dC), $\varepsilon_{246} = 9.4\times10^3\ M^{-1}\cdot cm^{-1}$ (in bases) for oligo(dI), $\varepsilon_{264} = 8.5\times10^3\ M^{-1}\cdot cm^{-1}$ (in bases) for poly(dT) and oligo(dT), $\varepsilon_{257} = 9.6\times10^3\ M^{-1}\cdot cm^{-1}$ (in bases) for poly(dA/dεA) and $\varepsilon_{260} = 1.54\times10^4\ M^{-1}\cdot cm^{-1}$ for ATPγS.

Experimental Conditions—The experiments were mainly performed at 20°C in a buffer containing 20 mM sodium phosphate, 50 mM NaCl, and 4 mM $MgCl_2$, pH 6.8, except for the experiments on the analysis of association kinetics, which were performed in 20 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.4. This condition was chosen to slow down the reaction for ease of observation. However, the same conclusion was obtained under other experimental conditions, including lower pH and higher temperature (35°C). The concentration of ATPγS was usually 50 μM. The concentration of RecA was 6 μM for fluorescence measurements and 2 μM for linear dichroism (LD) measurements. Since each RecA subunit is known to cover three bases of both the first-associated and the secondly associated polynucleotides (*12, 22*), the polynucleotides were added to give a polynucleotide/RecA ratio of 3 bases per subunit for both of the DNAs. The addition of the second polynucleotide was made 1 h after the start of incubation of ATPγS-RecA with the first polynucleotide.

The association was followed in terms of the increase in anisotropy of fluorescence of poly(dA/dεA) immediately after mixing of equivolumes of poly(dA/dεA) and polynucleotide-RecA-ATPγS complex solutions. The dissociation of the complex was induced by increasing the salt concentration. Aliquots of concentrated (4 M) NaCl solution were added in a stepwise way to the complex solution and the fluorescence anisotropy was measured. In some cases, the kinetics was monitored as described for the association measurements.

Fluorescence Measurements—Fluorescence intensity and fluorescence polarization anisotropy were measured in an RF-777 spectrofluorometer (Jasco) equipped with an automatic anisotropy measurement device (ADP301, Jasco). The excitation wavelength was 320 nm (bandwidth 5 nm) for selective excitation of etheno bases. Emission was observed at 410 nm (bandwidth 20 nm). For the kinetic measurements, the modification in anisotropy was estimated by measuring the horizontally polarized element upon excitation with horizontally polarized light. This avoids the need for change of orientation of the polarizer and therefore allows continuous measurement. For other anisotropy determinations, 25 measurements of 0.5 s were averaged. All measurements were made in a 0.4 cm × 1 cm quartz cell at 20.0°C.

Flow Linear Dichroism Measurements—LD was measured in a modified J-500 spectropolarimeter (Jasco) (*25*). The samples were oriented in an outer rotating Couette cell (*21*) at a shear force of 600 s^{-1}. At this mild shear, no significant LD signal arises from uncomplexed polynucleotides because of the high flexibility of the latter (*21, 22, 25*). The signal from free RecA is also practically negligible (*26*). Thus any LD signal appearing upon the addition of polynucleotide can be regarded as originating from complex formation.

RESULTS

Association of Second DNA—The fluorescent, partly etheno-modified poly(dA) [poly(dA/dεA)] was added, as the second DNA molecule, to various preformed RecA-oligonucleotide complexes. No significant change in intensity was observed, but the association kinetics could be followed in terms of the increase in fluorescence anisotropy of poly(dA/dεA), upon binding to the RecA-DNA fiber. The association kinetics of the second DNA is fast, being completed within 100 s, and independent of the base composition of the first DNA (Fig. 1). This is in contrast to the very slow RecA association to the first DNA, which requires more than 20 min for completion of the reaction (Fig. 1). The absence of complementarity apparently does not prevent the association of the second DNA to the RecA-DNA fibrous complex.

Dissociation of Second DNA from RecA—The stability of the RecA-$(DNA)_2$ complexes was assessed by increasing the NaCl concentration. Salt usually destabilizes DNA-protein complexes by decreasing the association rate as a result of ionic shielding of the electrostatic field of the DNA phosphates (*20*). This prevents the reassociation and facilitates analysis of the dissociation step of the complex.

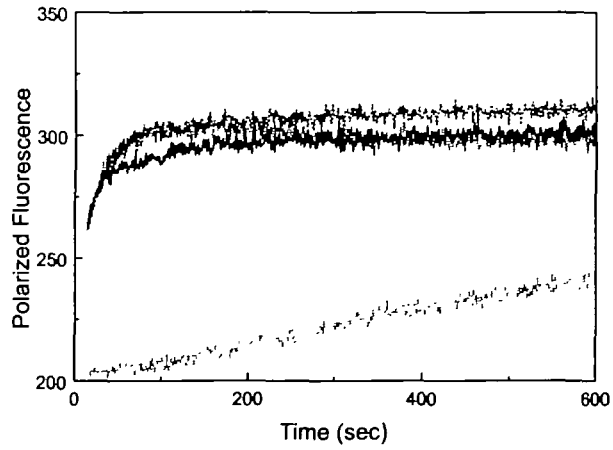

Fig. 1. **Polarized fluorescence change of poly(dA/dεA) monitoring association of a second DNA strand with preformed ATPγS-RecA-ssDNA complexes.** The polarized fluorescence was measured at 410 nm with vertically positioned polarizers for excitation and emission for the detection of anisotropy change. The kinetics were measured under a low salt condition (without NaCl) by mixing poly(dA/dεA) (18 μM in bases) and RecA (6 μM) bound to oligo(dA) (broken line), oligo(dC) (dotted line), or oligo(dT) (continuous line) in the presence of ATPγS. Slow binding of poly(dA/dεA) to the first site of RecA is also shown (thin dotted line).

In fact, the association of second DNA to RecA becomes slower under higher salt conditions. At 300 mM NaCl concentration, no significant binding of poly(dA/dεA) to the RecA-oligo(dT) complex was observed (not shown). This shows that the dissociation of second DNA can be observed under this salt condition.

Upon the addition of salt, the fluorescence anisotropy of poly(dA/dεA) bound to a RecA complex with non complementary DNA [oligo(dA), oligo(dC), or oligo(dI)] was decreased, reaching that of free poly(dA/dεA) at 0.3 M NaCl (Fig. 2B). This indicates that the dissociation of non-complementary second DNA from the RecA filament occurs. Complete dissociation was observed to occur at around 0.3 M NaCl. Kinetic analysis showed that the dissociation is fast, being completed within the mixing time (less than 20 s) (not shown). By contrast, when poly(dA/dεA) was bound to the preformed ATPγS-RecA-oligo(dT) complex, its anisotropy was not modified by addition of NaCl up to 1 M (Fig. 2B). The complementary second DNA was not dissociated by salt. Since there is no reassociation of second DNA at this salt concentration, the result shows that the dissociation rate of the second DNA from RecA filament is extremely slow, when the second DNA is complementary to the first DNA. The dissociation rate thus strongly depends upon the sequence complementarity, in contrast to the association rate.

Very similar results were obtained when polynucleotides, instead of oligonucleotides, were used as the first DNA in the complex with RecA (data not shown). The secondly added DNA is always dissociated from the RecA fiber when it is not complementary to the primary DNA. In one case with oligo(dC), the anisotropy value of poly(dA/dεA) remained larger than that of uncomplexed polynucleotide (Fig. 2B). This is probably because the binding of oligo(dC) to the first site of RecA is not efficient and a part of poly(dA/dεA) binds to the primary binding site and is not dissociated.

The DNA that is bound to the primary site of RecA, in the presence of ATPγS, was not dissociated even at 1 M NaCl. The fluorescence anisotropy of poly(dA/dεA) in the ATPγS-RecA-poly(dA/dεA) complex was not affected by NaCl (Fig. 2A). The dissociation of the primary DNA did not occur even when a non-complementary second DNA was bound to the RecA filament. The fluorescence characteristics of poly(dεA) bound to the first site were not significantly affected by addition of NaCl up to 1 M, regardless of the presence or absence of complementary or non complementary second polynucleotide (data not shown).

Linear Dichroism Measurements—In order to confirm our conclusions with unmodified DNA, we performed LD measurements on some polynucleotide samples (polynucleotides were used in order to gain good flow orientation). The LD amplitudes in the nucleobase absorption region of ATPγS-RecA-poly(dT)-poly(dT) and ATPγS-RecA-poly(dT)-poly(dA) complexes were very weak, in contrast to that of the ATPγS-RecA-poly(dT) complex (Fig. 3), as has been reported (*27*). The association of the second DNA results in a strongly impaired orientation of the RecA fiber, probably because it bridges different fibers, but the orientation is practically completely recovered if the second strand

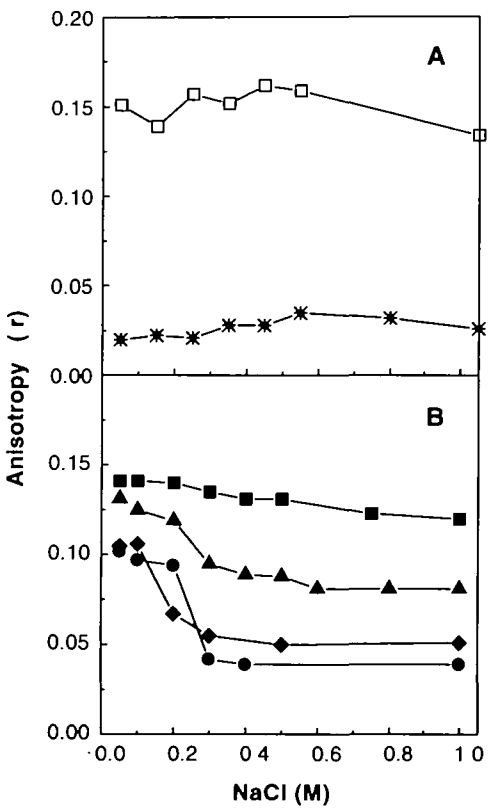

Fig. 2. **Effect of sequence complementarity on the salt-induced dissociation of RecA-DNA complex.** Fluorescence anisotropy of poly(dA/dεA) bound to RecA-oligo(dA) (●), RecA-oligo(dC) (▲), RecA-oligo(dI) (◆), or RecA-oligo(dT) (■) complex was measured at various salt concentrations (panel B). Panel A shows for comparison the variations of anisotropy of RecA-poly(dA/dεA) complex without any additional DNA (□) and of free poly(dA/dεA) in the absence of RecA (*).

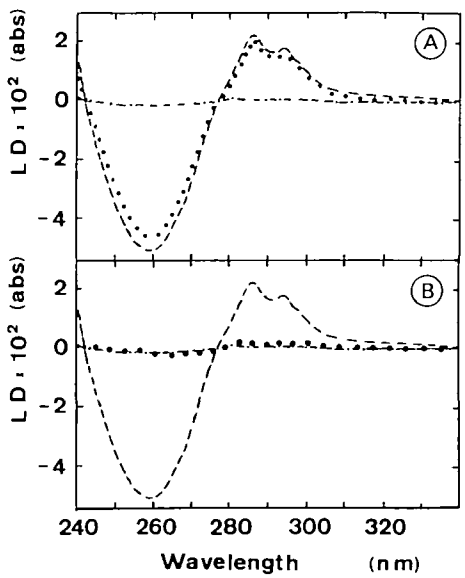

Fig. 3. **Salt effects on linear dichroism spectra of RecA-DNA complexes.** The spectra of RecA-oligo(dT)-oligo(dT) (panel A) and RecA-oligo(dT)-oligo(dA) complexes (panel B) before (—·—) and after (·····) addition of 0.5 M NaCl are shown along with the spectrum of RecA-oligo(dT) complex (- - -). All complexes were formed in the presence of ATPγS.

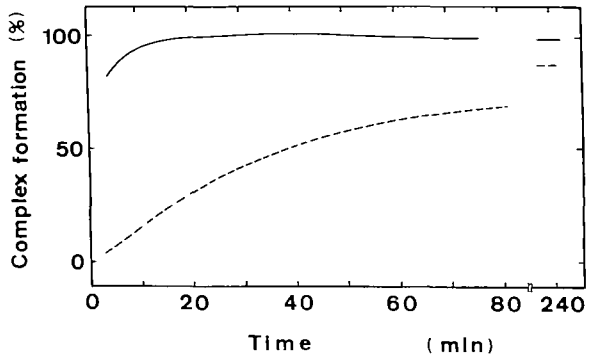

Fig. 4. **Association of double-stranded DNA to the preformed ATPγS-RecA-poly(dT) complex.** The association kinetics was monitored in terms of the change in the LD signal at 260 nm (continuous line) and compared with the association of the same dsDNA (calf thymus DNA) to the ATPγS-RecA complex without ssDNA (broken line).

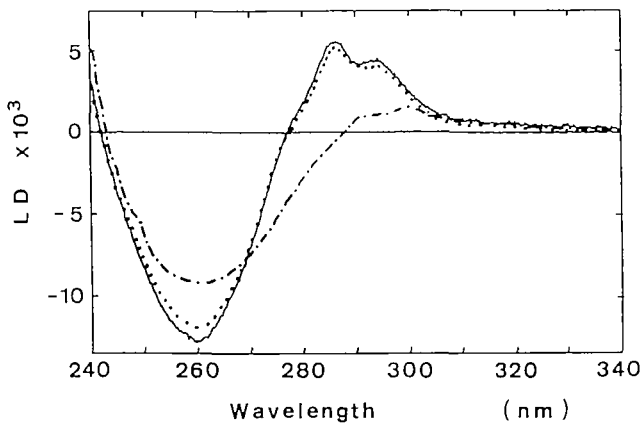

Fig. 5. **Dissociation of non-homologous double-stranded DNA from the RecA filament by salt.** Association and dissociation of calf thymus dsDNA to and from RecA were monitored by means of linear dichroism measurements. ----: ATPγS-RecA-poly(dT) complex; -·-·-: after addition of dsDNA to the ATPγS-RecA-poly(dT) complex; ·····: after addition of 0.5 M NaCl to the above mixture.

dissociates. Thus, the addition of 0.5 M NaCl to the complex formed with two poly(dT) chains restored the LD signal, and a spectrum similar to that of ATPγS-RecA-poly(dT) indicates that the second DNA had dissociated (Fig. 3A). Also poly(dC) as the second DNA was found to dissociate from ATPγS-RecA-poly(dT) at high salt concentration (data not shown). By contrast, the addition of NaCl to the ATPγS-RecA complex formed with one poly(dT) and one poly(dA) did not significantly modify the LD signal (Fig. 3B), which remained very small. Hence, the dissociation of the second DNA is confirmed not to occur when it is complementary to the first DNA strand. The same conclusion was reached from the results obtained when using poly(dA) or poly(dεA) as the first DNA (data not shown). In the case of ATPγS-RecA-poly(dC) complex, however, the LD signal was too small to provide any useful data.

Some preliminary experiments with dsDNA as a secondly binding DNA were also performed. Calf thymus DNA was added to the preformed ATPγS-RecA-poly(dT) complex and the association was followed in terms of the change

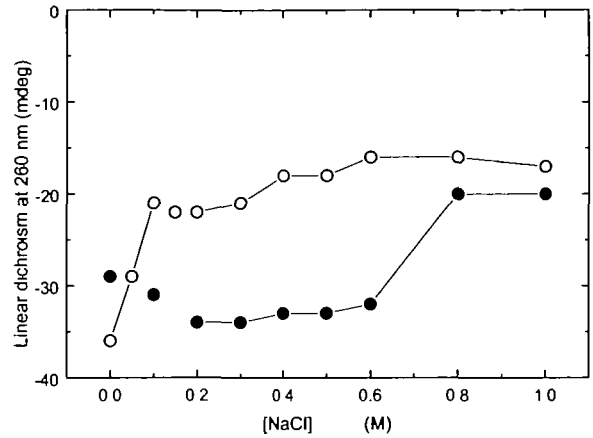

Fig. 6. **Effect of sequence complementarity on the salt-induced dissociation of RecA-DNA complex.** The linear dichroism signal at 260 nm of oligo(dT)-RecA-poly(dA):poly(dT) (●) and oligo(dT)-RecA-calf thymus DNA (○) complexes was measured as the salt concentration was increased. The concentration of RecA was 5 μM.

in the LD signal at 260 nm (Fig. 4). The association was much faster than that of dsDNA to uncomplexed RecA (*i.e.*, binding to the first site), and was almost completed within 10 min. Since calf thymus DNA should not have any significant homology with poly(dT), the results show that sequence complementarity is not required for efficient binding of dsDNA to the second site of RecA. However, the complex is not stable. The addition of 0.5 M NaCl restored an LD spectrum very similar to that of the RecA-poly(dT) complex before addition of dsDNA (Fig. 5). Double-stranded DNA is concluded to be dissociated from RecA when it is not complementary to the first DNA, just as in the case of ssDNA.

We have also performed the experiment using poly(dT):poly(dA) duplex, complementary to poly(dT), as a second DNA. The complex formed by addition of poly(dT):poly(dA) to the ATPγS-RecA-oligo(dT) complex was stable. The LD signal of the complex was not modified by addition of up to 0.6 M NaCl (Fig. 6). By contrast, the LD signal of ATPγS-RecA-oligo(dT)-calf thymus DNA complex was decreased at around 0.2 M NaCl. The dissociation of non-complementary second DNA occurs at a lower salt concentration. Our results also show that the dissociation of dsDNA from the second site of RecA occurs at lower salt concentration than that of ssDNA (*cf.* Figs. 2B and 6), indicating a weaker interaction of dsDNA. This observation is in accord with that reported by Mazin and Kowalczykowski (*28*).

DISCUSSION

The effects of sequence complementarity of an entering or leaving second DNA molecule in ATPγS-RecA-DNA complexes have been investigated in order to understand the mechanism of the search for homologous (or complementary) sequences of DNA by RecA. The results clearly show that the association of the second DNA is fast, even when the second DNA strand is not complementary to the primary bound DNA. By contrast, the dissociation depends upon complementarity: a complementary second DNA does not dissociate while a non-complementary one does. This

observation indicates that the dissociation step could play a crucial role in the discrimination of complementary from non complementary DNA.

The dissociation of the second DNA from RecA is fast and occurs under rather mild, physiological conditions. Although we could not observe the dissociation under a very low salt condition, where the *in vitro* strand exchange reaction is performed, this is certainly due to quick reassociation: dissociation probably occurs even under such conditions. The dissociation occurs in the absence of ATP hydrolysis and does not require any energy. This contrasts with the complete lack of dissociation of the first DNA even at 1 M NaCl and indicates that the binding mode of the second DNA in the RecA complex filament is quite different from that of the first DNA. This suggests the presence of two distinct DNA-binding sites in RecA.

The lack of dissociation of the complementary DNA strand observed here may be due to extra stabilization by transient DNA-DNA contacts by base-pairing in the RecA filament (*29*). Rao and Radding have reported recognition between identical sequences of DNA by RecA-ssDNA complex ("self-recognition") (*30*). By contrast, we did not observe any stable interactions either between poly(dA) and a preformed RecA-poly(dA) complex or between poly(dT) and RecA-poly(dT) complex. There is no "self-recognition" in the case of homopolymer. This could indicate either that the homopolymers-RecA complex has particular structures or that the DNA sequence used by the above authors has some particularity. Further analyses using other DNA sequences support the latter explanation (*28, 31*).

Association of any DNA with the RecA-ssDNA fiber, irrespective of sequence, probably plays a role analogous to that of the unspecific DNA binding of sequence-specific DNA-binding proteins, such as the repressor of the lactose operon. These proteins first bind weakly to any part of DNA and, thereafter, by a one-dimensional diffusion mechanism, relatively quickly find their specific target (*32, 33*). In the case of RecA protein too, such a weak "unspecific" binding, possibly due to non-specific interactions with the ribophosphate backbone, may allow the RecA-ssDNA complex to move relatively quickly along the second DNA molecule. Dissociation and reassociation may occur repeatedly until the RecA-ssDNA complex finds the complementary sequence. This would limit the search to one dimension of the DNA coil instead of a three-dimensional search in solution, and correspondingly greatly increase the reaction rate.

We have so far restricted ourselves mainly to examination of the interactions of single-stranded oligo- and polynucleotides. However, our preliminary experiment with dsDNA suggests that a mechanism similar to the one proposed may also apply to the search for homology in double-stranded DNA. Müller *et al.* observed that non-complementary dsDNA does not form a stable complex with RecA-ssDNA (*14*), supporting this notion of a fast-dissociation recognition mechanism.

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