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Genetics

Strands hybridize in postreplicative adenovirus overlap recombination

(intermolecular reassociation/nonreciprocal recombination/mismatch correction/precise gene fusion)

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ABSTRACT **We describe a postreplicative mechanism for adenovirus overlap recombination. An adenovirus minichromosome system was used to study overlap recombination driven by adenovirus DNA replication. Crossing-over appeared to occur equally at, but not within, the borders of the overlap between partner molecules. We propose that recombination in the minichromosome system proceeds through an intermediate formed by direct hybridization of complementary sequences on displaced strands generated by adenovirus-specific DNA replication. Some, but not all, heterologous regions in the intermediate are susceptible to mismatch correction. This pathway is intrinsically nonreciprocal and differs significantly from other adenovirus recombinational mechanisms that have been described previously.**

Overlap recombination assembles complete adenovirus genomes from overlapping terminal fragments (1–4). It is normally a prereplicative process mediated by homologous recombination (3). We have used an adenovirus minichromosome system (5, 6) to study overlap recombination driven by adenovirus DNA replication. We propose that, in contrast to the prereplicative mechanism (3), postreplicative overlap recombination proceeds by direct hybridization of complementary sequences on displaced strands to form a heteroduplex intermediate spanning the entire overlap. Crossovers are limited to the borders of the overlap by DNA repair synthesis during conversion of the intermediate into a duplex molecule. Adenovirus origins are thus regenerated at both ends of the intermediate. Terminal nonhomology at the boundaries of the overlap is efficiently removed by mismatch correction. Large-scale heterology within the overlap is not altered, but segregates equally upon subsequent rounds of adenovirus-specific DNA replication. This pathway is intrinsically nonreciprocal, differs from other recombinational mechanisms that have been described previously, and incorporates the requirement for efficient strand-displacement replication by both partner molecules. We discuss the significance of the postreplicative mechanism for overlap recombination in the cycle of adenovirus DNA replication.

MATERIALS AND METHODS

Reagents and Enzymes. ^{32}P-labeled deoxyribonucleotides (3000 Ci/mmol; 1 Ci = 37 GBq) were from New England Nuclear. Assorted restriction endonucleases, *Escherichia coli* DNA polymerase I, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim, or Pharmacia. Pronase was from Calbiochem. All enzymes were used as recommended by the suppliers.

Cells and Virus. *E. coli* C600 was grown, transformed, and selected by standard procedures (7). The 293 human cell line (8) was grown in Dulbecco's modified Eagle's medium (GIBCO) with 5% fetal calf serum. Adenovirus type 2 was grown in HeLa cells. The HeLa cell line was grown in Joklik's minimal essential medium (GIBCO) containing 5% fetal calf serum. Virus and viral DNA were isolated and purified by previously published methods (9).

Transfection. Line 293 cells were transfected according to the standard DNA–calcium phosphate coprecipitation method (10). In addition, cells were shocked for 1 min with 25% (vol/vol) glycerol 4 hr after transfection. Each 60-mm dish was inoculated with 4×10^5 cells 24 hr before transfection. Transfections utilized 3 μg of each plasmid DNA, 3 μg of helper adenovirus 2 DNA, and sufficient salmon sperm DNA to make 15 μg of total DNA per 60-mm dish.

Extraction and Analysis of DNA. DNA was isolated from 293 cells 57 hr after transfection (except as noted) and analyzed by Southern blot hybridization using ^{32}P-labeled pBR322 DNA (specific activity $> 1 \times 10^8$ cpm/μg) as a probe as described previously (6).

RESULTS

Replication-Driven Overlap Recombination. Recombination between overlapping subgenomic adenovirus fragments is ordinarily a prereplicative event since viral DNA replication requires origins at both chromosomal ends (11) (for review, see ref. 12). We have used an adenovirus minichromosome system (5, 6) to study overlap recombination driven by adenovirus DNA replication. Clone 7 and pXD6 have, respectively, a single left or right adenovirus origin; neither plasmid contains inverted repeat sequences (Fig. 1). *Eco*RI-linearized plasmids transfected singly with helper adenovirus DNA synthesized only a single strand during strand-displacement replication since each molecule became resistant to both *Dpn* I and *Mbo* I cleavage (data not shown; *Dpn* I cuts only input plasmid DNA with GATC sites methylated on both strands; *Mbo* I cuts only replicated DNA with GATC sites unmethylated on both newly synthesized strands). Fig. 2 (lane 1) shows that when *Eco*RI-linearized clone 7 and pXD6 were together transfected with helper, a new band appeared which had the size (6.7 kb) expected for a recombinant formed between the two input plasmids. Since the recombinant was *Dpn* I resistant but *Mbo* I sensitive (data not shown), the recombination event created adenovirus origins at both ends of the molecule, allowing both strands to participate in replication. In the absence of helper, doubly

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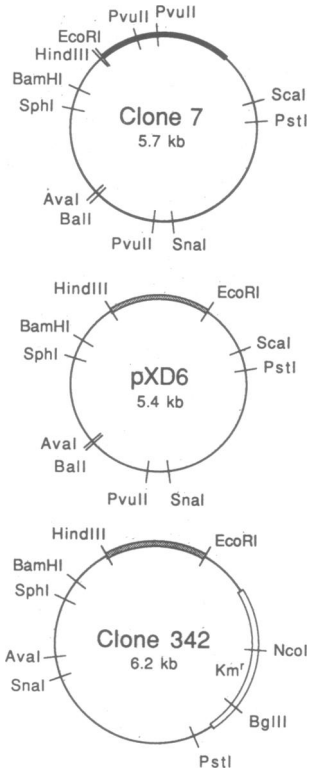

FIG. 1. Maps of plasmids clone 7, pXD6, and clone 342. pBR322 sequences are indicated by the thin line. The thick black line in clone 7 represents the left-most 1.3-kilobase-pair (kb) adenovirus *Xba* I fragment, and the thick shaded line in pXD6 and clone 342 is the right-most 1.0-kb adenovirus *Hin*dIII fragment. An adenovirus replication origin is located on each of these fragments immediately adjacent to the *Eco*RI site. The direction of adenovirus-specific replication is clockwise from the *Eco*RI site on clone 7 and counterclockwise from the *Eco*RI sites on pXD6 and clone 342. pXD6 and clone 342 both lack a 29-base-pair (bp) fragment found on clone 7 between the *Eco*RI and *Hin*dIII sites. Clone 7 lacks roughly 50 bp found on pXD6 and clone 342 just clockwise from the *Eco*RI site. Clone 342 differs from pXD6 by the deletion of a 0.6-kb *Bal* I–*Pvu* II fragment and the insertion into the *Sca* I site of a 1.5-kb fragment (open box) carrying a gene conferring resistance to kanamycin (Km^r).

transfected cells did not yield a recombinant (Fig. 2, lane 2). Recombination was also not detected if one of the partner plasmids lacked an adenovirus origin (data not shown), even though the expected recombinant would have had inverted repeats allowing subsequent regeneration of viral origins at both ends of the molecule (5, 6). Minichromosomes, too small to be packaged, are limited to a single lytic cycle. Fig. 3 presents the kinetics of appearance of the recombinant and shows that it accumulated late in the cycle after the onset of viral DNA replication. It was not detected at early times (lanes 1–3) but appeared 35 hr after transfection or later (lanes 4–7). Since the recombinant was not detected in the gel without prior Pronase digestion of the DNA (lane 8), adenovirus preterminal protein, the primer for viral DNA replication, is presumably attached to the ends of the molecule (5) [covalent adenovirus DNA-terminal protein complexes do not enter agarose gels during electrophoresis (13)]. These results taken together suggest two potential roles for adenovirus DNA replication: first, strand-displacement synthesis by both partners may be required to create the recombinant (see below); and second, further replication of the recombinant is necessary to amplify the copy number to a detectable level.

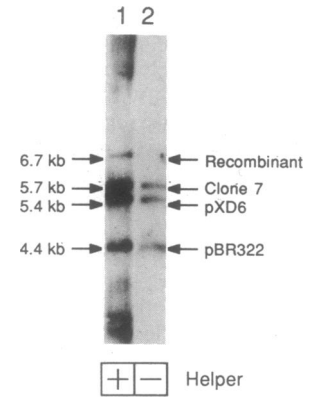

FIG. 2. Detection of a 6.7-kb recombinant molecule. DNA was isolated 57 hr after cotransfection of *Eco*RI-cut clone 7 and *Eco*RI-cut pXD6 with (lane 1) or without (lane 2) helper adenovirus DNA and was analyzed by Southern blot hybridization. All transfections had *Eco*RI-cut pBR322 DNA as an internal marker. The sizes of molecules are indicated in kb.

Segregation of Markers. We designed a genetic cross with specially constructed plasmids to follow the segregation of markers. Clone 342 (Fig. 1) was derived from pXD6 by deleting 0.6 kb between the *Bal* I and *Pvu* II sites and inserting a 1.5-kb fragment carrying a gene for kanamycin resistance into the *Sca* I site. Fig. 4 diagrams the three regions of homology shared between clones 7 and 342. Recombination within the three crossover regions should generate unique recombinants, designated A, B, and C, each distinguishable by size. Fig. 5 (lane 7) shows that, when clones 7 and 342 were transfected together with helper, only recombinants A and C were detected in addition to the parental input molecules. Both recombinant bands had equal intensities, indicating equivalent yields. The identities of the bands were further verified by cleavage with *Bgl* II (lane 6): neither clone 7 nor recombinant C was cut as expected, but both clone 342 and recombinant A were trimmed to identical 4.5-kb molecules. No *Bgl* II-resistant DNA was observed with a size between 5.7 kb and 6.7 kb, eliminating the possibility that the missing recombinant B (6.0 kb) comigrated with clone 342 (6.2 kb).

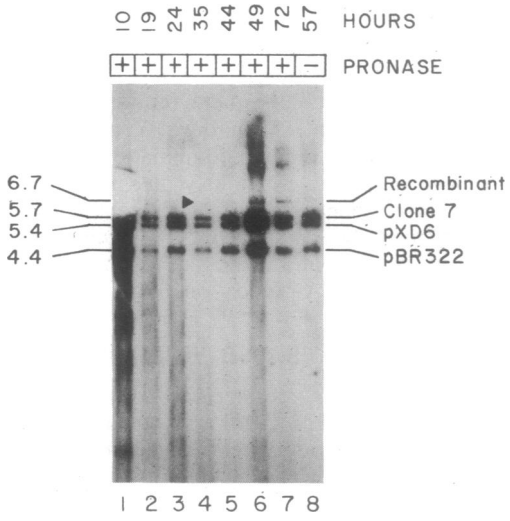

FIG. 3. Time course of the appearance of the 6.7-kb recombinant molecule. DNA was isolated at the indicated hours after cotransfection with *Eco*RI-cut clone 7, *Eco*RI-cut pXD6, and helper adenovirus DNA. Southern blot analysis was performed. *Eco*RI-cut pBR322 DNA served as an internal marker. All samples, except the one analyzed in lane 8, were digested with Pronase prior to gel electrophoresis. The black triangle indicates the presence of the 6.7-kb recombinant in lane 4. Molecular sizes are in kb.

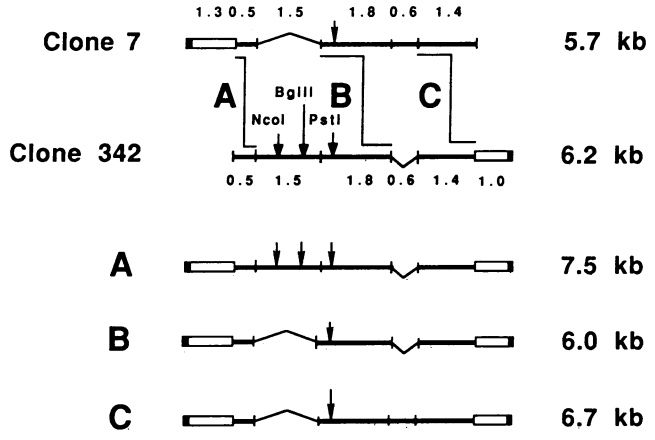

FIG. 4. Structures predicted for homologous recombination between clones 7 and 342. Sequences missing in one plasmid relative to the other are symbolized by bent thin lines. Certain restriction endonuclease sites are indicated by arrows on all clones, but they are identified by name only on clone 342 (see Fig. 1). Intervals (with sizes in kb) along each plasmid are marked by small vertical lines. Crossovers (thin lines) in regions of homology (A, B, or C) shared between the plasmids produce recombinants respectively named A, B, and C.

Recombination appears limited to the outermost of the three regions of homology spanning the overlap. We could, however, force crossovers in region B by removing region A from clone 342 with *Pst* I (Fig. 5, lane 5) or *Nco* I (Fig. 5, lane 3) prior to transfection with clone 7. As diagrammed in Fig. 4, *Pst* I deletes region A, 1.5 kb of neighboring heterologous DNA, and 0.2 kb of region B from clone 342, whereas *Nco* I removes only region A plus 0.4 kb of the 1.5-kb heterologous DNA. Fig. 5 (lanes 3 and 5) shows that in both experiments recombinants B and C, but not recombinant A, appeared in equal amounts. Furthermore, recombinant B was *Bgl* II resistant as expected (Fig. 5, lanes 2 and 4). Since recombinant B was produced in equal yield to recombinant C when the left border of the B crossover region had 0.9 kb of adjacent heterologous DNA (Fig. 5, lanes 2 and 3) or perfectly homologous DNA (Fig. 5, lanes 4 and 5), the previous inability to observe crossovers in region B (Fig. 5, lanes 6 and 7) was not due to polarity (14) induced by extensive flanking nonhomologous regions. These results also eliminate strand invasion (15) and double-strand-break-stimulated (16, 17) mechanisms of recombination, since terminal nonhomologous stretches, even as small as 40 bp, inhibit recombination by these pathways in yeast (18), *Xenopus* oocytes (19–21), and mouse cells (22).

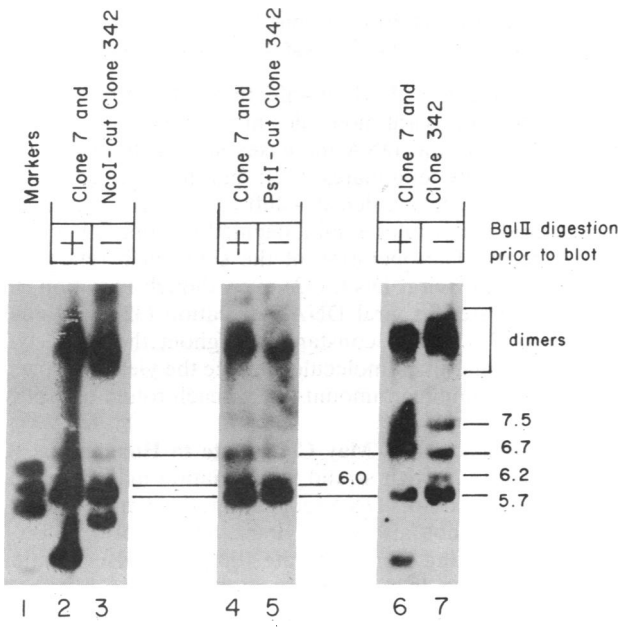

FIG. 5. Analysis of recombination between clone 7 and clone 342. All transfections included helper adenovirus DNA. DNA was isolated 57 hr after transfection. Some samples (lanes 2, 4, and 6) were cut with *Bgl* II prior to Southern blot analysis. Sizes are given in kb. Lane 1, marker DNA molecules. Lanes 2 and 3, *Eco*RI-cut clone 7 transfected with *Eco*RI- and *Nco* I-cut clone 342 (*Nco* I-cut clone 342 is 5.3 kb in lane 3; after *Bgl* II digestion, *Nco* I-cut clone 342 is 4.5 kb in lane 2). Lanes 4 and 5, *Eco*RI-cut clone 7 transfected with *Eco*RI- and *Pst* I-cut clone 342 (*Pst* I-cut clone 342 has a size of 4.0 kb and has run off the gel in both lanes). Lanes 6 and 7, *Eco*RI-cut clone 7 transfected with *Eco*RI-cut clone 342 (both clone 342 and recombinant A are cleaved by *Bgl* II to identical 4.5-kb molecules in lane 6).

Another Mode of Overlap Recombination. We propose a mechanism for postreplicative overlap recombination, detailed in Fig. 6, which exhibits several salient features: (*i*) It is driven by adenovirus-directed strand-displacement replication. (*ii*) Only one strand, the displaced strand, from each parental molecule participates in the formation of a heteroduplex intermediate (the pathway is therefore intrinsically nonreciprocal). (*iii*) The heteroduplex arises from direct intermolecular hybridization of displaced strands, so single strands need not sequentially invade a duplex to form a recombinational intermediate (the process does not manifest polarity). (*iv*) The intermediate structure is initially duplex only within the overlap. (*v*) Any heterologous sequences form single-stranded loops within the overlap or remain as unpaired 3′ or 5′ ends if located externally to the overlap. (*vi*) Unpaired 3′ ends, but not 5′ ends, are excised exonucleolytically from the heteroduplex. (*vii*) Large-scale internal nonhomologous regions (e.g., large deletion or insertion loops) remain intact. (*viii*) Apparent crossovers at the outermost borders of the overlap are a consequence of repair DNA synthesis exclusively at those sites. (*ix*) DNA repair regenerates adenovirus origins at both ends of the heteroduplex molecule. (*x*) Subsequent rounds of adenovirus-directed DNA replication segregate and amplify two recombinant molecules arising equally from each strand of the repaired heteroduplex. The suggested pathway accounts for the structures of all the recombinants observed between clones 7 and 342 (Fig. 6*A*), clone 7 and *Pst* I-cut clone 342 (Fig. 6*B*), and clone 7 and *Nco* I-cut clone 342 (Fig. 6*C*).

Not all mismatches in the heteroduplex intermediate appear to be susceptible to correction. Efficient exonucleolytic removal of unpaired 3′ and 5′ sequences from transfected heteroduplexes has already been documented in mammalian cells (23). Unpaired 3′ sequences are lost from the heteroduplex intermediate: an unpaired 3′ terminus 900 nucleotides long (Fig. 6*C*) or a perfectly matched 3′ end (Fig. 6*B*) served equally effectively as primers for DNA repair synthesis to form recombinant B (Fig. 5, lanes 3 and 5). Further experiments have confirmed the efficient exonucleolytic removal of unpaired 3′ sequences as long as 4000 nucleotides (data not shown). Perhaps adenovirus DNA polymerase, with a potent 3′-exonuclease activity (24), removes unpaired 3′ ends prior to DNA repair synthesis. However, the adenovirus preterminal protein (Fig. 3, lane 8) presumably protects the 5′ ends of displaced plasmid strands from exonucleolytic attack (25, 26). Large single-stranded loops in the heteroduplex intermediate also do not seem to be targets for correction, since the parental orders of markers within the overlap were recovered in the recombinants without rearrangement (Fig. 5, lane 7; Fig. 6*A*). Heteroduplex loops ranging in size from 8 to 283 nucleotides are efficiently repaired both in yeast (27) and in monkey cells (23, 28). Repair is biased heavily in favor of the nonlooped strand (28), and it is presumably initiated by

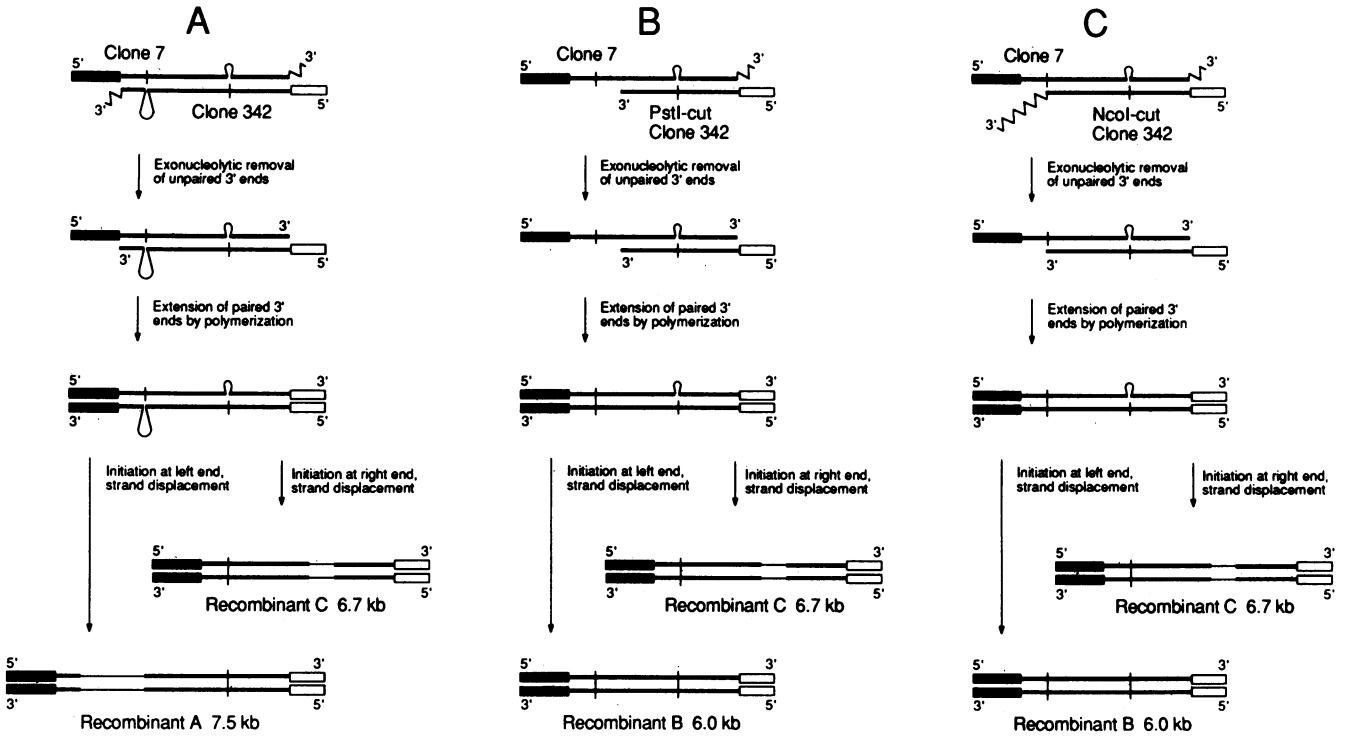

FIG. 6. Schematic diagram of the intermolecular hybridization of complementary displaced strands generated during adenovirus-directed DNA replication of clone 7 and clone 342. Filled and empty boxes represent respectively the left and right ends of adenovirus DNA. The vertical lines mark the boundaries of homologous regions. Complementary sequences (thick lines) on displaced strands from input plasmids hybridize to create a heteroduplex intermediate with internal nonhomologous loops (thin lines) and unpaired 3′ tails (jagged lines). Removal of unpaired 3′ tails together with subsequent DNA repair synthesis produces a heteroduplex with adenovirus replication origins at both ends. Further rounds of adenovirus-specific replication from either end of the heteroduplex segregate equal yields of "recombinants" retaining unrearranged internal nonhomologous regions of the input plasmids. (*A*) The repaired heteroduplex between *Eco*RI-cut clone 7 and *Eco*RI-cut clone 342 segregates recombinants A and C. (*B*) The repaired heteroduplex between *Eco*RI-cut clone 7 and *Eco*RI- and *Pst* I-cut clone 342 segregates recombinants B and C. (*C*) The repaired heteroduplex between *Eco*RI-cut clone 7 and *Eco*RI- and *Nco* I-cut clone 342 segregates recombinants B and C.

cleaving loops endonucleolytically. It is possible that endonucleases fail to attack large loops coated with the adenovirus DNA-binding protein, a protein essential for viral DNA replication (12). Estimates for the size of the DNA-binding protein site range from 3 to 11 bases (29–31). Recent studies have shown that little loops (4 bases), presumably too small to be protected by DNA-binding protein, are subject to mismatch correction in the adenovirus minichromosome system (data not shown).

DISCUSSION

Two quite dissimilar mechanisms have now been documented for adenovirus overlap recombination: a prereplicative mode occurring by homologous recombination with distance- and position-dependent segregation of genetic markers within the overlap (3), and a postreplicative process involving intermolecular hybridization of displaced strands as described in this paper. We argue below that interstrand hybridization may play a fundamental role in normal adenovirus DNA replication.

Strand Reassociation Short-Circuits Semiconservative DNA Replication. Parental strands are displaced by adenovirus DNA replication (12). The nonreplicative reassociation of displaced strands rejoins complementary parental strands back into "parental-like" duplexes (more correctly, heteroduplexes). Such molecules "look" unreplicated, but in fact they constitute one of several products of strand-displacement replication. Consequently, interstrand hybridization acts as a shunt in the semiconservative synthesis of viral DNA. The shunt has been observed experimentally (32, 33). The reassociation of strands is a concentration-dependent bimolecular reaction. Early in infection, when there are few genomes per cell, viral molecules are synthesized semiconservatively. As viral DNA increases later in the cycle, the shunt eventually dominates (32): parental molecules no longer "chase" during density-shift experiments, and CsCl density gradient profiles exhibit decidedly abnormal density-shift patterns. The operation of the shunt also retards the accumulation of viral DNA (32) even though initiation, the rate-limiting step in viral DNA replication (32), and chain elongation (34) remain constant throughout the entire lytic cycle. "Parental-like" molecules reduce the yield of progeny DNA by an equimolar amount during each round of replication.

Strand Reassociation May Contribute to Homologous Recombination. Although strand reassociation may be a basic pathway in adenovirus DNA replication, the consequences of its action are not always obvious. The hybridization of strands originating from separate, but otherwise identical, molecules can be demonstrated only by density-shift experiments (32, 33). This explains why strand reassociation is not "apparent" in prereplicative overlap recombination (3): the only molecule capable of replicating in this case is the initial recombinant assembled nonreplicatively from subgenomic fragments. Even when genetically distinguishable viral molecules replicate together, several factors probably limit the contribution of strand reassociation to recombination. First, interstrand hybridization functions late in the lytic cycle. Second, as shown above, not all mismatches (especially extensive nonterminal heterologous regions) in heteroduplexes are susceptible to correction. Both the formation and resolution of heteroduplexes leading to recombinant molecules are therefore as yet difficult to predict even qualita-

tively. However, the high frequency of supernumerary crossovers in adenovirus recombinants (35, 36) may be due, at least in part, to interstrand hybridization coupled with specific mismatch correction.

Postreplicative Overlap Recombination as a Genetic Engineering Technique. Adenovirus DNA replication, DNA amplification *in vivo* with consecutive cycles of strand displacement and reassociation, resembles the *in vitro* polymerase chain reaction technique (37). Postreplicative overlap recombination, like polymerase chain reaction (38), creates precise junctions when molecules are fused (Fig. 6). The minimum length of complementary sequences required for interstrand hybridization is not yet known, but it exceeds 89 bp (data not shown). Postreplicative overlap recombination may therefore prove useful for assembling, manipulating, and amplifying DNA sequences 36 kb or longer.

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