Intramolecular Recombination between Transfected Repeated Sequences in Mammalian cells Is Nonconservative

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When plasmids carrying a fragmented gene with segments present as direct repeats are introduced into mammalian cells, recombination or gene conversion between the repeated sequences can reconstruct the gene. Intramolecular recombination leads to the deletion of the intervening sequences and the loss of one copy of the repeat. This process is known to be stimulated by double-strand breaks. Two current models for recombination in eucaryotic cells propose that the reaction is initiated by double-strand breaks, but differ in their predictions as to the fate of the intervening sequences. One model suggests that these sequences are always lost, while the other indicates that the reaction will be conservative as a function of the position of the double-strand break. We have constructed a plasmid in which two overlapping portions of the simian virus 40 early region, which contains the origin and T-antigen gene, are present as direct repeats separated by sequences containing a plasmid with a simian virus 40 origin of replication. Recombination across the repeated segments could produce a plasmid with an origin of replication and/or a plasmid with a gene for a functional T-antigen which would drive the replication of both. Introduction of this construct into African green monkey kidney cells, without coinfection, establishes a condition in which the products of the recombination or gene conversion can be interpreted unambiguously. We find that the majority of the reconstruction reactions are nonconservative.

Recombination and gene conversion in mammalian cells have been studied by many groups who have monitored the reconstruction of selective genes after infection with appropriately constructed viral or plasmid substrates (1, 3, 5–7, 11, 12, 17, 20–22, 24, 25, 27). The results of these experiments indicate that cells efficiently support both intra- and intermolecular recombination and gene conversion. Intramolecular recombination, the particular concern of this report, can be modeled with plasmid constructions in which duplicated regions of a fragmented (or otherwise defective) gene are present as direct repeats separated by some intervening sequences. Recombination between the repeated sequences produces a functional gene while deleting the intervening sequences (4, 18). In experiments of this type, the focus is on the appearance of the active gene, and generally the fate of the intervening sequences is undetermined. Although there is limited experimental evidence on this point, the molecular models of recombination proposed by several groups do make specific predictions as to the fate of these sequences. Those models which suggest that recombination is initiated by nicks in a strand in one (15) or both (10) regions of homology predict that crossover resolution of the resultant Holliday junctions yields two smaller plasmids, each with one copy of the repeated sequence. Thus, the reaction, whatever the precise mechanism, would be conservative, i.e., the intervening sequence and both copies of the repeated element survive the reaction.

The relevance of these models to intramolecular recombination of homologous sequences transfected into mammalian cells is uncertain in the light of studies which show that the reaction is greatly stimulated by appropriately placed double-strand breaks (11, 12). Two current models describe recombination pathways that are initiated by a double-strand cut (12, 23). Both suggest that the initial cleavage is followed by exonuclease digestion at both termini to reveal 3′ single strands. In the model of Szostak et al. (23) the exonucleolytic activity is limited and exposes single strands homologous to the second repeat which is still in duplex form. Invasion of the duplex by the single strand is followed by a series of events which may be resolved with or without a crossover event. In the case of the intramolecular reaction, crossing over would yield two smaller plasmids. If the resolution proceeds without crossing over, it is possible to have conversion of mismatched sequences while the original size of the plasmid is maintained. If the initial cleavage occurs within one of the homology regions, then the exonucleolytic action immediately reveals single strands homologous to the other copy of the repeat, and no sequences need be lost in subsequent events. Under these circumstances the reaction would be conservative. If the initial cleavage occurs outside the homology region, the sequences between the cleavage site and the repeated sequences would be lost. This model suggests that the reaction would be conservative or nonconservative, depending on the position of the cleavage.

Sternberg and his colleagues have proposed that the exonucleolytic activity proceeds until homology is revealed in both resultant single strands (12). Pairing between strands yields a complex on which, after the action of endonucleases, gap-filling polymerases, and ligases, the gene is reconstructed. In this model all productive cleavages regardless of location lead to the loss of sequences, including one copy of the repeat, during reconstruction. Thus, this model is always nonconservative, and a gene conversion event with retention of the original plasmid size cannot occur.

We are interested in modeling conservative, homologous recombination in mammalian cells. Accordingly, it seemed useful to inquire whether the intramolecular reaction is conservative, i.e., do the sequences between the repeats (and a copy of the repeat) survive? We have designed an experiment, based on the classical illustration of this reac-

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reaction is defective. Gene conversion could convert the XbaI site back to the wild-type PstI site. Recombination across the repeated sequences could produce pST (5.7 kb) or pK (7.5 kb) or both.

MATERIALS AND METHODS

Cells. The African green monkey kidney (AGMK) cell lines CV-1 and COS-1 (8) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Plasmid construction and infection of cells. Plasmids were constructed using standard procedures.

Cells were infected with appropriate plasmids, using DEAE-dextran as a facilitator (14). The cells were seeded in 150-mm culture dishes so as to be approximately 50% confluent the next day. After washing with phosphate-buffered saline and TBS buffer (25 mM Tris hydrochloride, pH 7.3, 10 mM NaCl, 1 mM Na2HPO4), 1 ml of TBS, 500 μg of DEAE-dextran, and the indicated amount of plasmid DNA were added. The cells were incubated for 10 min at 37°C and then washed with TBS containing 1.0 mM CaCl2 and 0.3 mM MgCl2, then with phosphate-buffered saline. The cells were fed with Dulbecco modified Eagle medium supplemented with 2% fetal calf serum. After 44 to 48 h, they were extracted by the method of Hirt (9), and the DNA in the supernatant was further purified and digested with DpnI to remove the unreplicated input plasmid DNA (16). The DNA was then electrophoresed and blotted, and the identity of the replicated plasmids was determined by hybridization using plasmid pXP as the probe.

RESULTS

The products of intramolecular recombination between homologous sequences on the same plasmid can be modeled with the plasmids shown in Fig. 1. When the homologous sequences are present as direct repeats, then a simple rendering of the textbook schematic diagram provides two possible outcomes for the resolution of the intermediate complex. If crossing over occurs, two smaller plasmids will be formed. If resolution proceeds without crossover, then the original size plasmid is recovered, although if there is a mismatch in the homologous sequences, there is the possibility of conversion of the heteroduplex formed in the intermediate. Thus, a plasmid with the same size as the starting material, but with identical repeated sequences, may be formed.

No specific molecular mechanism is implied by this model, but there are clear predictions as to the possible products of the reaction. We have designed an experiment and constructed a plasmid to ask whether both smaller plasmids are formed during the reconstruction of the fragmented, partially duplicated gene, or whether gene conversion can occur to yield a starting size, “convertant” plasmid. The structure of this plasmid, pXP, is shown in Fig. 1. The plasmid has an entire copy of the simian virus 40 (SV40) early region (3,000 base pairs [bp], the HpaII-BamHI fragment) which includes the enhancer, the origin of replication, and the T-antigen gene. This T-antigen gene is defective because of the insertion of an XbaI linker at the PstI site. The region of the viral
genome from TaqI to BamHI (2,200 bp) is present again as a direct repeat on the plasmid. Although the sequences in this fragment are wild type, they are not sufficient to code for a functional T-antigen. Thus, the plasmid as constructed does not carry a wild-type T-antigen gene. Recombination or conversion between the repeated elements is necessary to form a functional gene coding for a wild-type T-antigen which will drive replication. Between the two repeats we inserted another plasmid which contains an SV40 origin of replication, the gene for the bacterial enzyme galactokinase, and those sequences of the pBR322 derivative pML2 (13) necessary for replication and resistance to ampicillin in bacteria.

Conservative recombination across the T-antigen homology regions would give rise to two product plasmids, one coding for a functional T-antigen, which would drive the replication of both plasmids. If the process were nonconservative, that is, if only one product were formed, then only the fully replication-competent plasmid (pST) would survive. If the other plasmid (pK) were formed in a nonconservative fashion, it could not support its own replication and would not appear as a replication product. If gene conversion without crossing over happened, a plasmid with the same size as the starting material, but with a wild-type T-antigen gene, could be produced. There are, of course, a variety of other events which would occur but would not produce a wild-type T-antigen gene and so would not be detected (e.g., recombination across the repeated ampicillin resistance genes). The relative mobilities of the three anticipated products are shown in the gel pattern of Fig. 2.

An unambiguous interpretation of the pattern of product formation in this experiment would be possible only if an infected cell contained no more than one copy of the starting plasmid. That is, the transfection must proceed without coinfection. We have established the conditions of transfection that comply with this restriction in the following way.

Two plasmids were constructed (Fig. 3). One contains the entire wild-type early region of SV40, including the enhancer, the origin, and a functional T-antigen gene. This plasmid is fully replication competent. The other plasmid contains an SV40 origin of replication and the gene for galactokinase, which can be used to distinguish the two plasmids in microbiological assays. The plasmids are sufficiently different in size that they can be identified by electrophoretic mobility. These two plasmids are similar to the two products predicted from conservative recombination between the T-antigen repeats on the pXP plasmid (Fig. 1).

The plasmids were mixed at an equimolar ratio, and serial dilutions of the mixture were prepared. CV-1 cells were infected with the dilutions, using DEAE-dextran as a facilitator (14). After 2 days, the cells were extracted by the method of Hirt (9), the DNA in the supernatant was purified, input DNA was eliminated by cleavage with DpnI (16), the plasmids were electrophoresed, and the gel was blotted. In the experiment shown in Fig. 3, the plasmids were linearized by EcoRI digestion to facilitate interpretation.

Figure 3 illustrates two extremes of our results. When approximately 100 ng of each plasmid was present in the solution applied to the cells, coinfection occurred and both plasmids were recovered. (Complete coinfection occurred when 200 to 500 ng of each plasmid was applied to the cells.) When the cells were exposed to approximately 1 ng of each plasmid, only the fully replication-competent plasmid was detected. Intermediate concentrations of the plasmid mixture yielded progressively lower relative amounts of the pK plasmid (not shown). We conclude that when the cells are exposed to the plasmid at 1 ng/ml under these conditions they are not detectably coinfected.

**Low-copy infection of cells with pXP.** CV-1 cells were infected with 1 ng of pXP per ml per dish. Since the molecular weight of this plasmid is almost twice that of the plasmids used to establish the conditions for the low-copy infection, this is a conservative condition for the infection. After 48 h, the cells were harvested and extracted as before. The hybridization pattern of the replicated products of the recombination is shown in Fig. 4. It is clear that the dominant product was that with the reconstructed T-antigen gene, the fully replication-competent plasmid (pST). The results of this experiment suggest that the intramolecular recombination reaction was primarily nonconservative; that is, in the majority of the reactions only one product plasmid was formed from a given starting plasmid. Since only the pST product is fully replication competent, that is the only product which could be detected.

**Infection of COS-1 cells with pXP.** Although the results of the preceding experiment may be interpreted as suggesting

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**FIG. 2.** Relative mobilities of pXP, pK, and pST. pK and pST were recovered from a Hirt extract of COS-1 cells infected with pXP by transformation of *Escherichia coli* HB101 followed by screening colonies for the two plasmids.

**FIG. 3.** Infection without coinfection. The plasmids pMLSV40 (6 kb) (similar to pST) and pML0galk (5.1 kb) (similar to pK) were mixed such that they were equimolar, and dilutions were prepared of the mixture. CV-1 cells were infected with the individual dilutions, and the plasmids were recovered after 48 h and analyzed by electrophoresis and hybridization of the blotted gels. Lane 1. Cells were infected with 100 ng of pMLSV40 (a) and 83 ng of pML0galk (b). Lane 2. Result of coinfection with 1 ng of pMLSV40 and 0.83 ng of pML0galk. Lane 3. Long exposure of lane 2. The dark smear adjacent to lane 3 is from lane 1 after long exposure (the negative was reversed to facilitate comparison of the lanes).
that the recombination is largely nonconservative, there are alternative explanations for the result. The first possibility is that there is some inherent, unappreciated difficulty in the formation of the plasmid, regardless of the mechanism of the pathway. We have considered this possibility by infecting COS-1 cells which contain an endogenous T-antigen gene. In this background any plasmid with an SV40 origin of replication will replicate, and so a variety of recombination products plus the starting material should appear. The results of this experiment are shown in Fig. 5A. The pattern includes the starting plasmid pXP, both pST and pK, and a plasmid which was the result of recombination across the two repeats of the β-lactamase gene in pXP (above pK, at 8,700 bp). The greater amount of the pST plasmid relative to the other products is due to the arrangement of sequences on the pXP plasmid. The pK portion of the starting plasmid presents a larger target for the initial cleavage event than does the pST side. The smallest target for a viable recombination event is that which lies between the two copies of the ampicillin resistance genes (see below).

Another explanation for the results of the infection of CV-1 cells with pXP (Fig. 4) is that there is some problem with the replication of the plasmid once formed. Although this question was anticipated in the coinfection experiments of Fig. 3, we recovered the products pST and pK from the Hirt extract of the COS-1 cell infection and purified them. We then infected CV-1 cells with the two plasmids under conditions of coinfection (Fig. 5B). (The plasmids were linearized before electrophoresis to facilitate comparison.) The data from these experiments indicate that the predicted products of recombination between the T-antigen repeated sequences on pXP can form, and if present in a CV-1 cell together, both will replicate. Thus, if the pK plasmid were generated in the recombination reaction, it would have been recovered. The most likely explanation for our failure to detect significant levels of this plasmid is that it was not formed during the reaction which produced the pST plasmid.

Infection with linearized pXP. Intramolecular recombination between homologous sequences on constructions of the pXP type is stimulated by appropriately placed double-strand breaks (12). Cleavage of transfected DNA in vivo has been demonstrated by several groups (2, 19, 25). Two current models for recombination in eucaryotes propose that the process is initiated by a double-strand break. Although the Sternberg model proposes that the recombination pathway is always nonconservative (12), that of Szostak et al. suggests that the process is conservative as a function of the position of cleavage (23). Consideration of the structure of pXP in the light of this model indicates that a conservative reaction could result from initial cleavages in one or another of the repeat sequences (2,200 × 2 = 4,400 bp), but if the cleavage were in the intervening region (5,200 bp containing the galK gene), the reaction would be nonconservative. (Cleavages on the other side of the plasmid would always be unproductive because the unduplicated N-terminal portion of the T-antigen gene would be destroyed during the process.

FIG. 4. Recovery of pST after low-copy infection with pXP. CV-1 cells were infected with 1 ng of pXP, and the replicated plasmids (DpnI resistant [16]) were displayed by electrophoresis and hybridization. The positions of the possible products, pST, pK, and starting plasmid (pXP), are shown. The smaller fragments are from the residual input pXP plasmid after DpnI cleavage. The film was exposed for 5 days.

FIG. 5. (A) Infection of COS-1 cells with pXP. The starting plasmid, pXP, and the products of recombination, pST and pK, were recovered. Densitometry of the original X-ray film indicated that there was 1.8 times as much pST as pK. The band marked with only an arrow is the product of recombination across the duplicated ampicillin resistance genes. (B) High-copy coinfection of CV-1 cells with pST (100 ng) and pK (130 ng). Before electrophoresis, the replicated plasmids were linearized to facilitate comparison.

FIG. 6. Infection of CV-1 cells with linearized pXP. Cells were infected with 1 ng of pXP linearized at the XbaI site. The positions of the superhelical pXP, pK, and pST are shown. The small fragments are from the input pXP after DpnI treatment. The film was exposed for 2 days. Note the intensity of the pST signal relative to the DpnI fragments in this figure compared to Fig. 4.
FIG. 7. Two pathways for reconstruction of the T-antigen gene. Both models initiate with a double-strand break. (Left) Szostak et al. (23) propose that mild exonuclease digestion reveals single strands which invade the duplex in the other homology region. Holliday junctions are established, and crossover resolution yields two product plasmids. (Right) Stemberg and colleagues (12) suggest that the exonuclease digestion is extensive and that reconstruction occurs by pairing of homologous single-strand regions. Digestion of nonhybridized single strands, repair synthesis, and ligation yield one product plasmid.
of revealing the single strands with homology to the other repeat.) Thus, more than 50% of the initial cleavage events in the cell would give rise to a nonconservative reaction even if the pathway described by Szostak et al. were followed (23). Furthermore, if there were bias in the cleavage site such that the intervening region with the galK gene were favored over the repeated sequences, there would be a higher frequency of nonconservative reactions. To deal with these questions directly, we repeated the CV-1 cell infection with pXP linearized at the single XbaI site which lies 670 bp from the end of one of the T-antigen repeats. Limited exonuclease digestion of this linear molecule in the cell, as called for by Szostak et al. (23), should permit the formation of the intermediates described in their model, and the reaction should then be conservative. CV-1 cells were infected with 1 ng of the linearized plasmid, and the experiment was carried out as before (Fig. 6). As predicted by both models, we recovered a strong signal at the position of pST but were unable to detect the other product of a conservative cross-over (pK) or the "gene converted" starting size plasmid. The recombination was again nonconservative.

DISCUSSION

The recombination reaction modeled in our experiment is known to be stimulated by appropriately placed double-strand breaks (14). Consequently, it seems appropriate to discuss the results in terms of two current models for recombination, both of which involve a double-strand break as the initiating event (Fig. 7). As discussed above, the model of Szostak et al. predicts a conservative reaction as a function of the site of the initial cleavage (23). Thus, in the infection with the plasmid linearized in one of the homology regions by cleavage at the XbaI site, the pK plasmid should appear as frequently as the plasmid which is fully replication competent, pST. This model also predicts, for this experiment, that if resolution of the Holliday junctions proceeds without crossing over, then the gap which began at the XbaI site would be repaired against wild-type sequences and a replication-competent pXP plasmid could be formed. These predictions were not realized in the experiments described here.

The model proposed by Sternberg and his colleagues (12) indicates that the sequences which lie between the two homology regions will be lost regardless of the position of the cleavage. Furthermore, only one copy of the repeated sequence would survive the reconstruction of the T-antigen gene. Our data are in agreement with these predictions and thus consistent with this model. Since our experiment is designed to display the products of the reaction, our results do not yield information about the intermediates formed during the recombination process and thus are silent regarding the validity of other features of the Sternberg model. An interesting variant of this model has been described recently (26). Furthermore, with respect to the model of Szostak et al., it is important to note that interpretation of our data is limited by the specific features of the experiment; we have studied intramolecular recombination between homologous sequences in transfected DNA. The requirement for the gene reconstruction to occur before replication can begin permits the copy number of the plasmid in an infected cell to be controlled and establishes a situation in which the appearance of specific products can be unambiguously interpreted. Thus, as noted by others (25), replication is not necessary and the reconstruction probably occurs very early in the infection, perhaps before the input plasmid is assembled into chromatin. These qualifications do not permit more general conclusions about the applicability of the double-strand break repair model for recombination in mammalian cells.

A key feature of the model of Szostak et al. is the call for "limited exonuclease digestion" after the double-strand cleavage. It may be that the product of the limited exonuclease digestion in our experiment is simply too short lived to participate in the invasion of the still double-stranded second homology region. If the competition between further exonuclease digestion and invasion of the second homology region favors the exonuclease, then most of the time the molecule will be converted to a structure appropriate for the intermediates proposed by the Sternberg group (12). Similarly, gene conversion, which has been reported by others in a somewhat different system (5), may also be precluded. If the plasmid were assembled into chromatin, then exonuclease digestion might be sufficiently "limited" to permit the intermediates proposed by Szostak et al. to form. It is also important to note that the double-strand break repair model was originally described in terms of intermolecular recombination. This type of reaction is not modeled in our experiment. (However, modifications of the model to explain the intramolecular mating type switching in Saccharomyces cerevisiae are discussed by Szostak et al. [23].)

It seems likely that conservative recombination between homologous sequences does not occur in mammalian cells and that specific assays can be designed to detect it. However, we suggest that constructions designed to measure intramolecular recombination do not report the conservative reaction. Our results are in agreement with those of Wake et al. (25), who infected cells with heterodimers of SV40 under conditions similar to those described here and found mixed plaques to be infrequent. We have infected cells with constructions in which portions of the T-antigen gene were present as inverted repeats. Intramolecular recombination between these elements would invert the sequences and reconstruct the T-antigen gene. We have not detected this inversion reaction. It remains to be seen whether rearrangements of this sort can occur in sequences integrated into the genome.

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