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Replicative Hybrid of T4 Bacteriophage DNA

BEVERLY S. EMANUEL

Department of Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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Hybrid density replicative T4 DNA was isolated from CsCl, sheared, and reanalyzed in CsCl. The results rule out a branched model for T4 DNA replication and confirm that T4 DNA replicates to a conventional, semiconservative, colinear hybrid.

Hybrid is an intermediate of T4 DNA replication which appears during a density labeling experiment. A light parental DNA molecule which has acquired (during synthesis in the presence of 5-bromodeoxy-uridine [5BU] a complement of heavy progeny DNA equivalent in mass to its own initial mass will band in CsCl density gradient at the hybrid density location (i.e., halfway between the location of pure light and fully 5BU-substituted heavy reference DNA). Therefore, for the purpose of this discussion, hybrid is defined as a replicative intermediate of T4 DNA which is composed of equal proportions of parental and progeny density material and which operationally bands in CsCl halfway between the parental and progeny density locations. Insight into the molecular configuration of this replicative intermediate can help to discriminate between certain proposed models for DNA replication. Consider first the hybrid produced at the end of one round of conventional semiconservative replication of the entire parental molecule in a density-shift experiment (Fig. 1A). This hybrid would consist of a colinear association of one parental and one progeny density DNA strand, a one-dimensional duplex. Shearing a hybrid of this molecular configuration would produce only hybrid density fragments. Thus, sheared "colinear hybrid" would reband homogeneously at the original hybrid density location in CsCl. Consider alternatively the molecular configuration of the hybrid density intermediate produced in a density shift experiment as a result of replication according to a model which calls for repeated reinitiation of replication at one specific site prior to the conclusion of one full round of replication, Werner's model (5, 6). All early replicative intermediates would be branched according to the model, and the hybrid density intermediate generated in accordance with Werner's observed distance of 0.15 phage equivalent units between growing points would resemble the two-dimensional branched structure (Fig. 1B). The molecule with this configuration would be composed of equal proportions of parental and progeny density material and hence would band in CsCl at the hybrid density location. However, not only would it be branched, but it would be only half replicated. Hence, it would contain an unreplicated parental density portion on one end representing 50% of the total parental DNA. If, on the other hand, one considers the progeny DNA, 50% of it would reside in pure progeny density branches (Fig. 1B, branches 2, 3, 4). Shearing this "branched hybrid" would release 50% of the total parental DNA (solid line) as light, unreplicated parental density parental density fragments, and 50% as hybrid density replicated fragments. Conversely, while 50% of the total progeny material (broken line) would reside, upon shearing, in these hybrid density fragments, the remaining 50% of the progeny material would reside in fragments derived from the branches of pure progeny density material (Fig. 1B, branches 2, 3, 4). It is also important at this point to note that this branched hybrid density intermediate would be a very short-lived replicative intermediate as a result of the simultaneous action of the multiple growing points. As growing points would proceed along in replication, the molecule would become saturated with growing points and would resemble Fig. 1b. This molecule would be composed of at least 80% progeny density material and hence would band in CsCl close to the progeny density location.

To discriminate between these two models, T4 hybrid DNA was isolated from CsCl, sheared to 0.125 molecular size and reanalyzed in CsCl. It will be shown that the experimental results confirm a colinear, unbranched association of equal quantities of parental and progeny den-

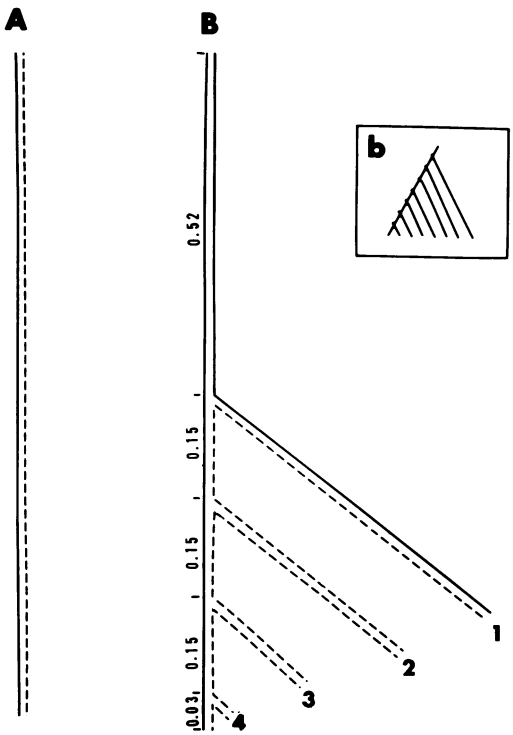

FIG. 1. *Two hybrids. (A) The hybrid density molecule produced by conventional semiconservative replication. (B) Hybrid density molecule resulting from branched replication according to Werner. Insert (b) in the figure is from Werner's paper. The detailed molecule (B) was constructed from his data which calls for reinitiation every 0.15 φ equivalent unit (eu). The four branches measure: 1 = 0.48 φ eu, 2 = 0.33 φ eu, 3 = 0.18 φ eu, 4 = 0.03 φ eu. Branches 2, 3, and 4 are pure progeny density. Symbols: ——, parental; -----, progeny.*

sity material as the structure for the hybrid density moiety which one observes in CsCl density gradient analysis of replicative T4 DNA.

Experimental details, materials, and methods have been described elsewhere (B. S. Emanuel, Ph.D. thesis, Univ. of Pennsylvania, Philadelphia, 1972). *E. coli* B23 was grown to a concentration of 3×10^8 cells per ml for two generations in 5BU-substituted TCG medium. Cells were infected with a multiplicity of infection of 10.0 of specific activity = 3.0 mCi/mg of P, light ^{32}P-labeled T4 wild-type phage. At various times after infection, DNA was extracted by the sodium dodecyl sulfate (SDS)-Pronase-phenol method (3). The efficiency of recovery of trichloroacetic acid-precipitable parental label was invariably 100 ± 10%. The extracts were analyzed in alkaline sucrose gradients and CsCl density gradients. The light, ^{32}P-labeled parental DNA extracted 6 min after infection cosediments in an alkaline sucrose gradient with integral ^{3}H-labeled T4 reference DNA. Thus the parental DNA, 6 min after infection, is integral. The 6-min extract was supplemented with ^{3}H-labeled heavy and light reference DNA and subjected to CsCl density gradient analysis. The results of this analysis reveal that most of the parental DNA has replicated and bands at the hybrid density location (Fig. 2A). Thirty-five percent of the parental label remains at the light density location, and this represents DNA which will never replicate. This observation comes from samples taken at later times after infection but is not documented here. The hybrid fractions denoted by the arrows in Fig. 2A were pooled, dialyzed, and used for reanalysis in CsCl (i) without any further treatment (Fig. 2B), and (ii) after shearing to 0.125 molecular size (Fig. 2C), both samples being supplemented with light and heavy ^{3}H-labeled reference DNA. The 0.125 molecular size was confirmed by alkaline sucrose gradient analysis. The isolated material rebands at the hybrid density location both in the unsheared and in the sheared form (Fig. 2B, C). Shearing the isolated hybrid density material releases some fragments of pure parental density which comprise about 10 to 15% of the total parental label. This 10 to 15% is between 3 to 5 times less than the 50% expected for Werner's model of replication. It probably arises as a consequence of the large number of fractions included in the isolation. Since DNA if homogeneously labeled does not band in an infinitely sharp configuration but has a σ (standard deviation attributed to dispersion in the gradient) of 0.032 (4), the isolation of any fraction will tend to include molecules of reasonably similar density. Thus the obtained figure of 10 to 15% parental density corresponds fairly well to the observed σ but differs more than threefold from the 50% expected from shearing of a branched hybrid. In addition, the isolation was intentionally performed with a bias toward the light parental density fractions in the hybrid peak, thus including some molecules which have not completed one full round of replication.

It has been shown that if chloramphenicol (CM) is added at 5 min after infection (a time of CM addition which allows replication but not recombination) a hybrid density intermediate accumulates (2). Hybrid persists for prolonged periods of incubation in chloramphenicol (up to 60 min) and has been shown to be actively involved in replication (i.e., actively replacing an integral progeny strand with a

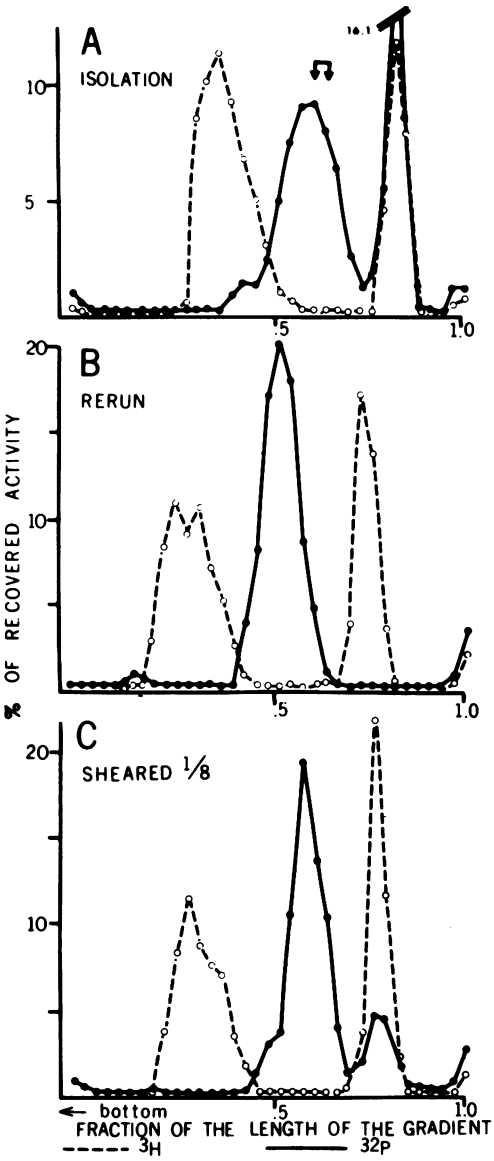

FIG. 2. *CsCl density gradient analysis of extracted intracellular DNA and hybrid moiety isolated 6 min after infection of heavy bacteria with light, radioactive ^{32}P bacteriophage. E. coli B23 was grown in substituted (heavy) TCG for 2 generations to a concentration of 3 × 10^8 cells/ml. Cells were infected with light ^{32}P-labeled (specific activity = 3 mCi/mg of P) T4 bacteriophage at a multiplicity of infection of 10 phage per bacterium. At 6 min after infection, cells were chilled in 0.15 M NaCl-15 mM EDTA (pH 7.6). Intracellular DNA was extracted by the SDS-Pronase-phenol method. (A) Extracted DNA was fractionated by CsCl density gradient centrifugation in the presence of ^3H-labeled light and heavy reference DNA from T4. The fractions were collected from the CsCl centrifugation into shell vials, and a sample of each fraction was counted. The solid line represents the ^{32}P, and the dashed line represents the ^3H reference. The hybrid density fractions denoted by the arrows were pooled and dialyzed against 0.15 M NaCl-15 mM sodium citrate. (B) A sample of the isolated hybrid was analyzed by CsCl density gradient centrifugation in the presence of ^3H light and heavy reference DNA. (C) A sample of the isolated hybrid was sheared to 0.125 molecular size. The size was confirmed by sucrose gradient sedimentation. The sheared hybrid was analyzed by CsCl density gradient analysis with ^3H-labeled light and heavy reference DNA.*

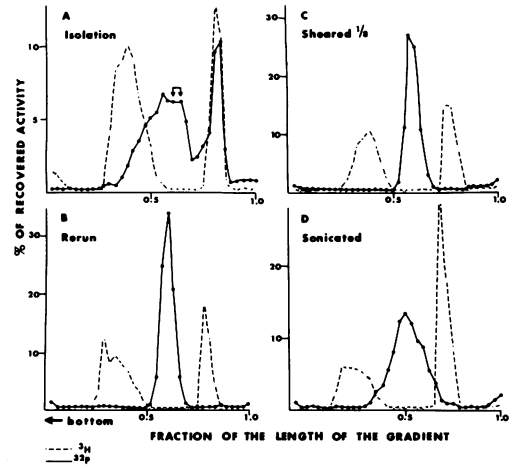

FIG. 3. *CsCl density gradient analysis of extracted intracellular DNA and the hybrid density moiety isolated 45 min after infection of heavy bacteria with light ^{32}P-labeled bacteriophage. E. coli B23 was grown for 2 generations in heavy medium to a concentration of 3 × 10^8 cells/ml. Cells were infected with light ^{32}P-labeled (specific activity = 3.0 mCi/mg) T4 bacteriophage at a multiplicity of infection of 10. Chloramphenicol (100 μg/ml) was added to the culture 5 min after infection. At 45 min after infection, cells were chilled in 0.15 M NaCl-15 mM EDTA (pH 7.6), and intracellular DNA was extracted by the SDS-Pronase-phenol method. (A) Extracted DNA was fractionated by CsCl density gradient centrifugation in the presence of ^3H-labeled light and heavy reference DNA from T4. The fractions were collected from CsCl into shell vials, and a sample of each fraction was counted. The hybrid density fractions denoted by the arrows were pooled and dialyzed against 0.15 M NaCl-15 mM sodium citrate. (B) A sample of the isolated hybrid was analyzed by CsCl density gradient centrifugation in the presence of ^3H light and heavy reference DNA. (C) A sample of the isolated hybrid was sheared to 0.125 molecular size and analyzed by CsCl density gradient centrifugation with added ^3H-labeled light and heavy reference DNA. (D) A sample of the isolated hybrid was fragmented by sonic treatment. The sonicated hybrid was analyzed by CsCl density gradient centrifugation in the presence of ^3H-labeled light and heavy reference DNA.*

new one; reference 2). If this hybrid has completed more than one full round of colinear replication during prolonged incubation in CM, it should not contain a conservative parental density contribution, and indeed the hybrid would have a colinear structure. At 5 min after infection, a sample of the previously described infected culture was treated with chloramphenicol (100 μg/ml). The culture was then incubated for 45 min, and the DNA was extracted by the SDS-Pronase-phenol method. The extract was supplemented with ^{3}H-labeled light and heavy reference DNA and subjected to CsCl density gradient analysis (Fig. 3A). The hybrid fractions (arrows) were pooled and dialyzed and (i) reanalyzed in CsCl with no further treatment (Fig. 3B), (ii) sheared to 0.125 molecular size (Fig. 3C), and (iii) sonicated (Fig. 3D) all with the appropriate ^{3}H-labeled heavy and light references added. It is obvious that the hybrid produced upon prolonged incubation in the presence of CM releases no pure parental density fragments after shearing or even after sonication. When one considers only the persistence of a hybrid moiety of replicative parental DNA in the presence of CM (added at 5 min), one immediately appreciates that the structure is most likely colinear throughout its entire length, since a parental moiety which would persists as a half-replicated, branched intermediate actively replacing its progeny strands is difficult to imagine.

In a parallel experiment, the identical heavy host was infected with a multiplicity of infection of 10.0 cold, light T4 phage. The newly synthesized progeny DNA was labeled by the uptake of ^{3}H-labeled adenine. DNA was extracted by the SDS-Pronase-phenol method, with no losses of progeny-labeled material. The extract was subjected to CsCl density-gradient analysis, having been supplemented with the appropriate heavy and light ^{32}P-labeled reference DNA. All of the progeny DNA label bands at the hybrid density location (Fig. 4A). It is important in the context of this discussion to observe (Fig. 4A) that there is no pure progeny-labeled heavy material which could represent branches sheared or stripped from the replicative DNA. The hybrid density fractions (arrows) were pooled and dialyzed and (i) reanalyzed in CsCl without any further treatment (Fig. 4B), (ii) sheared to 0.125 molecular size (Fig. 4C), and (iii) sonicated (Fig. 4D) all with the appropriate ^{32}P-labeled heavy and light reference DNA added. Results reveal that all of the isolated hybrid rebands at the hybrid density location, and that all of the fragments of sheared hybrid reband at the hybrid density location. Sonication of isolated hybrid releases about 10 to 15% of the progeny label as pure

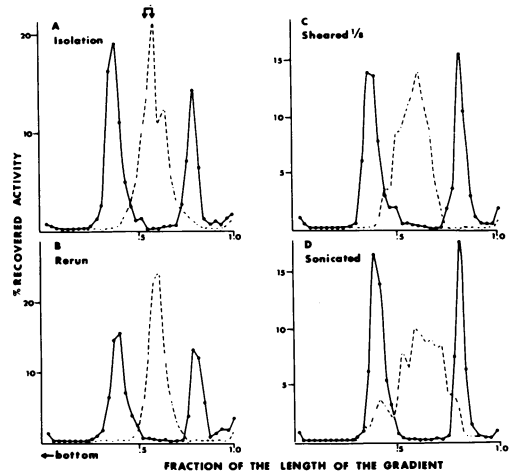

FIG. 4. *CsCl density gradient analysis of extracted intracellular DNA and hybrid moiety isolated 6 min after infection by heavy bacteria with light, nonradioactive bacteriophage. E. coli B23 was grown in 5BU-substituted (heavy) TCG for two generations to a concentration of 3 × 10^8 cells/ml. Cells were infected with nonradioactive, light T4 bacteriophage at a multiplicity of infection of 10 phage per bacterium. At 3 min after infection, ^{3}H-adenine was added to the culture at a specific activity of 30 mCi/mg of adenine. Cells were chilled in 0.15 M NaCl-15 mM EDTA (pH 7.6) 6 min after infection, and intracellular DNA was extracted by the SDS-Pronase-phenol method. The extracts were dialyzed for 4 h against 0.15 M NaCl-15 mM sodium citrate. (A) The dialyzed extract was fractionated by CsCl density gradient centrifugation in the presence of ^{32}P-labeled light and heavy reference DNA from T4. The fractions from the CsCl centrifugation were collected into shell vials. A sample of each fraction was precipitated with 0.3 M Cl_3CCOOH (trichloroacetic acid). The fractions were then incubated with 1.0 M KOH for 18 h at 37 C, neutralized, and precipitated with trichloroacetic acid. After washing, the fractions were dissolved and counted. Note that all of the ^{3}H progeny-labeled material bands at the hybrid density location and not at the pure heavy progeny density. The hybrid density fractions denoted by the arrows were pooled and dialyzed against 0.15 M NaCl-15 mM sodium citrate. (B) A sample of the isolated hybrid was analyzed by CsCl density-gradient centrifugation with ^{32}P-labeled light and heavy reference DNA. The fractions were digested with alkali, neutralized, precipitated with trichloroacetic acid and counted. (C) A sample of the isolated hybrid was sheared to 0.125 molecular size and analyzed in CsCl with added ^{32}P light and heavy reference DNA. The fractions were treated with alkali as previously described. (D) A sample of the isolated hybrid was sonicated and analyzed in CsCl with ^{32}P-labeled light and heavy reference DNA. The fractions were treated with alkali as previously described.*

progeny density fragments, again probably due to the bias in selecting the hybrid moiety closer to the progeny density location and the sigma for CsCl distribution. Knowing (i) that the parental strands were size one; (ii) that no losses were encountered during the extraction procedure; and (iii) that no pure progeny moiety appeared during the primary isolation (thus contradicting the possibility of breaking off the pure progeny density branches), we are left with the conclusion that hybrid is a colinear association of parental and progeny density material, which is, for the most part, unbranched, and must somehow have the configuration of conventional semiconservative hybrid (Fig. 1A). Replication by a branching mechanism would demand that 50% of the progeny material in a hybrid density intermediate be present as pure progeny density material, and 50% of the parental material be present as pure parental density material. This has not been observed. The identical experiments performed either with reverse density labeling or at a lower temperature (25 C) yield the identical results, i.e., no release of unreplicated pure parental and no release of pure progeny density material from isolated hybrid. This leads us to the conclusion that a branching mechanism for T4 DNA replication must either be excluded or considered as a very infrequent phenomenon. In conclusion, we must emphasize that this observed pattern of replication leading to a colinear unbranched hybrid may apply only during the time prior to the onset of recombination. In addition, the production of such a hybrid is not inconsistent with the initiation of DNA replication at multiple sites as proposed by Delius, Howe, and Kozinski (1), as long as re-initiation at the same site (or loop) is restricted. Indeed, Delius, Howe, and Kozinski (1) have observed some evidence for secondary initiation, but they reported that the phenomenon of re-initiation is rather infrequent and amounts to only 1.5% of the total number of replicative loops observed. Thus, replication of T4 phage DNA is semiconservative and proceeds with very little, if any, secondary initiation (or branching) until the entire molecule has been completely replicated.

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