Proc. Natl. Acad. Sci. USA
Vol. 76, No. 3, pp. 1084–1088, March 1979
Biochemistry

Biochemical assay designed to detect formation of recombination intermediates *in vitro*

(plasmid DNA/*in vitro* genetic recombination/figure 8s and chi forms/*lac* repressor and operator/filter binding assay)

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Communicated by I. Robert Lehman, December 6, 1978

ABSTRACT **A biochemical assay that is designed to detect recombination intermediates formed *in vitro* is described. The assay measures the fusion of two essentially homologous plasmids, one of which is radioactively labeled and the other of which carries several copies of the *lac* operator. The fusion product is radioactive and can be bound to a nitrocellulose filter by *lac* repressor. This assay for genome fusion is rapid and readily applicable to the many fractions that result during enzyme purification. The fused product is not destroyed in the assay and may be recovered from the filter for further analysis by electron microscopy. The product is then seen to consist of figure 8 structures that can be cleaved by the restriction enzyme *Eco*RI to give chi forms, structures similar to those recovered from recombination-proficient cells. It is expected that this assay will be useful in the purification of the "recombinase-type" activity detected in crude cell lysates. To demonstrate this point, the assay was applied to the protein fractions recovered from a molecular sieve column. The results indicate that the fusion activity has an apparent molecular weight of 50,000–100,000.**

The process of DNA recombination is usually observed through a genetic analysis of progeny recombinant chromosomes. More recently, however, by using the electron microscope it has become possible to observe individual DNA molecules that appear to be in the midst of genetic recombination. For the bacteriophages ϕX, G4, and λ, and also for bacterial plasmids, dimer-size DNA forms have been observed which have the appearance of a figure 8 (1–5). This geometry suggests two genomes fused at a region of DNA homology (see Fig. 1), an interpretation that has been confirmed by linearizing the structures with a restriction enzyme. This treatment converts the figure 8 structures into bilaterally symmetric dimers shaped like the Greek letter χ (4, 14). In each χ form there are two pairs of arms of equal length, which indicates that the two component genomes are in fact held together at a region of DNA homology.

How the fused structure is formed is not yet clear. Two current proposals for the formation of the recombination intermediate are shown in Fig. 1. To distinguish between these mechanisms, or to discover the true mechanism, it will be necessary to purify recombination enzymes from cells and study them *in vitro*. At the present time, considered at the level of enzymology, generalized DNA recombination is basically unsolved. However, just as the enzymology of DNA replication has yielded to biochemical analysis in the last decade, so too it is reasonable to be optimistic that the steps involved in the process of recombination can be made to occur individually in the test tube.

We have been able to prepare an *in vitro* system from *Escherichia coli* in which double-stranded circular DNA molecules are fused to give structures with the same basic characteristics as plasmid recombination intermediates recovered from intact cells (12, 13). Specifically, monomer-size plasmid DNA rings are incubated in gently lysed extracts of bacterial cells, aliquots being withdrawn over a period of hours for analysis by electron microscopy. During the incubation an increasing number of monomer rings are fused to give figure 8 structures. These structures are convertible to χ forms by

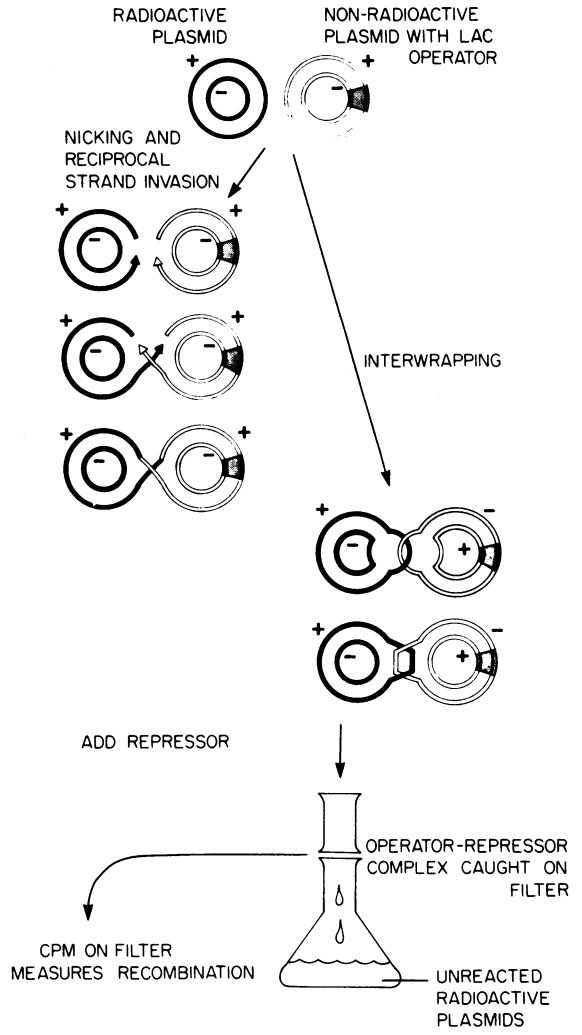

FIG. 1. Filter binding assay designed to detect recombination intermediates formed *in vitro*. Two plasmids which are almost entirely homologous are incubated with an *Escherichia coli* cell extract. The first plasmid is radioactive (shown black) and the second is a nonradioactive form that carries *lac* operator DNA (shown stippled). If the plasmids become fused, either by a nick and reciprocal strand exchange mechanism (*left*) (for discussions, see refs. 4 and also 6–8) or by an interwrapping mechanism (*right*) (9–13), then both radioactive label and *lac* operator sites will be joined in the same DNA molecule. Such composite structures can be trapped on a nitrocellulose filter by *lac* repressor.

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linearization with *Eco*RI. The development of the figure 8s and derived χ forms has been observed in >40 independent cell extracts and provides a reproducible assay for the formation *in vitro* of structures that have the appearance of recombination intermediates (12, 13).

Although the electron microscope affords an excellent assay for the fusion of genomes *in vitro* because of the specificity with which the products of an incubation can be analyzed, it would not be easy to apply this technique to the many samples that arise as the proteins of a cell are fractionated—for example, across chromatographic columns. Therefore, we have developed and report here an assay for recombination that is designed to overcome this difficulty. In essence, two nearly identical but separately assayable DNA molecules are given the opportunity to become fused *in vitro*. The basic feature of the assay is to use one set of input plasmid DNA molecules that are radioactively labeled but are not further detectable unless they become joined to a second DNA molecule. The second plasmid has the same nucleotide sequence as the first but has been supplemented through recombinant DNA techniques to contain multiple copies of the *lac* operator. The presence of the *lac* operator DNA will allow this plasmid to be bound to a membrane filter in the presence of the *lac* repressor protein (15).

To assay for genome fusion, the two plasmids are incubated together in a cell extract, then exposed to repressor, and passed through a nitrocellulose filter. The filter is expected to retain the repressor protein and, through this interaction, any DNA molecules to which the repressor is bound. If a reaction has occurred *in vitro*, fusing a radioactive plasmid and an operator-containing plasmid, the binding of the operator half of the structure will result in radioactivity also being retained on the filter.

This assay for recombination *in vitro* is potentially sensitive and rapid and, because it does not destroy the fused structures, allows their subsequent recovery from the filter and further analysis, for example by electron microscopy. We anticipate that the assay may also be useful for studying other types of genome fusion events—for instance, the site-specific recombination involved in prophage integration and the movements of transposable genetic elements.

Controls

Operator-Containing Plasmid. The first plasmid used in the genome fusion assay is a pMB-9 vehicle into which the *lac* operator has been cloned. The particular plasmid chosen for this study carries multiple copies of the *lac* operator, a situation designed to enhance its potential for specific binding to the filter. Fig. 2*A* shows the retention on the filter of the complex formed between the *lac* repressor and the operator-containing plasmid (which, for the purpose of this control, has been labeled with [^{3}H]thymidine). In the absence of repressor, less than 1% of the operator-containing plasmids adhere to the filter. However, in the presence of increasing amounts of repressor the percentage of plasmids that are bound rises to a plateau level of about 50%.

To adapt the assay to measure recombination *in vitro*, the radioactivity is "removed" from the operator-containing plasmid and instead used in a homologous plasmid that contains no *lac* operator sites.

Radioactive Plasmid. The second plasmid used in the fusion assay is identical to the first except that it lacks operator sites and is heavily labeled with [^{3}H]thymidine. As shown in Fig. 2*B*, this radioactive plasmid will not, by itself, bind to the filter. The background level of binding is less than 1% and is not increased by the presence of repressor. Based on these controls, it can be expected that the radioactivity of the second plasmid will only be retained on the filter if this plasmid becomes fused to an operator-containing plasmid (as in Fig. 1).

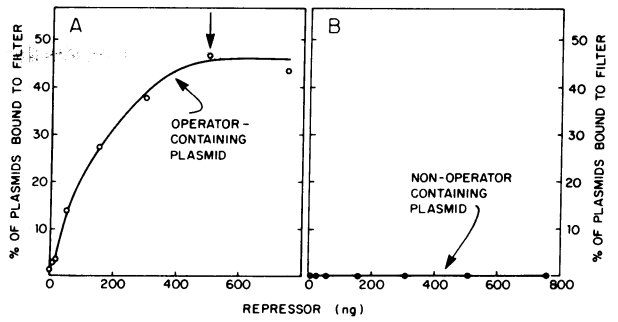

FIG. 2. Many proteins adhere to nitrocellulose filters and, in so doing, bind a nucleic acid molecule to which they are attached (15–19). (*A*) This filter binding assay applied to the repressor-mediated binding of a plasmid DNA molecule containing the *lac* operator—specifically, the plasmid pMB-9 carrying multiple copies of the *lac* operator. (This plasmid was constructed by Ronald Ogata and Walter Gilbert to study *lac* repressor–operator contacts; their analysis of the 660-base-pair insert with restriction enzymes has confirmed the existence of four and possibly more operator sites.)

Monomer-size DNA rings were obtained by transfecting the plasmid into the Rec A$^-$ *E. coli* strain WD 5260. Because, in Rec A$^-$ cells, monomers cannot recombine to generate a spectrum of multimeric species, it was possible to recover an individual transfected cell that had received and replicated a monomer-size plasmid (5). The clone resulting from this transfectant was used to prepare (20) a population of plasmids (>99% monomers by electron microscopy). Because the Rec A$^-$ strain was thymine-requiring, it was possible (for this control) to label the plasmid with radioactivity; the specific activity of the plasmid after labeling with [^{3}H]thymidine can reach 400,000 cpm/μg.

Binding assays were carried out essentially as described by Riggs *et al.* (15). In anticipation of the fact that in the actual experiment the plasmid DNA would have to be recovered from an enzyme reaction mixture, we performed the filter binding assay on DNA that was not placed directly in a buffer but was first mixed with a cell lysate. Plasmid DNA (10 μg) was placed with 100 μg of cellular proteins in 1 ml of buffer A (10 mM Tris, pH 7.5/10 mM KCl/0.2 mM EDTA). The mixture was immediately extracted sequentially with 250 μl of chloroform/isoamyl alcohol, 24:1 (vol/vol), and 250 μl of phenol, and the final aqueous phase was monitored to determine the recovery of the input radioactive plasmids (routinely 80–85%). The purified aqueous phase was then supplemented with $MgCl_2$, dithiothreitol, and bovine serum albumin to yield the final binding buffer (10 mM Tris, pH 7.5/10 mM KCl/10 mM $MgCl_2$/0.2 mM EDTA/1 mM dithiothreitol containing 50 μg of bovine serum albumin per ml). The repurified plasmid DNA was distributed into tubes, each of which received 0.5 μg of plasmid and then an amount of repressor ranging from 0 to 750 ng (the phosphocellulose fraction, 90% pure, as in ref. 21). After 40 sec, sodium pyrophosphate was added to a concentration of 20 mM in order to decrease nonspecific binding. Each mixture was then filtered dropwise through a prepared nitrocellulose filter under low negative pressure. The filters were turned over, washed with 5 ml of binding buffer (lacking albumin but containing 20 mM sodium pyrophosphate), dried, and assayed for radioactivity.

The filters (Schleicher & Schuell, BA 85) were prepared for the binding assay by preincubation for 5–10 min in 500 mM potassium hydroxide followed by washing in distilled water and finally soaking for at least 20 min in binding buffer (lacking albumin but containing 20 mM sodium pyrophosphate and 20 μg of sonicated calf thymus DNA per ml).

In the presence of increasing amounts of repressor, up to 50% of the operator-containing plasmids are bound to the filter. The arrow at 500 ng shows the amount of repressor used in the binding experiments shown in Figs. 3, 5, and 6.

(*B*) The other (radioactive) plasmid to be used in the genome-fusion assay is pM B-9 labeled to a specific activity of 400,000 cpm/μg. This plasmid does not bind (<0.4%) to nitrocellulose filters in the presence of *lac* repressor.

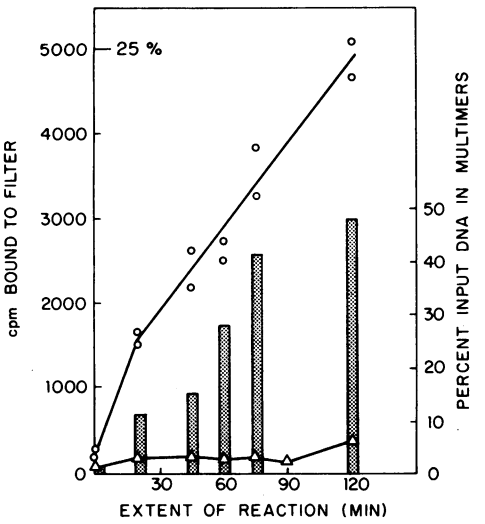

FIG. 3. Genome fusion assay. A lysate was prepared from a recombination-proficient strain of *E. coli* (MM 294) by lysozyme digestion and freeze–thaw treatment. After a high-speed clearing spin, the resulting supernatant was fractionated with ammonium sulfate (200–300 g/liter) to yield a pellet that, upon dialysis, was active in fusing unit plasmid genomes into multimeric structures (12, 13). The buffered enzyme fraction was supplemented with 10 mM Mg^{2+}, 2.5 mM spermidine, and 3 mM 2-mercaptoethanol and then incubated with roughly equal amounts of the two monomer-size plasmids—radioactive pM B-9 and nonradioactive pM B-9 carrying *lac* operator DNA. The concentration of cellular proteins in the reaction mixture (generally 20 μl) was 2 mg/ml and the combined concentration of plasmid substrate molecules was 150 μg/ml. Aliquots (3 μl) were withdrawn into 200 μl of buffer A and the filter binding assay was performed as described in the legend to Fig. 2. Over time, about 25% of the radioactivity initially present in one of the input monomer plasmids (pM B-9) was converted to a form that could adhere to a nitrocellulose filter in the presence of *lac* repressor (○). No radioactivity was bound in the absence of repressor (△). Bars, multimeric forms quantitated by electron microscopy.

Application of the assay to detect genome fusion in a crude cell extract

Fig. 3 shows the results of applying the filter binding assay to the product of an *in vitro* recombination system that fuses homologous DNA molecules. A reaction mixture containing enzymes from *E. coli* was prepared (as described in refs. 12 and 13) and supplemented with monomer forms of the two plasmids. At various times, aliquots of the reaction mixture were withdrawn and deproteinized as described in the legend to Fig. 2. Repressor was then added to the DNA from each aliquot, and the mixtures were passed through nitrocellulose filters. At time 0, essentially no radioactivity was retained on the filter. This was expected because the radioactive plasmids do not contain operator DNA and have no means of binding to the filter. However, with increasing time, the reaction mixture converted radioactivity into a form that could be bound to the filter by repressor (Fig. 3, upper curve). By 20 min, 7% of the available radioactivity was retained on the filter. By 60 min, 13% of the radioactivity was retained. This binding is totally dependent on repressor (Fig. 3, lower curve).

The design of the assay leads one to expect that the retention of radioactivity on the filter represents the formation of figure 8 structures. This can be tested directly.

(*i*) An electron microscopic analysis of the same time points gave a curve that is essentially identical to the one obtained with the biochemical assay (Fig. 3, bars).

(*ii*) Furthermore, it is possible to examine directly the material that has bound to the filter. The filter is exposed to a solution containing isopropylthiogalactoside (1 mM in 10 mM Tris/1 mM EDTA, pH 8.5; final volume, 1 ml), an analog of the small signal molecule that causes the repressor to release its hold on the operator DNA. This allows >99% elution of the bound plasmids from the filter. Electron microscopy of the released DNA showed figure 8 structures. In a typical analysis (of 500 molecules from the 60-min time point) there were 458 monomers (unreacted operator-containing plasmids) and 42 figure 8s and higher multimers (Fig. 4 *upper*) accounting for 30% of the mass of the DNA. Upon digestion with *Eco*RI, a number of χ forms roughly equivalent to the number of multimers was observed (Fig. 4 *lower*).

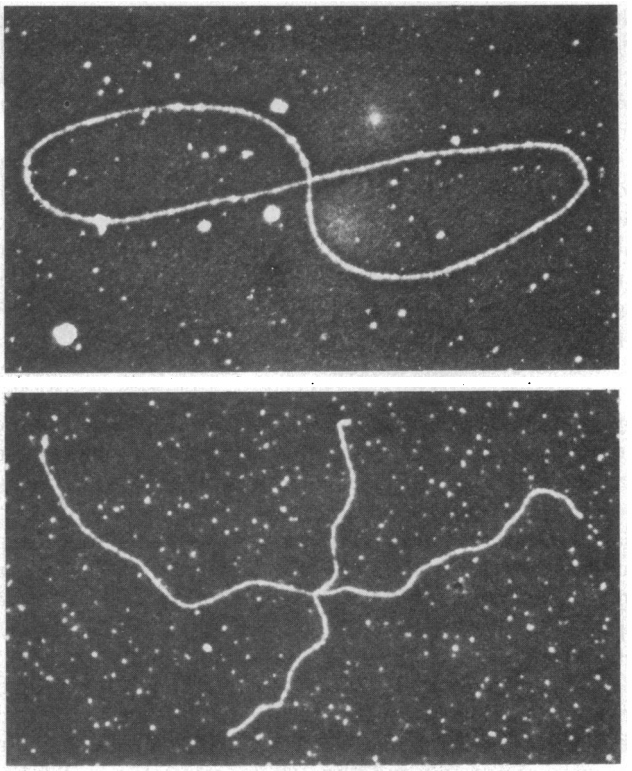

FIG. 4. Electron microscopy of the DNA molecules bound to the filter by repressor and eluted with isopropylthiogalactoside. The input plasmids were generally 80–90% supercoiled (the rest were relaxed circles). The product multimers were sometimes supercoiled (in one or both elements) and sometimes relaxed. Electron microscopy was carried out as described in ref. 22, with the DNA being rendered visible simply by staining with uranyl acetate.

Comparison of Filter Binding Assay and Electron Microscopic Assay. The basic difference between the biochemical curve and the electron microscopic curve lies not in the final result but in the time and effort needed to obtain it. As long as the genome-fusion reaction is high (for example, ≥20% multimer formation), one is able to quantitate the activity by using the electron microscope to scan a few hundred molecules. The preparation and counting of a sample requires about an hour. However, if the fusion activity is low (for example, 5%, as might occur after dilution on chromatographic columns) the electron microscopic assay becomes more difficult. It is then necessary to scan 1000 or more molecules in order to observe a statistically significant 50 multimers. In contrast, with the filter binding assay one can easily begin with 10^5 cpm in the input radioactive plasmid, and a potential 5% reaction then yields binding of several thousand cpm above a nonspecific background of about 500 cpm. Thus, with the biochemical assay, the time required for analysis is relatively short and is not increased by low enzyme activity—in a matter of a few hours, 50 or more reaction mixtures can be examined.

Application of the assay to enzyme fractions derived from a molecular sieve column

The data of Fig. 3 measure genome fusion in an enzyme fraction that had been purified only by differential ammonium sulfate fractionation. To demonstrate the potential usefulness of the filter binding assay in enzyme purification, we took this relatively crude protein preparation and passed it over a molecular sieve column, assaying the resulting 60 fractions for their ability to fuse monomer plasmid genomes *in vitro*.

The column resin chosen was Bio-Gel P-150 which, as shown in Fig. 5*A*, was capable of resolving three marker proteins into distinct peaks: immunoglobulin G (150,000), hemoglobin (63,000), and cytochrome *c* (12,500).

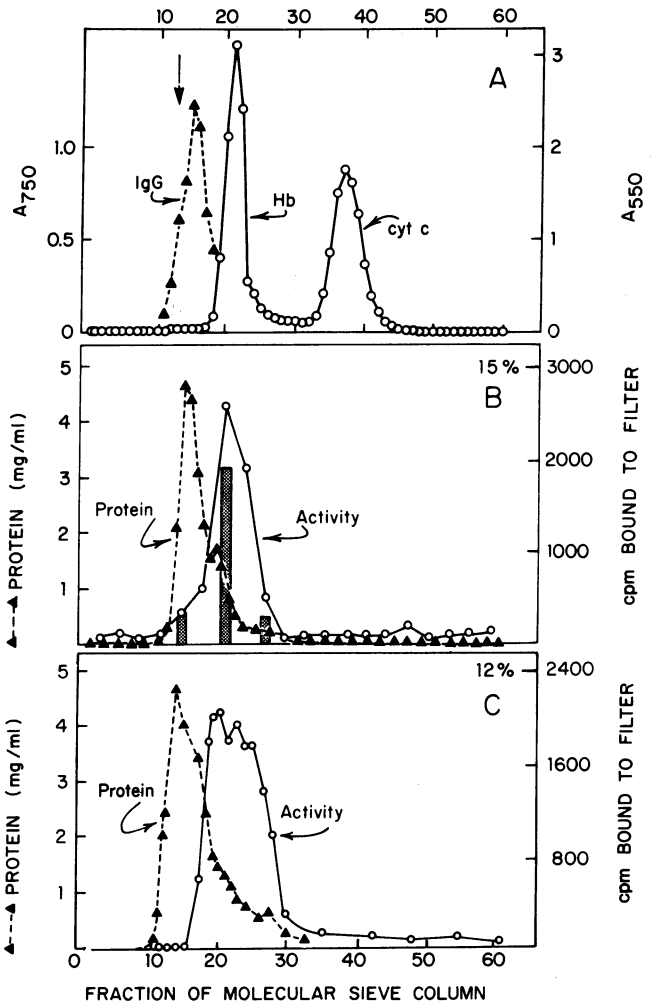

FIG. 5. Application of the filter binding assay: detection of fusion activity after purification on a molecular sieve column. About 25 mg of an ammonium sulfate-derived protein fraction that demonstrated an ability to fuse monomer plasmids *in vitro* (Figs. 3 and 4) was applied to a Bio-Gel P-150 molecular sieve column (1.2 × 30 cm). The sample was loaded in 800 μl of 50 mM potassium phosphate, pH 7.4/20% (wt/vol) glycerol/1 mM 2-mercaptoethanol and was eluted in the same buffer. The column was run at 4°C with a flow rate of about 2.5 ml/hr; 60 fractions were collected and protein concentrations (▲) were determined by the method of Lowry, with bovine serum albumin as a standard. Aliquots from each three successive fractions were pooled and assayed for their ability to support genome fusion *in vitro* (a 2-hr reaction with 400 μg of protein and 150 μg of substrate plasmid DNA per ml). Plasmid fusion was tested by the filter binding assay (○) and also by electron microscopy (vertical bars). (*B*) Results obtained from a lysate of Rec A^+ cells; the activity by both assays eluted in the region of fraction 20. (*C*) The fusion activity also could be recovered from Rec A^- MM 152 cells. (*A*) Marker proteins.

An assay of the fusion activity passed over the same column gave the curve shown in Fig. 5*B* and led to the following conclusions: (*i*) the DNA fusion activity remained viable, (*ii*) the peak of the activity appeared in the included volume and eluted at approximately the same place as hemoglobin, (*iii*) if two or more components were required for the reaction, they were all of about the same size, and (*iv*) if two or more fusion activities exist they were not resolved.

Effect of the *RecA* Locus. When we previously analyzed crude lysates from recombination-deficient, Rec A^- cells (MM-152) for their ability to fuse plasmid genomes *in vitro*, we generally found that the fusion activity was decreased to $\frac{1}{4}$th and often to $\frac{1}{10}$th (12). However, it has been difficult to draw a firm conclusion from this negative result because the substrate plasmids sometimes undergo degradation during their incubation in the Rec A^- extracts. A clear result is obtained, however, by applying the Rec A^- extract to the molecular sieve column and assaying the resulting fractions. Nucleases evidently are removed because DNA degradation is no longer a problem. The profile shown in Fig. 5*C* is obtained, which indicates that a fusion activity can be purified from Rec A^- extracts.

Because an activity is unambiguously observable after purification of Rec A^- extracts, we lack a genetic basis for concluding that the fusion activity we are studying is a physiological recombinase. Nonetheless, this would seem to be indicated because the extracts are fusing DNA molecules at a region of homology, which is a logical first step in genetic recombination. We are proceeding under the working hypothesis that the fusion activity we observe is a DNA recombinase involved in generalized reciprocal recombination but are aware of the possibility that such deliberate pairing of homologous DNA might be an aspect of some form of DNA repair or, working in reverse, be used for the segregation of two progeny chromosomes after a round of DNA replication.

Characterization of the Fusion Activity. Based on the results of the molecular sieve chromatography shown in Fig. 5, the approximate molecular weight of the fusion activity is judged to be 50,000–100,000. This size range includes several potentially relevant enzymes of nucleic acid metabolism. Two enzymes are of particular interest with respect to the interwrapping initiation mechanism shown in Fig. 1. These are the DNA untwisting enzyme ω (ref. 23; see also refs. 11 and 24) and DNA gyrase which also has an untwisting activity (25). The molecular weights of ω and of the components of gyrase are 110,000, 100,000, and 90,000, respectively. Although the fusion activity elutes close to hemoglobin (molecular weight, 63,000) the resolution offered by the column does not allow the conclusion that the activity is distinct from either ω or gyrase. It is possible, however, to test directly for the involvement of these enzymes by using the antibiotics oxolinic acid and coumermycin in one instance and *N*-ethylmaleimide in the other.

The requirement for gyrase, or either of its subunits, in the fusion reaction was tested by assaying the activity of the pooled peak fractions from Fig. 5*B* in the presence of oxolinic acid and coumermycin. These two antibiotics inhibit the α and β subunits of gyrase (26, 27) but did not prevent genome fusion (Fig. 6*B*).

To test for the involvement of ω, the reaction was carried out in the presence of the sulfhydryl blocking reagent *N*-ethylmaleimide. The ω protein is known to be resistant to *N*-ethylmaleimide (26). However, the fusion activity was completely abolished (Fig. 6*C*). The data thus indicate that ω cannot be solely responsible for the figure 8 structures formed *in vitro*.

In sum, it would appear that the fusion activity (potential recombinase) observed *in vitro* depends on other, perhaps novel, enzymes. The most certain route to understanding the fusion activity and its potential role in recombination will de-

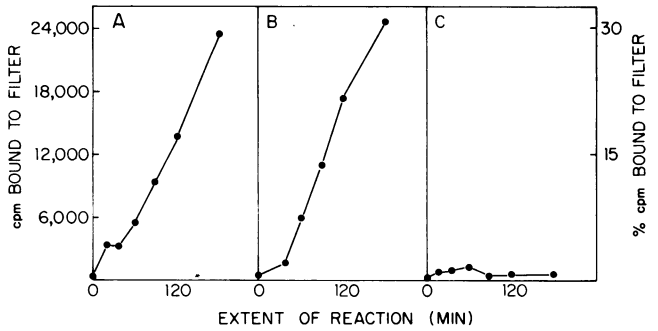

FIG. 6. In three parallel reaction mixtures, the fusion activity recovered from Fig. 5*B* (fractions 19–22) was incubated with radioactive and operator-containing plasmids. Genome fusion was measured by the filter binding assay. (*A*) Control. (*B*) Activity in the presence of oxolinic acid (100 μg/ml) and coumermycin (15 μg/ml), inhibitors of DNA gyrase. (*C*) Activity in the presence of 10 mM *N*-ethylmaleimide. This was the only treatment that inhibited the reaction and is significant because the ω protein is not inhibited by *N*-ethylmaleimide. That the *N*-ethylmaleimide inhibition was not due to interference with the filter binding assay was determined by a parallel electron microscopic analysis of the reaction mixture: only monomer-size plasmids were observed. In each case the enzyme fraction was incubated with the putative inhibitor for 4 min at 37°C prior to the addition of DNA. The reaction mixtures were 100 μl, and 12-μl aliquots were taken for the filter binding assay.

pend upon enzyme purification. This should be possible by using the assay described in this paper and established techniques of protein chemistry.

Origin of the assay

The key reagent in the filter binding assay is readily available because of the isolation of the *lac* repressor by Gilbert and Müller-Hill (21, 28). In particular, the construction of bacterial strains that overproduce the repressor (2–3% of the cell protein) allows the purification of the repressor in 100-mg amounts. The filter binding assay described in this paper uses the repressor in 100-ng amounts.

Although original in its application to the study of genetic recombination, the filter binding assay derives from three sources. In 1964, Leder and Nirenberg (16) discovered that ribosomes would adhere to nitrocellulose filters while holding triplet codons and their cognate transfer RNA molecules. In this way, more than half of the genetic code was determined. Jones and Berg (17) in 1966 extended the filter binding approach to study the interaction of proteins with DNA. They incubated bacteriophage T7 DNA with RNA polymerase in order to determine the number of binding elements (promoters) present in the viral chromosome. Under the conditions used by Jones and Berg, neither the protein nor the DNA would, by itself, adhere to the filter, and it seemed possible that a special polymerase-induced denaturation of the DNA was responsible for creating a complex that would bind. It was subsequently found, however, that when the magnesium concentration was increased many cellular proteins could be retained on a membrane filter, along with any nucleic acid elements bound to them (15, 18). Thus, in the ensuing period, the filter binding assay could be extended to the study of a number of protein–nucleic acid interactions, including the association between amino-acyl tRNA synthetases and tRNA (18) and the interaction between the *lac* repressor and the *lac* operator (15). The assay described in this paper is an extension of this process. In adapting the filter binding assay to study recombination, we are basically using a protein–nucleic acid interaction to assay for a DNA–DNA interaction.

We thank Dr. Ronald Ogata for his generous gift of *lac* repressor protein and Dr. John Wolfson for his careful reading of the manuscript. The work of our laboratory is made possible by Research Grants GM 17088 from the National Institutes of Health and NP 57 from the American Cancer Society and by the Dreyfus Teacher-Scholar Fund.

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