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Homologous pairing between single-stranded DNA immobilized on a nitrocellulose membrane and duplex DNA is specific for RecA activity in bacterial crude extract

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ABSTRACT

Reaction between a circular single stranded and a linear double stranded DNA molecule (ssDNA and dsDNA) provides an efficient system to study recombination mediated by RecA protein. However, classical assays using reaction in solution require highly purified enzymes. This limits biochemical studies of mutant RecA proteins from *Escherichia coli* or of RecA proteins from other organisms. We describe here an assay that is specific for RecA activity even in bacterial crude extracts. In this assay, the ssDNA is bound to a nitrocellulose membrane, proteins are loaded on this membrane and it is then incubated with a labeled homologous dsDNA. Joint molecules are visualized by autoradiography. We have shown that, despite the reduced mobility of the DNA due to its binding to the membrane, RecA protein is able to promote formation of stable plectonemic joints, in a homology dependent manner. Fourteen other proteins involved in DNA metabolism were checked and did not produce a signal in our assay. Moreover, in Dot blot analysis as well as after native electrophoresis and electrotransfer on a ssDNA coated membrane, production of a signal was strictly dependent on the presence of active RecA protein in the bacterial crude extracts used. We named this assay Pairing On Membrane blot (POM blot).

INTRODUCTION

Homologous recombination is involved in basic biological processes resulting in genomic rearrangements, DNA repair and replication. In *Escherichia coli*, RecA protein is essential for general genetic recombination. *In vitro*, reaction of circular single-stranded DNA (ssDNA) with a linear duplex DNA (dsDNA) provides an efficient model to study the different phases of the process: i) RecA protein binds to the ssDNA to form a helical nucleoprotein filament; ii) the nucleoprotein filament aggregates the duplex DNA in a network independent of sequence homology; iii) the DNA molecules are aligned and paired to form homology dependent joint molecules; iv) a polar strand displacement, requiring torsional motion, then branch migration, leads to formation of a new heteroduplex (for reviews see 1, 2, 3, 4).

In classical assays, the two DNAs are incubated with RecA protein in solution and products are analyzed, after incubation, by agarose gel electrophoresis (5) or filter binding assay (6, 7). However these powerful assays require a highly purified RecA protein. Indeed traces of other contaminating activities (exonuclease, histones, helicase) can either completely destroy the DNA or product artifactual results. Therefore the assays in solution cannot be used with crude protein extracts, a fact which limits in the biochemical study of mutant RecA proteins that have not been purified from *Escherichia coli*. Moreover, recombination activity cannot be detected after the initial purification steps of RecA-like proteins from other organisms.

We have devised a new assay (Figure 1) to analyze RecA mediated homologous pairing with pure RecA protein as well as with crude protein extracts. This assay allows separation of the nucleoprotein filament formation phase from the step of joint molecule formation.

RecA protein is able to promote the annealing of two complementary ssDNA molecules. A common approach in Southern and Dot blot experiments, is to hybridize two complementary ssDNA molecules one of which bound on a nitrocellulose membrane. This raises the possibility that RecA protein might be able to promote homologous pairing between a ssDNA bound on a nitrocellulose membrane and an incoming duplex DNA; i.e., in a condition of reduced mobility and torsional capabilities resulting from the physical binding of the ssDNA to the membrane.

We have demonstrated that pure RecA protein is able to promote formation of stable homologous plectonemic joints on a membrane. To assess the specificity of this test we checked different pure proteins involved in DNA metabolism. The specificity of the reaction for RecA activity was confirmed using extracts from different bacterial strains.

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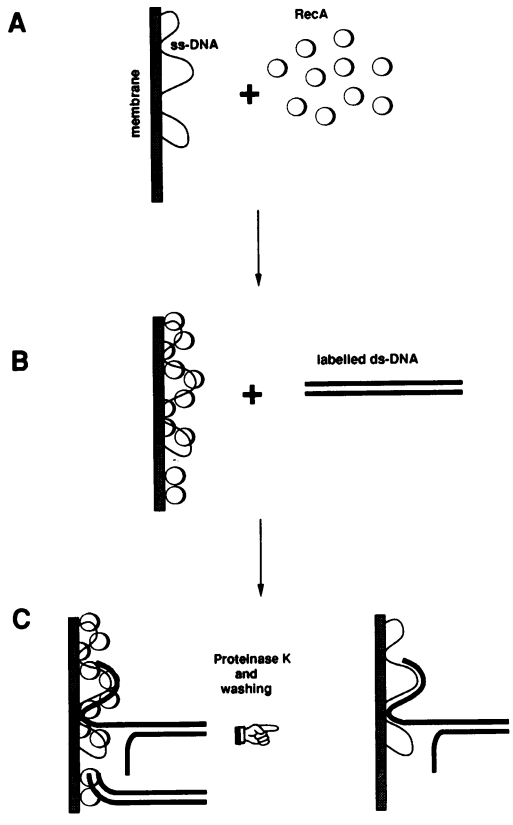

Figure 1. Scheme of the pairing on membrane assay (POM Blot). RecA protein was loaded (using a Dot blot apparatus), on a nitrocellulose membrane coated with ssDNA. After saturation with skimmed milk, the membrane was incubated with a homologous labeled duplex DNA. DNA−protein complexes unrelated to homologous pairing, nonspecific fixation of the probe onto the filter and to RecA itself were removed by proteinase K treatment followed by an extensive washing of the membrane. The efficiency of homologous pairing promoted by RecA protein was then detected by autoradiography.

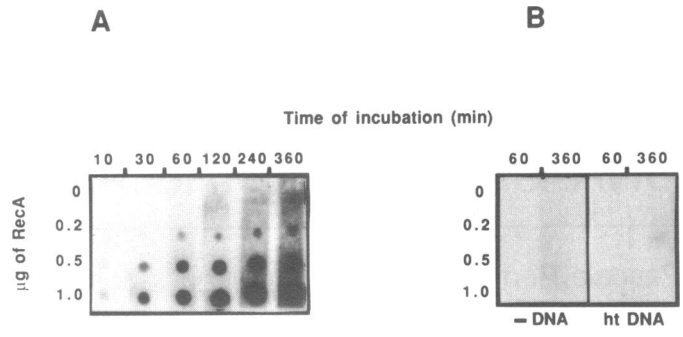

Figure 2. Homologous pairing reaction with RecA nucleoprotein filament bound onto nitrocellulose. **A)** Membranes were coated with ssDNA homologous to the probe and blotted with RecA protein. DNA substrates were ss-M13mp8 and ds-M13mp8 cleaved by BamHI restriction endonuclease. **B)** Membranes without DNA (−DNA) or coated with the heterologous ssDNA ϕX174 phage (ht); the probe was BamHI cleaved duplex M13mp8 DNA. Autoradiography exposure time was the same than in A.

MATERIALS AND METHODS

Enzymes and DNA

RecA protein was a generous gift of Dr C.Radding or was obtained from Pharmacia. Histones, restriction endonucleases, T4 DNA polymerase, polymerase I large fragment and T4 DNA ligase were purchased from Boehringer Mannheim. Exo III, Exo VII, λ Exo, Topo I and Gyrase were purchased from Gibco-BRL. DNA polymerase I, AMV reverse transcriptase and *E.coli* SSB were obtained from Pharmacia. Dnase I was obtained from Promega.

M13 mp8 duplex and ssDNA were prepared as described (8). Duplex DNA was labeled with ^{32}P, by filling in the 5′ protruding ends using polymerase I large fragment, as described (8).

Assay for homologous pairing on membrane

The whole surface of a moist nitrocellulose membrane (Hybond C-super, Amersham, UK or Schleicher & Schuell BAS 85) was coated with a solution of ssDNA (0.5 $\mu g/cm^2$) in 2×SSC. DNA fixation was achieved by heating in a vacuum oven for 2h at 80°C. Using a Dot blot apparatus, RecA protein diluted in buffer A (33 mM Tris HCl pH 7.5; 1 mM $MgCl_2$; 2 mM dithiothreitol, 0.5 mM ATPγS), was loaded on ssDNA coated-membranes previously soaked in the same buffer at 37°C. Membranes were then saturated for 1 h at 37°C with a boiled solution of 5% skimmed milk in buffer A, and rinsed in the same buffer without milk. The pairing reaction was performed by incubating the membrane in a sealed plastic bag with 200 µl/Dot of labeled homologous duplex DNA in buffer B (33 mM Tris HCl pH 7.5; 12 mM $MgCl_2$; 2 mM dithiothreitol, 0.1 mM ATPγS). The probe was used at a concentration of 1 µg/ml with a specific activity of 10^6 cpm/µg. After incubation, the membranes were treated with proteinase K (0.1 mg/ml) for 1h at 37°C and then successively washed in 20×SSC; 10×SSC, 0.1% SDS and 2×SSC, 0,1% SDS for 15 min at 65°C for each wash. The reaction products were visualized by autoradiography.

Escherichia coli K12 strains and crude extract preparation

Escherichia coli strains used were:

- GY7648: *omp T hsdS* ΔrecA306 (9).
- GY7671 was GY7648 strain containing plasmid pGY7671 (9). Plasmid pGY7671 carries the *recA* gene cloned into plasmid pET3 under the control of the T7 phage promoter (10). Phage λ int lacI lacpUV5 lacZ::IT7, which carries the T7 polymerase gene under *lac* promoter control was used to control *recA* expression from plasmid pGY7671. The expression of *recA* by IPTG was induced as described (9). These strains were kindly provided by Dr R.Devoret's laboratory.

Crude extracts were prepared as already described (9). Total protein concentration was estimated using the Biorad Protein Assay and RecA protein concentration was measured by western blot using a polyclonal antibody (9).

Native polyacrylamide gel electrophoresis and electrotransfer

5% acrylamide/bisacrylamide (29.2/0.8) gels were prepared in buffer (25mM Tris/0.19 M glycine/5% isopropanol at pH 8.3). Pre-run, run and electrotransfer were performed in the same buffer. After one hour of pre-run at 100 volts, the samples were loaded and run 40 minutes at 100 volts. Electrotransfer on a prewetted ssDNA coated nitrocellulose membrane (see above) was performed at 70 volts for 30 minutes. Membranes were then washed three times, 5 minutes each, in buffer A (see above) and the homologous pairing assay was performed as described above.

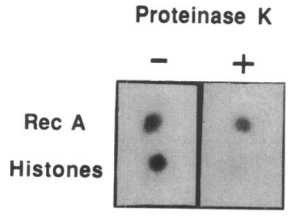

Figure 3. Effect of the proteinase K treatment on homologous pairing. Reactions were performed for 2 hours as described in Figure 2, except for the presence (+) or absence (−) of proteinase K treatment. 1 μg of RecA protein and 1 μg of histones were loaded on each membrane.

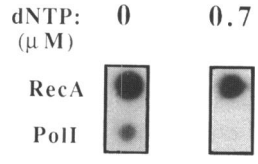

Figure 4. Effect of increasing concentration of cold dNTPs on the signal produced by pure RecA protein or PolI. 2μg of RecA protein was loaded in each condition. 0.1 unit of PolI was loaded in each condition. Concentration in cold dNTPs (in μM each) is indicated on the top of the picture.

RESULTS

Pure RecA protein

The amount of labelled dsDNA retained on the membrane increased as a function of the amount of loaded RecA protein and the duration of incubation (Figure 2A). These results indicate that our assay monitors the activity of RecA protein. The fact that no signal was obtained when RecA protein was omitted indicates that labeled duplex DNA was unable to interact with ssDNA by itself. No signal was detected without DNA on the filter or in the presence of heterologous DNA (Figure 2B), confirming that the radioactive signal detected was due to stable interactions between ssDNA and the homologous duplex DNA. However, the kinetics of the pairing reaction seem to be slower on the membrane, compared to that in classical assays in solution; the maximum efficiency of joint molecule formation was reached between 120 and 240 minutes on the membrane (Figure 2A) instead of the few minutes required under the free condition of solution (for reviews see 1, 2, 3, 4, and personal data). Since our different batches of RecA protein showed kinetics of reaction in solution similar to that classically published (data not shown), the present results can not be explained by some alteration in the RecA protein we used. Reduction of mobility and/or accessibility of the ssDNA on the membrane may account for these slow kinetics.

Since RecA protein is able to promote DNA aggregation and thus to bind two DNA molecules without promoting pairing (11), we have verified that the observed signal was due to DNA/DNA interactions. For this purpose, we have compared the effect of deproteinization treatment (Figure 1) on signals obtained with RecA protein or with histones, which are known to exhibit a strong affinity for DNA. Without proteinase K treatment both proteins produced a signal despite extensive washing with 3M NaCl (20×SSC). When the membranes were treated with proteinase K, the signal obtained with RecA was only slightly affected, whereas the signal due to the histones completely disappeared (Figure 3). Therefore, the signal observed in the RecA treated membrane resulted from DNA/DNA interactions and not from DNA/protein interactions. The resistance of the joint molecules to proteinase K treatment, temperature washing (65°C) and the requirement for sequence homology, indicates that RecA protein can catalyze the formation of stable plectonemic joints on the membrane.

Table 1. Pure protein checked in the assay

Proteins	Amount	Signal	
Nucleases:			
− Exo III	1 U	−	
	2 U	−	
− Exo VII	1 U	−	
	2 U	−	
−λ Exo	1 U	−	
	2 U	−	
−DNase I	1 mU	−	
	2 mU	−	
Ligase:			
− T4 DNA ligase	1 U	−	
	2 U	−	
Topoisomerases:			
− Topo I	1 U	−	
	2 U	−	
−Gyrase	1 U	−	
	2 U	−	
Polymerases:		−dNTPs*	+dNTPs
−AMV Reverse transcriptase	1 U	−	−
	2 U	−	−
− T4 DNA pol.	1 U	−	−
	2 U	−	−
− PolI Klenow	1 U	−	−
	2 U	−	−
−Polymerase I	1 U	+	−
	2 U	+	−
DNA binding proteins:		−Prot. K**	+ProtK.
− E.coli SSB	0.1μg	−	−
	0.5μg	−	−
− Histones	0.5μg	+	−
	2 μg	+	−
− E.coli HU	0.5μg	+	−
	1 μg	+	−

+ = positive signal. − = no signal. Incubations were performed with dNTPs except when specified(*) and membranes were treated with proteinase K except when noted (**)

Different pure DNA binding proteins

We have checked the ability of pure proteins involved in DNA metabolism for their ability to produce a signal in our assay (Table 1). Polymerase I from *E.coli* produced a signal whereas polymerase I large fragment, lacking 5′ to 3′ exonuclease activity, did not (Figure 4). However the addition of cold dNTPs during incubation, totally inhibits the production of a radioactive signal by polymerase I (Figure 4). This suggests that it is the proof-reading activity of PolI that generate the artifactual signal.

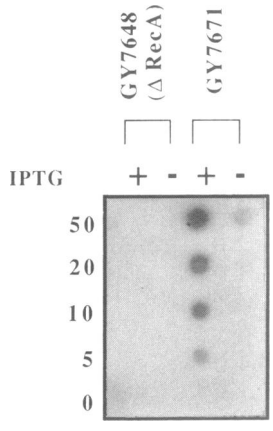

Figure 5. Analysis of bacterial crude extracts in Dot blot. The amount (in μg) of protein loaded is indicated on the side of the picture. The bacterial strains are indicated on the top of the picture. +: extracts from IPTG induced bacteria; −: extracts from bacteria non IPTG induced.

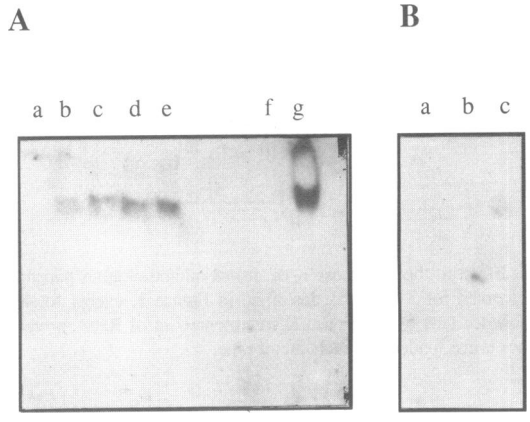

Figure 6. Analysis of bacterial crude extracts in native PAGE. **A)** migration from the − to the + pole. a, b, c, d, e lanes respectively correspond to 0, 1, 2, 3 and 4 μg of pure RecA protein. f: 100 μg of protein from crude extract GY7648 (Δ*recA*) treated with IPTG; g: 100 μg of protein from crude extract GY7671 (with inducible *recA* gene) treated with IPTG. **B)** migration from the + to the − pole. a: 4 μg of pure RecA protein; b: 100 μg of protein from extract GY7671 treated with IPTG; c: 100 μg of protein from extract GY7648 treated with IPTG.

Therefore, cold dNTPs were systematically added during incubation for experiments with bacterial extracts.

Exonucleases that could generate ssDNA tails on the probe, complementary to the ssDNA bound on the membrane, were tested. None of them produced a signal (data not shown).

DNA binding proteins such as Histones, HU or SSB did not produce a signal in our conditions after proteinase K treatment (Data not shown).

Bacterial crude extracts in Dot blot

Using the conditions defined above, we have checked the specificity of the assay with bacterial crude extracts. To do this, we blotted the bacterial proteins onto a ssDNA coated membrane which has first been saturated with milk. Under these conditions, the proteins retained on the membrane were mainly DNA binding proteins, effectively constituting an enrichment step for RecA protein in the mixture bound.

We used Δ*recA* bacteria containing a plasmid carrying the *recA* gene under IPTG control (strain GY7671) or containing the same plasmid without the *recA* gene (strain GY7648). In absence of IPTG induction, the amount of RecA protein due to the leakiness of the promoter, is comparable to the basal level of RecA protein in wild type bacteria without SOS induction (9). After treatment with IPTG, the amount of RecA protein is 20 to 40 fold increased and represents 1 to 2% of the total amount of proteins.

In our assay, pairing activity was detected in extracts from GY7671 but not in extracts from GY7648 (*recA*). In the absence of IPTG induction, a positive signal was produced when 50 μg or more of proteins extracted from GY7671 were loaded; whereas 5 μg of protein from IPTG treated GY7671 strain were sufficient to give a positive signal. The intensity of the signal increased with the amount of protein extract loaded (Figure 5). Similar results were obtained when keeping protein concentration constant by adding RecA free extract to the RecA containing extracts (data not shown). These results indicate that the signal observed in our assay is specific for RecA and that the intensity of this signal depends on the amount of RecA protein present in the bacterial extract.

Analysis in native polyacrylamide gel electrophoresis

We adapted the test to proteins separated by native polyacrylamide gel electrophoresis and electrotransferred to ssDNA coated membrane.

After electrophoresis, electrotransfer and incubation in our conditions (see methods), pure RecA protein produced a signal depending on the amount of loaded protein (Figure 6A). When RecA protein was omitted no band was seen. An activity band began to be detected with 0.5 μg of RecA protein. A unique band showing approximately the same migration pattern as pure RecA protein was recorded in wild type extract. Interestingly, Δ*recA* bacterial crude extract did not produced any signal (Figure 6A). When the DNA on the membrane was heterologous with regard to the duplex probe, no signal was detected neither with wild type extract nor with extracts from Δ*recA* bacteria (data not shown).

In native gels, proteins migrate according to of their molecular weight and of their charge which can be modified by the pH of the buffer. Native RecA protein migrated from the − to the + pole at pH8.3 (Figure 6A). To screen all the proteins contained in the crude extract, we have examined the pattern in the opposite direction i.e; from the + to the − pole. No proteins positively charged at pH8.3 were able to produce a signal (Figure 6B).

DISCUSSION

The assay we present here can be useful to test the ability of pure protein or crude extracts to promote joint molecules.

Using pure RecA protein, we showed that joint molecules formed on the membrane were DNA homology dependent, stable to deproteinization treatment and to temperature (65°C). These data indicate that stable plectonemic joints were formed on the membrane. This raises the question of the mobility requirement for RecA nucleoprotein filament forming joint molecules. Indeed, taking into account the helicity of the two recombining elements, different models propose a rotation of the nucleoprotein filament

and/or of the duplex about one another for pairing and strand exchange (1, 3). In this respect, some experiments suggest that the invasion of the duplex DNA by the ssDNA requires a rotation of both duplex and RecA nucleoprotein filament, each around its longitudinal axis (12). The torsional stress generated by this process, separating DNA strands could allow strand exchange to pass through heterologous inserts (13). In the assay presented here, the structure of ssDNA on the membrane and the average number of binding points remain unknown. Nevertheless, the ssDNA must have at least one binding point on the membrane and despite this condition of reduced mobility and torsional capacities, RecA protein remains able to promote homologous pairing. Therefore, this assay can be useful in addressing questions regarding torsional motion during strand exchange.

Another application of the assay presented here, is that it allows to separation of the nucleoprotein filament formation phase from the pairing step. Indeed, RecA protein can be loaded on the membrane under defined conditions, the membrane can be washed and then incubation with the dsDNA can be performed under other conditions. Requirements for cofactors, in each step, and buffers conditions can be independently studied.

Finally, this assay can be employed with crude extracts since it seems to be specific for RecA activity even in presence of other proteins. Different pure proteins able to act on the DNA were tested for their ability to produce a radioactive spot in our conditions. None of them produced a signal when cold dNTPs were added. When the cold dNTPs were omitted in the incubation mix, pure DNA polymerase I from *Escherichia coli* (PolI), gave a signal. This suggests that the radioactive spot observed with PolI results from a polymerization activity using traces of unincorporated labeled dNTPs contaminating the duplex probe. The fact that in the same conditions, neither T4 DNA polymerase nor PolI large fragment produced a signal, indicates that the 5′ to 3′ exonuclease activity of PolI is required to produce a signal. This exonuclease activity could create a 3′ single-stranded tail which able to anneal to complementary ssDNA on the membrane to serve as a primer for polymerization; this would create labeled heteroduplex long enough to resist washing. Thus, the observation that the PolI signal can be abolished by addition of cold dNTPs is in agreement with this hypothesis. Using these conditions, the specificity of our assay was assessed by analyzing bacterial crude extracts in Dot blot and after gel electrophoresis. In the native conditions we used, all the potential artifactual activities could be present in the crude extract, but only RecA protein was required to produce a signal. Exonuclease activities have often been described to produce heteroduplex. Indeed, partial degradation of the dsDNA would produce single-stranded tails that could naturally anneal complementary ssDNA resulting in an apparent joint molecule. This artifact does not seem to be recorded in our assay. Some ssDNA nucleases should first digest the DNA on the membrane during the saturation step; since DNA on the membrane is required, this would result in absence of signal. Moreover, in the reaction on membrane, the ss-tailed DNA, produced by exonucleases, could anneal complementary ssDNA fixed on the whole membrane. This type of reaction would increase the background but would not produce a discrete band. All this could limit the possible artifact in bacterial crude extracts, especially in gel electrophoresis analysis.

In conclusion, we have defined some experimental conditions allowing the use of the present assay to study the activity of RecA protein either pure or in bacterial crude extracts. This assay could represent a useful tool for preliminary analysis of mutant RecA protein from *Escherichia coli* or of RecA protein from other bacterial strains. Moreover, adaptation of this assay to extracts from other organisms is in progress. Since this assay detects homologous pairing, we named it Pairing On Membrane blot (POM blot).

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