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PCR-Directed Formation of Viral Hybrids *in Vitro*

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When constructing viruses that have desired hybrid phenotypes, anticipated difficulties include the nonviability of many, possibly most, of the hybrid genomes that can be constructed by incorporation of DNA fragments. Therefore, many different hybrid genomes may have to be constructed in order to find one that is viable. To perform this combinatorial work in a single experiment, we have used bacteriophage T7-infected cell extracts to transfer DNA *in vitro.* In an extract, we have incubated T7 DNA, together with DNA obtained by polymerase chain reaction (PCR) amplification of the gene (gene 17) for the tail fiber of the T7-related bacteriophage, T3. After *in vitro* packaging of DNA in the extract, hybrid progeny bacteriophage were detected by probing with a T3-specific oligonucleotide; hybrids are found at a frequency of 0.1%. By determination of the nucleotide sequence of the entire gene 17 of 14 independently isolated hybrids, both right and left ends of the PCR fragment are found to be truncated in all hybrids. For all 14 hybrids, the right end is in the same location; the left end is found at 3 different locations. The nonrandom location of the ends is explained by selection among different inserts for viability; that is, most of the hybrid genomes are nonviable. Some hybrids acquire from T3 the desirable phenotype of nonadherence to agarose gels during agarose gel electrophoresis. © 1997 Academic Press

INTRODUCTION

To transfer a phenotype from a donor to a recipient organism while minimally changing the recipient, a DNA fragment from the donor organism is inserted in the genome of the recipient. Insertion can be accomplished by either *in vitro* cleavage and rejoining (reviewed in Sambrook *et al.*, 1989; Alberts *et al.*, 1994) or *in vivo* recombination (Rubinstein *et al.*, 1993; Sands *et al.*, 1994; Degryse *et al.*, 1995). However, not all hybrid genomes will yield a viable hybrid organism. For example, deleterious nucleotide sequences may be transferred; the identity of these nucleotide sequences may not be known in advance. *In vivo* recombination can help solve this problem by truncating a transferring DNA fragment, thereby possibly removing deleterious nucleotide sequences. In the case of bacteriophages, the use of *in vivo* recombination appears not to be optimal, because *in vivo* recombination of uncloned DNA fragments would be made difficult by the requirement for the fragment to cross both the membranes and the cell wall of a bacteriophage-infected cell. In the case of bacteriophage T7, use of an *in vitro* analogue to *in vivo* recombination is an alternative suggested by the observation that (1) T7-infected cells can be made to package DNA *in vitro* with efficiency over 20% (Son *et al.*, 1988), and (2) related extracts are recombinogenic (Roeder and Sadowski, 1979; Masker, 1992). Thus, in the present study, attempts were made to construct hybrid T7 genomes by incubation of mature T7 DNA, together with a fragment of DNA from the T7-related bacteriophage, T3. After successful use of the procedure, analysis of several hybrids was performed to determine whether the transfer had the characteristics expected of a process that had produced (undetected) nonviable genomes, in addition to the viable genomes detected.

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MATERIALS AND METHODS

Bacteriophages and bacteria

Bacteriophages T3 (Hausmann strain) and T7 amber mutant in gene 17 ($T7_{17}$; mutant 8) were received from Dr. F. W. Studier (Studier, 1969, 1979). Double mutants used for making DNA packaging extracts were constructed by use of both standard genetic crosses and the single amber mutants of Studier (1969); some of the double mutants have been previously used (Son *et al.*, 1988). Double mutants will be labeled by the number (Studier and Dunn, 1983) of the mutant genes; for example, a bacteriophage with an amber mutation in both gene 4 and gene 9 will be called $T7_{4,9}$. *Escherichia coli* 011′ was the permissive host for amber mutants; *E. coli* BB/1 was both the nonpermissive host for amber mutants and the host for wild-type bacteriophages T3 and T7. For the preparation of purified bacteriophages, aerated cultures were infected in 2 × LB medium (20 g tryptone, 10 g yeast extract, 5 g NaCl) at 30°. Bacteriophage particles were purified from either one or six liter lysates, by use of both centrifugation in a cesium chloride step gradient and buoyant density centrifugation in a cesium

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chloride density gradient, followed by dialysis against storage buffer: 0.2 *M* NaCl, 0.01 *M* Tris–Cl, pH 7.4, 0.001 *M* $MgCl_2$. DNA was obtained from purified bacteriophage particles by extraction with phenol (Serwer *et al.*, 1983). To determine the concentration of bacteriophage preparations, OD_{260} was measured (Bancroft and Freifelder, 1970).

Polymerase chain reaction

To amplify T3 gene 17, the polymerase chain reaction (PCR)[2] was used. PCR was performed by use of both standard procedures (PCR core reagent kit from Perkin–Elmer Cetus Instruments, Norwalk, CT) and the following primers: 5′-AGAGGCGAAATAATCTTCTCCC-3′ and 5′-CCTTTGATGCTTATTACACGTCC-3′. Thirty-five of the following cycles were used during PCR: 96° for 60 sec; 64° for 60 sec; 70° for 300 sec. The primers bracket a 1.687-kb region that extends both 55 nucleotides to the left of the initiation codon of T3 gene 17 and 13 nucleotides to the right of the termination codon of T3 gene 17.

Production of hybrid bacteriophages

To insert portions of T3 gene 17 in the gene 17 of a complete T7 genome, the following were incubated at 30° for 90 min in an extract of T7-infected cells (20 μl): DNA from $T7_{17}$ (2 μl of 580 μg/ml) and the T3 gene 17 PCR fragment (2 μl of 240 μg/ml). When indicated, either the PCR fragment or the T7 DNA was completely digested with restriction endonuclease *AlwI*, an enzyme that produces DNA fragments that have a single base 5′ extension. The *AlwI* digestion was performed by the procedure recommended by the manufacturer (New England Biolabs, Beverly, MA). The extract used was a mixed extract that consisted of two different extracts (equal volumes), each prepared by the procedure of Son *et al.* (1988). The following mixed extracts were used (a mixed extract is identified by the infecting bacteriophages used to prepare each of the component extracts): (1) $T7_{4,9}$ + $T7_{5,19}$, or (2) $T7_{4,9}$ + $T7_{5,17}$. The mixture used is identified in Table 1. Because the above incubation in a mixed extract both incorporates a DNA fragment into a mature genome and packages the hybrid genome to form an infective bacteriophage *in vitro,* the processes involved will be collectively called *in vitro* recombination–packaging.

After *in vitro* recombination–packaging, images of plaques formed by progeny bacteriophages were recorded by video capture (Griess *et al.*, 1995) of light scattering. To identify hybrid bacteriophage plaques, the contents of plaques were transferred to a nitrocellulose filter. The filter was probed at 53 ± 0.5°; the filter was washed at 57 ± 0.5°, by use of the following oligonucleotide that had been ^{32}P-labeled by use of polynucleotide kinase (Son *et al.*, 1993): 5′-TCAGGCAGTTGTAGCCACTGAT-3′. If the first nucleotide in the initiation codon of T3 gene 17 is called number 1, then this oligonucleotide probe is complementary to nucleotides 1305–1326 on the 5′ left-oriented strand of Dunn and Studier (1983). After probing, the nitrocellulose filter was subjected to autoradiography.

Agarose gel electrophoresis of intact bacteriophage particles

To perform analytical one-dimensional nondenaturing gel electrophoresis, samples were prepared by diluting bacteriophage particles suspended in storage buffer (13 μl) with a solution of 6% sucrose, 400 μg/ml bromophenol blue (12 μl). One-dimensional electrophoresis was performed through submerged, horizontal gels made of Seakem LE agarose (FMC Bioproducts, Rockland, ME). Gels were cast in Tris/Mg electrophoresis buffer: 0.09 *M* Tris–acetate, pH 8.4, 0.001 *M* $MgCl_2$. After casting of a gel at room temperature (22 ± 3°C), a diluted sample was layered in a sample well; electrophoresis was performed at 2 V/cm, room temperature, for the time indicated in the figure legends.

To determine electrophoretic mobility (μ) as a function of gel concentration, nine gels of different concentrations (running gels) were embedded within a single frame of Seakem LE agarose in Tris/Mg electrophoresis buffer (the procedure is described in Serwer, 1980); electrophoresis of a sample was performed simultaneously through all nine running gels at 2 V/cm. The temperature was controlled ±0.5° at 23° by use of two Peltier cells (Serwer and Dunn, 1990). The use of embedded gels minimizes differences in both electrical potential gradient and temperature among the gels used to determine μ as a function of gel concentration.

To determine whether particles were adhering to the gel during nondenaturing gel electrophoresis, a first-dimensional electrophoresis was followed by a second-dimensional electrophoresis performed by use of the same conditions for both dimensions (1.5% Seakem LE agarose in Tris/Mg electrophoresis buffer; 23 ± 3°, 2 V/cm). If and usually only if adherence is absent, the gel profile has all particles on a straight line that passes through the origin of electrophoresis (Serwer *et al.*, 1986). After either one- or two-dimensional nondenaturing gel electrophoresis, bacteriophages were detected by staining with both the DNA-specific stain, ethidium, and the protein-specific stain, Coomassie blue (Serwer *et al.*, 1986).

When determined during gel electrophoresis, values of μ depend primarily on two characteristics of a spherical particle that does not adhere to the gel: (1) the average electrical surface charge density, a characteristic propor-

[2] Abbreviations used: PCR, polymerase chain reaction; μ, electrophoretic mobility; μ_0', μ extrapolated to a gel concentration of 0; μ_0, μ_0' corrected for electro-osmosis; SDS, sodium dodecyl sulfate.

TABLE 1

Characterization of Hybrids

Name of bacteriophage[a]	Extract, DNA used for isolation[b]	$-\mu_o{}^c$ (cm^2/V.S × 10^{-4}) ± 0.1
A1 Hybrid	1: $T7_{4,9}$ + $T7_{5,19}$ No digestion	0.64 0.57
A1 Hybrid	9: $T7_{4,9}$ + $T7_{5,17}$ *Alw*I (T3 PCR)	
A1 Hybrid	1: $T7_{4,9}$ + $T7_{5,17}$ *Alw*I ($T7_{17}$)	
A2 Hybrid	1: $T7_{4,9}$ + $T7_{5,17}$ *Alw*I (T3 PCR)	0.55[d]
B1 Hybrid	1: $T7_{4,9}$ + $T7_{5,17}$ *Alw*I (T3 PCR)	
B2 Hybrid	1: $T7_{4,9}$ + $T7_{5,17}$ *Alw*I (T3 PCR + $T7_{17}$)	
T3	—	0.92

[a] Class A hybrids formed bands during nondenaturing electrophoresis through a gel formed by underivatized (Seakem LE) agarose; class B hybrids formed a broad zone, like that of wild-type T7, during the same analysis. The numbers indicate the order of isolation. The plaques of B hybrids did not differ in size from the plaques of wild-type T7; the plaques of both the A1 hybrid (legend to Fig. 1) and the A2 hybrid were slightly larger.

[b] The number of independent isolations is indicated for each mutant; after this number, the extract used for *in vitro* recombination–packaging is indicated (Materials and Methods); finally, the DNAs are indicated that were *Alw*I-digested.

[c] Determined from a plot of μ vs gel concentration, according to procedures described under Materials and Methods. The A1 hybrid has two μ_o's, one for each band.

[d] Even though it is smaller than the experimental error for determining μ_o, the difference in μ_o between the A2 hybrid and both A1 hybrid particles has been confirmed by co-electrophoresis in uniform-concentration slab gels (see Fig. 4).

tional to μ that is both extrapolated to a gel concentration of 0 (μ_0') and corrected for electro-osmosis (μ_0; Serwer, 1983) and (2) the radius (or effective radius, if a particle's shape is not precisely spherical; Shaw, 1969; Serwer and Pichler, 1978). The slope of a semilogarithmic μ vs gel concentration plot increases in magnitude as the effective radius of a particle increases (reviewed in Tietz, 1987). The radius of both T7 and T3 wild-type bacteriophages is 30.1 nm (Stroud *et al.,* 1981; Serwer *et al.,* 1983).

To perform preparative agarose gel electrophoresis of bacteriophage particles, one-dimensional electrophoresis was performed through a 0.9% Seakem LE agarose gel cast in electrophoresis buffer; electrophoresis was performed at 2 V/cm, room temperature, for 24 hr. Particles were detected by visual observation of light scattering; the amount of sample was 140 μg. After preparative electrophoresis, fractions were collected by excising pieces of gel from the regions indicated in the text.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed by use of procedures described in Studier (1973). A 12.5% polyacrylamide gel was used. Staining was performed by use of either Coomassie blue (Serwer *et al.,* 1983) or silver (Wray *et al.,* 1981). When analysis was performed of particles in a piece of agarose gel from a preparative agarose gel electrophoresis, the particles were eluted from the gel by diffusion, before analysis.

Restriction endonuclease analysis

By use of conditions recommended by the manufacturer (New England Biolabs), the purified DNA of bacteriophages was digested by one of the following restriction endonucleases: *Hpa*I, *Ssp*I, *Sfi*I, *Msc*I, and *Bgl*I. The digested DNA (100 ng in 20 μl) was added to the following solution (10 μl): 0.003 *M* sodium EDTA, pH 7.4, 20% sucrose, 800 μg/ml bromphenol blue. This mixture was layered in the sample well of a horizontal submerged agarose slab gel, cast in Tris/EDTA electrophoresis buffer: 0.09 *M* Tris–acetate, pH 8.4, 0.001 *M* EDTA. Electrophoresis was performed at room temperature; the time, electrical potential gradient, and gel concentration are indicated in figure legends.

Determination of nucleotide and amino acid sequences

Nucleotide sequences were determined by use of cycle sequencing (Murray, 1989; Carothers *et al.,* 1989) with Vent(exo$^-$) DNA polymerase, used according to the instructions of the manufacturer (New England Biolabs) with 5′-^{33}P-end-labeled primers. To reduce compression of sequencing ladders, 7-deaza-dGTP was used, instead of dGTP. Amino acid sequences were determined by the use of both the nucleotide sequence and the standard

genetic code. On either side of an insertion point of T3 DNA in T7 DNA, both DNA strands were sequenced. When compared to the T3 gene 17 nucleotide sequence that was previously obtained (Yamada *et al.,* 1986) for the Luria T3 strain (T3 strains are reviewed in Studier, 1979), the sequence obtained here with the Hausmann T3 strain (Fig. 3a) differs at 44 positions. Of these differences, 16 were part of two-member palindromes; the T3 sequence at these palindromes was determined twice on each strand. The sequence of $T7_{17}$ agreed with the published (Dunn and Studier, 1983) sequence of wild-type T7, except at the position of the amber codon; the position of the amber codon is indicated under Results. All sequences reported here were obtained in the present study.

RESULTS

Construction of hybrids

In vitro recombination–packaging was used to construct a viable T7 genome that had incorporated a fragment of T3 DNA. The T3 fragment was a PCR fragment that encompassed the T3 (and T7) gene that encodes the tail fiber (gene 17; Dunn and Studier, 1983). This PCR fragment had the capacity of transferring to T7 the following desired tail fiber-associated phenotype of T3: nonadherence to agarose gels during agarose gel electrophoresis. Tail fiber-dependent adherence to agarose gels causes undesirable spreading of gel electrophoretic bands of intact (infective) T7, but not T3 (Serwer and Hayes, 1982; Serwer *et al.,* 1983). To increase the probability that the observed progeny of the *in vitro* reaction have incorporated the T3 PCR fragment, the mature-length T7 DNA was obtained from $T7_{17}$; progeny were plated on a host nonpermissive for amber mutants. After plating either T3 (Fig. 1a), T7 (Fig. 1b), or the progeny of the *in vitro* reaction (Fig. 1c), plaques were first transferred to a nitrocellulose filter and then probed with a ^{32}P-labeled oligonucleotide 22-mer (Materials and Methods) that was completely homologous to a sequence within T3 gene 17, but had a two base pair mismatch with the corresponding sequence of bacteriophage T7. This probe produced a signal from T3 plaques (Fig. 1d) that was higher than the signal from T7 plaques (Fig. 1e). Quantification, by use of a phosphorimager, revealed the ratio of the two signals to be 20 ± 3. Of the plaques formed by progeny of the *in vitro* extract, most yielded a T7-like signal after probing. However, some plaques yielded a higher, T3-like signal (Fig. 1f; a plaque with a T3-like signal is indicated by an arrow). When four independent experiments were performed, each producing 1500 randomly screened plaques, the number of plaques with a T3-like signal was 1;2;2;1. Based on its nondenaturing gel electrophoretic phenotype described below, the hybrid isolated in Fig. 1 will be called a class A hybrid.

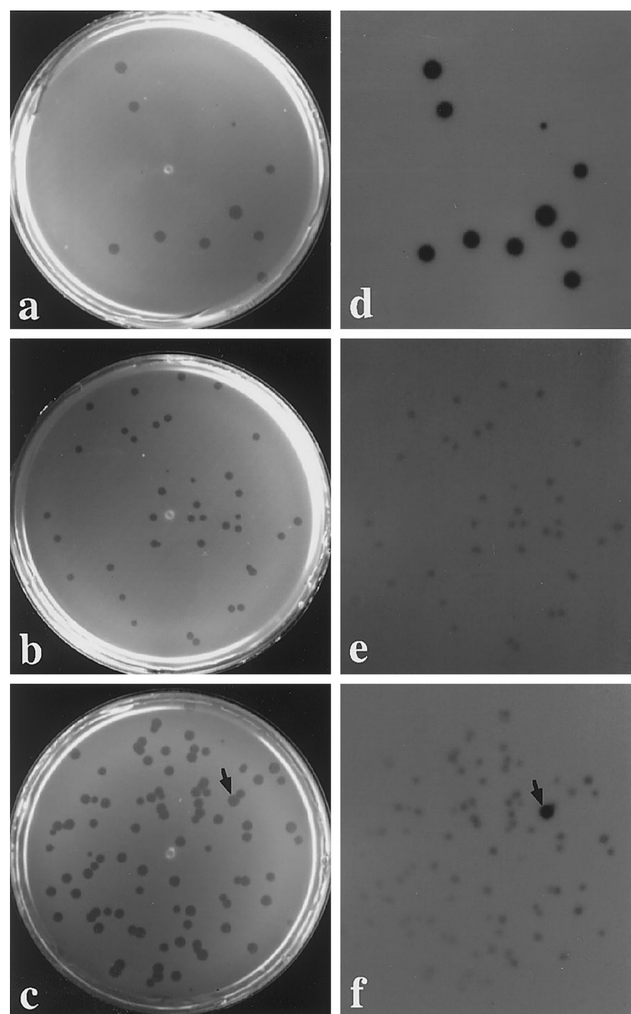

FIG. 1. Production of a hybrid bacteriophage. After incubation of both the T3 PCR fragment and intact $T7_{17}$ DNA together in a $T7_{4,9}$ + $T7_{5,19}$ extract, plaques formed by the progeny of the *in vitro* recombination–packaging were both transferred and probed. Light scattering in shown from plaques formed by the following: (a) bacteriophage T3, (b) bacteriophage T7, and (c) the progeny of *in vitro* recombination–packaging. After probing, autoradiography of the nitrocellulose filter is shown for the following: (d) T3, (e) T7 and (f) progeny of *in vitro* recombination–packaging. As seen qualitatively, side-by-side quantitative comparison (diameters of 150 plaques measured) revealed that T3 plaques were larger by a factor of 1.37 ± 0.12 than T7 plaques; the hybrid plaques were larger by a smaller factor, 1.18 ± 0.11. Because the hybrid is shown to undergo less binding than T7 to agarose, the slightly larger plaque size of the hybrid is explained by reduced binding to agar during formation of a plaque. Agarose is a subfraction of agar (reviewed in Serwer, 1983). The comparatively large size of the T3 plaques appears to be only partially explained by reduced binding to agar.

In an attempt to both increase the efficiency of transfer and force variability in the hybrid isolated, *in vitro* transfer was also attempted after cutting either the T3 PCR fragment or the mature T7 DNA with restriction endonuclease *AlwI*, an enzyme that cuts only in gene 17 (for both T3 and T7, the cleavage sites are indicated by arrowheads in Fig. 3). When the donor T3 PCR fragment was *AlwI*-digested before *in vitro* recombination–packaging, the

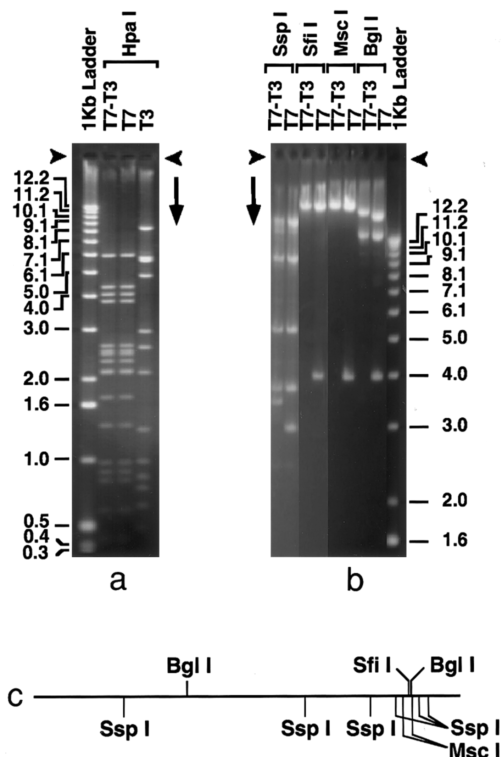

FIG. 2. Restriction endonuclease analysis of a hybrid bacteriophage. Restriction endonuclease digests (the restriction endonuclease is indicated above each lane) were analyzed by agarose gel electrophoresis for T7, T3 (indicated above a lane), or the hybrid isolated in Fig. 1 (indicated by T7-T3 above the corresponding lanes). The conditions of electrophoresis were (a) 1.2% agarose, 1.0 V/cm, 20 hr, and (b) 0.7% agarose, 0.7 V/cm, 22 hr. The arrow indicates the direction of electrophoresis; the arrowheads indicate the origins of electrophoresis. (c) A map of restriction endonuclease cleavage sites in wild-type T7 DNA.

efficiency of hybrid formation was 0.7%, the highest efficiency that has yet been observed. Although a hybrid was observed when the recipient $T7_{17}$ DNA was *Alw*I-digested, the frequency was low enough to be difficult to measure (<0.1%). A single hybrid was isolated when both the T7 genome and the T3 PCR fragment were digested with *Alw*I. For further studies, hybrids were subjected to two successive clonal isolations; the hybrid bacteriophage particles were purified.

The genotype of hybrids

To determine whether hybrid genomes had, as expected, primarily a T7 nucleotide sequence, DNA from a purified hybrid bacteriophage was digested with restriction endonuclease *Hpa*I. This enzyme cleaves T7 DNA at 18 sites, only one of which is in gene 17 (Rosenberg *et al.,* 1979). For the *Hpa*I fragments of the hybrid of Fig. 1, agarose gel electrophoresis revealed a pattern (T7-T3 lane in Fig. 2a) that was indistinguishable from the pattern for T7 (T7 lane in Fig. 2a); the *Hpa*I pattern for T3 (T3 lane in Fig. 2a) was different from the *Hpa*I pattern for T7. This analysis was repeated by use of four other restriction endonucleases that cleave in T7 gene 17, *Ssp*I, *Sfi*I, *Msc*I, and *Bgl*I. For all of these latter restriction endonucleases, a band present in the digest of T7 DNA was absent from the digest of the hybrid DNA (appropriate lanes under enzymes indicated in Fig. 2b). As found for the hybrid analyzed in Fig. 2, for each of the other hybrids isolated (hybrids are listed in Table 1), the *Hpa*I pattern was that of T7. Cleavage in gene 17 by the other restriction endonucleases occurred if and only if the DNA sequence presented below had the known recognition sequence for the restriction endonuclease (data not shown). Thus, all of the hybrid genomes have a nucleotide sequence that is T7, except for an alteration, presumably an insertion of T3 DNA, in T7 gene 17.

To determine whether a hybrid genome had a T3 insert in T7 gene 17, the nucleotide sequence of gene 17 was determined. Although most nucleotides in T7 gene 17 (Dunn and Studier, 1983) are the same as their counterparts in T3 gene 17 (Yamada *et al.,* 1986), some are different. These nonidentical nucleotides serve as markers for the region of the hybrid genome derived from T3. If the hybrid has the T3 version of a nucleotide in a position of nonidentical nucleotides, then the nucleotide at this position is called T3-specific; if the hybrid has the T7 version, then the nucleotide at this position is called T7-specific. A region that has several T3-specific nucleotides, but no T7-specific nucleotides, will be called a T3 block; a T7 block is similarly defined. All nucleotides of a T3 block are assumed to have been transferred to the hybrid via the PCR fragment, even though most of these nucleotides are the same for T7 as they are for T3. By use of this convention, the only ambiguous region is the string of nucleotides between a T7-specific nucleotide from one block and a T3-specific nucleotide from a neighboring block. The source of this string of nucleotides (T3 PCR fragment or T7 genome) cannot be determined, thereby limiting resolution in the determination of the position of crossover.

For comparison with the nucleotide sequence of hybrids, the nucleotide sequence of the gene 17 of both $T7_{17}$ and T3 was determined. In the top row of Fig. 3a, the nucleotide sequence of $T7_{17}$ gene 17 is presented. In the second row, the nucleotide sequence of T3 gene 17 is presented, but only at positions for which the T3 nucleotide differs from the corresponding T7 nucleotide (i.e., the T3-specific nucleotides are presented). Presumably because of strain differences, the T3 nucleotide sequence differs slightly from the sequence reported in Yamada *et al.* (1986) (see Materials and Methods). If a T3 nucleotide does not have a T7 counterpart, a dash replaces a letter in the T7 sequence; if a T7 nucleotide does not have a T3 counterpart, a dash is placed above the T7 nucleotide. The numbering system used for all DNAs places position 1 at the first nucleotide of the initiation codon of gene 17 (position 34,623 in the nucleotide sequence of Dunn and Studier, 1983). The direction

a

```
ATGGCTAACGTAATTAAAACCGTTTTGACTTACCAGTTAGATGGCTCCAATCGTGATTTTAATATCCCGTTTGAGTATCTAGCCCGTAAGTTCGTAGTGG   100  T7_17
                                                                                                 A          T3
TAACTCTTATTGGTGTAGACCGAAAGGTCCTTACGATTAATACAGACTATCGCTTTGCTACACGTACTACTATCTCTCTGACAAAGGCTTGGGGTCCAGC   200  T7_17
   C        C        C                  G     C T      G        C    A T C                                  T3
CGATGGCTACACGACCATCGAGTTACGTCGAGTAACCTCCACTACCGACCGATTGGTTGACTTTACGGATGGTTCAATCCTCCGCGCGTATGACCTTAAC   300  T7_17
G      A    T              C              A                                       T                         T3
       ※
GTCGCTTAGATTCAAACGATGCACGTAGCGGAAGAGGCCCGTGACCTCACTACGGATACTATCGGTGTCAATAACGATGGTCACTTGGATGCTCGTGGTC   400  T7_17
      C                                                  G  T    C  A          T        T                   T3
GTCGAATTGTGAACCTAGCGAACGCCGTGGATGACCGCGATGCTGTTCCGTTTGGTCAACTAAAGACCATGAACCAGAACTCATGGCAAGCACGTAATGA   500  T7_17
           T              T               C                     T                    G     G G\_ _ _/       T3
AGCCTTACAGTTCCGTAATGAGGCTGAGACTTTCAGAAACCAAGCGGAGGGCTTTAAGAACGAGTCCAGTACCAACGCTACGAACACAAAGCAGTGGCGC        600  T7_17
G  AC                                   T   A     TT       T     G     T                            A         T3
G  AC                                   T   A     TT       T     G     T                            A         A1
G  AC                                   T   A     TT       T     G     T                            A         A2
GATGAGACCAAGGGTTTCCGAGACGAAGCCAAGCGGTTCAAGAATACGGCTGGTCAATACGCTACATCTGCTGGGAACTCTGCTTCCGCTGCGCATCAAT        700  T7_17
    G  T  T GC     T     G   A                                                    A T                         T3
    G  T  T GC     T     G   A                                                    A T                         A1
    G  T  T GC     T     G   A                                                    A T                         A2
CTGAGGTAAACGCTGAGAACTCTGCCACAGCATCCGCTAACTCTGCTCATTTGGCAGAACAGCAAGCAGACCGTGCGGAACGTGAGGCAGACAAGCTGGA        800  T7_17
                         C  T     G A  G       GA            A  C                    A             G          T3
                         C  T     G A  G       GA            A  C                    A             G          A1
                         C  T     G A  G       GA            A  C                   GA             G          A2
                         A  T                                                                                 B1
                         A  T                                                                                 B2
AAATTACAATGGATTGGCTGGTGCAATTGATAAGGTAGATGGAACCAATGTGTACTGGAAAGGAAATATTCACGCTAACGGGCGCCTTTACATGACCACA        900  T7_17
G   TT      C               C G   G                             GG    C  T TG     A        C  T  T            T3
G   TT      C               C G   G                             GG    C  T TG     A        C  T  T            A1
G   TT      C               C G   G                             GG    C  T TG     A        C  T  T            A2
AACGGTTTTGACTGTGGCCAGTATCAACAGTTCTTTGGTGGTGTCACTAATCGTTACTCTGTCATGGAGTGGGGAGATGAGAACGGATGGCTGATGTATG       1000  T7_17
G T   C     T                               TCTG  GG                        T                    C            T3
G T   C     T                               TCTG  GG                        T                    C            A1
G T   C     T                               TCTG  GG                        T                    C            A2
TTCAACGTAGAGAGTGGACAACAGCGATAGGCGGTAACATCCAGTTAGTAGTAAACGGACAGATCATCACCCAAGGTGGAGCCATGACCGGTCAGCTAAA       1100  T7_17
                              T A         C            T     G         A     T    C   G                       T3
                              T A         C            T     G         A     T    C   G                       A1
                              T A         C            T     G         A     T    C   G                       A2
ATTGCAGAATGGGCATGTTCTTCAATTAGAGTCCGCATCCGACAAGGCGCACTATATTCTATCTAAAGATGGTAACAGGAATAACTGGTACATTGGTAGA       1200  T7_17
           A    CC   T C                        A                        A C                                  T3
           A    CC   T C                        A                        A C                                  A1
           A    CC   T C                        A                        A C                                  A2
                      \_ _ _ _ _ _ _ _ _ _ _ _/A                         A C                                  B1
GGGTCAGATAACAACAATGACTGTACCTTCCACTCCTATGTACATGGTACGACCTTAACACTCAAGCAGGACTATGCAGTAGTTAACAAACACTTCCACG       1300  T7_17
  A     ▲                                GT         A          C                      G                       T3
  A                                      GT         A          C                      G                       A1
  A                                      GT         A          C                      G                       A2
  A                                      GT         A          C                      G                       B1
  A                                      GT         A     G    C                      G                       B2
                                                     \_ _ _ _ /C                      G
TAGGTCAGGCCGTTGTGGCCACTGATGGTAATATTCAAGGTACTAAGTGGGGAGGTAAATGGCTGGATGCTTACCTACGTGACAGCTTCGTTGCGAAGTC      1400  T7_17
            A    A                                      G     T              AAC  T CT A    AA   A           T3
            A    A\_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _/                                                     A1
            A    A\_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _/                                                     A2
            A    A\_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _/                                                     B1
            A    A\_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _/                                                     B2
                                                                                       ▼
CAAGGCGTGGACTCAGGTGTGG--T-CTGGTAGTGCTGGC--GGTGGG-GTAAG-TGT--GACTG-----TTTCACAGGATCTCCGCTTCCGCAATATCT      1486  T7_17
A T  C        A  A  GC G   C     G A TTACA    A G G T C CA     ATACTC C_    _  CT  G  A          C  A         T3
GGATTAAGTGTGCCAACAACTCTTGGAACTTCTTCCGTACTGGCCCCGATGGAATCTACTTCATAGCCTCTGATGGTGGATGGTTACGATTCCAAATACA      1586  T7_17
        ACCAGA        A          _A     T  T  C  T          C TT AG C    C  T   C  AA     G                   T3
CTCCAACGGTCTCGGATTCAAGAATATTGCAGACAGTCGTTCAGTACCTAAT-GCAATCATGGTGGAGAACGAGTAA                              1662  T7_17
   T T CAGG T  T     C  A  G  T   A A GC ACT C  A     AGCC  A  G  T                                         1674  T3
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b

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MANVIKTVLTYQLDGSNRDFNIPFEYLARKFVVVTLIGVDRKALTINTDYRFATRTTISLTKAWGPADGYTTIELRRVTSTTDRLVDFTDGSILRAYDLN   100  T7
                                                   A                                                         T3
VAQIQTMHVAEEARDLTTDTIGVNNDGHLDARGRRIVNLANAVDDRDAVPFGQLKTMNQNSWQARNEALQFRNEAETFRNQAEGFKNESSTNATNTKQWR   200  T7
         A                                                                  T V    G                         T3
                                                                            T V    G                         A1
DETKGFRDEAKRFKNTAGQYATSAGNSASAAHQSEVNAENSATASANSAHLAEQQADRAEREADKLENYNGLAGAIDKVDGTNVYWKGNIHANGRLYMTT   300  T7
 AN S   EQ            T           A   N   H    E   GF      R        G  V    LS                               T3
 AN S   EQ            T           A   N   H    E   GF      R        G  V    LS                               A1
                                               G                                                             A2
                             TT                                                                              B1
                             TT                                                                              B2
NGFDCGQYQQFFGGVTNRYSVMEWGDENGWLMYVQRREWTTAIGGNIQLVVNGQIITQGGAMTGQLKLQNGHVLQLESASDKAHYILSKDGNRNNWYIGR   400  T7
D          SAG         H         D       H  A  D   P      A Y     Q                                          T3
D          SAG         H         D       H  A  D   P      A Y     Q                                          A1
                                                                  Q                                          B1
GSINNNDCTFHSYVHGTTLTLKQDYAVVNKHFHVGQAVVATDGNIQGTKWGGKWLDAYIRDSFVAKSKAWTQVWSGSAGGGV-SVTVSQDLRFRNIWIKC   500  T7
          Y N    P       R                               N TY K TM     AAAS  SYMGGGSQTDT  HRTCDSATY          T3
          Y N    P       R                                                                                   A1
          Y N    P       R                                                                                   B1
                 P       R                                                                                   B2
ANNSWNFFRTGPDGIYFIASD---GGWLRFQIHSNGLGFKNIADSRSVPNAIMVENE                                             553  T7
GLRPETTIG  SSELVLTVSTSFQPRR   K      RV      RDAT  I A  DV                                              557  T3
```

FIG. 3. The nucleotide and amino acid sequences. (a) In the first line, for $T7_{17}$, the complete nucleotide sequence of gene 17 is shown; in the second line, a T3 nucleotide is shown if it differs from the corresponding $T7_{17}$ nucleotide; in subsequent lines, a hybrid nucleotide is shown if it differs from the corresponding $T7_{17}$ nucleotide (the hybrid name is indicated at the right). The complete gene 17 of all bacteriophages indicated at the right was sequenced here. The position of the amber mutation in $T7_{17}$ DNA is indicated by an asterisk; T7 blocks for the A1 and A2 hybrids are indicated by boldface letters in the $T7_{17}$ sequence. For each hybrid, the horizontal, dashed lines indicate the string of nucleotides between a T3 block and a T7 block. The restriction endonuclease *AlwI* cleavage sites are indicated by an arrowhead for both T3 and T7 DNA.

of numbering is left to right in the genetic map (Studier and Dunn, 1983); the sequence of the 5′-left oriented strand is given.

In the third row of Fig. 3a, the nucleotide sequence of the type A hybrid isolated in Fig. 1 (to be called the A1 hybrid) is presented, but only at positions for which the hybrid nucleotide differs from the T7 nucleotide. For this hybrid, inspection of Fig. 3a reveals that, with one exception, the nucleotides form the following three blocks: A T7 block is at both ends of gene 17 (boldface letters in the T7 sequence of Fig. 3a); a T3 block is inserted in between the two T7 blocks. The nucleotides between the T3 block and either T7 block are indicated by a horizontal dashed line in Fig. 3a. In gene 17 of the A1 hybrid, all non-T7 nucleotides are T3-specific nucleotides. Thus, during insertion, the PCR fragment was apparently truncated at both ends and inserted, without additional changes in nucleotide sequence. The exception to the division of the hybrid sequence into three blocks is at the position of the amber mutation in gene 17 (position 307; asterisk in Fig. 3a). At position 307, the amber mutation was reverted by the presence of a T3-specific nucleotide (T → C; thereby converting the amber codon to a codon for glutamine). However, this (and only this) one T3-specific nucleotide was not part of the T3 block. This latter observation is explained by the assumption that, in addition to insertion of a segment of the PCR fragment, a second event has occurred to change the nucleotide sequence of the $T7_{17}$ genome; this second event probably is reversion of the amber mutation.

In an attempt to obtain hybrids that had a different insert, a T3 PCR fragment (from an independent PCR) was digested with restriction endonuclease *Alw*I before *in vitro* recombination–packaging. However, the A1 hybrid was again isolated. Nine of 11 independent isolates yielded a hybrid that had a gene 17 nucleotide sequence identical to that determined for the A1 hybrid in Fig. 3a. Because the T3 PCR fragment had been cleaved internally to the A1 insert and the cleavage caused an increase in recombination–packaging efficiency (see previous section), these results suggest that (1) the PCR fragment had been rejoined either before or during incorporation, and (2) selective pressure was exerted for this rejoining.

Of the remaining two hybrids isolated after *Alw*I digestion of the T3 PCR fragment, the first (A2 hybrid) had the same collection of both T7-specific and T3-specific nucleotides that were present in the A1 hybrid. However, a single nucleotide (G, indicated in italics at position 785 in Fig. 3a) was neither a T7-specific nor a T3-specific nucleotide. Apparently, an independent mutation was introduced in the A2 hybrid. The second of the remaining hybrids (B1 in Fig. 3a) had an insert with a right end indistinguishable from the right end of the type A hybrids. However, the leftmost T3-specific nucleotide was at position 1152 (Fig. 3a), closer to the rightmost T3-specific nucleotide than it was for the type A hybrids. This new hybrid is placed in class B because of phenotypic characteristics to be described below. From an independent experiment in which both $T7_{17}$ DNA and the T3 PCR fragment had been *Alw*I-digested, a second class B hybrid (B2 hybrid in Fig. 3a and Table 1) was isolated. The B2 hybrid had an insert even shorter than the insert of the B1 hybrid (left end at position 1268; Fig. 3a). Both the B1 and the B2 hybrids had independent mutations within a T7 block (indicated by letters in italics at positions 724 and 728); the B1 hybrid had an additional mutation at position 1260 (indicated in italics in Fig. 3a).

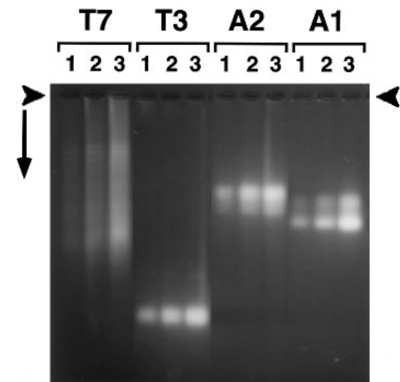

FIG. 4. Nondenaturing agarose gel electrophoresis. Electrophoresis was performed for 28 hr through a 1.2% gel. The bacteriophage is indicated at the top (A1 and A2 indicate hybrids described in Table 1). The amount of bacteriophage was (in μg) (lane 1) 0.15, (lane 2) 0.45, (lane 3) 1.4. The arrow indicates the direction of electrophoresis; the arrowheads indicate the origins of electrophoresis.

The nucleotide sequences of Fig. 3a were used to obtain the amino acid sequences of the protein product of gene 17 (p17) for each of the hybrids. These results are shown in Fig. 3b. Because of a shorter C-terminal region, the T7 p17 is four amino acids shorter than the T3 p17. The mutational changes (i.e., those not explained by insertion of a T3 nucleotide sequence within T7 gene 17) all produced changes in amino acid sequence, except for the change at position 1260 in the B2 hybrid (Fig. 3b).

The nondenaturing gel electrophoretic phenotype of the hybrids

To determine whether either the A1 or the A2 hybrid bacteriophage had the T3 agarose gel-adherence phenotype, nondenaturing agarose gel electrophoresis was performed of intact particles of both a hybrid bacteriophage and each of the two parents, T7 and T3. After electrophoresis through a 1.2% agarose gel cast in Tris/Mg electrophoresis buffer, staining with the DNA-specific stain, ethidium, revealed that bacteriophage T3 formed a band whose position did not vary as the amount of T3 increased from 0.15 to 1.4 μg (lanes marked T3 in Fig. 4). In contrast, bacteriophage T7 formed a broad zone

when used at the same concentrations (lanes marked T7 in Fig. 4). The hybrids did not form the broad zone formed by T7; the A1 hybrid formed two distinguishable, but partially overlapping, bands, both of which were closer to the origin than the band formed by T3 (Fig. 4; lanes marked A1). The A2 hybrid formed a band even closer to the origin of electrophoresis (Fig. 4; lanes marked A2). In Fig. 4, the band of the A2 hybrid is significantly skewed to the origin-distal side. This skewing was reproducible, suggesting the presence of at least a second, unresolved band for the A2 hybrid. Because the broadening of the zone occupied by T7 particles is caused by adherence to the agarose gel (Serwer and Hayes, 1982), the formation of bands by the A hybrids is explained by the assumption that the gel-nonadherence phenotype of T3 has been transferred to both the A1 and the A2 hybrids. That no irreversible adherence occurred for the A1 hybrid was confirmed by the following two-dimensional electrophoresis: electrophoresis in both dimensions was the same (Materials and Methods). The mobility in the first dimension was identical to the mobility in the second (data not shown).

The experiment of Fig. 4 was repeated for the B1 and B2 hybrids. In contrast to the A1 and A2 hybrids, both the B1 and the B2 hybrids yielded results indistinguishable from those obtained for bacteriophage T7 in Fig. 4 (data not shown). That is, the gel-nonadherence phenotype was not transferred to the class B hybrids. Thus, the determinant(s) for the gel-nonadherence phenotype is between nucleotides 501 and 1125 (Fig. 3a).

To determine why A1 hybrid particles formed two bands during nondenaturing gel electrophoresis, μ was determined as a function of gel concentration for both band-forming particles. When μ was extrapolated to a gel concentration of 0, the two band-forming A1 hybrid particles were found to differ in μ_0' and, therefore, μ_0 (plots indicated by A1 in Fig. 5). Thus, these particles also differ in average electrical surface charge density. Because the slopes of the two A1 plots in Fig. 5 do not significantly differ, the effective radii of the two band-forming A1 hybrid particles are the same (±5%). Because these two particles differ in μ_0, but not in effective radius, they do not differ by state of aggregation. For a μ vs gel concentration plot of a bacteriophage dimer, the slope would be greater than the slopes in Fig. 5 (Serwer, 1980). By this reasoning, the single A2 hybrid particle also does not detectably differ in effective radius from the two A1 hybrid particles; it does, however, differ significantly in μ_0 from both of the A1 hybrid particles (Table 1). The difference in μ_0 (more negative for the A1 hybrid) is opposite in sign to the difference that is predicted from the difference in the net charge on amino acid side chains; the A2 hybrid has one more negative charge per p17 (glutamic acid at position 262) than the A1 hybrid has (glycine at position 262). Thus, the difference in μ_0 must be caused by movement of electrically charged amino

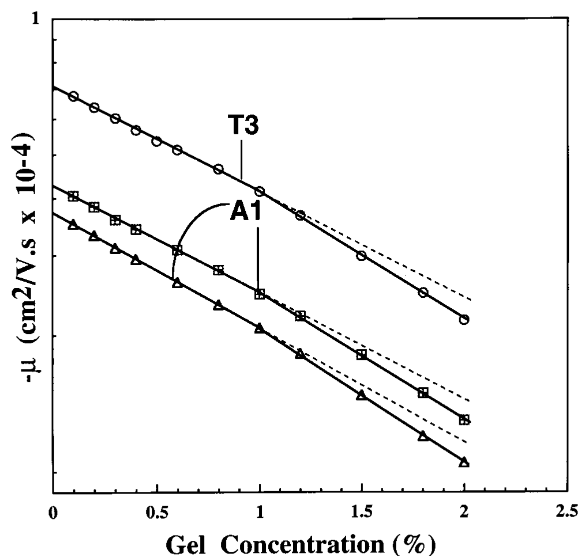

FIG. 5. Quantitative analysis of μ for the A1 hybrid. By use of procedures described under Materials and Methods (time of electrophoresis-22 hr), μ was determined as a function of gel concentration for both bacteriophage T3 and the two electrophoretically distinguished particles of the A1 hybrid (plots are labeled in the figure); all three particles had been in a single mixture, so that only one set of running gels was used. Semilogarithmic μ vs gel concentration plots for all three particles are presented.

acid side chains either to or from the surface, not by a change in the total number of side chains that are electrically charged.

Protein composition and infectivity of bacteriophage particles

The specific infectivity of the purified A1 hybrid bacteriophage was 0.34 ± 0.08 plaque-forming units per bacteriophage particle (11 independent preparations), not significantly different from that for T7 (0.40); the A2, B1, and B2 hybrids were comparably infective. Nonetheless, the absence from a hybrid was possible for some of the six p17-containing tail fibers shown (Steven *et al.*, 1988) to be on wild-type bacteriophage T7. To determine whether the intact hybrid bacteriophages had the amount of p17 present on either T7 or T3, the proteins of these three bacteriophages were analyzed by use of SDS–polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. Each T7 protein has a T3 analog, although some differences exist in the mobility of analogous proteins. The T3 p17 (lane marked T3 in Fig. 6) migrated 3.5% more rapidly than the T7 p17 (lane marked T7 in Fig. 6). Apparently, the greater length of the T3 p17 did not compensate for some other (unknown) difference that caused T3 p17 to migrate more rapidly than T7 p17. Both A hybrids had a p17 that migrated like T3 p17; both B hybrids had a p17 that migrated like T7 p17 during SDS–polyacrylamide gel electrophoresis (lanes marked by hybrid names in Fig. 6). In confirmation of the qualita-

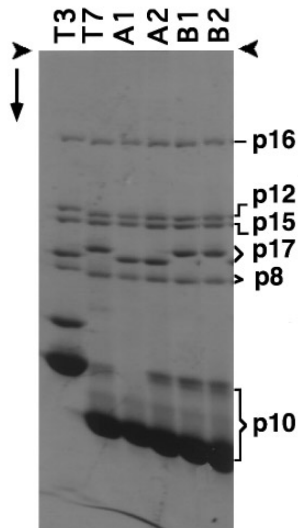

FIG. 6. SDS–polyacrylamide gel electrophoresis. The bacteriophage indicated above each lane was subjected to SDS–polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. The arrow indicates the direction of electrophoresis; the arrowheads indicate the origins of electrophoresis.

tive conclusion drawn from inspection of Fig. 6, densitometry revealed that, in relation to p8 (the protein that connects the T7 head to its tail), the amount of p17 in the hybrids was the same as the amount of p17 in either T7 or T3 (±10%).

To compare directly the protein compositions of the particles that form each of the two A1 hybrid bands in Fig. 4, a preparative separation of these two particles was performed by agarose gel electrophoresis; most infectivity was lost for both particles during electrophoresis. After this separation, SDS–polyacrylamide gel electrophoresis, followed by silver staining, was performed on the separated particles. The result was no significant difference in the amount of p17 between the particles that had formed the origin-proximal band and the particles that had formed the origin-distal band during agarose gel electrophoresis (data not shown). For reasons not known, internal proteins p14, p15, and p16 were lost (probably cleaved) for both particles during preparative electrophoresis. The results for p17 indicate that a difference in the amount of p17 is not the cause of the difference in the μ of the two A1 hybrid particles.

DISCUSSION

Bacteriophage hybrids have previously been constructed by *in vivo* recombination of either two whole genomes in a mixed infection or a whole genome and a plasmid-associated cloned gene in a single infection. These hybrids include T7-T3 hybrids in various combinations (Yamagishi *et al.,* 1985; see also Campbell *et al.,* 1978). Hybrid formation of bacteriophage tail fiber genes also occurs in the wild (Sandmeier, 1994). However, formation of hybrids by *in vivo* recombination with a plasmid requires construction of the plasmid-containing strain. Formation of hybrids by *in vivo* recombination of whole genomes yields inserts that are not restricted to a specific region of the genome. To achieve specificity, a DNA donor fragment is sometimes inserted in a recipient genome *in vitro.* The procedure used here is of the latter type. Although performed *in vitro,* the procedure used here potentially achieves the following advantage of *in vivo* recombination: formation of recombinants that differ from each other in the amount of DNA inserted. If this advantage is achieved, then viable inserts can be obtained, even if most inserts are nonviable because of the insertion of deleterious nucleotide sequences. If, as is usually the case, the deleterious sequences are not known in advance, the procedure presented here potentially reduces the number of experiments that must be performed in order to isolate hybrids. That is, the extract used for insertion of DNA fragments has the potential to perform the combinatorial work.

To help determine whether the extract was performing combinatorial work during the formation of the hybrids described here, the nucleotide sequence was determined for several independently isolated hybrids. For all of these hybrids, the inserted DNA was found to be truncated at both ends. The length of the inserted fragment was between 50 and 817 nucleotides. Although T7 actively recombines DNA during its infection of *E. coli,* no evidence exists for the occurrence of T7 site-specific recombination (Roeder *et al.,* 1979; Masker, 1992). Assuming, therefore, that the hybrids were not formed by T7 site-specific recombination, then the following observations indicate that the viable hybrid genomes detected were accompanied by other hybrid genomes that were nonviable: (1) The right end of all inserts was the same, even when restriction endonuclease-cut DNA was used for *in vitro* recombination–packaging, in an attempt to favor the formation of hybrids that had a right end closer to the right of gene 17. (2) The left end of all inserts was to the right of the amber codon of $T7_{17}$, even though the procedure used selected in favor of inserts that include the amber codon. In analogy to results previously obtained for a gene that encodes a component of the bacteriophage T2 tail fiber (gene 36; Riede *et al.,* 1985), apparently a barrier exists to extending the insert either to the left or to the right of the insertion points of the two A hybrids isolated here. In the case of the rightward extension, a source of the barrier could be the loss of the reading frame during protein synthesis beyond the point of the first T7 nucleotide that does not have a T3 counterpart (nucleotide 1423; see Fig. 3a). In the case of the leftward extension, a source of the barrier is not deduced by inspection of the nucleotide sequence.

To help understand both the barrier to leftward extension of T3 inserts in T7 gene 17 and the phenotypes of the A and B hybrids, the structure of the T7 tail fiber is

considered. The T7 tail fiber is a parallel triple-stranded assembly of p17. In addition, the T7 tail fiber has the following features that can be correlated with the amino acid sequence of p17 (Steven *et al.,* 1988): (a) N-terminal end of p17 attached to the tail; C-terminal (distal) end free in solution, (b) a kink near its middle (approximately amino acid 280), (c) four thickened regions (nodules) on the C-terminal side of the kink, (d) a strong disposition for an α helical coiled-coil at amino acids 226–280, and (e) a strong disposition for β-sheet structure at both the N-terminal (capsid-proximal) and the C-terminal ends. In both A hybrids, i.e., the hybrids with the longest insert, the N-terminal end of the transferred T3 amino acid sequence is approximately at the N-terminal end of the region that has a disposition for α helical coiled-coil; the C-terminal end of all inserts is at the position of the third nodule C-terminal to the kink. The leftmost left end of the insert is, therefore, at the junction of the capsid-proximal region and the α helical coiled-coil region. This observation suggests the hypothesis that a barrier to leftward extension of inserts is the incompatibility of the N-terminal T3 p17 amino acid sequence with the interaction of the T7 tail and the hybrid tail fiber.

Although the phenotype transferred here was nonadherence to agarose gels during agarose gel electrophoresis, presumably many other desired phenotypes can be transferred among related bacteriophages, by use of *in vitro* recombination–packaging. The nonadherent A hybrids are useful for the gel electrophoretic analysis of bacteriophage particles during the study of either *in vivo* assembly (example: Serwer *et al.,* 1983) or *in vitro* assembly (examples: Son *et al.,* 1993; Cerritelli *et al.,* 1996).

An unexpected characteristic of the A1 hybrid was the formation of two bands during the nondenaturing agarose gel electrophoresis of intact bacteriophage. Analysis by use of quantitative agarose gel electrophoresis revealed that the two A1 hybrid particles differed significantly in μ_0, but not in effective radius. The μ_0 of the more rapidly migrating A1 hybrid particle was 0.70 times the μ_0 of bacteriophage T3; for wild-type bacteriophages T7 and T3, this ratio was previously (Serwer *et al.,* 1983) found to be 0.78 when a phosphate-based electrophoresis buffer was used at pH 7.4. For the two particles of the A1 hybrid, the cause of the difference in μ_0 is not known. Because the μ_0 of the (tail-free) T7 capsid decreases in magnitude as the T7 procapsid is converted to the mature T7 capsid during DNA packaging (Serwer and Pichler, 1978), one hypothesis is that the two electrophoretically distinguishable particles of the A1 hybrid bacteriophage differ in the structure of the outer shell of the capsid. By this hypothesis, the hybrid tail fiber causes a change in the structure of the outer shell of T7. If so, this change could be related to changes that occur when T7 injects its DNA at the beginning of its infection of a host cell. Further investigation of the variability of the structure of the A1 hybrid is in progress.

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