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Replication and Recombination in Adenovirus-Infected Cells Are Temporally and Functionally Related

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We have studied the temporal and functional relationships between DNA replication and recombination in adenovirus-infected cells by using Southern blot hybridization to detect recombinant products among intracellular viral genomes. The data show that recombination can be detected soon after DNA replication has commenced and that the proportion of recombinant products increases thereafter. To determine the functional relationship between DNA replication and recombination, replication was blocked with the protein synthesis inhibitor anisomycin, the replication inhibitor cytosine arabinoside, and conditionally lethal mutations in either the virus-specified DNA-binding protein or the DNA polymerase. All treatments that directly or indirectly blocked DNA replication caused a delay in the appearance of recombinant products and a marked decline in their abundance relative to products of parental genotype. These data strongly suggest that DNA replication and recombination are interrelated, either because both processes share functions or because DNA structures produced by replication are suitable substrates for recombination. In addition, we have shown that some recombination function(s) is intrinsically thermolabile at 40.9°C, even in wild-type crosses, since the appearance of recombinant products is delayed and their extent is reduced compared with that from crosses performed at 39.9°C.

The processes of DNA replication and recombination frequently occur contemporaneously during the course of the replicative cycle of both bacteriophages (15, 21) and animal viruses (28). This temporal coincidence could arise, in theory, from any or all of the following causes: (i) independent functions necessary for one or the other process may be expressed and become active simultaneously, (ii) some polypeptides may be utilized by both pathways, and (iii) DNA replication intermediates may provide substrates that are especially attractive to the machinery responsible for recombination. Therefore, a thorough investigation of the causes of the temporal coincidence and the functional relatedness of replication and recombination can be expected to reveal important details of the mechanisms by which genomes exchange information in any viral system.

In a previous study we demonstrated that recombination between human adenoviruses begins at some point during the eclipse phase, continues throughout the rise period of an infectious cycle, and is accompanied by progressive rearrangement of unselected markers (28). Furthermore, in superinfection studies, it was shown that cells retained the capacity to recombine genomes that were introduced late in the infectious cycle with those that were already present. Those studies suggested that the functions responsible for recombination were not temporally partitioned to the early phase of the viral replicative cycle (16). Independent evidence suggesting that recombination occurs during the DNA-replicative phase of the adenovirus infectious cycle comes from electron microscopic studies, which demonstrated interactions between full-length duplexes and replicative intermediates (27). Taken together, these data show that for adenoviruses, recombination is at least in part contemporaneous with DNA replication.

In this manuscript, we describe experiments designed to determine the time in eclipse at which recombination could first be detected. Next, having shown that the onset of recombination appears to be contemporaneous with that of DNA replication, we determined the effects of inhibiting DNA replication on the appearance of recombinant products. We employed three approaches to block DNA replication: treatment with the potent protein synthesis inhibitor anisomycin, treatment with the DNA replication inhibitor cytosine arabinoside, and the use of temperature-sensitive mutations in the adenovirus-specified DNA-binding protein and DNA polymerase genes. All such disruptions of normal DNA replication severely delayed the appearance of recombinant products. In addition, we showed that at 40.9°C, in cells infected with wild-type virus, recombination is delayed, despite active DNA replication. These results imply that one of the components of the recombination machinery is intrinsically thermolabile.

MATERIALS AND METHODS

Cells. Human KB cells were used as the host for all the recombination experiments, production of virus stocks, and titration by fluorescent focus assay. Plaque assays were performed on HeLa cells. Details of the methods may be found in Young and Silverstein (28) and references therein.

Viruses. The assay for recombination requires viruses that are differentially marked so that crossing over between two restriction site alleles can be scored (11). The alleles at the *Bam*HI sites at map units (m.u.) 29.3 and 42.0 are characteristic of adenovirus 2 but are missing from adenovirus 5 (19). The various pairs of parental viruses have the sites in *trans* or in *cis*, but recombination between them yields diagnostic recombinant fragments that can be detected in the pool of intracellular DNA by Southern blot hybridization with the appropriate probe (28). The geometries of the two types of crossovers that we scored are presented in Fig. 1, and the relevant genotypes and derivation of each parent are presented in Table 1.

Virological methods. Most of the virological methods used have been described previously (28). The various specific

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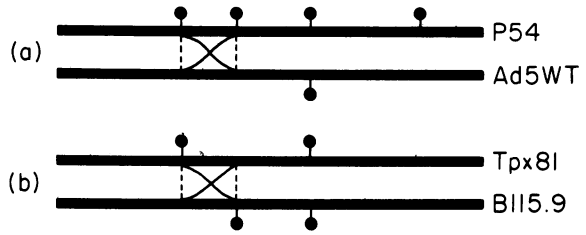

FIG. 1. Genomes of the viruses used in the crosses. In cross (a), recombination between the two *Bam*HI restriction sites at 29.3 and 42.0 m.u. generated two recombinant products, each containing a single leftward recognition site for the enzyme. The cloned probe containing adenovirus 2 sequences between 29.3 and 42.0 m.u. hybridized to parental fragments extending from 29.3 to 42.0 (P54) and from 0 to 59.5 m.u. (from wild-type adenovirus 5 [Ad5WT]) and to fragments of recombinant products extending from 0 to 42.0 and from 29.3 to 59.5 m.u. The crosses involving *ts*125 and *ts*149 were set up with restriction sites identical to those in cross (a). In cross (b), the probe hybridized to parental fragments extending from 29.3 to 59.5 (from Tpx81) and from 0 to 42.0 m.u. (from B115.9) and to recombinant fragments extending from 0 to 59.5 and from 29.3 to 42.0 m.u. Symbols: ♦, *Bam*HI sites; ≀, crossovers occurring somewhere in the interval from 29.3 to 42.0 m.u.

manipulations performed on the infected cells can be found in each figure legend. When cells were infected with temperature-sensitive mutants at the restrictive temperature, they were always suspended in medium warmed to the appropriate temperature after adsorption of virus. Incubation temperatures were monitored with a thermometer traceable to a National Bureau of Standards reference that was accurate to ±0.1°C.

Inhibitors. Anisomycin, kindly provided by Nathan Belcher of Pfizer Inc., Groton, Conn., was prepared as a 10 mM solution in ethyl alcohol and used at a final concentration of 100 μM. This concentration of drug reduces the incorporation of radioactive amino acids to <0.5% of normal levels (13). Cytosine 1-β-D-arabinofuranoside (Ara-C) was obtained from Sigma Chemical Co., St. Louis, Mo. A stock solution (2 mg/ml) was prepared in distilled water and sterilized by passage through a 0.22-μ filter; Ara-C was used at a final concentration of 20 μg/ml. Cytidine was obtained from the same source and prepared and used in the same fashion as Ara-C.

DNA filter hybridization. DNA was extracted from infected cells as previously described (28) except that treatment with RNase was omitted. The nucleic acid samples from infected cells were digested with 5 U of enzyme per μg of nucleic acid for 2 h at 37°C and then electrophoresed through 0.6% agarose gels prepared in 40 mM Tris–4mM CH_3COONa–1 mM EDTA (pH 7.9) at 40 V for 16 h. The gel was treated with mild acid to facilitate the transfer of high-molecular-weight DNA (25) and then treated sequentially with 500 mM NaOH and 20× SSC (1× SSC is 150 mM NaCl, 15 mM $Na_3C_6H_5O_7$) containing 100 mM Tris–100 mM HCl. The DNA in the gel was then transferred to nitrocellulose and prepared for blot hybridization (20) as previously described (26). A *Bam*HI fragment from adenovirus 2 encompassing m.u. 29.3 to 42.0 was cloned into pBR322 and used as the probe for detection of recombinant bands after nick translation with four ^{32}P-labeled deoxyribonucleotide triphosphates (14). Hybridizations were performed in 4× SSC–5% dextran sulfate–10 mM EDTA for 20 h at 68°C. The filters were sequentially washed for 10 min each at 68°C in solutions containing 2×, 1×, and 0.5× SSC and then placed between two sheets of plastic foil and exposed to X-ray film (Kodak XAR or DuPont Cronex 2DC) at −90°C with DuPont Cronex intensifying screens. The films were developed with an automatic developer (Kodak X-Omat).

RESULTS

Time course of recombination during infection. In a previous study, we demonstrated that recombination between viral genomes occurred during the eclipse phase of the replicative cycle (28). Recombinant molecules were detected among the background of input parental and replicating daughter molecules by isolating intracellular DNA, cleaving it with *Bam*HI, and analyzing the products of digestion by Southern blot hybridization. These experiments showed clearly that recombination was a continual process during the late phase of the adenovirus replicative cycle, because the fraction of the products that was indicative of recombination increased with time. However, these results did not establish whether recombination occurred before, at, or after the onset of DNA replication. A classical technique which would directly establish whether recombination between parental molecules occurred before or during DNA replication involves the use of density transfer experiments. In such experiments, both parental and daughter molecules are isolated and analyzed for the presence of diagnostic recombinant bands. Our attempts to apply this technique to our system, by crossing viruses whose genomes were substituted with bromodeoxyuridine, were unsuccessful. Novel bands that did not correspond to either input parental sequences or known recombinant products were observed, making interpretation of the data problematic. Instead, we chose to determine the earliest time in eclipse at which recombination could be detected and to compare this with the onset of DNA replication. Our aim was to detect very low levels of recombination. Consequently, viruses were constructed so that truly recombinant bands could easily be distinguished from partial digestion products. The cross in Fig. 1b, in which the *Bam*HI sites at 29.3 and 42.0 m.u. are in *trans*, allows a recombinant product fragment extending from 29.3 to 42.0 m.u. to be produced. This band is diagnostic for recombination, whereas the reciprocal fragment which extends from 0 to 59.5 m.u. could result from partial digestion of complete genomic DNA.

KB cells in suspension culture were infected with ca. 100 PFU of each parent; samples were withdrawn at intervals, and the intracellular DNA was extracted. After digestion with *Bam*HI, the DNA was displayed on an agarose gel and then analyzed by Southern blot hybridization for the presence of bands that were diagnostic for parental and recombinant genomes. The autoradiogram of the hybridized blot

TABLE 1. Viral strains used

Strain	Segregating markers[a]		Relevant genetic markers	Lesion	References
	*Bam*HI 29.3	*Bam*HI 42.0			
Ad5WT	−	−			4
P54	+	+			16
B115.9	−	+			[b]
Tpx81	+	−			16
H5*ts*125	−	−	*ts*125	DBP	4,12,23
17	+	+	*ts*125	DBP	16
H5*ts*149	−	−	*ts*149	Pol	7,10,22
I_0	+	+	*ts*149	Pol	16

[a] Only those restriction endonuclease sites whose rearrangement was monitored by Southern blot hybridization are tabulated. + and − indicate the presence or absence of the respective sites.

[b] Munz and Young, Virology, in press.

shows that a novel band diagnostic for recombination could be detected as early as 9 h postinfection (Fig. 2). Further exposure of this and other gels failed to reveal the presence of recombinant bands in either the 7- or 8-h samples. From the intensities of the parental bands, it is also clear that DNA replication had begun between 7 and 9 h postinfection. In addition, we note that the intensities of the recombinant product bands relative to the genotypically parental bands in the 24 h sample were much higher than at 9 h, confirming our previous observation that recombination continues into the late phase of the replicative cycle.

Anisomycin blocks recombination. In the previous section, we showed that the onset of DNA replication and the first appearance of recombinant products are closely linked in time. One possibility consistent with this observation is that replication itself is necessary or important for one or more stages of recombination. If this is the case, blocking DNA replication should inhibit or abolish recombination between input parental genomes. Our first attempt to test this hypothesis involved the use of the potent protein synthesis inhibitor anisomycin.

A concentrated suspension of KB cells was prepared and divided into separate cultures. Anisomycin was added 0.5 h before infection to one sample, at infection to another, and at various times thereafter to others. All cultures were incubated at 37°C for 24 h after infection; the cells were harvested, and intracellular DNA was isolated from them to determine whether any recombination had occurred. The results of this analysis can be summarized as follows (Fig. 3). In cultures treated with anisomycin early and continuously, no recombination was observed. This was the case for all cultures to which the drug was added before 5 h postinfection. If, however, addition of drug was delayed until later periods of time, increasing amounts of recombinant products were detected. Concomitant with this increase, the total yield of DNA increased, as judged by the intensity of the hybridization signal. This suggests that the block to protein synthesis at 6 or 7 h occurred after the onset of DNA replication, because the addition of anisomycin is expected to inhibit replication completely. The effects of anisomycin were reversible, since cultures that were treated from 0.5 h before infection and then washed and suspended in medium without drug at 4 or 7 h postinfection demonstrated an ability to synthesize DNA and recombine genomes when assayed after 24 h. These data are consistent with the hypothesis that DNA replication is a potentiating step in recombination but of course do not exclude the possibility that other viral early functions expressed between the time of infection and 5 h postinfection are necessary for recombination. Nor do they exclude the possibility of a role for rapidly turning over host functions in recombination.

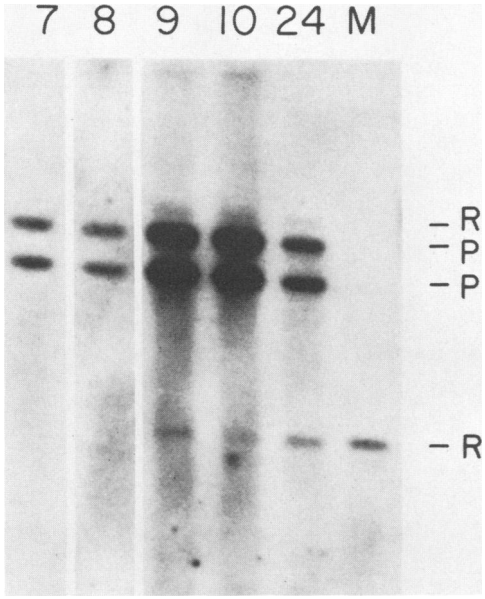

FIG. 2. Time course of recombination. KB cells were infected as described in the text with 100 focus-forming units of Tpx81 and B115.9 [cross (b) of Fig. 1] per cell. Samples were removed at intervals and processed for analysis by blot hybridization. Each lane of the gel contained 2.0 μg of nucleic acid except the 24-h sample, which contained 100 ng. The numbers above the lanes refer to the time (hours) postinfection; M refers to a sample from cells infected with P54. When cleaved with *Bam*HI, P54 DNA generated a fragment from 29.3 to 42.0 m.u. which was detected by the probe and is diagnostic of recombination in cross (b). The viral replication cycle was still in eclipse at 10 h, but by 24 h, it had produced 2.9×10^3 viruses per cell. P, Parental bands; and R, recombinant bands.

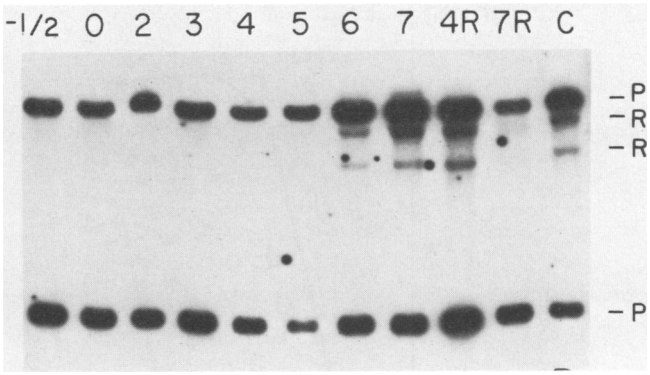

FIG. 3. Effects of anisomycin upon recombination. KB cells were centrifuged, suspended at 2×10^6 cells/ml, and distributed to 3 spinner flasks. One culture received anisomycin; the other two received an equivalent volume of ethanol. All three were incubated at 37°C for 0.5 h, after which anisomycin was added to the second culture and 100 PFU of P54 and wild-type adenovirus 5 virus [cross (a)] was added to all three. At 2 h postinfection, all three were diluted to 2×10^5 cells/ml; the correct concentration of anisomycin was maintained. At 2, 3, 4, 5, 6, and 7 h postinfection, samples were withdrawn from the untreated culture, supplemented with anisomycin, and incubated. At 4 and 7 h, samples from the 0.5-h preinfection culture were withdrawn, harvested, washed, and suspended in medium lacking anisomycin. All cultures were harvested at 24 h and processed for analysis by blot hybridization. The numbers above each lane refer to the time (hours) before or after infection at which anisomycin was added. Suffix R refers to the time of removal of anisomycin. An equal amount of intracellular DNA was loaded onto each lane except for the untreated controls and the reversed cultures, all of which received 10-fold less. P, Parental bands; and R, recombinant bands.

The appearance of recombinant products in wild-type infections blocked with Ara-C. The use of a protein synthesis inhibitor to block DNA replication is necessarily indirect and affects the synthesis of proteins other than those required for replication. To examine the role of replication more directly, we chose to use Ara-C as an inhibitor of DNA replication. This drug has been shown to be a potent inhibitor of virus DNA replication in cell culture, provided that it is repeatedly added to the medium (9). Moreover, early virus-specified mRNAs and polypeptides accumulate in the presence of this drug (9). Therefore, if any viral functions operate exclusively

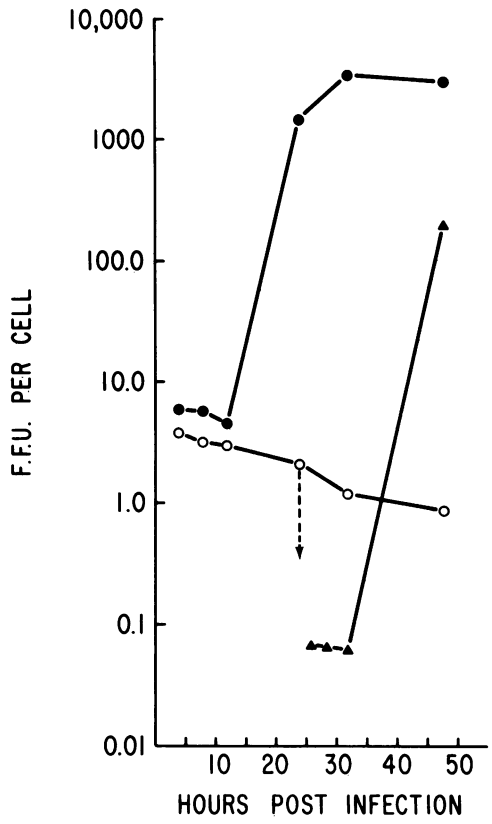

FIG. 4. Viral replication curves in cultures treated with Ara-C. KB cells were infected with 100 focus-forming units of Tpx81 and B115.9 [cross (b)] per cell. After adsorption, cells were harvested, suspended at 2×10^5 cells/ml, and divided into two cultures, to one of which 20 μg of Ara-C per ml was added. Samples were removed at intervals, and infectious virus was titrated by fluorescent focus assay. At 24 h postinoculation, one part of the culture treated with Ara-C was reversed by addition of cytidine, centrifugation, and suspension in medium containing 20 μg of cytidine per ml. Symbols: ●, untreated culture, ○; culture treated with Ara-C; and ▲, cytidine-reversed culture.

on the recombination pathway, they may have the opportunity to do so in the blocked cross. If, on the other hand, recombination requires actively replicating templates, no recombination will be observed until the block is removed. Furthermore, if recombinational activities accumulate before the removal of the block, the onset of replication may trigger a burst of recombination.

KB cells were infected, and one part of the culture was maintained in medium containing Ara-C. At 24 h, a portion of this culture was removed; cytidine was added, and the cells were centrifuged and suspended in medium containing cytidine to reverse the action of the drug. This culture was incubated for an additional 24 h, and samples were withdrawn at intervals for analysis by Southern blot hybridization.

Figure 4 shows the viral replicative cycles for the three cultures. The repetitive addition of Ara-C prevented the virus from replicating extensively, although the high background of unadsorbed virus prevents us from ruling out a limited amount of viral production. The reversal was effective in allowing replication to begin. Note that the background level of unadsorbed virus was considerably lower than in the culture treated with Ara-C, presumably because the extra centrifugation and suspension in fresh medium lowered the concentration of unadsorbed virus. Figure 5 presents the blot hybridization profiles of DNA extracted from the three cultures. In the untreated culture, we detected a distinct recombinant band at 12 but not at 8 h postinfection; DNA replication had initiated at some point within this interval. In the culture treated with Ara-C, a small amount of recombination was detected at 24 h, but at 32 and 48 h, when some DNA replication had taken place, more recombinant product was apparent. Since Ara-C was added at 10-h intervals, the appearance of DNA replication was unexpected, but it does not alter the major conclusion that blocking DNA replication slows down the appearance and reduces the extent of recombination. In the reversed culture, DNA replication began no later than 4.5 h after suspension in cytidine-containing medium and proceeded to a level higher than was detected in the untreated control. In the autoradiograms, equivalent amounts of nucleic acid were electrophoresed from the two 48-h samples. Recombination proceeded in concert with replication, but bands indicative of recombination did not appear to increase dramatically compared with bands of parental genotype. One may conclude that the accumulation of early gene products up to 24 h postinfection, particularly those necessary for DNA replication, allowed replication to occur synchronously and rapidly after the Ara-C block was reversed; however, specific recombinational functions did not appear to accumulate and act rapidly once the block to replication was removed.

Recombination in crosses employing *ts* DNA replication mutants. The results with Ara-C outlined above indicate that DNA replication is probably an important potentiating event in recombination. If this is so, then *ts* mutations located in

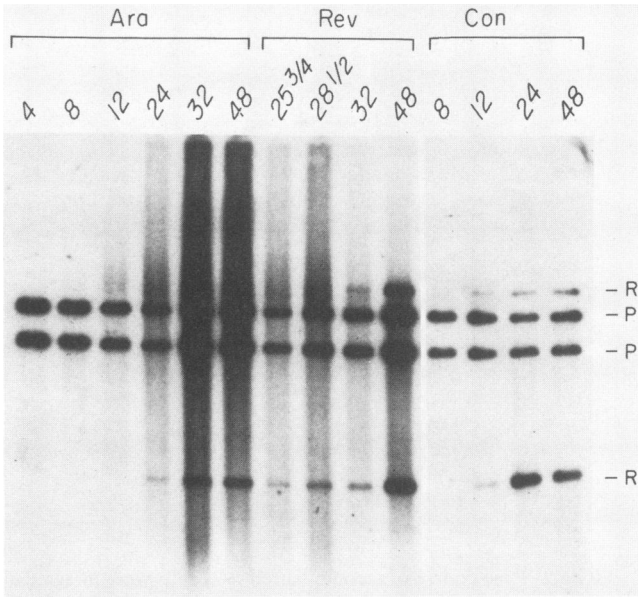

FIG. 5. Effects of Ara-C treatment upon recombination. Samples from the cultures shown in Fig. 4 were prepared for analysis by Southern blot hybridization as described in the text. The autoradiogram is blocked into three sections: Con, control cultures; Ara, samples removed from drug treated cultures; and Rev, reversed culture. The numbers above the lanes refer to the times postinfection at which the samples were taken. The amount of nucleic acid analyzed in each lane was as follows. Ara (all samples): 1.2 μg. Con: 8, 1.2 μg; 12, 240 ng; 24 and 48, 120 ng. Rev: 25.7 and 28.5, 1.2 μg; 32, 240 ng; 48, 120 ng. P, Parental bands; R, recombinant bands.

functions essential for adenovirus DNA replication should fail to recombine at the restrictive temperature. Two complementation groups of DNA$^-$ *ts* mutants exist, one containing lesions in the 72K DNA-binding protein (4, 12, 23) and the other in the adenovirus-specific DNA polymerase (7, 10, 22). The two alleles chosen for the crosses were H5*ts*125 (DBP$^-$) and H5*ts*149 (pol$^-$), respectively. Each infection was designed as a homoallelic cross but with a distinctive pair of *Bam*HI markers (Fig. 1a).

KB cells were infected with pairs of virus containing either *ts*125, *ts*149, or wild-type alleles. Virus was absorbed for 2 h at 36°C, and the cells were then centrifuged, suspended in prewarmed medium, and incubated in a water bath at 39.9°C. Samples were withdrawn at 5, 10, and 24 h postinfection and examined for the presence of recombinant products. In the wild-type control culture, recombination was detected in the 5-h sample, and by 24 h, extensive recombination and replication had occurred (Fig. 6A). In contrast, no recombination was detected in the cells infected with *ts* mutants until 24 h postinfection. The extent was limited, and the band intensities in the autoradiogram indicate that a limited amount of replication (perhaps a two- to threefold increase) had taken place. This was not caused by the presence of *ts*$^+$ revertants in the culture, which were estimated to be present at a frequency of $<10^{-5}$ and were not enriched during the incubation (data not shown). The conclusion from these data is that blocking DNA replication delays the onset and the extent of recombination when either the DNA-binding protein or the polymerase is defective. We might also suggest that the limited amount of recombination that was detected was associated with an equally limited amount of replication that took place at 39.9°C.

The effects of nonphysiological temperatures upon recombination. In the experiments performed at 39.9°C, it could be argued that the appearance of recombinant bands in the cells infected with *ts* mutants was linked to the small degree of "leakiness" observed in DNA replication. If conditions could be found in which this leakiness was abolished, then recombination should likewise disappear. In other studies, 41°C has been used as the nonpermissive temperature for experiments with H5*ts*125, as DNA synthesis was shown to stop rapidly upon shift up from the permissive temperature (2, 6). Accordingly, we chose to use 40.9°C for one set of cultures, in parallel with the 39.9°C set described in the previous section, to determine if this more stringent condition blocked both DNA replication and recombination.

After adsorption at 36°C for 2 h, the cultures were divided in two; one was incubated at 39.9°C as just described, and the other was incubated at 40.9°C. Samples were withdrawn at the same times and processed for analysis by Southern blot hybridization (Fig. 6B). In both mutant-infected cultures, replication was completely abolished and so was recombination. But the more striking observation is that in the wild-type infection, recombination was delayed and was not apparent at 10 h postinfection, and the extent of recombination after 24 h was not as great as that in the sample incubated at 39.9°C. DNA replication did not appear to be affected in either its time of onset or its extent. This result strongly suggests that there is an intrinsically thermolabile function in the infected cell, a function that either directly or indirectly affects the process of viral recombination. In a separate experiment, we confirmed the effect of incubation at the elevated temperature; the mutant crosses showed no recombination, and the wild-type control showed a delayed onset (about 12 h postinfection) of recombination (data not shown). We also note that the data from mutant crosses at

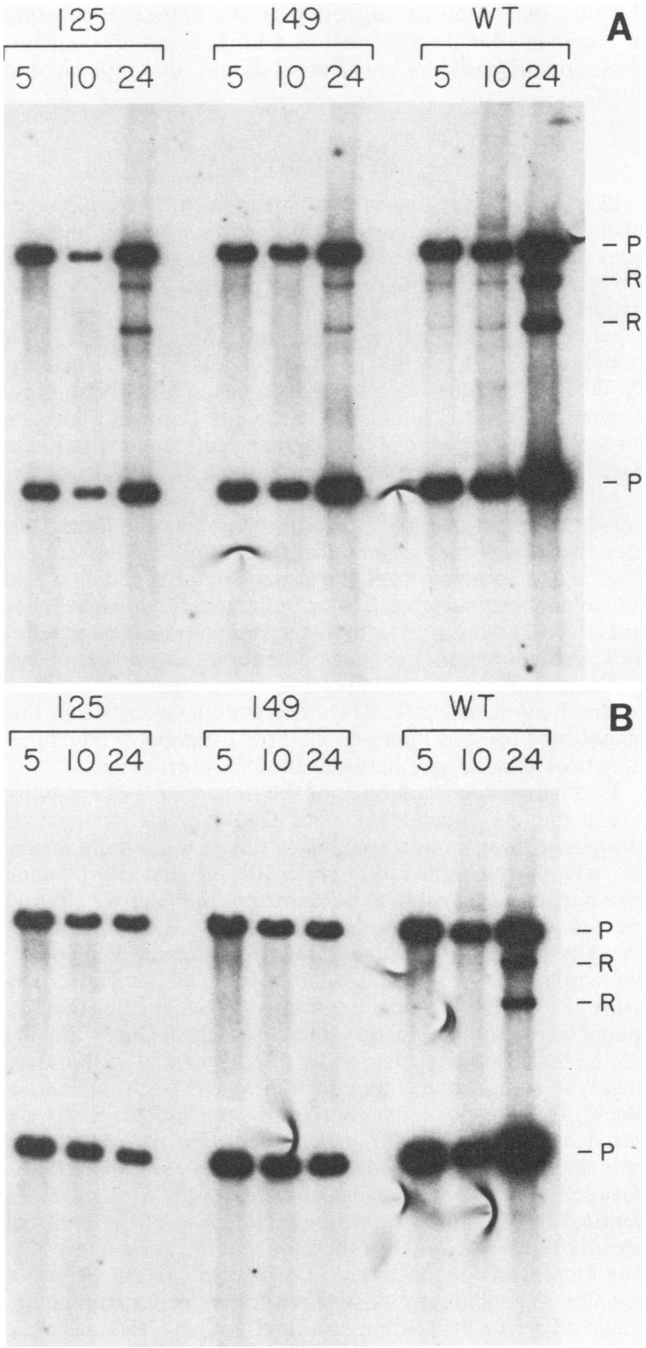

FIG. 6. Effects of mutations in the adenovirus DBP and DNA polymerase genes upon recombination. KB cells were infected with mutant or wild-type genomes in the cross (a) configuration (Fig. 1) at an MOI of 250 focus-forming units of each parent. After 2 h of adsorption at 36°C, cells were centrifuged at room temperature, suspended in prewarmed medium, and incubated at either 39.9°C or 40.9°C. Samples were taken at intervals for both fluorescent focus and recombination assays. (A) Incubation at 39.9°C. No viral production above the eclipse value was detected in either mutant cross. The wild-type infection yielded 270 focus-forming units per cell at 24 h. (B) Incubation at 40.9°C. No viral production was observed in any of the crosses. The amounts of nucleic acid analyzed in each lane were as follows. Mutant crosses (all time points): 1.2 μg. Wild type: 5, 1.2 ug; 10, 240 ng; and 24, 120 ng. The numbers above the lanes refer to times postinfection at which the samples were removed. P: Parental bands; and R, recombinant bands.

the two temperatures suggest that the limited replication observed at 39.9°C was almost certainly a result of leakiness, since contaminating ts^+ revertants should have replicated at 40°9C.

DISCUSSION

In organisms ranging in complexity from bacteriophages to the fungi, there are several pathways whereby homologous recombination leads to genetic rearrangement. It seems likely that a similar situation holds for adenovirus recombination and that we can expect diverse mechanisms to play a role in the production of recombinants after infection (28) or transfection with partial genomes (8, 24; F. C. Volkert, Ph.D. thesis, Columbia University, New York, 1983). Nevertheless, despite the diversity, major pathways can be discerned that play the predominant role in recombination in particular organisms. Such is the case in bacteriophage T4, in which the ends of molecules produced by replication invade recipient duplexes to form recombination intermediates that are in turn origins for new rounds of replication. This major pathway links the processes of replication and recombination and produces specific genetic consequences (reviewed by Mosig [15]). In this system, replication potentiates recombination. It is our contention that in adenovirus also, replication is an important component in a major recombination pathway. The experiments described in this manuscript present data which show a temporal and functional correspondence between these two processes.

Two independent analyses of the time course of recombination and its coincidence with DNA replication demonstrate that these events are closely linked temporally during the adenovirus replicative cycle (Fig. 2 and 5). In each instance it was possible to demonstrate that shortly after an increase in the amount of virus DNA could be detected it was possible to find evidence for the presence of recombinant molecules within the pool of intracellular DNA. Because the relative proportion of novel DNA species that are indicative of recombination increased throughout the life cycle, these studies also show that genomes of parental genotype continue to recombine during the DNA replication phase. This conclusion supports previous findings that were based on genetic (16, 28) and physical (28) tests. Taken in toto, these experiments demonstrate that recombination is not restricted to a prereplicative phase of the viral cycle, in contrast to pseudorabies virus (1). Because of the limits of sensitivity associated with the blot hybridization assay that was employed for the analysis of recombination, it is not possible to exclude the possibility that some recombination occurred before replication of virus DNA. Nevertheless, the bulk occurred after the initiation of rounds of DNA replication.

The apparently contemporaneous onset of recombination and replication could result from one or a combination of reasons as mentioned above. These might be classified into two categories: one in which the two events are functionally or structurally coupled or both, and one in which the coincidence is merely temporal. To differentiate between these possibilities, we attempted to uncouple the processes of replication and recombination by employing a variety of means to block DNA replication. Inhibition of protein synthesis until as late as 4 h postinfection blocked both DNA replication and recombination (Fig. 3). When the inhibitor was added at 5 h, a limited amount of recombination could be detected at 24 h. As the addition of the inhibitor was delayed further into the replication cycle, more recombination was detected. On the basis of changes in the intensity of the hybridization signal from the bands of parental genotype, we estimate that in this experiment (Fig. 3), DNA synthesis initiated somewhere between 5 and 6 h postinfection. These results rule out the possibility that recombination is mediated solely by stable cellular products and strongly suggest a role in recombination for a virus-specified product(s) synthesized before 5 h postinfection.

The experiment in which DNA replication was blocked by the addition of Ara-C demonstrated that delaying the onset of replication delayed the appearance of recombinant genomes (Fig. 5). Reversal of the block allowed replication to proceed after a short lag, and recombination likewise became more apparent. Early gene products accumulate in the presence of Ara-C (9). This observation, in conjunction with the data from Fig. 5, suggests that, although they are necessary, early products by themselves are not sufficient to allow viral recombination to proceed efficiently. Some further points should be made about the data. (i) Despite the replenishment of the levels of Ara-C at 10-h intervals, a limited amount of DNA replication occurred. To what extent the observed recombination depended on this small level of DNA synthesis is not clear. (ii) Treatment with Ara-C leads to the production of a distinct background of hybridizable material, particularly noticeable in DNA from the 32- and 48-h samples. The origin of this material is unknown. (iii) After reversal, there was a marked increase, compared with the untreated control, in the total amount of DNA that accumulated after 48 h. We suspect that this results from the synchronization of initiation of DNA replication as a result of accumulation of high levels of the relevant viral polypeptides.

The use of inhibitory drugs to study biological processes has obvious disadvantages, so we considered it necessary to use conditional lethal mutants that were temperature sensitive in DNA replicative functions to study the relationship between replication and recombination. Mutations in the adenovirus-specified DNA polymerase (7, 10, 22) and DNA-binding protein (4, 23) are available and have been transferred to genetic backgrounds that are suitable for recombination studies (16). When cells were infected at 39.9°C (a temperature generally held to be nonpermissive) with a pair of viruses that were allelic with respect to the mutations but not with respect to the disposition of restriction endonuclease sites, a limited amount of recombination was detected after 24 h (Fig. 6A). However, a limited amount of replication had also taken place, almost certainly reflecting leakiness of the mutations and not the presence of ts^+ revertants. To curb the leakiness, a more stringent temperature, 40.9°C, was employed. Mutant viruses crossed under these conditions did not replicate their DNA, and bands indicative of recombination were not detected by blot hybridization (Fig. 6B). The most striking result of this experiment was that in the control crosses, recombination was delayed and never reached the levels seen in the culture incubated at 39.9°C, despite the fact that more virus DNA accumulated at the increased temperature. Thus it appears that a partially thermolabile component, distinct from those involved in replication, is required for recombination. Others have reported thermosensitive functions in adenovirus replication, namely, at assembly (18) and in the inhibition of host DNA replication (6). To what degree these observations and the one we report are related is as yet unknown.

Taken together, the data on blocking DNA replication suggest a role for replication in the potentiation of recombination. We have considered two possibilities. One is the

relatively trivial explanation that the genome pool before replication is too small to allow recombination to occur or to be detected by hybridization. We think this is unlikely, because recombination is readily detected even when replication is severely limited. Furthermore, in a separate experiment with a pair of *ts*125 mutants incubated at 39.9°C, we examined the effect of varying the input multiplicity of infection (MOI) on recombination. Cells were infected at MOIs of 10 + 10, 50 + 50, and 250 + 250. No viral production was observed, but as with the experiment presented in Fig. 6A, a very limited amount of DNA replication took place at all MOIs. Even at a combined MOI of as little as 20 PFU/cell, recombinant bands could be detected (data not shown). We are confident, therefore, that the absence of recombination in several of the experiments reported here reflects either no recombination or extremely low levels of recombination and that genome pool size is not a limiting factor.

An alternative explanation is that replication and recombination are related as a consequence of sharing functions or by virtue of structural features such as the production and processing of single-stranded sequences as first suggested by Flint et al. (5). Our data are entirely consistent with this explanation. Any treatment leading to the abolition of DNA synthesis delays or reduces recombination. However, our data do not distinguish among the possibilities: (i) the two processes may share functions, such as the DNA-binding protein, DNA polymerase, or cellular polypeptides: (ii) viral replication may be required to produce single strands that are the substrates for the recombination machinery; and (iii) both functions and DNA structures may be required. If replication and recombination are truly interdigitated, then addressing the functions that are required to effect reciprocal exchange in adenovirus-infected cells will prove difficult. Nevertheless, with the development of systems capable of replicating adenovirus DNA in vitro both in crude nuclear extracts (3) and in a purified system (17), it may be possible to distinguish those functions required for both replication and recombination from those required only for the latter. A simple hybridization assay for detecting recombination by the rearrangement of restriction markers, as described here, will be invaluable for this task.

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