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In Vitro Recombination of Bacteriophage T7 DNA Detected by a Direct Physical Assay

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We developed a simple, direct, physical assay to detect genetic recombination of bacteriophage T7 DNA in vitro. In this assay two mature T7 DNA molecules, each having a unique restriction enzyme site, are incubated in the presence of a cell-free extract from T7-infected *Escherichia coli* cells. After extraction of the DNA, restriction enzyme digestion, and agarose gel electrophoresis, genetic recombination is detected by the appearance of a novel recombinant DNA band. Recombination frequencies as high as 13% have been observed. We used this assay to determine the genetic requirements for in vitro recombination. In agreement with results obtained previously with a biological assay, T7 recombination in vitro appears to proceed via two distinct pathways.

In recent years our understanding of the process of genetic recombination has increased markedly (for a review, see reference 1). This progress is in part attributable to studies of recombination mechanisms in bacteria and bacteriophages. Bacteriophage T7 is an attractive system for the study of DNA metabolism and genetic regulation. This phage has been extensively characterized genetically (4, 22, 23), and its genome consists of a double-stranded DNA molecule which codes for about 50 proteins. The entire genomic DNA molecule has recently been sequenced (J. Dunn and F. W. Studier, J. Mol. Biol., in press).

Genetic recombination of T7 has been studied in vivo (3, 5, 7–9, 19, 20, 24, 25). From these studies, it was concluded that T7 recombination in vivo requires the products of T7 gene 3 (endonuclease), gene 4 (DNA primase), gene 5 (DNA polymerase), and gene 6 (exonuclease). Recombination is independent of the host and phage ligase, DNA packaging, and maturation functions. To understand the molecular mechanisms of genetic recombination, we have been studying this process in vitro.

In vitro genetic recombination of T7 DNA was first observed by using a recombination-packaging assay (18). Recombination between endogenous and exogenous DNAs could be demonstrated by both genetic and physical means (13, 16, 18, 26). In a subsequent refinement of this assay, an in vitro recombination-packaging system was developed, which was independent of the level of endogenous DNA (15). It was found that T7 recombination in vitro could proceed via three pathways. The first was the wild-type pathway, in which all T7 gene functions were operative. The second was the endonuclease pathway, which required gene 3 (endonuclease) and gene 5 (DNA polymerase) functions but was inhibited by the gene 4 (DNA primase) function. The third pathway was the exonuclease pathway, which required the gene 6 exonuclease.

Although this assay allowed detection of recombination between two exogenously added DNA molecules, it proved to be of limited use in defining the mechanisms of in vitro recombination. Because the recombination reaction involved incubations with two crude cell extracts, followed by plating for viable phage progeny, one could not draw definitive conclusions concerning the relative contributions of each step toward the recombination process (17).

Because our ultimate aim is to study the molecular mechanism of T7 recombination by fractionating the process into separate components, a direct physical assay which allows us to study in vitro recombination is desirable. In this paper, we describe the development of such an assay and its application in determining the genetic requirements for T7 recombination in vitro.

MATERIALS AND METHODS

Bacteria and bacteriophages. The bacterial and phage strains used were those described previously (13).

Preparation of infected cell extracts. T7 mutant-infected cell extracts were prepared by infecting *Escherichia coli* B with T7 phages that carried amber mutations in the defective genes (for example, T7 3^--infected cell extract was made from a T7 *3am* infection, and 4^- extract was made from a T7 *4am* infec-

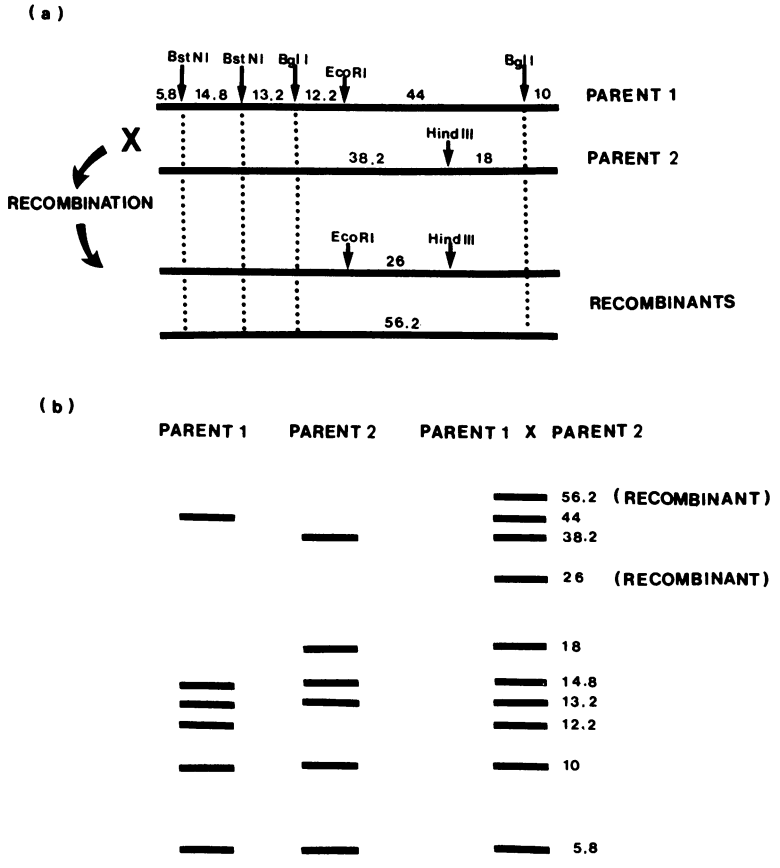

FIG. 1. Scheme for the physical assay of genetic recombination. Two T7 mutant DNAs are used as substrates in the assay. The first mutant contains a unique *Eco*RI site at position 46% from the left end of the genome (the DNA is called *s*RI [sensitive to *Eco*RI]), and the second contains a unique *Hin*dIII site at 72% (*sHin*dIII$_2$). Thus, these two unique restriction sites can serve as recombination markers, and recombination between them generates a recombinant DNA which contains both sites (a). This can be detected after restriction digestion and gel electrophoresis by the appearance of a newly formed 26% restriction fragment (b). The reciprocally recombined molecule which contains neither restriction site can also be detected by the presence of a 56% fragment. *Bst*NI and *Bgl*I are used in the assay in addition to *Hin*dIII and *Eco*RI. *Bst*NI is used to digest selectively the *E. coli* chromosomal DNA, and *Bgl*I is used to resolve the 26% recombinant fragment from the 28% parental fragment.

tion, etc.), except for the following infections. T7 3$^-$ 4$^-$ 5$^-$-infected cell extract was made from a T7 3*am*4*am*5*am*9*am*19*ts* infection, and T7 3$^-$ 4$^-$ 5$^-$ 6$^-$-infected cell extract was made from a T7 3*am*4*am*-5*am*6*am*19*ts* infection. Since both gene 9 (prohead scaffolding protein [14]) and gene 19 (DNA maturation protein [2]) are not involved in genetic recombination (3), the absence of these gene products from infected cell extracts should not affect recombination. For convenience, references to these phages below omit mention of the 9*am* and 19*ts* mutations.

E. coli B was infected with T7 phage at a multiplicity of infection of 5, as described previously (15). The infected cells were then chilled and centrifuged at 4,000 × *g* for 5 min. The pellet was suspended in 1/200 volume of T7 diluent (20 mM Tris-hydrochloride [pH 7.4], 10 mM $MgSO_4$, 0.5% NaCl, 10 μg of gelatin per ml) and incubated with 500 μg of lysozyme per ml at 0°C for 30 min. The concentrated cells were frozen in dry ice and then thawed at 30°C before use. Infected cells could be stored at −90°C for at least 2 weeks.

In vitro recombination assay. Infected cell extracts were thawed at 30°C and centrifuged at 37,000 × *g* for 15 min. Then 10 μl of the supernatant was added to an equal volume of T7 diluent and 3 μg of T7 DNAs (equal amounts of *s*RI and *sHin*dIII$_2$ DNA). Each reaction mixture was supplemented with 1 mM ATP and the four deoxynucleoside triphosphates (0.1 mM each). The mixture was incubated at 30°C for 60 min, and then the reaction was stopped by adding EDTA and Sarkosyl (5) along with 100 μg of proteinase K per ml. After 2 h at 42°C, the DNA was extracted with phenol, precipitated with ethanol, and digested with *Bgl*I, *Bst*NI, *Eco*RI, and *Hin*dIII (5). One-half of the reaction mixture was then subjected to agarose gel electrophoresis (5).

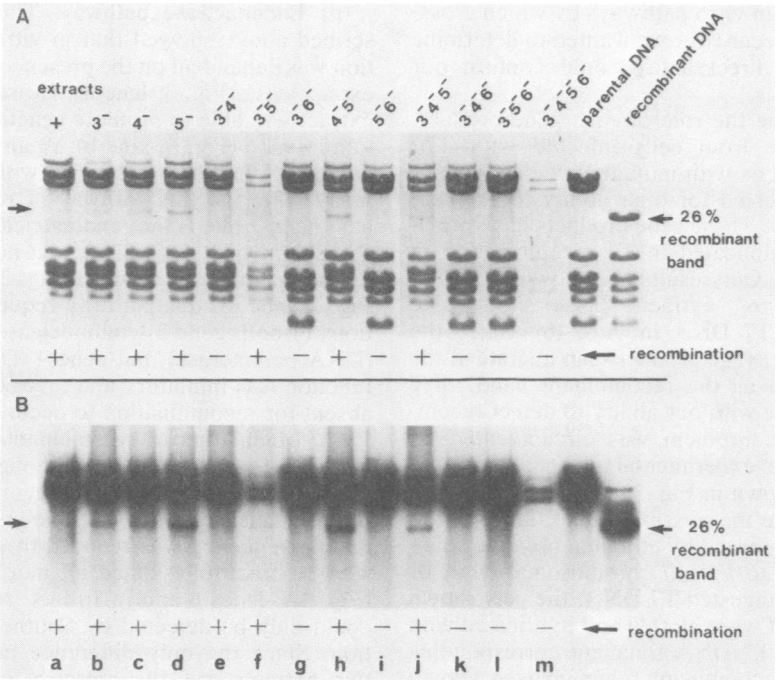

FIG. 2. In vitro recombination promoted by T7 mutant extracts. Single- and multiple-mutant extracts were prepared as described in the text. The abilities of these extracts to promote recombination were assayed as described in the legend to Fig. 1. Recombination is indicated by the presence of the 26% recombinant band, as determined by ethidium bromide staining (A). Since the T7 mutants used to prepare the infected cell extracts did not contain either restriction marker, the 56% band could not be used to score for recombination due to the presence of parental DNA in the extract. To confirm the results, the same gel was blotted onto a nitrocellulose filter and hybridized with a DNA probe which corresponded to the 26% recombinant fragment (B). The results were consistent with the results obtained in (A), except with the 3^- 4^- extract. Since ethidium bromide staining allows detection of recombination frequencies only greater than 1.0%, this implies that the 3^- 4^- extract promoted less than 1.0% recombination.

Filter hybridization. To prepare a suitable probe, T7 *s*RI*s*HindIII$_2$ DNA was digested with *Eco*RI and HindIII, and the DNA fragment bounded by the two restriction sites was recovered from an agarose gel by electroelution and labeled in vitro (12).

The agarose gels were blotted onto nitrocellulose sheets (21) and hybridized with a nick-translated DNA probe in a solution containing 0.9 M NaCl, 0.05 M $NaPO_4$ (pH 7.0), 5 mM EDTA, 0.3% sodium dodecyl sulfate, 30 μg of denatured calf thymus DNA per ml, and a heat-denatured ^{32}P-labeled DNA fragment (2×10^5 cpm/ml). Hybridization was done at 65°C for 16 h, and the filter was washed as described elsewhere (5).

RESULTS

Rationale of recombination assay. We have previously shown the feasibility of using restriction enzyme site polymorphisms to study genetic recombination of T7 DNA in vivo (5). In this study we used the same approach to assay T7 recombination in vitro. A change in linkage between unique restriction sites can be detected by agarose gel electrophoresis without the need to resort to packaging of DNA into viable phage particles (15). The rationale for the assay is summarized in Fig. 1.

As determined by control experiments, this assay was sensitive enough to detect as little as 1% recombination by using only ethidium bromide staining and UV illumination. The sensitivity of this assay could be increased another 10-fold by Southern hybridization with a ^{32}P-labeled DNA probe (5). However, the sensitivity could not be further enhanced by using ^{32}P-labeled probes of higher specific activity or longer exposures of Southern blots due to background noise from random DNA degradation by the crude extracts.

Recombination mediated by various T7 mutant extracts. We previously used a similar assay to determine the genetic requirements for T7 recombination in vivo (5). Therefore, it was of interest to determine whether the genetic requirements for in vitro recombination were consistent with the in vivo requirements. Furthermore, since our previous results based on the two-stage biological assay suggested the exis-

tence of three in vitro pathways by which crossing over can occur (15), we wanted to determine whether this direct assay could confirm our previous results.

To determine the role of each gene product, extracts made from cells infected with $T7^+$ phage, as well as with mutants in genes 3, 4, 5, and 6, were tested for their ability to promote recombination. These gene products have previously been implicated in T7 recombination in vivo (3, 5, 9). Our results are shown in Fig. 2. Certain gene 6^- extracts cause site-specific cleavages of T7 DNA in vitro (6). Since the anomalous cleavage products can migrate at the same position as the recombinant band, they might interfere with our ability to detect recombination. This problem was circumvented by modifying the experimental protocol, and the results are shown in Fig. 3.

To eliminate the possibility that the recombinant bands observed by ethidium bromide staining were due to *E. coli* chromosomal DNA or incompletely digested T7 DNA, the gels shown in Fig. 3 and 4 were blotted and hybridized with a ^{32}P-labeled T7 DNA fragment corresponding to the 26% recombinant fragment (see above) (Fig. 2B).

Recombination frequencies were estimated by densitometric scanning of a photographic negative of the ethidium bromide-stained gel. The percentage of recombination was then calculated from the area under the peaks. (A concern in using this type of analysis is that this method only gives a rough estimate due to the logarithmic response of photographic film to exposure [10, 11]. However, since it is convenient and our control experiments seemed to show a linear correlation between the traced area and the molecular weights, we nevertheless used the procedure for approximating the frequency of recombination.) The results are summarized in Table 1.

Multiple pathways of in vitro recombination. (i) Exonuclease pathway. We first studied the effect of single mutations in genes 3, 4, 5, and 6 on in vitro recombination. These experiments showed that extracts made from gene 3^-, 4^-, or 5^- mutant-infected cells each promoted efficient recombination (Fig. 2, lanes b through d), whereas a gene 6^- mutant-infected cell extract did not (Fig. 3, lane a). Furthermore, 3^- 4^- 5^- extracts promoted recombination, but the 3^- 4^- 5^- 6^- extract did not (Fig. 2, lanes j and m). These results suggested that of genes 3, 4, 5, and 6, only the gene 6 product was needed for in vitro recombination. This is consistent with our previous findings, and in accordance with our initial nomenclature (15), we designate recombination promoted by a 3^- 4^- 5^- extract as the exonuclease pathway.

(ii) Endonuclease pathway. The results described above showed that in vitro recombination was dependent on the presence of the gene 6 exonuclease (Fig. 3, lane a). However, a 4^- 6^- extract was able to promote genetic recombination in vitro (Fig. 3, lane b). Again, this finding confirmed the finding obtained with a biological assay (15), and this pathway of recombination has been called the endonuclease pathway. Since neither a 3^- 4^- 6^- extract nor a 4^- 5^- 6^- extract gave recombination (Fig. 2, lane k, and Fig. 3, lane c), this pathway required the functions of both gene 3 (endonuclease) and gene 5 (DNA polymerase), but gene 4 (DNA primase) function was inhibitory and therefore had to be absent for recombination to occur.

(iii) Modulation of recombination activity by the gene 4 and 5 proteins. Although the gene 6 exonuclease could mediate recombination by the exonuclease pathway (Table 1), the recombination frequency was reduced from 8% in a 3^- 4^- 5^- extract to less than 1% in a 3^- 4^- extract (Fig. 2A, lanes e and j). In fact, recombination could only be detected by Southern hybridization. Since the only difference between these two extracts was the presence of the gene 5

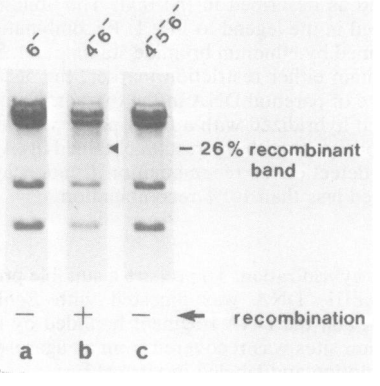

FIG. 3. In vitro recombination promoted by T7 6^-, 4^- 6^-, and 4^- 5^- 6^- mutant-infected cell extracts. Since some T7 6^- mutants exhibit site-specific DNA cleavages in vivo and in vitro (6), these DNA cleavage fragments interfered with our detection of recombination. To assay for recombination, we modified our procedure as follows. DNA was incubated and extracted as described in the text and then subjected to restriction digestion with only *Bst*NI and *Bgl*I. The digested mixture was then separated by electrophoresis on a low-melting-point agarose gel. This allowed the fast-migrating fragments (which included any anomalous cleavage products) to be separated from the slowly migrating 56% DNA band, which arose from the *Bgl*I cleavages (see Fig. 1). The latter band was then excised and recovered after heating and phenol extraction. This DNA was then digested with *Hin*dIII and *Eco*RI and subjected to electrophoresis, as before. Only the ethidium bromide-stained gel is shown.

DNA polymerase in the 3^- 4^- extract, this finding could be interpreted to mean that the gene 5 function was inhibitory to the exonuclease pathway. When the recombination catalyzed by the 3^- 4^- extract was compared with that catalyzed by the 3^- extract (Fig. 2, lane b), in which the gene 4 DNA primase was functional, the recombination frequency returned to the wild-type level. This implies that the presence of the gene 4 protein can relieve the reduced recombination rate in 3^- 4^- extracts.

Our hypothesis that there are at least two pathways for in vitro recombination is consistent with other observations (Fig. 2 and Table 1). Both a 3^- 5^- extract and a 4^- 5^- extract promoted recombination because the gene 6 exonuclease was present. On the other hand, the 3^- 6^-, 5^- 6^- and 3^- 5^- 6^- extracts did not catalyze recombination because essential components from both the exonuclease pathway (gene 6 exonuclease) and the endonuclease pathway (gene 3 endonuclease and gene 5 DNA polymerase) were absent.

Other requirements. In vitro recombination was dependent on T7 infection since uninfected *E. coli* extracts did not promote recombination. Furthermore, recombination was independent of the *E. coli recA* function since extracts from T7-infected *recA* cells still promoted efficient recombination (data not shown). Since T7 codes for a function which inactivates the host *recBC* nuclease (27), we think it improbable that the *recBC* enzyme plays a role in our system.

We routinely added ATP and four deoxynucleoside triphosphates to our reaction mixtures to avoid the extensive degradation of exogenous DNA which occurs upon incubation with certain T7-infected cell extracts. For example, when incubated with a gene 5^- extract in the absence of ATP, the DNA underwent extensive degradation. DNA ligase was also an important component in preventing extensive degradation of the DNA substrate since the triphosphates were ineffective in preventing DNA degradation if phage ligase (gene 1.3) was mutated in addition to gene 5. It is likely that the gene 6 exonuclease was largely responsible for this extensive degradation since the DNA was stable in a gene 6^- extract (data not shown).

TABLE 1. Abilities of various mutant-infected cell extracts to promote recombination[a]

T7 extract	Recombination	Recombination frequency (%)
Wild type	+	4.1
3^-	+	5.2
4^-	+	7.2
5^-	+	12.9
6^-	−	<0.1
3^- 4^-	−	<1.0
3^- 5^-	+	5.2
3^- 6^-	−	<0.1
4^- 5^-	+	13.6
4^- 6^-	+	4.4
5^- 6^-	−	<0.1
3^- 4^- 5^-	+	8.3
3^- 4^- 6^-	−	<0.1
3^- 5^- 6^-	−	<0.1
4^- 5^- 6^-	−	<0.1
3^- 4^- 5^- 6^-	−	<0.1

[a] The in vitro genetic recombination promoted by various single- and multiple-mutant extracts was determined. The results were derived from Fig. 2 and 3. To quantitate the amount of recombination mediated by the infected cell extracts, the negative films of the ethidium bromide-stained gels (see Fig. 2A) were analyzed by densitometric scanning. The recombination frequencies were calculated by using the following formula: recombination frequency = [(area under the 26% band × 100/26)/(area under the 18% band × 100/18)] × 100.

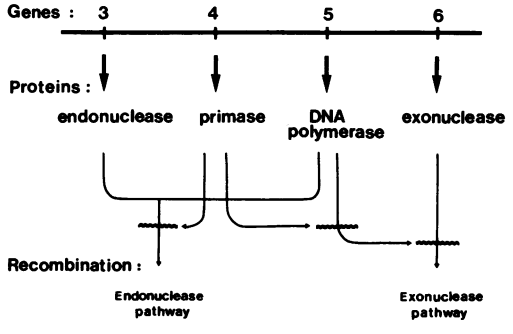

FIG. 4. Modulation of recombination by both T7 gene 4 and T7 gene 5 products. In vitro recombination can be achieved via two pathways. The first of these is the exonuclease pathway, which requires only gene 6 exonuclease. The second is the endonuclease pathway, which requires both gene 3 (endonuclease) and gene 5 (DNA polymerase) functions. The exonuclease pathway can be inhibited by the DNA polymerase. This inhibition is relieved by the presence of the gene 4 primase. The primase can also inhibit the endonuclease pathway.

DISCUSSION

We have developed a simple physical assay to study T7 general recombination. This assay uses two T7 DNAs as substrates, each having a unique restriction endonuclease site as a marker. Recombination between these two markers is detected by restriction digestion, followed by gel electrophoresis. This assay is capable of detecting as little as 0.1% recombination when it is coupled with Southern hybridization.

We used this assay to study the involvement of T7 genes in recombination in vitro. Our results in studies with crude extracts suggest that recombination may proceed via at least two pathways in vitro. The first of these is the endonulcease pathway, which requires the products of genes 3 (endonuclease) and 5 (DNA polymerase) but is inhibited by gene 4 (primase). The second is the exonuclease pathway, which requires the product of gene 6 (exonuclease) but not the products of genes 3, 4, and 5. Gene 5 function is inhibitory in this pathway, but this inhibition can be relieved by the action of the gene 4 protein.

Our findings are consistent with previous results obtained with a two-stage biological assay (15). However, the physical assay described here appears to have advantages over the previous assay in that recombination products are detected directly without requiring any packaging or plating step. This is important in that it is not possible to estimate the extents of the contributions that these intermediate steps make toward the final recombination product (17). The fact that the two assays yielded similar results substantiates the proposal that there are at least two pathways for in vitro recombination of T7 DNA.

Because T7 recombination in vivo absolutely requires the functions of genes 3, 4, 5, and 6, we still do not understand the relationship of the endonuclease and exonuclease pathways to recombination in vivo. Whereas we have implicated the gene 3 endonuclease, gene 5 polymerase, and gene 6 exonuclease in in vitro recombination, the gene 4 primase has a paradoxical role; it stimulates exonuclease-mediated recombination by antagonizing the gene 5 polymerase, but it also inhibits the recombination carried out via the endonuclease pathway. It is probable that in wild-type-infected cell extracts these apparently antagonistic functions are delicately balanced to give efficient genetic recombination and that whenever one component is altered, recombination is also affected.

We are currently using this assay to define the mechanisms by which recombination of T7 DNA occurs in vitro.

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